Experimente zur Generierung einer knockout Maus für Untersuchungen zur Funktion des *Plasmodium chabaudi* induzierbaren *imap38* Gens.

I n a u g u r a l – D i s s e r t a t i o n

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To my Father

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1. Introduction

1.1. Malaria: problem definition

Malaria is one of the top three killers among communicable diseases. There are 300 to 500 million clinical cases every year and between one and three million deaths, mostly of children, are attributable to this disease (Sachs, 2002). To symptoms of acute stage malaria belong not only fever and severe anaemia. *P. falciparum* parasites sequester in various organs, including heart, lung, brain, liver, kidney, subcutaneous tissues and placenta, causing such terrible complications as cerebral anaemia, metabolic acidosis, hypoglycaemia and respiratory distress (Miller, 2002). Infants and young children particularly suffer from life-threatening anaemia, older children from an induced coma (Marsh& Snow,1999), and primagravid women from severe disease related to placental sequestration (Ricke *et al.,* 2000). There are several immune responses that restrict parasite growth in humans, but the parasite persists. People infected repeatedly by malaria develop 'naturally acquired immunity' (NAI), which protects against clinical diseases. Nevertheless, if their parasites are eliminated through radical drug cure, these individuals can become re-infected, which indicates that NAI does not include absolute anti-infection immunity (Hoffman *et al.*, 1987). Due to this observation, drug cure is not optimal against malaria, neither insecticides do prevent transmission: use of anti-malaria chemotherapeutics and insecticides results in survival of resistant malaria strains and mosquito populations (Greenwood, 2002). Even after many exposures to malaria, humans are not refractory to malaria parasites, but develop clinical immunity that prevents symptomatic disease. This type of immunity limits the outbreak of the disease. Although the individuals may carry low numbers of parasites, they do not develop into a symptomatic infection. (Miller, 2002) Vaccination seems to be the best way to prevent malaria. Malaria vaccines are feasible. Immunisation with irradiated sporozoites protects or partially protects humans from being infected by sporozoites (Clyde, 1990; Egan *et al.*, 1993; Rieckmann, 1979). Immunisation studies performed over the past 15 years show that vaccines already in hand can protect against malaria infection in animal models and in humans, but efficiency of these vaccines is still too low and the duration of protection still too short to be of practical value (Richie, 2002). The main complications are that different stages of the parasite express different antigens and that many parasite proteins exhibit polymorphism, which potentially limits the effectiveness of any vaccine not incorporating distinct variants of antigen (Volkman, 2002). In 2002 the complete genomic sequences of *Anopheles gambiae* (Holt *et al*, 2002) and *P. falciparum* (Gardner *et al*, 2002)

have been determined and annotated (Hoffman, 2002b). Proteome of all stages of *P. falciparum* was analysed (Lasonder *et al.*, 2002). Human genome was completed in 2003 (Little, 2003). Following the publication of the malaria parasite's genome sequence and the beginnings of relevant proteomics, research tools are now available that could bring huge efforts to the long-term fight against malaria. It is expected that already in 7-23 years we will have anti-malaria vaccine (Long, 2002). But meanwhile every 40 seconds a child dies of malaria. Another aspect of the problem is that malaria and other tropical diseases have major influences on the economic development of Africa (Sachs, 2002). Malaria alone decreases the economical growth of African countries for more then 1% per year (WHO, Geneva, 2002). However malaria progressive invades new regions favourable to its development (Martens, 1999). Vectors require specific ecosystems for survival and reproduction. These ecosystems are influenced by numerous factors, many of which are climatically controlled. Changes in any of these factors will affect the survival and hence the distribution of vectors. A permanent change in one of the abiotic factors may lead to alteration in the equilibrium of the ecosystem, resulting in the creation of less favourable vector habitats. Malaria distribution is mostly determined by minimum temperature required for plasmodium life stage changes (for *P. falciparum* it is about 18°C (Barry, 1992)). The global warming determined by human activities will bring 2200 million persons in risk of a malaria infection till 2050 due to increasing of areas suitable for malaria (Beniston, 2002). If the trend will be the same, area suitable for malaria survival will move to the north. Spending on malaria research and development are $100 mln and $ 500 mln per year (Long, 2002) Maybe this is not a too high price for malaria prevention.

1.2. Mouse as a model for malaria research

To study malaria and develop an effective anti-malaria vaccine, adequate animal models are necessary. The closest relatives of humans – higher primates, are the best models due to their high similarity to humans. For ethical reasons and because of large costs of experiments use of these animals is extremely rare - practically only in pre-clinical trials. Mice are very common, less expensive and good known models in many biological experiments. It is known a lot about mouse genetic, immunology, behaviour and diseases. There are many inbred strains with well defined characteristics. Till now hundreds of knock-out, knock-in and other genetically modified mice have been generated allowing investigation of gene functions during infection. Many inborn and infectious diseases have a similar

pathology in mice and humans. All these considerations make mice the best alternative to human and primate experiments.

There are several mouse models that can be used to study malaria pathogenesis (Li *et al.*, 2001). Until recently, *P. berghei* infection has been the major model for study of pathogenesis of cerebral malaria (CM), and *P. chabaudi* and *P. yoelii* infections have been used to study the pathogenic processes involved in hypoglycaemia, anaemia and other clinical signs of the malaria infection (Li, 2001). One of the most adequate analogues for human infection with *P. falciparum* is mouse infection with *P. chabaudi*. In both cases erythrocytes (normocytes) but not reticulocytes are invaded and destroyed by parasites. Though both *P. falciparum* and *P. chabaudi* sequester on the endothelia of notkapillari venuola, they differ in tissue tropism: while strains of *P. falciparum* sequester in brain, liver and placenta, *P. chabaudi* appears to be restricted predominantly to the liver (Gilks *el al*., 1990; Phillips *et al*., 1994). Both pathogens show sequestration of schizonts and trophozoits in tissues (Sinden *et al*., 1989; Philips, *et al*. 1994). Some data show that *P. chabaudi*–infected erythrocytes adhered to brain vessels in mice can also be found using electron microscopy scanning (Mota *et al*., 2000). *P. chabaudi* also does not form knobs on the surface of the host cells (Cox, *et al*., 1987). *P. chabaudi* shows a frequency of antigen variation from 1.3×10^{-2} to 4.3×10^{-4} that is similar to that observed for *P. falciparum* (Brannan *et al*., 1994). *P. chabaudi* infection in its natural host, *Thamnomys rutilans* rats, as well as in laboratory mice lead to synchronic erythrocyte infections with cycle durations of 24 h (Carter & Walliker, 1975). These observations show that mouse infection with *P. chabaudi* is a useful system for research on protective immunity and host-parasite interactions.

However, although some severe complications found in human occur also in mice, the pathogenic mechanism can be different. Therefore, extrapolation from mouse to human studies should be done with care.

The life cycle of *Plasmodium* is a complex with distinct phases, where each stage is characterized by the expression of stage specific proteins. Therefore, different immune effector mechanisms with different specificities are required for the elimination of these various forms. Innate immunity components such as acute phase proteins, complement, phagocyting macrophages, and NK cells are responsible for clearance of malaria infection (Mohan *et al*., 1997). Nearly all complications of malaria infection are related to erythrocytic stages of malaria life cycle change. Erythrocytes contain no antigen-processing machinery and also do not express major histocompatibility complex class II or class I. Therefore infected erythrocytes or free merozoites cannot be a target of specific effector T cells (Li *et*

al., 2001). Bone marrow-derived dendritic cells (DC) are thought to be able to respond directly to pathogens without a second activating signal (Basu *et al*., 2000; Von Stebut *et al*., 1998). It was shown that *P. chabaudi* parasites are capable of rapidly activating DC to produce cytokines including IL-12 and to up-regulate costimulatory molecules such as CD40 and CD86 independently of T cells, NK cells and IFN-γ (Seixas et a., 2001). Additionally DC process antigen and present it to T cell. IL-12 has been detected in the plasma of *P. chabaudi* infected mice as early as 2 days after infection (Mohan and Stevenson, 1998). IL-12 activates NK cells to produce IFN-γ (Scharton-Kersten &Sher, 1997). NK cells activity is an important part of infection clearance: mice with high NK activity are generally resistant to parasites such as *P. chabaudi* (Mohan *et al.*, 1997). Furthermore IL-12 is a pre-requisite for the development of $CD4^+$ Th1 cells upon interaction with APC. $CD4^+$ T cells are critical for controlling erythrocytic stage parasites (Amant&Good, 1997; Kumar *et al.*, 1989; Meding&Langhorne, 1991; Podoba&Stevenson, 1991). They do not limit parasite growth directly but achieve this effect by activating other effector cells to eliminate the parasite. The two major subsets of $CD4^+$ cells are Th1 and Th2, although Th0 and Th3 have also been described (Groux *et al.*, 1997; Mosmann *et al.*, 1986). In humans and mice *Plasmodium* activated γδT cells expand during and following an acute malaria infection and are involved in parasite control or in the pathology of the infection (Goodier *et al.*, 1995; Seixas& Langhorne, 1999). They are able to secrete IFN-γ and TNF-α (Goodier *et al.*, 1995) and human γδT cells can inhibit growth of *P. falciparum in vitro* (Elloso *et al.,* 1994). The parasite factors responsible for stimulation of γδT cells have not been fully characterized, but host proteins induced by infection or released by damaged cells are thought to be involved (Boismenu&Havran, 1997).

Th1 and Th2 cells themselves cross-regulate the differentiation and activity of each other via cytokines produced (Constant&Bottomly, 1997). Th1 cells produce IL-2, IFN-γ, and TNF-β and so initiate cell-mediated immune responses via macrophage activation. IFN-γ is important in initial phase of infection. One of the major functions of this cytokine is macrophage activation with consequent production of TNF-α, IL-1, IL-6 and soluble mediators (NO, reactive oxygen species (ROS)). IFN-γ also regulates the production of opsonizing, cytophilic antibodies such as IgG2a in the mouse (Coffman *et al.*, 1993) and is associated with inflammation. TNF-α exerts its effect indirectly by inducing anti-parasitic factors, but not necessarily NO (role of NO and ROS in parasite killing is still controversial (Nussler *et al.*, 1991; Amante&Good, 1997)). The level of TNF-α in serum correlates with severity of disease, a higher capacity of blood leucocytes to produce TNF-α *in vitro* was

associated with faster reduction of fever, accelerated cure and good prognosis during *P. falciparum* infection (Mordmuller *et al.*, 1997). The mechanism of its action is still far from being clear. In *P. chabaudi chabaudi*, as a primary infection progresses there is a shift in the pattern of the $CD4^+$ T cell response (Langhorne *et al.*, 1990; Langhorne&Simon, 1989). After three weeks of infection, the predominant $CD4^+$ cells are Th2. Th2 cells can mediate their effect by providing help to B lymphocytes for the production of antibodies (Coffman *et al.*, 1989). Th2 cells produce IL-4, -5, -6, and -13 and regulate antibody-dependent responses. They also produce IL-10, which has anti-inflammatory properties and can antagonize Th1 responses inhibiting macrophage activation (Moore *et al.*, 1993). The switch from Th1 to Th2 cells activation seems to take place as parasite levels are decreasing and thus antigen dose is reduced. The factors and mechanisms involved in this switch have not been fully identified. The immune responses driven by Th1 and Th2 cells are also influenced by Th3 cells whose main function is suppression of the immune responses mediated by Th1 and Th2 (Groux *et al.*, 1997) through production of transforming growth factor-β (TGF-β). TGF-β is a regulator of inflammation, which would be important for the balance between control and elimination of parasites and prevention of pathology. Levels of TGF-β seem to be inversely correlated with severity of malaria, suggesting that its role may be down-regulating the production of pro-inflammatory cytokines (Omer & Riley, 1998; Wenisch *et al.*, 1995).

Clearance of infection is complicated by parasite evasion of the host defences: growth in protected location, polyclonal activation of lymphocytes, antigenic variation, sequestration and preferential induction of antibodies without protective function (Li, 2001). Certain strains of *P. falciparum* have been shown to inhibit the maturation of DC and their antigen-presentation capability (Urban *et al.*, 1999). This might be an explanation for the immune suppression described in malaria infections. Presentation to T cells in the absence of the correct costimulation may induce energy, apoptosis, and clonal deletion (Hirunpetcharat&Good, 1998).

Some rodent malarias are lethal only in certain strains of mice. This allows investigating the mechanisms leading to susceptibility or resistance to infection (Wunderlich *et al.*, 1988; Stevenson, 1989; Jacobs *et al.*, 1996). Several loci, which are genetically linked to resistance to either *P. c.chabaudi* or *P.c.adami* (in A/J, C3He/J and SJL/J mice) have been mapped to chromosome 8 and 9 (Foote *et al.*, 1997; Fortin *et al.*, 1997). The proteins encoded by genes on these chromosomes are poorly characterized but are linked to genes coding for several erythrocyte membrane proteins that may be involved in parasite invasion of red cells. Resistance and susceptibility of mice to *P.chabaudi* is also influenced by gender. Increased

mortality of male A/J mice in comparison to female may be related to the male hormone, testosterone (Foote *et al.*, 1997; Fortin *et al.*, 1997; Stevenson, 1989). Castrated male mice are more resistant to malaria (Wunderlich *et al.*, 1991) and treatment of female mice with testosterone makes them more susceptible to malaria (Schmitt-Wrede *et al.*, 1991; Mossmann *et al.*, 1997). Since testosterone has been shown to suppress inflammatory responses by down regulation of IFN-γ production of $CD4^+$ cells (Paavonen, 1994) and TNF-α and IL-12 production by macrophages (Benten *et al.*, 1993; Chao *et al.*, 1995), it is possible that male mice have less pronounced proinflammatory responses than female mice early in infection. Thus, they cannot initiate an effective early response that controls the parasite making them unable to control a primary blood stage infection. On the other hand, enhanced inflammatory responses lead to a more severe disease and increased mortality without an increase in parasitemia. For example, female IL10-KO mice are more susceptible to a lethal infection and suffer a greater hypoglycaemia and loss of body weight (Li *et al.*, 1999). Death in this model is related to the effects of the cytokines IFN-γ and TNF-α and not to an inability to control early parasitemia (Li *et al.*, 1999; Linke *et al.*, 1996). Therefore the pathology associated with *P. chabaudi* seems to be caused not only by high parasitemias but also by the cytokine responses of the host.

The spleen plays a central role in the immune response against malaria. Splenectomy leads to increasing susceptibility against malaria caused by *P. falciparum* and *P. chabaudi* infections (Wyler *et al.*, 1979; Wyler, 1983). Infections with *P. c. chabaudi* AS in splenectomized C57BL/6 mice have chronic character (Yap & Stevenson, 1994). For unknown reasons, transfer of antibodies obtained from immune mice is not sufficient to produce protective immunity in splenectomized mice, but transfer of activated B memory cells from immune mice is. To activate naive B cells for antibody production, a signal from T cells is necessary. But neither naive nor memory T cells are able to excrete these signals outside of the spleen. Dramatic spleen enlargement during malaria infection is on one hand due to the immigration of cells from blood, on the other hand to activation and expansion of B cells as well as to erythropoesis. While hematopoesis during malaria infection is suppressed in bone marrow, spleen takes over the erythropoetic function of bone marrow (Weiss *et al.*, 1989; Villeval *et al.*, 1990; Mohan & Stevenson, 1998). Morphological changes in the spleen also contribute strong defence mechanism against malaria. During infection of mice with *P. yoelii* (Weiss *et al.*, 1986) and *P. chabaudi adami* (Alves *et al.*, 1996) stromal fibroblasts in the spleen (barrier cells) expand and built barrier-forming complexes which mechanically protect erythropoethic islands and non-mature lymphocytes from circulating parasites.

Filtration capacity of the spleen in this period is significantly lower then in naive mice, because most of the blood flows directly from arterioles in the venous sinus (Wyler, 1983; Weiss *et al.*, 1986; Weiss, 1989). Later on, release of reticulocytes and effector cells in the bloodstream causes a decrease of parasitemy and anaemy. Filtration capacity of the spleen at this stage normalizes, but destroyed erythrocytes cannot bypass endothelial cells. They stay in the pulpa of the spleen, where they are phagocytozed by activated macrophages.

1.3. *Imap*-genes family

IMAP38 belongs to a family of immune-associated proteins. These proteins are highly conserved between mice, human, rats and plants. Typical for *imap* genes is molecular organisation: several exons with a largest (80-90%) last exon, and a large first intron. All members of the family have ATP/GTP-binding motif in the sequence; some of characterised proteins show low GTP-binding activity. This family is presented in mice by 8 genes (seven of which are annotated in Ensembl database) clustered in mouse genome in chromosome 6B within 130 kbp region, in human genome within a 300 kb interval at 7q36.1 (Fig. 1) (MacMurray, 2002). Homologues genes are also found in rats and plants. The first member of immune associated proteins family, AIG, was characterized in plant *Arabidopsis thaliana*. *Aig1* is induced after infection with bacterium *Pseudomonas syringae* (Reuber&Ausubel, 1996).

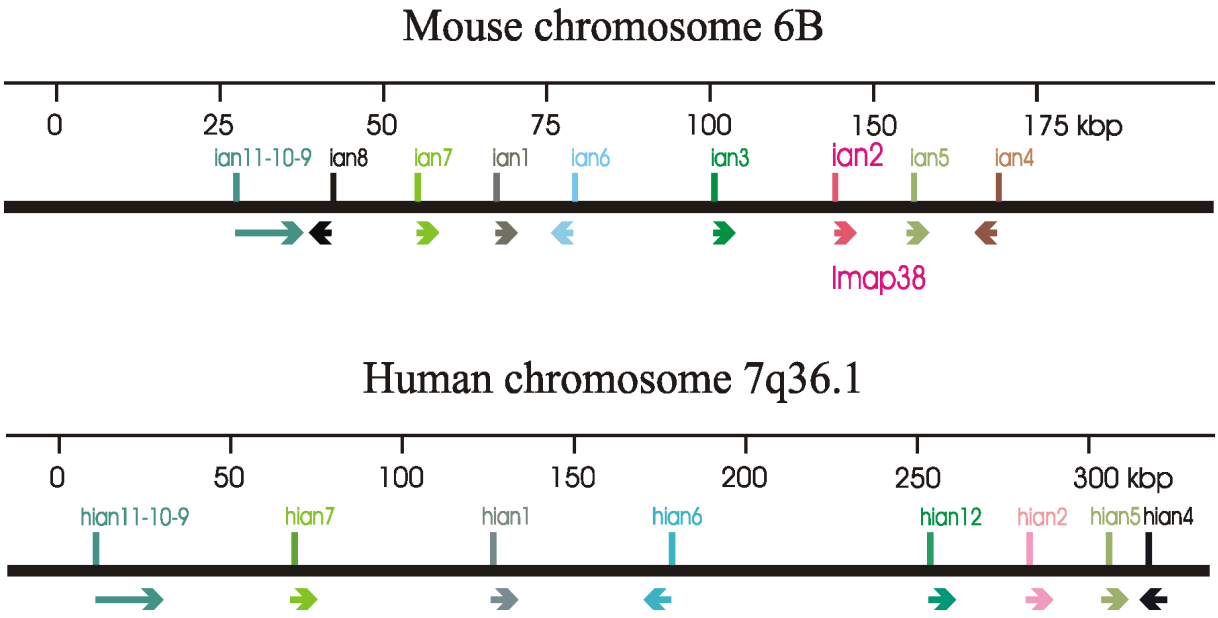


Fig. 1. Location of mouse and human AIG-like genes family. *Ian8* and *Hian4* are pseudogenes.

IMAP38 is a first protein of mouse *Aig*-similar gene family. It was identified using differential screening (Krücken *et al.*, 1997). Genomic clones containing the complete

sequence of the *imap38* gene were obtained from C57BL/10 mice by genomic PCR and from 129/Ola mice by screening of a cosmid library (Krücken *et al.*, 1999). *imap38* is a single copy gene localized on the chromosome 6B in mice. It was believed, that gene consists of a single exon and encodes 2 overlapping open reading frames. The proteins encoded in these two ORFs were named IMAP38-1 and IMAP38-*2*. IMAP38-1 has nuclear localisation which allows suggestion of a regulatory function of the protein. IMAP38-2 is localized on the ER. It contains GTP-binding domain, which shows a weak GTP-binding activity (Stamm, 2002). After genome project was finished, it has become clear that *imap38* consists of 3 exons and contains ATP/GTP-binding motif and transmembrane domain. Expression of *imap38* gene was determined in mouse spleen (in macrophages and B-cells, very low in T-cells) after infection with *Plasmodium chabaudi*. On the seventh day after infection (shortly before peak of parasitemia) *imap38* is induced on the high level. Immune mice show 50-times stronger expression than non-infected mice. On this level *imap38* expression is maintained even 13 weeks after infection at a point when parasites are not present in blood any more for 3-5 weeks (Krücken *et al.*, 1997). Different mice strains with C57BL/6 and C57BL/10 background express *imap38*-mRNA at low levels. Mice with BALB and DBA background express *imap38* at very low level even 7 days after infection. *P. chabaudi, P. falciparum, Trypanosoma cruzi* and *T. congolense* induce expression of *imap38 in vivo* after infection (Krücken, 1999). In *P. chabaudi* susceptible strain C57BL/10ScCr expression of *imap38* is uninducible by parasite lysates. Inducability of *imap38* in the spleen cells is controlled by non-H2-background, not by H2-genes or gender of the mice. In C57BL/6 and C57BL/10 mice inducability of *imap38* correlates with formation of protective immunity (Krücken *et al.*, 1999). *Ex vivo*, induction of the gene was never successful neither in spleen primary cultures nor in murine macrophage cell lines. For induction of *imap38* parasite and spleen specifical factors are necessary. Testosterone levels and genes of H2 complex do not play any role in inducability of *imap38*. Microarray analysis has shown 6-fold overexpression of *imap38* during P53-mediated apoptosis, but it stays unclear yet if *imap38* plays a pro- or antiapoptotic role (Kannan *et al.*, 2001).

In mouse genome all *Aig1*-like genes homologues to *imap38*. They all are clustered inside of 130 kbp in the locus B on the chromosome 6. Not all of them are characterized. *Ian1* (immune-associated nucleotide) has a single ORF consisting of 3 exons and encodes a 42 kDa large proteins. It is expressed in lymphatic organs especially in thymus and only in T- and B-lymphocytes, not in macrophages. Analysis of protein expression at various stages of thymocyte development links mIAN-1 to CD3 mediated selection events, suggesting that it

represents a key player of thymocyte development and that it participates to peripheral specific immune responses (Poirier, 1999). *Ian4* possesses two not-overlapping ORFs. The larger of the encoded proteins (301 aa) is similar to *Ian1* and has a weak GTP-binding activity (Daheron *et al.*, 2001). *Ian4* was preferentially expressed in the spleen at very low level, but was not found in any other lymphoid tissue. It is preferentially expressed in haematopoietic precursor 32D cells after transfection with a *Bcr/Abl* oncogene expressing vector (Daheron *et al.*, 2001) and was shown to play important role during apoptosis (Pandarpurkar *et al.*, 2003). IMAP4 is localized on endoplasmatic reticulum, Golgi and plasma membrane. Down regulation of *Imap4* expression after *P.chabaudi* infection was shown recently (Paunel A.N. 2002, diploma work). *mIan5* and *hIan5* are homologous to a rat *Ian5* that consists of three exons and contains two overlapping ORFs. *rIan5* is 308 aa large and is expressed in rat spleen, thymus, and lymph nodes, suggesting that *rIan5* may be a key factor in T-cell development (MacMurray, 2002). Expression of *Imap5* is suppressed during *P. chabaudi* infection.

In humans *himap1* is a homologue of *imap38* (*mIan2*) and is situated together with other eight similar proteins in locus 7q32-36. *himap1* (as well as *himap2,-3,-4,-5*) consists of three exons from which the first is non-coding, the second contains translation start and the third contains the rest of protein information. HIMAP1 which is localized at the endoplasmatic reticulum is expressed in different human tissues. Higher levels were determined in spleen and lymph nodes. HIMAP1 has no GTP-binding activity (Stamm, 2002). *Himap4* encodes a 38kDa protein and has been determined in spleen, leukocytes, peripheral blood (T- and B-lymphocytes) at lower levels in thymus, intestine, and ovary (Cambot, 2002). *Himap5* has three exons transcribed into two alternative splice products himap5a (encodes 32,9kDa protein) and himap5b (encodes a 24, 1 kDa protein). *Himap6* and *himap7* are built from single exons. *Himap6* consists of 1141 bp and encodes 34, 5 kDa protein, *himap7* is 795 bp long and encodes a 26,9 kDa protein. HIMAP9 is a 38,4 kDa protein with nuclear localization. *Himap9* is found to be expressed in different organs and tissues such as spleen, heart, skeletal muscles, placenta, and lungs (Müller, 2002; diploma work). So *Ian*-genes are highly conservative proteins which show large similarity of molecular organisation and in all known cases their expression correlates with immune responses against different pathogens in different plants and animals. They are very interesting in studying of defence mechanisms against malaria infection.

1.4. Creation of genetically modified mice

The mouse as a very similar to human and good characterized mammalian serves as a common tool in gene and disease investigation (Bogue, 2003; Buer&Balling, 2003). Hundreds of inbred strains performing a homogenous genetic background were generated since the beginning of 20th century (MGI web site). Much is known about mouse genetic and gene function. Many processes are well characterized on the molecular level. Now the draft mouse genome sequence is available and transcriptome analysis is performed (Okazaki, 2002). Furthermore bioinformatical resources present wide range of information related to gene and structure prediction, phylogenetic comparison and many other useful tools (Mount, 2001). In these conditions modifying gene function *in vivo* inside of developing or living organisms has become a standard method. During the last decade, conventional transgenic and gene knock-out technologies have become invaluable experimental tools for modelling genetic disorders, assigning functions to genes, evaluating drugs and toxins, and other purposes in basic and applied research (Bockamp, 2002; Thyagarajan, 2003). Routinely modification of gene function in mice is commonly performed in two ways: mutation of specific DNA locus (classical approach termed gene targeting) and gene silencing on the post-transcriptional level (RNAi technology termed knock-down (Shramke&Allshire, 2003)).

Today, genetically modified mice are routinely obtained by injection of foreign DNA into one cell embryo (transgenic mice) or are transfected in totipotent (Feng *et al.*, 2002) embryonic stem (ES) cells (gene targeting) which are later aggregated with mouse embryo giving rise to a chimeric mouse (Tohrres&Kuhn, 1997; Hogan *et al.*, 2001). If simply overexpression of desired protein is required, *transgenic mice* can be generated by injection of expressing vector in pronucleus of one cell embryo. The injected expression vector integrates randomly in the genome. Injected embryos are then transferred into pseudo pregnant mice and are ready for analysis and experiments after birth. Such animals usually contain several tandem copies of the DNA construct per genome and phenotype can be strong influenced by disruption of sequences at integration sites. Therefore, gene targeting is a more accurate tool for investigation of gene function. Gene targeting begins with transfection of targeting construct into ES cells followed by selection of clones containing a single integrated vector at the target site. ES cells of these clones are then aggregated with or injected in the early mouse embryos. In such a system, ES cells containing the targeted allele as well as wild type cells from the injected embryo contribute to the germ line resulting in the generation of chimeras. If modified ES cells are contributing to germ line tissues, some of the chimeras' progeny will be heterozygous for a desired mutation. Homozygous animals can be obtained by mating of

heterozygous progeny. Unfortunately, each step of this procedure can be the last for each particular experiment. There are some common reasons for failure of experiments: ES cells are not stabile, they tend to differentiate during culturing or they obtain chromosomal defects; for each ES cell line only the embryos of certain mouse strains are compatible; chimeras are often sterile or give no germ line transmission; mutation of genes important in embryo- or ontogenesis are often lethal. Many other factors which are not described in literature can also be a reason and maybe they will forever stay unknown. In this work a classical gene targeting combined with site-specific excision of the *Neo*^{r} selection marker by Cre recombinase was made.

The use of site-specific recombinase systems has revolutionized ability to genetically manipulate embryonic stem cells and mice. Two recombination systems are established in mice (Lakso *et al.*, 1992; Orban *et al.*, 1992; Gu *et al*., 1994; Dymecki, 1996): Cre-loxP system from bacteriophage P1 and Flp-FRT system from the budding yeast *Saccharomyces cerevisiae.* Both Cre and Flp are members of the λ integrase super family of site-specific recombinases (Argos *et al.*, 1986) that cleave DNA at a distinct target sequence and then ligate it to the cleaved DNA of a second identical site to generate a contiguous strand. No cofactors or accessory proteins are required, making recombinase systems quite adaptable for use in a wide variety of heterozygous environments. Target sites (for Cre-recombinase LoxP, for Flp-recombinase FRT) are 34 bp long and are asymmetric (Stark *et al.*, 1992). Recombinase reactions can be used in generation of insertions and deletions: removal of selection marker genes or gene coding sequences for small genes, engineering a subtle mutations, swapping sequences, conditional gene (in)activation, conditional gene repair, generation of gene differences and for gene mapping and mutant identification; inversions: conditional gene expression, model human diseases associated with chromosomal inversions and chromosome loss; translocations: model human diseases associated with chromosomal translocations, generation of mitotic clones in drosophila; integrations: isogenic gene transfer systems (Joyner, 2001). The critical step responsible for successfulness of the whole experiment is generation of targeting construct serving to introduce desirable mutation in targeted locus. A targeting *vector* is designed to recombine with and mutate a specific chromosomal locus. The general event leading to introduction of foreign DNA into genome inside of ES cell is homologous recombination. Minimal components of targeting vector are sequences homologous with the desired chromosomal integration site and selection marker (Torres & Kuhn, 1997; Joyner, 2001). Additional components (loxP sites, foreign functional DNA sequences, reporter genes) can be present in targeting vector and introduced in the

chromosome. Two vector design types can be used for targeting in mammalian cells, giving rise for all the other targeting constructs: replacement and insertion vectors (Torres & Kuhn, 1997). In replacement vector homology arms flank a targeted sequence from both sides. Final recombinant allele for replacement vector is a consequence of double reciprocal recombination which takes place between the homology arms of vector and the chromosomal sequences. Targeted allele contains all the vector components which are flanked on both sites with homology sequences. Since targeting frequency is low, selection markers must be included in the vector. Positive selectable marker in targeting vector can serve two functions. First, it acts as a selection marker to isolate rare transfected cells that have stably integrated DNA. Second, it serves as a mutagen, for instance if it is inserted into the coding exon of a gene or replaces coding exons. Negative selection marker is usually situated in plasmid backbone outside of homology arms and makes lethal random integration of targeting construct which occurs via recombination outside of homology arms or via concatamer formation (Torres & Kuhn, 1997; Joyner, 2001).

