Aus dem

Max-Planck-Institut für Psychiatrie

Direktor: Prof. Dr. rer. nat. Dr. med. Florian Holsboer

Genetic Targeting of Cre Recombinase to the Murine ACTH Receptor Locus

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Vorgelegt von

Florian Riese

aus

Hannover

Jahr

2009

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. rer. nat. Dr. med. Florian Holsboer
Mitberichterstatter:	Prof. Dr. Rainer Rupprecht Priv. Doz. Dr. Christoph Auernhammer
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. rer. nat. Jan Deussing
Dekan:	Prof. Dr. med. Dr. h. c. M. Reiser, FACR, FRCR
Tag der mündlichen Prüfung:	19.03.2009

CONTENTS

1 Introduction

1.1 The Hypothalamic-Pituitary-Adrenocortical Axis

1.1.1 Overview of Function

All organisms strive towards maintaining their homeostasis, i.e. the dynamic equilibrium of their internal milieus that is essential for survival. The challenge of homeostasis by internal or external factors is classically referred to as “stress”. “Stress reaction” is the response of an organism to stress and spans from local biochemistry to global behavior. In mice and men, the sympathetic nervous system and the hypothalamic-pituitary-adrenocortical (HPA) system are the central regulators of the stress response (de Kloet et al., 2005). The HPA axis is formed by three main structures: the hypothalamus, the pituitary and the adrenal gland.

The hypothalamus is located below the thalamus along the walls of the third ventricle and has multiple roles in the maintenance of homeostasis. For the mediation of the stress response, parvocellular neurons from the paraventricular part of the hypothalamus are of crucial importance. They secrete corticotropin-releasing hormone (CRH, also CRF: corticotropin-releasing factor) into the pituitary portal vessels. The pituitary gland is situated in the sella turcica and consists of three lobes, an anterior, an intermediate and a posterior lobe. Upon CRH stimulation, endocrine cells from the anterior lobe of the pituitary release adrenocorticotropic hormone (ACTH) into the general circulation. With the blood flow, ACTH reaches the adrenal glands which are located on the upper pole of the kidneys. The adrenal glands comprise two organ compartments, the adrenal medulla and the adrenal cortex. While the medulla forms part of the sympathetic nervous system, the cortex is the body’s major source of steroid hormones. Of these, mineralocorticoids are synthezised in the glomerular layer, glucocorticoids in the fasciculate layer and adrenal androgens in the reticulate layer of the human adrenal cortex. ACTH stimulation results in release of adrenal glucocorticoids that are key mediators of stress response (Bear et al., 1996).

Glucocorticoids, of which cortisol is of most importance in humans, exert their pleiotropic effects on metabolism, the immune system and cognitive function by binding to cytosolic glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). These receptors regulate the transcriptional activity of genes by binding to glucocorticoid-response elements (GREs) in promoter regions. In contrast, rapid actions of glucocorticoids are transduced by membrane bound receptors (Dallman, 2005;Norman et al., 2004). In the brain, glucocorticoid-regulated genes are involved in neuronal metabolism, connectivity and synaptic transmission (de Kloet et al., 2005). The release of adrenal glucocorticoids is tightly controlled by negative feedback mechanisms within the HPA axis (see figure I1).

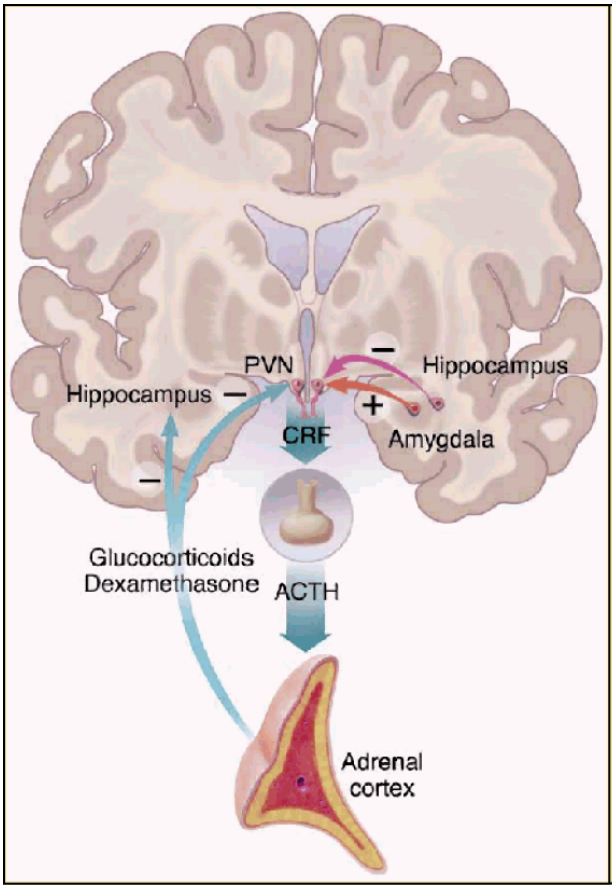

Figure I1: The classical model of HPA axis regulation.

CRF (also termed "CRH") is produced by neurons of the paraventricular nucleus of the hypothalamus. It reaches the pituitary via its portal vessel system and stimulates the release of ACTH. ACTH is released into the bloodstream and exerts its effects on adrenal glucocorticoid secretion.

In addition to many other effects, glucocorticoids and synthetic glucocorticoid receptor agonists such as dexamethasone inhibit CRF and ACTH synthesis and release, thereby constituting negative feedback control loops.

Not depicted are the direct influence of CRF on the adrenal bypassing the pituitary and the ultra-short feedback of glucocorticoids on the adrenal cortex.

Figure from Nestler et al., 2002

While structure and function of the HPA axis are essentially the same in mouse and human, the mouse lacks expression of steroid 17α-hydroxylase (CYP17) in the adrenal, an enzyme essential for the generation of cortisol and androgens. Therefore the main glucocorticoid in the mouse is not cortisol but corticosterone and adrenal androgens are not synthezised (Parker and Schimmer, 2001). How the HPA axis and

more specifically, how the adrenal cortex and adrenal glucocorticoids, are involved in the pathophysiology of depression will be described in the following section.

