universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Generation of floxed STOP TYK2 and floxed STOP STAT1 mice“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2017 / Vienna, 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Molecular Microbiology, Microbial Ecology and Immunobiology
Betreut von / Supervisor:	Univ.-Prof. Dr. Thomas Decker

Acknowledgements

First of all, I want to thank Prof. Thomas Decker for giving me the opportunity to work on this project. I really appreciated working in your lab, thank you a lot for your supervision.
I also want to thank Prof. Mathias Müller, whose lab collaborated on this project, and I hope the floxed STOP TYK2 mouse will be useful for your studies.
I also must not forget to express my gratitude to Emilio Casanova. The alleles and the cloning strategy were designed in close collaboration with him, so thank you very much for a lot of advice, for answering every e-mail and proofreading sequences.

Many thanks to my colleagues from the lab: Andrea, Katrin, Daniel, Duygu and Birgit. It was a great time. But especially I want to thank Roland for being my supervisor: Thank you a lot for your support and your patience throughout this year.
I would also like to thank the Baccarini, Kovarik and Versteeg lab for always being kind and helpful. Special thanks to Botond, Christian, Tania, Stefanie and Georg for admitting me as some kind of part of their lab. Thank you for caring for me.

Last but not least, if not most of all, I want to thank my parents. Thank you so much for supporting me. Not only during my master's thesis, but also during my whole life so far.

Table of contents

1 Introduction

1.1 The Immune system

1.1.1 Principles of innate and adaptive immunity

To protect the body effectively against pathogens and diseases, the immune system has to fulfill four tasks. The first is immunological recognition, the ability to detect the presence of an infection, carried out by white blood cells of the innate immune system and lymphocytes of the adaptive immune system. The second is to contain the infection and, if possible, eliminate it. This task is undertaken by immune effector functions such as the complement system of blood proteins, antibodies produced by lymphocytes and destructive capacities of lymphocytes and other white blood cells. The third task is immune regulation, or more precisely self-regulation of immune responses. Failed regulation can lead to allergies and autoimmune diseases. The fourth and last task is immunological memory, a unique feature of the adaptive immune system to protect the body against recurring infections [1].

The innate immune system provides the first line of defense, consisting of cellular and biochemical mechanisms. In addition to physical and chemical barriers, such as epithelial cells and antimicrobial chemicals produced on epithelial surfaces, it comprises phagocytic cells, such as macrophages and neutrophils, natural killer (NK) cells and dendritic cells. Other components are blood proteins, including members of the component system, and cytokines, proteins that coordinate and regulate important functions of the innate immunity. Adaptive immunity, in contrast, develops as a response to infection and adapts to it. It is able to recognize a large number of microbial and nonmicrobial substances and even distinguish between closely related microbes and molecules. Its main component are lymphocytes and their secreted products, such as antibodies. Antibodies recognize antigens, foreign substances that induce specific immune responses. While the innate immunity provides and effective initial defense against infections, many pathogens have evolved to resist that system. In that case, the adaptive immunity is required to provide more powerful mechanisms. However, there are many connections between the innate and the adaptive immune system, and both are part of an integrated system of host defense in which various cells and molecules function cooperatively [1,2].

1.1.2 Cells of the Immune system

Both innate and adaptive immunity depend on the activities of white blood cells or leukocytes. Phagocytes, antigen-presenting cells (APCs), lymphocytes and various other leukocytes function to eliminate antigens [2,1].

Phagocytes, such as neutrophils and macrophages, recruit cells to the site of infection. They recognize and are activated by microbes, leading to their ingestion and destruction by the process of phagocytosis. Phagocytes also communicate with other cells by direct contact and secreted proteins to regulate immune responses [2,1].

Neutrophils are the most numerous cells in innate immune responses and mediate the earliest phases of inflammatory reactions. They efficiently destroy microorganisms in intracellular vesicles filled with degradative enzymes such as lysozyme, collagenase and elastase. Neutrophils circulate in the blood for only six hours, and can migrate to sites of infection within a few hours after microbial entry. If not recruited, they undergo apoptosis and are usually phagocytosed by resident macrophages [2,1].

Macrophages are the mature form of monocytes and are resident in almost all tissues. They are relatively long-lived cells and perform several important functions in innate and adaptive immunity. Besides their major function of ingesting pathogens, they also ingest dead host cells and cell debris as part of the cleaning up process after infection or injury. Macrophages secrete proteins, called cytokines, that bind to signaling receptors and thereby instruct other cells to respond and contribute to host defense. They can also serve as APCs, specialized cells to capture antigens. They display these antigens to lymphocytes and provide signals for their proliferation and differentiation [2,1].

Macrophages and B cells present antigens to T-lymphocytes in different types of immune responses, but the most important APCs are dendritic cells. Most dendritic cells have finger-like processes, like dendrites of nerve cells. Similar to macrophages, they express receptors that recognize molecules made by microbes and respond by secreting cytokines. Conventional dendritic cells in skin, mucosa and organ parenchyma can become mobile and migrate to lymph nodes where they display antigens to T-lymphocytes. Thus, these cells are an important link between innate and adaptive immunity, functioning in both immune responses [2,1].

Three additional cell types that play a role in innate and adaptive immunity are mast cells, basophils and eosinophils. All three types have cytoplasmic granules filled with various inflammatory and antimicrobial mediators. They are also involved in immune responses that

protect against helminths and that cause allergic diseases. Mast cells are bone-marrow derived and are present in the skin and epithelial mucosa. They are believed to play a role in protecting the internal surfaces of the body against pathogens. Basophils and eosinophils are blood granulocytes that express granules containing various enzymes and toxic proteins. These are harmful to parasitic cell walls, but in allergic immune responses their effects on host tissues are rather damaging than protective [2,1].

A unique cell type of and key component the adaptive immune system are lymphocytes, cells that express clonally distributed antigen receptors, each with a fine specificity for a different antigenic determinant. In absence of infection, most lymphocytes that are circulating in the body are small, featureless cells. These cells, not yet activated by an antigen, are called naïve lymphocytes. Lymphocytes that met an antigen, become activated and differentiate into fully functional lymphocytes are called effector lymphocytes [2,1].

The two main types of lymphocytes are B cells (B lymphocytes) and T cells (T lymphocytes), each with quite different roles in the adaptive immunity and distinct types of antigen receptors. B cells that bind an antigen with the B cell receptor (BCR) on their surface proliferate and differentiate into plasma cells. Plasma cells produce antibodies, the secreted form of the BCR with an identical antigen specificity. Thus, the antigen becomes the target of the antibodies, thereby promoting ingestion and destruction by phagocytes. Antibody molecules as a class are known as Immunoglobulins [2,1].

T cells that bind an antigen with their T cell receptor (TCR) proliferate and differentiate into one of three types of effector T cells. Cytotoxic T cells (CTLs) kill cells that are infected with viruses or other intracellular pathogens. Helper T cells provide essential signals that influence the behavior and activity of other cells, like the antibody production of antigen stimulated B cells or the ability of macrophages to become more efficient in killing pathogens. The last type are regulatory T cells that suppress the activity of other lymphocytes, thus helping to regulate immune responses [2,1].

One additional population of lymphocytes are Natural killer (NK) cells, that recognize and kill abnormal cells. NK cells respond to the presence of an infection, but are not specific for an antigen, thus being an important part of the innate immunity by holding viral infections in check before the adaptive immunity kicks in [2,1].

During the process of an immune response, some of the activated B cells and T cells differentiate into memory cells. Immunological memory is an important feature of the

adaptive immune response, leading to a long-lasting immunity that can follow exposure to disease or vaccination. After a second contact to an antigen, memory cells will readily differentiate into effector cells and maintain host defense [2,1].

1.1.3 Receptors of the Immune system

The innate immune system has to recognize molecular structures that are characteristic of microbial pathogens. These microbial substances stimulating the innate immunity are called pathogen-associated molecular patterns (PAMPs). Different classes of pathogens express different PAMPs, such as mannose-rich oligosaccharides, peptidoglycans, lipopolysaccharides (LPS) and unmethylated CpG DNA. In addition to PAMPs, the innate immunity also recognizes endogenous molecules that are produced or released from damaged and dying host cells. These molecules, called danger-associated molecular patterns (DAMPs), are produced as a result of cell damage caused by infection or injury. Both PAMPs and DAMPs are recognized by receptors generally known as pattern recognition receptors (PRRs). They allow the body to distinguish between self and non-self. PRRs are mainly expressed by macrophages, neutrophils and dendritic cells. They are linked to intracellular signal transduction pathways, thus activating various cellular responses. There are four main classes of PRRs, which differ in structure and specificity: Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs) [2,1].

Toll was originally identified as a *Drosophila* gene involved in embryogenesis of the fruit fly, but discovery of antimicrobial responses mediated by the protein led to the identification of mammalian homologues, termed Toll-like receptors. There are ten known TLRs in humans and thirteen in mice. TLRs are type I integral membrane glycoproteins, containing leucin-rich repeats flanked by characteristic cysteine-rich motifs in their extracellular regions that are involved in ligand binding. In their cytoplasmic tails, they contain a Toll/IL-1 receptor (TIR) homology domain which is essential for signaling. TLR1, TLR2, TLR4, TKR5 and TLR6 are expressed on the plasma membrane, while TLR3, TLR7, TLR8 and TLR9 are mainly expressed on endosomes. Ligands recognized by the different TLRs are structurally diverse and include products of all classes of microorganisms e.g. LPS and flagellin of bacteria, double- and single-stranded RNA of viruses and mannose polysaccharides of fungi. Besides these microbial products, TLRs are also involved in responses to endogenous molecules, e.g. HSPs (heat shock

proteins) and HMGB1 (high-mobility group box 1), both intracellular proteins becoming extracellular when released from injured or dying cells [2,1].

NLRs are a family of more than 20 different cytosolic proteins named after NOD (nucleotide oligomerization domain-containing protein). Typical NLRs contain a c-terminal leucine-rich repeat domain similar to the ones in TLRs, that senses presence of a ligand, a centrally located NACHT domain allowing the receptors to form oligomers and an N-terminal effector domain that recruits other proteins to form signaling complexes. Subfamilies of NLRs can be distinguished due to this effector domain, called CARD (caspase recruitment domain), Pyrin or BIR (baculovirus inhibitor of apoptosis protein repeat) domain. NOD1 and NOD2, members of the CARD domain subfamily, respond to bacterial cell wall peptidoglycans and are important in innate immunity against bacterial pathogens in the gastrointestinal tract, such as *Helicobacter pylori* and *Listeria monocytogenes*. Other members of the NLR family, such as NLRP1, NLRP3, and NLRC4/IPAF, are components of the inflammasome, leading to the activation of inflammatory caspases e.g. Caspase-1 [2,1].

RLRs are cytosolic receptors of viral RNA, responding to viral nucleic acids by inducing the production of antiviral type I interferons. RIG-I (retinoic-acid inducible gene I) and MDA5 (melanoma differentiation-associated gene 5) are the two best characterized RLRs. Both contain two N-terminal CARD domains for the interaction with other signaling proteins, and a RNA-helicase domain that bind to viral RNAs. They display different specificities for viral RNA, partly based on the length of the RNA genome. RIG-I recognizes single-stranded (ss)RNA, distinguishing between eukaryotic and viral RNA due to 5' triphosphate, and short blunt-ended double-stranded (ds)RNA. MDA5, in contrast, only recognizes long dsRNA. RLR signaling activates NF-κB and initiates signaling events leading to IRF3 and IRF7 activation, thus inducing the production of Type I interferons [2,1].

CLRs are membrane bound receptors with a C-type lectin-like domain (CTLD) consisting of two protein loops that are stabilized by disulfide bridges, whereas the second loop contains the ligand binding site. CLRs recognize a wide range of ligands, such as carbohydrates, proteins, and lipids of both pathogens and self. Examples for members of the CLR family are Dectin-1 and Dectin-2, which preferentially bind to microbial organisms and induce pro-inflammatory signaling. Other CLRs sense tissue damage and are important for cell homeostasis [3].

1.1.4 Cytokines

Cytokines are a large and heterogenous group of secreted proteins, which are produced by many different cell types. They mediate and regulate all aspects of innate and adaptive immunity. They can act in an autocrine manner by affecting the cells that released them, in a paracrine manner by affecting the adjacent cells, or in an endocrine manner and affect the behavior of distant cells when entering the blood circulation. Many of them were arbitrarily named based on one of the biological activities they were discovered to have, e.g. Tumor necrosis factor and interferons, others are called interleukins followed by a number (e.g. IL-1), because they were thought to be produced by and acting on leukocytes. Cytokines are usually not stored as performed molecules, but their synthesis is initiated by new gene transcription in consequence of cellular activation. Their synthesis is transient, just as their messenger (m)RNA is unstable and often rapidly degraded. While one cytokine can have multiple biological effects, called pleiotropism, many of them are said to be redundant due to having the same actions and functions. The structural, functional and genetic relations between cytokines and their receptors suggest that they may have diversified in parallel during the evolution of specialized effector functions. There are many different families, grouped by their structure. Important ones are the IL-1 family, the very large hematopoietin superfamily, the TNF family and the type I interferons. Interferon receptors are a small family of heterodimeric receptors signaling through the JAK/STAT pathway and activating different combinations of STATs (signal transducers and activators of transcription) with different effects [2,1].

Since Interferons (IFNs) and their signaling pathway are targeted in this project, they are explained more precisely in the following chapter.

1.2 Interferon signaling

1.2.1 Interferons and their receptors

Around 60 years ago, IFN was discovered as an agent that inhibited the replication of influenza virus. Due to this interference, it was called Interferon. Nowadays, the IFN family is recognized as a key component of the innate immune response and the first line of defense against viral infections.[4] These widely expressed cytokines induce a cell-intrinsic antiviral state, activate proinflammatory responses and have growth-inhibitory and immunomodulatory effects [5,6]. They stimulate or inhibit up to 300 different genes encoding proteins involved in both innate and adaptive immunity. Various types of IFNs are used for treatment of viral infections, inflammatory diseases and other malignancies. Besides their role in inflammasome activation, intestinal homeostasis and inflammatory diseases, they were also found to be overexpressed in several autoimmune disorders, such as systemic lupus erythematosus, polymyositis or rheumatoid arthritis [7,8,9].

IFNs have been grouped into three different types, based on structural features, receptor usage and biological activities: type I, II and III, the latter not discovered until 2003. While there are various kinds of type I IFNs (IFN-α, IFN-β, -ω, -ε, -κ) in humans and mice, there is only one type II IFN (IFN-γ) and three type III IFNs (IFN-λ1, -λ2, -λ3) [8,10]. Actions of IFNs are mediated by three receptor complexes, whereby a heterodimer of IFN- α receptor 1 (IFNAR1) and IFNAR2 binds to type I IFNs, and a heterodimer of interleukin 10 receptor 2 (IL-10R2) and IFN-λ receptor 1 (IFNLR1) binds to the three subtypes of IFN-λ. The type II IFN receptor complex however consist of a tetramer of two IFN-γ receptor 2 (IFNGR2) chains and two IFNGR1 chains, binding to IFN-γ (see **Figure 2**) [4].

In addition to their antiviral activity, type I IFNs increase cellular immunity regarding NK and T cell activation. They impact on cells at the interface of innate and adaptive immunity, such as macrophages and dendritic cells, by increasing antigen presentation. Despite their generally protective function against viral infections, defense of bacterial pathogens can be both increased or decreased by type I IFNs. While they have protective effects in *Chlamydia pneumoniae*, *Legionella pneumophila*, *Salmonella typhimurium* and *Streptococcus* infections, their production is associated with decreased innate immunity in *Listeria monocytogenes*, *Francisella tularensis*, and *Mycobacterium tuberculosis* infections. Type I IFNs also contribute to immunosurveillance against cancer [11].

IFN-γ mainly targets macrophages and T cells, where it induces transcription factors and influence cell differentiation. In macrophage biology, it provides cell-autonomous antimicrobial activity via upregulation of microbicidal gene products. This impact on macrophage activation results from synergizing or antagonizing effects of different cytokines, growth factors, and PAMP signaling pathways, leading to antimicrobial and antitumor effects. IFN-γ upregulates chemokines and adhesion molecules, and directs cells to the sites of inflammation [11].

Although IFN-λ is signaling through a different receptor complex than type I IFNs, it stimulates the same pathway, described in detail in the next subchapter. IFN-λ produces similar biological changes in target cells, such as antiviral, antiproliferative, and antitumor activity. However, due to receptor distribution it leads to different organismic responses, which is mainly on cells of epithelial origin [11].

1.2.2 JAK/STAT signaling pathway

After uncovering IFNs and their ability to instruct gene expression in human cells, a logical progression of empirical findings lead to the discovery of the JAK/STAT pathway. Nowadays, this pathway is regarded as a central communication node of the immune system, operating downstream of more than 50 cytokines and growth factors. In mammals, it is mediated by four kinases of the Janus kinases (JAK) family (JAK1, JAK2, JAK3 and TYK2) and seven transcription factors of the signal transducer and activator of transcription (STAT) family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). In the canonical pathway, JAKs are composed of four domains: the FERM (protein 4.1, Ezrin, Radixin, Moesin) domain, the SH2 (Src homology 2) -like domain, the pseudokinase domain and the kinase domain. The FERM and the SH2-like domains both mediate interactions with upstream receptors, while the FERM domain also promotes kinase function. In addition, the pseudokinase domain limits unwarranted kinase activity, and the kinase domain contains the activation loop tyrosines and the catalytic elements for tyrosine-phosphorylation of the receptors. STATs are composed of seven domains: The N-terminal domain, the coiled-coil domain, the DNA-binding domain, the linker domain, the SH2 domain, the transactivation domain and the C-terminal domain. The N-terminal domain and the coiled-coil domain are involved in protein-protein interactions, while the coiled-coil domain also contains nuclear-localization signals. The DNA-binding domain directly interfaces with DNA and contains nuclear import-export signals. The linker

domain is a structurally important domain promoting transcriptional activity. The SH2 domain mediates dimerization and, like the SH2-like domain in JAKs, interacts with upstream receptors. The transactivation domain contains phosphorylated tyrosine residues necessary for canonical signaling, and the C-terminal domain contains serine-phosphorylated residues supporting both canonical and non-canonical functions (**Figure 1**) [12].