1.5. Aim of the work

Characterized in our laboratory gene *imap38* (Krücken *et al.*, 1999) is induced by malaria infection. Function of *imap38* is supposed to be related to immune response to malaria. Aim of this work was generation of *imap38* knockout mouse to create a tool for *in vivo* investigation of *imap38* function. C57BL/6 mice which survive malaria infection with *P. chabaudi* and express high levels of *imap38* are taken as background. To obtain targeted deletion of *imap 38* targeting construct containing self excision ACN cassette must be generated.

2. Materials and methods

2.1.1 Chemicals.

Agar; yeast extract; Trypton, Peptone: Difco Laboratories, Detroit, U.S.A.
Agarose: SeaKem®LE Agarose for gel electrophoresis, BIOZYM Diagnostic, Oldendorf, Germany
Ampicilin: Serva, Heidelberg, Germany
Anaesthetics Ketanest, Rompun: Bayer, Leverkusen, Germany
Chemicals for molecular biology: BIOZYM Diagnostic, Oldendorf; Roche, Mannheim; Fermentas, St. Leon Rot; Pharmacia, Freiburg; Promega, Heidelberg; Serva, Heidelberg; Sigma, Deisenhofen; Stratagene; Heidelberg; USB, Bad Homburg, Germany; Invitrogen, Leek, Netherlands
hCG: Sigma, Deisenhofen, Germany
dNTP PCR Nucleotide mix 10 mM each dNTP: Roche, Mannheim, Germany
Ethidium bromide 1% (10 mg/ml) stock: Roth, Karlsruhe, Germany
Kanamicin: Serva, Heidelberg, Germany
Light oil, embryo tested: Sigma, Deisenhofen, Germany
Long Ranger® Gel Solution: CAMBREX, Rockland ME, USA
Mineral Oil for molecular biology: Sigma, Deisenhofen, Germany
PMSG, Sigma, Deisenhofen, Germany
Radiochemicals: ICN, Meckenheim; Braunschweig, Germany
Other chemicals (p. a.): Acros Organics, Geel, Belgium; Baker, J. T., Deventer, Nederland; Bio-Rad, München; Biozym Diagnostik, Hameln; Fluka, Neu-Ulm; Grüssing GmbH, Filsum; Janssen Chimica, Geel, Belgien; Merck, Darmstadt; National Diagnostics, Atlanta, Georgia, U.S.A.; Riedel-de Häen, Seelze; Roth, Karlsruhe; Serva, Heidelberg; Sigma, Deisenhofen, Fluka, Neu Ulm; Merck, Darmstadt; Riedel-de Häen,Seelze; Roth, Karlsruhe; Serva, Heidelberg; Sigma, Deisenhofen, Germany.
Roti®-Phenol for separation of DNA/RNA: Roth, Karlsruhe, Germany
Sterile filter: Nunc, Wiesbaden, Germany
Streptomycin/Penicillin Solution: Sigma, Deisenhofen, Germany
Ultra Pure SequaGel®XR: National Diagnostics, Atlanta, U.S.A.

2.1.2 Kits

Expand High Fidelity PCR system, Roche Molecular Biochemicals, Mannheim, Germany
InvisorbSpin Tissue Mini Kit, Invitek, Berlin-Buch, Germany
Megaprime DNA Labeling Kit; Thermosequenase fluorescent labeled primer cycle sequencing Kit with 7-deazadGTP: Amersham, Braunschweig, Germany
NUCLEOBOND®AX Midi Kit, Macherey-Nagel, Düren, Germany
NucleoSpin®extract, Macherey-Nagel, Düren, Germany
NucleoSpin®plasmid, Macherey-Nagel, Düren, Germany
pcDNA3.1/V5-His TOPO®TA Expression kit, Invitrogen, Leek, Netherlands
pGEM®T Easy Vector System, Promega, Madison WI, USA
TOPO TA Cloning®Kit pCR®2.1-TOPO®Vector, Invitrogen, Leek, Netherlands
Triple Master™ PCR system, Eppendorf, Hamburg, Germany
Qiagen Blood and Cell Culture Genomic DNA Purification Kit: Qiagen, Hilden, Germany
Qiagen-Spin pre-Mini, Midi-Plasmid isolation kits, Qiagen GmbH, Hilden, Germany
QIAquickTM Spin Nucleotide Removal Kit, Qiagen GmbH, Hilden, Germany
QIAGEN Plasmid Maxi kit, Qiagen GmbH, Hilden, Germany
ThermoSequenase™Primer Cycle Sequencing kit, Braunschweig, Germany
VenorGeM® Mycoplasma Detection kit, Minerva BioLabs, Berlin, Germany

DNA molecular weight markers- pUC Mix, λ/HindIII, λ/HindIII+EcoRI, λMix, Fermentas
6× loading buffer, Fermentas GmbH, St.Leon, Germany

2.1.3 Enzymes.

Cre Recombinase, New England BioLabs® GmbH, Frankfurt am Main, Germany
Restriction endonucleases: Fermentas, St Leon; New England BioLabs, Frankfurt am Main; Stratagene, Heidelberg; Germany
RNAse A, Roche, Mannheim, Germany
Proteinase K, Roche, Mannheim, Germany
Shrimp Alkaline Phosphatase, USB, Bad Homburg, Germany
Taq DNA polymerase in storage buffer B, 5 U/µl, Promega, Heidelberg, Germany
Taq DNA polymerase, Eppendorf, Hamburg, Germany
T4 DNA ligase, Fermentas, St. Leon, Germany

2.1.4 Solutions.

Anesthetic solution for mice
1 ml 50 mg/ml Ketanest (Ketavet), 4 ml 0.9% NaCl, 0.25ml 2% Rompun sterile mixed, kept at +4°C in 15 ml Falcon tube

APS (10%) 10% ammonium peroxodisulfate in H_2O, aliquoted, stored at -20°C

Blotting solution 3 M NaCl; 8 mM NaOH, pH 11.40-11.45 in H_2O

Denaturation solution 0.5 M NaOH, 1.5 M NaCl

Denhardts (50×) 5 g Ficoll 400; 5 g Polyvinylpyrrolidon; 5 g BSA (Fraction V) ad 500 ml H_2O

EDTA (0.5 M) 232.6 g ad 1l H_2O, pH 8.0

IPTG 2 g IPTG in 10 ml of H_2O, filtered through a 0.22 µm filter, aliquoted and stored at -20°C

Hybridization solution 6 × SSC; 2 × Denhardts; 0.5 % SDS; 100 µg/ml denaturated salmon sperm DNA

LB-agar LB-medium with 1.5% agar

LB-medium 10 g trypton, 5 g yeast extract, 5 g NaCl, add H_2O to 1 l, adjust pH to 7.5

Lysis buffer for ES cells 10 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% Sarcosyl, 0.4 mg/ml freshly added proteinase K

NaPi 39.0 ml 0.2 M NaH_2PO_4, 61.0 ml 0.2 M Na_2HPO_4; pH 7.0

PBS (1×) 8 g NaCl; 0.2 g KCl; 1 g $Na_2HPO_4 \times 2\ H_2O$; 0.15 g $NaH_2PO_4 \times H_2O$; 0.2 g KH_2PO_4 ad 1 l H_2O, pH 7.2-7,4

Proteinase K	20 mg/ml stock solution in water, keep aliquots at -20°C
Sequencing gel	32 ml SequaGel XR Monomer solution (19:1 Acrilamide to Bisacrilamide); 8 ml SequaGel XR Puffer; 300 µl 10 % APS
SNET	20 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH8.0; 400 mM NaCl; 1% w/v SDS
S.O.C.-medium	2% trypton, 0.5% yeast extract, 10 mM $MgSO_4 \times 7\ H_2O$, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2 \times 6\ H_2O$, 20 mM glucose in H_2O
SSC (20×)	175.4 g NaCl, 88.2 g sodium citrate, adjust pH to 7.2, add H_2O up to 1l, treated with DEPC, sterilized by autoclave
TAE buffer (40×)	1.6 M Tris; 1.33 M Sodium acetate; 0.04 M EDTA in H_2O, pH 8.0
TB medium	12 g trypton, 24 g yeast and 4 ml glycerol dissolve, autoclave and cool to room temperature. 2.3 g KH_2PO_4, 12.5g K_2HPO_4 dissolve in 100 ml water, autoclave and cool to room temperature. Add the phosphate buffer solution to the media solution and mix thoroughly
TBE (5×)	54 g Tris-base, 70 g boracic acid, 3.4 g EDTA, add H_2O up to1 l
TE buffer	10 mM Tris-HCl, pH 7.4, 1 mM EDTA
X-gal stock solution	X-gal 50 mg, add 1 ml N, N-dimethylformamid

2.1.5 Primers:
2.1.5.1 Primers for PCR without modification, MWG-Biotech AG, Ebersberg, Germany

Name	**Sequence from 5' to 3' end**
short-arm-up-claI	ATC GAT ACT AGG ATA AGA ATA AGC CAC CAT GCT TAA C
short-arm-lo-SrfI-ClaI	ATC GAT GCC CGG GCC CTG GCT CCC AAG CGG CTG TCA GCG CCC TGG
imap-long arm-low	GGA TCC GCA GAT GCT CCT GCT GAC GTT TCA CAA
imap-long-arm-up	CTC GAG TTC CGG AGT GTA CAG CCA CCA GGC TTC TG
both-arms-Lo1	GAA TTA ACT GGG TAT CAG TAT TTG GAG TCA GGG
both-arms-Up1	AGC TAG GTA GAT AAA GCC CTC TGC TTG TA
both-arms-Lo2	GCT CGA CAT TGG GTG GAA ACA TTC CAG GCC
UP-deleted	GAA CCC TGG TGT CTC AAT TCA GCA TCA GGT
UP-targeted	CCC TCA CTC AGA GCT TTA CCA ATG CCC TC
UP-wild	ACT CTG GTC TCT GGC AGG TTT CTG AGA GAC
imap-P1-3'	GAC TCT GAG CAC CAG AGC CTG CTT CTG TAA TCC CA
imap-P1-5'	AAT TGA GGG TCG GCG ACA GCA TAG AGT GGG
P2-very-new-up	GAT CCC CTG GAA TTG GAG TTG TGA ATT GAT

Name	Sequence from 5' to 3' end
P2-very-new-lo	CTG TGA TCA GGA GGA TGA TGC GAA ACG ACC
LO-Mouse	TCT ATT GTC AGG AAA GTA TAC TTG GCT CTC T
Lo-tails	AGG AAG AGA GAT GAA AGG AGG GAA GGA CAG T

2.1.5.2 Primers with 5'-IRD800-modification for sequencing, LI-COR, MWG, Ebersberg, Germany

Name	Sequence from 5' to 3' end
M13 Forward	GTA AAA CGA CGG CCA G
M13 Reverse	CAG GAA ACA GCT ATG AC
loxP1	CCT CGT GCT TTA CGG TAT CGC CGC T
loxP2	GCA GAG GGC TTT ATC TAC CTA GCT T

2.1.6 Vectors

pACN (Bunting *et al.*, 1999) kindly provided by F.Edenhoffer, University of Cologne, Germany
pCR®2.1-TOPO®Vector, Invitrogen, Leek, Netherlands.
pEasyFlox (Chui *et al.*, 1997) kindly provided by F.Edenhoffer, University of Cologne, Germany
pGEM®-T Easy Vector System, Promega, Madison WI, USA
pLox2+, New England BioLabs, Frankfurt am Main, Germany

2.1.7 Bacterial strains

Library Efficiency®DH5α™ Competent cells, Invitrogen, Germany
MAX Efficiency®STBL2™ competent cells, Invitrogen, Germany
TOP10F' chemically competent cells, Invitrogen, Leek, Netherlands
XL10-Gold®Ultracompetent Cells, Stratagene, Heidelberg, Germany

2.1.8 Medium and chemicals for ES and EF cell culture

DMEM (no sodium Pyruvate, 4500 mg glucose), DMEM with glutamax (no sodium Pyruvate, 4500 mg glucose, with pyridoxine), Fetal calf serum (FCS) ES cell tested, L-glutamine 100 mM, Non-essential amino acids 100 mM, RPMI without phenol red, Trypsin-EDTA (10×), Sodium pyruvate 100 mM, Gelatine 2% solution, PBS, GANC, GIBCO BRL, Karlsruhe, Germany
β-mercaptoethanol, Merck, Darmstadt, Germany
G418 (Geneticin) sulfate, Sterile-Filtrated Aqueous Solution, Calbiochem-Novabiochem, La Jolla, CA, U.S.A.
LIF is a supernatant from transfected CHO cells line 8/24 70 LIFD from Genetic Institute, University of Cologne
Mineral oil, embryo tested, Sigma, Deisenhofen, Germany
Mitomycin C (MMC) powder, Sigma, Deisenhofen, Germany
Trypsin-EDTA for feeder preparation: 0.05% trypsin, 0.02% EDTA in PBS

2.1.9 Materials for cell culture

1 ml syringes with 25-gauge needles
Cuvette for electroporator, GIBCO BRL
8-place manifold aspirator (Drummont Scientific Co.)
Petri dishes 15 cm, 60 mm for cell culture, multiwell plates, one time pipettes, 170 × 11mm tubes: Greiner, Solingen; Waldeck, Münster; Germany
Petri dishes 10 cm for cell culture, 50, 15 ml tubes, Falcon

2.1.10 Devices

DNA sequencer model 4000, MWG-Biotech AG, Leverkusen, Germany
Electroporator, Capacitance Extender und Gene Pulser, Bio-Rad, München, Germany
Electrophoresis Power Supply – EPS 301, Amersham, Braunschweig; POWER PAC, BioRad, München, Germany
PTC-200™, PTC 100™ Mini cycler™, MJ research, BIOZYM, Hess. Oldendorf, Germany
Scintillation counter Beckman LS 6000 SC, Beckman, Stavanger, Norway
Micromanipulators type CellTramAir, Eppendorf, Hamburg, Germany
Microscope type Axiovert10, Zeiss, Jena, Germany
Microscope type Labovert and micromanipulators, Leitz, Wetzlar, Germany
Injection needles, Eppendorf, Hamburg, Germany

2.2 General methods

2.2.1 Plasmid DNA preparation

Isolation of plasmid DNA from 5 ml of overnight culture was performed using NucleoSpin®plasmid Kit (Macherey-Nagel, Düren, Germany) or Qiagen-Spin pre-Mini - Plasmid isolation kit (Qiagen, Hilden, Germany). For Midi preparations of DNA from 100 ml NUCLEOBOND®AX Midi Kit (Macherey-Nagel, Düren, Germany) or Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany) were used. For Maxi preparation of targeting vector QIAGEN Plasmid Maxi kit (Qiagen, Hilden, Germany) was used.

2.2.2.1 Purification of genomic DNA from embryonic stem cells

Embryonic stem cells were washed twice with PBS, trypsinized and after neutralization of trypsine with medium centrifuged down in 15 ml Falcon tube. Cells were resuspended in SNET containing 40 µg/ml proteinase K. After overnight incubation at 56°C lysate was extracted once with phenol, then with phenol-chlorophorm and washed with chlorophorm. DNA was precipitated with 1/10 volume 3 M NaAc and 2 volumes ethanol, reprecipitated with 10 volumes ethanol, washed with 70% ethanol and dissolved in TE pH 8.0.

2.2.2.2 Purification of genomic DNA from tissue

Genomic DNA from mouse spleen was purified using Qiagen Blood and Cell Culture Genomic DNA Purification Kit (Qiagen, Hilden, Germany), from mouse tails using InvisorbSpin Tissue Mini Kit (Invitek, Berlin-Buch, Germany).

2.2.3 Measurement of DNA concentration

DNA concentration was defined through measurement of absorption at 260 nm, 280 nm and 320 mn. According to Sambrook *et al.* (1989) Absorption at 260 nm correlates with concentration of nucleic acids, at 280 to protein contain, at 320 dispersion. For high molecular nucleic acids 1 unit A_{260} is equivalent to 50 µg/ml. Purity of DNA is defined through quotient A_{260}/A_{280} and must be more than 1.8.

2.2.4 Polymerase chain reaction (PCR)

All PCR-reactions were performed in PTC-200™ gradient cycler, PTC™ 150-16, PTC 100™ or PTC 150-25 MiniCycler (MJ Research, BIOZYM, Hess. Oldendorf). Expand High Fidelity PCR system (Roche, Mannheim, Germany) or Triple Master™ PCR system (Eppendorf, Hamburg, Germany) were used to perform reactions according to instructions of manufacturer. Typical PCR reaction mixture contained 5 µl of Expand High Fidelity 10 × PCR buffer with magnesium chloride or Tuning buffer with magnesium chloride, 0.2 mM dNTP mix, 10 pmol upstream primer, 10 pmol downstream primer, 10 – 400 ng DNA, 0.5 µl Expand High Fidelity enzyme or 0.6 µl of Triple master Polymerase mix, millipore water till 50 µl. Reaction mixture was overlaid with 30 µl mineral oil (Sigma, Deisenhofen, Germany). Reaction was performed in 0.2 thin wall tubes (BIOZYM Diagnostic, Oldendorf, Germany)

2.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook *et al.* 1989.
For resolving of restricted genomic DNA 0.8% agarose gel with 1×TAE was used and low voltage (<1 V/cm) was applied. For other applications gel concentrations of 0.7-2 % in 1×TBE were determined according to fragment sizes to be resolved and 3-5 V/cm was applied. Ethidium bromide was added to gel to a final concentration of 0.5 µg/ml for staining

of DNA. DNA solutions were mixed with 6×Loading buffer (Fermentas, St.Leon, Germany) before loading on the gel. DNA molecular weight markers pUC Mix, λ/HindIII, λ/HindIII+EcoRI, λMix (Fermentas, St.Leon, Germany) served for definition of DNA fragment sizes.

2.2.6 Restriction of DNA from different sources

Restriction endonucleases (Fermentas, St. Leon, New England BioLabs, Frankfurt am Main; Roche, Mannheim; Stratagene, Heidelberg, Germany) were used for digestion of plasmid and genomic DNA under conditions recommended by manufacturer. Concentration of DNA in reaction mixture was always less than 0.2 µg/µl. Before addition of restriction endonucleases into the reaction mixture, genomic DNA was allowed to stay at room temperature in 1× reaction buffer for 2-3 h to obtain homogenous solution of genomic DNA and prevent incomplete or unspecific digestion.

2.2.7 Purification of DNA fragment by agarose gel electrophoresis

Electrophoresis of DNA fragment was performed in 1×TBE agarose gel. DNA fragments were visualized under long wave UV-light and gel slice containing DNA fragment was cut out of the gel. Purification of DNA from agarose was performed using Quiaquick or NucleoSpin®extract (Macherey-Nagel, Düren, Germany) or QIAquick™ Spin Nucleotide Removal Kit (Qiagen, Hilden, Germany) purification kits according to manufacturer instructions. If DNA was used for salt-sensitive application, it was eluted from the column with pre-warmed (50°C) MilliPore water instead of elution buffer. DNA quantity definition was performed by comparison of band intensities between 1, 2 and 3 µl aliquots of purified DNA fragment and band of similar intensity in DNA molecular weight marker after agarose gel electrophoresis.

2.2.8 DNA sequencing and sequence analysis

Sequencing of cloned DNA was performed using the chain termination method (Sanger *et al.* 1977).

„Thermosequenase fluorescent labeled primer cycle sequencing“ Kit with 7-deaza-dGTP (Amersham, Braunschweig, Germany) was used for making sequence reactions. 1.5 µg

plasmid DNA, 2 - 4 pmol 5'- IRD800-labelled primer were used for preparation of master mix. Reaction was performed in PTS 100 thermocycler (MJ Research, BIOZYM, Hess. Oldendorf) as follows 94°C for 2 min, then 35 cycles 95°C for 30 s, 55°C for 30 s, 72°C for 1min. IRD800-labelled DNA fragments were resolved in 40 cm long and 0.25 mm thin gel at 1500 V and 50°C with 1 × TBE. Sequencing using 5'- IRD800-labelled primers was performed using the automatic DNA-sequencer Licor 4000 (MWG, Eberberg, Germany). Infrared stain-labeled DNA fragments were resolved in gels consisting out of 6% modified polyacrilamid-gelmatrix (Ultra Pure SequaGel®XR, National Diagnostics, Atlanta, U.S.A.) or Long Ranger® Gel Solution (CAMBREX, Rockland ME, USA) with 7 M urea in 1 × TBE and detected by Laser-Photomultiplier-Unit. For detection and reading of infrared signals was used software BaseImagIR, Version 2.20, from Licor (MWG, Ebersberg, Germany), for analysis of sequences software package PC/Gene, Version 6.8 (IntelliGenetics, Mountain View, U.S.A.) and software package GCG, Version 7.1, Genetics Computer Group Inc. (Madison, WI, U.S.A.)

Comparisons between sequences were performed using „BLAST2 sequences" software (Tatusova&Madden, 1999) at National Institute of Health home page www.ncbi.nlm.nih.gov/BLAST/. In addition, sequences were analyzed using FASTA-Algorithms (Lipman &Pearson, 1985; Pearson&Lipman, 1988) in EMBL- and SWISSPROT databases.

2.2.9 Cloning of PCR products into T-vectors

Taq-DNA-polymerase and some other DNA-polymerases produce 3'-overhanging A on the blunt end of dsDNA. This allows efficient ligation of PCR products in T-vectors carrying 3'-overhanging T on the linearization site. A number of suppliers offer ready-to-use T-vectors premixed with ligation buffers and enzymes. Ligations into T-vectors were performed according to protocols of supplier. 4 µl (100-600 ng) of PCR product were used for ligation. Ligation time 5-30 min according to the size of insert. In TOP10F' competent cells (Invitrogen, Leek, Netherlands) as recommended by manufacturer. Blue-white test for definition of colonies containing insert was performed for pCR2.1 vector (32 µl of 50 mg/ml X-gal and 40 µl of 100 mM IPTG per 10 cm plate). *E. coli* transformants were analyzed for insert specifity and orientation by PCR of colonies (2.2.11) and restriction analysis of plasmid DNA.

2.2.10 Cloning of DNA fragment into vector.

Sticky-end ligation was usually performed. Vector was cut with appropriate restriction endonucleases and dephosphorilated using shrimp alkaline phosphatase. Fragment of DNA to be cloned was cut out with corresponding restriction endonucleases. Purification and quantity definition of insert and vector were made by electrophoresis (2.2.7). Three ligation mixtures were always made for each vector-insert pair: ligation mixture usually contained 100 ng of vector and insert in molar ratio to vector from 1:2 till 1:10 in different tubes, 1µl T4-ligase buffer with ATP and 1 µl T4-DNA ligase (Fermentas, St. Leon, Netherlands) in total volume of 10 µl. Reaction was mixed according to manufacturer instructions and incubated overnight at +7° C. 5 µl of ligation mixture were used for transformation into competent *E.coli*. No heat inactivation prior to transformation was performed. Next day after transformation, *E.coli* colonies were analyzed for insert specifity and orientation by restriction analysis or PCR amplification of transformants (2.2.11).

2.2.11 Direct identification of positive transformants using PCR

PCR cocktail containing 5 µl of 10×Taq-polymerase buffer (Promega, Madison WI, USA), 3 µl 25 mM $MgCl_2$, 1 µl dNTP mix (Roche, Mannheim, Germany), 10 pmol downstream primer outside insert, 10 pmol upstream primer inside/another side of insert, water ad 50 µl was prepared. 10 colonies were picked and resuspended individually in 20 µl of PCR cocktail. A patch plate was made to preserve the clones by overnight culture. PCR mixtures are overlaid with mineral oil and incubated first for 10 min at 94°C to inactivate nucleases and destroy the cells. Then 30 cycles were performed as follows: 94°C for 20 s, 55°C for 30 s, and 72°C for 1-3 min depending on insert size, final extension at 72°C for 10 min. PCR products were visualized in 1% agarose gel with 1×TBE.

2.3 Generation of targeting construct (designated as pTV)

2.3.1 Cloning of short homology arm

The short homology arm was amplified using primers containing recognition sites for restriction endonuclease SrfI site for linearization of Targeting vector and ClaI: *short-arm-up-ClaI* 5' -ATC GAT ACT AGG ATA AGA ATA AGC CAC CAT GCT TAA C- 3' and

downstream *short-arm-lo-SrfI-ClaI* 5'- ATC GAT GCC CGG GCC CTG GCT CCC AAG CGG CTG TCA GCG CCC TGG -3'. The reaction mixture contained 200 ng cosmid clone MPGc121K12460Q2 DNA and was performed using Expand™High Fidelity system (Roche, Mannheim, Germany) (2.2.4). Amplification was performed as follows: 94°C for 2 min, hot start, then 35 cycles as follows 94°C for 40s, 50°C for 45s, 72°C for 1 min 30 s, final elongation 15 min at 72°C. PCR products were purified by electrophoresis (2.2.7) and cloned into pCR®2.1-TOPO®Vector (2.2.9). Inserts were confirmed by restriction analysis. The short homology arm was then cut out with ClaI and cloned into ClaI restriction site of pEasyFlox (2.2.10). The resulting vector was designated pEasyFlox/ShortArm.

2.3.2 Cloning of long homology arm

In the plasmid pEasyFlox/ShortArm the neomycin resistance gene was replaced with the long homology arm at BamHI and XhoI sites resulting in generation of pEasyFlox/(Short+Long arms) vector. Long homology arm was amplified using primers: *imap-long-arm-up* 5'- CTC GAG TTC CGG AGT GTA CAG CCA CCA GGC TTC TG -3' containing an XhoI recognition site and *imap-long arm-low* 5'- GGA TCC GCA GAT GCT CCT GCT GAC GTT TCA CAA -3' containing BamHI recognition site. PCR reaction contained 100 ng female C57BL/10 mouse genomic DNA and was performed using Expand™ High Fidelity PCR system (Roche, Mannheim, Germany) (2.2.4). Amplification program: initial denaturation 94°C for 2 min, then 5 cycles 94°C for 20s, 56°C for 30s, 68°C for 4 min 30s, then 25 cycles 94°C for 20s, 60°C for 30s, 68°C for 4 min 30 s and final elongation step 72°C for 8 min. The PCR product was cloned into pCR 2.1 TOPO®TA cloning vector (2.2.9), insert was identified by restriction analysis. Long Arm was cloned into pEasyFlox/ShortArm between BamHI and XhoI sites (2.2.10). Presence and direction of long arm was determined by amplifying of DNA directly from bacterial colonies (2.2.11) using primers *both-arms-Lo1* 5'-GAA TTA ACT GGG TAT CAG TAT TTG GAG TCA GGG-3' and *both arms-up1* 5'- AGC TAG GTA GAT AAA GCC CTC TGC TTG TA -3'.

2.3.3 Cloning of ACN cassette

ACN self-excision cassette was cut out of pACN with XhoI, purified by electrophoresis (2.2.7). ACN cassette with sticky ends to be cloned was first filled-in with Taq-DNA polymerase in reaction volume 100 µl (0.2 mM dNTP, 1 µl Taq-DNA polymerase, 10 µl 10

×PCR buffer (Promega, Madison WI, USA), 1.5 mM $MgCl_2$ (Promega, Madison WI, USA) at 72°C for 30 min without initial denaturation step. Filled-in fragment was purified from reaction mixture using NucleoSpin®extract (Macherey-Nagel, Düren, Germany) purification kit. Ligation mixture contained 5µl of 2×Rapid ligation buffer, 1 µl pGEM®-T EasyVector, 4 µl (200-600 ng) of purified insert, 1 µl T4 DNA ligase (Fermentas, St. Leon, Germany). Ligation was performed overnight at +7°C, then 4 µl of reaction mixture were used for transformation of TOP10F' competent cells. ACN cassette was cut out from pGEM T-Easy/ACN and ligated into NotI site of pEasyFlox/(Short+Long arm) vector. Ligation mixture contained 100 ng of vector, insert in molar ratio to vector from 1:5, 2,5 µl T4-ligase buffer with ATP and 1 µl T4-DNA ligase in a volume of 25 µl and incubated at 23°C for 1h, then at 16°C for 1,5 h. For transformation 10µl and 5 µl of reaction mixture were used. MAX Efficiency®STBL2™ competent cells were used according to manufacturer instructions. Constructs were confirmed by restriction analysis and PCR (2.2.11). Expand High Fidelity PCR (Roche, Mannheim, Germany) system was used with primers *UP-targeted* 5'- CCC TCA CTC AGA GCT TTA CCA ATG CCC TC -3' complementary to ACN cassette and *LO-mouse* 5'-TCT ATT GTC AGG AAA GTA TAC TTG GCT CTC T -3' complementary to short homology arm. Amplification program: 94°C for 2 min, 30 cycles as 94°C for 20s, 56°C for 30s, 72°C for 4 min 20 s, final elongation at 72°C for 12 min. Visualization of products was performed in 1% agarose gel 1×TBE. Ready targeting construct was designated as pTV.

2.3.4 Testing of targeting construct

LoxP sites sequences of pTV were confirmed by sequencing using 5'-IRD800-modified primers: loxP1 5'- CCT CGT GCT TTA CGG TAT CGC CGC T -3' and loxP2 5'- GCA GAG GGC TTT ATC TAC CTA GCT T -3' (2.2.8) and by in vitro recombination test using Cre-recombinase (2.3.5).

2.3.5 Cre-mediated recombination in vitro

Reaction mixture for Cre-mediated recombination contained 126 µg of pTV linearized with SmaI, 5 µl 10×buffer for Cre-recombinase and 0, 1, 2, 5 or 10 U of Cre recombinase in total volume of 50 µl. Control reaction contained 250 ng of pLox2+ linearized vector in 1 × Cre-recombinase buffer and 0 or 2 U of Cre-recombinase. After 4 h incubation at 37°C reaction

mixtures were heated at 70°C for 10 min to inactivate Cre-recombinase. Excision of ACN cassette was visualized by electrophoresis in 1 % agarose gel with 1×TBE at 5 V/cm (2.2.5).