1.1.2 The HPA Axis and Depression

Major depressive disorder (MDD) is a chronic, recurring and potentially life-threatening illness. According to the WHO world health report 2002 it accounts for 4.4 percent of the total overall disease burden, which is similar to the contributions of ischemic heart disease or diarrheal diseases (Mann, 2005). Besides being a highly disabling condition in itself, MDD patients have an increased risk of death from suicide, accidents, respiratory disorders, stroke and heart disease. Conversely, effective treatment of depression lowers the risk of suicide and improves the outcome after stroke and acute myocardial infarction (Mann, 2005). From the psychiatric as well as the somatic medical perspective, adequate treatment for depression therefore seems highly indicated. However, current treatment options are still not optimal as the molecular pathology of depression remains largely unknown despite extensive research efforts. Amongst others, contributions of several neurotransmitter systems including the monoaminergic system, neurotrophic factors, the endogenous opioid and cannabinoid systems and several neuropeptides have been demonstrated (Ebmeier et al., 2006) as well as differences in intracellular signaling cascades (Manji et al., 2001) up to the recent discussion of a possible role for chromatin organization (Berton and Nestler, 2006). However, by far the most robust and well-studied somatic changes in depression are changes in HPA hormone secretion.

Morphologically, an increase in adrenal volume as measured by computed tomography has been reported in depressed patients which was reversible by antidepressant-treatment (Nemeroff et al., 1992;Rubin et al., 1995). Paraclinical evidence for a feedback impairment in HPA axis regulation comes from abnormalities in a variety of functional neuroendocrine tests such as the combined dexamethasone suppression/CRH stimulation test (Holsboer, 2000;Holsboer, 2001). Indeed, HPA axis hyperreactivity was even found to be a predictor for relapse into a depressive episode within the six months following hospital discharge (Zobel et al., 1999).

Recently, the use of the GR antagonist mifepristone in the treatment of psychotic depression has been successfully tested in a small randomized, placebo-controlled, double-blinded study, providing additional clinical evidence for the importance of HPA signaling in depression (Flores et al., 2006).

On the level of preclinical research, multiple mouse models with either disrupted or increased expression of key HPA axis molecules have been developed to experimentally elucidate HPA axis functioning (Keck et al., 2005). These models have become increasingly more sophisticated and have reached the level of tissue specificity. An example for such an approach with respect to glucocorticoid signaling is the mouse model of conditional gene disruption of the glucocorticoid receptor (GR) in the forebrain. Even though the GR is left intact in the hypothalamus and the pituitary, GR disruption in forebrain in this model nonetheless leads to impaired negative feedback regulation of the HPA axis and increased depression-like behavior in behavioral paradigms (Boyle et al., 2005). Forebrain specific GR overexpression on the other hand results in increased anxiety-like behavior and an overall increased emotional lability (Wei et al., 2004).

A direct involvement of the adrenal gland in the pathology of depression can therefore be deduced both from abnormalities in cortisol secretion in patients as well as from findings in animal models. Notably, regulation of adrenal glucocorticoid secretion is more complex than postulated in the classical model, where cortisol is released upon ACTH stimulation (see figure I1) (Ehrhart-Bornstein et al., 1998). It is now known that glucocorticoids enhance the expression of steroidogenic enzymes in the adrenal constituting an ultra-short feed-forward loop (Feltus et al., 2002). On the other hand, corticosterone release after ACTH administration is dependent on CRH signaling via CRHR1, as was shown in CRHR1 knock-out mice (Muller et al., 2001;Timpl et al., 1998). The latter findings directly point toward the existence of an intra-adrenal regulatory CRH system.

In conclusion, the adrenal is receiving increasing attention in psychiatry and somatic medicine especially as evidence about the connection between major depressive disorder and somatic illnesses such as coronary heart disease is accumulating (Joynt

et al., 2003). As glucocorticoids exert their effect on a multitude of central as well as peripheral targets, they could represent the missing link between the different pathologies (Brown et al., 2004). Deepening our understanding of function and regulation of the central steroid-hormone-producing organ, the adrenal gland, will therefore be of great value. For this purpose, constructs for the germline-transmissable genetic manipulation of mice are generated in this thesis and applied to the mouse, that allow advanced functional genetic experiments in the murine adrenal. How mice contribute to our knowledge of psychiatric disorders will be outlined in the following section.

1.2 Genetic Mouse Models in Psychiatric Research

The existence of a genetic component in the pathogenesis and maintenance of psychiatric disorders has long been recognized from twin and adoption studies. Nowadays, genetic linkage analysis even allows the precise identification of genetic loci involved in a pathology (Inoue and Lupski, 2003). In combination with information on the human genome sequence this represents an invaluable resource for understanding human disease processes. It is however for technical and ethical reasons impossible to systematically manipulate genes in humans. In order to learn about human gene function we must therefore take advantage of model organisms such as the mouse. Mice in somatic medical and psychiatric research allow a variety of experiments including genetic engineering and behavioral testing. Compared to invertebrate model organisms, mice show a much wider range of social and emotional behaviors that are essential for the understanding of psychiatric illness. Genetically engineered mice can be rigorously tested to study the effects of a genetic mutation on the animal's behavior, cognition, physiology and response to pharmacological agents (Bucan and Abel, 2002;Seong et al., 2002).

As fellow mammals, mice and humans possess a similar anatomy and physiology. Furthermore, genomic analysis indicates that a mouse gene equivalent, an orthologue, exists for about 99,5% of human disease genes, amongst which the ones with neurological function exhibit the highest grade of evolutionary conservation

(Huang et al., 2004). In addition, murine genes are often located on the chromosomes in a syntenic manner, i.e. in regions with the identical chromosomal arrangement of genes as in humans. This high level of genetic homology underlines the theory that humans differ from other mammals rather by the complexity of gene regulation than by the number or composition of their genes.