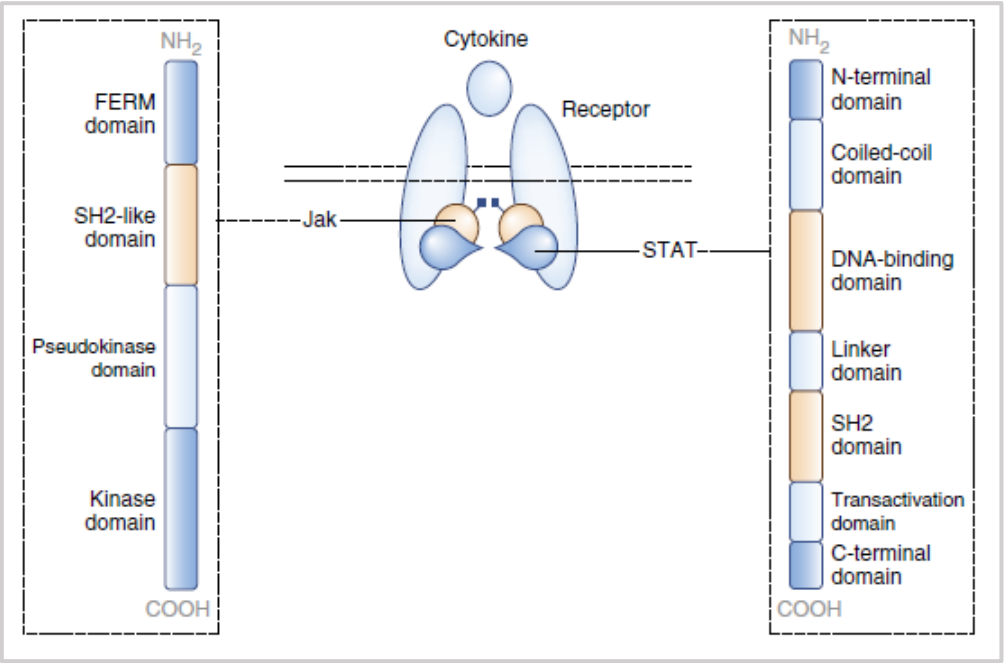


Figure 1 Protein structure of JAKs and STATs [12]. *The JAK family consists of four members, JAK1, JAK2, JAK3 and TYK2, each composed of four domains, the FERM (protein 4.1, Ezrin, Radixin, Moesin) domain, the SH2 (Src homology 2) -like domain, the pseudokinase domain and the kinase domain. The STAT family consists of seven members, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6, each composed of seven domains, the N-terminal domain, the coiled-coil domain, the DNA-binding domain, the linker domain, the SH2 domain, the transactivation domain and the C-terminal domain. Functions of each domain are explained in the text.*

In the canonical JAK/STAT signaling, cytokines initially associate with their corresponding transmembrane receptors. Oligomerization of the receptors leads to trans-activation of the JAKs and phosphorylation of the cytoplasmic tails of the receptors, creating a docking site for STATs. Recruitment of STATs is followed by their tyrosine-phosphorylation, mediated by JAKs. This results in STAT dimerization and nuclear translocation, followed by DNA binding and gene transcription (**Figure 2**).

In case of type I IFNs, after binding to IFNAR1/IFNAR2 receptor complex, the pre-associated JAK1 and TYK2 initiate signal transduction upon phosphorylation of the receptor. This leads to

the recruitment and phosphorylation of STAT1 and STAT2, which form heterodimers and associate with the IFN-regulatory factor 9 (IRF9), forming the complex IFN-stimulated gene factor 3 (ISGF3). After translocating to the nucleus, the complex binds to sequence elements termed IFN-stimulated response elements (ISRE) and modulates the expression of a group of more than 300 genes referred to as IFN-stimulated genes (ISGs), e.g. *ISG15* (IFN-stimulated protein of 15 kDa), *Mx* (myxovirus resistance), *OAS* (2',5'-oligoadenylate synthetase) and *PKR* (protein kinase R) [4,10,12].

In the type II IFN pathway, IFN-γ binds to the IFNGR2/IFNGR1 receptor complex with pre-associated JAK1 and JAK2. Phosphorylation leads to recruitment of STAT1, forming a homodimer termed IFN-γ activation factor (GAF), which translocates to the nucleus, binds to the IFN-γ activated sequence (GAS) promotor and induces ISGs [4,10,12].

The type III IFN pathway proceeds similar to the type I IFN pathway. Type III IFNs bind to the IL-10R2/IFNLR1 complex with pre-associated JAK1 and TYK2, ultimately resulting in the ISGF3 complex and the induction of ISGs [4,10,12].

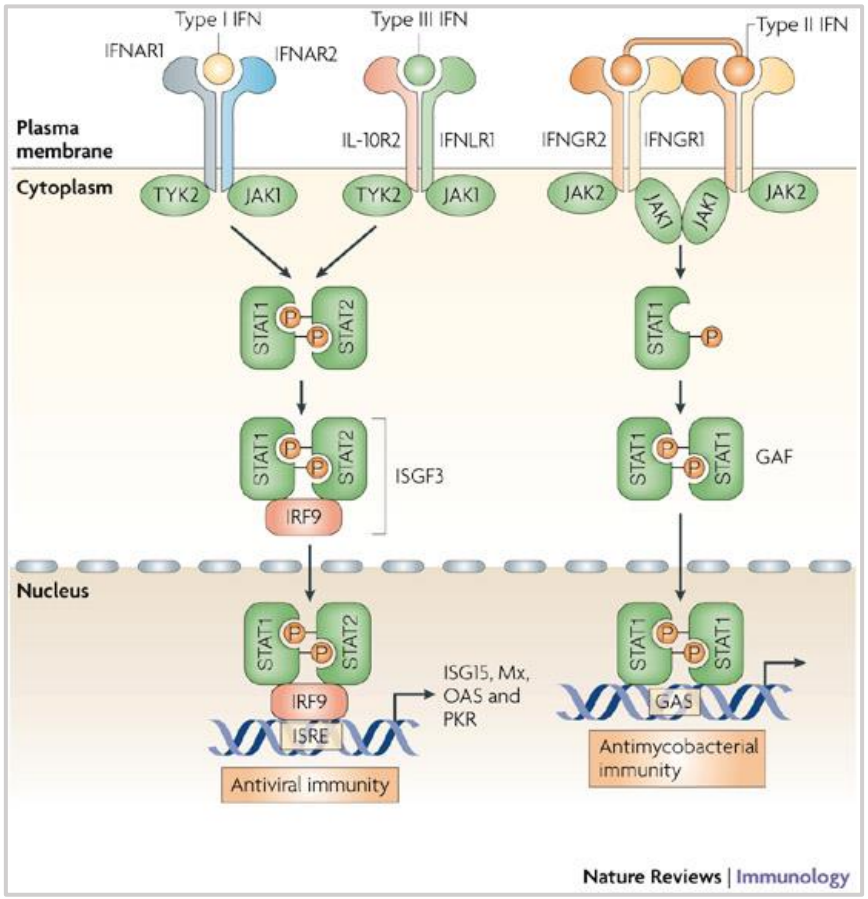


Figure 2 Canonical JAK/STAT signaling pathway [4]. *Actions of interferons (IFNs) are mediated by three different receptor complexes. A heterodimer of IFN-α receptor 1 (IFNAR1) and IFNAR2 binds to type I IFNs, a heterodimer of interleukin*

10 receptor 2 (IL-10R2) and IFN-λ receptor 1 (IFNLR1) binds to the three subtypes of IFN-λ and a tetramer of two IFN-γ receptor 2 (IFNGR2) chains and two IFNGR1 chains binds to type II IFN-γ. After binding of the IFNs to their corresponding receptors, pre-associated Janus Kinases (JAKs) initiate signal transduction by phosphorylation of the receptors and recruitment and phosphorylation of signal transducers and activators of transcription (STATs). In case of type I and II IFNs, STAT1 and STAT2 form heterodimers and associate with IFN-regulatory factor 9 (IRF9) to form the complex IFN-stimulated gene factor 3 (ISGF3). In case of IFN-λ, STAT1 forms homodimers, termed IFN-λ activation factor (GAF). These complexes translocate to the nucleus, followed by DNA binding and transcription of IFN-stimulated genes from IFN-stimulated response elements (ISRE) or IFN-γ activated sequence (GAS) promotor elements. ISG15: IFN-stimulated protein of 15 kDa. Mx: myxovirus resistance OAS: 2',5'-oligoadenylate synthetase. PKR: protein kinase R.

Although the large majority of IFN-induced gene expression is mediated by canonical pathways, increasing evidence indicates the formation of alternative, non-canonical complexes. While the canonical model posits that type I and type III IFNs employ STAT1/STAT2 heterodimers and type II IFN employs STAT1/STAT1 homodimers, numerous other examples have been reported, such as STAT1/STAT3 and STAT1/STAT4 heterodimers. Function of these complexes remain largely unexplored, as well as the importance of tetramers. Tetramerization of STAT1 was reported to be required for anti-microbial activities of type II IFNs but not for anti-viral activities of type I IFNs. Another tetramer was shown to be formed by STAT5, playing a role in NK cell homeostasis, regulatory T cell function and cytotoxic T cell proliferation [12]. Examples for other non-canonical STAT complexes are STAT1/IRF9 and STAT2/IRF9, each like a ISGF3 complex lacking the other STAT. Both of these complexes are important in *Legionella pneumophila* resistance, while STAT1/IRF9 also plays a role in colitis (when induced by IFN-γ), and STAT2/IRF9 also plays a role in Flavivirus resistance. Furthermore, there is the possibility of unphosphorylated (U-)STATs, controlling organelle metabolism and function in mitochondria and Golgi apparatus [11]. U-STAT1 was shown to inhibit nuclear translocation of tyrosine-phosphorylated STAT2 in the type I IFN response and to contribute to innate antibacterial immunity against *Listeria monocytogenes* and *Legionella pneumophila* [13]. Recently reports also indicate functions of kinase-inactive mutants of JAK2 and TYK2, both exerting non-canonical roles not requiring their kinase activity. While kinase-dead TYK2 functions in NK cytotoxicity and mitochondrial respiration, kinase-dead JAK2 is important for the stability of the IFN-γ receptor and the residual IFN-γ response [11].

Since the genes for TYK2 and STAT1 are the main targets of this project, these two members of the JAK/STAT signaling pathway are described in detail in the following subchapters, mainly focused on their canonical functions.

1.2.3 TYK2

The non-receptor Tyrosine kinase 2, which was originally described in 1990, was the first member of the JAK family to be genetically linked to IFN responses [14,15]. While human *Tyk2* is located on chromosome 19, murine *Tyk2* is located on chromosome 9. Compared to the other JAKs, TYK2 is a large protein with a molecular weight of approximately 130 kDa [16]. It is activated by phosphorylation of tyrosine 1051/1055 in humans and tyrosine 1047/1048 in mice, respectively. Although several other amino acid residues were reported to undergo phosphorylation on serine or tyrosine, their function so far remains unknown. Up to now, only one case of ubiquitination, on lysine, has been identified for TYK2 in humans and mice, and there is no known case of SUMOylation. Like all JAKs, TYK2 is negatively regulated by phosphatases and suppressors of cytokine signaling (SOCS), induced in a negative feedback loop in the JAK/STAT pathway. There are eight members of the SOCS family, two of them (SOCS1 and SOCS3) known to inhibit TYK2. Besides its role in signaling downstream of the receptors IFNAR1 and IL-10R2 described above, it has been shown to associate with three other receptor chains: interleukin 12 receptor β1 (IL-12Rβ1), interleukin 13 receptor α1 (IL-13Rα1) and glycoprotein 130 (gp130) [17]. Therefore, TYK2 is phosphorylated upon binding of type I IFNs [18,19], the IL-10/IL-20 families of cytokines [20,21], the IL-12 family [22], IL-4/IL-13 [23,24] and the IL-6/gp130 family of cytokines [25,26] to their corresponding receptor complexes. Accumulated data from cells with a loss of function of TYK2 indicates that the protein contributes to type I and III IFNs, IL-12, IL-13, IL-22 and IL-23 signaling. Its role in signaling of IL-10 and IL-6 family members still remains unclear [17].

The first patient with homozygous TYK2 deletion was described in 2006 [27]. In this case, a premature stop codon resulted in a loss of TYK2, leading to recurring viral, fungal and mycobacterial infections. Cells of the patient showed impaired type I IFN, IL-6, IL-19, IL-12 and IL-23 signaling. The patient also suffered from autosomal recessive hyper IgE syndrome (HIES), although screening of other HIES patients revealed that TYK2 deficiency is not a common cause of this disease [28]. Following this, seven other TYK2-deficient patients were identified, all suffering from viral and/or mycobacterial infections, but neither from HIES, nor from candiasis or inflammatory diseases. Analysis of signaling pathways revealed impaired responses to all TYK2 activating cytokines mentioned above, except to IL-6 [29,30,31]. Since data from patients

with mutant or lacking TYK2 is constantly increasing, valid murine models for the translation into pathophysiology and clinical setting are indispensable.

Three different groups have reported TYK2-deficient mice (*Tyk2*$^{-/-}$) [32,33,34]. Although they display no pathological phenotypes under conventional housing conditions, they are highly sensitive to infections and show defective tumor surveillance [35]. The high susceptibility to viral, bacterial and parasitic infections has been mainly attributed to a reduced type I IFN response and impaired IL-12-dependent IFN-γ production [36]. *Tyk2*$^{-/-}$ mice fail to clear vaccinia virus and to raise an effective CTL response after lymphocytic choriomeningitis virus challenge [32,35]. When it comes to bacterial infections, such as *Listeria monocytogenes* and *Escherichia coli*, they display increased bacterial load and reduced survival. They are also more sensitive to protozoan pathogens, as infection with *Leishmania major* leads to more severe skin lesions [35]. As mentioned above, *Tyk2*$^{-/-}$ mice show defects in tumor surveillance. TYK2 was linked to cancer after these mice developed hematopoietic malignancies after transplantation and induction of tumors. These tumor models also showed an impaired cytotoxicity of NK and T cells, suggesting TYK2 to be an important regulator of NK and T cell mediated tumor surveillance, such as in adenocarcinoma and T cell lymphoma cells [37,38]. *Tyk2*$^{-/-}$ mice were also used in various colitis models, like DSS-induced colitis. Besides significant weight loss, they displayed more severe colitis symptoms compared to WT mice, such as massive destruction of the intestinal epithelium and a high amount of infiltrating inflammatory cells [36].

Despite these harmful effects on the immune system, absence of TYK2 was shown to be beneficial in some cases, and leading to increased resistance against inflammatory and autoimmune diseases. *Tyk2*$^{-/-}$ mice survive high-dose LPS-induced endotoxin shock and show normal systemic levels of pro-inflammatory cytokines. However, their IFN-γ production is once more strongly impaired. TYK2-deficient mice also show reduced infiltration of inflammatory cells during development of experimental autoimmune encephalomyelitis [35].

To uncover cell type-specific functions of TYK2 in vivo, floxed mice (*Tyk2*$^{fl/fl}$) containing a conditional *Tyk2* null allele were crossed to different Cre mouse lines. *Tyk2*$^{fl/fl}$ mice were generated by using the third exon of *Tyk2*, flanked by two *LoxP* sites and a neomycin resistance cassette. Since this exon contains a start codon, Cre-mediated deletion (see chapter **1.2**) results in the loss of TYK2. These mice revealed that myeloid TYK2 highly contributes to defense against murine cytomegalovirus. Although it is an important regulator of tumor surveillance, such as in adenocarcinoma and T cell lymphoma, absence of the protein in

exclusively myeloid, dendritic or T cells did not impair the process [39]. In DSS-induced colitis, the major STAT3-activating cytokine IL-22 was shown to be significantly reduced in TYK2-deficient intestinal epithelial cells [36].

Future work with ubiquitous and conditional TYK2-deficient mice, tissues and cells is necessary to further investigate the role and functions of TYK2 and to validate the growing number of inhibitors for therapeutic intervention [40,41,42], thus pointing out the utility of floxed STOP TYK2 mice.

1.2.4 STAT1

Signal transducer and activator of transcription 1 was first described in 1993, but due to its molecular weight of 91 kDa it was initially named STAT91 [43]. Human *Stat1* is located on chromosome 2, while murine *Stat1* is located on chromosome 1 [44,45]. The protein is mainly activated by phosphorylation of tyrosine 701 and serine 727 [46,47]. Phosphorylation of tyrosine 701 is done by JAKs, leading to STAT1 activation and translocation to the nucleus. Serine phosphorylation, in response to cellular stress and upon IFN stimulation, leads to full transcriptional activation [48,49]. STAT1 can also be modified by SUMO, which increases its solubility and in this way tyrosine dephosphorylation, leading to a decreased responsiveness to IFN-γ [11]. STAT1 exists in two isoforms, STAT1α and STAT1β, which are generated by alternative splicing. STAT1β lacks part of the C-terminally transactivation domain (see **Figure 1**), including the serine 727 phosphorylation site. Due to this truncation, it was initially assumed to be transcriptionally inactive and a competitive inhibitor of STAT1α. By now, STAT1β has been shown to be able to drive transcription in response to IFN-γ, even though delayed and at reduced levels in comparison to STAT1α [46,48]. STAT1, as its name implies, transduces signals from cytoplasmic domains and transmembrane receptors to the nucleus. There it regulates gene expression and modulates various cellular processes, such as proliferation, differentiation and cell death. STAT1 transduces anti-proliferative effects of all types of IFNs, like cell cycle inhibition, sensitization to apoptotic stimuli, and induction of different forms of cell death. Besides IFNs, STAT1 transduces activities of IL-21, IL-27 and IL-35 [48].

In humans, various degrees of loss of function of STAT1 have been identified. Patients with compete autosomal recessive STAT1-deficiency suffer from recurrent viral and mycobacterial infections, often with lethal consequence. Heterozygous autosomal recessive deficiency leads

to similar, but milder clinical symptoms with more positive prognosis. Both of these forms of deficiency cause impaired IFN responses. In case of an autosomal dominant loss of function, patients suffer from mycobacterial diseases and show reduced responses to IFN-γ and IL-27 [50,51]. Several mutations have been reported impairing the phosphorylation of tyrosine 701, like an autosomal dominant mutation in the SH2 domain that also leads to impaired responses to IFN-γ and IL-27 [52,53]. In another case, a heterozygous Y701C mutation was identified, where tyrosine 701 is replaced with phenylalanine, leading to an abolished phosphorylation and impaired responses to IFN-γ and IFN-α. Although the patient did not suffer from mycobacterial infections and several viral infections followed a normal clinical course, he was diagnosed with Mendelian susceptibility to mycobacterial disease and multifocal osteomyelitis [54]. However, most inborn errors of STAT1 lead to autosomal dominant gain-of-function mutations. Patients show a broad clinical phenotype, such as recurrent *Candida* infections and autoimmune disorders [50,55,56]. Similar to TYK2, murine models targeting STAT1 have enabled important and often unexpected discoveries related to human immunology, and are indispensable for further studies.

