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Für Nana

“It’s never Lupus!”

Hugh Laurie, 2006

House M.D. S03e09 (“Finding Judas”)

Danksagungen

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Abstract

(i) Erythropoiesis strictly depends on signal transduction via the *erythropoietin receptor* (EpoR) – *Janus kinase 2* (Jak2) – *signal transducer and activator of transcription 5* (Stat5) axis, regulating proliferation, differentiation and survival. Using mice completely lacking Stat5 we demonstrated that these animals suffer from microcytic anemia due to reduced expression of the anti-apoptotic proteins Bcl-x_L and Mcl-1 resulting in enhanced apoptosis. Moreover, transferrin receptor-1 (TfR-1) cell surface levels were decreased >2-fold on erythroid cells of $Stat5^{-/-}$ animals. This reduction could be attributed to reduced transcription of TfR-1 mRNA and reduced expression of iron regulatory protein 2 (IRP-2), the major regulatory molecule of TfR-1 mRNA stability in erythroid cells. Finally, both genes were demonstrated to be direct transcriptional targets of Stat5. This established an unexpected mechanistic link between EpoR/Jak/Stat signaling and iron metabolism, processes absolutely essential for erythropoiesis and life.

(ii) Deletion of EpoR or Jak2 causes embryonic lethality as a result of defective erythropoiesis. The contribution of distinct EpoR/Jak2-induced signaling pathways to functional erythropoiesis is incompletely understood. We demonstrated that sole expression of a constitutively activated Stat5a mutant (cS5) was sufficient to overcome proliferation defects of $Jak2^{-/-}$ and $EpoR^{-/-}$ cells in an Epo independent manner. Transplantation of $Jak2^{-/-}$ fetal liver cells transduced with cS5, into irradiated mice gave rise to mature erythroid and myeloid cells of donor origin up to 6 months after transplantation. In conclusion, we demonstrated that activated Stat5 is a critical downstream effector of Jak2 in erythropoiesis/myelopoiesis.

(iii & iv) Stat5 has been implicated in lymphoid development and leukemic transformation. Most studies addressing these aspects have, however, so far employed "hypomorphic" Stat5-knock out mice still expressing N-terminally truncated Stat5 ($Stat5^{\Delta N/\Delta N}$ mice). We reanalyzed lymphoid development in $Stat5^{-/-}$ mice with a complete deletion of the Stat5a/b gene locus. $CD8^+$-T-lymphocytes, γδ T-cell receptor positive T-cells as well as $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T-cells were completely absent in $Stat5^{-/-}$ animals. Furthermore FoxP3, the transcription factor strictly required for regulatory T-cell polarization was shown to be a direct target gene of Stat5. Additionally, B-cell maturation was abrogated at the pre–pro-B-cell stage in $Stat5^{-/-}$ mice. Most strikingly however, $Stat5^{-/-}$ cells were resistant to leukemic transformation *in vitro* and leukemia development *in vivo*, induced by Abelson oncogenes (bcr/abl p185 & v-abl).

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1 Introduction

1.1 Hematopoiesis

Hematopoiesis (derived from ancient greek: *haima* - blood; *poiesis* – to make) is the process of formation of all cellular blood components. Blood is a very complex organ, consisting of multiple specialized cell types. The vast majority of cells in blood are *erythrocytes* (red blood cells; approx. 4-6x10^6 cells/µL human blood) whose functions are tissue oxygen transport and carbon dioxide removal. *Thrombocytes* (platelets; approx. 2-5x10^5 platelets/µL human blood) are responsible for blood coagulation (blood clotting) to prevent blood loss in case of blood vessel damage. The category of *leukocytes,* the cellular guardians of our body, can be subdivided into *granulocytes, lymphocytes* and *monocytes* (white blood cells; approx. 4-10x10^3 cells/µL human blood). Their multifaceted task is to clear and destroy old and/or defective cells as well as to fight pathogens.

In tissues with high cellular turnover (e.g. blood) stem cells are pivotal for lifelong maintenance of organ function. *Hematopoietic stem cells* (HSCs), residing in the bone marrow (BM) of adults, are responsible for the permanent daily production of all mature blood lineages (Weissman 2000).

1.1.1 Ontogeny of the hematopoietic system in the mouse

Already more than 100 years ago the observation of the close temporal and spatial relationship between blood and endothelial cells during embryogenesis led to the hypothesis of a common ancestral cell, called *hemangioblast* (His 1900). This hemangioblast was believed to reside in the yolk sac (Haar and Ackermann 1971; Ferkowicz and Yoder 2005) and to spawn both, the hematopoietic as well as the endothelial lineage, during embryonic development. Although a primary allocation of hemangioblasts to the yolk sac was backed by early studies (Murray 1932; Shalaby et al. 1995; Shalaby et al. 1997), Gordon Keller and colleagues were able to provide experimental evidence for the existence of such a cell type *in-vitro* in embryoid-body differentiation cultures (Choi et al. 1998) and more recently also *in-vivo* at the mid-streak stage of gastrulation in the ventral mesoderm of the developing mouse embryo (Huber et al. 2004). Despite these findings, formal proof of the hemangioblast hypothesis is still missing, since this would require direct demonstration of a single cell

(hemangioblast) dividing asymmetrically and giving rise to both, hematopoietic stem cells as well as endothelial stem cells *in-vivo*. Nevertheless one can conclude that the first cellular commitment towards a hematopoietic fate starts in the ventral mesoderm briefly after the initiation of gastrulation (for a review see Murry and Keller 2008).

The first wave ("primitive") of embryonic blood formation takes place in the yolk sac (Figure 1) between embryonic days E7.25–E9.0 (Wong et al. 1986; Palis et al. 1999). Immature primitive erythroid cells rapidly gather into so-called blood islands, and become enveloped by endothelial cells (Haar and Ackermann 1971; Ferkowicz and Yoder 2005) enter the circulation and support proper tissue oxygenation of the rapidly growing embryo (Wong et al. 1986). In contrast to adult-type erythroid cells, primitive red blood cells retain their nucleus longer and express adult-type as well as embryonic globin chains. The second wave of embryonic blood formation in the yolk sac between E8.25 and E10.5 generates erythroid progenitors which colonize the fetal liver (Figure 1) at E9.5 and initiate adult-type ("definitive") erythropoiesis, which readily displaces primitive erythropoiesis (Palis et al. 1999; for a review see (Palis 2008). Of note, primitive erythroid cells are present in the embryo already prior to the development of fetal hematopoietic stem cells, which become the source of all blood lineages throughout later stages of embryogenesis.

It is well established that the *aorta-gonad-mesonephros* (AGM) region (Figure 1), which is composed of the dorsal aorta, its surrounding mesenchyme and the urogenital ridges is a source for definitive HSCs (Muller et al. 1994; Cumano et al. 1996; Medvinsky and Dzierzak 1996). It was suggested that so-called "hemogenic endothelial cells" at the ventral wall of the dorsal aorta bud off HSCs (North et al. 1999; North et al. 2002). HSC activity is technically defined by their capability to reconstitute an entire hematopoietic system of a recipient upon transplantation, and single cell suspensions of E11 AGM cells indeed displayed significant HSC engraftment activity in adult recipients (Muller et al. 1994).

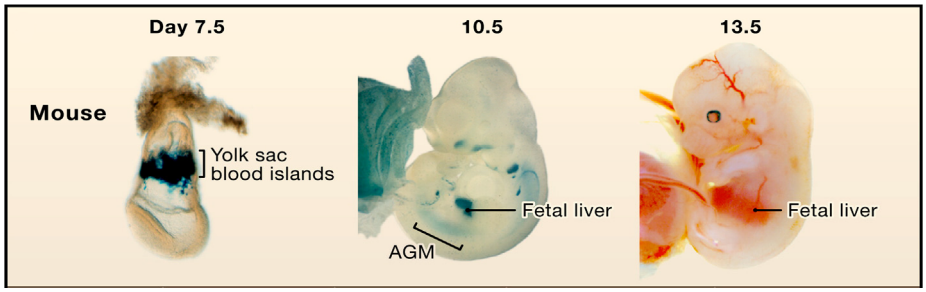

Figure 1: Sites of embryonic and fetal hematopoiesis. Hematopoiesis first occurs in the yolk sac (YS) blood islands and later at the aorta-gonad-mesonephros (AGM) region, placenta and fetal liver (FL). YS blood islands are visualized by knockin of a lacZ-reporter into the Gata-1 locus. AGM and FL are visualized by knockin of a lacZ-reporter into the Runx-1 locus. (adapted from Orkin & Zon, - Cell 2008)

Additional HSC activity was detected in the placenta of mouse embryos suggesting the placenta as additional niche, in which fetal HSCs could develop (Gekas et al. 2005; Ottersbach and Dzierzak 2005; Figure 2). Until recently, however it had remained unclear whether the placenta only provided a suitable microenvironment to support expansion of AGM derived HSCs flushed to the placenta via the circulation, or if it was also a site of genuine *de novo* HSC-genesis. This question was recently clarified by an elegant study utilizing mice deficient for the Na^{+}/Ca^{2+} exchanger Ncx1 (Rhodes et al. 2008), which lack heartbeat and hence are not able to establish blood circulation (Koushik et al. 2001). Interestingly, $Ncx1^{-/-}$ mice exhibited fetal HSCs (defined as $CD41^{+}$ $Runx1^{+}$ cells) in the large vessels of the placenta, although at reduced numbers compared to controls (Rhodes et al. 2008). These findings established the placenta as a site of *de novo* HSC-genesis, as well as a supportive niche for AGM derived fetal HSCs.

The AGM region has been widely viewed as the principal site for HSC production during vertebrate development. Accordingly, the yolk sac has often been demoted to a inferior position, despite older experiments which had suggested it as a source for HSCs. Whole cultures of E7.5 embryos from which the yolk sac was removed (the exclusive site of hematopoiesis by this time), showed complete absence of hematopoietic cells in the fetal liver after several days in culture (Moore and Metcalf 1970). This indicated the exclusive presence of definitive hematopoietic stem/progenitor cells in the yolk sac. More recent work further strengthened this notion by using mice expressing tamoxifen-inducible Cre recombinase under the regulation of the Runx1 promoter (Samokhvalov et al. 2007), to activate the expression of a floxed-stop-lacZ reportergene at the time of interest. Administration of tamoxifen to pregnant female mice at a particular developmental window permitted to follow the fate of cells expressing Runx1 at the time of hormone treatment. At E7.5 Runx1 is exclusively expressed in the yolk sac. Tamoxifen-treatment of embryos at this time resulted in permanent staining of adult hematopoietic cells, suggesting the presence of definitive hematopoietic stem cells in the yolk sac (Samokhvalov et al. 2007). The authors interpreted the data to argue for the yolk sac as a site of HSC-formation prior to the AGM. Although several other studies also suggested HSCs to originate in the yolk sac (Moore and Metcalf 1970; Weissman et al. 1978), direct evidence is still elusive. As already mentioned, HSCs are defined by their capacity to reconstitute the hematopoietic system of myeloablated adult recipients. Yolk sac progenitors however can only contribute to adult hematopoiesis if injected directly into the fetal liver of newborn mice (Yoder et al. 1997). From these findings, one might conclude, that yolk sac contains mesodermal precursor cells, committed to become

hematopoietic stem cells after migration to the AGM, placenta or fetal liver, but no transplantable genuine HSCs.

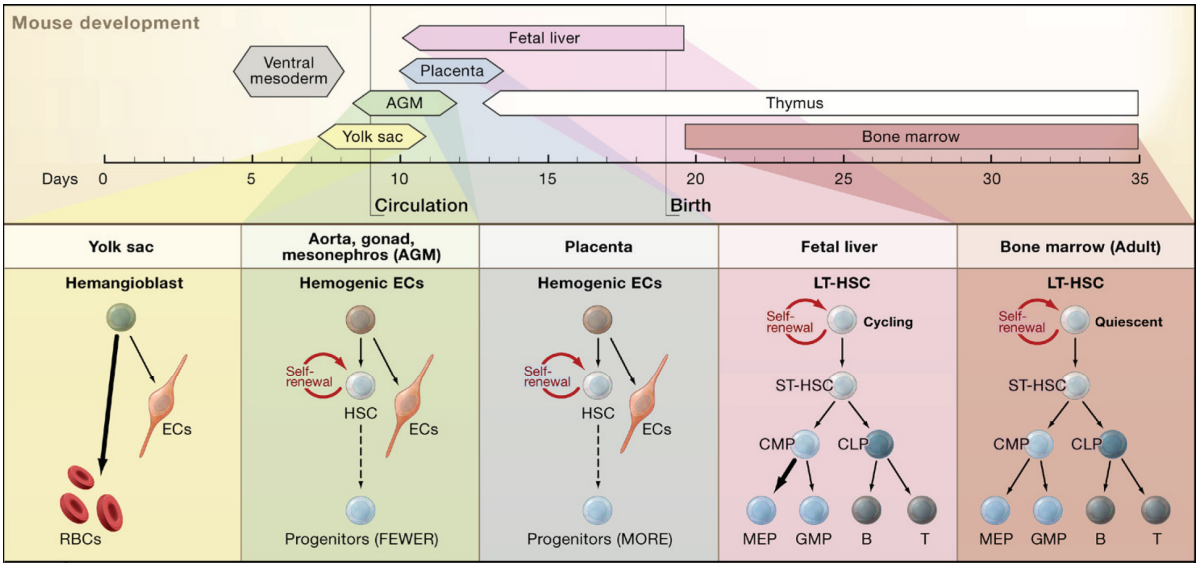

Figure 2: Development of the hematopoietic system of the mouse. Hematopoietic stem cells (HSCs) are derived from ventral mesoderm. Sequential sites of hematopoiesis during development include the yolk sac, the aorta-gonad-mesonephros (AGM) region, the fetal liver, placenta, and finally the bone marrow. The properties of HSCs in each site differ, presumably reflecting diverse niches that support HSC expansion and/or differentiation and intrinsic characteristics of HSCs at each stage. The hemangioblast of the yolk sac is proposed to give rise to both blood and endothelial cells (ECs). The next region of hematopoiesis is the AGM. It has been proposed that the AGM forms hemogenic ECs in the ventral wall of the aorta that give rise to HSCs. Significant numbers of HSCs are also found in mouse placenta. Placental HSCs could arise through *de novo* generation or colonization upon circulation, or both. The relative contribution of each of the above sites to the final pool of adult HSCs remains largely unknown. Subsequent definitive hematopoiesis involves colonization of fetal liver, thymus, spleen, and ultimately the bone marrow. In definitive hematopoiesis, long-term HSCs (LT-HSCs) give rise to short-term HSCs (ST-HSCs). ST-HSCs produce common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CLPs are the source of committed precursors of B and T lymphocytes, whereas CMPs give rise to megakaryocyte/erythroid progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). GMPs give rise to the committed precursors of mast cells, eosinophils,neutrophils, and macrophages. (adapted from Cell SnapShot: Hematopoiesis by Orkin & Zon 2008)

Following their *de novo* generation in the (yolk sac), dorsal aorta and placenta, nascent fetal HSCs colonize the developing fetal liver which henceforward serves as the main organ of HSC expansion and differentiation from E11.5 until birth (Figure 2). It is generally accepted that the fetal liver is no site of *de novo* HSC formation but rather acts as niche supporting the massive HSC expansion required prior to the seeding of the adult hematopoietic organs, the bone marrow, spleen and thymus (Orkin and Zon 2008). Accordingly, fetal HSCs residing in the fetal liver differ substantially from adult-type HSCs in the bone marrow with respect to their cell cycle status. Whereas adult-type HSCs are primarily in the G_0- phase of the cell cycle under homeostatic conditions (Passegue et al. 2005; Passegue and Wagers 2006; Warren and Rossi 2008), fetal HSCs are massively cycling to produce sufficient cells to inoculate the adult hematopoietic organs (Bowie et al. 2006; Kim et al. 2007). Around birth, fetal HSCs colonize the bone marrow and expand in numbers

for about another 4 weeks (Bowie et al. 2006; Kim et al. 2007). Quiescently residing in their niche in the bone marrow of adult long bones, adult type HSCs divide only infrequently but continue to give rise to all hematopoietic lineages through out a lifetime.

Starting with commitment of cells in the ventral mesoderm towards a hematopoietic fate, the journey of the developing hematopoietic stem cell is a very complex trip, with various pit stops probably required for proper maturation, in the (yolk sac), AGM region, placenta and fetal liver until finally settling down in the adult bone marrow around birth (Figure 2).

1.1.2 Early hematopoietic lineage commitment and differentiation

All adult hematopoietic cells are derived from the hematopoietic stem cell. The immunophenotypic identification and subsequent purification of hematopoietic stem cells (Spangrude et al. 1988) opened the door for drawing a hierarchical lineage map based on the existence of isolatable, increasingly lineage-restricted progenitors.

The complete multipotent activity of mouse bone marrow resides within a small population of cells which according to their immuno-phenotype, can be defined as negative (or low) for all hematopoietic lineage markers (lin^-) and double positive for Sca-1 and c-Kit (LSK; Ikuta and Weissman 1992). Using additional cell surface markers the LSK-fraction could be broken down further into (i) *long-term repopulating hematopoietic stem cells* (LT-HSCs; cell surface marker combinations to highly enrich for LT-HSCs are shown in Figure 3), the only cell population capable of long-term multi-lineage reconstitution, (ii) into a rather heterogeneous population of so-called *short-term repopulating hematopoietic stem cells* (ST-HSC) and (iii) *multipotent progenitors* (MPP; Osawa et al. 1996; Randall et al. 1996). Transplantation of purified ST-HSCs or MPP into myeloablated hosts results in multilineage reconstitution, although only for several weeks. There were attempts to define functional differences between ST-HSCs and MPPs, which came up with minor differences in magnitude and duration of engraftment (Morrison and Weissman 1994; Morrison et al. 1997). So far, however no clear-cut phenotypic or functional characterization of the ST-HSC versus MPP could be achived, therefore discrimination between ST-HSC and MPP remains arbitrary.

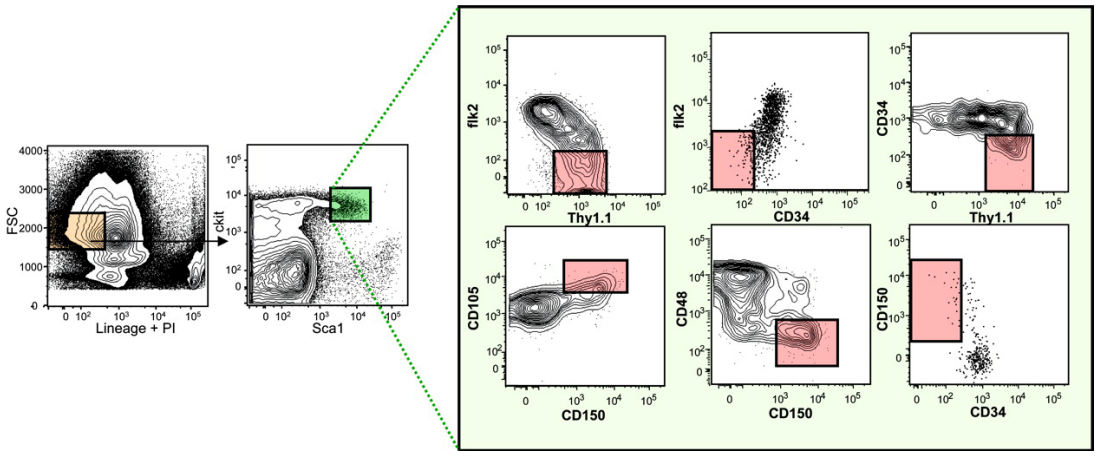

Figure 3: Cell surface stainings of the murine LSK compartment. In bone marrow, all HSC activity is found within the lineage-negative (orange box: negative for antigens found on mature blood cells including B220, Mac1, Gr-1, Il7Rα, Ter119, CD3, CD4, CD8) and Sca1^{high} and c-kit^{high} fractions (small green box). Because only ~1 in 30 LSK cells is a long-term multilineage reconstituting HSC, additional cell surface markers are used to enrich for HSCs as illustrated in the expanded green box. These include positive cell surface markers such as Thy1.1, CD105 (Endoglin), and CD150 (Slamf1), in addition to negative markers like CD34, CD48, and flk2. (adapted from Bryder et al. – Am J Pathol 2006)

The MPP was thought to give rise to lymphoid lineage-committed progenitors (*common lymphoid progenitor*, CLP; Kondo et al. 1997) as well as myeloid lineage-committed progenitors (*common myeloid progenitor*, CMP; Akashi et al. 2000; Figure 4A). This classical text-book view remained broadly accepted since all mature blood cells were assigned either to the lymphoid or the myeloid lineage. The former was comprised of B-, T- and NK-cells, and the latter consisted of granulocytes, monocytes, erythrocytes, megakaryocytes and mast cells. While the CLP was demonstrated to directly give rise to B- and T-cell progenitors (Kondo et al. 1997), the CMP was shown to differentiate into a megakaryocyte/erythroid progenitor (MEP) or a granulocyte/monocyte progenitor (GMP), which then could give rise to unilineage committed progenitors with either megakaryocytic, erythroid or myeloid fates (Akashi et al. 2000; Traver et al. 2001; Figure 4A).

This traditional symmetric, view of hematopoietic lineage commitment became increasingly challenged over the last years. Using mice expressing GFP under control of the *Rag1* (recombination activating gene 1) promoter, Ingrashi and colleagues described a fraction of LSK cells (~5%) expressing GFP (Igarashi et al. 2001; Igarashi et al. 2002). This population was shown to display potent T-, B-, and NK-cell potential with only weak myeloid colony-forming activity, and thus was named *early lymphocyte precursor* (ELP). Interestingly, ELPs reside within the MPP population, upstream of the CLP. The concept of the CLP giving rise to B- and T- cell progenitors was further challenged by the finding that the predominant thymus seeding cells do not resemble the characteristics of a CLP but are

more similar to earlier hematopoietic progenitors, probably the ELP (Bhandoola et al. 2007). The ELP was then suggested to give rise to an *early T-cell progenitor* (ETP), which then progresses through T-cell maturation during its migration through the thymus (Petrie and Zuniga-Pflucker 2007). Two recent studies further contributed to the now obligate reconsideration of the classical lineage tree by demonstrating, that ETPs still retained robust myeloid differentiation potential *in vivo* (Bell and Bhandoola 2008; Wada et al. 2008).

All these observations pointed towards an unexpected heterogeneity within the MPP compartment and proposed that lineage commitment could take place prior to the CLP and CMP stages. Thorough analyses of the MPP compartment revealed that Flt3^{hi} (*Fms-like tyrosine kinase 3*, also known as Flk2) MPPs completely lacked the potential to differentiate into megakaryocytic or erythroid cells (MegE), while retaining robust myelo/lymphoid reconstituting capability (Adolfsson et al. 2005; Figure 4B). The characterization of this population, termed *lymphoid-primed multipotent progenitor* (LMPP), was quite surprising since it had been widely accepted that myeloid and erythroid lineages descended from a common ancestral cell - the CMP. In contrast, Forsberg and colleagues claimed that Flt3^{hi}(Flk2^{hi}) MPPs still exhibited low but reproducible MegE differentiation potential (Forsberg et al. 2006). Whether or not the LMPP has MegE potential is still a matter of ongoing discussion.

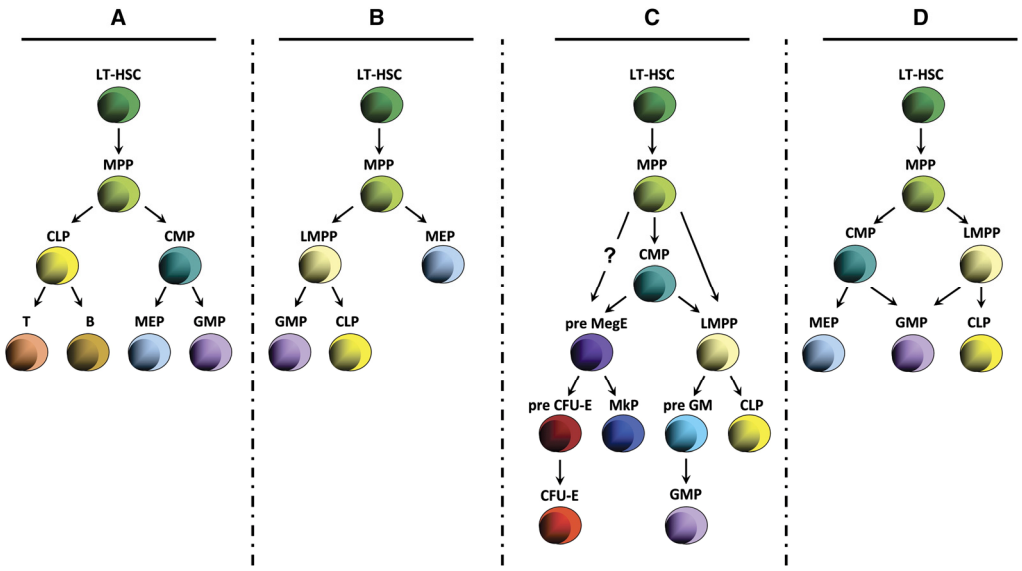

Figure 4: Map of early adult hematopoiesis

A Model proposed by Kondo et al. (1997) & Akashi et al. (2000)
B Model proposed by Adolfson et al. (2005)
C Model proposed by Pronk et al. (2007)
D Model proposed by Arinobu et al. (2007)
(adapted from C. Murre - Cell Stem Cell 2007)

In a further attempt to clarify the developmental paths of hematopoietic progenitors, Pronk and co-workers employed a set of new cell surface markers (i.e. CD150, CD105, CD41), not used before to characterize progenitor populations (Pronk et al. 2007). Combining results from transplantation, *in vitro* methylcellulose as well as clonal lineage marker expression experiments, a novel set of intermediate progenitors was outlined. It displayed a range cells with granulocyte/monocyte, erythroid and megakaryocyte potential (i.e. Pre-MegE; Pre-CFU-E; MkP; Pre-GM; GMP; Figure 4C), contributing to the increasing complexity of the hematopoietic lineage tree.

In a similar approach, Arinobu and colleagues combined classical cell surface marker straining for hematopoietic progenitors with reporter mice expressing GFP or dsRed under the control of the endogenous GATA-1 or Pu.1 promoter (Arinobu et al. 2007). The zinc finger transcription factor GATA-1 is strictly required for erythroid and megakaryocyte development (Fujiwara et al. 1996), while the Ets-family transcription factor Pu.1 is essential to promote granulocyte/macrophage and lymphoid development (Scott et al. 1994). On the one hand a subpopulation of MPPs with high expression of Pu.1 exhibiting both, lymphoid and granulocyte/macrophage but no Meg/E differentiation potential, reminiscent to the LMPP (Figure 4D). On the other hand, hematopoietic progenitors expressing substantial levels of GATA-1 developed into CMPs. In brief, Arinobou and co-workers suggested that macrophages and granulocytes could develop from both, LMPPs or CMPs.

This does not seem to be the end of the story yet. Apparently, there are many roads that can be taken towards one or the other cell fate, important however is to find and characterize the highways. Another interesting question is whether or not these highways are true one-way routes. Two recent studies provided compelling evidence that the direction of differentiation routes may not be irreversible. Over-expression of the myeloid transcription factors C/EBPα (*CAAT/enhancer binding protein* α) or Pu.1 in fully committed pre-T-cells resulted in trans-differentiation into genuine macrophages in the case of C/EBP alpha and into myeloid dendritic cells with Pu.1 (Laiosa et al. 2006). In a similar study, Cobaleda and co-workers demonstrated that deletion of Pax5 in mature peripheral B-cells permitted retro-differentiation into an early, uncommitted progenitor, which could then be differentiated into functional mature T-cells *in vivo* (Cobaleda et al. 2007).

1.2 Erythropoiesis

Erythropoiesis (derived from ancient greek: *erythros* - red; *poiesis* – to make) is the maturation of red blood cells (erythrocytes). Adult humans possess 2-3x10^{13} erythrocytes under homeostatic conditions, which translates into 4-6x10^{6} cells per microliter blood. Adult erythrocytes are generated in the bone marrow of the long bones at a rate of 2x10^{6} cells per second. The diameter of an adult red blood cell is 5-6µm, and comprises on average 270x10^{6} molecules of hemoglobin carrying 4 heme groups for oxygen transport. Thus a single erythrocyte can transport up to 10^{9} O_2 (oxygen) molecules. The principal task of red blood cells is to transport oxygen from the lung to peripheral tissues. Therefore mammalian erythrocytes have evolved as biconcave discs to optimize the cell shape for the exchange of oxygen with its surroundings and also to render them flexible to make them fit even through the smallest capillaries, where they release their cargo.

1.2.1 Ontogeny of erythropoiesis

Primitive erythropoiesis starts with the formation of mesodermal cells, which migrate through the primitive streak and contribute to the emergence of yolk sac and placenta. Soon after the onset of gastrulation (approx. E7.25), immature primitive erythroid cells rapidly group in the yolk sac into structures termed blood islands. These islands become enveloped by endothelial cells, which also form the initial vascular plexus of the yolk sac (Ferkowicz and Yoder 2005; Murry and Keller 2008). Circulation of immature primitive erythroblasts (EryP) in the mouse yolk sac begins at E8.25 soon after the first embryonic heartbeat (McGrath et al. 2003; Lucitti et al. 2007). During the following 8 days primitive erythroid cells undergo certain maturation steps, comprising a limited number of cell divisions, accumulation of embryonic and adult-type hemoglobin, gradual decrease in cell size, nuclear condensation, and ultimately enucleation (Fantoni et al. 1969; Kingsley et al. 2004; Fraser et al. 2007; Figure 5). Concomitantly with enucleation of primitive erythroblasts, a transient population of very small “cells” with a rim of εγ-globin positive cytoplasm can be found in the circulation of mouse embryos. These “cells” where named *pyrenocytes* (from ancient greek: *pyren* – pit of a stone fruit) and actually represent the extruded nuclei of primitive late stage erythroblasts (McGrath et al. 2008).

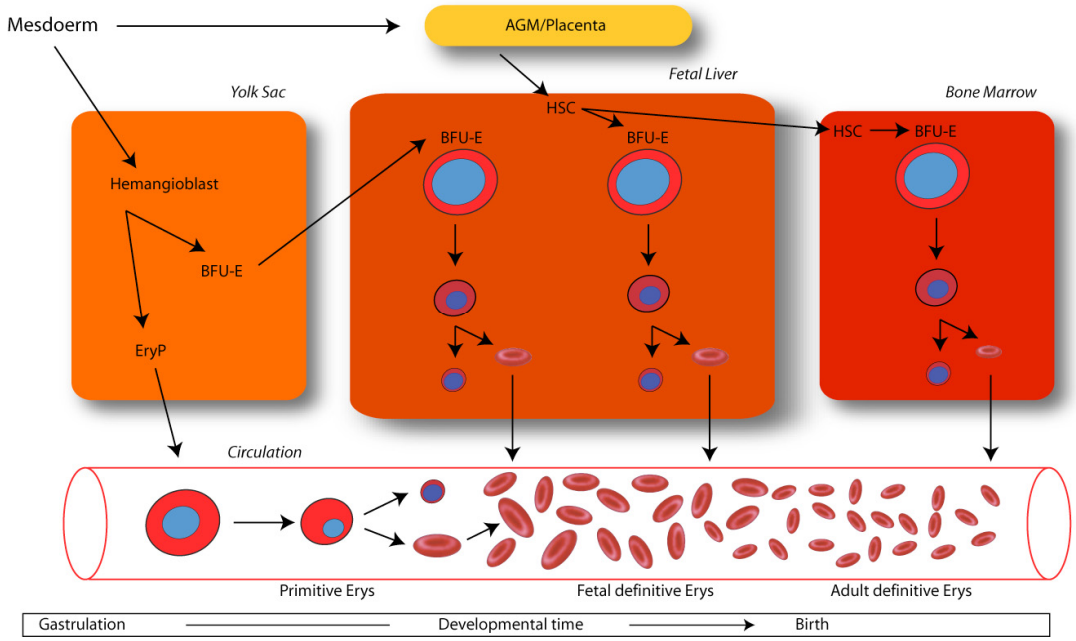

Figure 5: Ontogeny of erythroid cells in the mammalian embryo (see text for details)

Partially concurrent with the first wave of primitive erythropoiesis in the yolk sac, between E7.25-E9.0, a second wave of adult-type erythroid progenitors (*Blast Forming Unit-Erythroid*; BFU-E) emerges and expands in the yolk sac between E8.25-E10.5. Although primitive erythroid cells sufficiently fulfill critical functions for the early developing embryo, the rapidly growing fetus requires more erythrocytes to catch up with the increasing demand for oxygen (Palis et al. 1999). Around E9.5, adult-type erythroid progenitors derived from the yolk sac as well as fetal HSCs derived from the AGM region and/or placenta colonize the developing fetal liver, which remains the prime organ of erythropoiesis until birth (Palis 2008; Figure 5; see also Figure 1). Soon after, BFU-Es and the more mature CFU-Es (*Colony Forming Unit-Erythroid*) proliferate exponentially for several days, concomitantly differentiating into mature erythrocytes (Kurata et al. 1998). Around birth, a third wave of definitive erythroid progenitors colonizes the newly formed bone marrow, the lifelong major site of adult mammalian erythropoiesis (Palis 2008; Figure 5).

1.2.2 Erythroid versus myeloid lineage commitment

As early hematopoietic progenitors leave their multipotential state, they undergo distinct lineage choices, frequently governed by cross-antagonizing transcription factors with different sets of differentiation promoting target genes simultaneously blocking each others activity

(Loose and Patient 2006; Swiers et al. 2006; Loose et al. 2007). In the classical hierarchical model of hematopoiesis as outlined above it was thought that a binary decision at the level of a common myeloid progenitor predestined progenitor cells either towards megakaryocyte/erythroid (MEP) or granulocyte/monocyte (GMP) differentiation (Akashi et al. 2000; Figure 6; see also Figure 4A). This decision is governed by the mutual antagonistic relationship between GATA-1 and PU.1.

GATA-1 plays a central role in erythroid development and was first identified by its ability to bind functionally important sequences in the locus control region of globin genes (Evans and Felsenfeld 1989; Martin et al. 1989). GATA-1 knock out mice die during mid-gestation from severe anemia due to erythroid maturation arrest at the proerythroblast stage (Fujiwara et al. 1996). Pu.1 is essential for granulocytic, monocytic as well as lymphoid development (Hromas et al. 1993; Scott et al. 1994; McKercher et al. 1996). Elevated levels of GATA-1 in the CMP drive differentiation into the megakaryocytic/erythroid lineage whereas higher expression of Pu.1 promotes development of myeloid cells (Liew et al. 2006; Figure 6).

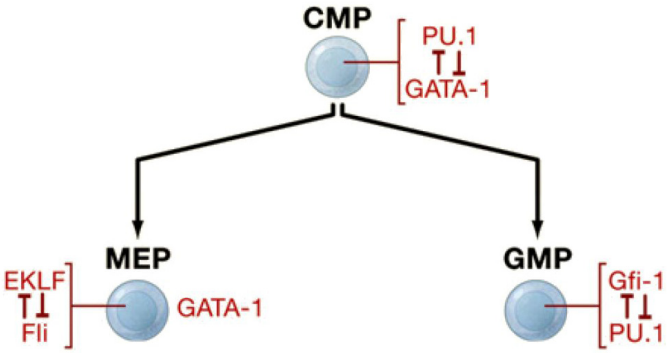

Figure 6: Erythroid versus myeloid lineage specification.
(adapted from Orkin & Zon, - Cell 2008)

At the molecular level, GATA-1 suppresses myeloid differentiation by binding to the Ets domain of Pu.1, thereby blocking its DNA binding activity as well as interfering with its interaction with c-Jun, resulting in loss of Pu.1 target gene expression (Loose and Patient 2006; Loose et al. 2007). Conversely the Ets domain of Pu.1 binds the C-terminal zinc finger of GATA-1 thus antagonizing GATA-1 DNA binding (Rekhtman et al. 1999; Liew et al. 2006). Pu.1 also inhibits the CBP/p300-mediated acetylation of GATA-1, which is required for proper GATA-1 chromatin occupancy *in vivo* (Hong et al. 2002; Lamonica et al. 2006). Furthermore it was suggested that Pu.1-mediated inhibition of erythropoiesis involves recruitment of a co-repressor complex consisting of *retinoblastoma protein* (pRb), a histone methyltransferase (Suv39H) and binding of *heterochromatin protein 1a* (HP1a), resulting in a

repressed chromatin state of certain GATA-1 target genes (Rekhtman et al. 2003; Stopka et al. 2005).

1.2.3 Erythroid differentiation

The next lineage determining switch on the way to a red cell takes place at the level of the MEP, in which again transcriptional cross-antagonism between transcription factors EKLF (*erythroid Kruppel-like factor*; Miller and Bieker 1993) and the Ets family member Fli-1 (*friend leukemia virus integration-1*; Ben-David et al. 1990) govern the development of either becoming BFU-E or BFU-meg (*Burst Forming Unit-megakaryocyte*; Starck et al. 2003; Figure 7). EKLF is crucial for erythropoiesis, in particular as it is required for transcription of the adult β-globin gene (Nuez et al. 1995; Perkins et al. 1995), but is also involved in the expression of heme biosynthesis genes (Drissen et al. 2005; Hodge et al. 2006). Fli-1 has been shown to repress the activity of EKLF on the β-globin promoter (Starck et al. 2003). This prevents the expression of additional genes important in terminal erythroid maturation and favors differentiation towards a megakaryocytic fate. *Vice versa* EKLF suppresses the activity of Fli-1 on the *megakaryocyte glycoprotein IX* (GPIX) promoter, further underscoring the functional cross antagonism between EKLF and Fli-1. Summing up, higher expression levels of EKLF favor erythroid differentiation whereas elevated Fli-1 levels favor megakaryocytic maturation.

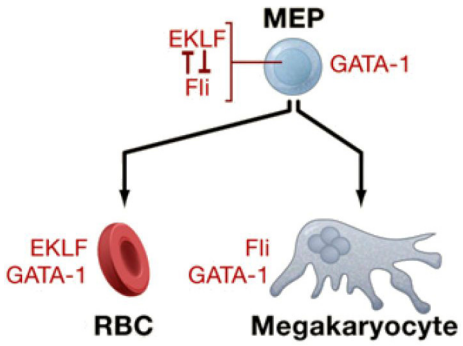

Figure 7: Erythroid versus megakaryocytic lineage choice.
(adapted from Orkin & Zon, - Cell 2008)

Persistent expression of EKLF and GATA-1 in MEPs leads to differentiation into BFU-Es, which represent the first unilineage-committed progenitors of the erythroid lineage. BFU-Es give rise to approximately 500 red cells in semisolid culture medium within 6-10 days (Iscove and Sieber 1975). Developmentally, BFU-Es are followed by the more mature CFU-Es (Stephenson et al. 1971), which form colonies of 8-32 red cells in colony assays within 2-3 days. The formation of CFU-Es and all subsequent maturation steps are strictly

dependent on the glycohormone *erythropoietin* (Epo; see below). CFU-Es are followed by the erythroblasts, which sequentially form *pro-erythroblasts*, *basophilic erythroblasts*, *polychromatic erythroblasts* and *orthochromatic erythroblasts* (Figure 8). All these cell types can be distinguished morphologically as they undergo changes characteristic of terminal erythropoiesis, i.e. hemoglobin accumulation, cell size decrease and chromatin condensation. The final step of red cell maturation is the extrusion of the highly condensed nucleus, which gives rise to enucleated *reticulocytes* (Figure 8; Palis and Segel 1998). Reticulocytes enter the bloodstream, where it takes them about one more day to finish maturation into bi-concave-shaped erythrocytes.

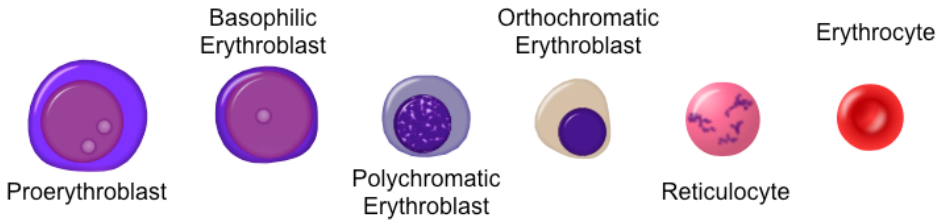

Figure 8: Terminal steps of erythroid differentiation.
Erythroid maturation progresses from left to right.
(adapted from Rad A. - Wikipedia Commons 2006)

Terminal erythropoiesis occurs in highly specialized microenviromental niches known as erythroblastic islands (Bessis 1958), found in fetal liver, bone marrow and spleen. They consist of a central macrophage encircled by maturing erythroid cells (Figure 9; Chasis and Mohandas 2008). In human bone marrow the number of erythroblasts per island ranges from 5-30 cells (Lee et al. 1988). Erythroblastic islands are in general localized throughout the whole bone marrow, but approximately 50% of all islands are close to sinusoids, i.e. fenestrated capillary-like blood vessels (Yokoyama et al. 2003). It is speculated that these islands are not stationary elements but migrate towards sinusoids to release mature reticulocytes into circulation (Yokoyama et al. 2003). This view is strengthened by the observation that erythroblastic islands close to sinusoids bear more mature erythroid cells than their distant counterparts.

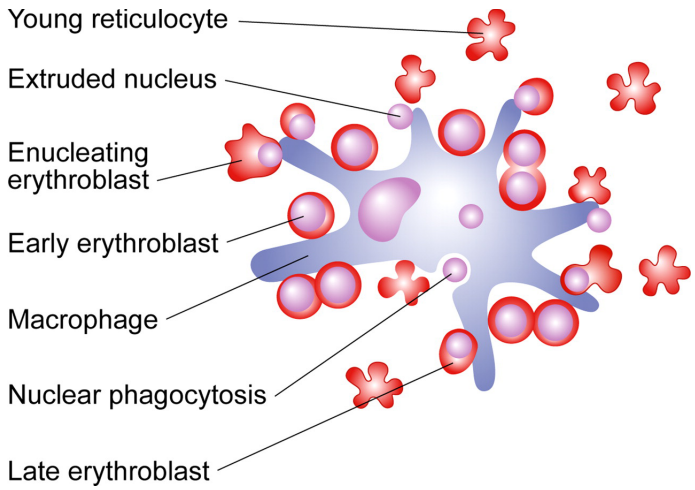

Figure 9: Illustration of an erythroblastic island. Early-stage erythroblasts are depicted as large cells with centrally located nuclei; more mature erythroblasts are shown as smaller cells containing nuclei located adjacent to plasma membranes. Expelled nuclei are phagocytosed by the central macrophage. Reticulocytes are illustrated multi-lobulated cells, which are initially attached to the macrophage surface and later detach. (adapted from Chassis et al. – Blood 2008)

During definitive erythropoiesis, erythroblasts express various adhesion molecules, mediating erythroblast/erythroblast or erythroblast/macrophage interactions as well as attachment to extracellular matrix components like fibronectin or laminin (Chasis and Mohandas 2008). The first cell adhesion molecule identified on erythroblasts as well as on macrophages was Emp (*erythroblast macrophage protein*; Hanspal and Hanspal 1994). It mediates erythroblast/macrophage binding via homophilic interactions (Hanspal and Hanspal 1994). Inhibition of this interaction using an anti-Emp antibody leads to a decrease in proliferation, maturation, enucleation and even an increase in apoptosis (Hanspal et al. 1998). In accordance, Emp knock out-mice display severe anemia and die perinatally (Soni et al. 2006).

Besides acting as anchor for developing erythroblasts and providing survival or differentiation-promoting signals, the central macrophage has been implicated in phagocytosing the expelled nucleus of terminally maturing erythroblasts (Yoshida et al. 2005; Figure 9). Immediate phagocytosis of extruded nuclei seems to be a protective mechanism, as suggested by a study using DNase II knock out mice (Kawane et al. 2001). In wild type mice ingested nuclear DNA of expelled nuclei is quickly degraded by central macrophages, whereas the DNA degradation defects of DNase II knock out macrophages resulted in significantly fewer central macrophages and severe anemia (Kawane et al. 2001). Recently Leimberg and collegues proposed a new function for central macrophages. They suggested a direct transport of iron from macrophages to erythroblasts (Leimberg et al. 2008). Under

transferrin-free culture conditions iron-loaded ferritin synthesized by central macrophages was exocytosed and taken up by the attached erythroblasts. Subsequently, iron was released from ferritin and contributed to heme synthesis of the erythroblast (Leimberg et al. 2008). Taken together the interaction between erythroblasts and macrophages is important for survival, maturation and potentially even iron metabolism of red cells.

1.2.4 EpoR signaling in erythroid cells

Whereas early erythroid lineage commitment is controlled by numerous transcription factors and their binding partners (e.g. GATA-1; EKLF; Cantor and Orkin 2002), late stage differentiation from CFU-Es to mature erythrocytes is strictly regulated by Epo (for a review see Richmond et al. 2005). Epo is the crucial regulator of red blood cell production and merges survival, differentiation and proliferation signals essential for erythroid cell development in fetal liver, bone marrow or spleen (Richmond et al. 2005). Epo is produced in the kidney where its synthesis is regulated by oxygen tension via the action of *hypoxia inducible factor-2α* (HIF-2α; Gruber et al. 2007). Under normoxic conditions, HIF-2α is hydroxylated by a prolyl-hydroxylase which results in its recognition by *von-Hippel–Lindau* (VHL) factor and ensuing degradation. Under reduced oxygen or iron concentrations, HIF-2α is stabilized, functions as transcription factor and drives expression of Epo (Zhu et al. 2002).

The *erythropoietin receptor* (EpoR) is a member of the cytokine receptor super-family (D'Andrea et al. 1989), primarily expressed on developing erythroid progenitors and to a lesser extant, on (cardio)myocytes, cortical neurons, ovary and breast epithelia (Richmond et al. 2005). Recently, EpoR receptor activity in hepatocytes was demonstrated to suppress expression of the iron metabolism controlling hormone *hepcidin* (see below; Pinto et al. 2008). EpoR is thought to subsist in a latent dimeric-state even prior to ligand binding (Wojchowski et al. 2006) but undergoes conformational changes upon Epo binding that activate the pre-associated, tyrosine kinase *Janus Kinase 2* (Jak2; Witthuhn et al. 1993). Thereupon Jak2 and potentially other cytoplasmic tyrosine kinases phosphorylate several tyrosine residues in the cytoplasmic domain of EpoR dimers that act as docking site for the transcription factor *Signal transducer and activator of transcription 5* (Stat5; Wakao et al. 1994), which in turn is also activated via Jak2-mediated tyrosine phosphorylation (Wakao et al. 1995; Quelle et al. 1996; Figure 10). *[For a detailed review of Stat5 functions in hematopoiesis please see section 1.4]*. Activating phosphorylation of Stat1 and Stat3 by

EpoR/Jak2 has also been reported (Kirito et al. 2002; Kirito et al. 2002; Halupa et al. 2005), but the relevance in erythropoiesis is less well understood.

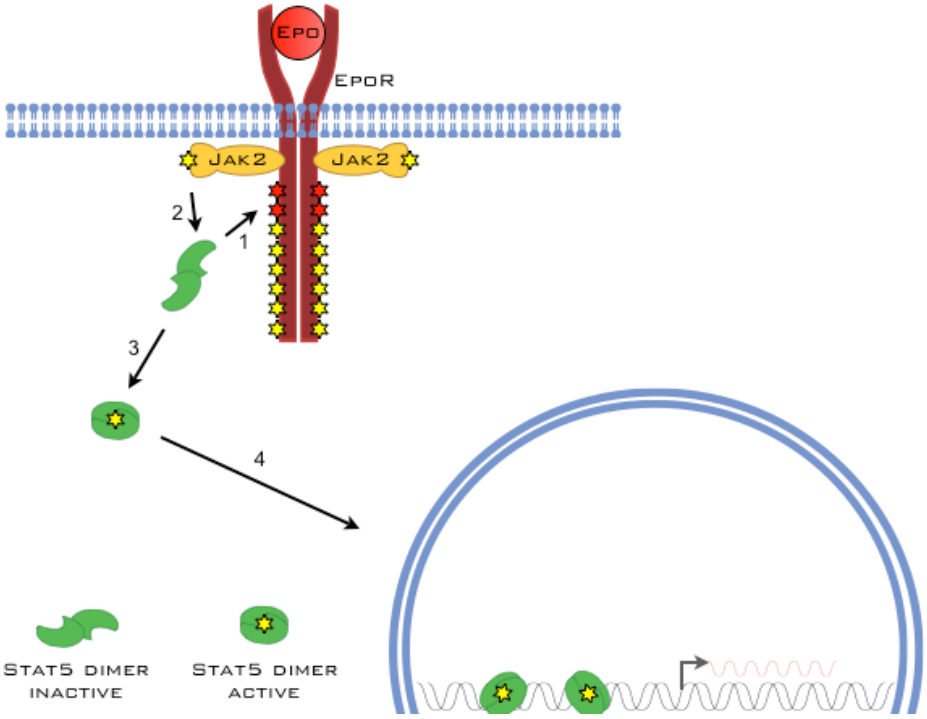

Figure 10: Jak-Stat signaling induced by Epo. Phosphorylated tyrosine residues are illustrated as yellow asterisks. Stat5 tyrosine binding sites Y343 and Y401 are highlighted in red. Inactive Stat5 dimers bind to phosphorylated tyrosines 343 and/or 401 of the EpoR (1), become phosphorylated by Jak2 (2) leading to conformational rearrangements resulting in active Stat5 dimers (3), which translocate to the nucleus (4) to activate their respective target genes.

Alongside with the prominent Stat5-activation, Epo/EpoR also activates *extracellular regulated kinase 1/2* (Erk1/2; Menon et al. 2006), *Jun kinase* (Jnk), *mitogen-activated protein kinase (MAPK) p38* (Nagata et al. 1998) and *phophatidylinositol 3 kinase* (PI 3-kinase; Miura et al. 1994).

Many growth factors and cytokines activate the small G-protein Ras (*Rat sarcoma*) by recruiting Grb2-Sos (*growth factor receptor-bound protein 2 / Son of sevenless*) to their receptors. The complex of Grb2/Sos is recruited to the EpoR where Grb2 either binds directly to Y464 (Barber et al. 1997) or indirectly via association with SHIP-1 (*SH2-containing inositol 5-phophatase-1*; Mason et al. 2000). Epo/EpoR induced Ras activation stimulates the canonical MAPK signaling cascade resulting in activation of Erk1/2 and is believed to be important for the contribution of Epo-signaling to proliferation (Haq et al. 2002).

Epo induced activation of Jnk and p38 has been demonstrated in several cases (Wojchowski et al. 1999; Jacobs-Helber et al. 2000; Haq et al. 2002), but the modes of activation and functions in erythropoiesis are still elusive. Nevertheless mice devoid of the

p38 isoform p38α, die due to severe fetal anemia putatively as a result of reduced Epo expression (Tamura et al. 2000).

Epo stimulation activates PI 3-kinase signaling either by recruiting the p85 regulatory subunit directly to EpoR Y479 or indirectly via *GRB2-associated proteins 1 or 2* (Gab1, Gab2), or *insulin-receptor substrate 2* (IRS2; Wojchowski et al. 1999). Since mice deficient for p85 showed strongly reduced BFU-E as well as CFU-E numbers, it was reasoned that PI 3-kinase signaling was required for cell survival in erythroid cells (Bao et al. 1999; Huddleston et al. 2003). PI 3-kinase activates *Protein kinase B* (PKB)/Akt which in turn modulates the activity of its downstream target substrate, the transcription factor *forkhead box O3A* (Foxo3a) which appears to have important functions in erythropoiesis (Kashii et al. 2000; Bouscary et al. 2003; Bakker et al. 2007). Foxo3a activity is negatively regulated by phosphorylation (Huang and Tindall 2007). The levels of active non-phosphorylated Foxo3a increase during erythroid maturation, which results in cell cycle exit and terminal differentiation (Bakker et al. 2004). This is in agreement with the cyclin-dependent kinase inhibitor $p27^{Kip1}$ being a target gene of Foxo3a. Activation of PI 3-K signaling in primary human erythroblasts reduced expression levels of $p27^{Kip1}$ (Bouscary et al. 2003). Mice devoid of Foxo3a are anemic and display elevated reticulocyte counts, indicative for compromised erythropoiesis (Castrillon et al. 2003). A recent study also suggested a protective role of Foxo3a in erythroid cells against reactive oxygen species that might be byproducts of heme synthesis (Marinkovic et al. 2007).

The Src-family kinase *Lyn*, and the Tec-family kinase *Btk* have also been shown to be substrates of Epo signaling. Lyn-deficient erythroblasts express reduced levels of GATA-1, EKLF and Stat5 compared to wild-type cells and display increased extramedullary hematopoiesis in the spleen and develop anemia with age (Ingley et al., 2005). Btk is activated following Epo stimulation. Erythroid cells from mice lacking Btk display enhanced erythroid differentiation when cultured in media supporting self-renewal. Erythroblasts lacking Btk show lowered levels of EpoR-, Jak2-, and Stat5 phosphorylation upon exposure to Epo (Schmidt et al. 2004). The exact position of Src- and Tec-family kinases in the EpoR signal transduction network is still elusive, but present data suggest that Jak2 and Lyn are upstream of Btk and that the latter might be required to integrate signaling inputs converging from EpoR and *stem cell factor* (SCF)-induced *c-Kit* activation (von Lindern et al. 2004).

1.3 Iron metabolism

Iron metabolism can basically be subsumed as the sum of all chemical/enzymatic reactions maintaining iron homeostasis (balance between iron uptake and loss) of a life form. Iron (Fe; latin: *ferrum*) is essential for life, due to its unique ability to serve as electron donor as well as electron acceptor. For this particular reason however, free cellular iron is toxic since it catalyzes the conversion of hydrogen peroxide into free radicals (*Fenton's* reaction). Free radicals cause severe cell damage, eventually leading to cell death. To prevent such kind of damage all life forms have evolved cytochromes that coordinately bind iron to limit its harmful abilities but still allow cells to utilize its specific functions for their benefit. One prominent example is the electron transport during oxidative phosphorylation in the respiratory chain. In vertebrates however, the most abundant iron-chelating molecule is heme. Heme is the prosthetic group of myoglobin or hemoglobin consisting of one iron atom in the center of the porphyrin ring. As major protein of erythrocytes, hemoglobin transports oxygen from the lung to all peripheral tissues.

1.3.1 Cellular iron homeostasis

Cellular iron homeostasis requires tight control of intestinal iron uptake, transport in blood, (temporal) intracellular storage as well as export towards cells with particular demand for the metal. To exert these duties, vertebrates have evolved a number of highly specialized mechanisms. In contrast to previous sections describing timelines and conditions around mouse hematopoiesis, the following chapters on regulation of iron metabolism apply to mouse and human systems similarly.

Extracellular iron circulates in blood plasma bound to *transferrin* (Tf; Schade and Caroline 1946), which keeps iron in "solution" and nonreactive. Mammalian Tf molecules have two similar iron-binding lobes each capable of holding one iron atom (Cheng et al. 2004). Tf delivers iron to respective cell surface receptors termed *transferrin receptor* (TfR-1, also known as CD71). TfR-1 is present on every proliferating cell and of course more abundantly on developing erythroid progenitors, due to their tremendous requirement for iron to enable efficient heme synthesis. TfR-1 binds diferric transferrin and internalizes it through receptor-mediated endocytosis. The resulting endosomes become acidified through proton influx, entailing conformational changes in Tf as well as TfR-1, leading to the liberation of *ferric iron* atoms (Fe^{3+}). After reduction via the endosomal ferrireductase *STEAP3 ferrous*

iron (Fe^{2+}) is released from the endosome via *divalent metal transporter 1* (DMT1; Fleming et al. 1997; Gunshin et al. 1997; Fleming et al. 1998). Endosomes containing apo-Tf and TfR-1 are subsequently recycled to the cell surface, resulting in release of Tf back into the circulation. This process became a paradigm in cell biology termed *transferrin cycle* (Figure 11; Dautry-Varsat 1986; Aisen 2004; Hentze et al. 2004; Andrews 2008). The immediate fate of iron freed from the endosome is still not well understood. In erythroid progenitors however the bulk of iron is used for heme synthesis. Since the first one and last three steps of heme biosynthesis take place in the lumen of mitochondria (Ajioka et al. 2006), including the final insertion of iron into protoporphyrin IX by *ferrochelatase* (Ajioka et al. 2006), iron must pass through the mitochondrial membrane. This transport is mediated via the mitochondrial iron importer *mitoferrin* (Shaw et al. 2006). Excess cytosolic iron is stored in a cage-like heteropolymer consisting of 24 subunits of H- (heavy or heart) and L- (light or liver) *ferritin* (Figure 11), which can hold up to 4500 iron atoms as insoluble $Fe(OH)_3$, or *hemosiderin*, for permanent detoxification (Harrison et al. 1967).

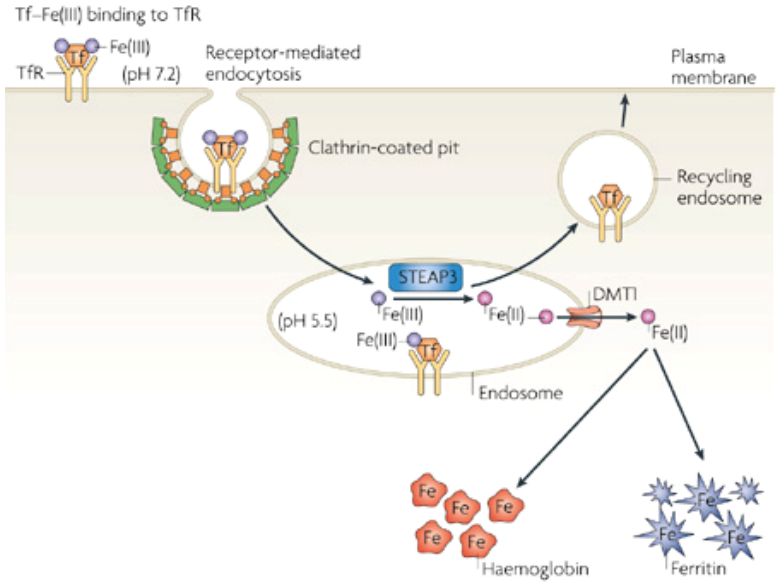

Figure 11: The transferrin cycle (see text for details)
(adapted from Domenico et al. – Nat. Rev. Mol. Cell Biology 2008)

Intracellular iron homeostasis is maintained via an elegant post-transcriptional regulatory feedback mechanism. In the late 1980s several groups independently described highly conserved regions in the *5´untranslated regions* (UTRs) of both the H- and L-ferritin mRNAs (Aziz and Munro 1987; Hentze et al. 1987) and the 3´UTR of TfR-1 mRNA (Mullner and Kuhn 1988). Thermodynamic predictions indicated that these sequences should form stable RNA hairpins which soon were experimentally verified and termed *iron responsive*

elements (IREs; Casey et al. 1988). Shortly thereafter, cytoplasmic proteins, called *iron regulatory proteins* (IRPs) recognizing and specifically binding to IREs were described (Hentze et al. 1987; Caughman et al. 1988; Leibold and Munro 1988; Mullner et al. 1989). In terms of regulating TfR-1 transcript levels IRP-1+2 were shown to bind TfR-1 mRNA IREs with high affinity under low intracellular iron concentrations (Figure 12). This binding selectively stabilizes the TfR-1 mRNA, ensuring elevated cell surface expression and iron uptake (Hentze et al. 2004; Pantopoulos 2004; Rouault 2006). On the contrary, intracellular iron excess structurally converted IRP-1 to cytosolic aconitase (catalyzing isomerization of citrate to iso-citrate) (Haile et al. 1992), while IRP-2 was degraded by proteasome (Guo et al. 1995). Thus, both proteins no longer bound to IREs, resulting in strongly reduced TfR-1 mRNA stability, reduced cell surface expression, and diminished Tf-iron uptake (Koeller et al. 1989; Mullner et al. 1989; Binder et al. 1994; Figure 12). For H-&L-ferritin mRNAs the situation is completely opposite since the ferritin IREs are located upstream of the start codon for protein translation. IRP binding under low iron sterically blocks recruitment of the small ribosomal subunit thus abrogating translation initiation (Muckenthaler et al. 1998). High iron conditions reverse this translational repression and lead to accumulation of ferritin subunits to provide sufficient iron storage / detoxification capacity. Other mRNAs coding for important molecules in iron metabolism have been shown to have either 5´IREs (e.g. ferroportin, erythroid iso-form of aminolevulinic acid synthase) or 3´IREs (e.g. DMT1; Leipuviene and Theil 2007)

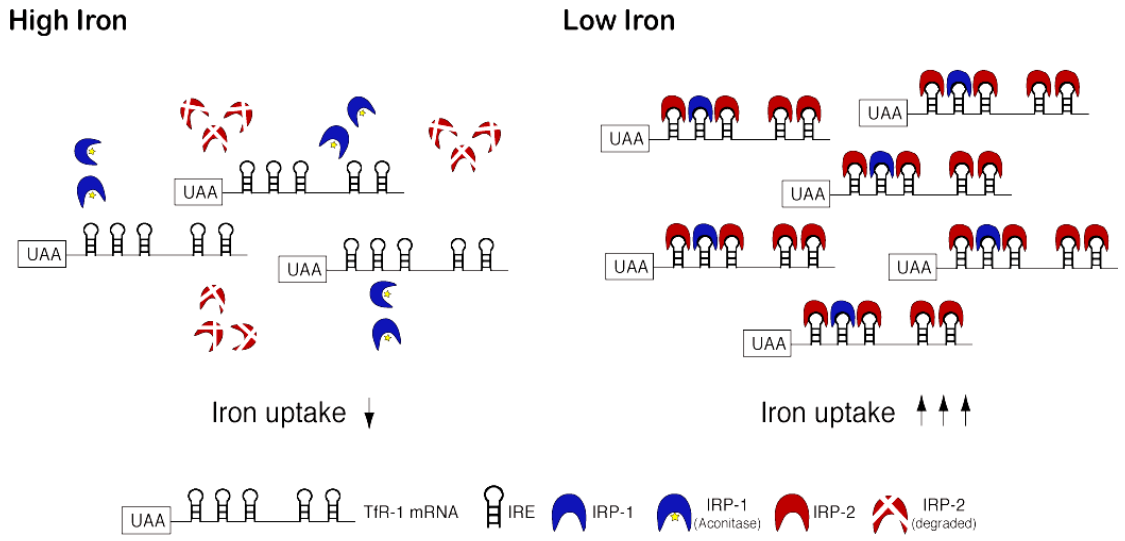

Figure 12: Regulation of TfR-1 mRNA stability. In iron replete cells, IRP-1 is converted into cytosolic aconitase (catalyzes isomerization of citrate to iso-citrate in the citric acid cycle and exhibits no mRNA binding affinity; yellow asterisk) and IRP-2 is degraded. Therefore both cannot bind to IREs in the 3´UTR of TfR-1 mRNA. Free unprotected IREs in turn enhance degradation rates of TfR-1 mRNA, resulting in reduced iron uptake. In iron depleted cells, IRP-1+2 bind to the respective IREs, thereby stabilizing TfR-1 mRNA, resulting in increased iron uptake.

1.3.2 Systemic iron homeostasis

Systemic iron homeostasis is the control of iron balance throughout the entire body. This includes mechanisms governing intestinal iron uptake or mobilization of liver / macrophage iron stores to satisfy erythropoietic needs. Since (i) iron enters the body exclusively through the diet, and since (ii) there is no regulated excretion of iron through liver or kidney, iron balance must be primarily regulated at the level of intestinal absorption. Human duodenal enterocytes approximately absorb 1–2mg dietary iron per day employing a plasma-membrane form of DMT-1 in a tightly controlled intake process. Subsequently they convey iron to blood plasma by transcytosis using *ferroportin*, where it becomes immediately bound by apo-Tf and delivered mainly to liver and other peripheral tissues.

Effete erythrocytes are phagocytosed by macrophages of the reticuloendothelial system, which degrade hemoglobin and recycle iron back into plasma via ferroportin the only known mammalian iron exporter yet (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000) or directly deliver it to the liver at a combined rate of approximately 20–30 mg day per day. If dietary iron is absorbed or released into plasma at levels exceeding organismal demand, excess, non-transferrin-bound iron is deposited in the liver parenchyma (Figure 13; Hentze et al. 2004; Andrews 2008; De Domenico et al. 2008), which under pathological conditions like hemochromatosis can lead to eventual organ damage.

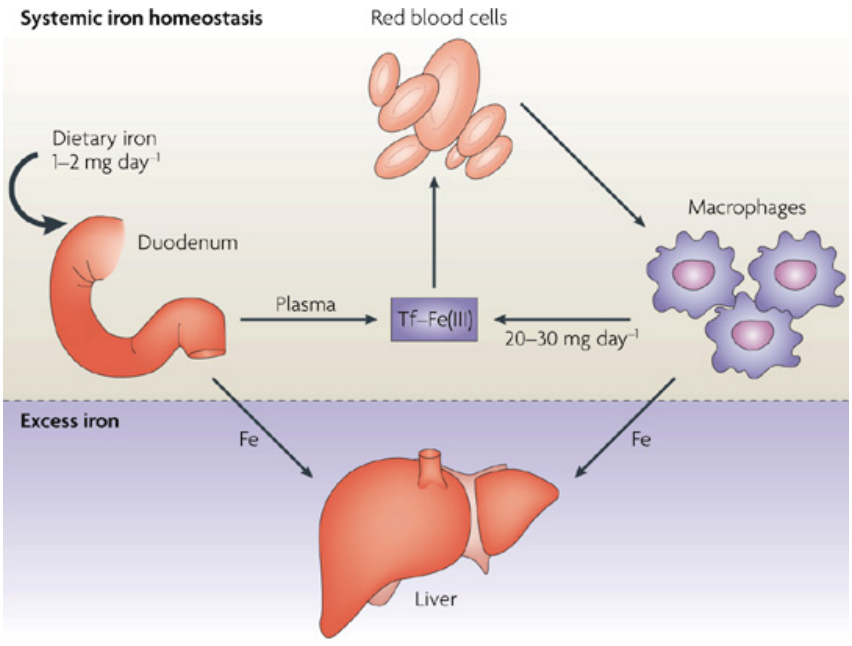

Figure 13: The iron cycle (see text for details)
(adapted from Domenico et al. – Nat. Rev. Mol. Cell Biology 2008)

More precisely, iron absorption commences in the proximal side of the duodenum, in which *enterocytes* are arranged in villi, projecting into the intestinal lumen to maximize the absorption area (Figure 14). Most dietary non-heme iron is in the ferric (Fe^{3+}) state and therefore must be reduced prior to entry into enterocytes. This reduction is performed by a ferrireductase called *DCYTB* (also known as *CYBRD1*) on the apical surface of the duodenal mucosa (Gunshin et al. 2005). Fe^{2+} subsequently enters the enterocytes via the intestinal isoform of DMT1 (Hubert and Hentze 2002; Lam-Yuk-Tseung et al. 2005). Once inside the cells, a small portion of Fe^{2+} is employed for metabolism or storage whereas the largest part is exported into plasma via basolateral ferroportin. In conjunction with ferroportin-mediated export, a multicopper-ferroxidase called *hephaestin* oxidizes Fe^{2+} to ferric iron, which thereupon is loaded to apo-Tf in the plasma, turning the iron cycle (Figures 13 & 14).

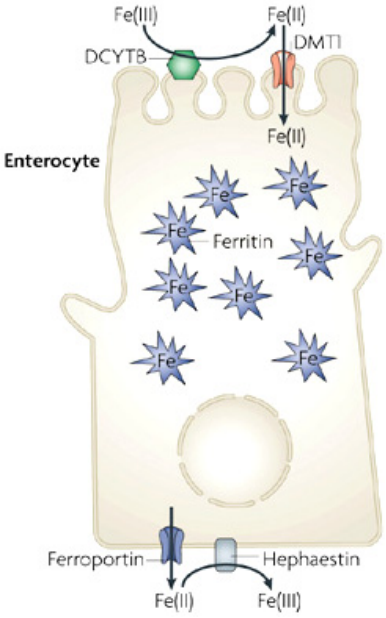

Figure 14: Intestinal iron absorption. Ferric iron (Fe(III)) in the diet is converted to ferrous iron (Fe(II)) by DCYTB on the apical surface of enterocytes. Fe(II) is then transported into enterocytes through DMT1. Fe(II) in enterocytes can be incorporated into the cytosolic iron-storage molecule ferritin or can be transported across the basolateral surface of enterocytes into the plasma by ferroportin. Fe(II) is subsequently converted to Fe(III), by hephaestin (adapted from Domenico et al. – Nat. Rev. Mol. Cell Biology 2008)

Systemic iron homeostasis requires accurate control of intestinal iron absorption, effective iron utilization for erythropoiesis, efficient iron recycling of senescent erythrocytes and controlled storage of iron by macrophages and hepatocytes. Whereas erythroid iron usage is primarily determined by the iron uptake-efficiency of the transferrin cycle, intestinal absorption, iron recycling and iron storage are controlled systematically and in a coordinated manner. A specific hormone termed *hepcidin* (Krause et al. 2000; Park et al. 2001; Pigeon et al. 2001), orchestrates the fine-tuning of systemic iron balance. Hepcidin is produced by the

liver in response to high serum iron conditions, is secreted into plasma and binds to ferroportin on the basolateral side of enterocytes as well as to ferroportin on macrophages, triggering internalization and ubiquitin-mediated degradation of ferroportin (Nemeth et al. 2004; De Domenico et al. 2007), resulting in a net reduction of serum iron levels, i.e. lowered transferrin saturation (Figure 15).

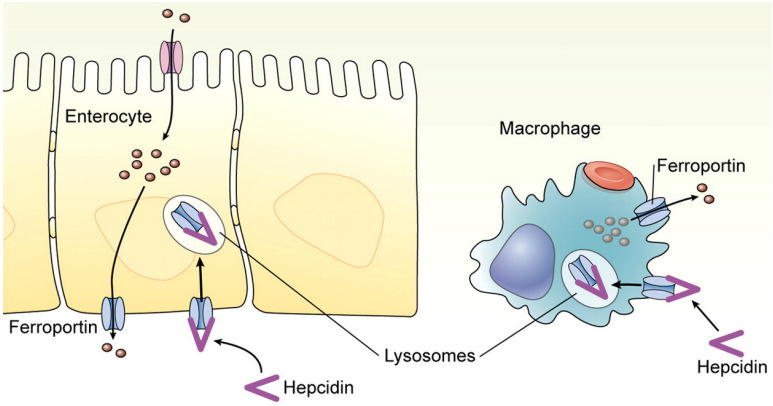

Figure 15: Hepcidin's mode of action. Hepcidin targets ferroportin in enterocytes as well as macrophages, triggering its internalization and lysosomal degradation. (adapted from Andrews N. – Blood 2008)

Regulation of hepcidin expression primarily occurs at the level of transcription and is modulated by anemia, hypoxia, inflammation and the status of iron stores (Nicolas et al. 2002). Under normal conditions, basal hepcidin expression is enabled via a *bone morphogenetic protein* (BMP)/SMAD pathway in conjunction with a membrane protein called *hemojuvelin* acting as a BMP-co-receptor (Wang et al. 2005; Babitt et al. 2006). Of note mutations in either, hepcidin or hemojuvelin lead to severe iron overload diseases such as juveline hemochromatosis (Roetto et al. 2003; Papanikolaou et al. 2004). Hepcidin also exhibits anti-microbial function: Since all invasive microorganisms require iron for proliferation, inflammatory cytokines such as *interleukin-6* induce hepcidin expression in a Stat3-dependant manner, resulting in reduced plasma iron abundance (Wrighting and Andrews 2006) and thus contributing to the innate immune response.

Hepcidin levels are also down regulated in cases such as hypoxia of compromised erythropoiesis. Hypoxia decreases hepcidin expression, resulting in increased iron export into plasma (Nicolas et al. 2002). Under normoxic conditions, hydroxylation of HIF-1/2 results in recognition by, VHL-factor, targeting it for degradation. In the absence of oxygen or iron, however, stabilized HIF proteins function as transcription factors suppressing hepcidin expression (Yoon et al. 2006; Peyssonnaux et al. 2007). Finally, also Epo can repress hepcidin function (Pinto et al. 2008): Elevated Epo levels, frequently being a response to compromised erythropoiesis result in activation of the transcription factor C/EBPα that transcriptionally represses the hepcidin gene in hepatocytes (Pinto et al. 2008).

[Besides multiple other pathologies involving human iron metabolism the following section introduces one particular condition with relevance to the work presented in chapter 3.1.1]

1.3.3 Iron deficiency anemia

Iron deficiency anemia is a major public health problem, with estimated 3 billion people affected worldwide. The symptoms of iron deficiency anemia are pallor (due to reduced oxyhemoglobin in skin), fatigue, weakness and hair-loss; in severe cases even *dyspnea* (breathing problems). Paradoxically also behavioral symptoms such as obsessive food cravings (*pica*) or lightheadness have been described (Cook 1994). The disease usually progresses rather slowly and therefore remains often unrecognized. Anemia might be diagnosed by its symptoms, but is most frequently detected by routine blood tests, which generally include a complete blood count (CBC). Characteristics of anemia are low hemoglobin (HGB) or hematocrit (HCT) values. *Hypochromic microcytic anemia* (iron deficiency anemia) in particular is specifically characterized by reduction in the values of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), paired with morphological changes of erythrocytes in peripheral blood smears (larger central pallor; Guyatt et al. 1990). Serum ferritin levels are also implicated as a sensitive test to diagnose iron deficiency anemia (Guyatt et al. 1990).

Iron deficiency anemia is subdivided into acquired- or genetic-iron deficiency anemia. The vast majority of iron deficiencies are acquired, due to blood loss, insufficient dietary iron uptake or both in conjunction (Brady 2007). If diagnosed, acquired iron deficiency, can usually be corrected by iron supplementation in form of $FeSO_4$ or ferrous gluconate.

In human several rare genetic alterations in genes involved in iron metabolism have been reported and linked to iron deficiency anemias. Mutations in the genes encoding DMT1 (*SLC11A2*) or *glutaredoxin 5* (*GLRX5*) are associated with autosomal recessive hypochromic, microcytic anemia (Camaschella et al. 2007). The patients display common alterations in erythroid morphology and CBC, but also exhibit hepatic iron overload, which is not fully understood (Mims et al. 2005; Priwitzerova et al. 2005; Beaumont et al. 2006; Iolascon et al. 2006; Lam-Yuk-Tseung et al. 2006). Another iron deficiency disorder called *hypotransferrinemia* has been reported which bears mutations in the Tf gene itself. The altered Tf structure interferes with iron delivery to erythroid progenitors and results in a massive accumulation of intestinal iron (Heilmeyer et al. 1961; Goya et al. 1972). Deficiency of the plasma protein *ceruloplasmin* was also reported to result in iron deficiency anemia. This mutation leads to the loss of ceruloplasmin's ferroxidase activity required to mobilize stored iron (Harris et al. 1995).

1.4 Stat5 in hematopoietic homeostasis and disease

A modified version of the following section of the ***Introduction*** has also been submitted for publication as book chapter in "*JAK STAT Pathway in disease*", edited by Anastasis Stephanou and published by Landes Bioscience 2008 (http://www.eurekah.com/chapter/4038).

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1.4.1 Stat5 in hematopoietic stem cells and progenitors

Tissues with high cell turnover require pluripotent stem cells to maintain life-long organ homeostasis. In case of the hematopoietic system, hematopoietic stem cells (HSCs) residing in the bone marrow are able to ensure permanent production of all mature blood lineages (Weissman 2000). HSCs can be highly enriched by sorting for the cell surface marker combination lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK; Figure 16). HSCs eventually give rise to multi-lineage progenitors, restricted to either the myeloid (common myeloid progenitor; CMP; (Akashi et al. 2000) or lymphoid compartment (common lymphoid progenitor; CLP; Kondo et al. 1997; Figure 16). Among other factors, stem cell factor (SCF), *thrombopoietin* (Tpo), *interleukin* (IL)-3, *Flt-3-ligand* (Flt-3L) and their receptors c-kit, *c-mpl*, *IL-3R* and Flt-3 have been implicated to support proliferation and survival of LSKs or multi-lineage progenitors (Blank et al. 2008). The receptors mentioned all share the crucial downstream effector transcription factors Stat5a and Stat5b (Stat5).

First results on the function of Stat5 in HSCs and multi-lineage progenitors were compromised by the original hypomorphic Stat5 knock out (Teglund et al. 1998). Later studies revealed that these animals still expressed significant levels of N-terminally truncated Stat5 proteins (Sexl et al. 2000; Cui et al. 2004; Hoelbl et al. 2006; Yao et al. 2006; Engblom et al. 2007; Yao et al. 2007; Kornfeld et al. 2008). Therefore, the corresponding animals can rather be regarded as knock-in mice for mutant Stat5 proteins with loss of the N-terminal domain. Today, the "old" animal model is referred to as Stat5$^{\Delta N/\Delta N}$ mouse (Teglund et al. 1998). In 2004, "true null protein" Stat5 knock out mice (Stat5$^{-/-}$) became available (Cui et al. 2004), in which the complete locus for Stat5a and Stat5b was flanked with loxP sites allowing a 110 kb deletion upon Cre recombinase action, either in whole animals or specific tissues (Hoelbl et al. 2006; Yao et al. 2006; Yao et al. 2007).

Although most work addressing the role of Stat5 in HSCs and multi-lineage progenitors was performed with $Stat5^{\Delta N/\Delta N}$ mice, interesting results were obtained and in general phenotypes were aggravated in fully Stat5-deficient mice (Bunting et al. 2002; Yao et al. 2006; Bunting 2007; Li et al. 2007). $Stat5^{\Delta N/\Delta N}$ HSCs displayed impaired re-population capability in both serial as well as competitive reconstitution assays (Bunting et al. 2002; Snow et al. 2002). Impaired re-population was neither due to reduced HSC numbers nor due to defects in homing, as revealed by direct quantitation experiments (Bunting et al. 2002; Snow et al. 2002). This already suggested that Stat5 is rather supporting self-renewal of HSCs than survival or homing. The interpretation was strengthened by competitive reconstitution assays comparing engrafting capability of each, wt, $Stat5^{\Delta N/\Delta N}$, and $Stat5^{-/-}$ fetal liver cells, demonstrating a more drastic re-population defect of $Stat5^{-/-}$ compared with $Stat5^{\Delta N/\Delta N}$ HSCs (Li et al. 2007). Analysis of the $Stat5^{\Delta N/\Delta N}$ multi-lineage progenitor compartment revealed a massive reduction in progenitor numbers, as judged by *spleen colony-forming units* (CFU-S) assays: $Stat5^{\Delta N/\Delta N}$ multi-lineage progenitors gave rise to fewer and smaller CFU-S (Bunting et al. 2002; Snow et al. 2002). Furthermore, flow cytometry for the apoptosis marker AnnexinV displayed increased cell death of $Stat5^{\Delta N/\Delta N}$ lin^-Sca1^- progenitors (Snow et al. 2002). Thus, Stat5 apparently has an important function in survival of lineage-restricted progenitors.

Whether the loss of Stat5 affects CMPs, CLPs, or both has not yet been analyzed in murine models. Future studies should address this important question, since it has consequences for lymphoid or myeloid hyper- versus hypo-proliferation and potential side effects of eventual future therapies targeting Stat5. For example, lentivirus-mediated RNA interference against Stat5 in human $CD34^+CD38^-$ cells derived from umbilical cord blood (resembling murine LSK cells) revealed a 3.25-fold reduction in stem cell number and a 3.9-fold reduction in progenitor cell abundance as determined by long-term culture-initiating cell assays and colony-forming cell assays, respectively (Scherr et al. 2006; Schepers et al. 2007). Taken together, data from both, mouse and human, demonstrate that Stat5 is an important positive regulator for hematopoietic stem / progenitor cell fitness.

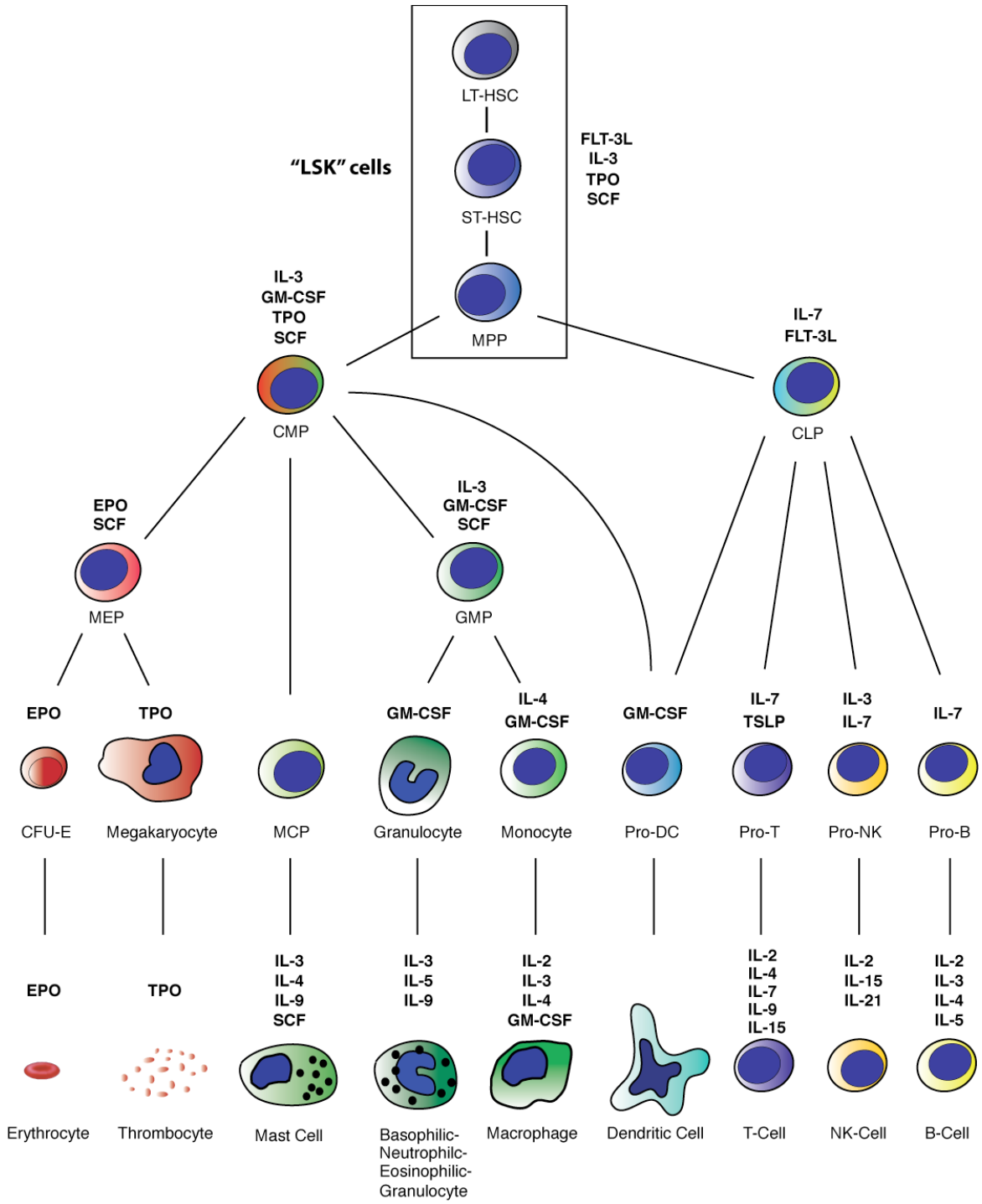

Figure 16: Simplified schematic diagram of the linear hierarchy of hematopoietic cells. Cytokines and growth factors denoted in this illustration are all implicated in activation of Stat5 and to either promote survival, proliferation or differentiation of their respective cell types they have been allocated to. Long-term reconstituting hematopoietic stem cells (LT-HSCs) divide to give a phenocopy of themselves (self-renewal) and a more committed ST-HSC (short-term reconstituting hematopoietic stem cell). ST-HSCs give rise to multi-potent progenitors (MPPs). These 3 cell populations are subsumed as "LSK" cells (boxed cells) according to their cell surface marker phenotype (lineage$^-$ sca-1$^+$ c-kit$^+$). Multipotent progenitor cells produce the lineage specific common lymphoid progenitor (CLP), which differentiates into all mature lymphoid cells, as well as the common myeloid progenitor (CMP), which differentiates into the megakaryocytic-erythroid progenitor (MEP) as well as the granulocytic-monocytic progenitor (GMP). The MEP and the GMP give rise to all mature cells of the erythroid and myeloid lineage.

