universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

“The role of hypoxia inducible factor in iron-deficient megakaryopoiesis”

Verfasserin

Kathrin Lippert

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Studienkennzahl lt. Studienblatt:	A 441
Studienrichtung lt. Studienblatt:	Diplomstudium Genetik – Mikrobiologie
Betreuer:	Univ.-Prof. Dr. Christoph Gasche

Wien, 2013

Acknowledgements

Hereby I would like to thank all the lab members for their warm support and for making this study possible.

My special thanks to…

Vineeta, for supervising me, for sharing her experience and for creating a friendly and relaxed working atmosphere.

Dr. Gasche, for the opportunity to be a lab member, for letting me participate in such an exciting and challenging project and for his helpful suggestions.

Rayko, for his help concerning work progress and theoretical uncertainties and for giving my work the final touch. I could always count on his professional experience.

Michaela, for methodical help and technical support and for always helping me with words and deeds.

Thanks to my family for support and encouragement.

A special thank to Manuel for always listening, helping and encouraging me throughout the whole process of working and writing on my thesis.

Table of contents

Table of figures

1 Introduction

Iron deficiency (ID) is the most common nutritional deficiency worldwide (1). Iron deficiency can lead to anemia – so-called iron deficiency anemia (IDA) – a condition presenting with symptoms like fatigue, headache, dyspnea and palpitations, thereby affecting health, quality of life and working capacity (2). Fifty percent of all cases of anemia are assumed to be due to ID – affecting more than 12% of the world population (3). Nevertheless, even in patients who are not anemic, ID has negative effects on health, physical capacity, work performance, cognitive performance and behavior (4). In addition, ID is often associated with a mild to moderate reactive thrombocytosis (about $500x10^9$/L). Furthermore cases of considerable thrombocytosis (~$1000x10^9$/L) in ID are documented (5), which can eventually lead to thrombosis (6;7;8;9;10;11). In cancer patients high platelet counts were shown to be a risk factor for venous thromboembolism (12).

Due to dietary restriction, malabsorption and intestinal bleeding, inflammatory bowel disease (IBD) is often associated with ID and anemia (2), but also with secondary thrombosis (13). Thromboembolic events are also more common in IBD patients compared to the healthy population (14). This increased risk might be contributed to the active inflammation but possibly also to thrombocytosis. Studies show that the number of platelets is positively correlated with disease activity (15). Apart from their function in homeostasis, platelets do have a significant role in mucosal inflammation (13). Thrombocytosis in IBD is essentially explained by the enhanced production of proinflammatory cytokines, for instance Interleukin-6 (IL6) or Interleukin-11 (IL11), which may stimulate platelet production (16). However, clinical studies, in vitro studies and studies in animal models suggest that ID itself triggers platelet production.

In a recently published retrospective study by our group it was shown that intravenous or oral treatment of patients with iron, suffering from IBD associated with anemia and thrombocytosis, led to normalization of platelet counts (17). The study represents initial evidence that ID acts directly on megakaryopoiesis to enhance platelet production. These results were confirmed by a following randomized controlled trial (ThromboVIT) showing that the mean platelet counts decreased in patients with IDA in IBD after treatment with intravenous iron (18). Several animal studies, including a study by our lab, support the finding that the iron status is associated with the count of platelets: animals on low iron diet presented with high platelet numbers (19;20;21;22). Additionally, injections of iron led to decreased platelet counts and decreased platelet production in anemic rats (23). Finally, studies in a

recently established in vitro model to study megakaryopoiesis under ID indicate a direct influence of ID on the ploidy level of megakaryocytic cell lines (24).

However, although there are many indicators for a direct involvement of ID in megakaryopoiesis, the underlying mechanism has not been clarified yet. Transcription factors play an important role in megakaryocytic differentiation. This study aims to elucidate the possible role of the transcription factor hypoxia inducible factor (HIF) in megakaryopoiesis under ID by studying the process in an in vitro model.

1.1 Development of platelets

In addition to their important role in homeostasis, platelets are involved in many pathophysiological processes including thrombosis, inflammation and immunity (25). Platelets are anucleate cells and they arise from polyploid MKs. Humans produce approximately $1x10^{11}$ platelets per day, but in cases of higher demand the production may increase more than 20-fold (26).

1.1.1 Megakaryopoiesis and thrombopoiesis

Cellular aspects

Megakaryopoiesis is a multi-stage process in which pluripotent hematopoietic stem cells (HSC) differentiate into mature megakaryocytes (MKs) which have the ability to produce platelets. Two major cell-lineages may arise from HSCs: the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). The latter has the ability to differentiate into the granulocyte/macrophage progenitor (GMP) and the megakaryocyte/erythroid progenitor (MEP), but there is also evidence that MEPs can differentiate directly from HSCs (27;28). MEPs are bipotent cells either differentiating into the erythroid or into the megakaryocytic lineage. Cells committed to the megakaryocytic lineage differentiate into the burst forming-unit megakaryocytes (BFU-MK), subsequently evolve into colony forming-unit megakaryocytes (CFU-MK) and become immature MKs or megakaryoblasts (29) (Fig. 1).

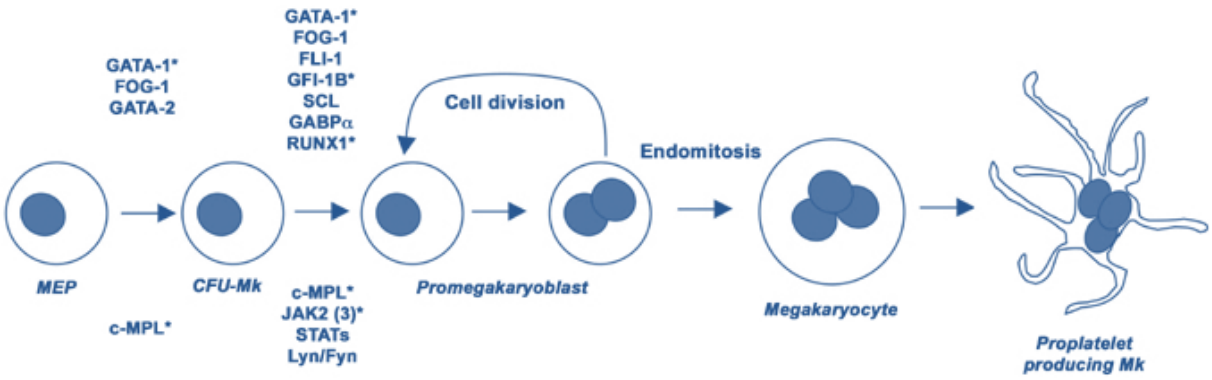


Fig.1: Megakaryocytic differentiation (30)

The hallmarks of megakaryocytic differentiation are

- endomitosis,
- expression of megakaryocytic surface markers,
- synthesis of megakaryocyte-specific proteins such as Platelet factor-4 (PF4) and von Willebrand factor (vWf), which are finally stored in the alpha granules,
- maturation of the cytoplasma including formation of lysosomes, dense granules and alpha-granules
- and development of the demarcation membrane system (DMS), a complex membrane system (29).

The exact production and release process of platelets has not been established yet. There are two main models explaining the final step of thrombopoiesis: the "cytoplasmic fragmentation model" and the "proplatelet model". In the "cytoplasmic fragmentation" or "platelet territories model" platelets are already pre-packed within the mature MKs and form platelet territories, which are finally released. Today there is a tendency to support the "proplatelet model", in which mature MKs form long cytoplasmic extensions, the so-called proplatelets. Within these proplatelets platelets are packed and assembled de novo (Fig. 2) (31).

Initially MKs are localized in the osteoblastic niche in the marrow. Maturing MKs migrate to the sub-endothelium region near venous sinusoids where they start to form proplatelets. The process of proplatelet formation is driven by microtubules, which translocate to the cell cortex, when the cell starts to form the pseudopodial structures. Ran binding protein 10 (RanBP10) was suggested to be a central regulator organizing and stabilizing microtubules during the process of proplatelet formation (32). The DMS ensures the necessary amount of

membrane to allow the generation of proplatelets. Megakaryocytes have the ability to cross the endothelial barrier and finally release platelets and proplatelets. Alternatively, MKs release the platelets directly into the blood stream (33). It is suggested that extracellular matrix proteins are involved in the regulation of proplatelet development. In a review of 2006 the authors propose a model in which the osteoblastic niche contains predominately factors, which inhibit the formation of proplatelets, whereas proteins in the vascular niche like Laminin, Fibrinogen and Type IV collagen trigger the proplatelet formation (34). There is evidence that the generation of platelets continues in the peripheral blood and the release of platelets is facilitated by shear forces in pulmonary arterioles (32). Although platelets do not have a nucleus and are thought to be completely differentiated there are studies showing that platelets have the capacity to synthesize proteins (35) and to produce progeny (36).

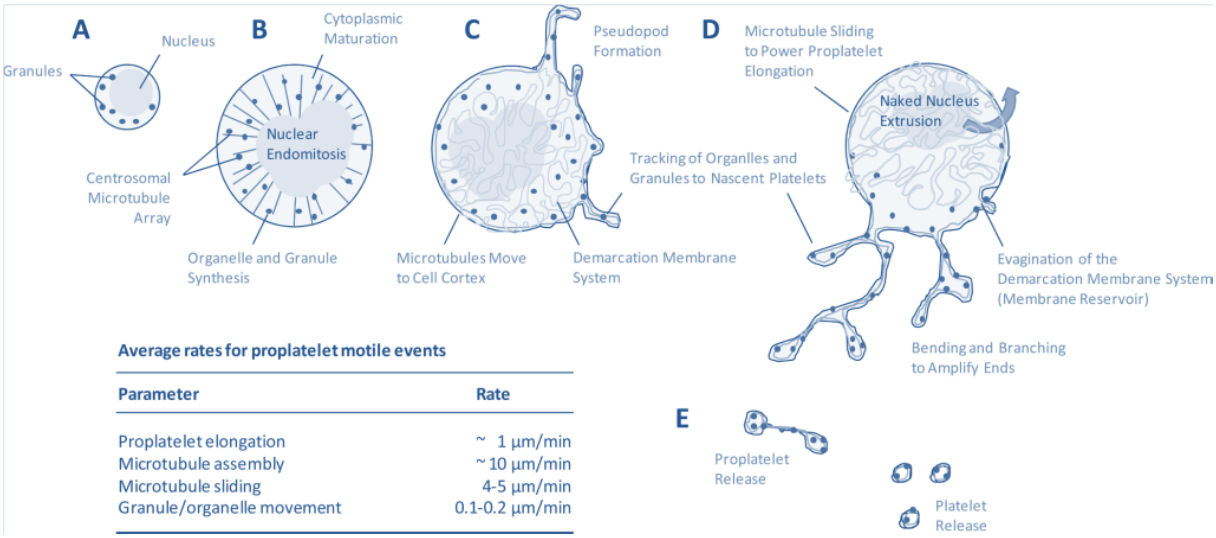


Fig.2: Platelet formation (32)

Differentiation markers

The expression of CD41 (GlycoproteinIIb or alphaIIb-Integrin) and CD61 (beta3-Integrin chain) is considered as an early marker for the megakaryocytic lineage (26) and functions as membrane receptor for Fibrinogen, Fibronectin, Vitronectin and vWF (29). As MKs mature they also express CD42 (GlycoproteinIB) which is a multimer consisting of CD42a (GlycoproteinIX), CD42b (CDIbeta alpha), CD42c (GlycoproteinIbbeta) and CD42d (GlycoproteinV). Initially CD42a, CD42b and CD42c form a complex on the cell surface whereas CD42d appears later on the cell surface (37). CD42 acts as a receptor for the vWf. The Thrombopoietin (TPO) receptor c-Mpl is upregulated during the megakaryocytic differentiation. Early 2N and 4N MKs were shown to express high levels of vWf (38). The

erythroid marker Glycophorin A (GpA) is lost during differentiation of MEPs into primitive megakaryocytic lineages and is probably the earliest marker of megakaryocytic differentiation (39). Moreover, MKs are documented to downregulate the transferrin receptor1 (TfR1 or CD71) during their maturation (40).

In contrast, cells differentiating into the erythroid lineage increase expression of TfR1, lose CD41 but express CD36 (Thrombospondin receptor), GpA and ultimately Globin (26).

Endomitosis and cell cycle

Although polyploidy is often seen as a consequence of cellular stress, it can also occur as a physiological process as it is the case in megakaryopoiesis (41). The reason why MKs undergo endomitosis is still not completely understood. As high ploidy is associated with increased cell size, mRNA and protein production (42) endomitosis might be an efficient mechanism to extend the metabolic output without performing all aspects of cellular division. There are studies saying that the state of ploidy determines the reactivity of the platelets produced. It is still contradictory if a state of higher ploidy leads to an increased platelet output (43;44).

Mitosis and endomitosis follow the same pattern until the late anaphase. After the initiation of the cleavage furrow cells undergoing diploid mitosis run through cytokinesis and complete division whereas MKs show regression of the cleavage furrow leading to the characteristic multi-lobulated nucleus. Cells then re-enter G1 as polyploid cells. Until now there has not been an exact explanation how furrow regression is accomplished (45;46). However, there are studies showing that in MKs the contractile ring is not functioning properly as there is no accumulation of nonmuscle Myosin IIA. Additionally the Rho/Rock pathway is inhibited (47).

A difference in cleavage furrow formation of early MKs and more mature MKs could be determined. Early MKs with a low ploidy level have the ability to form a furrow which later regresses, whereas the high ploidy MKs may not form the furrow at all (46;48). Survivin, a mitotic regulator - essential for proper chromosome segregation and cytokinesis in mitotic cells - was shown to be rarely localized in midzone microtubule spindles of high-ploidy MKs (49).

In diploid cells cell cycle checkpoints including licensing factors ensure that the genome is replicated exactly once per mitotic division. How polyploid cells escape to undergo apoptosis

and how endomitosis is regulated are still open questions. Many studies aimed at learning more about the regulation of the endomitotic cell cycle and studied important regulators of cell cycles, the cyclins (50). Cyclin D3 - a G1 phase cyclin - is highly expressed in MKs undergoing endomitosis. Mice overexpressing cyclin D3 showed an increased level of megakaryocytic polyploidy (51). Mice overexpressing cyclin D1 in MKs showed moderately increased ploidy (52). Another study in MKs lacking GATA1 showed that overexpression of Cyclin D1 was sufficient to rescue polyploidization, but not terminal differentiation. Cyclin E also seems to play an important role in regulating the endomitotic process: studies on primary MKs showed an upregulated cyclin E level after TPO treatment. Mice overexpressing cyclin E - specifically in megakaryocytes - showed an increased ploidy level (44).

1.1.2 Regulation of megakaryopoiesis by cytokines

The most important cytokine during megakaryocytic differentiation is TPO, regulating all aspects of megakaryopoiesis by stimulating the survival of HSCs, the expression of specific megakaryocytic differentiation markers, endomitosis, cytoplasmic reorganization and formation of the demarcation membranes. TPO belongs to the four-helix bundle family of cytokines and is produced in the liver, but also in kidneys and the bone marrow. The cytokine acts by binding to its receptor c-Mpl, which is expressed in HSCs, primarily in megakaryocytic cells (50).

Binding of TPO to the homodimeric receptor c-Mpl leads to phosphorylation and activation of the associated tyrosine kinase Janus kinase 2 (JAK2). JAK2 subsequently phosphorylates the signal transducer and activator of transcription (STAT) - specifically STAT3, 5a, 5b and 1 - and thereby induces the expression of p21, Bcl-xL and Cyclin D1. In addition to STAT JAK2 activates the mitogen-activated protein kinase (MAPK) cascade and the small GTPase RAS, which finally leads to the activation of extracellular signal-related kinase 1/2 (ERK 1/2). ERK1/2 is required for maturation of megakaryocytic progenitor cells. Additionally the Phosphoinositol-3-kinase (PI3K) pathway is activated which stimulates the survival and the proliferation of MKs by inducing AKT which in turn promotes anti-apoptotic signals (50). Studies showed that TPO acts on the expression of heterodimeric homeodomain proteins and on the HIF pathway (53).

In order to maintain the homeostatic balance, several mechanisms ensure the regulation of TPO. After binding TPO, c-Mpl gets internalized and degraded. Furthermore, platelets

generate an auto-regulatory loop: they possess highly affine c-Mpl-receptors leading to the removal of circulating TPO (54). The activated JAK/STAT pathway leads to induced transcription of the Suppressor of cytokine signaling (SOCS) assuring the negative regulation of TPO levels (50).

Animal studies emphasize the important role of TPO during megakaryocytic differentiation. A particular study showed that TPO deficient mice had diminished platelet and MK counts. Furthermore the ploidy level of the MKs was reduced (55). Similar results were obtained from mice lacking the TPO receptor c-Mpl: the animals produced strongly decreased numbers of MKs and platelets (56;57). In humans mutations in the c-Mpl gene leading to an impaired TPO signaling pathway are known to lead to congenital amegakaryocytic thrombocytopenia (58). In addition, the opposite effect could be shown: myeloproliferative disorders can arise due to mutations of the c-Mpl causing sustained TPO signaling. Some of the megakaryocytic cell lines, which are derived from myeloproliferative disorders are known to have a V617 mutation in the JAK2 gene leading to a constitutively activated TPO signaling (59;60).

The chemokine Stromal derived factor-1 (SDF1) with its main receptor CXC-motif-chemokine-receptor 4 (CXCR4) plays an important role by boosting the migration of maturing MKs from the bone marrow towards the vascular niche. It is suggested that CXCR4/SDF1 have a negative effect on RhoA signaling, and induce thereby polyploidy and the formation of proplatelets (33). In combination with TPO and SDF1, SCF (stem cell factor) is known to stimulate megakaryopoiesis through its receptor c-kit, being the only cytokine that is able to sustain a low level platelet production in c-Mpl knockout mice (61).

Besides TPO, SDF1 and SCF, IL6 and IL11 influence MK differentiation to a minor extend. The erythropoietic stimulator EPO is also involved in the maturation of MKs, but the exact role has not been clarified yet (33).

Interferon alpha belongs to the negative regulators of megakaryopoiesis: the cytokine inhibits the generation of the transcription factors GATA1 and NF-E2 and hence prevents the late differentiation steps of MKs. Interferon gamma has the opposite effect: it activates STAT1 (a downstream target of GATA1) and induces polyploidization and maturation of MKs (33).

1.1.3 Regulation of megakaryopoiesis by transcription factors

Under the influence of TPO and certain transcription factors MEPs differentiate into MKs. Transcription factors, which are involved in megakaryopoiesis, can be classified into two types: those, which regulate the differentiation process (e.g. GATA1), and those, which regulate the proplatelet formation (e.g. NF-E2).

The transcription factor GATA1, a zinc-finger DNA binding protein, which forms a heterodimer with FOG1 (Friend of GATA1), is essentially involved in megakaryocytic and erythroid differentiation (62). GATA1 activates specific megakaryocytic genes such as Glycoproteins, the TPO receptor and platelet factor 4 (29) and promotes polyploidization by activating STAT1 (33). While GATA1 and FOG1 promote the maturation of MKs and erythrocytes, they inhibit myeloid differentiation (50). The knockout of GATA1 in mice is embryonically lethal due to severe anemia, whereas a specific knockdown of GATA1 in MKs leads to a low level of platelets and an increased number of immature MKs (63).

Fli1 (Friend leukemia integration 1) belongs to the Ets gene family, characterized by a highly conserved DNA binding element, the Ets domain. Fli1 proved to be essential for megakaryopoiesis, as it activates MK-specific genes like GATA1, GlycoproteinIIb, GlycoproteinVI, GlycoproteinIX and GlycoproteinIb, as well as c-Mpl on transcriptional level. Like in GATA1, mice with a knockout of Fli1 die at embryonic stage. It is suggested that this Ets-family member supports megakaryocytic differentiation, while preventing erythropoiesis (29).

In comparison to Fli1, the expression of c-Myb - a proto-oncogene - promotes erythropoietic differentiation and its expression level is reduced during megakaryocytic maturation. It was shown that the micro RNA miR-150 has a negative effect on c-Myb expression during megakaryopoiesis (50).

The expression of Runx1 is increased in cells, which undergo megakaryopoiesis and decreased during erythropoiesis. Deletion of Runx1 in mice is embryonically lethal, the knockdown in MKs leads to small MKs with a lower degree of ploidy and decreased platelet counts (29). Runx1 was shown to interact with other megakaryocytic factors like Fli1 and GATA1 (50).

The transcription factor nuclear factor-erythroid 2 (NF-E2) plays an important role in terminal thrombopoiesis as it is regulating the expression of genes involved in the late stage of platelet

generation. These genes include beta-tubulin, Thromboxane synthase and Rab27b. Complete knockout of NF-E2 in mice was shown to be neonatally lethal due to thrombocytopenia. These mice had increased levels of MKs, which were not able to differentiate into platelets (29).

A transcription factor with a yet unknown function in megakaryopoiesis is the hypoxia inducible factor (HIF). The investigation of the role of HIF in megakaryopoiesis is an important aim of this study.

1.2 Hypoxia inducible factor

Many critical processes in mammals are dependent on oxygen, therefore it is important to maintain oxygen homeostasis. The most important regulator in this tightly regulated mechanism is HIF (64). Genes having a role in oxygen transport, glucose uptake and glycolysis as well as angiogenesis are regulated by HIF (65).

1.2.1 Structure and different forms of HIF

HIF alpha and ARNT (aryl hydrocarbon nuclear translocator, also known as HIF1 beta) form the heterodimeric transcription factor HIF. In contrast to ARNT, which is constitutively expressed, the expression of the HIF alphas is controlled by oxygen levels. HIF binds to the HIF response element (HREs), a core DNA motive (G/ACGTG) in the promoter region of target genes (66). Three different isoforms of HIF alpha are known: HIF1 alpha, HIF2 alpha (also endothelial PAS, HIF-like factor, HIF related factor and member of PAS super family 2 (MOP2)) and HIF3 alpha with several splice variants. HIF1 alpha and HIF2 alpha have 48% amino acid sequence identity (64).

All HIF alphas as well as ARNT belong to the basic helix-loop-helix (bHLH)/PAS protein family. The bHLH domain consists of two amphipathic alpha helices, separated by a loop of variable length, forming the primary dimerisation interface between family members. HIFs have a Per/Arnt/Sim-domain (PAS), 200-300 amino acids, consisting of two loosely conserved, largely hydrophobic regions of approximately 50 amino acids, called PAS A and PAS B. They represent a secondary dimerisation interface between family members. Additionally HIF1 and HIF2 alpha contain two transactivation domains (TADs), separated by an inhibitory domain, which is responsible for normoxic repression of TAD activity. An

oxygen-dependent degradation domain (ODDD) is overlapping with the amino-terminal TAD and leads to normoxic instability of the HIF alpha proteins (Fig. 3) (64).

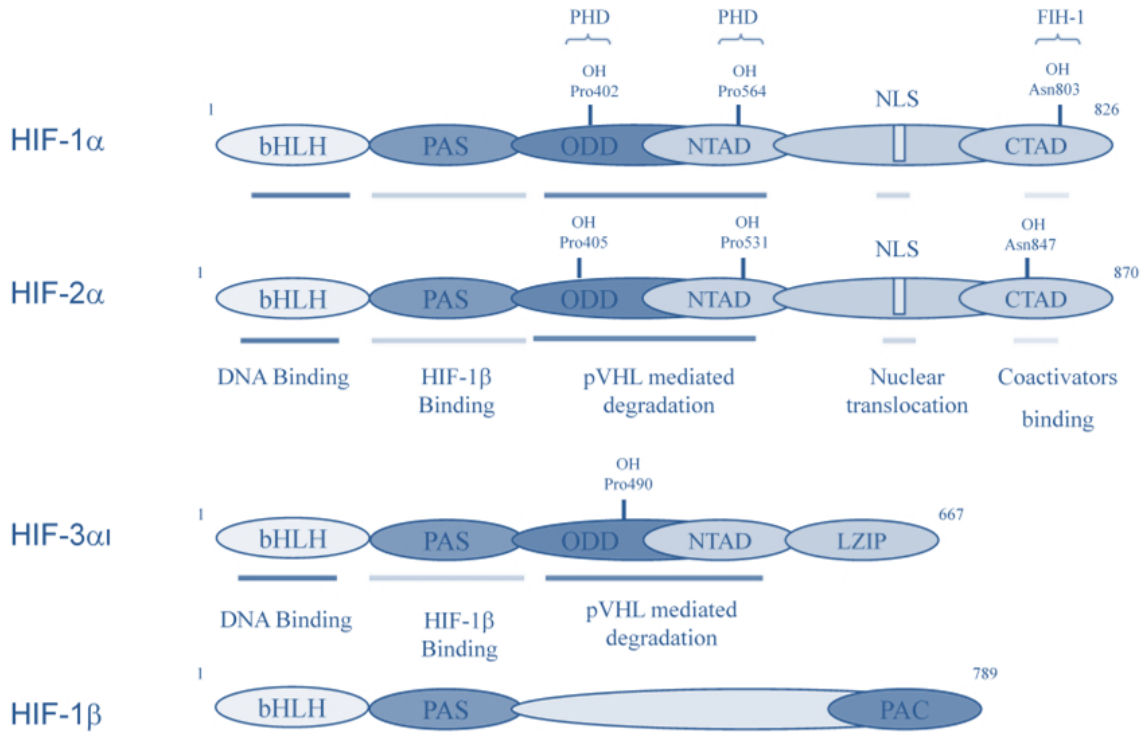


Fig.3: Structure and different forms of HIF (67)

1.2.2 Regulation of HIF

The HIF alpha RNA is expressed ubiquitously and independently of the oxygen supply, but the HIF alpha protein is not detectable under normoxic (21% O2) conditions. The regulation of HIF includes post-translational modifications of the transcription factor leading to an increased protein stability on the one hand and an increased transactivation capacity on the other hand (64).