Two different types of knockout mice for STAT1 have been generated, one leading to a truncated and inactive protein [57] and the other one leading to a complete loss [58]. STAT1-deficient mice (*Stat1*$^{-/-}$) are highly susceptible to various pathogens, due to their lack of type I, II and III IFN responses [57,58]. They showed high susceptibility to many viruses, except (+) single -stranded RNA dengue virus and (-) single-stranded measles virus, and they succumb to intracellular bacteria, such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*, and parasites, such as *Toxoplasma gondii* and *Leishmania major* [11] These mice were also hyper-sensitive to certain inflammatory pathologies, such as EAE, a phenotype that is so far not apparent in human patients [12,59]. STAT1 is widely considered to be a tumor suppressor, since *Stat1*$^{-/-}$ mice develop more methylcholantrene-induced tumors with shorter latency than WT mice, assumably due to an impaired IFN-γ-dependent tumor surveillance. In absence of the tumor suppressor p53, loss of STAT1 also decreases the latency of spontaneous tumor formation and even broadens the tumor spectrum [60]. However, there is growing evidence that STAT1 can also act as an tumor promotor, like in case of v-abl-induced B-lymphoid leukemia where *Stat1*$^{-/-}$ mice are partially protected from the disease [37].

To uncover cell type-specific functions of STAT1 in vivo, floxed mice (Stat1$^{fl/fl}$) containing a conditional *Stat1* null allele were crossed to different Cre mouse lines. The *Stat1*$^{fl/fl}$ allele was

generated in a similar procedure as the complete loss *Stat1*$^{-/-}$ model [58], by using the exons 6 to 10, flanked by *LoxP* sites. At the 3' end of exon 10, a floxed Neomycin resistance cassette was added to the construct. The exons are part of the coiled-coil domain and the DNA binding domain of STAT1 (see **Figure 1**), resulting in a functionally inactive protein (after Cre-mediated deletion, see chapter **1.2**) [47]. This restricted loss of STAT1 revealed strikingly different and unique roles of the protein in different cell types. Myeloid STAT1, especially in macrophages, is essential for a protective innate immunity, and mice lacking STAT1 in these cells fail to clear even low doses of *Listeria monocytogenes*. In contrast, absence of the protein in T cells leads to an increased survival and resistance against the bacteria. Absence of STAT1 in dendritic cells did not affect the phenotype of the mice. Additionally, bone-marrow chimeric mice revealed that STAT1 in non-hematopoietic cells seems to have a minor role in innate resistance, since mice lacking STAT1 in these cells showed only a minimal reduction of the bacterial load. However, in bone marrow-derived cells, the loss of STAT1 is linked to a loss of resistance [61].

As with TYK2, future work with ubiquitous and conditional STAT1-deficient mice, tissues and cells is necessary to further investigate the role and functions of STAT1 in innate and adaptive immunity and supporting the discovery of inhibitors and modulators for therapeutic intervention [62], thus pointing out the utility of floxed STOP STAT1 mice.

1.3 Design of a floxed STOP cassette

1.3.1 Cre/LoxP system

The Cre/LoxP System is a widely used, site-specific gene targeting approach. It derives from the bacteriophage P1, and was first described in 1981 [63]. Cre, a recombinase belonging to the integrase family, recognizes a 34 bp - long sequence motif called LoxP [64]. If a DNA sequence is flanked by two LoxP sites in the same orientation, Cre cuts out the sequence and the remaining ends rebind, leaving one single LoxP site. If a sequence is flanked by two LoxP sites in opposite orientation, it is inverted by Cre (**Figure 3**). For conditional gene targeting, Cre can be expressed via transient transfection of a vector into the target cells or by crossing a Cre-transgene into a mutant animal, leading to a cell-type-specific or inducible expression [65].

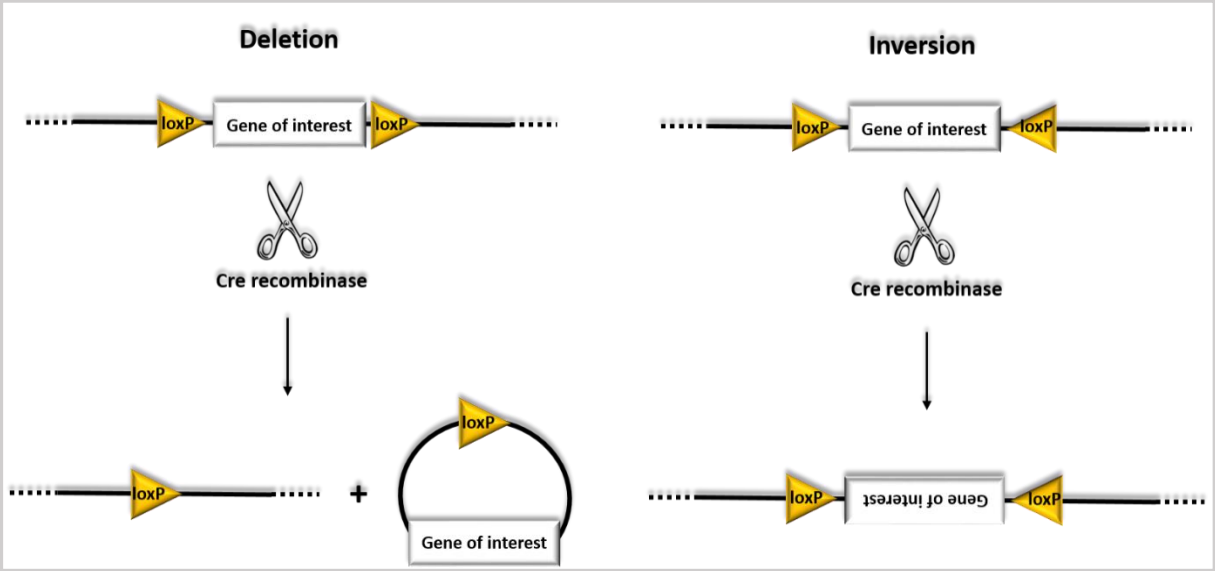


Figure 3 Orientation of LoxP sites determines way of modification. *If a DNA sequence is flanked by two LoxP sites in the same orientation, Cre cuts out the sequence and the remaining ends rebind, leaving one single LoxP site. If a sequence is flanked by two LoxP sites in opposite orientation, it is inverted by Cre.*

Using the Cre/LoxP System for floxed STOP mice works in a very similar manner. The only difference is the target, since not a gene but a STOP cassette is flanked by the two LoxP sites. Without Cre, the target gene is interrupted by the STOP signal. This inhibits gene expression and consequently production of the protein. By inducing Cre, the STOP signal is excised and the rebound sequence should lead to gene expression and production of a functional protein (**Figure 4**).

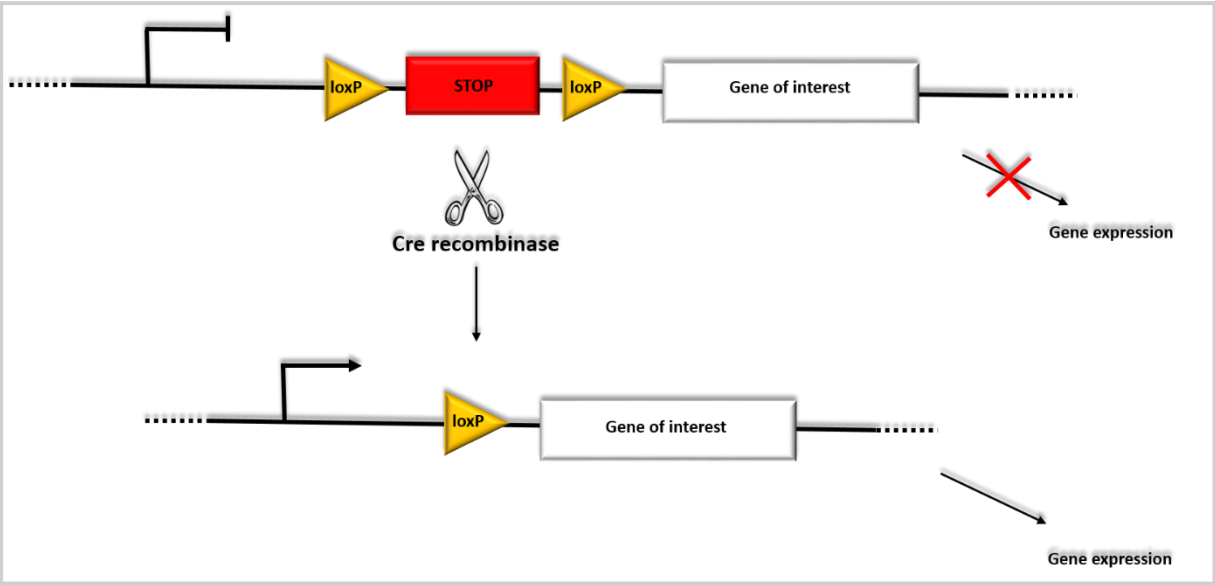


Figure 4 Functional principle of a floxed STOP cassette. *The STOP cassette is inserted upstream of the gene of interest, flanked by two LoxP sites. Without Cre recombinase, the gene is interrupted by the STOP signal. This inhibits gene expression and consequently production of the protein. By inducing Cre, the STOP signal is excised and the rebound sequence should lead to gene expression and production of a functional protein.*

1.3.2 STOP signals

Two sequence elements were chosen to generate the STOP cassette in this project. On the one hand, a synthetic polyadenylation signal with a transcriptional terminator site (pA-tT) was incorporated. Addition of a poly(A) tail is essential to produce mature mRNA and to export it from the nucleus, inhibit its molecular degradation in the cytoplasm, and for translation. Therefore, polyadenylation is necessary for proper gene expression. Mutant poly(A) signals could inhibit mRNA polyadenylation and disrupt transcriptional termination, resulting in unstable, and often elongated mRNAs. In the STOP cassette, the pA-tT signal is supposed to promote polymerase drop-off and abort transcription [66,67,68].

On the other hand, the nucleotide sequence of the last exon of the homeodomain transcription factor Engrailed-2 (en2) was used. The gene was discovered in *Drosophila*, where it plays an important role during segmentation development and is required for the formation posterior compartments. Different mutations in the mouse homolog lead to various developmental defects, frequently with lethal consequences. The human homolog encodes homeodomain-containing proteins, and is associated with pattern formation during development of the central nervous system [69,70]. In the generation of gene traps, the nucleotide sequence of the en2 splice acceptor has been used historically, as it was shown to increase their efficiency [71,72].

The final STOP cassette consists of the last exon of en2 followed by the pA-tT signal, and should result in the inhibition of gene expression. To prevent expression of any functionally relevant, truncated transcripts, the cassette is inserted upstream of the stretch of DNA deleted in the conditional knock out. For TYK2, the deleted stretch was exon 3, hence the STOP cassette is inserted between exon 1 and exon 2. For STAT1, exons 6 to 10 were deleted, thus the cassette is inserted between exon 4 and exon 5. It is assumed that the insertion sites do not interrupt any known regulatory elements and is not within a repetitive sequence element [73].

1.3.3 Neomycin resistance cassette

The neo cassette is a common feature in generating conditional knock outs, used for selection in embryonic stem cells. It is also very common to flank it with LoxP sites, in order to remove it by transient expression of the Cre recombinase [39,47,65,74]. Both constructs for floxed STOP mice do neither contain additional LoxP sites nor FRT (flippase recognition target) sites to remove the neo cassette. Since transcription is inhibited either way when the STOP cassette is inserted, neo should not affect gene expression. After induction of Cre recombinase, the whole floxed cassette containing neo is removed, and does not remain in the genome.

1.4 Red/ET Recombination system

Red/ET Recombination is a method that allows various modifications of DNA molecules at any chosen position. It enables the engineering of very large DNA fragments, like bacterial artificial chromosomes (BACs) or the *Escherichia coli* chromosome, without the use of restriction enzymes. This method relies on homologous recombination in *E. coli*, but has a way higher efficiency than the natural event [75,76].

Homologous recombination is a DNA repair mechanism that has common features in all cells. It only takes place between DNA molecules that share extensive stretches of similar sequences, called homologies. These homologies engage base-pairing between a single strand from one DNA molecule with the complementary single strand from the other one. In its simplest form, homologous recombination repair a double strand break of a DNA duplex by using the complementary strand of the intact duplex as a template. A nuclease chews back the ends of the broken DNA and creates singe-stranded 3' overhangs. Through a so-called strand exchange or strand invasion, these 3' ends search for homologous sequences in the template duplex by base-pairing. After the strand exchange, a DNA polymerase extends the invading strand by using the template information and restores the damaged DNA. The repair process is completed by strand displacement, further repair synthesis and ligation. Besides its role of in DNA repair, homologous recombination is also crucial for meiosis [77].

Since the sequences of these homologies can be chosen freely, recombination can take place at any chosen position in a precise and specific manner.

To increase the efficiency of homologous recombination in *E. coli*, Red/ET recombination utilizes proteins of the λ-phage, consequently the technique is often also referred to as λ-mediated recombination. λ-phages have, besides their lytic life cycle, also a lysogenic life cycle in which they integrate their own genome into the host genome at a specific site, using homologous recombination. The two main responsible enzymes for this reaction are called Redα and Redβ. Redα is a 5' – 3' exonuclease and Red β is a DNA binding protein that promotes annealing. Redα digests the 5' end of a DNA from a double-strand break, or rather the end of a linear stretch of DNA, creating a single-stranded, 3' ended overhang. Redβ binds to this overhang, leading to a protein-nucleic acid filament that aligns with homologous DNA. After the alignment, the 3' end acts as a primer for DNA replication (**Figure 5**) [78,79].

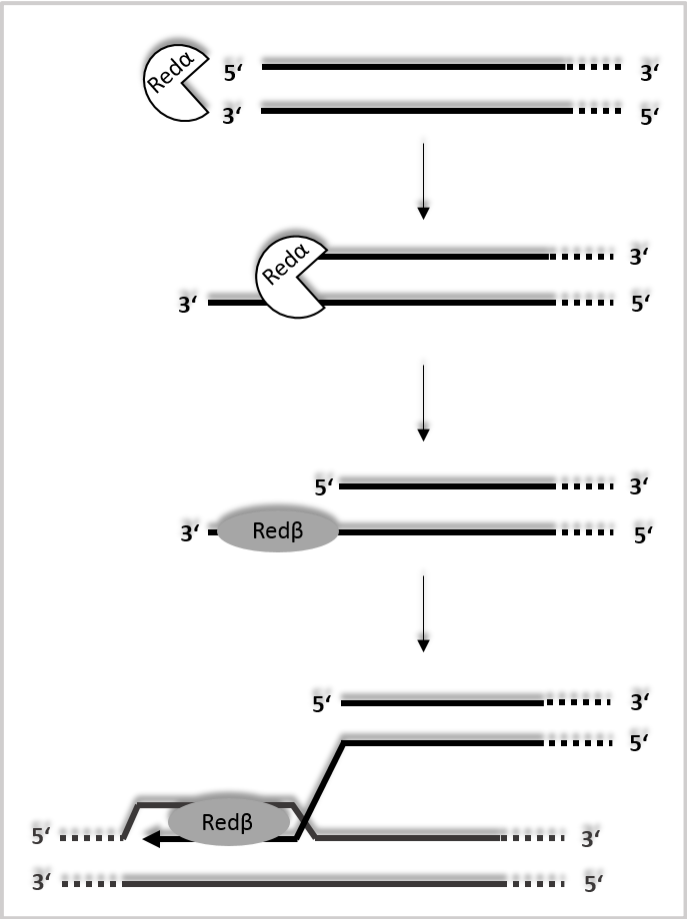


Figure 5 Functional principle of Redα and Redβ. *Redα is a 5' – 3' exonuclease that digests the 5' end of DNA from a double-strand break, or rather the end of a linear stretch of DNA, creating a single-stranded, 3' ended overhang. Redβ is a DNA binding protein that binds to this overhang, leading to a protein-nucleic acid filament that aligns with homologous DNA. After the alignment, the 3' end acts as a primer for DNA replication.*

Recombination is also assisted by expression of the *redγ* gene. The protein Gam inhibits the *E. coli* exonuclease RecBCD, which is involved in the repair of double-stranded DNA breaks and the degradation of λ-phage DNA [78,79].

Redα and Redβ are inducibly expressed from the plasmid pSC101, in this way transferrable to any *E. coli* strain. pSC101 is a low-copy-number plasmid containing a temperature-sensitive origin of replication due to the protein RepA [78,79]. RepA binds to directly repeated sequences within the origin, and it is required for plasmid replication and partitioning during cell division [80,81,82,83]. To keep the plasmid, cells have to be cultured at 30°C. At the restrictive temperature of 37°C, RepA becomes non-functional and plasmids containing this origin can no longer propagate [84]. The plasmid also carries the λ-phage-derived *redγβα* operon, which is expressed

under the control of the pBAD promotor, and a Tetracyclin resistance cassette. The pBAD promotor is regulated by the transcriptional regulator AraC. The AraC dimer inhibits transcription in the presence of glucose, but can form a complex with L-arabinose to allow transcription initiation, thus making the pBAD promotor arabinose-inducible [85]. Since the transcribed phage proteins are not temperature-sensitive, cells can be cultured between 37°C and 42°C after addition of arabinose. However, the recombination window is limited due to the transient expression of the Red proteins. Since *redγ* expression has been shown to be cytotoxic under some conditions and limit the efficiency of recombination, it is necessary to strictly regulate expression of *redγ* simultaneously with expression of *redα* and *redβ* [78,79].

Red/ET recombination is applicable to various kinds of modifications like insertions, deletions, replacements and to subcloning. Although the methodical approaches are very similar, there are some essential theoretical differences (**Figure 6**). For an insertion, deletion or replacement, the homology arms define the integration site, and an existing replication-competent molecule is modified. To create a linear DNA construct, the forward oligonucleotide is designed to have a 5' end homologous to a stretch of DNA directly upstream of the insertion site. The reverse oligonucleotide has a 5' end homologous to the stretch of DNA directly downstream of the insertion site, but in reverse complement orientation [79].

For a subcloning, the homology arms define the region that is to be copied into the plasmid. To design oligonucleotides, the DNA sequence on the 3' end of this region should be homologous to the 5' end of the forward oligonucleotide. The reverse oligonucleotide has a 5' end homologous to the 5' end of the region, but in reverse complement orientation [78].