Abbreviations: CFU-E (colony forming unit erythroid) DC (dendritic cell) EPO (erythropoietin), GM-CSF (granulocyte-macrophage colony stimulating factor), IL (interleukin), MCP (mast cell progenitor), SCF (stem cell factor), TPO (thrombopoietin), TSLP (thymic stromal lymphopoietin)

1.4.2 Stat5 in erythropoiesis and myelopoiesis

$Stat5^{\Delta N/\Delta N}$ mouse embryos were described to be anemic which was attributed to increased apoptosis of erythroid progenitor cells in the fetal liver, due to decreased expression of the anti-apoptotic gene *Bcl-x_L* (Socolovsky et al. 1999). This phenotype could be completely rescued by ectopic expression of Bcl-x_L (Dolznig et al. 2006) *in vitro*. Adult $Stat5^{\Delta N/\Delta N}$ mice showed no overt erythroid defects during steady state erythropoiesis. However, under erythrolytic stress induced by phenylhydrazine, $Stat5^{\Delta N/\Delta N}$ mice exhibited a massive delay in erythroid recovery (Socolovsky et al. 2001).

Complete deletion of both *Stat5* genes did not reveal abnormalities in early embryo development but lead to significant deviations from expected Mendelian ratios in later stages of embryogenesis. More importantly and in contrast to $Stat5^{\Delta N/\Delta N}$ mice, $Stat5^{-/-}$ animals died between E16.5-birth (Cui et al. 2004; Kerenyi et al. 2008), except for 1-2% (instead of 25%) of survivors on an Sv129 x C57Bl/6 mixed background. Puzzling but quite common in Jak-Stat-Socs knock out mouse model systems, loss of Stat5 genes caused elevated expression and tyrosine phosphorylation of Stat1 and Stat3 in $Stat5^{-/-}$ fetal liver-derived hematopoietic progenitors (MAK, RM, and EWM, unpublished), suggesting compensatory mechanisms (Murray 2007). The few surviving mice died around 5 weeks, likely due to l severe autoimmunity caused by a lack of regulatory T-cells (Hoelbl et al. 2006; Yao et al. 2007). The vast majority of Stat5-deficient embryos developed severe erythroid defects, particularly on pure genetic backgrounds such as C57Bl/6 or Balb/c. They displayed reduced hematocrits, massive anemia and defects in erythroid iron metabolism (Kerenyi et al. 2008; Zhu et al. 2008). The pivotal role of Stat5 in erythropoiesis was further supported by recent findings: (i) Persistent activation of Stat5 in $Jak2^{-/-}$ as well as $EpoR^{-/-}$ fetal liver-derived erythroid progenitors could significantly restore BFU-E and CFU-E colony formation *in vitro* and erythropoiesis *in vivo* upon transplantation of cells into irradiated recipients (Grebien et al. 2008). (ii) Gene knock out mouse studies displayed a complete failure of erythroid engraftment upon transplantation of $Jak2^{-/-}$ (Neubauer et al. 1998; Parganas et al. 1998), $EpoR^{-/-}$ (Wu et al. 1995) or $Stat5^{-/-}$ fetal liver cells into lethally irradiated wt mice (Li et al. 2007).

Stat5 is also a key molecule for myelopoiesis (reviewed in Coffer et al. 2000). Compared to wt, $Stat5^{\Delta N/\Delta N}$ bone marrow formed less than 50% of myeloid colonies in response to IL-3, GM-CSF, SCF, or Flt-3L factor combinations *in vitro* (Teglund et al. 1998; Bunting et al. 2002). Moreover, $Stat5^{\Delta N/\Delta N}$ mice displayed thrombocytopenia, due to defective

TPO signaling (Bradley et al. 2002; Bunting et al. 2002; Snow et al. 2002). These data were corroborated by observations on increased apoptosis of $Stat5^{\Delta N/\Delta N}$ bone marrow cells undergoing IL-3- or GM-CSF-induced myeloid differentiation in suspension cultures (Kieslinger et al. 2000). Apoptosis of Stat5-deficient myeloblasts was attributed to loss of *Bcl-x_L* expression, reminiscent of the erythroid situation (Kieslinger et al. 2000). $Stat5^{\Delta N/\Delta N}$ bone marrow cells also failed to develop into Th_2 cytokine-producing eosinophilic granulocytes in response to IL-5 (Zhu et al. 2004). In line, ectopic expression of a dominant-negative Stat5 variant (Stat5Δ750; Moriggl et al. 1996) in human $CD34^+$ cord blood cells blocked eosinophil differentiation (Buitenhuis et al. 2003). New studies on complete Stat5 deletion revealed a role of Stat5 in G-CSF signaling and for development of inflammatory M2 macrophages (Xiao et al. 2008). Interestingly, Stat5 can also act as negative regulator of granulopoiesis: $Stat5^{\Delta N/\Delta N}$ mice displayed mild neutrophilia but had a 25-fold increase in serum G-CSF levels (Fievez et al. 2007). This was attributed to negative feedback of Stat5 on the *G-CSF* promoter in liver endothelial cells. Finally a recent study also demonstrated an essential role for Stat5 in myeloid leukemia induced by truncated a G-CSF receptor (Liu et al. 2008).

In addition, Stat5 is also essential for mast cell development. $Stat5^{\Delta N/\Delta N}$ mice display massively reduced mast cell numbers, due to aberrant expression of *Bcl-2, Bcl-x_L, cyclin A2* and *B1* (Shelburne et al. 2003). Stat5 is not only important for mast cell survival and -proliferation but was even implicated to play a role in their effector function, as it was activated upon IgE cross-linkage (Barnstein et al. 2006). Accordingly, $Stat5^{\Delta N/\Delta N}$ mast cells displayed defects in degranulation and leukotrien B4 production (Barnstein et al. 2006). Mast cells from $Stat5^{-/-}$ fetal livers were even more severely affected in cytokine-dependent proliferation but could be efficiently rescued with both, wt or $Stat5a^{\Delta N/\Delta N}$ (Li et al. 2007).

1.4.3 Stat5 in lymphopoiesis

Stat5 is activated by all lymphoid cytokines that use the common gamma chain (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) and exerts important functions in lymphopoiesis such as regulation of proliferation, survival and differentiation (Figure 16).

B-cell development is strictly dependent on IL-7-signaling, as evident from *IL-7* or *IL-7 receptor* (*IL-7Rα*) knock out mice, which completely lack mature B-cells due to a differentiation block at the pre-pro B-cell stage (Kikuchi et al. 2005). Since Stat5 is efficiently

activated by IL-7 (Foxwell et al. 1995; van der Plas et al. 1996; Goetz et al. 2004), it was anticipated that Stat5-deficiency would display similar defects. Interestingly, expression of a constitutively active *Stat5b* allele complemented B-cell development in *IL-7Rα*$^{-/-}$ mice (Goetz et al. 2005). Unexpectedly, *Stat5*$^{\Delta N/\Delta N}$ mice did produce mature peripheral B-cells, although in reduced numbers. This was originally attributed to an incomplete block at the stage of early pro B-cell differentiation (Sexl et al. 2000). These results were extended by recent studies using complete Stat5 knock out mice (Hoelbl et al. 2006; Yao et al. 2006) and by *in vivo* experiments employing B-cell specific deletion of Stat5 (CD19cre; Dai et al. 2007). IL-7 stimulation of immature lymphocytes also led to a robust Stat3 activation, which might explain why IL-7- or IL-7Rα-deficiency had a more drastic phenotype. Thus, the exact function of Stat5 downstream of IL-7 is still under debate. It is not clear whether Stat5 solely promotes B-cell survival or if it is also required for lineage commitment. But the fact that increased apoptosis of in IL-7Rα-deficient B-cells could not be overcome by expression of a *Bcl-x*$_L$ transgene (Goetz et al. 2005), argued for a developmental role of Stat5. Since Bcl-x$_L$ is not expressed in wt B-cells, one should keep in mind that other antiapoptotic genes such as Mcl-1, which has been described to be important for survival of lymphocytes (Opferman et al. 2003), might be able to overcome this defect. Several studies nevertheless implicated Stat5 in transcriptional control of *EBF-1* and/or *Pax-5,* (Hirokawa et al. 2003; Goetz et al. 2005; Dai et al. 2007) both necessary for B-cell development. Stat5$^{-/-}$ B-cell progenitors, however, do only partially phenocopy the defects of *IL-7*-, *IL-7Rα*–, *Pax-5*- or *EBF-1*-deficient mice in VDJ-immunoglobulin rearrangement or the surprising capacity of B-cells to retrodifferentiate to T lymphocytes (Cobaleda et al. 2007; Cobaleda et al. 2007). Apparently, B cell commitment and the regulation of Pax5 is a more complex scenario, which does not solely depend on linear cytokine-transcription factor regulation like IL-7-IL-7R-Jak1/3-Stat5. Hence Stat5 might induce only genes important for survival and proliferation downstream of IL-7Rα without a direct function in B-cell lineage determination.

Considering the importance of IL-2, IL-4, and IL-7 in T-cell development, analysis of the T-lymphocyte compartment in Stat5$^{\Delta N/\Delta N}$ mice revealed a surprisingly mild phenotype. There was a slight reduction of peripheral CD8$^+$ T-cells accompanied by a lack of functional NK cells(Moriggl et al. 1999). CD4$^+$ and CD8$^+$ T-cells failed to proliferate in response to IL-2 or IL-4 even in combination with full T-cell receptor activation and co-stimulation (Moriggl et al. 1999; Moriggl et al. 1999). Typical activation-specific genes like *D-type cyclins, c-myc, Pim-1, Socs1-3,* or *Bcl-x*$_L$ were not induced and the T-cells remained arrested in G1, although IFN-gamma production was normal (Moriggl et al. 1999; Moriggl et al. 1999; Gatzka et al.

2006). Interestingly, $Stat5^{\Delta N/\Delta N}$ T-cells displayed an activated/memory phenotype ($CD44^{high}$ $CD62L^{low}$; Moriggl et al. 1999), suggesting a disturbed negative regulation. Consequently, the mice developed autoimmune disease accompanied by reduction of peripheral $CD4^+CD25^+$ regulatory T-cells (T_{reg}); at that time FoxP3 was not yet known as a T_{reg} specific marker (Antov et al. 2003; Snow et al. 2003; Kang et al. 2004). Given the necessity of (Stat5-mediated) IL-2 signaling in T_{reg} development and maintenance, it was reasoned that Stat5 might not be needed for T_{reg} development but rather for maintenance (Antov et al. 2003; Snow et al. 2003). And since IL-2 signaling activated not only Jak/Stat signaling but also Ras-MAPK and PI3-K, in consequence the latter pathways were considered to be required for T_{reg} development.

Likewise as for B-cells, analysis of mice harboring the complete Stat5 knock out revealed more drastic T-cell phenotypes. While $CD8^+$ as well as $CD4^-CD8^-\gamma\delta TCR^+$ T-cell numbers were strongly reduced, $CD4^+CD25^+FoxP3^+$ T_{regs} were completely absent (Hoelbl et al. 2006; Yao et al. 2006; Yao et al. 2007), similar results were also seen in conditional $Stat5^{flLckCre}$ mice. Furthermore, *FoxP3*, which is necessary and sufficient for polarization of naïve $CD4^+$ T-cells to fully functional regulatory T-cells, was identified as a direct transcriptional target of Stat5 (Zorn et al. 2006; Burchill et al. 2007; Yao et al. 2007). These findings finally demonstrated that Stat5 was indeed required for T_{reg} maintenance as well as for their development and polarization.

Recently, it was proposed that differentiation of naïve $CD4^+$ T-cells towards the T-helper-17 (Th_{17}) lineage concomitantly antagonizes T-cell polarization towards $CD4^+CD25^+FoxP3^+$ T_{regs}, and *vice versa* (Bettelli et al. 2006). In line with this, mice with T-cell specific Stat5 deletion (using $Stat5^{fl/flCD4Cre}$) were almost completely devoid of T_{regs} but actually did exhibit increased numbers of Th_{17} cells (Laurence et al. 2007; Yao et al. 2007). In contrast, conditional deletion of Stat3, which has no effect on regulatory T-lymphocyte abundance, led to loss of Th_{17} cells. These studies convincingly established the intrinsic requirements for Stat5 in polarization towards the T_{reg}- and Stat3 towards the Th_{17} helper cell lineage (Laurence et al. 2007; Yao et al. 2007), and a reciprocal relationship between Stat3 and Stat5 during T-cell development. Finally, Stat5 was also demonstrated to play a role for T-helper cell lineage commitment, since it suppresses Th_1 differentiation and controls Th_2 cytokine production (Cote-Sierra et al. 2004; Takatori et al. 2005).

1.4.4 Stat5 as a master regulator of hematopoietic cancers?

So far, Stat5 has been described as master regulator of hematopoiesis, but to which extent is elevated Stat5 signaling critically associated with leukemia or lymphoma formation? Persistent activation of Stat5 indeed has been observed in a broad spectrum of human hematologic malignancies, including chronic myelogenous leukemia (CML), erythroleukemia, acute lymphocytic leukemia (ALL), myelo-proliferative diseases (MPDs) like polycythemia vera, thrombocytopenia, idiopathic myelofibrosis, or mastocytosis. Moreover, several studies reported that distinct types of T- and B-cell lymphomas were associated with persistent Stat5 activity (Joliot et al. 2006; Nagy et al. 2006; Martini et al. 2008; Table 1). Introduction of Stat5 gain-of-function mutants by retroviral integration and transplant models or massive over-expression of wt *Stat5a* and *Stat5b* genes (Tsuruyama et al. 2002; Kelly et al. 2003; Bessette et al. 2008) in transgenic mouse models demonstrated that Stat5 can promote factor-independent proliferation (Grebien et al. 2008) and tumor initiation in virtually all hematopoietic cell types (Moriggl et al. 2005). Thus, by standard definition, P-Y-Stat5 can be viewed as an onco-protein. Importantly, several studies with human leukemic cells have shown that Stat5 is also involved in tumor cell maintenance, a feature described as oncogene dependence (Weinstein 2002; Ye et al. 2006; Schepers et al. 2007).

Disease	Cell type	Oncogene/Mutation
Leukaemia		
erythroleukemia	erythroleukemia / blast cells	Bcr-Abl; Bcr-Jak2
chronic myelocytic leukemia (CML)	granulocytes	Bcr-Abl(p210); v-Abl; Bcr-Jak2;
acute lymphocytic leukemia (ALL)	B- or T-lymphocytes	Bcr-Abl(p185); Tel-Jak2; Tel-Abl; EML1-Abl
acute myelocytic leukemia (AML)	myeloid cells	Flt3-ITD; c-KitD816V; Bcr-Abl; Jak3-A572V
megakaryocytic leukemia	megakaryocytes	Jak3-A572V
Lymphomas		
Sézary syndrome (cutanous T-cell lymphoma)	T-cells	IL-2R mutations
anaplastic large cell lymphoma (ALCL)	T-cells	Tel-Jak2; Tel-Abl; NPM1/ALK*
B-cell lymphoma	B-cells	v-Abl; Bcr-Abl
HTLV-I-dependent T-cell leukemia/lymphoma	T-cells	IL-2Rα overexpression
Clonal myeloproliferative disorders		
(EMS)/stem cell leukemia-lymphoma syndrome	myeloid progenitor cell	ZNF198-FGFR1
polycythemia vera	erythrocytes	Jak2-V617F; Jak2 Exon12
essential thrombocythemia	megakaryocytes	Jak2-V617F
idiopathic myelofibrosis	megakaryocytes	Jak2-V617F
severe congenital neutropenia	promyelocyte / myelocyte	nonsense mutation in CSF3R (G-CSFR)
chronic myelo-monocytic leukemia (CMML)	monocytes	Tel-PDGFRβ
mastocytosis	mast cells / basophils	c-Kit D816V; Bcr-Abl; FIP1L1-PDGFRα

HTLV human T-cell lymphoma virus; * NPM1/ALK down-regulates Stat5

Table 1: Stat5 activation in hematopoietic malignancies

Constitutive activation of Stat5 can occur either due to activating mutations or chromosomal translocations in genes of upstream kinases, generating fusion tyrosine kinases, which are particularly frequent in leukemias. Alternatively, persistent Stat5 activation can also be associated with amplification of essential upstream signaling components such as growth factors or cytokines, or over-expression of their cognate receptor (Figure 17). The ensuing amplification of Stat5 signaling drives cells into sustained proliferation, protects against apoptotic signals, leads to evasion from the immune system and finally can render cells almost immortal. All these processes of course do not only depend on persistent activation of the Jak-Stat pathway alone, but are associated with additional oncogene mutations and amplifications or silencing of tumor suppressors. Only the combined action of activated oncogenes and silenced tumor suppressors and likely in synergy with P-Y-Stat5, causes tumor development. It is still a challenge for future studies to identify disease specific combinations of these players, since most work so far focused either on Stat5 alone or the most common genetic modifications. Generally, activation of Stat5 by mutations in upstream tyrosine kinases is mainly associated with increased proliferation or survival of hematopoietic cells. This is of course mainly due to activation of Stat5 target genes (Schuringa et al. 2004; Gatzka et al. 2006). These can be either involved in cell cycle progression (e.g. *IL-2Rα*, *D-type cyclins, c-Myc, oncostatin M, IL-7Rα, IL-3Rβ, ALS, IGF-1, Pim kinases, epidermal growth factor-receptor, prolactin receptor*), survival (e.g. *A1, Mcl-1, Bcl-2, Bcl-x_L, survivin*), negative feedback inhibition of tyrosine kinase signaling pathways (e.g. *CIS, Socs1-3*), lymphocyte function (e.g. *FoxP3, CD25, TCRγ/δ rearrangement region, perforin, lymphotoxin-α, Pax5, EBF, Glut1*) or co-factor regulation (e.g. *Cited2*).

1.4.5 Stat5 and transforming tyrosine kinases activated by chromosomal rearrangements

De-regulated tyrosine kinase activity promoting leukemogenesis frequently results from chromosomal breakage-and-reunion events causing gene fusions. Several examples involving translocated tyrosine kinases are known to result in activation of Stat5 (Figure 17a). The probably most prominent and best studied Stat5 activating fusion kinase is *Bcr-Abl*, the protein product of the Philadelphia chromosome (Ph^+ t(9;22); Shtivelman et al. 1985), which is responsible for >90% of CML, 25-30% of adult and 2-10% of childhood ALL (Hermans et al. 1987), and rare cases of AML. The reciprocal t(9;22) translocation involves different exon sets of the *bcr* gene (breakpoint cluster region; (Groffen et al. 1984) fused to a common subset of exons from the *abl* gene, generating two alternative chimeric oncogene products –

p190 and p210 (Chan et al. 1987; Lichty et al. 1998). p210 is responsible for CML, whereas p190 results almost exclusively in adult ALL, and very rarely causes AML. The first exon of *bcr*, retained in both isoforms, appears essential for constitutive activation of the Abl tyrosine kinase, which leads to factor-independent proliferation and transformation of hematopoietic cells (Ilaria and Van Etten 1996). Carlesso and co-workers were the first to demonstrate persistent Stat5 activity in human Ph^+ CML cell lines (Carlesso et al. 1996). Additional studies provided further evidence that Stat5 is indeed essential for transformation and leukemogenesis (de Groot et al. 1999; Sillaber et al. 2000; Hoelbl et al. 2006; Scherr et al. 2006). The mechanism of Stat5 activation by Bcr-Abl was described either as direct, without requirement for phosphorylation via Jak kinases (Carlesso et al. 1996; Ilaria and Van Etten 1996) or indirect, involving tyrosine phosphorylation by Jak2 (Samanta et al. 2006) or Src-family kinases (Nieborowska-Skorska et al. 1999). One prominent example of a target gene exhibiting increased expression levels along the Bcr-Abl-Stat5 axis is the anti-apoptotic gene *Bcl-X_L,* whose relevance for hematopoietic development is well documented (de Groot et al. 1999; Nieborowska-Skorska et al. 1999; Gesbert and Griffin 2000; Donato et al. 2001).

Therapy of CML is based on inhibition of Bcr-Abl kinase activity. Inhibitors like *Imatinib-mesylate* (*Gleevec*) or the newer compounds *Dasatinib* and *Nilotinib* cause efficient inhibition of CML progression, in close association with suppressed Stat5 activity. This in turn leads to a reduction in expression of genes like *Rad51, D-type cyclins, c-Myc, Mcl-1* or *Bcl-X_L,* resulting in selective apoptosis of Bcr-Abl expressing cells (Druker et al. 1996; Nieborowska-Skorska et al. 1999; Horita et al. 2000; Skorski 2002; Aichberger et al. 2006). The relevance of Stat5 in Bcr-Abl induced leukemia progression was underscored by animal studies demonstrating that even the absence of Stat5a alone (Ye et al. 2006) or the absence one allele of Stat5a/b ($Stat5^{+/-}$) already reduced the incidence of CML, while $Stat5^{-/-}$ fetal liver cells were even completely resistant to transformation (Hoelbl et al. 2006). Cain and co-workers obtained similar results, supporting a dosage effect of Stat5 (Cain et al. 2007).

Also members of the Jak kinase-family (comprising Jak1-3 and Tyk2), which are the "classical" upstream regulators of Stat activity, can be affected by chromosomal rearrangements leading to aberrant signaling. Such translocations are rare but were reported in human leukemias, suggesting a direct Jak-Stat-mediated leukemic process. The leukemia-associated *Tel(Etv6)-Jak2* fusion protein is formed by fusion of the oligomerization domain of the Ets-family transcription factor (Tel) to the catalytic domain of Jak2 (Lacronique et al. 1997). Tel-domain-mediated oligomerization then leads to constitutive tyrosine kinase activity (Schwaller et al. 1998; Ho et al. 1999), resulting in constitutive Stat activation

(Schwaller et al. 1998; Carron et al. 2000). *In vitro,* persistent activation of the Jak-Stat pathway by a Tel-Jak2 fusion protein rendered murine hematopoietic IL-3-dependent Ba/F3 cells growth factor-independent (Lacronique et al. 2000), which was abrogated upon expression of dominant-negative Stat5a (Lacronique et al. 2000). *In vivo,* Tel-Jak2 transgenic mice developed T-cell leukemia, in association with constitutive activation of Stat5 and Stat1 (Carron et al. 2000). Also the involvement of Stat5 activation, induced by Tel-Jak2, in the development of myelo- and lympho-proliferative disease could be demonstrated in a murine transplant model (Schwaller et al. 2000).

1.4.6 Mutated or amplified Jak kinases affecting Stat5 activity

Enhanced Stat5 activation has been observed in clonal myelo-proliferative disorders like *polycythemia vera* (PV), *essential thrombocythemia* (ET), and *idiopathic myelofibrosis* (IM; James et al. 2005; Aboudola et al. 2007). These diseases originate from multipotent progenitors capable of giving rise to erythroid and as well as myeloid cells. A high proportion of patients were found to carry a dominant gain-of-function mutation (*Jak2-V617F*) in the negative regulatory Jak-homology-2 (JH2) domain of Jak2 (Baxter et al. 2005; James et al. 2005; Kralovics et al. 2005; Levine et al. 2005; Tefferi et al. 2005; Levine et al. 2007; Morgan and Gilliland 2008). This mutation was associated with constitutive phosphorylation of Jak2 and ensuing activation of Stat5 (Baxter et al. 2005; James et al. 2005; Kralovics et al. 2005; Levine et al. 2005; Figure 17c). Constitutive tyrosine phosphorylation activity of Jak2 promoted cytokine hypersensitivity (particularly towards Epo; Ugo et al. 2004; Veselovska et al. 2008) and induced erythrocytosis in a mouse model (Bumm et al. 2006; Lacout et al. 2006; Wernig et al. 2006; Zaleskas et al. 2006). Mice expressing Jak2-V617F under its endogenous promoter exhibited differential expansion of hematopoietic lineages, depending on both, presence and abundance of mutated Jak2 (Tiedt et al. 2007; Shide et al. 2008). PV patients with the Jak2-V617F mutation showed an increased number of hematopoietic stem cells with aberrant erythroid potential in peripheral blood. This phenotype was potently inhibited by the Jak2 inhibitors AG490 (Jamieson et al. 2006) or by the specific Jak2-V617F inhibitor TG101358 (Wernig et al. 2008).

An additional Jak2 gain-of-function mutant (K539L) was described in patients with Jak2-V617F-negative PV or idiopathic erythrocytosis. In Ba/F3 cells expressing murine Epo-R, the K539L mutation increased phosphorylation of Jak2 and ERK1/2, when compared with cells transduced by wt Jak2 or Jak2-V617F, rendering the cells growth factor-independent

(Scott et al. 2007). In mice, *Jak2-K539L* also led to a myelo-proliferative phenotype, including erythrocytosis (Scott et al. 2007). Furthermore, Jak2-V617F and another Jak2 mutation (K607N; in the pseudo-kinase domain) were also observed in a subset of AML patients (Lee et al. 2006).

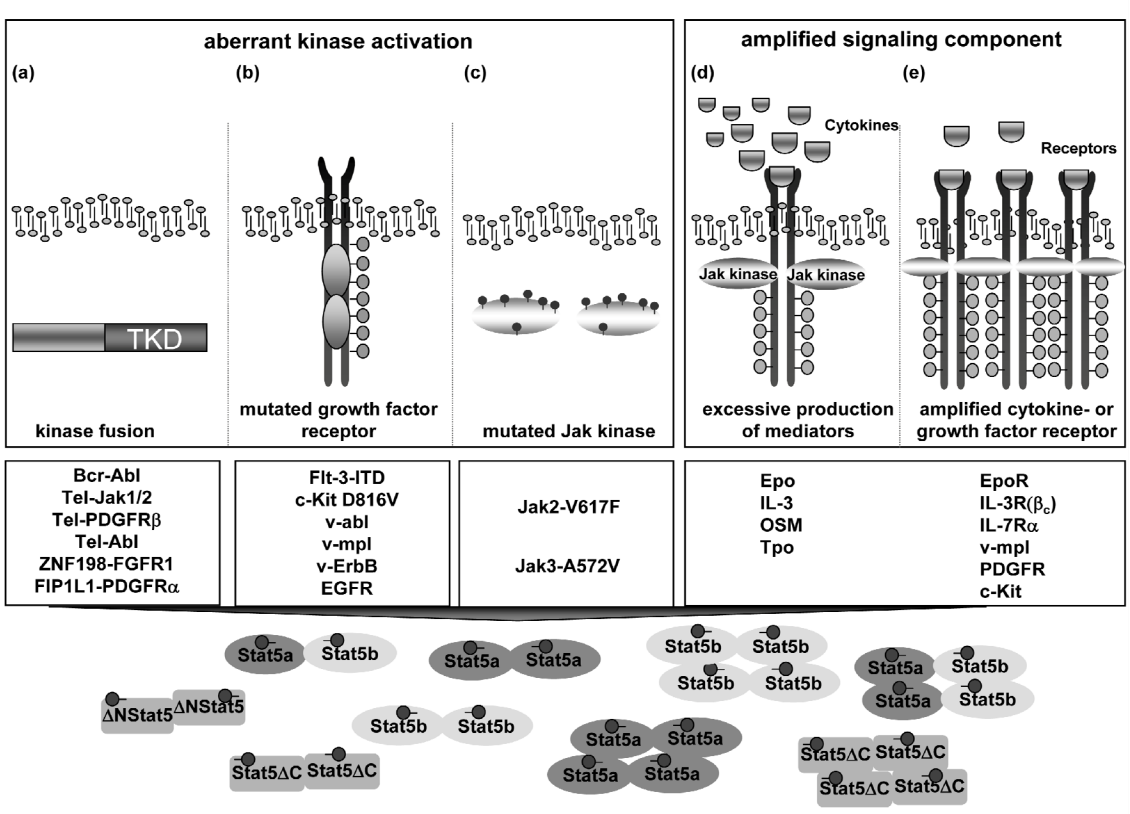

Figure 17: Mutations and expression change leading to persistent Stat activation. Persistent activation of STAT5 has been observed in a broad spectrum of human hematologic malignancies. It may be attributed to **(a)** translocations generating fusion tyrosine kinases, **(b)** activating mutations in growth factor receptors, **(c)** activating mutations in upstream kinases, or alternatively be due to an amplification of a signaling component such as **(d)** autocrine growth factor production or **(e)** receptor over-expression. This leads to hyper-activation of Stat5 downstream targets resulting in increased cell proliferation, cell survival, or reduced apoptosis. Multiple phosphorylated Stat5 proteins can form dimers or oligomers on DNA molecules. Moreover, secondary post-translational modifications (e.g. P-Ser/P-Thr), different

1.4.7 Mutated growth factor receptors in Jak-Stat signaling

Amplification, over-expression, or somatic mutations of the class III receptor tyrosine kinases (RTKs) Flt-3, c-Kit, FMS, and PDGF-R can result in increased receptor signaling, eventually leading to tumorigenesis (Figure 17b+e). Especially the activation loops of these RTKs form a hotspot for activating mutations, due to common structural characteristics.

Mutations in Flt-3 represent a common genetic lesion in AML but are rare in adult ALL (Armstrong et al. 2004). Activation of Flt-3 due to internal duplications in the juxta-

membrane domain, the most frequent and best characterized type of mutation, is found in 20-25% of AML patients (Nakao et al. 1996; Kottaridis et al. 2001; Meshinchi et al. 2001). Point and deletion mutations in the activation loop of the protein tyrosine kinase domain were described in ~7% of AML cases (Abu-Duhier et al. 2001; Yamamoto et al. 2001). Flt-3 internal-tandem-duplication mutations (*Flt-3-ITD*) cluster in the juxta-membrane domain. These alterations cause constitutive activation of the receptor, which becomes phosphorylated independent of ligand binding, and transforms hematopoietic cell lines to growth factor-independent proliferation. Aberrant signals emerging from Flt-3-ITD include activation of Stat5 (Zhang et al. 2000; Bunting et al. 2007), and repression of myeloid transcription factors Pu.1 and C/EBP-α. The mechanism of Stat5 activation by Flt-3-ITD is independent of Src- or Jak kinases as Stat5 was shown to be a direct target of Flt-3 (Zhang et al. 2000; Choudhary et al. 2007). Although Flt-3-ITD was sufficient to induce MPD in a murine bone marrow transplant model, the AML phenotype observed in humans could not be recapitulated (Kelly et al. 2002).

c-Kit (also designated CD117), the RTK for stem cell factor, is required for normal hematopoiesis, melanogenesis, and gametogenesis (Ikuta et al. 1992; Besmer et al. 1993). Point mutations in this receptor were described to result in ligand-independent tyrosine kinase activity and auto-phosphorylation, which in turn lead to stimulation of downstream signaling pathways and uncontrolled cell proliferation. Consequently, mutations in the *c-Kit* gene are known to induce mast cell leukemia and AML. They cluster in two distinct regions, the juxta-membrane domain and the activation loop. Activating point mutations in c-Kit, mainly D816V (Fritsche-Polanz et al. 2001; Valent et al. 2003; Wimazal et al. 2004; Horny et al. 2007), have been also linked to systemic mastocytosis (Furitsu et al. 1993). Mastocytosis is often transient and limited in children but persistent or progressive in adults. Occurrence of somatic c-Kit mutations correlates with severity of disease. They are found in a high number of adult sporadic mastocytosis patients as well as in children at risk for extensive or persistent disease (Longley et al. 1999). One explanation for increased mast cell numbers in tissues of patients with mastocytosis is offered by enhanced chemotaxis of CD117-positive cells derived from CD34/CD117 double-positive precursors (Taylor et al. 2001). Harir and co-workers demonstrated that also a gain-of-function mutant of Stat5 could promote mast cell disease in mouse bone marrow transplant models (Harir et al. 2008).

1.4.8 Amplified Signals

Excessive production of cytokines, amplification of their receptor, as well as autocrine cytokine stimulation, are additional mechanisms to cause hyper-activation of downstream signal transducers (Figure 17d&e). Several heterozygous mutations in the Epo-R with increased sensitivity towards Epo were described to result in strong activation of Jak2 & Stat5 (Watowich et al. 1999) and ensuing *autosomal-dominant erythrocytosis-1* (ECYT-1; de la Chapelle et al. 1993; Kralovics et al. 1997). The majority of ECYT-1 Epo-R mutants lacked the C-terminal negative regulatory domain (Kralovics et al. 1997). The potential for such truncations in the pathogenesis of human erythroleukemia was also revealed by characterization of a 3´-end deletion in the Epo-R gene of a cell line over-expressing Epo-R and proliferating in response to Epo (Ward et al. 1992). Also a point mutation in the mouse Epo-R gene resulted in constitutive activation, leading to erythrocytosis and splenomegaly (Longmore and Lodish 1991). In some AML patients, wt c-Kit as well as non-mutated Flt-3 was found over-expressed and activated. For Flt-3, there was also *in vitro* evidence that an autocrine loop promotes growth and survival of AML blasts (Zheng et al. 2004).

1.4.9 Constitutively activated mutants of Stat5 inducing transformation

Stat5 is activated by various onco-proteins with tyrosine kinase activity and its involvement in tyrosine kinase-mediated leukemogenesis is known to be crucial. Direct evidence that constitutive activation of Stat5 by itself can be sufficient to cause cell transformation was obtained by analysis of the Stat5 mutants Stat5-1*6 (Onishi et al. 1998) and $cS5^F$ (Moriggl et al. 2005). These constitutively active variants were capable of inducing MPD and multi-lineage leukemia in transplanted mice (Schwaller et al. 2000; Moriggl et al. 2005; Harir et al. 2007). Moreover, $cS5^F$ relieved cytokine-dependence and prolonged the persistence of Stat5 signaling in response to growth factors or cytokines (Harir et al. 2007; Grebien et al. 2008). Interestingly, constitutively active Stat5a-1*6 could promote senescence in fibroblasts, similar to oncogenic Ras (Mallette et al. 2007; Mallette et al. 2007). A potential role of $cS5^F$ in senescence is currently under investigation (G. Ferbeyre, personal communication).

1.5 Stat5 as target for diagnosis and treatment of hematopoietic disorders

As pointed out above, expression and activity of *Stat5* genes and proteins could be of substantial diagnostic value, e.g. in CML. Quantitative analyses of Stat5 mRNA, protein and activity levels, however, have been neglected so far although they influence the response to cytokines, growth factors or aberrant tyrosine kinases. Determination of P-Y-Stat5 activity status is no standard procedure in current clinical leukemia staging, despite the availability of suitable immuno-staining protocols with monoclonal antibodies (Nevalainen et al. 2004; Harir et al. 2007).

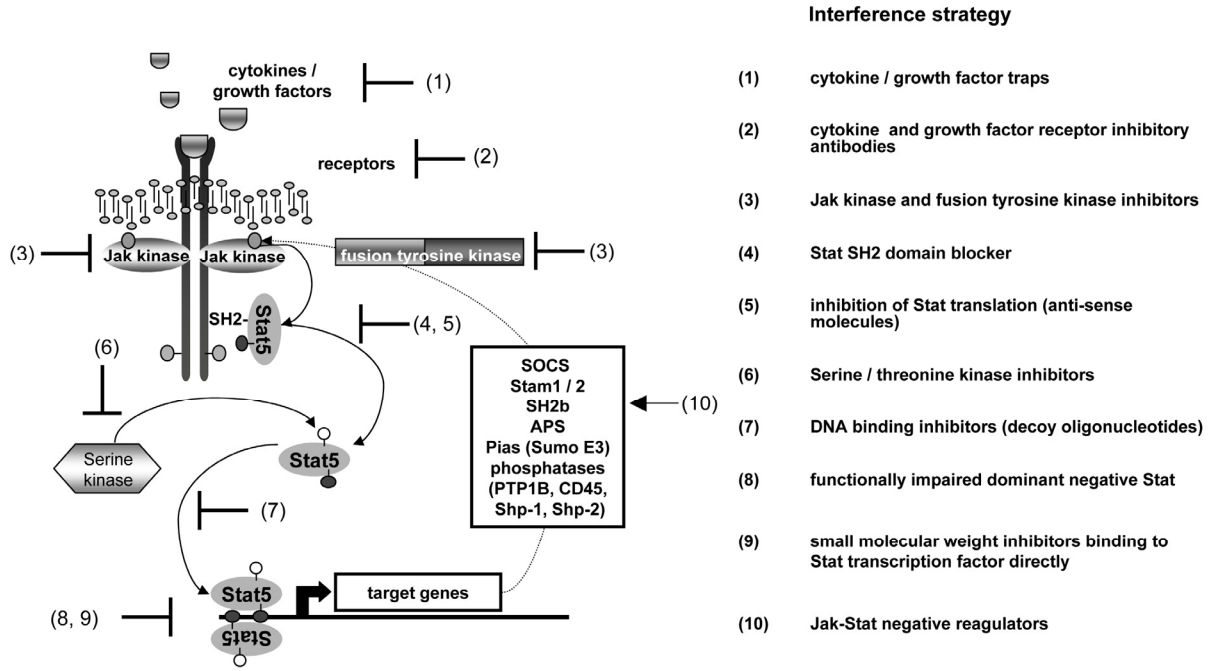

Figure 18: Strategies for targeting Stat5 for anti-leukemia therapy. The increasing understanding of the pathways leading to persistent Stat5 activation provides important insights into the potential therapeutic use of inhibitor compounds. There are several steps in the activation pathway of Stat5, which could potentially serve as direct or indirect targets for therapeutic intervention. These include **(1)** cytokine or growth factor depletion, **(2)** blocking cytokine receptors by receptor antagonists or antibodies **(3)** inhibition of Jak or fusion tyrosine kinases, **(4)** direct interference with Stat function by blocking receptor recruitment via the SH2-interaction domain **(5)** blocking Stat transcription by anti-sense molecules, **(6)** inhibition of serine kinases, **(7)** inhibition of the transcriptional activity of Stat5 by the use of small molecules that interfere with DNA binding such as decoy oligonucleotides, **(8)** functionally inactive dominant negative Stats, **(9)** small molecular weight inhibitors that bind directly to Stat transcription factors, or **(10)** targeting negative regulators of Stats.

1.5.1 Implications of Stat5 inhibition for leukemia intervention

In many manuscripts dealing with research on Stat5, discussion sections frequently end with the claim that Stat5 is a candidate for chemotherapy intervention in leukemia treatment. Indeed, there might be several steps in the activation pathway of Stat5, which could potentially serve as direct or indirect targets for therapeutic intervention (Figure 18). Unfortunately, as not to trip over the rope of oversimplification, at least three important aspects need clarification before Stat5 can be considered as a potential drug target.

First of all, Stat5 must be shown to actually drive the malignant behavior of leukemic cells. It is essential to demonstrate that Stat5 is not only sufficient for tumor initiation but also required for leukemia maintenance. As mentioned below, genetic analyses of leukemia models could help to address that issue in the future; at present no detailed studies are available.

Second, selective targeting of Stat5 proteins by pharmacological intervention must be feasible. Among the seven Stat-family members, Stat5a and b are the two most closely related; differences are restricted to the C-terminal trans-activation domain. Thus, targeting could aim at the corresponding regions of either both or individual Stat5 proteins. ATP binding pockets of kinases are commonly smaller than transcription factor protein-protein or protein-DNA interaction surfaces. This might exacerbate targeting of the latter with small molecular weight compounds. Of note, there are several reports documenting successful inhibition of e.g. Stat3, Gli-1, or TCF-4 transcription factors with small molecules (Lauth et al. 2007). These mostly mimic amino acid surfaces and interfere with their binding properties (Tan et al. 2006; Huang et al. 2007). Like for Gli-1, micro-RNAs could also provide an alternative approach, given that limitations of RNA stability and cell-specific delivery can be overcome (Tsuda et al. 2006).

Third, inhibition of Stat5 must not exhibit deadly side effects on non-transformed cells. Unfortunately, this is not the case: Stat5-deficient mice are completely devoid of mature B-lymphocytes, NK-cells, γδ–T-cells and regulatory $CD4^+CD25^+$ $FoxP3^+$ T-cells. While the loss of mature B- and NK- cells leads to severe impairment of the immune system, loss of regulatory T-cells results in severe autoimmune reactions from the remaining immune system. Furthermore, Stat5-deficiency results in strong impairment of fetal erythropoiesis, although adult red cell formation may be less affected. Together, there might be a therapeutic window for use of Stat5 inhibitors but care will be needed to determine to which extent Stat5 activity may be reduced for tackling leukemia without causing unacceptable side affects.

Lets consider the best-studied example, which would be CML and the therapeutic potential of targeting Stat5 in this particular disease. The sensitivity of CML to the Bcr-Abl tyrosine kinase inhibitors used in conventional chemotherapy is due to Bcr-Abl oncogene addiction (Weinstein 2002). Interestingly, Pimozide, a known chemotherapeutic agent, was identified to block P-Y-Stat5 activity in synergy with Bcr-Abl kinase inhibitors (Nelson et al., Abstract 2953, ASH meeting, Atlanta 2007). The detailed mechanism of Pimozide action, however, remains puzzling. Ongoing research in animal models tries to resolve the question whether Jak2 and/or Stat5 are only required for the initial transformation phase of Bcr-Abl induced CML or if these molecules are also needed for the maintenance of fully progressed CML. These considerations entail genetic evaluations of the therapeutic potential of targeting Stat5a/b proteins. Technically, these studies should be based on mice bearing floxed Stat5 or floxed Jak2 alleles intercrossed with mice carrying inducible Cre recombinase. Analysis of the disease phenotype of CML mouse models after Cre-mediated deletion of Stat5 or Jak2 might impact on decisions for future therapeutic intervention strategies converging on the Jak-Stat pathway.

2 Aims of this work

The principal objective of this thesis was to scrutinize the functions of Stat5 in erythropoiesis, lymphopoiesis and leukemia. As outlined in the introduction, the prominent transcription factor Stat5 can be activated by a plethora of cytokines and growth factors, but most of the previous work concerning the consequences of Stat5 ablation in hematopoiesis was performed using genetically modified mice retaining a hypomorphic Stat5 allele, still expressing N-terminally truncated Stat5 and therefore presenting incomplete cellular and organismal phenotypes. My thesis contributed to the clarification of Stat5 function in erythropoiesis, lymphopoiesis and leukemia by characterizing the hematopoietic phenotypes of mice completely devoid of Stat5 at the genetic, cell biological and molecular level.

This thesis comprises four core pieces of work:

(i) Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1

(published in ***Blood,*** 2008 August; Epub ahead of print)

(ii) Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2

(published in ***Blood,*** 2008 May 1;111(9):4511-22)

(iii) Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation

(published in ***Blood,*** 2006 Jun 15;107(12):4898-906)

(iv) Nonredundant roles for Stat5a/b in directly regulating Foxp3

(published in ***Blood,*** 2007 May 15;109(10):4368-75)

*[For a detailed list of all publications please see the **Results** section]*

(i) Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1

Marc A. Kerenyi; Florian Grebien; Helmuth Gehart; Manfred Schifrer; Matthias Artaker; Boris Kovacic; Hartmut Beug; Richard Moriggl and Ernst W. Müllner

(published in ***Blood,*** 2008 August; Epub ahead of print)

This publication describes for the first time a detailed characterization of the erythroid phenotype of mice completely devoid of Stat5. Analysis of these mice revealed that these animals suffer from hypochromic microcytic anemia due to reduced expression of the anti-apoptotic proteins Bcl-x_L and Mcl-1 resulting in increased apoptosis. Further more we demonstrate that cellular iron uptake was compromised since transferrin receptor-1 (TfR-1) cell surface levels were decreased >2-fold on erythroid cells of $Stat5^{-/-}$ animals. This reduction was attributed to reduced transcription of TfR-1 mRNA and diminished expression of iron regulatory protein 2 (IRP-2), the major regulator of TfR-1 mRNA stability in erythroid cells. Both genes were demonstrated to be direct transcriptional targets of Stat5. This established an unexpected mechanistic link between EpoR/Jak/Stat signaling and iron metabolism, processes absolutely essential for erythropoiesis and life.

(ii) Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2

Florian Grebien, **Marc A. Kerenyi**, Boris Kovacic, Thomas Kolbe, Verena Becker, Helmut Dolznig, Klaus Pfeffer, Ursula Klingmüller, Mathias Müller, Hartmut Beug, Ernst W. Müllner and Richard Moriggl

(published in ***Blood,*** 2008 May 1;111(9):4511-22)

This publication demonstrates for the first time that activated Stat5 is sufficient to enable erythropoiesis in Jak2- EpoR- deficient erythroid cells. Using a constitutive active mutant of Stat5a (cS5) we show that persistent activation of Stat5a substitutes for external Epo signaling in WT, $Jak2^{-/-}$ and $EpoR^{-/-}$ cells. Moreover, $Jak2^{-/-}$ fetal liver cells expressing cS5 contributed to erythropoiesis *in vivo* upon transplantation up to 6 month. Additionally we outlined that Jak2 plays a role in SCF-induced c-Kit signaling and that cS5 can be activated via c-Kit in erythroid cells.

(iii) Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation

Andrea Hoelbl, Boris Kovacic, **Marc A. Kerenyi**, Olivia Simma, Wolfgang Warsch, Yongzhi Cui, Hartmut Beug, Lothar Hennighausen, Richard Moriggl, and Veronika Sexl

(published in ***Blood,*** 2006 Jun 15;107(12):4898-906)

This piece of work demonstrates the essential function of Stat5 in lymphopoiesis. We analyzed and compared lymphoid compartments of $Stat5^{-/-}$, $Stat5^{\Delta N/\Delta N}$ and wt mice. $Stat5^{-/-}$ as well as $Stat5^{fl/fl}$ lck-cre transgenic animals mice nearly lacked all $CD8^+$ T lymphocytes in contrast to $Stat5^{\Delta N/\Delta N}$ mice. While γδ T-cell receptor–positive cells were expressed at normal levels in $Stat5^{\Delta N/\Delta N}$ mice, they were completely absent in $Stat5^{-/-}$ animals. Moreover, B-cell maturation was abrogated at the pre–pro-B-cell stage in $Stat5^{-/-}$ mice, whereas $Stat5^{\Delta N/\Delta N}$ B-lymphoid cells developed to pro-B-cell stage. Most strikingly, however was the observation that $Stat5^{-/-}$ cells were resistant to leukemic transformation in methylcellulose assays as well as to leukemia development *in vivo*, induced by Abelson oncogenes.

(iv) Nonredundant roles for Stat5a/b in directly regulating Foxp3

Zhengju Yao, Yuka Kanno, **Marc A. Kerenyi**, Geoffrey Stephens, Lydia Duran, Wendy T. Watford, Arian Laurence, Gertraud W. Robinson, Ethan M. Shevach, Richard Moriggl, Lothar Hennighausen, Changyou Wu and John J. O'Shea

(published in ***Blood,*** 2007 May 15;109(10):4368-75)

This work demonstrates that Stat5 is strictly required for regulatory T-cell (T_{reg}) development. Regulatory T-cells are pivotal to confine immune responses. The importance of Stat5 versus Stat3 in T_{reg} development was unclear. Using mice bearing a complete deletion of the Stat5a/b locus ($Stat5^{-/-}$) we observed a dramatic reduction in $CD4^+CD25^+Foxp3^+$ regulatory T-cells. Furthermore we could show that Stat5 is required for induction of Foxp3 expression and bound directly to the Foxp3 gene *in vivo*. Conversely, reduction of Stat3 in T-cells did not alter the numbers of regulatory T-cells in the thymus or spleen. Interestingly, we discovered that Stat3 was required for IL-6–dependent down-regulation of Foxp3. Therefore, we concluded that Stat5 has an essential role in regulating regulatory T-cells and that Stat3 and Stat5 appear to have opposing roles in the regulation of Foxp3.

3 Results

Publications are sorted in chronologically descending order from 2008 - 2006

3.1 Original articles

3.1.1 Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1

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blood

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Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1

Marc A Kerenyi, Florian Grebien, Helmuth Gehart, Manfred Schifrer, Matthias Artaker, Boris Kovacic, Hartmut Beug, Richard Moriggl and Ernst W Mullner

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Stat 5 Regulates Cellular Iron Uptake of Erythroid Cells via IRP-2 and TfR-1

Running title: Stat5 regulates iron uptake into erythroid cells

Marc A. Kerenyi[1#]; Florian Grebien[1#*]; Helmuth Gehart[1]; Manfred Schifrer[1]; Matthias Artaker[1]; Boris Kovacic[2]; Hartmut Beug[2]; Richard Moriggl[3] and Ernst W. Müllner[1&]

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Keywords: Stat5, erythropoietin, iron, apoptosis, iron regulatory protein, transferrin receptor

Abbreviations: Bcl-x_L, B-cell leukemia/lymphoma-x_L; Epo, erythropoietin; EpoR, erythropoietin receptor; Hct, hematocrit; Hgb, hemoglobin content; IRP-1&2, iron regulatory protein 1&2; Jak2, Janus kinase 2; MCH, mean corpuscular hemoglobin; Mcl-1, myeloid cell leukemia-1; MCV, mean corpuscular volume; qPCR, quantitative PCR; RBC; red blood cell count; Stat5, signal transducer and activator of transcription 5; TfR-1, transferrin receptor 1;

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Abstract

Erythropoiesis strictly depends on signal transduction through the erythropoietin receptor (EpoR) – Janus kinase 2 (Jak2) – signal transducer and activator of transcription 5 (Stat5) axis, regulating proliferation, differentiation and survival. The exact role of the transcription factor Stat5 in erythropoiesis remained puzzling, however, since the first Stat5-deficient mice carried a hypomorphic *Stat5* allele, impeding full phenotypical analysis. Using mice completely lacking *Stat5* – displaying early lethality – we demonstrate that these animals suffer from microcytic anemia due to reduced expression of the anti-apoptotic proteins Bcl-x_L and Mcl-1 followed by enhanced apoptosis. Moreover, transferrin receptor-1 (TfR-1) cell surface levels on erythroid cells were >2-fold decreased on erythroid cells of $Stat5^{-/-}$ animals. This reduction could be attributed to reduced transcription of TfR-1 mRNA and iron regulatory protein 2 (IRP-2), the major translational regulator of TfR-1 mRNA stability in erythroid cells. Both genes were demonstrated to be direct transcriptional targets of Stat5. This establishes an unexpected mechanistic link between EpoR/Jak/Stat signaling and iron metabolism, processes absolutely essential for erythropoiesis and life.

Introduction

Erythroid cell formation needs to be tightly regulated to maintain proper tissue oxygenation. Although in humans, about 10^{11} red cells are produced every day, total red cell numbers are kept within a narrow range in bone marrow, spleen and fetal liver. While early erythroid lineage commitment is controlled by numerous transcription factors and their binding partners (like GATA-1, FOG-1 and EKLF-1)[1], later stage differentiation from erythroblasts to mature erythrocytes is strictly regulated by erythropoietin (Epo)[2].

Epo induces dimerization of erythropoietin receptor (EpoR), which results in juxtaposition and activation of the receptor-associated Janus Kinase 2 (Jak2). Jak2 subsequently phosphorylates several tyrosine residues in the cytoplasmic tail of EpoR, which in turn acts as docking sites for signaling molecules such as PI3-K[3], MAPK[4], PKC[5] and PLC-gamma[6]. A central pathway of EpoR signaling is the activation of the transcription factor Signal Transducer and Activator of Transcription 5 (Stat5)[7,8]. Upon EpoR phosphorylation, Stat5 molecules are tyrosine-phosphorylated by Jak2 and translocate to the nucleus. This leads to activation of Stat5 target genes such as *Pim-1, c-myc, Bcl-x_L, D-type cyclins, oncostatin M* and *SOCS* members that play important roles in differentiation, proliferation, apoptosis and other processes[9-15].

The importance of Epo signaling is evidenced by the phenotypes of $Epo^{-/-}$, $EpoR^{-/-}$ and $Jak2^{-/-}$ mice, which die *in utero* at embryonic day E13.5 due to defects in erythropoiesis. Fetal livers of $Jak2^{-/-}$ animals completely lack BFU-E (burst forming unit-erythroid) and CFU-E (colony forming unit-erythroid) progenitors. In line with this, Epo- and EpoR-deficient embryos also fail to develop mature erythroid cells *in vivo*[16-18]. Interestingly, mice expressing a truncated form of EpoR ($EpoR_H$), which solely activates Stat5 and lacks all critical phosphotyrosine sites required to activate other signaling pathways, exhibited no strong erythroid phenotype[15,19], suggesting Stat5 as a critical component of the EpoR signaling pathway. Un-

expectedly, however, mice expressing an EpoR mutant additionally lacking the binding site for Stat5 ($EpoR_{HM}$), displayed no overt erythroid phenotype except under stress conditions[15,19]. Moreover, the mice initially reported to be deficient for Stat5a and Stat5b were viable and had a surprisingly mild erythroid phenotype[7,20,21]. Although showing fetal anemia, adult animals only displayed mild defects in recovery from phenylhydrazine-induced erythrolytic stress. This was explained by increased apoptosis in early erythroid progenitors, due to lack of Stat5-induced expression of the anti-apoptotic gene *Bcl-x*$_L$[21]. Later, however, these initial Stat5 knockout animals – now referred to as $Stat5^{\Delta N/\Delta N}$ – were found to express a N-terminally truncated protein able to bind DNA and to activate a subset of Stat5 target genes[14,22-25]. In contrast to $Stat5^{\Delta N/\Delta N}$, the recently described *bona fide* Stat5a/b null knockout mice (referred to as $Stat5^{-/-}$)[8] die during gestation or at latest (<2%) shortly after birth. Complete ablation of Stat5 resulted in severe defects in generation of all lymphoid lineages[23,24,26]. However, an accurate analysis of the erythroid compartment from these mice is still missing.

Maturing erythroid progenitors depend on large amounts of bio-available iron (humans require 20mg iron complexed to transferrin per day) to enable efficient heme synthesis. Cellular uptake of iron-loaded transferrin is mediated by transferrin receptor-1 (TfR-1, also called CD71)[27], followed by receptor internalization. Accordingly, maturing erythroid cells express high levels of TfR-1. However, as excess iron would lead to oxidative damage, expression of proteins involved in iron uptake, storage and utilization is tightly controlled. In case of TfR-1, this occurs primarily at the level of TfR-1 mRNA stability[28] and to a lesser extent at the transcriptional level[29]. At low iron conditions, trans-acting iron regulatory proteins (IRP1+2) bind to conserved hairpin structures (iron responsive elements; IREs) in the 3´-untranslated region (UTR) of TfR-1 mRNA, which selectively stabilizes this mRNA and ensures proper TfR-1 cell surface expression and iron uptake[30-32]. Upon iron excess, IRP-1 is converted to a cytosolic aconitase catalyzing isomerization of citrate to isocitrate[33] while IRP-2 is degraded by the proteasome[34]. Thus, both proteins no longer bind to IREs, resulting in

strongly reduced TfR-1 mRNA stability which leads to reduced TfR-1 cell surface expression and iron uptake[35-37]. Recently generated knockout mouse models for IRP-1 and IRP-2 suggested IRP-2 as the master regulator of IRE-regulated mRNAs, as ablation of IRP-2 led to a decrease in TfR-1 expression and microcytic anemia while IRP-1 knockout animals had no overt phenotype[38,39].

Here we show that Stat5$^{-/-}$ embryos suffer from severe microcytic anemia, a disease mostly associated with iron deficiency and characterized by small-sized red blood cells. In Stat5-deficent animals this anemia had two causes: Firstly, fetal livers were much smaller in knockout embryos, due to a strong increase of apoptosis in the erythron. We demonstrate that the anti-apoptotic proteins Mcl-1 and Bcl-x_L were largely absent in Stat5$^{-/-}$ cells, but ectopic expression of Mcl-1 complemented the survival defect of Stat5$^{-/-}$ erythroid cells. Secondly and more importantly, we demonstrate for the first time a direct link between Stat5 and iron metabolism. In the absence of Stat5, IRP-2 expression was strongly decreased, resulting in >2-fold lower cell surface expression of TfR-1 and thus strongly reduced iron uptake in erythroid cells. Together, the high levels of apoptosis and impaired iron uptake caused severe microcytic anemia and probably contributed to the death of Stat5$^{-/-}$ embryos.

Materials and Methods

Cell culture and retroviral infections

Stat5$^{+/-}$ mice[8] were maintained under pathogen-free conditions and bred on a mixed background (C57Bl/6xSv129F1) to obtain Stat5$^{-/-}$ embryos. For the determination of blood indices heparinized blood was measured in a Vet animal blood counter (Scil Animal Care). Serum Epo-levels were determined using a *Quantikine* mouse-erythropoietin ELISA-kit (R&D Systems) according to the manufactures protocol, measured on a Victor^{3}V 1420 multilabel counter (Perkin Elmer). All animal experiments described in our manuscript were performed in accordance with Austrian and European laws and under approval of the ethical and animal protection committees of the Veterinary University of Vienna.

Fetal livers of E13.5 mouse embryos (Stat5$^{-/-}$ and wild type; =wt) were resuspended in serum-free medium (StemPro-34^{TM}, Invitrogen). In brief, for self-renewal[40], cells were seeded into medium containing 2U/mL human recombinant Epo (Erypo, Cilag AG), murine recombinant stem cell factor (SCF; 100ng/mL, R&D Systems), 10^{-6}M dexamethasone (Dex, Sigma), and insulin-like growth factor 1 (IGF-1; 40ng/mL, Promega). The resulting erythroblast cultures were expanded by daily partial medium changes and addition of fresh factors, keeping cell densities between 2-4x10^6 cells/mL. Proliferation kinetics and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System)[40]. For retroviral infections, fetal liver erythroblasts were co-cultured for 72h with retrovirus-producing fibroblast cell lines selected for high virus production[14]. Infection efficiency was 75-95%, as measured by flow cytometry for GFP expression. Photomicrographs were taken using an Axiovert 10 microscope (Zeiss) equipped with a 63x oil-immersion objective lens (numerical aperture 44-07-61). Images are presented at 630x magnification.

Flow cytometry

Cultured erythroblasts or single cell suspensions of freshly isolated fetal livers were stained with fluorescence-conjugated antibodies (all Becton Dickinson Biosciences; =BD) against Ter-119 (PE-conjugated) and TfR-1 (biotinylated) for *in vivo* erythroid development analyses. Annexin V (APC-conjugated) staining was performed according to the manufacturers' instructions. For *in vivo* proliferation assays, pregnant mice were injected with 80mg 5-bromo-2-deoxyuridine (BrdU) per kg body mass. After 1h, embryos were isolated, fetal liver cells fixed, and stained with anti-BrdU-FITC plus Ter119-PE, following the manufacturer's protocol (BrdU flow kit, BD). Samples were analyzed on a FACS-Calibur flow cytometer (BD). Where indicated, $Ter119^{+}$ cells were isolated by magnetic cell sorting using AutoMACS (Miltenyi Biotech).

Western blot analysis

Antibodies used for Western blotting: anti-mouse ERK1/2, anti-horse ferritin H, anti-mouse actin (all Sigma), anti-mouse TfR-1 (BioSource), anti-mouse Bcl-$x_{L,}$ anti-mouse PCNA, anti-mouse Stat5 (all BD Transduction Laboratories), anti-mouse Mcl-1 (Abcam), anti-mouse eIF4E, anti-mouse eIF2-alpha , pSer-eIF2-alpha (all Cell Signaling), anti-rat IRP-1[41], anti IRP-2[39].

Chromatin immuno-precipitation

Chromatin immunoprecipitation was performed as in[42]. $2x10^{7}$ primary erythroblasts were stimulated with 10U Epo/mL for 30min. Cells were fixed with 1% formaldehyde for 30min. DNA was sonicated using a Bandelin Sonoplus GM70 sonicator (cycle count 30%; power 45%; 6x30 sec). DNA fragments were recovered using anti-mouse Stat5ab (C20, Santa Cruz) or anti-mouse Stat5 (N20, Santa Cruz). Recovered DNA fragments were directly used for PCR analysis.

Quantitative Real Time PCR

RNA was isolated using RNeasy Mini Kit (Quiagen). RNA integrity was checked with the Agilent Bioanalyzer (Agilent). 2.5 µg RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Real Time PCR was performed on an Eppendorf Mastercycler RealPlex using RealMasterMix (Eppendorf) and SYBR Green. Results were quantified using the "Delta Delta C(T) method"[43].

Statistical analyses

Statistical analyses were performed using Excel 2004 (Microsoft). Student's t-test was used to calculate p-values (two-tailed). p-values: $p \leq 0.05$, one asterisk; $p \leq 0.01$, two asterisks. Data are presented as mean values ± standard deviation.

Results

Stat5$^{-/-}$ embryos are severely anemic

Embryos lacking the entire *Stat5* locus (i.e. *Stat5a/b*) die during gestation or at latest perinatally (~99%) with severe defects in diverse hematopoietic lineages[23,24]. The previously demonstrated pivotal role of Stat5 in Epo-signaling[19-22] prompted us to analyze the function of Stat5 in erythropoiesis in detail. E13.5 Stat5$^{-/-}$ embryos and newborn Stat5$^{-/-}$ animals appeared paler than their wt littermates, particularly in the fetal liver region (Figure 1A). The relative abundance of erythroid cells in Stat5$^{-/-}$ fetal livers (~80% of all cells) remained unchanged, as determined by staining for the pan-erythroid marker Ter119 followed by flow cytometry (Figure 1B, left). Yet, the size of Stat5$^{-/-}$ fetal livers in E13.5 was visibly reduced and total fetal liver cellularity was decreased by 50% (n=6), corresponding to a similar reduction in the total number of erythroid cells (Figure 1B, right). Since anemia causes elevation of Epo levels[44] to counteract hypoxia, sera from E16.5 or newborn wt and Stat5$^{-/-}$ animals were analyzed for Epo levels. These were highly elevated in Stat5$^{-/-}$ versus wt embryos (3.8±0.6-fold; n=5), newborn animals showed an even higher elevation (35.2±5.1-fold; n=5; Figure 1C). This strongly suggested that Stat5-deficient animals suffer from severe anemia. To determine the specific type of anemia, blood from E16.5 wt versus Stat5$^{-/-}$ animals was analyzed (Table 1). Red blood cell counts of E16.5 Stat5$^{-/-}$ embryos was lowered to 1,0±0.3x10^6/mm^3, in contrast to 2,4±0,4x10^6/mm^3 in wt embryos. In line with this, hematocrit (Hct) of E16.5 Stat5$^{-/-}$ embryos was reduced to 9,8±0,7% as compared to 31.4±1,8% in wt embryos. Likewise, mean corpuscular volume (MCV), hemoglobin content (Hgb) and mean corpuscular hemoglobin (MCH) of E16.5 Stat5$^{-/-}$ blood was strongly reduced. These effects also were clearly visible in blood smears, showing hypochromic microcytic erythrocytes (Supplementary Figure 1A+B).

An additional cause for early lethality and high serum Epo levels could have been a lung defect leading to reduction in red cell oxygenation. Analysis of tissue sections from wt

and Stat5$^{-/-}$ newborn animals, however, did not reveal any histological differences (not shown). Taken together, Stat5$^{-/-}$ mice suffer from microcytic anemia.

Loss of Stat5 causes enhanced apoptosis in the fetal liver

Hypomorphic Stat5$^{\Delta N/\Delta N}$ mice displayed enhanced erythroid cell death, attributed to reduced expression of the anti-apoptotic protein Bcl-x_L[20,21]. To determine apoptosis in Stat5$^{-/-}$ mice, freshly isolated fetal liver cells were stained for Ter119 and apoptosis assessed by Annexin V staining. In Stat5$^{-/-}$ embryos, the frequency of Annexin V-positive cells was >2-fold enhanced, regardless of developmental stage (Figure 2A). In line with this, Stat5$^{-/-}$ fetal liver cells showed a >6-fold reduction of erythroid colony numbers in CFU-E assays irrespective of Epo concentrations, supporting the notion that lack of functional Stat5 reduces cell survival (Figure 2B). In contrast, no significant alterations were observed in BFU-E assays (Supplementary Figure 1C).

To assess potential differences in viability of Stat5-deficient erythroid cells in an adult (bone marrow) versus an embryonic (fetal liver) microenvironment, short term transplantation experiments were performed. Equal amounts of sorted GFP-expressing wt- or Stat5$^{-/-}$ pro-erythroblasts cultured from fetal livers (c-Kit^{+}/TfR-1^{high}/Ter119^{-} cells[15]) were injected into lethally irradiated wt recipients (Figure 2C). This setup was chosen to circumvent any influence of the well-known re-population defect of Stat5-deficient hematopoietic stem cells[45-47]. Three days after transplantation, bone marrow cells were harvested and scored for GFP-positive mature Ter119^{+} erythroid cells, as previously reported[48]. In line with the preceding experiments, a 5-fold reduction in abundance of transplanted mature Stat5$^{-/-}$ versus wt cells was determined in the adult microenvironment (bone marrow; Figure 2D). Moreover, these data indicated a cell-autonomous survival defect of Stat5$^{-/-}$ erythroid cells.

Stat5-deficiency leads to reduced expression of anti-apoptotic proteins

Consistent with the apoptotic phenotype described above, $Ter119^+$ cells from $Stat5^{-/-}$ fetal livers showed decreased levels of Bcl-x_L as compared to wt cells (Figure 3A, left). Nevertheless, Bcl-x_L protein levels in $Stat5^{-/-}$ cells were still ~50% of wt. This prompted us to analyze the expression of other anti-apoptotic *Bcl-2* family members. We focused on Mcl-1 for three reasons: (i) Mcl-1 is up-regulated during early erythroid commitment in human cells[49], (ii) its bone marrow-specific ablation reduces blood formation[50] and (iii) it appears to be regulated by Stat5[51-53]. Indeed, Mcl-1 protein and mRNA expression were drastically reduced in Stat5-deficient cells (Figure 3A, right; Figure 3B). To assess if *Mcl-1* is an Epo-inducible Stat5-regulated gene, primary wt and $Stat5^{-/-}$ erythroblasts were factor-deprived for 2h and subsequently re-stimulated with Epo for 30min. Quantitative PCR (qPCR) revealed a 3.5-fold increase in Mcl-1 mRNA in Epo-stimulated cells which was abrogated in Stat5-deficient cells (Figure 3C).

To test if exogenous Mcl-1 provides protection against apoptosis to erythroid cells, primary wt or $Stat5^{-/-}$ fetal liver cells were transduced with retroviral constructs encoding *GFP*, *Bcl-x_L* or *Mcl-1*. Erythroblasts were cultivated for 48h in the presence or absence of Epo and apoptosis was determined by flow cytometry. Ectopic expression of either Mcl-1 or Bcl-x_L completely prevented apoptosis of wt as well as Stat5 knockout erythroblasts upon Epo withdrawal (Figure 3D).

The decrease of fetal liver size and cellularity in $Stat5^{-/-}$ embryos could also have been due to reduced proliferation of erythroid cells, as suggested by the known ability of Stat5 to enhance expression of proliferation-promoting genes such as *c-Myc, Cyclin D2* and *D3 or oncostatin M*[14,23,54,55]. Cell division kinetics of erythroid cells *in vivo* and *in vitro*, however, were similar in $Stat5^{-/-}$ and wt cells (Supplementary Figure 2, Supplementary Text).

Taken together, Sta5-deficient fetal liver erythroid cells were massively apoptotic. This effect could be attributed to reduction of Bcl-x_L levels together with complete loss of Mcl-1, translating into massive decrease of fetal liver cellularity.

TfR-1 expression is strongly reduced in Stat5$^{-/-}$ erythroid cells

To analyze if Stat5-deficient mice had a defect in erythroid lineage commitment, wt and Stat5$^{-/-}$ fetal livers were analyzed for the presence of Megakaryocytic-Erythroid Progenitors[56,57] (MEP), the first erythroid-committed progenitor detectable by flow cytometry. Interestingly, we observed a two-fold increase of the MEP compartment in Stat5-deficient fetal livers (Supplementary Figure 3), suggesting a compensatory attempt to counteract the increased erythroid cell death during definitive erythropoiesis.

To determine whether the anemia in Stat5$^{-/-}$ embryos was due to a defect in erythroid differentiation, fetal liver cells were analyzed for erythroid markers Ter119 and TfR-1. This combination allows staging of maturing erythroid cells from immature progenitors (TfR-1^{low} Ter119^{low}) over an intermediate stage (TfR-1^{high} Ter119^{high}) to late orthochromatophilic erythroblasts (TfR-1^{neg} Ter119^{high}; Figure 4A, left; gates R1 to R5, increasing maturity[58]). Stat5-deficient and wt fetal livers contained cells of all differentiation stages at indistinguishable frequencies (Figure 4A). For detailed morphological analysis, wt and Stat5$^{-/-}$ fetal liver cells were sorted according to their cell surface marker phenotype (R2-R5), spun onto glass slides and subsequently stained with either May-Grunwald Giemsa or Benzidine-Wright Giemsa (Supplementary Figure 4). No apparent morphological differences in maturity between wt and Stat5$^{-/-}$ cells were observed. Thus, the reduction in fetal liver cellularity (Figure 1B) was probably not due to differentiation arrest at a distinct step of maturation. We did, however, observe a reproducible decrease in TfR-1 cell surface expression, which prompted us to align the gating strategy accordingly.

Accumulation of hemoglobin is the hallmark of terminal erythropoiesis, requiring an enormous up-regulation of iron intake via increased expression of TfR-1. Quantification of TfR-1 levels in Stat5-deficicient versus wt cells by flow cytometry revealed a >2-fold reduction in knockout cells (Figure 4B). This was confirmed at the mRNA level (Figure 4C) and further corroborated by Western blot analysis of wt, $Stat5^{+/-}$ and $Stat5^{-/-}$ fetal liver cell lysates (Figure 4D+E). A recent report described functional Stat5 binding sites (GAS elements) in the first intron of the TfR-1 gene, using an erythroleukemic cell line expressing a constitutively active Stat5 variant [59]. To corroborate these data, we decided to analyze DNA binding of endogenous Stat5 to these elements in primary fetal liver erythroblasts after Epo-stimulation by chromatin immunoprecipitation (Figure 4F). Indeed, DNA binding of Stat5 to all three sites analyzed was confirmed and apparently resulted in an Epo-induced increase of TfR-1 mRNA as quantified by qPCR (Figure 4G). As expected from the well-known inverse relation in expression of TfR-1 to the iron-storage protein ferritin[30], $Stat5^{-/-}$ cells showed elevated levels of ferritin protein (Supplementary Figure 5).

As a direct consequence of reduced TfR-1 cell surface expression, we observed a significant reduction of intracellular iron (~40%) in freshly isolated $Stat5^{-/-}$ fetal livers as measured by atomic absorption spectrometry (Figure 4H), further supporting the idea of altered iron metabolism in $Stat5^{-/-}$ cells. Reduced iron availability leads to a drop in heme synthesis[60], known to result in activation of heme-regulated inhibitor (HRI)[61]. This kinase, via inactivation of translation initiation factor eIF2-alpha, throttles expression of globins to ensure that heme, alpha- and beta-globin are always synthesized at the appropriate ratio of 4:2:2[61]. To test if this regulatory circuit was disturbed in $Stat5^{-/-}$ erythroid cells, abundance of globin mRNAs in lysates from erythroid cells sorted out of wt or $Stat5^{-/-}$ fetal livers were analyzed by qPCR. Indeed, relative levels of both globin mRNAs were significantly reduced in $Stat5^{-/-}$ cells; moreover, eIF2-alpha showed the expected increase in phosphorylation (Supplementary Fig-

ure 6). In summary, these data demonstrated that Stat5$^{-/-}$ erythroid cells were severely iron-deficient.

IRP-2 expression and mRNA binding activity is reduced in Stat5-deficient cells

Stabilization of TfR-1 mRNA by binding of IRP-1 and IRP-2 is considered the pre-dominant mechanism to satisfy the iron demand of proliferating cells[30,31]. A possible activation of IRPs by Epo has been discussed[62,63]. Accordingly, Western blot analyses for IRP-1 and IRP-2 from lysates of wt and Stat5$^{-/-}$ primary erythroblasts revealed a striking, 5-fold down-regulation of IRP-2 in Stat5 knockout cells (Figure 5A+B, right), accompanied by a 2-fold up-regulation in IRP-1 expression. IRP-1+2 mRNA levels were similarly changed in Stat5-deficent cells (data not shown). Determination of IRP-2 RNA-binding in Stat5$^{-/-}$ cells using *in vitro* transcribed, radioactively labeled IRE probes in electrophoretic mobility shift assays (EMSA, Figure 5C) showed a similar decrease of IRP-2 activity (Figure 5D). Given the important role of IRP-2 in TfR-1 expression in erythroid cells[38,39], these data strongly suggested that the decrease of IRP-2 was an additional cause for reduction of TfR-1 cell surface expression.

IRP-2 is a direct transcriptional target of Stat5

To test a possible direct role of Stat5 in regulating *IRP-2* expression, we analyzed the *IRP-2* promoter in detail. A region 1030-1100bp upstream of the transcriptional start site contained one perfect Stat5 DNA-binding site ($TTCN_3GAA$)[64] plus two adjacent low-affinity Stat5 response elements with a mismatch in one half-site of the inverted repeat (Figure 6A). Annotated Stat5 sites[24,64] together with the IRP-2 sequence I suggested that the latter fulfilled bioinformatic criteria for perfect Stat5 binding (Figure 6B).

To test if there was a direct transcriptional induction of *IRP-2* by Stat5, the 2kb fragment of the *IRP-2* promoter upstream of the predicted transcription start site, comprising all three putative Stat5 response elements (REs), was inserted into a firefly-luciferase reporter

gene construct. 293T cells were co-transfected with Stat5a and EpoR cDNAs together with the respective reporter construct. Transfected cells were stimulated with Epo or left untreated. Epo-treated cells displayed a significant increase of luminescence over untreated controls (Figure 6C). Direct Epo-induced expression of endogenous *IRP-2* and *oncostatin M* (a bona-fide Stat5 target gene) was demonstrated in murine erythroid leukemia cells: Following 3h of factor deprivation, cells were re-stimulated for 1h with Epo and mRNA expression levels determined by qPCR. Epo stimulation induced expression of IRP-2 as well as oncostatin M about 3-fold (Figure 6D).

To further substantiate that IRP-2 is a direct target of Stat5, EMSAs were performed. 293T cells were co-transfected with constructs encoding EpoR and murine Stat5a. Transfected cells were Epo-stimulated or left untreated. Extracts were subsequently subjected to EMSAs using radiolabeled oligonucleotides encompassing either the newly identified Stat5-RE I of the IRP-2 promoter or a well-described Stat5-RE probe from the bovine β-casein promoter as positive control. For super-shifts, a serum directed against the C-terminus of Stat5 was added to the oligonucleotide/lysate mixture. Stat5-DNA complexes were clearly evident in Epo-stimulated extracts, using both, the IRP-2-I or the β-casein probe, as these complexes were readily super-shifted upon addition of anti-Stat5 serum (Figure 6E). Similar results were obtained using Epo-stimulated cells transfected with murine Stat5b (data not shown).

To test if Stat5 recognizes one of these putative DNA-binding sites *in vivo*, we finally performed chromatin-immunoprecipitation (ChIP) experiments using two different anti-sera directed against N- or C-terminal epitopes in Stat5. PCR analysis of immuno-precipititated Stat5-DNA complexes from Epo-stimulated primary wt erythroblasts yielded a PCR product representing Stat5-binding sites in the *IRP-2* promoter in both specific Stat5 ChIPs, but not in control IgG ChIP experiments (Figure 6F).

Together, these results indicated that Stat5 is directly involved in the control of *TfR-1* transcription as well as in the modulation of its mRNA stability by regulating expression of *IRP-2*.

Discussion

In this paper cooperating mechanisms underlying the erythroid defect leading to microcytic anemia in Stat5$^{-/-}$ mice were uncovered, demonstrating a novel direct link between the EpoR-Stat5 axis and regulation of iron metabolism *in vivo*. First, Stat5$^{-/-}$ fetal livers showed reduced cellularity due to massively enhanced apoptosis of maturing erythroid cells, apparently caused by defective expression of the anti-apoptotic genes *Mcl-1* and *Bcl-x$_L$*. Second, Stat5$^{-/-}$ erythroid cells exhibited reduced expression of IRP-2 and TfR-1, resulting in a large decrease of TfR-1 cell surface expression, iron uptake and globin synthesis. Together, these mechanisms appear to be sufficient to explain the severe anemia of Stat5$^{-/-}$ animals.

Complete ablation of Stat5 leads to early lethality

None of the conditional Stat5 knockout models created so far in multiple cell types such as hemangioblasts (Stat5fl/fl Tie2-Cre)[59] B-cells (CD19-Cre)[65], T-cells (CD4-Cre, Lck-Cre)[23,24], hepatocytes (albumin-Cre, albumin-alpha-fetoprotein-Cre)[25,66], pancreatic β-cells/hypothalamus (Rip-Cre)[67], endocrine/exocrine pancreas progenitors (Pdx1-Cre)[67], or skeletal-muscle (Myf5-Cre)[68] die during fetal development. In contrast, ablation of Stat5 in the entire organism resulted in mortality[8] during gestation or at latest shortly after birth. Since Epo$^{-/-}$, EpoR$^{-/-}$ and Jak2$^{-/-}$ mice all die *in utero* at E13.5 due to defects in definitive erythropoiesis and given the prominent role of Stat5 in EpoR-signaling, it was unexpected that a few Stat5$^{-/-}$ embryos developed to term.

There are several possible explanations for the discrepancy in phenotypes. First, we detected high levels of pY-Stat1 and pY-Stat3 in Stat5-deficient cultivated primary erythroblasts as well as in lysates from freshly isolated fetal livers but not in wt counterparts (MAK, FG, unpublished). This is in line with increased pY-Stat1 and pY-Stat3 levels found upon liver-specific Stat5 deletion[66,69,70]. Since Stat3 and Stat5 response elements are similar[64], increased activation of Stat3 might partially compensate for loss of Stat5. Second, the anemia of

Stat5$^{-/-}$ embryos led to a compensatory elevation of Epo levels in the serum, which was most pronounced in the few newborn animals. This might contribute to prolonged survival mediated by hyper-activation of Stat5-independent EpoR signaling. Third, Stat5-deficient erythroid cells exhibited elevated levels of phosphorylated eIF2-alpha, indicative for an active "integrated stress response" (ISR), presumably via the kinase heme-regulated inhibitor (HRI)[71]. In mouse models for the red blood cell disorders erythropoietic protoporphyria and beta-thalassemia, ablation of HRI exacerbated the phenotype of these diseases[71]. Thus, the modulation of translational efficiency to balance heme and globin production could represent another protective mechanism accounting for the "mild" erythroid phenotype of Stat5$^{-/-}$ animals. Nevertheless, the ultimate reason for the early death of the animals remains to be determined.

Stat5 is not essential for erythroid differentiation

Earlier studies addressing the role of Stat5 (i.e. Stat5a+b) in erythropoiesis were performed with *Stat5*$^{\Delta N/\Delta N}$ animals. These mice are born, viable and show only a mild erythroid phenotype[20,21]. *Stat5*$^{\Delta N/\Delta N}$ animals express a N-terminally-truncated Stat5, which still activates target genes[22]. Here we used Stat5$^{-/-}$ mice lacking the entire *Stat5a/b*-locus[8]. Animals lacking other components of Epo signaling upstream of Stat5 (Epo, EpoR or Jak2)[2], all die *in utero* around E13.5, due to a block in definitive erythropoiesis. If Stat5 was the only crucial target of this pathway, full Stat5 knockout animals should show a similarly severe phenotype. Indeed, Stat5-deficient animals display erythroid defects and die at latest after birth. Although Stat5 is essential for differentiation of other hematopoietic lineages like maturation of pre-pro- to pro-B cells[23,24], or in formation of FoxP3^{+} regulatory T-cells[26], the observed block in erythroid maturation *in vivo* was not complete. The presence of erythroid cells at all developmental stages in Stat5$^{-/-}$ embryos strongly argued against an absolutely essential function of Stat5 in erythroid development. Nevertheless, there were defects in hemoglobinization of Stat5$^{-/-}$

erythroid cells, which may have several causes. For instance, it could decrease through direct defects in the erythroid differentiation program and/or through a secondary response to iron deficiency.

Involvement of Stat5 in iron metabolism

The most striking observation in the peripheral blood morphology of Stat5$^{-/-}$ animals was an apparent microcytic hypochromic anemia. This type of anemia, characterized by decreased mean corpuscular volume and reduced mean cell hemoglobin, is frequently associated with iron deficiency. Thus it was tempting to investigate the molecular players involved.

The normal adaptive response to low iron is the well-characterized feedback regulation that increases TfR-1 mRNA stability upon binding of IRPs to its 3'-UTR (Figure 6). In Stat5$^{-/-}$ cells, this response apparently was impeded as delineated from the reduced expression of TfR-1, which in turn was the direct result of decreased IRP-2 protein levels. This mechanistic link was further substantiated by the measured reduction in total intracellular iron in Stat5$^{-/-}$ fetal livers, finally resulting in decreased globin mRNA expression. It remained unclear, however, whether a connection between Stat5 and IRP-2 expression existed. The promoter region of the *IRP-2* gene contains three adjacent potential binding sites for Stat5, which indeed turned out to be functional. Moreover, qPCR showed reduced IRP-2 mRNA abundance in the absence of Stat5. Thus, one could envision a chain of events in Stat5-deficient erythroid cells, starting with decreased IRP-2 and TfR-1 expression, resulting in a net decrease of TfR-1 mRNA stability and abundance, followed by diminished TfR-1 surface expression. The consequence would be insufficient iron uptake (even in iron-depleted cells), ultimately leading to decreased heme synthesis, activation of the integrated stress response pathway and reduced globin mRNA translation. Interestingly, no functional compensation for low IRP-2 levels by the highly homologous IRP-1 protein was observed. This is in line with *in vivo* data from corresponding IRP-1 or IRP-2 knockout animals[38,39], which indicated that IRP-2 is the predomi-

nant regulatory factor modulating TfR-1 mRNA stability. While ablation of *IRP-1* produced no overt phenotype, loss of *IRP-2* resulted in hypochromic microcytic anemia due to reduced TfR-1 expression, a phenotype reminiscent to the one of Stat5 knockout animals described here. Accordingly, lowering the expression of TfR-1 by 50% led to a similar phenotype, as TfR-$1^{+/-}$ mice also displayed the same type of anemia[74]. It should be mentioned, however, also other conditions are known to result in microcytosis, including ablation of the genuine Stat5-target Pim-1[9].

Involvement of Stats in iron metabolism might even be a more general mechanism. Hepcidin[75,76], the dominant regulator of dietary iron absorption in enterocytes and iron release from macrophages, is a direct Stat3 target gene[75,76]: Upon infection, the inflammatory cytokine IL-6 promotes hepcidin expression via Stat3, trapping iron in macrophages, resulting in decreased plasma iron concentrations. Hepcidin expression is decreased by hypoxia and anemia, directly responding to increased Epo levels[75-77]. Thus its regulation in the anemia resulting from Stat5-deficiency may be of interest in future studies.

Stat5$^{-/-}$ fetal liver cells exhibit high levels of apoptosis

In erythropoiesis, up-regulation of Bcl-x_L was found to be defective in Stat5$^{\Delta N/\Delta N}$ erythroid cells[20,21]. Other studies, however, suggested that Bcl-x_L prevents apoptosis only of late stage erythroblasts[11,78] but not directly via EpoR[78]. Upon re-addressing this question in mice that are fully devoid of Stat5, we observed a reduction in Bcl-x_L levels of about 50% in fetal liver erythroid cells. Furthermore, Mcl-1 expression in Stat5$^{-/-}$ erythroid cells was analyzed, based on the finding that this *Bcl-2* gene family member could be a Stat5 target gene[52,53,79]. Indeed, Mcl-1 was completely absent in Stat5$^{-/-}$ fetal liver cells whereas Epo-stimulation of wt primary erythroblasts led to a 3.5-fold increase of Mcl-1 mRNA. Furthermore, re-introduction of Mcl-1 or Bcl-x_L completely prevented apoptosis of wt as well as Stat5 knockout erythroblasts upon Epo withdrawal.

Besides the finding that Mcl-1 is a Stat5-dependent Epo target gene, down-regulation of Mcl-1 in Stat5$^{-/-}$ erythroid cells could also occur through an additional mechanism, which is induced through iron deficiency in Stat5 knockout animals. Recently it was suggested that Mcl-1 levels decrease after activation of the phospho-eIF2-alpha-mediated ISR pathway already mentioned above[80-82]. eIF2-alpha-phosphorylation can be induced by different kinases in response to several stress stimuli[83], including HRI. Iron deficiency activates HRI, which in turn phosphorylates eIF2-alpha on its inhibitory Ser51, resulting in global reduction of mRNA translation[61], which immediately affects Mcl-1, as it is a highly unstable protein[84]. The observed reduced iron levels, together with elevated eIF2-alpha phosphorylation in Stat5$^{-/-}$ primary erythroblasts suggested an active ISR in Stat5$^{-/-}$ cells. Hence, loss of Stat5 could lead to a direct decrease of Mcl-1 mRNA, but alternatively also to a down-regulation of Mcl-1 protein due to iron deficiency-induced ISR. Taken together, the apoptosis in Stat5$^{-/-}$ fetal livers most probably reflects a composite effect of reduced levels of Bcl-x_L and loss of Mcl-1.