A putative gene function is usually deduced from a phenotype that derives from either excessive or abolished function of the gene of interest, i.e. from either gain-of-function or loss-of-function experiments. In principle, this genetic approach offers much higher specificity than the classical pharmacological experiments based on small-molecule compounds as single genes are precisely targeted. From the experimenters point of view, the availability of inbred mouse strains that diminish genetic background heterogeneity on experimental read-out variables has proven particularly useful (Tecott, 2003). Furthermore, mice are excellent experimental animals as their reproductive rate is high and their physical size is low, allowing the maintenance of large groups of animals (Sung et al., 2004). Finally, the online availability of the complete mouse genome sequence permits convenient experimental design and bioinformatic comparison with the human genome sequence.

A variety of different mouse models have been established revolutionizing our understanding of the pathophysiology of many diseases. In psychiatry, the range of models covers the entire spectrum of disease. Phenotypes mirroring such diverse entities as Brunner's syndrome (Brunner et al., 1993;Cases et al., 1995), severe language disorder (Lai et al., 2001;Shu et al., 2005), trichotillomania (Greer and Capecchi, 2002) and narcolepsy (Chemelli et al., 1999) could be generated by modification of single genes in the mouse. Moreover, complex syndromatical disorders like substance dependence, schizophrenia and depression have also been modelled with great success (for reviews see (Cryan and Holmes, 2005;Cryan and Mombereau, 2004;Ellenbroek, 2003;Nestler, 2000;Seong et al., 2002).

Although mice serve as valid models for many aspects, they cannot possibly mimic all the complex facettes of human psychiatric disease. Above all, the common

multifactorial genesis of many psychiatric disorders, involving environmental variables exerting their effects on the background of a complex genetic susceptibility, can so far only be partly accounted for. Current rodent models are therefore best used to examine certain disease sub-features, so called “endophenotypes”, i.e. measurable biological traits that are associated with target behavioral phenotypes (Hasler et al., 2004).

Modelling DSM-IV symptoms of major depressive disorder in mice	
Symptom	**Possible modelling paradigm**
Markedly diminished interest or pleasure in everyday activities (anhedonia)	Reduced intracranial self-stimulation or ad-libitum sucrose intake
Marked changes in appetite or weight	Abnormal loss of weight after exposure to chronic stressors
Insomnia or excessive sleeping	Abnormal sleep architecture in the EEG
Psychomotor agitation or slowness of movement	Alterations in locomotor activity and motor function
Fatigue or loss of energy	Reduced activity in home cage, tread mill running activity or nest building
Indecisiveness or diminished ability to think or concentrate	Deficits in working or spatial memory and impaired sustained attention
Difficulty performing even minor tasks, e. g. leading to poor personal hygiene	Poor coat condition during chronic mild stress
Depressed mood	Cannot be modelled
Recurrent thoughts of death or suicide	Cannot be modelled
Feelings of worthlessness or excessive or inappropiate guilt	Cannot be modelled

Table 1: Modelling DSM-IV symptoms of major depressive disorder in mice (adapted from Cryan and Holmes, 2005)

As table 1 shows, mice lack some of the unique human functions such as awareness of one’s self or suicidality, whose impairments form integral parts of psychiatric

disorders, or as Keck and colleagues put it "… it is now very unlikely that we will ever be able to diagnose a rodent according to the algorithms given by ICD-10 or DSM-IV" (Keck et al., 2005). When compared to rodents, disparities in cortical and other neural structures enable humans to react with a wider range of behavior in a given situation and may also increase their adaptive capacity towards a genetic modification. On the other hand, the neurobehavioral consequences of certain genetic mutations may be more readily detectable in humans due to the availability of self-report data and the stringent functional requirements of our highly complex human society (Tecott, 2003). In conclusion, despite species-inherent limitations, genetic mouse models have proven highly useful for somatic medical and psychiatric research. How the mouse genome can be manipulated for research purposes and what strategies were chosen for this thesis will be described in the next section.

1.3 Manipulating the Mouse Genome

1.3.1 The Phenotype-Based Approach

The historic roots of selective mouse breeding for desired phenotypes such as specific coat colors date back to 18th century China and Japan where collectors held mice as pets. This custom was adopted in Europe and imported mice from the Far-East were bred to local mice. In the beginning of the 20th century, pioneers of mouse genetics, such as Castle and Little, recognized the applicability of the Mendelian laws for coat color inheritance in the mouse. Their increasing demand for laboratory mice led to the establishment of the first mouse-breeding farm by Abbie Lathrop in Granby, Massachusetts. The mouse inbred lines founded by Lathrop are derived from crosses of imported East-Asian "fancy" mice and European mice and represent the origin of most strains of modern inbred laboratory mice (Wade and Daly, 2005).

Today, the phenotype-based approach of manipulating the mouse genome as in the past still relies on the same principles of selection and interbreeding of mice with particular phenotypes of interest. It is then however complemented by a genetic analysis in order to identify the genetic modifications contributing to the observed phenotype. The LAB-M and HAB-M mice are one example for this strategy in

psychiatric research. These mice were selectively bred for either low- or high-anxiety-related behavior which led to the discovery of glyoxalase-I as protein marker in trait anxiety (Kromer et al., 2005). On a larger scale, the phenotypic approach is pursued by n-ethyl-n-nitrosourea (ENU) mutagenesis projects. ENU treatment induces random genomic point mutations at a high rate. ENU-mice that show a phenotype of interest are selectively bred and the genetic locus involved is mapped (Keays and Nolan, 2003). ENU mutagenesis enabled the functional characterization of the clock gene which is fundamental for circadian rhythmicity (Vitaterna et al., 1994). Clock also forms part of a group of genes in which certain polymorphisms are associated with seasonal affective disorder (Johansson et al., 2003). It also modifies exploratory behavior in mice, a parameter indirectly reflecting anxiety (Easton et al., 2003). Interestingly, it has recently been shown by the more elaborate approach of cre/lox mediated site-directed mutagenesis that even in the absence of clock certain circadian systems remain intact (Debruyne et al., 2006). This example points to the crucial importance of tissue-specific gene disruptions to study gene function as will be discussed later.