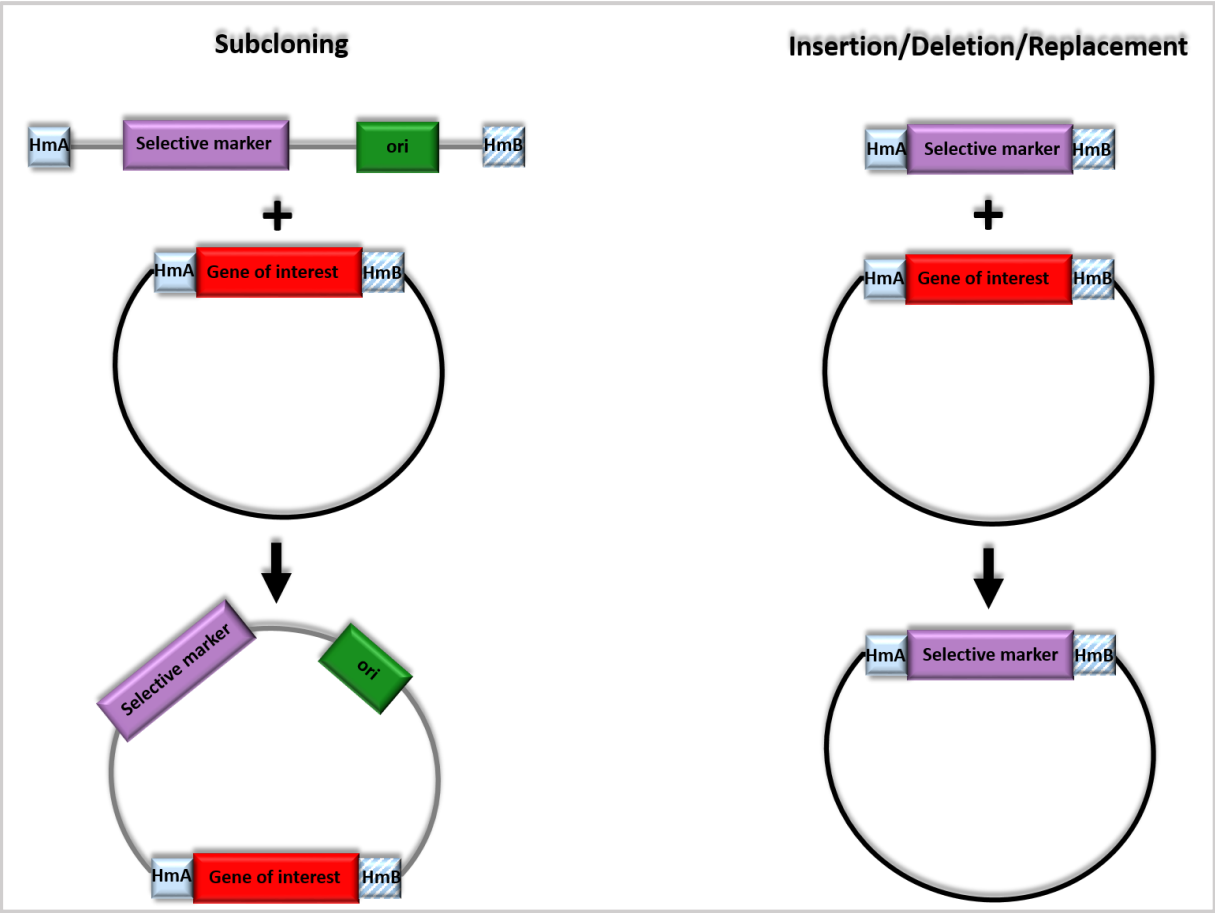


Figure 6 Differences between BAC Subcloning and BAC Modification. *For subcloning, the homology arms (HmA/B) define the region (Gene of interest) that is to be copied into the linear plasmid, containing a selective marker and an origin of replication (ori). For an insertion, deletion or replacement, the homology arms (HmA/B) define the integration site (Gene of interest), and an existing replication-competent molecule is modified with the help of a selective marker.*

2 Aims

This project deals with the generation of two different DNA constructs for transgenic mice. The aim was to generate mice in which the expression of TYK2 or STAT1 is abrogated by the insertion of a floxed STOP cassette, but can be restored to wildtype levels via inducible or tissue-specific expression of the Cre recombinase.

Without Cre recombinase, these mice are expected to show the same phenotype as *Tyk2*$^{-/-}$ and *Stat1*$^{-/-}$ mice, whereas Cre-mediated deletion results in a nearly wildtype allele (**Figure 7**).

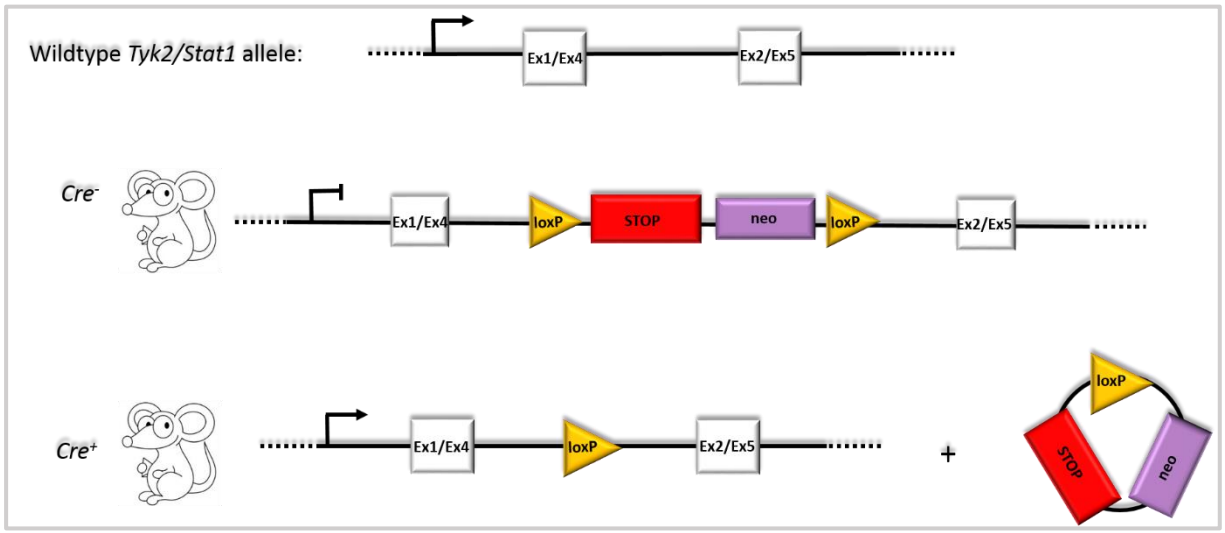


Figure 7 Floxed STOP TYK2 or floxed STOP STAT1 mouse with and without Cre expression. *The floxed STOP cassette, consisting of STOP signals (STOP) and a neomycin resistance cassette (neo), flanked by two LoxP sites, is integrated in the Tyk2 or Stat1 gene. In case of Tyk2, it is integrated between exon 1 and exon 2, in case of Stat1 between exon 4 and exon 5. Without Cre recombinase (Cre^{-}), the floxed STOP cassette interrupts gene expression, and these mice are expected to show the same phenotype as Tyk2$^{-/-}$ and Stat1$^{-/-}$ mice. Cre-mediated deletion (Cre^{+}) of the cassette results in a nearly wildtype allele with a single remaining LoxP site, allowing tissue-specific expression of Tyk2 or Stat1. The rest of the STOP cassette is degraded in the cell.*

Floxed STOP TYK2 and floxed STOP STAT1 mice are expected to complement former insights gained with conditional knockouts and to further investigate the role of the two proteins in innate immunity. Since macrophages are key players in innate immunity, an interesting topic would be what level of defense is provided exclusively by them when activated through TYK2 or STAT1. Floxed STOP TYK2 mice could also be used to compare development and resolution of an induced tumor and to allot a role for TYK2 in these two processes. Furthermore, an attractive topic would also be a comparison of necessity and sufficiency of the protein in different cell types. Floxed STOP STAT1 mice could be used to uncouple the role of STAT1 in

homeostasis and during infection, and to determine its function in non-hematopoietic cells. These are only few ideas to use the mice generated in this project.

3 Materials and Methods

3.1 Vectors

The plasmids pBluscript KS used for subcloning and as vector for the final construct, pR6K used as a source for the last exon of en2 as well as pA-tT signal, pPGK-Neo used as a source for the neomycin resistance cassette as well as the loxP sites and pSC101, used for the transient expression of the λ-phage-derived *redγβα* operon were kindly provided by Prof. Dr. Emilio Casanova (Ludwig Boltzmann Institute for Cancer Research, Medical University Vienna). Maps of the respective plasmids are shown in chapter **9.**, **Figure 18 – 20**.

The BAC clones containing the mouse *Tyk2* (clone number RP23-361H17) and *Stat1* (clone number RP23-140M10) loci were ordered from the BACPAC Resources Center at the Children's Hospital Oakland Research Institute in Oakland, California.

3.2 Primer

The primers used in this study are listed table 1. All primers were ordered from Sigma-Aldrich.

en2 fw	ATATGAGCTCGAATTCAACCCTTTCCCACACCAC
en2 rev	ATATCTCGAGTCTGCGTTCTTCTTCTTTGGTTTTCGGG
pA tT fw	ATATCTCGAGTCTAGATAAGTAATGATCATAATCAGCCATATC
pA tT rev	ATATCTGCAGGAATTCCTCCTGCAACATGAATATTAGAATTTTC
Tyk2 200bp 5’ fw	ATATGCGGCCGCTTAATTATTTATGAATAAATCCCTAGCCCAAAAG
Tyk2 200bp 5’ rev	ATATGTTTAAACAAGCTTCGGTAGAGAGGGAGGAACTC
Tyk2 200bp 3’ fw	ATATGGCGCGCAAGCTTATGCATCTCCCACCTAACACTTCCTGC
Tyk2 200bp 3’ rev	ATATGGCGCGCCGCGGCCGCCTTTTCCAGTACTTAGAACCGGTAAC
Tyk2 KS rec fw	GCCTCCCAAGTGCTGGGACTAAAGGAATACACCATGACTGCCTGGCTTCCGCGGCCGCTCCCCCGGGCTGCAGGAATTC
Tyk2 KS rec rev rev	ACATGTCAGCTCACAGCCACCTGCAACCCCAGTTGCAAGGGATCTGATGGGCGGCCGCCACCGCGGTGGAGC
Stat1 200bp 5’ fw	ATATGCGGCCGCAGGTTTGGCTCCTTCAAGC
Stat1 200bp 5’ rev	ATATGTTTAAACGGATCCTTCATTTCTGTTAAAATGAGAAAATTCTGC
Stat1 200bp 3’ fw	ATATGGCGCGCCACTAGTCAACCAAGCATTTCACCC
Stat1 200bp 3’ rev	ATATGGCGCGCCGCGGCCGCGTTTTCACTGTCAAAGCTATTTTTGC
Stat1 KS rec fw	TGTCTGCACGCATCAGAGCCTCTGAGGGGATATATTTTGCTATCAGCGTCGCGGCCGCCACCGCGGTGGAGC

Stat1 KS rec rev	TCACTGGAACTTGAAGGTTTGCCCGCTTTTGAGCTCCTGTTAGAAAGTCCGCGGCCGCTCCCCCGGGCTGCAGGAATTC
KSrec Stat new 5’	CTCTGGACCCTACAGCGCCGGGCATATAGCCTAAGTGTTACTGAGCACAGGCGGCCGCCACCGCGGTGGAGC
KSrec Stat new 3’	ATGGCCTGTGGAGAGCTAGCGTTGAGCCTCCCAGATGAGGAGGGTTGAGCGCGGCCGCTCCCCCGGGCTGCAGGAATTC
KSrec Stat 100 5’	TGTCTGCACGCATCAGAGCCTCTGAGGGGATATATTTTGCTATCAGCGTCCTCTGGACCCTACAGCGCCGGGCATATAGCCTAAGTGTTAGCGGCCGCCACCGCGGTGGAGC
KSrec Stat 100 3’	CACTGGAACTTGAAGGTTTGCCCGCTTTTGAGCTCCTGTTAGAAAGTCCATGGCCTGTGGAGAGCTAGCGTTGAGCCTCCCAGATGAGGAGCGGCCGCTCCCCCGGGCTGCAGGAATTC
Stat 8kb fw Xma 3	ATATCCCGGGGCGGCCGCGCAAGCTTCCTGTGGAAAGTTCATG
Stat 8kb rev Spe 3	ATATACTAGTGCGGCCGCCCTTCAAGTTCCAGTGACCAGC
8kb Stat fw 4 Xma	ATATATCCCGGGGCGGCCGCGTAACACTTAGGCTATATGCCCGGC
8kb Stat rev 4 Not	ATATTGCGGCCGCGCAAAGTGCTCAACCCTCCTC
Stat 8kb fw Spe 4	ATATATACTAGTCGGCCGCGTAACACTTAGGCTATATGCCCGGC

Table 1 Primers used in this project, sequence 5’-3’.

3.3 Preparation of competent *Escherichia coli*

E. coli Top10 cells were originally purchased from Thermo Fisher and were rendered chemically competent as follows: One colony was inoculated in 20 ml LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.5 - 8) and incubated overnight at 37 °C. On the next day, 200 µl of the overnight culture were inoculated into 20 ml fresh LB medium and allowed to grow at 37°C until they reached a OD600 of 0.45 – 0.5. After cooling them on ice for 5 minutes, they were centrifuged at 4°C at 3000 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 5 ml of TFBI buffer (30 mM KOAc, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% glycerol, pH 5.8). After incubation for 30 - 60 minutes on ice, the cells were again centrifuged at 4°C at 2000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 2 ml of pre-cooled TFBII buffer (10 mM H-MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol, pH 7.0). The cells were then aliquoted in 100 µl, frozen in liquid nitrogen and stored at - 80°C.

3.4 PCR

All PCRs were performed using the Q5 High Fidelity DNA Polymerase from New England BioLabs (NEB). 10 - 50 ng of the template were used in each reaction. Other reaction components were used as follows:

- 10 µl Q5 reaction buffer (5x)
- 1 µl dNTPs (10mM)
- 1 µl primer fw (10 µM)
- 1 µl primer rev (10 µM)
- 1 µl Q5 Polymerase

Nuclease-free water was added to a final volume of 50 µl per reaction.

All PCRs were performed on an Eppendorf cycler. In the initial denaturation step they were heat activated at 98 °C for 30 seconds, and then cycled 35 times using a denaturation step of 98 °C for 30 seconds. In the annealing step of 30 seconds, temperature varied between 50 °C and 72 °C, the appropriate annealing temperature for each primer pair was determined with the help of the NEB Tm calculator. The time of the extension step of 72°C was calculated according to the length of the amplicon, 40seconds per kb. After a final 2 minutes long extension step at 72 °C, PCRs were held at 4 °C.

3.5 Manipulation of recombinant DNA

All restriction enzymes were purchased from Thermo Scientific or NEB. T4 ligase and alkaline phospatase were purchased from Thermo Scientific. All enzymes were used according to manufacturer's instructions, unless otherwise noted.

Plasmids were isolated from *E. coli* using QUIAGEN plasmid maxi prep kit or Thermo Scientific GeneJET Plasmid Miniprep kit, according to manufacturer's instructions.

DNA was isloated from agarose gels (1% agarose in 1x TAE, unless otherwise indicated) using the QUIAQuick gel extraction kit according to manufacturer's instructions.

DNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific).

3.6 Transformation

1 µl of plasmid DNA or 1 µl of ligation mix were added to 100 µl of Top10 competent cells thawed on ice. After 20 minutes of incubation on ice, a heat shock at 42 °C was performed for 80 seconds. 900 µl of fresh LB medium was added to the cells, followed by a regeneration period at 37°C for 1 hour at 300 rpm.

After 3 minutes of centrifugation at 10.000 rpm, the supernatant was discarded and the pellet was resuspended in 100 µl nuclease-free water and plated onto a 10 cm LB agar plate containing appropriate antibiotics. Plates were then incubated overnight at 37 °C.

3.7 Red/ET Recombination

One colony containing the circular target DNA and the plasmid pSC101 was inoculated in 5 ml LB containing appropriate antibiotics and allowed to grow overnight at 28 °C. On the next day, 1 ml of the culture was inoculated into 100 ml fresh LB media containing appropriate antibiotics. The culture was allowed to grow at 28 °C until OD600 reached 0.2. L-Arabinose (Sigma Aldrich) was added to a final concentration of 0,5%, then the growth temperature was switched to 37 °C until OD600 reached 0.5.

The next steps were carried out at 4 °C, using pre-cooled reagents, pipet tips and tubes. The culture was centrifuged at 4000 rpm for 5 minutes. Supernatant was discarded, and the pellet was washed twice with 10 ml nuclease-free water. The pellet was transferred to an Eppendorf tube and again washed twice with 500 µl nuclease-free water, with centrifugation at 10.000 rpm for 3 minutes. The pellet was again resuspended in nuclease-free water and 200 µl were transferred into an electroporation cuvette. Approximately 100 ng of linear DNA that should be recombined was pipetted into the cuvette and the electroporation was performed in an MicroPulser Electroporator (BioRad) at 1,8 V, 200 Ω and 25µF.

The cells were transferred from the cuvette into a fresh Eppendorf tube. 1 ml fresh LB was added and the bacteria were allowed to recover at 37 °C for 60 - 90 minutes. After incubation, the culture was centrifuged and the pellet was resuspended in nuclease-free water. 100 µl of the cells were plated onto a 10 cm LB agar plate containing appropriate antibiotics.

3.8 Sequencing

All Sequencing results were ordered via the sequencing service Ready2Run from LGC Genomics. The reaction mix contained approximately 100 ng of plasmid DNA and two sequencing primers with a concentration of 5 µM each.

4 Results

4.1 Cloning Strategy

The cloning strategy consists of three parts: In the first step, the STOP cassette is assembled via a conventional cloning approach, applying restriction endonucleases and ligations. In the second step, approximately 8 kb of the *Tyk2* or the *Stat1* locus are isolated from a BAC and subcloned into a plasmid. These 8kb will ultimately serve as approximately 4 kb long homology arms 5' and 3' of the STOP cassette to allow recombination into the mouse genome. In the third step, the STOP cassette is inserted into the 8 kb to generate the final construct, a floxed STOP cassette flanked by approximately 4 kb long stretches of DNA homologous to *Tyk2* or *Stat1*. In both of these steps, Red/ET recombination was applied. Insertion of the constructs into mouse embryonic stem cells and the final generation of the floxed STOP mice will be done at the University of Veterinary Medicine in Vienna.

4.2 Assembly of the STOP cassette

The two functional components of the STOP cassette, pA-tT and en2, were amplified from pR6K using the primers pA tT fw, pA tT rev, en2 fw and en2 rev, adding XhoI restriction sites to the 5' end of pA-tT and the 3' end of en2 and EcoRI restriction sites to the 3' end of pA-tT and the 5' end of en2. The resulting amplicons were digested with XhoI and EcoRI and purified from a 1% agarose gel. The vector pBluescript KS was digested with XhoI and EcoRI and treated with alkaline phosphatase to avoid religation. The digested vector and inserts were ligated in a ratio of 1:5:5 before being transformed into *E. coli* (see **Figure 8A** for a graphical representation of the construct). Candidate clones able to grow on LB-ampicillin (100 µg/ml) plates were picked and plasmid DNA isolated via miniprep. To ascertain correct insertion, an analytical digest with EcoRI was performed (**Figure 8B**) and a promising clone was chosen to continue.