This contribution should help to clarify the long-discussed role of Stat5 in erythropoiesis *in vivo*: We identify Stat5 as a key factor regulating erythroid iron metabolism *in vivo* and, additionally, link the anti-apoptotic machinery of erythroid cells with their iron uptake system.

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Author Contribution

MAK, FG, HG, MS, MA and BK conducted experiments. MAK and EWM designed experiments and interpreted results. HB contributed essential reagents and worked on the draft of the manuscript. MAK, RM and EWM wrote the paper.

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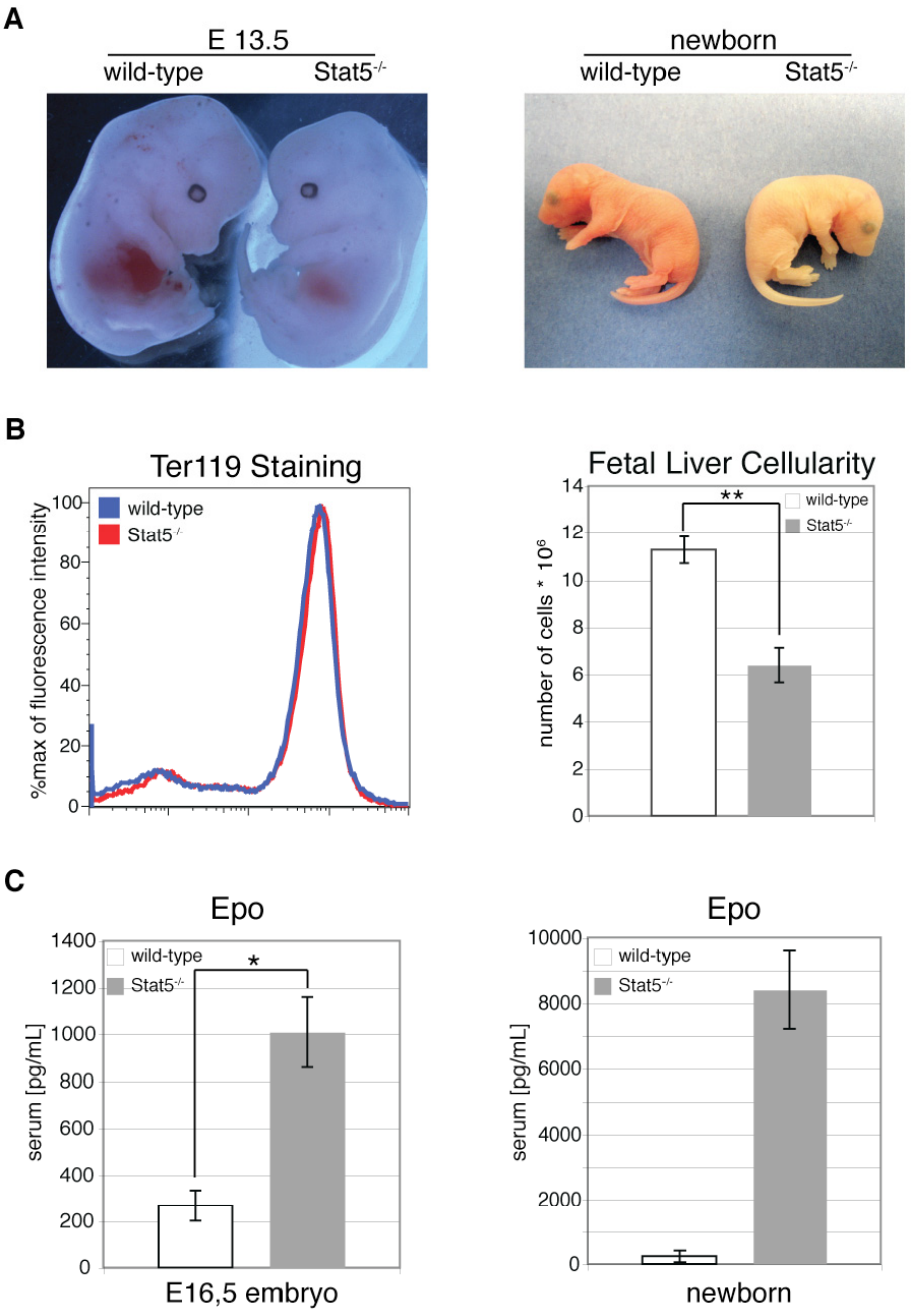
E 16.5 embryos	wild-type	Stat5-/-
RBC (10^6/mm^3)	2.4±0.4	1.0±0.3**
Hct (%)	31.4±1.8	9.8±0.7**
Hgb (g/dL)	9.1±1.2	2.3±0.7**
MCV (μm^3)	128.2±3.7	95.9±5.2**
MCH (pg)	37.5±0.3	23.1±0.7**

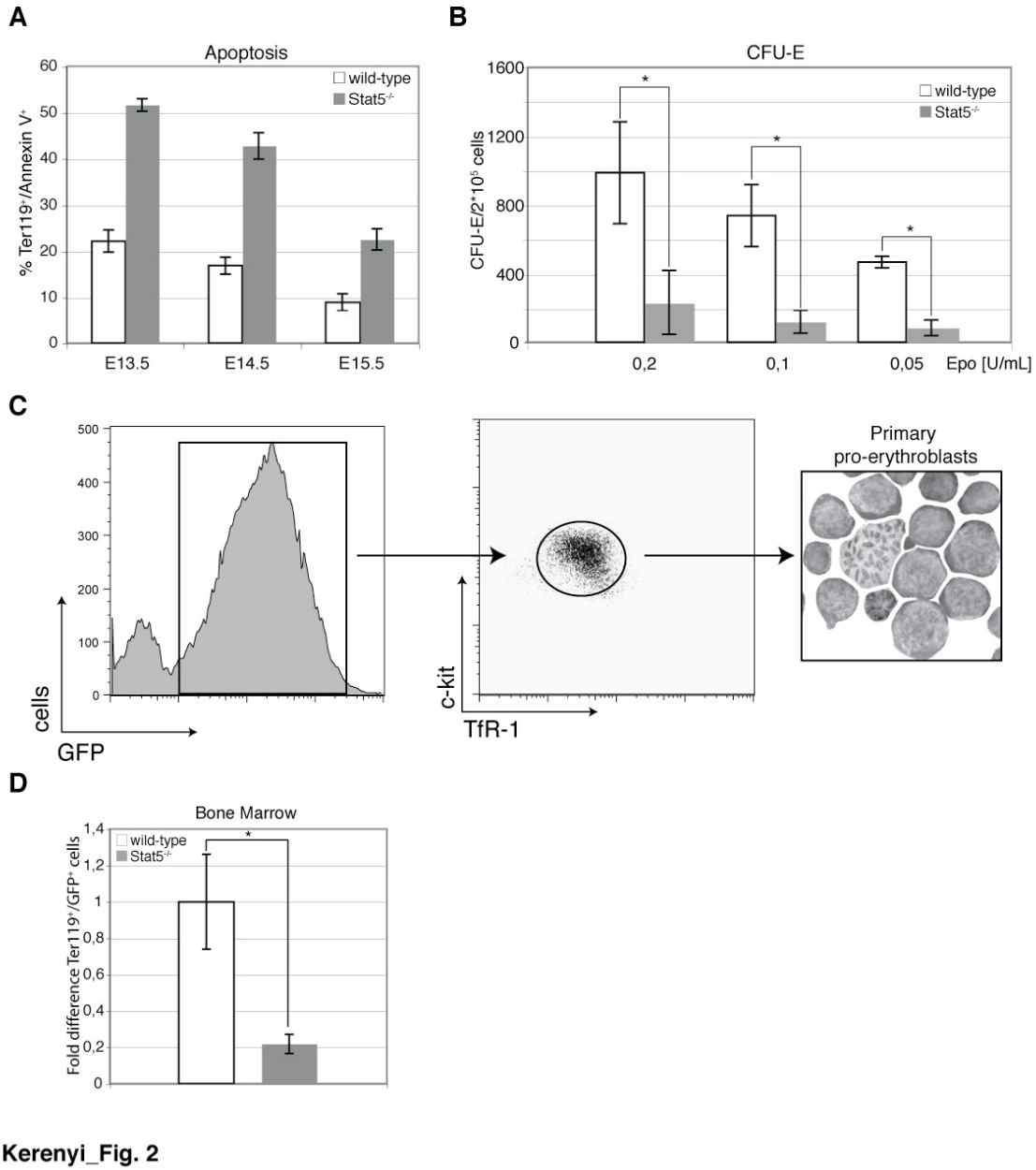
Table 1. $Stat5^{-/-}$ embryos mice display severe microcytosis. Blood indices of E16.5 $Stat5^{-/-}$ embryos (data are presented as mean ± SEM; n=15 each genotype). RBC, red blood cell count; Hct, hematocrit; Hgb, hemoglobin content; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

Figures and Figure Legends



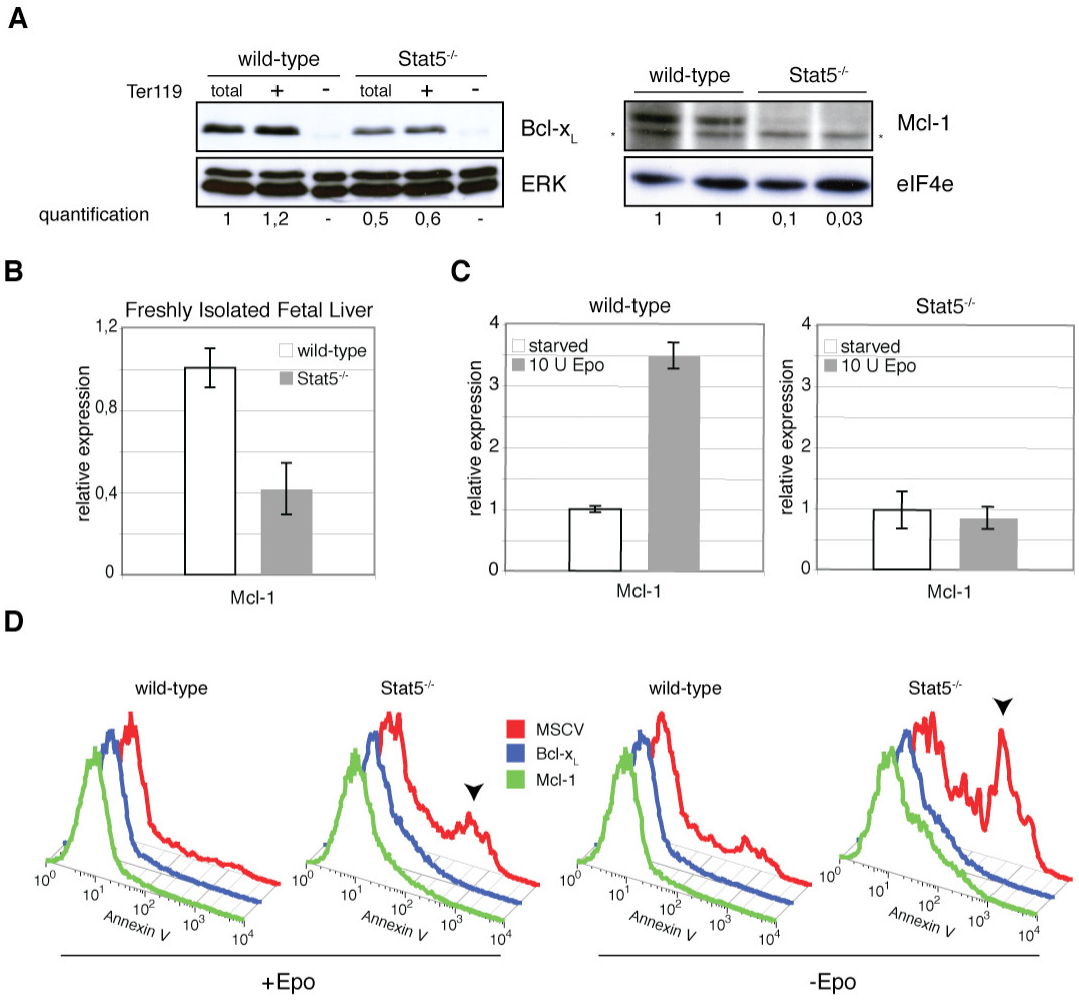
Kerenyi_Fig. 1

Figure 1. Stat5$^{-/-}$ embryos are severely anemic. (A) Wt and Stat5$^{-/-}$ E13.5 embryos (left) and newborn animals (right). (B) Ter119-positive erythroid cells (left) and fetal liver cellularity (right; data are presented as mean ± SD; n=6) of wt versus Stat5$^{-/-}$ fetal livers. (C) ELISA for Epo from serum of wt and knockout E16.5 embryos and newborns (data are presented as mean ± SD; n=5).



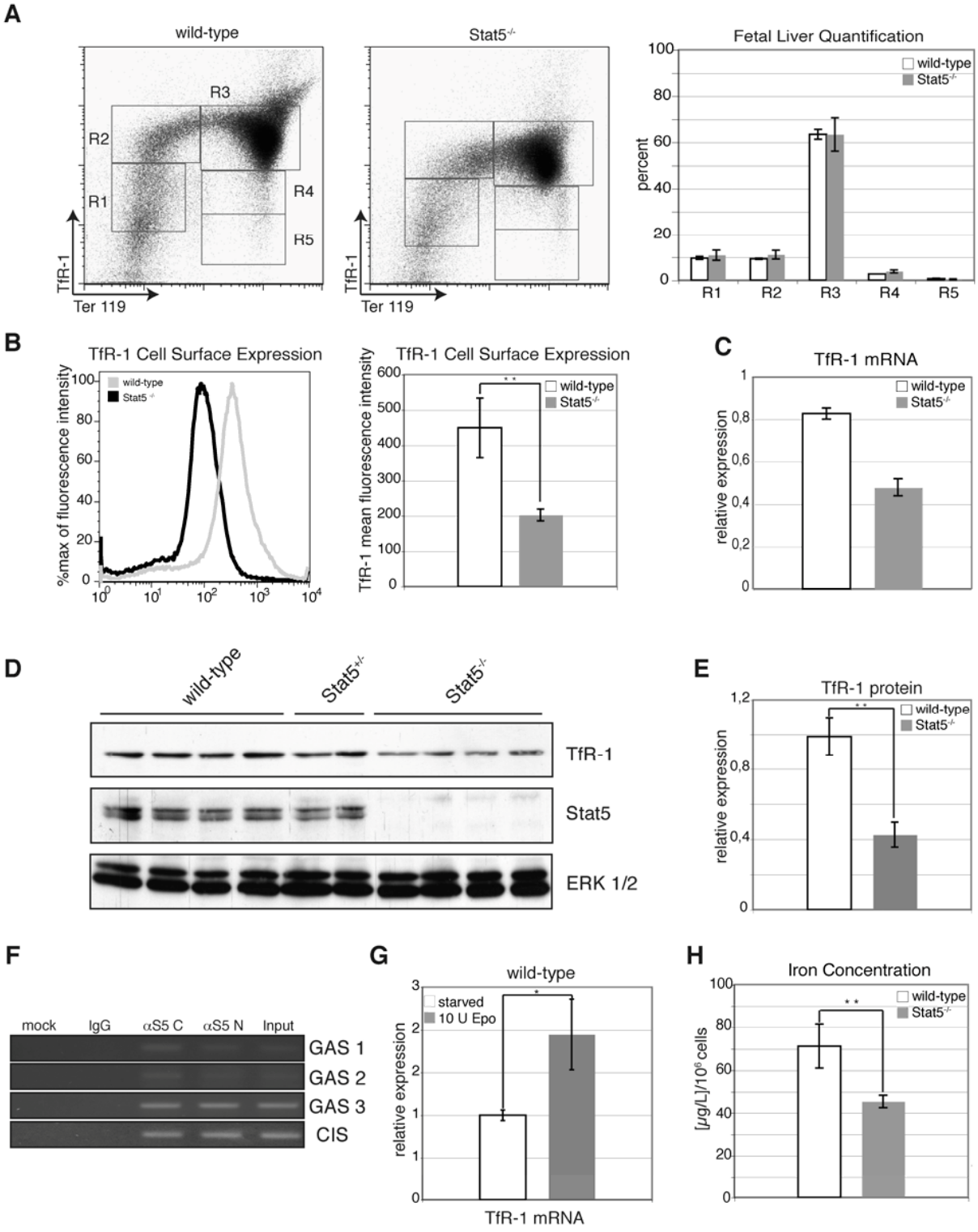
Kerenyi_Fig. 2

Figure 2. Loss of Stat5 results in increased levels of apoptosis in fetal liver cells. (A) Freshly isolated fetal livers from E13.5-E15.5 were stained for Ter119 and Annexin V to determine rates of apoptosis (data are presented as mean ± SD; n=3) for each genotype and time point. (B) CFU-E colonies derived from wt or Stat5$^{-/-}$ fetal liver cells using the indicated Epo concentrations (data are presented as mean ± SD; n=4). (C) E13.5 fetal liver cells of wt and Stat5$^{-/-}$ embryos were infected with a retrovirus encoding GFP. TfR-1^{high}/c-Kit^{+}/GFP^{+} cells were isolated by FACS after seven days under self-renewal conditions (cytospin, right panel). (D) 1.5x10^{7} of TfR-1^{high}/c-Kit^{+}/GFP^{+} cells were injected into the tail vein of lethally irradiated mice (950 rads) and Ter119^{+}/GFP^{+} bone marrow cells scored three days later (mean ± SD; n=4).



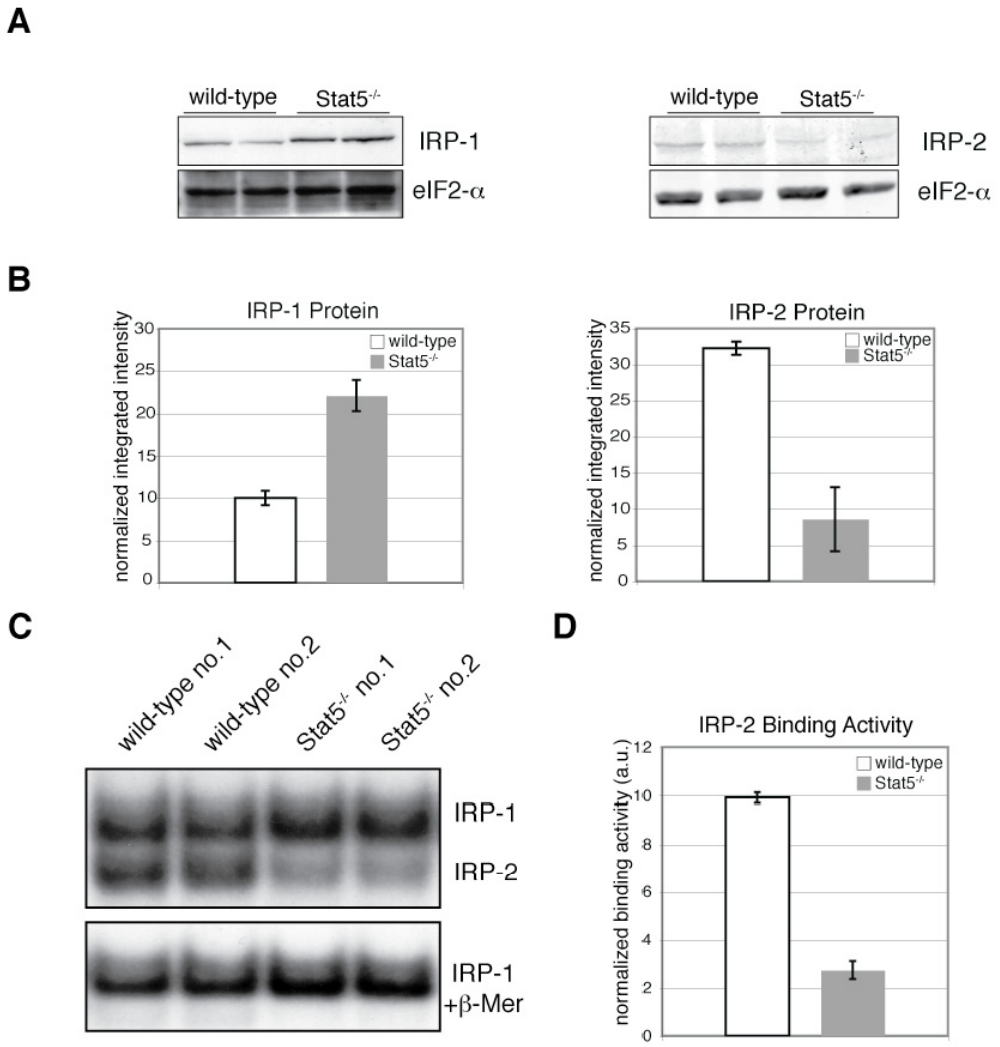
Kerenyi_Fig. 3

Figure 3. Loss of the anti-apoptotic protein Mcl1 in Stat5$^{-/-}$ fetal liver. (A) Western blot of lysates from freshly isolated fetal livers or Ter119^{+} and Ter119^{-} sub-fractions (separated using magnetic beads; see Material and Methods) for Bcl-x_L. (left; ERK, loading control). Western blot for Mcl-1 of two individual freshly isolated fetal livers of each genotype (right; eIF4E, loading control). (B) Quantitative PCR analysis for Mcl-1 mRNA isolated from the Ter119^{+} sub-fraction of freshly isolated fetal liver cells (data are presented as mean ± SD; n=3) (C) Wt and Stat5$^{-/-}$ primary erythroblasts expanded for 5 days under self renewal conditions (see Material and Methods) were deprived of factors for 3h followed by a 30min re-stimulation with 10U/mL Epo. qPCR analysis for Mcl-1 (representative experiment; error-bars are SD of experimental triplicates) (D) Wt or Stat5$^{-/-}$ fetal liver cells were infected with retroviruses encoding GFP alone (MSCV), or Bcl-x_L plus GFP, or Mcl-1 plus GFP from bi-cistronic constructs. After retroviral infection (72h), primary erythroblasts were cultivated for another 48h under self-renewal conditions (see Methods) in the presence or absence of Epo. Rates of apoptosis were determined by flow cytometry for Annexin V. One representative set of histograms from three independently performed experiments of GFP-gated Annexin V positive cells at 48 hours of treatment is depicted. Arrowheads indicate increased levels of apoptosis.



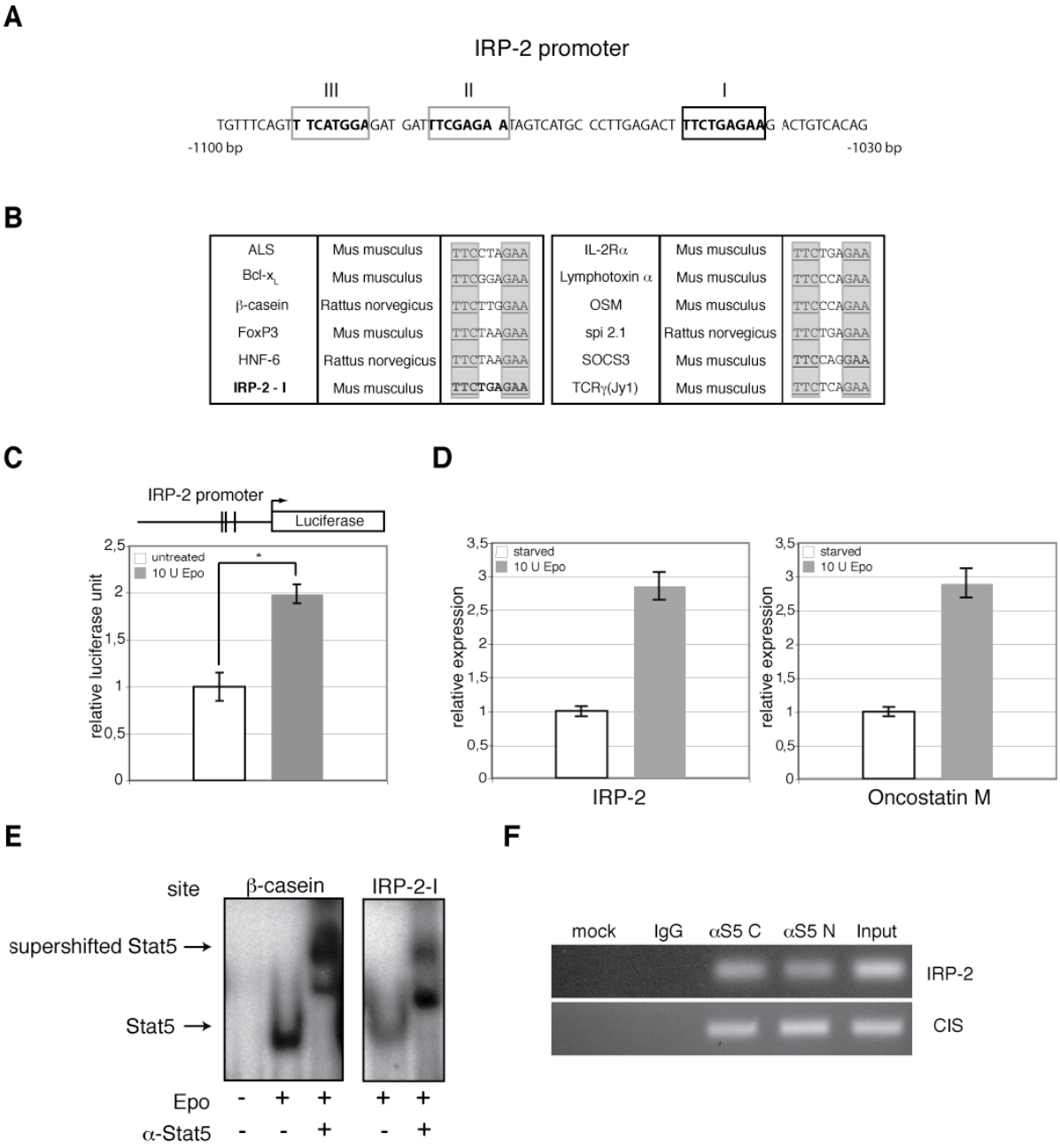
Kerenyi_Fig. 4

Figure 4. Cell surface expression of TfR-1 is strongly reduced in Stat5$^{-/-}$ erythroid progenitors. (A) Representative flow cytometry histograms of E13.5 wt and Stat5$^{-/-}$ fetal liver cells stained for the erythroid markers TfR-1 and Ter119 (left). The sequence from gate R1 (TfR-1^{low}Ter119^{low}) to gate R5 (TfR-1^{-}Ter119^{high}) represents development from the most immature erythroid progenitors (late BFU-E; CFU-E) to mature erythroid cells (orthochromatic erythroblasts; reticulocytes)[58]. Quantification of gates R1-R5 (data are presented as mean ± SD; n=4) (right). (B) Cell surface expression of TfR-1 of Terr119^{high} gated wt (blue line) or Stat5$^{-/-}$ (red line) fetal liver cells (left). Quantification of TfR-1 cell surface expression of wt and Stat5$^{-/-}$ fetal livers (right; data are presented as mean ± SD; n=4). (C) Expression of TfR-1 mRNA from lysates of freshly isolated wt or Stat5$^{-/-}$ fetal liver cells (data are presented as mean ± SD; n=3). Expression was normalized on HPRT levels. (D) Western blot analysis of freshly isolated wt and Stat5$^{-/-}$ fetal liver cell lysates for TfR-1. ERK was used as loading control. (E) Densitometric quantification of TfR-1 Western blot in 3D. (F) Primary wt fetal liver erythroblasts were stimulated with Epo for 30min and ChIP for Stat5 was performed. DNA from Epo-stimulated primary wt erythroblasts was recovered using two different anti-sera directed against N- or C-terminal epitopes (alphaS5 C, alphaS5 N). Specific PCR products from Stat5-binding sites GAS 1, GAS 2, and GAS 3 in *TfR-1* intron 1[59] were only obtained with Stat5-specific antibodies but not with control IgGs. PCR for the genuine Stat5 site in the *CIS* promoter was used as positive control. (G) Primary wt fetal liver-derived erythroblasts were factor depleted for 2.5h followed by a 1.5h of Epo stimulation (10U/mL). TfR-1 mRNA expression was scored by qPCR normalized on HPRT (data are presented as mean ± SD; n=4). (H) Iron concentration in freshly isolated fetal liver lysates determined via atomic absorption spectrometry (data are presented as mean ± SD; n=4; see Material and Methods).



Kerenyi_Fig. 5

Figure 5. Stat5-deficient erythroid cells display reduced *IRP-2* expression and mRNA binding activity. (A) Western blot analysis of primary wt and Stat5$^{-/-}$ erythroblast lysates for IRP-1 (left) and IRP-2 (right) (B) Quantification of Western blot analysis from (A) (data are presented as mean ± SD). Samples were normalized on eIF4E as levels and quantified using the Odyssey infrared imaging system. (C) Two representative lysates each, of wt and Stat5$^{-/-}$ erythroblasts, were subjected to RNA-EMSAs for IRP-1 and IRP-2 using an IRE-RNA probe corresponding to the IRE of mouse ferritin heavy chain[85] (for experimental details see Supplementary Methods). (D) Quantification of IRP-2 binding activity (data are presented as mean ± SD; n=4).



Kerenyi_ Fig. 6

Figure 6. Loss of Stat5 directly decreases IRP-2 gene expression. (A) Sequence of the IRP-2 promoter -1030bp to –1100bp upstream of the transcription start, showing one perfect GAS site (boxed in black) and two GAS sites with one mismatch (grey) (B) Multiple perfect Stat5 sites taken from[26,64], together with the IRP-2-I. (C) Luciferase reporter assay using a DNA fragment ranging from 2kb immediately upstream of the predicted IRP-2 transcription start site. Vertical lines indicate the approximate positions of the putative Stat5 response elements. 293T cells were co-transfected with constructs encoding IRP-2-firefly-luciferase, renilla-luciferase, Stat5a and EpoR. Cells were treated with 10U/mL Epo or left untreated, and luminescence scored 3h later. Transfection efficiencies were normalized to renilla-luciferase activity. (D) Epo-dependent induction of endogenous *IRP-2* and *oncostatin M* analyzed via quantitative PCR in murine erythroid leukemia cells serum deprived for 3h followed by stimulation with 10U/mL Epo (1h). Quantitative PCR was normalized on HPRT. (E) 293T cells were co-transfected with constructs for EpoR and wt Stat5a, followed by 30min stimulation with 10U Epo/mL. Whole cell extracts of these cells were subjected to EMSAs using either the IRP-2-I oligonucleotide (left), or β-casein oligonucleotide as positive control (right). Respective arrows indicate Stat5 DNA complexes and Stat5 DNA complex super-shifts. (F) Primary wt fetal liver erythroblasts were stimulated with Epo for 30min and ChIP for Stat5 was performed. Recovered DNA was analyzed for the presence of promoters of IRP-2 and CIS (positive control) by PCR.

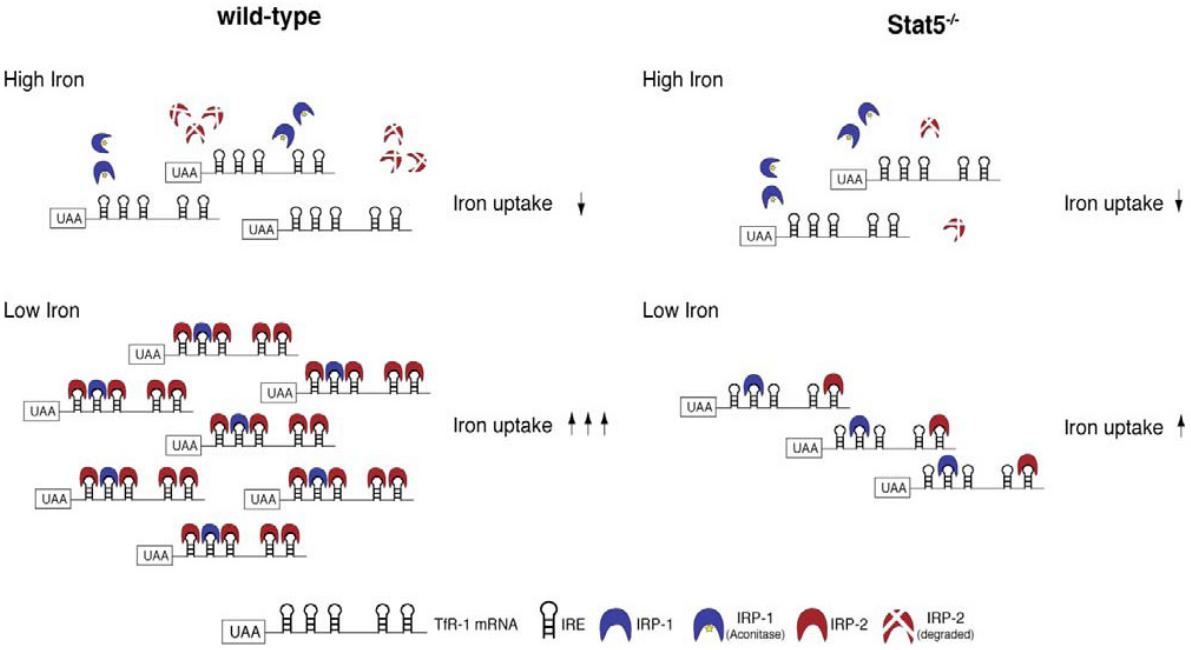


Figure 7. Model for involvement of Stat5 in iron uptake. (wild-type) In iron replete cells, IRP-1 is converted into cytosolic aconitase (catalyzes isomerization of citrate to isocitrate in the citric acid cycle and exhibits no mRNA binding affinity; yellow asterisk) and IRP-2 is degraded. Therefore both cannot bind to IREs in the 3´UTR of TfR-1 mRNA. Free unprotected IREs in turn enhance degradation rates of TfR-1 mRNA, resulting in reduced iron uptake. In iron depleted cells, IRP-1+2 bind to the respective IREs, thereby stabilizing TfR-1 mRNA, resulting in increased iron uptake. ($Stat5^{-/-}$) Due to lack of Stat5, basal TfR-1 transcript abundance is reduced in comparison to wild type cells. In addition, Stat5 deficiency further results in decreased levels of IRP-2 and, in consequence, a reduction of binding to IREs in the 3´UTR of TfR-1 mRNA and decreased transcript stabilization. Together, this constitutes a double-negative effect on erythroid iron uptake even in a situation of high iron demand, as in iron deficiency anemia.

3.1.2 Oncogenic Kit controls neoplastic mast cell growth through a Stat5/PI 3-kinase signaling cascade

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Oncogenic Kit controls neoplastic mast cell growth through a Stat5/PI 3-kinase signaling cascade

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Stat5 signaling in neoplastic mast cells

Abstract

The D816V-mutated variant of Kit triggers multiple signaling pathways and is considered essential for malignant transformation in mast cell (MC) neoplasms. We here describe that constitutive activation of the Stat5-PI3K-Akt-cascade controls neoplastic MC development. Retrovirally transduced active Stat5 ($cS5^F$) was found to trigger PI3K and Akt activation, and to transform murine bone marrow progenitors into tissue-infiltrating MC. Primary neoplastic Kit $D816V^+$ MC in patients with mastocytosis also displayed activated Stat5, which was found to localize to the cytoplasm and to form a signaling-complex with PI3K, with consecutive Akt activation. Finally, the knock-down of either Stat5 or Akt activity resulted in growth inhibition of neoplastic Kit $D816V^+$ MC. These data suggest that a downstream Stat5-PI3K-Akt signaling-cascade is essential for Kit D816V-mediated growth and survival of neoplastic MC.

Introduction

Mast cells are critical effector cells in innate and acquired immunity[1,2]. Under various circumstances and pathologic conditions, mast cells increase in number and accumulate in various tissues and organs. In many cases, reactive mast cell hyperplasia is found[1]. However, mast cells (mast cell progenitors) may also undergo neoplastic transformation [3, 4]. Disorders that lead to enhanced proliferation and/or accumulation of neoplastic mast cells are well-defined by WHO criteria [3-6].

Mast cells are derived from pluripotent hematopoietic cells in the bone marrow and undergo terminal maturation in their ultimate tissue destinations under the influence of stem cell factor, also known as Kit ligand [7-9]. Studies in mast cell-deficient mouse strains displaying mutations in the *stem cell factor* (SCF) gene or the gene encoding the SCF receptor, *c-Kit*, as well as activating *c-Kit* mutations that are considered to represent major transforming hits in mastocytosis, underline the importance of SCF and Kit for mast cell development [10-16]. Binding of SCF to Kit induces activation of various signaling molecules including phospholipase C, the Src family tyrosine kinase, the scaffolding molecule Gab2, the MAP Kinases Erk1/2, the JAK tyrosine kinase, the Phosphatidyl-inositol 3-kinase (PI3K), and the Stat transcription factors [17-19]. Lessons from gene deletion studies in mice have indicated that PI3K, Gab2, and Stat5 play a critical role in mast cell development and function, suggesting that these molecules may represent important downstream effectors of c-Kit signaling [20-22]. Moreover recent data have shown that Stat5 and Gab2 are also required for signaling via the high affinity IgE receptor FcεRI that plays a critical role in mast cell function and allergic response [23,24].

Besides their physiological role in mast cells, accumulating evidence suggests that persistent Stat5 and PI3K activation is frequently found in hematopoietic neoplasms and solid tumors [25, 26]. It has also been described that disease-related oncogenic tyrosine kinases like Tel-Jak2,

Bcr-Abl, Tel-PDGFRβ, mutated Kit or Flt3 receptors, and the Jak2 (V617F) mutant, detectable in most myeloproliferative disorders (MPD), induce constitutive activation of Stat5, PI3K and its downstream effector, the serine threonine kinase Akt [27-35]. Moreover, Stat5 proteins were found to be required for Tel-Jak2 and Bcr-Abl-induced MPD [36-38], and other studies have demonstrated the requirement of the PI3K/Akt pathway and Gab2 for Bcr-Abl-induced transformation[28,39]. Direct evidence for the involvement of Stat5 in hematopoietic cell transformation came from the use of constitutively active Stat5 mutants Stat51*6 and $cS5^F$ that are capable to induce a MPD and a multi-lineage leukemia in transplanted mice[36, 40]. We have recently shown that the leukemogenic effect of $cS5^F$ is coupled with its capacity to activate the PI3K/Akt signaling pathway in the cytoplasm of neoplastic cells through complex formation with p85, the regulatory subunit of the PI3K, and Gab2 [41, 42].

We asked in the current study, whether persistent Stat5 and Akt signaling contribute to the transformation of mast cells in mastocytosis. The results of our study show that constitutively activated Stat5 and the subsequent Akt-activation promote abnormal development of mast cells *in vivo* and *in vitro*. In addition, we show that Stat5 and Akt are constitutively phosphorylated in neoplastic mast cells isolated from in patients with $KitD816V^+$ systemic mastocytosis, and that in these cells, activated cytoplasmic Stat5 proteins associate with PI3K. Inhibition of Stat5 or Akt activity by shRNA or transducible, dominant negative recombinant TAT fusion proteins of Stat5 or Akt were found to abrogate the growth of neoplastic mast cells expressing the oncogenic KitD816V mutant. In contrast, transduction of a TAT fusion protein containing the $cS5^F$ mutant promoted SCF-induced hematopoietic stem cell (HSC) expansion and mast cell development.

Collectively, these data suggest that activated cytoplasmic Stat5 is an important downstream effector molecule of oncogenic Kit kinase activation, and that Stat5 oncogenic properties in mast cells may rely on the interaction with the PI3K/Akt kinase pathway.

Materials and Methods

Animals, primary cell isolation and cell culture

Introduction of recombinant retroviruses carrying $cS5^F$ and IRES-EGFP (Green Florescent Protein) or the IRES-EGFP vector alone in murine BM cells and mice transplantation were done as previously described [40]. Bone marrow was harvested from hind limbs of leukemic and control mice 6 weeks post-transplantation. BM cells from $cS5^F$-transplanted mice were grown for 24 hours in RPMI 1640 with 10% Fetal Calf Serum (FCS) and SCF (10 ng/ml, Valbiotech) while GFPv BM cells from control mice were grown in the same medium supplemented with IL-3 (10 ng/ml). The next day, GFP^+ cells were sorted by flow cytometry and cultured in the same medium as above for 6 weeks. All mouse experiments were done in accordance with the Ludwig Boltzman Institute for Cancer Research's institutional policies.

Umbilical cord blood samples were collected from full-term deliveries after informed consent was obtained from donors according to the Declaration of Helsinki. Cord blood was diluted with phosphate-buffered saline (PBS) supplemented with 5 mM EDTA. Mononuclear cells were collected after standard separation on Ficoll gradient. Purification of Human $CD34^+$ cells was performed with an immunomagnetic bead separation Kit (MiniMACS, Milteneyi Biotec) according to the manufacturer's instructions. The purity of the enriched CD34+ cells was about 95%. Cells were then cultured in RPMI medium supplemented with 10% FCS, 2 mM L-Glutamine, 10 UI Penicillin, 10 μg/ml Streptomycin and rhSCF (10 ng/ml, Valbiotech). The human mast cell leukemia cell line HMC-1[43] was kindly provided by Dr. Jospeph H. Butterfield (Mayo Clinic, Rochester, MA). In this study subclone HMC-1.2 carrying KIT D816V was analyzed. HMC-1 cells were cultured in Iscove's medium supplemented with 10% FCS, 2 mM L-Glutamine, 10 UI Penicillin, 10 μg/ml Streptomycin.

Patients

Twenty patients with systemic mastocytosis (SM) and 3 control cases (normal bone marrow = bm) were examined. Mastocytosis was diagnosed according to established criteria [3]. In the SM-group, 15 patients had indolent SM (ISM), 2 had smouldering SM (SSM), 2 aggressive SM (ASM), and 1 had mast cell leukemia (MCL). 15 patient data were assembled in Table 1. Informed consent was obtained before bone marrow biopsies were taken. The study was approved by the institutional review board of the Medical University of Vienna and was conducted in accordance with the Declaration of Helsinki.

Plasmids and reagents

The coding regions of wtStat5a, $cS5^F$ and dnStat5a (Stat5aΔ749), wtAkt and dnAkt (K179M) were amplified by PCR and cloned at the KpnI/EcoRI (Stat5) or NcoI/EcoRI sites (Akt) of the bacterial expression vector pTAT-HA. The LY294002 PI3K inhibitor and the calcium ionophore A23187 were purchased from Sigma, and the multikinase inhibitor PKC412 that blocks the TK activity of wild type- and mutant Kit, from LC Laboratories (Woburn, MA).

Lentiviral vectors and transduction.

Sence and antisense oligonucleotides (5'-GGAGAACCTCGTGTTCCTG-3') from human *Stat5a and Stat5b* coding region were annealed and introduced in pSuper plasmid 3' of the *polIII* H1 promoter. *polIII* H1 promoter-shRNA *Stat5* DNA fragment was then subcloned in the pTRIP/ΔU36EF1α encoding the green fluorescent protein (GFP) lentiviral vector, as previously described [44]. A shRNA directed against luciferase protein was used as control (Luc). Production of both shRNA-*Stat5* and shRNA-*Luc* lentiviral vectors was performed as previously described[44]. 10^6/ml of HMC-1 cells were incubated with the lentiviral particles at a multiplicity of infection (MOI) of 10 for 24 hrs and then intensively washed. Cells were then

maintained in Iscove's medium during 2 days and GFP^+ cells were next sorted by FACS and cultured in the same medium for 9 days.

Purification of recombinant TAT-Stat5 and Tat-Akt proteins

The TAT-Stat5 and TAT-Akt fusion proteins were produced and purified as previously described [42]. A concentration of 10 μM was routinely prepared for each individual TAT-Stat5 and TAT-Akt preparation.

Flow cytometry analysis

Expression of cell surface molecules was analyzed by flow cytometry. $5x10^5$ cells were incubated for 30 min on ice in the dark with the following PE-conjugated monoclonal antibodies: anti-human or -mouse FcεRI (eBioscience), anti-human or -mouse CD117 (BD Bioscience), anti-human CD34 and with isotype control PE conjugated antibodies. After washes in PBS-3% BSA- 0,1% Sodium azide, expression of these antigens was analyzed by FACS (Coulter Epics Elite cytometer) and evaluated as the percentage of positive cells. For flow cytometric detection of intracellular P-Y-Stat5, HMC-1 cells were incubated in control medium or medium containing PKC412 (1 μM) for 4 hours. Then, cells were fixed in formaldehyde (1.6%), permeabilized by ice-cold methanol (-20°C, 10 minutes), washed in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), and stained with an Alexa488-conjugated monoclonal antibody against P-Y-Stat5, i.e. Anti-Phospho-Stat5(Y694):Alexa Fluor® 488 (BD Biosciences Pharmingen, CA) or an isotype-matched control antibody (mIgG1-Alexa Fluor® 488, BD Biosciences, Pharmingen, San Jose, CA) for 15 minutes at room temperature (RT). Cells were then washed in PBS/0.1% BSA, and analyzed by FACS.

Immunoprecipitation, Western blotting and antibodies

Cells were lysed in Laemmli's buffer (0.0625M pH 6.8, SDS 2% β-Mercaptoethanol 5%, glycerol 10%). Equal amounts of each protein sample were separated by electrophoresis on

SDS-PAGE and blotted onto nitrocellulose membrane (BioRad). Blots were incubated as indicated with antibodies raised against the following proteins: Stat5 (Transduction Laboratories), HA (Roche), P-$Y^{694/699}$-Stat5a/b, P-Ser^{473}-Akt (Cell Signaling Technology), Akt (Santa Cruz biotechnology), p85 (UBI). Membranes were developed with the ECL chemiluminescence detection system (Amersham Pharmacia biotech) using specific peroxidase conjugated anti-IgG antibodies. Immunoprecipitation experiments and subcellular fractionation were performed as previously described [41,42].

Immunohistochemistry and immunocytochemistry

Immunohistochemistry was performed on serial consecutive sections from paraffin-embedded, formalin-fixed tissues using the indirect immunoperoxidase staining technique as described [45, 46]. Either patient bone marrow biopsies or murine stomach mucosa and submucosa sections of vGFP and $cS5^F$-transplanted mice were stained. Sections were pre-treated in 10 mM citrate buffer (pH 6.0) either in a microwave oven (α-tryptase) or steamer (all others) prior to staining with antibodies. Endogenous peroxidase was blocked by PBS/H_2O_2. Tissues were treated with avidin/biotin blocker (Vector Laboratories) and Super Block (ID Labs) to block unspecific protein staining. Mouse-on-mouse-blocking solution (Vector Laboratories) was applied on sections stained with mouse primary antibodies. For immunhistochemical analysis, sections were stained with anti-tryptase antibody G3 (Chemicon, Temecula; 1:500), anti-P-Y-Stat5a/b antibody AX-1 with or without blocking peptide (Advantex Bioreagents; 1:1,000), and anti-P-S^{473}-Akt with or without blocking peptide (P-S-Akt, #3787, Cell Signaling; 1:100) diluted in PBS (pH 7.4)/1 % BSA. G3 or AX-1 antibodies were applied for 1 hour at RT, anti-P-S-Akt for 20 hours at 4 °C. Subsequently, slides were washed and incubated with a biotinylated anti-polyvalent IgG (ID Labs) for 10 minutes at RT, washed and exposed to streptavidin-horseradish peroxidase complex for 10 minutes (ID Labs). AEC (Sigma) was used as chromogen. Slides were

counterstained in Mayer´s Hemalaun and mounted in Aquatex. To display organotypic structures tissue sections were stained with hemalaun and eosin (H&E). To control for cytoplasmic localization of P-Y-Stat5 staining, a different antigen retrieval protocol was used. A high pH retrieval buffer (Ventana Medical Systems; pH= 8.0) was applied for 15 minutes at 95°C. Sections were incubated with the AX1 antibody at the dilution of 1:500 for 1 hour at room temperature. Subsequent antibody reactivity was detected as described above. Quantification of immunohistochemical stainings was performed with the HistoQuest analysis software (TissueGnostics; www.tissuegnostics.com) using an average of four fields of view. The cutoff value for background staining was chosen utilizing the forward/backward gating tool of the HistoQuest software. Images were captured with a PixeLINK camera and the corresponding acquisition software on a Zeiss Imager Z.1 (magnification of 400x), at a color temperature of 1300K. For details on the quantification method see Supplementary Figure 3. For immunocytochemical analysis, cells were spun on cytospin slides prior to staining with the AX1 or P-S-Akt antibodies. Slides were counterstained with Mayer's Hemalaun.

Histochemical staining

cS5^{F}-BMMC or human mast cells were spun on cytospin slides, fixed with Carnoy's fluid and incubated for 15 min with toluidine blue. For the degranulation experiments, cS5^{F}-BMMC were incubated with or without 2 μg/ml of mouse IgE overnight at 37°C, washed, and incubated with 10 μg/ml of affinity-purified anti-mouse Ig (H+L) for 30 minutes at 37°C prior to staining with toluidine blue.

Results

Constitutive activation of Stat5 induces abnormal development of murine mast cells in vivo and in vitro.

Retroviral transduction of $cS5^F$ in murine bone marrow (BM) cells leads to development of multilineage leukemia within 4 weeks post transplantation in lethally irradiated wild-type recipient mice[40]. Disease is obvious in the spleen, bone marrow, and liver, but we found in addition, that the stomach wall and colon are strongly inflamed (Supplementary Figure 1A) and infiltrated with tryptase positive mast cells in tissue sections compared to control GFPv-transplanted mice (Figure 1). Staining with an anti-P-Y-Stat5 antibody showed that these mast cells abundantly express phosphorylated Stat5. We also observed a higher number of tryptase positive cells expressing P-Y-Stat5 in stomach and large bowel submucosa and mucosa tissues (Figure 1A and Supplementary Figures 1B and 2). In addition to mast cell infiltrates, we also detected an infiltration of intestinal walls by inflammatory cells of lymphoid and myeloid origin. These cells were also found to express P-Y-Stat5. Transplantation of BM cells transduced with wtStat5a or GFP-vector controls did not cause detectable leukocyte accumulations or mast cell infiltrates in the gastrointestinal tract.

Since oncogenic $cS5^F$ proteins activate the PI3K/Akt pathway in bone marrow cells of transplanted leukemic mice [42], the expression of the phosphorylated form of Akt in $cS5^F$-stomach mucosa and submucosa tissues was analyzed (Figure 1A). Staining with anti-P^{ser473}-Akt (P-S-Akt) antibody showed that $cS5^F$-expressing mast cells also express P-S-Akt. All data were confirmed by direct quantification of immunohistochemical stainings using the HistoQuest analysis software (Figure 1B, Supplementary Figure 3). Furthermore, higher magnification of the $cS5^F$-stomach submucosa section showed that $cS5^F$ is mainly detected in the cytoplasm, which is in line with our previous observation that constitutively active Stat5 has a predominant cytoplasmic localization in myeloid leukemias (Supplementary Figure 2).

The cytoplasmic localization of phosphorylated Stat5 was also confirmed with a different staining protocol with high pH antigen retrieval [47] (Supplementary Figure 2).

cS5^F-induced proliferation of murine bone marrow cells and subsequent mast cell development is dependent on c-Kit receptor and PI3K activation.

Mast cells originally arise from hematopoietic progenitors in the bone marrow [7-9]. We isolated GFP positive bone marrow (GFP$^+$-BM) cells from different cS5^F-transplanted mice, six weeks post-transplantation, to analyze the growth factor requirement of primary mast cells *in vitro*. We first addressed whether activation of Kit is required to support cS5^F-induced BM cell growth. cS5^F-BM cells were incubated at the indicated times with SCF or Flt3L (Fetal liver tyrosine kinase-3 Ligand) as control, and cell proliferation was determined (Figure 2A). Respective results showed that growth of cS5^F-BM cells required the continuous presence of SCF but not Flt3L. We also found that persistent phosphorylation of cS5^F is dependent on Kit activity in these primary leukemic cells (Figure 2B). To investigate whether expression of cS5^F could trigger mast cell differentiation *in vitro*, freshly isolated GFP$^+$-BM cells from cS5^F-transplanted mice were grown in culture in the presence of SCF for 2 weeks. Expression of FcεRI and c-Kit receptors were then analyzed by flow cytometry. Interestingly, 96% of GFP$^+$-cS5^F-BM cells expressed FcεRI and c-Kit surface receptors (Figure 2C). Almost 100% of the cells contained toluidine blue positive granules confirming that they were mast cells (Figure 2D). It is well established that antigen-induced cross-linking of IgE bound to FcεRI initiates mast cell activation and granule mediator release [48]. We determined by toluidine blue staining whether cS5^F-BM derived mast cells (cS5^F-BMMC) could be activated to release granule content by cross-linking IgE-receptors. Anti-IgE treatment of cS5^F-BMMC or treatment with the calcium ionophore A23187 induced degranulation of these cells (Figure 2D). These findings suggest that activation of cS5^F supports the development of functional

mast cells induced by SCF. These results are of interest, as development of normal murine mast cells from their bone marrow progenitors is believed to require the presence of IL-3, not only SCF [49] (Supplementary Figure 4A and 4B). Thus, our data suggest that expression of $cS5^F$ by-passes the requirement of IL-3. Recent data on activation of the IgE receptor implied the involvement of an autocrine IL-3 loop[50], but we were not able to detect IL-3 mRNA or protein in our $cS5^F$-BM-derived mast cell model (Supplementary Figure 4C and 4D), excluding an IL-3-autocrine loop. In addition and in sharp contrast to $cS5^F$-BMMC, we were not able to detect the phosphorylation of Stat5 upon stimulation with SCF in normal GFPv-BMMC indicating that persistent activation of $cS5^F$ proteins via SCF stimulation can replace IL-3 as trigger of mast cell development (Supplementary Figure 4E). We then asked whether activation of PI3K is also required for $cS5^F$-induced mast cell growth and survival. $cS5^F$-BMMC and control GFPv-BMMC were left untreated or were treated with the PI3K inhibitor LY294002 (1 µM) for 3 days, and the percentage of viable cells was then determined (Figure 2E). Growth of $cS5^F$-BMMC was completely abrogated by LY294002 at a concentration that did not affect GFPv-BMMC proliferation indicating that activation of the PI3K/Akt pathway is necessary to sustain the proliferation and survival of $cS5^F$-BMMC.

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Stat5 and Akt are constitutively activated in neoplastic mast cells in patients with mastocytosis.

Activating mutations of *Kit* are considered essential for the development of mastocytosis [3, 14, 15, 51]. To investigate whether mastocytosis is also associated with constitutive Stat5 and Akt activation, immunohistochemical analysis was performed on bone marrow sections obtained from patients with $KitD816V^+$ systemic mastocytosis (SM) with anti-P-Y-Stat5, P-S-Akt and anti-tryptase antibodies. In normal bone marrow, activated Stat5 was mainly detected in megakaryocytes and in some myeloid progenitor cells. By contrast, in the bone marrow in

SM, numerous mast cell infiltrates were detected and more than 95% of all spindle-shaped cells (mast cells) were found to be tryptase$^+$ cells and to express P-Y-Stat5 as determined by immunohistochemistry (same bone marrow and same patients) without any difference among patients (Figure 3A). In particular, in serial sections obtained from the same patients, more than 80% of the spindle-shaped cells (mast cells) were found to react with the anti- P-Y-Stat5 antibody AX-1. Similarly, we found that neoplastic mast cells in SM in the serial sections examined frequently express P-S-Akt (Figure 3B). Activated Stat5 and activated Akt were detectable in neoplastic mast cells in all patients examined, including five with indolent systemic mastocytosis (ISM), 2 with smouldering SM (SSM), 1 with aggressive SM, and 1 patient with mast cell leukemia (MCL), all classified according to WHO criteria (3) (Table 1). We did not stain for P-S-Akt in ASM and MCL due to a lack of material. A most intriguing finding was that both P-Y-Stat5 and P-S-Akt could be localized predominantly to the cytoplasmic compartment of neoplastic mast cells in all mastocytosis patients.

Cytoplasmic localization of P-Y-Stat5 and P-S-Akt in neoplastic mast cells.

We next extended our analysis to isolated neoplastic mast cells from a patient with mast cell leukemia. The human mast cell leukemia cell line HMC-1 is known to display the oncogenic Kit mutant D816V [43]. Both types of malignant mast cells expressed P-Y-Stat5 and P-S-Akt in these staining experiments (Figures 4A, 4B and 4C). The specificity of the immunostainings could be confirmed by using blocking phospho-peptides and by Western blot analysis (Figure 4B; Supplementary Figure 6). Confirming our immunohistochemical staining results, P-Y-Stat5 was primarily detected in the cytoplasm of leukemic mast cells although a significant fraction of P-Y-Stat5 was also found in the nucleus of HMC-1 cells. Next, cell fractionation experiments were made to better quantify and analyze nuclear versus cytoplasmic P-Y-Stat5. Only a minor fraction colocalized to the nucleus, whereas cytoplasmic P-Y-Stat5a and P-Y-

Stat5b was much more abundant (Figures 4D and 4E). Cell fractionation was controlled by cytoplasmic Raf-1 and nuclear Topoisomerase-I. Nuclear P-Y-Stat5 proteins remain transcriptionally active and contribute to growth of HMC-1 cells (Supplementary Figure 5). Finally, we were able to show by flow cytometry that persistent activation of Stat5 in HMC-1 cells is dependent on KitD816V TK activity since PKC412, a Kit TK inhibitor abrogated the expression of phosphorylated Stat5 in these neoplastic mast cells (Figure 4F). Collectively, these data suggest an important function of P-Y-Stat5 downstream of KitD816V in the cytoplasm of human neoplastic mast cells supporting our results with oncogenic $cS5^F$-transplanted mice.

Proliferation of neoplastic mast cells requires activation of Stat5.

We next determined whether activation of Stat5 was necessary for the growth of neoplastic human mast cells (HMC-1). For this purpose, we directly transduced recombinant Stat5 proteins fused to the protein transduction domain of the HIV TAT protein into mast cells. We first aimed to analyze the effect on mast cell growth of TAT -Stat5 proteins. Three TAT-Stat5 fusion proteins (Figure 5A) were applied: the wild type form (TAT-wtStat5a), a constitutively active Stat5 mutant (TAT-$cS5^F$) and a Stat5 mutant that lacks the transactivation domain (TAT-dnStat5a). These Stat5 variants were generated and purified from bacterial extracts (Figure 5B). Transduction efficiency of the three different TAT-Stat5 proteins in HMC-1 cells was verified by Western blotting with anti-HA and anti-Stat5 antisera (Figure 5C). Efficient and equal transduction of the three proteins was observed. Their effect on growth of HMC-1 cells was determined. Cells were left untransfected or were transduced with the TAT-Stat5 fusion proteins for 9 days, and the number of living cells was then evaluated by trypan blue exclusion (Figure 5D). Transduction of TAT-dnStat5 proteins abolished the growth of HMC-1 cells in these experiments, whereas transduction of TAT-$cS5^F$ and TAT-Stat5 proteins

slightly increased their proliferation (Figure 5D). To further substitute our findings we used knock-down of expression of Stat5 in HMC-1 cells. For that purpose we developed and used lentiviral vectors expressing Stat5 shRNA. HMC-1 cells were transduced with recombinant lentivirus expressing Stat5 shRNA (or luciferase shRNA as control), and thereafter Stat5 expression and cell growth were determined. In these experiments we found that inhibition of Stat5 (Stat5a and Stat5b) expression completely blocked HMC-1 cell growth (Figures 5E and 5F). Taken together, these data provide evidence that growth of neoplastic mast cells requires persistent Stat5 activity.

c-Kit signaling promotes the growth of human hematopoietic progenitor cells and mast cell development through Stat5.

SCF-binding to the Kit receptor induced proliferation of human $CD34^+$ cells and mast cell development [7-9, 52]. Furthermore, murine mast cell development and function are abrogated in *stat5a/b*$^{\Delta N}$ mice[22]. Little is known so far about the role of Stat5 in SCF-induced human mast cell development. We first determined whether SCF could activate Stat5 in human $CD34^+$ cells. Phosphorylation and nuclear localization of Stat5 following binding of SCF to Kit was strong, as shown by both Western blot and immunocytochemistry analysis with an anti-P-Y-Stat5 antibody, confirming that SCF signals through Stat5 in human $CD34^+$ cells (Figure 6A). Similar results were obtained with the SCF-dependent human mast cell line LAD-2 (Supplementary Figure 4F). We then transduced human $CD34^+$ cells with the different TAT-Stat5 fusion proteins to analyse the role of Stat5. Expression of the three TAT-Stat5 proteins in transduced $CD34^+$ cells was detected 12 hours after addition to the cell cultures (Figure 6B). The levels of transduced TAT-Stat5 proteins were equivalent to endogenous Stat5 and remained stable for at least 48 hours (not shown). TAT-Stat5 proteins (10 nM) were therefore added every 48 hours to the $CD34^+$ cell cultures in order to evaluate their biological activities

(Figure 6C). In the absence of SCF, none of the fusion proteins were able to promote the growth of $CD34^+$ cells (data not shown). In contrast, the numbers of $CD34^+$ cells transduced with the TAT-wtStat5a and TAT-$cS5^F$ were significantly enhanced in the presence of SCF (10 ng/ml), whereas no growth promotion was seen with the TAT-dnStat5a-transduced cells. TAT-wtStat5a and TAT-$cS5^F$ were similar in their ability to promote growth of $CD34^+$ cells during the first 10 days of culture. However, after this period, we found a growth-advantage of $CD34^+$ cells transduced with TAT-$cS5^F$ (Figure 6C). We were able to expand these transduced cells for at least 7 weeks in similar growth rates then obtained in three week cultures. In addition, the TAT-wtStat5a and the TAT-$cS5^F$ fusion proteins were found to promote SCF-induced mast cell development as evidenced by expression of markers specific for the mast cell lineage, including tryptase and FcεRI, the high affinity IgE receptor. Cell surface expression of FcεRI was determined by flow cytometry 30-days after transduction of TAT-wtStat5a or TAT-$cS5^F$ proteins in $CD34^+$ cells grown in presence of SCF. We consistently found that transduction of TAT-wtStat5a or TAT-$cS5^F$ proteins increased the percentage of cells expressing FcεRI as compared to non-transduced cells (25,1 % versus 11,9% of total cells, Figure 6E). FcεRI expression was approximately two fold elevated upon TAT-$cS5^F$ transduction (~45%) compared to TAT-wtStat5a. Cells transduced with TAT-$cS5^F$ show the presence of metochromatic granules after staining with toluidine blue. Immunocytochemical analysis with anti-tryptase antibody revealed that almost 80% of these cells were tryptase positive (Figure 6E). Overall, these data provide evidence that SCF-mediated expansion of human hematopoietic progenitors and mast cell development require P-Y-Stat5 activity.

Stat5 associates with PI3K in human neoplastic mast cells and regulates cell growth through Akt kinase.

Formation of a complex between Stat5 and PI3K, and the consecutive activation of Akt, apparently promotes neoplastic mast cell development *in vitro* and *in vivo* in $cS5^{F}$-transplanted mice. We therefore determined whether such a complex would also be present in neoplastic HMC-1 cells. For this purpose, Stat5 was immunoprecipitated from HMC-1 cell extracts, and the presence of co-immunoprecipitated p85 was assessed by Western blot analysis (Figure 7A). Results from two different immunoprecipitation experiments demonstrated that p85 associated with Stat5 in these neoplastic mast cells. The specificity of this interaction was confirmed by performing the reverse experiment and we were also able to show the presence of Stat5 in the p85 immunoprecipitates (Figure 7A, right panel).

We recently reported the successful use of recombinant TAT-wtAkt (wild type) and TAT-dnAkt (dominant negative) proteins to demonstrate the crucial role of Akt in the transforming properties of constitutively active Stat5 proteins[42]. Thus, we freshly purified these two recombinant proteins to analyse the requirement of Akt in the proliferation of HMC-1 cells. In particular, cells were incubated with TAT-wtAkt or TAT-dnAkt proteins (100 nM) for 3, 6, or 9 days, and then, cell growth was determined. Transduction of TAT-dnAkt inhibited the proliferation of HMC-1 cells while TAT-wtAkt was without any detectable effects (Figure 7B). In control Western blot experiments with anti-HA and anti-Akt antibodies, we showed that both TAT-Akt fusion proteins were efficiently transduced into HMC-1 cells (Figure 7C).

We next analyzed whether inhibition of both Stat5 and Akt activities could synergistically increase the growth inhibitory response in neoplastic mast cells. In these experiments, HMC-1 cells were incubated with recombinant TAT-Stat5 (wt or dn), TAT-Akt (wt or dn) proteins, or a mixture of both proteins before determining cell growth (Figure 7D). Results showed that transduction of TAT-dnStat5 or TAT-dnAkt alone inhibited HMC-1 cell growth, but addition of both proteins did not further increase the growth inhibitory effect. Similar results were obtained by incubating HMC-1 cells with the PI3K inhibitor LY294002 and the TAT-dnStat5

protein (data not shown). We next determined the effect of transduced TAT-dnStat5 or TAT-cS5^F on the levels of phosphorylated Akt in HMC-1 cells. We found that while TAT-cS5^F increases Akt phosphorylation, transduction of TAT-dnStat5 in HMC-1 cells inhibited Akt phosphorylation, indicating that Stat5 regulates Akt activity in HMC-1 cells. These data were also confirmed through the use of pharmacological inhibitors. In fact, addition of LY294002 did not result in decreased Stat5 activation. As expected and in line with our data above, Akt phosphorylation was abrogated when using an inhibitor of Stat5 activation (Supplementary Figure 6). Collectively, these data indicate that constitutive activation of Stat5 and Akt are interconnected to promote mast cell growth via a shared signaling pathway triggered by oncogenic Kit receptors.

Discussion

Activating mutations of tyrosine kinases are often associated with the development of hematologic malignancies. For instance, Bcr-Abl and point mutations in Jak2 kinase (Jak2V617F) or in c-Kit (KitD816V) are responsible for myeloproliferative and mast cell proliferative disorders. These oncogenic tyrosine kinases activate distinct signaling pathways that play a role in cell growth and survival [28, 29, 33-35]. Activation of the PI3K/Akt pathway and Stat5 are commonly induced by these oncogenes, and their constitutive activation may be sufficient to transform hematopoietic cells. Here, we demonstrate that persistent activation of Stat5 and Akt in bone marrow cells of $cS5^{F}$-transplanted mice results in an increased growth of mast cells *in vivo* and enhanced Kit-dependent development of mast cells *in vitro*. Most importantly, we provide evidence that constitutive activation of Stat5 and Akt are detectable in neoplastic mast cells in patients suffering from systemic mastocytosis and mast cell leukemia. We also show that P-Y-Stat5 and P-S-Akt proteins promote mast cell growth suggesting that persistent Stat5 and Akt activation have an important role in mastocytosis. This observation was supported by our finding that abrogation of Stat5 or Akt activity is followed by inhibition of growth of neoplastic mast cells expressing the oncogenic KitD816V mutant.

We have recently shown that activated Stat5 is primarily located in the cytoplasm of leukemic cells in myeloid leukemias [42]. In the present study, we confirmed these findings also to neoplastic mast cells of mice and men. These findings suggest that besides its nuclear function as a transcription factor, the highly expressed and tyrosine phosphorylated Stat5 might also have an important role as an effector or signaling adaptor in the cytoplasm to amplify (e.g. Kit-dependent) oncogenic signaling. In fact, based on our results, we hypothesize that cytoplasmic Stat5, and the Stat5/PI3K complex with consecutive activation of Akt, play an important role in malignant growth of mast cells. P-Y-Stat5 interacted with the cytoplasmic

scaffolding adapter Gab2 in HMC-1 cells and expression of a Gab2 mutant deficient in PI3K binding inhibited HMC-1 cell growth (Supplementary Figure 7). Our data from neoplastic mast cells are in line with data from myeloid leukemias, where we found that phosphorylated Stat5 proteins form a signaling complex with the p85 regulatory subunit and Gab2[41, 42]. Thus, Stat5, Gab2 and PI3K/Akt may act in concert via a common (Kit-dependent) oncogenic signaling pathway in neoplastic mast cells. The essential role for Stat5, Gab2 and PI3K expression in mast cells development and function came from knock out mouse analysis [20-24, 53, 54]. Interestingly, mice deficient in p85 or Gab2, have a profound defect in gastrointestinal mast cells, while development of mast cells in other tissues remained unaffected [20]. These data are consistent with our observations that $cS5^F$-induced PI3K activation (via formation of a Stat5/Gab2/p85 signaling complex) promotes in particular the development of gastrointestinal mast cells.

In contrast to our data, it was recently shown that neoplastic mast cells in systemic mastocytosis display nuclear P-Y-Stat5[47]. On the other hand, we and others have shown that cytoplasmic P-Y-Stat5 can be detected in the cytoplasmic compartment in AML[42,55]. The reason for the discrepancy concerning nuclear as opposed to cytoplasmic P-Y-Stat5 detection in neoplastic mast cells remains unknown. One reason may be the differences in the staining protocols applied. In fact, depending on the technique applied, the nuclear or the cytoplasmic portion of P-Y-Stat5 may be optimally visualized. This would also be in line with the hypothesis that P-Y-Stat5 in neoplastic (mast) cells can fulfil important functions in both the cell nucleus and the cytoplasm. Based on our data in HMC-1 cells, an attractive hypothesis would be that both the DNA-binding- and PI3K/Akt-triggering activities contribute to the oncogenic potential of Stat5. Irrespective, our data suggest that KitD816V signals via P-Y-Stat5 in neoplastic mast cells. It is questionable if Stat5 is also an important signalling effector

in further diseases associated with oncogenic *Kit* mutations like in gastrointestinal stromal tumors (GIST) or acute myeloid leukemias.

Despite the important role of mast cells in immunity, allergic response or inflammatory diseases models in mice, signaling properties of human mast cells or signaling effectors that are involved in human mast cell development are still poorly understood. It was therefore important to demonstrate here that activation and tyrosine phosphorylation of Stat5 following SCF binding to c-Kit is required to promote expansion of human hematopoietic progenitors and consecutive mast cell development. It was previously shown that human mast cells arise from a pluripotent $CD34^+$ progenitor cell population that also give rise to eosinophils, basophils, monocytes and neutrophils[7,9, 56]. Thus it is conceivable that the growth-promoting effect of the TAT-Stat5 proteins on $CD34^+$ cells affects the entire cell population. Importantly, our data also suggest that deregulated Stat5 activity due to oncogenic c-Kit signaling promotes neoplastic cell development in part through an uncontrolled mitogenic effect on Hematopoietic Stem Cells (HSCs). This idea is also supported by recent work demonstrating a critical role of Stat5 in HSCs self-renewal and/or expansion in mammals [57-59]. The activation of Stat5 in myeloid diseases and mast cell neoplasms suggests that small inhibitor molecules targeting Stat5 or Akt might be relevant for the treatment of patients suffering from these malignancies. Such novel targeted drugs may be of special interest for those cases who are resistant against conventional kinase inhibitors, which has been described in CML but also in patients with mastocytosis [60-62]. In fact, although new Kit-targeting drugs have been described to counteract growth of neoplastic mast cells in most patients [33, 63, 64], resistance may develop within short time [62, 65]. Therefore, drugs targeting the Stat5-Akt axis are an alternative and complementary strategy in the treatment of systemic mastocytosis.

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Author contribution

NH, CB, KB, KS, RK, SM, LK,MK and SY designed and performed research,VG, JG, ID contributed analytical tools and analyzed data, KL analyzed data and wrote the paper, PV, RM and FG designed research, analyzed data and wrote the paper.

Conflict of Interest Disclosure: The authors declare to have no conflict of interest

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Figure legends

Figure 1: ***Constitutive activation of Stat5 induces mast cell hyperplasia and leukemic cell infiltrates in the gastrointestinal tract of transplanted mice.***

A) Immunohistochemical analysis of stomach walls of $cS5^F$-transplanted mice versus vGFP-transplanted control mice (n>6 mice analyzed in each group and a representative example is shown). In order to detect neoplastic mast cells, sections of the stomach mucosa and submucosa of vGFP- and $cS5^F$-transplanted mice were immunostained with an anti-tryptase antibody (a-f). The presence of $tyrosine^{694/695}$ phosphorylated Stat5 (P-Y-Stat5; g-l) and $serine^{473}$ phosphorylated Akt (P-S-Akt; m-r) was analyzed with specific antibodies on consecutive tissue sections (400x magnification). An overview of each staining on consecutive sections is shown in the two middle panels (c+d, i+j, o+p, u+v) for $cS5^F$-transplanted mice and vGFP-transplanted control mice (25x and 100x magnifications, respectively). H&E staining is shown for organotypic structure comparison (s-x).

B) Quantification of P-Y-Stat5, tryptase, and P-S-Akt stainigs was performed on consecutive sections using HistoQuest analysis software (results shown in the scattergram plots are the mean of 4 fields of view for each staining). The cut-off values for background staining were chosen manually utilizing the forward/backward gating tool of the HistoQuest software.

Figure 2: ***SCF-dependent activation of $cS5^F$ promotes the development of murine mast cells in vitro.***

A) Bone marrow (BM) cells from $cS5^F$-transplanted mice were cultured in presence of SCF (10 ng/ml) or Flt3L (10 ng/ml) and viable cells were enumerated daily using the trypan blue dye exclusion method.

B) $cS5^F$-BM cells were deprived of SCF for 3 hours and then re-stimulated for the indicated times. Cell extracts were prepared and analyzed by Western blot with anti-P-Y-Stat5 and anti-Stat5 antibodies.

C) Two weeks-old $cS5^F$-BM cells grown in culture in presence of SCF were analyzed for expression of FcεRI and c-Kit by flow cytometry.

D) $cS5^F$-bone marrow derived mast cells (cS5F-BMMC) were incubated with anti-IgE alone or/and IgE. Alternatively cells were treated with DMSO or A23187 and stained with toluidine blue.

E) $cS5^F$-BMMC and GFPv-BMMC were treated or not with LY294002 (1 μM) for 3 days and the percentage of viable cells was determined daily with the trypan blue dye exclusion assay. Results shown are representatives of three independent experiments.

Figure 3: ***Immunohistochemical detection of P-Y-Stat5 and P-S-Akt in neoplastic human mast cells.***

A) Adjacent bone marrow (BM) sections from a patient with indolent systemic mastocytosis (ISM) and normal bone marrow sections (normal BM) were stained with an antibody against tryptase for mast cell detection (brownish staining) and an antibody against P-Y-Stat5 (brownish staining).

B) Similarly, bone marrow sections from a patient with indolent systemic mastocytosis were stained with an antibody against P-Ser^{473}-Akt antibody. Higher magnification of the Immunostaining (400x) is also shown.

Figure 4: ***Detection and localization of P-Y-Stat5 and P-S-Akt in isolated neoplastic mast cells.***

A) Expression of P-Y-Stat5 and tryptase was also evaluated by immunocytochemistry in isolated neoplastic mast cells (MCL: isolated neoplastic mast cells from a patient with mast cell leukaemia) and normal isolated bone marrow cells: BM

B) Detection of P-Y-Stat5 and P-S-Akt in neoplastic mast cells obtained from patients with mast cell leukaemia (MCL) in the absence or presence of a blocking phospho-peptide.

C) Immunodetection of P-Y-Stat5, P-S-Akt and tryptase in the human HMC-1 neoplastic mast cell line. The P-S-Akt staining was also performed in presence of a blocking phospho-peptide D+E). Cytoplasmic and nuclear extracts (CE and NE) were prepared from HMC-1 cells and analysed by Western blotting using the indicated antibodies.

F) Detection of cytoplasmic P-Y-Stat5 in HMC-1 cells by FACS. Cells were cultured in control medium (solid line, open graph) or 1 μM PKC412 (solid line, grey graph) at 37°C for 4 hours followed by FACS using an antibody against P-Y-Stat5. The dotted line represents the buffer control. The isotype control exhibited a slight shift compared to the buffer control, but did not change after exposure to PKC412 (not shown).

Representatives of three independent experiments are shown.

Figure 5: ***Biological effects of Stat5 proteins on neoplastic mast cell growth.***

A) Schematic representation of TAT-Stat5 proteins. DBD: DNA Binding Domain; SH2: Src-Homology Domain 2; TAD: Transactivation Domain.

B) Purity of recombinant TAT-Stat5 proteins was assessed by Coomassie blue staining on SDS-PAGE. L,bacterial lysate; E, Eluate fraction.

C) HMC-1 cells were transduced with the different TAT-Stat5 proteins (10 nM) during 24 hours. Lysates from transduced $cS5^{F}$-BM cells were prepared and analyzed by Western blotting with the indicated antibodies.

D) HMC-1 cells were transduced or not with 10 nM of the different TAT-Stat5 proteins during 9 days and the number of living cells was determined every 3 days using the trypan blue dye assay. Results are the mean of three independent experiments.

E) HMC-1 cells were transduced with recombinant lentiviruses expressing a Stat5 shRNA or a luciferase shRNA as control. GFP^{+} cells were sorted by flow cytometry and cultured in normal medium for 9 days. Cell extracts were then prepared and analyzed by Western blot with indicated antibodies.

F) The number of viable GFP^+ cells expressing Stat5 or Luciferase shRNAs was also enumerated every three days. Representatives of two independent experiments are shown.

Figure 6: ***Analysis of Stat5 function in human $CD34^+$ cells via c-Kit signaling***.

A) Purified human $CD34^+$ cells from umbilical cord blood were stimulated with recombinant SCF (10 ng/ml) or IL-3 (10 ng/ml) during 30 min. Tyrosine phosphorylation of Stat5 was evaluated by Western Blot analysis using an anti P-Y-Stat5 antibody. SCF-mediated activation of Stat5 in $CD34^+$ cells was also analyzed by immunocytochemistry with an anti-P-Y-Stat5 (AX1) antibody.

B) Human $CD34^+$ cells were transduced or not (PBS) with the different TAT-Stat5 proteins (10 nM) for the indicated times. After extensive washes, the presence of recombinant TAT-Stat5 proteins in CD34+ cells were analyzed by Western blotting with the indicated antibodies.

C) 50 x 10^3 $CD34^+$ cells (n=4) were cultured in presence of SCF (10 ng/ml) during 20 days. TAT-Stat5 proteins (10 nM) were added or left away (PBS) every 2 days in culture and cells were enumerated every five days.

D) Transduced cells grown during 30 days in presence of SCF were analyzed for expression of FcεR1 by flow cytometry. Immunocytochemical analysis (IC) was also performed on TAT-$cS5^F$ protein-transduced cells with an anti-tryptase antibody.

E) The presence of metachromatic granules was detected after staining with toluidine blue. Results shown are representative of four independent experiments.

Figure 7: *P-Y-Stat5 interacts with PI 3-kinase in HMC-1 cells.*

A) Stat5 (left panel) or p85 (right panel) was immunoprecipitated from HMC-1 cell extracts with specific or isotype control antibodies. The presence of p85 (left panel) or Stat5 (right panel) in the immunoprecipitates was detected by Western blot.

B) HMC-1 cells were also transduced with 100 nM of TAT-wtAkt or TAT-dnAkt during 9 days as above and the number of living cells was determined every 3 days using the trypan blue dye assay. Results are the mean of three independent experiments.

C) Lysates from transduced HMC-1 cells were analyzed by Western Blotting using anti-HA and anti-Akt antibodies.

D) HMC-1 cells were transduced or not (NaCl) with the TAT-wtStat5, TAT-dnStat5, TAT-wtAkt, TAT-dnAkt fusion proteins or a mixture of TAT-wtStat5/TAT-wtAkt or TAT-dnStat5/TAT-dnAkt proteins (ratio1:1) during 3 days. Cell growth was determined using the trypan blue dye assay.

E) Extracts from HMC-1 cells, either untreated (NaCl) or transduced with TAT-cS5^{F} and TAT-dnStat5 recombinant proteins during 6 days were analyzed by Western blot with anti-P-S-Akt and anti-Akt antibodies. Representatives of three independent experiments are shown.

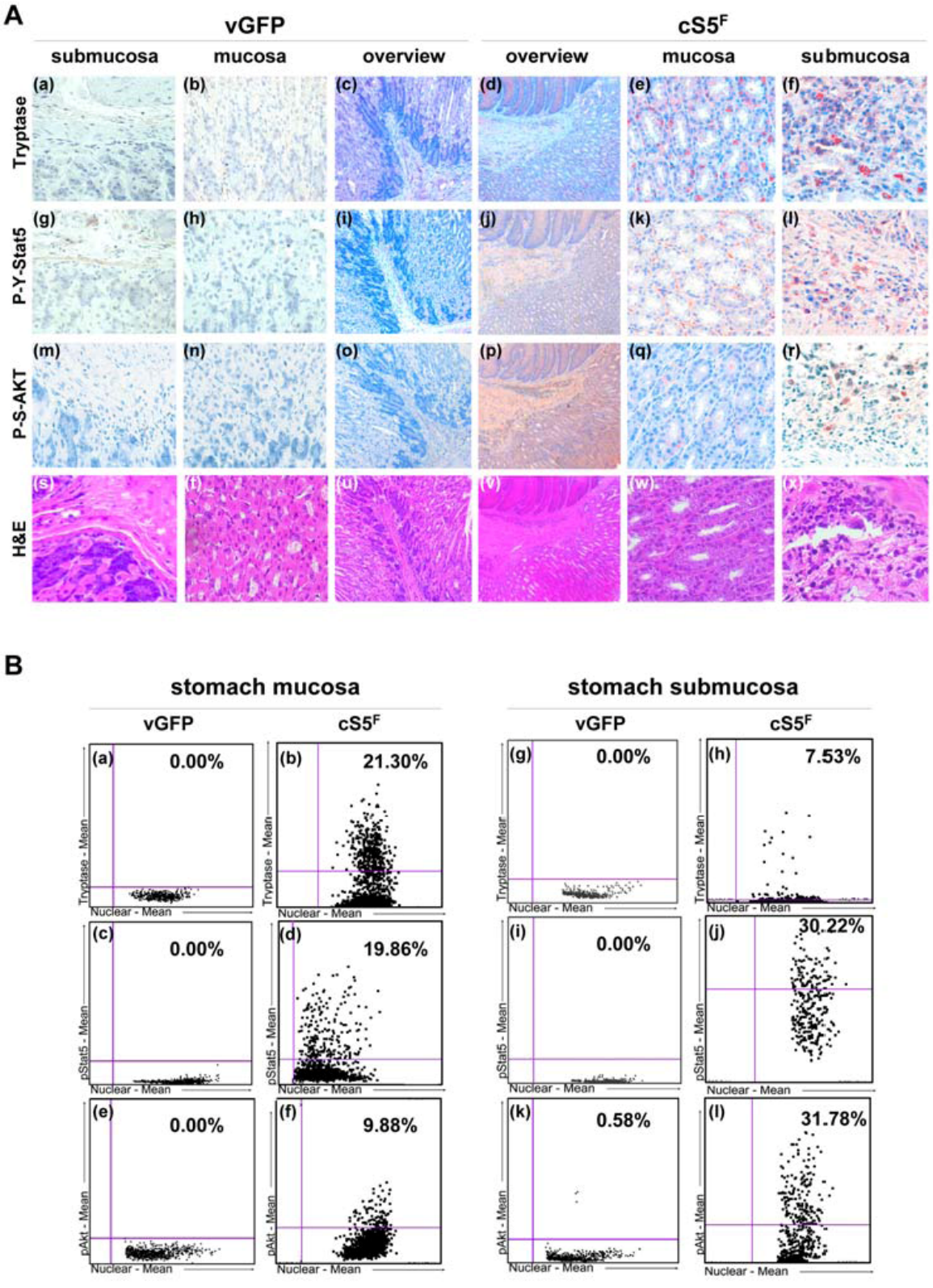
Table 1

Patient characteristics

no#	diagnosis	age	f/m	serum tryptase (ng/ml)	% infiltration of BM with MC	MC positive for: P-Y-Stat5	MC positive for: P-S-Akt
1	SSM*	47	f	153	25	+	+
2	SSM*	34	m	937	50	+	+
3	ISM*	66	f	17	2	+	+
4	ISM*	39	m	17	5	+	+
5	ISM*	70	m	28	2	+	+
6	ISM*	64	m	164	15	+	n.d.
7	ISM*	62	f	26	10	+	n.d.
8	ISM*	53	m	50	15	+	n.d.
9	ISM*	48	f	54	5	+	n.d.
10	ISM*	43	m	20	5	+	+
11	ISM*	32	f	18	5	+	n.d.
12	ISM*	32	m	36	5	+	+
13	ISM*	30	m	148	10	+	n.d.
14	ASM*	40	f	n.d.	5	+	n.d.
15	MCL*	60	m	285	70	+	n.d.