1.3.2 Transgenic Mice

The term “transgenic mouse” as employed in this work refers to a mouse that is derived entirely from the successive cell divisions of a fertilized one-cell egg into which foreign DNA is introduced by microinjection (Palmiter et al., 1982). The foreign DNA constructs usually consist of a gene of interest linked to promoter and regulatory sequences that direct the spatial and temporal pattern of transgene expression. After microinjection, integration of foreign into host DNA occurs at random. The manipulated oocytes are surgically transferred into the uteri of foster mothers, which give birth to offspring entirely derived from the engineered oocyte. As the resulting transgenic mice carry the genetic modification in every cell including their germ cells, they can subsequently be used as founding breeders to transmit the genomic modification into the next generations (Wells and Murphy, 2003). Notably, integration by transgenesis is an additive process: The host genome gains new information. Therefore transgenesis is per se a more feasible technique to conduct gain-of-function rather than loss-of-function experiments. One of many prominent

examples for the transgenic approach to model a human neuropsychiatric disease was the generation of the first mouse model for the polyglutamine expansion disease Huntington's chorea (Mangiarini et al., 1996). The generation of transgenic mutations for all existing genes is currently pursued by "gene trapping" through large international consortia (Schnutgen et al., 2005).

A limitation of the transgenic approach is the essentially random integration into the genomic DNA of the recipient egg. Therefore the undesired disruption of a native gene may confound the interpretability of the resulting phenotype. However, the random nature of transgene integration may as well lead to serendipitous insights into the function of unintentionally mutated genes as in the case of the MAOA gene (Cases et al., 1995). Multiple transgene integration as head-to-tail repeats in a single integration site is a second caveat. Depending on the number of integrants this will result in differing expression levels. Gene dosage is furthermore affected by the sequence environment of the integration site: If integration takes place in a highly transcribed genetic region, it is likely that the level of transgene transcription is also high and vice versa (al-Shawi et al., 1990). Moreover, completely unexpected tissue patterns of transgene expression may arise due to local modulators of transgene promoter activity depending on the site of integration (Tan, 1991). Such positional and copy number effects can be minimized by using bacterial artificial chromosomes (BACs) or similar transgene constructs as presented in this work (Heintz, 2001).

1.3.3 Gene Targeting

"Gene targeting" refers to DNA integration via homologous recombination between a specifically designed targeting construct and a DNA sequence of interest. The development of mouse gene targeting in the late 1980s by the groups of Evans, Smithies and Capecchi marks an enormous extension of the tool set for genetic manipulations. Gene targeting allows the precise integration of an engineered genetic construct into a predetermined position of the mouse genome. This technique has been most frequently used to generate "knock-out" mice, i.e. mice in which the function of an endogenous gene is selectively disrupted by the insertion of an exogenous construct.