The dioxygenases prolyl hydroxylase domains (PHDs) are responsible for the catalyzation of prolyl hydroxylation reactions in the ODDD (prolyl 402 and 564 in HIF1 alpha, prolyl 405 and 531 in HIF2 alpha) of HIF alphas. Oxygen, iron, 2-oxoglutarate and ascorbate (68) are required cofactors for this reaction (66). Hydroxylation of these residues is a recognition signal for the tumor suppressor von-Hippel-Lindau (VHL), an E3 ubiquitin ligase that supports Lys48 linked ubiquitination and final degradation by proteasomes (68).

In the original model, PHDs are the direct sensors of oxygen levels: as oxygen levels are decreased during hypoxia, it is not longer available for PHDs to act as a cofactor. Subsequently PHDs cannot hydroxylate HIF, VHL cannot recognize HIF and HIF is not degraded. HIF alpha then translocates to the nucleus, binds to ARNT and acts as a transcription factor on target genes (65).

Emerging evidence suggests that the mitochondrial electron transport chain plays a role in oxygen sensing. There are two theories about this involvement: 1) the "oxygen consumption model" suggesting that mitochondria consume all the oxygen during hypoxia, leaving the rest of the cell "anoxic" and 2) a model where reactive oxygen species (ROS), generated during mitochondrial electron transport chain in hypoxia, acts as a signaling molecule to stabilize HIF (69). Nitric oxide, ROS and tricarboxylic acid cycle intermediates like succinate and fumarate have been shown to prevent the enzymatic activity of PHDs. It is suggested that iron bound to PHD is oxidized by ROS, as it was shown to inhibit the catalytic function of PHD directly. The mitochondrial deacetylase sirtuin-3 (SIRT3) is also involved in HIF alpha regulation: the protein hinders the production of ROS, thereby supporting the enzymatic activity of PHDs and the proteasomal degradation of HIF alpha (70).

As already mentioned HIF1 and HIF2 alpha both contain an N-terminal and a C-terminal transactivation domain. The factor inhibiting HIF 1 (FIH1) - a dioxygenase - catalyzes the hydroxylation of asparaginyl in the most C-terminal transactivation domain of HIF alpha (67). If not hydroxylated, TADs have the ability to bind to the coactivators p300/CBP (Creb-binding protein), a histone acetyl-transferase, which leads to the activated transcription of target genes in hypoxia (64). Very recently a study of Masson et al. revealed FIH to be even more sensitive to oxidative stress than PHDs. Future studies will determine the particular role and relevance of ROS in hypoxic signaling (71).

The protein sentrin-specific protease 3 (SENP3) has the capacity to de-SUMOylate p300 under oxidative stress, thus facilitating the interaction between p300 and HIF1 alpha and enhancing the transcription of HIF target genes. In the case of p300/CBP-associated factor (PCAF) acetylation of HIF1 alpha leads to the same effect. In addition interaction of HIF1 alpha and p300 is promoted by Pontin, a chromatin-remodeling factor in hypoxic conditions (68).

CITED2 and CITED4 were found to bind p300, thereby blocking the interaction of HIF1 alpha and p300 and inhibiting the transactivation of HIF1 alpha. It is suggested that CITED2

has a central role in a negative feedback loop to inhibit HIF-transactivation, as hypoxia is inducing CITED2 expression (68).

Sirtuin-1 (SIRT1) was shown to act as a regulator of HIF alphas by deacetylating HIF1 alpha (72) and HIF2 alpha (73). The deacetylation causes an inhibition of HIF alpha activity due to inhibited recruitment of p300. SIRT1 itself was reported to be regulated positively by HIF1 and HIF2 alpha under low oxygen levels by Chen et al. 2011 (74). Therefore the authors propose that SIRT1 is involved in positive- and negative feedback loops, which contribute to HIF regulation. SIRT6 might also influence HIF1 alpha expression, though the mechanism behind that phenomenon has not been clarified yet (Fig. 4) (75).

PKM2 (pyruvate kinase muscle isozyme 2) recently turned out to be another coactivator of HIF alpha by binding directly to the transcription factor and thereby stabilizing the binding of HIF alpha to HREs. This interaction was shown to facilitate the transactivation of HRE containing genes. PHD3 mediated prolyl hydroxylation of PKM2 led to even more effective transactivation. Furthermore HIF1 but not HIF2 alpha induces the expression of PKM2, proposing that the two proteins are acting in a positive feedback loop under hypoxic conditions (68).

On mRNA level HIF alpha was shown to be positively regulated by STAT3 and to be a mammalian target of rapamycin (mTOR). mTOR was also identified to have a positive effect on the expression of the HIF alpha coactivator PKM2 (68).

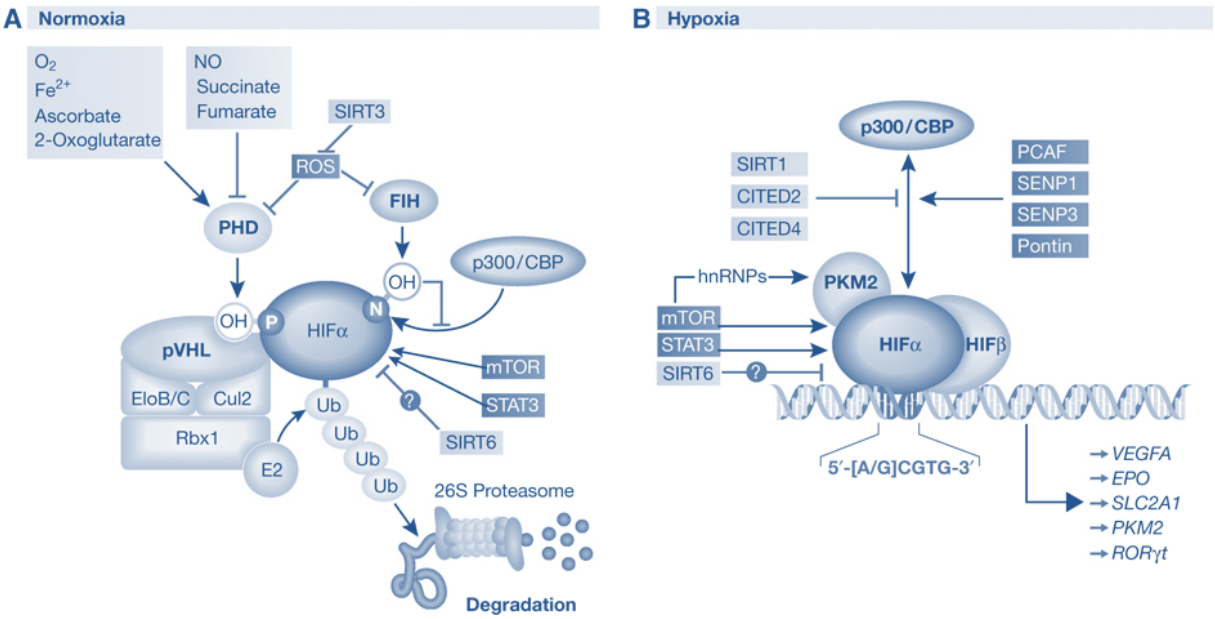


Fig.4: Regulation of HIF (68)

1.2.3 HIF target genes

Functionally three categories of HIF target genes can be distinguished roughly:

- proteins involved in erythropoietic development like erythropoietin (EPO), transferrin, transferrin receptor or Heme oxygenase to ensure oxygen supply in tissues during hypoxia
- proteins which are important for oxygen supply in tissues in situ like vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS)
- proteins necessary for adapting the cellular metabolism to hypoxic conditions like glucose transporter-1 and the most part of glycolytic enzymes (76)

Amongst others VEGF, glucose transporter-1, adipose differentiation-related protein and adrenomedullin have been described to be common targets of HIF1 alpha and HIF2 alpha (77;78). As already indicated in the case of SIRT1, HIF1 alpha and HIF2 alpha are not always regulated in the same way. Taken together with the fact that the two subunits do also show distinct expression patterns - HIF1 alpha is expressed ubiquitously, HIF2 alpha only in particular tissues (endothelium, kidney, lung, heart, and small intestine) - it is likely that they do have distinct biological effects. Knockout studies in mice underscore this assumption as both HIF1 alpha-/- and HIF2 alpha-/- knockouts are embryonically lethal. However, the reasons of this lethality are different. The knockdown of HIF1 alpha leads to death of embryos because of cardiac and vascular defects, whereas HIF2 alpha -/- embryo mice die somewhat later as a consequence of bradycardia, vascular defects and incomplete lung maturation (79).

Enzymes involved in the glycolytic pathway are known to be HIF1 alpha targets (77) as well as BCL2/adenovirus E1B-interacting protein 1 (NIP3/Bnip3), a protein promoting apoptosis (80). In contrast, the regulation of EPO production in the liver is mediated by HIF2 alpha (81). Additionally Oct4 (Octamer binding transcription factor 4), a protein important for the capacity of self-renewal of stem cells, has been described to be a HIF2 alpha target. CyclinD1 and transforming growth factor alpha were shown to be regulated by HIF2 alpha (82) and also in the context of fatty acid storage HIF2 alpha seems to be an important regulator of transcription. Studies in endothelial cells identified HIF1 alpha to be important for VEGF

expression, migration and proliferation of these cells and HIF2 alpha to be necessary for endothelial cells to adhere to the extracellular matrix (83).

Besides the important function of HIF to adapt to changing oxygen tension HIF does also have an impact on the regulation during iron homeostasis. The HIF pathway is involved in sensing iron levels, as well as in regulating iron homeostasis actively (see 1.3.5) (84).

Some of the proteins involved in megakaryocytic differentiation have been shown to be HIF targets. Two examples are the low-density lipoprotein receptor related protein 1 (LRP1) and the C-X-C chemokine receptor type 4 (CXCR4): the former protein is associated with the negative regulator platelet factor 4 (PF4) on MKs and is maximally expressed on polyploid and large MKs while it is neither expressed in megakaryocyte-erythrocyte progenitor cells nor in platelets (85). CXCR4 is the main receptor of SDF1, a homing receptor that is important for migration of megakaryocytes from the bone marrow into the vascular niche (see 1.1.2) and was shown to be an HIF1 alpha target (53). During megakaryocytic differentiation the expression of the receptor increases (33). VEGF, a well-established HIF target, is known for its angiogenic function. Additionally it was found to be involved in early hematopoiesis as well as in maturation stages of megakaryocytic progenitor cells (53).

1.3 Iron

The ability to accept and donate electrons makes iron a very important component of enzymes dealing with oxygen transport, cellular respiration and DNA synthesis (86). Consequences of iron deficiency include cellular growth arrest and cell death. However, free iron can lead to the formation of hydroxyl or lipid radicals, causing damage on all organic molecules including nucleic acids, proteins, and lipid membranes (87). Therefore it is very important to control iron homeostasis tightly (86).

1.3.1 Iron homeostasis

As iron excretion from the body is not a regulatory mechanism in mammals (88), homeostasis has to be controlled by intestinal iron uptake and by binding free iron to scavenging proteins like transferrin and ferritin in order to limit radical formation.

Food contains mostly Heme or inorganic ferric iron, which can be absorbed by intestinal uptake in the duodenum by enterocytes. Ferric iron is reduced by ferric reductases, e.g. DcytB

(duodenal cytochrome B) to ferrous iron, which is able to pass the apical membran of enterocytes via the DMT1 (divalent metal transporter 1). Heme gets into the cell through HCP1 (89;90), where it is subsequently degraded through HO1 (Heme oxygenase) to release iron. Intracellular iron is exported into the circulation via ferroportin, oxidized to Fe^{3+} by Hephaestin (multicopper oxidase) and subsequently scavenged by transferrin (91).

The most common pathway for iron uptake is via the Transferrin receptor 1 (TfR1). TfR1 is expressed ubiquitously, binds with high affinity to iron-bound transferrin and the whole complex gets internalized. The early endosome is acidified and leads to iron release (92). Subsequently members of the STEAP family of metalloreductases (93;94) reduce iron to Fe^{2+} and iron is transported into the cytosol via DMT1. Apo-transferrin and TfR1 translocate back to the cell surface (95).

Once in the cell, iron can be used for the synthesis of Heme or iron sulphur cluster (ISC) within the mitochondria (Fig. 5) (96). Intracellular excess iron can be stored in the form of cytosolic ferritin, exported via ferroportin or in the case of Heme via the Heme transporter FLVCR.

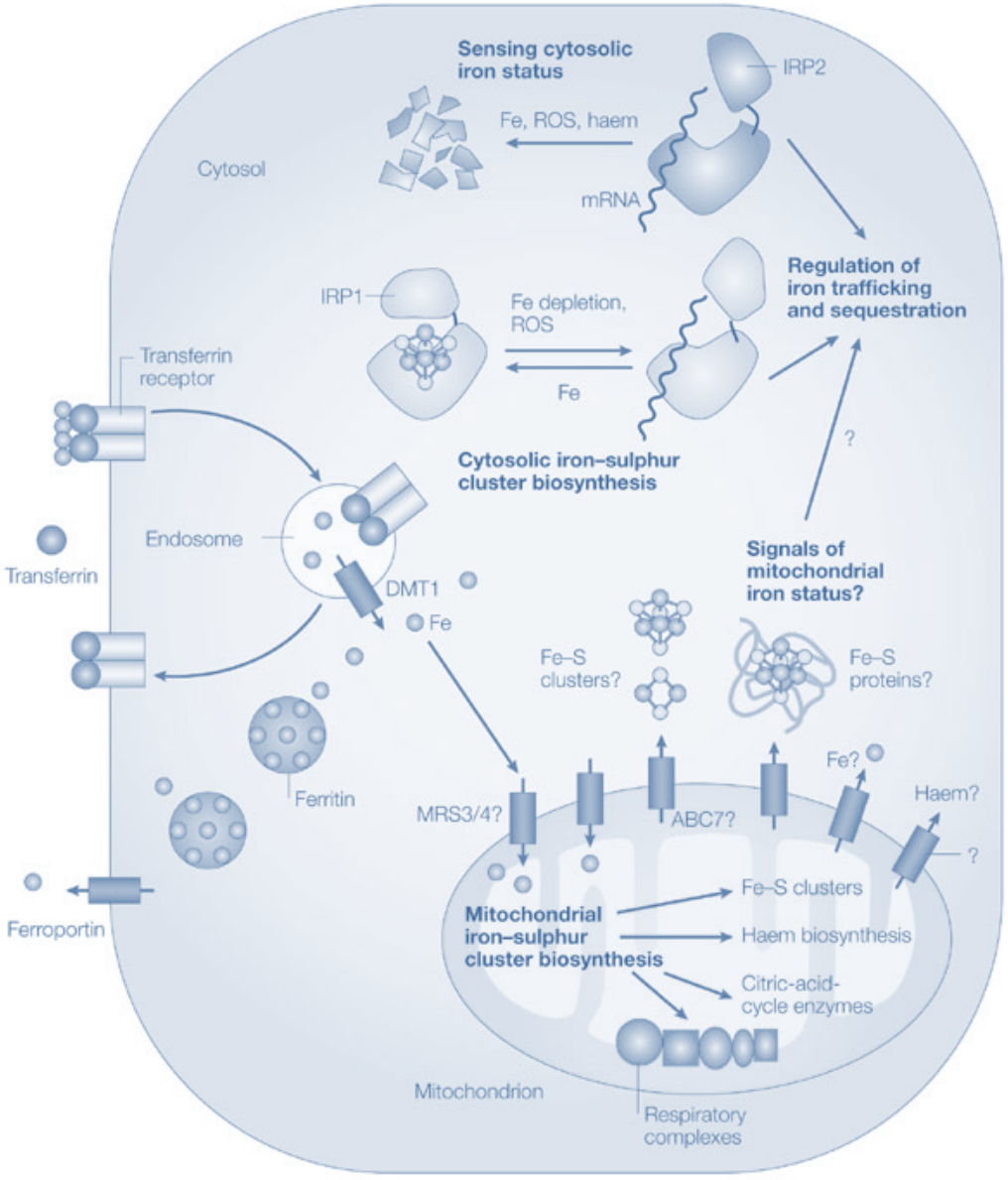


Fig.5: Iron homeostasis (96)

1.3.2 Systemic iron regulation by Hepcidin

Hepcidin, which is produced by hepatocytes, has a central role in systemic iron regulation: it binds to ferroportin on the surface of cells and thereby propagates its internalization, ubiquitination and lysosomal degradation. This process leads to blocked iron absorption from enterocytes, blocked iron recycling from macrophages and blocked mobilization of iron stored in the liver, resulting in low plasma iron levels, whereas iron levels in organs and cells remain high (95;97).

The regulation of Hepcidin is influenced by the availability of iron stores in hepatocytes, by erythropoiesis, by inflammatory signals and by hypoxia (95).

Excess iron leads to the upregulation of Hepcidin expression, thereby preventing iron overload. On the contrary, in ID Hepcidin expression is downregulated. This facilitates dietary iron absorption and enhances the accessibility of iron from storage organs (91).

Bone morphogenetic protein 6 (BMP6) is a member of the TGFbeta super family (97) and plays an important role in Hepcidin regulation dependent on iron availability: the protein is positively regulated by iron and functions as an iron sensor. It forms a complex with the BMP coreceptor haemojuvelin (HJV) and binds to type I (Alk2 and Alk3) and type II (ACTRIIA) receptors. Together they promote the phosphorylation and activation of receptor activated SMAD (R-SMAD) proteins leading subsequently to an active transcriptional complex of SMAD4. A soluble form of HJV was discovered, which competes with HJV bound to the hepatocytic membrane for BMP and has a negative effect on Hepcidin expression (98).

Erythropoietic signals have an influence on Hepcidin transcription independently from iron availability. This fact possibly serves as a mechanism to ensure sufficient iron supply for erythropoiesis. Erythroid precursors release the soluble factors GDF15 (growth differentiation factor 15) and TWSG1 (twisted gastrulation protein 1), which were shown to inhibit the transcription of Hepcidin. However, the complete molecular mechanism is still an open question (91;99).

Like erythropoietic signals proinflammatory cytokines, such as IL1 (100) and IL6 (101), induce Hepcidin expression independently from the supply of iron. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is activated by IL6, leading to the binding of STAT to a STAT binding motif near the transcription start site of the Hepcidin promoter and thereby induces Hepcidin transcription. This pathway acts in synergy with the BMP signaling cascade via SMAD4 (102). It is thought that cytokine-induced Hepcidin transcription is a strategy to limit iron availability to microorganisms (91).

Hypoxia or chemically induced stabilization of HIF1 and HIF2 in the liver was shown to decrease Hepcidin expression (84) suggesting a role of iron dependent PHDs in sensing hepatic iron. A direct binding of HIF to the Hepcidin promoter has not been shown yet (95).

1.3.3 Cellular iron regulation by the IRE/IRP system

On cellular level, iron homeostasis is ensured posttranscriptionally by the two orthologous RNA-binding proteins, iron regulatory protein 1 and 2 (IRP1 and IRP2), which bind to conserved cis-regulatory hairpin structures - termed iron responsive elements (IRE) - in the 5' and 3' untranslated regions (UTRs) of target genes (95). Translation initiation is inhibited when IRP1 or IRP2 bind to the single 5' UTR IRE of mRNAs, whereas IRP1 or IRP2 binding to multiple IRE motifs in the 3' UTR of mRNAs hinders the endonucleolytic cleavage and degradation of the mRNA (87;95). In summary, the IRE/IRP system ensures that genes, which are important for iron storage, export and utilization are downregulated and proteins involved in iron uptake are upregulated in an iron deficient state. Additionally IREs were found in the 3' UTR of CDC14A (cell division cycle 14 homolog A), CDC42BPA (CDC42 binding protein kinase alpha) and HAO1 (Hydroxyacid oxidase 1) with yet undetermined relevance (103;104;105).

Cellular iron levels are responsible for the capacity of IRP binding to IREs. However, IRP1 and IRP2 sense cellular iron levels differently. In iron replete cells IRP1 is bound to a cubane 4Fe-4S cluster, which prevents the mRNA binding ability of IRP1 (87;106). IRP1 bound to the 4Fe-4S cluster has then the ability to act as a cytosolic aconitase. Iron deficiency leads to disassembly of 4Fe-4S from IRP1 and subsequently IRP1 is able to bind to IRE (95). IRP2 binds to FBXL5 (F-box and Leucin repeat protein 5) in iron replete cells. Together with SKP-Cul1-F-box E3 ligase complex FBXL5 binding leads to proteasomal degradation of IRP2. FBXL5 senses the cellular iron levels by a Hemerythrin-like domain, which binds iron directly, leading to stabilized FBXL5. In iron deficient cells no iron is bound to Hemerythrin, FBXL5 is degraded and IRP2 can bind to the target mRNA (107;108).

Beside iron, oxygen levels do also have an influence on the binding capacity of IRP1 and 2 to IREs: in hypoxic conditions IRP1 is inactivated, while IRP2 is stabilized. Together with ROS and the phosphorylation state of IRP 1 and 2, oxygen levels are thought to allow the fine-tuning of IRP/IRE regulation, independently from iron levels (95). This regulation system gets even more complex considering that the stability of TfR1 mRNA (e.g. in erythroid progenitors) is not solely dependent on iron levels and the IRE/IRP (109) and that the Ferroportin mRNA of erythroid and duodenal precursor cells do not possess an IRE. It is thought that this phenomenon ensures erythropoiesis restriction and facilitated iron uptake in the intestine in states of ID (110).

1.3.4 Iron deficiency

As described above iron is a critical component of several important cellular functions. Therefore it is obvious that ID leads to restrictions on multiple cellular mechanisms like proliferations, growth and differentiation of cells. In order to adapt to the state of ID and to ensure facilitated utilization of iron, the cellular metabolism is altered. This alteration involves the already mentioned IRE/IRP system, the HIF pathway but also the redox balance of cells leading to modified free radical production, which in turn has an effect on the JNK kinase pathway, p53 and the NFkappaB. As another consequence of iron deficiency the production of ISC and Heme is blocked which leads to an altered mitochondrial metabolism. The lack of ISCs induces impaired enzyme function during DNA synthesis and repair. In addition, cytochromes containing this cofactor are affected. Heme deficiency compromises multiple enzymes, which contain Heme as a cofactor. Additionally signaling pathways involving Heme as a signaling molecule are affected (Fig. 6)(91;111).