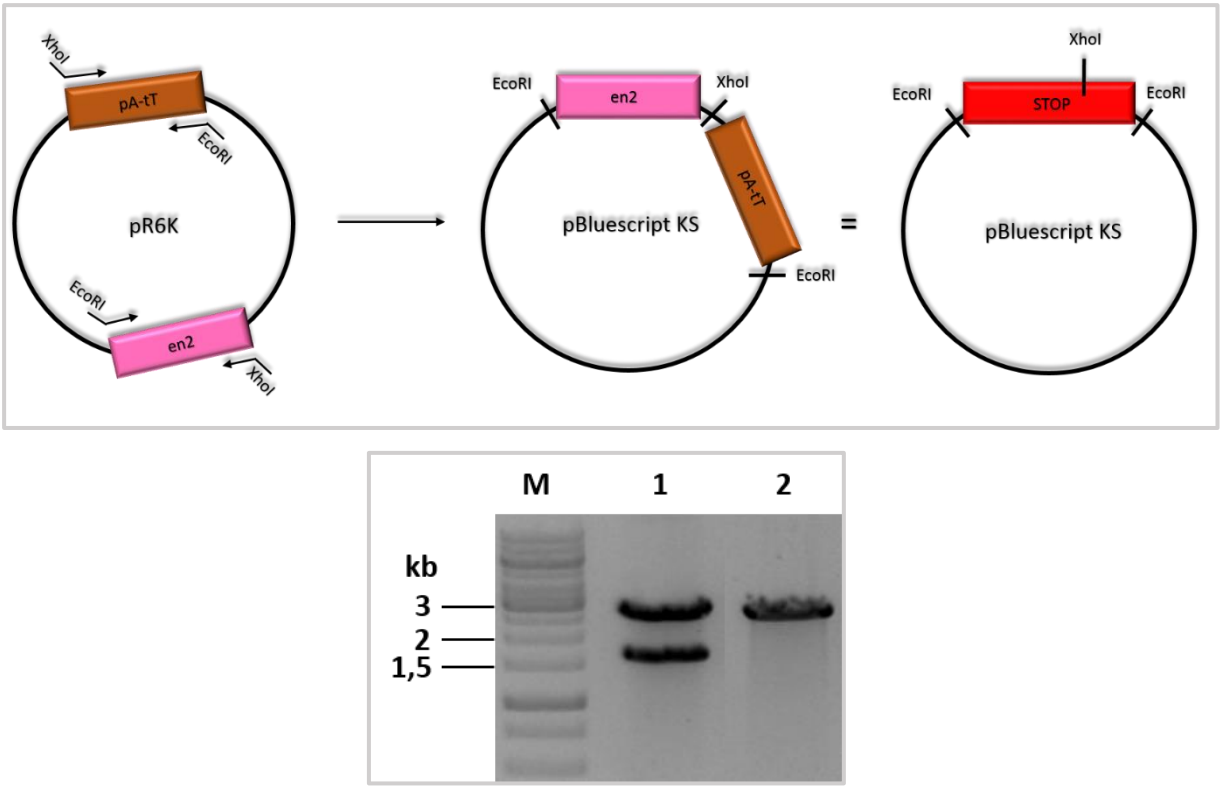


Figure 8 A (above) Amplification of the STOP signals pA-tT and en2*. The synthetic polyadenylation signal with the transcriptional terminator (pA-tT) and the last exon of the gene Engrailed-2 (en2) were amplified from pR6K. XhoI restriction sites were added to the 5' end of pA-tT and the 3' end of en2 and EcoRI restriction sites were added to the 3' end of pA-tT and the 5' end of en2. The resulting amplicons were digested with XhoI and EcoRI and ligated into the vector pBluescript KS.* ***B (below) Analytical gel of pBluescript KS digested with EcoRI.*** *M: Marker. 1: pBluescript KS (3 kb) containing the STOP signals (1,6 kb). 2: Religation of pBluescript KS (3 kb).*

Next, the correctly assembled STOP signals were subcloned into the vector pPGK-Neo. This plasmid contains two LoxP signals flanking a neomycin resistance cassette (neo), which will be helpful for the selection of embryonal stem cells that successfully insert the transgene. The STOP signals were cut out of the pBluescript KS - variant generated in the previous step with EcoRI, while pPGK-Neo was digested with MfeI, an isocaudomer of EcoRI, followed by treatment with alkaline phosphatase to avoid religation. Both constructs were purified from a 1% agarose gel and ligated in a vector to insert ratio of 1:5 (see **Figure 9A** for a graphical representation of the construct). Candidate clones able to grow on LB-ampicillin (100µg/ml) and -kanamycin (50 µg/ml) plates were picked and plasmid DNA isolated via miniprep. An analytical digest using XhoI and EcoRI was performed to ascertain correct assembly of the desired construct: the STOP cassette and the neomycin resistance cassette flanked by two LoxP sites (**Figure 9B**). Correct clones were confirmed by sequencing.

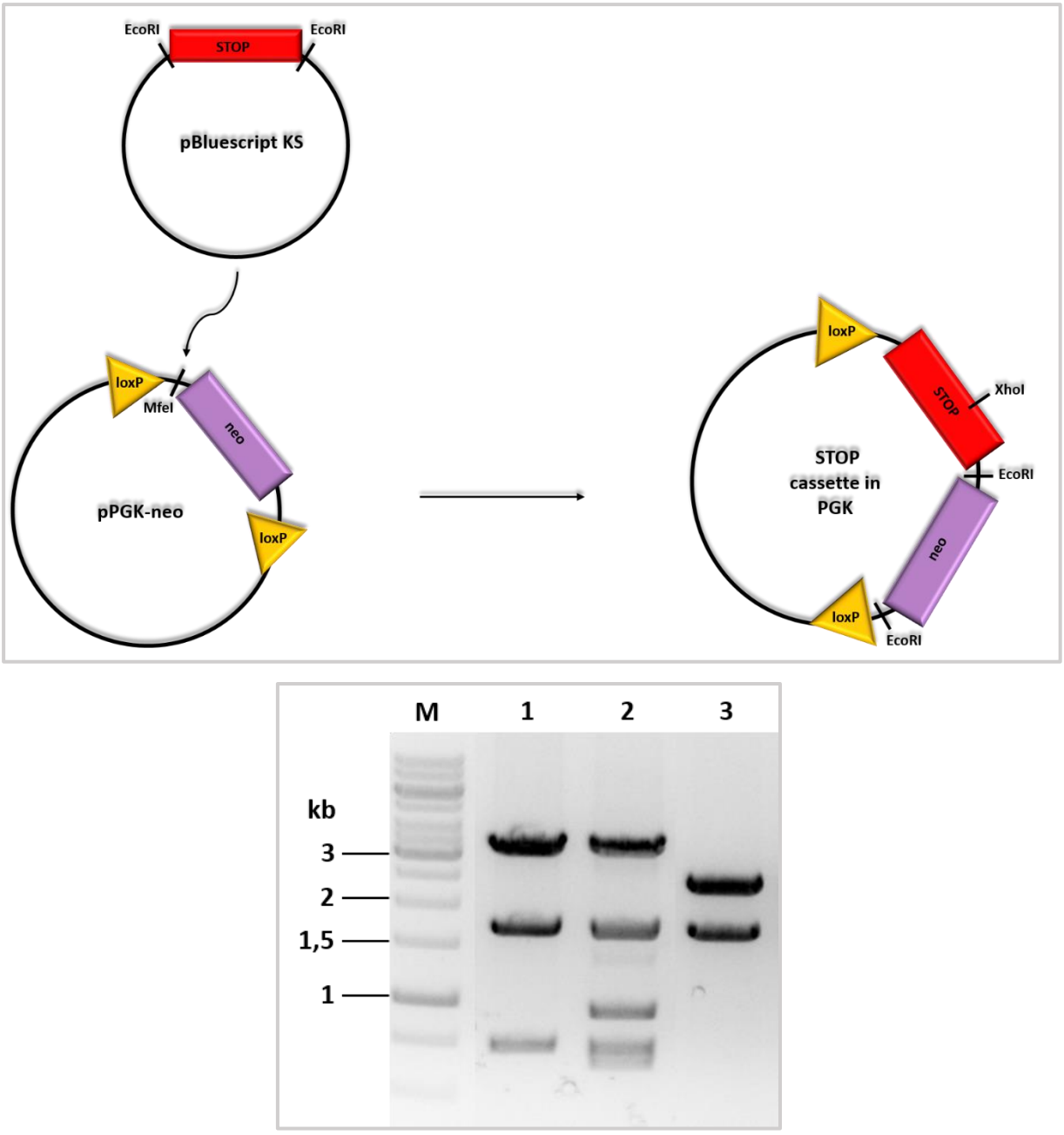


Figure 9A (above) Supplement of the STOP signals with LoxP sites and a neomycin resistance cassette. *STOP signals were cut out of pBluescript KS with EcoRI, and ligated into the vector pPGK-Neo, which was digested with the EcoRI isocaudomer MfeI. Since the plasmid pPGK-Neo contains two LoxP signals flanking a neomycin resistance cassette (neo), the subcloning results in the (floxed) STOP cassette in PGK.* ***B (below) Analytical gel of STOP cassette in PGK, digested with EcoRI and XhoI.*** *M: Marker. 1: STOP signals inserted in correct orientation. The lower band represents part of STOP (~ 700 bp), the middle band represents neo (~ 1,7 kb) and the upper band represents the rest of the plasmid, together with the LoxP signals and the other part of STOP (~ 3,4 kb). 2: False insertion of STOP signals (double inserted). Lower band represents part of one STOP (~ 700 bp), the one over it the other part of the second STOP (~ 900 bp). The middle band represents two pieces of DNA, neo (~ 1,7 kb) and the remaining parts of the two STOPs, adding up to an inverted STOP (~ 1,6 kb). The upper band represents the rest of the plasmid, together with the LoxP signals and part of STOP (~ 3,4 kb). 3: Religation of pPGK-Neo. The lower band represents neo (~ 1,7 kb), the upper one the rest of the plasmid (~ 2,5 kb).*

Amplification of the Stop signals and subcloning into pPGK-Neo was performed by Roland Tschismarov.

To insert the STOP cassette into the stretch of approximately 8 kb, it needs to be flanked by 200 bp long homologies directly 5' and 3' of the insertion site in the *Tyk2* or *Stat1* locus, respectively. After insertion, these 8 kb will serve as approximately 4 kb long homologies of the STOP cassette necessary for homologous recombination in mouse embryonic stem cells (see chapter **4.1**)

To generate the 5' homology region, 200 bp directly upstream of the insertion site were amplified from the appropriate BAC clone using the primers Tyk2 200bp 5’ fw and Tyk2 200 bp 5’ rev for *Tyk2* and Stat1 200 bp 5’ fw and Stat1 200 bp 5’ rev for *Stat1*, adding a NotI site at the 5' end and a PmeI site at the 3' end. The PCR product as well as the vector pPGK- Neo modified to contain the Stop cassette (STOP cassette in pPGK- Neo, see **Figure 9A**) were digested with NotI and PmeI, the plasmid was treated with alkaline phosphatase and both constructs were purified from a 1% agarose gel. Vector and insert were ligated at a ratio of 1:5 (see **Figure 10A** for a graphical representation of the construct) and transformed into *E. coli*. Candidate clones able to grow on LB-kanamycin (50 µg/ml) plates were picked and plasmid DNA isolated via miniprep, followed by an analytical digest with NotI and XhoI (**Figure 10B**).

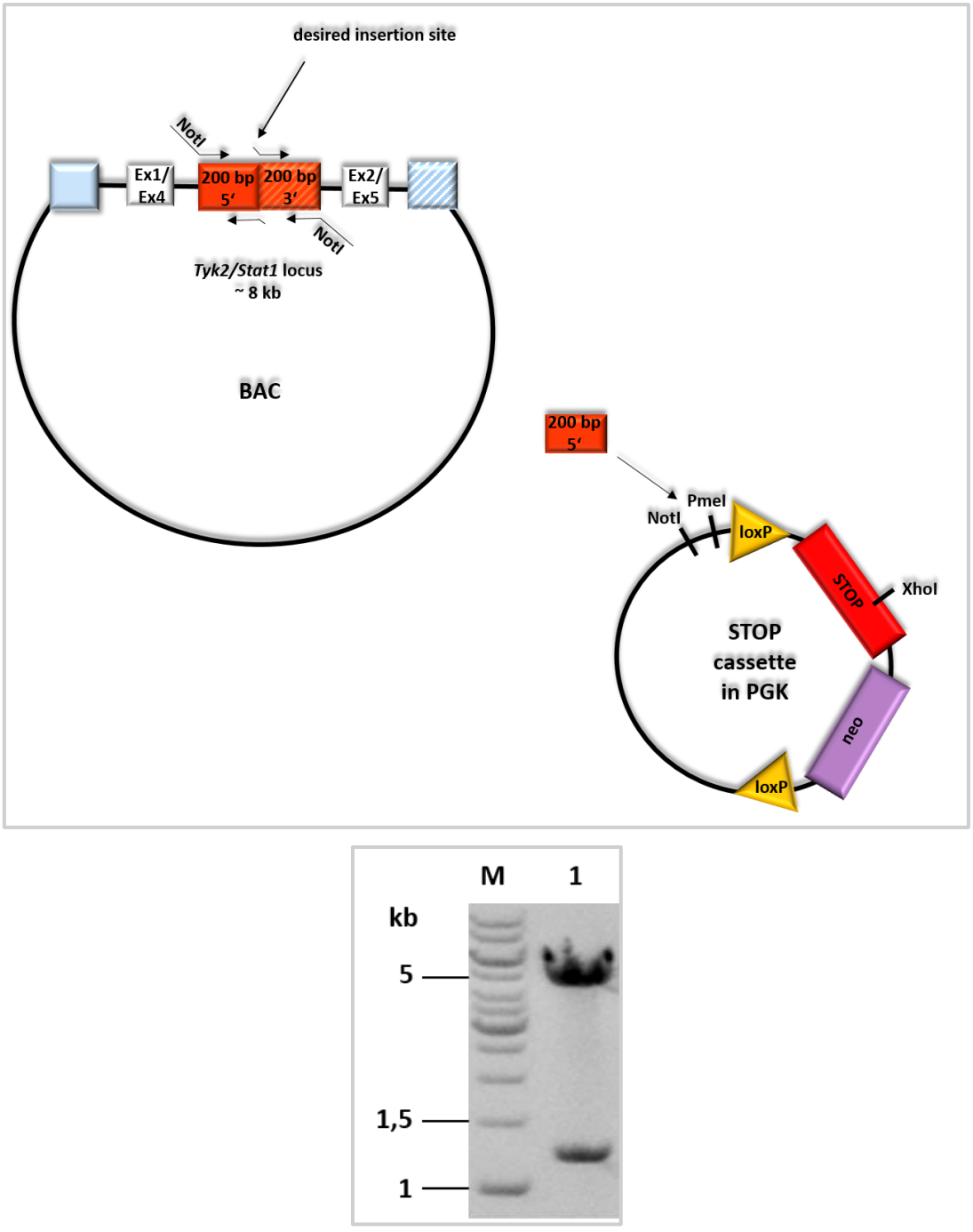
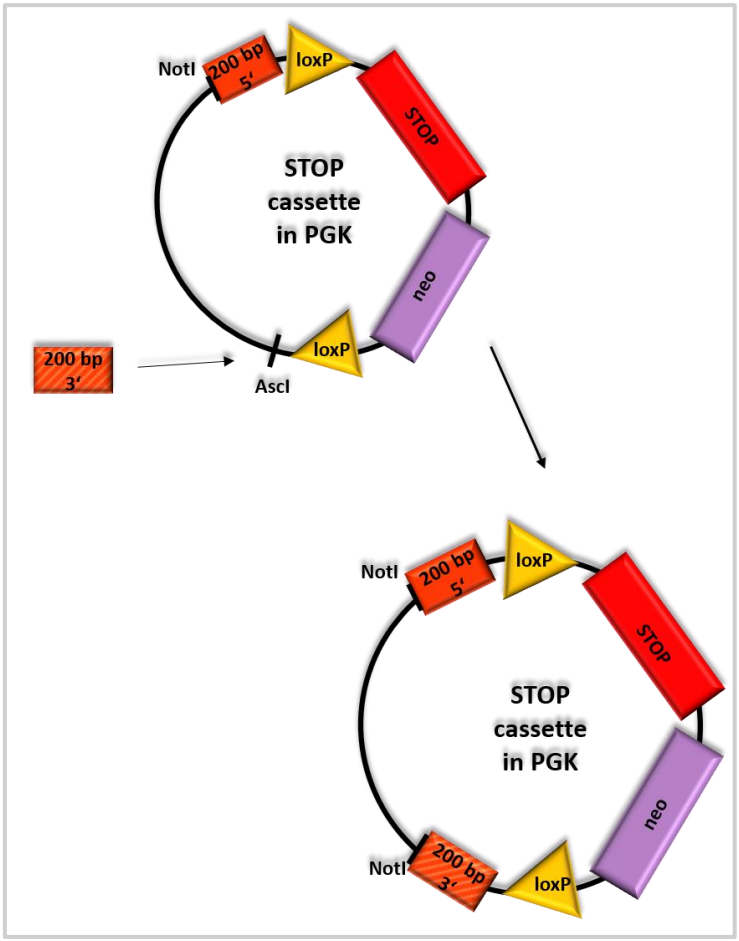


Figure 10A (above) Amplification of 200 bp homologies and 5' insertion into STOP cassette in PGK. *200bp directly upstream of the insertion site were amplified from the appropriate BAC clone, adding a NotI site at the 5' end and a PmeI site at the 3' end. The PCR product was digested with NotI and PmeI, and ligated into the vector pPGK- Neo containing the floxed (flanked by LoxP sites) STOP cassette (STOP cassette in pPGK-Neo, see Figure 9A). Ex1/4: Exon 1 in Tyk2 and Exon 4 in Stat1. Ex2/5: Exon 2 in Tyk2 and Exon 5 in Stat1. 200 bp 5' and 3': Homologies required for the second recombination step. STOP: Stop signals to abort transcription. Neo: neomycin resistance cassette.* ***B (below) Analytical gel of 200 bp 5' inserted into STOP cassette in PGK, digested with NotI and XhoI.*** *M: Marker. 1: 200 bp inserted in STOP cassette in PGK. The lower band represents part of STOP together with one LoxP signal (~ 1,3 kb), the upper band the rest of the plasmid, together with the other LoxP site, neo and the other part of STOP (~ 4,7 kb).*

To generate the 3' homology region, 200 bp directly downstream of the insertion site were amplified from the appropriate BAC clone using the primers Tyk2 200 bp 3’ fw and Tyk2 200 bp 3’ rev for *Tyk2* and Stat1 200 bp 3’ fw and Stat1 200 bp 3’ rev for *Stat1*, adding an AscI site at both ends. The PCR product as well as the vector pPGK- Neo modified to contain the STOP cassette and the 200 bp 5’ (STOP cassette in pPGK-Neo, see **Figure 10A**) were digested with AscI, the plasmid was treated with alkaline phosphatase and both constructs were purified from an 1% agarose gel. Vector and insert were ligated at a ratio of 1:5 (see **Figure 11A** for a graphical representation of the construct) and transformed into *E. coli*. Candidate clones able to grow on LB-kanamycin (50 µg/ml) plates were picked and plasmid DNA isolated via miniprep, followed by an analytical digest with NotI (**Figure 11B**).