2. 4 ES cell culture

2.4.1 ES and EF cells

ES cells of line Bruce4, derived from C57BL/6 male mouse embryo were kindly provided by the Laboratory of Molecular Genetics, University of Cologne, Germany.
EF cells were recovered from embryonic fibroblasts of mice embryos containing neomycin resistance gene (see 2.4.5).

2.4.2 ES medium

500 ml DMEM (without sodium pyruvate, 4500 mg glucose, with pyridoxine), 90 ml (final 15%) of ES pretested and heat-inactivated (56°C for 1 h) FCS, 6 ml 100 mM Sodium Pyruvate, 6 ml 100 mM L-glutamine, 6 ml 100 mM non-essential amino acids, 1.2 ml LIF, 0.6 ml 0.1 M β-mercaptoethanol. LIF (leukaemia inhibitory factor) is supernatant from transfected CHO cells line 8/24 70 LIFD(A) from Genetics Institute of Cologne. For selection of transfected cells 0.6 ml of G418 (71% activity) was added to 600 ml ES medium.

2.4.3 EF medium

500 ml DMEM with glutamax (without sodium Pyruvate, 4500 mg glucose, with pyridoxine), 60ml FCS, 6ml non-essential amino acids, 6 ml 100mM sodium pyruvate.

2.4.4 Freezing medium

2 ×freezing medium: 20% DMSO, 80% FCS.
1 × freezing medium: 10% DMSO, 90% FCS.

2.4.5 Preparing mouse embryo fibroblasts

Mouse embryos (possessing neomycin resistance gene) 12.5-14.5 days post coitus were dissected and placed in a 10 cm plastic petri dish containing DMEM without serum. Limbs were removed and internal organs were scooped out. The upper part of the head containing

brain was removed. The carcasses were washed three times by transferring in new petri dish containing sterile medium. Then embryos were placed into a 10 cm petri dish, the rest of medium was removed. Embryos were minced with sterile scissors until they were the consistency of sludge. The minced embryos were placed into a 100 ml glass retort containing approximately 20 ml trypsine-EDTA for feeder preparation, 200 µl DNase I (10 mg/ml stock), sterile stripping bar and 5 ml glass beads. Retort was placed in rack on a stirring plate and incubated at 37°C for 30 min. Additional 10 ml trypsin-EDTA for feeder preparation were added and suspension was stirred for another 30 min. Procedure was repeated one more time. Cell suspension was decanted into a new 50 ml tube containing 4 ml of FCS to stop trypsine activity. Retort with glass beads was washed twice with 5 ml DMEM with 10% FCS and added to the 50 ml tube. If the cell pellet was viscous, 200 µl DNase I (10mg/ml) were added and cell suspension was incubated for 30 min at 37°C. Cell pellet was centrifuged at 1200 rpm for 5 min. Pellet was resuspended in 10 ml DMEM with 10% FCS. Viable cells were counted. Yield is about 5×10^7 to 10^8 viable cells from ten embryos. Cells were plated 5×10^6 cells per 150 mm tissue culture dish in 25 ml DMEM with 10% FCS and cultured overnight at 37°C (5% CO_2 in air). Next day medium was changed to remove a debris. After two to three days cells formed a confluent monolayer. Each plate was trypsinized and re-plated into five 150 mm plates. When plates were confluent, 1 stock from each 15 cm plate was made (2.4.9). This stocks are named EF0, meaning passage 0.

2.4.6 EF cell culture

Embryonic Feeder cells were used as a supporting layer for ES cell colonies. EF cells were maintained in EF medium. Medium was changed every day. To expand EF cells EF0 stock (2.4.5) was thawed on the 15 cm plate. Each passage or freezing-thawing cycle makes EF cells one level older. The culture was split 2 times in ratio 1:3 on the 15 cm plates till EF2 to obtain maximal cell number. Then EF2 cells were either split one more time (till EF3) and MMC treated to use immediately or EF2 stocks were made (2.4.9) and kept in liquid nitrogen until use. EF2 stocks were thawed (they become EF3) 3 days before use, allowed to reach confluence and treated with MMC (2.4.7). EF3 cells should not be split or frozen any more. They must be treated with MMC to use for ES cells or discarded. EF4 cells can loose their ability to adhere to the plate and all ES cell colonies growing on the full confluence monolayer of MMC-treated EF cells will be lost.

2.4.7 MMC treatment of EF cells

To arrest growth of EF cells mitomycine C (MMC, Sigma, Deisenhofen, Germany) destroying microtubules of mitotic spindle was used. Alternatively high dose of γ-irradiation can be applied. 2 mg MMC were resuspended in 2 ml PBS and stored at +4°C. Medium on the EF3 cells was replaced with EF medium containing 100 µl of MMC suspension per 10 ml medium. Cells were incubated in CO_2 incubator for 2-4 h. Then cells were trypsinized and split onto gelatine coated plates or medium was replaced with EF medium and the cells were split next day. Alternatively EF3 were treated with MMC without splitting. In this case EF2 stock was thawed on the gelatinized plates. After full confluence was reached, cells were treated with MMC for 2 - 4 h. Medium containing MMC was removed and cells were washed twice with PBS, once with EF medium before plating of ES cells on them. MMC-treated cells older then 7 days were avoided to use. Older MMC-treated or EF4-derived cells can spontaneously detach from the plate and all ES cell colonies growing on the EF monolayer will be lost. Number of MMC treated EF cells in different plates which is necessary to make full confluent is shown in Table 1.

Table1

Costar plates	**Surface area (cm^2)**	**Total surface area (cm^2)**	**EF feeder cells per plate**
150 mm dish	148		9×10^6
100 mm dish	55		3×10^6
60 mm dish	21		1.2×10^6
35 mm dish	8		0.45×10^6
6 well plate	9.5	57	3×10^6
12 well plate	3.8	45.6	2.5×10^6
24 well plate	1.9	45.6	2.5×10^6
48 well plate	0.8	38.4	2.1×10^6
96 well plate	0.32	30.72	1.7×10^6

2.4.8 ES cell culture

C57BL/6 – derived embryonic stem (ES) cell line Bruce4 was maintained in ES medium on the full confluent monolayer of MMC-treated EF cells. Alternatively cells can grow on the

gelatinized plates (i.e. for DNA preparation) but in this case they tend more to differentiate. Medium was changed daily to prevent differentiation. Exception is the first day after thawing when medium was not changed, to enhance cell survival. Cells were fed before transfection or injection manipulations. Cells were split 1:3 (~2×10^6 per 10 cm plate) at every 3 days. Number of cells must never exceed ~1.5×10^7 per 10 cm plate. 1×Trypsine-EDTA in PBS was used for splitting and single cell suspension preparation of ES cells.

2.4.9 Preparation of ES, EF cells stocks.

Sub confluent ES cells or full confluent EF cells were washed twice with PBS, 1×Trypsin-EDTA solution in PBS was added to the cells. After less then 5 min in CO_2 incubator at 37°C the equal volume of medium was added to the plate to neutralize trypsin solution. Single cell suspension was prepared by vigorous pipetting up and down. Then cells were transferred into 50 ml Falcon tube, centrifuged at 1200 rpm for 10 min and resuspended in 1×Freezing medium. From each plate 3 stocks were prepared to thaw later one stock on one plate of the same size. Stocks were frozen gradually in Styrofoam box first at -20°C for 2h, then at -80°C for 24-48 h and finally stored in liquid nitrogen.

2.4.10 Transfection of ES cells

Before transfection the targeting vector pTV was linearized with SrfI (Stratagene, Heidelberg, Germany) restriction endonuclease to improve efficiency of homologous recombination. 100µg of Targeting vector in a total volume 600 µl were digested overnight. After restriction DNA was extracted from reaction mixture with Roti®-Phenol (Roth, Karlsruhe, Germany), then phenol chloroform (1:1) (Roth, Karlsruhe, Germany) and washed once with chlorophorm. DNA was precipitated with 2 volumes ethanol, pelleted by centrifuging, washed with 70% ethanol and kept under 70% ethanol until a day of transfection. On the day of transfection DNA was pelleted, washed with 70% ethanol, air dried under sterile hood and dissolved in RPMI without phenol red (GIBCO BRL, Karlsruhe, Germany) till final concentration 0.1 µg/µl. 3 h before transfection ES cells were fed with fresh ES medium. ES cells were trypsinized and resuspended in RPMI without phenol red at 2.5×10^7 cells/ml. Two transfections were performed in parallel. For one transfection 400 µl of DNA solution were combined with 400 µl of cell suspension in electroporation cuvette, gently taped to mix and incubated for 5 min at room temperature. Then cuvette was placed into electroporation

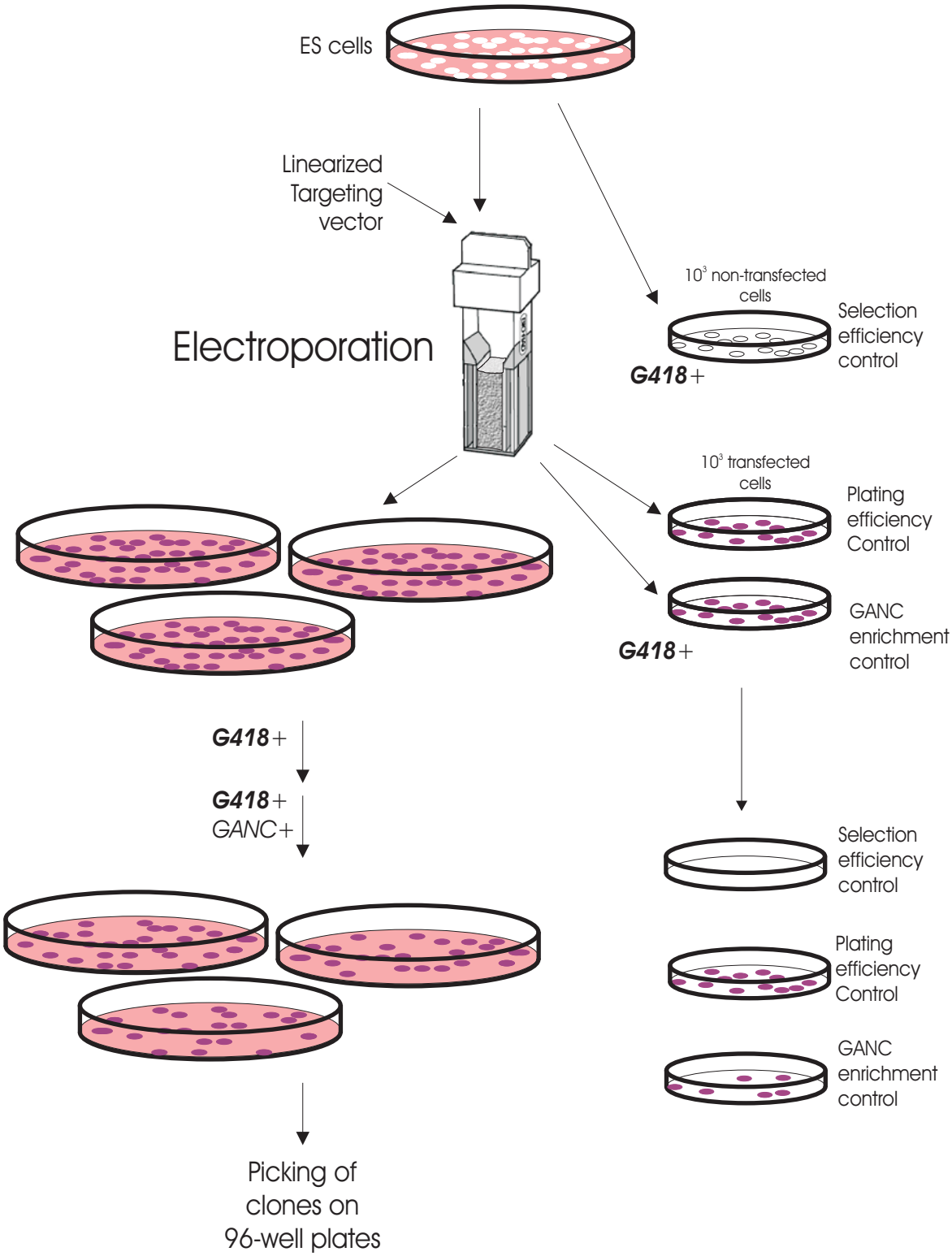


Fig. 1. Electroporation of ES cells. Pink designates feeder layer on the plates. Non-transfected ES cells are shown as white colonies, transfected shown as purple colonies. Transfected plates and control plates are shown before and after selection.

chamber and a single pulse at 230 V, 500 μF was applied. After 5 min incubation at room temperature transfected ES cells were resuspended in 30 ml ES medium. From this suspension controls were made and the rest of cells was plated onto three 10 cm plates

containing EF cells washed with ES medium. Next day 71% relative activity G418 was diluted in fresh ES medium 1:1000 and applied to the transfected cells. On the fifth day negative selection was began. GANC was stored as 0.2 M solution at -20°C, thawed directly before preparation of medium and added to G418-containing ES medium ad 2×10^{-6} M GANC. Each day for changing of medium fresh GANC-containing medium was prepared. After 3 days of GANC selection resistant colonies were picked. The following controls were performed for each transfection (Fig1): a) non-transfected 10^3 cells on 6 cm gelatinized plate were maintained with G418 to control selection specificity, b) 10^3 transfected cells on 6 cm gelatinized plate were maintained without selection agents to control plating efficiency, c) 10^3 transfected cells on the 6 cm gelatinized plate were selected only with G418 and on the day 5 counted to control GANC enrichment.

2.4.11 Picking of ES cell colonies in 96-well plates

For all the manipulations with 96-well plates multichannel pipettor (Eppendorf, Hamburg, Germany) and disposable tips were used. U-shaped 96-well plate with 25 µl of trypsin-EDTA per well was prepared. Plate with resistant colonies were washed twice with PBS and filled with 10 ml PBS. Using a stereo microscope, a 20 µl micropipettor and a sterile disposable tips individual colonies were picked in a small volume of PBS (5-10 µl) and transferred to individual wells of the trypsin-EDTA containing 96-well plate. Picking can not continue longer then 30 min after the first ES cell colony was put in trypsin. After 30 min picking or if plate is full 96-well plate was loaded for 3-5 min into incubator, then 100 µl of ES medium was added in each well. ES cell colonies were dissociated by vigorously pipetting up and down using multichannel pipettor and disposable tips. Some wells were controlled under the microscope to be really single cell suspension. Cell suspension from each well was transferred into the respective well of the flat bottomed 96-well plate with MMC-treated feeder cells and 100 µl/well of fresh ES medium with G418. ES cells in 10 cm plates were not allowed to stay in PBS longer as 1h as it can result in cell death or differentiation. After ~40 min of picking PBS was removed and the other colonies were picked next two days.

2.4.12 Freezing ES clones in 96-well plates

ES cells are allowed to grow to sub-confluence in feeder cell containing 96-well plates. 2-4 h before freezing they were fed with fresh ES medium. Medium from ES cells was aspirated

using multichannel 8-place manifold aspirator (Drummont Scientific Co.) and cells were washed twice with 100 µl PBS and 50 µl of 1 × Trypsin-EDTA solution were added in each well. Cells were remaining in incubator for 3-5 min. Then 50 µl of 2 × freezing medium was added in each well. All the manipulations from this point were performed very fast, because long incubation in DMSO at room temperature can lead to differentiation or damaging of the ES cells and must be avoided. ES colonies were dissociated in medium by vigorous pipetting up and down and overlaid with 100 µl of sterile mineral oil (embryo tested, Sigma, Deisenhofen, Germany) to prevent evaporation during storage at -70°C. 96-well plate was sealed with parafilm, placed in Styrofoam box and stored at – 70°C until analysis of DNA was completed. Plates older then 2 months give no rise for cells suitable for injection into mouse embryo.

2.4.13 Purification and restriction of ES cell genomic DNA in 96-well plates

ES cells on the gelatinized 96-well plates were allowed to reach full confluent. They were washed twice with PBS and either proceeded directly or PBS was aspirated and plates were frozen at -20°C. To each well were added 50µl ES-lysis buffer with proteinase K. Plate was transferred into a pre-warmed humidified chamber and incubated overnight at 56°C. Then box was cooled at room temperature for 1h and 100 µl of 100% ethanol were added in each well and cell lysate was allowed to precipitate at room temperature for 2 h. Wells were checked visually to contain a filamentous pellet of DNA. After this plate was inverted gently on the paper towels to discard ethanol. In the same way DNA precipitate was washed twice with 200 µl of 70% ethanol. The plate was inverted with bottom up and DNA was air dried for 10 min. Then 40 µl of restriction mix (1× Restriction buffer, 1mM spermidine, 1 mM DTT, 100 µg/ml BSA, 50 µg/ml RNAse A, 70-100 U of highly-concentrated restriction enzyme per reaction) were added to each well. 96-well plates with digestion mixes were incubated overnight at appropriate temperature in humidified chamber. Next day digestion mixtures were loaded on the agarose gel for southern blot analysis.

2.4.14 Expansion of ES clones (Fig 2)

Cells were allowed to grow for 3 days after thawing from 96-well plate. Then they were split 1:3. For this, each well was washed twice with 100 µl PBS, then 50 µl trypsin-EDTA solution were added to the cells and the cells were incubated for 3-5 min in the incubator. 100

µl of ES medium was added into each well, colonies were dissociated by vigorous pipetting and content was split in three feeder cell containing 96-well plates. From these plates two were frozen at -70°C and one after 3 days was split in 3 gelatinized flat bottomed 96-well plates for DNA preparation. After analysis of DNA candidate clones containing targeted allele were thawed in 96-well plates and transferred into 12-well plates. After 3 days in

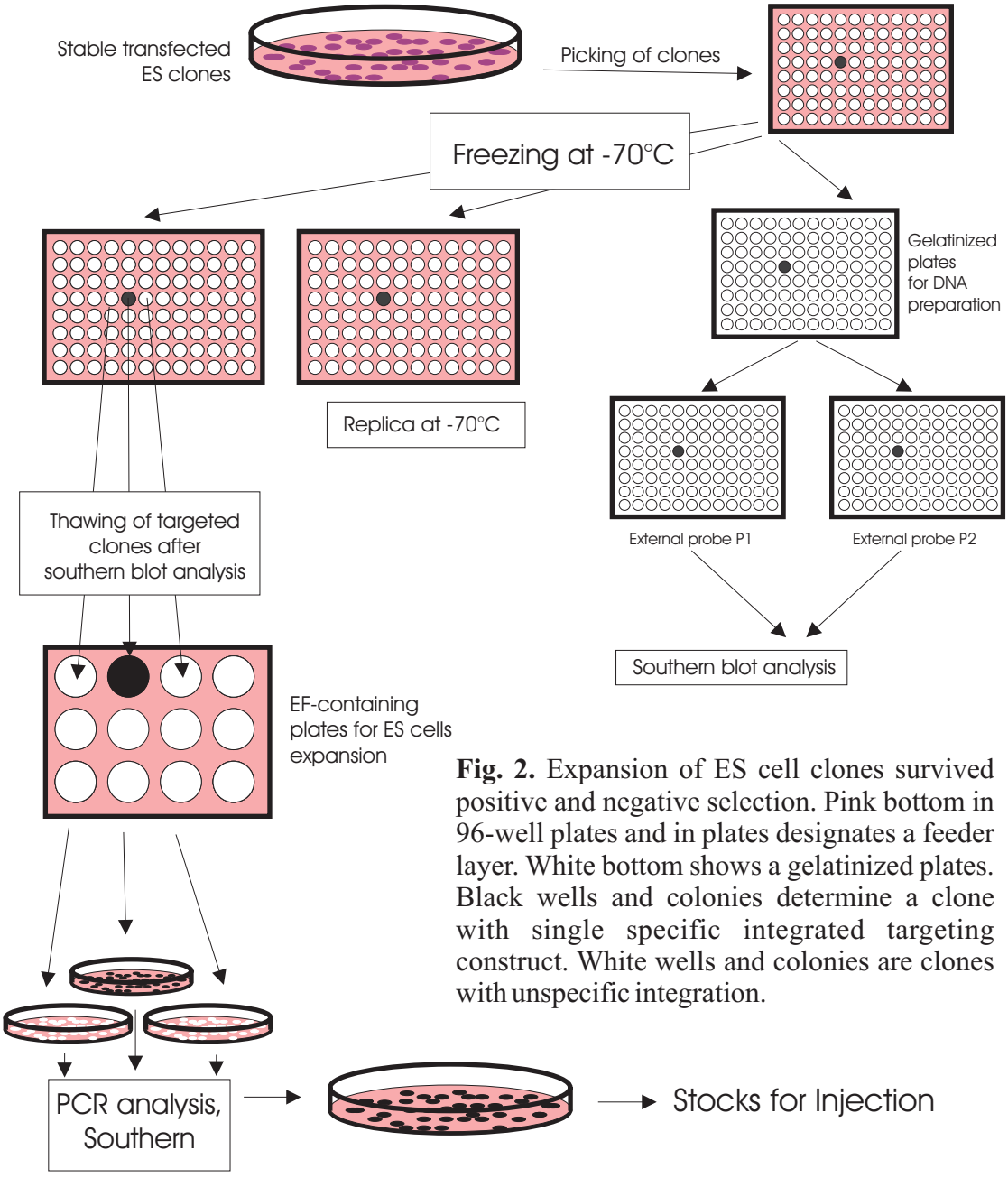


Fig. 2. Expansion of ES cell clones survived positive and negative selection. Pink bottom in 96-well plates and in plates designates a feeder layer. White bottom shows a gelatinized plates. Black wells and colonies determine a clone with single specific integrated targeting construct. White wells and colonies are clones with unspecific integration.

culture they were trypsinized and split into 6cm plates, ½ of the cells was used for DNA purification (2.2.2.1) and PCR analysis (2.6.5) to confirm, that right clones were taken from 96-well plate. After 3 days cells from 6 cm plates were trypsinized and transferred in 10 cm plates. From this 10 cm plates stocks for injection were made.

2.4.15 Thawing ES clones from 96-well plates

Warmed up to 40°C sterile water was pored in a clean tray in the laminar flow hood. Frozen 96-well plate was removed from -70°C, transported fast on dry ice, parafilm was removed and plate was placed on the surface of the water. Care was taken not to allow water to enter into the wells. After content of wells was thawed, each selected clone was transferred into 15 ml tube containing 2 ml of ES medium. In addition, each well was washed with 200 µl of ES medium to make sure that all the cells are transferred in the 15 ml tube. ES cells were spun down at 250g for 5 min, supernatant was discarded, cells were resuspended in 1 ml of ES medium and transferred into individual wells of a 12-well plate containing EF cells.

2.5 Production of germ-line chimeras by injection of ES cells into mouse blastocysts

2.5.1 Medium for embryo recovery and injection

All stocks for M2, M16, injection medium and BSA keep at -20°C for not longer as 4 months.

Salt mix: 1.78g KCl, 0.81g KH_2PO_4 1.46g $MgSO_4 \times 7H_2O$, 5.00g Glucose, 0.31g Penicillin, 0.25g Streptomycin were mixed in a 50ml falcon tube, ddH_2O was filled ad 25 ml. 21.75g of sodium lactate or aliquot 60% syrup (= 16.45ml) were solved in ddH_2O ad 50 ml in another 50 ml falcon tube. The salt-mix was added to the sodium lactate. Water was added until a total volume of 50ml.

Stock A (10 ×): 2.77g NaCl was weighted out in a 50ml Falcon-Tubes and solved each in 45ml ddH_2O add 5ml of the Salt Mix to each tube with 45ml NaCl-solution. The solutions of these 10 falcon tubes were united to one 750ml tissue culture flask and filter sterilized. 12ml aliquots were made.

Stock B for M2 (10×): 0.85 g $NaHCO_3$ were weighted out in a 50 ml Falcon tube and solved in 50 ml ddH_2O. 10ml aliquots were put into five 50 ml Falcon tubes and 40 ml ddH_2O were added to each of these five tubes. The solutions of these tubes were united to one 250 ml tissue culture flask and filter sterilized. 11 ml aliquots were made.

Stock B for M16 (10×): Weight up 1.05 g $NaHCO_3$ and solve in 50 ml, filter sterilize, make 1.25 ml aliquots.

Stock C(10×): to 1.68 ml of 100 mM sodium pyruvate were added 48.32 ml water in five 50 ml Falcon tubes. Solutions from 5 tubes were united in one culture flask and filtered through 0.22µm Millipore filter, 12 ml aliquots were made.

Stock D (10×)	0.65g $CaCl_2 \times 2H_2O$ were dissolved in 50 ml water and aliquoted in five 50 ml Falcon tubes. 40 ml water was added to each tube. Then solutions were united all in 250 ml culture flask and filtered through 0.22µm Millipore filter, 12 ml aliquots were made.
Stock E (10×)	0.05 g phenol red were weighted out in 15 ml Falcon tubes and solved in 10 ml water. 0.5 ml of this solution was added to five 50 ml Falcon tubes and made up ad 50 ml with ddH_2O, united all to one 250 ml tissue culture flask and filter sterilized. 12 ml aliquots were made.
Stock F for M2 (10×)	8.76 ml 1 M Hepes buffer was measured out in five 50 ml Falcon tubes. 41.24 ml ddH_2O was added to each tube. Solutions were united all to one 250 ml tissue culture flask and filter sterilized, 12 ml aliquots were made.
BSA for M2 (10×)	5.0g BSA was weighted out in five 50 ml Falcon tubes and solved in 50 ml ddH_2O. To dissolve were left at 37°C for up to 1-6 h, shaking should be avoided. Care was taken that no clumps remained before continue. All solutions were united in one 250 ml tissue culture flask and filter sterilized. To 2 ml of this solution 18 ml ddH_2O was added in new 50 ml tubes.
BSA for M16 (10×)	1.5g of BSA were weighted out in one 50 ml Falcon tube, solved in 50 ml ddH_2O. To dissolve were left at 37°C for up to 1-6 h, shaking should be avoided. Care was taken that no clumps remained before continue. 13.3ml aliquots of the solution were put to each of three 50 ml tubes, 36.7 ml ddH_2O was added to each tube. All solutions were united in one 250 ml tissue culture flask and filter sterilized. Aliquots 5 ml were made in 15 ml Falcon tubes.
M2 medium	The following components were combined in tube with BSA for M2: 5 ml stock A, 5 ml stock B for M2, 1 ml stock C, 1 ml stock D, 1 ml stock E. pH was checked and readjusted if necessary to 7.2-7.4 (until the salmon pink color) with 1 M NaOH. Medium was filter sterilized and aliquoted into 15 ml polypropylene tubes. Medium is stored at +4°C for up to 14 days.
M16 medium	The following components were combined in the tube with BSA for M16:1 ml stock A, 1 ml stock B for M16, 1 ml stock C, 1 ml stock D, 1 ml stock E. Solution is filter sterilized and aliquoted in Eppendorf tubes. Medium can be stored at +4°C for up to 14 days.
Injection medium	50 ml M2 + 0.0075 g DNAse (Sigma, Deisenhofen, Germany). Medium was filter sterilized and kept in 6 ml aliquots at -20°C up to 1 month.
KSOM	Specialty Media, Phillipsburg, USA

2.5.2 Mice strains

Mice of inbred strains C57BL/6, 129sv, CB20, C^{2J} and non-inbred NMRI were used in experiments. They were housed in plastic cages, and a standard diet for C57BL/6, CB20 (Nohrlin, Bad Salzufen, Germany) or Global diet (Harlan, Borchen, Germany) for strains NMRI, 129sv, C57BL/6 and water were available *ad libitum.* Mice were bred under specific pathogen-free conditions in animal facilities. The experiments were approved by the state authorities and followed German law on animal protection.

2.5.3 Super ovulation

10 U of pregnant mare serum gonagotropin (PMSG) was injected intraperitoneally to NMRI and 129sv mice at 14.00 h on the day one and 2-10 IU of human chorionic gonadotropin (hCG) at 12.00 on day three. On the day three females were put together with fertile stud males. This mice were used for morula recovery (2.5 day post coitus). On the 3.5 day the mice were used for blastocysts recovery.

2.5.4 Setting up matings

To obtain embryos 3 different mice systems were used in experiments: CB20, NMRI and 129sv × C57BL/6. On day 0 maitings between fertile males and females were set up. In NMRI and 129sv mice super ovulation was induced before matings. Next day after mating, day 1, plug positive mice were set together in a different cage and were used later for collection of embryos. Additional matings between females and vasectomized males were set up for the production of pseudo pregnant females. For this NMRI mice or C57BL/6×Balb/c females were used. On day 2 females mated with sterile males were checked for plugs and those which were mated were placed in a separate cage. They were used on the day 3 at 2,5 days of pregnancy as recipients for the injected blastocysts.

2.5.5 Recovery of embryos for injection

Two different methods were applied to obtain mouse blastocysts. For NMRI and 129sv ×C57BL/6 morulas were washed out from oviduct on the 2.5 day. Morulas were allowed to develop in blastocysts during overnight incubation in M16 medium at 37°C, 5% CO_2 in

humidified atmosphere. This blastocysts were taken next noon for injection. For CB20 blastocysts were obtained from the uterus of mice on the 3.5 day. To wash the embryos from the mice reproductive tract mice on the third (for morulas recovery) or on the forth (for blastocysts recovery) day of pregnancy were killed. Reproductive tract was isolated from mice, spread in the sterile 6 cm Petri dish and cut – for morula recovery at the upper part of uterus, for blastocysts recovery at the utero-tubal junction and across the cervix to expose entrances to both uterine horns. Using a syringe needle the parts of reproductive tract were flushed several times with approximately 0.5 ml of KSOM medium by inserting needle into the cervical end of oviduct/uterus opening. Embryos were washed 3 times by transferring into fresh drops of M2 medium in sterile Petri dish using mouth controlled transfer pipette. Washing of embryos and control of embryos quality was performed under the stereomicroscope.