BM, bone marrow; MC, mast cells; SSM, smouldering systemic mastocytosis; ISM, indolent systemic mastocytosis; ASM, aggressive systemic mastocytosis; MCL, mast cell leukemia; f/m, sex; n.d., not determined

**All patients examined exhibited the Kit D816V as determined by RT-PCR and RFLP analysis*


A
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stomach mucosa
stomach submucosa
vGFP
cS5F
vGFP
cS5F
0.00%
21.30%
0.00%
7.53%
0.00%
19.86%
0.00%
30.22%
0.00%
9.88%
0.58%
31.78%
Tryptase - Mean
pStat5 - Mean
pAkt - Mean
Nuclear - Mean

Figure 1

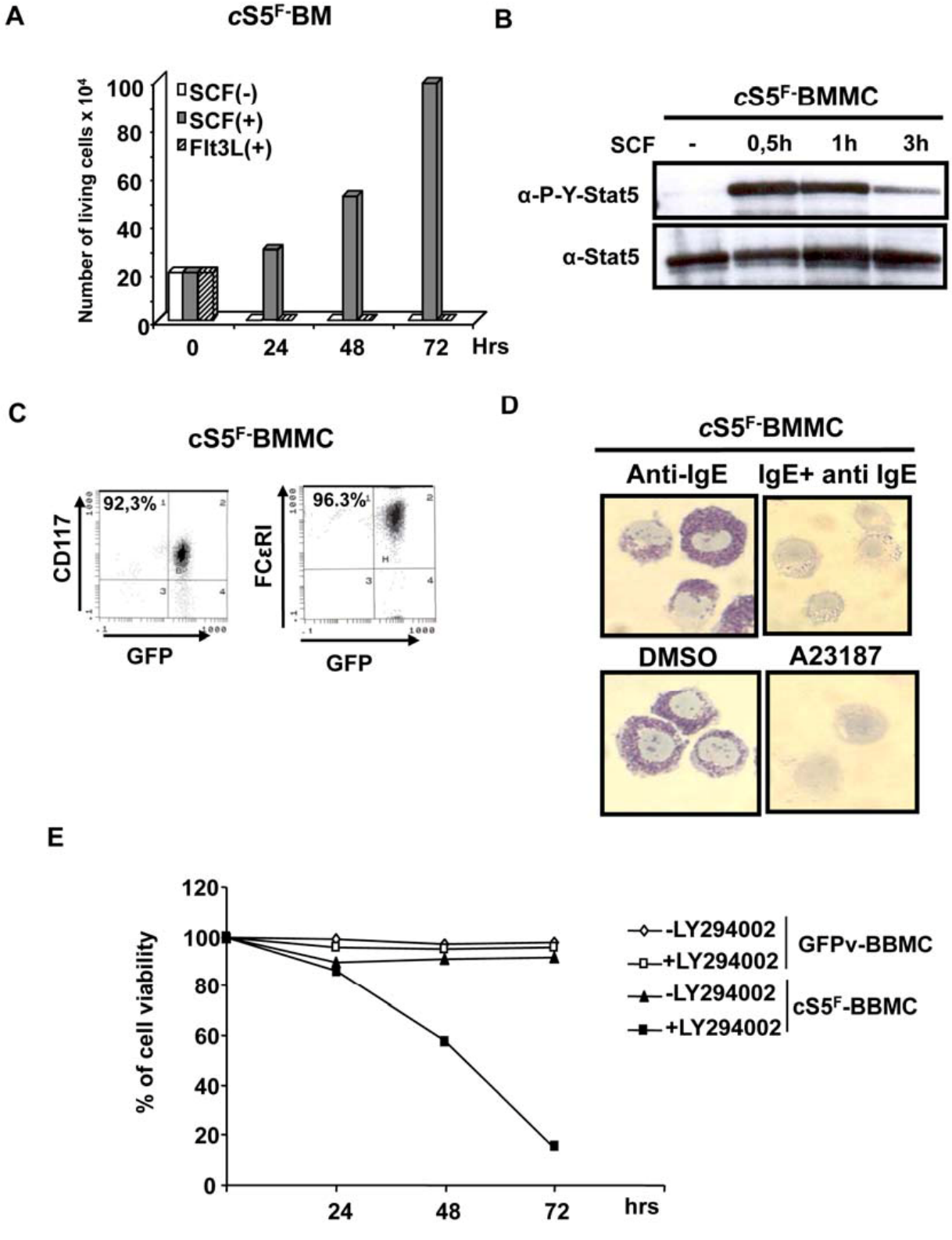
A
cS5F-BM
Number of living cells x 10⁴
100
80
60
40
20
0
SCF(-)
SCF(+)
Flt3L(+)
0
24
48
72
Hrs
B
cS5F-BMMC
SCF
-
0,5h
1h
3h
α-P-Y-Stat5
α-Stat5
C
cS5F-BMMC
92,3%
96.3%
CD117
FCεRI
GFP
GFP
D
cS5F-BMMC
Anti-IgE
IgE+ anti IgE
DMSO
A23187
E
% of cell viability
120
100
80
60
40
20
0
24
48
72
hrs
-LY294002
+LY294002
GFPv-BBMC
-LY294002
+LY294002
cS5F-BBMC

Figure 2

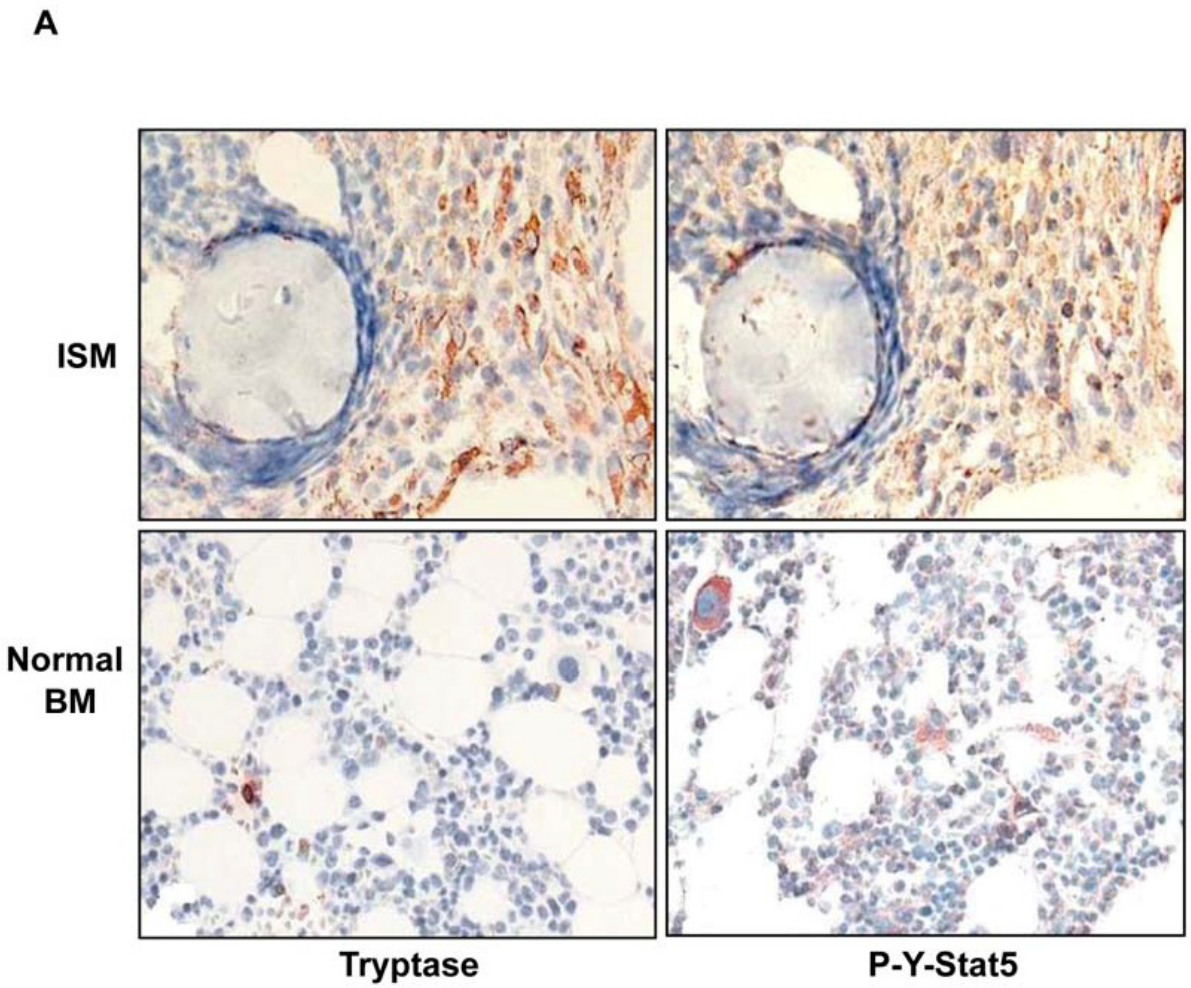

Figure 3

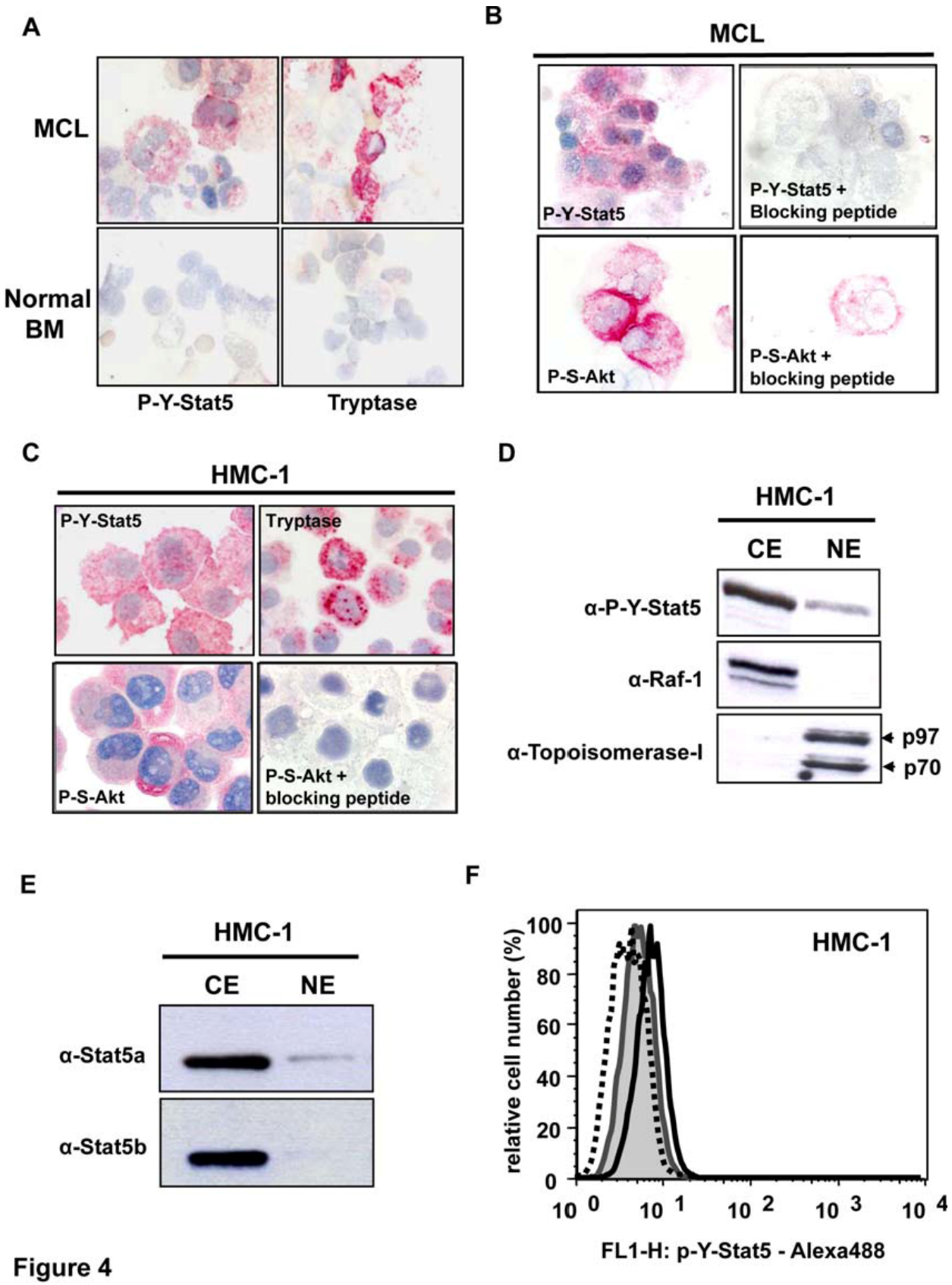
A
MCL
Normal BM
P-Y-Stat5
Tryptase
B
MCL
P-Y-Stat5
P-Y-Stat5 + Blocking peptide
P-S-Akt
P-S-Akt + blocking peptide
C
HMC-1
P-Y-Stat5
Tryptase
P-S-Akt
P-S-Akt + blocking peptide
D
HMC-1
CE
NE
α-P-Y-Stat5
α-Raf-1
α-Topoisomerase-I
p97
p70
E
HMC-1
CE
NE
α-Stat5a
α-Stat5b
F
HMC-1
relative cell number (%)
100
80
60
40
20
0
10 0
10 1
10 2
10 3
10 4
FL1-H: p-Y-Stat5 - Alexa488

Figure 4

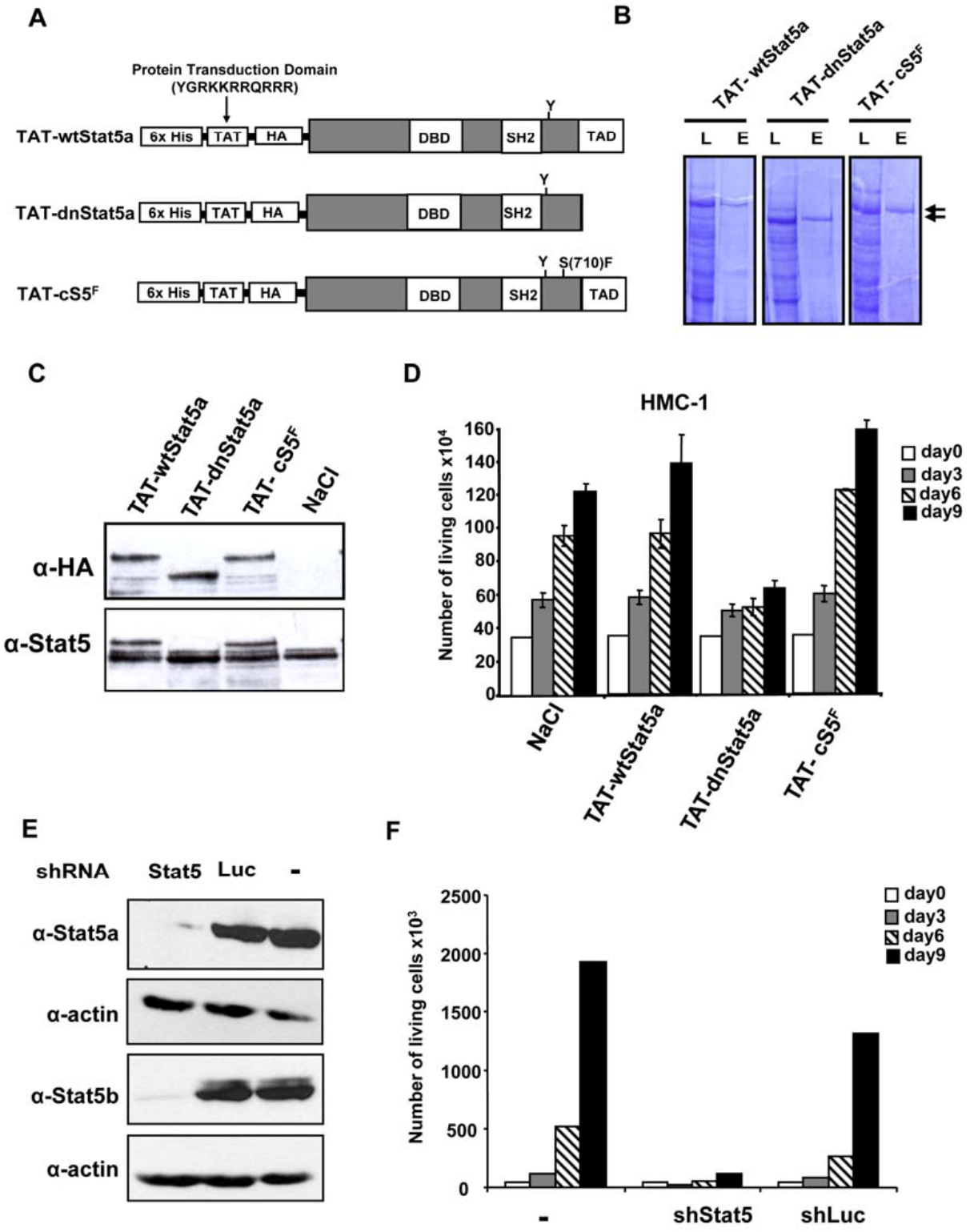
A
Protein Transduction Domain
(YGRKKRRQRRR)
TAT-wtStat5a
6x His
TAT
HA
DBD
SH2
Y
TAD
TAT-dnStat5a
TAT-cS5F
S(710)F
B
TAT- wtStat5a
TAT-dnStat5a
TAT- cS5F
L E L E L E
C
TAT-wtStat5a
TAT-dnStat5a
TAT- cS5F
NaCl
α-HA
α-Stat5
D
HMC-1
Number of living cells x10⁴
day0
day3
day6
day9
NaCl
TAT-wtStat5a
TAT-dnStat5a
TAT- cS5F
E
shRNA
Stat5
Luc
-
α-Stat5a
α-actin
α-Stat5b
α-actin
F
Number of living cells x10³
shStat5
shLuc

Figure 5

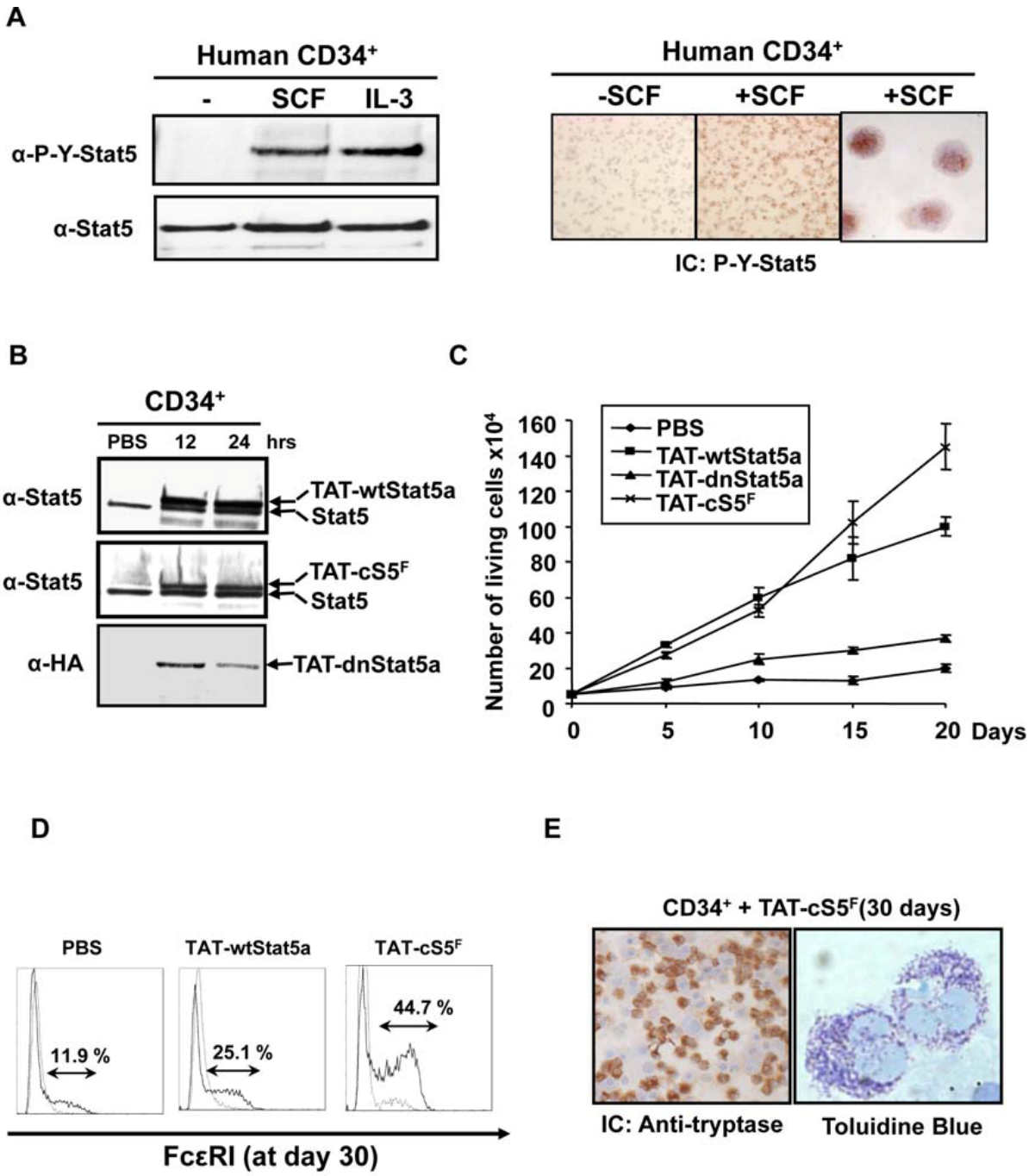
A
Human CD34+
- SCF IL-3
α-P-Y-Stat5
α-Stat5
Human CD34+
-SCF +SCF +SCF
IC: P-Y-Stat5
B
CD34+
PBS 12 24 hrs
α-Stat5
TAT-wtStat5a
Stat5
α-Stat5
TAT-cS5F
Stat5
α-HA
TAT-dnStat5a
C
Number of living cells x10^4
160
140
120
100
80
60
40
20
0
PBS
TAT-wtStat5a
TAT-dnStat5a
TAT-cS5F
0 5 10 15 20 Days
D
PBS
TAT-wtStat5a
TAT-cS5F
11.9 %
25.1 %
44.7 %
FcεRI (at day 30)
E
CD34+ + TAT-cS5F(30 days)
IC: Anti-tryptase
Toluidine Blue

Figure 6

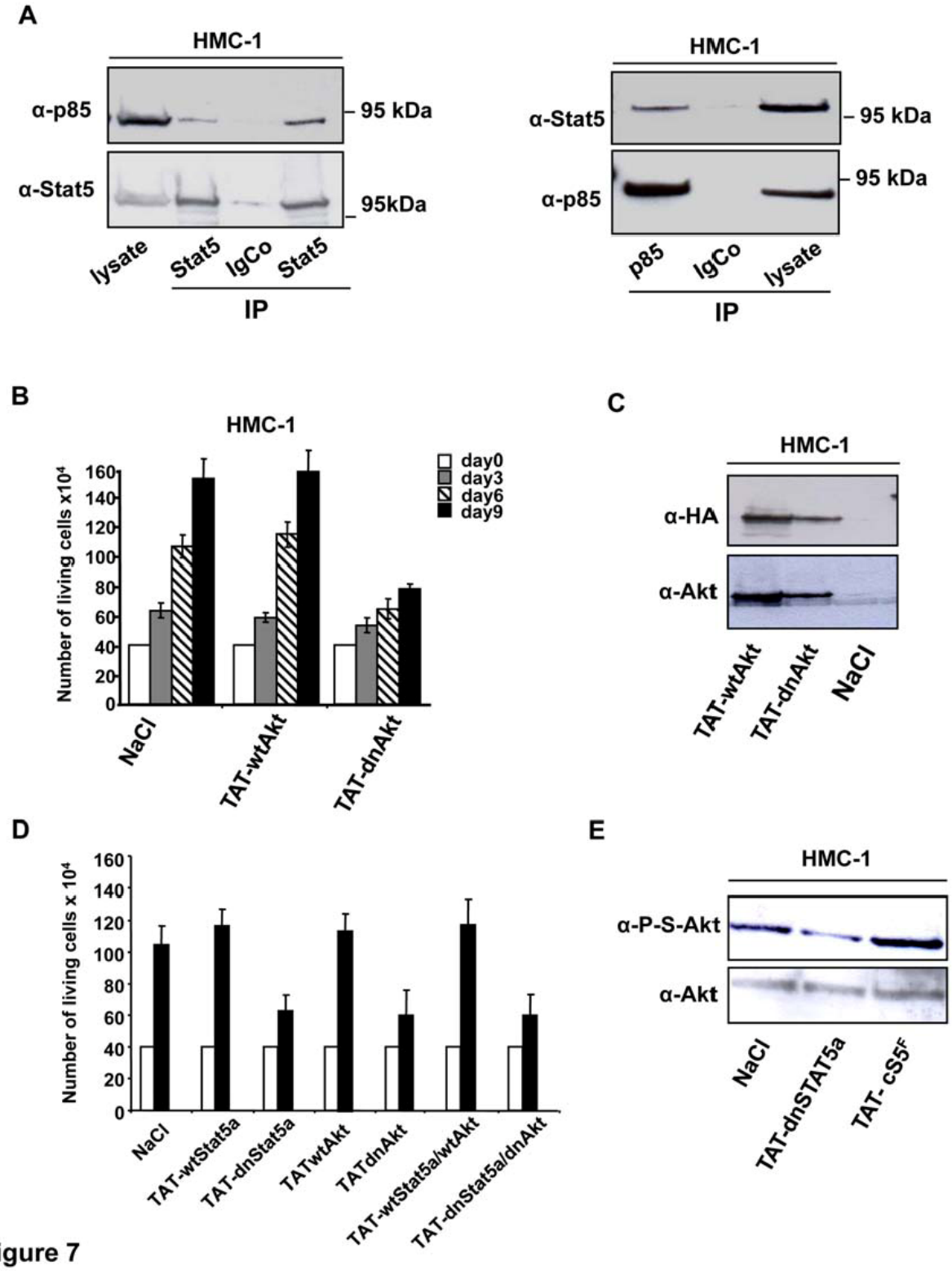
A
HMC-1
α-p85
95 kDa
α-Stat5
95kDa
lysate
Stat5
IgCo
Stat5
IP
HMC-1
α-Stat5
95 kDa
α-p85
95 kDa
p85
IgCo
lysate
IP
B
HMC-1
Number of living cells x10⁴
day0
day3
day6
day9
NaCl
TAT-wtAkt
TAT-dnAkt
C
HMC-1
α-HA
α-Akt
TAT-wtAkt
TAT-dnAkt
NaCl
D
Number of living cells x 10⁴
NaCl
TAT-wtStat5a
TAT-dnStat5a
TATwtAkt
TATdnAkt
TAT-wtStat5a/wtAkt
TAT-dnStat5a/dnAkt
E
HMC-1
α-P-S-Akt
α-Akt
NaCl
TAT-dnSTAT5a
TAT- cS5F

Figure 7

3.1.3 Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2

Florian Grebien[1], **Marc A. Kerenyi**[1], Boris Kovacic[2], Thomas Kolbe[3,4], Verena Becker[5], Helmut Dolznig[6], Klaus Pfeffer[7], Ursula Klingmüller[5], Mathias Müller[3,8], Hartmut Beug[2], Ernst W. Müllner[1,10] and Richard Moriggl[9,10]

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HEMATOPOIESIS AND STEM CELLS

Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2

Florian Grebien,[1] Marc A. Kerenyi,[1] Boris Kovacic,[2] Thomas Kolbe,[3,4] Verena Becker,[5] Helmut Dolznig,[6] Klaus Pfeffer,[7] Ursula Klingmüller,[5] Mathias Müller,[3,8] Hartmut Beug,[2] *Ernst W. Müllner,[1] and *Richard Moriggl[9]

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Erythropoiesis requires erythropoietin (Epo) and stem cell factor (SCF) signaling via their receptors EpoR and c-Kit. EpoR, like many other receptors involved in hematopoiesis, acts via the kinase Jak2. Deletion of EpoR or Janus kinase 2 (Jak2) causes embryonic lethality as a result of defective erythropoiesis. The contribution of distinct EpoR/Jak2-induced signaling pathways (mitogen-activated protein kinase, phosphatidylinositol 3-kinase, signal transducer and activator of transcription 5 [Stat5]) to functional erythropoiesis is incompletely understood. Here we demonstrate that expression of a constitutively activated Stat5a mutant (cS5) was sufficient to relieve the proliferation defect of Jak2$^{-/-}$ and EpoR$^{-/-}$ cells in an Epo-independent manner. In addition, tamoxifen-induced DNA binding of a Stat5a–estrogen receptor (ER)* fusion construct enabled erythropoiesis in the absence of Epo. Furthermore, c-Kit was able to enhance signaling through the Jak2-Stat5 axis, particularly in lymphoid and myeloid progenitors. Although abundance of hematopoietic stem cells was 2.5-fold reduced in Jak2$^{-/-}$ fetal livers, transplantation of Jak2$^{-/-}$-cS5 fetal liver cells into irradiated mice gave rise to mature erythroid and myeloid cells of donor origin up to 6 months after transplantation. Cytokine- and c-Kit pathways do not function independently of each other in hematopoiesis but cooperate to attain full Jak2/Stat5 activation. In conclusion, activated Stat5 is a critical downstream effector of Jak2 in erythropoiesis/myelopoiesis, and Jak2 functionally links cytokine- with c-Kit-receptor tyrosine kinase signaling. (Blood. 2008;111:4511-4522)

Introduction

Erythropoiesis is a tightly controlled process in bone marrow and spleen of adult mammals and in the fetal liver of embryos that produces highly variable erythrocyte numbers during fetal development and in diseases such as anemia, induced by, for example, hypoxia or blood loss. Erythroid maturation proceeds through burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) stages, the latter cell type dividing 4 to 5 times while maturing into erythrocytes in response to erythropoietin (Epo).

Epo is strictly required for erythropoiesis, promoting survival and late maturation stages.[1] Ligand-induced Epo-receptor (EpoR) dimerization triggers activation of the pre-associated kinase Jak2, which then phosphorylates tyrosine residues in the cytoplasmic tail of EpoR. These phosphotyrosines serve as docking sites for SH2-domain containing proteins, leading to activation of various signaling pathways, including phosphatidylinositol 3-kinase (PI3-K),[2] mitogen-activated protein kinase,[3] protein kinase C,[4] and phospholipase C-γ.[5] A central pathway in EpoR signaling, however, is the activation of the transcription factor known as signal transducer and activator of transcription 5 (Stat5).[6-8] Upon phosphorylation, Stat5 dimers translocate to the nucleus, bind to cognate elements in various promoters, and activate transcription. Stat5-mediated functions regulate cell proliferation, differentiation, apoptosis, and other processes. Several important Stat5 target genes, such as *Pim*, *c-Myc*, *OncostatinM*, *Bcl-xL*, *SOCS*, or *D-type cyclins* are required for functional erythropoiesis.[9-14]

The requirement of Epo signaling pathway components for erythropoiesis is evident from mice deficient for Epo, EpoR, or Jak2. All mutant animals die in utero at embryonic day 13.5 (E13.5) because of a failure of erythropoiesis; BFU-E and CFU-E progenitors are completely absent from the fetal liver.[15-17] The absolute requirement for Jak2 to transduce EpoR signals was recently substantiated by mutational analyses of EpoR domains: all EpoR mutants unable to bind Jak2 were nonfunctional.[18]

Knowledge about signaling downstream of Epo is still limited. Exogenous Bcr-Abl rescued the erythroid defect of Jak2$^{-/-}$ fetal liver cells in vitro.[19] Likewise, a dominant-active mutant of Akt restored erythroid differentiation in Jak2-deficient erythroid progenitors.[20]

At first, Stat5 seemed to be nonessential for erythropoiesis, in that original Stat5ab$^{-/-}$ mice were viable and showed no overt erythroid defects.[8] Later, these mice were found to display fetal anemia and elevated rates of apoptosis of erythroid cells as a result of a failure in Bcl-xL up-regulation.[21,22] A recently generated complete knockout of Stat5ab, however, is perinatally lethal and

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*E.W.M. and R.M. contributed equally to this work.

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highly anemic in utero.[6] Conversely, mice expressing truncated EpoR-mutants that retain solely the ability to activate Stat5 but lack all other tyrosines critical for activation of other signaling pathways live normally.[23] These animals display only mild phenotypes in recovery from erythropoietic stress. Additional mutation of Tyr343 (required for Stat5 activation), however, strongly affected stress erythropoiesis.[24]

This wide range of erythroid phenotypes of mice mutated in the EpoR/Jak2/Stat5 axis prompted us to clarify the role of Stat5 in erythropoiesis. To this purpose, we introduced a hyperactivatable mutant of Stat5a (cS5, S711F[25]) into EpoR$^{-/-}$ and Jak2$^{-/-}$ hematopoietic cells. Tyrosine-phosphorylated, DNA-bound cS5 complemented the proliferation defect of the mutant cells, enabling self-renewal and erythroid differentiation in the absence of Epo signals. Likewise, 4-hydroxy-tamoxifen (4-OH-T)-induced activation of a Stat5a–estrogen receptor (ER)* fusion construct was sufficient to replace Epo in erythropoiesis. Jak2-deficient fetal liver cells also showed defects in myelopoiesis and massively decreased responses to SCF, SCF + IL-3, or SCF + IL-7. Expression of cS5 in Jak2$^{-/-}$ cells partially corrected these proliferation defects in vitro. Moreover, Jak2$^{-/-}$-cS5 cells efficiently contributed to the erythroid and myeloid lineages in vivo upon transplantation. cS5-mediated rescue of myeloid and erythroid lineages was strictly dependent on c-Kit signaling and Jak2.

Methods

All animal experiments were performed in accordance with Austrian and European laws and under approval of the ethical and animal protection committees.

Cell culture and retroviral infections

E12.5 fetal liver cells from Jak2$^{-/-}$, EpoR$^{-/-}$, and wild-type (WT) embryos were isolated and cultivated as described previously.[26] For a detailed description of isolation, retroviral infection, and culture of primary erythroblasts; see Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) 293T cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA). Transient transfections were done with Lipofectamine 2000 (Invitrogen). Colony assays of retrovirally transduced cells were performed in triplicate as described[17] using MethoCult M3234 (StemCell Technologies, Vancouver, BC). The Bcr-Abl-inhibitor imatinib (Gleevec; inhibiting also c-Kit) was used at 10 μmol/L.

Plasmids

The cS5 mutant of mouse Stat5a (S711F) was used.[27] In the murine Stat5a cDNA, however, the serine residue lies at amino acid position 710 instead of 711 as initially reported by Onishi et al.[25] For clarity, the initial nomenclature was retained here. For the generation of Stat5-ER* constructs, a point-mutated ligand binding domain of the estrogen receptor[28] was fused in frame to the C terminus of Stat5a, or cS5 or Stat5aΔ749,[7] after digestion with SacII. The Stat5a-EE/AA and Stat5a-Y_{694}F mutants were described by Wang et al.[29] Both mutations were introduced into cS5 by polymerase chain reaction (PCR) mutagenesis or cassette exchange.

All constructs were cloned into the retroviral vector pMSCV-IRES-GFP (Clontech, Mountain View, CA) and verified by sequencing. Ecotropic, replication incompetent gp + E86 producers were generated as described previously[27] and selected for high virus titer production by fluorescence-activated cell sorting (FACS).

Transplantation of fetal liver-derived hematopoietic progenitors

Freshly isolated E12.5 WT and Jak2$^{-/-}$ fetal liver cells (C57Bl/6, CD45.2) were cocultured with retrovirus-producing cells in DMEM, 15% FCS, stem cell factor (SCF; 200 ng/mL), interleukin-6 (IL-6, 50ng/mL; R&D Systems, Minneapolis, MN) and IL-3 (25 ng/mL; R&D Systems) for 72 hours. Hematopoietic cells (1-2 × 10^6) were injected into 4- to 6-week-old mice (B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ; Ly-5.1; CD45.1) after sublethal irradiation (750 rad) via the tail vein. Engraftment of injected cells was monitored by FACS analysis of peripheral blood starting 4 weeks after transplantation at regular intervals.

Flow cytometry

Cultured erythroblasts or single cell suspensions of spleen and bone marrow cells from mice 6 months after transplantation were stained with fluorescence-conjugated antibodies against Ter-119, CD71, GR-1, Mac-1, c-Kit, Sca-1, CD19, B220, CD4, and CD8 (all from BD Biosciences, San Jose, CA). Annexin V staining was performed according to the manufacturer's instructions (BD Biosciences). Samples were analyzed on a FACScalibur flow cytometer (BD Biosciences).

Cytokine stimulation, Western blot analysis, and DNA binding assays

Cultured erythroblasts were starved for 3 hours in plain DMEM and subsequently stimulated for 10 minutes with Epo (10 U/mL) or SCF (100 ng/mL) or with SCF plus imatinib (10 μmol/L). 293T cells transiently transfected with the murine pXM-EpoR expression vector and different Stat5 constructs were stimulated with Epo (50 U/mL) and/or 4-OH-T (50 nM) for 30 minutes. Sample preparation and Western blotting was performed according to standard techniques. Antibodies used for Western blotting were anti-phospho-Stat5ab (Millipore, Billerica, MA), anti-Stat5ab (BD Biosciences), anti-Jak2 (Cell Signaling Technology, Danvers, MA), anti-EpoR (Santa Cruz Biotechnology, Santa Cruz, CA), anti-extracellular signal-regulated kinase 1/2 (Sigma-Aldrich, St Louis, MO), and anti-Actin (Sigma-Aldrich). Stat5-EMSAs were performed as described previously.[7]

Reporter gene assays

Self-renewing primary WT and Jak2$^{-/-}$ erythroblasts were transfected in triplicate with 2.5 μg of luciferase reporter constructs containing the β-casein-promoter (β-casein-luc)[30] or the promoter of the IL-2R-α gene (IL-2R-α-luc)[31] along with 0.5 μg of pRL-TK2 (Promega, Madison, WI) using the Nucleofector technology (program U-08; Amaxa Biosystems, Gaithersburg, MD). Cells were stimulated with Epo (50 U/mL) for 6 hours after transfection or left untreated. Luciferase activity was measured 12 hours after transfection.

Quantitative PCR

RNA was isolated using TRIzol (Invitrogen). RNA integrity was checked with a Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA (2.5 μg) was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed on an Eppendorf RealPlex cycler using RealMasterMix (Eppendorf North America, New York, NY) and SYBR Green. The sequences of primers used can be found in Document S1.

Results

Persistent Stat5 activation complemented the proliferation defect of EpoR$^{-/-}$ and Jak2$^{-/-}$ erythroid cells

Primary WT erythroblasts cultivated in vitro undergo limited self-renewal in response to SCF, Epo, and dexamethasone (Dex).[26] Such expanding progenitors behave like primary erythroid cells from young mice, expressing only adult hemoglobins.[32] Fetal liver-derived cells from WT, EpoR$^{-/-}$, or Jak2$^{-/-}$ E12.5 embryos were tested for outgrowth of immature erythroblasts in the presence of SCF, Epo, and Dex (hence termed "self-renewal conditions"). WT fetal liver erythroblasts proliferated exponentially for 15 days (Figure 1A left), whereas EpoR$^{-/-}$ and Jak2$^{-/-}$

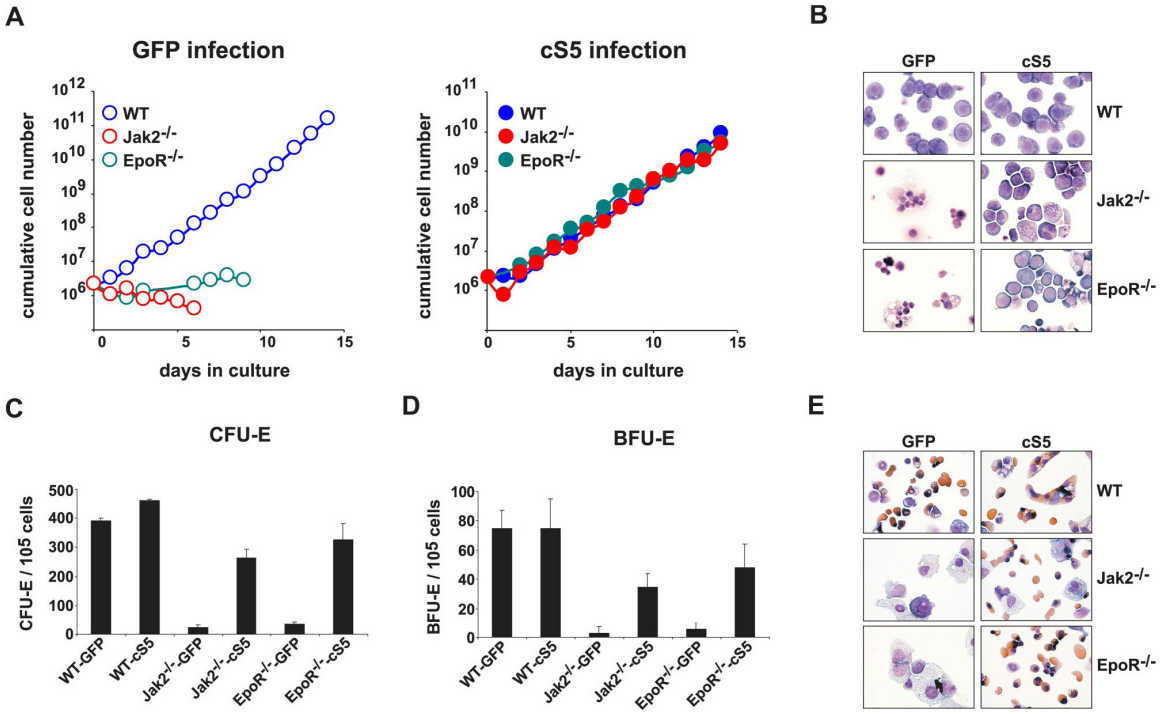

Figure 1. Expression of cS5 rescues proliferation and differentiation of EpoR$^{-/-}$ and Jak2$^{-/-}$ erythroid cells. (A) E12.5 WT, EpoR$^{-/-}$, and Jak2$^{-/-}$ fetal liver cells infected with retroviruses encoding GFP (left panel) or cS5 (right panel) were cultivated in proliferation medium and cumulative cell numbers calculated after daily determination of growth rates. Data plotted for 1 typical experiment of 4. (B) Cytospins were prepared at day 6 from erythroid cultures shown in (A) and stained for hemoglobin (brownish color) plus histologic dyes. WT, Jak2$^{-/-}$, and EpoR$^{-/-}$ fetal liver cells expressing cS5 or GFP were subjected to CFU-E- (C) or BFU-E assays (D), and acid benzidine–positive colonies were scored at day 2 (CFU-E) or day 8 (BFU-E), respectively. (E) Cytospins of cells retrieved from the CFU-E assays in (C), stained with hematoxylin/eosin and for hemoglobin (brownish color).

fetal liver cells failed to do so and disintegrated (Figure 1B). To clarify the role of Stat5 in Epo signaling, we used the Stat5a mutant S711F (cS5), which is persistently tyrosine-phosphorylated and exhibits enhanced chromatin binding activity.[27] Retroviral transduction of EpoR$^{-/-}$ and Jak2$^{-/-}$ cells with cS5 rescued the severe proliferation defect of mutant cells. All cS5-transduced cell types could be expanded for more than 2 weeks with identical proliferation kinetics (Figure 1A right). WT and cS5-transduced mutant cells showed a typical pro-erythroblast phenotype after 6 days of expansion in cytospins (Figure 1B), whereas uninfected control cells disintegrated. Complementation was confirmed by surface marker analysis after 9 days, which showed coexpression of c-Kit and CD71 together with low levels of Ter119. All cultures contained low numbers of immature progenitors (c-Kit^{+}Sca-1^{+}), whereas T- and B-lymphoid cells were absent (Table S1). Although after 3 days, approximately 65% of immature progenitors were retrovirally infected under the conditions used (data not shown), cS5 did not induce proliferation of multipotent cells, because these would have contributed to both erythroid and other myeloid lineages.

To confirm cS5-dependent erythropoiesis of EpoR$^{-/-}$ and Jak2$^{-/-}$ cells, colony-forming assays for committed erythroid progenitors (BFU-E, CFU-E) were performed. In WT cells, cS5 expression did not alter the numbers of benzidine-positive BFU-E or CFU-E colonies compared with green fluorescent protein (GFP)–vector infected controls (Figure 1C,D). Conversely, the low incidence of CFU-E and BFU-E colonies in EpoR$^{-/-}$ and Jak2$^{-/-}$ cells transduced with a GFP vector control could be raised approximately 10-fold upon expression of cS5, reaching levels comparable with those of WT cultures. Remarkably, Jak2$^{-/-}$-cS5 and EpoR$^{-/-}$-cS5 colonies showed the same, typical morphology as WT colonies (Figure S1) and contained mature, enucleated, highly hemoglobinated erythrocytes, similar to WT samples (Figure 1E).

In contrast to cS5, retrovirally transduced WT Stat5a or Bcl-xL failed to induce efficient erythroid colony formation in EpoR$^{-/-}$ and Jak2$^{-/-}$ cells (Figure S2). Thus, activated Stat5 was required and sufficient to overcome the proliferation defect and erythroid colony formation potential of EpoR- and Jak2-deficient cells.

cS5 expression allowed Epo-independent erythropoiesis

Next we sought to determine whether cS5 activity was sufficient to substitute for Epo signaling in erythropoiesis. WT, EpoR$^{-/-}$, and Jak2$^{-/-}$ cells expressing cS5 were kept in Epo-free medium for 6 days. cS5-expressing WT, EpoR$^{-/-}$, or Jak2$^{-/-}$ cells continued to proliferate in the absence of Epo (Figure 2A) without detectable cell disintegration (Figure 2B). WT controls expressing GFP ceased to proliferate after 3 to 4 days (Figure 2A). Dead cells (Figure 2B arrows) and pyknotic nuclei (Figure 2B arrowheads) became visible in cytospins after 4 days without Epo. Withdrawal of SCF rapidly induced terminal differentiation and/or apoptosis in all cell types, indicating that cS5 could not substitute for SCF-signaling (data not shown).

In CFU-E assays, Epo withdrawal prevented colony formation of WT cells. In contrast, cS5-expressing WT, EpoR$^{-/-}$, and Jak2$^{-/-}$ cells formed erythroid colonies at more than 100-fold

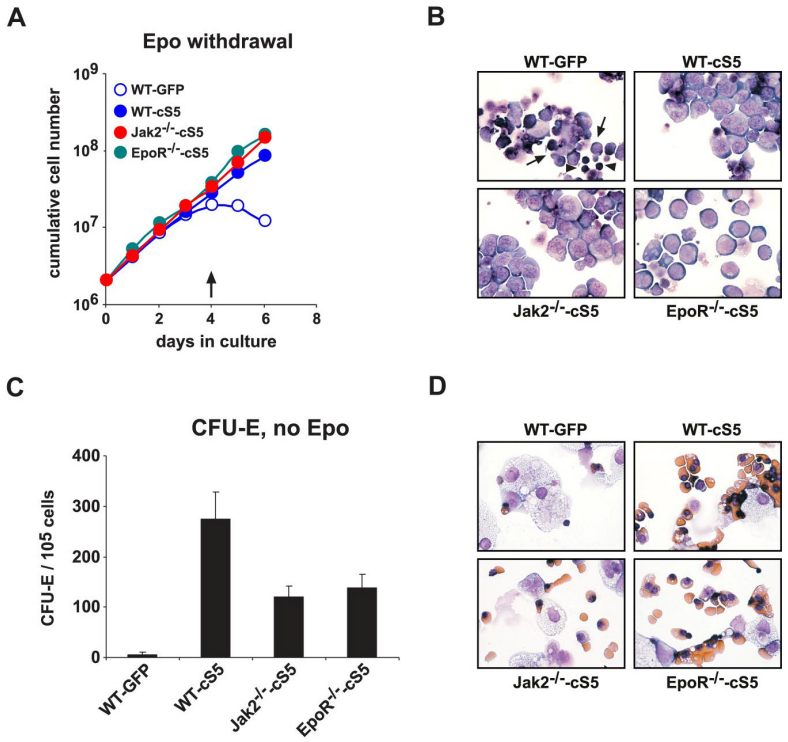

Figure 2. cS5 expression allows erythroid development in the absence of Epo. (A) E12.5 WT, EpoR$^{-/-}$, and Jak2$^{-/-}$ fetal liver cells were infected with retroviruses encoding GFP or cS5 and cultivated in proliferation medium without Epo for 6 days. Cumulative cell numbers are shown for one representative experiments of 3. (B) Cytospins were prepared from cultures shown in panel A at day 4 (→ in A) and stained with hematoxylin/eosin and benzidine. → indicates apoptotic cells; ▶, pyknotic nuclei. (C) WT fetal liver cells expressing cS5 or GFP, or Jak2$^{-/-}$ and EpoR$^{-/-}$ cells expressing cS5, were subjected to CFU-E assays in the absence of Epo and acid benzidine–positive colonies scored at day 2. (D) Cytospins of cells retrieved from the CFU-E assays in (C), stained with hematoxylin/eosin and for hemoglobin (brownish).

higher frequency under the same conditions (Figure 2C). Epo-independent CFU-E formation was more efficient in WT cS5 cells than EpoR$^{-/-}$-cS5 or Jak2$^{-/-}$-cS5 cells. Cytospins of cells retrieved from the CFU-E assays revealed the presence of normoblasts and enucleating erythrocytes in cS5 expressing WT, Jak2$^{-/-}$, and EpoR$^{-/-}$ cells but not in WT GFP cultures (Figure 2D). Apparently, Stat5 activity via cS5 can partially but effectively substitute for Epo signals in WT, EpoR$^{-/-}$, and Jak2$^{-/-}$ cells during both erythroid progenitor proliferation and terminal differentiation.

cS5 induced Epo-independent Stat5 reporter gene expression but its activity was dependent on tyrosine phosphorylation and DNA-binding

To test whether Epo-independent erythropoiesis in cS5-expressing cells was really due to transcriptional activity of cS5, we performed promoter reporter experiments, using a previously described construct containing a Stat5-responsive part of the IL-2Rα gene enhancer[31] (Figure 3A, right). Primary WT GFP, WT cS5, and Jak2$^{-/-}$-cS5 erythroblasts were transfected with IL-2Rα-luc, and the cells were stimulated with Epo 5 hours before harvest or left untreated. WT GFP cells showed a modest induction of reporter gene expression upon stimulation with Epo (Figure 3A). However, in cS5-expressing WT and Jak2$^{-/-}$ erythroblasts, we observed a much higher induction of the reporter gene expression, even in the absence of Epo stimulation (Figure 3A). Similar results were obtained using a different reporter construct, containing the Stat5-responsive part of the β-casein promoter[30] (data not shown). This indicates that cS5 indeed was able to activate gene expression in WT and Jak2$^{-/-}$ erythroid cells.

To prove that the cS5-mediated rescue of erythropoiesis in Jak2$^{-/-}$ and EpoR$^{-/-}$ cells was indeed dependent on transcriptional activation of DNA-bound, tyrosine-phosphorylated Stat5 complexes, 2 additional cS5-derived constructs were generated (Figure 3B). In the mutant cS5-EE/AA, 2 glutamic acid residues (Glu437/Glu438) in the DNA-binding domain of cS5 are mutated to alanine, resulting in a tyrosine-phosphorylated cS5 molecule unable to bind to DNA (Figure 3C,D). Conversely, the Y694F mutation in cS5 prevented phosphorylation of the critical tyrosine required for dimerization and DNA binding in transient transfections of 293T cells (Figure 3C,D). Expression of cS5-EE/AA and cS5-Y694F in primary WT fetal liver erythroblasts did not significantly alter CFU-E formation compared with expression of cS5 or GFP (data not shown). In CFU-E assays of Jak2$^{-/-}$ cells expressing GFP, cS5, or the mutants cS5-EE/AA and cS5-Y694F, however, only expression of cS5 resulted in a significant increase in erythroid colony formation (Figure 3E). The same results were obtained using EpoR$^{-/-}$ cells (Figure 3F). Thus, tyrosine-phosphorylation and DNA-binding functions of cS5 are required to allow erythropoiesis of Jak2$^{-/-}$ and EpoR$^{-/-}$ cells.

Induced activation of Stat5 could replace Epo in erythropoiesis

Because cS5 is a leukemogenic protein with enhanced signaling capacity, we generated constructs consisting of Stat5a or cS5 fused to ER*, a point-mutated ligand-binding domain of the estrogen receptor.[33] In the respective fusion proteins (Stat5a-ER*, cS5-ER*; Figure 4A), dimerization, nuclear translocation, DNA binding, and transcriptional activity can be triggered by 4-hydroxy-tamoxifen (4-OH-T).[28] It is noteworthy that the cS5-dependent hyperactivation of PI3-K signaling[34] was not induced by cS5-ER* compared with Stat5a-ER* (Figure S3A). 4-OH-T or Epo induced specific binding of Stat5a-ER* as well as cS5-ER* to a β-casein Stat5 DNA response element, whereas 4-OH-T + Epo strongly enhanced Stat5a-ER* DNA binding (Figure S3B). Retrovirally transduced Stat5a-ER*

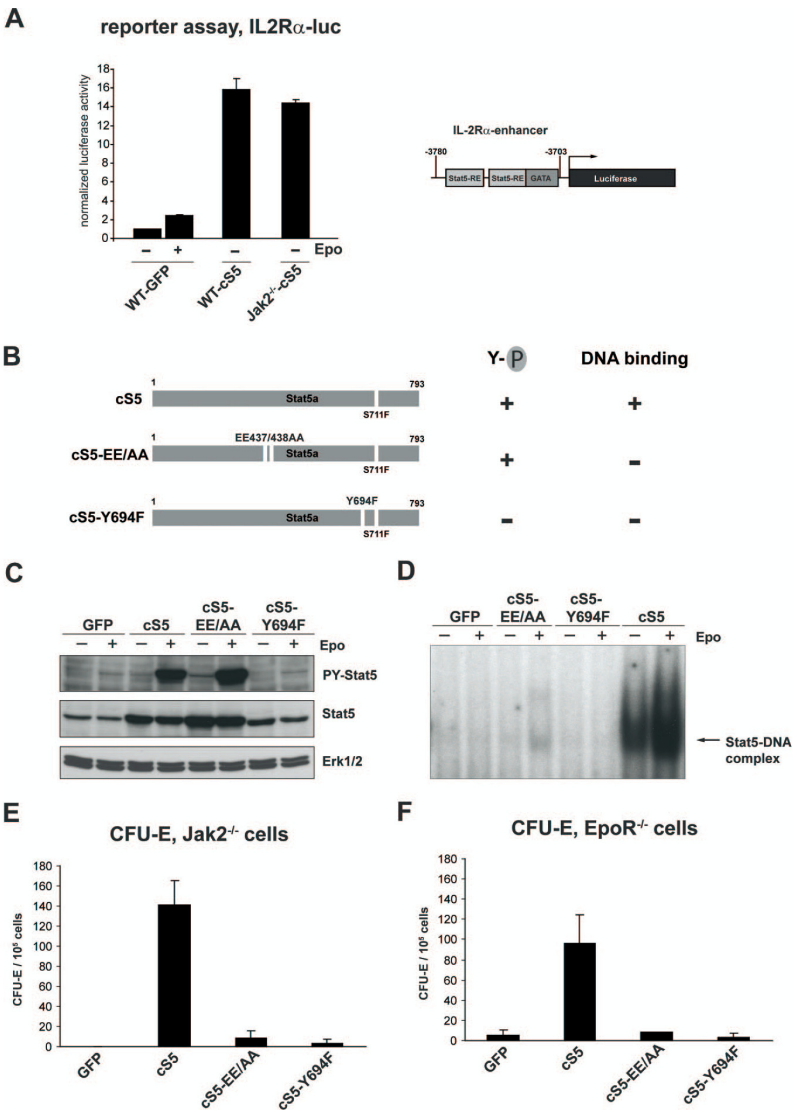

Figure 3. cS5 allows Epo-independent induction of a Stat5-responsive reporter construct but requires tyrosine phosphorylation and DNA-binding for activity. (A) Self renewing WT GFP and cS5-expressing WT and Jak2$^{-/-}$ erythroblasts were transfected with IL-2R-α-Luc. Before harvesting, cells were stimulated with Epo for 5 hours (+) or left untreated (–). Luciferase expression was measured 12 hours after transfection. Right, schematic representation of the promoter enhancer element from the IL-2R-α gene containing 2 Stat5 response elements (Stat5-RE) fused to the luciferase gene (IL-2R-α-Luc). (B) Schematic representation of the mutants used. 293T cells were transiently transfected with different cS5 constructs, together with a murine EpoR cDNA. Twenty-four hours later, cells were left untreated or treated for 30 minutes with Epo. Extracts were analyzed for tyrosine-phosphorylated Stat5 and total Stat5 by Western blot (C) and DNA-binding of transfected Stat5 constructs by EMSAs on a β-casein-specific promoter sequence (D). Jak2$^{-/-}$ (E) and EpoR$^{-/-}$ (F) fetal liver cells were infected with retroviruses encoding GFP, cS5, cS5-EE/AA, or cS5-Y694F and subjected to CFU-E assays. Acid benzidine–positive colonies were scored at day 2.

underwent Epo-induced tyrosine phosphorylation in primary WT fetal liver erythroblasts, as shown by Western blot analysis (Figure 4B). An inducible dominant-negative Stat5-ER* fusion protein (Stat5aΔ749-ER*, Figure 4A[7]) was not tolerated in primary erythroid cells, resulting in strong negative selection against cells expressing this construct (data not shown).

Using primary erythroblasts expressing Stat5a-ER* or GFP, we tested whether 4-OH-T–induced activation of WT Stat5 could substitute for Epo in erythroid progenitor expansion. Under self-renewal conditions, both Stat5a-ER*- and GFP-expressing cells proliferated with identical kinetics (Figure 4C left) and equally low rates of apoptosis (annexin V staining, day 6, Figure 4D left). Replacement of Epo with 4-OH-T under the same conditions allowed sustained proliferation of Stat5a-ER* expressing cells, but not of GFP-control cells (Figure 4C middle), consistent with corresponding apoptotic indices (~10% vs > 50% annexin V–positive cells; Figure 4D middle). In the absence of Epo and 4-OH-T, both cell types gradually ceased to proliferate and underwent cell death (Figure 4C,D right). Cultures of fetal liver cells expressing cS5-ER* instead of Stat5a-ER* behaved similar in these experiments (data not shown).

Induction of terminal erythroid differentiation by 4-OH-T-activated Stat5a-ER* was analyzed in CFU-E assays in the presence of 4-OH-T instead of Epo. Stat5a-ER*– but not GFP-expressing cells formed CFU-E colonies (Figure 4E), which in both cases mainly consisted of mature normoblasts and erythrocytes as seen in cytospins of cells recovered from CFU-E assays and stained for hemoglobin (Figure 4F).

Taken together, 4-OH-T–induced activation of Stat5a-ER* was able to significantly substitute for Epo signaling in erythropoiesis.

A role for Jak2 in c-Kit signaling in hematopoietic progenitors

In addition to EpoR, Jak2 interacts with a variety of other cytokine receptors. Thus, we tested whether cS5 would alleviate Jak2 deficiency also in other lineages. cS5- or GFP-expressing WT or Jak2$^{-/-}$ fetal liver cells were subjected to colony-forming assays in the presence of granulocyte macrophage–colony-stimulating factor

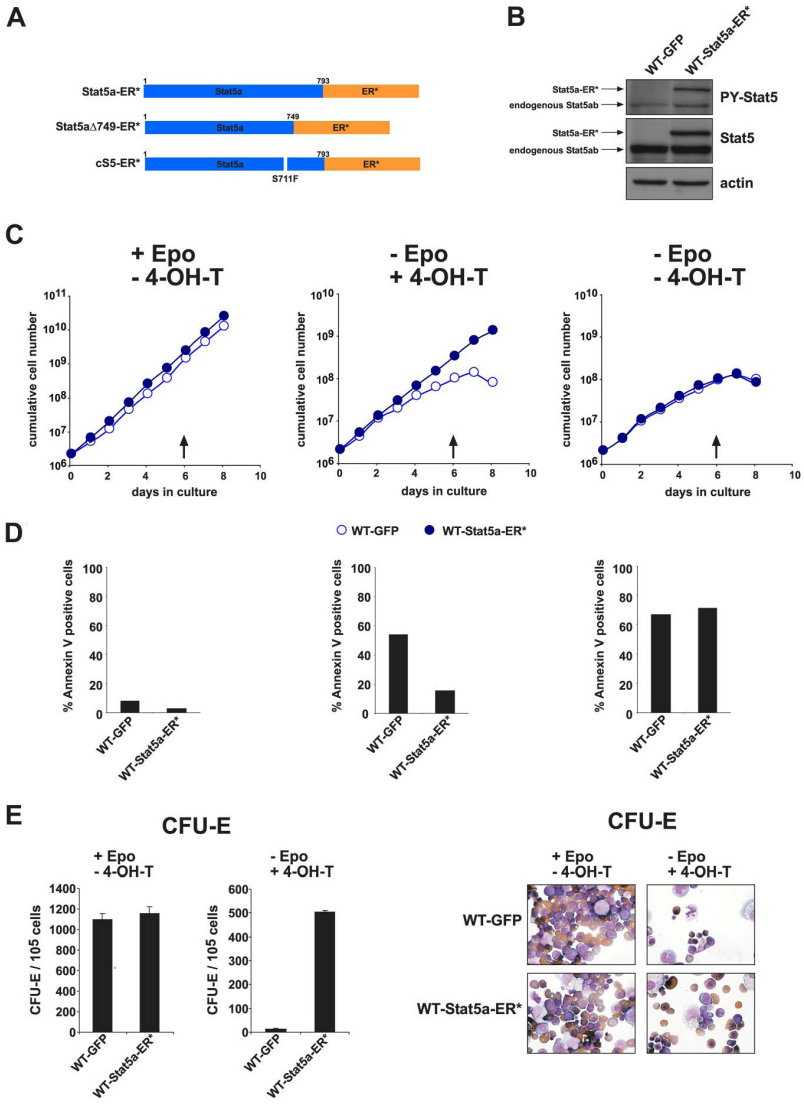

Figure 4. 4-Hydroxy-tamoxifen-induced Stat5a-ER* activation replaced Epo in erythropoiesis. (A) Scheme of the 4-OH-T-inducible Stat5-ER* constructs used, encoding fusion proteins of WT Stat5a, cS5, or Stat5aΔ749 with ER* (see "Methods"). (B) Western blot analysis of phosphorylated Stat5 (P-Y-Stat5), and total Stat5 protein in WT fetal liver erythroblasts expressing GFP or Stat5a-ER*. The larger protein recognized by P-Y-Stat5 and Stat5 antibodies corresponds to Stat5a-ER*. Actin, loading control. (C) Cumulative cell numbers (1 representative experiment of 3) of proliferating primary erythroblast cultures expressing Stat5a-ER* or GFP determined in the presence of Epo (+ EPO, −4-OH-T, left, normal self-renewal conditions), the presence of 4-OH-T (5 nM) instead of Epo (−Epo, + 4-OH-T, middle) and without Epo and 4-OH-T (right). (D) Percentage of apoptotic cells of cultures in panel C at day 6 (arrows in panel C) as analyzed by annexin V staining. (E) WT fetal liver cells expressing Stat5a-ER* or GFP were subjected to CFU-E assays in the presence of Epo (left panels) or 4-OH-T (50 nM) instead of Epo (right panels). Acid benzidine–positive colonies were scored at day 2. (F) Cytospins of cells retrieved from the CFU-E assays in panel E and stained with hematoxylin/eosin and for hemoglobin (brownish color).

(GM-CSF). Jak2$^{-/-}$-GFP cells yielded 5.2-fold lower colony numbers than WT GFP cells, whereas expression of cS5 increased colony numbers 2.6-fold in Jak2$^{-/-}$ cells (Table 1). Therefore, activation of Stat5 may be sufficient to augment myeloid differentiation in absence of Jak2.

Immature blood cells of all lineages require c-Kit signaling for proliferation and/or differentiation, mainly visible in cooperation with other hematopoietic cytokines.[35-37] We therefore analyzed whether loss of Jak2 would affect colony formation induced by SCF alone or in combination with other cytokines. Jak2-deficient

Table 1. Colony formation ability of fetal liver hematopoietic cells in response to various cytokines and growth factors is dependent on Jak2 and Stat5

Cytokine	WT GFP	WT cS5	Jak2$^{-/-}$-GFP	Jak2$^{-/-}$-cS5	EpoR$^{-/-}$-GFP	EpoR$^{-/-}$-cS5
Epo	392 ± 8	461 ± 5†	24 ± 8	264 ± 29†	35 ± 9	325 ± 57†
Epo + IL-3	75 ± 12	75 ± 20‡	3 ± 5	35 ± 9†	5 ± 5	48 ± 16†
GM-CSF	293 ± 26	432 ± 32†	56 ± 8	144 ± 29†	nd	nd
SCF	304 ± 8	352 ± 21*	109 ± 12	173 ± 20†	nd	nd
SCF + IL-3	448 ± 8	528 ± 28†	139 ± 12	219 ± 28†	nd	nd
SCF + IL-7	360 ± 14	408 ± 32‡	149 ± 20	216 ± 16†	nd	nd
None	0	0 ± 1	1	1 ± 1	1	0 ± 1

SCF-induced colonies represent immature progenitors and mast cells, SCF + IL-3 and SCF + IL-7 induce colony formation of myeloid and lymphoid progenitors (data not shown).

nd indicates not determined.

*$P < .05$.

†$P < .01$.

‡Not significant.

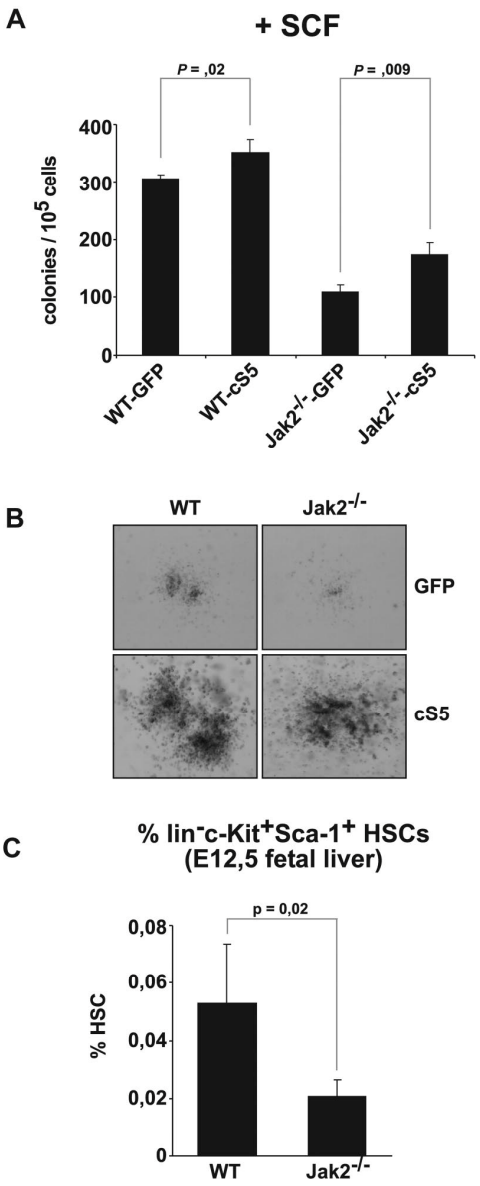

Figure 5. c-Kit signaling depends on Jak2 and is modulated by Stat5. (A) WT and Jak2$^{-/-}$ fetal liver cells transduced with GFP or cS5 were subjected to colony assays supplemented with SCF (50 ng/mL). Colonies were scored at day 8. (B) Photographs of colonies from (A). Representative pictures of 4 for each condition are shown. (C) E12.5 WT and Jak2$^{-/-}$ fetal livers analyzed for HSC content (lin$^-$c-Kit$^+$Sca-1$^+$ cells) by flow cytometry. The percentage of HSCs is depicted (n = 4).

fetal liver cells indeed showed a 2.8-fold reduction in SCF-dependent colony formation compared with WT cells (Figure 5A), in line with previous findings.[17,38] Jak2$^{-/-}$-cS5 cells generated significantly more colonies than Jak2$^{-/-}$-GFP cells (1.6 fold; Figure 5A). The size of SCF-dependent colonies was strongly reduced in Jak2$^{-/-}$-GFP cells but massively increased by cS5 in both WT and Jak2 mutant cells (Figure 5B). Colony formation of early myeloid and lymphoid progenitors induced by SCF + IL-3 and SCF + IL-7, respectively, was 3.2- and 2.5-fold reduced in Jak2-deficient cells (Table 1). Again, cS5 expression led to more than 1.6-fold increased colony numbers when expressed in Jak2$^{-/-}$ cells, whereas WT cells showed a similar but weaker increase (Table 1).

Hematopoietic stem cells (HSCs; lin$^-$c-Kit$^+$ Sca-1$^+$ cells) also depend on SCF for proliferation.[39] Because c-Kit function might be linked with Jak2, we tested whether Jak2 deficiency would affect HSC numbers. Indeed, HSC numbers were approximately 70% lower in Jak2$^{-/-}$ fetal livers than in WT fetal livers (n = 4; Figure 5C) as determined by flow cytometry in freshly prepared fetal livers of E12.5 embryos. These observations argue that Jak2 has an essential function in hematopoietic stem cells.

Complementation of Jak2 deficiency in vivo

The finding that Jak2 is not only necessary for erythropoiesis but also important for expansion of c-Kit–responsive immature hematopoietic progenitors and HSCs prompted us to analyze whether Jak2$^{-/-}$ cells expressing cS5 were capable of long-term repopulation of erythromyeloid lineages in vivo. Freshly isolated fetal liver cells from WT and Jak2$^{-/-}$ embryos were transduced with cS5. Equal cell numbers were injected into irradiated recipient mice 72 hours later. To rule out that Jak2$^{-/-}$ fetal livers generated lower numbers of HSCs during the retroviral infection period, we determined the amounts of GFP-positive, long-term (LT) HSCs 72 hours after retroviral infection. Thy1.1^{low}/Flt3$^-$ LT-HSCs are the sole cells capable of long-term reconstitution of transplanted mice.[40] We found that LT HSCs were efficiently infected by the retrovirus, leading to an equal presence of GFP$^+$ LT HSCs in WT cS5 and Jak2$^{-/-}$-cS5 cultures (1667 ± 451 GFP$^+$ LT HSC per 10^6 WT cS5 cells; 1967 ± 351 GFP$^+$ LT HSC per 10^6 Jak2$^{-/-}$-cS5 cells; n = 3, data not shown).

Use of congenic mice expressing CD45.1 allowed detection of CD45.2$^+$ donor cells among the host cells.[41] Because cS5-expressing fetal liver cells cause leukemia,[27] onset of the disease was strongly delayed by transplanting reduced numbers of cS5-transduced fetal liver cells into sublethally rather than lethally irradiated mice. Indeed, all mice in which the transplanted Jak2$^{-/-}$-cS5 cells were engrafted (n = 6) remained disease-free for more than 6 months and did not develop leukemia.

Transplanted mice were regularly monitored for transplant-derived, GFP-CD45.2 double-positive cells in peripheral blood. Animals were killed 6 months after transplantation and assayed for the presence of GFP$^+$Ter119$^+$ erythroid cells in bone marrow and spleen and for GFP$^+$GR-1$^+$ and/or GFP$^+$Mac-1$^+$ myeloid cells in peripheral blood and bone marrow. Significant numbers of transplant-derived, GFP$^+$Ter119$^+$ double-positive cells were observed in spleen and bone marrow of mice receiving WT cS5 cells

Table 2. Hematopoietic repopulation is influenced by Jak2

Transplant	Mice engrafted	Engraftment of GFP$^+$CD45.2$^+$ cells in peripheral blood % (range)	Erythroid (mice with GFP$^+$Ter119$^+$ cells)	Granulocytic (mice with GFP$^+$GR-1$^+$ cells)	Macrophage (mice with GFP$^+$Mac-1$^+$ cells)
WT-cS5	10 (10)	14.8 (4-40)	9 (10)	10 (10)	5 (10)
Jak2$^{-/-}$-cS5	6 (8)	8.6 (1-23)	4 (6)	6 (6)	2 (6)

GFP$^+$CD45.2$^+$ cells correspond to transplant-derived, cS5-expressing cells. Transplant-derived erythroid cells were scored in spleen and bone marrow, and GFP$^+$ myeloid (GR-1$^+$ and Mac-1$^+$) cells were measured in peripheral blood and bone marrow.

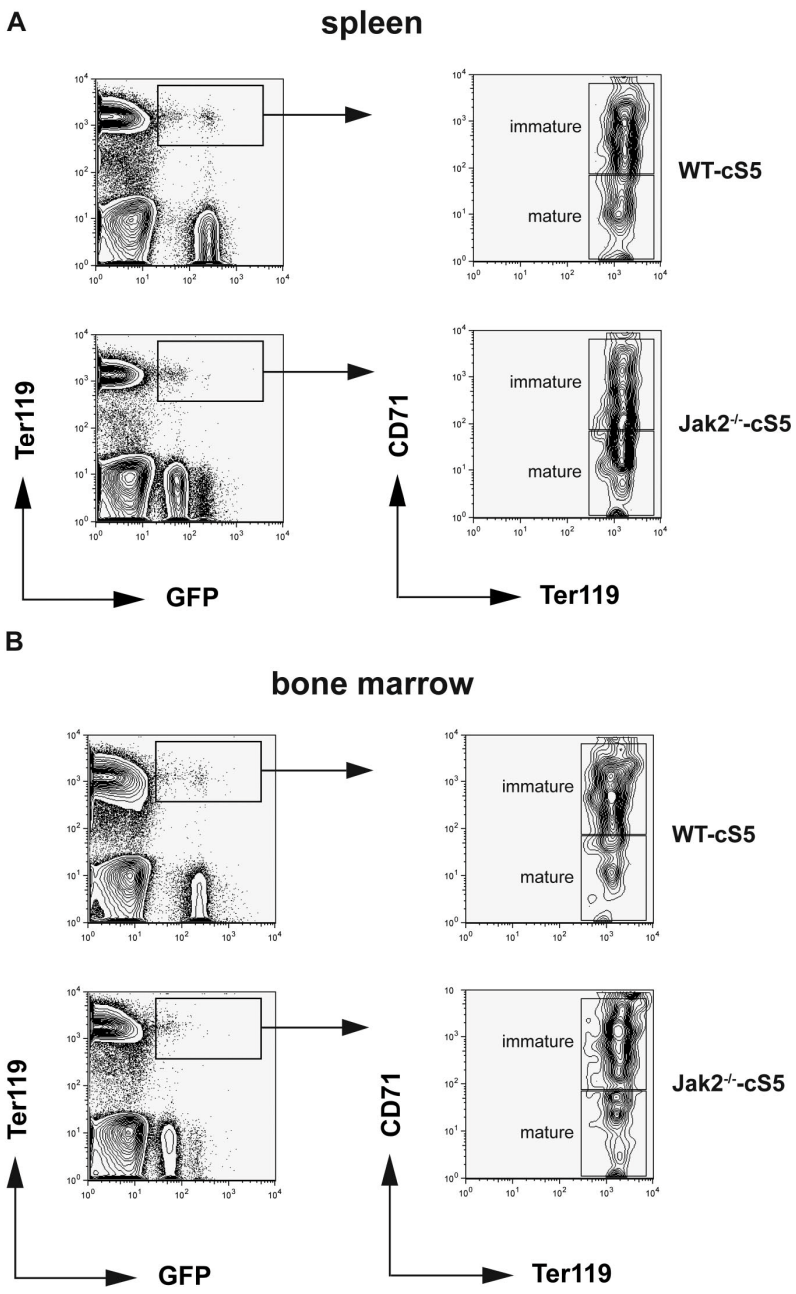

Figure 6. Jak2$^{-/-}$ cells expressing cS5 undergo erythroid differentiation in vivo. Equal numbers of cS5-transduced E12.5 WT and Jak2$^{-/-}$ fetal liver cells were injected into sublethally irradiated mice. Six months after transplantation, spleen (A) and bone marrow (B) of engrafted animals were monitored for GFP-positive erythroid cells. FACS plots for Ter119 and GFP (left) indicate transplant-derived double-positive erythroid cells. Gated cells (boxed) were further analyzed for CD71 and Ter119 (right) to discriminate between immature (CD71^{high}Ter119^{pos}) and mature (CD71^{low}Ter119^{pos}) erythroid cells.

(9 of 10 animals) as well as Jak2$^{-/-}$-cS5 (4 of 6 mice, Table 2, Figure 6A,B top and bottom left panels). This indicated that cS5 could partially but efficiently substitute for Jak2 in erythroid differentiation in vivo. To characterize transplant-derived erythroid cells for different stages of maturation, GFP$^+$Ter119$^+$ cells were gated for high versus low CD71 expression, allowing us to distinguish mature (Ter119$^+$CD71^{low}) from more immature erythroid progenitors (Ter119$^+$CD71$^+$). In both spleen and bone marrow, transplanted Jak2$^{-/-}$-cS5 as well as WT cS5 cells gave rise to multiple stages of erythroid cells in vivo, ranging from CD71$^+$Ter119$^+$ immature, basophilic erythroblasts to almost mature CD71$^-$Ter119$^+$ orthochromatophilic erythroblasts[22] (Figure 6A,B right panels).

Likewise, animals successfully engrafted with WT cS5 cells displayed robust contribution of GFP$^+$ cells to mature myeloid lineages (GFP$^+$GR-1$^+$ granulocytes, 10 of 10 mice; GFP$^+$Mac-1$^+$ monocytes/macrophages, 5 of 10 mice; Table 2) in peripheral blood and bone marrow (Figure S4 right panels and data not shown). It is noteworthy that Jak2$^{-/-}$-cS5 transplanted mice showed a significant contribution to the Mac-1$^+$- (2 of 6 animals with GFP$^+$ cells) and GR-1$^+$-compartments (6 of 6, Table 2; Figure S4 right panels), both in peripheral blood and bone marrow (Table 2 and data not shown). Thus, in GM-CSF-dependent myelopoiesis in vivo, cS5 can substitute for the lack of Jak2 to a significant extent.

It should be noted that overall engraftment was clearly lower in Jak2$^{-/-}$-cS5 transplanted mice (6 of 8, 8.6% engraftment) than in

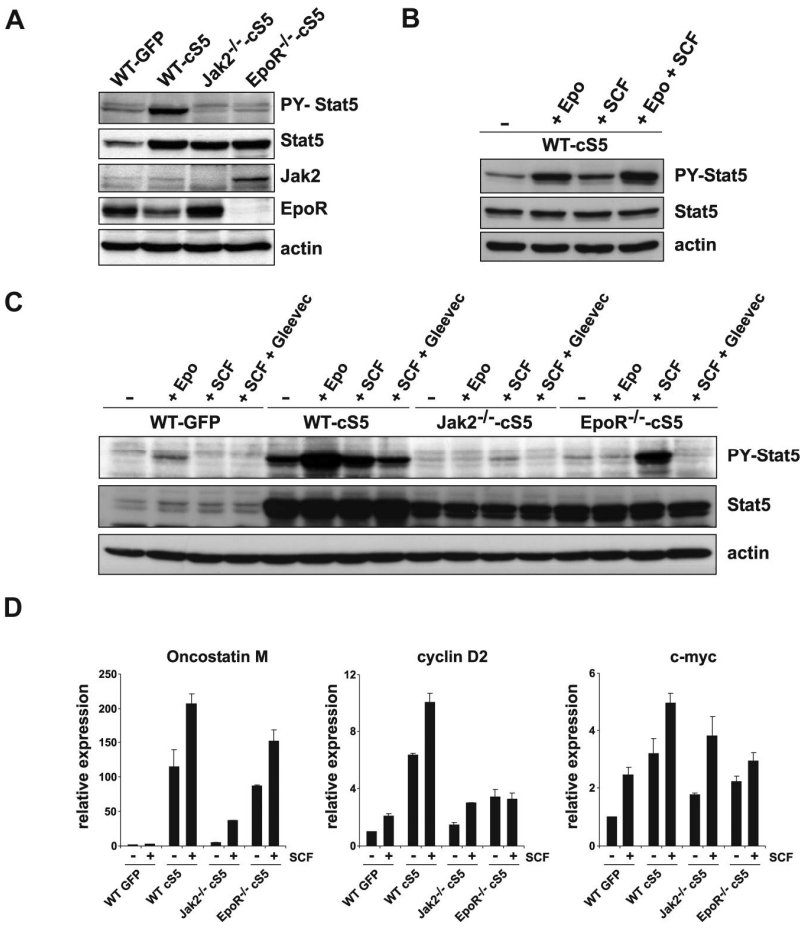

Figure 7. Jak kinase(s) and c-Kit cooperate in cS5 activation. (A) Lysates from self-renewing WT GFP, WT cS5, Jak2$^{-/-}$-cS5, and EpoR$^{-/-}$-cS5 cultures were analyzed for P-Y-Stat5, total Stat5, Jak2, and EpoR protein levels. Actin, loading control. (B) cS5-expressing WT erythroblasts were starved for 3 hours (−) and subsequently stimulated with Epo, SCF, or Epo + SCF for 10 minutes. Lysates were analyzed for P-Y-Stat5 and total Stat5 protein. Erk1/2, loading control. (C) WT erythroblasts expressing GFP or cS5, and Jak2$^{-/-}$ and EpoR$^{-/-}$ cells expressing cS5 were starved for 3 hours (−) and subsequently stimulated with Epo, SCF, or SCF + imatinib (10 μmol/L) for 10 minutes. Lysates were analyzed for P-Y-Stat5 and total Stat5 protein. Erk1/2, loading control. (D) WT GFP, WT cS5, Jak2$^{-/-}$-cS5, and EpoR$^{-/-}$-cS5 cultures were starved for 3 hours (−) and subsequently restimulated with SCF for 2 hours (+). Expression of the Stat5 target genes *oncostatinM*, *cyclin D2*, and c-*myc* was assessed by real-time PCR.

respective WT cS5 controls (10 of 10, 14.8% engraftment, Table 2). Likewise, contribution to erythroid (4 of 6) and macrophage lineages (2 of 6) was clearly reduced in Jak2$^{-/-}$-cS5 transplanted animals than in control WT cS5 mice (9 of 10 and 5 of 10, Table 2).

In summary, Jak2$^{-/-}$ cells expressing cS5 contribute to both erythroid and myeloid lineages upon transplantation, albeit with lower efficiency than WT cS5 cells. Furthermore, Jak2$^{-/-}$-cS5 cells were able to generate mature CD71^{-}Ter119^{+} erythroid cells. Thus, the severe defects resulting from Jak2 deficiency in erythro- and myelopoiesis can be efficiently but not completely corrected upon expression of cS5 in vivo.

cS5 phosphorylation required Jak2 or c-Kit kinase activity

We next tested the tyrosine phosphorylation status of WT Stat5 in self-renewing primary erythroblasts. WT cells expressing GFP exhibited low P-Y-Stat5 levels under self-renewal conditions[42] (Figure 7A). As expected, proliferating WT cells expressing cS5 displayed strongly increased P-Y-Stat5 abundance. It is noteworthy that Jak2$^{-/-}$-cS5 and EpoR$^{-/-}$-cS5 cells showed only modest P-Y-Stat5 levels, similar to those of WT GFP cells, although cS5 protein was similarly overexpressed in WT, Jak2$^{-/-}$, and EpoR$^{-/-}$ cells.

Thus, although the EpoR-Jak2 axis is the main pathway leading to Stat5 activation, the clearly detectable basal P-Y-Stat5 levels and, more importantly, the resulting strongly Stat5 activation in cells devoid of EpoR or Jak2, must originate from another tyrosine kinase. Because we found a role for Jak2 in c-Kit signaling, we reasoned that c-Kit might be critical for cS5 phosphorylation. cS5-expressing erythroblasts were stimulated with Epo, SCF, or Epo + SCF (see Document S1). Epo induced high P-Y-Stat5 levels, but SCF stimulation was also able to induce significant Stat5 phosphorylation (Figure 7B). Strikingly, the combination of Epo and SCF induced the highest P-Y-Stat5 levels, indicating that both c-Kit and EpoR could contribute to Stat5 activation. To elucidate the molecules involved in SCF-induced Stat5 activation, the previous experiment was repeated with WT GFP, WT cS5, EpoR$^{-/-}$-cS5, and Jak2$^{-/-}$-cS5 cells in the presence or absence of the Bcr-Abl inhibitor imatinib, which also inhibits c-Kit kinase activity. Analysis of P-Y-Stat5 levels revealed that Epo but not SCF stimulation induced significant levels of P-Y-Stat5 in WT GFP cells (Figure 7C). In contrast, WT cS5 cells showed high levels of P-Y-Stat5 after starvation, further increased by Epo. Jak2$^{-/-}$-cS5 cells showed lower expression levels of cS5 protein than WT cS5 or EpoR$^{-/-}$-cS5 cells. As expected, no significant P-Y-Stat5 was found after starvation or Epo stimulation. SCF stimulation induced significant Stat5-phosphorylation that could be blocked by imatinib treatment. In EpoR$^{-/-}$-cS5 cells, basal P-Y-Stat5 levels were unexpectedly high (similar to that in Jak2$^{-/-}$-cS5 cells after SCF stimulation) and did not disappear upon starvation. SCF strongly increased P-Y Stat5 levels, which were reduced back to basal levels by imatinib, whereas, as expected, Epo had no effect. Thus, SCF-activated c-Kit was able to induce strong tyrosine-phosphorylation of cS5 in the absence of EpoR, suggesting that

Jak2 contributed to both basal and c-Kit-enhanced cS5 phosphorylation in EpoR$^{-/-}$-cS5 cells.