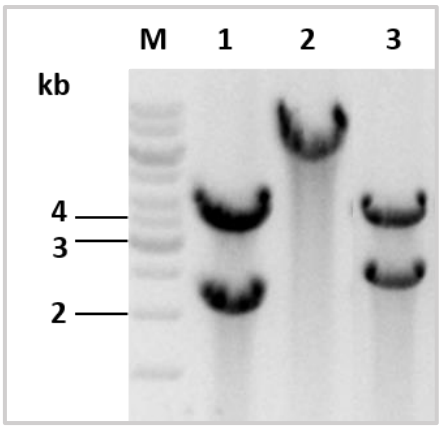


Figure 11A (above) Insertion of 200 bp 3' into STOP cassette in PGK. *200bp directly downstream of the insertion site were amplified from the appropriate BAC clone, adding an AscI site at both ends. The PCR product was digested with AscI and ligated into the vector pPGK-Neo containing the floxed (flanked by Loxp signals) STOP cassette and the 200 bp 5' (STOP cassette in pPGK-Neo, see Figure 10A). STOP: Stop signals to abort transcription. Neo: neomycin resistance cassette. 200 bp 5' and 3': Homologies required for the second recombination step.* ***B (below) Analytical gel of 200 bp 3' inserted into STOP cassette in PGK, digested with NotI.*** *M: Marker. 1: 200 bp inserted in correct orientation. The upper band represents the floxed STOP cassette together with 200 bp 5' and 3' (~ 4 kb), the lower one the rest of the plasmid (~2,2 kb). 2: Religation of STOP cassette in PGK (~ 6 kb). 3: 200 bp inserted in incorrect orientation. The upper band represents the floxed STOP*

cassette with 200 bp 5' (~ 3,8 kb), the lower one the rest of the plasmid with 200 bp 3' (~ 2,4 kb).

4.3 Generation of the recombination backbone "rec construct"

To generate the long homology arms needed for homologous recombination in mouse embryonic stem cells, approximately 8 kb of genomic DNA surrounding the desired insertion sites needed to be subcloned into a plasmid. Due to the length of this piece of DNA, a Red/ET recombination approach was chosen to avoid the need for unique restriction sites. First, a linear stretch of DNA containing an origin of replication (ori) and an antibiotic resistance gene was required to act as a backbone, flanked by small stretches of DNA homologous to the very 5' and 3' ends of the 8 kb.

The recombination backbone, termed "rec construct", was directly amplified from the vector pBluescript KS using the primers Tyk2 KS rec fw and Tyk2 KS rec rev for *Tyk2* and Stat1 KS rec fw and Stat1 KS rec rev for *Stat1*, adding 50 bp long homologies and a NotI site at both ends (see **Figure 12** for a graphical representation of the construct). Due to a high carryover of circular template DNA, pBluescript KS was linearized with BamHI and XbaI before performing the PCR and the amount of template was strongly reduced from 600 ng to 10 ng. The PCR product was then digested with DpnI, which cleaves methylated and hemimethylated, but not PCR-amplified DNA,[86] and purified from an 1% agarose gel. "Rec constructs" and 50 bp homologies of the BAC were verified by sequencing. Functionality of DpnI was confirmed by digestion of pBluescript KS.

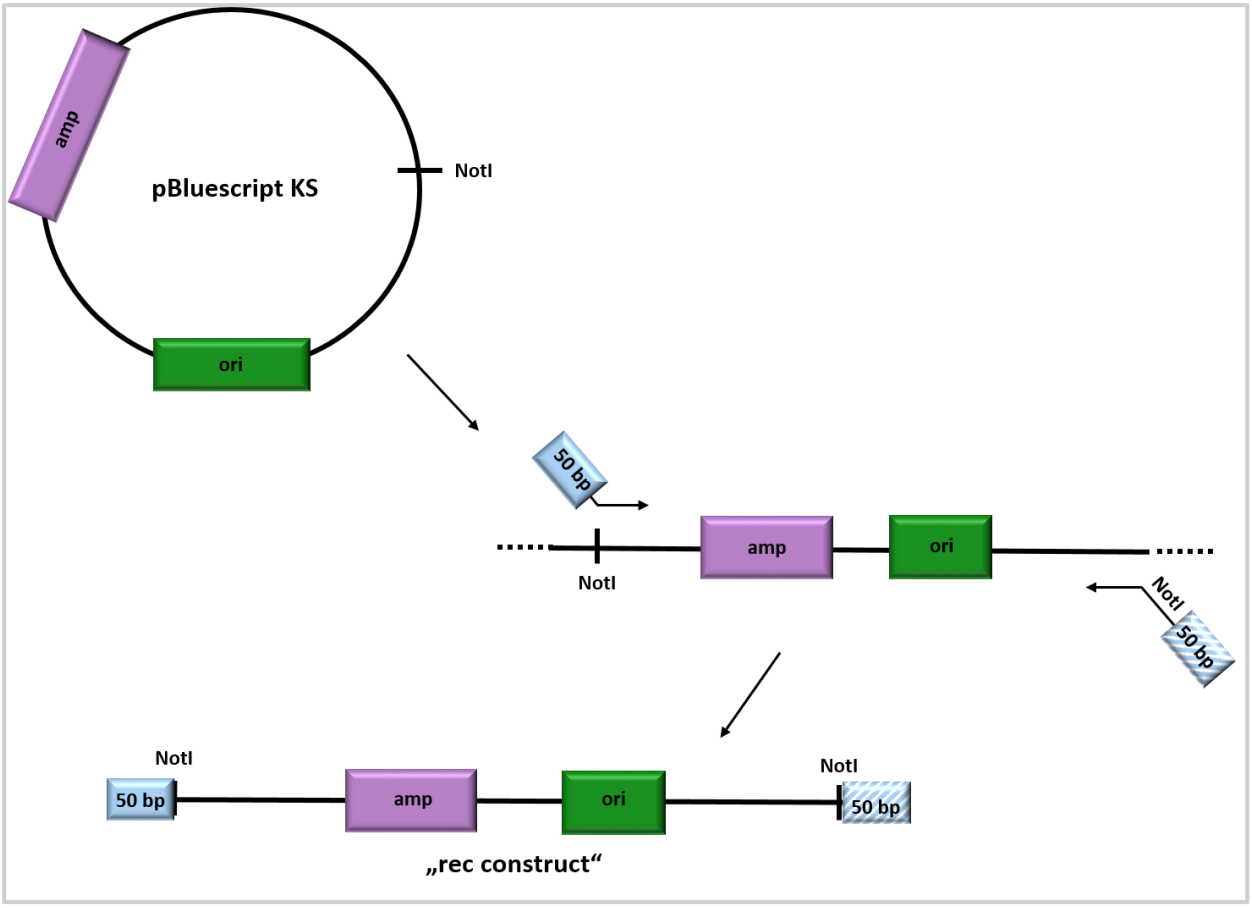


Figure 12 Amplification of recombination backbone "rec construct". *The "rec construct" was directly amplified from the vector pBluescript KS, adding 50 bp long stretches of DNA homologous to the Tyk2 or the Stat1 locus, and a NotI site at both ends. 50 bp: Homologies required for the first recombination step. Amp: Ampicillin resistance. Ori: Origin of replication.*

4.4 Floxed STOP TYK2

4.4.1 Isolation of 8 kb of the *Tyk2* locus

To combine the "rec construct" with 8 kb of genomic DNA surrounding the *Tyk2* locus, Red/ET recomination was performed as described in chapter **3.7**. The "rec construct" was electroporated into *E. coli* carrying the BAC RP23-361H17, containing the *Tyk2* locus, and the plasmid pSC101, containing the λ-phage-derived *redγβα* operon. BACs were controlled via genotyping PCR and pSC101 was verified via restriction digest. Upon induction of the phage proteins, the bacteria could recombine a plasmid termed "KSrec", containing 8 kb of the Tyk2 locus and the pBluescript KS backbone (see **Figure 13A** for a graphical representation of the construct). Several candidate clones able to grow on LB-ampicillin (100 µg/ml) plates were picked and plasmid DNA isolated via miniprep. An analytical digest using NotI was carried out to select correctly recombined candidate clones (**Figure 13B**). Screening of eighty clones revealed correct banding patterns for nine clones, six of them yielding enough plasmid DNA

for sequencing. Five of those were shown to be free of errors, and one of them was chosen for subsequent steps.

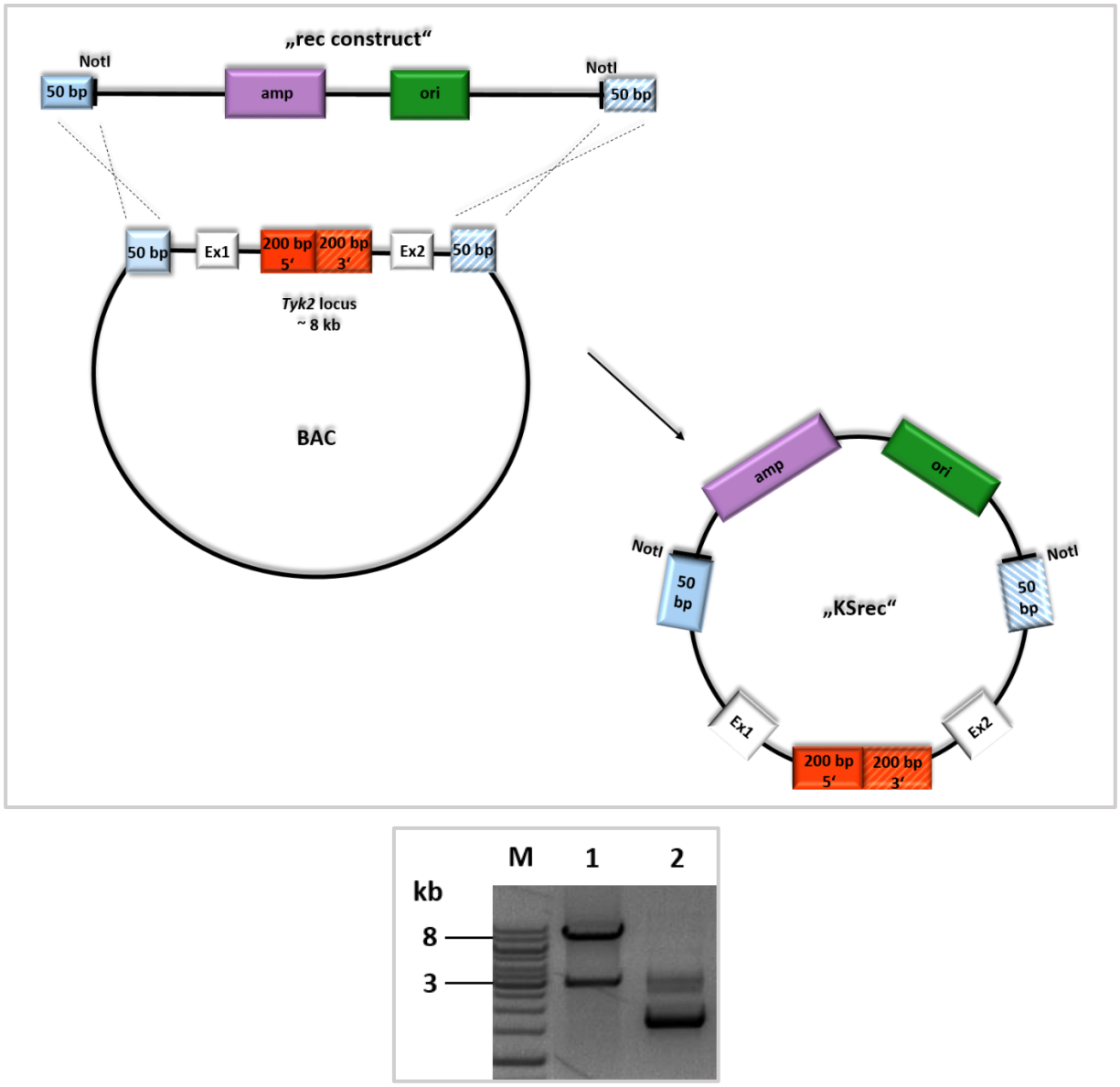
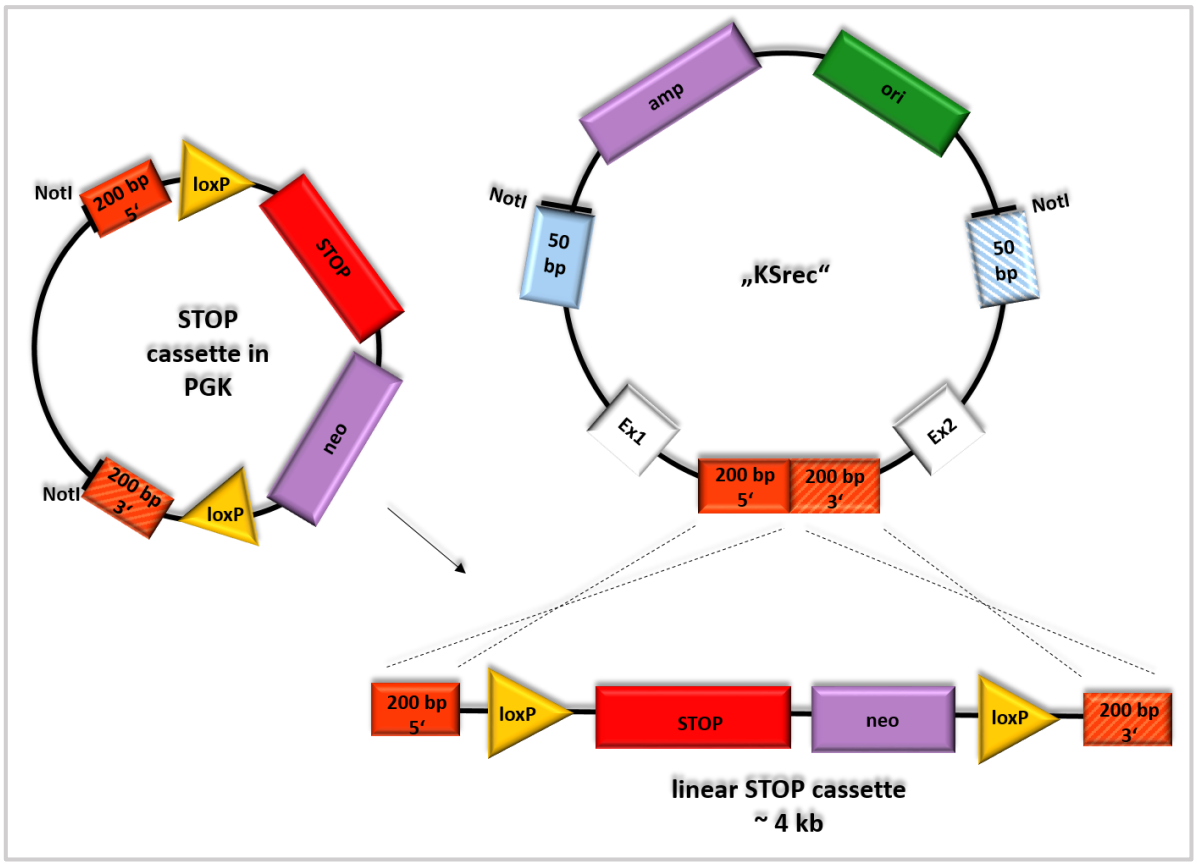


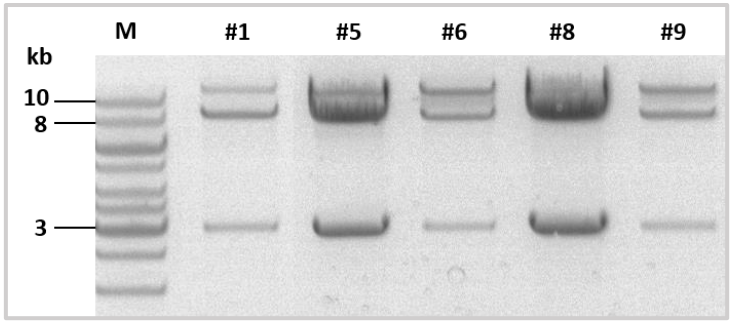
Figure 13A (above) Isolation of 8 kb of the Tyk2 locus and subcloning into “rec construct”. *The “rec construct” was electroporated into E. coli carrying BAC DNA with the Tyk2 locus as well as the plasmid pSC101 and treated as described in chapter 3.7 to initiate Red/ET recombination. The resulting plasmid was termed “KSrec”. 50 bp: Homologies required for this recombination step. Amp: Ampicillin resistance. Ori: Origin of replication. Ex1 and Ex2: Exon 1 and Exon 2. 200 bp 5’ and 3’: Homologies required for the next recombination step.* ***B (below) Analytical gel of “KSrec” candidates digested with NotI.*** *M: Marker. 1: Successful recombination, 8 kb of Tyk2 locus subcloned into “rec construct” (~ 3 kb). 2: Failed recombination.*

4.4.2 Insertion of the STOP cassette into “KSrec”

To linearize the STOP cassette prior to recombination, it was digested with NotI over night and purified from a 1% agarose gel. The linear cassette and “KSrec”, one of the five clones that was chosen for subsequent steps (see chapter **4.4.1**), were electroporated into *E. coli* containing pSC101 in ratios of 1:1 and 1:2 and treated as described in chapter **3.7** to initiate recombination (see **Figure 14A** for a graphical representation). Plasmids from several clones able to grow on LB-ampicillin (100 µg/ml) and -kanamycin (50 µg/ml) were isolated via miniprep and an analytical digest with NotI was performed to identify correctly recombined constructs (**Figure 14B**).

Since the desired recombined construct can co-exist with unmodified "KSrec" in *E. coli*, promising candidates were re-transformed to decrease the likelihood of having cells containing a mixture of plasmids. Four clones for each of five candidates were miniprepped and digested with NotI. Fifteen of those twenty candidates showed bands representing a correctly recombined construct (**Figure 14C**) Eleven of those clones yielded enough plasmid DNA for sequencing, two of which (#6-1 and #9-3) were found to be free of errors.