2.5.6 ES cell preparation for blastocysts injection

Before injection cells were checked for mycoplasma contamination (2.6.4). Stock of ES cells was thawed onto 10 cm plate containing feeder cells. Sub-confluent cells were split in three 10 cm plates containing EF cells as follows: 2×10^6 cells, 1×10^6 cells, 5×10^5 cells per 10 cm plate. On the day of injection ES cells were fed with fresh ES medium one hour before manipulations with cells. Then ES cells were trypsinized and resuspended in plate using a 5 ml one-time pipette to obtain single cell suspension. Cells were spun down at 1200 rpm for 10 min in 50 ml falcon tube, resuspended in 10 ml ES medium and plated on the 10 cm gelatinized plate. After 35 min in CO_2 incubator at 37°C with 10% CO_2 the feeder cells are already strongly attached to the gelatine plate, but the ES cells are either loosely attached on the plate or floating on the supernatant. The medium containing poorer performing non-adhering ES cells was gathered in 15 ml tube N1. Then 10 ml of medium were added to the plate and loosely attached ES cells were re-suspended by vigorously pipetting up and down. This suspension of ES cells was gathered in 15 ml tube N2. Cells were spun down at 1200 rpm for 5 min and resuspended in 400 µl of Injection medium. Cells were placed on ice for 1 h and then used for injection. Fraction 2 was usually contains viable and quite homogenous in size and shape ES cells and was used for injection. While in the fraction1 (depending on the quality of ES cells cultures and the trypsinization procedure) some cells can be dead or dying.

2.5.7 Injection of ES cells into mouse embryos

For injection of CB20 blastocysts phase, bright field, or differential interference contrast microscope with micromanipulators was used. Home-made glass blastocyst-holder needles and commercial or home-made glass injection needles were applied. ES cells were prepared in the morning of injection day and kept on the ice in 15 ml tube at least for 1 h before injection and till the end of procedure. Using a drown-out, mouth controlled Pasteur pipette under the manipulation microscope directly before injection ES cells and blastocysts were washed 4 times by transferring from one drop of M16 medium into another in the 6 cm Petri dish. Then blastocysts and a single cell suspension of ES cells are introduced into drops of medium covered with embryo tested light oil (Sigma, Deisenhofen, Germany) in the manipulation chamber. Using micromanipulators 5-7 ES cells for NMRI, C^{2J} and 129sv × C57BL/6 blastocysts and 15 ES cells for CB20 blastocysts were pipetted up into injection needle. ES cells taken for injection must be small, round-shaped. Then blastocysts were picked with a holding pipette by soaking and positioned so that the inner cell mass of blastocyst can be seen in profile against the holding needle opening. Injection needle was introduced through the ectoderm wall of blastocyst and ES cells were expelled into the blastocoelic cavity. Finally pipette was removed. Morulas of C^{2J} strain which were not developed in blastocysts after overnight incubation in KSOM medium were also injected with ES cells. Morulas were fixed with the holding needle and 4 ES cells were injected under the zona pellucida of morula. When all the embryos in the injection chamber were injected with ES cells, they were transferred in 6 cm Petri dish with drops of M16 culture medium under heavy liquid parafilm. For 1-3 h embryos were kept in an incubator at 37°C with 5% CO_2 to allow cells to rearrange inside of embryo. Then embryos were transferred into pseudo pregnant hosts.

2.5.8 Embryo transfer

Pseudo pregnant females NMRI or C57BL/6 × Blab/c obtained from matings with vasectomized males were used for transfer of injected embryos on the 2.5-3 day of pseudo pregnancy. Mice were anaesthetized with 10 µl/g of anesthetic solution. Window of complete anesthesia is small and all the manipulations must be performed fast. The back of mouse was cleaned with ethanol. 1cm long transverse incision in the skin at the level of the first lumbar vertebra was made. The skin incision was slid laterally until the right ovarian fat pad and

ovary were visible through peritoneal wall. Then 3 mm incision in the peritoneum was made and the fat pad, ovary and oviduct were grasped with a blunt forceps and pulled out through the opening in the direction midline. The ovary was stabilized outside of peritoneum by clamping with a small sepaphim clip. Embryos were washed 3 times with M16 medium then soaked into mouth-controlled transfer pipette. To mark transfer of embryos air bubbles were used. Namely during soaking of embryos into transfer pipette one little air bubble was soaked before medium drop with embryos and two bubbles divided from each other by little drop of M16 medium. Pipette tip was inserted into the ostium of oviduct and embryos were blown in along with air bubbles on either side of them. The marker air bubbles were then visible in the first turn of oviduct. Reproductive tract was carefully replaced into the peritoneum. Incision was closed with a small wound clip.

2.5.9 Detection and qualification of chimerism

Chimeric animals derived from CB20 or NMRI blastocysts were determined by coat color of older than one week pups. Namely black spots derived from injected ES cells were seen on the white background coming from blastocysts. If ES clone contribute more then 50-60 % of the coat in a series of chimeras, then it was considered as one quite likely contributing to the germ line at a similar level. Generally all chimeric animals showing 40-50 % of chimerism and more were taken in matings. Chimeric animals derived from 129sv × C57BL/6 were genotyped by PCR analysis of mouse tail DNA (2.6.5).

2.5.10 Maintaining a targeting mutation

Chimeric animals 7-8 weeks old were mated with wild type mice (strain C57BL/6). F1 mice with black coat color were analyzed for possessing of targeted allele. Presence of targeted allele was proved by PCR analysis of mouse tail DNA (2.6.5) and southern blot analysis (2.6.1).

2.6 Genotyping of ES clones and mice

2.6.1 Generation of probes for southern blot analysis of ES genomic DNA

External probes P1 and P2 were amplified from mouse genomic DNA derived from C57BL/10 female mice. Expand High Fidelity PCR system (Roche, Mannheim, Germany)

(2.2.4) was used with primer pair *imap-P1-5'* 5'- GTGAAT TGA GGG TCG GCG ACA GCA TAG AGT GGG - and *imap-P1-3'* 5'- GAC TCT GAG CAC CAG AGC CTG CTT CTG TAA TCC CA -3' for P1 and *P2-very-new-up* 5'-GAT CCC CTG GAA TTG GAG TTG TGA ATT GAT –3' and *P2-very-new-lo* 5'- CTG TGA TCA GGA GGA TGA TGC GAA ACG ACC –3' for P2. Programs of amplification were for P1: 94°C for 2 min then 30 cycles 94°C for 30s, 58°C for 1 min, 72°C for 1 min, final elongation 72°C for 8 min; for P2: 94°C for 2 min, 35 cycles 94°C for 15s, 60°C for 30s, 72°C for 1 min, final elongation 72°C for 8 min. PCR products were purified by electrophoresis in agarose gel (2.2.6) and cloned into pCR 2.1 TOPO TA vector (2.2.9). Probes for hybridization were digested out of the vector with BstXI for P1, with HindIII plus XhoI restriction endonucleases for P2 and purified by electrophoresis. Quantity was estimated by electrophoresis in 1% agarose gel.

Internal Neo probe was cut out from pACN vector with restriction endonucleases BamHI and NcoI. 586 bp long fragment corresponding a part of neomycin resistance gene and filled-in with Taq-polymerase. Reaction mixture contained 0.2 mM dNTP, 1 µl Taq-DNA polymerase, 10 µl 10 ×PCR buffer (Promega, Madison WI, USA), 1.5 mM $MgCl_2$ (Promega, Madison WI, USA) in reaction volume 100 µl and was incubated at 72°C for 30 min without initial denaturation step. Filled-in fragment was purified from reaction mixture using NucleoSpin®extract (Macherey-Nagel, Düren, Germany) purification kit. Then fragment was dephosphorilated with Shrimp alkaline phosphatase (SAP). Reaction mixture contained 10 µl 10 × SAP buffer, all the filled-in DNA fragment, 2U of SAP. After 2h of vial incubation at 37°C 3U of SAP were added to reaction mixture and incubation continued for 2 h. Then fragment was purified using NucleoSpin®extract (Macherey Nagel, Düren, Germany) and 4 µl were used for ligation into pCR®2.1-TOPO®Vector (Invitrogen, Leek, Netherlands) according to manufacturer protocol. 2.5 µl of ligation mixture were used for transformed in TOP10F' competent *E.coli* cells. For hybridization neo probe was cut out of vector with EcoRI restriction endonuclease, purified and quantified by electrophoresis (2.2.7) in 1% agarose gel.

2.6.2 Radioactive labeling of DNA

Radioactive labeling of probes for southern blot with [α-^{32}P]dCTP was performed through "random priming" as described by Feinberg und Vogelstein. Megaprime DNA Labeling Kit (Amersham, Braunschweig, Germany) was used according to manufacturer instructions. Reaction mixture contained 30 ng of DNA probe and 100 µCi (ICN, 10 µCi/µl; specific activity: 3000 Ci/mmol) in reaction volume of 100 µl. Non-incorporated [α-^{32}P]dCTP was

removed using NucleoSpin®extract (Macherey Nagel, Düren, Germany) according to manufacturer instructions. Rate of incorporation was measured in Beckmann LS 6000 SC scintillation counter by Cherenkov-counts of 1 µl aliquot of sample. Before hybridization probes were denaturated for 5 min at 94°C, chilled on ice, then added to hybridization solution.

2.6.3 Transfer of DNA on membrane and hybridization „Southern Blot“

Digested DNA was resolved in 0.8 % agarose gels with 1×TAE buffer overnight at <1V/cm. Transferred on Nylon membrane Hybond-N (Amersham, Braunschweig, Germany) according to the protocol "Downward Alkaline Capillary Transfer" (Chomczynsk, 1992). Namely agarose gel after electrophoresis was denaturated in denaturation solution for 30 min by gentle agitation. Then a down streaming blot was built using blotting solution for DNA transfer. After 2h of blotting membrane was neutralized in 0.2 M NaPi and finally binding of DNA to membrane was improved by backing at 80°C for 20 min. Membrane was preincubated for 2 h in hybridization solution for Southern at 65°C and then heat-denaturated radioactive labeled probe (2.6.2) was added to the hybridization solution. After overnight hybridization at 65 °C membrane was washed for 15 min in 2 × SSC, 0,1% SDS two times for 15 min and in 0,1% SSC, 0,1% SDS two times for 15 min. Membrane was exposed to Kodak BioMAX MS film with Kodak BioMAX MS intensifying screen at -80°C for 1-14 days.

2.6.4 Mycoplasma test

PCR-based mycoplasma tests with internal control were performed using VenorGeM® Mycoplasma detection kit (Minerva BioLabs, Berlin, Germany). 50-150 ng of ES cell genomic DNA or of cell supernatant/medium were used for reaction. 100 µl cell supernatant/ medium were heated at 95°C for 5 min and centrifuged briefly, 2 µl were used for amplification. PCR was performed according to manufacturer instructions using Expand High Fidelity PCR system (Roche, Mannheim, Germany) in 50 µl volume. Amplification program was 94°C for 2 min, 55°C for 2 min, 72°C for 2 min then 34 times 94°C for 30s, 55°C for 30s, 72°C for 1 min, final extension 72°C for 4 min. Resulting PCR products were resolved in 2% agarose gel with 1 × TBE at 3 V/cm for 1h.

2.6.5 PCR genotyping of mice and ES cells

DNA was purified from tails of F1 mice from mating of chimeric animals with C57BL/6 using, InvisorbSpin Tissue Mini Kit (Invitek, Berlin-Buch, Germany). The downstream primer was always *Lo- tails* 5'-AGG AAG AGA GAT GAA AGG AGG GAA GGA CAG T -3', the upstream primers were *UP-wild* 5'- ACT CTG GTC TCT GGC AGG TTT CTG AGA GAC -3', *UP- targeted* 5'- CCC TCA CTC AGA GCT TTA CCA ATG CCC TC -3' and *UP-deleted* 5'- GAA CCC TGG TGT CTC AAT TCA GCA TCA GGT -3'. Taq DNA polymerase (Eppendorf, Hamburg, Germany) was used for mouse tails testing. Primers *UP-wild, UP-targeted, UP-deleted* and *Lo-tails* were combined in one tube. Reaction volume was 50 µl. 200 ng of tail DNA was taken in amplification. PCR program was 94°C for 2 min, then 30 cycles 94°C for 15 s, 60°C for 30s, 72°C for 1 min were performed, 72°C for 8 min. Products were analyzed in 1% agarose gel with 1×TBE. For analyzing DNA from ES cell clones positive in southern blot analysis primer pair *UP-targeted* 5'-CCC TCA CTC AGA GCT TTA CCA ATG CCC TC -3', *imap-P1-3'* 5'-GAC TCT GAG CAC CAG AGC CTG CTT CTG TAA TCC CA-3' was used in amplification with Expand High Fidelity PCR system(Roche, Mannheim, Germany). PCR product was purified through electrophoresis (2.2.7), cloned into pCR2.1 TOPO TA vector (2.2.9) and sequenced from both sides (2.2.8) using 5'-IRD800-modified primers *M13 Forward* 5'-GTA AAA CGA CGG CCA G-3' and *M13 Reversed* 5'-CAG GAA ACA GCT ATG AC-3'.

3. Results

3.1. Scheme of targeting

Imap38 (gi28559036) is a single copy gene localized on the chromosome 6 locus B (48722102-48724085 bp) in the mouse genome (Krücken *et al.*, 1997, 1999). Seven other genes belong to a family of AIG related genes. They are situated in the same locus within 130 kbp (Fig.1A,B). There are two variants of the imap38 (NM175860, NM008376) which differ in splicing but encode the same 33.4 kDa protein (NP032402). The transcript includes 43 bp of exon 2, 860 bp of exon3 and 1081 bp intron. Analysis in Ensembl (Clamp *et al.*, 2003) and PROSITE (Bairoch *et al.*, 1991) databases have shown the large exon to encode ATP/GTP binding motif (P-loop) (Saraste *et al.*, 1990) near the IMAP38 N-end and transmembrane domain on the C-terminus (Fig. 1C,D). This structure of the last large exon is typic for many members of the *imap* family. Deletion of the larger exon which encodes functional domains was the strategy to knock out *imap38*. Such deletion woud lead to synthesis of transcript which can not be translated into functional protein and influence phenotype. The nearest neighbours of *imap38* are situated far enough so that their structure would not be influenced by this deletion (Fig. 1B). For generation of *imap38* deficient mouse a Cre-LoxP recombination system was used (Orban *et al.*, 1992; Lasko *et al.*, 1992; Gu *et al.*, 1993). The targeting vector (pTV) was introduced into C57BL/6-derived ES cell line Bruce4 (Kontgen *et al.*, 1993). Inside ES cells pTV was integrated in the genome through homologous recombination so that *imap38* gene was replaced (Fig. 2A,B) by the self-excision ACN-cassette (Bunting *et al.*, 1999). This cassette (Fig. 3) contains two genes between two directly orientated LoxP sites. First, Cre recombinase gene under control of the testis-specific intragenic promoter of the murine angiotensin-converting enzyme (tACE). Second, the selectable marker neomicine rasistance gene *Neo*^{r} linked to *tACE-Cre*.

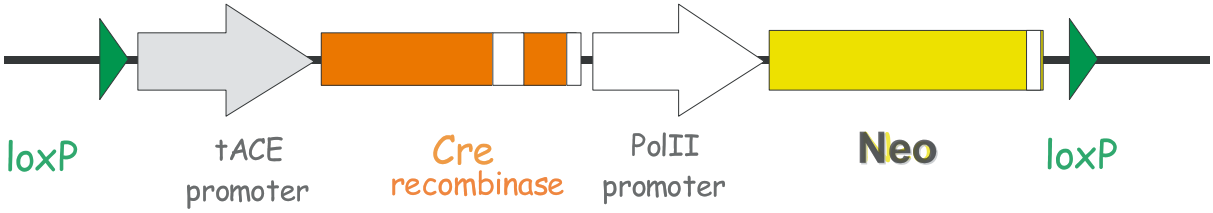


Fig. 3. Self-excision cassette, ACN. The testis-specific elements from the mouse *ACE* gene (grey arrow) are followed by a modified Cre structural gene (orange) with the minimal polyadenylation signal from HSV-TK. SV40 T-antigen derived intron (white box) is inserted into the Cre gene to prevent in-frame translation and subsequent self-excision during propagation of the vector in bacteria. The *Neo*^{r} gene (yellow box) is controlled by a promoter from the mouse RNA polymerase II gene and on the 3' end minimal polyadenylation signal from HSV-TK is placed. These elements are flanked with two directly orientated LoxP sites (green triangles).

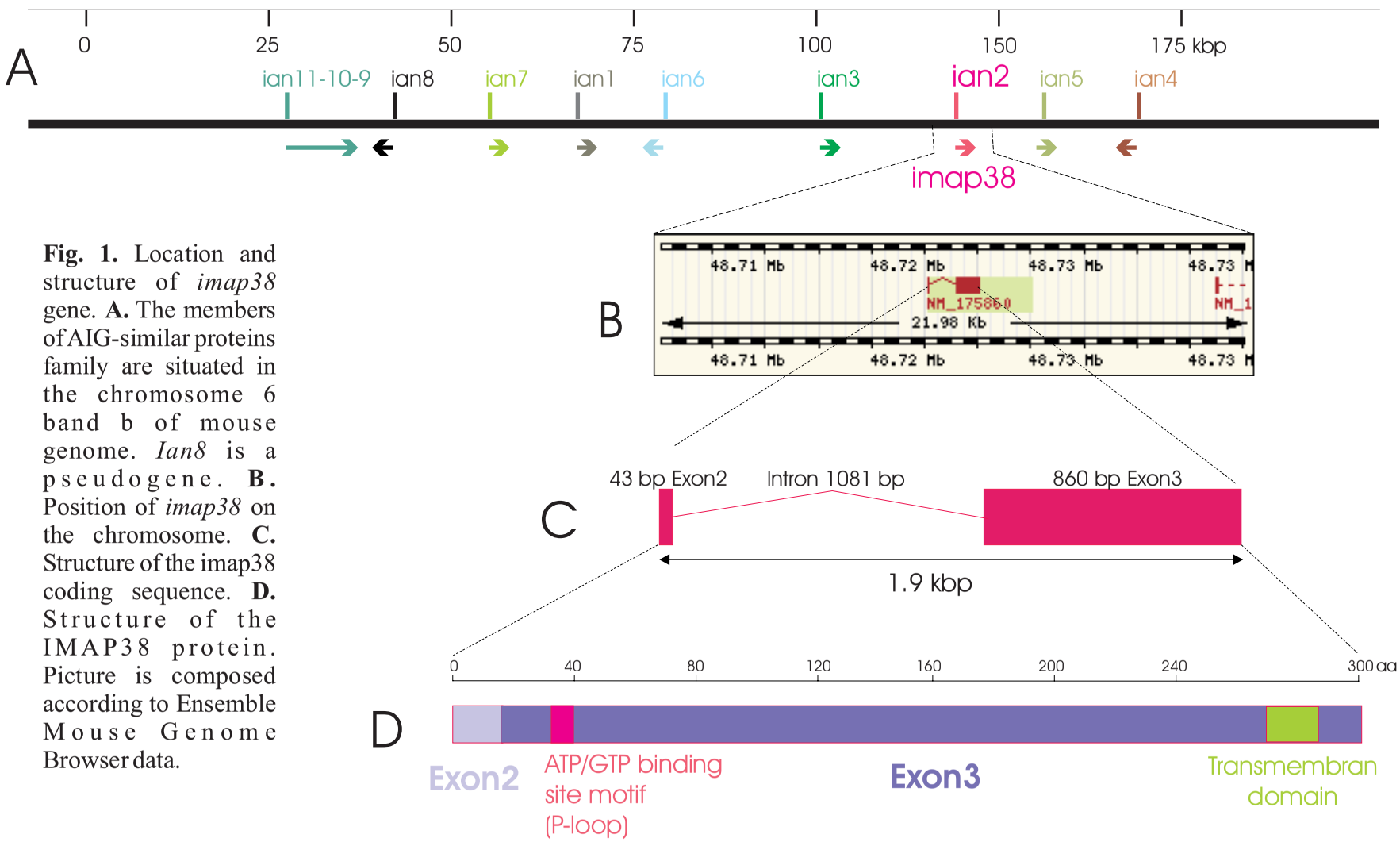


Fig. 1. Location and structure of *imap38* gene. **A.** The members of AIG-similar proteins family are situated in the chromosome 6 band b of mouse genome. *Ian8* is a pseudogene. **B.** Position of *imap38* on the chromosome. **C.** Structure of the imap38 coding sequence. **D.** Structure of the IMAP38 protein. Picture is composed according to Ensemble Mouse Genome Browser data.

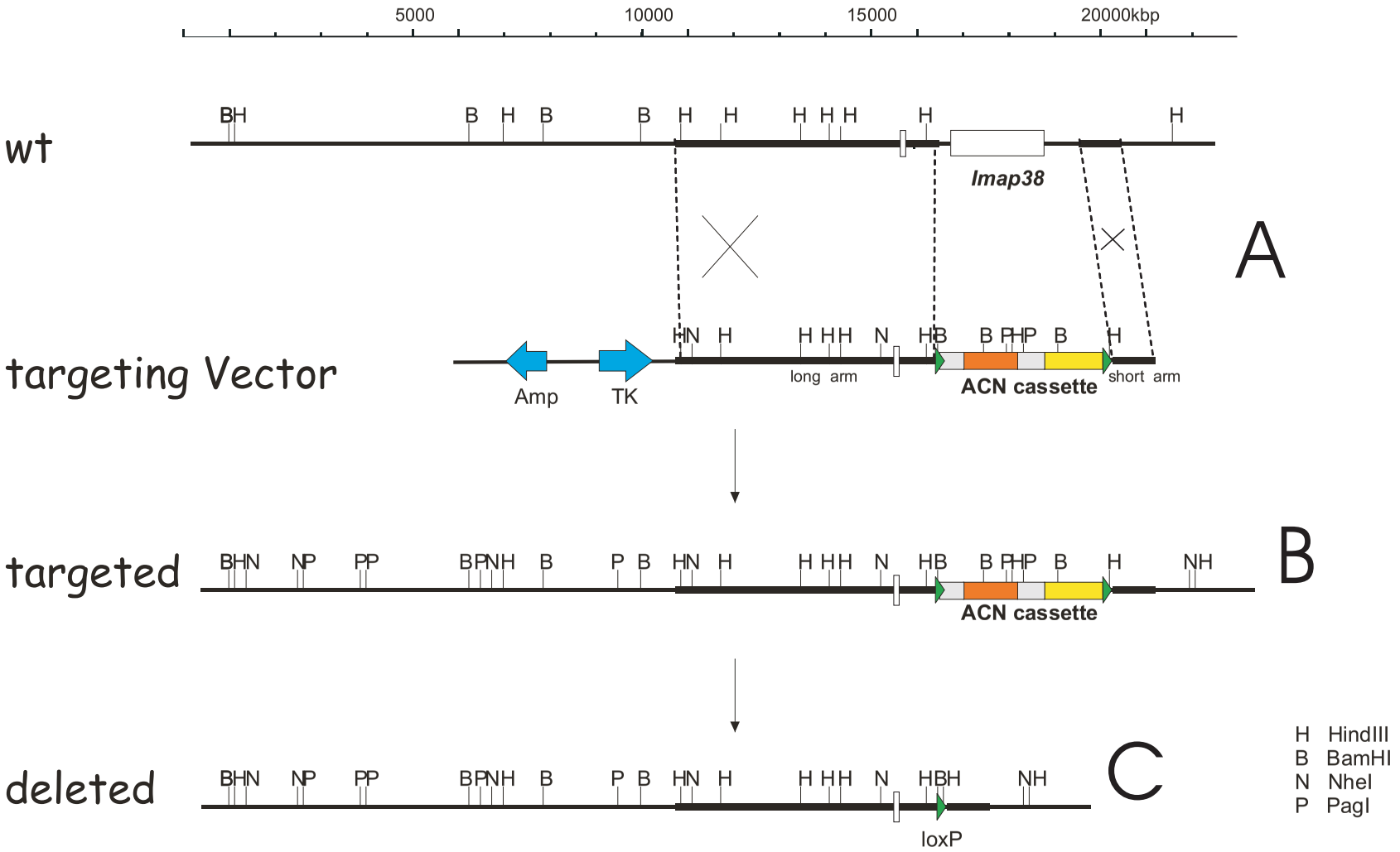


Fig. 2. Scheme of targeting. **A.** Two rounds of homologous recombination between the wild type allele and targeting vector. **B.** Targeted allele generated by homologous recombination. **C.** Deleted allele after self excision of ACN cassette in testis. White boxes show two coding exons of *imap38* gene. Long and short homology arms of the targeting vector and homologous regions inside of allele are shown as a thick lanes. Amp - ampiciline resistance gene. TK thymidine kinase gene. Green triangles show LoxP sites. Recognition sites for restriction endonucleases HindIII, BamHI, NheI, PagI are marked with first letter.

The tACE promoter is activated in spematogonia during spermatogenesis (Langford *et al*., 1991) leading to germ line specific synthesis of Cre recombinase. This fresh synthesized recombinase will cut the ACN cassette out. The ACN self-excision system was previously tested in deletion of some genes (Bunting *et al.*, 1999). So introduction of the ACN cassette in a specifical locus in ES cells results in generation of ES cell clones heterozygous for an allele containing the ACN cassette instead of *imap38*. These clones were injected in mouse blastocysts. The animals developed from injected embryos are chimeric for wild type and ES cells derived tissues. The ES cells can contribute in the germ line where during spermatogenesis self excision occurs generating a deleted allele (Fig. 2C). Male chimeras can transmit the deleted allele through their sperm derived from ES cells to F1 progeny and produce mice heterozygous for *imap38*-lacking allele. Mice homozygous for the deleted allele can be obtained by mating of the heterozygous mice with each other.

3.2. Generation of targeting vector

A targeting replacement vector pTV was constructed according to the general considerations (Torres&Kuhn, 1997). The ACN cassette was cut out from pACN vector (Bunting *et al.*, 1999) and positioned between two PCR-amplified homology arms. The long homology arm is situated on the 5'end, is 6 kbp long enabling specificity of binding and efficient homologous recombination (Hasty *et al.*, 1991). The short homology arm on the 3' end of ACN cassette is 1 kbp long to make possible PCR testing of the ES cell clones. These homology arms are equal to the sequences surrounding *imap38* gene (Fig. 2A). Two events of homological recombination taking place inside of the arms lead to replacement of the last exon with ACN cassette (Fig. 2B). Besides, pTV contains a negative selctive marker (TK-Thymidine kinase gene from pEasyFlox vector (Chui *et al.*, 1997)) before the long homology arm. TK makes cells sensetive to GANC. Negative selection with GANC allows deminishing the number of clones with a random integrated targeted construct (Torres&Kuhn, 1997; Joyner, A, 2001).
Sequences of the targeting vector intermediates were proven at every stage by restriction and PCR analysis, as well as by sequensing of homology arms. The restriction pattern of final vector is shown in Fig. 4. All band sizes correspond to awaited. Both LoxP sites in pTV were cut out from the pACN together with the whole ACN cassette making very unprobable any changes in the sequence. But to ensure functionality of the both loxP sites additional tests were performed. LoxP sites were sequenced in both directions and *in vitro* Cre-mediated recombination of the linearized pTV was performed. Fig. 5 shows increasing quantity of recombination products in responce to increasing concentrations of Cre recombinase in the

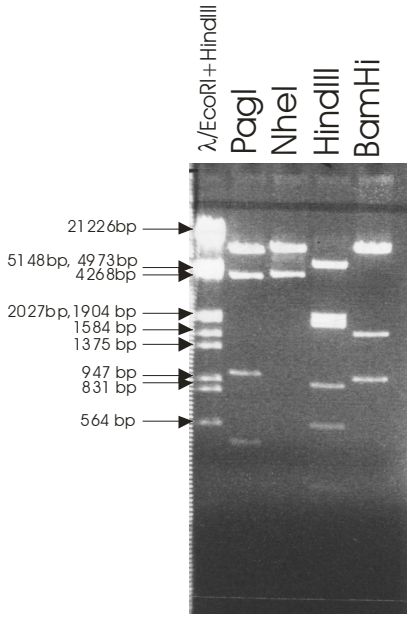


Fig. 4. Restriction analysis of pTV.

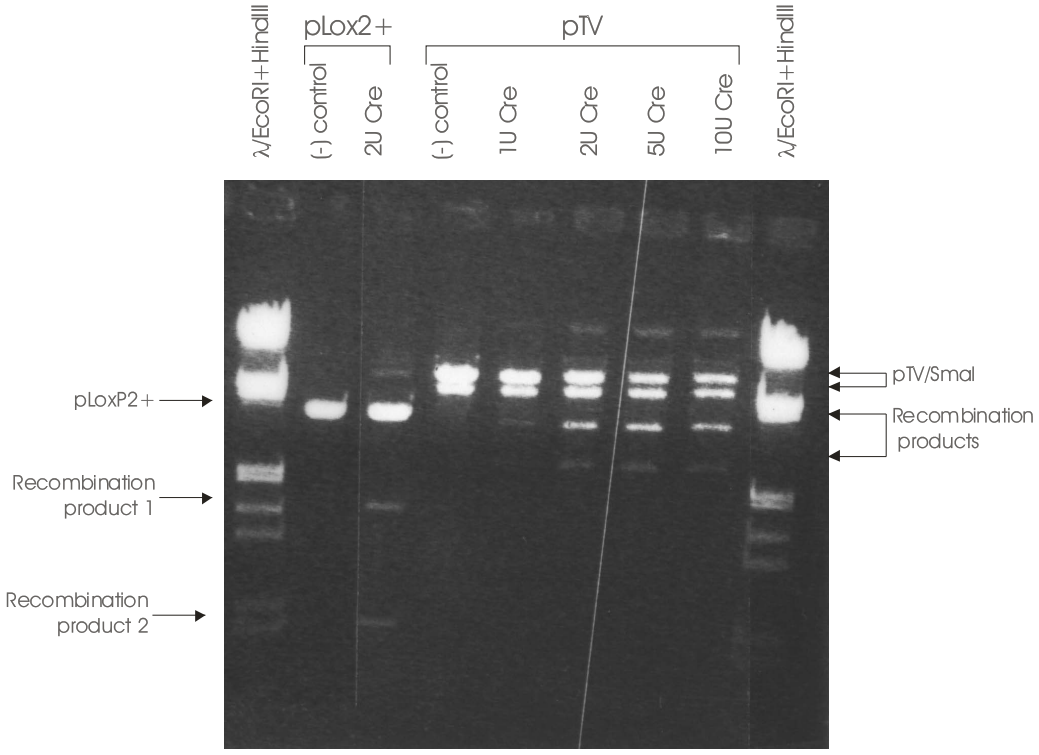


Fig. 5. Checking of LoxP sites of the targeting vector pTV functionality by *in vitro* Cre mediated recombination. As a control commercially available vector pLoxP2+ was used. Different concentrations of Cre recombinase from 2U to 10U were taken for the recombination of pTV digested with SmaI. Concentration-dependent increase of the bands intensity corresponding to recombination products accumulation can be seen.

reaction mixture. This concentration dependence makes evident the accumulation of the products due to recombination. So LoxP sites from pTV were shown to contain no mutation and to be functional *in vitro*.