To confirm that c-Kit could functionally activate cS5, we determined SCF-dependent gene expression of known Stat5 target genes. Self-renewing WT GFP, WT cS5, EpoR$^{-/-}$-cS5, and Jak2$^{-/-}$-cS5 cells were starved for 3 hours and subsequently restimulated with SCF or left untreated. In the absence of SCF, oncostatin M (OSM), cyclin D2, and c-myc mRNAs were already expressed at significantly elevated levels in WT cS5, EpoR$^{-/-}$-cS5, and Jak2$^{-/-}$-cS5 cells compared with WT GFP cells. It is noteworthy that all cS5-expressing cell types showed a further upregulation of Stat5 target genes in response to SCF (Figure 7D).

These data provide direct molecular evidence that the SCF-activated c-Kit tyrosine kinase was able to cause cS5 phosphorylation and target gene transcription, which to a large extent depends on the presence of Jak2.

Discussion

Activated Stat5 modulates diverse cellular processes, including induction of proliferation, suppression of apoptosis, and promotion or inhibition of differentiation, particularly in cells of the hematopoietic lineage. This output can vary extensively, depending on cell type and stages of maturity. Here, we showed that expression of activated Stat5 was sufficient to allow erythropoiesis in vitro and in vivo, both upon ablation of the EpoR or Jak2 or in the absence of Epo-signaling (Figure S5A). Moreover, our data clearly implicate the c-Kit pathway in Jak2/Stat5 activation in immature hematopoietic cells (Figure S5B).

Activated Stat5 was an essential target of Epo signaling in erythroid cells

The role of Stat5 in promoting erythropoiesis is well recognized but remained controversial because of different phenotypes of Stat5-deficient mouse models, either retaining a hypomorphic Stat5 allele[21,22,42] or representing a complete knockout.[6] Until now, however, it remained unclear whether or not Stat5 would be an essential downstream target of EpoR and Jak2 in erythropoiesis.

To test this possibility, we expressed cS5, a persistently activated Stat5a mutant (S711F,[27]), in primary fetal liver–derived hematopoietic progenitors of WT, Jak2$^{-/-}$, and EpoR$^{-/-}$ embryos. cS5 allowed proliferation and terminal differentiation of erythroid cells from Jak2- and EpoR-deficient embryos in vitro. Both EpoR$^{-/-}$-cS5 and Jak2$^{-/-}$-cS5 cells were able to produce mature CFU-E and BFU-E colonies of normal appearance. The observed effect of cS5 was completely dependent on tyrosine-phosphorylation and DNA-binding ability. cS5 did not require endogenous Stat5 proteins for constitutive activity, because cS5 readily transformed Stat5 mutant cells (Moriggl et al[27] and data not shown). In line, only the cS5 protein was persistently tyrosine-phosphorylated in the absence of cytokines in cS5-expressing WT cells, whereas stimulation with IL-3 induced activation of both endogenous WT Stat5 as well as exogenous cS5 proteins.[34]

Remarkably, cS5 was reported to cause activation of PI3-K signaling via binding to the adaptor protein Gab2.[34] PI3-K signaling, in turn, is required for erythroid renewal.[43] To rule out such possible indirect effects of cS5 on erythropoiesis, we used a 4-OH-T–inducible WT Stat5a-ER* construct, which has little effect on PI3-K activation. Consistent with a direct role for Stat5 proteins in promoting erythropoiesis, primary erythroblasts expressing WT Stat5a-ER* were able to proliferate and terminally differentiate upon replacement of Epo with 4-OH-T. Thereby it could be excluded that the observed genetic complementation of Jak2$^{-/-}$ and EpoR$^{-/-}$ cells with cS5 was due to effects caused by the particular mutation in cS5. We therefore concluded that tyrosine-phosphorylated, transcriptionally active Stat5 was essential and sufficient to enable terminal erythropoiesis.

The initially reported Stat5a/b double-knockout mice (Stat5$^{\Delta N}$) showed surprisingly mild hematopoietic phenotypes, especially with respect to Epo-signaling.[8] These mice still expressed a hypomorphic, N-terminally truncated Stat5 allele.[42,44] In contrast, complete ablation of Stat5a/b (Stat5^{null}) resulted in embryonic lethality.[6] On a mixed Sv129 × C57Bl/6 genetic background, however, approximately 1% of animals survived up to 6 weeks.[45] Thus, even the complete lack of Stat5 proteins resulted in a less severe erythroid phenotype than shown by EpoR$^{-/-}$ or Jak2$^{-/-}$ mice.[6] This could be due to additional signaling pathways, activated by Epo-EpoR-Jak2 besides Stat5 activation. Alternatively, other Stat protein family members might compensate for the absence of Stat5.[46] In Stat5^{null} but not WT erythroblasts, we indeed observed strong Stat1- and Stat3 tyrosine phosphorylation and DNA-binding (F.G. and M.A.K., unpublished observations). A final proof for redundancy among Stat family members will have to await additional experiments, including erythroid-specific Stat1/3/5 knockout mouse models.

One important Stat5 target in erythroid cells is the antiapoptotic protein Bcl-xL.[21,22] Bcl-xL–deficient mice display severe erythroid defects,[12] whereas Bcl-xL overexpression in erythroid cells allows Epo-independent maturation.[47,48] It is likely, however, that there are additional functions of the EpoR/Jak2/Stat5 axis in erythropoiesis. Recently, roles for Epo regulation of cell cycle progression[49] and cell adhesion[50] were indeed proposed. In line with these findings, cS5 but not Bcl-xL rescued renewal and alleviated Epo-dependence of primary erythroid cells of WT as well as EpoR$^{-/-}$ or Jak2$^{-/-}$ fetal livers. Likewise, expression of another hyperactive Stat5 variant was sufficient to allow Epo-independent colony formation of human erythroid cells,[48] and human CD34^{+} cells expressing a constitutive active Stat5 mutant showed enhanced self-renewal with increased erythroid commitment of transduced cells.[51] Besides their requirement for Epo-signaling in erythropoiesis, Jak2 and Stat5 are similarly essential in other hematopoietic lineages. Constitutive activation of Stat5 was recently shown to abrogate cytokine dependence for proliferation of several hematopoietic lineages.[34,52] Here, we show that GM-CSF–dependent myelopoiesis decreased more than 5-fold in Jak2-deficient versus WT fetal liver cells in vitro, whereas cS5 expression in Jak2$^{-/-}$ fetal liver cells robustly increased GM-CSF–dependent colony formation. This suggests that P-Y-Stat5 is necessary to allow functional myeloid differentiation in absence of Jak2. Together with the ability of activated Stat5 to promote renewal and differentiation of erythroid cells in the absence of Epo/EpoR/Jak2 signaling, this suggests a considerable amount of conservation among distinct cytokine receptor/Jak/Stat5 signaling pathways.

Jak2 was required for efficient c-Kit signaling during hematopoietic development

Hematopoietic progenitors and HSCs depend on SCF/c-Kit signaling for proliferation. In addition, erythropoiesis depends on both Epo/EpoR/Jak2- and SCF/c-Kit signals. This raised the question whether or not Jak2 participated in c-Kit signaling. Direct activation of Jak kinases by the tyrosine kinase activity of c-Kit remains controversial,[53,54] although a direct connection between EpoR and

c-Kit signaling modules was demonstrated.[55-57] A role for Jak2 in SCF-dependent colony formation and differentiation was also described in mast cells.[17,38] We consistently observed that Jak2-deficient fetal liver cells were impaired for SCF-dependent colony formation in several immature hematopoietic lineages. Jak2$^{-/-}$ cells not only failed to efficiently respond to SCF alone (favoring immature hematopoietic and mast cells) but were also less responsive to SCF + IL-3 and SCF + IL-7, promoting myeloid and lymphoid progenitor development, respectively. Again, cS5 expression partially reversed these defects in cytokine-dependent colony formation of Jak2$^{-/-}$ cells. These data suggest that c-Kit–dependent Stat5 activation via Jak2 mainly occurs in immature, primary hematopoietic cells (Figure S5B). SCF alone induced robust Stat5 phosphorylation in WT cS5 cells, which was further enhanced by the combination of Epo and SCF. Furthermore, Stat5 activation was also induced upon SCF stimulation in EpoR$^{-/-}$-cS5 and Jak2$^{-/-}$-cS5 cells. SCF-dependent activation of cS5 was completely dependent on the kinase activity of c-Kit, because it was blocked by the c-Kit inhibitor imatinib. The contribution of (other) Jak kinases in this process is likely because EpoR$^{-/-}$-cS5- and Jak2$^{-/-}$-cS5 cells were clearly more susceptible to a pan-Jak inhibitor than WT GFP and WT cS5 cells (data not shown). Alternatively, the c-Kit-dependent cS5 activation could occur in a complex "EpoR-c-Kit-signalosome," harboring different scaffold proteins and signal transducers.

Together, these interpretations are consistent with a model in which EpoR and c-Kit cooperate to activate the Jak-Stat pathway (Figure S5B), perhaps by interaction of the respective signaling modules upon receptor activation.[55,56,58] Such interactions could reflect the in vivo situation more closely than anticipated.

The role of Jak2 in c-Kit signaling might also have mechanistic implications for the biology of the Jak2V617F mutation in patients with polycythemia vera.[59-62] Because cells from respective patients respond more strongly to SCF,[63] it is conceivable that Jak2V617F is more susceptible to SCF signaling and thus also to Stat5 activation.

cS5 compensated for loss of Jak2 in mouse hematopoiesis in vivo

It is noteworthy that Jak2$^{-/-}$-cS5 cells gave rise to mature erythroid and myeloid cells in vivo upon transplantation of freshly transduced fetal liver cells in mice. To avoid development of the aggressive leukemia induced by cS5,[27] we used competitive repopulation conditions (ie, low amounts of fetal liver cells injected into sublethally irradiated mice). Under those conditions, all mice stayed healthy for longer than 6 months. Transplantation of higher cell numbers into lethally irradiated mice failed, because Jak2$^{-/-}$-cS5 cells did not provide radioprotection, whereas transplanted WT cS5 cells induced the expected leukemia.

It is noteworthy that Jak2$^{-/-}$-cS5 cells differentiated into mature CD71^{-}Ter119^{+} erythroid cells in vivo. This is of particular interest because erythroid cells of this stage were undetectable in fetal livers of Jak2$^{-/-}$ embryos. Attempts of transplanting Jak2$^{-/-}$-GFP cells failed because of the inability of those mutant cells to proliferate in vitro. Thus, we never obtained sufficient numbers of viable cells for transplantation experiments after the retroviral infection period (data not shown).

Despite the observed functional erythropoiesis of Jak2$^{-/-}$-cS5 cells in vivo, we observed a profound long-term repopulation defect of the Jak2 mutant cells. In line with this, transplantation of equal numbers of GFP^{+}Jak2$^{-/-}$-cS5 versus WT cS5 LT HSCs always resulted in a lower degree of chimerism in animals receiving Jak2$^{-/-}$-cS5 cells. Although all animals infused with WT cS5 cells maintained abundant GFP^{+} cells in their peripheral blood, 75% of mice receiving Jak2$^{-/-}$-cS5 cells were successfully engrafted, yet showed lower degrees of chimerism; some even lacked erythroid and/or macrophage-like cells.

Here we demonstrated that activation of Stat5 allowed in vitro and in vivo erythropoiesis and myelopoiesis in the absence of EpoR or Jak2. Note that the receptor tyrosine kinase c-Kit exhibited a significant degree of cross-talk with the Jak2-Stat5 axis to promote hematopoiesis (Figure S5B). Our data suggest that contributions from cytokine and growth factor signaling pathways converge on Jak2-Stat5 activation. This may represent a principle shared by different hematopoietic lineages or even multipotent progenitors and HSCs, to ensure efficient hematopoiesis as well as its strict regulation under different physiologic or pathologic conditions.

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Authorship

Contribution: F.G., R.M., and H.B. designed research. F.G., M.A.K., B.K., R.M., and H.D. performed research and collected and analyzed data. T.K., V.B., U.K., K.P., and M.M. provided mouse lines and fetal liver cells. F.G. wrote the manuscript with the help of E.W.M., H.B., and R.M.

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3.1.4 Nonredundant roles for Stat5a/b in directly regulating Foxp3

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Nonredundant roles for Stat5a/b in directly regulating *Foxp3*

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Stats (signal transducers and activators of transcription) regulate multiple aspects of T-cell fate. T regulatory (Treg) cells are a critical subset that limits immune responses, but the relative importance of Stat5a/b versus Stat3 for Treg cell development has been contentious. We observed that peripheral $CD25^+CD4^+$ T cells were reduced in *Stat5*$^{\Delta N}$ mice; however, the levels of Foxp3, a transcription factor that is critical for Treg cells, were normal in splenic $CD4^+$ T cells even though they were reduced in the thymus. In contrast, complete deletion of Stat5a/b (*Stat5*$^{-/-}$) resulted in dramatic reduction in CD25- or Foxp3-expressing $CD4^+$ T cells. An intrinsic requirement was demonstrated by reduction of Stat5a/b in CD4-expressing cells and by stem cell transplantation using *Stat5*$^{-/-}$ fetal liver cells. Stat5a/b were also required for optimal induction of Foxp3 in vitro and bound directly to the *Foxp3* gene. Reduction of Stat3 in T cells did not reduce the numbers of Treg cells in the thymus or spleen; however, Stat3 was required for IL-6–dependent down-regulation of Foxp3. Therefore, we conclude that Stat5a/b have an essential, nonredundant role in regulating Treg cells, and that Stat3 and Stat5a/b appear to have opposing roles in the regulation of Foxp3. (Blood. 2007;109: 4368-4375)

Introduction

The development and differentiation of immune cells is carefully orchestrated by an array of cytokines. Signal transducers and activators of transcription (Stats) represent a small but critical family of transcription factors that play important roles in transmitting cytokine signals. Consequently, Stats are critical for immunoregulation and the development of immune cells.[1,2] Stat5a and Stat5b are two closely related proteins that have overlapping functions with respect to lymphoid development and differentiation.[3,4] Gene targeting of Stat5a and Stat5b (collectively referred to as Stat5), results in impairment in the development of T, B, and natural killer (NK) cells.[5-7] In mice in which the amino termini of Stat5a and Stat5b are deleted (denoted as *Stat5*$^{\Delta N}$ mice), major disruption of various immune cell parameters was noted.[8,9] However, residual Stat5 function permits T cell development, albeit suboptimally.[10] This contrasts with the complete absence of Stat5a/b, which results in dramatic reduction in thymocyte numbers, in part due to effects on lymphoid stem cell function.[5]

T regulatory (Treg) cells comprise a population of cells enriched in $CD4^+CD25^+$ T cells that suppresses T-cell proliferation and function and attenuates immune responses against self- or nonself-antigens.[11-13] Naturally arising Treg cells are produced in the thymus as a functionally distinct T-cell subpopulation, whereas adaptive Treg cells are induced from naive T cells after antigen exposure in the periphery.[14-17] In classic studies, mice develop organ-specific autoimmune disease following neonatal thymectomy, which is corrected by reconstitution with $CD4^+CD25^+$ T cells.[13] The essential role of Treg cells in maintaining tolerance has been confirmed by findings that defective function of this subset is a feature of many models of autoimmunity.[18]

More recently, it was discovered independently by several groups that a subset of $CD4^+CD25^+$ T cells expresses the transcription factor Foxp3, which is necessary and sufficient for Treg cell development and function.[19-22] Foxp3 is highly conserved in mice and humans. Mutation of Foxp3 in mice (scurfy) results in early autoimmune disease,[23] whereas mutations of human Foxp3 are associated with a disorder known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX).[24] In mice, Foxp3 is a reliable marker for the Treg lineage.

Multiple lines of evidence have indicated that IL-2 is an important growth factor for Treg development and maintenance. Mice lacking IL-2 or its receptor subunits, IL-2Rα (CD25) and IL-2Rβ (CD122), have deficits in $CD4^+CD25^+$ Treg cells and develop autoimmune disease similar to *Foxp3*$^{-/-}$ mice.[25-27] However, IL-2 is dispensable for Treg cell development, as some Foxp3-expressing cells are present in *Il2*$^{-/-}$ and *Il2ra*$^{-/-}$ mice, suggesting the involvement of other cytokines.[28] In vitro culture of $CD4^+$ T cells with transforming growth factor-β1 (TGF-β1) can promote the generation of $Foxp3^+$ Treg cells from naive $CD4^+$ T cells. In contrast, in vitro culture of $CD4^+$ T cells with TGF-β1 and IL-6 promotes the differentiation of inflammatory T helper 17 (Th17) cells and suppresses Treg cells.[29]

A first step in IL-2 signaling is the activation of the Janus kinase, Jak3, which associates with the IL-2Rγ chain (CD132), also known as the common gamma chain (γc).[30] Jak3 and γc are

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essential for Treg cell development and maintenance, as *Jak3*$^{-/-}$ and *Il2rg*$^{-/-}$ mice lack CD25 and Foxp3 expression in the thymus and spleen.[28,31] Activation of Jaks results in phosphorylation of Stats, and Stat5a/b are the most prominent Stats activated by IL-2.[32] *Stat5*$^{\Delta N}$ mice, which express N-terminally–truncated Stat5 proteins, have reduced numbers of CD25$^+$CD4$^+$ cells in the periphery and autoimmunity, although assessment of these mice has led to conflicting conclusions regarding the importance of Stat5 in Treg cell development.[33-35] Interpretation of these studies is further complicated by the finding that *Stat5*$^{\Delta N}$ mice expressed truncated Stat5 proteins that are partially functional; as such, these actually represent hypomorphic Stat5 alleles.[5,6] As noted, the residual function in *Stat5*$^{\Delta N}$ mice was illustrated by comparing immune defects in these mice with those of a different model of Stat5a/b deletion in which both Stat5a and Stat5b were completely deleted.[5] However, the role of Stat5 in regulating Foxp3 was not assessed, and other studies have argued for a role of Stat3 in regulating Foxp3.[36,37] This suggests that Stat3 and Stat5 may play redundant roles in regulating this transcription factor. These findings prompted reassessment of the roles of Stat5 and Stat3 in Treg cell development and maintenance with focus on their effects on Foxp3 expression.

In this report, we compared and contrasted Treg cell development in *Stat5*$^{\Delta N}$ mice and *Stat5a/b*$^{-/-}$ (called *Stat5*$^{-/-}$) mice. Stat5 was demonstrated to be critical for both Treg cell development and maintenance and critical for Foxp3 expression. The intrinsic requirement for Stat5a/b in Treg cells was evident through the use of tissue-specific Stat5 deletion and stem cell transplantation experiments. Moreover, Stat5 directly binds the *Foxp3* gene. In contrast, reduction of Stat3 in T cells did not reduce the numbers of Treg cells in the thymus or spleen. However, the ability of IL-6 to down-regulate Foxp3 expression was greatly attenuated in Stat3-deleted T cells. Thus, Stat5a/b have an essential and direct positive role in regulating Foxp3 and Treg cells. Although Stat3 is not essential for the development or maintenance of Treg cells, it does appear to have an important role in mediating IL-6 signals to attenuate Foxp3 expression.

Materials and methods

Mice

Stat5a/b$^{-/-}$ (*Stat5*$^{-/-}$), *Stat5a/b*$^{fl/-}$, *CD4cre* (*Stat5*$^{fl/-}$, *CD4cre*), and *Stat5*$^{\Delta N}$ mice were described previously[5-7] and housed at NIH under approved protocols. *Stat5*$^{fl/-}$, *CD4cre*, *Yfp* mice were generated by crossing *Stat5*$^{fl/-}$, *CD4cre* with *Yfp* indicator mice (ROSA26–stop-floxed–YFP reporter mice; Jackson Laboratory, Bar Harbor, ME). Cre-mediated deletion was monitored by yellow fluorescent protein (YFP) expression from a "ROSA26–stop-floxed–YFP" reporter. *Stat3*$^{fl/fl}$ mice were bred with mice expressing Cre under the control of the MMTV (*MMTV-Cre*) to produce *Stat3*$^{fl/fl}$, *MMTV-Cre* mice.[38] CD45.1 congenic *Rag2*$^{-/-}$, *Jak3*$^{-/-}$, and *Il2rg*$^{-/-(Y)}$ mice were obtained from Taconic Farms (Hudson, NY). Animals were handled and housed in accordance with the guidelines of the NIH Animal Care and Use Committee.

Antibodies

Biotin-, FITC-, PE-, Per-CP–, PE-Cy5.5–, and APC-conjugated antibodies to mouse CD4, CD25, CD8, CD62L, CD44, and CD122 were purchased from BD Biosciences (San Jose, CA). The mouse Foxp3 staining kit was purchased from eBioscience (San Diego, CA).

Cell preparation, DNA, RNA, and protein expression analysis

CD4$^+$ single-positive (SP) thymocytes and splenocytes from *Stat5*$^{fl/fl}$ mice and YFP$^+$CD4$^+$ SP thymocytes and splenocytes from *Stat5*$^{fl/-}$, *CD4cre*, *Yfp* mice were sorted using a Moflo cell sorter (Dako Cytomation, Glostrup, Denmark). The purity of the sorted cells was greater than 99%. These sorted cells were subjected to DNA, RNA, and protein analysis essentially as described before.[5] In brief, DNA was purified using the DNA easy kit from Qiagen (Valencia, CA). RNA was prepared with Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). For real-time polymerase chain reaction (PCR), cDNA was generated using a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA). The primers and probes for real-time PCR were purchased from Applied Biosystems (Applied Biosystems). Real-time (RT)–PCR was performed on ABI PRISM 7700 (Applied Biosystems, Foster City, CA).

Cell culture

CD25$^-$CD4$^+$ splenic T cells from *Stat5*$^{fl/fl}$ mice, YFP$^+$CD25$^-$CD4$^+$ splenic T cells from *Stat5*$^{fl/-}$, *CD4cre*, *Yfp* mice or *Stat3*$^{fl/fl}$ mice and *Stat3*$^{fl/fl}$, *MMTVcre* mice were isolated by the Moflo cell sorter and cultured for 3 days with plate-bound anti-CD3 (5 μg/mL) and anti-CD28 (5 μg/mL) (BD PharMingen, San Diego, CA) plus TGF-β1 (5 ng/mL, PeproTech, Rocky Hill, NJ), hIL-2 (100 U/mL; provided by National Cancer Institute [NCI]–Frederick, MD) and IL-6 (10 ng/mL; PeproTech) as indicated, with or without anti–murine IL-2 antibody (20 μg/mL; R&D Systems, Minneapolis, MN) in complete RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and 2 mM β-mercaptoethanol.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed as previously described.[39] CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were sorted from thymi and spleens and stimulated with IL-2 (100 U/mL) for 1 hour. Formaldehyde (final concentration, 1%) was then added to cross-link proteins and DNA. The cell lysates were sonicated and immunoprecipitated with normal rabbit serum (Upstate Biotechnology, Charlottesville, VA), α-Stat5 (R&D Systems), and α-Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA). The immunopreciptated DNA was eluted and amplified by real-time PCR using an ABI 7700 (Applied Biosystems). Values were normalized to corresponding input control and are expressed as fold enrichment relative to normal rabbit serum for each experiment. The sequences specific primers and probes used for amplification of the *Foxp3* gene surrounding putative Stat binding sites were as follows: site in I, 5′-CCTCCTGGAAACCTGTGTCAC-3′, 5′-AACTTGGTCAGAGAGGTGGCA-3′, and 5′-6FAM-TACCCCTCATTTACTTATC-3′; site in II, 5′-CTTCTGGGAGCCAGCCATT-3′, 5′-GCTGTACTCCCCCCACAAATT-3′, and 5′-6FAM-TGAGACTCTCTGATTCTGT-3′; sites in III, 5′-ACAACAGGGCCCAGATGTAGA-3′, 5′-GGAGGTTGTTTCTGGGACATAGA-3′, and 5′-6FAM-CCCGATAGGAAAACA-3′. The primers and probe used for irrelevant IV are 5′-CACCAAAGGCTGGAAGCCT-3′, 5′-CAGACGAGCCTCCACAGAGTT-3′, and 5′-6FAM-CCGTGCCTTGTCAGG-3′.

Stem cell transplants

Single-cell suspensions were generated from E14.5 *Stat5*$^{+/+}$ or *Stat5*$^{-/-}$ fetal livers, and cells (2×10^6) were injected into tail veins of lethally irradiated (9 Gy [900 rad]) Rag2$^{-/-}$ CD45.1 congenic recipient mice housed under pathogen-free conditions with acidified water as previously described.[5] At 7 to 8 weeks later, tissues were harvested. Thymi and spleens were analyzed by flow cytometry for donor-derived CD45.2$^+$ cells.

Results

Stat5a/b are critical for thymic development of Treg cells

Previous studies using *Stat5*$^{\Delta N}$ mice have reached conflicting conclusions regarding requirement for Stat5a/b in thymic Treg cell

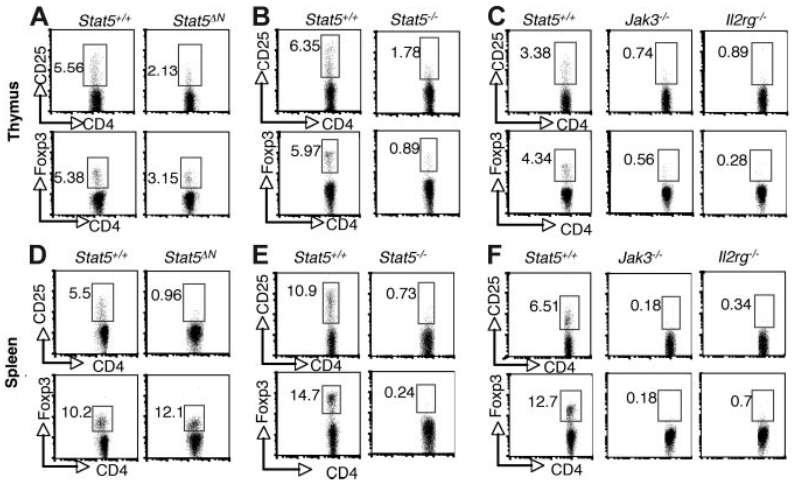

Figure 1. Stat5a/b are critical for the generation of thymic Foxp3$^+$ CD4$^+$ T cells and maintenance of peripheral Foxp3$^+$ CD4 T$^+$ cells. CD25 and Foxp3 expression were analyzed by flow cytometry in CD4 SP thymocytes (A-C) and splenocytes (D-F) from 4- to 6-week-old *Stat5*$^{+/+}$ and *Stat5*$^{\Delta N}$ mice (A,D), *Stat5*$^{-/-}$ mice (B,E), and *Jak3*$^{-/-}$ and *Il2rg*$^{-/-}$ mice (C,F). Numbers indicate the percentage of CD25+ or FoxP3+ cells delineated by the rectangles.

development, but because of the aforementioned limitations with this animal model, we sought to revisit this issue.[34,35] As shown in Figure 1, the proportion of CD4$^+$ CD25$^+$ cells in thymi from *Stat5*$^{\Delta N}$ mice was substantially reduced compared with wild-type (WT) littermates (Figure 1A). This is consistent with the well-documented effect of Stat5 on CD25 expression.[40] In addition, the proportion of CD4$^+$ Foxp3$^+$ thymocytes in *Stat5*$^{\Delta N}$ mice was reduced (Figure 1A; bottom panels). Furthermore, the level Foxp3 expression, as assessed by mean fluorescence intensity (MFI), was also reduced in *Stat5*$^{\Delta N}$ by approximately 20% compared with that of controls. The specificity of Foxp3 staining was documented by comparison with isotype control (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). As the cellularity of the thymus in adult *Stat5*$^{\Delta N}$ mice is roughly comparable with that of WT mice, the absolute numbers of Foxp3$^+$ CD4 SP and CD25$^+$ CD4 SP thymocytes were also reduced.[8,10] In interpreting data using *Stat5*$^{\Delta N}$ mice, one needs to bear in mind that truncated Stat5a/b proteins are expressed. However, *Stat5*$^{-/-}$ mice in which the entire *Stat5a* and *Stat5b* loci were deleted die perinatally, but a small numbers of mice (approximately 2%) survive for 6 to 8 weeks after birth.[5-7] *Stat5*$^{-/-}$ viable mice exhibited marked reductions in the proportions and absolute numbers of CD25- and Foxp3-expressing CD4 SP thymocytes (Figure 1B). In fact, the reductions in CD25- and Foxp3-expressing cells from Stat5$^{-/-}$ mice were comparable to that observed in *Jak3*$^{-/-}$ and *Il2rg*$^{-/-(Y)}$ mice (Figure 1C). CD4 SP thymocytes were generated (albeit in substantially lower numbers) in these mice; nonetheless, there was profound reduction in Foxp3-expressing cells.[28,31] The presence of Treg cells in *Stat5*$^{\Delta N}$ mice is likely due to the residual activity of the truncated Stat5 protein rather than Stat5-independent development.

Stat5a/b are critical for peripheral Foxp3 expression and Treg cell maintenance

Previous studies using *Stat5*$^{\Delta N}$ mice noted a substantial reduction of CD25$^+$CD4$^+$ T cells in the periphery; however, expression of Foxp3 was not examined.[33,34] As shown in Figure 1D, the proportion of Foxp3-expressing CD4$^+$ T cells was not reduced in 4- to 5-week-old *Stat5*$^{\Delta N}$ mice, despite the dramatic reduction in CD25-expressing cells. Since the cellularity of spleens from *Stat5*$^{\Delta N}$ mice is comparable with that of WT mice at this young age,[8] the absolute number of Foxp3$^+$ Treg cells was normal in *Stat5*$^{\Delta N}$ mice, which might lead one to conclude that peripheral Foxp3 expression does not require Stat5a/b. In contrast, though, the proportions of Foxp3-expressing and CD25-expressing splenic CD4 T cells were both markedly reduced in *Stat5*$^{-/-}$ mice (Figure 1E). This is consistent with the notion that Stat5a/b are essential for Treg cell maintenance, and the expression of Foxp3 in *Stat5*$^{\Delta N}$ mice is the result of residual Stat5 expression. The loss of peripheral Foxp3 expression was again comparable with that seen in spleens from *Jak3*$^{-/-}$ and *Il2rg*$^{-/-}$ mice (Figure 1F).

Intrinsic requirement for Stat5a/b but not Stat3 for Treg cells

While these data indicate a requirement for Stat5a/b in Foxp3 expression in the thymus and periphery, Stat5a/b are lacking in all tissues from *Stat5*$^{-/-}$ viable mice (data not shown), and an intrinsic requirement for Stat5a/b in T cells cannot be inferred. We therefore approached this problem in two ways: (1) stem cell transplantation using Stat5$^{-/-}$ precursors; and (2) tissue-specific deletion of Stat5 using transgenic expression of Cre.

Because Stat5 deficiency is usually lethal (approximately 98%) and affects multiple cell lineages and pathways, we first reconstituted irradiated *Rag2*$^{-/-}$ recipient mice with *Stat5*$^{-/-}$ fetal liver cells to analyze Treg cell development. As noted, reconstitution with *Stat5*$^{-/-}$ precursors is inefficient due to defective hematopoietic/lymphoid stem cell functions.[5,41] Nonetheless, T-cell development with production of SP thymocytes does occur.[5] However, no CD25$^+$ or Foxp3$^+$ cells were detected in the CD4$^+$ population of thymocytes from mice that received transplants (Figure 2A; right panel). In contrast, these cells were readily detected when normal stem cells were transplanted into *Rag2*$^{-/-}$ recipients (Figure 2A; left panel). CD25$^+$ or Foxp3$^+$ CD4$^+$ T cells were also absent in spleens of *Rag2*$^{-/-}$ recipient mice that received transplants of *Stat5*$^{-/-}$ precursors (Figure 2B), consistent with what we observed in viable *Stat5*$^{-/-}$ mice.

We also approached the issue of an intrinsic requirement for Stat5 by selectively reducing Stat5a/b levels in T cells by breeding *Stat5*$^{fl/-}$ mice with *CD4 cre* mice.[5] This approach had advantages, but also had some significant limitations in that Stat5 levels were reduced but not totally absent. To monitor Cre-mediated deletion, we also introduced YFP into the mice by breeding *Stat5*$^{fl/-}$, *CD4cre* mice with indicator mice in which the gene encoding YFP was inserted into the Rosa locus (ROSA26–stop-floxed–YFP reporter mice). Flow cytometric analysis of thymocytes from 4- to 5-week-old *Stat5*$^{fl/-}$, *CD4cre*, *Yfp*$^+$ mice showed normal proportions of

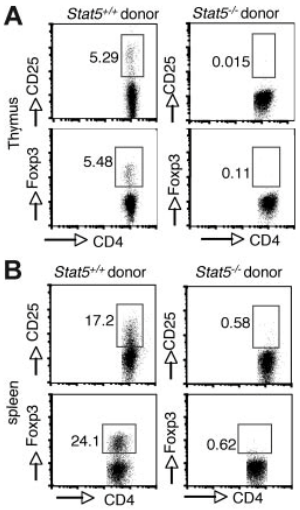

Figure 2. Intrinsic requirement of Stat5a/b for generation of Foxp3⁺ CD4 T cells. Irradiated C57BL/6 *Rag2*$^{-/-}$ CD45.1⁺ congenic mice were reconstituted with 2 × 10⁶ total fetal liver cells from CD45.2⁺ WT (left panels) or Stat5$^{-/-}$ (right panels) mice. After reconstitution (8 weeks), cell populations in the thymus (A) and spleen (B) were analyzed. Donor-derived CD4 SP T cells were analyzed for CD25 and Foxp3 expression. Numbers indicate the percentage of CD25⁺ or FoxP3⁺ cells delineated by the rectangles.

CD4 and CD8 thymocytes.[5] Cre-mediated gene deletion as assessed by YFP expression was apparent in 90% of CD4⁺CD8⁺ double-positive (DP) thymocytes (data not shown), and in 98% of CD4⁺ SP thymocytes (Figure S2A). In sorted YFP⁺ CD4 SP thymocytes, genomic deletion of the Stat5a/b loci was observed as previously published.[5] In YFP⁺ CD4⁺ cells, the levels of Stat5a/b mRNA were reduced to approximately 10%, and the levels of Stat5 protein were reduced to 20% to 30% (Figure S2B-C); not surprisingly, in YFP⁻ cells, the levels of Stat5 were even greater. Thus, the presence of residual Stat5 protein is a caveat that needs to be considered in interpreting experiments using these cells.

Examination of thymi from these mice revealed that the percentages of Foxp3- and CD25-expressing CD4 SP thymocytes were reduced by about 50% compared with that of *Stat5* $^{fl/fl}$ or *Stat5*$^{fl/-}$ littermates (5.18% and 5.65% versus 2.52% and 2.81% for Foxp3⁺CD4⁺ and CD25⁺CD4⁺ cells respectively; Figure 3A,C). The absolute numbers of Foxp3⁺CD4 SP thymocytes were also reduced by 50% compared with that of WT (Figure 3E), since the numbers of total thymocytes and proportions of CD4 SP T cells were normal in *Stat5*$^{fl/-}$, *CD4cre, Yfp* mice (Figure 3B,D). In the spleen, 97% of the T cells were YFP⁺, and Stat5 protein levels were reduced by approximately 70% (Figure S2A,C). Flow cytometric analysis of the splenic CD4⁺ T cells showed that the percentage of CD25⁺ CD4⁺ T cells was reduced (Figure 4A), and the proportion of Foxp3-expressing cells from *Stat5*$^{fl/-}$, *CD4cre, Yfp* mice was modestly and consistently reduced compared with that in *Stat5*$^{fl/fl}$ or *Stat5*$^{fl/-}$ mice (Figure 4A,C). The reduction in CD25-expressing cells was more dramatic than that of Foxp3-expressing cells, and in fact, most of the Foxp3-expressing CD4⁺ T cells from *Stat5*$^{fl/-}$, *CD4cre, Yfp* mice were CD25^{dim} (Figure 4A; bottom panel). The total numbers of splenic CD4⁺ T cells in *Stat5*$^{fl/-}$, *CD4cre, Yfp* mice were roughly half of that seen in control mice (Figure 4B,D); as a result, the absolute number of Foxp3⁺CD4⁺ T cells in *Stat5*$^{fl/-}$, *CD4cre, Yfp* mice was reduced by approximately 60% (Figure 4E). These results contrast with the near absence of Treg cells found in *Stat5*$^{-/-}$ mice. While this could be interpreted to suggest that extrinsic Stat5 expression contributes to the loss of Treg cells, in view of the stem cell transplantation experiments, we would argue that the modest reductions of Treg cells in *Stat5*$^{fl/-}$, *CD4cre, Yfp* mice are more likely due to persistence of Stat5 expression in this model of tissue-specific deletion. Thus, these results further demonstrate the importance of Stat5 in both Treg cell development and maintenance. Consistent with the reduction in Foxp3⁺CD4⁺ T cells, we noted that systemic autoimmune disease was evident in *Stat5*$^{fl/-}$, *CD4cre, Yfp* mice (Figure S3), similar to what has been observed in Stat5$^{\Delta N}$ mice.[33]

Recent studies using transient transfection have suggested that Stat3 and Stat5a/b may both positively regulate Foxp3.[36] The reduction in Foxp3⁺ CD4⁺ T cells in the thymus and periphery of Stat5$^{-/-}$ mice argues against an essential role for Stat3 in regulating Foxp3 under normal circumstances. Nonetheless, it was important to formally document whether Stat3 was a significant contributor to the regulation of Foxp3. In *Stat3*$^{fl/fl}$, *MMTVCre* mice, Stat3 mRNA levels were reduced to approximately 16% of normal levels in CD4⁺ T cells (Figure S4A). Examination of thymi and spleens from these mice revealed that the proportion and absolute numbers of Foxp3⁺ CD4⁺ T cells were normal (Figure 5).

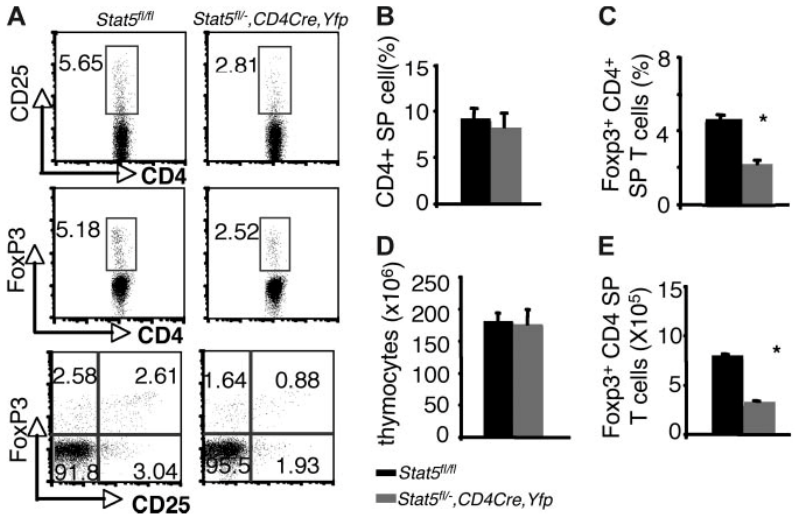

Figure 3. Reduction of thymic Foxp3⁺ CD4⁺ T cells with tissue-specific diminution of Stat5a/b levels. (A) CD25 and Foxp3 expression were assessed on sorted CD4 SP thymocytes from *Stat5*$^{fl/fl}$ mice and YFP⁺CD4 SP thymocytes from *Stat5*$^{fl/-}$, *CD4Cre, Yfp* mice. (B) Average proportion of CD4 SP thymocytes. (C) Mean percentage of Foxp3⁺ CD4⁺ T cells. (D) Average total numbers of thymocytes. (E) Absolute numbers of Foxp3⁺ CD4⁺ T cells. Means ± SE (n = 6) are shown; *$P < .01$ as determined by Student t test. Numbers indicate the percentage of CD25⁺ or FoxP3⁺ cells delineated by the rectangles.

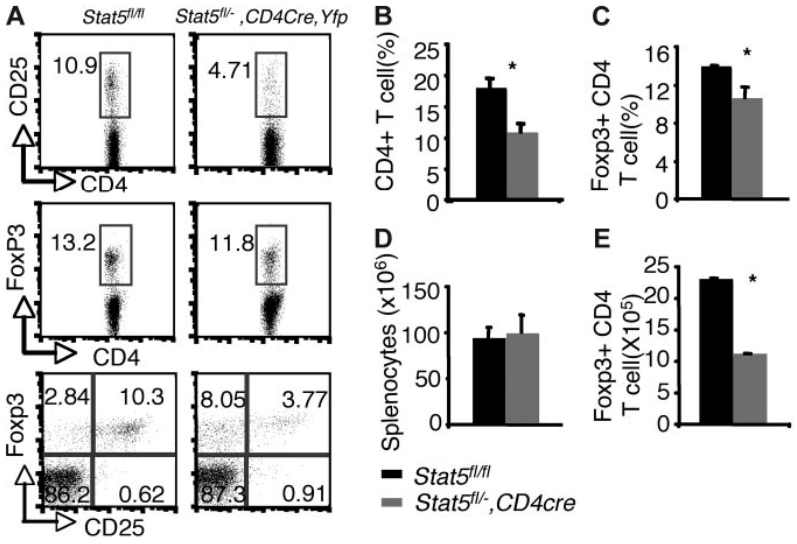

Figure 4. Reduction of peripheral Foxp3⁺ CD4⁺ T cells with tissue-specific decrease of Stat5a/b levels. (A) CD25 and Foxp3 expression were analyzed in sorted CD4⁺ SP splenocytes from *Stat5$^{fl/fl}$* mice and YFP⁺CD4⁺ SP splenocytes from *Stat5$^{fl/-}$*, *CD4Cre*, *Yfp* mice. Numbers indicate the percentage of CD25⁺ or FoxP3⁺ cells delineated by the rectangles. (B) Average proportion of CD4⁺ SP splenocytes. (C) Mean percentage of Foxp3⁺ CD4⁺ T cells. (D) Average total numbers of splenocytes. (E) Absolute numbers of Foxp3⁺ CD4 T cells. Means ± SE (n = 6) are shown (*$P < .01$).

Stat5 and Stat3 have opposing effects on cytokine-dependent FoxP3 regulation

Despite the persistence of residual Stat5 protein expression in *Stat5$^{fl/-}$*, *CD4cre*, *Yfp* mice, this system was more amenable to analyzing T cells with reduced Stat5 levels. Previous studies have shown that Foxp3 can be induced in vitro by addition of exogenous TGF-β1.[29,42,43] We therefore assessed the in vitro induction of Foxp3 in YFP⁺ CD25⁻CD4⁺ T cells from *Stat5$^{fl/-}$*, *CD4cre*, *Yfp* mice. As shown in Figure 6A, CD25⁻CD4⁺ T cells expressed little Foxp3 prior to stimulation. Stimulation with anti-CD3, anti-CD28, and TGF-β1 was a potent inducer of Foxp3 in WT cells (Figure 6A). When endogenous IL-2 production was neutralized with anti–mIL-2 antibody, the percentage of Foxp3⁺ CD4⁺ T cells induced by TGF-β1 was markedly reduced. Conversely, addition of exogenous hIL-2 enhanced the generation of Foxp3⁺ CD4⁺ T cells. However, under all conditions, the proportion of Foxp3⁺ cells was markedly reduced when cells from *Stat5 $^{fl/-}$*, *CD4cre*, *Yfp* mice were used, and few Foxp3-expressing cells were induced even with addition of exogenous IL-2. Moreover, the level of induction of Foxp3 indicated by MFI was also lower, only 35% of that in wild-type cells, even under optimal conditions. Thus, despite the limitations of residual, low-level Stat5 expression in T cells from *Stat5 $^{fl/-}$*, *CD4cre*, *Yfp* mice, this system clearly supports the importance of Stat5a/b in Foxp3 regulation.

TGF-β1 in combination with IL-2 promotes in vitro differentiation of Treg cells and enhances Foxp3 expression, whereas IL-6 inhibits Foxp3 expression.[29,44] To determine the role of Stat3 in mediating this effect of IL-6, we next analyzed Treg cell differentiation in CD25⁻CD4⁺ cells from *Stat3$^{fl/fl}$*, *MMTVcre* mice. As shown in Figure 6B, Foxp3 levels were induced to comparable levels in CD25⁻CD4⁺ T cells from *Stat3$^{fl/fl}$*, *MMTVcre* mice and WT mice. However, when IL-6 was added to cultures of WT T cells, the induction of Foxp3 was blocked, consistent with previously published data.[29] In contrast, addition of IL-6 to cultures of T cells

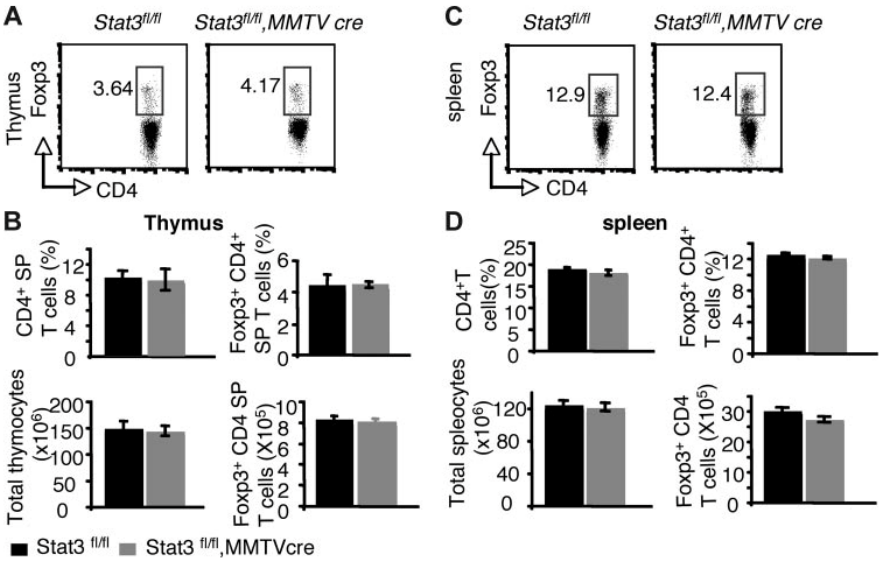

Figure 5. Reduction of Stat3 levels in CD4 T cells has no effect on Foxp3 expression from both thymi and spleens. (A) Foxp3 expression was analyzed in CD4 SP thymocytes by flow cytometry. (B) The mean proportion of CD4 SP thymocytes, the mean percentage of Foxp3⁺ CD4⁺ T cells, the average total numbers of thymocytes, and the absolute numbers of Foxp3⁺ CD4 T cells in thymocytes are shown. (C) Foxp3 expression was analyzed in CD4⁺ splenocytes by flow cytometry. (D) The average proportion of CD4⁺ SP splenocytes, the mean percentage of Foxp3⁺ CD4⁺ T cells, the average total numbers of splenocytes, and the absolute numbers of Foxp3⁺ CD4 T cells are shown. Means ± SE (n = 5) are shown. In panels A and C, numbers indicate the percentage of FoxP3⁺ cells delineated by the rectangles.

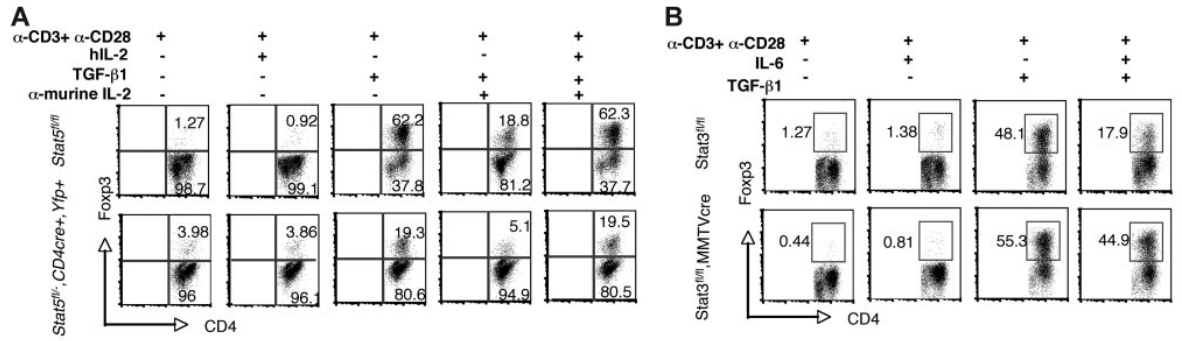

Figure 6. Stat5a/b are important for the in vitro induction of Foxp3. (A) Sorted splenic CD25$^-$CD4$^+$ T cells from *Stat5$^{fl/fl}$* and YFP$^+$ CD25$^-$CD4$^+$ T cells from *Stat5^{fl-}*, *CD4Cre*, *Yfp*$^+$ mice were cultured with plate-bound anti-CD3 and anti-CD28 combined with or without TGF-β1, IL-2 (100 U/mL), and/or anti–IL-2 antibody as indicated. Representative of 3 experiments. Numbers in quadrants indicate FoxP3$^+$ or FoxP3$^-$ cells. (B) Sorted splenic CD25$^-$CD4$^+$ T cells from *Stat3$^{fl/fl}$* and *Stat3$^{fl/fl}$*, *MMTVcre* mice were cultured with plate-bound anti-CD3 and anti-CD28 combined with or without TGF-β1 and IL-6 as indicated. Numbers indicate the percentage of FoxP3$^+$ cells delineated by the rectangles.

from *Stat3$^{fl/fl}$*, *MMTVcre* mice failed to block the up-regulation of Foxp3 (Figures 6B, S4). We interpret these data to indicate that Stat5a/b are critical in vivo and in vitro for enhancing Foxp3 expression. In contrast, Stat3 is not required for induction of Foxp3; however, it does appear to be critical for mediating IL-6–dependent negative regulation of this key transcription factor.

Foxp3 is a direct target of Stat5

The present data suggest that Stat5a/b are critical for promoting Foxp3 expression in vivo and in vitro, likely through their role in mediating IL-2 signals, but it was possible that actions of Stat5a/b may not be direct. However, multiple consensus Stat-binding sites are present in the mouse *Foxp3* gene promoter region (I, II) and the first intron (III). The most highly conserved sites are in the first intron (Figure 7). Stat5 binding to the native *Foxp3* gene in murine primary cells was assessed using sorted CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ SP thymocytes and chromatin immunoprecipitation with anti-Stat5 antibodies. Subsequent real-time PCR amplification of the *Foxp3* gene surrounding 3 putative Stat binding sites showed significant IL-2–inducible Stat5 binding. As a control, we also

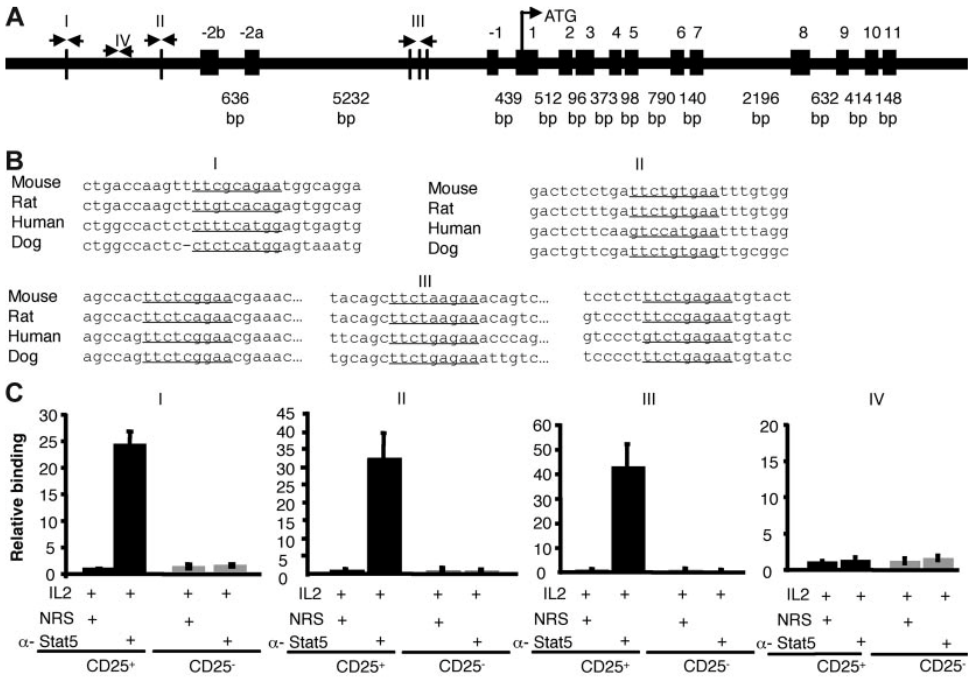

Figure 7. Stat5 binds the *Foxp3* gene. (A) Schematic of the mouse *Foxp3* gene. Vertical lines depict potential Stat binding sites in the first intron and the putative promoter (I, II, III). Site IV does not contain a Stat-binding site and was used as a control. (B) Stat-binding sites in the mouse *Foxp3* gene are underlined and aligned with sequences from other species. Site I is located between 006010267 and 006010275 in the mouse genome (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid = 83 436 504&clade = vertebrate&org = Mouse&db = mm7). Site II is located between 006012112 and 006012120. Intronic sites designated III are located between 006017209 and 006017217, 006017406 and 006017414, and 006017523 and 006017531. All sequences are from the sense strand; note that the previous Stat-binding site sequences identified by Zorn et al[36] were from the antisense strand, although the same sites were interrogated in our analysis. (C) Sorted thymic CD25+CD4 SP (■) and CD25−CD4 SP (▤) T cells were treated with IL-2 for 1 hour. Proteins and DNA were cross-linked with formaldehyde, cells were lysed, and DNA was sheared. Chromatin immunoprecipitation was performed using either normal rabbit serum or anti-Stat5 antibody. Quantification of immunoprecipitated DNA fragments was performed by real-time PCR using primers and probes for sites I, II, III, and the irrelevant site IV. Values were normalized to corresponding input control and are expressed as fold enrichment relative to normal rabbit serum for each experiment. Means ± SE are shown.

assessed a segment that does not contain consensus Stat binding sites, but found that this segment was not amplified with anti-Stat5 immunoprecipitation (Figure 7C). Stat5 binding to the *Foxp3* gene was also not detected in $CD25^{-}CD4^{+}$ SP cells, but was demonstrable in $CD25^{+}CD4^{+}$ splenocytes (data not shown). These data suggest that Stat5 may play a direct role in regulating *Foxp3* transcription.

Because IL-6–mediated inhibition of Foxp3 expression was Stat3 dependent, we also assessed whether we could also detect direct binding of Stat3 to the *Foxp3* locus. However, IL-6–dependent binding of Stat3 to the regions of *Foxp3* gene to which Stat5 binds was marginal (Figure S4C). That is, in contrast to Stat5, which enriched the binding regions by more than 20-fold, the same regions were enriched by less than 4-fold with anti-Stat3 antibody compared with normal rabbit serum. This is of interest because the binding sites for Stat3 and Stat5 binding are thought to be quite similar.[45] It is notable therefore that Stat5 evidently binds the *Foxp3* gene well, whereas Stat3 binds this locus poorly despite the clear Stat3-dependent functional effects on Foxp3 in T cells.

Discussion

In this study, we explored the role of Stat5a/b in regulating Foxp3 expression and Treg cells. Our data indicate that Stat5a/b are critical for both the development and maintenance of Treg cells. This appears to be an intrinsic requirement for Stat5a/b in Treg cells and is likely mediated through direct effects of Stat5a/b on the transcription of the *Foxp3* gene, which has multiple Stat-binding sites.

Stat5a/b have long been recognized to mediate IL-2 signals, and the importance of IL-2 in Treg cell development and maintenance has been established by a number of approaches. IL-2 signaling not only leads to the activation of Jaks and Stats, but also Ras-MAPK and PI3K-AKT pathways. Jak3 and γc are essential for Treg cells, but previous studies using CD25 as a marker in $Stat5^{\Delta N}$ mice led to the conclusion that Stat5 is required for peripheral Treg cell maintenance but not for Treg cell development.[34] This would suggest that Stat5 might not be the key factor regulating Foxp3 expression, or that it was redundant with other factors. However, it is now clear that $Stat5^{\Delta N}$ mice have residual Stat5 function that may support $Foxp3^{+}$ $CD4^{+}$ T-cell development. Indeed, analysis of mice completely deficient in Stat5 showed that $Foxp3^{+}$ cells were severely reduced (Figures 1,3) documenting the criticality of this transcription factor for Foxp3 expression. Of note, we found reduced but not absent Treg cells in both thymi and spleens of $Stat5^{fl/-}$, *CD4cre, Yfp* mice, but, like cells from $Stat5^{\Delta N}$ mice, some residual Stat5 protein is also present in T cells from $Stat5^{fl/-}$, *CD4cre, Yfp* mice. We interpret these data to suggest that low levels of Stat5 in $Stat5^{\Delta N}$ and $Stat5^{fl/-}$, *CD4cre* mice permit Foxp3 expression, even in circumstances where CD25 levels are dramatically reduced. We have found other circumstances where selective pressure for Stat5 expression allows for escape of cells in which the Stat5 genes are not deleted. We believe that this is simply a limitation of this system and needs to be carefully considered in assessing phenotypes using Cre-mediated deletion.

Because of its role in binding IL-2, CD25 is a critical factor in regulating Foxp3. Thus it was possible that the importance of Stat5 for Treg cells was primarily due to its requirement in regulating CD25 expression rather than directly regulating Foxp3. However, we found that in vitro induction of Foxp3 was poor in Stat5-deficient cells, despite levels of IL-2 that would obviate the need for CD25. While regulation of CD25 may contribute to the poor expression of Foxp3, we believe our data also argue for a more direct role independent of effects on CD25 expression. In addition, analysis of $Il2^{-/-}$ and $Il2ra^{-/-}$ ($CD25^{-/-}$) mice showed that the impairment of Foxp3 expression is less severe than what was observed in mice lacking *Jak3, Il2rg*, or *Stat5*.[28] This also suggests that other γc cytokines, which signal predominantly through Stat5, may also play a role in promoting Foxp3 expression and Treg cell development.

Previous studies have noted two consensus Stat-binding elements located in intron 1 of the human *FOXP3* gene.[36] Gain-of-function Stat5 and Stat3 alleles were also found to *trans*-activate a *FOXP3* reporter construct in transient transfection assays.[36] This led to the conclusion that IL-2–induced Foxp3 expression is mediated through both Stat5 and Stat3, and both of the Stats might play positive roles in regulating Foxp3 expression. Similarly, constitutive Stat3 activation was observed in malignant T cells that expressed the NPM/ALK fusion protein. These T cells express IL-10, TGF-β, and Foxp3, and have immunosuppressive properties reminiscent of Treg cells.[37] These authors attributed this phenotype to constitutive activation of Stat3,[37] but Stat5 is also constitutively activated in this setting.[46] However, we found that reduction of Stat3 levels in T cells did not have a major effect on Foxp3 expression in $Stat3^{fl/fl}$, *MMTVcre* mice, indicating that Stat3 and Stat5 do not have redundant roles in Foxp3 regulation in normal mice. Indeed, IL-6–dependent Stat3 activation was suggested to inhibit Treg development during allergic airway inflammation.[44] Recently, it was shown that in vitro stimulation of $CD4^{+}$ T cells with IL-6 and TGF-β1 down-regulated Foxp3 expression.[29] Our data demonstrate that the inhibitory effect of IL-6 on Foxp3 expression is dependent on Stat3 by using Stat3-deficient T cells.

In addition, our data clearly indicate that Stat5a/b bind to the murine *Foxp3* gene, implying a direct role of Stat5 in regulating Foxp3 transcription. In contrast, Stat3 binding to these sites in the *Foxp3* locus was marginal. Given that IL-6 inhibits Foxp3 expression in Stat3-dependent manner, Stat3 must either bind to the *Foxp3* locus at sites distinct from the Stat5 binding sites, or it must act indirectly. Regardless, it is clear that Stat5a/b and Stat3 function distinctly in regulating Foxp3, and it will be important to elucidate precisely how they mediate cytokine effects. It will be of great interest to define how they interact with other transcription factors and coactivators and to determine their ability to influence epigenetic modifications of the *Foxp3* locus.

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Authorship

Contribution: Z.Y. was the prinicipal participant designing and performing research, analyzing data, and writing the manuscript; Y.K., G.S., M.K., and L.D. performed research and analyzed data; Y.K. and W.T.W. performed research and prepared the manuscript; A.L., G.W.R., and L.H. provided materials; E.M.S. and R.M.

analyzed data and participated in the preparation of the manuscript; and J.J.O'S. and C.W. were responsible for the overall study and wrote the manuscript.

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3.1.5 Constitutive activation of Stat5 promotes its cytoplasmic localization and associates with PI3-kinase in myeloid leukemia

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NEOPLASIA

Constitutive activation of Stat5 promotes its cytoplasmic localization and association with PI3-kinase in myeloid leukemias

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Persistent activation of Stat5 is frequently found in hematologic neoplasms. Studies conducted with constitutively active Stat5 mutants (Stat51*6 and cS5^F) have shown that deregulated Stat5 activity promotes leukemogenesis. To investigate the oncogenic properties of these mutants, we used cS5^F-expressing bone marrow cells which induce a multilineage leukemia when transplanted into recipient mice. Here, we show by immunocytochemistry that cS5^F is localized mainly in the cytoplasmic compartment of leukemic cells, suggesting that the transforming nature of cS5^F may be associated with a cytoplasmic function. In support of this hypothesis, we found that cS5^F forms a complex with the p85 subunit of the phosphatidylinositol 3-kinase (PI3-K) and the scaffolding adapter Gab2 in leukemic bone marrow cells, resulting in the activation of Akt/PKB, a crucial downstream target of PI3-K. By using transducible TAT-Gab2 or TAT-Akt recombinant proteins, we were able to demonstrate that activation of the PI3-kinase/Akt pathway by cS5^F molecules through Gab2 is essential for induction of cell growth. We also found that persistently phosphorylated Stat5 in primary cells from patients with myeloid leukemias has a cytoplasmic localization. These data suggest that oncogenic Stat5 proteins exert dual transforming capabilities not only as transcriptional activators but also as cytoplasmic signaling effectors. (Blood. 2007;109:1678-1686)

Introduction

Stat5A and Stat5B transcription factors are important mediators of cytokine-induced cell survival and proliferation.[1] There is a large body of evidence indicating that they play crucial roles in hematopoiesis in humans and mice. Stat5a/b$^{-/-}$ mice have multiple hematopoietic defects which affect the proliferation and/or survival of both lymphoid and myeloid lineages.[2-7] In addition, Stat5 proteins regulate the growth of hematopoietic progenitor cells, and a recent report has suggested that Stat5 might be involved in self-renewal of human CD34$^+$ progenitor cells.[8,9]

Deregulation of the Jak-Stat signaling pathway, particularly Stat3 and Stat5, was reported in many different types of cancer, including hematopoietic neoplasms.[10,11] Persistent activation of these transcription factors is frequently found in many tumor cells, most probably as a consequence of deregulated tyrosine kinase activity. Importantly, Stat5 is a common target for different oncoproteins with tyrosine kinase activity such as Tel-Jak2, Bcr-Abl, the mutated forms of Flt3 and c-Kit, and the Jak2V617F mutant which has been recently characterized in various myeloproliferative disorders.[12-16] Furthermore, it was shown that Stat5 plays a critical role in Bcr-Abl– and Tel-Jak2–induced leukemia.[17-19] The most direct evidence that constitutive activation of Stat5 is an important causative event in cell transformation came from the analysis of the Stat5 mutants, Stat5A1*6, Stat5B1*6, and cS5^F. These proteins with mutations at residues $H_{299}\rightarrow R$ and $S_{711/716}\rightarrow F$ (Stat5A1*6) or with the single mutation $S_{711}\rightarrow F$ (cS5^F) possess constitutive tyrosine phosphorylation and DNA binding activity and are capable of transforming cell lines to growth factor independence.[20] Stat5A1*6 can also induce a fatal myeloproliferative disease in mice, whereas cS5^F has been shown to induce a multilineage leukemia and to restore the defective phenotype of Stat5a/b-deficient mice, closely mimicking wt Stat5 function.[20,21]

Comprehensive analysis of gene-deleted mice as well as dominant-negative approaches shed light onto the mechanisms by which Stat5 controls cell growth and survival of hematopoietic cells. In particular, Stat5 has been shown to regulate expression of genes involved in cell survival and cell-cycle progression, such as *Bcl-x$_L$*, *A1* and D type cyclins, as well as genes encoding cytokines or growth factors, such as *Osm* or *Igf-1*, and the proto-oncogene *Pim-1*.[2,22-26] Thus, persistent transcriptional activation of these genes by Stat51*6 or cS5^F transcription factors may be the sole consequence for the oncogenic properties of these mutants, a hypothesis that awaits experimental evidence. Several studies suggested that Stat5 proteins may control cellular processes independently of their nuclear functions, through interactions with various signaling molecules. Accordingly, the tyrosine-phosphorylated forms of Stat5 were shown to interact with the SH2-SH3–containing adapter CrkL, the scaffolding adapter Gab, and the PI3-K.[27-29] We previously reported that activation of the PI3-K–signaling cascade plays an important role in Stat51*6-induced cell growth and survival via the scaffolding adapter Gab2.[30,31] It is well

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established that PI3-K function requires the activation of the serine threonine kinase Akt.[32] Akt regulates the activity of a number of substrates involved in cell apoptosis or proliferation such as Bad, Forkhead, NFκB, and GSk3β.[33,34] Akt is the cellular homologue of the v-akt oncogene, and there is strong evidence that inappropriate activation of the PI3-K/Akt pathway contributes to the development of cancers.[35] Altogether, these different data argue that oncogenic activation of Stat5 triggers its association with PI3-kinase and that activation of this pathway may be involved in the leukemic potential of constitutively active Stat5 molecules. By using bone marrow (BM) cells from mice that received a transplant of $cS5^F$-expressing cells, we demonstrated for the first time that $cS5^F$ is essentially localized in the cytoplasm of primary leukemic cells. We also found that $cS5^F$ forms a signaling complex with the PI3-K and the scaffolding adapter Gab2 which results in the activation of Akt in primary leukemic cells. We were able to show that Gab2 and Akt play a critical role in $cS5^F$-induced cell growth through the use of transducible TAT fusion proteins. In addition, we demonstrated that persistent Stat5 phosphorylation is detected mainly in the cytoplasm of primary cells from patients with chronic myeloid leukemia (CML) and patients with acute myeloid leukemia (AML). Collectively, these data indicate that oncogenic activation of Stat5 affects its cellular localization and function and that activation of the PI3-kinase/Akt pathway via an interaction with p85 and Gab2 is implicated in Stat5-induced leukemia.

Patients, materials, and methods

Animals, primary cell isolation, retroviral infection

Bone marrow (BM) was harvested from hind limbs of 6-week-old male mice (C57/B6 × Sv129j F1). Freshly isolated BM cells were preactivated for 48 hours in medium containing IL-3 (25 ng/mL), IL-6 (50 ng/mL), and SCF (10 ng/mL). Cells were then cocultured on irradiated (1.5 Gy) semiconfluent ecotropic producer cell lines for 48 hours in the presence of 6 μg/mL Polybrene. Generation of retroviral packaging cell lines carrying the $cS5^F$ mutant or the GFPv was described elsewhere.[2] Lethally irradiated wt female (1 Gy) recipients were then reconstituted with the transduced BM cells by tail vein injection (4×10^6 cells/mouse). Mice that received a transplant were checked for disease onset through blood analysis.

Patients

Four patients with acute myeloid leukemia (AML; FAB-subtype: M2, M3, M6, M7), 3 patients with chronic myeloid leukemia (CML; chronic phase, cp, n = 1; myeloid blast phase, pb, n = 1; megakaryoblast crisis, n = 1), and 1 control patient (no hematologic neoplasm, normal bone marrow) were examined. Diagnosis and classification of cases were performed according to established criteria.[36] This study was approved by the institutional review board (Medical University of Vienna) and conducted in accordance with the declaration of Helsinki. Informed consent was obtained before bone marrow biopsies were taken.

Cell culture, plasmids, and reagents

BM cells from mice that received a transplant of $cS5^F$ were grown for 24 hours in RPMI 1640 with 10% fetal calf serum (FCS) and SCF (10 ng/mL; Valbiotech, Paris, France), and GFPv BM cells were grown in the same medium supplemented with IL-3 (10 ng/mL). The next day, GFP^+ cells were sorted by flow cytometry and cultured in their respective medium for 6 weeks.

Ku-812 cells were grown in RPMI 1640 medium (Life Technologies, Cergy-Pontoise, France) containing 10% FCS, 2 mM L-glutamine (10 U/mL), and penicillin/streptomycin (10 μg/mL) at 37°C with 5% CO_2. The LY294002 PI3-K inhibitor and imatinib mesylate were purchased from Sigma (St Quentin Fallavier, France) and Novartis (Basel, Switzerland), respectively.

The coding regions of Akt and Akt (K_{179}→M), Gab2, and Gab2-3YF were amplified by polymerase chain reaction (PCR) and cloned at *Nco*I and *Eco*RI sites of the bacterial expression vector pTAT-HA (for the Akt cDNAs) or at the *Kpn*I-*Xho*I sites (for the Gab2 cDNAs).

Fluorescence-activated cell sorting (FACS) analysis

Cells were incubated 20 minutes with the following phycoerythrin-conjugated antibodies: CD117, CD34, Mac-1, Gr-1, Ter-119, Sca-1, CD19, and Thy1.2 (BD Biosciences, le Pont de Claix, France) and analyzed by flow cytometry (Elite; Becton Dickinson, Le Pont de Claix, France).

Immunohistochemistry and immunocytochemistry

Immunohistochemistry was performed on sections prepared from paraffin-embedded formalin-fixed BM specimens using the indirect immunoperoxidase staining technique as described.[37,38] Endogenous peroxidase was blocked by methanol/H_2O_2. Prior to staining with anti–P-Y-Stat5a/b antibody, sections were pretreated by microwave oven. Sections were stained with an anti–P-Y-Stat5a/b antibody AX-1 (Advantex Bioreagents, Conroe TX) (1.2 μg/mL) diluted in 0.05 M Tris-buffered saline (TBS, pH 7.5) plus 1% BSA for 20 hours. Then slides were washed and incubated with biotinylated goat anti–mouse IgG for 30 minutes, washed, and exposed to streptavidin-peroxidase complex. AEC was used as chromogen. Slides were counterstained in Mayer Hemalun. For immunocytochemical analysis, cells were spun on cytospin slides prior to staining with the AX1 antibody. DAB was used as chromogen. Slides were counterstained with Mayer Hemalun. Acquisition of micrographs was performed by an Olympus DP11 camera connected to an Olympus microscope equipped with 40×/0.85 (Figure 7A,C) and 100×/1.35 (Figure 3A) Uplan-Apo objective lenses (Olympus, Hamburg, Germany). Images were acquired with Photoshop CS2 software version 9.0 (Adobe Systems, San Jose, CA) and were processed with PowerPoint software (Microsoft, Redmond, WA).

Purification of TAT fusion proteins

Purification of TAT fusion proteins was performed as previously described[39] with the following modifications: BL21 (DE3) pLysS bacteria (Stratagene, Amsterdam, The Netherlands) expressing the TAT fusion proteins were cultured in LB broth medium containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37°C. Protein expression was induced by addition of 1 mM IPTG at 37°C during 4 hours. Cells were harvested and sonicated in buffer A (8 M urea, 20 mM HEPES [pH = 8.0], and 500 mM NaCl). Lysates were clarified by centrifugation at 15 600*g* for 4 minutes at 4°C. The supernatants containing the recombinant TAT fusion proteins were equilibrated in 30 mM imidazole and then applied to Ni-NTa agarose columns (Qiagen, Courtabeuf, France). Columns were washed with 6 bed volumes of buffer B (4 M urea, 20 mM HEPES [pH 8.0], 500 mM NaCl, and 30 mM imidazole). After washing, bound proteins were eluted with buffer B containing 250 mM imidazole. Eluates containing the purified proteins were dialyzed against PBS or (0.9% NaCl) with 4 buffer changes overnight at 4°C. The purity and concentration of TAT fusion proteins was assessed by Coomassie blue–stained SDS–polyacrylamide gel electrophoresis (PAGE) using standard concentrations of BSA. Stability and biologic activity of TAT fusion proteins were determined in Ba/F3 cells expressing the constitutively active form Stat51*6 (See Figures S1-S6, available on the *Blood* website; see the Figure link at the top of the online article).

In vitro proliferation studies

Cells were incubated as triplicates in flat-bottom 24-well plates at a density of 2×10^4 cells/well in medium supplemented with the different TAT fusion proteins at the indicated concentration for 16, 24, or 48 hours. The percentage of living cells was evaluated using the trypan blue dye exclusion assay.

Subcellular fractionation

Cells were lysed in hypotonic buffer (20 mM HEPES, 10 mM KCl, 1 mM EDTA, 0.2% NP40, 10% glycerol, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 mM phenylmethylsulfonide fluoril, and 1 mM Na_2VO_4) and centrifuged for 5 minutes at 800*g* (Eppendorf centrifuge). Supernatants (cytoplasmic fraction) were frozen at −70°C. Pelleted nuclei were resuspended in hypertonic buffer (Hypotonic buffer plus 350 mM NaCl), and proteins extracts were prepared by constant agitation during 30 minutes at 4°C. Debris was removed by centrifugation, and nuclear extracts were frozen at −70°C.

Immunoprecipitations, Western blotting, and antibodies

Cells were lysed in Laemmli buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol). Equal amounts of each protein sample were separated by electrophoresis on SDS-PAGE and blotted onto nitrocellulose membrane (Bio-Rad, Munich, Germany). Blots were incubated as indicated with antibodies raised against the following proteins: Actin, Akt, Gab2, Raf-1, and Lamin C (Santa Cruz Biotechnology, Santa Cruz, CA), $p27^{kip,1}$ Stat5 (Transduction Laboratories, Lexington, KY), Bim (Affinity BioReagents, Golden, CO), HA (Roche, Basel, Switzerland), P-Stat5 or P-Akt^{Ser473} (Cell Signaling Technology, Boston, MA), and topoisomerase I. The blots were developed with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Orsay, France) using specific peroxidase-conjugated anti-IgG Abs. Immunoprecipitation experiments were performed as previously described.[27]

Results

Mice that received a transplant of $cS5^F$ as a model to characterize the transforming properties of Stat5

Mice that received a transplant of $cS5^F$ have been previously reported to develop a multilineage leukemia as a result of enhanced and sustained Stat5 tetramer DNA binding complexes.[21] It remained questionable how $cS5^F$ proteins transduce signals to transform hematopoietic cells and if the predominant function can be attributed only to the transcriptional activity of the oncogenic mutant. In the present model, BM cells were infected with a retrovirus carrying the $cS5^F$ mutant or the GFP vector (GFPv) alone, analyzed for infection efficiency by FACS and injected into lethally irradiated wt mice. Eight mice that received a transplant of $cS5^F$ and 4 mice that received a transplant of GFPv were analyzed. All 8 $cS5^F$ mice were fully reconstituted 4 weeks after transplantation. They developed multilineage leukemia after 4 weeks, whereas no disease was seen in the 4 control mice (Figure 1A). None of the $cS5^F$-expressing mice survived more than 8 weeks after transplantation, whereas all control mice remained disease free. Thus, in our experiments we chose to analyze diseased mice that received a transplant of $cS5^F$ at 6 weeks after transplantation (Figure 1A). Blood from these animals always contained a remarkable increase in white blood cell counts as compared with controls that was associated with splenomegaly and mesenterial lymphadenopathy (Figure 1B-C). Persistent activation of Stat5 was demonstrated by Western blotting in extracts from $cS5^F$-transformed splenic (SP) and BM cells using anti–phospho-Y^{694}-Stat5 (P-Y-Stat5) antisera (Figure 1D, lanes 3, 4, 8, 9). Extracts from unstimulated or IL-3–stimulated Ba/F3 cells were also loaded as control (lanes 1 and 2). By contrast no phosphorylation was detected in the splenocytes or BM cells of control GFPv mice (lanes 5, 6, 7).

In vitro proliferation of $cS5^F$-expressing BM cells

Proliferation and survival of BM progenitors are influenced by various hematopoietic growth factors, including stem-cell factor

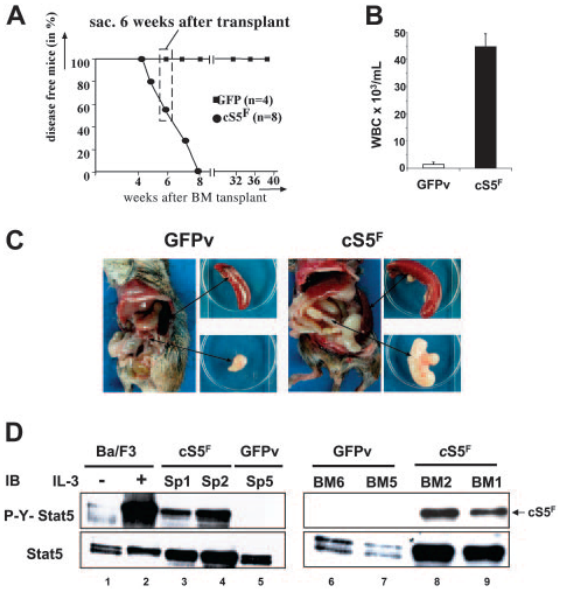

Figure 1. Leukemogenic mice that received a transplant of $cS5^F$ as a model to characterize the transforming properties of Stat5. (A) Kaplan-Meier plot of mice that received a transplant of $cS5^F$ (n = 9) versus mice that received a transplant of GFPv (n = 4). Mice that received a transplant of $cS5^F$ died within 7 weeks after transplantation, while control GFPv mice remain disease free (followed for more then 10 months after transplantation). Thus, experimental mice that received a transplant of $cS5^F$ were killed and analyzed 6 weeks after transplantation for all following experiments (dashed box). (B) White blood cell counts from mice that received a transplant of $cS5^F$ (n = 9) and from GFPv mice (n = 4). Error bars represent SE. (C) Typical organic alterations of mice that received a transplant of $cS5^F$ and control GFPv mice that were killed at 6 weeks after transplantation. Leukemic mice that received a transplant of $cS5^F$ have a unique pathologic appearance with massively enlarged mesenterial lymph nodes (lymphadenopathy) and splenomegaly. (D) Splenic- and BM-cell extracts from 2 mice that received a transplant of $cS5^F$ (Sp1, Sp2, BM1, BM2) or from control GFPv mice (Sp5, BM5, BM6) were analyzed by Western blotting with the indicated antibodies. Cell lysates from Ba/F3 cells unstimulated or stimulated with IL-3 for 30 minutes were also included as controls.

(SCF) and IL-3. Because persistent Stat5 signaling was shown to relieve the growth factor dependence of cell lines, we then analyzed the growth factor requirement of $cS5^F$-expressing BM cells. GFP^+ BM cells of 2 leukemic mice that received a transplant of $cS5^F$ or of 2 control GFPv mice were isolated and cultured in a medium containing SCF. In these conditions, $cS5^F$-GFP^+ BM cells (BM1 and BM2) were able to grow, whereas control GFP^+ BM cells (BM5 and BM6) did not grow (Figure 2). Addition of IL-3 restored the proliferating capacities of control GFP^+ BM cells. However, we observed that the presence of SCF was absolutely required for the growth of these $cS5^F$-transformed primary cells, indicating that expression of this mutant partially relieved their cytokine (IL-3) dependence. Note, the growth rate of GFPv BM cells cultured in presence of SCF, and IL-3 was always higher than the growth rate of cS5F BM cells cultured in the presence of SCF alone (Table 1).

$cS5^F$ promotes the growth of hematopoietic progenitor cells in vitro

Freshly isolated BM cells from animals that received a transplant of $cS5^F$ (n = 4) were subjected to lineage markers and GFP expression by FACS analysis (Table 1). The lineage markers included Ter119 (erythroid); Gr-1/Mac-1 (myeloid); CD19 and Thy-1.2 (B and T lineages); and combinations of CD34, Sca-1, and CD117

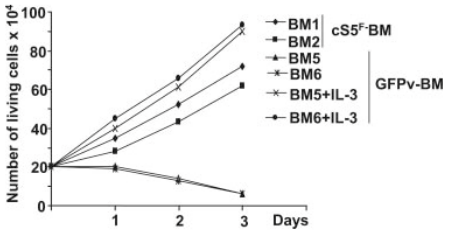

Figure 2. In vitro proliferation of cS5^F-transformed bone marrow cells. Sorted GFP+ BM cells from 2 cS5^F-grafted mice (BM1 and BM2) or from 2 control GFPv mice (BM5 and BM6) were cultured in the presence of SCF (10 ng/mL). In addition to SCF, IL-3 (10 ng/mL) was added to BM5- and BM6-cell cultures. Cells were counted daily using the trypan blue dye exclusion method.

(c-Kit), typical markers for immature multipotent cells or hematopoetic stem cells (HSCs). The majority of cS5^F-GFP$^+$ BM cells was Gr-1– and Mac-1–positive myeloid cells and around 20% of GFP$^+$ BM cells were of B- or T-lymphoid origin. A fraction of cS5^F-GFP$^+$ BM cells also expressed markers typical for primitive multipotent cells. Following 1 week of culture, the myeloid- and lymphoid-specific markers disappeared from the cS5^F-GFP$^+$ BM cells. By contrast, GFP$^+$ cells expressing the immature markers CD34, Sca-1, and CD117 were amplified, indicating that cS5^F promotes the growth of immature progenitor cells. These data support the recent findings that the multilineage cell expansion observed in these mice arose from immature cells transformed by cS5^F.[21]

Cytoplasmic localization of cS5^F in transformed BM cells

It was previously shown that phosphorylated forms of Stat5 interact with various cytoplasmic signaling effectors.[27-29] We therefore addressed the question whether cS5^F may have a cytoplasmic function. We first determined the localization of cS5^F in transformed BM cells by immunocytochemistry with an anti–phospho-Y^{694}-Stat5 antibody before and after stimulation with IL-3. Results clearly showed (Figure 3A) that constitutively active cS5^F mutant is mainly localized in the cytoplasm of BM cells, whereas it is found in the nucleus after stimulation with IL-3. We also analyzed the localization of P-Y-Stat5 in control GFPv BM cells stimulated or not with IL-3. No phosphorylated Stat5 was detected in IL-3–deprived cells, whereas IL-3 treatment of GFPv cells induced the appearance of nuclear P-Y-Stat5. To confirm the specific cytoplasmic localization of cS5^F, we prepared cytoplasmic and nuclear extracts of cS5^F BM cells from 3 different leukemic mice, and the presence of P-Y-Stat5 in both fractions was analyzed by Western blotting with an anti–P-Y-Stat5 antibody (Figure 3B). P-Y-Stat5 was detected in the cytoplasmic fraction of cS5^F BM cells, whereas it was mainly found in the nucleus after stimulation with IL-3. The purity of the extracts was controlled by Western blot analysis for the cytoplasmic ser/thr kinase Raf-1 and the nuclear protein Lamin C. Altogether, these results support the idea that cS5^F has a cytoplasmic signaling function that might play an important role in cS5^F-induced leukemia.

Critical role of the Gab2/PI3-K/Akt pathway in cS5^F-mediated cell transformation

We have previously demonstrated that tyrosine-phosphorylated Stat5 is able to interact with both the PI3-K regulatory subunit p85 and the Gab2-scaffolding adapter in Ba/F3 cells.[30,31] We therefore determined whether cS5^F also interacts with PI3-K and Gab2 in the primary leukemic cells from diseased mice. For this purpose, cell lysates obtained from 2 different cS5^F-expressing BM cells (BM1 and BM2) were immunoprecipitated with anti-Stat5 or isotype-matched control antibodies. The coimmunoprecipitations of the p85 regulatory subunit of PI3-K and Gab2 were next evaluated by immunoblotting (Figure 4A). Results showed that both p85 and Gab2 were associated with cS5^F in primary leukemic-cell extracts. BM-cell lysates of mice that received a transplant with cS5^F or control GFPv mice were next analyzed by immunoblotting with anti–P^{ser473}-Akt and anti-Akt antibodies (Figure 4B, top). A weak phosphorylation of Akt was found in GFPv BM cells, whereas the level of phosphorylated Akt was much higher in cS5^F-expressing BM cells, indicating that Akt phosphorylation was significantly induced by cS5^F in these primary leukemic cells. IL-3 treatment of GFPv BM cells and cS5F BM cells did not further increase the level of phosphorylated Akt (Figure 4B, lower panel) but retained the capacity to activate Stat5 (see Figure S5A, available on the *Blood* website; click on the Supplemental Figures link at the top of the online article).