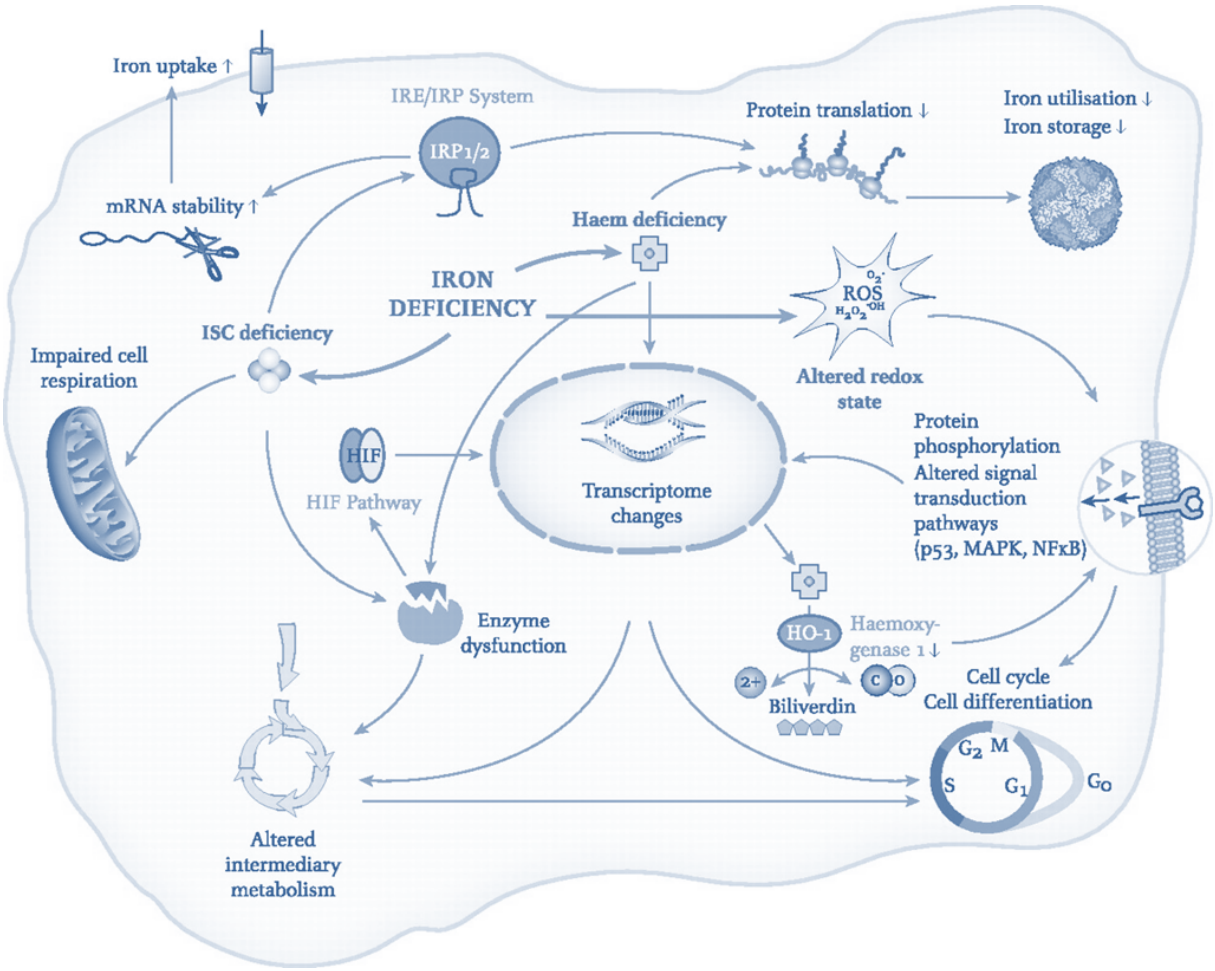


Fig.6: Signaling in iron deficiency (91)

The underlying molecular mechanism of how MKs are affected by ID has not been clarified yet - although already in the 1970s increased platelet counts and increased size of MKs with no effects on the number of MKs were observed in iron deficient rats (19;23). As discussed earlier, polyploidy could be a strategy to increase platelet output without performing all aspects of mitosis (33), suggesting that a higher state of polyploidy could lead to increased production of platelets. The iron deficient rat model, established in our laboratory, with high counts of platelets and observed changes in the bone marrow led to the hypothesis that enhanced megakaryocytic differentiation was the cause for the state of thrombocytosis (22). First studies in megakaryocytic cell lines emphasized this suggestion by showing increased polyploidy under iron deficient conditions (24;110).

However, the involvement of the transcription factor HIF during regulation of iron deficiency and also during megakaryopoiesis, as described below, makes HIF an interesting factor to study concerning its role during the regulation of iron deficient megakaryopoiesis.

1.3.5 Iron homeostasis and HIF

As already mentioned, PHDs play an important role during the regulation of HIF. The activity of PHDs is not only dependent on the availability of oxygen but also on the availability of iron: this fact gives PHDs the ability to sense intracellular iron levels. In ID PHDs cannot perform the hydroxylation of HIF alpha and the protein is stabilized, then acting itself as a transcription factor for several target genes. In addition, Peyssonnaux et al. were able to show that HIF negatively regulates Hepcidin (84). Even more importantly, HIF2 alpha but not HIF1 alpha was shown to be stabilized in enterocytes under ID, inducing thereby the expression of DMT1 and DcytB (112;113). Another evidence that the HIF pathway and iron metabolism are closely connected was found 2007 by Sanchez et al. identifying an IRE in the 5' UTR of the HIF2 alpha mRNA (114). The significance of this IRE is not known yet, but it is suggested to be a mechanism limiting the generation of erythrocytes induced by hypoxia in states of iron deficiency. EPO was shown to be induced by HIF in hypoxia to enhance erythropoiesis (115) and it was supposed that it thereby inhibits the expression of Hepcidin indirectly (116;76).

A HRE element was found in the genes of transferrin and TfR1 indicating that they are HIF targets (117;118;119). Well known targets of HIF1 are HO1 (120) and Coeruloplasmin (121) ensuring the recycling of iron in states of hypoxia in tissues. In summary, three strategies are pursued by activation of the HIF pathway in order to increase the bioavailability of iron: the

intestinal iron absorption is enhanced, iron recycling is stimulated and the production of Hepcidin is inhibited (91).

2 Hypothesis

Many pieces of evidence indicate that ID and thrombocytosis correlate positively. However, the mechanism behind this phenomenon has not been clarified yet. The high prevalence of ID all over the world emphasizes the importance to understand the molecular mechanism behind that incidence in order to develop and apply more targeted therapies in the future. In the case of patients who show ID associated thrombocytosis combined with IBD these targeted therapies would be even more critical.

Theoretically ID can influence megakaryopoiesis at all differentiation levels to cause finally increased counts of platelets:

- MEPs: by inhibiting differentiation of erythrocytes and thereby inducing a shift towards the MK lineage
- megakaryocytic progenitor cells: by enhancing their proliferation or accelerating their maturation
- maturing MKs: by increasing their polyploidy and thereby increasing their capacity to produce platelets
- thrombopoiesis: by enhancing the production of proplatelets and platelet shedding

The important role of transcription factors as regulators during megakaryopoiesis has already been described in the introduction. Preliminary data in Dami cells, a megakaryocytic cell line (110), and in the new established in vitro model of megakaryopoiesis under ID (24) indicate a state of increased polyploidy under ID. This observation led to the assumption that ID leads to a changed regulation of megakaryopoiesis, possibly caused by an altered expression of transcription factors. The fact that HIF is regulated by an iron sensitive PHD and the fact that it is involved in iron homeostasis and in megakaryopoiesis (see introduction) make HIF a potential transcription factor playing a role in iron deficient megakaryopoiesis.

We hypothesize that HIF1 alpha and HIF2 alpha are involved in the regulation of iron deficiency induced MK differentiation including endomitosis. We expect that these proteins are expressed in cells of the MK lineage, show nuclear localization and change the expression

pattern parallel to DNA ploidy and iron content. Furthermore we assume that HIF1 alpha and HIF2 alpha play a differential role during the process of iron deficient megakaryopoiesis.

3 Aim

The aim of this study is to identify the role of HIF in the regulation of iron deficiency induced megakaryopoiesis by investigating the expression pattern of HIF and its target genes in megakaryocytic cell lines under iron deficiency and iron repletion. To verify the role of HIF in megakaryocytic maturation, we analyze megakaryocytic differentiation markers and HIF target genes in megakaryocytic cell lines overexpressing HIF1 alpha and HIF2 alpha.

4 Materials and Methods

4.1 Cell lines, culture conditions and treatments

The experiments are based on the megakaryocytic cell lines CMK and HEL (DSMZ, Germany). The cell line CMK is derived from the peripheral blood of a patient with Down's syndrome and acute megakaryoblastic leukemia. CMK cells express megakaryocytic markers (GlycoproteinsIIa/IIIb and IB and Plt-1) as well as erythropoietic (Glycophorin A) and myeloid (MY4, MY7 and MY9) markers (122). HEL is established from the peripheral blood of a 30-year old man with erythroleukemia after treatment for Hodgkin lymphoma. Cells express markers of all three myeloid lineages: granulocyte, erythrocyte and MK antigens (123). The cell line carries the JAK2 V617F mutation (59).

Cell lines were grown in suspension in RPMI-Medium (Gibco, U.K.) supplemented with 10% FCS and 1% Penicillin Streptomycin (Gibco). One week before the start of experiments involving iron deficiency, cells were transferred in the serum-free medium Panserin 401 (PAN Biotech, Aidenbach, Germany) supplemented with 1% Penicillin Streptomycin for adaption. To induce iron deficiency, cells were transferred into the customized iron-free and transferrin-free Panserin 401S medium (PAN Biotech, Aidenbach, Germany), supplemented with 1.25% Panserin 401. Alternatively, iron deficiency was induced by adding Deferoxamine (Sigma, D9533) to Panserin 401 at a concentration of 25 or 50 µM.

PMA (phorbol-12-myristate-13-acetate) is a well-established inducer of megakaryopoiesis in human megakaryocytic cell lines like HEL and CMK (124). Cells treated with this phorbol ester show increased expression of megakaryocytic differentiation markers, increased

polyploidization and multi-lobulated nuclei, increased cell-to-cell and cell-to-substrate adhesion, as well as a strongly reduced proliferation (125;126;127). The regulation of gene expression by PMA is based on its capacity to induce protein kinase C (PKC)(126;128) and on subsequent activation of the ERK1/2 and the MAP kinase pathway (127;129;130).

Cells treated with PMA were kept in Panserin 401 with 20nM PMA (Sigma Aldrich, St. Louis, U.S.A) for one hour, were washed three times in PBS and then plated in Panserin 401.

4.2 Western Blotting

Cells were lysed in RIPA-buffer (whole cell extract). To separate nuclear and cytoplasmic fraction cells were lysed in the cytosol extraction buffer (CEB: 10mM HEPES pH7.5, 3mM $MgCl_2$, 14mM KCl, 5% glycerol, 1mM DTT, 1x protease inhibitors) for 10 minutes. By adding 0.2% NP-40 cell lysis was completed. Cells were vortexed for 10 seconds and a centrifugation step at 8600g for 2 minutes at 4°C followed. The supernatant representing the cytosolic fraction was transferred into a new tube and stored afterwards. The remaining pellet was washed three times in CEB and lysed in the nuclear extraction buffer (NEB: 10mM HEPES pH7.5, 3 mM $MgCl_2$, 400mM NaCl, 5% glycerol, 1 mM DTT, 1x protease inhibitors) and set on ice for 30 minutes. A centrifugation at 16100g for 30 minutes on 4°C followed. The supernatant, which represents the nuclear fraction, was transferred into a new tube and stored.

Approximately 30µg of protein were separated on a 10% polyacrylamide gel (BIS-Tris 10% gel, Invitrogen) and transferred onto a PVDF membrane (Immobilon, Millipore).We used rabbit/mouse polyclonal antibody against human HIF1 alpha (Novus Biologicals NB100-449 and NB100-105), rabbit antibody against human HIF2 alpha (Novus Biologicals, NB100-122), mouse antibody against human alpha tubulin (Abcam, ab7291), anti-human ferritin (Sigma-Aldrich, F5012) and rabbit polyclonal antibody against fibrillarin (Abcam, ab5821). IR dye conjugated secondary antibodies were used and detected by utilizing the Odyssey Imager.

4.3 Real-time-PCR

4.3.1 RNA Isolation

We collected around 3 million cells per condition, washed them and lysed them in 1 ml TRIzol® (Invitrogen). After 5 minutes at room temperature the samples were snap frozen in liquid nitrogen and stored at -80°C. Samples were thawed on ice and 200 µl chloroform (Merck, New Jersey, USA) were added. Then the samples were shaken for approximately 30 seconds and left on room temperature for 5 minutes. A centrifugation at 12000 g for 15 minutes at 4°C followed. The upper aqueous phase was transferred to a fresh tube and 500 µl isopropanol (Sigma) were added. Samples were incubated for 10 minutes on room temperature and spun down at 1200 g for 10 minutes at 4°C. Following the careful removal of the supernatant 500 µl of 100% ethanol (VWR, Germany) were added and samples were pelleted at 7500 g for 10 minutes at 4°C. The supernatant was carefully removed and 500 µl of 75% ethanol were added. A centrifugation at 7500 g for 10 minutes at 4°C followed. The supernatant was removed, the pellet was dried for approximately 8 minutes and resuspended in 20 µl RNase free water (Ambion, Texas, USA). To allow complete dissolving, samples were incubated at 60°C for 10 minutes and RNA concentration was measured on Nanodrop 1000 (PeqLab, Erlangen, Germany). The samples were stored on -80°C until they were analyzed.

4.3.2 cDNA synthesis

400ng of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). After thawing of components on ice the 2x Reverse Transcription Master Mix was prepared (per reaction): 2µL 10x RT Buffer, 0.8µL 25x dNTP Mix (100 mM), 2µL 10x RT Random Primers, 1µL MultiScribe^{TM} Reverse Transcriptase, 4.2µL Nuclease-free H_2O and stored on ice. 10µL RNA/Nuclease-free H_2O mix was added to 10µL 2x Reverse Transcription Master mix and loaded into the thermal cycler (Step 1: 25°C for 10min; Step 2: 37°C for 120min; Step 3: 85°C for 5min; Step 4: 4°C ∞). The cDNA was stored at 4°C until it was analyzed.

4.3.3 qRT-PCR

Real-time-PCR was performed on the 7500 Fast Real-Time PCR System using SYBR Green kit FAST (Qiagen) for differentially regulated genes with the specific primer pairs (GAPDH, HIF1 alpha, HIF2 alpha, TfR-1, CXCR4, LRP1, VEGF, Quantitect Primer Assay, Qiagen). 10µL Fast SYBR® Green Master Mix were mixed with 1µL cDNA, 1µL forward primer, 1µL reverse primer and 7µL Nuclease-free water. Thermal cycling conditions: AmpliTaq® Fast DNA Polymerase, UP Activation: 95°C for 20sec; Denature: 95°C for 3sec; Anneal/Extend: 60°C for 20sec; 40 cycles. To analyze the data Applied Biosystems® 7500 Fast Software was used.

4.4 Cell transfection

For overexpression studies 2,5µg of pcDNA3 with inserted HIF1 alpha or HIF2 alpha (kind gifts from Dr. Thilo Hagen) were used respectively. Cells were transfected by electroporation (Amaxa Nucleofector Kit, Lonza) and incubated for 48 hours in RPMI medium. To test transfection efficiency, cells were cotransfected with 2µg pmaxGFP® Vector (Lonza) and flow cytometry analysis on Lab Quanta SC flow cytometer (Beckman Coulter, Fullerton, CA) was performed to screen for GFP positive cells. Transfection was verified by RT-PCR and by western blotting for the transfected construct. The megakaryocytic cell lines were transfected with the empty construct for control.

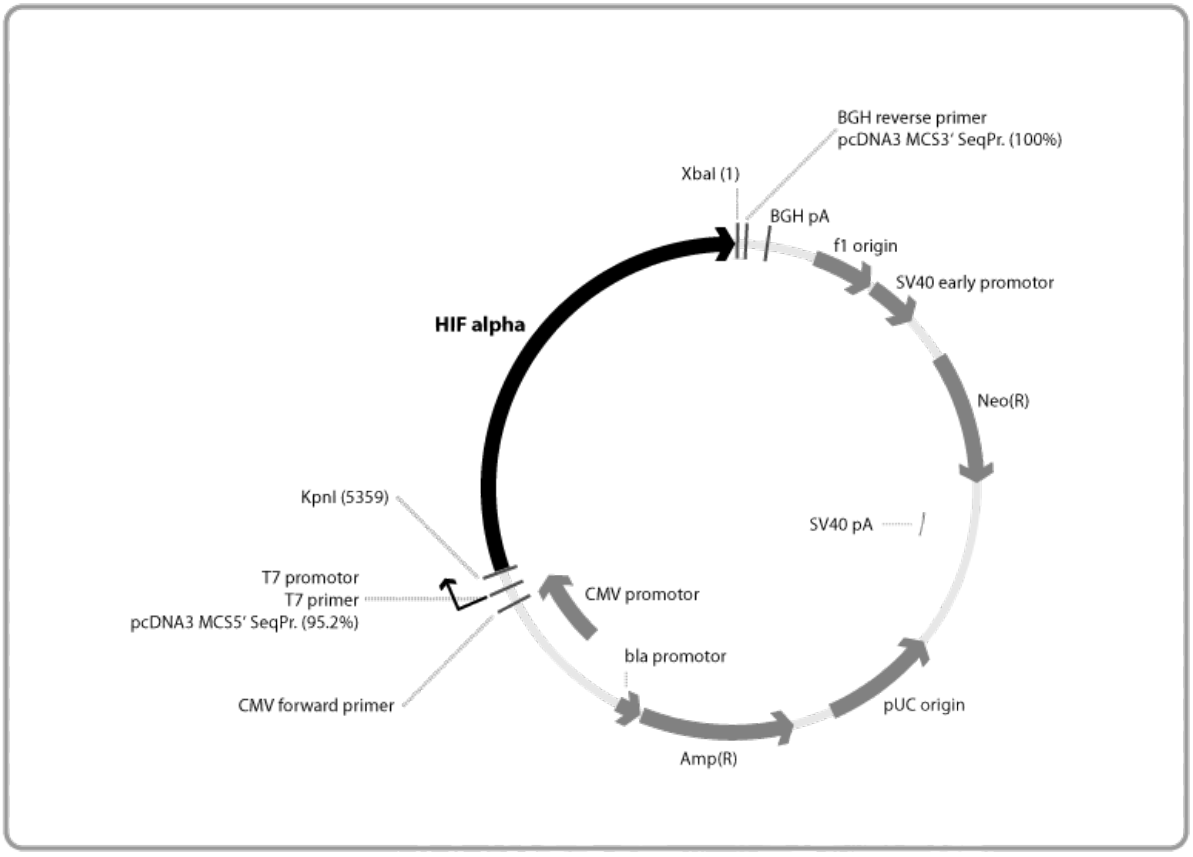


Fig. 7: Plasmid map

4.5 Surface differentiation marker and Transferrin Receptor 1 staining

To determine megakaryocytic differentiation, approximately $2x10^5$ cells were blocked in 125 µl of 20% FCS in PBS for 10 minutes and after adding 2.5 µl antibody (PE Mouse Anti-Human CD42a, BD Pharmingen, 558819; PC7 Mouse Anti-Human CD41, Beckman Coulter, 6607115; FITC Mouse Anti Human CD61, BD Pharmingen, 557291) or 1 µl antibody (PE-CyTM5 Mouse Anti-Human CD235a = Glycophorin A, BD Pharmingen, 561776), cells were incubated for 30 minutes in the dark at room temperature. Cells were analyzed via flow cytometry on Lab Quanta SC flow cytometer (Beckman Coulter, Fullerton, CA) via Cell Lab Quanta Analysis software and the various median fluorescences of cells were compared under defined conditions. To determine the induction of iron deficiency approximately $2x10^5$ cells were blocked in 125 µl 20% FCS in PBS for 10 minutes. After adding 2.5 µl antibody (FITC Mouse Anti-Human CD71, BD Pharmingen 561939) cells were incubated on room temperature for 30 min and analyzed via flow cytometry. The median fluorescences of cells have been compared under defined conditions.

4.6 DNA staining and analysis of ploidy

To determine MK ploidy we measured Hoechst 33342 (Invitrogen, California) staining via flow cytometry on the Cell Lab Quanta SC flow cytometer. Cells were washed, resuspended in 500 µl of PBS and 2 µl of Hoechst (1:4 in PBS) were added. After 1 hour of incubation at 37°C cells were measured via flow cytometry and the data was analyzed via Cell Lab Quanta Analysis software.

4.7 Statistics

Unless mentioned otherwise, error bars in graphs represent standard deviation. For all statistical tests, a P-value below 0.05 was considered significant. Metric variables were analyzed using unpaired t-tests (for variables with two groups) or ANOVA followed by Dunnett's test for post-hoc multiple comparison.

5 Results

5.1 Establishment of growth conditions and readouts

The cell lines HEL and CMK were grown under iron replete and iron deficient conditions over the course of 4 or 7 days, respectively. We used two different ways to induce iron deficiency:

- Panserin 401S (1.25% v/v Panserin 401 in Panserin 401S)
- Addition of the iron chelator Deferoxamine (DFO) at a concentration of 25 and 50 µM

Finally cells were pulse-treated with 20nM PMA for one hour (see methods) as a positive control.

5.1.1 TfR1 expression in ID and PMA treatment

In order to determine the induction of ID TfR1 expression was evaluated on mRNA level and on surface expression level.

Surface TfR1 expression in ID and PMA treatment

Both cell lines showed an enhanced median TfR1 fluorescence in iron-deficient conditions at day 2 (Fig. 8).

In CMK ID induced by Panserin 401S led to a slight upregulation of TfR1 at day 2 compared to untreated cells, a trend which was diminished at day 4 (due to missing data, statistical significance could not be tested) (Fig. 8A). DFO treated cells showed a significant upregulation of the receptor at day 2 in both concentrations ($P = 0.002$ and $P = 0.007$, respectively) in contrast to the untreated and the PMA-treated conditions. However, at day 4 we found opposite results. In comparison to the untreated group TfR1 was downregulated significantly in iron-deficient cells ($P < 0.001$ in both conditions) and PMA-treated cells ($P < 0.031$) (Fig. 8B).

HEL showed a similar picture: Panserin 401S induced iron deficiency led to an upregulation of TfR1 at day 2. As in CMK this trend diminished at day 4 (Fig. 8C). The median TfR1 fluorescence of cells treated with 25µM DFO was higher than the fluorescence of the untreated group. The higher concentration of DFO showed similar results as the untreated group. However, at both days PMA-treated cells showed a low level of TfR1 (Fig. 8D). Due to missing data, the statistical significance could not be analyzed.

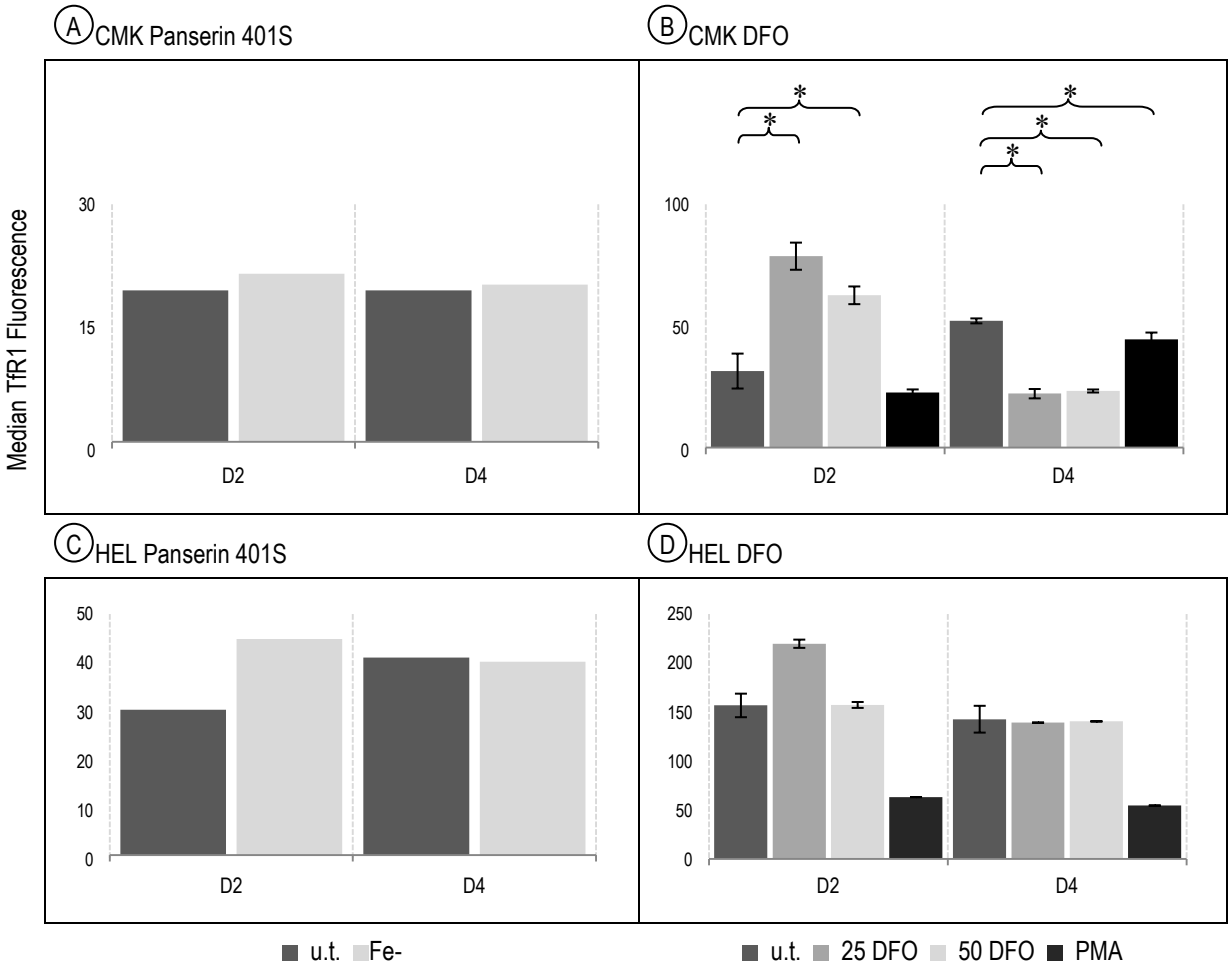


Fig. 8: Surface TfR1

Median Fluorescence of TfR1 positive (A, B) CMK and (C, D) HEL cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Asterisks indicate a P-value < 0.05 compared to untreated cells.

mRNA TfR1 expression in iron deficiency and PMA treatment

Alterations of TfR1 mRNA were analyzed by real-time-PCR in untreated, iron deficient and PMA treated cells (Fig.9).