3.3. Generation and analysis of the targeted ES cell clones

pTV was linearized inside of the vector backbone and transfected in Bruce4 ES cells. Plating efficiency controls (cells were plated after transfection and grew without selection, 2.4.10) have shown that more then 66-78 % of the cells after transfection have attached to the plate and have formed colonies. This demonstrates that electroporation has not led to drastic decrease of ES cells survival. The transfected ES clones have undergone positive and negative selection. Non-transfected cells treated with G418 (selection specificity control, 2.4.10) have all died showing efficiancy of selection. Control plates with transfected cells selected only with G418 for 5 days (GANC enrichment, 2.4.10) showed that 0.003-0.005% of the plated cells have survived positive selection and contain the integrated targeting construct.

Two rounds of transfection were performed. Clones survived in the positive-negative selection were analysed using southern blot and PCR. Southern blot strategy includes analysis of the clones with two external probes and one internal (Fig. 6). An external probe P1 is situated after the 3' end of the short arm. Hybridisation of genomic DNA digested with HindIII with P1 results in 5.3 kbp product corresponding to wild type allele and 1.8 kb corresponding to the targeted allele. An external probe P2 is situated before the 5' end of the long homology arm. When P2 hybridizes with BamHI restriced genomic DNA the wild type allele gives a band more then 20 kbp, targeted of 6.5 kbp. Before apply in experiment, the external probes were tested for specificity through hybridization with the mouse genomic DNA digested with several restriction endonucleases (Fig. 7A,B). Hybridisation with the external probes allows to determine clones containing the targeting construct which has specifically replaced *imap38*. Southern blot screening of the clones resistant to positive-negative selection was performed. From the 251 clones from the first targeting 2 were positive (C5 and D7). From second targeting 5 out of 360 clones were positive (Fig. 8). In addition, PCR analysis (Fig. 6, Table1) was established. Three upstream primers targeted to the *imap38* coding region (*UP-wild*), to the ACN cassette (*UP-targeted*) and to the 3'end of the long homology arm (*UP-deleted*) were designed. They can be combined with two downstream primers: one situated immediately after the 3'end of the short homology region (*imap-P1-3'*) and second within the short homology arm near the 5'end (*Lo-tails*) (Fig. 6, table1).

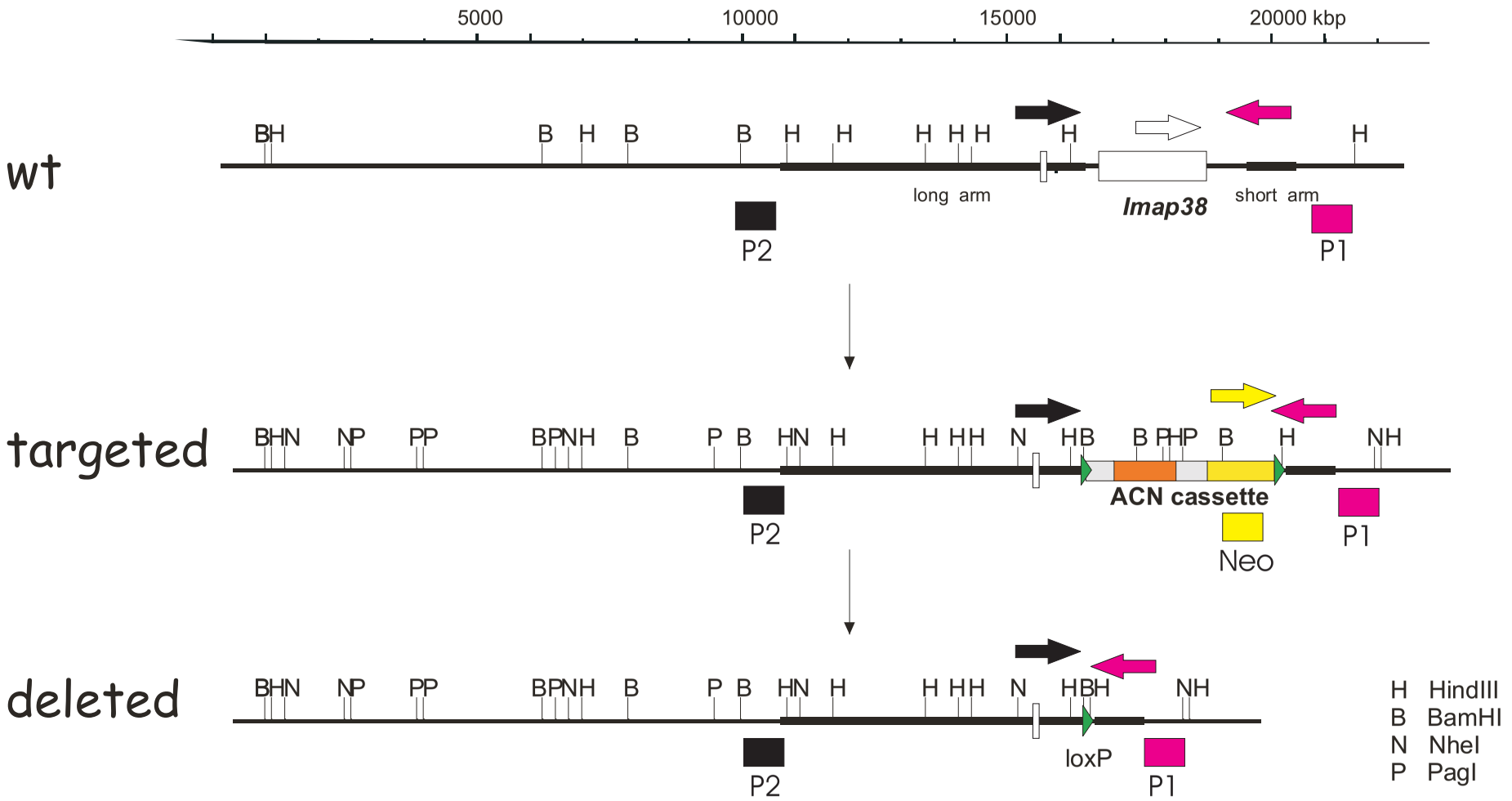


Fig . 6. PCR and southern analysis strategy. PCR primers are shown as arrows: *UP-deleted*, *UP-targeted*, *UP-wild*, *Lo-tails* or *imapP1-3'* primer. Southern blot probes are shown as boxes: external probe *P1*, external probe *P2*, internal probe *Neo*.

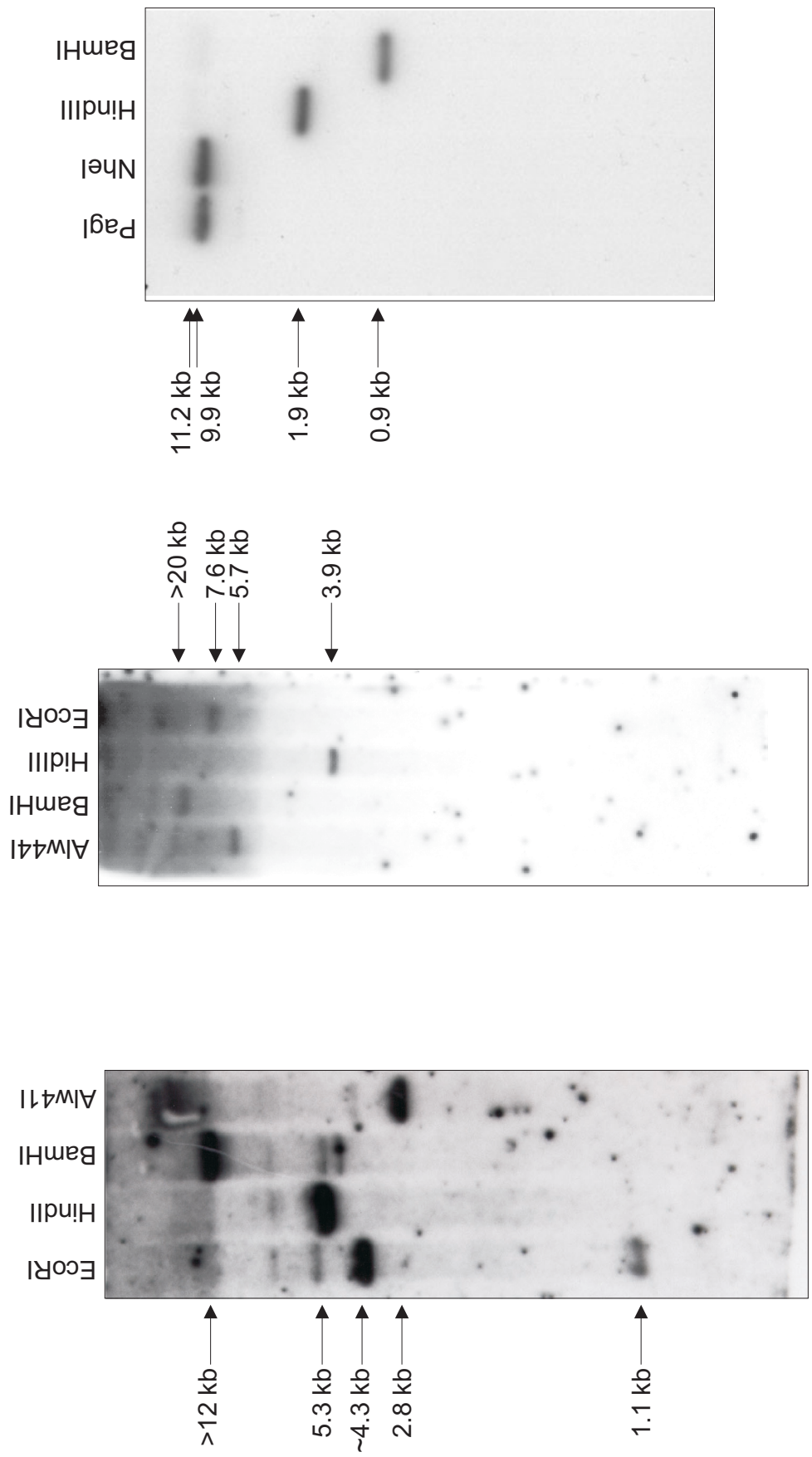


Fig. 7. Checking of probes for Southern blot analysis. **A.** External probe P1 hybridized with C57BL/10 mouse genomic DNA. **B.** External probe P2 hybridized with mouse C57BL/10 genomic DNA. **C.** Internal probe Neo hybridized with pTV. All the product sizes correspond to predicted.

Table. 1. PCR primers and product sizes for ES cell clones and mice DNA analysis.

Upstream primer	Downstream primer					
	Lo-tails ←			*imap-P1-3'* ←		
	wild type allele	**targeted allele**	**deleted allele**	**wild type allele**	**targeted allele**	**deleted allele**
UP-wild ⇨	787 bp	-	-	2.6 kbp	-	-
UP-targeted →	-	606 bp	-	-	2.4 kbp	-
UP-deleted →	3.3 kbp	4.1 kbp	376 bp	5.1 kbp	5.8 kbp	2.1 kbp

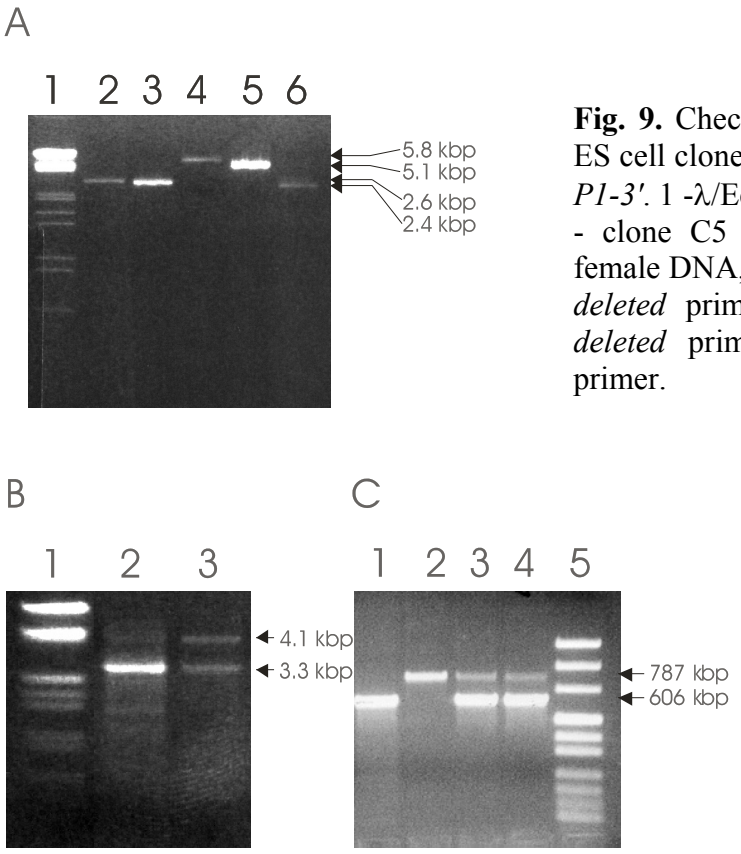


Fig. 9. Checking of the primers for PCR analysis of ES cell clones and mice. **A.** Downstream primer *imap-P1-3'*. 1 -λ/EcoRI+HindIII molecular weight marker; 2 - clone C5 DNA, *UP-wild* primer; 3 - C57BL/10 female DNA, *UP-wild* primer; 4 - clone C5 DNA, *UP-deleted* primer; 5 - C57BL/10 female DNA, *UP-deleted* primer; 6 - clone C5 DNA *UP-targeted* primer.

B, C Downstream primer is *Lo-tails*. **B.** 1 - λ/EcoRI+HindIII; 2 – wild type mouse tail DNA, *UP-deleted*; 3 - D7 clone DNA, *UP-deleted*. **C.** Amplification of D7 clone DNA: 1 - *UP-targeted*; 2 - *UP-wild*; 3 - *UP- wild* and *UP-targeted* in one tube; 4 - *UP-wild*, *UP-targeted*, *UP-deleted* in one tube; 5 - DNA molecular weight marker pUCMix.

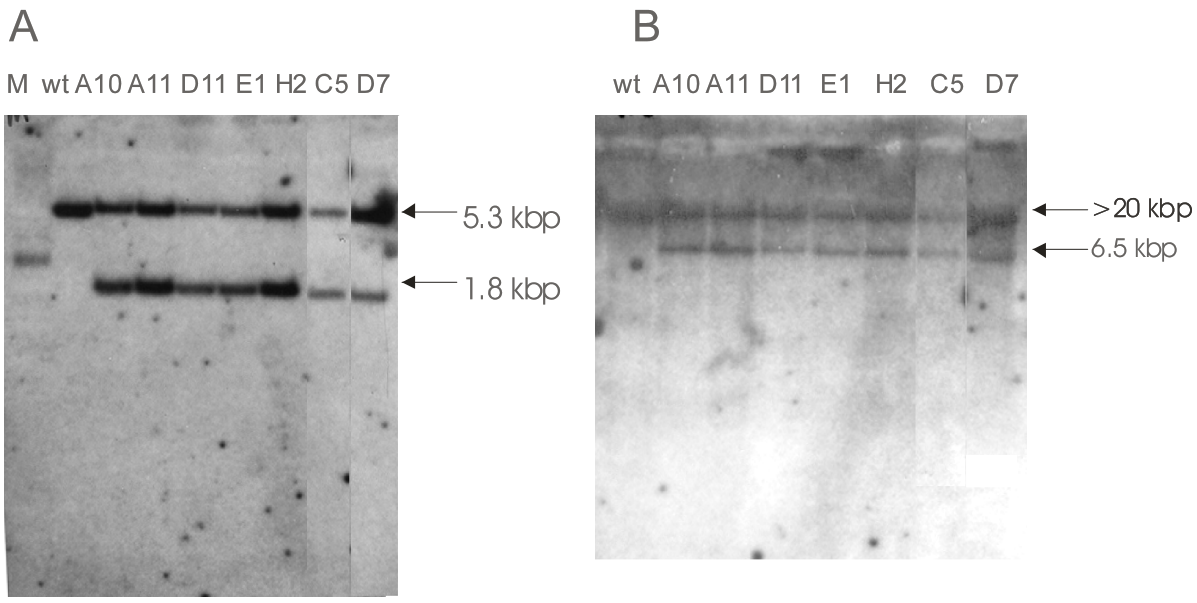


Fig. 8. Southern blot analysis of all 7 ES cell clones. **A.** External probe P1. Genomic DNA was digested with HindIII. 1.8 kbp band corresponds to targeted allele. **B.** External probe P2. Genomic DNA was digested with BamHI. Band of 6.5 kbp corresponding to the targeted allele is seen in all the ES clones. wt – wild type DNA of C57BL/10 mouse, M- DNA molecular weight marker λ/EcoRI+HindIII

The both primer combinations allow to amplify specific products (Fig. 9). All three upstream primers and *Lo-tails* can be combined in one tube to detect all allele combinations in mouse tail DNA (Fig. 9C). For two clones (C5 and D7) obtained in the first round of targeting the upstream primer situated inside of ACN cassette and downstream after 3'end of short homology arm were used (Fig. 10).

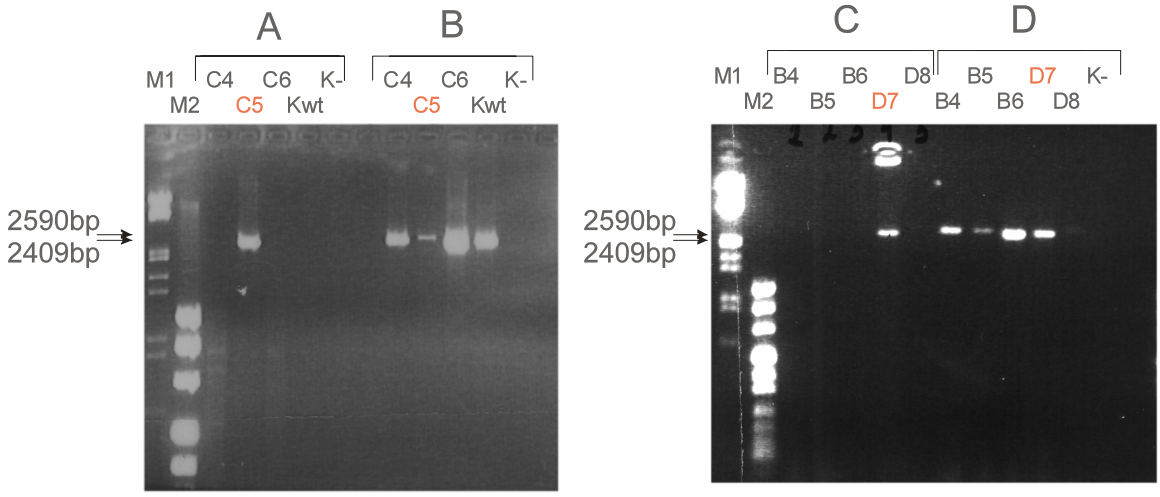


Fig. 10. PCR analysis of ES clones. A, C – primer pair *UP-targeted* and *imap-P1-3'*. Only the clones C5 and D7 positive in southern blot analysis give a band corresponding to a targeting allele. B,D – primer pair *UP-wild, imap-P1-3'* as a control of amplification. Kwt – wild type mouse genomic DNA was taken as a control of amplification. (K-) no DNA in reaction mixture. M1 – λ/EcoRI+HindIII, M2 – pUCMix DNA molecular weight markers.

PCR products of right length were obtained. Their sequence is identical on the one side to the ACN cassette, on the other side to the genomic DNA sequence. For the rest of clones obtained in second round of targeting PCR analysis was performed only to ensure that the right clones were expanded from 96-well plate (Fig. 11).

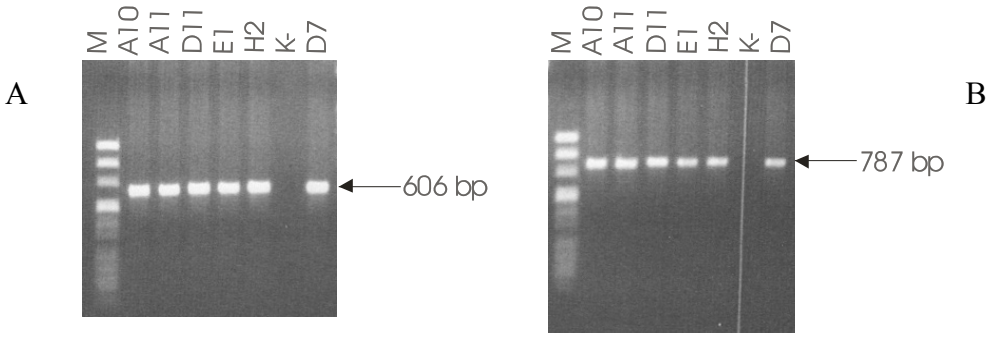


Fig. 11. PCR analysis of the targeted clones positive in southern blot. **A.** Primer pair *UP-targeted*, *Lo-tails* was used. A10 - H2 ES cell clones, (K-) wild type mouse genomic DNA C57BL/10 female mouse; D7 clone DNA, a positive control of amplification; M – pUCMix molecular weight marker. **B.** Primer pair *UP-wild*, *Lo-tails* was used. A10 - H2 ES cell clones; (K-) water control; D7 clone DNA, a positive control of amplification; M - pUC Mix molecular weight marker.

In this way specific integration was proven, but it was also necessary to check, whether only a single pTV has integrated in each of the candidate clones. For that southern blot analysis with the internal Neo probe was performed. Internal probe Neo is a part of coding sequence of the neomicine resistance gene and was cut out from pTV before cloning into pCR2.1TOPO vector (Fig. 6; 2.6.1). It guarantees identity of probe sequence to the sequence of the targeted allele. Specificity of the Neo probe was checked by hybridization with pTV digested with different restriction endonucleases (Fig. 7C). Hybridization of Neo probe for all the clones resulted in a single band in the autoradiography (Fig. 12). It is a clear evidence that all the clones do contain a single integrant.

All the seven ES clones were proven to be suitable for injection into blastocysts. First round of targeting resulted in generation of 2 clones applicable for injection out of 251, second in 5 out of 360 clones. Correspondingly a rate of targeting lies between 1/120 and 1/70. The last test to be performed before injection of the ES cells in blastocysts is a mycoplasma contamination test. Mycoplasma contamination of the ES cells is one of the well known reasons of germ line transmission failure (Robertson, 1987). PCR based mycoplasma tests are sensitive and rapid. All the targeted clones were tested with PCR before preparation of the stocks for injection, no clones were mycoplasma contaminated, all were taken in injections (Fig. 13).

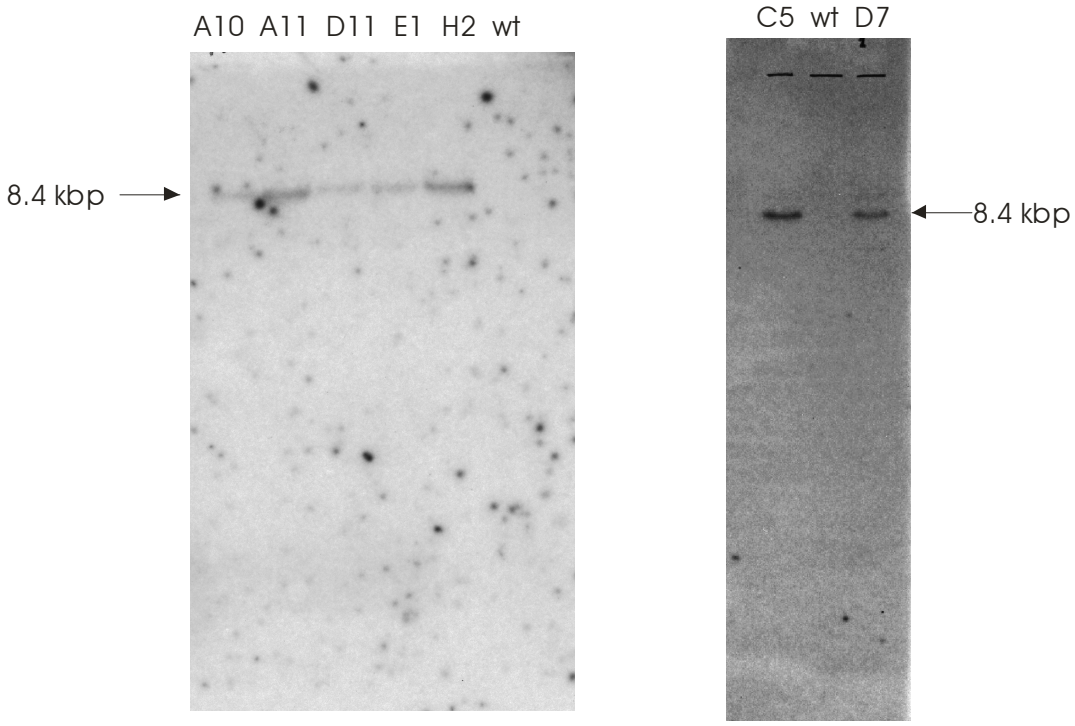


Fig. 12. Southern blot analysis of ES cell clones using internal probe Neo. wt - negative control - wild type genomic DNA from C57BL/10 female mouse. A single band of 8.4 kbp corresponding to the targeted allele is seen for each ES clone.

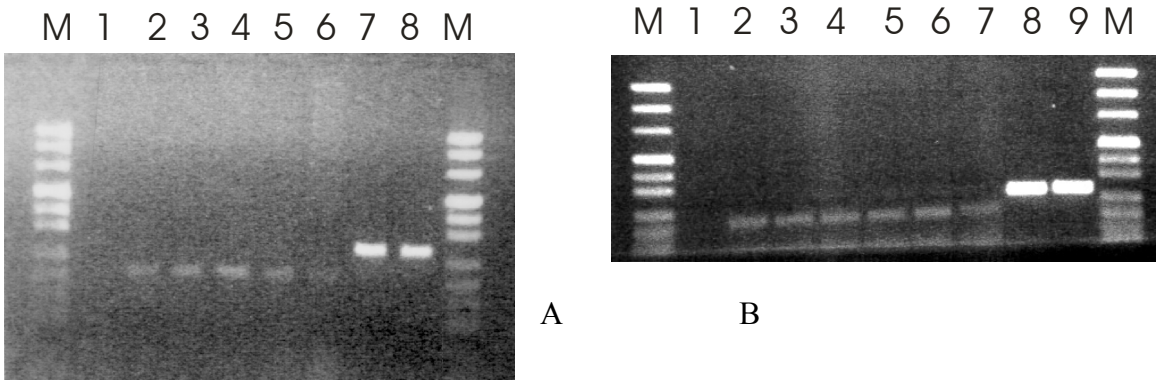


Fig. 13. Mycoplasma contamination test. ES cell clones genomic DNA was analysed. **A.** Clones C5 and D7. 1 no DNA control; 2 internal control only; 3 internal control + 50 ng C5 DNA; 4 internal control + 50 ng D7 DNA; 5 internal control + 150 ng C5 DNA; 6 internal control + 150 ng D7 DNA; 7 internal + positive controls; 8 positive control only. **B.** The other clones. 1 no DNA control; 2 internal control + 200 ng A10 DNA; 3 internal control + 200 ng A11 DNA; 4 internal control + 200 ng D11 DNA; 5 internal control + 200 ng E1 DNA; 6 internal control + 200 ng H2 DNA; 7 internal control only; 8 internal + positive control; 9 positive control only. M molecular weight marker pUC Mix.

3.4. Injections of mouse embryos and transfer

C57BL/6 mice survive malaria (Li *et al.*, 2001) and show high expression of *imap38* in responce to infection and in immune mice (Krücken *et al.*, 1999). *imap38* knockout mice were planed to be used in the experiments related to malaria infection. That is why C57BL/6 derived ES cell line Bruce4 was chosen for *imap38* targeting. Bruce4 ES cells are homozygous in nonagouti locus (a/a), and give rise to mice with black coat. c^{2J} strain is identical to C57BL/6. The only difference is a mutated Tyr gene changing C57BL/6 phenotype from black to albino. Therefore isogenic c^{2J} blastocysts are ideal for Bruce4 ES cells. Injections of Bruce4 cells in CB20 (A/A), NMRI (A/a) or c^{2J} (a/a) blastocycsts have given white-black chimeras. These systems allow to determine chimerism rate and germ line transmission according to a coat colour. For some clones black C57BL/6 or agouti 129sv×C57BL/6 blastocysts were used. In this case chimerism rate can not be determined according to a coat colour. All the mice born after injections were mated and all the black F1 progeny were genotyped. All obtained ES cell clones were used for injections into mouse blastocysts. Details of injections and transfer as well as obtained chimeras are listed in table 2.