We next determined whether activation of the PI3-K is required for cS5^F-induced cell growth and survival. For this purpose, we used the pharmacologic PI3-K inhibitor LY294002. cS5^F BM cells cultured in the presence of SCF or control GFPv BM cells grown in the presence of SCF and IL-3 were treated with increasing concentrations (ranging from 1 to 10 μM) of LY294002 (Figure 4C). Growth arrest of the cS5^F-expressing BM cells was observed with 1 μM LY294002 after 48 hours of culture, whereas 10 μM was required to inhibit the growth of GFPv BM cells, indicating that cS5^F BM cells were more sensitive to the inhibition of PI3-K than were control cells. Collectively, our results suggest that cS5^F molecules form a signaling complex with Gab2 and PI3-K to promote the phosphorylation of Akt.

Recombinant TAT fusion proteins that interfere with PI3-K signaling block cS5^F-induced cell growth and Akt phosphorylation

To investigate the role of Gab2 and Akt in the transforming properties of cS5^F, we generated different recombinant TAT-Gab2 or TAT-Akt fusion proteins (Figure 5A). The use of TAT fusion proteins is an appealing approach to transduce cell lines or primary cells.[39,40] Adding them to the culture medium requires, however, biochemical testing for each individual fusion protein purification which had to be done freshly for each individual experiment. We first analyzed the role of Gab2 in cS5^F-induced cell growth, and, for this, we generated a TAT-wtGab2 and a TAT-Gab2-3YF deficient in PI3-kinase binding in which the 3 tyrosine residues (Y,^{452}, Y^{476}, and

Table 1. cS5^F promotes the growth of hematopoietic progenitor cells

cS5^F-BM*	Thy1.2	CD19	Mac-1	Gr-1	Ter119	CD117	CD34	Sca-1
Day 0	9.5 ± 1	12.25 ± 5.5	44.5 ± 8.4	44.25 ± 8.8	15.5 ± 1.9	15.75 ± 1.7	5.5 ± 1.3	9.25 ± 0.96
Day 7	0.375 ± 0.03	2.45 ± 0.07	0.9 ± 0.8	6.3 ± 1	5.9 ± 0.7	99.25 ± 0.7	43.6 ± 7.39	56.8 ± 4.1

Bone marrow cells from cS5^F leukemic mice (n = 4) were subjected to FACS analysis using indicated markers before (day 0) and after 1 week (day 7) of culture in medium containing SCF (10 ng/mL). Results are expressed as the percentage of positive cell markers among GFP-positive cells (mean ± SD).

*n = 4.

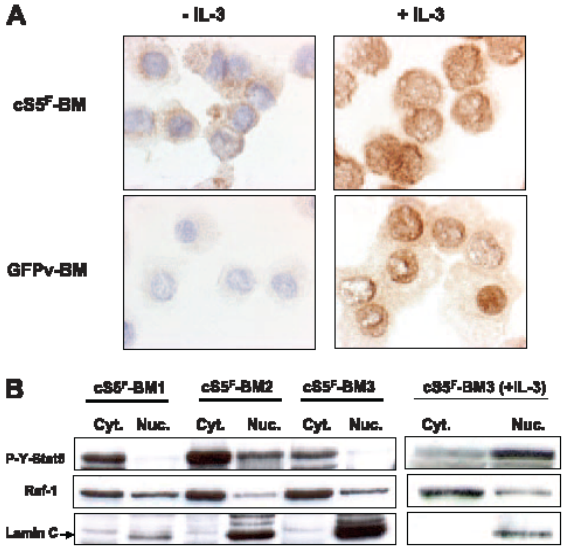

Figure 3. Cytoplasmic localization of cS5F in bone marrow cells. (A) Immunocytochemical detection of phosphorylated Stat5 in cS5F-expressing BM cells and control GFPv BM cells stimulated or not with IL-3. In each case, cells were spun on cytospin slides and analyzed by indirect immunocytochemistry using an anti–P-Y-Stat5 antibody (AX1). (B) The localization of cS5F was also determined by Western blot analysis using cytoplasmic and nuclear extracts of unstimulated or IL-3–stimulated cS5F BM cells from 3 different mice that received a transplant.

Y584) were changed to phenylalanine (Figure 5A). It was shown that mutations of these 3 tyrosine residues inhibit activation of the PI3-K/Akt pathway in cytokine-stimulated cells.[41] TAT-wtGab2 and TAT-Gab2-3YF proteins were purified and quantified as described in "Patients, materials, and methods" (data not shown).

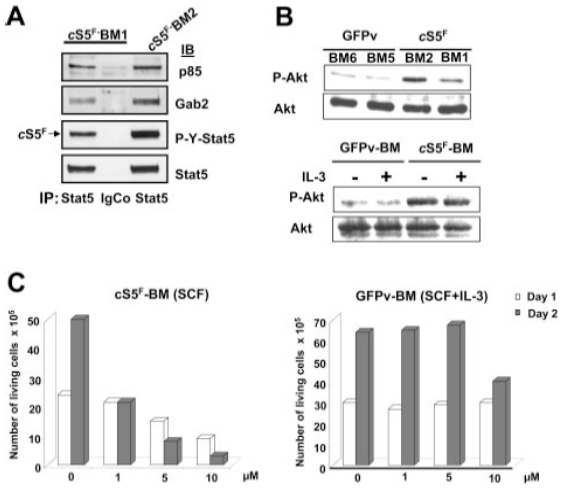

Figure 4. cS5F interacts with Gab2 and PI3-K and induces Akt activation in transformed bone marrow cells. (A) Stat5 was immunoprecipitated from 2 cS5F-expressing BM-cell lysates (BM1 and BM2). Subsequently, the content in p85 and Gab2 proteins was evaluated in the Stat5 immunoprecipitates by Western blotting using the indicated antisera. Cell lysates were also immunoprecipitated with an isotypic control IgG antibody. (B) BM-cell lysates from 2 mice that received a transplant of cS5F or from 2 control GFPv mice (top) and lysates from GFPv BM and cS5F BM cells stimulated or not with IL-3 (10 ng/mL) for 30 minutes (bottom) were analyzed by Western blotting with the indicated antibodies. (C) cS5F-expressing BM and control GFPv BM cells cultured in the presence of SCF and SCF + IL-3, respectively, were incubated or not with different concentrations of LY294002 for 48 hours. The number of living cells was determined daily using the trypan blue dye exclusion method. Results are the mean of 3 independent experiments.

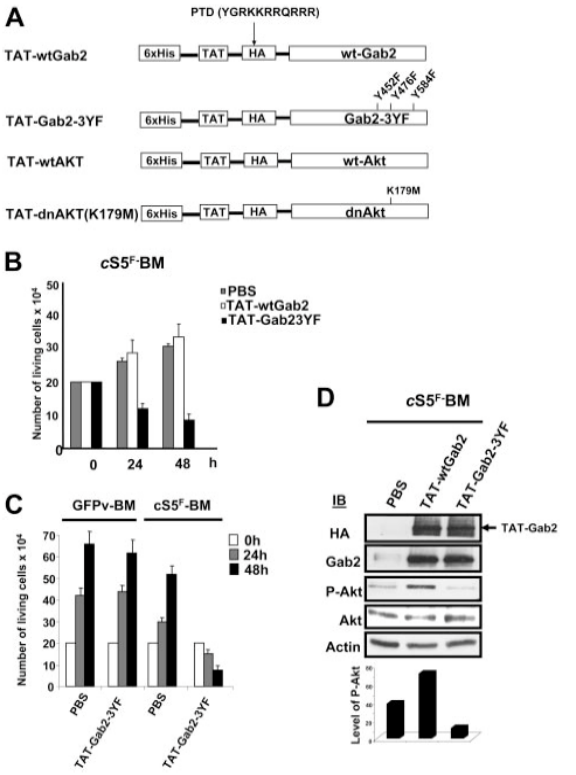

Figure 5. Effects of recombinant TAT-Gab2 fusion proteins on cS5F-induced cell growth and Akt phosphorylation. (A) Schematic representation of TAT-Akt and TAT-Gab2 constructs. The different cDNAs were introduced into the bacterial expression vector pTAT-HA. Resulting recombinant Akt (wt and dn) and Gab2 (wt and Gab23YF) proteins were fused in their N-terminal part to a 6 ×His-Tag followed by the protein transduction domain (PTD) of the TAT protein and a HA tag sequence. (B) cS5F BM cells were transduced or not with 100 nM of the different TAT-Gab2 proteins during 48 hours, and the number of living cells was determined daily using the trypan blue dye assay. Results are the mean of 3 independent experiments performed with 2 independent cS5F BM-cell cultures. (C) GFPv BM cells and cS5F BM cells were transduced with 100 nM TAT-Gab23YF fusion protein, and growth of the cells was determined daily. Results are the mean of 3 independent experiments. (D) After extensive washes, lysates from transduced cS5F BM cells were prepared and analyzed by Western blotting with the indicated antibodies. Densitometric analysis was also performed to determine the ratio of P-Akt/Akt in the different samples (bottom).

cS5F BM cells from 3 different mice that received a transplant were transduced or not with the TAT-Gab2 fusion proteins (100 nM) for 24 and 48 hours, and the number of living cells was estimated by the trypan blue dye exclusion method (Figure 5B). Transduction of TAT-Gab2-3YF protein abolished the growth of the cells, whereas transduction of TAT-wtGab2 slightly increased their proliferation. Transduction efficiency of the different TAT-Gab2 proteins was evaluated in cS5F-expressing BM cells by Western blot analysis with anti-HA and anti-Gab2 antibodies (Figure 5D). Equal transduction of both proteins was observed. To verify the specific effect of the TAT-Gab2-3YF fusion protein, control GFPv BM cells grown in the presence of SCF and IL-3 or cS5F BM cells were transduced with the TAT-Gab2-3YF protein (100 nM), and cell growth was determined next (Figure 5C). The TAT-Gab2-3YF protein clearly inhibited the proliferation of cS5F BM cells, whereas control GFPv BM cells remained insensitive. Western blot analysis with anti-HA and anti-Gab2 antibodies showed that both types of cells were equally and efficiently transduced (data not shown). We also determined whether TAT-Gab2 proteins could alter the PI3-K activity in cS5F BM cells by analyzing the phosphorylation level of

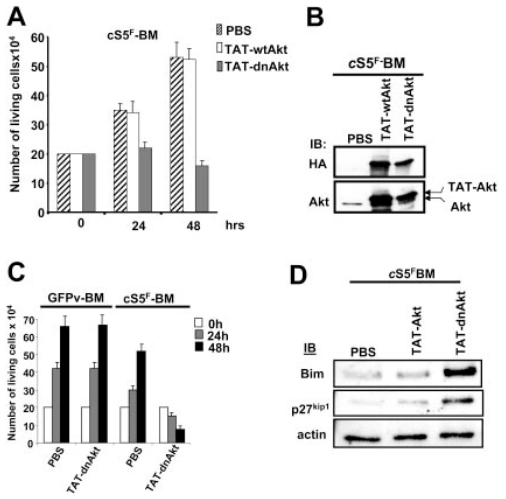

Figure 6. Specific inhibition of cS5^F-induced cell growth by the recombinant TAT-dnAkt protein. (A) cS5^F BM cells were grown in the presence of PBS, TAT-wtAkt, or TAT-dnAkt fusion proteins (100 nM), and the cells were counted daily using the trypan blue dye assay. Results are the mean of 3 independent experiments. (B) cS5^F BM cells were transduced with the different TAT-Akt fusion proteins during 24 hours, and the presence of the recombinant proteins was detected by immunoblotting with the indicated antibodies. (C) GFPv BM cells and cS5^F BM cells were transduced with 100 nM TAT-dnAkt fusion protein, and the number of living cells was enumerated daily. Results are the mean of 3 independent experiments. (D) Expression of Bim and p27^{kip1} was evaluated by Western blotting in cS5^F-expressing BM cells transduced or not with recombinant TAT-Akt.

Akt. Although phosphorylation of Akt was clearly enhanced following transduction with TAT wtGab2, it sharply decreased Akt activation in TAT-Gab2-3YF–treated cells (Figure 5D). Similarly, a wild-type (wt) or a dominant-negative (dn) form of Akt containing a point mutation that inactivates its kinase activity (K_{179}→M) were fused to the protein transduction domain of the TAT HIV protein (Figure 5A). Resulting TAT-Akt fusion proteins were produced, purified, and quantified exactly like TAT-Gab2 fusion proteins (see Figure S1). The biologic effects of TAT-Akt proteins were then evaluated on cS5^F-transformed BM cells. Primary cells were incubated with the different TAT-Akt fusions, and cell growth was determined in a time course experiment. Transduction of TAT-dnAkt completely inhibited the proliferation of cS5^F BM cells, whereas TAT-wtAkt was without any detectable effects on cell growth (Figure 6A). Similar results were obtained after transduction of the TAT-Akt fusion proteins in Ba/F3 cells (Figure S2). Western blot analysis with anti-HA and anti-Akt antibodies showed that both TAT-Akt fusion proteins were equally transduced in cS5^F-expressing BM cells (Figure 6B). We also analyzed the effect of the TAT-dnAkt fusion protein on the growth of GFPv-BM cells (Figure 6C). Although GFPv BM cells and cS5^F BM cells were equally and efficiently transduced (data not shown), results clearly showed that transduction of the TAT-dnAkt fusion protein had no effect on GFPv BM cell growth, arguing again that cS5^F BM cells but not GFPv BM cells were highly sensitive to inhibition of the PI3-kinase/Akt pathway. It was previously shown that Akt modulates the phosphorylation of the Forkhead family members.[33] Inhibition of Akt activity promotes the nuclear translocation and transcriptional activity of these proteins. Accordingly, we found that transduction of TAT-dnAkt but not TAT-wtAkt inhibited FKHR (FoxO1A) phosphorylation (see Figure S3). Bim and p27^{kip1} are among the target genes of the Forkhead transcription factors, and they execute critical roles in apoptosis or inhibition of cell-cycle progression.[42,43] We therefore determined whether TAT-dnAkt affected the transcriptional activity of Forkhead transcription factors and analyzed expression of Bim and p27^{kip1} by immunoblotting in cS5^F-expressing BM cells transduced with the different TAT-Akt proteins (Figure 6D). Expression of both Bim and p27^{kip1} was up-regulated in cells transduced with the TAT-dnAkt but not with the TAT-wtAkt. These data clearly indicate that TAT-dnAkt is capable to specifically inhibit the phosphorylation of FKHR in primary transformed cS5^F-expressing BM cells, thereby inducing its transcriptional target genes. These results also indicate that Akt plays an important regulatory role in the cS5^F-induced cell growth and survival by down-modulating Bim and p27^{Kip1} molecules.

Constitutively phosphorylated Stat5 proteins are present in the cytoplasm of human neoplastic cells from patients with chronic myeloid leukemia (CML) and patients with acute myeloid leukemia (AML)

It was previously shown that Stat5 is constitutively active in leukemic cells from patients with CML and patients with AML.[10] We then analyzed by immunohistochemistry the localization of P-Y-Stat5 in neoplastic cells from patients with CML at different phases of the disease progression (Figure 7A). In the normal bone marrow (BM), P-Y-Stat5 was detectable in the cytoplasm of

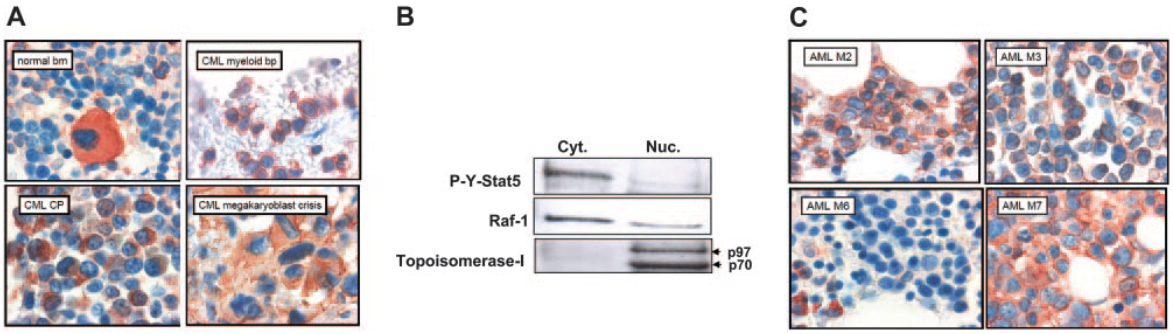

Figure 7. Cytoplasmic localization of phosphorylated Stat5 in myeloid leukemia. (A) Immunohistochemical detection of P-Y-Stat5 in neoplastic cells from patients with CML. P-Y-Stat5 immunostaining was realized on normal bone marrow (BM), on neoplastic cells from CML in chronic phase (CML-cp), in blast phase (CML-pb), and in megakaryoblast crisis. (B) Cytoplasmic and nuclear extracts were prepared from leukemic cells of a patient with CML and analyzed by Western blot with the indicated antibodies. (C) Immunohistochemical detection of P-Y-Stat5 in AML blasts. P-Y-Stat5 immunostaining was performed with bone marrow sections from patients AML-M2, -M3, -M6, and -M7.

megakaryocytes and some immature myeloid cells, whereas mature granulocytes and mature erythrocytes did not contain substantial amounts of P-Y-Stat5. In chronic-phase CML (CML-cp), an increase in P-Y-Stat5–positive myeloid progenitors was found when compared with normal BM. Blast cells in patients with CML blast phase (CML-pb) were also found to react with the anti–P-Y-Stat5 antibody. In both cases, P-Y-Stat5 was predominantly detected in the cytoplasm of the leukemic cells. The most abundant staining reaction was seen in a patient with CML with megakaryoblast crisis. To confirm the cytoplasmic localization of P-Y-Stat5, cytoplasmic and nuclear fractions of cells from a patient with CML were analyzed by Western blotting using an anti–P-Y-Stat5 antibody. As shown Figure 7B, P-Y-Stat5 was exclusively localized in the cytoplasm of these leukemic cells. The purity of the cellular fractions was controlled by a Western analysis for the cytoplasmic Raf-1 kinase and the nuclear Topoisomerase I p97/p70 proteins. Bone marrow sections obtained from patients with AML-M2, -M3, -M6, and -M7 were also stained with anti–P-Y-Stat5 antibody (Figure 7C). A clear staining of blast cells was found in those with AML-M2 and AML-M3, and in the patient with AML-M7, in whom blasts cells contained huge amounts of P-Y-Stat5. By contrast, erythroid progenitors were found to be P-Y-Stat5-low cells in one patient (AML-M6) closely reflecting the situation in the normal BM. Collectively, these results suggested that oncogenic activation of Stat5 promotes the cytoplasmic localization of these transcription factors in myeloid leukemias.

Discussion

Beside their physiologic role in hematopoietic-cell development and function, deregulated Stat5 activities promote leukemogenesis.[10] Engineered Stat5 mutants with constitutive activities are a good model to mimic persistent tyrosine kinase activation and to investigate the role of deregulated Stat5 activity in cancers that are susceptible to tyrosine kinase oncogenes. Mutant tyrosine kinases activate distinct signaling pathways (eg, Ras/Map kinase, PI3-K/Akt, NFκB) that play a role in cell growth and survival.[44-47] It is therefore crucial to determine the precise contribution of Stat5 molecules in the transforming process. Here, we have started to analyze cytoplasmic functions of Stat5 that bridge to PI3-K signaling both in a murine transplantation system with oncogenic Stat5 or in cells from patients with CML and patients with AML associated with a constitutive Stat5 activity. To address signaling properties of activated cytoplasmic Stat5 proteins, we used mainly a murine transplantation model of Stat5-induced leukemia.[21] Mice that received a transplant with BM cells expressing $cS5^F$ exhibited a multilineage hematopoietic-cell expansion that lead to severe leukemia within 4 weeks after transplantation. We found that contrary to normal hematopoietic progenitors, the in vitro growth and survival of $cS5^F$-transformed BM cells did not require IL-3, but SCF was essential for self-renewal of immature cell populations. The possibility of an autocrine loop of IL-3 secretion was excluded by the lack of alteration of cell growth in the presence of neutralizing anti–IL-3 antibodies (data not shown). These findings are reminiscent of the loss of IL-3 dependence of Stat5A1*6-expressing Ba/F3 cells.[20]

The present study demonstrates for the first time that a major fraction of constitutively active Stat5 molecules is cytoplasmic under steady state culture conditions in the presence of growth factors such as SCF or in leukemic patient samples. The cytoplasmic localization of P-Y-Stat5 is not restricted to CML and is also found in AML. We lack a full understanding of the cytoplasmic localization of P-Y-Stat5 in leukemic cells, but active Stat5 proteins are rapidly shuttling between nucleus and cytoplasm.[48] It is therefore conceivable that persistent phosphorylation of Stat5 may affect its subcellular localization. Alternatively, it is possible that translocation signals provided by activated cytokine receptors or signals that induce activation of tyrosine phosphatases are lacking in $cS5^F$ BM cells, thereby inducing accumulation of $cS5^F$ in the cytoplasm. Studies with kinetic experiments of Stat5 protein shuttling in and out of the nucleus on cytokine action in normal versus transformed cell pairs would reveal important insights. Irrespective, persistent transcriptional activity of Stat5 seems not to be the dominant form of oncogenic Stat5 molecules in myeloid-cell types. Our results support the concept that a large part of oncogenic Stat5 activity might not only involve a nuclear function but also a cytoplasmic function. The data are consistent with previous work showing that phosphorylated forms of Stat5 act as signaling effectors by interacting with various signaling molecules such as Crkl, Gab2, and PI3-kinase.[27-31] Accordingly, we also found that expression of $cS5^F$ in BM cells induced activation of the PI3-K/Akt pathway via an interaction with the scaffolding adapter Gab2 and p85, the regulatory subunit of the PI3-K. It will be difficult in future experiments to create mutants of Stat5 that do not interact with PI3-K signaling, because tyrosine phosphorylation of Stat5 is absolutely required for Gab2 and p85 interaction.[31] Importantly, the PI3-K inhibitor LY294002 completely blocked the growth of $cS5^F$ BM cells, but PI3-K activation is also achieved efficiently through activation of the c-Kit receptor and SCF was always present in the primary murine-cell cultures. Thus, activation of the PI3-K/Akt pathway was required for $cS5^F$-mediated cell transformation. We also addressed in detail how activation of the Gab2/PI3-kinase/Akt pathway is involved in the $cS5^F$-induced cell transformation. Activation of the PI3-K by tyrosine kinases following activation by extracellular stimuli or after oncogenic challenge occurs via distinct mechanisms. One of them implies the binding of p85 to the scaffolding adapters Gab1/2.[49] In such a scenario, tyrosine phosphorylation of Gab proteins allows recruitment of the p85 subunit via its SH2 domain and this leads to the allosteric activation of the catalytic subunit. Data from our laboratory are in line with this model and we demonstrate here by means of a recombinant and transducible TAT-Gab2-3YF fusion protein, a deficient p85 binding mutant, that Gab2 is absolutely necessary for $cS5^F$-induced cell growth and Akt phosphorylation. We also analyzed the downstream effects of Akt on $cS5^F$-induced cell growth by using transducible recombinant TAT fusion proteins fused to a wt or a dominant-negative form of Akt. Akt has been shown to directly phosphorylate and inactivate members of the Forkhead family which includes AFX, FKHR, and FKHRL1.[33,34] Accordingly, we found that transduction of the mutant TAT-dnAkt protein inhibits the phosphorylation of FKHR and up-regulates expression of Bim and $p27^{kip1}$ proteins in transformed bone marrow cells inhibiting cell growth (see also Figure S3). In regard to the transformation process induced via $cS5^F$ activity, the overall increase in Akt phosphorylation contributes to growth and survival advantages in the absence of cytokines. Therefore, signaling via the Akt kinase is an essential step in Stat5-induced leukemogenesis.

Persistent Stat5 and PI3-K activation were observed in cells expressing various oncogenic tyrosine kinases such as Bcr-Abl, and it was shown that both pathways contribute to the transformation process controlled by this oncoprotein.[13,47] Interestingly enough, Bcr-Abl is unable to transform and to activate the PI3/Akt pathway in primary myeloid cells isolated from *gab2*$^{-/-}$ mice, indicating

that Gab2 signaling via the PI3-K is an important step in Bcr-Abl–induced leukemogenesis.[50] Collectively, these data support our novel findings that formation of a cytoplasmic signaling complex among Stat5, Gab2, and PI3-K might be one of the key steps in cS5^F-evoked multilineage leukemia. A recent report documented lentivirus-mediated RNA interference to inhibit Stat5 or Gab2 protein expression, leading to diminished in vitro colony formation of CML colony-forming units (CFUs) pinpointing to an essential role of both proteins for Bcr-Abl transformation in line with knock-out studies of both molecules.[17,51]

Immunohistochemistry or nuclear versus cytoplasmic fractionation proved that persistent Stat5 activity is mainly present in the cytoplasm of leukemic cells isolated from patients with CML or from primary cS5^F-transformed cells. In addition, we also found that phosphorylated Stat5 proteins interact with PI3-kinase and Gab2 in human Bcr-Abl–expressing cell lines such as K562[30] and Ku812 (data not shown). Thus, Stat5 proteins might also play an integral part as cytoplasmic signaling effectors via its association with Gab2 and PI3-K in the development of human CML. It is possible that oncogenic activation of Stat5 may affect the ratio of nonphosphorylated/phosphorylated forms of Stat5 in both cellular compartments which could enhance the capacity of these molecules to interact with other signaling pathways, most importantly the PI3-K/Akt pathway. However, the cytoplasmic function of Stat5 is not the sole mechanism by which these proteins promote leukemogenesis.

Some previously identified target genes of Stat5 have been shown to contribute to the development of cancer. DNA binding and transcriptional activities play an important role in the transforming properties of Stat5. We have shown that tetramer formation is required for cS5^F-induced leukemia.[21] Formation of Stat5 tetramers results in DNA binding to low-affinity Stat5 binding sites and putative Stat5 tetramer target genes include *CIS*, *SPI2.1*, *CD25*, D type cyclins, *OSM*, or *IGF-1*.[21,52-54] The transduction of persistently activated forms of Stat5 that are tetramerization deficient do not cause leukemia when transplanted in mice.[21] Nonetheless, we were able to stain for localization of tetramerization-deficient Stat5 mutants that are persistently active from transfected Ba/F3 cells followed by FACS sorting and immunocytochemistry. cS5^F and the tetramer-deficient mutants cS5^{F}W37A and cS5^{F}Δ136 proteins were found to have a predominant cytoplasmic localization under IL-3–deprived conditions in Ba/F3 cells, again IL-3 stimulation caused nuclear translocation and increased tyrosine phosphorylation of the mutants (see Figure S4; data not shown). Because the tetramer-deficient but constitutively active Stat5 mutants are not able to induce cell transformation, this would argue again that the cytoplasmic role of cS5^F is also not sufficient to transform cells. The data parallel the predominant cytoplasmic activation on persistent activation of mutant Stat5 in primary mouse cells and the findings from CML or AML patient samples. Our current hypothesis is that both cytoplasmic and nuclear functions are linked to the full transforming activity of cS5^F molecules or persistently active Stat5 proteins in leukemia.

In conclusion, both the transcriptional activity and the capacity to regulate the PI3-K/Akt activity are required for the full transforming properties through persistent Stat5 protein activation. Oncogenic activation of Stat5 revealed a surprising cytoplasmic localization and function. This report sheds light on the crucial role of the Gab2/PI3-K/Akt pathway to contribute to cancer progression through persistent Stat5 signaling.

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Authorship

Contribution: N.H., C.P., M.K., K.S., B.K., R.N., A.R., I.D., C.S., and M.B. designed and performed the research; V.G.-G. designed the research and wrote the paper; H.B. contributed analytical tools and analyzed the data; K.L. analyzed data and wrote the paper; P.V., R.M., and F.G. designed the research, analyzed data, and wrote the paper.

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3.1.6 Regulation of matrilysin expression in endothelium by fibroblast growth factor-2

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Regulation of matrilysin expression in endothelium by fibroblast growth factor-2 ☆

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Abstract

Matrilysin (MMP7) is a secreted matrix metalloproteinase, which contributes to angiogenesis by breaking down basement membranes. We show that the angiogenic factor FGF-2 induces MMP7 expression in human endothelial cells. The promoter contains a Lef/Tcf consensus sequence, but using wildtype or Lef/Tcf-mutated promoter constructs, FGF-2-induced MMP7 reporter activity is independent from Lef/Tcf sites. Instead, we show that overexpression of a dominant negative Stat3 mutant reduces FGF-2-mediated MMP7 promoter activity. However, Stat3 does not bind to the MMP7 promoter, but activates MMP7 gene expression indirectly via AP-1. This is confirmed by MMP7 promoter constructs with mutated AP-1 sites which did not respond to FGF-2 and by siRNAs against Stat1 and Stat3, which repressed FGF-2-induced MMP7 protein expression. In conclusion, we show that FGF-2-induced MMP7 expression in endothelium depends on AP-1 and FGF-2 signaling to AP-1 involves a Stat1/3-dependent pathway.

Keywords: Endothelium; Matrilysin; MMP7; FGF-2; Stat1; Stat3; AP-1; Lef/Tcf

Angiogenesis is the highly coordinated process of emerging new blood vessels from preexisting ones [1]. Beyond the "classical" activators of angiogenesis like FGFs or VEGFs several lines of evidence favor a role for Wnt signaling in vessel formation. Wnt proteins bind to members of the frizzled family and induce translocation of β-catenin into the nucleus resulting in Lef/Tcf/β-catenin complex formation and gene transcription. Examples are: (i) Wnt2$^{-/-}$ mice display defects in the yolk sac vasculature [2], (ii) mutations in frizzled-4 are associated with impaired retinal angiogenesis [3], and (iii) inhibition of β-catenin/Tcf complex formation decreases endothelial cell proliferation [4]. We have recently shown that also FGF-2 induces nuclear translocation of β-catenin [5]. Moreover, FGF-2-induced cyclin D1 expression depends on activation of Lef/Tcf [6], indicating that angiogenesis induced by FGF-2 involves target genes of the Wnt signaling pathway.

So far, about 70 Lef/Tcf target genes have been identified. Many of them have roles in the remodeling of blood vessels; among them are connexin-43 [7], cyclin D1 [8], fibronectin [9], VEGF [10], urokinase receptor [11], and MMP-26 [12]. Also the matrix metalloproteinase matrilysin (MMP-7) plays a role in angiogenesis, it is expressed in newly formed vessels adjacent to tumor cells [13] and anti-sense strategies targeting matrilysin inhibit angiogenesis [14,15]. Matrilysin has two Lef/Tcf-binding sites in the promoter

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region and purified Lef-1 as well as purified Tcf-4 have been shown to bind to these sequences in vitro [16,17]. This implicated that it is a Lef/Tcf target gene. However, overexpression of β-catenin in 293 cells was not sufficient for promoter activation [17]. Based on this knowledge we explored effects of FGF-2 on matrilysin expression in endothelial cells. We show that FGF-2 induces matrilysin expression, but promoter activation does not depend on Lef/Tcf. Instead, matrilysin expression by FGF-2 depends on transcription factors Stat1/Stat3 and subsequent activation of AP-1.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described [18]. Cells were used at passages 2–6. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Semiquantitative and quantitative real-time PCR. For quantitation of matrilysin mRNA, HUVEC were kept in serum-free medium containing 5% BSA for 12 h and then stimulated with FGF-2 (25 ng/ml) for indicated times. RNA was isolated and reverse transcribed as described [6]. Conditions and primers for semiquantitative PCR are described elsewhere [13]. Real-time PCR was carried out with a TaqMAN Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primer kits for GAPDH and matrilysin were purchased from Applied Biosystems (Assay-IDs are *Hs99999905 m1* for GAPDH and *Hs00159163 m1* for matrilysin). PCR was performed on an ABI Prism 7700 Sequence Detector using the following cycling conditions: 95 °C for 10 min followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). Relative expression of matrilysin was calculated using the formula $2^{C_t(\text{matrilysin})-C_t(\text{GAPDH})}$. The C_t value represents the cycle number at which the fluorescence passes a fixed threshold value. As a control for the correct size, PCR products were resolved by electrophoresis through a 2% agarose gel.

Plasmids, transfections, and reporter assays. The human matrilysin promoter containing 296 nucleotides upstream of the transcriptional start site, constructs with mutated Lef/Tcf site at positions (−109) and/or (−194) [17], and a construct with a mutated AP-1 site [19] were kind gifts from Dr. Lynn Matrisian (Vanderbilt University, Nashville, TN). The cyclin D1 promoter constructs contain a 1745 bp fragment of the human cyclin D1 promoter [6,8]. Wildtype Stat1, dominant negative Stat3 (which has a C-terminal deletion beginning from amino acid 684 [20]), and Stat3β were subcloned into pCR3.1 vector (Invitrogen). The pCIneo vector containing a degradation-resistant mutant of β-catenin (Δ45-β-cat) was a kind gift from Dr. Bert Vogelstein [21]. The peGFP-ΔN-Tcf-4 plasmid contains a fusion protein of GFP and a N-terminally deleted version of Tcf-4 (aa 1–31 deleted), which is able to bind to DNA, but not capable of binding β-catenin. Plasmid pUBTluc(AP-1) was used as an AP-1-reporter and was a kind gift of Dr. Rainer de Martin (Medical University of Vienna, Vienna). The pRL-TK plasmid coding for *Renilla* luciferase was purchased from Promega. Transfections of HUVEC and 293T cells were performed using calcium phosphate technique and lipofectamine 2000 (Invitrogen, Carlsbad), respectively. For luciferase assays, cells were kept in medium containing 5% BSA or stimulated with 5% BSA supplemented with FGF-2 (25 ng/ml) and heparin (5 U/ml). Nineteen hours later, cells were lysed in passive lysis buffer (Promega, Madison, WI) and *firefly* and *Renilla* luciferase activities were measured with a dual luciferase assay kit from Promega. For siRNA transfections HUVEC were transfected with 100 nM Stat1 and Stat3 siRNA (*SMART*pool reagent, Dharmacon) or with 100 nM luciferase GL3 duplex siRNA (Dharmacon, negative control) with Oligofectamine (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, protein extracts were prepared as described below.

Electrophoretic mobility shift assay. For Lef/Tcf-binding assays, nuclear extract preparation and electrophoretic mobility shift assays were performed as described previously [6]. For Stat3 and AP-1 EMSAs a modified protocol was used: cells were allowed to swell on ice in a buffer containing 20 mM KOH Hepes (pH 8), 0.5% Igepal CA-630, 1 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor. Thereafter, nuclei were collected and lysed in high-salt buffer (20 mM KOH Hepes (pH 8), 1 mM $MgCl_2$, 10 mM KCl, 450 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 20% gylcerol). Sequences of the matrilysin and the proximal tissue factor AP-1-binding elements are 5′-ACTCAAATGAGTCACCTATT-3′ and 5′-CTGGGGTGAGTCATCCCTT-3′, respectively. In supershift experiments, 1 μg of the following antibodies was used: anti-Stat3, anti-Stat1 (Upstate Biotechnology), anti-Lef-1, Anti Tcf-1, anti-Tcf-4 (ExAlpha), and anti-c-jun (Transduction Laboratories); anti-c-fos (Pharmingen).

Immunoblotting. Cells were lysed in RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 0.5% deoxycholic acid, 1% NP-40, and 0.1% SDS), supplemented with 1 mM DTT and protease inhibitor cocktail (Sigma). Protein concentration was measured with a Bradford protein determination kit (Bio-Rad). Fifteen micrograms of protein extract was resolved on 10% polyacrylamide gels and then blotted onto PVDF membranes. After blocking with non-fat dried milk (5%), incubation with first-step antibodies [anti-Stat3, anti-Stat1 (Upstate), anti-matrilysin (R&D), and anti-GSK3β (Transduction Laboratories)] was performed, followed by an incubation step with the second-step antibody (HRP-labeled anti-rabbit-IgG). Visualization was done by the enhanced chemiluminescence system (Amersham Biosciences).

Results

FGF-2 increases matrilysin mRNA and promoter activity independent from Lef/Tcf proteins in HUVEC

In resting HUVEC, matrilysin mRNA is expressed at low levels [13] (see also Fig. 1A). FGF-2 increased matrilysin transcripts in a time-dependent manner as determined by semi-quantitative RT-PCR (Fig. 1A, upper panel) and by quantitative real-time PCR (lower panel). To test if matrilysin is regulated by FGF-2 via Lef/Tcf, wildtype and mutated reporter constructs of the human matrilysin promoter (hMAT) were transfected into HUVEC. FGF-2 induced the wildtype matrilysin promoter about 2-fold (Fig. 1B). Promoter constructs with mutated Lef/Tcf sites at positions (−194) and/or (−109) relative to the transcriptional start site were equally induced by FGF-2. To further exclude a role for the Tcf sites, matrilysin promoter constructs were cotransfected with a degradation-resistant β-catenin mutant (Δ45β-cat), which did not induce matrilysin promoter expression (Fig. 1C). As a positive control for the β-catenin mutant, this construct was cotransfected with cyclin D1 promoter constructs. The wildtype but not the construct with mutated Lef/Tcf sites (CycD1 TOP and CycD1 FOP, respectively) responded to the β-catenin mutant (similar results were obtained in Cos-1 and SW480 cells; data not shown). We conclude that in endothelial cells FGF-2-induced matrilysin expression is independent of the Lef/Tcf/β-catenin pathway.

Tcf-4 binds weakly to the (−194) Lef/Tcf site and not to the (−109) Lef/Tcf site in the human matrilysin promoter

To analyze reasons for the unexpected non-function of matrilysin Lef/Tcf sites in response to FGF-2,

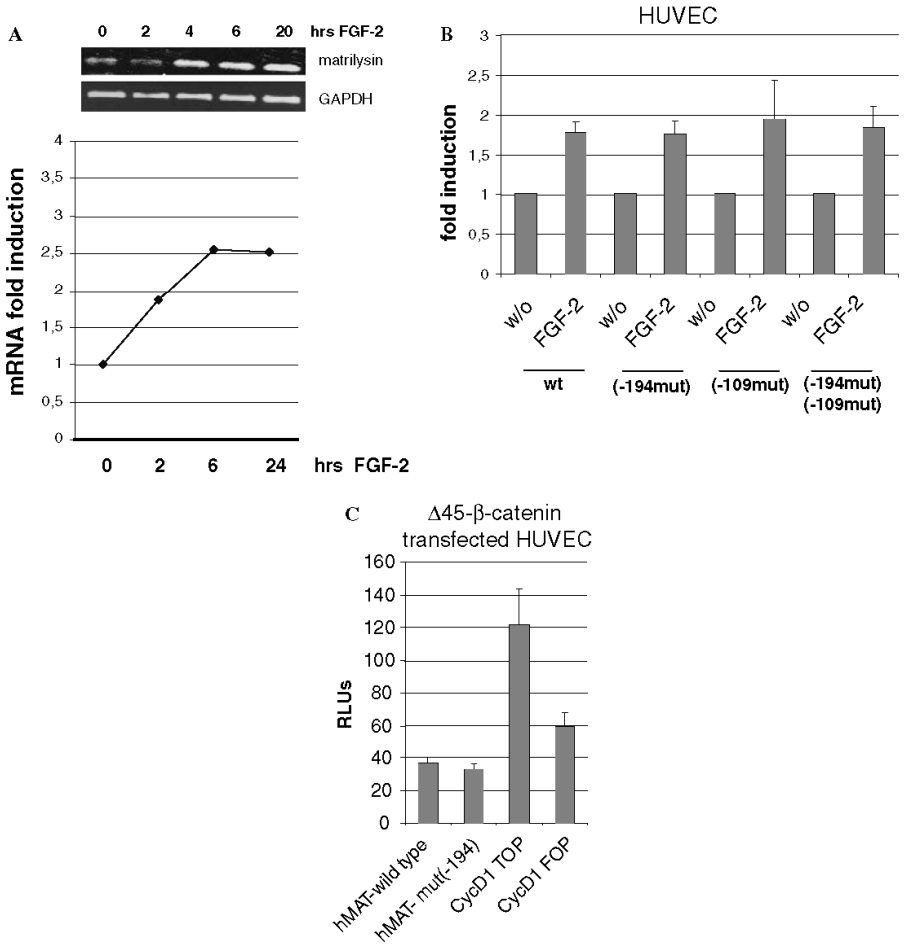

Fig. 1. FGF-2-induced matrilysin expression is independent from Lef/Tcf-binding sites within the promoter region. (A) Matrilysin PCR from HUVEC-derived cDNA. Cells were kept in serum-free medium overnight and then stimulated with FGF-2 (25 ng/ml) for the indicated times. Top panel, semiquantitative RT-PCR showing PCR products of matrilysin and GAPDH; lower panel, quantitative real-time PCR. Values represent the "fold induction" relative to the unstimulated control and were calculated as described in "Materials and methods." One representative of three experiments is shown. (B) Matrilysin promoter expression studies in HUVEC transfected with matrilysin promoter (wildtype or mutated Lef/Tcf sites as indicated), along with a *Renilla* luciferase as internal control. Following transfection, cells were cultivated in serum-free medium with or without FGF-2 for 19 h. Cells were lysed and luciferase activities were measured. Values are the fold increase relative to the unstimulated control and represent the matrilysin promoter activity corrected to the internal *Renilla* luciferase activity (mean ± SEM; at least three experiments in triplicate). (C) Cotransfection studies in HUVEC transfected with a degradation-resistant β-catenin mutant (constitutively active) and indicated reporter constructs (both wildtype or with mutated Lef/Tcf site as described in "Materials and methods"). The *Renilla* luciferase was included as internal control. Twenty-four hours later cells were lysed and analyzed as described in (B). (Mean ± SD; two experiments in triplicate).

electrophoretic mobility shift assays (EMSAs) were performed. Neither Lef-1 nor Tcf-1 bound to either of the two matrilysin Lef/Tcf sites (at −194 and at −109) (Fig. 2A). A very weak and incomplete supershift at both Lef/Tcf sites was seen with the anti-Tcf-4 antibody (Fig. 2A). The human matrilysin Lef/Tcf core sites differ from that of the murine matrilysin and the cyclin D1 sites in one base (A instead of a T, Table 1). Moreover, the core sequences have different surroundings. When using the Lef/Tcf site from the murine matrilysin promoter [22], a nearly complete supershift was observed with the anti-Tcf-4 antibody (Fig. 2B). To analyze the influence of the surrounding nucleotides, several mutated oligonucleotides (Table 1) were tested for Tcf-4-binding. Nuclear extracts were used from HUVEC or from 293T cells transfected with a GFP-ΔN-Tcf-4 fusion vector, which results in high levels of nuclear ΔN-Tcf-4. Tcf-4-binding was strongly enhanced, when the surrounding sequence at the 3′-end was changed to a GTT, but changes in the core sequence had no effect. The importance of the surrounding at the 3′-end was supported by the lack of Tcf-4 binding to Lef/Tcf sites from the c-jun

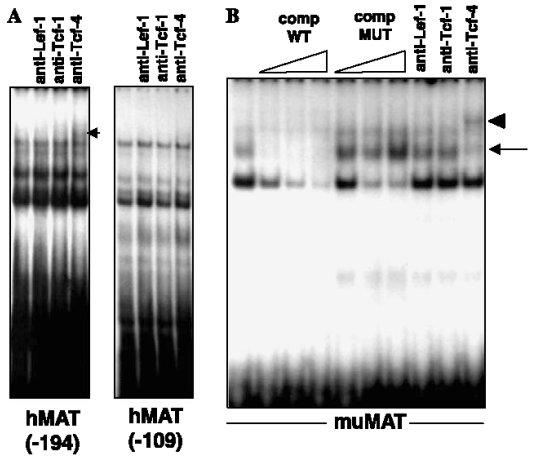

Fig. 2. EMSA showing insignificant Tcf-4 binding to Lef/Tcf sequences from the human matrilysin promoter. (A) Nuclear extracts from HUVEC were incubated with radio-labeled oligonucleotides comprising human matrilysin Lef/Tcf sites at position (−194) or position (−109). A weak and incomplete supershift was seen with the anti-Tcf-4 antibody and with both matrilysin Lef/Tcf probes (arrowhead). (B) The same nuclear extracts were incubated with an oligonucleotide containing the murine matrilysin Lef/Tcf site. Shown are competitions with an excess of wildtype and mutated unlabeled oligonucleotide as well as a nearly complete supershift with the anti-Tcf-4 antibody (shown by the arrow and the arrowhead, respectively).

and the E-cadherin promoter, which both have no CTT or GTT at the 3′-end and both did not bind Tcf-4.

Dominant-negative Stat3 reduces FGF-2-induced matrilysin promoter activity

Others have shown that signal transducer and activator of transcription-3 (Stat3) is involved in matrilysin induction by FGF-1 in a prostate carcinoma cell line [23,24] and that FGF-2 activates Stat3 in endothelial cells [25]. We therefore explored the possibility that FGF-2-induced matrilysin activation is Stat3-dependent. Due to difficulties in yielding high enough transfection efficiency in triple-transfected HUVEC, 293T cells were used. In these cells, FGF-2 induced the (−296) bp matrilysin promoter 3- to 4-fold. Cotransfection of a dominant-negative Stat3 mutant reduced FGF-2-induced promoter activity by about 60% (Fig. 3A). To test if this is a direct Stat3 effect, we searched the (−296) bp matrilysin promoter for potential Stat3-binding elements. Stat3 is promiscous in binding to promoter regions, hence it is difficult to search for a "high stringency" consensus. Using the minimal consensus sequence (TTNNNNAA [26,27]) we found two putative Stat-binding elements (puSBE1 and puSBE2) within the (−296) bp promoter region (Fig. 3B).

For EMSAs nuclear extracts from 293T cells were used, which were transfected with Stat3β, a naturally occurring splice form of Stat3, which strongly binds to the DNA but lacks the transactivation domain and acts thus dominantly negative [28]. As a positive control the Stat3 element from the human c-fos promoter (hSIE) was used as the DNA probe. We found a robust Stat3/DNA complex (Fig. 3C, arrow), which supershifted by addition of an anti-Stat3 antibody (arrowhead). Using puSBE1 no band was detectable. An unspecific band was seen with the puSBE2.

These results indicate: (i) that effects of the Stat3 dominant negative mutant were mediated indirectly and (ii) that FGF-2 activates Stat3. Indeed, in HUVEC FGF-2 strongly enhanced Stat1 and Stat3 binding to hSIE, which supershifted with the respective antibodies (Fig. 3D).

Stat1 and Stat3 influence FGF-2-induced AP-1 activation

The matrilysin promoter harbors an AP-1-binding site and FGF-2 is known to activate the transcription factor

Table 1
Summary of oligonucleotides used in EMSA studies, sequences, and relative binding activities of Tcf-4 derived from nuclear extracts from HUVEC or from 293T cells transfected with the ΔN-Tcf-4 construct

Oligonucleotides used for EMSA		Tcf-4 binding	
		HUVEC	293T ΔNTcf-4
hMAT(194)	gcaaaatc**CTTTGAA**agacaaatccctctcctt	+/−	++
hMAT(194)A->T	gcaaaatc**CTTTGAT**agacaaatccctctcctt	+/−	++
hMAT(194)short	atc**CTTTGAA**aga	−	−
hMAT(194)gtt	gcaaaatc**CTTTGAA**gttcaaatccctctcctt	+/−	+++
hMAT(194)gtt-short	gcaaaatc**CTTTGAA**gtt	++	++++
hMAT(109)	aaggagagggagtctacagaa**CTTTGAA**agtatgtg	−	+
hMAT(109)A->T	aaggagagggagtctacagaa**CTTTGAT**agtatgtg	−	+
hMAT(109)short	a**CTTTGAA**agtat	−	−
muMAT	ttctcagagcta**CTTTGAT**gtt	+++	++
Cyclin D1	ctctgccggg**CTTTGAT**cttgcttaacaaca	+++	+++
c-jun	gagagccc**CTTTGAG**acacct	−	−
E-cadherin	gaataaagtc**CTTTGTA**actccatg	−	+

Bold denotes, Lef/Tcf binding sites and underline denotes, mutated nucleotides.

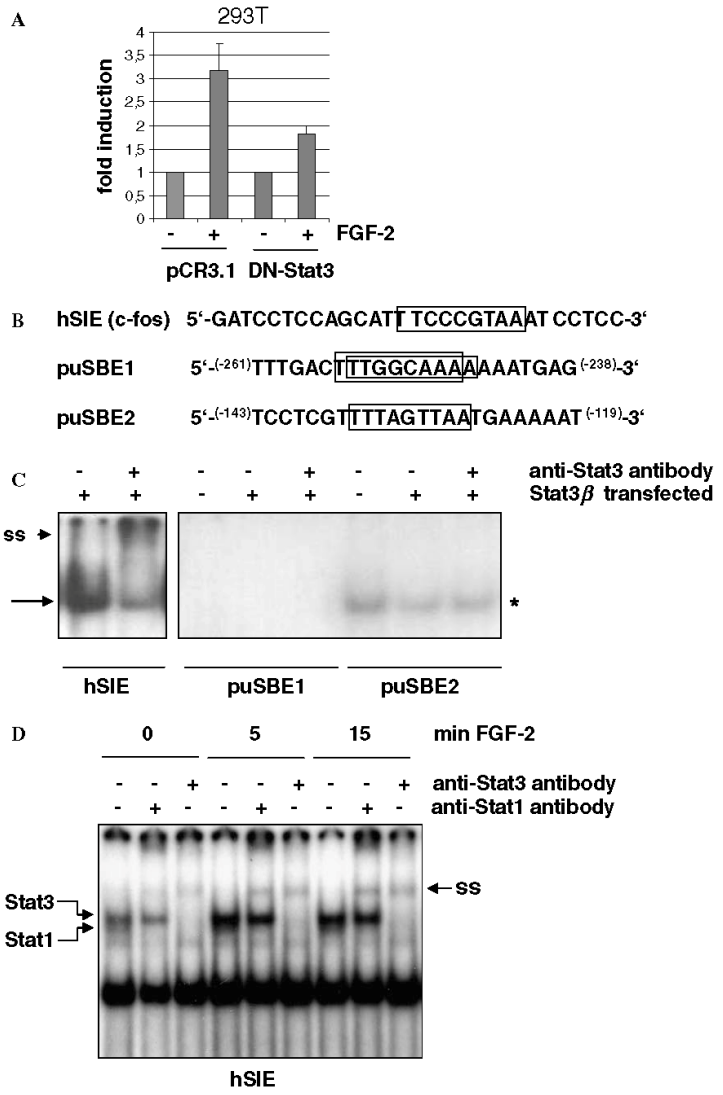

Fig. 3. A dominant-negative mutant of Stat3 reduces FGF-2-induced matrilysin promoter activity, but Stat3 does not bind to putative-binding elements within the matrilysin promoter. (A) 293T cells were cotransfected with the (−296) bp fragment of the matrilysin promoter together with a dominant-negative mutant of Stat3 or with a control vector (pCR3.1) and stimulated with FGF-2 for 19 h. (B) Sequences of Stat3-binding-elements (SBEs) from the c-fos promoter (hSIE) and of putative SBEs (puSBE1 and puSBE2) from the (−296) bp matrilysin promoter fragment. (C) EMSA showing Stat3β binding to the hSIE, but not to the puSBE1 and puSBE2. Nuclear extracts were from 293T cells or from 293T cells transiently transfected with Stat3β (which was used because it strongly binds to DNA). cDNA was incubated with radio-labeled oligonucleotides containing the Stat3-binding element from the c-fos promoter or the putative Stat3-binding elements from the matrilysin promoter. Stat3β bound to the hSIE (arrow). The Stat3/DNA complex was supershifted with an anti-Stat3 antibody (arrowhead). Using puSBE1 no band was detected at all, no specific protein/DNA binding was detected on puSBE2 (asterisk). (D) Nuclear extracts of HUVEC cultivated in serum-free medium with or without FGF-2 were incubated with the radio-labeled hSIE oligonucleotide and subjected to EMSA. Stat1/DNA as well as Stat3/DNA complexes were induced by FGF-2 after 5 and 15 min. The nature of the complexes was determined by addition of anti-Stat3- and anti-Stat1-antibodies, which resulted in supershifts (ss).

AP-1 [29]. We therefore analyzed FGF-2-induced expression of an AP-1 reporter construct in the presence of the dominant negative Stat3, which indeed reduced FGF-2-mediated AP-1-reporter activity (Fig. 4A). In order to test if FGF-2 also induces AP-1 binding to the matrilysin AP-1 site and if this is mediated through Stat3, we performed EMSAs. Following FGF-2 stimulation AP-1-binding to an oligonucleotide harboring the matrilysin AP-1 site was induced and this binding was strongly attenuated upon transfection with the dominant-negative Stat3β (Fig. 4B).

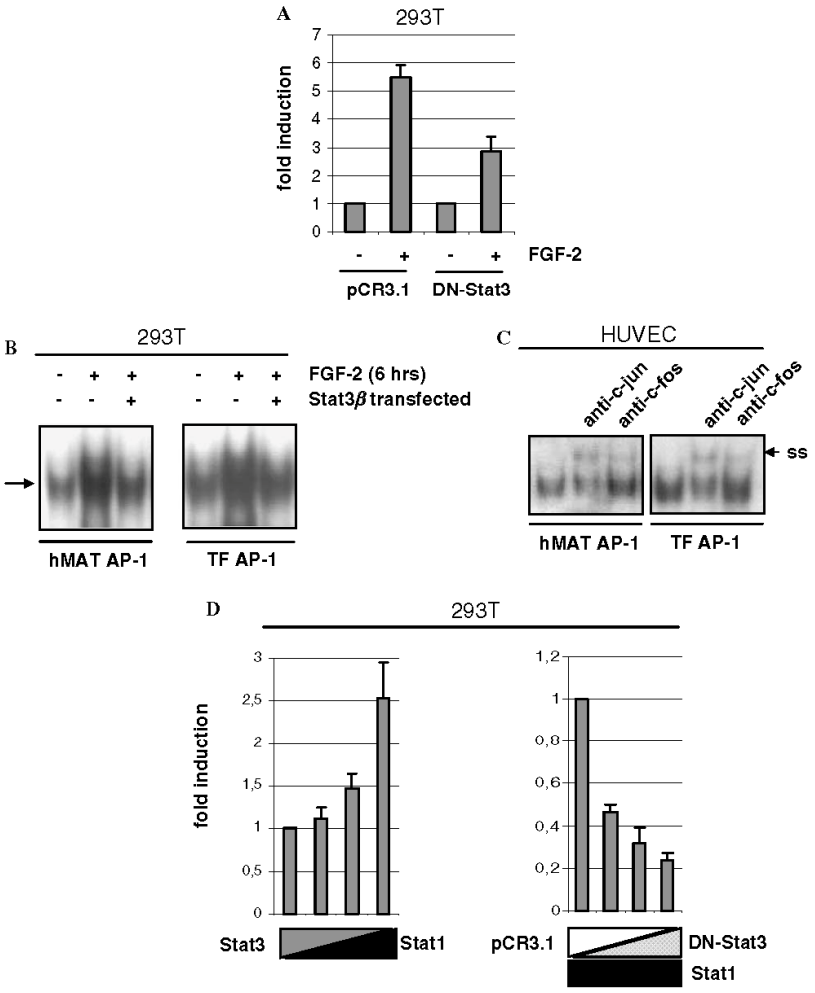

Fig. 4. Dominant negative Stat3 reduces AP-1-dependent transcription and protein binding to AP-1-binding sites. (A) 293T cells were cotransfected with an AP-1-reporter construct along with either pCR3.1 vector or pCR3.1 vector containing the dominant negative Stat3-mutant. Thereafter, cells were cultivated in serum-free medium with or without FGF-2 for 19 h. Values were corrected against the cotransfected *Renilla* luciferase and expressed as "fold induction" of the unstimulated basal level. (B) EMSA using nuclear extracts from 293T cells transfected with a control vector (peGFP) or with the dominant negative Stat3β construct. Probes were oligonucleotides containing AP-1 sites from the human matrilysin promoter (hMAT, left panel) or, as a positive control, from the human tissue factor promoter (TF, right panel). After 6 h of stimulation with FGF-2, AP-1-binding activity is enhanced (arrow). Upon transfection with the dominant negative Stat3β, AP-1 binding is strongly reduced on both, the hMAT as well as the tissue factor AP-1 site. (C) Nuclear extracts from FGF-2-stimulated HUVEC were used for supershift analyses using oligonucleotides containing AP-1-binding sites from the human matrilysin or from the tissue factor (TF) promoter. Supershifts (ss) with anti-c-jun and anti-c-fos antibodies are marked with an arrow. (D) Left panel, 293T cells were transfected with the AP-1-reporter plasmid (1 μg), together with increasing amounts of pCR3.1-Stat1 (0, 0.5, 1, and 2 μg) and decreasing amounts of pCR3.1-Stat3 (2, 1.5, 1, and 0 μg). Right panel, 293T cells were transfected with the AP-1-reporter plasmid (1 μg) and pCR3.1-Stat1 (1 μg) together with increasing amounts of dominant-negative Stat3 (0, 0.5, 1, 2 μg). The total DNA content was kept constant by supplementation of empty pCR3.1 plasmid. Expression levels (± SD) from three independent experiments, each performed in triplicate, are shown.

As a control the proximal AP-1 site from the tissue factor promoter was used [30], which gave identical results (Fig. 4B). We next used nuclear extracts from FGF-2-treated HUVEC in EMSA studies employing probes for hMAT and TF AP-1 and identified c-jun and c-fos binding to both oligonucleotides (Fig. 4C).

Stat3 heterodimerizes with Stat1 and Stat1 is known to be activated by FGF signaling [31]. Therefore, we investigated the effects of Stat1 and Stat3 overexpression on AP-1-dependent transcription. 293T cells were transfected with the AP-1-reporter construct together with Stat1 and Stat3. We found that increasing the amounts of transfected Stat1 dose-dependently increased AP-1-dependent reporter activity, whereas increased Stat3 had no effect (Fig. 4D, left panel). This is in seeming contrast to effects of the dominant negative Stat3 mutant (Fig. 4B). However, assuming

that nuclear Stat3 levels are not the rate-limiting factor, Stat1-induced AP-1 reporter activity should be inhibited by dominant negative Stat3. This is indeed shown in Fig. 4D, right panel. We conclude that Stat1/Stat3 heterodimers control AP-1-dependent transcription and the amount of nuclear Stat1 appears to be the rate-limiting factor.

FGF-2-induced matrilysin promoter activation depends on a functional AP-1 site

To confirm that AP-1 binding to the matrilysin promoter controls FGF-2-mediated matrilysin expression, cells were transfected with wildtype or mutated promoters. In the construct with the mutated AP-1 site, matrilysin promoter activity was abolished in HUVEC as well as in 293T cells (Fig. 5).

siRNAs against Stat1 and Stat3 abolish FGF-2-mediated induction of matrilysin protein

In order to prove the involvement of endogenous Stat1 and Stat3 in the FGF-2-mediated induction of matrilysin, we transfected HUVEC with siRNAs against these Stat proteins. Forty-eight hours after transfection protein extracts were prepared and SDS–PAGE was performed. Fig. 6 shows that siRNAs efficiently knocked down Stat1 and Stat3 expression in comparison to the unspecific GL3 control siRNA. Most importantly, this resulted in complete inhibition of FGF-2-mediated matrilysin protein expression. This confirms the role of Stat1/3 in FGF-2-induced matrilysin expression.

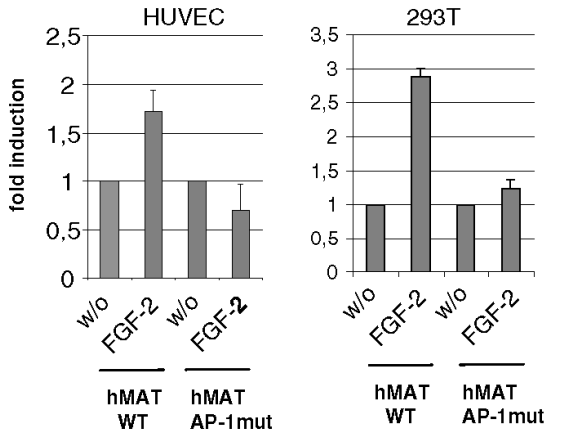

Fig. 5. FGF-2-induced matrilysin promoter activity depends on a functional AP-1 site. HUVEC (left panel) or 293T (right panel) were transfected with the human wildtype matrilysin promoter (hMAT WT) or with the human matrilysin promoter containing a mutated AP-1 site. Shown are "fold inductions" (±SD) of three independent experiments, corrected against the internal *Renilla* control plasmid.

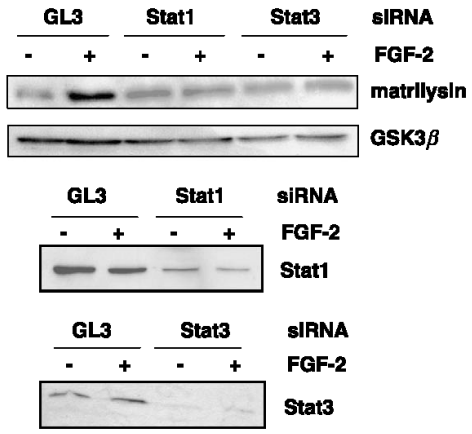

Fig. 6. Knockdown of endogenous Stat1 or Stat3 RNA abolishes FGF-2-induced upregulation of matrilysin. HUVEC were transfected with siRNAs against Stat1 and Stat3 or unspecific siRNA against luciferase (GL3). Twenty-four hours after transfection cells were cultured in serum-free medium with or without FGF-2 for 19 h and protein extracts were made. Western blotting was performed with antibodies against Stat1 and Stat3, matrilysin, and GSK3β as a control.

Discussion

In this study, we show that FGF-2 induces matrilysin expression in endothelial cells. The interest in the regulation of this matrix metalloproteinase is based on its implications in angiogenesis [13,15] and tumorigenesis [32]. Based on transfection studies in cancer cell lines, factors of the Lef/Tcf family, Stat3, AP-1, and members of the ETS family of proteins are candidates regulating matrilysin gene expression. For example, cotransfection of β-catenin, PEA3, and c-jun induces promoter activity [17,19]. In response to physiologic stimuli, an epidermal growth factor-induced pathway involving PEA3 and a FGF-1-induced pathway involving Stat3 have been described, however, again using cancer cells [33].

We wished to explore the mechanism of FGF-2-mediated matrilysin expression in endothelium. We have previously shown that FGF-2 induces Lef/Tcf-dependent transcription in this cell type [6]. However, analyzing the role of the matrilysin Lef/Tcf sites gave the unexpected result that promoters with wildtype or mutated Lef/Tcf sites responded equally to FGF-2. Furthermore, cotransfection with β-catenin was sine effectu, excluding a functional role of these sites for FGF-2-induced matrilysin expression. In attempt to explain this finding, EMSAs were performed, where Tcf-4 only insignificantly bound to the human matrilysin Lef/Tcf sites. This contrasts results with murine matrilysin and human cyclin D1 Lef/Tcf sites, which both strongly bound Tcf-4. In both promoters, the Lef/Tcf core sequence is CTTTGAT and differs from the human matrilysin promoter in one base (A → T), but exchanging this base in the matrilysin site did not restore

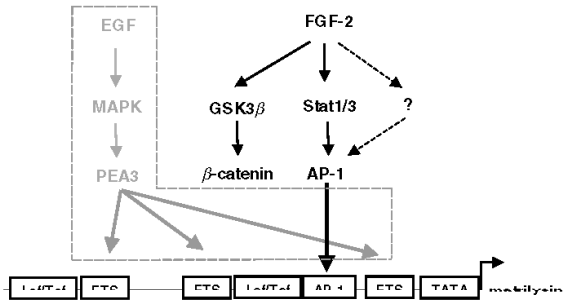

Fig. 7. A model of signal transduction from FGF-2 to the matrilysin promoter. Whereas epidermal growth factor was shown to induce matrilysin via the MAP kinase pathway and subsequent activation of transcription factor PEA3 [33], FGF-2 enhances Stat1/3 activity, which leads to increased binding of AP-1 on the matrilysin AP-1 site. FGF-2-induced reduction in GSK-3 activity and β-catenin activation [6] does not influence expression of matrilysin.

Tcf-4 binding. Instead, we found that the sequence surrounding the core influences Lef/Tcf binding, specifically a GTT mutation at the 3′-end of the Lef/Tcf site enhanced binding. This might be due to either an altered affinity of Tcf-4 itself or due to altered binding of co-activator or co-repressor elements, which participate in the Tcf-4/DNA complex [34].

To analyze a role of Stat3, we cotransfected a dominant-negative Stat3 mutant with the human matrilysin promoter and found FGF-2-mediated promoter activity decreased, but Stat3 binding to the matrilysin promoter was absent. Thus, we considered that Stat3 acts indirectly by activating other transcription factors. As a general finding, these experiments describe for the first time that FGF-2 enhances Stat1/Stat3 binding to DNA, which underscores recent data that FGF-2 phosphorylates Stat3 in endothelial cells [25] and that FGF signaling activates Stat1 [31].

We next explored the possibility that AP-1 is the target of Stat1/3. Matrilysin, like most matrix metalloproteinases, contains proximal AP-1-binding motifs in their promoter regions [32]. FGF-2 is known to induce AP-1-dependent transcription [29]. We show that Stat1/3 complexes were responsible for the FGF-2-induced AP-1-dependent transcription. Moreover, FGF-2-induced c-jun/c-fos binding to the matrilysin AP-1 site. Mutation of this site inhibited FGF-2-mediated matrilysin promoter activation. These findings demonstrate that Stat1/3 heterodimers activate AP-1 and FGF-2-mediated matrilysin expression depends on the AP-1 site. These results were confirmed by using siRNA technology. We show that endogenous Stat1 and Stat3 are crucial for the FGF-2-mediated expression of matrilysin protein in endothelial cells.

In summary, we are the first to report that matrilysin expression in endothelial cells is induced by FGF-2. This is independent from Lef/Tcf sites and regulated in an AP-1-dependent fashion (Fig. 7). These studies describe a novel pathway of FGF-2 induced activation of AP-1 with Stat1/3 proteins being interposed.

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3.1.7 Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation

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NEOPLASIA

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The Stat5 transcription factors Stat5a and Stat5b have been implicated in lymphoid development and transformation. Most studies have employed Stat5a/b-deficient mice where gene targeting disrupted the first protein-coding exon, resulting in the expression of N-terminally truncated forms of Stat5a/b (*Stat5a/b*$^{\Delta N/\Delta N}$ mice). We have now reanalyzed lymphoid development in *Stat5a/b*$^{null/null}$ mice having a complete deletion of the *Stat5a/b* gene locus. The few surviving *Stat5a/b*$^{null/null}$ mice lacked CD8$^+$ T lymphocytes. A massive reduction of CD8$^+$ T cells was also found in *Stat5a/b*$^{fl/fl}$ *lck-cre* transgenic animals. While γδ T-cell receptor–positive (γδTCR$^+$) cells were expressed at normal levels in *Stat5a/b*$^{\Delta N/\Delta N}$ mice, they were completely absent in *Stat5a/b*$^{null/null}$ animals. Moreover, B-cell maturation was abrogated at the pre–pro-B-cell stage in *Stat5a/b*$^{null/null}$ mice, whereas *Stat5a/b*$^{\Delta N/\Delta N}$ B-lymphoid cells developed to the early pro-B-cell stage. In vitro assays using fetal liver-cell cultures confirmed this observation. Most strikingly, *Stat5a/b*$^{null/null}$ cells were resistant to transformation and leukemia development induced by Abelson oncogenes, whereas *Stat5a/b*$^{\Delta N/\Delta N}$-derived cells readily transformed. These findings show distinct lymphoid defects for *Stat5a/b*$^{\Delta N/\Delta N}$ and *Stat5a/b*$^{null/null}$ mice and define a novel functional role for the N-termini of Stat5a/b in B-lymphoid transformation. (Blood. 2006;107:4898-4906)

Introduction

Stat molecules are part of a highly conserved signaling pathway involved in cell-fate decisions like differentiation, proliferation, and apoptosis.[1-3] The cytokines interleukin-2, -4, and -7 (IL-2, IL-4, IL-7) regulate important aspects of lymphoid development and are strong activators of the transcription factors Stat5a and Stat5b.[4] The importance of Stat5a/b for lymphoid cells is also underlined by the fact that constitutively activated Stat5a/b are found in several forms of lymphoid leukemia in mice and humans.[5-10] Gene knockouts have greatly contributed to our knowledge about Stat transcription factors because they allowed exploration of their physiologic and pathophysiologic functions.[11] So far, all studies investigating the role of Stat5a/b in lymphopoiesis employed gene-targeted mice still expressing a residual protein corresponding to an N-terminal deletion mutant (Stat5a/bΔN).[4,12-14] *Stat5a/b*$^{\Delta N/\Delta N}$ mice revealed surprisingly mild phenotypes in B- and T-cell development and function.

Characterization of the lymphoid compartment in *Stat5a/b*$^{\Delta N/\Delta N}$ mice showed a modest reduction of B- and T-lymphoid–cell numbers accompanied by a complete lack of natural killer (NK) cells and CD4$^+$CD25$^+$ suppressor T cells.[4,13,15] CD8$^+$ T cells were present but failed to respond to α-CD3 and IL-2.[4] Mature B-cell numbers in the periphery were also reduced due to an incomplete block at the early pro-B-cell developmental stage (Hardy fraction B).[13,14]

Mice lacking IL-7 or the IL-7R have a block at the earliest step of B-cell development at Hardy fraction A and lack mature B-lymphoid cells in the periphery.[16,17] Notably, B-cell development can be rescued in these mice by forced expression of a constitutively active Stat5a/b mutant.[17] In addition, transgenic mice expressing a constitutively active Stat5b (Stat5b-CA) have increased numbers of pro-B cells.[14] As Stat5a/b are critical components in the signaling cascade downstream of IL-7R, abrogation of Stat5a/b was predicted to result in a dramatic phenotype. Thus, the observations in *Stat5a/b*$^{\Delta N/\Delta N}$ mice were difficult to reconcile with the current understanding of signaling pathways controlling B-cell development.

Moreover, Stat5a/b transcription factors have been shown to play an important role in various T-cell developmental decisions. Transgenic *Stat5b-CA* mice display increased numbers of CD8$^+$ but not CD4$^+$ T cells.[18] This implicates Stat5b as an important regulator of CD4$^+$/CD8$^+$ lineage decision. Moreover, Stat5a/b DNA binding sites were found in regulatory regions of the T-cell receptor γ (TCRγ) gene locus, and *Stat5b-CA* mice displayed a modest increase in γδ T-cell numbers.[18,19] In *Stat5a/b*$^{\Delta N/\Delta N}$ mice, embryonic γδ T-cell development was severely affected, but numbers were rapidly restored after birth.[20] Therefore, the relevance for Stat5a/b in adult γδ thymopoiesis remained elusive.

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Another finding in *Stat5a/b*$^{\Delta N/\Delta N}$ mice was striking. Among many substrates that are phosphorylated downstream of the Abelson oncogene, Stat5a/b were originally described to be among the most strongly activated ones.[5,21-23] Therefore, Stat5a/b was considered potential critical factors in Ab-MuLV– and bcr/abl-mediated transformation. This hypothesis was substantiated by a plethora of in vitro data using various forms of dominant-negative Stat5a/b mutants.[24-27] Despite this evidence for an essential role for Stat5a/b in Abelson-induced transformation, *Stat5a/b*$^{\Delta N/\Delta N}$ mice were still susceptible to leukemia when challenged with Abelson oncogenes.[13]

Because of these inconsistencies and open questions, we set out to unveil the impact of Stat5a/b on lymphopoiesis and on Abelson-induced transformation using mice in which the entire Stat5a/b locus had been deleted (*Stat5a/b*$^{null/null}$ mice). Our experiments redefine the role of Stat5a/b in lymphoid development and we can clearly attribute a functional role to the truncated Stat5 proteins present in *Stat5a/b*$^{\Delta N/\Delta N}$ mice. We furthermore unravel the key role of Stat5a/b in Abelson-induced transformation.

Materials and methods

Mice

Stat5a/b$^{null/null}$, *Stat5a/b*$^{fl/fl}$, *lck-cre* (distal promoter) transgenic, *Stat5a/b*$^{\Delta N/\Delta N}$, and *Rag2*$^{-/-}$ mice were described previously[12,28-31] and were maintained at the Biomedical Research Institute, Medical University of Vienna, under specifically pathogen-free sterile conditions. The *Stat5a/b*$^{null/null}$, *Stat5a/b*$^{fl/fl}$, and *Stat5a/b*$^{\Delta N}$ mice were on a mixed 129/C57B/6 background. All animal experiments were carried out in accordance with protocols approved by Austrian law.

Flow cytometric analysis

Single-cell suspensions were preincubated with CD16/CD32 antibodies (BD Biosciences, San Jose, CA) to prevent nonspecific Fc-receptor–mediated binding. Subsequently, 5×10^5 cells were stained with monoclonal antibodies conjugated with fluorescent markers and analyzed by a FACScan flow cytometer using CellQuest Pro software (Becton Dickinson, Heidelberg, Germany). The antibodies used for determination of specific B-lineage maturation stages included the markers B220 (CD45R; RA3-6B2), CD43 (1B11), CD19 (1D3), BP-1 (6C3), IgM (R6-60.2), and IgD^b (IgH-5b; 217-170). For surface staining of T-lineage cells, antibodies directed against CD3 (ϵ chain; 145-2C11), CD4 (L3T4), CD8a (Ly-2; 53-6.7), TCRβ chain (H57-597), and γδ TCR (GL3) were used. Hematopoietic stem cell (HSC) staining was performed using a Mouse Lineage Panel kit and anti–sca-1 (Ly-6A/E; D7), anti–c-kit (CD117; 2B8), and anti-CD34 (RAM34) antibodies (all BD Pharmingen, Heidelberg, Germany).

Protein analysis and Western blotting

Splenic T cells were magnetic-activated cell sorted for Thy1.2$^+$ cells according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). Thy1.2$^+$ cells were separated using an autoMACS Instrument (Miltenyi Biotech). Cells were lysed in a buffer containing protease and phosphatase inhibitors (50 mM Hepes, pH 7.5; 0.1% Tween-20; 150 mM NaCl; 1 mM EDTA; 20 mM β-glycero-phosphate; 0.1 mM sodium vanadate; 1 mM sodium fluoride; 10 g/mL aprotinin; leupeptin; and 1 mM PMSF). Protein concentrations were determined using a BCA kit as recommended by the manufacturer (Pierce, Rockford, IL). Proteins (50-100 g) were separated on an 8% SDS polyacrylamide gel and transferred onto Immobilon membranes. Membranes were probed with anti-Stat5a/b (C-17; Santa Cruz Biotechnologies, Santa Cruz, CA) and anti–β-actin (clone Ac-54; Sigma, St Louis, MO) antibodies at dilutions of 1:500 and 1:2000, respectively.

T-cell stimulation

Splenic T cells were isolated from 3 12-week-old *Stat5a/b*$^{f/f}$, 3 *Stat5a/b*$^{f/f}$ *lck-cre*, and 4 *Stat5a/b*$^{\Delta N/\Delta N}$ mice. Splenic-cell solution was subjected to red blood cell lysis for 5 minutes using a lysis buffer containing 150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA (pH 7.3) and cultured in T-cell culture medium (RPMI 1640 containing 10% FBS; 10 mM Hepes, pH 7.0; 2 mM L-glutamine; [1 ×] nonessential amino acids; 1 mM sodium pyruvate; and 50 μM 2-mercaptoethanol) in the presence of 1 μg/mL anti-CD3 monoclonal antibody 145.2C11 (Pharmingen) and 1000 units/mL recombinant human IL-2 (Boehringer Mannheim, Mannheim, Germany).[4] Pellets were prepared before and after 4 hours of stimulation and subjected to RNA isolation.

RNA isolation and semiquantitative RT-PCR analysis

First-strand cDNA synthesis and PCR amplification were performed using a reverse transcriptase–polymerase chain reaction (RT-PCR) kit (GeneAmp RNA PCR kit; Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. The following primer sequences were used: pim-1, 5′-ACGTGGAGAAGGACCCGATTTCC-3′ and 5′-GATGTTTTCGTCCTTGATGTCGC-3′; cis, 5′-CTGCTGTGCATAGCCAAGACGTTC-3′ and 5′-CAGAGTTGGAAGGGGTACTGTCGG-3′; cyclin D2, 5′-AGAAGGGGCTAGCAGATGA-3′ and 5′-AGGATGATGAAGTGAACACA-3′; β-actin, 5′-CAGGTCCAGACCCAGGATGGC-3′ and 5′-ACTCCTATGTGGGTGACGAG-3′.

In vitro B-cell differentiation

Single-cell suspensions of fetal liver cells (embryonic day 14 [ED 14]) were prepared. The cells were maintained in RPMI medium containing 10% fetal calf serum (FCS), 100 U/mL penicillin/streptomycin, and 5 μM β-mercaptoethanol on an OP-9 fibroblast feeder layer. IL-7, Flt-3L, and SCF (each 10 ng/mL) were added every other day. Outgrowth of specific B-lineage cells was examined for 12 days by analyzing an aliquot of the suspension cells every other day by FACS.

Tissue culture conditions and virus preparation

Transformed fetal liver, bone marrow cells, and tumor-derived cell lines were maintained in RPMI medium containing 10% FCS, 100 U/mL penicillin/streptomycin, 5 μM β-mercaptoethanol, and 2 mM L-glutamine. GP+E86 cell lines (MSCV-*bcr/abl p185*-IRES-eGFP producer), A010 cells (Ab-MuLV producer), and mouse embryonic fibroblasts (MEFs) were maintained in DMEM medium containing 10% FCS, 100 U/mL penicillin/streptomycin, 5 μM β-mercaptoethanol, and 2 mM L-glutamine. A010 cells produce an ecotropic replication-deficient form of the Abelson virus and were a generous gift of Dr Naomi Rosenberg. For collection of the viral supernatant, A010 cells were plated in 100-mm dishes precoated with gelatin (1%) and grown to confluency. Supernatant was harvested every 8 hours for 40 hours, pooled, and filtered through a 0.45-μm filter, as described previously.[32]

Infections, in vitro transformation assays, and establishment of cell lines

For the preparation of fetal liver cells, *Stat5a/b*$^{null/+}$ animals were set up for breeding and vaginal plugs were checked daily. Fourteen days after conception, the pregnant animals were killed and fetal livers were prepared. The tail of the embryo was used for genotyping by PCR. Single-cell suspensions from fetal livers were infected for 1 hour with viral supernatant derived either from A010 cells or from GP+E86 bcr/abl p185-IRES-eGFP producer cell lines in the presence of 7 g/mL polybrene, as described previously.[13,32,33] Using the same procedure, single-cell suspensions of bone marrow of tibiae and femora of mice were infected. The cells were then maintained in complete RPMI medium or plated in cytokine-free methylcellulose at a density of 2.5×10^5 cells/mL in 35-mm dishes. After 10 days, cloning efficiency was evaluated by counting colonies by light microscopy (Leica Fluovert microscope, 4 × magnification; Heidelberg, Germany). Photographs of single colonies were taken using an Axiovert

200 microscope (ZEISS, Oberkochen, Germany; 40 ×/0.6 NA objective), a CoolSNAP fx camera (Visitron Systems, Puchheim, Germany), and MetaMorph software (Version 4.6; Molecular Devices, Downingtown, PA). The assays were performed in triplicates. Mock-infected cells did not result in growth factor–independent colonies. As a control, individual clones were picked and analyzed by flow cytometry for the expression of B-lineage markers (CD19, CD43). The ability to form cell lines was tested by transferring an aliquot of the infected cells (1×10^6) to growth factor–free medium. The medium was changed twice a week and the culture was observed for the outgrowth of stable cell lines.

Injection of Abelson-infected cells into Rag2$^{-/-}$ mice

For tail vein injections, 10^6 cells were resuspended in 200 μL of PBS and injected via tail vein into *Rag2*$^{-/-}$ mice. Prior to injection, the cells were infected with either Ab-MuLV or pMSCV-*bcr/abl p185*-IRES-eGFP retrovirus as described under "Infections, in vitro transformation assays, and establishment of cell lines." Sick mice were killed and analyzed for spleen weights, white blood cell counts, and the presence of leukemic cells in bone marrow, spleen, liver, and blood. The leukemic cells were also analyzed by flow cytometry and expressed the surface markers CD19 and CD43.

[^{3}H]thymidine incorporation

Cells were plated at a density of 2×10^5 cells in 96 round-bottom wells. [^{3}H]thymidine (0.1 μCi/well [0.0037 MBq/well]) was added 18 hours after plating, for another 12 hours.

Statistical analysis

Statistics were carried out using a paired *t* test, Mann-Whitney test, or a one-way analysis of variance (ANOVA) as appropriate. ANOVA was followed by a Tukey test. Differences in Kaplan-Meier plots were analyzed for statistical significance using the log-rank test.

Results

Stat5a/b are essential for CD8$^+$ and γδ T-cell development

Stat5a/b$^{\Delta N/\Delta N}$ mice on a mixed genetic background are viable and may survive up to 2 years in our mouse colony. However, about 40% of *Stat5a/b*$^{\Delta N/\Delta N}$ mice die due to an autoimmune phenotype caused by a significant reduction of CD4$^+$CD25$^+$ suppressor T cells within the first few months (Snow et al[15] and data not shown). In contrast, *Stat5a/b*$^{null/null}$ mice die perinatally[28] (Figure 1A). Although the cause of death is not known, severe anemia and reduced lung capacity are possibly contributing factors (Cui et al[28]; L.H., unpublished observation). Approximately 1% of the *Stat5a/b*$^{null/null}$ mice reach weaning age. These rare *Stat5a/b*$^{null/null}$ survivor mice are much smaller than their littermates, display a significantly reduced body weight, and die within the first 6 weeks after birth (Figure 1A). Stat5a/b$^{\Delta N/\Delta N}$ mice express N-terminally truncated Stat5a/b proteins that are found at significant levels in the lymphoid lineage (Figure S1, available on the *Blood* website; see the Supplemental Figures link at the top of the online article). We therefore monitored lymphoid development in the survivor mouse population. Five 4-week-old *Stat5a/b*$^{null/null}$ mice were killed and thymi and lymph nodes were subjected to flow cytometric analysis. Notably, thymi, spleen, and lymph nodes were smaller than would be expected from the body size of the mice. As depicted in Figure 1B, CD4$^-$CD8$^-$γδTCR$^+$ cells were present in wild-type, *Stat5a/b*$^{\Delta N/+}$, *Stat5a/b*$^{\Delta N/\Delta N}$, and *Stat5a/b*$^{null/+}$ mice but were completely absent in the thymic-cell suspension of *Stat5a/b*$^{null/null}$ mice. We stress that CD4$^-$CD8$^-$γδTCR$^+$ cells are present at significant levels in *Stat5a/b*$^{\Delta N/\Delta N}$ mice. Moreover, *Stat5a/b*$^{\Delta N/\Delta N}$ mice were reported to show a normal distribution of CD4$^+$ and CD8$^+$ T cells in the adult thymus, but *Stat5a/b*$^{\Delta N/\Delta N}$-derived CD8$^+$ cells show an inability to proliferate in response to IL-2.[4] Again, *Stat5a/b*$^{null/null}$ mice revealed a distinct phenotype. The thymus was reduced in size in relation to the body size and age of the mice. We found a significant reduction of CD8$^+$ T cells (2.5-fold). In the lymph nodes, the situation was even more pronounced; *Stat5a/b*$^{null/null}$ mice displayed a 12-fold reduction in CD8$^+$ T lymphocytes (Figure 1C).