In CMK, the iron deficient cells induced by Panserin 401S showed an significant upregulation of TfR1 at all days with a maximum increase at day 3 ($P = 0.001$) versus the untreated condition of the same day (Fig. 9A). DFO treated cells showed an upregulation at day 2 ($P < 0.001$ and $P = 0.001$, respectively) and day 4 ($P < 0.001$ in both conditions) compared to untreated cells. At day 4 TfR1 expression was down-regulated in PMA-treated cells ($P < 0.001$) (Fig. 9B).

In HEL, iron deficiency induced by Panserin 401S already displayed an significant increase of TfR1 expression at day 1 ($P = 0.003$) – showing a maximum increase at day 2 ($P < 0.001$). This trend diminished slightly at day 4 and day 7 ($P = 0.028$) (Fig. 9C). DFO induced iron deficiency led to a dose-dependent upregulation of TfR1 at day 2 ($P = 0.006$ and 0.002, respectively). At day 4, TfR1 was still significantly upregulated in comparison to the untreated cells ($P < 0.001$ and $P = 0.002$, respectively). In cells treated with PMA there was a downregulation of TfR1 at both days ($P = 0.029$ and 0.008, respectively) (Fig. 9D).

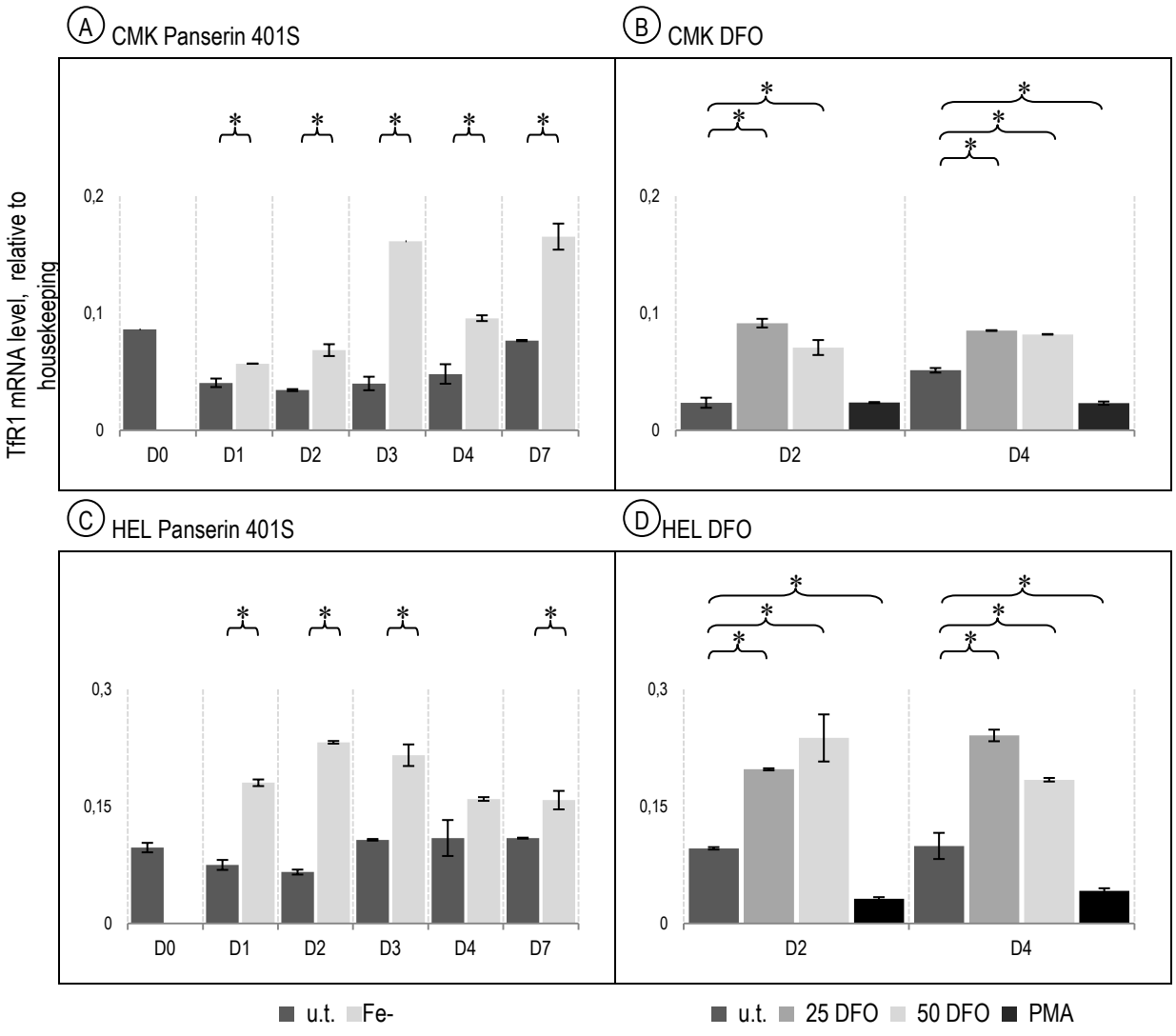


Fig. 9: TfR1 mRNA

qRT-PCR for TfR1 of (A, B) CMK and (C, D) HEL cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control. Asterisks indicate a P-value < 0.05 compared to untreated cells.

Altogether the results indicated an upregulation of TfR1 on both mRNA and protein level in iron deficiency with a maximum in CMK at day 3 and in HEL at day 2. The surface receptor staining and the real-time-PCR results were comparable. PMA treatment led to a downregulation of TfR1 in both cell lines.

5.1.2 Ferritin expression in ID and PMA treatment

To determine changes in the iron storage protein ferritin expression in untreated, iron-deficient and PMA-treated cells, the cells were grown under the three specific conditions. Whole cell lysate proteins were isolated and immunoblotted using a ferritin antibody.

In the cell line CMK a prominent downregulation of ferritin in iron deficient cells induced by Panserin 401S compared to the untreated cells was detected at day 4. To a lower degree this trend was already apparent at day 2 (Fig.10). In contrast to untreated and PMA treated cells, cells, which were treated with 25 µM DFO, showed a downregulation of ferritin at day 2 (Fig. 11).

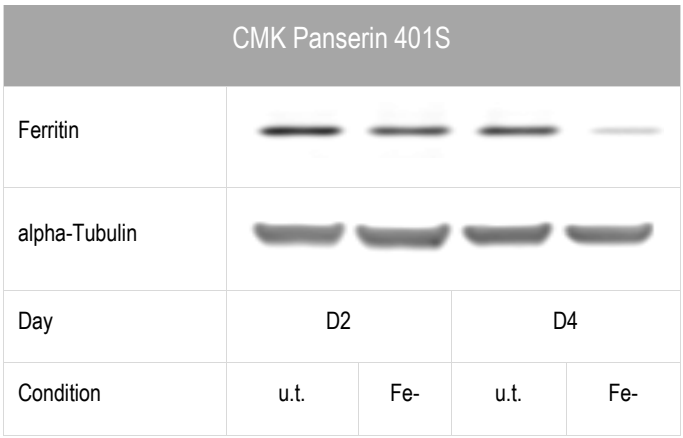


Fig.10: Ferritin protein CMK Panserin 401S

Protein expression of Ferritin in CMK cultured in 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-) for 4 days. Expression of alpha-Tubulin was used as a loading control.

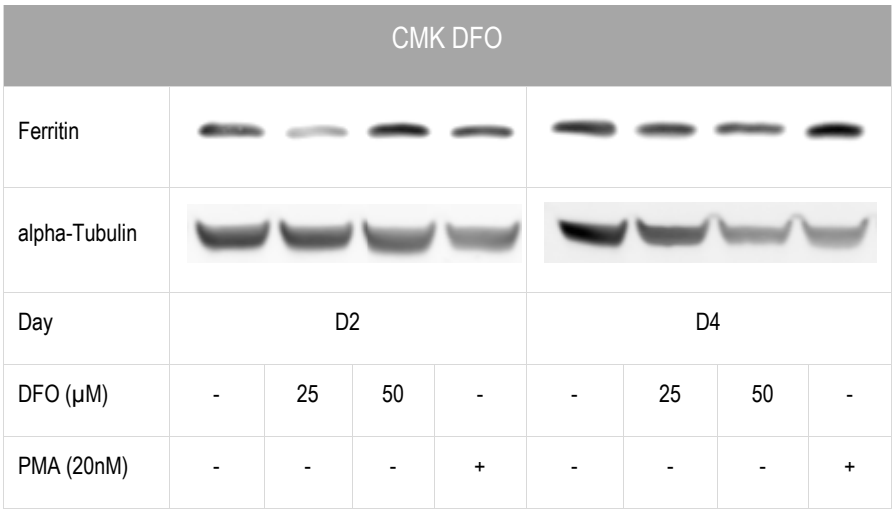


Fig.11: Ferritin protein CMK DFO

Protein expression of Ferritin in CMK cultured in Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Expression of alpha-Tubulin was used as a loading control.

We found similar results in HEL. In comparison to the untreated group iron deficient cells (Panserin 401S) expressed ferritin to a lower degree at day 2 and to an even lower degree at day 4 (Fig. 12). In DFO treated cells the downregulation trend of ferritin expression compared to untreated and PMA-treated cells was most prominent at day 4 (Fig. 13).

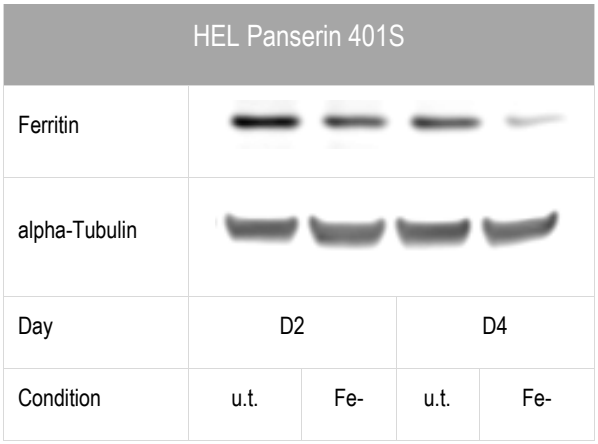


Fig.12: Ferritin protein HEL Panserin 401S

Protein expression of Ferritin in HEL cultured in 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-) for 4 days. Expression of alpha-Tubulin was used as a loading control.

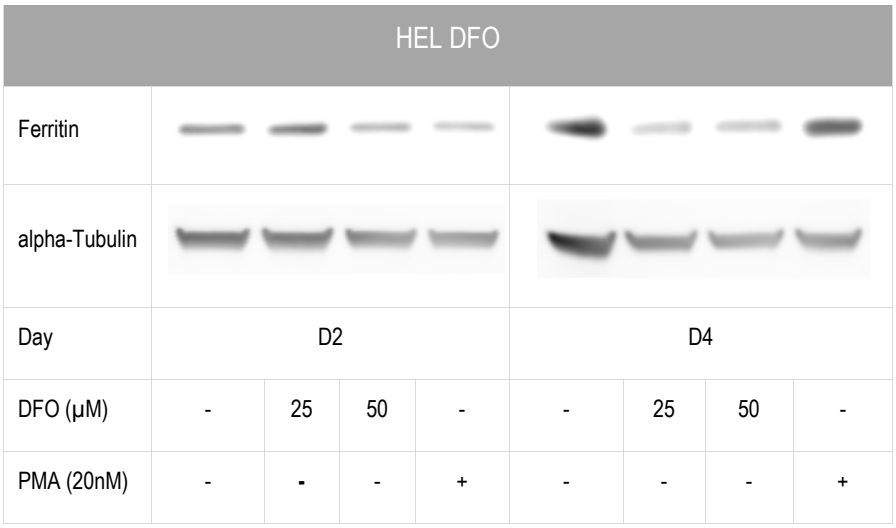


Fig.13: Ferritin protein HEL DFO

Protein expression of Ferritin in HEL cultured in Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Expression of alpha-Tubulin was used as a loading control.

5.1.3 Megakaryocytic differentiation in ID

To evaluate megakaryocytic differentiation in iron deficient and in PMA treated cells the median fluorescence of the megakaryocytic markers CD41, CD42a and CD61 and of the erythropoietic marker Glycophorin A (GpA) was measured (Fig. 14 and Fig. 15).

In CMK, the expression of the two megakaryocytic markers CD41 (Fig. 14A) and CD42a (Fig. 14B) did not change on treatment with DFO in comparison to untreated cells. In PMA treated cells the median fluorescence of CD41 was significantly increased ($P < 0.001$) at day 4. The median fluorescence of CD42 was slightly increased in cells treated with PMA at day 4, but did not reach a significant level. DFO treatment led to loss of the expression of the erythroid marker GpA at day 2 ($P = 0.045$ and 0.010, respectively) and day 4 ($P = 0.003$ and 0.003, respectively) and also PMA treatment led to a down-regulation of the erythroid surface marker at both days ($P = 0.004$ and 0.014, respectively) (Fig.14C).

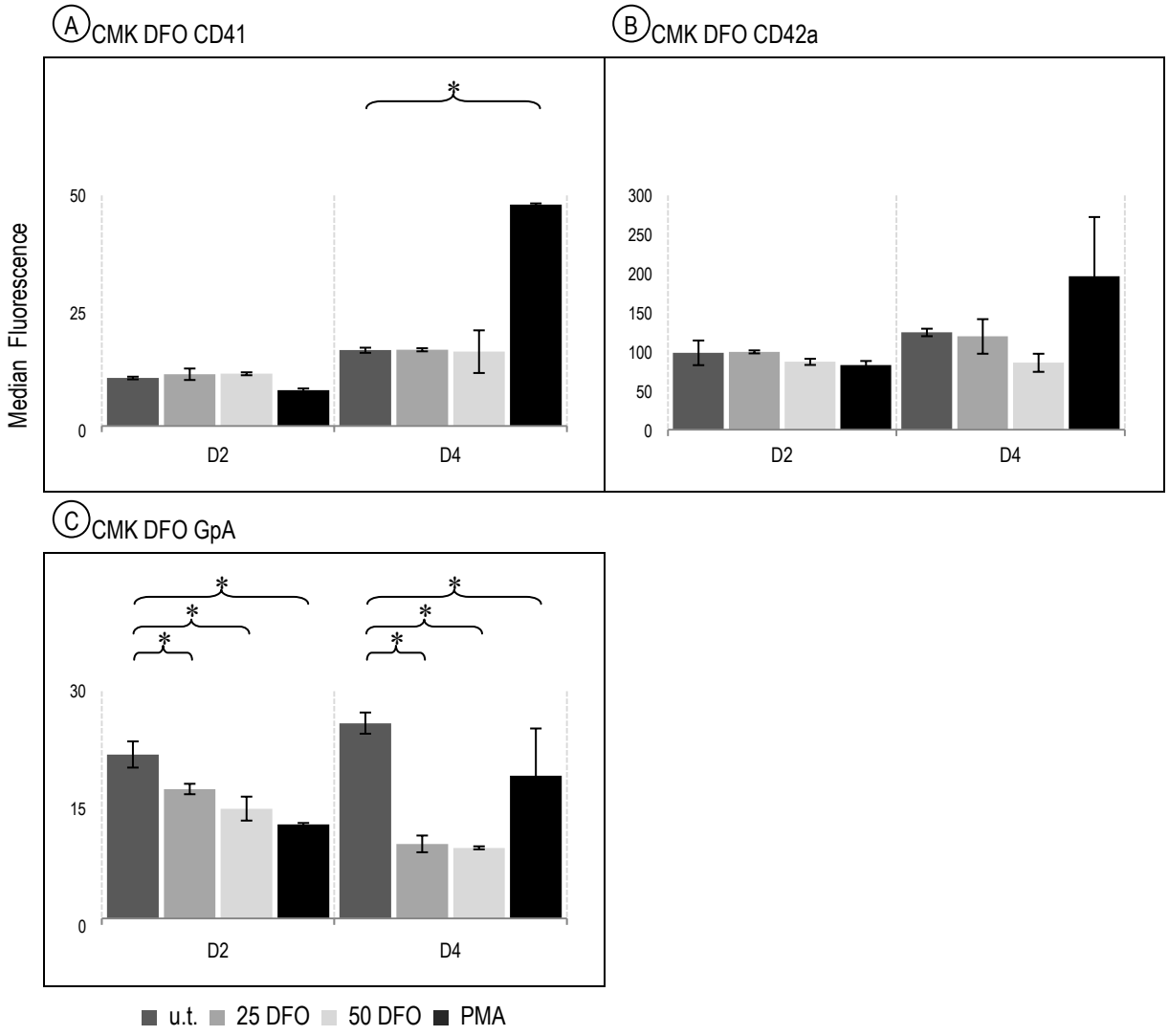


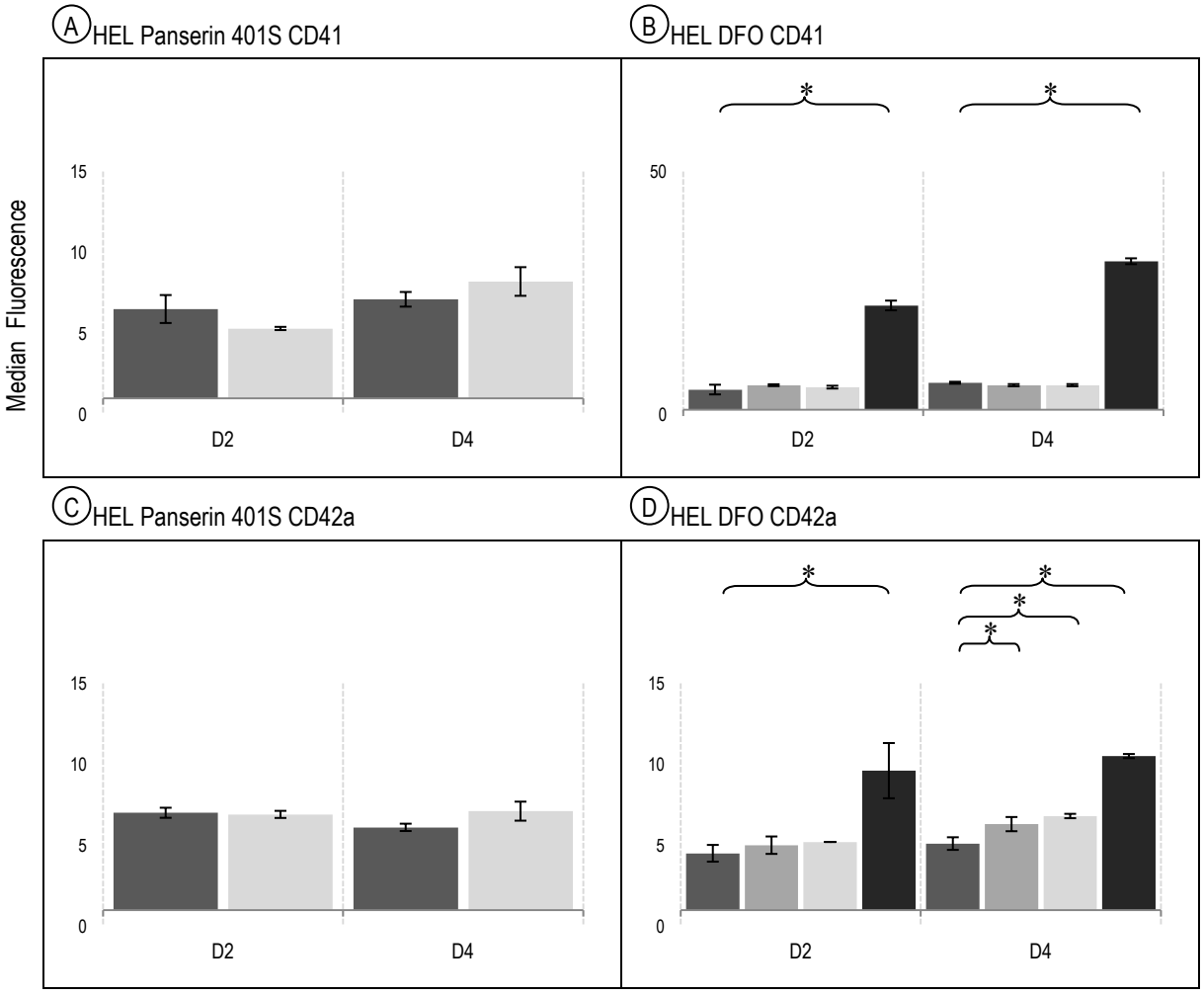
Fig. 14: Surface megakaryocytic markers CMK

Median Fluorescence of (A) CD41, (B) CD42a, and (C) GpA positive CMK cultured in Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Asterisks indicate a P-value < 0.05 compared to untreated cells.

The median fluorescence of CD41 in HEL did not change in iron deficient cells, which had been induced by Panserin 401S (Fig.15A) or DFO (Fig. 15B). Furthermore iron deficiency induced by Panserin 401S did not induce the expression of CD42a (Fig.15C). In DFO treated cells, the median fluorescence of CD42a increased ($P = 0.035$ and 0.010, respectively) in contrast to the untreated group at day 4 in a dose-dependent manner (Fig. 15D). At day 4 the median fluorescence of CD61 increased in iron deficient cells induced by Panserin 401S compared to untreated cells (Fig. 15E). In PMA treated cells the expression of CD41 ($P < 0.001$ at day 2 and 4) and CD42a ($P = 0.012$ at day 2 and $P < 0.001$ at day 4) increased

significantly over time. CD61 showed the same trend with PMA treatment, but did not reach a significant level.

In comparison to the untreated cells of the same day Panserin 401S induced iron deficiency led to a reduced median fluorescence of GpA at day 4 (Fig.15F). DFO treated cells showed reduced expression of GpA already at day 2 – a trend which reached a significant level at day 4 (P = 0.003 and 0.002, respectively) (Fig. 15G). Cells in Panserin 401S showed a similar pattern, but not at a significant level. In cells treated with PMA, the expression of GpA decreased (P = 0.007 at day 2 and 0.023 at day 4).



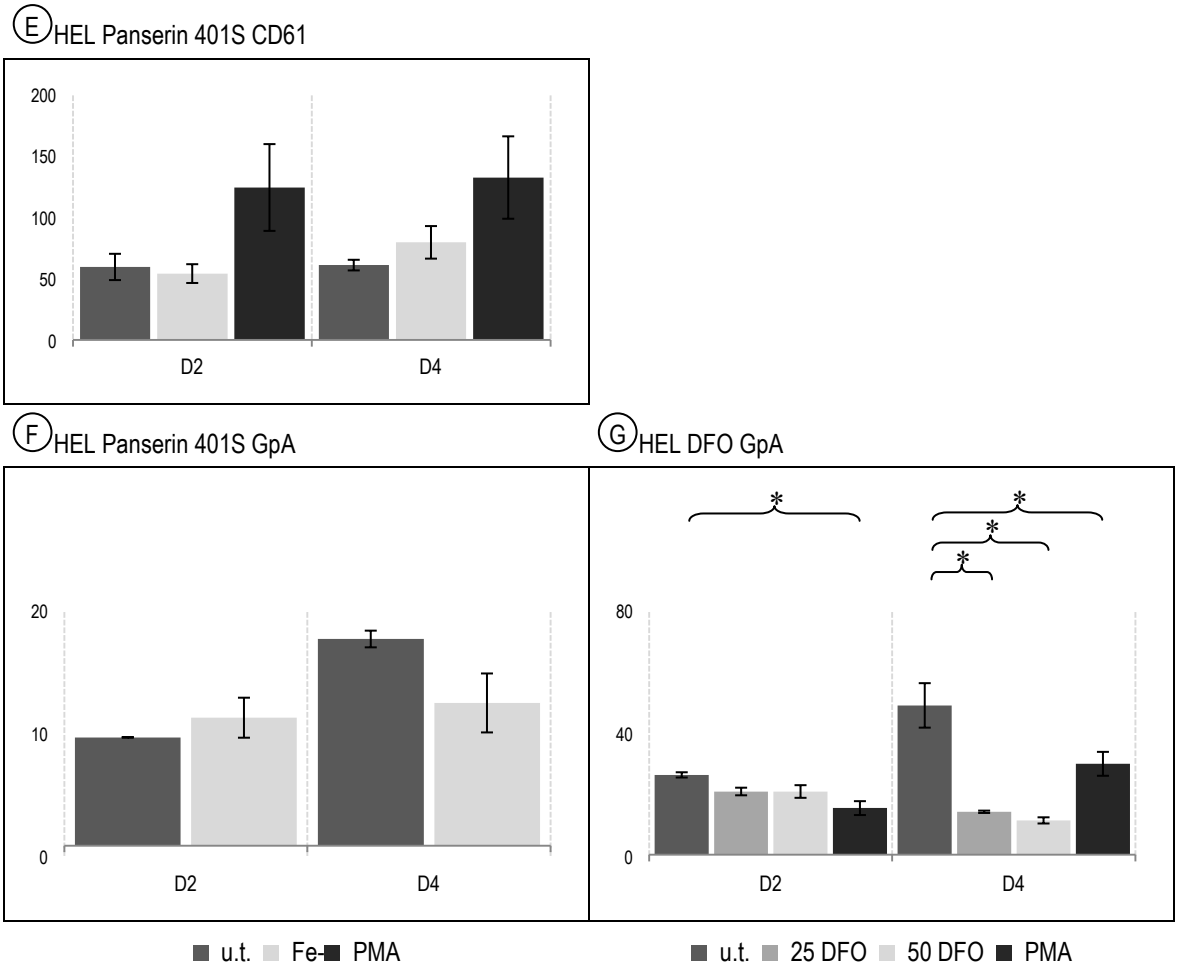


Fig. 15: Surface megakaryocytic markers HEL

Median Fluorescence of (A, B) CD41, (C, D) CD42a, (E) CD61, and (F, G) GpA positive HEL cultured in (A, C, E, F) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D, G) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Asterisks indicate a P-value < 0.05 compared to untreated cells.