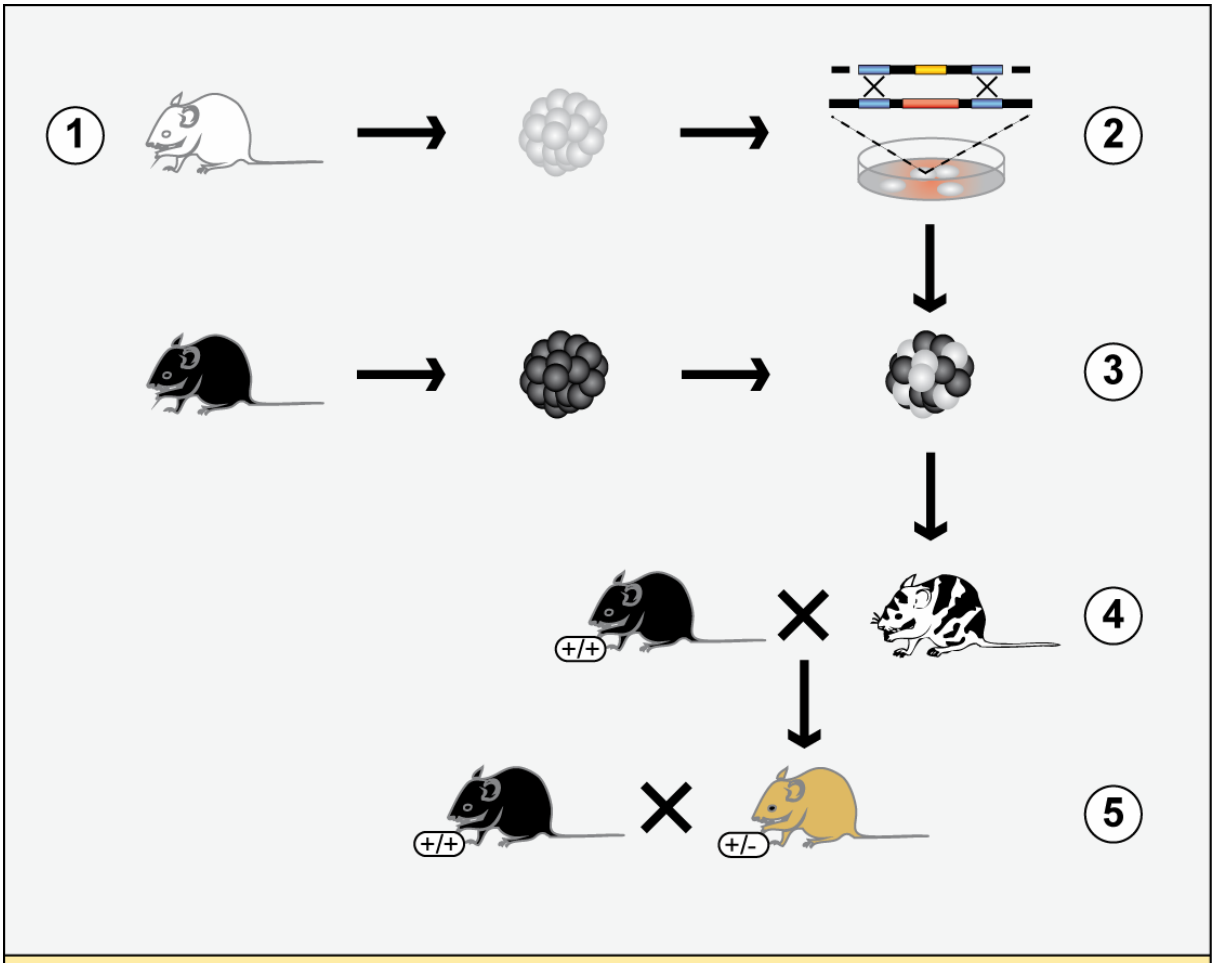

Figure I2: Gene targeting overview. 1) Murine embryonic stem cells are derived from a mouse with a determined coat colour. 2) A linearized targeting vector is electroporated into the stem cells. It contains sequences homologous to the wild-type host genome enabling homologous recombination between the targeting vector and one host chromosomal allele. 3) After selection for correct integration, the manipulated stem cells are aggregated with the morula of a mouse of a different coat colour. Alternatively, blastocyst injection of ES cells can be performed (not depicted). 4) The hybrid morula is transplanted into a foster mother that gives birth to a chimera, a mouse whose tissues are partly derived from wild-type, partly from recombinant stem cells. The amount of contribution of the two types can be estimated from the coat colour, as the fur is also derived from both sources. In case of germline transmission, crossing the chimera with a wild-type mouse (+/+) will result in 50% offspring that is heterozygous for the introduced mutation. All heterozygous mice of the F1 generation are agouti colored as alleles from both parental strains are present in all cells and therefore create an intermediate coat color phenotype. 5) To minimize strain influences on the phenotype, heterozygous mutant mice are then bred with wild-type mice for several generations. To obtain homozygous mutant mice, heterozygous mutant mice are interbred, which will result in 25% homozygous offspring (not depicted).

First step of the gene targeting procedure is the incorporation of a genetic construct into murine embryonic stem cells (ES cell) by exposure to an electric pulse. Once inside the nucleus of the ES cell, the external DNA is integrated into the specific genomic locus by the homologous recombination machinery of the ES cells. Stem cell clones with the desired mutation are selected and microinjected into wildtype mouse blastocysts or aggregated into wildtype morulae where they form mosaic embryoblasts, that in part derive from wildtype cells, in part from engineered cells.

These mosaic blastocysts are then transplanted into pseudo-pregnant mice that give birth to mosaic animals, commonly termed “chimeras”. In case the germ cells of these chimeras derive from the mutant cells, they can be used as founding breeders. Half of their offspring will be heterozygous for the desired mutation in every cell of their organism (see figure I2).

In our group, gene targeting was successfully used to elucidate the function of corticotropin releasing hormone receptor 1 (CRHR1). In this experiment, CRHR1 function was disrupted by replacing CRHR1 exons 5-7 by a neomycin resistance cassette. The resulting animals, devoid of CRHR1 in their entire organism, show pronounced reduction of ACTH and corticosterone release following stress, differences in adrenal morphology and reduced anxiety-related behavior (Smith et al., 1998;Timpl et al., 1998). Another classical example of gene targeting in psychiatric research are the presenilin-1 deficient mice generated by Shen and colleagues modelling a familial form of Alzheimer’s disease (Shen et al., 1997).

Besides the generation of such null mutations by introducing selectable marker cassettes, gene targeting permits the introduction of more subtle changes, such as point mutations as the wildtype alleles are being specifically replaced by an external DNA. With their α_1H101R mice, for example, McKernan and colleagues could show that the replacement of a single amino acid in the α_1 subunit of the $GABA_A$ receptor abolishes the sedative but not the anxiolytic effect of diazepam (McKernan et al., 2000). Such a “knock-in“ strategy even allows the introduction of genes that are entirely unrelated to the targeted wildtype gene as is in this work where a cre recombinase encoding gene is inserted into the adrenocorticotropin receptor locus. The cre transgene will therefore take advantage of the wildtype regulatory elements at the targeted locus, resulting in an expression pattern thought to equal the expression pattern of the receptor for adrenocorticotropic hormone.

As in the classic transgenic animals described earlier, a limitation of mice generated by gene targeting is that the introduced genetic modifications are present and possibly active from conception onwards, through embryonic development into adulthood. As a consequence, a resulting phenotype either reflects the adult function

of the gene or its role during development or a combination of both. In the most drastic case, an embryonic lethal phenotype of a targeted gene may preclude the study of a phenotype in the adult animal. As a consequence, “conditional” or “inducible” mutagenesis was developed, which permits initiation of transgene expression in a specific sub-population of cells and at specific time-points during or after embryonic development (Wells and Murphy, 2003).

1.3.4 Conditional Control of Gene Expression

“Conditional” and “inducible” control of gene expression, i.e. control in a cell-type-specific and/or time dependent manner, can be achieved either on DNA sequence level or on the level of RNA transcription. Employing these techniques, the tissue-specific function of genes can be studied independent of confounding effects of possible gene functions in other tissues. Transcriptional transactivation relies on either the lac operon (Scrable, 2002) or tetracycline (tet) based systems (Gossen et al., 1995) and is reversible as the DNA sequence itself remains unaltered. A recent example for the application of the tet system to conditionally model a psychiatric disease is the generation of mice overexpressing the dopamine receptor D2 in the striatum (Kellendonk et al., 2006), mimicking the single photon emission computed tomography (SPECT) observation of increased striatal D2 receptor occupancy in schizophrenic patients (bi-Dargham et al., 2000). Amongst other phenotypes, these mice have defects in working memory, a cognitive parameter associated with prefrontal cortex function, which is known to be impaired in schizophrenic patients. On DNA level, conditional control of gene expression is realized by the application of site-specific DNA recombinases such as cre, flp or ΦC31. Of these, cre recombinase has found by far the most widespread use in the mouse and is also used in this thesis (Branda and Dymecki, 2004;Kwan, 2002;Lewandoski, 2001;Nagy, 2000;Sorrell and Kolb, 2005;Thyagarajan et al., 2001).