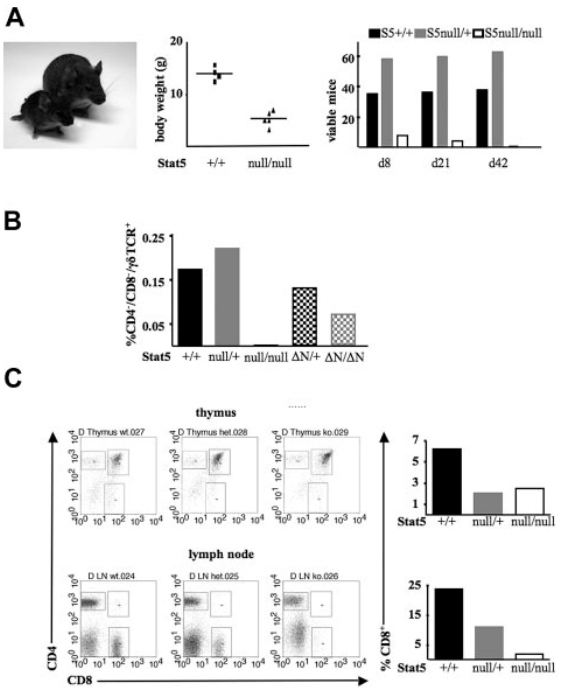

Figure 1. Impaired CD8$^+$ and γδ T-cell development in *Stat5a/b*$^{null/null}$ survivor mice. (A) Picture of a *Stat5a/b*$^{null/null}$ survivor mouse (left) compared with a *Stat5a/b*$^{+/+}$ littermate at the age of 4 weeks. Representative body weight values and numbers of *Stat5a/b*$^{null/null}$ mice and littermates surviving up to day 8, weaning (day 21), or day 42 are depicted. (B) Numbers of CD4$^-$/CD8$^-$/γδTCR$^+$ T lymphocytes in thymi of *Stat5a/b*$^{null/null}$, *Stat5a/b*$^{\Delta N/\Delta N}$, and respective littermate controls. (C) Flow cytometric analysis of CD4$^+$, CD8$^+$, and CD4$^+$/CD8$^+$ cells in thymi and lymph nodes of 5 *Stat5a/b*$^{null/null}$ survivors and 2 individual *Stat5a/b*$^{null/+}$ and 2 *Stat5a/b*$^{+/+}$ littermate controls. Data are summarized in bar graphs. Due to the small size of thymi and lymph nodes, cells were pooled and did therefore not allow generation of error bars (B-C).

To confirm cell autonomy of Stat5a/b in CD8$^+$ T-cell development, we generated *Stat5a/b*$^{f/f}$ *lck-cre* mice. These mice express the Cre-recombinase under the control of the distal promoter of the T-cell receptor–associated kinase Lck, which is first active at the double-negative (CD4$^-$CD8$^-$) stage.[30] T-cell lineage–specific deletion of Stat5a/b was confirmed by Western blot analysis of magnetic-activated cell separation (MACS)–sorted Thy1.2$^+$ splenic cells (Figure 2A). *Stat5a/b*$^{f/f}$ *lck-cre* mice displayed splenomegaly and lymphoid organ infiltration that was first detected at the age of 4 weeks. This phenotype is most probably due to the expected lack of suppressor T cells that was previously described as a consequence of Stat5a/b deficiency.[15] As observed in *Stat5a/b*$^{null/null}$ survivors, *Stat5a/b*$^{f/f}$ lck-cre mice showed a significant reduction of CD8$^+$ T cells in all organs analyzed (thymus, $P < .01$; peripheral blood, $P < .001$; spleen, $P < .01$; lymph node, $P < .05$; Figure 2B). The selective disappearance of CD8$^+$ cells is also indicated by the increased ratio of CD4$^+$ versus CD8$^+$ cells, as described in Table 1. In order to see whether a different induction of target genes might account for the differences in the phenotype of *Stat5a/b*$^{\Delta N/\Delta N}$

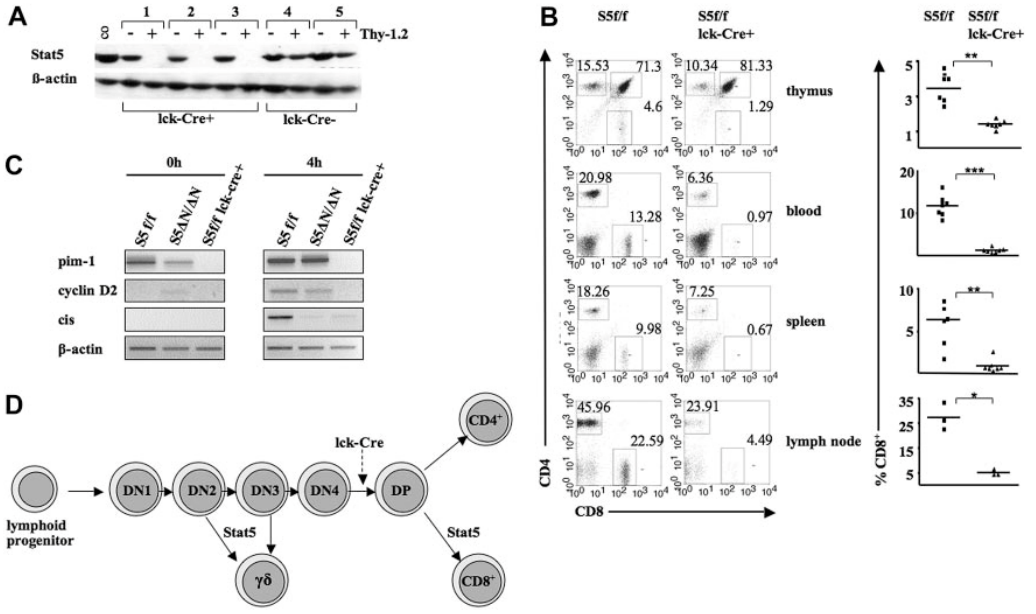

Figure 2. Impaired CD8⁺ T-cell development in *Stat5a/b$^{f/f}$ lck-cre* mice. (A) Splenic cells of 3 individual *Stat5a/b$^{f/f}$ lck-cre* (nos. 1-3) and 2 *Stat5a/b$^{f/f}$* (nos. 4-5) mice were magnetically sorted for Thy1.2⁺ cells, and Stat5a/b expression was assessed by Western blot analysis. (B) Representative flow cytometric profile of CD4⁺, CD8⁺, and CD4⁺/CD8⁺ cells in thymus, blood, spleen, and lymph nodes of *Stat5a/b$^{f/f}$ lck-cre* mice and *Stat5a/b$^{f/f}$* controls. Asterisks denote significant difference as determined by a paired *t* test. (C) Analysis of transcriptional expression of *pim-1*, *CIS*, and cyclin D2 genes by semiquantitative RT-PCR. Splenic T cells were stimulated for 4 hours with α-CD3 (1 μg/mL) and human IL-2 (hIL-2; 1000 U/mL) to induce Stat5a/b target gene transcription. (D) Schematic model for the role of Stat5a/b in T-cell developmental choices. Stages affected in *Stat5a/b$^{null/null}$* survivor mice and/or *Stat5a/b$^{f/f}$ lck-cre* mice are indicated. The time point of Cre-recombinase activation under the control of the distal lck promoter is also depicted.

and *Stat5a/b$^{null/null}$* mice, we stimulated splenic T cells with α-CD3 and IL-2, as depicted in Figure 2C. The Stat5a/b target genes *Pim1* and cyclin D2 were clearly expressed in *Stat5a/b$^{f/f}$* and *Stat5a/b$^{ΔN/ΔN}$* cells. Induction of these genes was not found in *Stat5a/b$^{null/null}$* T cells, indicating that Stat5a/bΔN are still capable of inducing some target genes. In contrast, expression of the suppressor of cytokine signaling (SOCS) gene family member CIS was lacking in both *Stat5a/b$^{ΔN/ΔN}$* and *Stat5a/b$^{f/f}$* lck-cre cells. The development of γδ T cells was not altered in these mice (data not shown). This was to be expected because transcription from the distal *lck* promoter occurs after the junction of γδ TCR⁺ cells. Taken together, these findings provide evidence that Stat5a/b are indispensable for CD8⁺ T-cell and γδ TCR⁺-cell homeostasis (Figure 2D scheme).

Stat5a/b are essential for the pre–pro-B to early pro-B-cell stage transition in vivo

Specific B-cell developmental stages can be distinguished by differential cell-surface expression of B220, CD43, CD19, BP-1, IgM, and IgD (Figure 3C schematic overview). Single fractions can be classified according to the Hardy nomenclature in pre–pro-B (B220⁺/CD43^{hi}/CD19⁻/BP-1⁻; fraction A), early pro-B (B220⁺/CD43^{hi}/CD19⁺/BP-1⁻; fraction B), late pro-B (B220⁺/CD43^{hi}/CD19⁺/BP-1⁺; fraction C), pre-B (B220⁺/CD43^{lo}/IgM⁻/IgD⁻; fraction D), immature (B220⁺/CD43^{lo}/IgM⁺/IgD⁻; fraction E), and mature (B220⁺/CD43^{lo}/IgM⁺/IgD⁺; fraction F) B cells.[34,35]

Table 1. Mean ratio of CD4⁺ to CD8⁺ T cells in thymi, peripheral blood, spleen, and lymph nodes of *Stat5a/b$^{f/f}$* and *Stat5a/b$^{f/f}$ lck-cre* mice

	Ratio of CD4⁺/CD8⁺ cells	
	S5f/f	S5f/f lck-cre
Thymus	4.1:1	11:1
Lymph node	1.9:1	5:1
Spleen	2.2:1	8:1
Peripheral blood	1.5:1	6:1

It had been shown in *Stat5a/b$^{ΔN/ΔN}$* mice that Stat5a/b are required for the transition from the early pro-B (Hardy fraction B) to the late pro-B-cell stage (Hardy fraction C).[14] We prepared bone marrow, spleen, and lymph nodes of 5 *Stat5a/b$^{null/null}$* survivor mice and their *Stat5a/b$^{null/+}$* and wild-type littermates. The numbers of pre–pro-B cells were comparable in all 3 groups. However, we failed to detect early and late pro-B cells in *Stat5a/b$^{null/null}$* mice in the bone marrow (22-fold and 40-fold reduction compared with *Stat5a/b$^{+/+}$*, respectively; Figure 3A). Accordingly, the numbers of mature B cells (Hardy fraction F) in spleen and lymph nodes were also significantly reduced compared with *Stat5a/b$^{+/+}$* controls (6.4-fold and 2.2-fold, respectively; Figure 3B). We emphasize that these percentages cannot be directly compared, since the total size and the cellularity of hematopoietic organs differ in wild-type and *Stat5a/b$^{null/null}$* animals. These data strongly argue for a role of Stat5a/b at the earliest steps of B-cell development (pre–pro-B cell to early pro-B-cell transition). However, the developmental block does not appear to be absolute, since few mature B-lymphoid cells are present in the periphery. Again, the phenotype in *Stat5a/b$^{null/null}$* mice is aggravated and very distinct from *Stat5a/b$^{ΔN/ΔN}$* mice, with a block occurring at an earlier B-cell developmental stage (as indicated in the scheme in Figure 3C).

In vitro B-cell differentiation of *Stat5a/b$^{null/null}$* fetal liver–derived cells

To further investigate the role of Stat5a/b for early B-cell development, we established a protocol that allows following B-cell

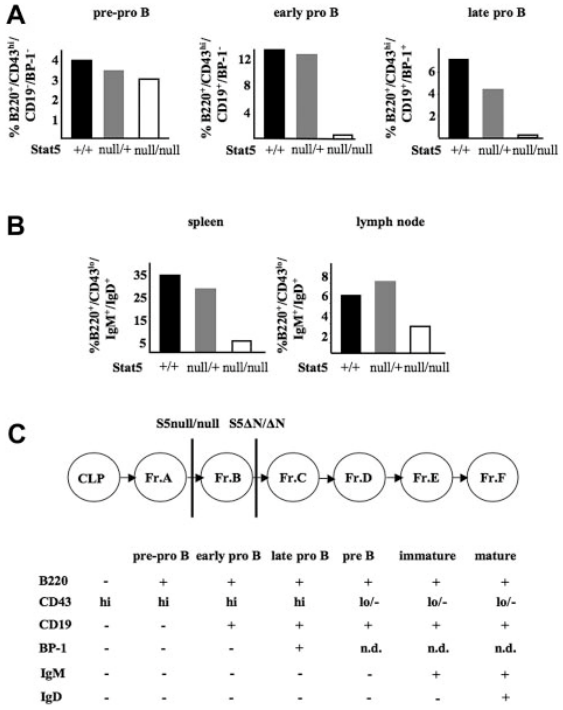

Figure 3. B-cell development is arrested at the pre–pro-B-cell stage in *Stat5a/b*$^{null/null}$ survivor mice. (A) Percentages of pre–pro-B, early pro-B, and late pro-B cells in bone marrow and (B) mature B cells in spleen and lymph node of 5 Stat5a/b$^{null/null}$ survivors compared with 2 *Stat5a/b*$^{null/+}$ and 2 *Stat5a/b*$^{+/+}$ controls. Due to the small body size, bone marrows were pooled and therefore did not allow generation of error bars. (C) Schematic model for maturation of B-cell developmental fractions A-F. As indicated, individual maturational stages were distinguished by differential surface expression of B220, CD43, CD19, BP-1, IgM, and IgD. The different blocks in *Stat5a/b*$^{\Delta N/\Delta N}$ and *Stat5a/b*$^{null/null}$ mice are indicated by vertical lines. CLP indicates common lymphoid progenitor.

differentiation of fetal liver–derived cells in vitro. First, we excluded that a reduction of *Stat5a/b*$^{null/null}$ HSCs causes any effects and determined the numbers of lin$^-$/c-kit$^+$/Sca-1$^+$ long term-HSCs (CD34$^-$) and short term-HSCs (CD34$^+$). Interestingly, *Stat5a/b*$^{null/null}$ fetal livers displayed comparable numbers of both populations (Figure S2A-S2B). We then cultured fetal liver cells (ED 14) of a *Stat5a/b*$^{null/+}$ intercross on an OP-9 fibroblast feeder layer in the continuous presence of IL-7, Flt-3L, and SCF (10 ng/mL each). Outgrowth of B-lineage cells was analyzed every second day by FACS analysis. Outgrowth kinetics of the single Hardy fractions reflected the observations made in the *Stat5a/b*$^{null/null}$ survivor mice (Figure 4). Fraction A was found comparable in cells of each genotype. Fraction B, C, E, and F cells were detectable in control cultures from day 6 on but were entirely missing in cultures derived from *Stat5a/b*$^{null/null}$ fetal livers. In these cultures, B-cell development was completely abrogated at fraction A and failed to proceed to any further maturation stages. We also performed an in vitro B-cell developmental assay only in the presence of IL-7 using an MEF feeder layer (10 ng/mL), confirming a critical role for Stat5a/b in the transition from Hardy fraction A to B (Figure S3).

Taken together, our data show that Stat5a/b are a critical transcription factor for the transition of pre–pro-B cells (fraction A) to the early pro-B-cell stage (fraction B) in adult and fetal hematopoiesis. Moreover, these findings indicate that the N-terminally truncated Stat5a/b proteins present in *Stat5a/b*$^{\Delta N/\Delta N}$ mice suffice to allow B-lymphoid cells to mature to the early pro-B-cell stage.

Stat5a/b are required for Ab-MuLV– and *bcr/abl p185*–induced transformation in vitro

A constitutive activation of Stat5a/b is found in a large variety of leukemias and lymphomas,[5,9,10,21,36] and constitutive activation of Stat5a/b suffice to induce a multi-lineage leukemia in mice.[36] Despite a broad experimental evidence for a role of Stat5a/b in lymphoid leukemia,[22,24,26,37,38] we have shown that *Stat5a/b*$^{\Delta N/\Delta N}$ mice developed Abelson-induced B-lymphoid leukemia with identical properties compared with wild-type littermate controls.[13] Bone marrow cells derived from *Stat5a/b*$^{\Delta N/\Delta N}$ mice were readily transformed by Abelson oncogenes and resulted in the outgrowth of stable cell lines.[13] We therefore repeated the transformation experiments with fetal livers and bone marrow from *Stat5a/b*$^{null/null}$ mice. Fetal liver cells (ED 14) were infected with Ab-MuLV and plated in growth factor–free methylcellulose. No colonies were detected in any of the *Stat5a/b*$^{null/null}$ fetal livers tested. A gene dosage effect was observed in *Stat5a/b*$^{null/+}$ fetal liver–derived cells, where we detected 50% to 60% of the growth factor–independent colonies compared with wild-type controls (Figure 5A-B). The retroviral constructs employed (Ab-MuLV and pMSCV-*bcr/abl p185*-IRES eGFP) result in the outgrowth of B-lymphoid colonies. This was confirmed by analyzing the colonies by flow cytometry for surface expression of CD43 and CD19. As expected, the transformed colonies were positive for both markers (Figure 5C).

A gene dosage effect was also observed regarding the proliferative capacity of *Stat5a/b*$^{+/+}$ and *Stat5a/b*$^{null/+}$ Abelson-transformed cell lines (Figure 5D). Transformation experiments were repeated with bone marrow of *Stat5a/b*$^{null/null}$ survivors and confirmed the lack of transformation ability: colony formation was completely

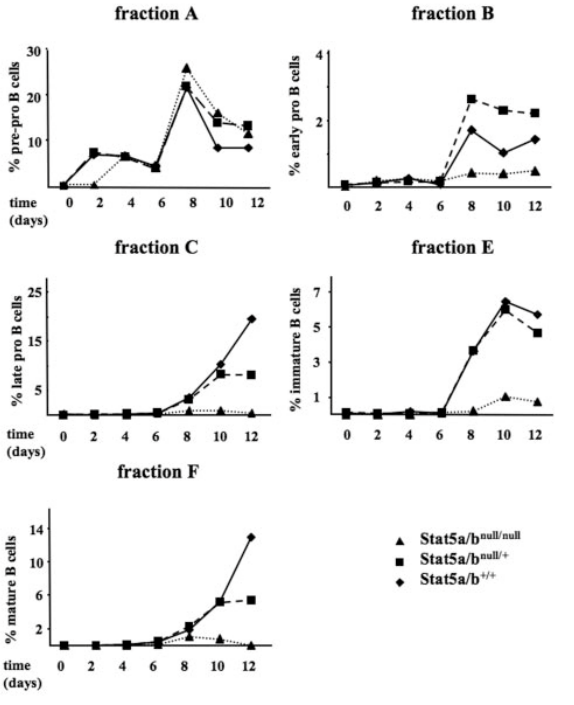

Figure 4. B-cell development is arrested at the pre–pro-B-cell stage in *Stat5a/b*$^{null/null}$ fetal liver–derived cultures. Fetal livers of 2 embryos of each genotype were pooled (ED 14) and cocultivated on an OP-9 fibroblast feeder layer in the presence of IL-7, Flt-3L, and SCF (10 ng/mL each). Outgrowth of pro-B-cell stages (fractions A-C), immature (fraction E), and mature (fraction F) B cells over a 12-day period is depicted.

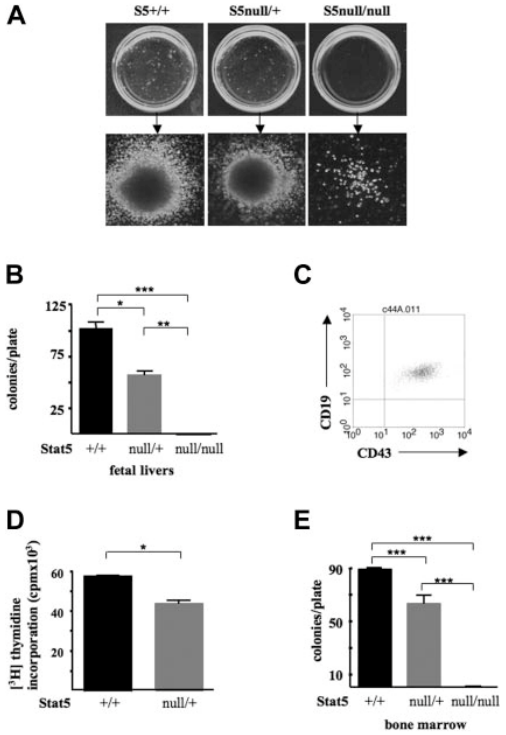

Figure 5. Abelson-induced transformation is dependent on Stat5a/b in vitro. (A) Ab-MuLV–induced colony formation of *Stat5a/b*$^{+/+}$, *Stat5a/b*$^{null/+}$, and *Stat5a/b*$^{null/null}$ fetal liver cells in methylcellulose. Single-colony pictures of each phenotype are depicted in the bottom panels. *Stat5a/b*$^{null/null}$ cells showed no ability to form growth factor–independent colonies. (D) Summary of data obtained from Ab-MuLV–induced colony formation assays represent means ± SEM of 4 embryos per genotype (each performed in triplicates). (C) Surface expression of B-lineage markers was verified by flow cytometric analysis (right; data of one representative CD19$^+$ CD43$^+$ colony is shown). (D) [^{3}H]thymidine incorporation of fetal liver–derived *Stat5a/b*$^{+/+}$ and *Stat5a/b*$^{null/+}$ Ab-MuLV–transformed cell lines. Stat5a/b-deficient fetal livers did not give rise to stable transformed cell lines. Data represent means ± SEM of 2 cell lines per genotype. cpm indicates counts per minute. (E) Ab-MuLV–induced colony formation of *Stat5a/b*$^{+/+}$ (n = 2), *Stat5a/b*$^{null/+}$ (n = 2), and *Stat5a/b*$^{null/null}$ (n = 5; pooled) survivor bone marrow cells in methylcellulose. *Stat5a/b*$^{null/null}$ survivor cells showed no ability to form growth factor–free colonies. Experiment was performed in triplicates. Asterisks denote significant differences as determined by a one-way ANOVA followed by a Tukey test (A,C) or a paired *t* test (B).

abrogated in *Stat5a/b*$^{null/null}$ cells (Figure 5E). Stable immortal Ab-MuLV–transformed cell lines were derived from wild-type and *Stat5a/b*$^{null/+}$ mice, but not a single cell line grew out from *Stat5a/b*$^{null/null}$ fetal livers or bone marrow (Table 2). These experiments indicate that Stat5a/b are required for Abelson-induced transformation but that Stat5a/bΔN suffice to support the transformation and immortalization process.

To control for this somewhat unusual observation, we performed several additional experiments that are summarized in Table 2. First, we repeated the transformation experiments side by side with bone marrow cells derived from *Stat5a/b*$^{\Delta N/\Delta N}$ and *Stat5a/b*$^{null/null}$ survivor mice. *Stat5a/b*$^{\Delta N/\Delta N}$ cells readily transformed, which resulted in the formation of growth factor–independent colonies and in the outgrowth of stable cell lines, but we failed to see signs of transformation when using *Stat5a/b*$^{null/null}$ cells.

We next reasoned that Ab-MuLV–induced transformation might target a distinct subset of B-cell precursors that were absent or present at low numbers in *Stat5a/b*$^{null/null}$ fetal liver cells or bone marrow. Hence, we employed a murine stem-cell virus encoding the *bcr/abl p185* retrovirus (pMSCV–*bcr/abl p185*-IRES-eGFP). MSCV infects murine hematopoietic stem cells, which are present at comparable numbers in *Stat5a/b*$^{null/null}$ fetal livers and controls (Figure S2). Cells derived from *Stat5a/b*$^{null/null}$ (fetal livers) or *Stat5a/b*$^{\Delta N/\Delta N}$ mice (bone marrow) were infected, the infection was controlled via FACS analysis, and the cells were subsequently plated in growth factor–free methylcellulose or transferred to growth factor–free medium. Again, cells derived from *Stat5a/b*$^{\Delta N/\Delta N}$ mice formed colonies and gave rise to cell lines, whereas *Stat5a/b*$^{null/null}$ cells failed to do so (Table 2).

Abelson-transformed *Stat5a/b*$^{null/null}$ cells fail to induce leukemia in vivo

One may speculate that the failure to transform *Stat5a/b*$^{null/null}$ cells might be compensated in vivo (eg, via cytokine-dependent activation of redundant signaling pathways). To test this, we first infected fetal livers with pMSCV–*bcr/abl p185*-IRES-eGFP and injected them via tail vein into 2 *Rag2*$^{-/-}$ mice each. Mice that had received wild-type bone marrow died from leukemia after 3 months, whereas the *Rag2*$^{-/-}$ mice that had received *Stat5a/b*$^{null/null}$ bone marrow survived in a disease-free state for at least 6 months (data not shown). Uninfected *Stat5a/b*$^{null/null}$ bone marrow was also injected into 2 *Rag2*$^{-/-}$ mice to verify that *Stat5a/b*$^{null/null}$ bone marrow did indeed have the capacity to reconstitute hematopoiesis, albeit to a lesser extent than control bone marrow (data not shown; J. O'Shea, NIH, Bethesda, MD, and L.H., oral communication). It is important to mention that *Stat5a/b*$^{null/null}$ fetal liver cells allowed the development of a few IgM$^+$ IgD$^+$ cells that were detected in the periphery. We next reasoned that the initial steps of transformation might be cytokine dependent or influenced by surrounding cells and that the environment within *Rag2*$^{-/-}$ mice only repopulated with *Stat5a/b*$^{null/null}$ marrow might prevent transformation in vivo.[39-41] To exclude this possibility we performed the following experiment. Bone marrow of 4 *Stat5a/b*$^{null/null}$ survivor mice was prepared and mixed with wild-type marrow derived from a littermate control at a ratio of 4:1. The cells were infected with Ab-MuLV retrovirus and again injected via tail vein into recipient *Rag2*$^{-/-}$ animals. As depicted in Figure 6A, mice that had received either *Stat5a/b*$^{+/+}$ or *Stat5a/b*$^{null/+}$ marrow succumbed to a B-lymphoid leukemia within 3 to 4 weeks. When administered mixed bone marrow that contained 80% of *Stat5a/b*$^{null/null}$ cells, *Rag2*$^{-/-}$ mice showed signs of a phenotypically identical disease with latency that was increased by 10 days ($P < .05$). The animals displayed splenomegaly and elevated white blood cell counts and

Table 2. Ability of fetal liver and bone marrow cells to form *bcr/abl p185*- or Ab-MuLV–induced colonies and stable cell lines

	Colony formation	Transformed cell lines
bcr/abl transformation		
S5$^{\Delta N/+}$, BM	Yes	Yes
S5$^{\Delta N/\Delta N}$, BM	Yes	Yes
S5$^{+/+}$, FL	Yes	Yes
S5$^{null/+}$, FL	Yes	Yes
S5$^{null/null}$, FL	No	No
Ab-MuLV transformation		
S5$^{\Delta N/+}$, BM	Yes	Yes
S5$^{\Delta N/\Delta N}$, BM	Yes	Yes
S5$^{+/+}$, FL	Yes	Yes
S5$^{null/+}$, FL	Yes	Yes
S5$^{null/null}$, FL	No	No
S5$^{+/+}$, BM	Yes	Yes
S5$^{null/+}$, BM	Yes	Yes
S5$^{null/null}$, BM	No	No

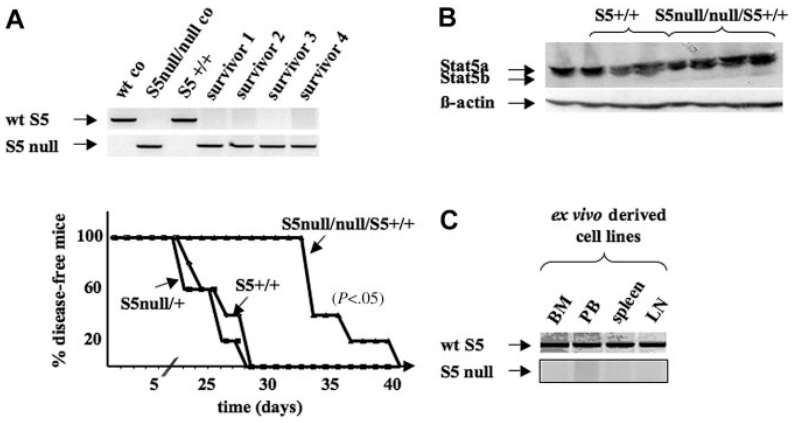

Figure 6. Abelson-induced transformation is dependent on Stat5 in vivo. (A) Kaplan-Meier plot of *Rag2*$^{-/-}$ mice that received a transplant of either *Stat5a/b*$^{+/+}$, *Stat5a/b*$^{null/+}$, or a 4:1 mixture of *Stat5a/b*$^{null/null}$ and *Stat5a/b*$^{+/+}$ freshly Ab-MuLV–transformed bone marrow cells (5 mice/group; 1 × 10^6 cells each mouse). Genotyping PCR analysis of mice used for 4:1 mixture is depicted. wt indicates wild type. (B) Immunoblotting for Stat5a/b of leukemic cells derived from bone marrow of *Rag2*$^{-/-}$ mice that received a transplant of either *Stat5a/b*$^{+/+}$ or a 4:1 mixture of *Stat5a/b*$^{null/null}$/*Stat5a/b*$^{+/+}$ bone marrow. (C) PCR analysis of ex vivo–derived cell lines. Representative data of bone marrow (BM)–, peripheral blood (PB)–, spleen-, and lymph node (LN)–derived leukemic cell lines of one *Rag2*$^{-/-}$ mouse that received a transplant of a 4:1 mixture of *Stat5a/b*$^{null/null}$/*Stat5a/b*$^{+/+}$ bone marrow cells, which was later killed. All cultures derived from *Rag2*$^{-/-}$ mice that received transplants of *Stat5a/b*$^{null/null}$/*Stat5a/b*$^{+/+}$ cells were *Stat5a/b*$^{+/+}$ as determined by PCR analysis.

spleen, bone marrow, and liver were infiltrated with CD19$^+$CD43$^+$ leukemic cells (Figure S4). Western blot analysis showed that all cells expressed Stat5a/b at comparable levels (Figure 6B). In addition, ex vivo–derived cell lines were established and analyzed by PCR (Figure 6C). All leukemic cells expressed Stat5a/b; no transformed cells derived from *Stat5a/b*$^{null/null}$ marrow were detectable. These experiments define Stat5a/b as essential transcription factors for Abelson-induced leukemia initiation and exclude the possibility that other signaling pathways compensate in vivo.

Discussion

The transcription factors Stat5a/b have been considered key regulators of immune functions and the lymphoid system.[10,12,14,15,42,43] Their relevance and importance are underlined by the fact that more than 1700 manuscripts have been published on Stat5a/b since their discovery. A major breakthrough was the generation of the first Stat5a/b knockout mouse in 1998 (*Stat5a/b*$^{\Delta N/\Delta N}$ mice) that served as a valuable tool for numerous studies and shed light on the multiple roles of Stat5a/b in the organism.[4,12,14,44] Despite the key role of IL-7–mediated Stat5a/b activation in lymphoid development, the phenotype of the *Stat5a/b*$^{\Delta N/\Delta N}$ mice in the lymphoid system is surprisingly moderate.[4,20] The most prominent effect is the complete absence of CD4$^+$CD25$^+$ suppressor T cells, leading to an autoimmune disease.[15]

Our present work provides compelling evidence that the function of Stat5a/b in lymphoid development and immune functions needs redefinition. Our findings prove that Stat5a/b are key regulators of early B-cell development and of CD8$^+$ and γδ T-lymphoid–cell generation. The difference between *Stat5a/b*$^{\Delta N/\Delta N}$ and *Stat5a/b*$^{null/null}$ mice implicitly defines separate roles for the N-terminally truncated Stat5a/b. Since deficiency in Stat5a/b was lethal,[28] one might also argue that disturbances at the locus occurred independently of the targeted deletion of *Stat5a/b*. We do exclude this possibility, since erythroid cells can be genetically complemented by wild-type Stat5a (M.A.K. and H.B., unpublished observations). Our attempts to complement *Stat5a/b*$^{null/null}$ HSCs with wild-type Stat5a to rescue lymphoid development continuously failed, most likely based on a severe defect of HSCs upon loss of Stat5a/b.[45-47] The vast majority of *Stat5a/b*$^{null/null}$ pups died rapidly after delivery. The rare survivors may have reflected outliers in the Gaussian distribution or a compensating adaptive change that occurred at low frequency. Regardless of the underlying basis, it is worth pointing out that the observations in these survivors were reproduced in the *Stat5a/b*$^{fl/fl}$ *lck-cre* mice. The analysis of T-cell development in *Stat5a/b*$^{fl/fl}$ lck-cre mice also excluded that *Stat5a/b*$^{null/null}$ thymic epithelial cells were responsible for the selective lack of CD8$^+$ cells. Finally, B-cell development was recapitulated in vitro by employing fetal hematopoietic progenitors. In this cell-culture system, the absence of Stat5a/b resulted in a complete block of B-cell development at that very stage predicted from the phenotype of the survivors (pre–pro-B-cell stage). Taken together, these data demonstrate that the observations obtained in the rare survivors are not confounded by an undefined adaptive escape phenomenon. They also provide formal proof for the interpretation that deficiency in Stat5a/b affects the lymphoid compartment by a cell-autonomous effect rather than an indirect effect mediated via abnormalities in stromal cells or thymic epithelium.

Hence, our observations fall in place with the predicted role of Stat5a/b in lymphopoiesis and are in perfect agreement with studies performed in other mouse models.[17,18,48] Goetz et al[49] recently showed that constitutively active Stat5b promotes B-cell development at the expense of early T-cell development in transgenic mice. The authors hypothesize that Stat5a/b serves as a switch, with Stat5a/b activation driving cells into the B-lymphoid lineage whereas a lack of Stat5a/b activation allows for the development of early T-lymphoid cells. Moreover, the block in B-cell development at the earliest step (Hardy fraction A) confirms the original concept that Stat5a/b are the relevant transcription factor downstream of IL-7 in early B-cell development.[17] It is also in line with an increased number of pro-B-cells in *Stat5b-CA* mice.[18]

Our experiments also lead to another important conclusion: the truncated proteins of Stat5a/b expressed in *Stat5a/b*$^{\Delta N/\Delta N}$ mice are able to partially rescue B-cell development and to allow for the development of γδTCR$^+$ and CD8$^+$ T cells. It is currently unclear how the truncated Stat5a/b operate but we know that the Stat5ΔN protein enters the nucleus and constitutively binds DNA (data not shown). We also know that, at least in T cells, the Stat5ΔN protein is able to induce some but not all Stat5a/b target genes (eg, cyclin D2, an important mediator of cell proliferation). Further analysis in different cell lineages will finally clarify which target genes can be activated or repressed by Stat5ΔN. In addition, Stat5a/b might act as a scaffold. It was recently shown that constitutively active Stat5a/b assemble in a complex with Gab2 to allow for activation of PI3K.[50,51] A potential protein docking function of Stat5a/b would be an alternative hypothesis to explain the differences observed in *Stat5a/b*$^{\Delta N/\Delta N}$ and *Stat5a/b*$^{null/null}$ cells.

Finally, we show that *Stat5a/b*$^{null/null}$ cells, in contrast to *Stat5a/b*$^{\Delta N/\Delta N}$ cells, fail to induce lymphoid leukemia in mice. The

truncated Stat5a/b proteins suffice to afford transformation as illustrated for Abelson oncogenes.[13] In each approach employed, growth factor–independent clones from *Stat5a/b*$^{\Delta N/\Delta N}$ cells grew out readily. In contrast, regardless of the experimental setup, we consistently failed to obtain a single colony or growth factor–independent clone from *Stat5a/b*$^{null/null}$ cells derived from either fetal livers or bone marrow. This key finding is of high clinical relevance because Stat proteins are potential candidates for drug targeting in the therapy of leukemia and other forms of cancer.[52,53] We have recently shown that constitutively active Stat5a/b induced a multi-lineage leukemia and that tetramer formation of Stat5a/b was crucial in this regard.[36] Apparently, N-terminally truncated Stat5a/b proteins that lack the tetramerization domain suffice to collaborate with at least some oncogenic tyrosine kinases, as proven here for the Abelson oncogenes. Therefore, a close definition of Stat5a/b functions in cancer progression is urgently needed. Our findings may therefore redirect therapeutic approaches that try to target Stat5a/b signaling in human leukemia. The importance of Stat5a/b in leukemia is further stressed by the fact that constitutively active mutations of Jak2 have recently been characterized as causative oncogenes in human leukemia.[54-57] Jak2 is a strong activator of Stat5a/b; hence, it is attractive to speculate that Stat5a/b are essential components in the signaling cascade underlying disease manifestation and progression in these patients. If this can be confirmed, Stat5a/b are likely to emerge as a potential target for therapeutic intervention.

Acknowledgments

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3.1.8 Erythroid progenitor renewal versus differentiation: genetic evidence for cell autonomous, essential functions of EpoR, Stat5 and the GR

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ORIGINAL ARTICLE

Erythroid progenitor renewal versus differentiation: genetic evidence for cell autonomous, essential functions of EpoR, Stat5 and the GR

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The balance between hematopoietic progenitor commitment and self-renewal versus differentiation is controlled by various transcriptional regulators cooperating with cytokine receptors. Disruption of this balance is increasingly recognized as important in the development of leukemia, by causing enhanced renewal and differentiation arrest. We studied regulation of renewal versus differentiation in primary murine erythroid progenitors that require cooperation of erythropoietin receptor (EpoR), the receptor tyrosine kinase c-Kit and a transcriptional regulator (glucocorticoid receptor; GR) for sustained renewal. However, mice defective for GR- ($GR^{dim/dim}$), EpoR- ($EpoR_H$) or STAT5ab function ($Stat5ab^{-/-}$) show no severe erythropoiesis defects *in vivo*. Using primary erythroblast cultures from these mutants, we present genetic evidence that functional GR, EpoR, and Stat5 are essential for erythroblast renewal *in vitro*. Cells from $GR^{dim/dim}$, $EpoR_H$, and $Stat5ab^{-/-}$ mice showed enhanced differentiation instead of renewal, causing accumulation of mature cells and gradual proliferation arrest. Stat5ab was additionally required for Epo-induced terminal differentiation: differentiating $Stat5ab^{-/-}$ erythroblasts underwent apoptosis instead of erythrocyte maturation, due to absent induction of the antiapoptotic protein Bcl-X_L. This defect could be fully rescued by exogenous Bcl-X_L. These data suggest that signaling molecules driving leukemic proliferation may also be essential for prolonged self-renewal of normal erythroid progenitors.
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Introduction

Pluripotent hematopoietic stem cells (HSCs) have continuously to decide between commitment, self-renewal and terminal differentiation to maintain proper numbers of all mature blood cell types. Originally, HSCs were thought to represent the only cells with self-renewal ability, and many human leukemias indeed involve mutated HSCs (Shet *et al.*, 2002). Recently, however, multi- or unipotent progenitors were also found to undergo normal or leukemic renewal (Blau *et al.*, 2001; Pardal *et al.*, 2003). This renewal is driven by normal or mutated cytokine receptors and receptor tyrosine kinases (RTKs), which cooperate with transcriptional and chromatin regulators as shown in culture (Schulte *et al.*, 2002; Carotta *et al.*, 2004; Von Lindern *et al.*, 2004) and human leukemia (Stirewalt and Radich, 2003; Tenen, 2003).

Erythropoiesis requires tight control of erythrocyte numbers but red cell production must increase rapidly in response to blood loss, hypoxia, or anemia. This flexibility is effected by tight regulation of erythroid progenitor renewal versus maturation via an interplay of cytokine- and nuclear receptors essential for stress erythropoiesis: Stem cell-factor (SCF) receptor (c-Kit) and erythropoietin (Epo) receptor cooperate with a nuclear hormone receptor (glucocorticoid receptor; GR) to induce prolonged expansion of primary avian, murine, and human erythroid progenitors (Bauer *et al.*, 1999; von Lindern *et al.*, 1999; Carotta *et al.*, 2004). These cells resemble proerythroblasts with properties of burst- and colony forming units-erythroid (BFU-E, CFU-Es) (Sawada *et al.*, 1990). In response to differentiation factors, they undergo *in vivo*-like ery-

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throid maturation (Dolznig *et al*., 1995, 2001; von Lindern *et al*., 2001). Owing to their close resemblance to *in vivo* progenitors, primary murine erythroblasts are particularly useful to analyse the balance between renewal and differentiation and its disturbance in leukemia at the molecular level, and are superior to established erythroid cell lines for such studies (Silva *et al*., 1996, 1999).

Culture models from avian erythroid and multipotent progenitors have already proven their value in analysing leukemic renewal (Beug *et al*., 1995; Schulte *et al*., 2002). Importantly, mutated or amplified RTKs (c-Kit; Flt-3) (Stirewalt and Radich, 2003) cooperating with transcriptional regulators (including mutated nuclear receptors) are increasingly implicated in human leukemias (He *et al*., 1998; Tenen, 2003).

Analysis of signaling pathways for erythroid progenitor renewal (Figure 1a) revealed that EpoR activates Stat5, probably cooperating with the GR (Doppler *et al*., 2000), whereas c-Kit activates the PI3K-pathway. Whereas PI3K-signaling was indispensable for erythroblast renewal, the requirement for Stat5 activation was only studied in differentiating myeloid progenitors, where it was essential for apoptosis protection via the antiapoptotic protein Bcl-X_L (Kieslinger *et al*., 2000). Interestingly, erythroid maturation also involves protection from apoptosis by Epo-dependent upregulation of Bcl-X_L (Motoyama *et al*., 1999; Dolznig *et al*., 2002).

Several transgenic mouse studies addressed how ablation of the above signal transducers would interfere with erythropoiesis *in vivo*. Deletion of the GR ($GR^{-/-}$) or a knocked-in mutation leading to a dimerization-defective protein ($GR^{dim/dim}$) prevented stress erythropoiesis (Bauer *et al*., 1999). Loss of c-Kit affected multiple progenitor types, including erythroid ones, leading to severe anemia (Broudy, 1997). Mice lacking EpoR or Jak2 are deficient for definitive erythropoiesis (Wu *et al*., 1995; Neubauer *et al*., 1998; Parganas *et al*., 1998). In contrast, knock-in mice expressing cytoplasmically truncated EpoR variants – binding Jak2 but lacking most ($EpoR_H$) or all ($EpoR_{HM}$) of signal transducer docking sites (Zang *et al*., 2001) – show largely normal erythropoiesis. Finally, $Stat5ab^{-/-}$ mice have no obvious defects in steady-state erythropoiesis except mild anemia (Socolovsky *et al*., 1999). Thus, the above-mentioned mouse models do not reflect the strict requirement of EpoR/Stat5- and GR-signaling for progenitor renewal in culture.

We therefore sought to obtain genetic evidence for essential, cell-autonomous roles of GR-signaling and EpoR-signaling (via Stat5) for renewal and apoptosis protection in cultured erythroid progenitors from genetically modified mice, as multiple, compensatory mechanisms might obscure defects in the respective erythroid progenitors *in vivo*. We show that primary erythroblasts from $GR^{-/-}$, $GR^{dim/dim}$, $EpoR_H$, $EpoR_{HM}$,

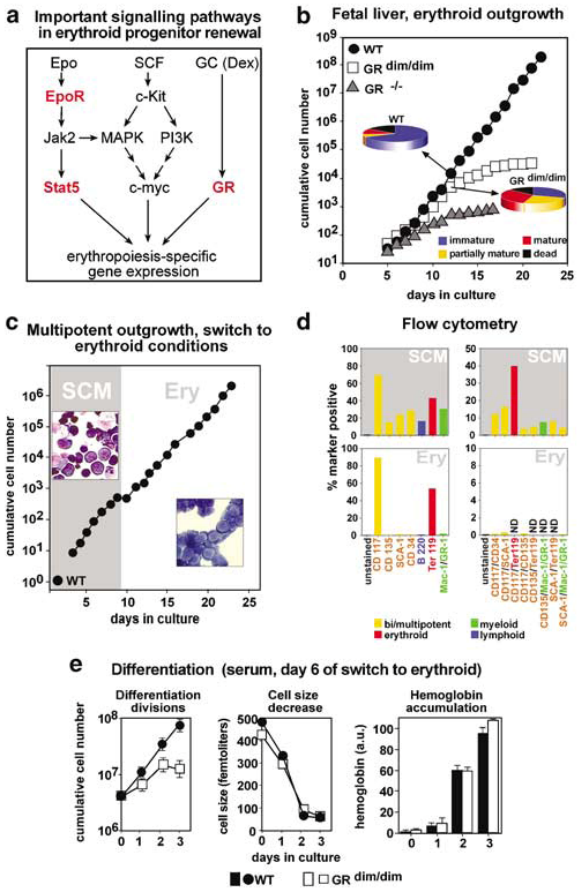

Figure 1 Pre-expansion of proliferation-defective $GR^{dim/dim}$-erythroblasts as multipotent cells yields erythroblasts defective for renewal but not for differentiation. (**a**) Signal transduction pathways important in erythropoiesis. Signaling molecules ablated or mutated in cells from genetically modified mice used in this study are indicated in red. (**b**) E12.5 fetal liver cells from $GR^{dim/dim}$ (white squares), $GR^{-/-}$ (gray triangles) or wild-type mice (WT, black circles) were cultivated in 'erythroid proliferation medium' and cumulative cell numbers determined daily. At day 12, aliquots were subjected to cytocentrifugation, stained and quantified for cells of increasing maturity (pie diagrams, blue, yellow, red) and apoptotic cells (black) as described (Kolbus *et al*., 2002). Cells ($\geqslant 300$) were counted per slide and mean values $\pm$s.d. calculated from at least three independent determinations. Percentages of immature/partially mature/mature/dead cells were $70 \pm 6/5 \pm 1/9 \pm 2/16 \pm 3$ for wild-type cells and $31 \pm 4/27 \pm 5/26 \pm 4/16 \pm 3$ for $GR^{dim/dim}$ erythroblasts. (**c**) Cells from wild-type mouse fetal livers were pre-expanded in 'stem cell mix' medium (gray area, 'SCM'), switched to erythroid conditions (day 9, white area, 'Ery') and cumulative cell numbers determined. Insets, cytospins from multipotent progenitors (top left) and cells switched to the erythroid lineage (day 17, bottom right). (**d**) Multipotent cells (top panels, day 9) and erythroblasts derived thereof (bottom panels, day 17, see panel (**c**) were subjected to flow cytometry. Percentages of cells single- (left) or double positive (right) for immature (green bars), erythroid (red bars), myeloid (green bars), and lymphoid surface markers (blue bars) detected by respective antibodies are shown. (**e**) Fetal liver cells from $GR^{dim/dim}$- (white symbols) and wild-type (WT) mice (black symbols) were expanded in SCM, switched to the erythroid lineage (day 0, not shown) and analysed for differentiation kinetics in serum-containing differentiation medium. Cumulative cell numbers (left panel; mean values $\pm$s.d., $n=3$), cell size (middle panel) and hemoglobin content (right panel) were determined at the times indicated.

and Stat5ab$^{-/-}$ mouse embryos cultured *ex vivo* under fully defined conditions indeed display distinct proliferation defects *in vitro*. These defects were due to increased terminal differentiation at the expense of renewal rather than to enhanced apoptosis. While differentiation of GR$^{-/-}$, GR$^{dim/dim}$, and EpoR$_H$ erythroblasts proceeded normally, Bcl-X$_L$ induction and survival of Stat5ab$^{-/-}$ cells was regulated in a complex fashion, involving Epo, serum factors, and compensatory upregulation of other Stat-family members.

Results

Impaired expansion of GR-defective erythroblasts

Erythroblasts from mice expressing a dimerization-defective GR (GR$^{dim/dim}$; Bauer *et al.*, 1999) showed impaired stress erythropoiesis *in vivo* and could not be expanded in culture (Reichardt *et al.*, 1998). While wild-type cells proliferated for >20 days, GR$^{dim/dim}$ erythroblasts stopped after 5 10 days and GR$^{-/-}$ cells could hardly be expanded at all (Figure 1b). At day 12, cytospin analysis of GR$^{dim/dim}$ cultures showed strongly increased numbers of mature cells and fewer immature cells than wild-type cultures. Dead cells were infrequent in both cultures. Therefore, the proliferation defect of GR$^{dim/dim}$ erythroblasts *in vitro* was due to increased differentiation at the expense of renewal. A similar, even more rapid increase in mature cells occurred in GR$^{-/-}$ erythroblasts (not shown).

Pre-expansion of proliferation-defective mutant erythroblasts as multipotent progenitors

Owing to their enhanced rate of spontaneous differentiation, GR-deficient erythroblasts could not be analysed for potential maturation defects. We thus tried to obtain homogenous, immature erythroblasts by pre-expansion of GR-defective multipotent progenitors. We expected that these cells would lack erythroid-specific defects and thus should be able to undergo commitment to the erythroid lineage in erythroid-specific proliferation medium (Schulte *et al.*, 2002). In establishing this procedure, wild-type fetal liver-derived cells could be expanded more than 10^6-fold (not shown) in serum-free 'stem cell medium' (SCM). Cytospin analyses after ~500-fold expansion (8 days) revealed >50% of cells with immature morphology, whereas the remainder consisted of maturing erythroblasts, monocytes/macrophages, neutrophils, eosinophils, and mast cells (Figure 1c). Flow cytometry of such cell populations revealed >20% of cells with markers typical of multipotent, immature progenitors (CD117/c-Kit, CD135/Flk2/Flt3, CD34, Sca-1), confirmed by coexpression of several immature markers plus the absence of lineage-specific markers on the same cells (Figure 1d). As expected, we also detected committed cells expressing erythroid (Ter119), myeloid (CD11b/Mac-1, GR-1), or lymphoid lineage markers (CD45R/B220).

From these wild-type cells, pure erythroid progenitors were generated by transfer into media containing SCF/Epo/Dex. After continuous proliferation for 5 7 days, populations of >90% pure erythroblasts were obtained as verified by cytospins (Figure 1c) and flow cytometry (CD117/c-Kit^{high}, Ter119^{low} Mac-1/GR-1^{low}, negative for CD135, CD34, and Sca-1; Figure 1d, lower panels). These erythroblasts could be expanded for ~2 weeks and induced to terminally differentiate at any time throughout their life span.

Applying this procedure to GR$^{dim/dim}$ fetal liver cells yielded similarly homogenous erythroid cultures, suitable for detailed analysis of differentiation kinetics. Purified GR$^{dim/dim}$ erythroblasts still underwent strongly increased spontaneous differentiation at the expense of renewal, showing the expected defect (as in Figure 1b, data not shown). Nevertheless, pre-expansion as multipotent cells yielded enough GR$^{dim/dim}$ erythroblasts with proliferation rate and size identical to wild-type cells to allow analysis of differentiation parameters. During terminal maturation, GR$^{dim/dim}$ erythroblasts showed normal kinetics of size decrease and hemoglobin accumulation as compared to that of wild-type cells, but displayed a reduced proliferation capacity (Figure 1e). Similar results were obtained in serum-containing- and serum-free differentiation media (see below and data not shown).

EpoR intracellular domain: required for renewal but not for differentiation

To address whether the EpoR cytoplasmic domain was required for erythroblast renewal, cells from mice lacking most (EpoR$_H$) or all (EpoR$_{HM}$) (Zang *et al.*, 2001) of this domain (schemes in Figure 2a) were analysed. EpoR$_H$ erythroblasts grown in 'erythroid proliferation medium' exhibited gradual proliferation arrest after 7 8 days (Figure 2a), caused by massively enhanced spontaneous differentiation under renewal conditions (data not shown). Again, pre-expansion of EpoR$_H$ fetal liver cells as multipotent progenitors and switch to erythroid conditions (Figure 2b, black arrow, day 6) yielded homogenous mass cultures of mutant erythroblasts for differentiation analysis (Figure 2b, gray arrow, day 12). In both serum-containing- and serum-free media, EpoR$_H$ cells showed the same maturation kinetics as that of wild-type cells, as judged by the number of differentiation divisions, size decrease, and hemoglobin accumulation (Figure 2c). Similar results were obtained with erythroblasts from EpoR$_{HM}$ mice. Thus, cytoplasmic docking sites for signal transducers were required for sustained erythroblast renewal but not for terminal differentiation, in line with EpoR$_H$/EpoR$_{HM}$ mice showing normal steady-state erythropoiesis.

Stat5ab: essential for erythroid progenitor renewal and terminal differentiation?

Activated Stat5 is a major downstream signal transducer of EpoR. We therefore addressed its potential role during erythroid progenitor renewal and differentiation, using fetal liver-derived erythroblasts of Stat5ab$^{-/-}$ mice (Teglund *et al.*, 1998). As these cells also showed a marked proliferation defect in 'erythroid proliferation medium', they were pre-expanded as multipotent

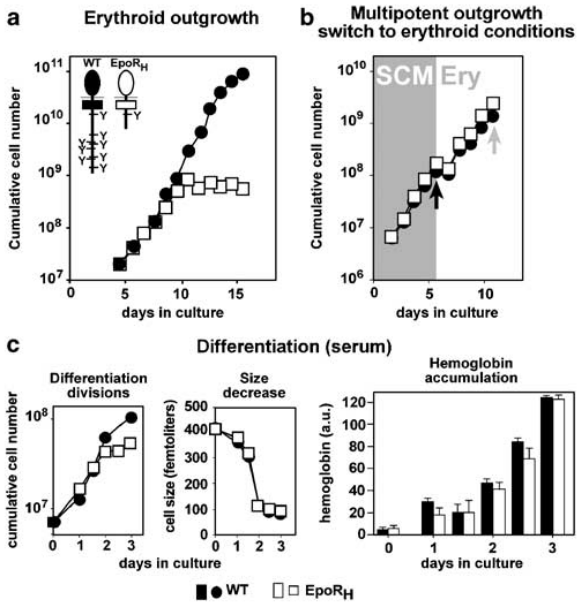

Figure 2 Erythroblasts expressing cytoplasmically truncated EpoR_H: impaired renewal but normal differentiation. (**a**) Fetal liver-derived cells from EpoR_H- (white symbols) and WT-mice (black) were cultivated in serum-free 'erythroid proliferation medium' and cumulative cell numbers determined as in Figure 1. Inset, scheme of mutated EpoR's used. (**b**) Cells as in (**a**) were pre-expanded in SCM (gray area, 'SCM') and switched to erythroid proliferation medium after 6 days (black arrow, white area, 'Ery'). (**c**) After expansion for another 6 days in (gray arrow in (**b**) cells were analysed for differentiation parameters as in Figure 1 (three separate experiments, representative data shown).

progenitors. The cultures proliferated slower than respective wild-type cells (Figure 3a; SCM), similar to avian myeloblasts expressing a dominant-negative Stat5ΔC mutant (Kieslinger *et al*., 2000). When switched to erythroid conditions (Figure 3a, black arrow, day 6), Stat5ab$^{-/-}$ cultures ceased to proliferate after about 8 days (='day 14' in Figure 3a), containing fewer immature cells whereas numbers of partially mature/mature cells were massively enhanced.

When seeded into standard, serum-containing differentiation medium 6 days after switching to erythroid conditions (Figure 3a, gray arrow, day 12), both Stat5ab$^{-/-}$ and wild-type cells exhibited similar differentiation kinetics (Figure 3b), with >75% of enucleated and nucleus-containing erythrocytes and <20% of dead/disintegrated cells in cytospins (Figure 3c). Unlike wild-type cells (Figure 3d and e, left panels) or GR$^{dim/dim}$ and EpoR_H cells, Stat5ab$^{-/-}$ erythroblasts in defined, serum-free differentiation medium died within 48 h (Figure 3d and e). They showed proliferation arrest after 24 h, aberrant size decrease, massively reduced hemoglobin accumulation (Figure 3d) and >75% of dead cells in cytospins (Figures 3e, right panels). These results suggest that functional Stat5 is required for both erythroblast renewal and differentiation under defined, serum-free conditions.

STAT5-dependent regulation of bcl-X_L transcription: Lineage-specific differences during differentiation and renewal

Earlier work had demonstrated an essential role of the antiapoptotic protein Bcl-X_L in protecting differentiating erythroid and myeloid progenitors from apoptosis (Kieslinger *et al*., 2000; Dolznig *et al*., 2002). In Epo-responsive Friend erythroleukemia cells (HCD57) and primary myeloid cells dependent on IL-3/GM-CSF, apoptosis protection correlated with direct, STAT5 mediated transcriptional activation of bcl-X_L (Socolovsky *et al*., 1999; Kieslinger *et al*., 2000). We therefore tested whether bcl-X_L was a direct target gene of Stat5 in proliferating or differentiating erythroblasts and whether ablation of Stat5ab would prevent upregulation of Bcl-X_L and survival in differentiating Stat5ab$^{-/-}$ cells. Surprisingly, Stat5ab$^{-/-}$ cells differentiating in serum-containing medium accumulated hemoglobin and upregulated Bcl-X_L like wild-type cells (Figure 4a, top), but failed to do so in fully defined, serum-free medium (Figure 4a bottom, see also Figure 3c and e). These unexpected findings prompted us to analyse the relationship between Stat5 and Bcl-X_L in more detail. In wild-type erythroblasts, total Stat5ab protein levels declined steadily during maturation (Figure 4b, top panel). Tyrosine phosphorylation and DNA-binding activity were transiently elevated during the first 12–24 h but declined to low levels after 36 h. In contrast, Bcl-X_L protein was not upregulated until 36 h after differentiation induction, when Stat5 activity had already declined (Figure 4b, bottom panel), suggesting that Stat5 would not directly activate transcription of bcl-X_L. To address this, proliferating wild-type erythroblasts were factor-deprived and restimulated with Epo, SCF or both. None of these cytokines induced bcl-X_L mRNA, whereas Epo and Epo+SCF clearly induced *cis* mRNA, a well-defined Stat5 target gene (Verdier *et al*., 1998). Furthermore, proliferating, immature wild type as well as Stat5ab$^{-/-}$ erythroblasts expressed similar levels of bcl-X_L mRNA after factor withdrawal and Epo restimulation (Figure 4c). Since bcl-X_L was directly induced by Stat5 in IL-3-stimulated wild type but not Stat5ab$^{-/-}$ myeloid cells (Kieslinger *et al*., 2000), bcl-X_L transcription in erythroid progenitors is probably coregulated by Stat5 plus other serum factors.

Exogenous Bcl-X_L rescues the differentiation but not the proliferation defect of Stat5ab$^{-/-}$ erythroblasts

Exogenous overexpression of Bcl-X_L enables terminal differentiation of primary erythroblasts in the absence of Epo (Dolznig *et al*., 2002). To test whether Bcl-X_L would also rescue the differentiation defect of Stat5ab$^{-/-}$ erythroblasts, cells were infected with a GFP-Bcl-X_L retroviral construct, expanded as multipotent progenitors and switched to the erythroid lineage. More than 90% of the infected cells stably expressed Bcl-X_L as shown by flow cytometry for GFP expression (data not shown). To analyse renewal, cells were cultivated in serum-free medium plus renewal factors (Epo/SCF/Dex), using empty vector-expressing cells as controls.

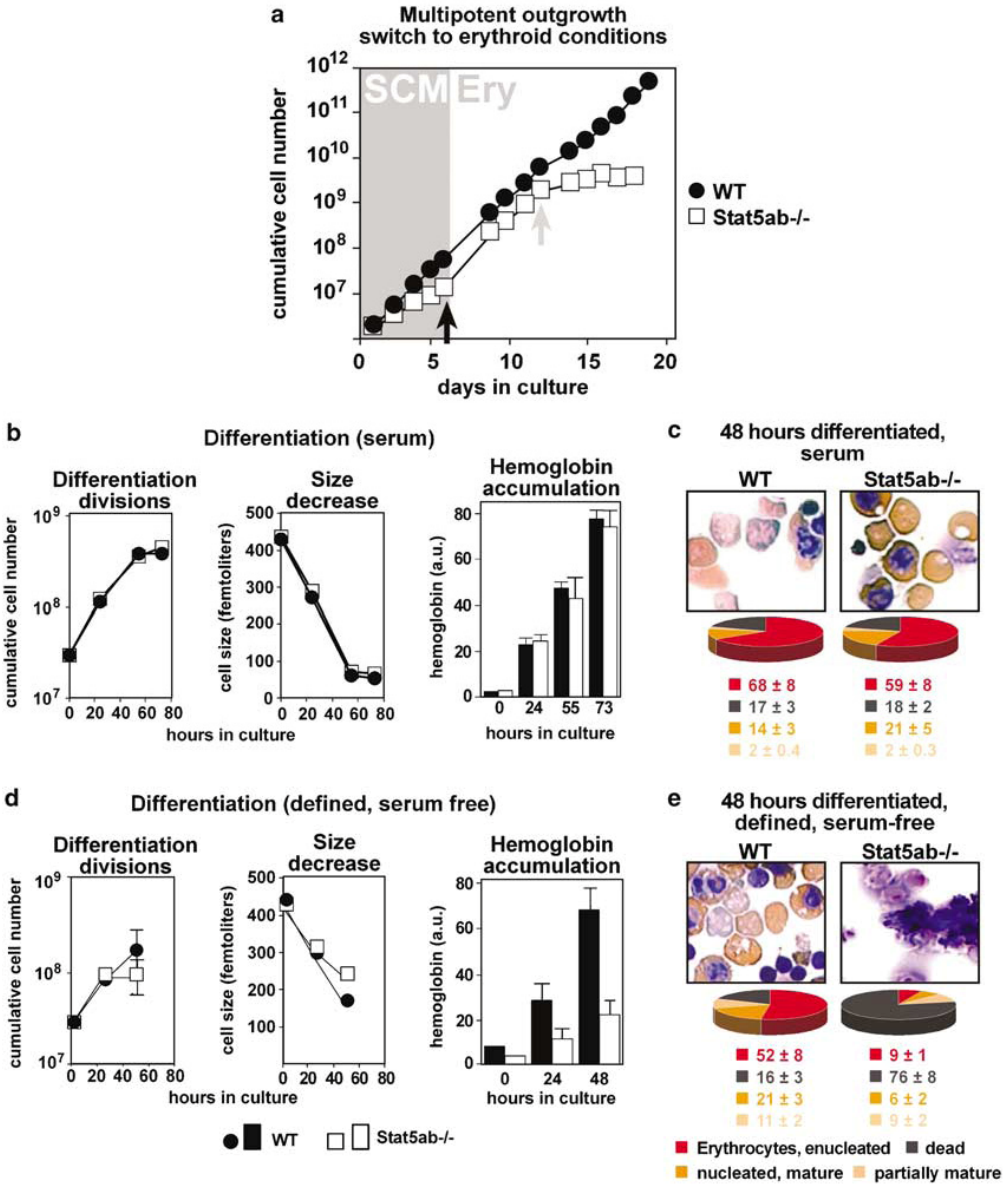

Figure 3 Erythroblasts from Stat5ab$^{-/-}$ mice: defects in renewal and terminal differentiation. (**a**) Fetal liver cells from Stat5ab$^{-/-}$ (white symbols) and wild-type mice (black) were expanded in SCM (gray area, 'SCM'), switched to erythroid proliferation medium (white area, 'Ery'), cultivated for another 6 days, and cumulative cell numbers were determined. (**b**, **d**) Stat5ab$^{-/-}$ and wild-type erythroid progenitors (day 12, gray arrow) analysed for differentiation parameters in both serum-containing (**b**) and defined, serum-free differentiation medium (**d**) as described in Figures 1 and 2 (error bars: s.d.s, $n=3$). (**c**, **e**) Top panels: Cytospins from (**b**) and (**d**) subjected to neutral benzidine/histological staining after 48 h. Bottom: quantitation with respect to partially mature/mature/enucleated erythrocytes (percentages $\pm$s.d.s, $n=3$).

Bcl-X_L-transduced and control Stat5ab$^{-/-}$ erythroblasts both showed enhanced differentiation at the expense of renewal (Figure 5b, top), leading to premature proliferation arrest, as revealed by reduced or absent increase in cell numbers (Figure 5a) and a strong reduction of metaphases in cytospins (Figure 5b, bottom right). Exogenous Bcl-X_L only slightly delayed growth arrest of renewing WT and Stat5 deficient cells, most likely due to enhanced survival of maturing cells (Figure 5b, bottom left). In contrast, wild-type cells proliferated exponentially (Figure 5a) as immature cells (Figure 5b), regardless of the presence or absence of exogenous Bcl-X_L.

We then analysed whether exogenous Bcl-X_L could rescue differentiation of Stat5ab$^{-/-}$ cells in defined, serum-free media. Even in the absence of Epo, Bcl-X_L-transduced

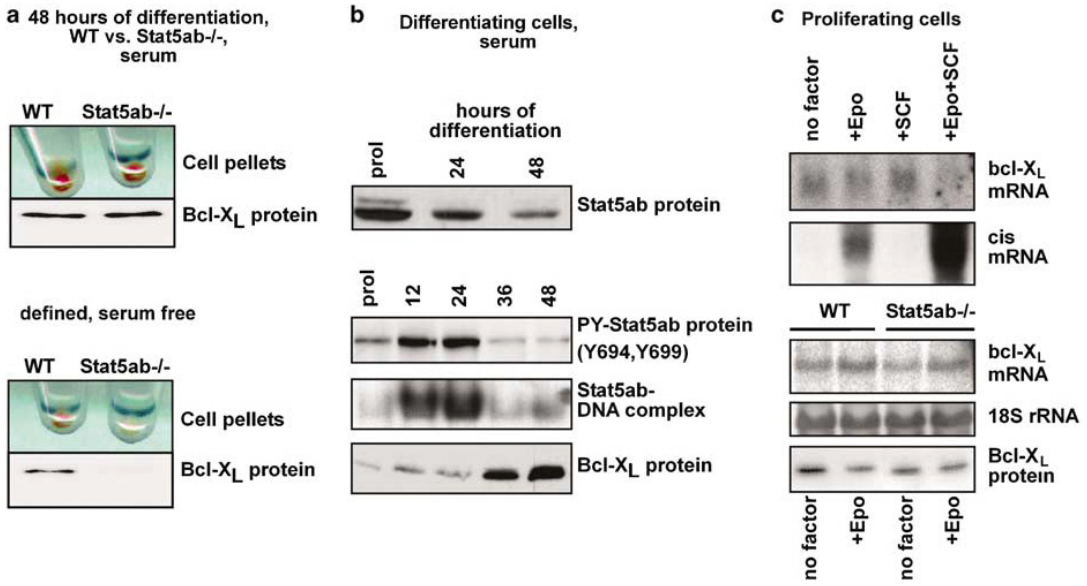

Figure 4 Bcl-X_L upregulation in Stat5ab$^{-/-}$ erythroblasts is dependent on serum and not subject to direct transcriptional activation by Epo-driven Stat5 activation. (**a**) Primary Stat5ab$^{-/-}$ and wild-type erythroblasts were induced to differentiate in serum-containing (upper panels) or defined, serum-free differentiation medium (lower). Respective cell pellets (viable cells only) show hemoglobinization (red color, top of panels) and upregulation of Ter119 expression (data not shown, Dolznig *et al.*, 2001). Lysates were analyzed for Bcl-X_L protein (bottom) (**b**) Erythroblasts were induced to differentiate in serum-containing differentiation medium and analyzed for total Stat5ab protein, tyrosine-phosphorylated Stat5ab, and Bcl-X_L at the times indicated. Stat5ab-DNA-binding activity was determined in EMSAs using extracts from the same preparations. (**c**) Upper panels: proliferating erythroblasts kept for 4 h without cytokines (no factor) and restimulated for 1 h with Epo (+Epo), SCF (+SCF) or both (+Epo+SCF) were analysed by Northern blotting for bcl-X_L mRNA and as positive control *cis* mRNA expression. Lower panels: primary mouse erythroblasts from wild-type (WT) or Stat5ab$^{-/-}$ fetal livers factor-depleted for 4 h, restimulated with Epo for 3 h and analysed for bcl-X_L-mRNA (loading control: 18S ribosomal RNA) and protein expression.

Stat5ab$^{-/-}$, and wild-type cells effectively underwent terminal differentiation in serum-free medium, as judged by histology and quantitative evaluation of cytospins (Figure 5c). Bcl-X_L also rescued the defective hemoglobin accumulation of Stat5ab$^{-/-}$ cells in medium plus Epo/Insulin (Figure 5d). Together with the results described above, this demonstrated that the inability of Stat5ab$^{-/-}$ cells to upregulate bcl-X_L during differentiation can be overcome by Stat5ab-independent pathways, induced by serum factors in cooperation with Epo.

Stat5ab$^{-/-}$ cells upregulate activated Stat3 and Stat1: compensation of Stat5ab$^{-/-}$ cell defects?

Asynchrony of Stat5ab activation versus Bcl-X_L expression, failure of Epo to increase basal levels of Bcl-X_L transcription in proliferating cells and serum-factor dependence of bcl-X_L upregulation in differentiating Stat5ab$^{-/-}$ cells all pointed to an involvement of Stat5ab-independent pathways. Furthermore, the relatively mild renewal defect in proliferating Stat5ab$^{-/-}$ cells was difficult to reconcile with the expected, complete defect in erythroblast outgrowth from EpoR$^{-/-}$ or Jak2$^{-/-}$ fetal livers (FG *et al*, unpublished data).

We therefore tested whether Stat5ab$^{-/-}$ cells might have residual Stat5ab activity and/or show enhanced expression/activation of other Stat family members. Phospho-Stat5-specific antibodies indeed detected high levels of a smaller, phosphorylated Stat5 protein in Stat5ab$^{-/-}$ cells 24 h after differentiation induction. Heterozygous Stat5ab$^{+/-}$ cells expressed the same small Stat5 together with apparently full-length wild-type Stat5, which in Stat5ab$^{+/+}$ cells was the only form detected (Figure 6a). Tyrosine phosphorylation of truncated Stat5 in Stat5ab$^{-/-}$ cells occurred with similar kinetics than that in wild-type cells (maximum after 24 h of differentiation) but was stronger than that in wild-type cells at all time points tested (Figure 6a), while total expression levels of truncated Stat5 were lower than those of Stat5 in wild-type cells and downregulation of the truncated protein during erythroid differentiation was delayed when compared to the kinetics of wild-type Stat5 expression. In line with this, we observed a several-fold increase in DNA-binding activity of the truncated Stat5 protein from Stat5$^{-/-}$ cells during erythroid differentiation in EMSA assays when compared to wild-type cells (Supplementary Figure S1). Thus, unlike the recently reported Stat5ab-knockout animals (Cui *et al*., 2004), the Stat5ab$^{-/-}$ cells used here express an N-terminally truncated but phosphorylated Stat5 in erythroblasts, which might retain some biological functions of wild-type protein (Supplementary Figure S1 and Moriggl *et al*., 2005).

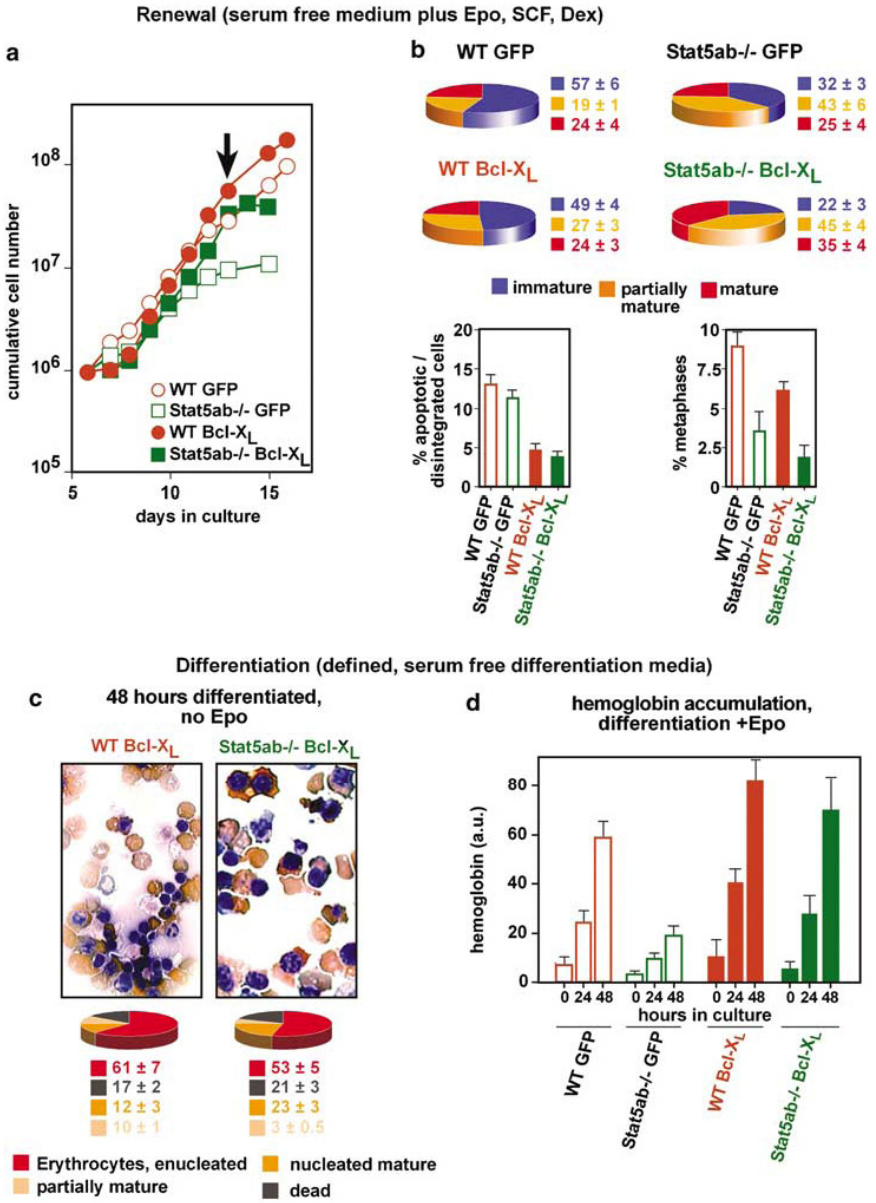

Figure 5 Retrovirally expressed Bcl-X_L rescues the differentiation but not the renewal defect of Stat5 $^{-/-}$ erythroblasts. Freshly isolated wild type or Stat5ab $^{-/-}$ fetal liver cells were infected with MSCV-IRES-GFP or MSCV-Bcl-X_L-IRES-GFP retroviral vectors (**a**) Cumulative cell numbers determined for Bcl-X_L expressing wild-type or Stat5ab $^{-/-}$ erythroblasts expanded in 'erythroid proliferation medium'. (**b**) Aliquots of proliferating cultures in (**a**) were subjected to cytospin analysis (arrow in **a**) for mature, partially mature, and immature cells as described for Figure 1 (top panels, viable cells only). The same cytospins were analysed for proportions of apoptotic/disintegrated cells and cells in mitosis, respectively (bottom) (**c**) The cell types shown in (**a**) and (**b**) were differentiated for 48 h in defined, serum-free medium lacking Epo. Cytospins (top) were quantified as in Figures 1 and 3 (percentages $\pm$ s.d.s, $n=3$). (**d**) Aliquots from wild-type or Stat5ab $^{-/-}$ erythroblasts expressing empty vector (WT GFP, Stat5ab $^{-/-}$) or exogenous Bcl-X_L (WT Bcl-X_L, Stat5ab $^{-/-}$ Bcl-X_L) were differentiated in fully defined medium plus Epo and analysed for hemoglobin content at the times indicated (error bars: s.d.s, $n=3$).

Stat5ab $^{-/-}$ and wild-type cells showed identical levels of Stat3 protein, which gradually decreased during differentiation. Interestingly, Stat3 was strongly tyrosine-phosphorylated in renewing Stat5ab $^{-/-}$ erythroblasts but not in wild-type cells (Figure 6b). This suggested that absence of Stat5 might promote compensatory Stat3 activation. We also observed strong upregulation of phosphorylated Stat1 in differentiating but not in proliferating Stat5ab $^{-/-}$ cells, which, however, was obviously insufficient to restore Bcl-X_L expression in

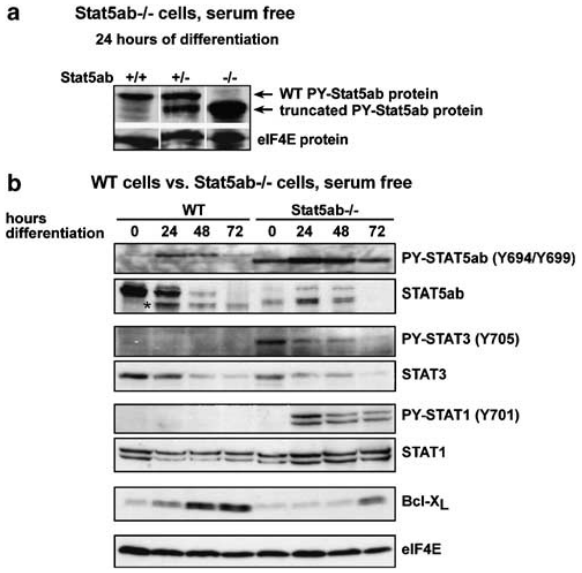

Figure 6 Selective activation of Stat3 and Stat1 in proliferating and differentiating Stat5ab$^{-/-}$ erythroblasts. (**a**) Primary fetal liver cells from wild-type, Stat5ab$^{+/-}$, and Stat5ab$^{-/-}$ mice were induced to differentiate in serum-free medium for 24 h and subjected to Western blot analysis for tyrosine-phosphorylated Stat5ab. (**b**) Primary wild-type- and Stat5ab$^{-/-}$ erythroblasts were induced to differentiate in defined serum-free medium for the times indicated and lysates were subjected to Western blot analysis for tyrosine phosphorylated Stat5, Stat3 and Stat1, as well as for total Stat5, Stat3, Stat1, and Bcl-X_L protein. Loading control, eIF4E levels, *, nonspecific band.

Stat5ab$^{-/-}$ cells (Figure 6b). These results raise the possibility that Stat3-signaling might play a role in partially rescuing the renewal defect of Stat5ab$^{-/-}$ erythroblasts, perhaps also improving survival during differentiation.

Discussion

It is increasingly recognized that abnormal self-renewal in human leukemia can be caused by cooperation of (mutated) transcriptional regulators – including nuclear receptors – with mutated/amplified receptor tyrosine kinases and/or constitutively active Stat5 or Stat3 (Stirewalt and Radich, 2003; Tenen, 2003; Yu and Jove, 2004; Ren, 2005). Here, we provide genetic evidence that members of the same protein families – the transcriptional regulator GR cooperating with EpoR and its key signal transducer Stat5 – plus the RTK c-Kit – are essential for sustained renewal of primary erythroid progenitors. Initially, this was not supported by analysis of respective mutant mice (GR$^{-/-}$, GR$^{dim/dim}$, EpoR$_H$, and Stat5ab$^{-/-}$), none of which showed major defects in steady-state erythropoiesis *in vivo* (Cole *et al*., 1995; Reichardt *et al*., 1998; Teglund *et al*., 1998; Zang *et al*., 2001). Using novel *in vitro* approaches to expand and differentiate primary mutant erythroblasts, we could show that all of these mutant cell types displayed enhanced differentiation at the expense of immature progenitor proliferation, gradually arresting expansion of the cultures. GR-function and EpoR cytoplasmic domain signaling were, however, dispensable for erythroid differentiation. In contrast, Stat5ab$^{-/-}$ erythroblasts failed to differentiate under fully defined, serum-free conditions, instead undergoing apoptosis due to defective up-regulation of bcl-X_L. This defect of Stat5ab$^{-/-}$ erythroblasts could be rescued by exogenous bcl-X_L or serum factors. Interestingly, Stat5ab$^{-/-}$ erythroblasts also exhibited increased activation of Stat3 and Stat1. These findings raise the possibility that other Stat family members might compensate for renewal- and survival defects of Stat5$^{-/-}$ erythroid progenitors.

Similar players and mechanisms in normal and oncogene-driven renewal?

The avian leukemia oncogenes v-ErbB and v-Sea – causing acute erythroleukemia in chicks (Beug *et al*., 1996; Bauer *et al*., 2001) – activate both Stat5- and PI3K-signaling pathways (von Lindern *et al*., 2001). Thus, these oncogenes can substitute for c-Kit-dependent PI3K activation and EpoR-dependent Stat5 activation in normal progenitors. Furthermore, v-Sea induces renewal in wild type, but not in Stat5$^{-/-}$ erythroblasts (HB, unpublished data). Apparently, these leukemia oncogenes utilize the same signaling pathways to induce renewal as those driving stress erythropoiesis in normal progenitors.

Both Stat5 and Stat3 are frequently upregulated and constitutively activated in human leukemia (Yu and Jove, 2004). A constitutively active Stat5 mutant causes multilineage leukemia after expression in Stat5$^{-/-}$ mouse bone marrow cells (Moriggl *et al*., 2005). Conversely, the human leukemia oncogene Tel-Jak2 (Lacronique *et al*., 1997) needs Stat5 to induce a myeloproliferative disease in transgenic mouse models (Schwaller *et al*., 2000). In our cell model, lack of Stat5 caused an (probably compensatory) activation of Stat3. This might explain the puzzling finding that EpoR$^{-/-}$ and Jak2$^{-/-}$ erythroblasts completely lack self-renewal ability (the respective fetal liver cells cannot be expanded *in vitro*), while Stat5$^{-/-}$ cells showed only a mild defect in renewal. In line with this, erythroblasts from mice completely lacking Stat5ab (Cui *et al*., 2004) showed even stronger compensatory upregulation of Stat3 during renewal, and exhibited no renewal defect at all (MK *et al*., unpublished data). These mice do not display an early embryonic lethal phenotype similar to EpoR- or Jak2-deficient animals, but die perinatally, probably due to other defects besides a failure of erythropoiesis. This further supports the idea of unknown compensatory mechanisms enabling functional erythropoiesis in the absence of Stat5ab *in vivo* (FG and MK, unpublished data). In trials to interfere with such compensatory mechanisms, cells expressing a dominant negative version of Stat5 (Stat5ΔC, lacking the transactivation domain and thus also interfering with Stat5 functions in complexes with other transcriptional regulators) were employed. These cells failed to undergo functional

erythroid differentiation and upregulation of Bcl-X_L even in serum-containing medium, where no defect was observed in Stat5$^{-/-}$ cells (see Supplementary Material, Figure S2 and respective experimental description).

Erythroid differentiation: mechanisms distinct from those controlling renewal?

Apparently, several molecular players essential for erythroblast renewal also function in terminal erythroid differentiation but act via different mechanisms in the two processes. For instance, terminal erythroid differentiation did not require signaling via the EpoR cytoplasmic domain *in vitro* or *in vivo*, which was, however, essential for renewal. Conversely, cytoplasmic truncation of the EpoR was reported to attenuate Epo-dependent proliferation and apoptosis in another study (Li *et al*., 2003). Similarly, GR function is essential for renewal, and even inhibitory for erythroid differentiation (Reichardt *et al*., 1998; von Lindern *et al*., 2001).

Distinct signaling pathways controlling erythroblast renewal versus differentiation were also evident for Stat5. The renewal defect of Stat5ab$^{-/-}$ cells was clearly due to increased terminal differentiation at the expense of renewal, rather than enhanced apoptosis, and could not be rescued by Bcl-X_L. In contrast, differentiating Stat5ab$^{-/-}$ erythroblasts failed to induce Bcl-X_L and did not survive in the absence of serum factors, which rescued both normal differentiation and bcl-X_L induction in serum-containing media. One possibility to explain these puzzling results is that Stat5ab$^{-/-}$, but not wild-type fetal liver erythroblasts, showed significant Stat3 tyrosine phosphorylation before and after differentiation induction. Therefore, Stat3 might contribute to survival and late Bcl-X_L upregulation during differentiation, either alone or in cooperation with truncated Stat5 protein still expressed in the Stat5ab$^{-/-}$ erythroid cells used.

Regulation of Bcl-X_L and apoptosis by Stat5: lineage-specific differences?

The complex regulation of bcl-X_L transcription by Stats plus serum factors indicates that Bcl-X_L induction in terminally differentiating, primary erythroid cells is not directly induced by cytokines but appears to be already 'on' due to Stat 5-independent pathways. In contrast, primary myeloid progenitors (Kieslinger *et al*., 2000), myeloid and lymphoid cell lines (Packham *et al*., 1998; Dumon *et al*., 1999; Silva *et al*., 1999) and erythroleukemic cell lines (Socolovsky *et al*., 1999), exhibit 'simple', direct transcriptional induction of bcl-X_L mRNA by activated Stat5. Apparently, control by Epo is not sufficient for erythropoiesis (requiring rapid modulation in response to stress or disease and generating $>10^{11}$ erythrocytes per day in man) but is modulated by multiple factors that activate Stat5, Stat3, or even Stat1. This might explain why human erythroleukemias are very rare or why Tel-Jak2, although perturbing both myeloid and erythroid differentiation (Onnebo *et al*., 2005), causes myelo- and lymphoproliferative disease but no erythroleukemia in mice (Schwaller *et al*., 2000).

Materials and methods

Cultivation of murine erythroid progenitors

Erythroid progenitors from fetal livers of E12.5 mouse embryos were cultivated as described (Dolznig *et al*., 2005), using serum-free 'erythroid proliferation medium' (StemPro-34™; Invitrogen), plus 2 U/ml human recombinant Epo ('Erypo', Cilag AG), 100 ng/ml murine recombinant SCF (R&D Systems), 10^{-6} M dexamethasone (Dex; Sigma) and 40 ng/ml human recombinant insulin-like-growth-factor-1 (IGF-1; Promega). Mice used were: wild-type, GR$^{dim/dim}$ (Reichardt *et al*., 1998), GR$^{-/-}$ (Cole *et al*., 1995), EpoR_H, EpoR_{HM} (Zang *et al*., 2001), and Stat5ab$^{-/-}$ (Teglund *et al*., 1998). Cell numbers and size distributions were determined using an electronic cell counter (CASY-1; Schärfe-System). Immortal mouse erythroblasts (clone I/11; p53-deficient) were cultivated as described (Dolznig *et al*., 2001).