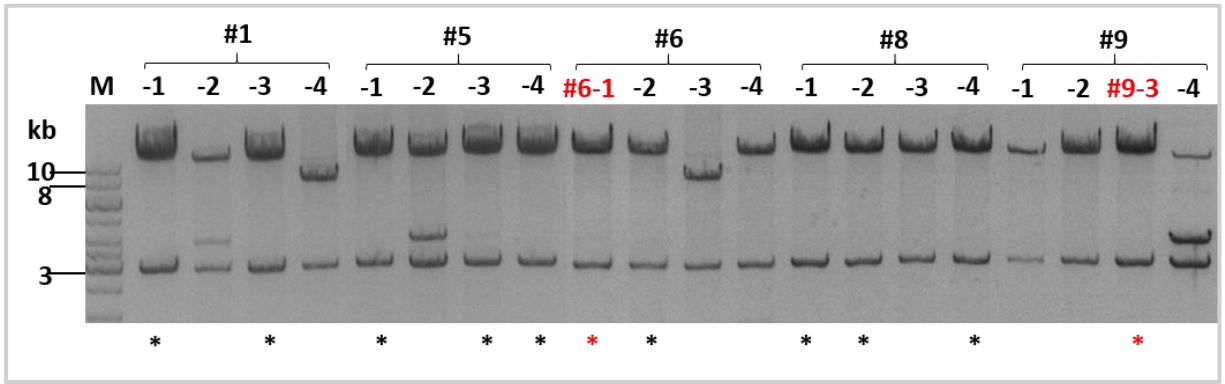


Figure 14A (above) Linearization of STOP cassette and recombination with "KSrec". *To linearize the floxed (flanked by LoxP signals) STOP cassette, it was digested with NotI. The linear cassette and "KSrec" were electroporated into E. coli containing pSC101 and treated as described in chapter 3.7 to initiate Red/ET recombination, resulting the final Stop cassette in KS (see Figure 15). STOP: Stop signals to abort transcription. Neo: neomycin resistance cassette. 200 bp 5' and 3': Homologies required for this recombination step. 50 bp: Homologies required for previous recombination. Amp: Ampicillin resistance. Ori: Origin of replication. Ex1 and Ex2: Exon 1 and Exon 2.* ***B (middle) Analytical gel of final STOP cassette in KS, digested with NotI.*** *M: Marker. #1 - #9: Candidates for Re-Transformation, containing a mixture of the recombined construct (~ 12 kb) and "KSrec" (~ 8 kb). The lower band (~ 3 kb) represents the pBluescript KS backbone.* ***C (below) Analytical gel of final STOP***

cassette in KS candidates after Re-Transformation. *Since the desired recombined construct can co-exist with unmodified "KSrec" in E. coli, candidates from Figure 14B were re-transformed to decrease the likelihood of having cells containing a mixture of plasmids. Four clones for each of the five candidates were miniprepped and digested with NotI. M: Marker. *: Candidates with correct banding pattern and enough DNA concentration for sequencing. Upper bands represent the floxed STOP cassette surrounded by 8 kb of Tyk2 (~ 12 kb), the lower bands represent pBluescript KS backbone (~ 3 kb). Red: Correct clones for floxed STOP Tyk2, confirmed by sequencing.*

After restriction digest with NotI, the linearized final STOP cassette separated from the pBluescript KS backbone can be recombined into the *Tyk2* locus of the mouse genome (**Figure 15**).

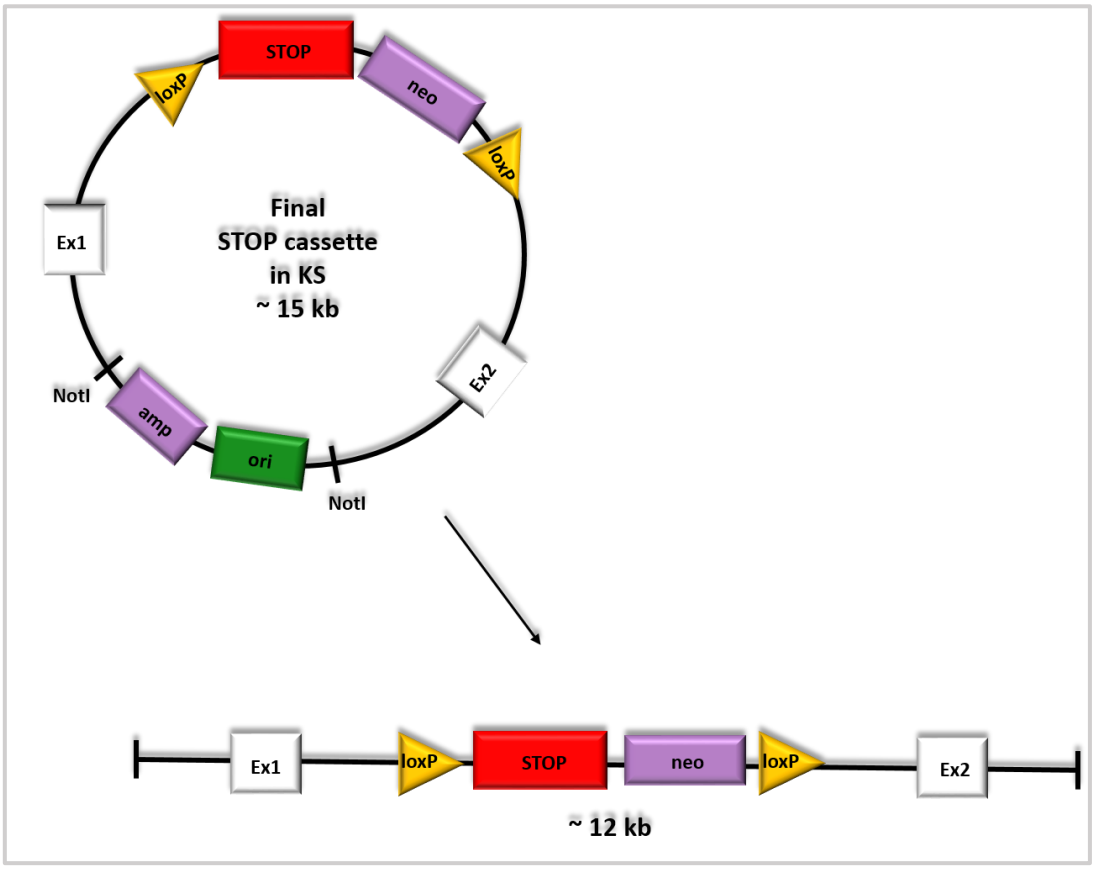


Figure 15 Linearization of floxed STOP Tyk2, ready for insertion into the mouse genome. *The floxed (flanked by LoxP signals) STOP cassette is separated from pBluescript KS backbone via NotI restriction digest. Ex1 and Ex2: Exon 1 and Exon 2. STOP: Stop signals to abort transcription. Neo: neomycin resistance cassette. Ori: Origin of replication. Amp: Ampicillin resistance.*

4.5 Floxed STOP STAT1

4.5.1 Isolation of 8 kb of the STAT1 locus

To combine the "rec construct" with 8 kb of genomic DNA surrounding the *Stat1* locus, Red/ET recomination was performed as described in chapter **3.7**. The “rec construct” was electroporated into *E. coli* carrying the BAC RP23-140M10, containing the *Stat1* locus, and the plasmid pSC101, containing the λ-phage-derived *redγβα* operon. Although the same adaptions and checks as for Tyk2 were applied on the STAT1 protocol, bacteria could not recombine the plasmid “KSrec”, containing 8 kb of the *Stat1* locus and the pBluescript KS backbone. Several candidate clones able to grow on LB-ampicillin (100 µg/ml) plates were picked and plasmid DNA isolated via miniprep. An analytical digest using NotI was carried out to select correctly recombined candidate clones, but screening of 179 clones revealed no correct banding patterns for any of those clones. Analytical gels still showed a high background of pBluescript KS (**Figure 16**). Homologies were modified in two different ways, by using another 50 bp lying outside of the former ones and by prolonging the former 50 bp up to 90 bp. “Rec constructs” were generated in the same way as described in chapter **4.3**. They were directly amplified from the vector pBluescript KS using the primers KSrec Stat new 5’ and KSrec Stat new 3’ for the construct containing the outer 50 bp and KSrec Stat 100 5’ and KSrec Stat 100 3’ for the construct containing the prolonged homologies. Additionally, the primers also introduced a NotI site at both ends of each construct. Red/ET recombination was performed as described above. Screening of 48 clones for the outer “new” 50 bp and 53 clones for the 90 bp homologies revealed no correct banding pattern (data not shown).

In total, 280 clones were screened in order to isolate the 8 kb Stat1 locus via Red/ET recombination.

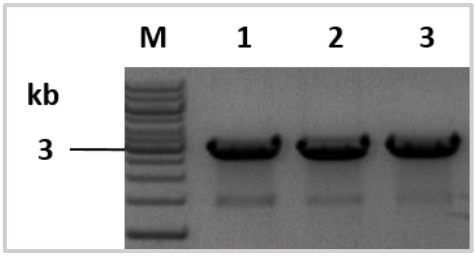


Figure 16 Analytical gel of "KSrec" candidates digested with NotI. *M: Marker. 1 – 3: Failed recombination, background of "rec construct" (~ 3 kb).*

4.5.2 Long-range PCR

Since the Isolation of the 8 kb long *Stat1* locus did not work via Red/ET recombination, an alternative cloning approach was applied (see **Figure 17A** for a graphical representation). The 8 kb of the *Stat1* locus surrounding the desired insertion site were amplified from BAC DNA using the primers Stat 8kb fw Xma 3 and Stat 8kb rev Spe 3, inserting a XmaI and a NotI site at the 5' end and SpeI and a NotI site at the 3' end. An analytical digest with XhoI was performed to ascertain the amplicon was correct (**Figure 17B**). The PCR product was digested with XmaI and SpeI and then PCR purified with the QIAquick PCR Purification Kit. The vector pBluescript KS was digested with XmaI and SpeI, treated with alkaline phosphatase and purified from a 1% agarose gel. Vector and insert were ligated at ratios of 1:1, 1:3, 3:1, 1:5 and 5:1 and transformed into *E. coli*. The few candidates able to grow on LB-ampicillin (100µg/ml) plates were picked and plasmid DNA isolated via miniprep, followed by an analytical digest with NotI. All analytical gels showed a background of pBluescript KS, but no successful ligation (**Figure 17C**). Ligation of the PCR product and pBluescript KS digested with NotI as described above was also ineffective.

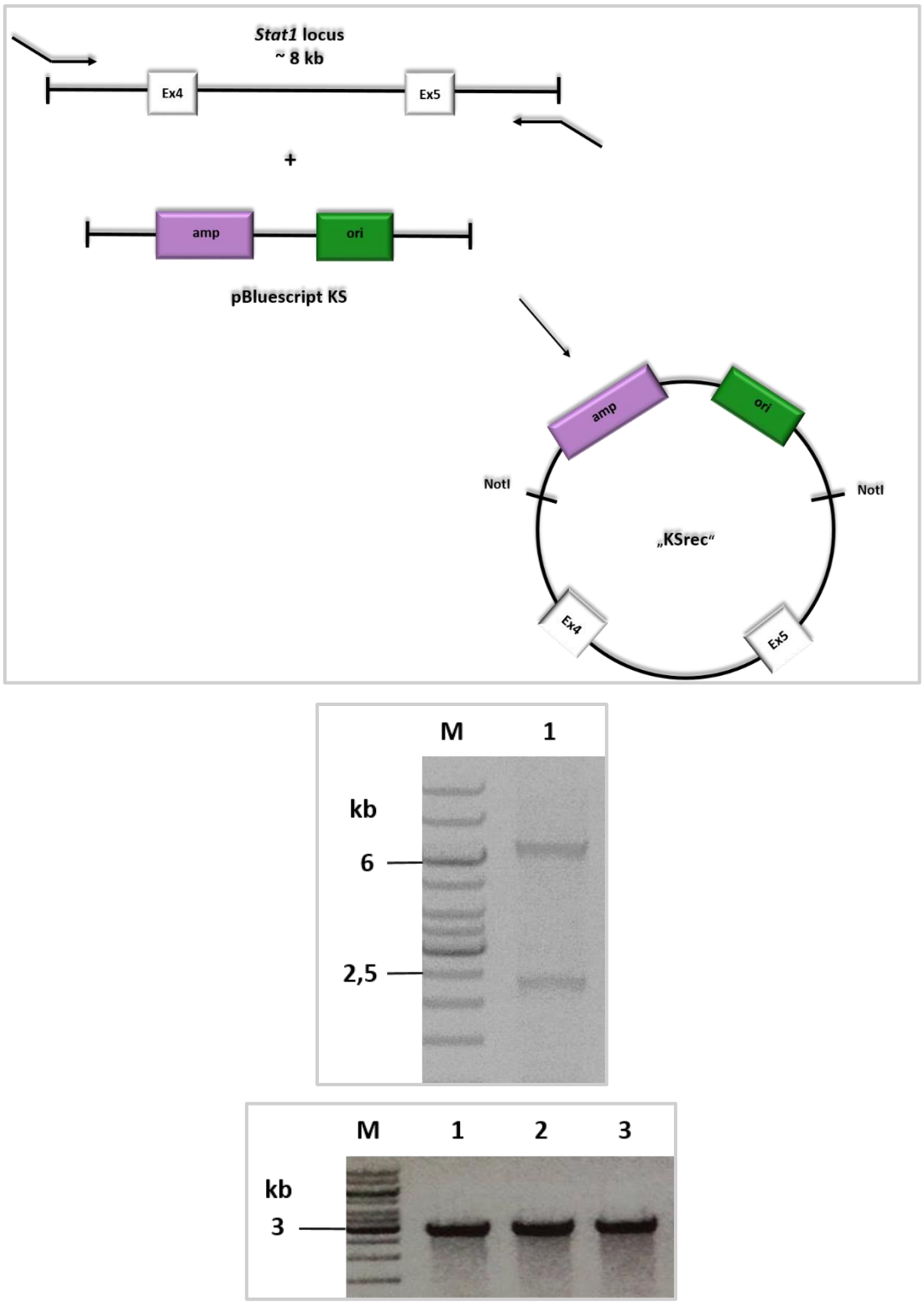


Figure 17A (above) Long-range PCR of 8 kb of Stat1, subcloned into pBluescript KS. *8 kb of Stat1 were amplified from BAC DNA by using different primer sets introducing a NotI site, followed by a XmaI or SpeI restriction site. The amplicon was digested with the appropriate restriction enzymes and ligated into pBluescript KS, resulting in a "KSrec"-like plasmid (see Figure 14A).* ***B (middle) Analytical gel of Long-range PCR of 8 kb of Stat1, digested with XhoI.*** *M: Marker. 1: 8 kb of Stat1 locus correctly cut by XhoI into two pieces of ~ 6,3 kb and ~ 2,3 kb.* ***C (below) Analytical gel of 8 kb of Stat1 ligated into pBluescript KS, digested with NotI.*** *M: Marker. 1 – 3 Religation of pBluescript KS (~ 3 kb).*

After screening several clones, two other sets of restriction sites were tested to obtain a more effective integration. For one set, BAC DNA was amplified by using the primers 8kb Stat fw4 Xma and 8kb Stat rev4 Not, inserting a XmaI and a NotI site at the 5' end and a single NotI site at the 3' end. For the other set, BAC DNA was amplified by using the primers Stat 8kb fw Spe 4 and 8kb Stat rev4 Not, inserting a SpeI and a NotI site at the 5' end and a single NotI site at the 3' end. Amplicons and the vector pBluescript KS were treated and ligated as described above, leading to similarly unsuccessful results. Adaptions of the ligation protocol as described in materials and methods were similarly ineffective: using a ligation temperature of 16°C, room temperature or 37°C and using 1 µl, 2 µl or 5 µl of the ligation mix for transformation.

Multiple attempts have been been made to isolate the 8 kb of the *Stat1* locus and to subclone them into pBluescript KS, without gaining the desired construct. Efforts by others to carry out this step even after the conclusion of the practical work for this thesis have been proven unsuccessful as well.

5 Discussion and Outlook

5.1 Floxed STOP TYK2

Two correct clones of the floxed STOP TYK2 construct were confirmed by sequencing and are theoretically ready for insertion into the mouse genome (see chapter **4.4.2**). The STOP cassette was designed from sequence elements that have been previously shown to stop transcription and is therefore expected to also work for TYK2 [66,67,68,71,72]. However, it will be necessary to validate its functional integrity. On the one hand, it should be efficient in preventing *Tyk2* expression and on the other hand, Cre recombinase should be able to restore *Tyk2* expression to wildtype levels. Moreover, introduction of the STOP cassette into intron 1 would theoretically still allow for the expression of a very short, truncated mRNA. To avoid any unspecific effects of such a message, the insertion site was chosen upstream of the region deleted in the well-established conditional knockout. However, the possible expression of such a transcript should still be assessed. In addition, Cre-mediated excision of the STOP cassette leaves behind a single LoxP site in intron 1. The insertion site was chosen because it does not contain any known regulatory motifs or repetitive sequence elements that could be indicative of transcription factor binding sites, making an effect of a 32bp insertion in this noncoding sequence unlikely. In case of STAT1, former studies showed that LoxP sites do not affect its transcriptional activity in conditional *Stat1* alleles, and a restoration to wildtype levels is entirely possible [47]. However, it will have to be tested if *Tyk2* expression is in any way affected after removal of the STOP cassette.

Lastly, it will have to be evaluated if the insertion of the transgene affects the expression of neighboring genes.

All the above issues suggest that the STOP cassette construct should be tested in an easy-to-use setup, before starting the cost- and labor- intensive process of homologous recombination in mouse embryonic stem cells. One approach would be to insert the transgene into a cell line using clustered regularly interspaced short palindromic repeats (CRISPR) and its associated protein-9 nuclease (Cas9). CRISPR/Cas9 derives from the prokaryotic adaptive immune system and serves for identification and disabling of invasive DNA, leading to one of two DNA repair pathways: non-homologous end-joining or homology-directed repair. The experimentally used system consists of the specific endonuclease Cas9 and a single guide RNA molecule (sgRNA). This sgRNA, which in turn consists of a target-specific CRISPR RNA (crRNA) and a trans-activating crRNA (tracerRNA), is complementary to a sequence inside the target DNA.

Cas9 cleaves double-stranded DNA site-specifically and therefore activates the double-strand break repair machinery of the cell. By supplying the floxed STOP cassette with its *Tyk2* homologies, this will lead to homology-directed repair [87,88].

Since the STOP cassette would need to be inserted in both alleles of *Tyk2* to be able to validate its efficiency, the use of a haploid mouse embryonic stem cell line would greatly simplify the experimental setup. Haploid model organisms only possess a single set of chromosomes, allowing an efficient disruption of gene functions and consequently unveiling recessive phenotypes. Haploid mouse embryonic stem cells have been used recently for insertion of gene cassettes via CRISPR/Cas9, utilizing their hemizygosity to facilitate genetic screening [89,90]. After this validation, the floxed STOP TYK2 construct can be inserted into the mouse genome to finally generate a floxed STOP TYK2 mouse.