3.5. Evaluation of chimerism rate

Chimeras obtained after injections in CB20, NMRI or c^{2J} blastocysts were evaluated according to the coat colour. For chimeras developed from injections in agouti (129sv×C57BL/6) and in black C57BL/6 blastocycsts the rate of chimerism was determined

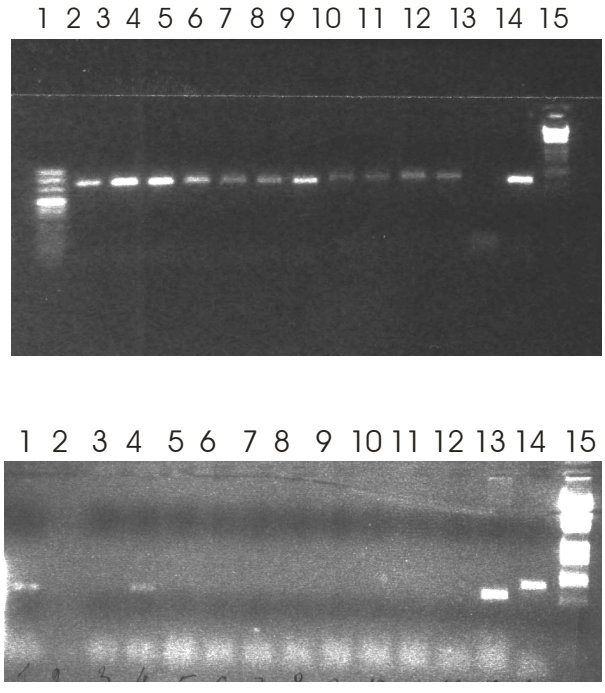
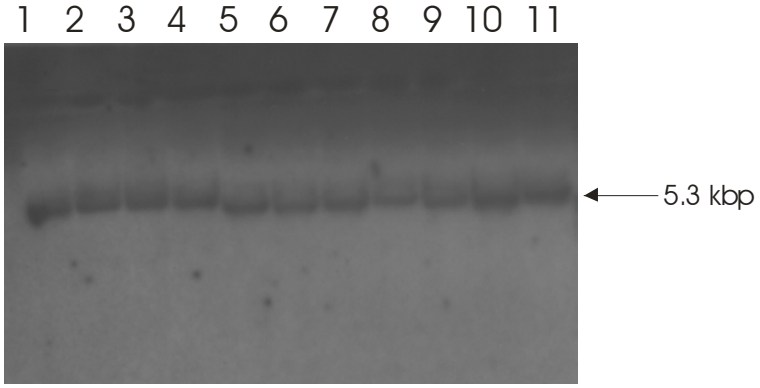


Fig. 14. PCR analysis of C5 clone chimeras. **A.** PCR of chimeras tail DNA with primer pair *UP-wild* and *Lo-tails*: 1- pUC Mix DNA molecular weight marker, 2-6 NMRI chimeras, 7-13 129×C57BL/6 chimeras, 13- water control, 14- control of amplification C57BL/6 genomic DNA, 15- λ/EcoRI+HindIII molecular weight marker. **B.** PCR of chimeras tail DNA with primer pair *UP-targeted* and *Lo-tails*: 1-4 NMRI chimeras, 5-11 129sv×C57BL/6 chimeras, 12- water control, 13- control of primers D7 genomic DNA; 14 – control of amplification C57BL/6 genomic DNA with *UP-wild* and *Lo-tails* primer pair, 15- λ/EcoRI+HindIII molecular weight marker.

by PCR and southern blot analysis of the tail DNA. Four NMRI chimeras were taken as a control. From eleven mice tested only two NMRI chimeras were positive for the targeted allele in PCR analysis (Fig. 14). None of them were positive according to southern blot analysis due to a lower sensitivity of the method in comparison with PCR (Fig. 15). Nevertheless all of the 129sv×C57BL/6-derived animals were taken in matings, because NMRI chimeras testing demonstrated irreliability of chimerism detection by PCR. On the other hand, a low percentage or no ES cell - derived tissues in tail is no evidence of their absence in testis.



3.6. Maiting of chimeras to maintain targeting mutation

A purpose of the present work is generation of *imap38* knockout mouse on C56BL/6 backgrownd for the experiments related with malaria infection in mice. That is why only mating of chimeras with C57BL/6 mice is suitable to eliminate the blastocyst background completely. Backcrossing with non C57BL/6 mice could lead to random variations in disease pattern making impossible correct controls and maybe decreasing inducibility of *imap38* in control mice.

3.7. Genotyping of F1

After crossing of all types of the chimeras with C57BL/6, heterozygous for deletion mouse can only be black, because it contains Bruce4 derived targeted allele from chimera (a) and C57BL/6 wild type allele from the mating partner (a). Deleted (or targeted) allele can be found only in the black (a/a) F1. That is why only the black pups were analysed. For PCR diagnostic in the same tube three upstream primers for wild type, targeted and deleted alleles and one downstream primer inside of short homology arm were mixed (Fig. 9C). Primer pair for the wild type allele was used as a positive control of amplification in each sample. If the control band was not seen, the mouse tail DNA was analysed by southern blot with the external probe P1. Results of testing are shown in table 3.

Table. 2. Injections of different clones and chimeras obtained

Clone	Date of injection and transfer	Number of blastocycts and type of blastocycts	Number of foster mothers made	Date of chimeras birth	Chimeras
C5	17.07.02	8 CB20	1	03.08	4 males, 2 survived
	18.07.02	8 CB20	1		
	24.07.02	14 CB20	2	-	-
	01.08.02	22 CB20	3	18.08.02	3 males
	02.08.03	20 CB20	3		
	11.02.03	40 NMRI	3	07.03.03	4
		26 129sv×C57BL6	2		7 mice give a litters, no targeted allele determined with PCR analysis
D7	13.02.03	26 NMRI	2	-	-
		29 129sv×C57BL6	2		
A11	29.07.03	60 C57BL/6	3	15.08.03	mice were eaten by mothers
E1	31.07.03	60 C57BL/6	3		5 mice born, are killed because of mistake of technical assistant
	23.09.03	45 c^{2J}	2	13.10.03	4
		60 C57BL/6	3		20
D11	25.09.03	65 C57BL/6	3	15.10.03	11

Table. 3. Results of testing of F1 obtained from chimeras' maiting

Clone	Blastocysts	Chimeras		F1	Test result
C5	CB20	5	#1	29	deleted allele absent
			#2	38	
			#3	-	
			#4	34	
			#5	-	
	NMRI	4	#1	33	
			#2	-	
			#3	6	
			#4	14	
	129sv×C57BL/6	7 mice born	#1	12	
			#2	13	
			#3	14	
			#4	-	
			#5	12	
			#6	19	
			#7	17	
D7	NMRI	no mice born	-	-	-
	129sv×C57BL/6				
A11	C57BL/6	no mice born	-	-	-
D11	C57BL/6	11	Injection 23.09.03		
E1	C57BL/6	6 mice born, killed by fault of TA	-	-	-
	c^{2J}	4	Injection 23.09.03		
	C57BL/6	20			
H2	ES cells were found to be differentiated during preparation for injection				

4. Discussion

The gene *Imap38* gene was found by differential screening (Krücken *et al.*, 1997) and characterized (Krücken *et al.*, 1999). It was shown that *imap38* is induced during malaria infection and is still expressed at the high level even 13 weeks after infection (Krücken *et al.*, 1997). *Imap38* belongs to a family of AIG-like proteins which were found in mouse, rat, human and plants. Functions of most other genes of the family are also not studied in details. *Aig1* is induced in plant *Arabidopsis thaliana* in response to the *Pseudomonas syringae* infection (Reuber *et al.*, 1996). *Ian1* takes part in thymus cells differentiation (Poirier *et al.*, 1999). *Ian4* is a mitochondrial outer membrane protein with GTP-binding activity that was found to be induced in hematopoetic precursor 32D cells in response to *Bcr/Abl* oncogene overexpression (Daheron *et al.*, 2001). A mutation in the rat *Ian4* gene results in severe T cell lymphopenia (Hornum *et al.*, 2002). The absence of *Ian4* in T cells causes mitochondrial disfunction, increased mitochondrial levels of stress-inducible chaperonins and a leucine-rich protein, and T cell-specific spontaneous apoptosis (Pandarpurkar *et al.*, 2003). This work was performed to generate an *imap38* knockout mouse as a tool for investigation of *imap38* function *in vivo* and for a wide range of experiments related to malaria. Conventional gene targeting was performed to delete the second exon of *imap38*. For this, a replacement type targeting vector containing a self-excision ACN cassette (Bunting *et al.*, 1999) was generated. The mouse strain C57BL/6 was chosen as background for knockout, because this strain can survive *P. chabaudi* infection, becomes immune, and shows a high level of *imap38* expression (Krücken *et al.*, 1999). C57BL/6 derived embryonic stem (ES) cell line Bruce4 (Kontgen *et al.*, 1993) was chosen for experiments. After two rounds of targeting seven ES cell clones containing a targeted allele where the *imap38* gene has been replaced with the self-excision ACN cassette were isolated and expanded. All these clones had normal morphology and were free of mycoplasma contamination. Six of them were used for blastocysts and morula injections. Different embryo stages were taken for injections: 3.5 days blastocysts, 3.5 days blastocysts developed *in vitro* from morulae and morulae. Altogether, ten injection rounds were performed in embryos derived from variety of mouse strains: CB20, 129sv×C57BL/6, C57BL/6, NMRI, and c^{2j}. Two out of six clones did not generate chimeras. Altogether from these injections 57 mice were born, 9 of which were black-white chimeras with a

chimerism rate of more than 40% according to the coat colour. To obtain mice heterozygous for the mutation, chimeras were mated with C57BL/6 mice. All black F1 progeny (241 pups) were genotyped, but none of them contained the deleted or targeted allele. Possible reasons for failure of germ line transmission will be discussed.

4.1. Genomic organization and chromosomal environment of *imap38* gene

Imap38 was first found by differential screening of transcriptomes of *P. chabaudi* infected against immune, reinfected with malaria C57BL/10 mice (Krücken *et al.*, 1997). The genomic sequence of *imap38* was then determined by screening of a 129/Ola mice cosmid library. *Imap38* was found to be intronless and encoding two different proteins. After draft sequence of mouse genome was available, these data were corrected and *imap38* is now known to contain three exons separated from each other by introns 2162 bp and 1081 bp. *Imap38* has two different transcripts generated by alternative splicing between first an second exons. Resulting transcripts differ in length (variant 2 is 6 bp shorter) and both encode the same 300 aa protein. Coding part of transcript contains a 43 bp of second exon and 860 bp of third exon. IMAP38 contains P-loop motif on this N-end and transmembrane domain on the C-terminus. The P-loop motif is annotated in Prosite database (Bairoch, 1991). It is a glycin-rich region, which typically forms a flexible loop between a beta strand and an alpha helix. This loop interacts with one of the phosphate groups of the nucleotide. The protein shows a weak GTP binding activity, no ATP binding activity was detected (Stamm *et al.*, 2002). The subcellular location assignment performed with programs PSORT (Nakai&Horton, 1999), TargetP (Emanuelsson *et al.*, 2000) and SignalP (Nielsen *et al.*, 1997) predicts *imap38* to be no secretory protein and not to contain any signaling sequences. According to these algorithms, localization is also less probable to be mitochondrial or nuclear. Gene organization is typical for many AIG-similar genes, which usually contain P-loop and a transmembrane region or coiled-coils. The functions of other genes have also not been studied completely yet (Poirier *et al.*, 1999; Hornum *et al.*, 2002; Cambot *et al.*, 2002; Reuber *et al.*, 1996). Expression of *imap38* is shown to be induced in spleen cells, mostly in B cells and macrophages, during malaria and long time after clearance of infection (Krücken *et al.*, 1999). During P53 mediated apoptosis, 6-times overexpression of *imap38* was detected

(Kannan *et al.*, 2001). However there is no direct evidence that *imap38* plays a pro- or antiapoptotic role. Apoptosis takes place in spleen cells during clearance of malaria infection only temporally (Helmby, 2000), while expression of *imap38* stays on the high level long after clearance of infection (Krücken *et al.*, 1999). All these data show no direct evidence that *imap38* is important for survival. So a conventional knockout should not lead to a lethal phenotype.

4.2. Choice of targeting strategy

There are several ways to knock out a gene. Routinely, modification of gene function in mice is commonly performed in two ways: mutation of a specific DNA locus (classical approach termed gene targeting) and gene silencing on the post-transcriptional level (RNAi technology termed knock-down) (Tiscornia *et al.*, 2003; Voorhoeve *et al.* 2003). RNAi allows avoiding germ line transmission as well as laborious and risk full steps of ES cell manipulation, injection of embryos with ES cells and waiting for mating results. Only injection of DNA into single cell embryos must be performed. On the other hand, genetically inhomogeneous populations of transgenic animals with different numbers of copies of RNAi expressing construct per genome must be examined in parallel. The phenotype can be influenced by modulation of function of other genes by disruption of their sequence with random integrated constructs. At the moment when the work has been started, application of siRNA mediated gene silencing in mammals was not established yet. For this reason, site-specific mutagenesis was regarded as the most promising method.

To knock out *imap38*, the third large exon containing the ATP-binding domain was deleted (Fig.1C,D). The *imap*-family genes are clustered within 130 kbp and are situated very close to each other. The neighbours of *imap38* are located 16.6 kbp away on the 5'end and 8.9 kbp away on the 3'end. Excision of *imap38* does not lead to disorder in the sequence of the other genes. According to classical scheme, a selectable marker (usually neomycin resistance gene) simply replaces a targeted gene in ES cells. Resulting targeted allele contributes in chimeric mouse when ES cells are injected in mouse embryo. Chimeras transmit the targeted allele through the germ line to their progeny. The disadvantage of the approach is that a strong promoter of the neomycin resistance gene can influence expression of the neighbouring genes. It is unacceptable for *imap*-genes situated very close to each other. The second generation

of targeting constructs for conventional gene knockout contains the same neomycin resistance gene flanked with loxP sites. Targeting construct is electroporated in ES cells and integrates at targeted locus. ES cells undergo selection and genotyping. Hereafter they are injected in mouse embryos and allowed to develop in mouse chimeric for embryo and ES cells derived tissues. If ES cells contribute to the germ line, some progeny of chimeras will be heterozygous for mutation. Then they are mated with a transgenic mouse expressing in all cells of the body. In the resulting F2, excision of the targeted gene happens in the cells expressing Cre recombinase. Next breeding step leads to mice homozygous for the mutation. Usually, to save one mating step in this case, Cre recombinase is transiently expressed in cell culture (Gu *et al.,* 1993). This procedure increases number of ES cells passages thereby decreases ES cells germ line transmitting competence. The use of ACN self-excision cassette allows saving one breeding step with a Cre expressing mouse at the same ES passage number. ACN cassette (Fig. 3 in results) contains, together with a neomycin resistance gene a Cre recombinase gene under control of the testis-specific promoter tACE (angiotensine converting element). In the first step *imap38* was replaced with the ACN cassette and this targeted allele has been delivered into chimeric animals. If ES cells contribute to the germ line, excision of ACN occurs already in male chimeric animal. At this stage an allele with an *imap38*-deletion can be transmitted to F1. Functionality of the ACN cassette and specifity of deletion *in vivo* was demonstrated before (Bunting *et al.*, 1999). An alternative way to generate knockout without germ line transmission is possible. After isolation of 39, X0 clones from targeted 40XY ES cells, the ES-cell derived males and females can be produced by tetraploid embryo complementation. The mice developed from such chimeric embryos are completely ES cell derived and after one step mating of 39, XO females with 40, XY males mice homozygous for mutation can be obtained (Eggan *et al.*, 2002). A disadvantage of this system is that the ES cell line must be younger as it would be necessary for traditional diploid embryo injection (Nagy, 1993). In addition, not every ES cell line can give rise to viable, fertile organisms consisting entirely of ES cell derived tissues (Nagy *et al.*, 1990).

4.3. Generation of the targeting construct

A targeting vector is designed to mutate a specific chromosomal locus. The targeting vector must contain homology arms, the sequences to be delivered into the locus and selection markers. Inside the homology arms recombination occurs, resulting in introduction of foreign sequence in the chromosome. Two types of the vector design can be used for targeting in mammalian cells, giving basis for all the other targeting constructs: replacement and insertion vectors (Torres & Kuhn, 1997). It was shown that an insertion vector can integrate at a 5- to 20-fold higher frequency than replacement vectors with the same homologous sequences (Hasty *et al.*, 1991). But this higher frequency of integration does not result in production of more specific integrants per total number of clones generated. Therefore, the screening procedure is more laborious. Homology arms in replacement vector correspond to regions flanking a targeted sequence (*imap38*). A minimum of 1 kb is necessary for correct and efficient targeting (Hasty *et al.*, 1992; Thomas *et al.*, 1992). Use of isogenic DNA for homology arms significantly improves the targeting efficiency (te Riele *et al.*, 1992). Also, efficiency of targeting significantly increases with increasing length of homology arms (Hasty *et al.*, 1991; Deng&Capecchi, 1992). For amplification of homology regions a polymerase mix with high level of specifity (Expand high Fidelity, Roche) was used. PCR amplification of C57BL/10 genomic DNA followed by partial sequencing was done to generate homology arms. Restriction and PCR analysis with partial sequencing was performed to verify the structure of vectors in the intermediate steps of pTV generation. The long homology arm was 6 kbp long to ensure specific recombination. The short homology arm was made of only 1 kbp to make PCR analysis of the specific integration possible. The high recombination efficiency of 1/120 to 1/70 observed in gene targeting of *imap38* serves as evidence that sequence of homology arms was correct and the length sufficient.

A single nucleotide mutation is enough to make Lox P site unsuitable for Cre recombinase (Langer *et al.*, 2002). The frequency of spontaneous mutagenesis in *E. coli* is relatively low in order of 10^{-10} per base per replication (Lindahl, 1993) but mutation can take place. After sequence of generated pTV was proven, LoxP sites were sequenced. Functionality of LoxP sites was tested *in vitro* through Cre mediated recombination. The same preparation of vector was taken in the first targeting experiment.

4.4. Generation of recombinant clones

It was decided to create *imap38* knockout mouse on a C57BL/6 genetic background. This is one of the common strains which are often used in malaria experiments due to the fact that it survives *P. chabaudi* malaria infection (Li *et al.*, 2001). This strain also shows *imap38* overexpression in response to malaria infection and also after clearance of infection (Krücken *et al.*, 1999). Therefore the C57BL/6–derived ES cell line Bruce4 (Kontgen *et al.*, 1993) was employed in targeting. C57BL/6 cell lines are known to be very karyotypic unstable, getting more often than the other cell lines chromosomal mutations. The criterion which definitively correlates with germ line transmission is the age of wild type ES cells taken for transfection. Optimally ES cell lines with less then 15 passages should be used for transfection (Longo *et al.*, 1997). The maximal germ line transmitting age for ES to the moment of injection cells is 32 passages. For parental wild type ES cells it corresponds to 26 passages to the moment of transfection (Fedorov *et al.*, 1997). Bruce4 cells used for targeting were 10 passages old, the clones of the first time targeting were 20 passages old, and the ones from the second were 18.

Common methods of DNA delivery in ES cells are electroporation (Hogan, 2002), retroviral transfection (Stewart *et al.*, 1985) and lipofection (Strauss and Jeanisch, 1992). Two rounds of targeting with two transfections each were performed during this work. Survival rates after electroporation were more then 60% according to plating efficiency controls. After first targeting the quantity of colonies surviving a positive/negative selection was extremely low: only 251 clones could be picked. Only 2 of them were positive for specific recombination and contained a single integrant. The second targeting resulted in normal cell density after selection. From 360 clones picked, 5 were suitable for injection. Such a difference can be explained by differences in DNA quality. First time QIAGEN maxi kit was used, second time Midi prep from Macherey Nagel. Photometrical characteristics of both preparations were equivalent, but any impurity could influence electroporation efficiency or recombination rate leading to a lower number of transformed colonies. Corresponding to the data of Matise *et al.* (2001), DNA isolated through some commercially available columns can be toxic to ES cells. They recommend double phenol-chlorophorm extraction after digestion and two times washing with 70% ethanol before electroporation. Before electroporation, pTV was linearized with SrfI.

Insufficient digestion was not the reason, because complete of digestion had been electrophoretically checked. The enzyme was removed by phenol-chloroform extraction; DNA was washed twice with ethanol. Any step in these manipulations could also play a role in decrease of transfection efficiency. However, the major organic contamination did probably not take place as time constants of electroporation which characterizes the electroporation process and depends heavily on electroporation buffer composition were normal (7.1 and 7.5 ms).

The first targeting resulted in only two clones. This low efficiency probably does not depend on position effects because the second round resulted in generation of higher number of targeted clones. Supposably poor DNA quality has led to drastic decrease in targeting efficiency. Therefore first targeting is not characteristic and the rate of specific integration was normal: 5 out of 360, keeping in mind rate of transfection lying between 1/120 and 1/70.

4.5. Recovery of embryos for injection

For embryo recovery young females were used. For different strains and animal facilities the optimal female age to obtain the maximum number of embryos differs slightly. Usually females at the age 3-8 weeks are mated. The necessary quantity of matings to recover enough embryos differs according to strain reproduction ability and type of mating. Natural matings require a bank of similar aged females. Normally the cyclus of females housed together is synchronized, so different cages with one or two days of oesterus cyclus difference should be prepared for this purpose. The stage of the oesterus cycle must be evaluated through examination of external vaginal changes. Considering this fact, natural matings are unattractive if a large number of embryos have to be obtained at a certain date. Superovulation permits to increase number of embryos per female. It was always performed in the experiments. This procedure includes sequential injection of PMSG and hCG leading to development of a large cohort of follicles. Hormone injections are followed by mating with trained males. Females which had not become pregnant in the first time were not used any more, because they need two weeks rest after superovulation to produce oocytes again. In addition, it is a fact that a second round of superovulation is usually unsuccessful because mice obviously become immune against foreign hormones (Joyner, 2001). The morphology of oocyte nuclei and

nucleoli was shown to be unusual after PMSG administration (Asseyet *et al.*, 1994). It was reported (Van der Auwera&D'Hooghe, 2001) that superovulation leads to delayed embryonic development *in vitro* and *in vivo*, an increased abnormal blastocysts formation, and pronounced fetal growth retardation. Another group mentions that the viability of oocytes obtained from superovulated mice does not differ from normal (Kovacs *et al.*, 1999). To enlarge harvest of blastocysts supplementary method was also used. Morulae were washed from the uterus on the afternoon of day 3 p.c. They were incubated overnight in KSOM medium (Erbach *et al.*, 1994) and on the following day 80-90% of them have been developed into blastocysts. Combination of superovulation with morula development *in vitro* has strongly increased the number of blastocysts available on the day of injection.

4.6. Production of chimeras

For delivery of ES cells into the mouse embryo several possibilities do exist. Nuclear transfer (McGrath *et al.*, 1983) of a single ES cell nucleus into one cell embryo is an even more laborious and less effective way than injection of blastocysts with ES cells. Regardless of the donor type, all the born clones suffer from serious phenotypic and gene expression abnormalities (Jeanisch *et al.*, 2002). This is related to both epigenetic changes of differentiated cells and high karyotypic and epigenetic instability of ES cells (Jeanisch *et al.*, 2002; Humpherys, 2001). However, clones obtained by nuclear transfer from ES cells in one cell embryos are significantly healthier than from adult cells (Jeanisch *et al.*, 2002).

Generation of chimeras that are able to transmit the mutated allele of ES cells through their germ line is the most common way. Generally, an inverse correlation between the extent of ES cell contribution and chimera viability exists with most ES cell lines (Joyner, 2001). Aggregation of ES cells with tetraploid embryo gives rise to fully ES cell derived embryos with tetraploid trophectoderm and primitive endoderm (Nagy *et al.*, 1990). Even fertile and genetically altered XO female were generated with this method (Eggan *et al.*, 2002). Unfortunately the overall success of such experiments is very low (Nagy *et al.*, 1993). Very often, the mice die shortly after birth. A possible reason for this is accumulation of genetic and epigenetic alterations in ES cell lines during culture. Another possibility is aggregation of ES cells with morula (Hogan, 2002). Contribution of ES cells to chimera is much higher as by

blastocysts injection due to the longer period during which they can proliferate and contribute into the inner cell mass after manipulation (Rossant&Spence, 1998). This results in higher lethality of chimeras due to higher ES cell contribution to tissues. This lethality therefore compensates higher chimeric embryos production efficiency. Morula aggregation is technically a less complicated method which does not require expensive equipment. It is a good alternative to blastocyst injection. However, overall efficiency of blastocyst injection is still higher. Since the experiments were performed in collaboration with lboratory of Molecular Immunology at University of Cologne and Transgene facility at MPI in Freiburg where this technique has already been established and commonly used, the decision was to perform blastocysts injection. ES cells quality is a critical factor for successful contribution of ES cells in germ line. ES cells must stay undifferentiated and have less than 50% cells with karyotypic abnormalities to contribute to germ line. The number of cells to be injected is an important factor to be mentioned. Due to the fact that the percentage of the cells contributing to the embryo cannot be predicted and quality of the ES cell population is inhomogeneous, it is difficult to determine how many cells have to be injected. Various opinions about this question exist. Usually in literature it is recommended to use 8-12 ES cells per blastocyst (Hogan, 2001; Schuster-Gossler *et al.*, 2001; Joyner, 2001). If the cells seems to be weak, it is possible to take more ES cells (up to 15) expecting that the weakest cells will be competed out by other ES and embryo cells. According to the experience of the transgenic service at the MPI Freiburg, injection of more than 5-7 cells leads to generation of chimeras with a large rate of chimerism which are less viable. Maybe this lower number of injected cells is only optimal for the 129- and Balb/c- derived ES cell lines usually used in MPI. The same number of Bruce4 cells for blastocysts injections was taken and some 40-60% chimeras were obtained with this modification of the method.

4.7. Choice of blastocysts

Due to up now unknown and uncharacterized reasons, injection of a certain ES cell line into blastocysts of different mice strains results in different chimera production efficiency and variable germ line transmission rates. CB20 blastocysts were proven to be compatible with the Bruce4 ES cell line. This strain has albino phenotype and definition of chimerism rate according to coat colour is possible. They

usually have a high reproductive potential. Shortly before the start of the experiments at the laboratory of Molecular Genetic (Cologne) some conditions of mice keeping were changed and only very few blastocysts were obtained for the first injections (Table 2). Later, for cost saving reasons, injections were performed in the MPI where a specialized animal facility and necessary equipment are available. At the MPI animal facility CB20 strain is not maintained at all. Balb/c embryos are known to be well compatible with C57BL/6 derived ES cells (Lemckert *et al.*, 1997; Hogan, 2001). However, the number of blastocysts obtained per mouse is very low and this strain is insensitive to hormonal treatment for superovulation (Pakrasi, 1991). More then 100 matings have to be set up to obtain enough blastocysts for the injection of only one clone. Deficit of personal and space in the animal facility made this experiment impossible. Albino strain FVB known to give extremely large harvest of embryos was also available at that moment. It was shown that although injection of C57BL/6 derived ES cell line R1 in FVB blastocysts resulted in generation of chimeras with high level of chimerism, only 1 of 14 chimeras was germ line transmitting. The same ES clone injected in c^{2J} blastocysts has given rise to 12 germ line transmitting chimeras (Schuster-Gossler *et al.*, 2001). MacGregor, (2002) reports that in both FVB<->C57BL/6 and C57BL/6<->FVB chimeras germ line was essentially FVB in composition. Reasons of such a strong selective advantage of FVB/N male germ cells over C57BL/6 remain unknown. 129-derived ES cell lines are known to be compatible with a number of random-bred mice (MF-1, CD-1, BlackSwiss) blastocysts. In combination with some C57BL/6-derived ES cell lines B6D2F1 blastocysts were successfully used. B6D2F1 is outbred F1 hybrid of C57BL/6 with DBA. This offers the possibility that outbred blastocysts can also be successfully used in combination with Bruce4. For two experiments (clones C5 and D7) NMRI mice were chosen. As outbred strain they have high reproduction potential and give a large number of viable blastocysts. NMRI mice are white and colour detection of chimerism is possible. In fact, four chimeras with rates of chimerism between 40-60% were obtained, but no black pup with the mutant *imap38* allele could be detected. So germ line transmission failed. In parallel outbred 129sv×C57BL/6 blastocysts were used. The strain 129sv is commonly used in combination with C57BL/6 in such experiments. The embryos have agouti phenotype and chimerism rate could not be determined from the coat colour. PCR and southern blot analysis of

129sv×C57BL/6<->Bruce4 mice gave no evidence of ES cell derived tissue. As far as only 50% of black-white chimeras with a high chimerism rate were positive in the PCR analysis and none was positive in southern, the probability that some of these 129sv×C57BL/6 derived mice are chimeric is still high. So all the 129sv×C57BL/6<->Bruce4 mice were mated. Until now no germ line transmission has been obtained. Possibly the blastocyst-derived outbred cells have better growth potential in comparison with outbred ES cells and they just contribute in embryo at higher level or are preferred during spermatogenesis. That is why inbred blastocysts were used in the following experiments. According to the Jackson Lab recommendations, a C57BL/6 derived mouse strain c^{2J} is an ideal candidate for injections. The only difference to C57BL/6 is a mutation in tyrosinase gene resulting in an albino phenotype on C57BL/6 background. So there is no reason to doubt about compatibility of this mouse strain with the Bruce4 cell line. The costs related with such injections are very high if the mice have to be bought. For this reason only one breeding pair was bought. During c^{2J} expansion, the clones A11 and E1 were injected in wild type C57BL/6 blastocysts. In this case no colour definition of chimerism rate or pups genotype is possible. This means that all mice obtained after injections have to be genotyped. Unfortunately C57BL/6 mice do not give good blastocysts yields from natural matings and plugged females do not always contain blastocysts (Hogan, 2002). More matings were set and superovulation was performed for this strain. PCR screening with several upstream primers in one tube allows saving time and reducing costs for this screening. C57BL/6 blastocysts leave no doubts about compatibility of ES cells-blastocysts system. All pups born from injections of clone A11 were rejected by their mothers. All six mice born from injections of clone E1 were killed by a mistake of a technician. Clone E1 was injected one more time in c^{2J} and C57BL/6 blastocysts. When expanding c^{2J} mouse strain, more then 60% of litters were usually males. At the appointment date only 11 six-week old females were available. They all were used in matings for embryo production. 45 embryos obtained from these females were injected. To use all c^{2J} embryos, morulae which were not developed in blastocysts after overnight incubation were also injected under zona pellucida. This technique was successfully used to produce ES cell chimeras, although it is known to give no clear advantage over blastocyst injection (Joyner, 2001). Unexpectedly, injections of clone E11 in c^{2J} blastocysts resulted in birth of only 4 mice which have low rate of

chimerism or even a wild type. The same clone injected in C57BL/6 blastocysts resulted in 20 healthy newborns. That allows speculation that lower rate of mice generated from injected blastocysts (8.8% for c^{2J} versus 33% for C57BL/6) is due to blastoysts quality or injection procedure.