Generation of erythroid progenitors from immature (multipotent) progenitors

Fetal livers of E12.5 mouse embryos from GR$^{dim/dim}$, GR$^{-/-}$, EpoR_H, EpoR_{HM}, and Stat5ab$^{-/-}$ mice were seeded at 4×10^6 cells/ml in 'SCM' medium, consisting of StemPro-34™ supplemented with SCF (100 ng/ml), IL-3 (2 ng/ml), IL-6 (0.5 ng/ml), Flk2/Flt3 ligand (10 ng/ml), GM-CSF (3 ng/ml), and IGF-1 (40 ng/ml; all from R&D), and 10^{-6} M Dex. Cultures were maintained between 4–7 $\times 10^6$ cells/ml by daily partial medium changes. Cells were purified by centrifugation through Ficoll (lymphocyte separation medium, 1.078 g/ml; Eurobio) when containing >20% of dead and/or differentiated cells.

To induce erythroid commitment, cells were transferred to 'erythroid proliferation medium' (2×10^6 cells/ml). After a lag phase of 1–2 days, exponential growth was re-established. Differentiating/dead cells were removed by Ficoll purification at day 4. After 6–7 days, cultures were subjected to cytofluorometry and cytospin analysis. Cell populations showing continuous proliferation and a clear erythroblast phenotype (>90% CD117^{high}, CD71^{high}, Ter119^{low}; Mac-1-, GR-1-, CD34-, and Sca-1-negative; see Figure 1d) were selected for further experiments.

Differentiation induction

Terminal differentiation in serum-containing or serum-free media supplemented with 10 U/ml Epo, insulin (10 ng/ml, Actrapid-HM), and the glucocorticoid-antagonist ZK112993 (3 μM) (Mikulits *et al*., 1995) was performed as described (von Lindern *et al*., 2001; Dolznig *et al*., 2005). Where indicated, differentiation was carried out in fully defined medium (Dolznig *et al*., 2002). Cell numbers, cell sizes, hemoglobin contents, and cytospin analyses were analysed as described (von Lindern *et al*., 2001). Quantitation of cytospins for immature, partially mature, mature and apoptotic erythroid cells was carried out as described (Kolbus *et al*., 2002).

Flow cytometry

Cells were stained with fluorescence-labeled antibodies (all PharMingen), against murine Ter119 (PE-labeled), Sca-1 (PE-labeled), GR-1 (PE-labeled), CD71 (PE-labeled), CD45R/B220 (APC-conjugated), CD135/Flk-2/Flt-3 (PE-labeled), CD11b/Mac-1 (PE-labeled), CD117/c-Kit (FITC-conjugated),

and CD34 (FITC-conjugated). Fluorescence-labeled antibodies against murine IgG were used as negative control. Surface marker expression was quantified by cytofluorometry (FacsScan, Becton Dickinson).

Retroviral infections
For infection of $p53^{-/-}$ (I/11) and primary erythroblasts from wild-type and $Stat5^{-/-}$ mice, a bicistronic retroviral vector (pMCSV) expressing human Bcl-X_L (Dumon *et al*., 1999) or murine Stat5ΔC (Moriggl *et al*., 1996), coupled to GFP via an IRES sequence, was used (Kieslinger *et al*., 2000). Infection efficiencies were determined by flow cytometry three days after infection. Stat5ΔC-transduced cells were subjected to FACS sorting (FACS-Vantage, Becton Dickinson).

Cytokine stimulation of erythroblasts
Immortal (I/11) as well as primary erythroblasts from wild type and mutant mice were incubated at 20×10^6 cells/ml in plain DMEM for 4 h at 37°C. Cells were then restimulated in StemPro34™ medium containing 10-fold-concentrated cytokines (1 μg/ml SCF, 20 U/ml Epo, or SCF + Epo) for 1 (mRNA-) or 3 h (protein analysis) and frozen in liquid nitrogen until further analysis.

Western blot analysis
Frozen cell pellets were lysed and subjected to SDS PAGE and Western Blot analysis, using antibodies against murine Bcl-X_L (Becton Dickinson), Stat5ab (Santa Cruz), tyrosine-phosphorylated Stat5ab (Upstate Biotechnology), Stat3 (Becton Dickinson), tyrosine-phosphorylated Stat3 (Cell Signaling Technologies), Stat1 (Santa Cruz), tyrosine-phosphorylated Stat1 (Cell Signaling), Erk1/2 (Sigma) and eIF4E (Cell Signaling).

RNA extraction and Northern analysis
Total RNA was prepared from $1–4 \times 10^7$ cytokine-stimulated or nonstimulated cells using Trizol (Sigma) and processed for Northern blot analysis using 10 μg of RNA per sample and labeled probes for murine bcl-X_L- or murine *cis* cDNA. Equal loading was confirmed by hybridizing with murine GAPDH cDNA (not shown).

Stat5/DNA bandshift analysis
Nuclear extracts from differentiating I/11 erythroblasts were incubated with 32p–labeled double-stranded DNA-oligonucleotides representing the Stat5 binding-site in the bcl-X_L-promoter (Socolovsky *et al*., 1999) or the Stat5 binding site in the beta-casein promoter (Moriggl *et al*., 1996) and subjected to electrophoretic mobility-shift assays (EMSAs) (Kieslinger *et al*., 2000).

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Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc)

3.2 Review articles

3.2.1 The different functions of Stat5 and chromatin alteration through Stat5 proteins

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Stat5 oligomerisation and cytoplasmic function

The different functions of Stat5 and chromatin alteration through Stat5 proteins

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1. ABSTRACT

Stat5 proteins modulate gene transcription upon cytokine- and growth factor action. Stat5a and Stat5b proteins alone are weak activators of transcription. They can modify chromatin organization through oligomerization and they act predominantly in co-operation and interaction with other proteins. The conservative view of exclusively nuclear functions of Stat5 was challenged by the observation of additional Stat5 effects in the cytoplasm, resulting in activation of the PI3K-Akt pathway. We summarize biological consequences of mutations in conserved domains of Stat5 or of deletions in the N- or C-terminal domains with impact on target gene transcription. Formation of higher-order oligomers is dramatically changed upon amino- or carboxyterminal deletions in Stat5 proteins. Mutations in or deletion of the Stat5 N-terminus leads to diminished leukemogenic potential of oncogenic Stat5, probably due to the inability to form Stat5 tetramers. The Stat5 N-terminal domain prevents persistent activation and can act as a DNA-docking platform for the glucocorticoid receptor (GR). The corresponding protocols should facilitate follow-up studies on Stat5 proteins and their contribution to normal physiological versus pathological processes through differential chromatin binding.

2. STAT5 ISOFORMS: THEIR ACTIVATION AND INVOLVEMENT IN DISEASE

Stat5 proteins play crucial roles in controlling physiological processes like hematopoiesis or hepatocyte function. Yet, unrestricted Stat5 activation leads to pathological conditions such as cancer promotion and -progression, myelo-proliferative diseases, inflammation, or auto-immunity. Too little Stat5 activity, however, also can cause diseases such as myeloid hypoplasia (anemia, thrombocytopenia), dwarfism, infertility, immuno-deficiency or metabolic syndromes (1-14). Thus, Stat5 activity needs to be tightly controlled, a demanding task given the broad spectrum of Stat5 functions in various parts of the organism. How and where Stat5 proteins exert their function and which post-translational modifications of Stat5 are actually necessary for proper function is still largely unknown, not least because other proteins interacting with Stat5 were not sufficiently taken into consideration until now.

Stat5a and Stat5b transcription factors are rapidly activated after stimulation of cells with different cytokines or growth factors in conjunction with the corresponding receptors. Subsequently, signals are transduced mainly through Jak1, Jak2 and Jak3 tyrosine kinases, which leads

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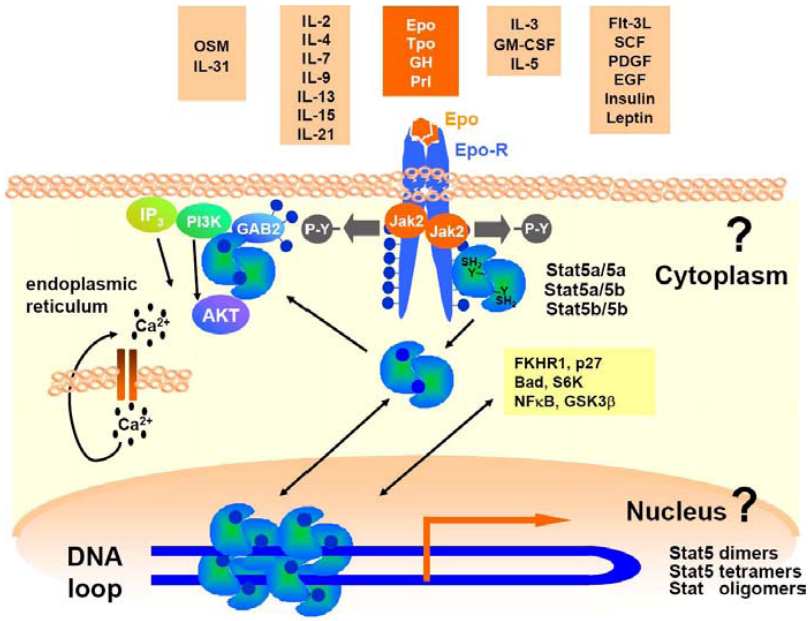

Figure 1. Cytokines, hormones or growth factors that activate Stat5. Cytokines can be grouped based on structural homologies, similar signal transduction pathways and shared receptor chains. The specific example shown refers to definitive erythropoiesis. Self renewal and differentiation of erythroid cells requires functional Epo signaling through its receptor EpoR and Jak2, leading to tyrosine-phosphorylated, transcriptionally active Stat5a and Stat5b proteins. Stat5 molecules involved were found to be dimers and to be attached to the receptor via the N-terminus prior to tyrosine phosphorylation (38). Efficient nuclear translocation and transcriptional activation of Stat5 requires tyrosine phosphorylated Stat5 proteins. Unrestrained Stat5 activation is found in the presence of many different transforming tyrosine kinases, which can contribute to diseases such as cancer. In contrast, too little Stat5 activation causes diseases like anemia. Recently, we found that Stat5 molecules also have an important function in the cytoplasm to activate the PI3K-Akt pathway. Stat5 molecules can form dimers, homo- or hetero-tetramers on DNA (so called oligomers), which can induce the formation of DNA loop structures.

to efficient nuclear translocation of Stat5 molecules (Figure 1). Stat5a and Stat5b proteins can form homo- or heterodimers. In addition, multiple shorter isoforms of Stat5a and Stat5b exist. Whether the latter arise from splicing, proteolytic processing or alternative start codons (Figure 1) is controversial (7, 15-20). Finally, Stat5 proteins are modified by post-translational modifications such as glycosylation, ubiquitinylation, serine/threonine phosphorylation and alternative tyrosine phosphorylation (18-22). Thus, Stat5 proteins can be found in many different homo- or heterodimeric complexes regulating differential gene expression. Overall, it is economical for different cell types to utilize these ancient transcription factors (homologues in dictyostelium or drosophila exist) to modulate the transcriptome in response to a variety of cytokine and growth factor challenges (Figure 1).

The observation that Stat5 proteins are prime targets of transforming tyrosine kinases in cancers was not unexpected, yet a recent surprise was that Stat5 proteins are essential to render malignant cells largely independent of external proliferation stimuli (Figure 1). The activation of Stat5 proteins by tyrosine kinases enables cancer cells to overcome cell cycle control and to survive within the cancer microenvironment. Many questions about the "the role of Stat5 in cancer" remain unanswered. It is unknown whether Stat5 activation in cancer contributes to immune escape, vascularization, metastasis or immortalization. Such roles were reported for activated Stat3, but in many cell types the functions of Stat3 and Stat5 proteins are distinct (23, 24). In general, it is well established that Stat5 plays an essential role in leukemogenesis. Whether it has a broader role in the emergence of solid tumors is not well studied. Data on the role of Stat5 in breast cancer are controversial (25-27), but lymphomas, liver cancer, prostate cancer, lung cancer, ovarian cancer or head and neck cancer were shown to require Stat5 proteins (28-33). Stat5 proteins are also implicated in infectious diseases, immune cell homeostasis or autoimmunity.

In general, proper function of Stat5 molecules is essential for all immune cells and controls e.g. lymphocyte development, NK cell activity, cytotoxic T cell function, T helper or suppressor/regulatory T cell function, mast cell-, platelet/megakaryocyte-, and macrophage responses or stress erythropoiesis (2, 5, 6, 14, 34-39). Most hematopoietic cytokines and growth factors signal through Stat5. In addition, cytokines and growth factors acting on epithelial and mesenchymal cell types as well as oncogenic tyrosine kinases can activate Stat5 (Figure 1). Thus, it is

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important to understand how Stat5 proteins are activated and how they modulate signal transduction, also under conditions when they are found to execute their function in the cytoplasm.

Recent work of several laboratories has demonstrated that activation of phosphoinositol-3-kinase (PI3K) and of the Akt pathway is induced by protein-protein interaction of Stat5 with the scaffold protein Gab2 (40, 41). Interestingly, Gab2- or Stat5-deficient fetal liver cells have both hematopoietic deficiencies due to diminished cytokine responses, although the phenotype of Gab2-deficiency is milder than in Stat5-deficiency (7, 42-44). This underlines the notion that Stat5 proteins have additional functions in the cytoplasm and are not mere nuclear transcription factors (40, 41).

Moreover, new data have shown that Stat5 regulates Akt activity in T cells, eventually involving also late or indirect transcriptional effects resulting in Akt activation (45, 46). It is currently unknown whether in leukemic cells Stat5 interacts directly with Gab2 (e.g. in primary CML cells, where endogenous Stat5 proteins are tyrosine phosphorylated and predominantly localized to the cytoplasm) to recruit the p85 subunit of PI3K, but tyrosine phosphorylation of Stat5 proteins is a prerequisite (41). Still, whether cytokine activated Stat5 or oncogenic Stat5 can activate Akt directly through a Gab2-p85-Stat5 interaction or indirectly through transcriptional effects remains controversial (40, 41, 46). It is possible that different mechanisms are involved in a cell type specific manner. In any case, Stat5 activation and tyrosine phosphorylation bridge to Akt activation. The two alternatives do not exclude each other. Direct protein-protein interaction or indirect transcriptional regulation might have the same result or target, both leading to enhanced Akt activity. In the case of IL-7-induced Glut1 induction through Stat5-Akt (46), IL-7 is very potent in activating Stat3 besides Stat5. Whether Stat3 also regulates Akt expression via a transcriptional mechanism was not tested and is questionable. In summary, oncogenic P-Y-Stat5 (as in CML patients or in mice transplanted with hematopoietic cells carrying oncogenic Stat5) is clearly cytoplasmically localized and very efficient in activating Akt through direct protein-protein interaction with Gab2 and p85 (40).

The ratio of nuclear versus cytoplasmic Stat3 protein is influenced by transforming tyrosine kinases (47), but the mechanism of nuclear-cytoplasmic shuttling of Stat5 in different cell types remains enigmatic (48). No conclusion can currently be drawn from the observation that activated Stat5 (P-Y-Stat5) is often found in the cytoplasm of transformed myeloid cells from CML or AML samples (40). We have extended these studies with additional patient samples and found that survival of myeloid cancer cells depends on the Stat5-PI3K-Akt axis (Gouilleux, Moriggl *et al.*, manuscript submitted).

One further hallmark of AML is the occurrence of an oncogenic variant of the FLT3 (FLT^{mut}) growth factor receptor, which leads to nuclear activation of Stat5 (49). Recent studies underscored the importance of Stat5 in FLT^{mut}-driven leukemogenesis via binding and activation of Rac1, an essential component of the NAPDH-oxidase. The latter was crucial for generation of endogenous reactive oxygen species (ROS), leading to genomic instability and increased DNA damage and thus might account for the observed poor prognosis of AML patients harboring $FLT3^{mut}$ receptors (50).

Cytokine and growth factor receptor signaling systems activate a large variety of downstream signaling molecules such as kinases, phosphatases, transcription factors or negative feedback regulators (e.g. SOCS/PIAS/STAM proteins). Stat5 transcription factors are highly and broadly expressed proteins found in these cellular signaling pathways. Expression levels of Stat5 proteins vary from cell type to cell type but can reach an abundance of 3,000-100,000 molecules per cell (51). Quantification of Stat5 proteins will be important in future studies and repression of Stat5 gene expression could be relevant in diseases.

In that context it is also important to mention that only a fraction of a Stat protein pool gets tyrosine phosphorylated (18, 52). ^{35}S-labeled cell extracts and pulse chase experiments with Stat1 and Stat5b revealed approximately 10-25% of the total Stat1 or Stat5b pool to be tyrosine phosphorylated upon cytokine action (18, 52). How many Stat molecules are activated might be cytokine and cell type specific, but quantitative studies even from cell lines are limited. P-Y-Stat proteins are recycled through tyrosine dephosphorylation (52), in contrast to the Jak kinase or cytokine receptor chains which are degraded. Recently, ubiquitination and proteasome-dependent degradation was postulated as an additional mechanism for inactivating Stat5a. Interestingly, nuclear Stat5a was mainly inactivated via ubiquitination and protein degradation whereas cytoplasmic Stat5a was dephosphorylated by abundantly available tyrosine phosphatases. This differential inactivation mechanism might argue for a distinct function of unphosphorylated nuclear Stat5a, as phosphorylation of nuclear Stat5a was not required for ubiquitination (53). Recently, Stat5a was also shown to be epigenetically silenced in NPM-ALK lymphomas (54). Usually, the limiting component during cytokine and growth factor receptor response (and even in transformed tyrosine kinase signaling) is the moderately expressed tyrosine kinase. There are exceptions to this rule, however, since BCR-ABL was calculated to be in a range of up to 100.000 molecules in K562 cells, a cell line derived from a human CML patient.

It should be emphasized again that Stat5 transcription factors are not only at the end of signaling cascades (40, 45, 46). Stat5 proteins are highly expressed signaling molecules with docking functions even when not activated. Cytoplasmic functions were also recognized for Stat1 and, as already mentioned, for Stat3 (55-57).

3. PHENOTYPES OF MOUSE MODELS WITH FULL OR PARTIAL DELETION OF STAT5

In the following we will provide an overview on "old" and "new" Stat5 knock out mouse models that have

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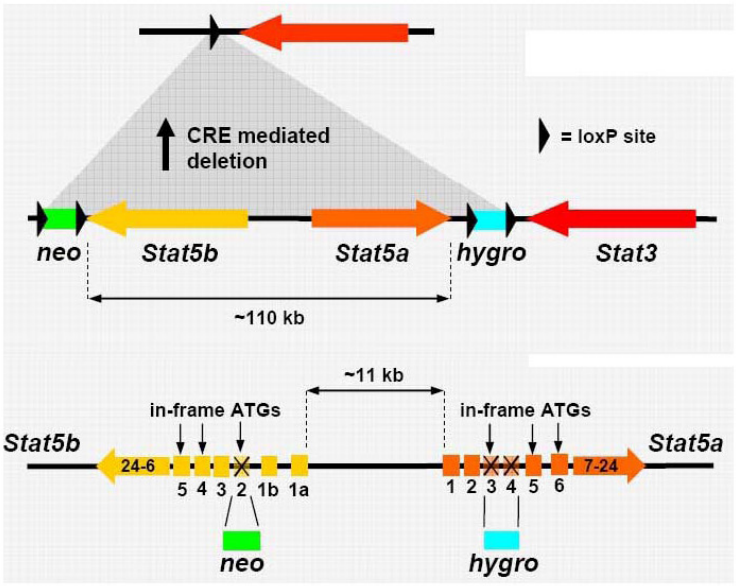

Figure 2. Chromosomal organization of the *Stat5* locus and strategies to generate knockout mouse models. Targeting strategies for complete or incomplete *Stat5a/b* deletion. The description of the different Stat5 mouse systems are originating from the following studies: (i) Complete gene deletion (s) of Stat5a, Stat5b or both: Gene deletion of Stat5a (8); Gene deletion of Stat5b (13); Gene deletion of Stat5a and Stat5b (1); or (ii) incomplete gene deletion (s): N-terminal gene deletion of Stat5a, N-terminal gene deletion of Stat5b and N-terminal gene deletions of Stat5a and Stat5b (12); Graphic representation of targeting strategies to generate single deletions of *Stat5a* or *Stat5b* is omitted, since the focus here is on the consequences of deleting both isoforms. Top panel: reproduced with permission from 1. Bottom panel" reproduced with permission from 12.

either no Stat5 proteins expressed (new) or that have a deletion in the Stat5-N terminus (old) (Figure 2). It is well understood that Stat5 molecules control a multitude of functions. The current picture is incomplete due to the multitude of Stat5 activities and due to the lack of more advanced mouse models (e.g. knock-in models). A further level of complexity originates from the broad expression (16) and activation pattern (58) of Stat5 proteins and their different specific functions in mammals. Here, we will not elaborate on studies targeting one single Stat5 isoform for deletion (8, 13), and focus instead on reports describing biological consequences of deleting both isoforms.

Old and new mouse models for Stat5a and Stat5b deletion display overlapping as well as distinct phenotypes. The generation of murine knock-out models for Stat5 proteins was complicated by the fact that the *Stat5a-Stat5b* locus spans only approximately 110 kb on chromosome 11 (in humans, the *Stat5* genes are localized in identical configuration on chromosome 17; Figure 2). The original Stat5 knock out mouse, now referred to as $Stat5^{\Delta N}$, was generated via double targeting ES cells at two different loci on chromosome 11. Neomycin- and hygromycin-resistance cassettes were inserted into the first coding exon of the *Stat5a* and *Stat5b* gene, respectively (Figure 2) (12). The resulting $Stat5^{\Delta N}$ mice were viable and were believed to represent true knock outs although they expressed N-terminally truncated Stat5 proteins at high levels. Surprisingly, N-terminally truncated Stat5a and Stat5b molecules were also found *in vivo* in wild type animals (7).

Many studies were based on the $Stat5^{\Delta N}$ mouse model (2-6, 9-12, 14). Today, these mice are regarded and described as a model to study functions of the N-terminal domain, particularly in the liver and in lymphocytes (3, 5, 14, 59). Apparently, the Stat5 N-terminus constitutes an important protein-protein interaction domain (Figure 3 to 4). $Stat5^{\Delta N}$ mRNA harbors intact Kozak sequences and in-frame start codons at amino acid position 103 or 137 of murine Stat5a or Stat5b (Figure 3). According to observed migration patterns in Western blots or in DNA binding assays, most likely the ATG at position 137 is predominantly used (3, 5, 7, 59). Amino acid M_{137} is conserved in all mammalian Stat5 proteins, in contrast to M_{103}, which is lacking in human Stat5a (Figure 4). $Stat5^{\Delta N}$ mice express both N-terminally truncated Stat5a and Stat5b molecules (2, 3, 5, 7, 14, 59). Interestingly and in contrast to endogenous Stat5, $Stat5^{\Delta N}$ proteins have lost the ability to physically interact with the glucocorticoid receptor (3, 60). It is currently questionable which parts of the phenotypes described in $Stat5^{\Delta N}$ mice are due to loss of Stat5-GR protein-protein interaction. Moreover, N-terminal truncation of Stat5a, but not of Stat5b renders the molecule persistently active (3, 61). This underlines a regulatory role of the N-terminus of Stat5a to prevent persistent tyrosine phosphorylation.

The complete knock out of Stat5a and Stat5b ($Stat5^{Null}$), was created in the laboratory of Lothar Henninghausen, who already had designed the complete knock-out of Stat5a (1, 62). The Cre-loxP technology used

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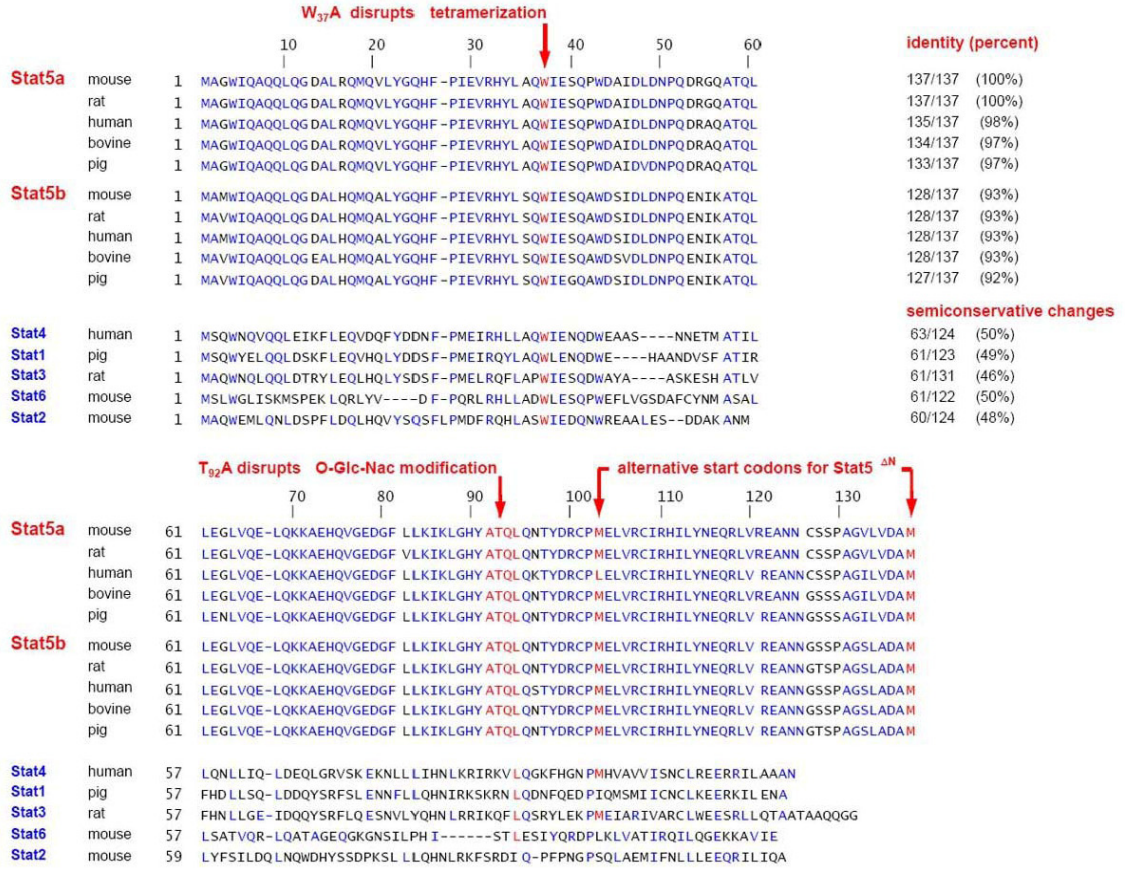

Figure 3. Identity and semi conservative amino acid changes. The N-termini of different Stat family member were compared. NCBI protein blast was used for similarity searches. Conserved amino acids in Stat5 N-termini are highlighted in bold. Mammalian species with closest homology to mouse Stat5a and Stat5b were used for comparison. Rodent, pig, bovine and human sequences were chosen, while ovine Stat5a was excluded from analysis. Ovine Stat5b was so far not completely sequenced and ovine Stat5a displayed significant differences to other mammalian Stat5 sequences in a stretch of 17 amino acids in the N-terminus. The amino acid motif containing the O-Glc-NAc modification at T_{92} is underlined. Moreover, the two alternative start codons M_{103} and M_{137} are underlined.

allowed deletion of the entire 110kb locus either in the germ line or via conditional deletion (Figure 3) (1, 59). Genetic complementation with a retrovirus encoding wild type Stat5a into the $Stat5^{Null}$ background restored critical functions, indicating that genome integrity was preserved despite the large deletion on chromosome 11 (Figure 2). Stat5 deletion during embryonic development was lethal, particularly on a pure C57Bl/6 or Balb/c genetic background. Surprisingly, mixed Sv129 x C57Bl/6 F1 mice developed to term, but over 97% died perinatally (1, 5, 59) . Recent data suggest that lethality in Stat5-deficient animals is due to anemia resulting from defects in iron metabolism of fetal erythroid cells and the ensuing elevated levels of apoptosis (Kerenyi *et al.*, manuscript submitted). Complete deletion of both Stat5 genes can result in activation of other Stat family members, a general phenomenon in Jak-Stat-SOCS gene deletion studies, complicating the interpretation of phenotypes (63).

Numerous conditional Stat5 knock out mouse models have already been created. To start with, Stat5 was deleted specifically in mammary epithelial cells (WAP-Cre) or ablated in a broader range of epithelial cell types (MMTV-Cre) (1), B-cells (CD19-Cre) (64), T lymphocytes (efficient recombination from CD4/CD8 double-positive cells via Lck-Cre; (5) less efficient in the $CD4^+$ compartment with CD4-Cre; (59)). In addition, Stat5 was deleted from hepatocytes plus biliary epithelial cells using albumin-alpha-fetoprotein-Cre (3), while liver-restricted deletion was achieved with Albumin-Cre (65), although less efficiently. Moreover, pancreatic beta-cells and hypothalamus (Rip-Cre) (66), endocrine and exocrine pancreas cells (Pdx1-Cre) (66), as well as skeletal-muscle (Myf5-Cre) (67) were targeted. Finally, transplant models using $Stat5^{Null}$ cells and the analysis of $Stat5^{Null}$ survivors unraveled new phenotypes in B- and T lymphocytes (5, 14, 59).

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From all studies listed above, it is clear that various cell types critically depend on Stat5 proteins for proliferation, differentiation or survival. Important functions in hematopoietic lineages were appreciated already for a long time but also mammary gland, ovary, prostate, testis, liver, bone or fat tissues depend on Stat5 proteins for full specification. Referring to hematopoiesis, already the repopulation capacity of hematopoietic stem cells depends to a large extent on Stat5 (7, 43, 44, 59). In more differentiated hematopoietic compartments, Stat5 is important for proper development of T-, NK- or B cells: The generation of $CD8^+$ T cells, gamma/delta T cells, regulatory T cells, but also T helper cells depends on Stat5 proteins (5, 14, 34, 59). Similarly, NK cell number and biological activity depend on Stat5 (35, 36, 39, 68), also B lymphocyte development and transformation are closely connected to Stat5 (5, 64). Moreover, Stat5 functions obviously are involved in definitive and stress erythropoiesis (2, 69), monocyte maturation and granulopoiesis (70), as well as activities of macrophages, eosinophilic granulocytes or mast cells (6, 71, 72). It is therefore not surprising that expression of oncogenic Stat5 variants or overexpression of Stat5 can lead to the development of leukemias, lymphomas or myelo-proliferative diseases (9, 28, 33, 61, 104).

4. STAT5 GAIN-OF-FUNCTION APPROACHES

Persistent Stat5 activation is known to be crucial for the persistent proliferation of various cancer cell types. Indeed, activation of Stat5 proteins is associated with the most frequently encountered transforming tyrosine kinases. The list of such persistently active kinase mutants or variants includes prominent examples like v-Abl, Jak2 ($V_{617}F$), Jak3 ($A_{572}V$), c-Kit ($D_{816}V$), Flt-3-ITD, v-ErbB or persistently activated EGFR. Another group of kinases aberrantly activating Stat5 is generated by chromosomal translocation events, exemplified by BCR-ABL, p185 and p210, TEL-ABL, TEL-PDGF, TEL-JAK2, TEL-Jak3, EML1-ABL or ZNF198-FGFR1. Accordingly, the first successful attempt to create a persistently active Stat5 gain-of-function mutant was the generation of a Stat5-tyrosine kinase fusion chimera, in which the Jak2 kinase domain was fused to the C-terminus of Stat5a, together with a strong transactivation domain in between (73). Transgenic mice expressing this construct developed breast cancer (25).

So far, only a limited number of studies addressed the important question whether Stat5 proteins are essential for transformation. So far, the requirement for the presence of Stat5 to induce transformation could be genetically proven only for BCR-ABL (5). Recently, we were able to show that a gain-of-function mutant of Stat5 was able to largely complement Jak2- or erythropoietin receptor deficiency during erythro- and myelopoiesis (69). The current view that too much Stat5 activity can indeed result in transformation of hematopoietic progenitors is best illustrated by the ability of one persistently activated Stat5 molecule to substitute for exogenous cytokine signaling and to cause multi-lineage leukemia. This well studied oncogenic variant is a point mutant of Stat5a, harboring a serine-to-phenylalanine mutation in the C-terminal domain, termed $cS5^F$ (or $S_{710}F$; Figure 3). Upon transduction of $cS5^F$ into wild type hematopoietic stem cells and subsequent transplantation into lethally irradiated wild type mice, leukemia develops within 4 weeks (40, 61).

The predecessor of $cS5^F$ had been another Stat5 mutant, designated caStat5a1*6, containing two independent mutations, $S_{710}F$ plus $H_{299}R$ in the DNA binding domain (74). This variant had originally been found in a screen for factor-independent proliferation of Ba/F3 cells, with which a PCR mutagenesis on Stat5 had been performed. CaStat5a1*6 conferred IL-3 independent growth to Ba/F3 cells. Both, the double mutant and the single point mutant $cS5^F$ used in our studies do not differ significantly in their biochemical properties: (i) They are constitutively activated through persistent tyrosine-phosphorylation, even after cytokine starvation. (ii) Both display hyper-activation upon cytokine stimulation and (iii) enhanced DNA binding (61). Thus, CaStat5a1*6 and $cS5^F$ could be termed "constitutively active" as well as "hyper-activatable". There is, however, one important difference: In contrast to $cS5^F$, caStat5a1*6 was unable to genetically complement $Stat5^{null}$- or $Stat5^{\Delta N}$ cells (61) (and data not shown), requiring full length, endogenous Stat5 proteins to exert its transforming function. In contrast, $cS5^F$ transformed $Stat5^{\Delta N}$ cells (61) as well as $Stat5^{null}$ hematopoietic cells, causing multi-lineage leukaemia upon transplantation of $cS5^F$-expressing $Stat5^{null}$ or $Stat5^{\Delta N}$ fetal liver-derived hematopoietic cells into irradiated mice (unpublished data). Moreover, we observed that $cS5^F$ was capable of transforming primary mast cells from Stat5-deficient animals. The mutant was also able to activate Stat5-reporter gene constructs in Cos7 cells which do not express any endogenous Stat5 (unpublished data). Together, these observations argue that $cS5^F$ does not require endogenous Stat5 protein and is structurally and functionally related to endogenous Stat5 since it genetically complements Stat5-deficiencies.

Mutations equivalent to caStat5a1*6 were also introduced into Stat5b to generate gain-of-function variants for the study of lymphopoiesis in corresponding transgenic mice (34, 39). Another screen for persistently active Stat5 mutants identified a point mutant in the SH_2 domain of Stat5a ($N_{642}H$), which is also a residue conserved in Stat5b. Since Stat5b is the only isoform expressed at significant levels in the liver, Stat5b ($N_{642}H$) was used for *in vivo* studies with hepatocytes (75).

In summary, it is recommendable to use the $cS5^F$ gain-of-function mutant rather than the double mutant caStat5a1*6, since $cS5^F$ was able to complement Stat5-, Jak2- or EpoR-deficiencies (61, 69). Contrarily, caStat5a1*6 required endogenous Stat5 for gain-of-function effects. Whether Stat5a ($N_{642}H$), Stat5b ($N_{642}H$) or the Stat5-tyrosine kinase fusion chimera can complement Stat5 deficiency is currently unknown. One transgenic mouse models with $cS5^F$ was reported, which resulted in B-cell lymphomas in absence of p53 (104).

Tyrosine phosphorylation, DNA binding activity and the C-terminal transactivation domain were essential

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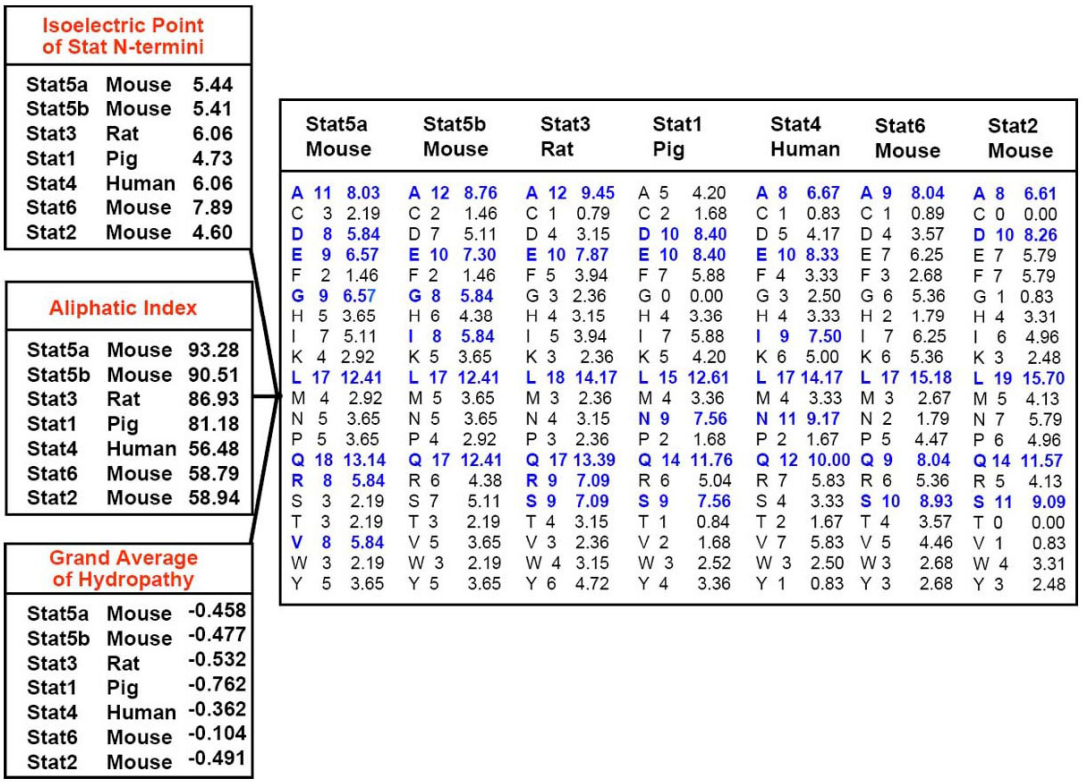

Figure 4. Amino acid composition and biochemical protein parameters. Protein characterization tools were used from Swiss-Prot/TrEMBL public databases to highlight distinction criteria of the N-termini of different Stat proteins. Specifically, the ProtParam program was used as a protein identification and analysis tool for the calculation of isoelectric points, aliphatic indices, grand averages of hydropathy and amino acid composition (103). The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). The GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the entire sequence. The amino acid composition is distinct among the different Stat N-termini. Amino acids with higher appearance of n=7 residues within the N-terminal domain are highlighted in bold and underlined.

for the ability of $cS5^F$ to substitute for myeloid growth factors or cytokines (69) (and Moriggl, unpublished data). We worked extensively with the $cS5^F$ oncogene to study transforming characteristics in immature and mature hematopoietic cell types (40, 69). Extracts of $cS5^F$ expressing cells displayed enhanced Stat5 chromatin binding activity. In addition, cytoplasmic $cS5^F$ efficiently activated the PI3K-Akt pathway. This led to the speculation that both, nuclear and cytoplasmic functions of $cS5^F$, might be required for full transforming ability. Moreover, $cS5^F$ relieved cytokine dependence and prolonged the duration of Stat5 signaling in response to growth factors or cytokines (40, 61, 69). Interestingly, the constitutively active caStat5a1*6 mutant promoted senescence in fibroblasts, similar to oncogenic Ras (76, 77). It is questionable whether the more physiologic $cS5^F$ mutant can promote senescence similarly. Knock in and inducible transgenic mouse models harboring the $cS5^F$ mutation are currently established to gain further insights into persistent cytokine and growth factor signaling through oncogenic Stat5 variants. These studies in immuno-competent adult transgenic mice will be important to illuminate how persistent Stat5 protein activation can lead to development and progression of cancers.

5. STAT5 PROTEIN-PROTEIN INTERACTIONS WITH FOCUS ON THE STAT5 N-TERMINUS

The changes in chromatin structure upon binding of Stat5 proteins are induced by the N-terminal tetramerization domain, whereas the C-terminal region is responsible for transactivation and also regulates P-Y-Stat5 turnover (Figure 5) (17, 18). For protein-protein interactions, both, the N- and C-terminal domains are of particular importance. These interactions influence signal transduction and transcription (Figure 5). Putative protein binding sites with NCoA/SRC, the TUDOR domain coactivators p100 and CBP/p300 cofactors were both mapped to the N- and/or C-terminal domains of Stat5 (78, 79). The N-terminal region contains a secondary

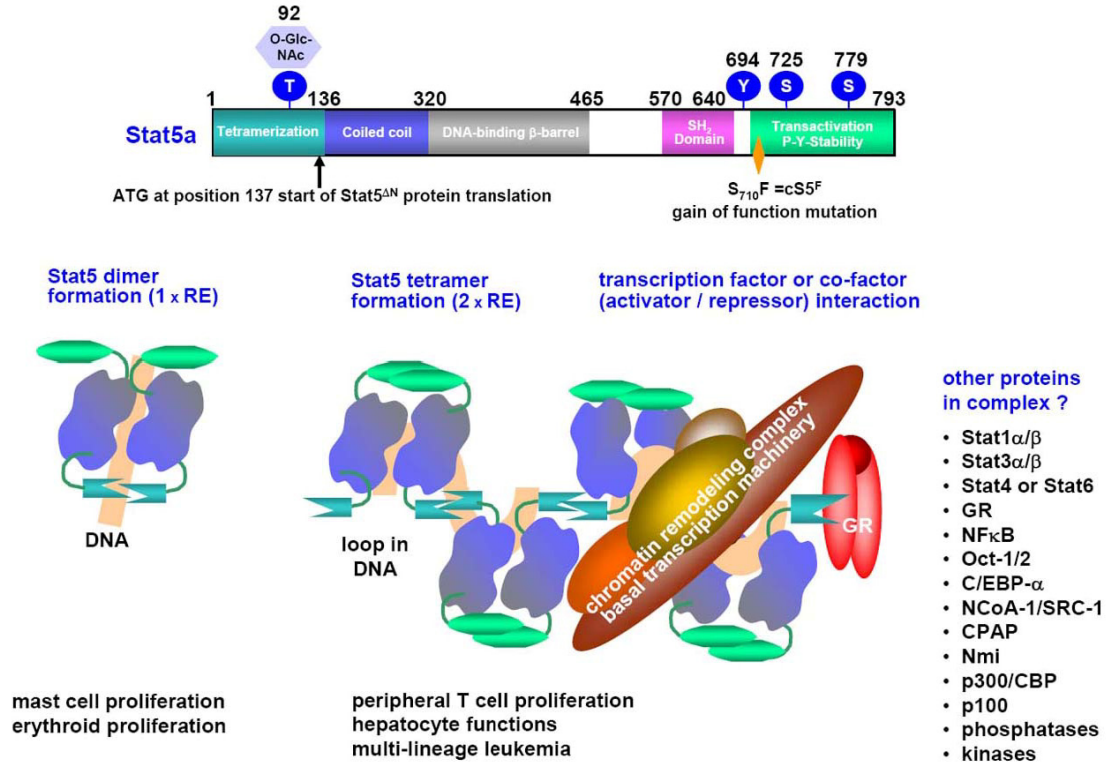

Figure 5. Stat5 domains and the $cS5^F$ gain-of-function mutant with a model for oligomerization. Diagram based on solved Stat structures (upper part) and models of oligomer formation (lower half). Structural data are available for Stat1 (87, 89, 92), Stat3 (93), Stat4 (88, 94, 95) and Stat5a (86). DNA loop formation by Stat5 tetramers is catalyzed by the N-terminal domains of Stat5 molecules. Stat5 tetramer formation is involved in regulation of Stat5 target gene expression in normal, cytokine and/or growth factor responsive cells, but also in transformation processes (61). The association and formation of Stat5 complexes with cofactors, co-repressors or other transcription factors is critical in the decision which genes are specifically activated or repressed. Multiple other proteins interacting with Stat5a and/or Stat5b on DNA were described as illustrated to the lower right.

modification at threonine 92 (O-Glc-NAc glycosylation), which is believed to be crucial for high affinity interaction with CBP/p300 (Figures 3 and 5) (80). The C-terminus of Stat5a and Stat5b is serine/threonine phosphorylated, most likely increasing the acidic blob of the amphiphatic alpha-helical transactivation domain core (17, 18). This core can interact with octamer transcription factors through a short amino acid motif similar to the one found in the octamer cofactors OBF-1 (BOB) and SNAP190 (81).

Additional interaction sites were mapped within the coiled-coil domain, the region regulating Stat5 nuclear versus cytoplasmic localization (48). Yeast-Two-Hybrid screens underlined the importance of the coiled-coil domain in interacting with the corepressor molecule SMRT (82) and the transactivator molecule Nmi (83). The SH_2 domain of Stat5 and its critical tyrosine residue are essential for tyrosine kinase contact, dimer specificity, nuclear translocation and attack by tyrosine phosphatases in conjunction with the carboxyl terminal domain.

In summary, the very N- and C-terminal domains of Stat5 proteins have multiple functions with respect to interaction with other proteins. Both domains are exposed on the surface of Stat5 dimers or tetramers as recognized by native DNA binding assays (EMSA) or antibody supershift experiments (Figure 6) (61, 84). Only antibodies directed against N- or C-terminal epitopes recognize Stat5 in native polyacrylamide gels or are able to immunoprecipitate Stat5 proteins efficiently, irrespective of their activation status (3, 36, 85). The postulated protein surface expression of the N- and C-terminus is in line with protein biology, as the N-terminus usually folds first and the C-terminus folds at the end of protein synthesis.

Compared to other Stat family members, Stat5a and Stat5b N-terminal domains show differences in amino acid composition, limited conservation, different isoelectric points, highest aliphatic indexes and distinct grand average of hydropathy (Figure 3 and 4). Only between Stat5a and Stat5b themselves, there is 93% amino acid conservation at the N-termini (Figure 4), while those of other Stat protein family members show less than 50% conservation (Figure

Stat5 oligomerisation and cytoplasmic function

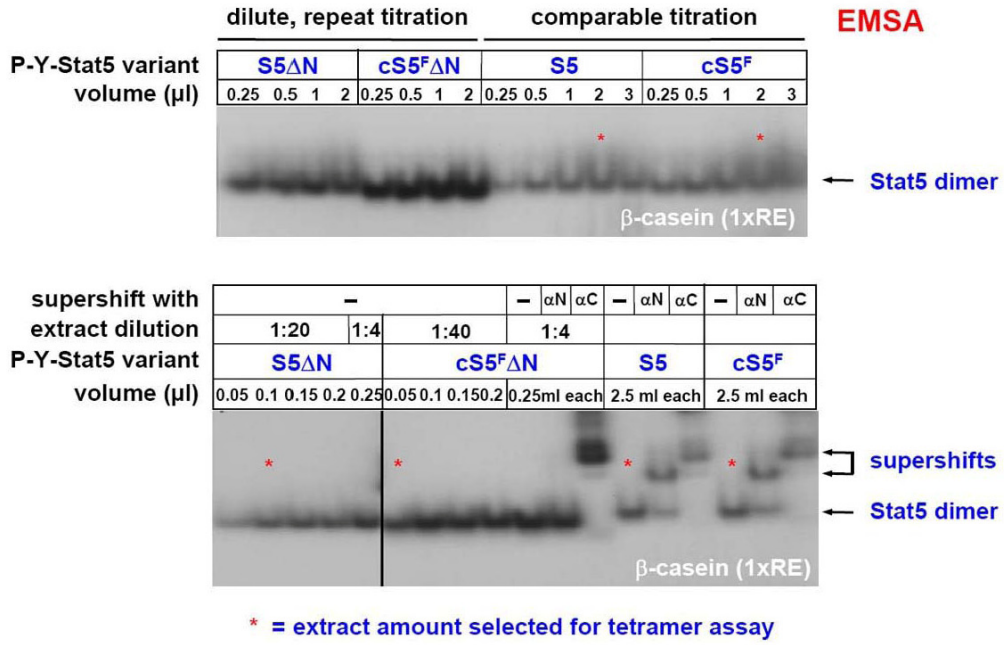

Figure 6. Comparison of Stat5a extracts to test them prior to tetramer stability assays. Dilutions of extracts have to be subjected to Stat5 EMSAs prior to the use of extracts in tetramer assays. After titration, four different volumes of protein extract for the indicated Stat5 variants (61) were used in tetramer assays (*). Equal activities have to be re-checked and loaded in an EMSA using the beta-casein site and Stat5 specific antisera for supershifting against the extreme N- and C-terminus to verify integrity of extracts.

3). Nevertheless, important conserved residues can be delineated throughout the entire N-terminus, in addition to unique amino acid motifs. Homologies among Stat family members with Stat5a and Stat5b N-terminal domains can be found particularly in the sequence of Stat4, followed by Stat1 and Stat3 (Figure 3). Stat1 and Stat4 N-termini were shown to assist in efficient oligomer formation, in contrast to activated Stat3, which we found to be a weak tetramer former when compared to the ability of Stat5 to tetramerize (R. Moriggl and A. Ecker, data not shown). Oligomers for Stat6 and Stat2 were not reported so far and the N terminal domains show only weak homology to the other family members.

Thus, extrapolations from these results onto other Stat family members have to be taken with caution. The Stat5 N-terminus was so far only modeled and not crystallized. So far, only unphosphorylated Stat5 dimers lacking the N- or C-terminus were crystallized (86). The structure of these unphosphorylated $Stat5^{\Delta N\Delta C}$ molecules reveals a high degree of similarity to non-phosphorylated $Stat1^{\Delta N\Delta C}$ dimers (87); In addition, $Stat5^{\Delta N\Delta C}$ also can form antiparallel dimers. This occurs through an interface consisting of the four alpha helix bundles (coiled-coil domain) and the beta-barrel (DNA binding domain) of the dimerization partners (Figure 5) (86). Structures of isolated Stat5a and Stat5b N-termini and their interaction with other proteins in complexes (e.g. with the GR or p300/CBP) are unresolved as yet. Further elucidation of these protein-protein interactions might become especially useful as a way to alter or inhibit Stat5 functions.

The N-terminal oligomerization domain of Stat is also involved in receptor docking and is important for pre-formation of dimers in absence of tyrosine phosphorylation (88, 89) Kinetics of nuclear shuttling of mutants devoid of the Stat5 N-terminus was not studied so far but efficient chromatin binding persisted upon deletion of the Stat5b N-terminus in hepatocytes as measured by CHIP assays *in vivo* (3). Moreover, mutants of Stat5 deficient in tetramerization (mutation of $W_{37}A$ or deletion of the first 136 amino acids) could translocate to the nucleus when cytoplasmic versus nuclear extracts were analyzed (40). Interestingly, deletion of the Stat5a N-terminus led to a constitutively active $Stat5a^{\Delta N}$ dimer, whereas deletion of the Stat5b N-terminus did not (3, 85). Thus, at least the Stat5a N-terminus inhibits auto-activation in the absence of cytokines or growth factors.

Importantly, we recently discovered that the Stat5 N-terminus serves as docking platform for binding of the GR (3), a known cofactor of Stat5 proteins (90, 91). The GR could provide the AF-1 and AF-2 transactivation domains, even when C-terminally truncated Stat5 molecules (lacking their transactivation domain) are bound to DNA (91). The result could be a stable DNA-bound complex of C-terminally truncated Stat5 and GR proteins that can moderately but not fully activate target gene transcription. DNA binding or dimerization of GR proteins

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was not needed for GR-Stat5 protein interaction. This was shown through the use of GR^{dim} mice (knock in mice expressing a mutated GR). The corresponding mutation resides in the second zinc finger domain, resulting in a loss of DNA binding capacity of the GR. GR^{dim} mice have an intact GR-Stat5 interaction and do not suffer from dwarfism, in contrast to $Stat5^{\Delta Hep}$, $GR^{\Delta Hep}$ or $Stat5^{\Delta N}$ mice that display dwarfism due to growth hormone defects (3, 85). From these results we concluded that the GR interacts with Stat5 through the N-terminus in a classic cofactor fashion.

It is evident from previous work and our current analyses of GR- and Stat5-transgenic mouse models that the molecular details of the Stat5-GR interaction are not restricted to a simple linear transcription factor model. A rather complex scenario appears to integrate positive versus negative transcriptional regulation, short- or long-term cytokine and hormone responses, dependent and interdependent signaling pathways in the cytoplasm or the nucleus (Kornfeld, Tuckermann, Friedbichler and Moriggl, unpublished data). Overall, the Stat5-GR axis is more important than previously anticipated and similar surprises might be revealed from closer studies on other Stat5-interacting proteins.

6. SIGNIFICANCE OF STAT5 TETRAMER FORMATION/OLIGOMERIZATION FOR ACTIVATION OF TARGET GENES

Further oligomeric protein-protein interactions can occur through the N-terminal domain of Stat5-containing homo- or hetero-oligomers, also called Stat5 tetramers (Figures 3 to 5). A simplified scheme of Stat5 tetramer formation is depicted in Figure 5, based on structural data of Stat family members. Detailed structural information is available for several Stat isoforms including Stat1 (87, 89, 92), Stat3 (93), Stat4 (88, 94, 95) and Stat5a (86). These 3D structure models have contributed to clarification of the molecular action and complex formation of DNA-bound Stat proteins and the mechanism for oligomerization through their N-termini. These studies were either based on protein crystal structures or 3D modeling, combined with mutational analyses to verify the structural predictions. We would like to mention that the high resolution structure of the crystallized Stat4 N-terminal domain was reinterpreted. Two alternate organizations were suggested but irrespective of this reinterpretation the N-termini apparently form stable protein-protein aggregates, like two interconnecting hooks (88, 94, 95).

Up to now, Stat5 proteins were mainly analyzed as transcription factors bound to a single high affinity DNA binding element, but this does most probably not reflect the situation *in vivo (*Figure 5). Stat5 proteins need to find their target sequences in open chromatin within minutes, where upon binding they modify chromatin structure. As already outlined above, Stat5 proteins do not only function in the nucleus, they also contribute to PI3K-Akt activation in the cytoplasm (Figure 1). Already several years after its discovery, it was recognized that Stat5 can influence the level of target gene expression through oligomers, so called Stat5 tetramers. In this case, two weak or high affinity Stat5 response elements in the promoter allow formation/recruitment of Stat5 tetramers onto DNA. These can even be two very weak Stat5 response elements (unpublished data). Multiple DNA/protein complexes were found upon analyses of patient samples using DNA probes with two Stat5 response elements, so called tetramer sites (61). These complexes contained Stat5 proteins and probably other unknown factors displaying high chromatin-binding affinity of their own.

One reason for the appearance of such diverse Stat5 protein complexes could be that Stat5 proteins from leukemic patient samples often show N- and/or C-terminal deletions. This can be explained by the observation that, in contrast to Stat3, Stat5 proteins are highly susceptible to proteolysis (unpublished data). The complexes from patients migrated differently and distinctly on native gels, as compared to controls. For analysis, patient extracts were first adjusted to equal Stat5 dimer DNA-binding activity and then employed in tetramer assays. The result was a strong enhancement of oligomer formation in leukemic extracts when compared to controls. Thus, it is likely that also other proteins or post-translational processing of Stat5 proteins might play a role in Stat5 oligomer complex formation (61). In summary, leukemic patient samples displayed an increased abundance of Stat5 tetramer complexes, which might not only be attributed to the mere presence of sufficient amounts of Stat5 protein.

A rare phenomenon in the world of transcription factors is the fact that Stat5 proteins can alter chromatin structure through oligomerization to redirect gene transcription upon cytokine action. Oligomerization is driven through the Stat5 N-terminal domain, where the GR and p300/CBP molecules can bind as cofactors. Both, p300/CBP and the GR are known to be strong chromatin modifiers. A significant part of the changes in chromatin structure is likely to be due to Stat5 proteins themselves. Unfortunately, currently no data are available on measurements or visualizations of Stat5 in complexes with these chromatin modifying proteins.

Until some time ago, the DNA binding activity of Stat5 proteins was mainly assessed by *in vitro* DNA binding electromobility shift assays (EMSA), frequently using the beta-casein milk protein gene response element (Figure 6) (17, 96). More recently, chromatin immunoprecipitation (ChIP) experiments using Stat5 proteins were performed in a limited set of cell lines or cell types *in vivo* (3, 58, 85). No global ChIP analysis was reported for Stat5 proteins based on their DNA binding preference so far, which might facilitate the analysis of binding site selection to understand if and where Stat5 proteins bind as dimers or oligomers. Such experiments could be elegantly performed using the different available genetic mouse models, since wild type Stat5, $Stat5^{\Delta N}$ (no tetramers) or conditional $Stat5^{NullCreX}$ mice would be available to study dimers and oligomers versus, as control, the results in the absence of Stat5.

Stat5 proteins were also shown to repress genes in a promoter- and (co)-repressor-dependent manner. Since

not many studies have been conducted on such mechanisms of gene repression by Stat5, we will rather mention a number of the most relevant Stat5 target genes with known and significant functions in the organism. Many Stat5-induced genes have two to four high-affinity binding sites in their promoter.

Prominent direct transcriptional targets of Stat5 (97) include (i) proteins important for cell cycle progression and cellular growth, together with cytokine- and growth factor-receptor signaling components (*IL-2R-alpha*, all three *D-type cyclins*, *c-Myc*, *OSM*, *ALS*, *IGF-1*, *Pim kinases*, *epidermal growth factor receptor*, *prolactin receptor*), (ii) tissue invasion (*MMP-1*, *MMP-3*, *Spi-2.1*), (iii) survival (*A1*, *Mcl-1*, *Bcl-2*, *Bcl-xL*, *survivin*), (iv) negative feedback inhibition in tyrosine kinase signaling pathways (*CIS*, *Socs-1*, *Socs-2*, *Socs-3*), (v) lymphocyte function (*FoxP3*, *CD25*, *TCR-gamma/delta rearrangement region*, *perforin*, *lymphotoxin-alpha*, *Pax5*, *EBF*, *Glut1*), (vi) cofactor regulation (*Cited2*), (vii) liver function (sexual dimorphic proteins like *p450 cytochrome* genes), (viii) major urinary proteins, (ix) ribosomal proteins, (x) acute phase response genes (such as *alpha2-macroglobulin*, *HNF-6*), but also (xi) genes involved in DNA damage repair (*Gadd45-gamma*, *Rad51*), (xii) reproduction or (xiii) mammary gland function and differentiation (*3beta-HSD*, *20alpha-HSD*, *alpha-*, *beta-* and *gamma-casein* genes, *beta-lactoglobulin*, *whey acidic protein*). We would like to close this necessarily rudimentary list of Stat5 target genes in the hope that it can be complemented by the characterization of additional prominent members in the near future.

7. METHODS TO STUDY DNA- AND CHROMATIN-BINDING PROPERTIES OF STAT5

The first two descriptions of higher order complex formation involving Stat proteins were published in 1996, describing the capability of Stat1 and Stat4 to form tetramers via their N-termini. The same was true for higher order oligomers while bound to DNA (92, 98). The first, a technically detailed biochemical study performed in the lab of James Darnell, revealed, how purified Stat1 would bind to DNA (92). Here, for the first time in Jak-Stat research, the migration behavior of Stat tetramer complexes was shown in EMSAs. The second paper, originating in the group of Tim Hoey, recognized differential binding site occupancies through oligomerization of heterologous expression of Stat1, Stat4 or Stat5 DNA-bound complexes, using the interferon-gamma promoter and DNA-footprinting as experimental read-out (98).

Subsequent studies performed with Stat5 proteins were pioneered by the laboratory of Warren Leonard (99-101). Here, complex formation of activated Stat5a proteins and its consequences for regulation of a 3'enhancer region of the *IL-2Ralpha chain* gene locus were delineated. The high affinity IL-2R-alpha chain (or CD25) constitutes an essential molecule of activated or regulatory T cells, whose functions are indeed controlled through Stat5 (5, 14, 36, 59, 100). A further approach focused on the *CIS* promoter and the multiple high and low affinity Stat5 elements therein (102).

Important mechanistic insights resulted from studies with the Stat4 N-terminus and subsequent extrapolation to other Stat family members. For instance, it became clear that the conserved $W_{37}A$ mutation resulted in unstable Stat proteins and that deletion of the N-terminus resulted in absence of tyrosine phosphorylation. As already mentioned, there are significant differences in the N-termini of Stat5a and Stat5b. Tyrosine phosphorylation of $Stat5a^{\Delta N}$ was persistent while that of $Stat5b^{\Delta N}$ is still inducible by cytokine (3, 61). In addition, the mutation $W_{37}A$ in Stat5a did not cause a chaotropic molecule, since Stat5a-$W_{37}A$ or $Stat5a^{\Delta N}$ were able to complement the phenotype of $Stat5^{Null}$ mast cells (K. Bunting, K. Friedbichler and R. Moriggl, unpublished data). Therefore, apparently, over-simplification by extrapolation of experiments performed with one particular Stat protein variant to all Stat family members can be drastically misleading. Each Stat protein, splice isoform, secondary protein modification, or proteolytically processed Stat protein might have individual chromatin binding behavior. This hypothesis is open for experimental extension and verification since additional studies on Stat5 and chromatin functions will be required in the future.

The differential tetramer formation of Stat proteins and their distinct ability to interact with specific sets of proteins leads to a great variety of transcriptional responses. Tetramer formation capability of distinct Stat family members might alter chromatin structures differentially. So far, only gene transcription rather then gene repression by Stat tetramers was reported, but the ability of Stat5 proteins to bind onto low affinity Stat5 sites should have consequences for differential cytokine responses via fine-tuning chromatin alterations.

In the following we present in detail a typical experiment using Stat5a variants with altered chromatin binding characteristic. A description of the corresponding mutants can be found in (61), and the extensive "Material and Methods" section below enables eventual follow up studies for questions regarding the "N-terminus of Stat5 and chromatin binding":

The first experiment before conducting the actual Stat5 tetramer assay is to normalize dimer-DNA binding activity of different Stat5 extracts on an EMSA gel. A typical example is shown for dilutions of extracts from cells expressing either wt Stat5a, $cS5^{F}$, or the tetramer-deficient mutants $Stat5a^{\Delta N}$ and $cS5^{F\Delta N}$, using the beta-casein single Stat5 response element (RE; Figure 6, upper part). This procedure has to be repeated until equal amounts of loaded Stat5 dimer activity can be observed (Figure 6, lower part). In addition, specificity of DNA binding activity in each extract needs to be assessed to verify integrity of protein samples. To this purpose, supershifts are performed using antibodies specific for epitopes in the Stat5 N- or C-terminal region (here obtained from Santa Cruz) (Figure 6, lower part). The interpretation of tetramer assays with

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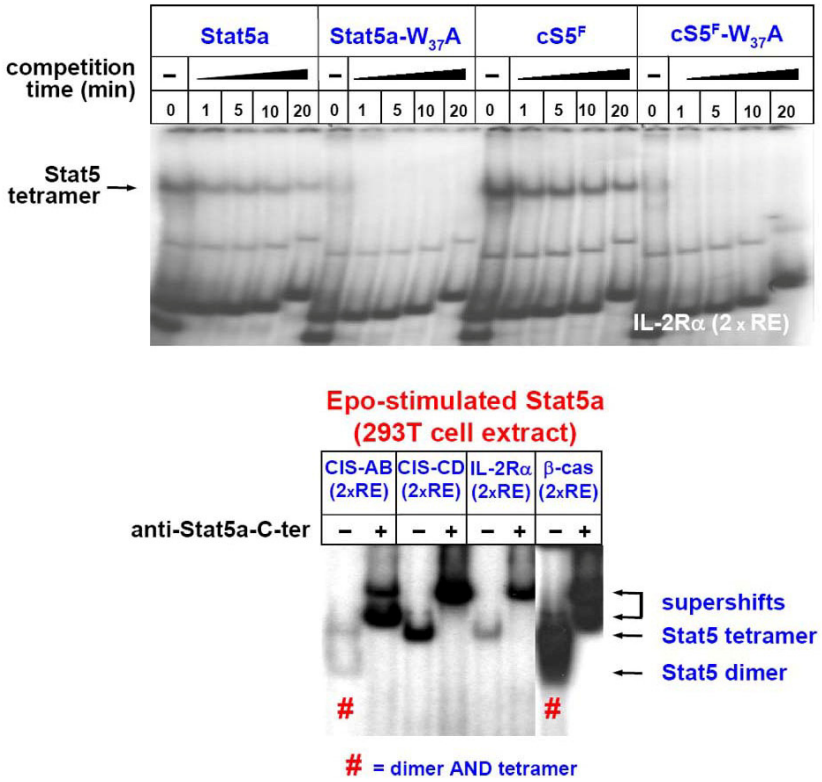

Figure 7. Tetramerization of Stat5 protein variants. Tetramer formation of the indicated Stat5a variants (61) was analyzed using 293T cell extracts stimulated with Epo (50 U/ml) using the IL-2R-alpha chain tetramer element (upper part). Four different tetramer DNA elements (CIS-AB, 2xRE; CIS-CD, 2xRE; IL-2R-alpha, 2xRE beta-casein, 2xRE) were used for incubation with a Stat5a extract from Epo-activated cells to demonstrate dimer and/or tetramer formation (lower part). "#" indicates formation of both dimers and tetramers.

different extracts is impossible without this titration and adjustment of DNA binding activity on a single RE.

Tetramer assays with wt Stat5a, $cS5^F$, or different tetramer-deficient mutants of Stat5a have been reported previously (61). Here, an unpublished experiment on the IL-2Ralpha chain tetramer element as a model is depicted (Figure 7, upper part). After mixing cell extracts and radioactive DNA oligonucleotide followed by brief centrifugation, equilibrium of the DNA-binding reaction is reached within two minutes at RT. The absence of unlabeled competitor DNA (0) represents the binding reaction under saturation conditions. The complexes of Stat5 proteins with labeled double stranded oligonucleotides are then competed with unlabelled double stranded oligonucleotides (2xRE) at 100fold molar excess and DNA off-on rates and oligomer complex stability are measured in EMSAs. Again, all reaction tubes containing extracts plus DNA are mixed vigorously to ensure homogenous distributions of protein, DNA and binding reaction components. Usually, a master mix for six reactions in one tube was prepared, and at specific time points equal amounts of extracts (here: 20 microliter) were loaded onto a continuously running native polyacrylamide gel. No pre-run of EMSA gels was required, but for better quality flushing of gel pockets with running buffer is recommended since chemicals remaining after acrylamide polymerization disturb the integrity of native complexes.

Quality of the extract is the crucial factor for complex formation and reduction of background. Samples with high Stat activity facilitate the analysis of oligomers and therefore, we frequently used transient transfection of Stat5 variants into 293T cells to enhance signal strength. We want to stress that also cytokine-stimulated cell lines or mouse tissues obtained after *in vivo* cytokine injection (growth hormone injected liver; IL-3 injected bone marrow cells; Epo stimulated erythroblasts, etc.) or samples from cancer patients work well provided sufficiently strong Stat5 activity can be detected on preliminary beta-casein bandshift assays.

In general, extracts from lymphoid cell types favor the formation of Stat5a/b higher order chromatin complexes of high affinity over dimer complexes. Until now, we did not observe tetramer complexes in myeloid cell types such as erythroid progenitors or Ba/F3 cells (R. Moriggl, unpublished). There is no straightforward

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explanation why samples from different hematopoietic cell types with Stat5 activity normalized on dimer sites are behaving so different in terms of biochemistry when employed in oligomerization assays. This is indeed surprising since extracts of fibroblasts or epithelial cell lines ectopically expressing Stat5a can form tetramers and also Stat5b proteins activated by growth hormone injection into mice *in vivo* do form efficient Stat5 oligomers.

We previously reported on the characterization of four different Stat5 tetramer elements (61) (Figure 7, lower part). Stat5a protein extracts isolated from Epo-stimulated 293T cells were incubated with these four tetramer sites, each of which contains two Stat5 REs either with high or weak affinity. Apparently, there must be alternatives in molecular composition, since three distinct DNA-binding complexes were observed with the same amount of extract and Stat5a activity (Figure 6). Two DNA elements displayed binding of Stat5 dimer- plus tetramer complexes (STD and CIS-AB), the two other elements displayed Stat5 tetramer complexes only (CD25 and CIS-CD) with extracts from Epo-stimulated 293T cells. Currently it is not understood how these differences in Stat5 oligomers bound to different Stat5 tetramer sites arise, but DNA structure conformation might be responsible (Figure 7).

8. SUMMARY AND PERSPECTIVE

The way how Stat5 proteins exert their distinct and specific functions in different cell types under various physiological and pathological conditions is still unclear to a large extent. It is possible that additional proteins functionally interacting with Stat5 still await detection or were at least not taken into sufficient consideration so far. The next years of research on Stat5 will have to address the question of composition of different protein complexes in which Stat5 proteins reside and through which they execute their specific functions. It is also open whether still other proteins interact with DNA-bound Stat5 oligomers. Unfortunately, no further reports on the chromatin binding properties of Stat5 were published.

So far the Stat5 N-terminus is known to influence three distinct signaling properties of Stat5:

1) The N-terminus of Stat5a blocks auto-activation by intrinsic tyrosine kinases within the cell. N-terminal deletion mutants of Stat5a are persistently tyrosine phosphorylated, possess constitutive DNA binding activity and exhibit robust transactivation potential.

2) The N-terminus of Stat5 is important for interaction with transcriptional cofactors like CBP and p300 and transcription factor interaction with nuclear hormone receptors like the GR.

3) The Stat5 N-terminus is required for tetramer formation (or oligomerization) through protein-protein interactions, which subsequently lead to higher order complex formation on chromatin. The N-termini of Stat5 stabilize two dimer interactions bound to Stat5 responsive elements in the vicinity of transcriptional regulatory regions.

Several important questions regarding the chromatin altering capability of Stat5 proteins are largely unanswered:

1) How efficiently and on which DNA loci can Stat5 proteins modify chromatin structure and thus transcriptional competence?

2) Can these structural changes be visualized?

3) Do other proteins interact with Stat5 in higher order DNA complexes and are these potential additional proteins cell type specific?

4) Are post-translational modifications of Stat5 proteins involved in altered chromatin binding or transcriptional capacity?

We currently do not understand the secondary protein modifications of Stat5 and how they influence DNA binding and complex formations on different natural DNA elements. Future work around the truly multi-facetted transcription factors and signaling mediators Stat5a and Stat5b will address their functions in a broader context.

9. MATERIALS AND METHODS

9.1. Transient transfection and preparation of whole cell extracts

Extracts of cells expressing different Stat5 variants were generated by transient transfection of 293T cells using the calcium phosphate precipitation method (4 microgram of *Stat5* cDNA in a pMSCV expression plasmid plus 2 microgram *EpoR* cDNA in pMSCV per 10 cm dish of 293T cells, 20-50% confluency). 48 hours post transfection, cells were harvested after a 30-min stimulation with 50 units/ml rhEpo. Culture dishes were rinsed twice with ice-cold PBS and cells scraped off and pelleted by centrifugation (1 min, 6,000 g). Cells were lysed by the addition of two pellet volumes of whole cell extract buffer (20 mM Hepes, pH 7.9; 20% glycerol; 50 mM KCl; 1 mM EDTA; 1 mM DTT (Sigma); 400 mM NaCl; 5 microgram/ml leupeptin (Roche); 0.2 units/ml aprotinin (Bayer); 1 mM PMSF (Roche); 5 mM Na_3VO_4; 10 mM NaF; 5 mM beta-glycerophosphate) and vigorous vortexing. Samples were frozen in liquid nitrogen, followed by thawing on ice. Freeze-thaw cycles were repeated 4 times. Subsequently, extracts were centrifuged at 15.000 g at 4°C for 20 minutes, and the supernatants used for EMSAs or Western blot assays or stored at –80°C until use.

9.2. Electrophoretic mobility shift assays (EMSAs)

DNA-Oligonucleotides were annealed at equimolar concentrations (>40 microM for each oligo) in 200 microliter using annealing buffer (10x = 0.625x PCR Buffer II (Roche); 9.4 mM MgCl2) by heating up to 95°C for 10 min, followed by slowly cooling down of samples to RT and an additional incubation on ice for another 10 min. Annealed oligos were then diluted to 2.5 microM for radioactive labeling. The labeling reaction (5 picomol of annealed ds-DNA in 20 microliter, containing 10 units of poly-nucleotide-kinase (Roche) and 5 microliter of gamma-

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ATP ^{32}P (Amersham; 8,000 cpm/microliter) was incubated at 37°C for 1 hour. Labeled oligo-nucleotides were purified on size exclusion columns (Micro Bio-Spin 6 chromatography columns; BioRad) and stored at -20°C until use.

For *in vitro* Stat5-DNA-binding assays, a 20 microliter reaction was prepared for each sample, containing 2 microliter BSA (10 mg/ml in 20 mM KPO_4, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol), 2 microliter poly-dI-dC (1 mg/ml; Roche), 4 microliter 5x binding buffer (50 mM Tris; 5 mM DTT; 1 mM PMSF; 0.5 mM EDTA; 25% Glycerol; 250 mM NaCl; 0.5% NPO_4) and 0.5-1 microliter labeled oligo-nucleotide probe (8,000 cpm/fM). Usually, 20-30 microgram of protein extract was used per reaction.

In the analysis of patient samples, low Stat5 activities are frequently encountered. In this case, up to 60 microgram of protein extracts can be loaded. In general, a maximum of 4 microliter of whole cell extract per 20 microliter of total EMSA sample volume should not be exceeded to ensure maintenance of proper salt concentrations. Extracts were incubated for approximately 5 min (equilibrium is reached within 2 min) at RT before gel loading. For supershift analyses, protein samples were pre-incubated for >2 min on ice with antibodies / antisera.

Complexes were separated on non-denaturating 4% acrylamide gels (BioRad; acrylamide / bis-acrylamide = 29 / 1) in 0.25x TBE and run in 0.25x TBE running buffer at 200 V for about 3 hours.

In the bandshift experiments the following high affinity Stat binding sites were used (Each of the Stat5 response elements (RE) listed contains an inverted repeat highlighted in bold). 1x response element for dimer assays: beta-casein (1xRE) 5′-3′ AGAT**TTCTAGGAA**TTCAATCC (binds Stat5/6 with high affinity, and Stat1/3/4 with very low affinity); 2x response elements for tetramer assays: CIS-AB (2xRE) 5′-3′ GAGTT**TTCCTGGAA**AAG**TTCTTGGAA**ATCTG; CIS-CD (2xRE):5′-3′ CGCGG**TTCTAGGAA**GATGAGGC**TTCCGGGAA**GGG CT; IL-2R-alpha (2xRE): 5′-3′ GTTTC**TTCTGAGAA**GTACCAGACAT**TTCTGATAA**G AGAG; beta-casein (2xRE): 5′-3′ AGAT**TTCTAGGAA**TTCAATC**TTCTAGGAA**TTCAAT C

9.3. Tetramer assays

For Stat5 oligomer assays, a classical approach (adapted from (92)) was used, relying on the stability of pre-formed DNA-Stat5 protein complexes on gamma-ATP ^{32}P-labeled Stat5 tetramer elements (see 2xRE above).

For Stat5 tetramer assays, the volume was scaled up to 120 microliter. The binding reaction was finished 2 min after gentle mixing, and the time point 0 was loaded (i.e. 20 microliter of the labeled tetramer assay mixture loaded onto the gel before applying voltage). Subsequently, 10 microliter of unlabeled competitor oligo-nucleotide were added, the reaction mixed, centrifuged and 20 microliter loaded after 1, 5, 10 and 20 minute (s) onto the gel continuously running at a constant 200 V. The cold competitor DNA was used in 100x molar excess over the radioactive labeled oligo. The use of large gels (15 cm x 20 cm, 2 mm thickness) facilitates handling significantly. Electrophoresis was continued until the orange-G dye front, usually loaded in an empty lane together with free probe, reached the lowest 5 cm of the gel. Orange-G migrates comparable to an oligo of tRNA size, which is close to the length of double stranded annealed Stat REs. After electrophoresis, the gel was transferred to chromatography paper (Whatman), dried on a vacuum gel drier at 80°C for two hours and subjected to autoradiography. Coated films (Kodak BioMax MR) were exposed over night or longer at -80°C.

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Curriculum Vitae

Name:	Marc André Kerényi
Date & Place of Birth:	24.April 1981; Vienna, Austria
Nationality:	Austrian

EDUCATION

- February 2005 – Fall 2008 — **Ph.D. training**
  Department of Medical Biochemistry Max F. Perutz Laboratories (MFPL)
  and
  Research Institute of Molecular Pathology (IMP):
  Joint supervisors:
  Prof. Ernst Müllner, Ph.D. & Prof. Hartmut Beug, Ph.D.
  Thesis Title:
  "Stat5 in Hematopoietic Development and Disease"

- January 2005 — Graduation in **Molecular Biology** at the University of Vienna, Austria, with **highest distinction**

- September 2004 — Accepted in the competitive **"Vienna Biocenter PhD-program"**; Selection 2004 (ranked 6th out of >200)

- November 2003-2004 — **Master thesis**
  Research Institute of Molecular Pathology (IMP):
  Supervisor: Prof. Hartmut Beug, Ph.D.
  Thesis Title:
  "Stat5 in Hematopoiesis: Gain and Loss of Function Studies"

- October 1999 - January 2005 — Undergraduate studies of **Molecular Biology** at the University of Vienna

1992 – 1999 — High School in Vienna, Austria

FURTHER RESEARCH EXPERIENCE:

- July – October 2003 — **Internship at Georgetown University Medical Center**
  Washington DC
  Supervisor: Prof. Hallgeir Rui, M.D. Ph.D.
  Project:
  Identification of Harakiri as a Stat5-regulated Gene

- February 2001-2004
- (part time) — **Research technician at Vienna General Hospital**
  (concomitantly with undergraduate studies)
  Supervisor: Prof. Christian Singer, M.D.
  Projects:
  Overexpression of Cadherin-11 in Malignant MaCas
  IGF II Overexpression in Herceptin Resistant Tumors
  EBAF a Putative Proto-Oncogene in Malignant EndoCas

abbreviations: MaCa: mama carcinoma; EndoCa: endometrium carcinoma

MISCELLANEOUS:

- February 2006 — "Top – Stipend" of the Austrian federal state of Lower-Austria,
- Since February 2006 — Member of the European Association for Cancer Research
- January 2006 — Supporting grant awarded for excellence to young scientists from the University of Vienna
- June 2001 - 2003 — Students representative of the branch of study Molecular Biology
- Since April 2000 — Member of the Austrian Society of Biochemistry and Molecular Biology (ÖGBM)

PUBLICATIONS:

Stat5 regulates cellular iron uptake via IRP-2 and TfR-1 in erythroid cells.
Kerenyi MA., Grebien F., Gehart H., Schiffrer M., Kovacic B., Artaker M., Beug H., Mullner EW.
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Oncogenic Kit controls neoplastic mast cell development through a cytoplasmic Stat5/PI 3-kinase signaling cascade.
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Kornfeld JW., Grebien F., **Kerenyi MA**., Friedbichler K., Kovacic B., Zankl B., Hoelbl A., Nivarti H., Beug H., Sexl V., Muller M., Mullner EW., Kenner L., Gouilleux F. and Moriggl R. **(Front Biosci. 2008 May 1;13:6237-54)**

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Yao Z.; Kanno Y., **Kerenyi M.,** Stephens G., Durant L., Watford WT., Laurence A., Robinson GW., Schevach EM., Moriggl R., Hennighausen L., Wu C., O'shea JJ. **(Blood. 2007 May 15;109(10):4368-75)**
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Harir N., Pecquet C., **Kerenyi MA**., Sonneck K., Kovacic B., Nyga R., Dhennin I., Regnier A., Gouilleux-Gruart V., Beug H., Lassoued K., Moriggl R., Gouilleux F. **(Blood. 2007 Feb 15;109(4):1678-86)**

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Holnthoner W., **Kerenyi MA**., Gröger M., Kratochvill F., Petzelbauer P. (**Biochem Biophys Res Commun. 2006 Apr 14;342(3):725-33)**

Clarifying the role of Stat5a/b in lymphoid development and Abelson induced transformation.
Hölbl A., Kovacic B., **Kerenyi MA.**, Simma O., Warsch W., Cui Y., Beug H., Hennighausen L., Moriggl R., Sexl V. **(Blood. 2006 Jun 15;107(12):4898-906)**
(Awarded with an **"Exceptional"** by the Faculty of 1000 Biology and with the **Hans-Horst Meyer Price** of the Austrian Society of Pharmacology)

Erythroid progenitor renewal versus differentiation: Genetic evidence for cell autonomous, essential functions of EpoR, Stat5 and the GR.
Dolznig H. , Grebien F., Deiner EM., Stangl K., Kolbus A., Habermann B., **Kerenyi MA**., Kieslinger M., Moriggl R., Beug H. and Müllner EW. **(Oncogene. 2006 May 11;25(20):2890-900)**

UNPUBLISHED WORK:

Loss of lamina-associated polypeptide 2-alpha expression in mice causes hyperproliferation of early progenitor cells in the skin and erythroid system.
Naetar N., Korbei B., **Kerenyi MA.,** Dorner D., Kozlov S., Kral R., Gotic I., Fuchs P., Bittner R., Stewart C. S., Foisner R. **(Nature Cell Biology, in revision)**

O-GlcNAc glycosylation of Stat5 is essential for its leukemogenic transforming properties.
Kerenyi MA., Grebien F., Kovacic B., Gouilleux F., van Deursen J.; Moriggl R., Beug H., Mullner EW. **(manuscript in preparation)**

Loss of transcription factors Stat5 and GR in hepatocytes leads to severe steatosis and peripheral lipodystrophy.
Kornfeld JW., Pospisilik A., Friedbichler K., Esterbauer H., Hasselblatt P., **Kerenyi MA**., Wagner KU., Engblom D., Haemmerle G., Kratky D., Kenner L., Terraciano L., Zechner R., Schütz G., Heim M., Moriggl R. **(manuscript in preparation)**

Glucocorticoid-induced changes in accessibility of specific genomic response elements to transcription factor Stat5.
LeBaron MJ., Neilson LM., **Kerenyi MA.,** Tran TH., Johnson KJ., Ryder A., Rui H. **(manuscript in preparation)**

BOOKS:

JAK STAT Pathway in Disease edited by Anastasis Stephanou
Chapter: Stat5 as hematopoietic gatekeeper and oncogene upon tyrosine kinase-induced transformation.
Friedbichler K., **Kerenyi MA.,** Mullner EW., Moriggl R. **(Landes Bioscience 2008, in press)**

INVITED TALKS:

Glycosylation of Stat5 is essential for its transforming properties

Kerenyi MA.

University of Philadelphia – Kimmel Cancer Center; Philadelphia, September 2006

Invited by Prof. Hallgeir Rui, M.D. Ph.D.

POSTER:

O-GlcNAc Glycosylation of Stat5 is Essential for its Transforming Properties.

Kerenyi MA., Grebien F., Kovacic B., Groner B., Gouilleux F., van Deursen J., Beug H., Moriggl R., and Mullner EW

-Society of Hematology and Stem cells 36th Annual Meeting, Hamburg, Germany, September 2007

-Keystone Symposia "Jaks and Stats and Immunity", Steamboat Springs CO, USA, January 2007

Persistent STAT5 Activation Supports Functional Erythropoiesis in the Absence of Jak2 and EpoR.

Grebien F., **Kerenyi, M.A**., Kovacic B., Kolbe T., Klingmueller U., Mueller M., Beug H. Moriggl R. and Mullner E.W.

-IMP/IMBA Recess, Vienna, Austria, October 2007

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Detection of Activated Stat5 in the Cytoplasm of Neoplastic Cells in Patients with AML, CML and Systemic Mastocytosis.

Sonneck K., Fritz R., Müllauer L., Gleixner K., Mayerhofer M., Florian S., **Kerenyi MA**., Sperr W., Sillaber C., Morrigl R., Valent P.

-The American Society of Hematology 48th Annual Meeting, Orlando, USA, December 2006

A Role for Stat3 in Erythropoiesis.

Kerenyi MA., Grebien F., Mullner EW., Beug H.

-IMP Recess (Research Symposium), Vienna, October 2005

Stat5 in Hematopoiesis: Gain and Loss of Function Studies.

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Harakiri a Prolactin-regulated Stat5 Gene in Breast Cancer Cells.

LeBaron MJ., **Kerenyi MA**., Rui H.

-Gordon Research Conferences on Prolactin, Ventura, CA, 01-06 February 2004