5.1.4 Alterations in ploidy under iron deficiency and PMA treatment

One characteristic of megakaryocytic differentiation is the polyploidization of cells. To investigate changes in ploidy under iron deficiency, HEL and CMK were analyzed via flow cytometry after DNA staining with Hoechst 33342. Particular attention was drawn to the >4N population, because this group reflects cells, which are definitely polyploid.

For both cell lines, CMK and HEL, a comparison of iron deficient cells – cultured in 1.25% Panserin 401 in Panserin 401S – to the untreated group showed an increase in the >4N population at day 7, but did not reach a significant level (Fig. 16A and C). However, DFO-

treated cells did not show this effect within 4 days. Only the treatment with PMA led to a slightly increased > 4N population (Fig. 16B and D).

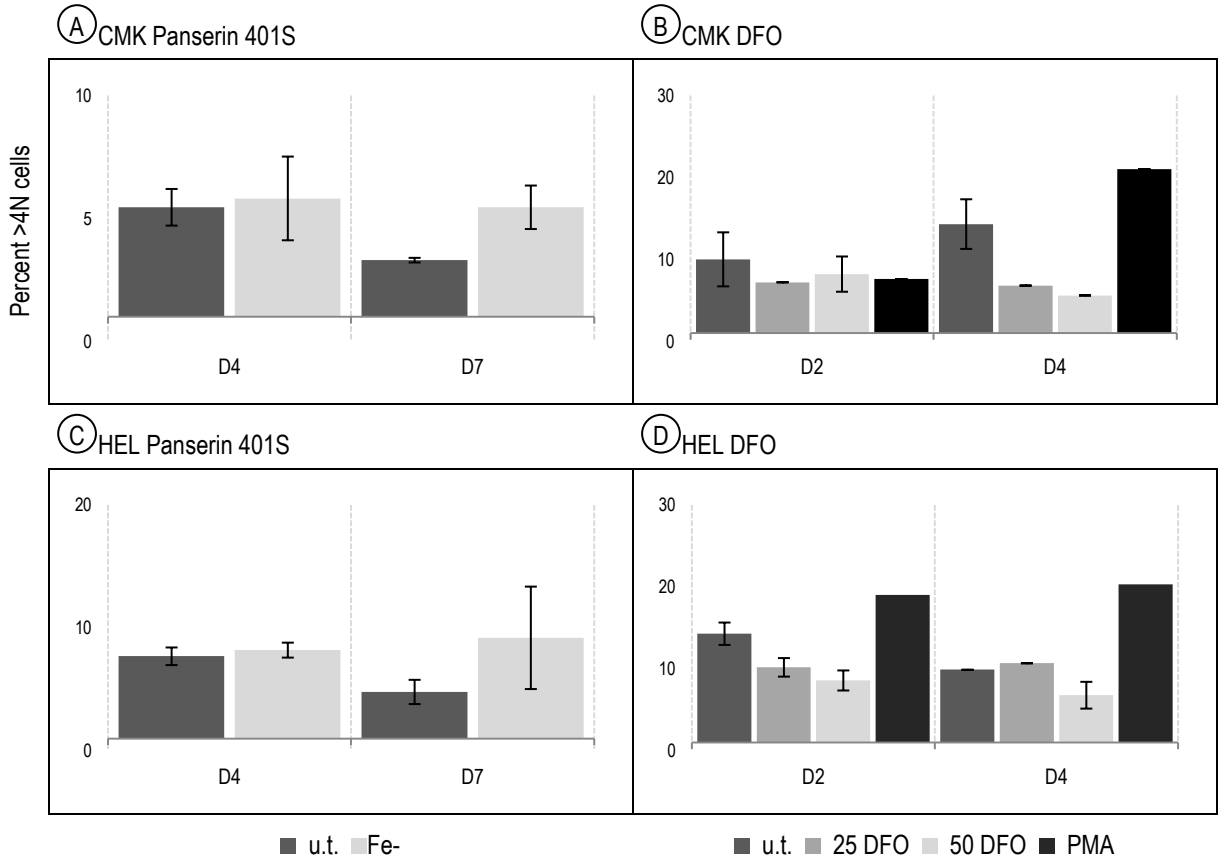


Fig. 16: Polyploidy

Cell cycle analysis by Hoechst 33342 staining and flow cytometry of (A, B) CMK and (C, D) HEL cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 or 7 days respectively.

5.2 Megakaryopoiesis and HIF

In order to determine changes in HIF expression under iron deficiency we examined expression levels of HIF1 alpha and HIF2 alpha on mRNA and HIF1 alpha on protein level. Due to the low expression level of HIF2 alpha protein it was not detectable on western blot.

5.2.1 HIF alpha mRNA expression under ID and PMA treatment

In CMK, the HIF1 alpha mRNA expression was undulated in Panserin 401S induced iron deficiency over the course of 7 days (Fig. 17A). DFO-treated cells did not show an upregulation of HIF1 alpha at mRNA level (Fig. 17B).

On the contrary, HIF2 alpha did not only show an upregulation at day 2 ($P = 0.018$), day 3 ($P = 0.002$) and day 4 ($P = 0.004$) in iron deficient cells induced by Panserin 401S (Fig.17C), but also DFO-treated cells showed an upregulation compared to untreated cells on day 2 ($P = 0.006$ in cells treated with 25µM DFO) and day 4 ($P < 0.001$ and $P = 0.001$, respectively) (Fig. 17D). However, PMA treatment led to a similar HIF1 alpha expression as DFO treatment, whereas the level of HIF2 alpha mRNA was decreased significantly after PMA treatment at day 2 ($P = 0.020$) and day 4 ($P = 0.005$).

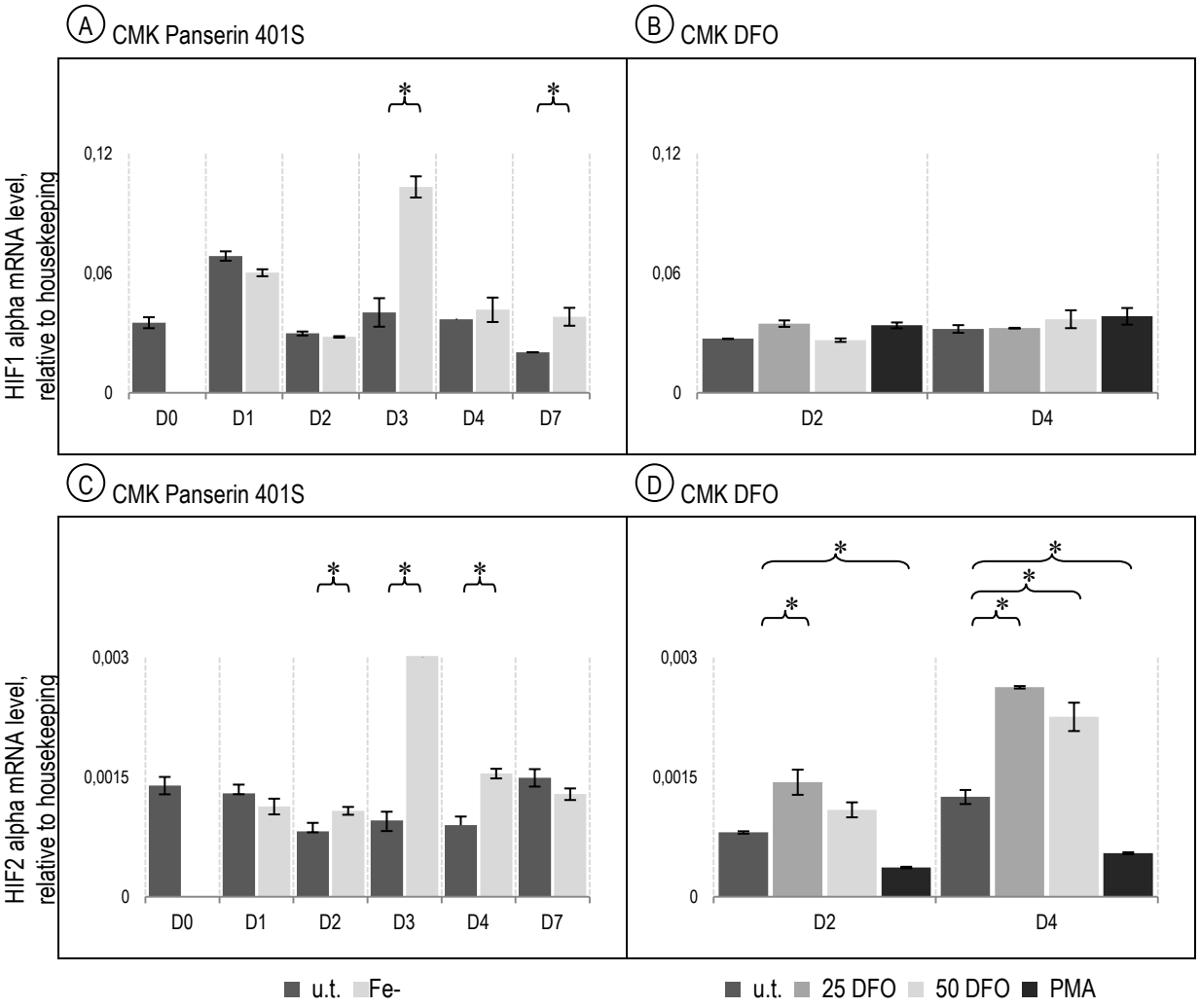


Fig. 17: HIF1 alpha and HIF2 alpha mRNA CMK

qRT-PCR for (A, B) HIF1 alpha and (C, D) HIF2 alpha of CMK cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control. Asterisks indicate a P-value < 0.05 compared to untreated cells.

In HEL, iron deficiency induced by Panserin 401S led to an increased mRNA level of HIF1 alpha at day 1 (P = 0.026), day 2 (P = 0.002) and day 7 versus untreated cells. However, HIF1 alpha was not altered at day 3 and day 4 (Fig. 18A). The HIF2 alpha mRNA level was enhanced significantly in iron deficient cells at day 1 (P = 0.046), day 3 (P = 0.002), day 4 (P= 0.010) and day 7 (P = 0.010) compared to untreated cells (Fig. 18C). DFO treated cells did not differ in HIF1 alpha expression at day 2, but at day 4 a significant upregultion of HIF1 alpha expression was observed in cell treated with 25 µM DFO (P = 0.012) and with 50µM

DFO (P = 0.023) (Fig. 18B). HIF2 alpha expression increased at day 4 (P < 0.001 in both conditons) compared to the untreated group (Fig. 18D).

In HEL, PMA treatment led to the increased expression of HIF1 alpha mRNA levels at day 4 (P = 0.039). Interestingly, the HIF2 alpha mRNA level decreased significantly (P = 0.005 at day 2 and P = 0.007 at day 4) in comparison to the untreated cells and DFO-treated cells.

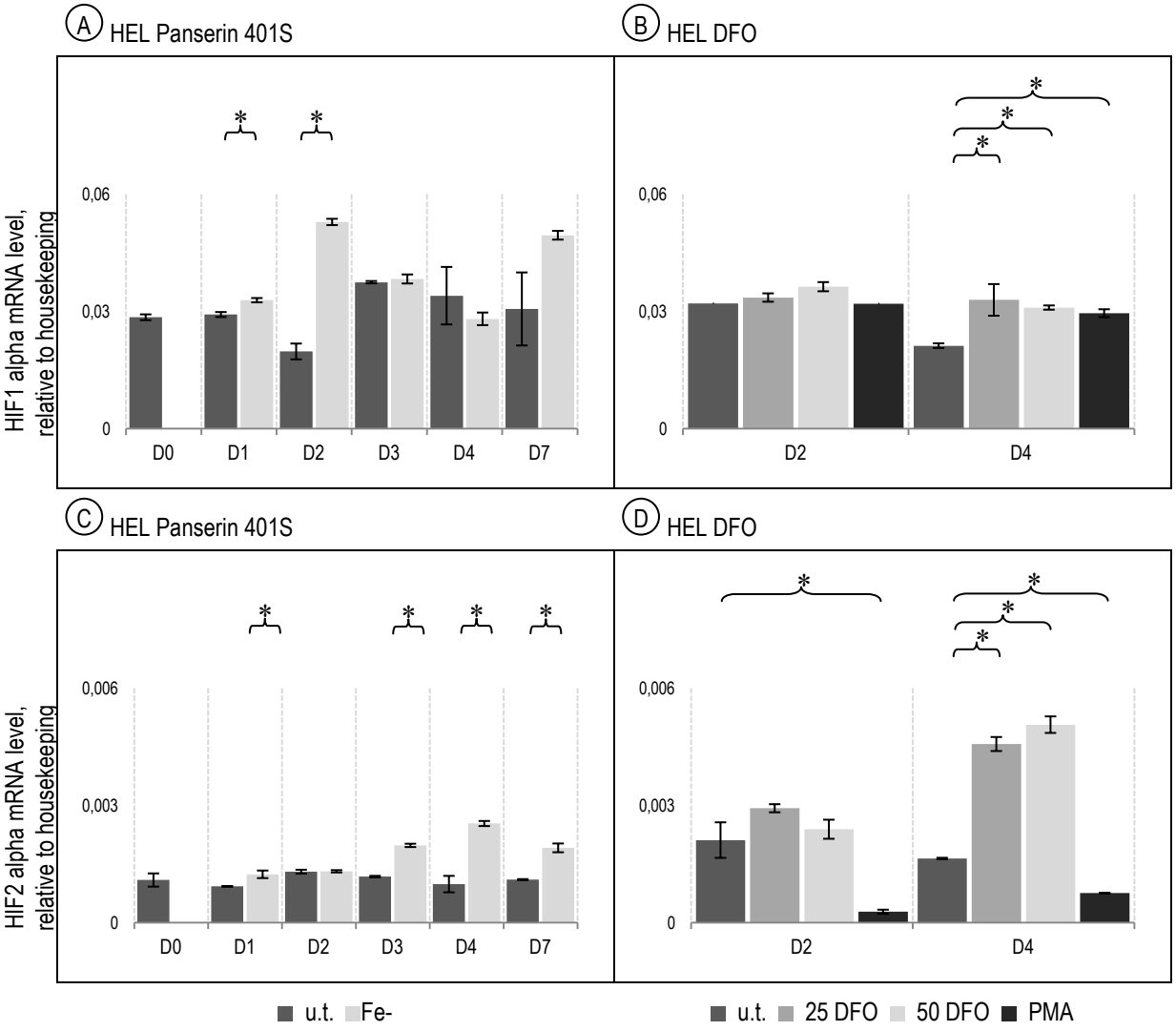


Fig. 18: HIF1 alpha and HIF2 alpha mRNA HEL

qRT-PCR for (A, B) HIF1 alpha and (C, D) HIF2 alpha of HEL cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control. Asterisks indicate a P-value < 0.05 compared to untreated cells.

In summary iron deficiency as well as PMA treatment seemed to influence HIF expression on mRNA level. However, HIF1 alpha expression was basically influenced by Panserin401S induced iron deficiency and only at specific time points, whereas the mRNA pattern of HIF2 alpha was more consistently changed in both induced iron deficiencies. In the two cell lines PMA treatment had a negative effect on the level of HIF2 alpha mRNA, but there was only a small effect on the HIF1 alpha mRNA level.

5.2.2 HIF1 alpha protein expression under ID and PMA treatment

Whole cell lysate

In total, the amount of HIF1 alpha protein was very low in the whole cell lysate. We were hardly able to detect any protein at the predicted size (120kDa), presumably because of very low concentrations due to HIF1 alpha protein degradation in normoxic conditions in the whole cell lysate (Fig. 19, 20 and 21).

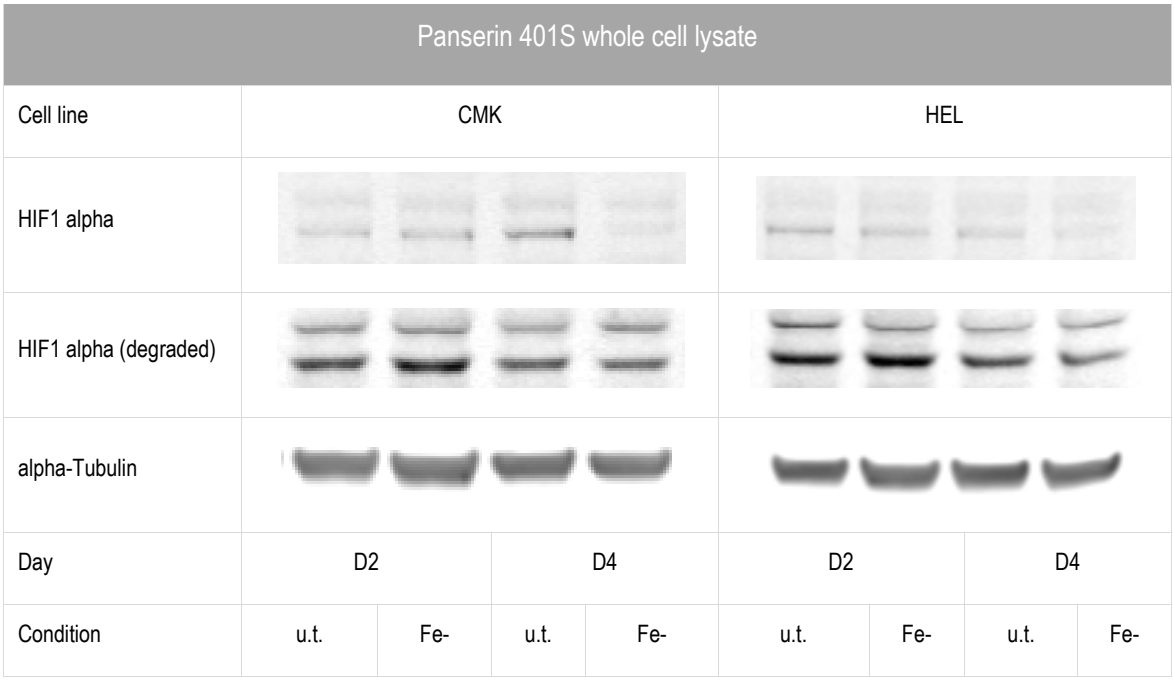


Fig.19: HIF1 alpha protein Panserin 401S whole cell lysate

Protein expression of HIF1 alpha in CMK and HEL cultured in 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-) for 4 days. Expression of alpha-Tubulin was used as a loading control.

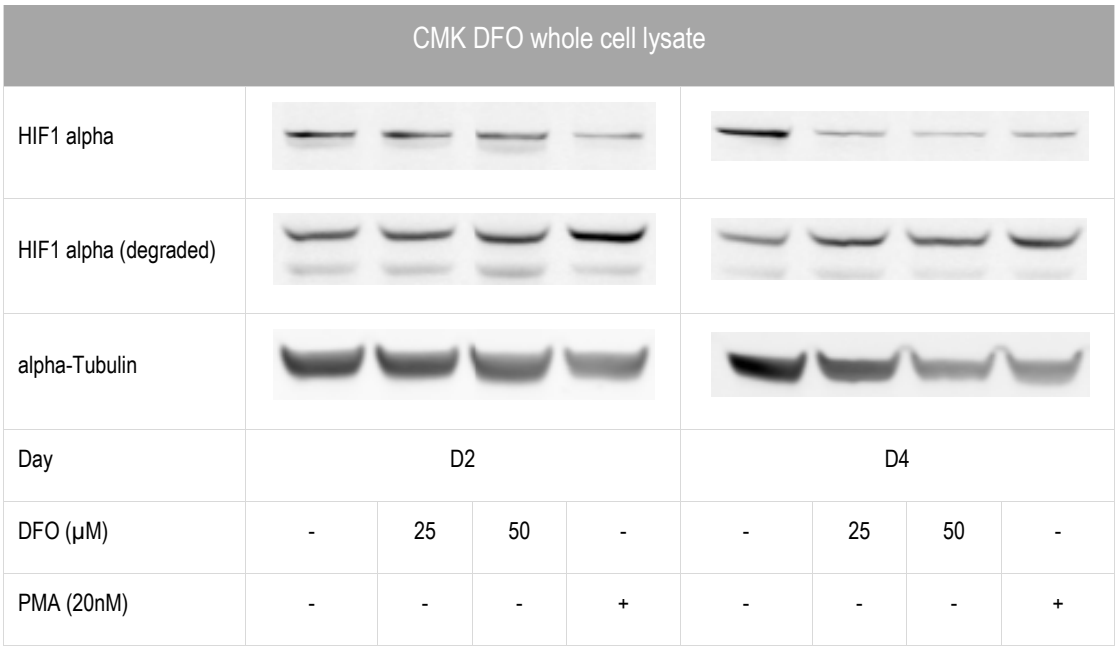


Fig.20: HIF1 alpha protein CMK DFO whole cell lysate

Protein expression of HIF1 alpha in CMK cultured in Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Expression of alpha-Tubulin was used as a loading control.

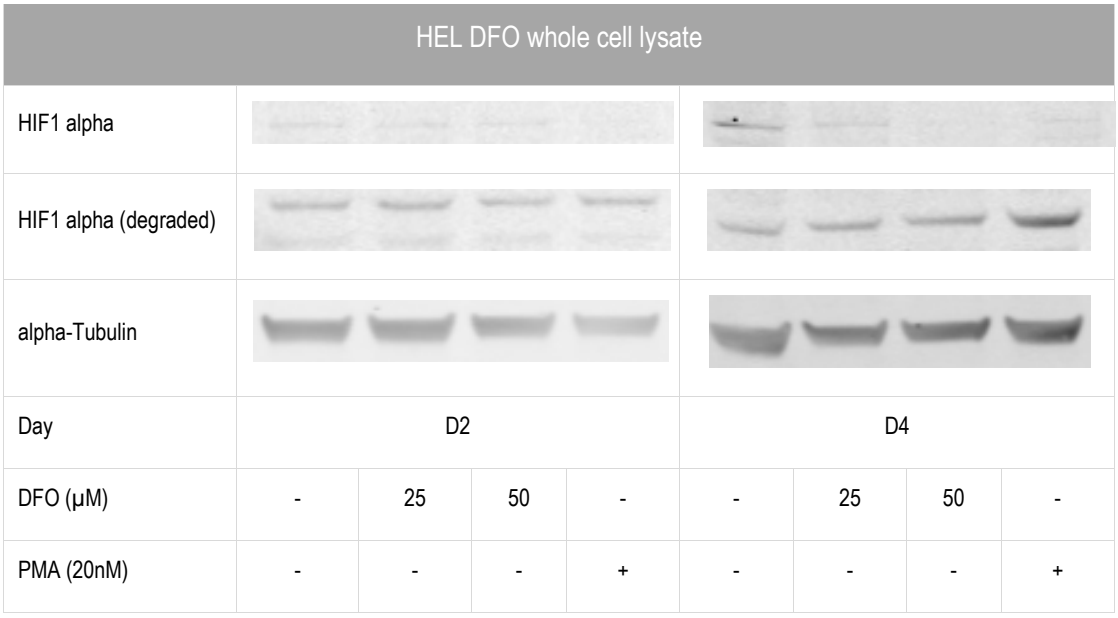


Fig.21: HIF1 alpha protein HEL DFO whole cell lysate

Protein expression of HIF1 alpha in HEL cultured in Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Expression of alpha-Tubulin was used as a loading control.

Nuclear/cytoplasmic fraction

The separation of the nuclear fraction led to a clear HIF1 alpha signal at the predicted size – accumulating at day 2 in iron-deficient (induced by Panserin401) and PMA treated HEL cells (Fig. 22 and 23).