1.4 The Cre/Lox System

1.4.1 Overview of Function

Cre/lox is a site-directed bacterial recombination system that enables DNA sequence modifications to be restricted to particular cell types and times of onset (Lewandoski, 2001;Nagy, 2000). Cre ("causes recombination") derives from phage P1 and belongs to the integrase family of site-specific DNA recombinases. Without the need for additional co-factors, cre recombinase catalyzes recombination between two of its recognition sites termed loxP sites ("locus of crossing over"). The enzyme recognizes the 34-bp loxP site (Hamilton and Abremski, 1984) by binding to its 13-bp inverted repeat elements that flank an 8-bp asymmetric core sequence (see figure I3).

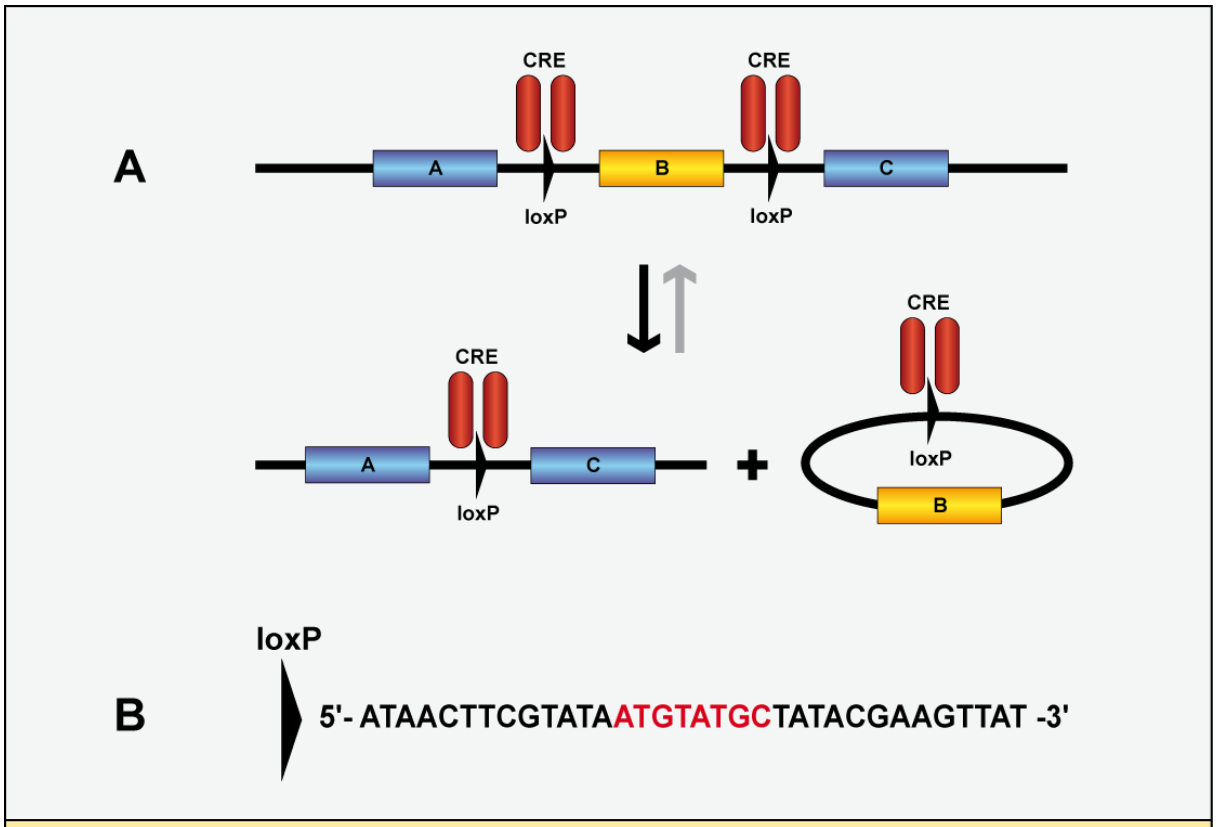

Figure I3: Mechanism of cre recombination A) Schematic representation of cre mediated DNA recombination between two loxP sites of the same orientation. In principle, the recombination strives towards a reaction equilibrium and is therefore reversible (indicated by arrows). However, as a consequence of the loss of the circular reaction product, the excision of the floxed sequence is irreversible (A,B and C represent exons of a fictive genomic locus). B) LoxP site, palindromic sequences in black, unidirectional core sequence in red.

Besides the classic loxP sites, several other cre recognition sequences have been identified (e.g. lox511, lox71) that are functionally similar to loxP (Langer et al.,

2002;Soukharev et al., 1999). The loxP core sequence is not involved in cre binding, but is the site of crossing over and provides directionality by its asymmetrical nature. Although cre-mediated recombination between two loxP sites in the same orientation is essentially a reversible process, it will effectively result in the deletion of the intervening sequence due to the loss of the circular reaction product (see figure I3). The binary cre/lox system therefore allows genetic manipulation with spatial precision as a recombination event can only take place in cells where both, cre protein and cre targeting sequences, are present. This robust, binary DNA recombination system has thus far been utilized in a variety of species, tissues and cell-types. The recent report on successful cre-mediated recombination in human embryonic stem cells furthermore points to a possible role in future gene-therapy approaches (Nolden et al., 2006).