4.8. Foster mothers and embryo transfer

To continue their development, injected embryos were transferred into pseudopregnant females. These pseudopregnant females were obtained after mating with trained vazectomized males, so that at the day of injection they displayed the hormonal profile of pregnant females. This allows acceptance of the transferred embryos. *In vitro* culture and manipulation have the effect of delaying the embryonic cell development (Van der Auwera&D'Hooghe, 2001). For this reason, 4 days old injected blastocysts were transferred into 3 days pseudopregnant foster mothers. The primary consideration by choosing foster mothers is the reproductive performance of the strain, including maternal behavior. The mice of outbred strains are usually taken because they are physically stronger and are better mothers. This means they are more tolerant to the unusual pups. Therefore, the risk that pups will be eaten is smaller than with inbred mothers. NMRI mice were always taken in experiments at MPI and F1 of CB20×Balb/c mating in University of Cologne. In both cases many chimeras were born and survived (Table 2).

4.9. Chimerism rate determination

There are several methods to determine chimerism rates. The easiest and most common way is to differentiate according to the coat colour. In this method evaluation of chimeric animals is necessarily subjective, but generally the degree of coat colour chimerism correlates with the degree of germ line contribution. Thus, it is awaited that chimeras with more than 40% of ES cell-derived black coat have good chances for germ line transmission (Thorres&Kuhn, 1997; Joyner, A, 2001). Spermatozoa recovered by uterine washing after mating with female mice can directly be analyzed with PCR for presence of targeted or deleted allele (Mann *et al.*, 1993). Another technique of chimerism detection is based on the isozyme differences between strains, such as the ubiquitous, dimeric enzyme glucose phosphate isomerase (GPI). GPI has three variants distinguishable by electrophoresis (Chapman *et al.*, 1972). Homozygous

animals show a single, homodimer band, while heterozygous have two homodimers for both alleles and a heterodimer which runs intermediate to the homodimers. Chimerism rate is usually determined through presence of two homodimers corresponding to ES cells and embryo cells. This method allows determining about 5% contribution of ES cells in the tissue (Joyner, 2001). For the described experiments this method was unfortunately not applicable, because diagnostic was desired for chimeras derived from heterozygous animals (129sv×C57BL/6) and in another case no difference existed between ES cells and embryos (C57BL/6). In other cases coat colour chimerism detection was possible.

4.10. Sex of chimeras and their fertility

Sex in mammals is determined through the sex of reproductive organs. In conclusion sex of chimeras is determined through sex of embryo and sex of ES cells and their proportion in reproductive organs. The sex of embryo is unknown whereas ES cell lines including Bruce4 are usually of male sex as XY cell lines are karyotypically more stabile. Female ES cell lines have the tendency to loose one X chromosome becoming karyotype XO. Females of such karyotype have reduced oocytes quantity (Burgoyne *et al.*, 1985). Another reason to use male ES cells is that female chimeras must first become pregnant to produce progeny. This requires 3 weeks; pups can be taken away from the mothers not before they are 4 weeks old. So the productivity of males which can be paired with several females during a short period of time is much higher. In case of male embryo there is no sex conflict with ES cells. In case of female embryo the sex of chimeras is determined by the quota of ES cells contributing to the gonad-forming tissues. Sex conversion is not always complete in XX<–>XY chimeras and sometimes results in infertile hermaphrodite or gynandromorph. Such organisms are usually sterile and can be discarded (Joyner, 2001). From injection of the same clone in NMRI blastocyst only one female was born. From injections of clone C5 into CB20×Balb/c blastocysts all together two hermaphrodites were born and discarded. All females and untypical males born from injections into C57BL/6 and C57BL/6×129sv were directly discarded.

4.11. Mutation maintaining scheme

To maintain the targeted allele different opportunities exist. Sometimes a random or genetically undefined background is sufficient for initial characterization of a mutation related phenotype. Then chimeras can be mated with mice strains which differ from ES cells strain. The offspring F1 will be much more vigour as the inbred one and will have better reproductive performance. From this point of view it is the best way to obtain animals homozygous for mutation soon and to maintain mutation. Later on backcrossing can be performed to transfer the allele to a certain inbred background. After ten generations of backcrossing the strain is 99.9% pure and considered congenic. Most of the donor strain is lost during the first few backcross generations. A strain is 93% pure after four generations and this can be enough for many purposes (Green, 1966). For later experiments on the function of *imap38* it is necessary to have a pure inbred background. First, phenotypic analysis is more straightforward due to less variability. Second, susceptibility to malaria and expression of *imap38* are strain-dependent. Mixing of background can lead to drastic changes in malaria infection appearance. No appropriate wild type controls in malaria experiments could be made for hybrid background. Pairing of chimeras with C57BL/6 inbred strain was the only way to delete the embryo genotype completely because for the further experiments it is necessary to obtain completely C57BL/6 derived knockout mice. Bruce4 cell line was first reported in 1993 (Kontgen *et al.*, 1993), cells taken in targeting were passage ten and it is improbable that cells have mutated much from the C57BL/6 genotype. Therefore C57BL/6 strain can still be considered as uniform genetic background for this cell line and can be taken in mating with Bruce4 chimeras to eliminate the embryo genotype completely.

4.12. Possible reasons of germ line transmission failure.

The population of ES cells of a single clone is usually very inhomogeneous. A clone which contains 50 – 100 % of normal metaphases contributes to germ line and is referred to as karyotypically normal (Longo *et al.*, 1997; Kondoh *et al.*, 1999). Chromosomal mutations happen very frequently in ES cells during culture. By simple genotyping of normal ES cells it was found that 1.7-2.7% of cells spontaneously loose their Y chromosome (Eggan *et al.*, 2002). If the cells acquire a trisomy 8, the mutant cell population gets a growth advantage in comparison with euploid cells. This leads

to selection of abnormal cells and depletion and eventual loss of euploid cells. ES cells with trisomy 8 grow fast but contribute rarely to the germ line (Liu *et al.*, 1997). Euploidy is gradually lost with increasing number of passages. It was found that ES cells older than 26 passages do not contribute to the germ line and the optimal age of parental ES cell lines is before 15 passages (Fedorov *et al.*, 1997), some outline the upper limit at 20 passages (Longo *et al.*, 1997). Other types of mutations can influence chimeras' fertility. Knocking out of *Ahch* gene, a mouse analogue of human *AHC* gene, results in disruption of spermatogenesis. Spontaneous duplication of the region Xp21 which harbours the *AHC* gene in human leads to development of female from 46XY individuals (Parker&Schimmer, 1998). If analogous duplication happens in ES cells, generated chimera will be sterile. Abnormal regulation of imprinted genes was shown to affect fetal growth (Bartholomei *et al.*, 1997; Reik *et al.*, 2001). But epigenetic instability of ES cells does not influence the routine generation of chimeric mice (Humpherys *et al.*, 2001; Labosky *et al.*, 1994). Differentiation of ES cells during culturing is one more common reason of germ line transmission failure. ES cells can differentiate during culturing because of medium composition, some physical and chemical factors, overgrowing of colonies, and too high colony density on the plate or asynchronous ES cells. Differentiated ES cells can usually be detected by a wrong colony form: not oval shaped colonies without distinct boundary, large, fibroblast-like boundary cells and large swimming ES cell aggregates occur. Sometimes differentiated cells have a right colony form and differentiation cannot be visually detected at all. For example, morphology of colonies for clone H2 was visually optimal, the cells were dealt in parallel with the other clones. The only difference was a slower growth from the 96-well plate. After preparation for injection the ES cells in suspension were larger as normal and less then 95% were shaped round. These symptoms are typical for differentiated cells. Therefore clone H2 was not taken for injection. DMSO is one of the agents leading to ES cell differentiation, so prolonged incubation of ES cells in freezing medium can initiate differentiation (Dinsmore *et al.*, 1996). Contrary to the common opinion that the ES cell differentiation is the main reason of germ line transmission failure, karyotype abnormalities really are the most frequent reason (Longo *et al.*, 1997). Mycoplasma contamination of ES cell culture can cause chromosome damage and reduce efficiency in obtaining chimeras (Joyner A., 2001). Moreover mycoplasma

contaminated ES cells even contributed to chimeras, give no germ line transmission (Robertson, 1987).

4.13. Outlook

At the moment none of the adult chimeras has given germ line transmission. The most probable germ line transmitting chimeras will be obtained from injections in c^{2J} and C57BL/6 blastocysts possessing equal genetic characteristics as Bruce4 ES cells. The existing chimeras should be mated untill loss of fertility or untill at least 50 pups have been analyzed, because there are known cases when the first heterozygous for desired mutation mouse appeared only in fifth litter.

If all chimeras obtained from all injections give no germ line transmission, it will be important to determine at which stage transfer of targeted or deleted allele fails. Targeted allele was identified in tail biopsy of black-white NMRI chimeras (Fig. 14B). This means that targeted allele participates in different tissues and is not lost. Presence of ES-derived sperm cells can be tested by PCR of the spermatozoa obtained by uterine washing directly after pairing with a female (Mann *et al.*, 1993). If *imap38*–deficient spermatozoa with deleted allele will be identified, chimera can be taken again in mating. First of all, chimeras should be mated with mice of outbred strain in hope to obtain a desired mutation on the vigour mixed background. If outbred heterozygous mice survive, conclusion can be made that C57BL/6 background is insufficient for *imap38* mutants' survival. In case when targeted or deleted allele is fixed in outbred strain, the mutation can later on be transferred in inbred strain by backcrossing (Green, 1966). If no *imap38*-deficient spermatozoa will be found, testis of several chimeras should be examined for the presence of targeted allele in any non-germ line tissues. This will clarify, if ES-derived cells were not contributed in testis at all or if *imap38* deficient allele was eliminated during spermatogenesis or from the germ line completely. *In situ* hybridization of chimeras' testis with ACN cassette-specific probe could give detailed information about the destiny of the targeted or deleted allele.

A little is known about *imap38* gene function. Expression of *imap38* is not limited to spleen cells. *imap38* expression was mentioned in thymus, lymph node, trachea of mice in large-scale transcriptome analysis (Su *et al.*, 2002; GSE97). Although it is supposed that *imap38* is not important for survival, a possibility that

gene is also important during embryonic development cannot be completely excluded. Different embryonic stages of chimeras' pups can be examined whether they possess deleted or targeted allele and possibly critical for survival stage can be determined. Inbred background can also influence phenotype and survival rate of chimeras' pups. Differences in mutant viability on different inbred backgrounds can vary strongly. For example in *mK8* gene targeting viability of mice on 129/sv or 129/sv×C57BL/6 background was 1.5%, while on the FVB/N background about 55% (Baribault *et al.*, 1994).

As homologous recombination in targeted locus is high and enough clones can be obtained from one round of electroporation, some other targeting experiments can be performed. It would be advisable to perform gene targeting one more time with another ES cell line of non C57BL/6 background. Alternatively, conditional targeting can be performed. Two ways could be rational for *imap38*. Induction of Cre recombinase expression by hormonal signal (Kellendonk, 1996) or drug (Corbel&Rossi, 2002) is not optimal as these stimuli can influence immune response in malaria experiments. Therefore, for *imap38* would be better to use Cre recombinase under control of tissue- or stage- specific promoter which turns excision only in desired tissue and/or in adult mice on (Lewandoski, 2001; Utomo *et al.*, 1999). Another reasonable opportunity would be to mate mice where *imap38* is just flanked by two loxP sites with different transgenic mice expressing Cre recombinase in different types of immune cells (Nagy lab, Cre transgenic database). In Nagy bank of Cre-expressing mice, mice with expression restricted to B cells and macrophages can be found. The array of mice deficient for *imap38* in different populations of T cells, B cells or macrophages would give rich basis for *imap38* function investigation and it's role in immune response (if any). It is also possible to generate ES cells homozygous for *imap38* deletion by increasing selection drug G418 concentration after transfection (Mortensen *et al.*, 1992). Introduction of a mutant *Neo* gene in targeting constructs permits dosage-dependent selection of hetero- or homozygous ES cell clones at lower G418 concentration (Yenofsky *et al.*, 1990). Obtained homozygous ES cell clones do still contribute to chimeras. The rate of clones with double specific integration would be very low, but obtained *imap38* deficient ES clones can be used for aggregation with tetraploid embryo. Such fusion results in generation of fetus where trophoblast and primitive endoderm are tetraploid and all tissues of developing mice come from

ES cells (Nagy *et al.*, 1990). Viability of such animals depends strongly on the ES cell line and its stability. However, cases are known that mice obtained in this way were even fertile (Eggan et al., 2002). If newborns are viable and fertile, knockout mouse is already generated. Otherwise, at least phenotype characterization of different embryonic stages and possibly even of adults can be performed. Recently, it was shown that mouse embryonic stem cells can develop into oogonia that enter meiosis, recruit adjacent cells to form follicle-like structures, and later develop into blastocysts. It has not been determined yet, if these blastocysts can develop into mice *in vivo*, and if the oocytes obtained in this manner can be fertilized and give rise to new organism (Hübner *et al.*, 2003). These data also leave room for hope that homozygous embryos bearing desired targeted mutation can probably be obtained directly from ES cell culture. Naturally, viability of such mice would be low. If the oocytes developed from ES clones containing desired mutation could be fertilized with normal sperm cells, then they could give rise directly to heterozygous animals. Spermatogonial cell lines able to differentiate *in vitro* and transmit their genotype to the progeny have been already established, but it is very difficult to transfect DNA into the primary cultures of spermatogonia and impossible to select them with drug *in vitro* (Feng *et al.*, 2002). Although retroviral transduction of primary male germ-line stem cells was successfully performed (Nagano *et al.*, 2001), it does not help to solve a problem of locus-specific foreign DNA integration in these cells. Apparently, this possibility to deliver targeting mutation in embryo needs a lot more time to be realized.

The best alternative to DNA excision is RNAi technology. It is much more gentle and less laborious than the classical approach. ES cell culture, chimera production and germ line transmission steps are completely excluded in this cost, time and work effective method. No mating steps are desired for this method. Post-transcriptional gene silencing is based on sequence-specific mRNA degradation with 21- or 22-nucleotide small interfering (si) RNAs (Zamore *et al.* 2000). These siRNAs are expressed under control of RNA polymerase III (PolIII) dependent H1- or U6-RNA gene promoters in culture of mammalian cells (Brummelkamp, 2002, Paddison, 2002). This degradation is very efficient and was successfully applied in mice (Tiscornia *et al.*, 2003; Voorhoeve *et al.* 2003). Generally, vectors expressing siRNAs are usually injected in pronuclei of fertilized oocytes. Mice developed from injected embryos express siRNAs in all cells. Recently, polymerase II-driven gene silencing

was shown in mice through expression of long dsRNA lacking cap and polyA tail in nucleus (Shinagawa and Shunsuke, 2003). This RNA cannot be transported from the nucleus into the cytoplasm. In this way, interferon response to long dsRNA in the cell is avoided. In the nucleus this dsRNA is processed into siRNAs which then moves into the cytosol to interact with mRNAs. In this way tissue-specific post-transcriptional gene targeting is possible. This means, that with the same work investment as for conventional knock-down a conditional *imap38* knock-down mouse can be created. Microinjection of siRNA-expressing vector in pronucleus of one cell embryo requires expensive equipment. For generation of transgenic mice linker based sperm-mediated gene transfer method (LB-SMGT) can be used. It enables the delivery of targeting vector in embryo during fertilization. The linker protein, mAbC (monoclonal antibody), is reactive to a surface antigen on sperm of pig, mouse, chicken, cow, goat, sheep and human. mAbC binds to DNA through ionic interaction allowing exogenous DNA to be linked specifically to sperm. After fertilization of the egg DNA was shown to be successfully integrated into the genome of pig and mouse offspring (Chang *et al.*, 2002).

5. Summary

The *imap38* gene was identified by differential screening and had previously been characterized in our laboratory. *Imap38* belongs to a family of AIG-like proteins, which were found in mice, rats, humans and plants. Mammal AIG-like genes are highly conservative in structure. Functions of other genes in the same family have not yet been studied in detail. *ian1* takes place in T-cell differentiation; *ian4* plays an important role during apoptosis in T-cells. *imap38* is supposed to be important in clearance of malaria infection. It was shown that *imap38* is induced during malaria infection and is still expressed at high level even 13 weeks after infection. Expression of *imap38* gene was determined in mouse spleen (in macrophages and B-cells, very low in T-cells) after infection with *Plasmodium chabaudi*. During P53-mediated apoptosis *in vitro* in a culture of mouse myeloid leukemic cell line LTR6, six-fold overexpression of *imap38* was detected. No stimuli led to induction of *imap38* in spleen cells *in vitro* or *ex vivo*. This work was performed to create an *imap38*-knockout mouse serving as a tool for *in vivo* investigation of *imap38* function. Investigation of *imap38* function can improve knowledge about immune response to malaria and make progress in antimalarial research. Mouse strain C57BL/6 was chosen as background for knockout, because it survives *P. chabaudi* malaria, becomes immune and expresses high levels of *imap38* during the acute phase and after clearance of the infection. *Imap38* transcript has a typical AIG-like gene structure consisting of two small exons and a last large exon separated from each other 2 and 1 kbp large introns. N-terminus of IMAP38 is encoded in 43 bp small part of the second exon, contains neither signalling sequences, nor functional domains. Deleting of the largest last exon encoding ATP-binding domain and transmembrane domain was the aim of the targeting, for which an improved classical targeting strategy was realized. Cre-mediated recombination was used to excise the resistance marker together with Cre recombinase gene during spermatogenesis. Targeting replacement type vector containing self-excision ACN cassette was constructed. Seven ES cell clones containing targeting allele were generated and injected in CB20, NMRI, C57BL/6, C57BL/6×129sv and c^{2J} blastocysts. 13 chimeras were obtained. From 241 chimeras' progeny analysed none possessed the deleted or targeted allele. Possible reasons and perspectives are discussed. An alternative approach in generation of *imap38* knockdown mice using RNAi might give a desired *imap38*-deficient mouse.

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7. References

Alves, H.J., Weidanz, W., Weiss, L. (1996). The spleen in murine *Plasmodium chabaudi adami* malaria: stromal cells, T lymphocytes, and haematopoiesis. *Am. J. Trop. Med. Hyg.* **55**, 370-378.

Amante, F.H., Good, M.F. (1997). Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. *Parasite Immunol.* **19**, 111-126.

Argos, P., Landy, A., Abremski, K., Egan, J.B., Haggard-Ljungquist, E., Hoess, R.H., Kahn, M.L., Kalionis, B., Narayana, S.V., Pierson, L.S. 3rd, et al. (1986). The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO J.* **5**, 433-440.

Assey, R.J., Hyttel, P., Roche, J.F., Boland, M. (1994). Oocyte structure and follicular steroid concentrations in superovulated versus unstimulated heifers. *Mol. Reprod. Dev.* **39**, 8-16.

Bairoch, A. (1991). PROSITE: a dictionary of sites and patterns in proteins. *Nucleic Acids Res.* **25**, 2241-2245.

Baribault, H., Wilson-Heiner, M., Muller, W., Penner, J., Bakhiet, N. (1997). Functional analysis of mouse keratin 8 in polyoma middle T-induced mammary gland tumours. *Transgenic Res.* **6**, 359-367.

Barry, R.G. (1992). Mountain weather and climate. *London: Routledge*.

Bartolomei, M.S., Tilghman, S.M. Genomic imprinting in mammals. (1997). *Annu. Rev. Genet.* **31**, 493-525.

Basu, A., Chakrabarti, G., Saha, A., Bandyopadhyay, S. (2000). Modulation of $CD11C^{+}$ splenic dendritic cell functions in murine visceral leishmaniasis: correlation with parasite replication in the spleen. *Immunology.* **99**, 305-313.

Beniston, M. (2002). Climatic change: possible impacts on human health. *SWISS MED WKLY.* **132**, 332–337.

Benten, W.P., Wunderlich, F., Herrmann, R., Kuhn-Velten, W.N. (1993). Testosterone-induced compared with oestradiol-induced immunosuppression against *Plasmodium chabaudi* malaria. *J. Endocrinol.* **139**, 487-494.

Bogue, C.W. (2003). Genetic Models in Applied Physiology. Functional genomics in the mouse: powerful techniques for unraveling the basis of human development and disease. *J. Appl. Physiol.* **94**, 2502-2509.

Boismenu, R., Havran, W.L. (1997). An innate view of gamma delta T cells. *Curr. Opin. Immunol.* **9**, 57-63.

Brannan, L. R., Turner, C. M., Phillips, R. S. (1994). Malaria parasites undergo antigenic variation at high rates *in vivo*. *Proc. R. Soc. Lond B. Biol. Sci.* **256**:71-75.

Brummelkamp, T.R., Bernards, R., Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science.* **5567**, 550-553.

Buchholz, F., Angrand, P.O., Stewart, A.F. (1998). Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat Biotechnol.* **16**, 657-662.

Buer, J., Balling, R. (2003). Mice, microbes and models of infection. *Nat. Rev. Genet.* **3**, 195-205.

Bunting, M., Bernstein, K.E., Greer, J.M., Capecchi, M.R., Thomas, K.R. Targeting genes for self-excision in the germ line. (1999). *Genes Dev.* **13**, 1524-1528.

Burgoyne, P.S., Baker, T.G. (1985). Perinatal oocyte loss in XO mice and its implications for the aetiology of gonadal dysgenesis in XO women. *J. Reprod. Fertil.* **75**, 633-645.

Cambot, M., Aresta, S., Kahn-Perlès, B., de Gunzburg, J. and Roméo, P.H. (2002). Human immune associated nucleotide 1: a member of a new guanosine triphosphate family expressed in resting T and B cells. *Blood.* **99**, 3293-3301.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* **6573**, 435-439.

Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G.J., Rosenquist, T.A. (2003). Germline transmission of RNAi in mice. *Nat. Struct. Biol.* **10**, 91-92.

Carter, R. and D. Walliker. (1975). New observations on the malaria parasites of rodents of the Central African Republic. *Ann.Trop.Med.Parasitol.* **69,**187-196.

Chang, K., Qian, J., Jiang, M., Liu, Y.H., Wu, M.C., Chen, C.D., Lai, C.K., Lo, H.L., Hsiao, C.T., Brown, L., Bolen, J. Jr., Huang, H.I., Ho, P.Y., Shih, P.Y., Yao, C.W., Lin, W.J., Chen, C.H., Wu, F.Y., Lin, Y.J., Xu, J., Wang, K. (2002). Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. *BMC Biotechnol.* **19**, 5.

Chao, T.C., Van Alten, P.J., Greager, J.A., Walter, R.J. (1995). Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cell. Immunol.* **160**, 43-49.

Chapman, V.M., Ansell, J.D., McLaren, A. (1972). Trophoblast giant cell differentiation in the mouse: expression of glucose phosphate isomerase (GPI-1) electrophoretic variants in transferred and chimeric embryos. *Dev. Biol.* **29**, 48-54.

Chui, D., Oh-Eda, M., Liao, Y.F., Panneerselvam, K., Lal, A., Marek, K.W., Freeze, H.H., Moremen, K.W., Fukuda, M.N., Marth, J.D. (1997). Alpha-mannosidase-II deficiency results in dyserythropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. *Cell.* **90**, 157-167.

Clamp, M., Andrews, D., Barker, D., Bevan, P., Cameron, G., Chen, Y., Clark, L., Cox, T., Cuff, J., Curwen, V., Down, T., Durbin, R., Eyras, E., Gilbert, J., Hammond, M., Hubbard, T., Kasprzyk, A., Keefe, D., Lehvaslaiho,

H., Iyer, V., Melsopp, C., Mongin, E., Pettett, R., Potter, S., Rust, A., Schmidt, E., Searle, S., Slater, G., Smith, J., Spooner, W., Stabenau, A., Stalker, J., Stupka, E., Ureta-Vidal, A., Vastrik, I., Birney, E. (2003). Ensembl 2002: accommodating comparative genomics. *Nucleic Acids Res.* **31**, 38-42.

Clyde, D. F. (1990). Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971–75. *Bull. World Health Organ.* **68**, 9–12.

Coffman, R.L., Lebman, D.A., Rothman, P. (1993). Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* **54**, 229-270.

Constant, S.L., Bottomly, K. (1997). Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu. Rev. Immunol.* **15**, 297-322.

Corbel, S.Y., Rossi, F.M. (2002). Latest developments and in vivo use of the Tet system: *ex vivo* and *in vivo* delivery of tetracycline-regulated genes. *Curr. Opin. Biotechnol.* **5**, 448-452.

Cox, J., Semoff, S., Hommel M. (1987). *Plasmodium chabaudi*: a rodent malaria model for *in vivo* and *in vitro* cytoadherence of malaria parasites in the absence of knobs. *Parasite Immunol.* **9,** 543-561.

Dahéron, L., Zenz, T., Siracusa, L.D., Brenner, C., Calabretta, B. (2001). Molecular cloning of *Ian4*: a BCR/ABL-induced gene that encodes an outer membrane mitochondrial protein with GTP-binding activity. *Nucleic Acids Research.* **29**, 1308-1316.

Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K., McMahon, A.P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **24**,1323-1326.

Deng, C., Capecchi, M.R. (1992). Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol. Cell. Biol.* **12**, 3365-3371.

Dinsmore, J., Ratliff, J., Deacon, T., Pakzaban, P., Jacoby, D., Galpern, W., Isacson. (1996). Embryonic stem cells differentiated *in vitro* as a novel source of cells for transplantation. *Cell Transplant.* **5**, 131-143.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A. Jr., Butel, J.S., Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature.* **6366**, 215-221.

Dymecki, S.M. (1996). Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc. Natl. Acad. Sci. U S A.* **93**, 6191-6196.

Egan, J.E., Hoffman, S.L., Haynes, J.D., Sadoff, J.C., Schneider, I., Grau, G.E., Hollingdale, M.R., Ballou, W.R., Gordon, D.M. (1993). Humoral immune responses in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. *Am. J. Trop. Med. Hyg.* **49,** 166–173.

Elloso, M.M., van der Heyde, H.C., vande Waa, J.A., Manning, D.D., Weidanz, W.P. (1994). Inhibition of *Plasmodium falciparum in vitro* by human gamma delta T cells. *J. Immunol.* **153**, 1187-1194.

Emanuelsson, O., Nielsen, H., Brunak, S., Gunnar von Heijne. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300**, 1005-1016.

Erbach, G.T., Lawitts, J.A., Papaioannou, V.E., Biggers, J.D. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol. Reprod.* **50**, 1027-1033.

Falkenroth, N. (2001): Molekulare Charakterisierung von *imap*-Genen der Maus. Diplomarbeit. Heinrich-Heine Universität, Düsseldorf.

Feinberg, A.P., Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Ana.l Biochem.* **137**, 266-267.

Feng, L.-X., Chen, Y., Dettin, L., Pera, R.A.R., Herr, J.C., Goldberg, E., Dym, M. (2002). Generation and *in vitro* differentiation of a spermatogonial cell line. *Science.* **297**, 392-395.

Foote, S.J., Burt, R.A., Baldwin, T.M., Presente, A., Roberts, A.W., Laural, Y.L., Lew, A.M., Marshall, V.M. (1997). Mouse loci for malaria-induced mortality and the control of parasitaemia. *Nat. Genet.* **17**, 380-381.

Fortin, A., Belouchi, A., Tam, M.F., Cardon, L., Skamene, E., Stevenson, M.M., Gros, P. (1997). Genetic control of blood parasitaemia in mouse malaria maps to chromosome 8. *Nat. Genet.* **17**, 382-383.

Friedrich, G., Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**, 1513-1523.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.-S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M.A., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Newbold, C., Davis, R.W., Fraser, C.M. and Barrell, B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature.* **419**, 498-511.

Gilks, C. F., Walliker, D., Newbold, C. I. (1990). Relationships between sequestration, antigenic variation and chronic parasitism in *Plasmodium chabaudi chabaudi*-a rodent malaria model. *Parasite Immunol.* **12**, 45-64.

Gondo, Y., Nakamura, K., Nakao, K., Sasaoka, T., Ito, K., Kimura, M., Katsuki, M. (1994). Gene replacement of the p53 gene with the lacZ gene in mouse embryonic stem cells and mice by using two steps of homologous recombination. *Biochem. Biophys. Res. Commun.* **202**, 830-837.

Goodier, M.R., Lundqvist, C., Hammarstrom, M.L., Troye-Blomberg, M., Langhorne, J. (1995). Cytokine profiles for human V gamma 9+ T cells stimulated by *Plasmodium falciparum*. *Parasite. Immunol.* **17**, 413-423.

Green, E. L. (1966). Biology of laboratory mouse. *McGraw-Hill Book Co., NY*, p.11.

Greenwood B. (2002). The molecular epidemology of malaria. *Tropic. Med. Internat. Health.* **7**, 1012-1021.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., Roncarolo, M.G. (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature.* **6652**, 737-742.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science.* **5168**, 103-106.

Gu, H., Zou, Y.R., Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell.* **73**, 1155.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A.B., Joyner, A.L. (1995). Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. *Science* **269**, 679-682.

Hasty, P., Rivera-Perez, J., Bradley, A. The length of homology required for gene targeting in embryonic stem cells. (1991). *Mol. Cell. Biol.* **11**, 5586-5591.

Hasuwa, H., Kaseda, K., Einarsdottir, T., Okabe, M. (2002). Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett.* **532**, 227-230.

Hay S.I, Cox J., Rogers D. J., Randolphß S. E.,Sternk D. I., Shanks G.D.¶, Myers M.F., Snow R.W. (2002). Climate change and the resurgence of malaria in the East African highlands. *Nature.* **415**, 905-909.

Helmby, H., Jonsson, G., Troye-Blomberg, M. (2000). Cellular changes and apoptosis in the spleens and peripheral blood of mice infected with blood-stage Plasmodium chabaudi chabaudi AS. *Infect. Immun.* **68**, 1485-1490.

Hirunpetcharat, C., Good, M.F. (1998). Deletion of Plasmodium berghei-specific CD4+ T cells adoptively transferred into recipient mice after challenge with homologous parasite. *Proc. Natl. Acad. Sci. U S A.* **95**, 1715-1720.

Hoffman, S.L., Oster, C.N., Plowe, C.V., Woollett, G.R., Beier, J.C., Chulay, J.D., Wirtz, R.A., Hollingdale, M.R., Mugambi, M. (1987). Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications. *Science.* **237,** 639–642.

Hoffman, S.L., Subramanian, G.M., Collins, F.H., Venter, J.K.. (2002). Plasmodium, human, Anopeles genomics and malaria. *Nature.* **415**, 702-709.