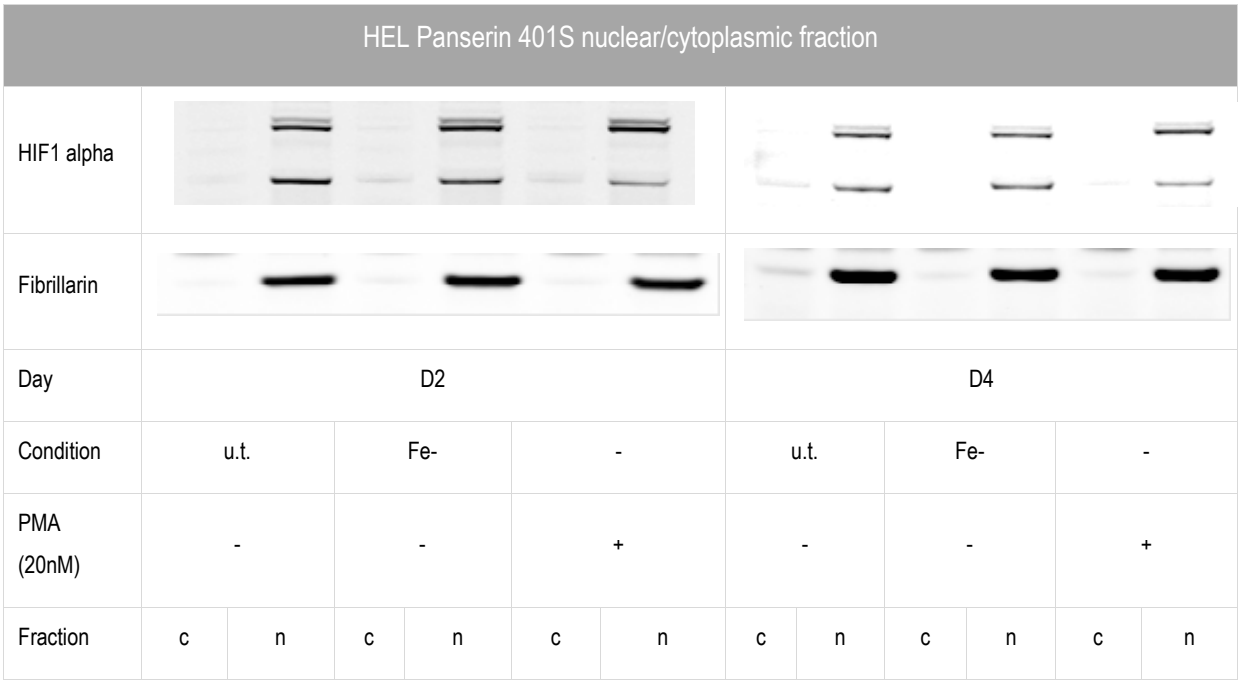


Fig. 22: HIF1 alpha protein HEL Panserin401S nuclear/cytoplasmic fraction

Nuclear and cytoplasmic protein expression of HIF1 alpha in HEL cultured in 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-) for 4 days. Expression of Fibrillarin was used as a loading control.

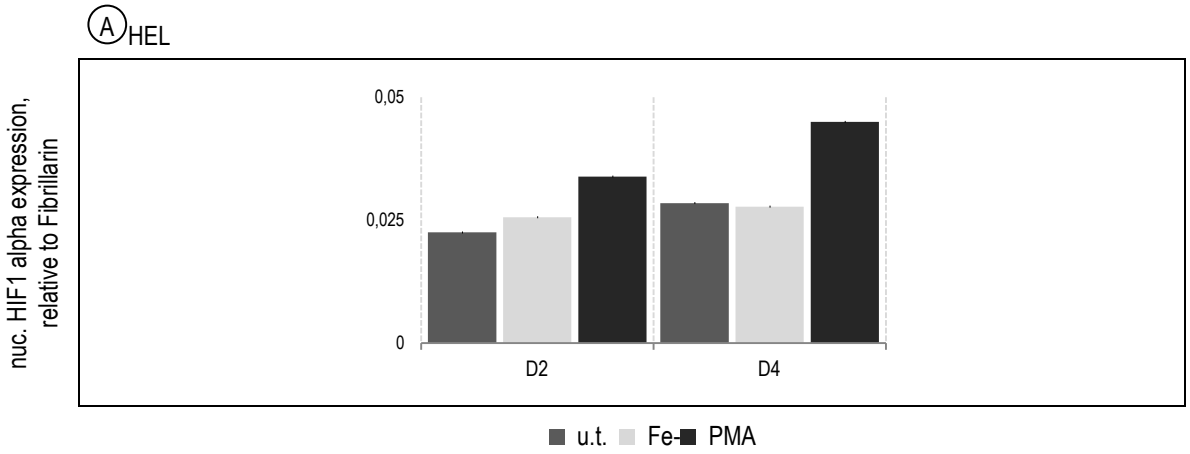


Fig. 23: HIF1 alpha protein HEL Panserin401S nuclear fraction densitometry

Nuclear protein expression of HIF1 alpha in HEL cultured in 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-) for 4 days normalized to Fibrillarin.

5.2.3 HIF target expression under ID and PMA treatment

After HIF1 alpha and HIF2 alpha have been identified to be possible regulators of megakaryopoiesis, which is induced by ID, we examined if HIF targets, which are involved in megakaryopoiesis (such as VEGF, CXCR4 and LRP1), show distinct expression patterns in untreated, iron deficient and PMA treated conditions on mRNA level by RT-PCR analysis.

CXCR4

In CMK, Panserin 401S induced iron deficiency ($P = 0.035$) and DFO treatment ($P < 0.001$ in both conditions) led to significantly increased mRNA levels of CXCR4 at day 4 (Fig. 24A and B).

In HEL, mRNA levels of the CXCR4 gene were increasing over time under both conditions – untreated and iron deficient (Panserin 401S induced). In DFO-treated cells a dose-dependent upregulation of CXCR4 mRNA could be distinguished (Fig. 24C and D) at day 4 ($P = 0.032$ and 0.003, respectively) in contrast to untreated cells. PMA treatment led to a significant upregulation of CXCR4 at day 2 ($P < 0.001$).

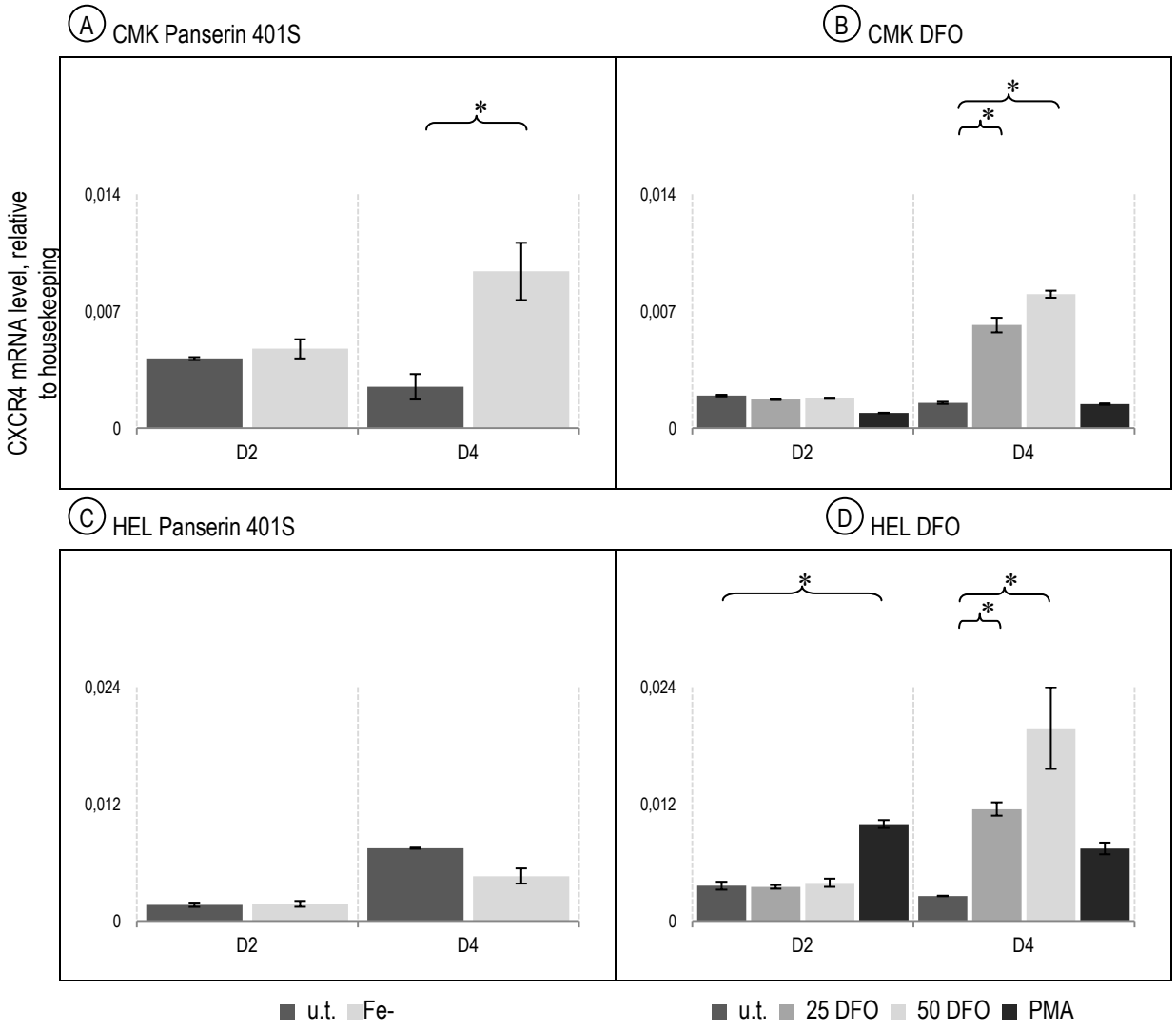


Fig. 24: CXCR4 mRNA

qRT-PCR for CXCR4 of (A, B) CMK and (C, D) HEL cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control. Asterisks indicate a P-value < 0.05 compared to untreated cells.

LRP1

Panserin 401S induced iron deficiency led to a slight upregulation of LRP1 at day 4 in CMK (Fig. 25A) – a trend which was more obvious in DFO treated cells, where a significant, dose-dependent effect was detected at day 4 ($P = 0.004$ and $P < 0.001$, respectively) (Fig. 25B). PMA treatment led to a significant upregulation at day 4 ($P = 0.020$) in comparison to the untreated group.

HEL cells cultured in 1.25% Panserin401 in Panserin401S did not show much difference in LRP1 expression compared to untreated cells (Fig. 25C). In contrast, DFO treatment (P = 0.010 and 0.034, respectively) and PMA treatment (P = 0.030) considerably increased the expression of LRP1 at day 4 on mRNA level (Fig. 25D).

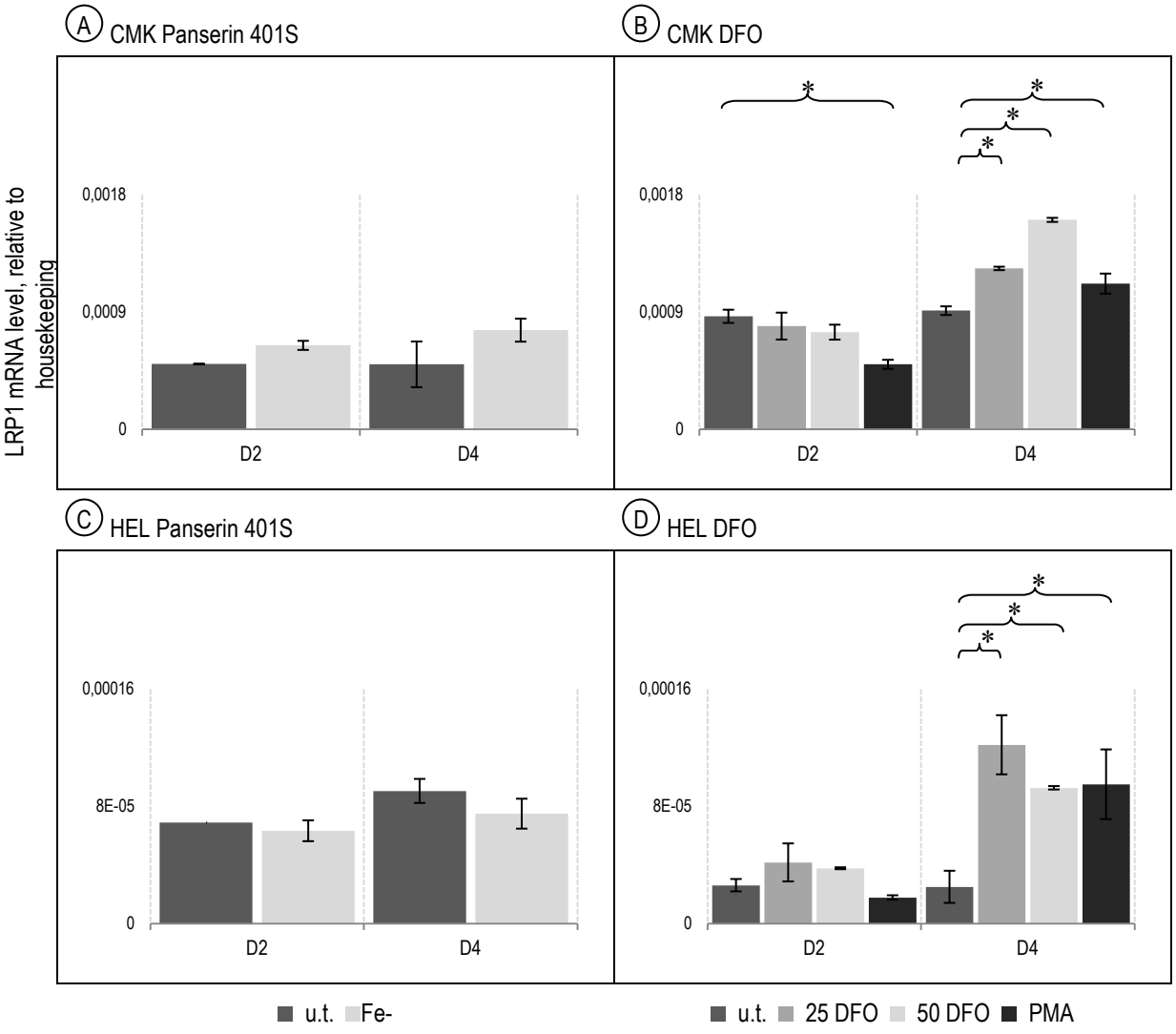


Fig. 25: LRP1 mRNA

qRT-PCR for LRP1 of (A, B) CMK and (C, D) HEL cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control. Asterisks indicate a P-value < 0.05 compared to untreated cells.

VEGF

In CMK, the expression of VEGF on mRNA level increased overtime – in Panserin 401S induced iron deficiency as well as in the untreated group (Fig. 26A). DFO treatment led to a dose-dependent upregulation of VEGF ($P < 0.001$ in both conditions) in comparison to the untreated group at day 4 (Fig. 26B). In PMA treated cells VEGF mRNA was upregulated at day 2 ($P < 0.001$) and day 4 ($P < 0.001$).

In HEL, Panserin401S induced iron deficiency did not lead to significant changes in VEGF mRNA levels. However, the untreated cells showed a quite high expression level of VEGF at day 4 (Fig. 26C). Similar to CMK, DFO treatment in HEL also led to a dose-dependent upregulation of VEGF mRNA in HEL – not so in the untreated group (Fig. 26D) – at day 4 ($P = 0.001$ in both conditions). PMA treatment led to a signifcant upregulation of VEGF ($P < 0.001$ at day 2 and day 4) on mRNA level.

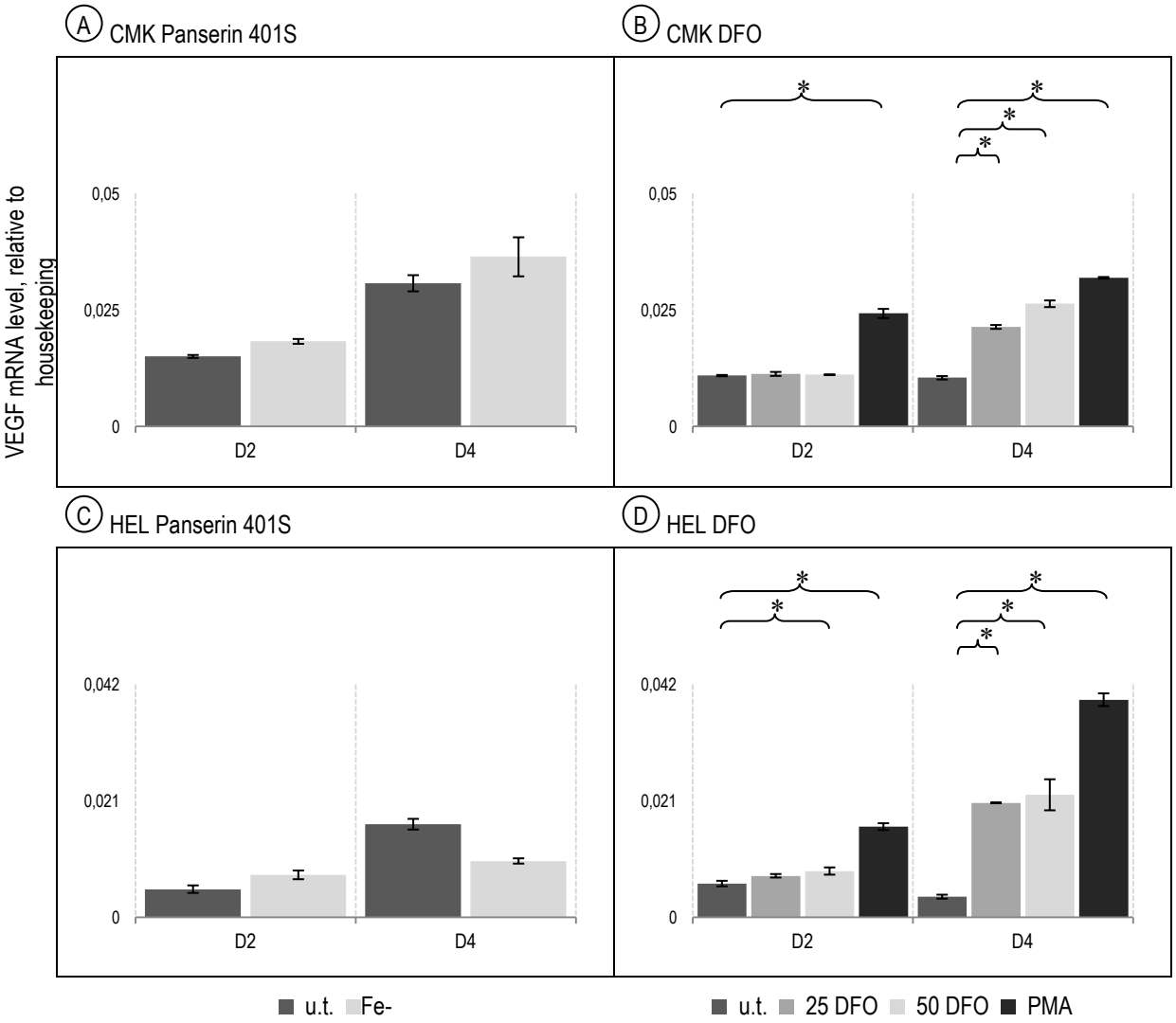


Fig. 26: VEGF mRNA

qRT-PCR for VEGF of (A, B) CMK and (C, D) HEL cultured in (A, C) 100% Panserin 401 (u.t.) and 1.25% v/v Panserin 401:401S (Fe-), and in (B, D) Panserin 401 medium (u.t.) supplemented with 25µM DFO (25 DFO), 50µM DFO (50 DFO) and 1 hour pulse-treated with 20nM PMA (PMA) for 4 days. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control. Asterisks indicate a P-value < 0.05 compared to untreated cells.

5.3 Overexpression of HIF

Our next aim was to investigate the specific role of HIF in megakaryopoiesis by overexpression of HIF1 and HIF2 alpha and determining changes in megakaryocytic differentiation and HIF target expression in comparison to untreated cells.

5.3.1 Transfection efficiency

After transient transfection of the cell line HEL with plasmids – containing HIF1 alpha, HIF2 alpha or no insert – the expression level of the respective protein was evaluated on mRNA and on protein level to confirm the successful overexpression after 48 hours.

The result was the confirmation of a successful overexpression of HIF1 alpha (Fig. 27A) and HIF2 alpha (Fig. 27B) on mRNA level and of HIF1 alpha on protein level (Fig. 28).

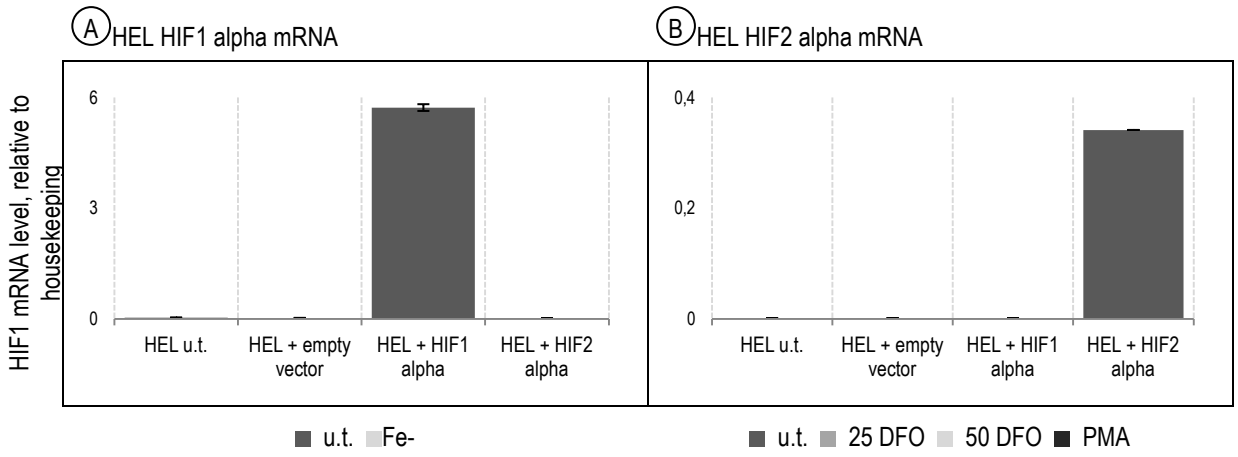


Fig. 27: HIF1 alpha and HIF2 alpha mRNA after HIF alpha overexpression

qRT-PCR for (A) HIF1 alpha and (B) HIF2 alpha of HEL cultured in RPMI and transfected with the indicated plasmids for 48hours. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control.

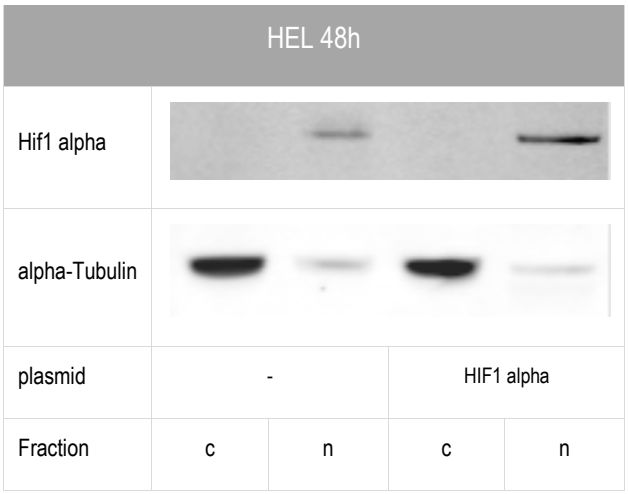


Fig.28: HIF1 alpha protein expression after HIF alpha overexpression

Protein expression of HIF1 alpha in HEL cultured in RPMI and transfected with plasmids containing HIF1 alpha for 48hours. Expression of alpha-Tubulin was used as a loading control.

5.3.2 Megakaryocytic marker expression in cells overexpressing HIF1 or HIF2 alpha

The expression levels of the markers CD41and CD61 were not altered in cells, which were overexpressing HIF1 or HIF2 alpha in comparison to the control groups (untreated cells and cells transfected with an empty vector, Fig. 29A and C).

In contrast, the expression of CD42a increased in cells, which were overexpressing HIF1 alpha ($P = 0.040$) or HIF2 alpha ($P = 0.028$), in contrast to cells transfected with the empty vector or untreated cells (Fig. 29B). The erythropoietic marker GpA was also affected by overexpression of HIF1 and HIF2 alpha. The expression was downregulated in these cells ($P < 0.001$ in cells overexpression HIF1 and HIF2 alpha) compared to the control cells (Fig. 29D).