5.2 Floxed STOP STAT1

Even after multiple attempts to subclone 8kb of the *Stat1* locus into pBluescript KS, the generation of the floxed STOP STAT1 construct was unsuccessful. This suggests that the chosen sequence might not be suitable for cloning, possibly due to secondary structures inhibiting ligation and/or recombination. Another reason might be an unknown toxicity associated with the sequence.

Consequently, follow-up work to generate a floxed STOP STAT1 mouse should employ an alternative strategy. Since the method of choice for the validation of the floxed STOP TYK2 cassette is CRISPR/Cas9, it also a suitable method to generate the floxed STOP STAT1 mouse itself, without using Red/ET recombination at all. CRISPR/Cas9 enables the generation of bi-allelic mutant cell lines in one single step, and therefore has become an efficient method in generating transgenic mice. On the one hand, mutations can be introduced into mouse embryonic stem cells by transient transfection of plasmids carrying Cas9, sgRNA and, if necessary, donor DNA. On the other hand, CRISPR/Cas9 components and the donor DNA, in that case the floxed STOP STAT1 cassette, can be introduced directly via microinjection into zygotes [91].

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7 Abstract

The immune system has to recognize a large number of pathogens and thereby activate defense mechanisms. To provide protection by the innate immune system, cells have to communicate with each other via cytokines and translate the microbial contact into gene expression. One important group of cytokines are the interferons (IFNs), more precisely the three subclasses IFN I, II and III. IFNs employ the JAK/STAT signaling pathway, where the Janus kinases (JAKs) phosphorylate STAT transcription factors which stimulate gene expression in the nucleus.

This project deals with the generation of two different DNA constructs for transgenic mice. The aim was to generate a mouse in which the Janus kinase TYK2 (Tyrosine kinase 2) or the transcription factor STAT1 (Signal transducer and activator of transcription 1) is not produced, but can be restored to wild-type levels via tissue-specific expression of the Cre recombinase. These mice are expected to complement the insights gained with conditional knock-outs for TYK2 and STAT1.

The idea was to assemble a floxed STOP cassette flanked by 4 kb long stretches of DNA homologous to the *Tyk2* or *Stat1* locus. This construct will then be introduced into mouse embryonic stem cells, to insert the STOP cassette in an intronic sequence at the very 5' end of the gene of interest. Two different stop signals were used for this: The last exon of the gene Engrailed-2 (en2) and a synthetic polyadenylation signal with a transcriptional terminator site (pA-tT). After the assembly of the cassette, 8 kb of the *Tyk2* or *Stat1* locus were isolated from a BAC (Bacterial artificial chromosome) and subcloned into the plasmid pBluescript KS. The STOP cassette was inserted roughly into the middle of these 8 kb. While the assembly of the STOP cassette was done by classical cloning, a homologous recombination approach was applied for the other steps, called Red/ET- or λ-mediated recombination.

The generation of the TYK2 construct was progressing nicely and is completed, but the λ-mediated recombination of the *Stat1* locus caused many problems and could not be finished. In case of TYK2, the floxed STOP cassette will be validated in a haploid mouse embryonic stem cell line with the help of a CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9) approach. Recombination of the floxed STOP TYK2 construct into the mouse genome and the actual generation of the transgenic mouse will be carried out at the University of Veterinary Medicine in Vienna.

To generate the STAT1 construct, a conventional cloning approach is applied to isolate the 8 kb locus via long-range PCR and subclone it into pBluescript KS. The STOP cassette itself will be again inserted into the locus via Red/ET recombination. Another possibility is also a CRISPR/Cas9 approach, where the floxed STOP cassette can be inserted directly into the mouse genome.

8 Zusammenfassung

Das Immunsystem muss eine große Anzahl an Pathogenen erkennen und dabei Verteidigungsmechanismen aktivieren. Um den Schutz durch die angeborene Immunität zu gewährleisten, müssen die Zellen untereinander durch Cytokine kommunizieren und den mikrobiellen Kontakt in eine Genexpression übersetzen. Eine wichtige Gruppe von Cytokinen sind die Interferone (IFNs), genauer gesagt die drei Subklassen IFN I, II und III. IFNs verwenden den JAK/STAT Signalweg, bei dem Janus Kinasen (JAKs) STAT Transkriptionsfaktoren phosphorylieren, die wiederum im Zellkern die Genexpression stimulieren.

Dieses Projekt behandelt die Entwicklung von zwei verschiedenen DNA Konstrukten für transgene Mäuse. Das Ziel war es, eine Maus zu generieren in der entweder die Janus kinase TYK2 (Tyrosine kinase 2) oder der Transkriptionsfaktor STAT1 (Signal transducer and activator of transcription) nicht produziert wird, jedoch jeweils mithilfe von zell-spezifischer Expression der Cre Rekombinase bis auf Wildtyp-Niveau wiederhergestellt werden kann. Es wird erwartet mit diesen Mäusen neue Erkenntnisse zu gewinnen, die die bereits mit den konditionalen Knock-outs für TYK2 und STAT1 erhaltenden ergänzen.

Die Idee war es, eine gefloxte STOP-Kassette herzustellen, die von zwei 4 kb langen DNA-Stücken flankiert wird, die homolog zum *Tyk2* bzw. *Stat1* Lokus sind. Dieses Konstrukt wird dann in embryonale Maus-Stammzellen eingebracht, um die STOP-Kassette in einer intronischen Sequenz am äußersten 5‘ Ende des adressierten Gens einzufügen. Hierfür wurden zwei verschiedene Stop-Signale verwendet: Das letzte Exon des Gens Engrailed-2 (en2) und ein synthetisches Polyadenylierungssignal mit einer transkriptionalen Terminationsstelle (pA-tT). Nach dem Zusammenfügen der STOP-Kassette wurden 8 kb des *Tyk2* bzw. *Stat1* Lokus aus einem BAC (Bacterial artificial chromosome) isoliert und in das Plasmid pBluescript KS subkloniert. Die STOP-Kassette wurde ungefähr in der Mitte der 8 kb eingefügt. Während das Zusammenstellen der STOP-Kassette durch klassisches Klonieren erfolgte, wurde für die anderen beiden Schritte eine Methode der homologen Rekombination angewandt, genannt Red/ET- bzw. λ-vermittelte Rekombination.

Die Entwicklung des TYK2 Konstrukts schritt sehr gut voran und ist fertiggestellt, aber die λ-vermittelte Rekombination des *Stat1* Lokus bereitete viele Probleme und konnte nicht abgeschlossen werden.

Im Fall von TYK2 wird die STOP-Kassette mithilfe eines CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9) Ansatzes in einer

haploiden embryonalen Maus-Stammzelllinie validiert werden. Die Rekombination des gefloxten STOP TYK2 Konstrukts in das Mausgenom und die eigentliche Herstellung der transgenen Maus wird an der Veterinärmedizinischen Universität Wien durchgeführt werden. Um das STAT1 Konstrukt zu generieren, wird eine herkömmliche Klonierungsstrategie verwendet, um den 8 kb Lokus mittels Long-Range PCR zu isolieren und in pBluescript KS zu subklonieren. Die STOP-Kassette selbst wird wieder mithilfe der Red/ET-Rekombination in den Lokus eingefügt werden. Eine andere Möglichkeit ist ebenfalls ein CRISPR/Cas9 Ansatz, wobei die gefloxte STOP-Kassette direkt ins Mausgenom eingefügt werden kann.

9 Appendix

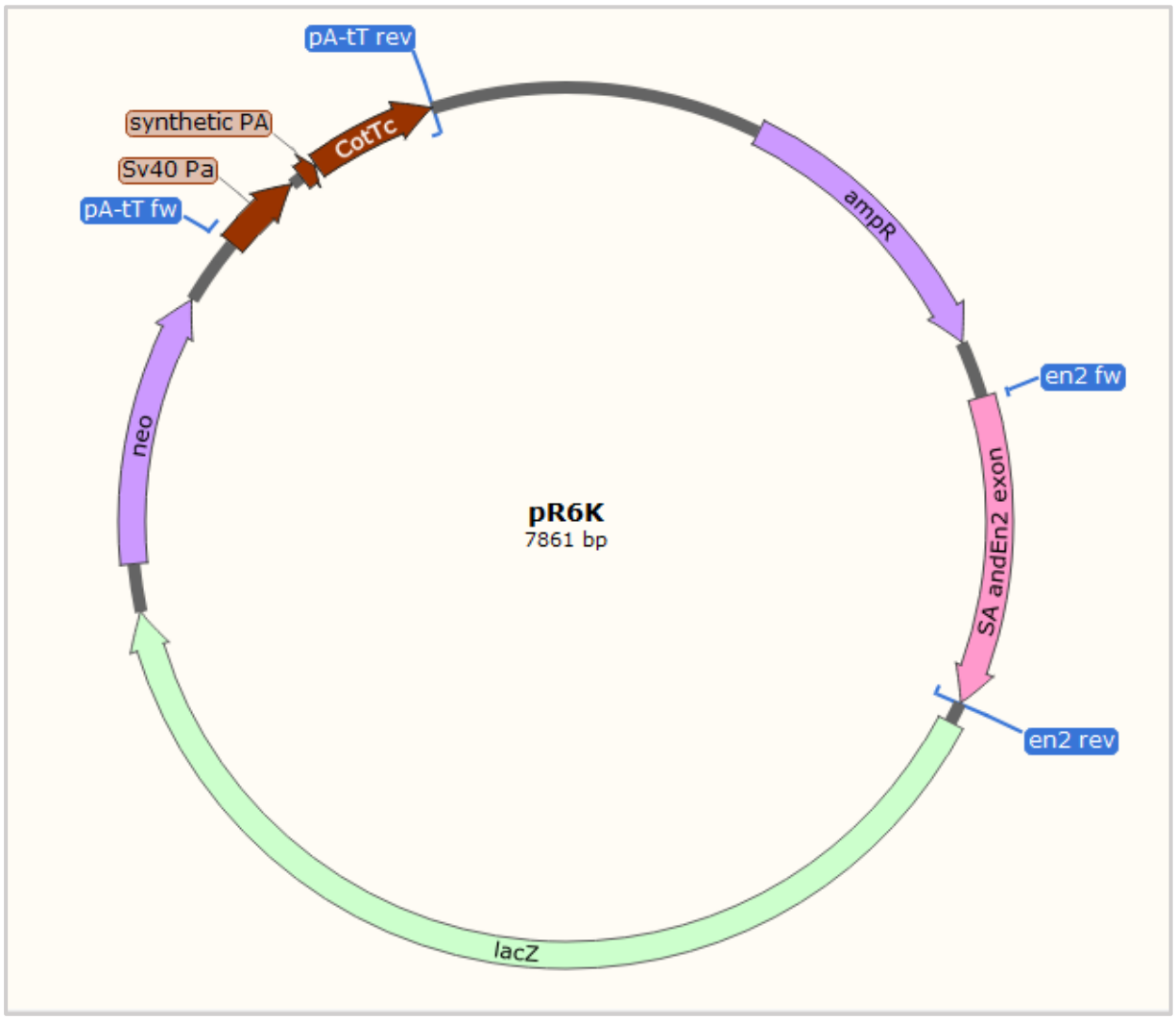

Figure 18 pR6K. *neo: Neomycin. Sv40 PA, synthetic PA and CotTc: synthetic polyadenylation signal with transcriptional terminator (pA-tT). ampR: Ampicillin resistance. SA andEn2 exon: Last exon of Engrailed-2 (en2). Blue: Primer to amplify pA-tT and en2.*

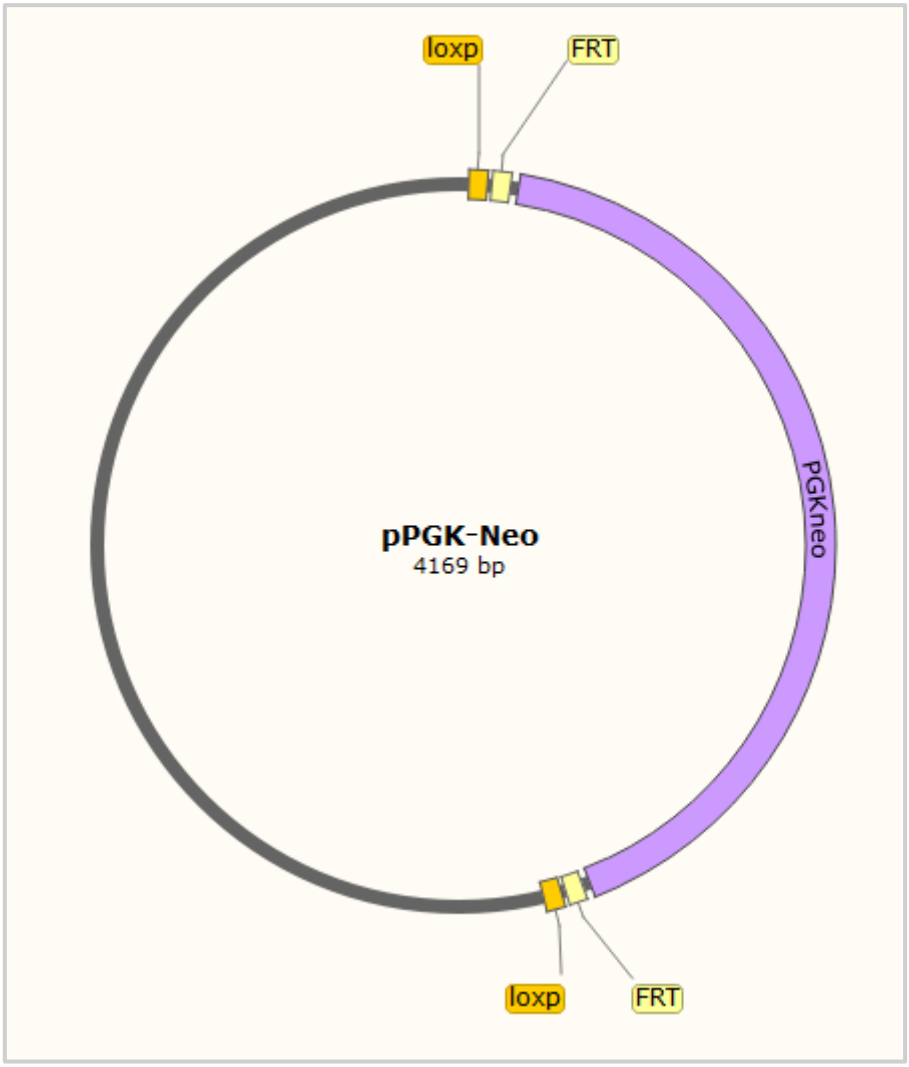

Figure 19 pPGK-Neo. *PGKneo: Neomycin resistance cassette. FRT: flippase recognition target.*

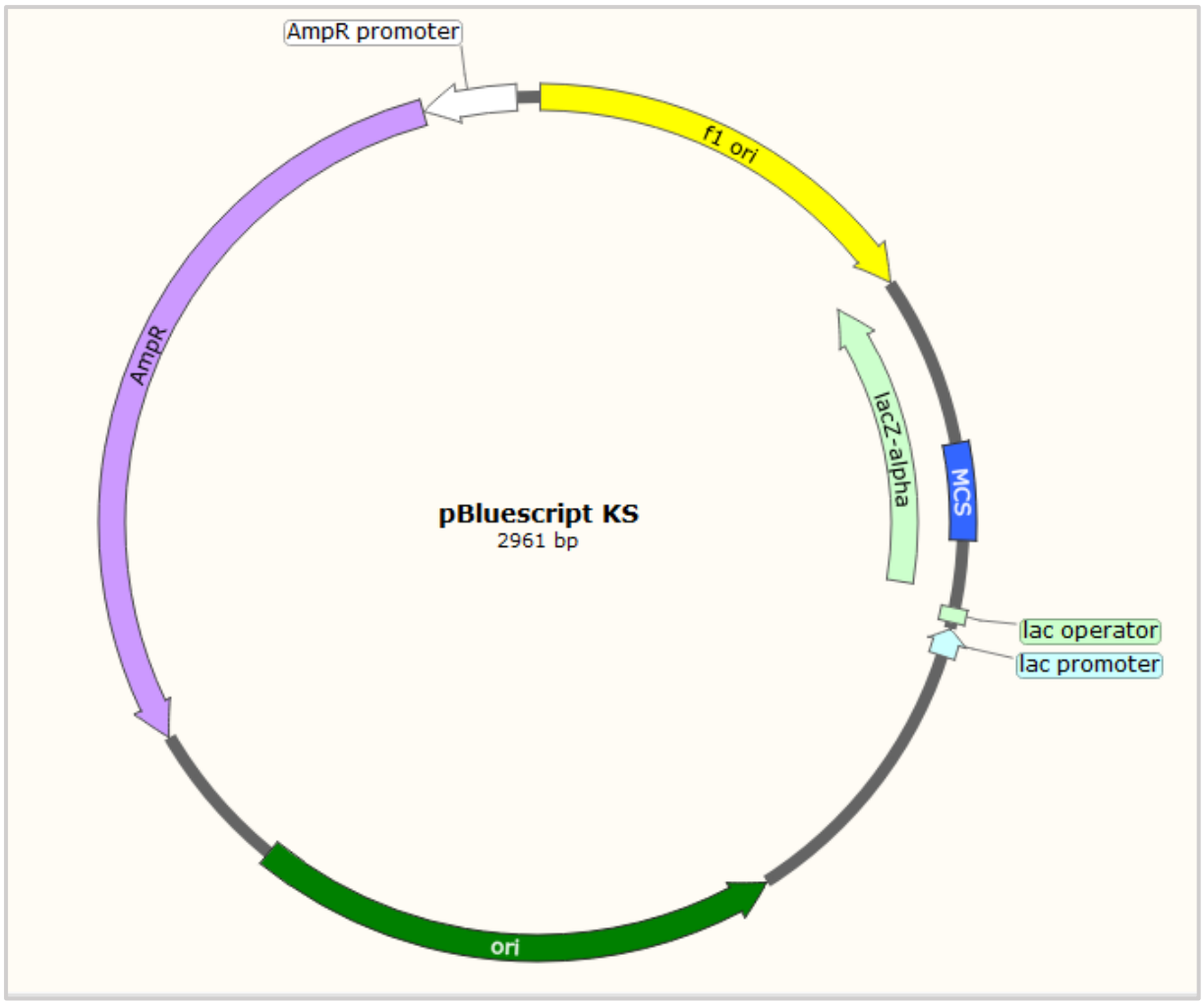

Figure 20 pBluescript KS. *AmpR: Ampicillin resistance. F1 ori: origin of replication of bacteriophage f1. MCS: Multiple cloning site. Ori: Origin of replication.*