1.4.2 Applications in Mouse

Originally applied to the mouse by Orban and colleagues (Orban et al., 1992) the cre/lox system initially found widespread use for the removal of selectable marker cassettes in classical knock-out strategies. These selectable marker cassettes influence the expression of genes at distances even greater than 100 kb (Pham et al., 1996). Meanwhile, the cre/lox system has also been successfully used to address a vast variety of other questions in mice including the effects of conditional gene inactivation (Gu et al., 1994) or gene over-expression (Lakso et al., 1992) and genomic rearrangement (Yu and Bradley, 2001). Recently, the cre/lox system was furthermore combined with the shRNA approach to generate inducible reductions of gene expression in mice (Yu and McMahon, 2006).

To achieve conditional gene inactivation by the cre/lox system in the mouse, an essential part of the gene of interest is loxP flanked (“floxed”) by gene targeting, ideally leaving the gene expression before cre-mediated recombination at wild-type level. The mouse line carrying the floxed allele is then interbred with a “cre-provider” line, i.e. a mouse line that was separately engineered to show a defined cre recombinase activity pattern. In their offspring, the floxed allele will be excised in the cells where cre recombinase is active (Nagy, 2000). Crossing one mouse line with a

floxed gene of interest to various cre lines with different activity profiles therefore elegantly allows to independently study the diverse functions of a single gene in multiple tissues. Conveniently, the activity and expression profiles of a large number of currently available cre lines are accessible through the online database at *http://nagy.mshri.on.ca/PubLinks/indexmain.php* (Nagy and Mar, 2001).

Using this approach, our group revealed the function of CRHR1 in limbic brain structures by generating a mouse line in which the CRHR1 gene was selectively disrupted in the anterior forebrain including the amygdala and striatum. For this purpose, mice were generated in which exons 9-13 of the CRHR1 were flanked by loxP sites and crossed with mice expressing cre recombinase under the control of the Camk2a promoter (Casanova et al., 2001). In this case, cre expression and subsequent CRHR1 deletion is restricted to the above-mentioned brain structures. As the pituitary gland is exempt from CRHR1 deletion, it could be shown that CRHR1 modulates anxiety-related behavior independent of HPA axis function (Muller et al., 2003).

1.4.3 Cell-type Specific Cre Expression

To provide cell-type specifity to cre recombinase as in the above-mentioned example of the forebrain specific Camk2a cre line, cell-type specific promoters are used to drive cre expression. However, adequate and well-defined, already-cloned promoters that would guarantee the desired expression pattern are not available for all cell-types. This problem can be circumvented using a knock-in strategy as in this thesis. The cre recombinase open reading frame (ORF) for this purpose replaces the ORF of an endogenous gene. For adrenocortical specificity, the endogenous gene encoding the receptor for adrenocorticotropic hormone (ACTHR) was chosen. As only the ORF of an endogenous gene is precisely replaced, all regulatory elements, such as promoter, enhancer and silencer elements, that normally restrict the expression of the endogenous gene to a certain cell-type will be left fully functional. Expression of an inserted, exogenous gene is therefore thought to mimic the expression of the endogenous gene.

A second way to ensure the presence of all regulatory sequence elements that are required for a cell-type specific expression of an exogenous gene is the use of large DNA vectors (e.g. BACs, cosmids etc.) as expression cassettes. This strategy was pursued in the second part of the thesis with the generation of a ACTHRcre cosmid construct. BACs (and cosmids) carrying most sequences of interests are readily available and can be used either for transgene generation via microinjection or for gene targeting in mouse ES cells (Liu et al., 2003). An additional benefit of BAC transgenes is that they are less prone to effects of chromosomal integration site on the levels of transgene expression, so called position effects. This is most likely due to the size of flanking DNA which is much larger than in traditional transgene constructs (Branda and Dymecki, 2004;Copeland et al., 2001;Heintz, 2001).

1.4.4 Inducible Cre Expression

While using the classical cre/lox system already permits cell-type specific genetic manipulations, the control over the temporal dimension of cre expression was added by the generation of inducible cre variants first reported by Kühn and colleagues in 1995 (Kuhn et al., 1995). To this end, several design strategies have been pursued, most of which are based on the fusion of cre recombinase to a steroid receptor ligand binding domain (LBD). These strategies take advantage of the nuclear localization capability of steroid receptor LBDs when bound to their ligand. The cre-LBD fusion protein is translated in the endoplasmatic reticulum and will remain in the cytoplasm, since the LBD forms a complex with heat shock protein 90 (hsp90) which precludes translocation into the nucleus. After binding the ligand, the cre-LBD/hsp90 complex dissociates, enabling nuclear translocation of cre. Only then cre mediated DNA recombination can occur, as cre and target DNA are now located in the same subcellular compartment (see figure I4) (Lewandoski, 2001).