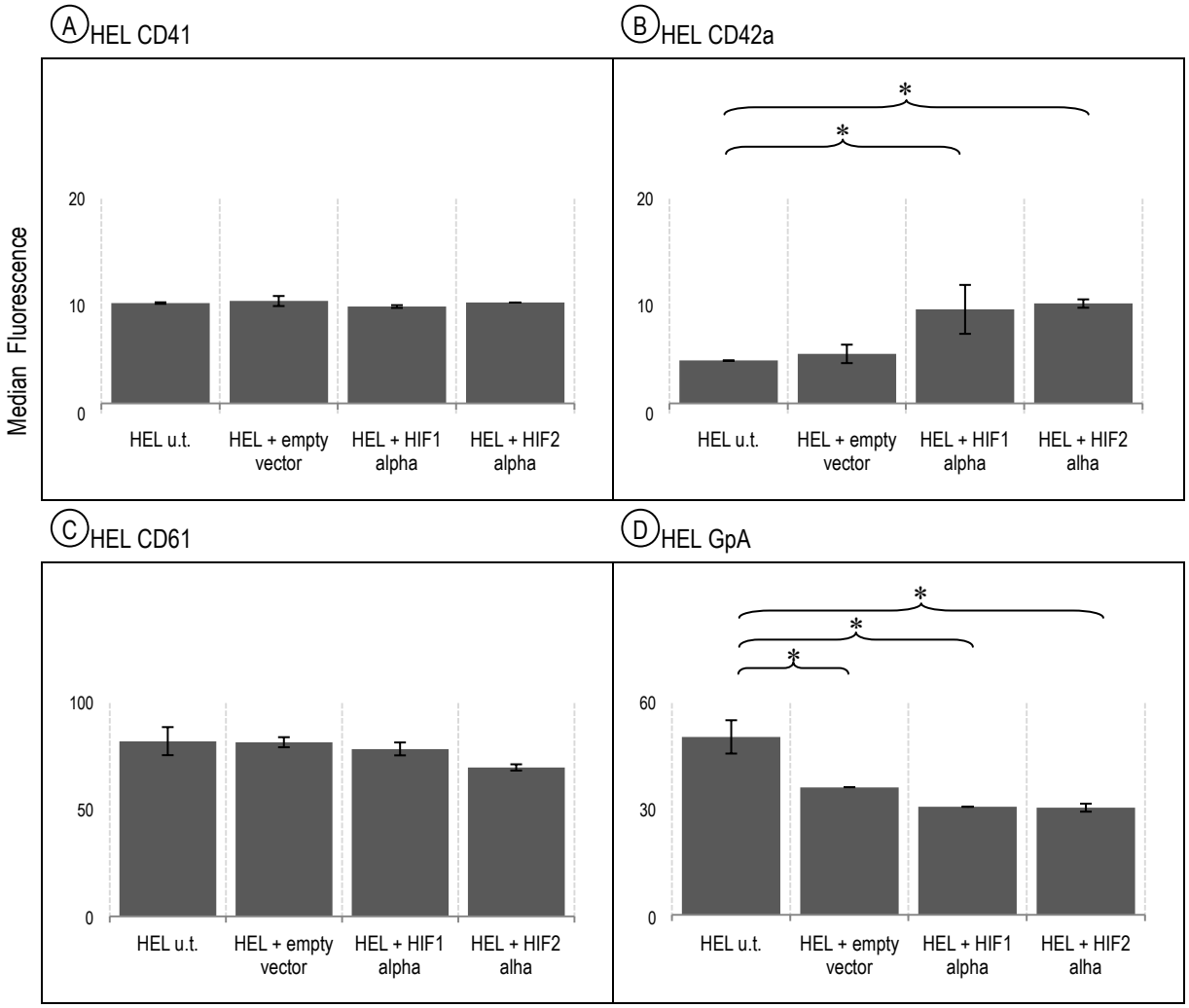


Fig. 29: Surface megakaryocytic markers after HIF alpha overexpression

Median Fluorescence of (A) CD41, (B) CD42a, (C) CD61, and (D) GpA positive HEL cultured in RPMI and transfected with the indicated plasmids for 48 hours. Asterisks indicate a p-Value < 0.05 compared to untreated cells.

5.3.3 HIF target expression in cells overexpressing HIF1 and HIF2 alpha

In cells overexpressing HIF1 alpha the mRNA level of CXCR4 was significantly increased (P = 0,015). In comparison to controls, overexpression of HIF2 alpha led to similar results as overexpression of HIF1 alpha, though the increase was not significant (Fig. 30A).

The mRNA levels of LRP1 were slightly affected by HIF overexpression: transfection with the HIF1 alpha plasmid and transfection with the HIF2 alpha plasmid showed an increase of LRP1 mRNA level – in both cases compared to untreated cells, but did not reach a significant level (Fig. 30B).

As opposed to CXCR4 and LRP1 the mRNA levels of VEGF were slightly downregulated in cells overexpressing HIF1 and HIF2 alpha on mRNA level (Fig. 30C).

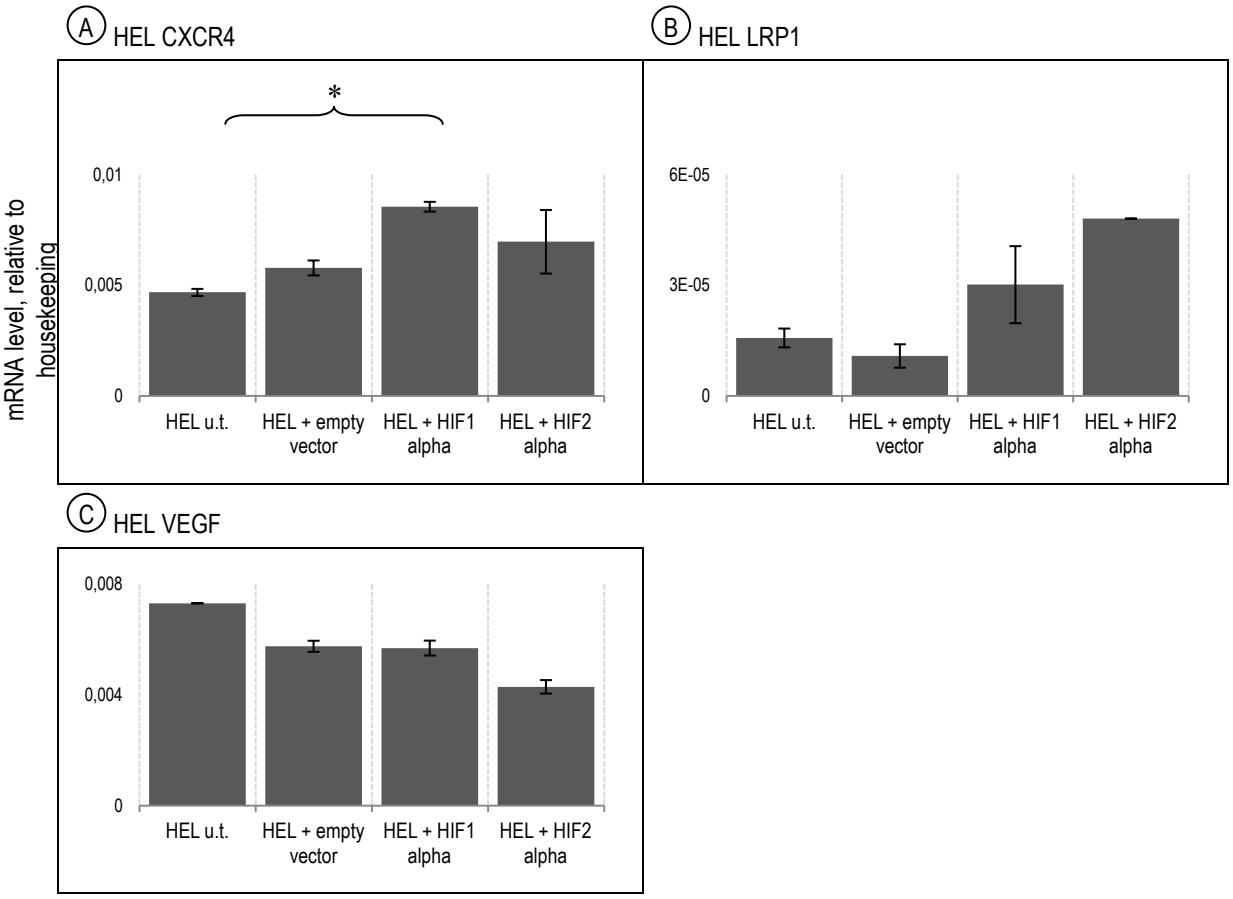


Fig. 30: CXCR4, LRP1 and VEGF mRNA after HIF alpha overexpression

qRT-PCR for (A) CXCR4 and (B) LRP1 and (C) VEGF of HEL cultured in RPMI and transfected with the indicated plasmids for 48hours. Bar graphs represent expression of mRNA levels quantified by normalization to GAPDH as an endogenous control. Asterisks indicate a P-value < 0.05 compared to untreated cells.

5.3.4 Alterations in ploidy in cells overexpressing HIF1 and HIF2 alpha

In terms of polyploidy, overexpression of HIF1 and HIF2 alpha did not lead to significant increases. Cells, which were overexpressing HIF1 alpha, showed a slightly increased >4N population, whereas the >4N population in cells transfected with the HIF2 alpha plasmid was comparable to untreated cells and cells transfected with the empty plasmid (Fig. 31).

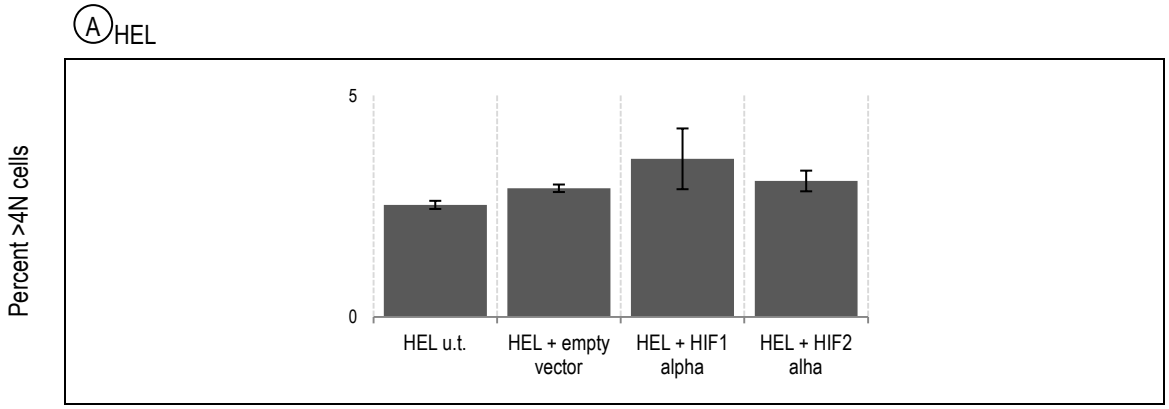


Fig. 31: Polyploid cells after HIF alpha overexpression

Cell cycle analysis by Hoechst 33342 staining and flow cytometry of HEL cultured in RPMI and transfected with the indicated plasmids for 48hours.

6 Discussion

The association of ID and thrombocytosis in patients is documented in many studies (see introduction), but only little is known about changes in megakaryocytic maturation induced by iron deficiency and the underlying molecular mechanisms. Elevated platelet counts and enhanced size of MKs have been observed in iron deficient rats already 1970, although the number of MKs was not increased. Similar observations have been made in an iron deficient rat model of our laboratory: elevated platelet counts and changes of the bone marrow led to the suggestion that the high platelet counts are the consequence of accelerated differentiation of MKs (22). Further studies in the megakaryocytic cell line Dami showed enhanced polyploidy under iron deficient conditions (110). The observations of this study concerning surface marker expression and polyploidy under conditions of ID, induced either by Panserin 401S or by DFO, are consistent with previous in vivo and in vitro results. However, the

molecular mechanisms behind this phenomenon have not been clarified yet and led to the establishment of an in vitro system to study iron deficient megakaryopoiesis (24).

In the present study, we induced ID in two different ways: by adding DFO to the media or by using the iron- and transferrin-free medium Panserin 401S. ID induced by Panserin 401 is developing slowly and could reflect the physiological situation better. However, as the manifestation of ID is very dependent on cell proliferation, it is firstly less pronounced and secondly more variable, which is confirmed by the TfR1 and ferritin results. DFO, on the other hand, has immediate action, is not dependent on several cell division cycles for ID to occur and allows to do dose response curves. However, DFO is a chemical with potentially other actions, it blocks all iron in the cells and does not allow the cell to adapt gradually to limited iron. These factors might be the reason why some responses are better observed with Panserin 401S (such as polyploidy) and others with DFO.

The aim of this study was to investigate the potential role of HIF as a regulator during megakaryopoiesis. We wanted to answer the question how HIF1 alpha and HIF2 alpha are regulated in our model of megakaryopoiesis of iron deficiency and if HIF is an essential regulator of megakaryopoiesis induced by ID.

In order to answer our questions we examined expression levels of HIF1 and HIF2 alpha on mRNA and of HIF1 alpha on protein level under iron deficient conditions in megakaryocytic cells lines. Additionally we investigated the expression of HIF target genes under the same conditions on mRNA level. In order to clarify to which extent HIF is involved in megakaryopoiesis we performed overexpression studies of HIF1 and HIF2 alpha and determined the expression of megakaryocytic maturation markers, the expression of HIF targets and the polyploidy level of these cells.

The first question was if HIF is overexpressed in megakaryopoiesis under ID and if there is a difference in the expression pattern of HIF1 and HIF2 alpha. As HIF1 and HIF2 alpha are both regulated primarily by protein stability, the investigation of the two factors on protein level is more significant. However, in order to exclude additional interfering control mechanisms on mRNA level and to determine differential expression patterns of HIF1 and HIF2 alphas under ID, the mRNA expression levels of the HIF alphas were also evaluated.

HIF1 alpha mRNA expression under iron deficient conditions compared to untreated cells showed strong undulations at each time point of mRNA collection in both cell lines, CMK

and HEL, and might not be biologically significant. The results for HIF2 alpha were more consistent as the upregulation in the ID condition was apparent at all days in both cell lines. Interestingly, PMA treatment led to a constant downregulation of HIF2 alpha. As PMA is not the natural inducer of megakaryopoiesis, it might turn on different pathways than the natural main regulator of megakaryopoiesis, TPO.

Studying physiological processes in cell lines is always limited. The addition of TPO to the cells had no effect on HEL and CMK (data not shown). HEL was shown to have a V617F mutation in JAK2 leading to constitutively activated TPO signaling. A general tendency of cell lines, which are obtained from myeloproliferative disorders, to constitutively signal through the TPO pathway is described in the literature (59). Future studies, which include primary cells or animal models, could help explain how ID affects TPO signaling and thereby megakaryopoiesis.

The total amount of HIF1 and HIF2 alpha protein is very low, as the proteins become degraded in the cytoplasm and are only stabilized in the nucleus. This makes HIF1 alpha very difficult to detect in the whole cell lysate. However, after separation of the nuclear and the cytoplasmic fraction western blot results indicate a slight upregulation of HIF1 alpha expression in the iron deficient group compared to untreated cells on protein level. HIF2 alpha protein is even less expressed in HEL and CMK and was not detectable on western blot.

We further examined the downstream activation of the HIF pathway by measuring the expression pattern of three HIF targets, CXCR4, LRP1 and VEGF in our in vitro model of iron deficient megakaryopoiesis. The three investigated HIF targets are involved in megakaryopoiesis and showed a trend of upregulation at day 4 in the DFO-induced iron deficient conditions. However, ID induced by Panserin 401S did not always have the same effect.

PMA treatment, representing a positive control, did not always lead to the same expression patterns of the HIF targets as ID, but as already mentioned PMA is not a natural inducer of megakaryopoiesis. The increased expression of CXCR4 and LRP1 after DFO-treatment indicate the more differentiated condition of the cells towards the megakaryocytic lineages and fits to the observed phenotype (e.g. GpA and TfR1 expression).

The next question we aimed to answer was, if the overexpression of HIF1 alpha and HIF2 alpha does induce megakaryocytic differentiation by evaluating megakaryocytic

differentiation markers (CD41, CD61, CD42a) and the erythropoietic differentiation marker GpA after transfecting cells with plasmids containing HIF1 alpha or HIF2 alpha. In the cell line HEL overexpression of HIF1 alpha as well as HIF2 alpha led to increased expression of CD42a, whereas the other markers CD41 and CD61 were not affected. The erythropoietic marker GpA was less expressed in these cells indicating megakaryocytic differentiation (39). These results suggest an association between the HIF alphas and iron deficient megakaryopoiesis as both ID and overexpression of HIF1 and HIF2 alpha lead to similar expression patterns of the megakaryocytic markers and the erythropoietic marker GpA indicating a differentiation towards the megakaryocytic lineage.

Another part of our HIF-overexpression study was the influence on the expression of HIF target genes. As in the case of the megakaryocytic markers, the HIF targets CXCR4 and LRP1 were affected similarly by overexpression of HIF1 and HIF2 alpha and under ID. Both proteins were upregulated in cells transfected with the HIF-containing plasmids. Overexpression of HIF1 alpha was not affecting VEGF mRNA levels, whereas overexpression of HIF2 alpha had an even downregulating effect on VEGF. Further studies in primary cells including siRNA experiments could verify, if the differentiation towards the megakaryocytic lineage induced by the overexpression of the HIF alphas, which we show by the downregulation of GpA, might be VEGF independent and if any of the downstream targets of HIF have a more specific role in this process.

Based on our results one could only speculate which one of the two HIF alphas is more important during megakaryopoiesis under ID. Both HIF alphas seem to affect megakaryopoiesis when overexpressed, but because only HIF1 alpha is detectible on protein level and is also more abundant on mRNA level it is more likely that HIF1 alpha is the regulator during this process. To confirm this, siRNA experiments for example in PMA treated cells could be performed to find out which HIF alpha is essential. Similar experiments in primary cells could help to determine if HIF affects TPO signaling. In the far future animal experiments with MK-specific knockouts would also be interesting and might help confirming the importance of HIF during megakaryopoiesis in ID.

7 References

(1) Milman N. Anemia - still a major health problem in many parts of the world! Ann Hematol 2011;90(4):369-77.

(2) Gasche C, Berstad A, Befrits R et al. Guidelines on the diagnosis and management of iron deficiency and anemia in inflammatory bowel diseases. Inflamm Bowel Dis 2007;13(12):1545-53.

(3) WHO. Worldwide prevalence of anaemia 1993-2005. 2010.

(4) DSM. Nutritional Anemia Guidebook. 2007.

(5) Dan K.Thrombocytosis in iron deficiency anemia. Intern Med 2005;44(10):1025-6.

(6) Kinoshita Y, Taniura S, Shishido H et al. Cerebral venous sinus thrombosis associated with iron deficiency: two case reports. Neurol Med Chir (Tokyo) 2006;46(12):589-93.

(7) Akins PT, Glenn S, Nemeth PM et al. Carotid artery thrombous associated with severe iron-deficiency anemia and thrombocytosis. Stroke 1996;27(5):1002-5.

(8) Benedict SL, Bonkowsky JL, Thompson JA et al. Cerebral ainovenous thrombosis in children: another reason to treat iron deficiency anemia. J Child Neurol 2004;19(7):526-31.

(9) Hartfield DS, Lowry NJ, Keene DL et al. Iron deficiency: a cause of stroke in infants and children. Pediatr Neurol 1997;16(1):50-3.

(10) Belman AL, Roque CT, Ancona R et al. Cerebral venous thrombosis in a child with iron deficiency anemia and thrombocytosis. *Stroke* 1990;**21**(3):488-93.

(11) Balci K, Utku U, Asil T et al. Deep cerebral vein thrombosis associated with iron deficiency anaemia in adults. *J Clin Neurosci* 2007;**14**(2):181-4.

(12) Simanek R, Vormittag R, Ay C et al. High platelet count associated with venous thromboembolism in cancer patients: results from the Vienna Cancer and Thrombosis Study (CATS). *J Thromb Haemost* 2010;**8**(1):114-20.

(13) Danese S, Motte Cd CL, Fiocchi C. Platelets in inflammatory bowel disease: clinical, pathogenic, and therapeutic implications. *Am J Gastroenterol*2004;**99**(5):938-45.

(14) Novacek G, Weltermann A, Sobala A et al. Inflammatory bowel disease is a risk factor for recurrent venous thromboembolism. *Gastroenterology* 2010;**139**(3):779-87, 787.

(15) Harries AD, Fitzsimons E, Fifield R et al. Platelet count: a simple measure activity in Crohn's disease. *Br Med J (Clin Res Ed)* 1983;**286**(6376):1476.

(16) Danese S, Papa A, Saibeni S et al. Inflammation and coagulation in inflammatory bowel disease: the clot thickens. *Am J Gastroenterol* 2007;**102**:174-86.

(17) Kulnigg-Dabsch S, Evstatiev R, Dejaco C et al. Effect of iron therapy on platelet counts in patients with inflammatory bowel disease associated anemia. *PLoS one* 2012;**7**(4):e34520.

(18) Kulnigg-Dabsch S, Schmid W, Howaldt S, Stein J, Mikisch O, Waldhör T, Evstatiev R, Kamali H, Volf I and Gasche C. Iron deficiency generates secondary thrombocytosis and platelet activation in IBD. The randomized, controlled thromboVIT trial. Accepted *IBD J* 2012.

(19) Choi SI, Simone JV, Jackson CW. Megakaryocytopoiesis in experimental iron deficiency anemia. *Blood* 1974;**43**(1):111-20.

(20) Garg SK, Weiner M. Thrombocyte and megathrombocyte kinetics during thrombocytosis induced by acute and chronic blood loss and by iron-deficiency diet. *Haemostasis* 1972;(1):121.

(21) Holter PH, Refsum HE. Postnatal anemia and thrombocytosis in suckling rabbits: influence of delayed weaning and iron supplies. *Pediatr Hematol Oncol* 1988;**5**(3):157-68.

(22) Evstatiev R, Bukaty A, Kulnigg S et al. Iron deficiency induces thrombocytosis in rats. *Gut* 2008;**57 (Suppl II):**A333.

(23) Choi SI, Simone JV. Platelet production in experimental iron deficiency anemia. *Blood* 1973;**42**(2):219-28.

(24) Jimenez K. Establishment of an in vitro system to study iron deficient megakaryopoiesis. Diploma thesis, Medical University Vienna, 2011.

(25) Spinelli SL, Maggirwar SB, Blumberg N et al. Nuclear emancipation: a platelet tour de force. *Sci Signal* 2010;**3**(144):e37.

(26) Kaushansky K. Historical review: megakaryopoiesis and thrombopoiesis. *Blood* 2008;**111**(3):981-6.

(27) Adolfsson J, Mansson R, Buza-Vidas N et al. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map of adult blood lineage commitment. *Cell* 2005;**121**(2):295-306.

(28) Forsberg EC, Serwold T, Kogan S et al. New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3 multipotent hematopoietic progenitors. *Cell* 2006;**126**(2):415-26.

(29) Szalai G, LaRue AC, Watson DK. Molecular mechanisms of megakaryopoiesis. *Cell Mol Life Sci* 2006;**63**(21):2460-76.

(30) Wickrema A, Crispino JD. Erythroid and megakaryocytic transformation. *Oncogene* 2007;**26**(47):6803-6815.

(31) Kosaki G. Platelet production by megakaryocytes: proplatelet theory justifies cytoplasmic fragmentation model. *Int J Hematol* 2008;**88**(3):255-67.

(32) Thon JN, Italiano JE. Platelet formation. *Semin Hematol* 2010;**47**(3)220-6.

(33) Bluteau D, Lordier L, Di Stefano A et al. Regulation of megakaryocyte maturation and platelet formation. *J Thromb Haemost* 2009;**7 Suppl 1**:227-34.

(34) Larson MK, Watson SP. A product of their environment: do megakaryocytes rely on extracellular cues for proplatelet formation? *Platelets* 2006;**17**(7):435-40.

(35) Schwertz H, Rowley JW, Tolley ND et al. Assessing protein synthesis by platelets. *Methods Mol Biol* 2012;**788**:141-53.

(36) Schwertz H, Koster S, Kahr WH et al. Anucleate platelets generate progeny. *Blood* 2010;**115**(18):3801-9.

(37) Lepage A, Leboef M, Cazenave JP et al. The alpha(IIb)beta(3) integrin and GPIb-V-XI complex identify distinct stages in the maturation of CD34(+) cord blood cells to megakaryocytes. *Blood* 2000;**96**(13):4169-77.

(38) Tomer A. Human marrow megakaryocyte differentiation: multiparameter correlative analysis identifies von Willebrand factor as a sensitive and distinctive marker for early (2N and 4N) megakaryocytes. *Blood* 2004;**104**(9):2722-7.

(39) Klimchenko O, Mori M, DiStefano A et al. A common bipotent progenitor generates the erythroid and megakaryocyte lineages in embryonic stem cell-derived primitive hematopoiesis. *Blood* 2009;**114**(8):1506-17.

(40) Wandzik K, Zahn C, Dassler K et al. Substantial changes of cellular iron homeostasis during megakaryocytic differentiation of K562 cells. Dev Growth Differ 2009;51(6):555-65.

(41) Ravid K, Lu J, Zimmet JM et al. Roads to polyploidy: The megakaryocyte example. *J Cell Physiol* 2002;**190**(1):7-20.