Hogan, B., Beddington, R., Costantini, F., Lacey, E. (2002). Manipulating mouse embryo: laboratory manual. *Cold Spring Harbor Laboratory Press.* 2nd edition.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.M.C., Wides, R., Salzberg, S.L., Loftus, B., Yandell, M., Majoros, W.H., Rusch, D.B., Lai, Z., Kraft, C.L., Abril, J.F., Anthouard, V., Arensburger, P., Atkinson, P.W., Baden, H., de Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chatuverdi, K., Christophides, G.K., Chrystal, M.A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, D.A., Flanigan, M., Grundschober- Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M.E., Hladun, S.L., Hogan, J.R., Hong, Y.S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J.-J., Lobo, N.F., Lopez, J.R., Malek, J.A., McIntosh, T.C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S.D., O'Brochta, D.A., Pfannkoch, C., Qi, R., Regier, M.A., Remington, K., Shao, H., Sharakhova, M.V., Sitter, C.D., Shetty, J., Smith, T.J., Strong, R., Sun, J., Thomasova, D., Ton, L.Q., Topalis, P., Tu, Z., Unger M.F., Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K.J., Wortman, J.R., Wu, M., Yao, A., Zdobnov, E.M., Zhang, H., Zhao, Q., Zhao, S., Zhu, S.C., Zhimulev, I., Coluzzi, M., della Torre, A., Roth, C.W., Louis, C., Kalush, F., Mural, R.J., Myers, E.W., Adams, M.D., Smith, H.O., Broder, S., Gardner, M.J., Fraser, C.M., Birney, E., Bork, P., Brey, P.T., Venter, J.C., Weissenbach, J., Kafatos, F.C., Collins, F.H., Hoffman, S.L. (2002), The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science.* **298**, 129-149.

Hornum, L., Romer, J., Markholst, H. (2002). The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. *Diabetes.* **51**, 1972-1979.

Hübner, K., Fuhrmann, G., Christenson, L.K., Kehler, J., Reinbold, R., De La Fuente, R., Wood, J., Strauss III, J.F., Boiani, M., Schöler, H.R. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science.* **300**, 1251-1256.

Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W.M. 3rd, Biniszkiewicz, D., Yanagimachi, R., Jaenisch, R. (2001). Epigenetic instability in ES cells and cloned mice. *Science.* **5527**, 95-97.

Jackson Lab: www.jax.org/index.html

Jacobs, P., Radzioch, D., Stevenson, M.M. (1996). A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. *Infect. Immun.* **64**, 535-541.

Jeanisch, R., Eggan, K., Humpherys, D., Rideout, W., Hochedlinger, K. (2002). Nuclear cloning, stem cells, and genomic reprogramming. *Cloning Stem Cells.* **4**, 389-396.

Jeanotte, L., Ruiz, J.C., Robertson, E.J. (1991). Low level of Hox1.3 gene expression does not preclude the use of promoterless vectors to generate a targeted gene disruption. *Mol. Cell. Biol.* **11**, 5578-5585.

Joyner, A.L. (2001). Gene targeting: a practical approach. *Oxford university press.*

Kannan, K., Kaminski, N., Rechavi, G., Jakob-Hirsch, J., Amariglio, N., Givol, D. (2001). DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of Apaf-1. *Oncogene.* **20**, 3449-3455.

Kellendonk, C., Tronche, F., Monaghan, A.P., Angrand, P.O., Stewart, F., Schutz, G. (1996). Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res.* **24**, 1404-1411.

Kilby, N.J., Snaith, M.R., Murray, J.A. (1993). Site-specific recombinases: tools for genome engineering. *Trends Genet.* **12**, 413-421.

Kim, D.G., Kang, H.M., Jang, S.K., Shin, H.S. (1992). Construction of a bifunctional mRNA in the mouse by using the internal ribosomal entry site of the encephalomyocarditis virus. *Mol. Cell. Biol.* **12**, 3636-3643.

Kondoh, G., Yamamoto, Y., Yoshida, K., Suzuki, Y., Osuka, S., Nakano, Y., Morita, T., Takeda, J. (1999). Easy assessment of ES cell clone potency for chimeric development and germ-line competency by an optimized aggregation method. *J. Biochem. Biophys. Methods.* **39**, 137-142.

Kontgen, F., Stewart, C.L. (1993a). Simple screening procedure to detect gene targeting events in embryonic stem cells. *Methods Enzymol.* **225**, 878-890.

Kontgen, F., Suss, G., Stewart, C., Steinmetz, M., Bluethmann, H. (1993b).Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. *Int. Immunol.* **5**, 957-964.

Kovacs, M.S., Lowe, L., Kuehn, M.R. (1993). Use of superovulated mice as embryo donors for ES cell injection chimeras. *Lab. Anim. Sci.* **43**, 91-93.

Krücken, J. (1999). Molekulare Charakterisierung und Expression des *Plasm o d iu m c h ab au d i*-induzierbaren *im ap 38*-Gens der Maus. Doktorarbeit. Düsseldorf.

Krücken, J., Schmitt-Wrede, H.-P., Markmann-Mulisch, U., Wunderlich, F. (1997). Novel gene expressed in spleen cells mediating acquired testosterone-resistant immunity to *Plasmodium chabaudi* malaria. *Biochem. Biophys. Res. Commun.* **230,** 167-170.

Krücken, J., stamm, O., Schmitt-wrede, H.P., Mincheva, A., Lichter, P., Wunderlich, F. (1999). Spleen-specific expression of the malaria inducible intronless mouse gene *imap38*. *J. Biol. Chem.* **274**, 24383-24391.

Kumar, S., Good, M.F., Dontfraid, F., Vinetz, J.M., Miller, L.H. (1989). Interdependence of CD4+ T cells and malarial spleen in immunity to *Plasmodium vinckei vinckei.* Relevance to vaccine development. *J. Immunol.* **143**, 2017-2023.

Kumar. S., Simons, J.P. (1993). The effects of terminal heterologies on gene targeting by insertion vectors in embryonic stem cells. *Nucleic Acids Res.* **21**, 1541-1548.

Labosky, P.A., Barlow, D.P., Hogan, B.L. (1994). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development.* **120**, 3197-3204.

Lakso, M., Sauer, B., Mosinger, B. Jr., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. U S A.* **89**, 6232-6236.

Langer, S.J., Ghafoori, A.P., Byrd, M., Leinwand, L. (2002). A genetic screen identifies novel non-compatible loxP sites. *Nucleic Acids Res.* **30**, 3067-3077.

Langford, K.G., Shai, S.-Y., Howard, T.E., Kovac, M.J., Overbeek, P.A., Bernstein, K.E. (1991). Transgenic mice demonstrate a testis-specific promoter for angiotensin-converting enzyme. *J. Biol. Chem.* **266,** 15559-15562.

Langhorne, J., Simon, B. (1989). Limiting dilution analysis of the T cell response to *Plasmodium chabaudi chabaudi* in mice. *Parasite Immunol.* **11**, 545-559.

Langhorne, J., Simon-Haarhaus, B., Meding, S.J. (1990). The role of CD4+ T cells in the protective immune response to *Plasmodium chabaudi in vivo*. *Immunol. Lett.* **25**, 101-107.

Lasonder E., Ishihama, Y., Andersen, J.S., Vermunt, A.M.W., Pain, A., Sauerwein, R. W., Eling M. C., Hall, N., Waters, A. P., Stunnenberg H.G., Mann, M. (2002). Analysis of the Plasmoium falciparum proteome by high-accuracy mass spectrometry. *Nature.* **419**, 537-542.

Lemckert, F.A., Sedgwick, J.D., Korner, H. (1997). Gene targeting in C57BL/6 ES cells. Successful germ line transmission using recipient BALB/c blastocysts developmentally matured *in vitro*. *Nucleic Acids Res.* **25**, 917-918.

Lewandoski, M. Conditional control of gene expression in the mouse. (2001). *Nature Reviews Genetics.* **2**, 743 -755.

Li, C., Corraliza, I., Langhorne, J. (1999). A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi chabaudi* infection in mice. *Infect. Immun.* **67**, 4435-4442.

Li, C., Seixas, E., Langhorne, J. (2001). Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. *Med. Microbiol. Immunol. (Berl.).* **189**,115-126.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature.* **6422**, 709-715.

Linke, A., Kuhn, R., Muller, W., Honarvar, N., Li, C., Langhorne, J. (1996). *Plasmodium chabaudi chabaudi*: differential susceptibility of gene-targeted mice deficient in IL-10 to an erythrocytic-stage infection. *Exp. Parasitol.* **84**, 253-263.

Lipman, D. J. and W. R. Pearson. (1985). Rapid and sensitive protein similarity searches. *Science.* **227,**1435-1441.

Little, J., Khoury, M.J., Bradley, L., Clyne, M., Gwinn, M., Lin, B., Lindegren, M.L., Yoon, P. (2003). The human genome project is complete. How do we develop a handle for the pump? *American J. epidemiology.* **157**, 667-673.

Liu, X., Wu, H., Loring, J., Hormuzdi, S., Disteche, C.M., Bornstein, P., Jaenisch, R. (1997). Trisomy eight in ES cells is a common potential problem in gene targeting and interferes with germ line transmission. *Dev. Dyn.* **209**, 85-91.

Long C.A., Hoffman S.L. (2002). Malaria-from infants to genomics to vaccines. *Science*. **297**, 345-347.

Longo, L., Bygrave, A., Grosveld, F.G., Pandolfi, P.P. (1997). The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. *Transgenic Res.* **6**, 321-328.

Lu, Z.H., Books, J.T., Kaufman, R.M., Ley, T.J. (2003). Long targeting arms do not increase the efficiency of homologous recombination in the beta-globin locus of murine embryonic stem cells. *Blood.* **102**, 1531-1533.

Mac Murray, A.J., Moralejo, D.H., KKKwitek, A.E., Rutledge, E.A., Yserloo, B.V., Gohlke, P., Speros, S.J., Snyder, B., Schaefer, J., Bieg, S., jiang, J., Ettinger, R.A., Fuller, J., Daniels, T.L., Pettersson, A., Orlebeke, K., Birren, B., Jaco, H.J., Lander, E.S., Lernmark,A. (2002). Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (*Ian*)-related gene. *Genome research.* **12**, 1029-1039.

MacGregor, G.R. (2002). An extreme bias in the germ line of XY C57BL/6<->XY FVB/N chimaeric mice. *Reproduction.* **124**, 377-386.

Mann, G.B., Fowler, K.J., Grail, D., Dunn, A.R. (1993). Identification of germ-line chimaeras by polymerase chain reaction and isoenzyme analysis of mouse spermatozoa. *J. Reprod. Fertil.* **99**, 505-512.

Mansour, S.L., Thomas, K.R., Deng, C.X., Capecchi, M.R. (1990). Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination. *Proc. Natl. Acad. Sci. U S A.* **87**, 7688-7692.

(2003). Malaria after genome. *Nature.* **421**, 97-98.

Marsh, K. & Snow, R. W. (1999). Malaria transmission and morbidity. *Parassitologia.* **41,** 241–246.

Martens, P., Kovats, R. S., Nijhof, S. (1999). Climate change and future populations at risk from malaria. *Global Iinv. Change.* **9**, 89-107.

McGrath, J., Solter, D. (1983). Nuclear transplantation in mouse embryos. *J. Exp. Zool.* **228**, 355-362.

Meding, S.J., Langhorne, J. (1991). CD4+ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi chabaudi*. *Eur. J. Immunol.* **21**, 1433-1438.

MGI (mouse Genome Informatics) web site http://www.informatics.jax.org/

Miller, L.H., Baruch, D.I., Marsh, K., Doumbo, O.K. (2002). The pathogenic basis of malaria. *Nature.* **415**, 673-679.

Mohan, K., Moulin P., Stevenson M. M. (1997). Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage *Plasmodium chabaudi* AS infection. *J Immunol.* **159**, 4990-4998.

Mohan, K., Stevenson, M.M. (1998). Interleukin-12 corrects severe anemia during blood-stage *Plasmodium chabaudi* AS in susceptible A/J mice. *Exp. Hematol.* **26**, 45-52.

Moore, K.W., O'Garra, A., de Waal Malefyt, R., Vieira, P., Mosmann, T.R. (1993). Interleukin-10. *Annu. Rev. Immunol.* **11**, 165-190.

Mordmuller, B.G., Metzger, W.G., Juillard, P., Brinkman, B.M., Verweij, C.L., Grau, G.E., Kremsner, P.G. (1997). Tumor necrosis factor in *Plasmodium falciparum* malaria: high plasma level is associated with fever, but high production capacity is associated with rapid fever clearance. *Eur. Cytokine Netw.* **8**, 29-35.

Mortensen, R.M., Conner, D.A., Chao, S., Geisterfer-Lowrance, A.A., Seidman, J.G. (1992). Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* **12**, 2391-2395.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348-2357.

Mossmann, H., Benten, W.P., Galanos, C., Freudenberg, M., Kuhn-Velten, W.N., Reinauer, H., Wunderlich, F. (1997). Dietary testosterone suppresses protective responsiveness to *Plasmodium chabaudi* malaria. *Life Sci.* **60**, 839-848.

Mota, M.M., Jarra, W., Hirst, E., Patnaik, P.K., Holder, A.A. (2000). *Plasmodium chabaudi*-infected erythrocytes adhere to CD36 and bind to microvascular endothelial cells in an organ-specific way. *Infect. Immun.* **68**, 4135-4144.

Mount, D.W. (2001). Bioinformatics. *Cold Spring Harbor Lab. Press.*

Müller, I.U. (2002). Molekulare Charakterisierung von *himap*-Genen. Diplomarbeit. Heinrich-Heine Universität, Düsseldorf.

Nagano, M., Ryu, B.Y., Brinster, C.J., Avarbock, M.R., Brinster, R.L. (2003). Maintenance of mouse male germ line stem cells in vitro. *Biol. Reprod.* **68**, 2207-2214.

Nagano, M., Ryu, B.Y., Brinster, C.J., Avarbock, M.R., Brinster, R.L. (2003). Maintenance of mouse male germ line stem cells *in vitro*. *Biol. Reprod.* **68**, 2207-2214.

Nagy lab, Cre transgenic database : http://www.mshri.on.ca/nagy/cre.htm

Nagy, A., Gocza, E., Diaz, E.M., Prideaux, V.R., Ivanyi, E., Markkula, M., Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development.* **110**, 815-821.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., Roder, J.C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U S A.* **90**, 8424-8428.

Nakai, K., Horton, P. (1999). PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **24**, 34-36.

Nielsen, H., Engelbrecht, J., Brunak, S., Gunnar von Heijne. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering.* **10**, 1-6.

Nussler, A., Drapier, J.C., Renia, L., Pied, S., Miltgen, F., Gentilini, M., Mazier, D. (1991). L-arginine-dependent destruction of intrahepatic malaria parasites in response to tumor necrosis factor and/or interleukin 6 stimulation. *Eur. J. Immunol.* **21**, 227-230.

Omer, F.M., Riley, E.M. (1998). Transforming growth factor beta production is inversely correlated with severity of murine malaria infection. *J. Exp. Med.* **188**, 39-48.

Orban, P.C,, Chui, D., Marth, J.D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. *Proc. Natl. Acad. Sci. U S A.* **89**, 6861-6865.

Paavonen, T. Hormonal regulation of immune responses. (1994). *Ann. Med.* **26**, 255-258.

Paddison, P.J., Caudy, A.A., Hannon, G.J. (2002). Stable suppression of gene expression by RNAi in mammalian cells. *Proc. Natl. Acad. Sci. U S A.* 99, 1443-1448.

Pakrasi, P.L. (1991). Pre-implantation embryo development in BALB/C ByJ mice. *Indian J. Exp. Biol.* **29**, 991-1001.

Pandarpurkar, M., Wilson-Fritch, L., Corvera, S., Markholst, H., Hornum, L., Greiner, D.L., Mordes, J.P., Rossini, A.A., Bortell, R. (2003). *Ian4* is required for mitochondrial integrity and T cell survival. *Proc. Natl. Acad. Sci. U S A.* **100**, 10382-10387.

Paunel, A. (2002), Charakterisierung von *imap*-Genen aus Maus und Mensch. Diplomarbeit. Heinrich-Heine Universität, Düsseldorf.

Pearson, W. R. and D. J. Lipman. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2444-2448.

Pelanda, R., Braun, U., Hobeika, E., Nussenzweig, M.C., Reth, M. (2002). B cell progenitors are arrested in maturation but have intact VDJ recombination in the absence of Ig-alpha and Ig-beta. *J. Immunol.* **169**, 865-872.

Phillips, R.L., Ernst, R.E., Brunk, B., Ivanova, N., Mahan, M.A., Deanehan, J.K., Moore, K.A., Overton, G.C., Lemischka, I.R. (2000). The genetic program of hematopoietic stem cells. *Science.* **5471**, 1635-1640.

Phillips, R.S., Mathers, K.E., Taylor-Robinson, A.W. (1994). T cells in immunity to *Plasmodium chabaudi chabaudi*: operation and regulation of different pathways of protection. *Res.Immunol.* **145**, 406-412.

Phillips, S. (1994). Effector mechanisms against asexual erythrocytic stages of *Plasmodium*. *Immunol. Lett.* **41**,109-114.

Podoba, J.E., Stevenson, M.M. (1991). CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage Plasmodium chabaudi AS. *Infect. Immun.* **59**, 51-58.

Poirier, G.M., Anderson, G., Huvar, A., Wagaman, P.C., Shuttleworth, J., Jenkinson, E., Jackson, M.R., Peterson, P.A., Erlander, M.G. (1999). Immune-associated nucleotide-1 (IAN-1) is a thymic selection marker and defines a novel gene family conserved in plants. *J. Immunol.* **163**, 4960-4969.

Poirier, G.M.C., Anderson, G., Huvar, A., Wagaman, P.C., Shuttleworth, J., Jenkinson, E., Jackson, M., Peterson, P.A., Erlander, M.G. (1999). Immune-associated nucleotide-1 (IAN-1) is a thymic selection marker and defines a novel gene family conserved in plants. *J. Immunol.* **163**, 4960-4969.

Reik, W., Walter, J. (2001). Evolution of imprinting mechanisms: the battle of the sexes begins in the zygote. *Nat. Genet.* **27**, 255-256.

Reuber, T.L. & Ausubel, F.M. (1996): Isolation of Arabidopsis genes that differentiate between resistance responses mediated by the RPS2 and RPM1 disease resistance genes. *Plant Cell.* **8,** 241-249.

Reuber, T.L., Ausubel, F.M. (1996). Isolation of Arabidopsis genes that differentiate between resistance responses mediated by the RPS2 and RPM1 disease resistance genes. *Plant. Cell.* **8**, 241-249.

Richie T.L., Saul, A. (2002). Progress and challenges for malaria vaccines. *Nature.* **415**, 694-701.

Ricke, C.H., Staalsoe, T., Koram, K., Akanmori, B.D., Riley, E.M., Theander, T.G., Hviid, L. (2000). Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. *J. Immunol.* **165,** 3309–3316.

Rieckmann, K. H., Beaudoin, R. L., Cassells, J. S., Sell, K. W. (1979).Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. *Bull. World Health Organ.* **57**, 261–265.

Rivolta, M.N., Halsall, A., Johnson, C.M., Tones, M.A., Holley, M.C. (2002). Transcript profiling of functionally related groups of genes during conditional differentiation of a mammalian cochlear hair cell line. *Genome Res.* **12**, 1091-1099.

Robertson, E.J. (1987). Teratocarcinomas and embryonic stem cells; A practical approach. *IRL Press at Oxford University Press*.

Rossant, J., Spence, A., Rossant, J. (1998). Chimeras and mosaics in mouse mutant analysis. *Trends Genet.* **14**, 358-363.

Sachs J., Malaney P. (2002) The economic and social burden of malaria. *Nature.* **415**, 680-685.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed., *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.*

Saraste, M., Sibbald, P.R., Wittinghofer, A. (1990). The P-loop--a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* **15**, 430-434.

Scharton-Kersten, T.M., Sher, A. (1997). Role of natural killer cells in innate resistance to protozoan infections. *Curr. Opin. Immunol.* **9**, 44-51.

Schipani, E. (2002). Conditional gene inactivation using cre recombinase. *Calcif. Tissue. Int.* **71**, 100-102.

Schmitt-Wrede, H.P., Fiebig, S., Wunderlich, F., Benten, W.P., Bettenhauser, U., Boden, K., Mossmann, H. (1991). Testosterone-induced susceptibility to Plasmodium chabaudi malaria: variant protein expression in functionally changed splenic non-T cells. *Mol. Cell. Endocrinol.* **76**, 207-214.

Schramke,V., Allshire, R. (2003). Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. *Science.* **5636**, 1069-1074.

Schroetel, R. (2002). Expression of *imap*-genes in human and mouse. Diplomarbeit. Heinrich-Heine Universität, Düsseldorf.

Schuster-Gossler, K., Lee, A.W., Lerner, C.P., Parker, H.J., Dyer, V.W., Scott, V.E., Gossler, A., Conover, J.C. (2001). Use of coisogenic host blastocysts for efficient establishment of germline chimeras with C57BL/6J ES cell lines. *Biotechniques.* **31**, 1022-1024, 1026.

Schwartzberg, P.L., Robertson, E.J., Goff, S.P. (1990). Targeted gene disruption of the endogenous c-abl locus by homologous recombination with DNA encoding a selectable fusion protein. *Proc. Natl. Acad. Sci. U S A.* **87**, 3210-3214.

Seixas, E., Cross, C., Quin, S., Langhorne, J. (2001). Direct activation of dendritic cells by the malaria parasite, *Plasmodium chabaudi chabaudi*. *Eur. J. Immunol.* **31**, 2970-2978.

Seixas, E.M., Langhorne, J. (1999). Gammadelta T cells contribute to control of chronic parasitemia in Plasmodium chabaudi infections in mice. *J. Immunol.* **162**, 2837-2841.

Shinagawa, T., Shunsuke, I. (2003). Generation of ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. *Genes&Development.* **17**, 1340-1345.

Sinden, R. E., Pudney, M., Marsh, K. (1989). The British Society for parasitology malaria. Malaria Meeting. *Parasitol. Today* 232 - 235.

Stamm, O., Kruecken, J., Schmitt-Wrede, H.P., Benten, W.P., Wunderlich, F. (2002). Human ortholog to mouse gene *imap38* encoding an ER-localizable G-protein belongs to a gene family clustered on chromosome 7q32-36. *Gene.* **282**, 159-167.

Stark, W.M., Boocock, M.R., Sherratt, D.J. (1992). Catalysis by site-specific recombinases. *Trends Genet.* **12**, 432-439.

Stevenson, M.M. (1989). Malaria: host response to infection.*CRC Press, Boca Raton.*

Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F., Abbondanzo, S.J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature.* **6390**, 76-79.

Stewart, C.L., Vanek, M., Wagner, E.F. (1985). Expression of foreign genes from retroviral vectors in mouse teratocarcinoma chimaeras. *EMBO J.* **30**, 3701-3709.

Strauss, W.M., Jaenisch, R. (1992). Molecular complementation of a collagen mutation in mammalian cells using yeast artificial chromosomes. *EMBO J.* **11**, 417-422.

Su, A.I., Cooke, M.P., Cing, K.A., Hakak, Y.H., Walker, J.R., Wiltshire, T., Orth, A.P., Vega, R.G., Sapinso, L.M., Moqrich, A., Patapoutian, A., Hampton, G.M., Schulz, P.G., Hogenesch, J.B. (2002). Large-scale analysis of the human and mouse transcriptomes. *Procl. Natl. Acad. Sci. USA.* **99**,4465-4470. (http://expression.gnf.org)

Taconic: http://www.taconic.com/anmodels/animlmod.htm#IDmice

Tatusova, T. A., Madden, T. L. (1999). BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett.* **174**, 247-250.

te Riele, H., Maandag, E.R., Berns, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc. Natl. Acad. Sci. U S A.* **89**, 5128-5132.

Thomas, K.R., Deng, C., Capecchi, M.R. (1992). High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. *Mol. Cell. Biol.* **12**, 2919-2923.

Thyagarajan, T., Totey, S., Danton, M.J., Kulkarni, A.B. (2003). Genetically altered mouse models: the good, the bad, and the ugly. *Crit. Rev. Oral. Biol. Med.* **14**, 154-174.

Tiscornia, G., Singer, O., Ikawa, M., Verma, I.M. (2003). A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci. U S A.* **100**, 1844-1848.

Torres, R.M. & Kuhn, R. (1997). Laboratory protocols for conditional gene targeting. *Oxford university press, Oxford.*

Urban, B.C., Ferguson, D.J., Pain, A., Willcox, N., Plebanski, M., Austyn, J.M., Roberts, D.J. (1999). *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature.* **6739**, 73-77.

Utomo, A.R., Nikitin, A.Y., Lee, W.H. (1999). Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nature Biotechnol.* **17**, 1091-1096.

Van der Auwera, I., D'Hooghe, T. (2001). Superovulation of female mice delays embryonic and fetal development. *Hum. Reprod.* **16**, 1237-1243.

Volkman, S. K., Hartl, D. L., Wirth, D.F., Nielsen, K.M., Choi, M., Batalov, S., Zhou, Y., Plouffe, D., Roch, K. G., Abagyan, R., Winzler, E.A. (2002). Excess polymorphisms in genes for membrane proteins in *Plasmodium falciparum*. *Science.* **298**, 216-218.

Von Stebut, E., Belkaid, Y., Jakob, T., sacks, D.L., Udey, M.C. (1998). Uptake of *Leischmania major* amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-leischmania immunity. *J. Exp.Med.* **188**, 1547-1552.

Voorhoeve, P.M., Agami, R. (2003). Knockdown stands up. *Trends Biotechnol.* **21**, 2-4.

Weiss, L. (1989). Mechanisms of splenic control of murine malaria: cellular reactions of the spleen in lethal (strain 17XL) *Plasmodium yoelii* malaria in BALB/c mice and the consequences of pre-infective splenectomy. *Am. J. Trop. Med. Hyg.* **41,** 144-160.

Weiss, L., Geduldig, U. and Weidanz, W. (1986). Mechanisms of splenic control of murine malaria: reticular cell activation and the development of a blood-spleen barrier. *Am. J. Anat.* **176,** 251-285.

Weiss, L., Johnson, J., Weidanz, W. (1989). Mechanisms of splenic control of murine malaria: tissue culture studies of the erythropoietic interplay of spleen, bone marrow, and blood in lethal (strain 17XL) Plasmodium yoelii malaria in BALB/c mice. *Am. J. Trop. Med. Hyg.* **41**, 135-143.

Wenisch, C., Parschalk, B., Burgmann, H., Looareesuwan, S., Graninger, W. (1995). Decreased serum levels of TGF-beta in patients with acute *Plasmodium falciparum* malaria. *J. Clin. Immunol.* **15**, 69-73.

WHO (2002). Report of the comission on macroeconomics and health organisation. *Geneva.*

Wunderlich, F., Marinovski, P., Benten, W.P., Schmitt-Wrede, H.P., Mossmann, H. (1991). Testosterone and other gonadal factor(s) restrict the efficacy of genes controlling resistance to *Plasmodium chabaudi* malaria. *Parasite Immunol.* **13**, 357-367.

Wunderlich, F., Mossmann, H., Helwig, M., Schillinger, G. (1988). Resistance to Plasmodium chabaudi in B10 mice: influence of the H-2 complex and testosterone. *Infect. Immun.* **56**, 2400-2406.

Wyler, D.J. (1983). Splenic functions in malaria. *Lymphology.* **16**, 121-127.

Wyler, D.J., Herrod, H.G., Weinbaum, F.I. (1979). Response of sensitized and unsensitized human lymphocyte subpopulations to *Plasmodium falciparum* antigens. *Infect. Immun.* **24**, 106-110.

Yap, G.S., Stevenson, M.M. (1994): Differential requirements for an intact spleen in induction and expression of B-cell-dependent immunity to *Plasmodium chabaudi AS*. *Infect. Immun.* **62**, 4219-4225.

Yenofsky, R.L., Fine, M., Pellow, J.W. (1990). A Mutant Neomycin Phosphotransferase II Gene Reduces the Resistance of Transformants to Antibiotic Selection Pressure. *Proc. Natl. Acad. Sci. U S A.* **87**, 3435-3439.

Zamore, P.D., Tuschl, T., Sharp, P.A., Bartel, D.P. (2000). RNAi:double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell.* **101**, 25-33.

8. Abbreviations

aa	amino acids
ACE	angiotensine converting enzyme
ACN	(Angiotensine converting enzyme, Cre, Neo) self-excision ACN cassette
Amp	ampiciline resistance gene
APC	antigen presenting cells
APS	ammonium peroxodisulfate
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
DC	dendritic cells
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EDTA	ethylene diaminetetraacetic acid
EF	embryonic feeder cells
EF N	embryonic feeder cells passage N (N=0, 1, 2, 3, 4)
ER	endoplasmatic reticulum
ES	embryonic stem (cells or medium)
F1	‘filial 1’ first generation
F2	‘filial 2’ second generation
FCS	fetal calf serum
g.l.t.	germ line transmission
G418	geneticine
GANC	gancyclovir
GTP	guanosine triphosphate
hCG	human horionic gonadotropin
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPTG	isopropylthiogalactoside
kbp	kilo base pairs
KO	knockout
LB-SMGT	linker based sperm-mediated gene transfer method
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
mAbC	monoclonal antibody C
MHC	major histocompatibility complex
min	minute (s)
MMC	mytomicin C
MPI	Max-Planck Insitute for Immunobiology in Freiburg, Germany
NAI	naturally acquired immunity
Neo^r	neomicine resistance gene
NK	natural killer cells
NO	nitric oxide
ORF	open reading frame
p.c.	post coitus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PMSG	pregnant mare serum gonadotropin
pTV	targeting vector for deletion of *imap38* containing ACN
RNA	ribonucleic acid
RNA	ribonucleotide acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rounds per minute
s	second (s)
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
SSC	saline-sodium citrate buffer
tACE	testis-specific promoter elements of angiotensine converting enzyme
TBE	Tris-Borat-EDTA buffer
TE	Tris-EDTA buffer
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TGF	transforming growth factor
TK	thymidine kinase
TNF	tumor necrosis factor
Tris	tris-(hydroxylmethyl) aminomethane
U	unit (s)
UV	ultra violet light

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