(42) Brodsky WY, Uryvaeva IV. Cell polyploidy: its relation to tissue growth and function. *Int Rev Cytol* 1977;**50**:275-332.

(43) Mattia G, Vulcano F, Milazzo L et al. Different ploidy levels of megakaryocytes generated from peripheral or cord blood CD34+ cells are correlated with different levels of platelet release. *Blood* 2002;**99**(3):888-97.

(44) Eliades A, Papadantonakis N, Ravid K. New roles for Cyclin E in megakaryocytic polyploidization. *J Biol Chem* 2010;**285**(24):18909-17.

(45) Geddis AE. Shedding light on endomitosis. *Blood* 2010;**116**(13):2202-3.

(46) Geddis AE, Fox NE, Tkachenko E et al. Endomitotic megakaryocytes that form a bipolar spindle exhibit cleavage furrow ingression followed by furrow regression. *Cell Cycle* 2007;**6**(4):455-60.

(47) Lordier L, Jalil A, Aurade F et al. Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and Rho/Rock signalling. *Blood* 2008;**112**(8):3164-74.

(48) Papadantonakis N, Makitalo M, McCrann DJ et al. Direct visualization of the endomitotic cell cycle in living megakaryocytes: differential patterns in low and high ploidy cells. *Cell Cycle* 2008;**7**(15):2352-6.

(49) McCrann DJ, Ravid K. Survivin localization during endomitosis of high ploidy mouse megakaryocytes. *Blood* 2012;**116**(12):2192-3.

(50) Geddis AE. Megakaryopoiesis. *Semin Hematol* 2010;**47**(3):212-9.

(51) Zimmet JM, Ladd D, Jackson CW et al. A role for cyclin D3 in the endomitotic cell cycle. Mol Cell Biol 1997;**17**(12):7248-59.

(52) Sun S, Zimmet JM, Toselli P et al. Overexpression of cyclin D1 moderately increases ploidy in megakaryocytes. *Haematologica* 2001;**86**(1):17-23.

(53) Kirito K, Kaushansky K. Transcriptional regulation of megakaryopoiesis: tpo signaling and nuclear factors. *Curr Opin Hematol* 2006;**13**(3):151-6.

(54) Kaushansky K. The molecular mechanisms that control thrombopoiesis. *J Clin Invest* 2005;**115**(12):3339-47.

(55) De Sauvage FJ, Carver-Moore K, Luoh SM et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med* 1996;**183**(2):651-6.

(56) Alexander WS, Roberts AW, Nicola NA et al. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* 1996;**87**(6):2162-70.

(57) Gurney AL, Carver-Moore K, de Sauvage FJ et al. Thrombocytopenia in c-mpl-deficient mice. *Science* 1994;**265**(5177):1445-7.

(58) Geddis AE. Congenital amegakaryocytic thrombocytopenia. *Pediatr Blood Cancer* 2011;**57**(2):199-203.

(59) Quentmeier H, MacLeod RA, Zaborski M et al. JAK2 V617F tyrosine kinase mutation in cell lines derived from myloproliferative disorders. *Leukemia* 2006;**20**(3):471-6.

(60) Kaushansky K. On the molecular origins of the chronic myeloproliferative disorders: it all makes sense. *Blood* 2005;**105**(11):4187-90.

(61) Minamiguchi H, Kimura T, Urata Y et al. Simultaneous signalling through c-mpl, c-kit and CXCR4 enhances the proliferation and differentiation of human megakaryocyte progenitors: possible roles of the PI3-K, PKC and MAPK pathways. *Br J Haematol* 2001;**115**(1):175-85.

(62) Chang AN, Cantor AB, Fujiwara Y et al. GATA-factor dependence of the multitype zinc-finger protein FOG-1 for its essential role in megakaryopoiesis. *Proc Natl Acad Sci USA* 2002;**99**(14):9237-42.

(63) Shivdasani RA, Fujiwara Y, McDevitt MA et al. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J* 1997;**16**(13):3965-73.

(64) Bracken CP, Whitelaw ML, Peet DJ. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cell Mol Life Sci* 2003;**60**(7):1376-93.

(65) Bárdos JI, Ashcroft M. Negative and positive regulation of HIF-1: A complex network. *Biochem Biophys Acta* 2005;**1755**(2):107-20.

(66) Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* 2004;**5**(5):343-54.

(67) Kenneth NS, Rocha S. Regulation of gene expression by hypoxia. *Biochem J* 2008;**414**(1):19-29.

(68) Greer SN, Metcalf JL, Wang Y et al. The updated biology of hypoxia-inducible factor. *EMBO J* 2012;**31**(11):1448-60.

(69) Klimova T, Chandel NS. Mitochondrial complex III regulates hypoxic activation of HIF. *Cell Death Differ* 2008;**15**(4):660-6.

(70) Finley LW, Carracedo A, Lee J et al. SIRT3 opposes reprogramming of cancer cell metabolism through HIF1 alpha destabilization. *Cancer Cell* 2011;**19**(3):416-28.

(71) Masson N, Singleton RS, Sekirnik R et al. The FIH hydroxylase is a cellular peroxide sensor that modulates HIF transcriptional activity. *EMBO Rep* 2012;**13**(3):251-7.

(72) Lim JH, Lee YM, Chun YS et al. Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. *Mol Cell* 2010;**38**(6):864-78.

(73) Dioum EM, Chen R, Alexander MS et al. Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. *Science* 2009;**324**(5932):1289-93.

(74) Chen R, Dioum EM, Hogg RT et al. Hypoxia increases sirtuin 1 expression in a hypoxia-inducible factor-dependent manner. *J Biol Chem* 2011;**286**(16):13869-78.

(75) Zhong L, D'Urso A, Toiber D et al. The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* 2010;**140**(2):280-93.

(76) Peyssonnaux C, Nizet V, Johnson RS. Role of the hypoxia inducible factors HIF in iron metabolism. *Cell Cycle* 2008;**7**(1):28-32.

(77) Hu CJ, Wang LY, Chodosh LA et al. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* 2003;**23**(24):9361-74.

(78) Hu CJ, Sataur A, Wang L et al. The N-terminal transactivation domain confers target gene specifity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. *Mol Biol Cell* 2007;**18**(11):4528-42.

(79) Huang LE, Bunn HF. Hypoxia-inducible factor and its biomedical relevance. *J Biol Chem* 2003;**278**(22):19575-8.

(80) Loboda A, Jozkowicz A, Dulak J. HIF-1 and HIF-2 transcription factors--similar but not identical. *Mol Cell* 2010;**29**(5):435-42.

(81) Rankin EB, Biju MP, Liu Q et al. Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *J Clin Invest* 2007;**117**(4):1068-77.

(82) Covello KL, Kehler J, Yu H et al. HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev* 2006;**20**(5):557-70.

(83) Majmundar AJ, Wong WJ, Simon MC. Hypoxia-Inducible factors and the response to hypoxic stress. *Mol Cell* 2010;**40**(2):294-309.

(84) Peyssonnaux C, Zinkernagel AS, Schuepbach RA et al. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). *J Clin Invest* 2007;**117**(7):1926-32.

(85) Lambert MP, Wang Y, Bdeir KH et al. Platelet factor 4 regulates megakaryopoiesis through low density lipoprotein receptor-related protein 1 (LRP1) on megakaryocytes. *Blood* 2009;**114**(11):2290-8.

(86) Emerit J, Beaumont C, Trivin F. Iron metabolism, free radicals, and oxidative injury. *Biomed Pharmacother* 2001;**55**(6):333-9.

(87) Muckenthaler MU, Galy B, Hentze MW. Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. *Annu Rev Nutr* 2008;28:197-213.

(88) Wang J, Pantopoulos K. Regulation of cellular iron metabolism. *Biochem J* 2011;**434**(3):365-81.

(89) Latunde-Dada GO, Takeuchi K, Simpson RJ et al. Haem carrier protein 1 (HCP1): expression and functional studies in cultured cells. *FEBS Lett* 2006;**58**0(30):6865-70.

(90) Shayeghi M, Latunde-Dada GO, Oakhill JS et al. Identification of an intestinal heme transporter. *Cell* 2005;**122**(5):789-801.

(91) Evstatiev R, Gasche C. Iron sensing and signalling. *Gut* 2012;**61**(6):933-52.

(92) Dautry-Varsat A, Chiechanover A, Lodish HF. pH and the recycling of transferring during receptor-mediated endocytosis. *Proc Natl Acad Sci USA* 1983;**80**(8):2258-62.

(93) Ohgmai RS, Campagna DR, Greer EL et al. Identification of a ferrireductase required for efficient transferring-dependent iron uptake in erythroid cells. *Nat Genet* 2005;**37**(11):1264-9.

(94) Ohgami RS, Campagna DR, McDonald A et al. The Steap proteins are metalloreductases. *Blood* 2006;**108**(4):1388-94.

(95) Hentze MW, Muckenthaler MU, Galy B et al. Two to tango: regulation of mammalian iron metabolism. *Cell* 2010;**142**(1):24-38.

(96) Rouault TA, Tong WH. Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat Rev Mol Cell Biol* 2005;**6**(4):345-51.

(97) Zhang AS, Enns CA. Iron homeostasis: recently identified proteins provide insight into novel control mechanisms. *J Biol Chem* 2009;**284**(2):711-5.

(98) Zhang AS, Anderson SA, Meyers KR et al. Evidence that inhibition of hemojuvelin shedding in response t iron is mediated through neogenin. *J Biol Chem* 2007;**282**(17):12547-56.

(99) Pak M, Lopez MA, Gabayan V et al. Suppression of hepcidin during anemia requires erythropoietic activity. *Blood* 2006;**108**(12):3730-5.

(100) Lee P, Peng H, Gelbart T et al. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proc *Natl Acad Sci* USA 2005;*102*(6):1906-10.

(101) Nemeth E, Rivera S, Gabayan V et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004;**113**(9):1271-6.

(102) Wang RH, Li C, Xu X et al. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metab* 2005;**2**(6):399-409.

(103) Sanchez M, Galy B, Dandekar T et al. Iron regulation and the cell cycle: identification of an iron-responsive element in the 3'-untranslated region of human cell division cycle 14A mRNA by a refined microarray-based screening strategy. *J Biol Chem* 2006;**281**(32):22865-74.

(104) Cmejla R, Petrak J, Cmejlova J. A novel iron responsive element in the 3'UTR of human MRCKalpha. *Biochem Biophys Res Commun* 2006;**34**(1):158-66.

(105) Recalcati S, Tacchini L, Alberghini A et al. Oxidative stress-mediated down-regulation of rat hydroxyacid oxidase1, a liver-specific peroxisomal enzyme. *Hepatology* 2003;**38**(5):1159-66.

(106) Walden WE, Selezneva AI, Dupuy J et al. Structure of dual function iron regulatory protein 1 complexed with ferritin IRE-RNA. *Science* 2006;**314**(5807):1903-8.

(107) Salahudeen AA, Thompson JW, Ruiz JC et al. An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science* 2009;**326**(5953):722-6.

(108) Vashisht AA, Zumbrennen KB, Huang X et al. Control of iron homeostasis by an iron-regulated ubiquitin ligase. *Science* 2009;**326**(5953):718-21.

(109) Schranzhofer M, Schifrer M, Cabrera JA et al. Remodeling the regulation of iron metabolism during erythroid differentiation to ensure efficient heme biosynthesis. *Blood* 2006;**107**(19):4159-67.

(110) Kulnigg S, Evstatiev R, Haymerle G et al. Iron deficiency increases DNA-ploidy of megakaryocytic cells and consecutive platelet counts in IBD. *Gut* 2008;**57(Suppl II)**:A337.

(111) Ghosh K. Non haematological effects of iron deficiency - a perspective. *Indian J Med Sci* 2006;**60**(1):30-7.

(112) Mastrogiannaki M, Matak P, Keith B et al. HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. *J Clin Invest* 2009;**119**(5):1159-66.

(113) Shah YM, Matsubara T, Ito S et al. Intestinal hypoxia-inducible transcription factors are essential for iron absorption following iron deficiency. *Cell Metab* 2009;**9**(2):152-64.

(114) Sanchez M, Galy B, Muckenthaler MU et al. Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. *Nat Struct Mol Biol* 2007;**14**(5):420-6.

(115) Wang GL, Semeza GL. General involvement of hypoxia-inducible transcription factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A* 1993;**90**(9):4304-8.

(116) Nemeth E, Ganz T. The role of hepcidin in iron metabolism. *Acta Haematol* 2009;**122**(2-3):78-86.

(117) Bianchi L, Taccini L, Cairo G. HIF-1 mediated activation of transferrin receptor gene transcription by iron chelation. *Nucleic Acids Res* 1999;**27**(21);4223-7.

(118) Lok CN, Ponka P. Identification of a hypoxia response element in the transferrin receptor gene. *J Biol Chem* 1999;**274**(34):24147-52.

(119) Rolfs A, Kvietikova I, Gassmann M et al. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *J Biol Chem* 1997;**272**(32):20055-62.

(120) Lee PJ, Jiang BH, Chin BY et al. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J Biol Chem* 1997;**272**(9):5375-81.

(121) Mukhopadhyay CK, Mazumder B, Fox PL. Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. *J Biol Chem* 2000;**275**(28):21048-54.

(122) Sato T, Fuse A, Eguchi M et al. Establishment of a human leukaemic cell line (CMK) with megakaryocytic characteristics from a Down's syndrome patient with acute megakaryoblastic leukaemia. *Br J Haematol* 1989;**72**(2):184-90.

(123) Martin P, Papayannopoulou T. HEL cells: a new human erythroleukemia cell line with spontaneous and induced globin expression. Science 1982;216(4551):1233-5.

(124) Baatout S, Chatelain B, Staquet P et al. Interaction between protein kinase C and actin in megakaryocyte polyploidization. *Anticancer Res* 1999;**19**(5B):4193-8.

(125) Komatsu N, Suda T, Moroi M et al. Growth and Differentiation of a Human Megakaryoblastic Cell Line, CMK. *Blood* 1989;**74**(1):42-8.

(126) Ballen KK, Ritchie AJ, Murphy C et al. Expression and activation of protein kinase C isoforms in a human megakaryocytic cell line. *Exp Hematol* 1996;**24**(13):1501-8.

(127) Herrera R, Hubbell S, Decker S et al. A role for the MEK/MAPK pathway in PMA-induced cell cycle arrest: modulation of megakaryocytic differentiation of K562 cells. *Exp cell Res* 1998;**238**(2):407-14.

(128) Niedel JE, Kuhn LJ, Vandenbark GR. Phorbol diester receptor copurifies with protein kinase C. *Proc Natl Acad Sci U S A* 1983;**80**(1):36-40.

(129) Séverin S, Ghevaert C, Mazharian A. The mitogen-activated protein kinase signaling pathways: role in megakaryocyte differentiation. *J Thromb Haemost* 2010;**8**(1):17-26.

(130) Melemed AS, Ryder JW, Vik TA. Activation of the mitogen-activated protein kinase pathway is involved in and sufficient for megakaryocytic differentiation of CMK cells. *Blood* 1997;**90**(9):3462-70.

8 Abbreviations

A	
ACD	Anemia of chronic disease
ARNT	Aryl hydrocarbon nuclear translocator

B	
BFU-MK	Burst forming unit megakaryocyte
BMP	Bone morphogenic protein
bHLH	Basic helix loop helix

C	
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CFU-MK	Colony forming unit megakaryocyte
CD41	GlycoproteinIIb; alphaIIb-integrin
CD61	Beta 3 integrin chain
CD42	Glycoprotein IB; vWF receptor
c-Mpl	Myeloproliferative leukemia virus oncogene; TPO receptor
CD36	Thrombospondin receptor
CXCR4	C-X-C chemokine receptor type 4
CDC14a	Cell division cycle 14 homolog A
CDC42BPA	CDC42 binding protein kinase alpha
CBP	Creb-binding protein

D	
DMS	Demarcation membrane system
DcytB	Duodenal cytochrome B
DMT1	Divalent metal transporter 1
DFO	Deferoxamine

E	
ERK	Extracellular signal-related kinase

EPO	Erythropoietin

F

FOG1	Friend of GATA1
Fli1	Friend leukemia integration 1
FBXL	F-box and leucin repeat protein 5
FIH1	Factor inhibiting HIF1
FLVCR	Feline leukemia virus subgroup C cellular receptor

G

GMP	Granuloloycte/macrophage progenitor
GpA	Glycophorin 1
GATA1	Erythroid transcription factor
GDF15	Growth differentiation factor 15

H

HAO 1	Hydroxyacid oxidase 1
HFE	Hereditary hemochromatosis protein
HIF1	Hypoxia inducible factor 1
HIF2	Hypoxia inducible factor 2, EPAS1
HJV	Haemojuvelin
HO1	Hemoxygenase 1
HRE	Hypoxia responsive element
HSC	Haematopoietic stem cell

I

ID	Iron deficiency
IDA	Iron deficiency anemia
IBD	Inflammatory bowel disease
IL6	Interleukin-6
IL11	Interleukin-11
ISC	Iron-sulfur cluster
IRIDA	Iron refractory iron deficiency anemia

IRP1	Iron responsive element 1
IRP2	Iron responsive element 2
IRE	Iron responsive protein
iNOS	Inducible nitric oxide synthase

J

JAK2	Janus kinase 2

L

LRP1	Low density lipoprotein receptor related protein 1

M

MK	Megakaryocyte
MEP	Megakaryoid-erythroid progenitor
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin

N

NF-E2	Nuclear factor (erythroid-derived) 2

O

ODDD	Oxygen-dependent degradation domain
Oct4	Octamer binding transcription factor 4

P

PF4	Platelet factor 4
PI3K	Phosphoinositol-3-kinase
PMA	Phorbol-12-myristate-13 acetate
PKC	Protein kinase C
PHD	Prolyl hydroxylase domains
PCAF	P300/CBP-associated factor
PAS	Per/Arnt/Sim-domain
PKM2	Pyruvate kinase muscle isozyme 2

R

RanBP10	Ran binding protein 10
RhoA	Ras homolog member A
ROS	Reactive oxygen species

S

STAT	Signal transducer and activator of transcription
SOCS	Suppressors of cytokine signaling
SDF1	Stromal cell-derived factor 1
SENP3	Sentrin-specific protease 3
SIRT	Sirtuin
SCF	Stem cell factor

T

TPO	Thrombopoietin
TfR1	Transferrin Receptor 1; CD71
TfR2	Transferrin Receptor 2
TAD	Transactivation domain
TWSG1	Twisted gastrulation homologue 1

U

UTR	Untranslated region

V

vWF	Von Willebrand factor
vHL	Von Hippel-Lindau
VEGF	Vascular endothelial growth factor

9 Appendix

9.1 Zusammenfassung (deutsch)

Titel:„Die Rolle des ‚hypoxia inducible factors' in Eisenmangel-induzierter Megakaryopoese"

Hintergrund: Chronisch entzündliche Darmerkrankungen (CED) werden oft mit Eisenmangel und einer erhöhten Anzahl von Blutplättchen in Verbindung gebracht. Diverse Studien zeigen, dass die Behandlung mit Eisensupplementen die Zahl der Blutplättchen normalisiert. Wir schlagen ein Modell vor, in dem Eisenmangel die Anzahl der Blutplättchen direkt durch die geförderte Differenzierung von Megakaryozyten beeinflusst. Auf molekularer Ebene könnte der ‚hypoxia inducible factor' (HIF) ein wichtiger Regulator von Eisenmangel-induzierter Megakaryopoese sein.

Ziele: Ziel dieser Arbeit war es den Einfluss von HIF auf den Prozess der Megakaryopoese unter eisenarmen Bedingungen anhand einer Analyse des Expressionsmusters von HIF1 alpha, HIF2 alpha und HIF Zielgenen und durch Überexpression der beiden Transkriptionsfaktoren in megakaryozytären Zelllinien zu untersuchen.

Methoden: Die megakaryozytären Zelllinien CMK und HEL wurden unter eisenarmen und eisenreichen Bedingungen kultiviert und analysiert. Die Expression von HIF1 alpha, HIF2 alpha und Zielgenen von HIF untersuchten wir auf mRNA Ebene durch ‚qRT PCR', die Expression von HIF1 alpha auf Proteinebene durch ‚Western blots'. Als Positivkontrolle induzierten wir die megakaryozytäre Differenzierung durch PMA. Nach der Transfektion der Zellen mit Plasmiden, die HIF1 oder HIF2 alpha enthielten, wurden Veränderungen hinsichtlich megakaryozytärer Differenzierung, Polyploidie und der Expression von HIF Zielgenen bestimmt.

Ergebnisse: Die durch Eisenmangel induzierte megakaryozytäre Differenzierung führte sowohl auf mRNA als auch auf Proteinebene zu einer Hochregulierung der HIF Expression. Die Expression von HIF Zielgenen CXCR4 und LRP1, beide involviert in Megakaryopoese, wurde unter eisenarmen Bedingungen auf mRNA Ebene signifikant hinauf reguliert. (HEL: CXCR4: $P = 0.032$ und $P = 0.003$ in DFO behandelten verglichen mit der Kontrolle; LRP1: $P = 0.010$ und 0.034 in DFO behandelten Zellen verglichen mit der Kontrolle).

Schlussfolgerungen: Diese Studie unterstreicht die These, dass HIF als Regulator während der Eisenmangel-induzierten Megakaryopoese agiert. Die Ergebnisse fördern das wissenschaftliche Verständnis für die molekularen Mechanismen während dieses Prozesses und könnten deshalb im Zuge vertiefender Studien zur Entwicklung von zielgerichteten Therapien für Patienten mit CED, Eisenmangel und Thrombozytose beitragen.

9.2 Abstract (English)

Title: "The role of hypoxia inducible factor in iron deficient megakaryopoiesis"

Background: Inflammatory bowel disease (IBD) is often associated with iron deficiency and increased platelet counts. Various studies show that the treatment based on supplemented iron normalizes the counts of platelets. We propose that iron deficiency affects platelet counts directly by inducing megakaryocytic differentiation. On molecular level the hypoxia inducible factor (HIF) might be an important regulator of iron deficient megakaryopoiesis.

Aims: The aim of this study was to investigate the influence of HIF on megakaryocytic maturation under iron deficiency by analyzing the expression pattern of HIF1 alpha, HIF2 alpha and HIF targets and by examining the overexpression of both transcription factors in megakaryocytic cell lines.

Methods: The megakaryocytic cell lines CMK and HEL were cultured under iron deficient and iron replete conditions and analyzed. We evaluated the expression of HIF1 alpha, HIF2 alpha and HIF target genes on mRNA level by real-time PCR and the expression of HIF1 alpha on protein level by western blots. As a positive control megakaryocytic differentiation was induced by PMA. After transfection of cells with plasmids containing HIF1 alpha or HIF2 alpha, changes in the megakaryocytic differentiation, polyploidy and expression of HIF targets were determined.

Results: Iron deficiency induced megakaryocytic differentiation was associated with an upregulation of HIF expression both at mRNA and protein level. The HIF targets CXCR4 and LRP1, both implicated in megakaryopoiesis, were upregulated significantly on mRNA level under iron deficient conditions. (HEL: CXCR4: $P = 0.032$ and $P = 0.003$ in DFO treated cells compared to control cells; HEL: LRP1: $P = 0.010$ and 0.034 in DFO treated compared to control cells).

Conclusions: The present study underlines, that HIF acts as a regulator during iron deficient megakaryopoiesis. The results help to improve our understanding of molecular mechanisms during this process and might contribute to develop more targeted therapies for patients with IBD, ID and thrombocytosis in the future.

9.3 Curriculum vitae

Personal data	
Name	Kathrin Lippert
Address	1080 Vienna, Buchfeldgasse 9/1/7
Telephone	0650/ 822 57 65
Email	kathrinlippert@yahoo.de
Date of Birth	1st of March 1985 in Vienna

Education	
2005-now	Studies in **Biology**, University of Vienna Branch of study „Genetics/Microbiology“ Focus on „Molecular Genetics & Pathology“
2004-2008	Studies in **Nutrition Science**, University of Vienna
1995-2003	Bundesgymnasium Nonntal, Salzburg
1991-1995	Elementary school, Vienna

Employment History	
3/2012-now	Dr. Michael Lippert, Surgeon, 2500 Baden near Vienna **Assistant** (part-time employment)
10/2010-02/2012	Christian Doppler Laboratory for molecular chemoprevention, Medical University of Vienna, 1090 Vienna **Diploma thesis,** Supervision: Prof. Christoph Gasche
09/2011	„Übung Molekulare Systematik“, Prof. Hans-Jürgen Busse **Tutorship**, supervision of undergraduate students
07/2008, 07/2007	Dr. Michael Lippert, Surgeon, 2500 Baden near Vienna **Assistant** (summer job)
07/2003-02/2004	La Houge Fouque Farm Hotel, GY7 9YF Guernsey, Channel Islands **Waitress** (full time employment)

Skills	
Language skills	English (good knowledge), Italian (basic knowledge)
Computer skills	MS-Office (good knowledge)

Soft skills
Teamwork, Reliability, Personal Responsibility, Cordiality