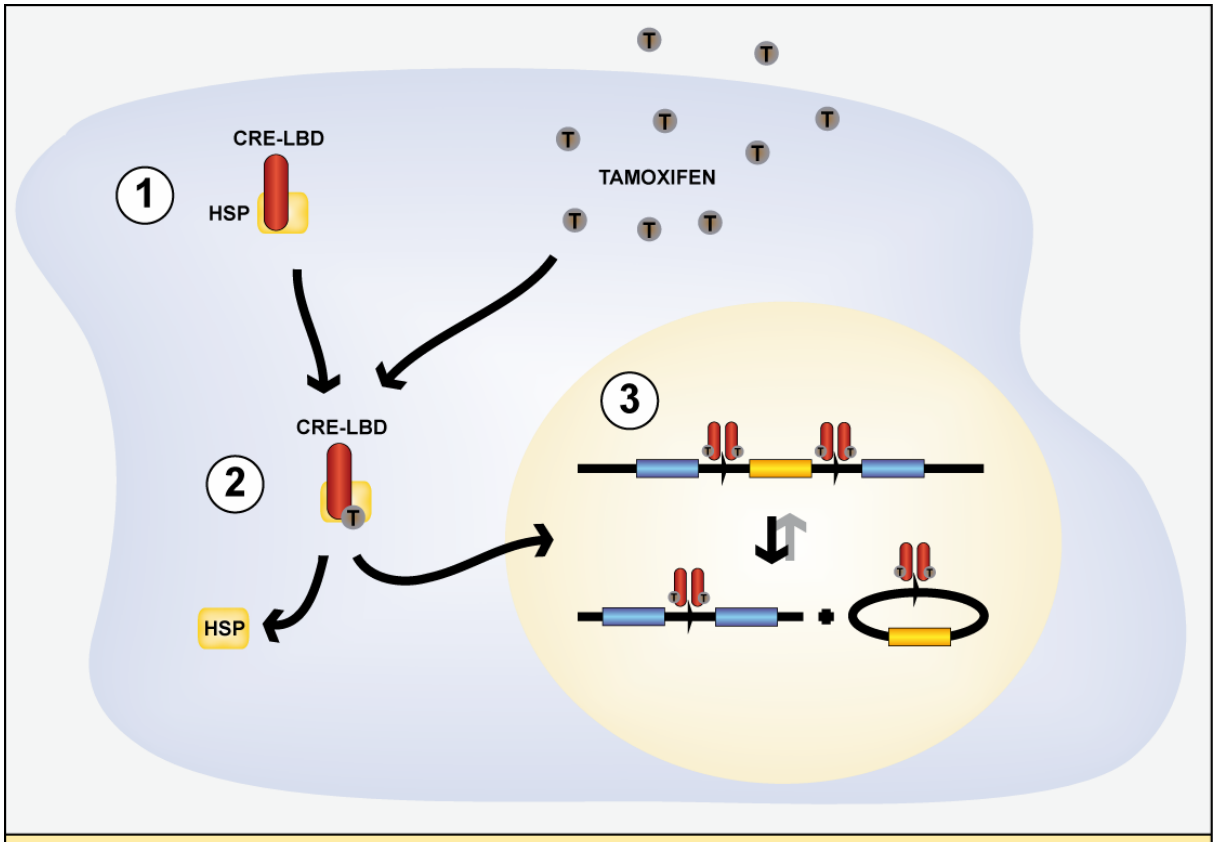

Figure I4: Induction of cre mediated recombination by tamoxifen 1) After translation in the endoplasmatic reticulum, the cre recombinase/steroid receptor ligand binding domain fusion-protein is trapped in the cytoplasm by formation of a complex with heat shock protein 90 (HSP). 2) Only upon exogenous tamoxifen administration the complex undergoes a conformational change and dissociates allowing nuclear translocation of CRE-LBD. 3) Once inside the same subcellular compartment as the target DNA sequences, cre-mediated recombination occurs.

A particularly successful strategy to achieve inducibility exploits the mutated LBD of the estrogen receptor fused to the c-terminus of cre recombinase (Brocard et al., 1997;Feil et al., 1996;Metzger et al., 1995;Schwenk et al., 1998). This mutated LBD does not bind endogenous steroids but synthetic ligands exclusively, lowering background activity induced by endogenous estrogen. CreERT2, first described by Feil and colleagues (Feil et al., 1997), is a cre recombinase fused to the human estrogen receptor LBD with three point-mutations (G400V/M543A/L544A). These point mutations render the LBD insensitive to estrogen binding, but allow a very high inducibility following tamoxifen administration even at comparatively low doses (Casanova et al., 2002;Feil et al., 1997).

For induction of recombination, tamoxifen, a selective estrogen receptor modulator widely used in the therapy of breast cancer (Jordan, 2003), can be administered either systemically or topically as shown by Vasioukhin and colleagues, who used tamoxifen-releasing dermal patches (Vasioukhin et al., 1999). Danielian and colleagues demonstrated that cre induction could also be achieved during embryonic development by tamoxifen administration to the mother (Danielian et al., 1998). The optimal amount and length of tamoxifen treatment for recombination induction varies depending on the cre line and the experimental question and has to be established specifically. Thus far, the CreERT2 variant has been expressed under a variety of tissue specific promoters resulting in specific expression in mouse skin (Indra et al., 1999), smooth muscle (Kuhbandner et al., 2000), adipose tissue (Imai et al., 2001), bone (Kim et al., 2004), glia cells (Leone et al., 2003) (Mori et al., 2006) (Hirrlinger et al., 2006) and melanocytes (Yajima et al., 2006).

1.4.5 Pitfalls of the Cre/Lox System

A number of inherent limitations have to be taken into consideration when using the cre/loxP system in the mouse. First of all, cre mediated deletion will not lead to a rapid onset phenotype as the cre sequence needs to be transcribed and translated. The resulting cre protein will excise the target sequence in a stochastic, time dependent manner and degradation of mRNA and protein of the target gene will require further time. However, as the recombination event is a once-for-all event, deletion frequency will accumulate over time (Nagy, 2000). This may result in decreased cell-type specificity of recombination over time as observed in the first brain-specific cre line, the CaMKIIα-cre line. Their cre activity was initially described to be specific for hippocampal CA1 cells but was later found to extend to additional forebrain regions (Fukaya et al., 2003;Tsien et al., 1996). A further point of note is that mosaic cre expression in various cells of the same tissue will result in a compound phenotype which will in part arise from cells in which recombination has taken place and in part from cells where cre is not active. This appears of particular importance in the case of secreted molecules like hormones (Lewandoski, 2001).

A major concern to the usefulness of inducible cre variants is the so-called “leakyness”, i.e. the cre background activity in the absence of the inducing molecule which could result in recombination at undesired timepoints. Unwanted nuclear translocation is hypothesized to be secondary to proteolytic cleavage of the LBD from the recombinase (Zhang et al., 1996) or secondary to alternative splicing of the cre-LBD RNA (Wunderlich et al., 2001). The tamoxifen-inducible CreERT2 used in our strategy has a very favourable ratio of background activity to inducibility as compared to other inducible cre variants (Indra et al., 1999) including the codon-usage improved variants iCreERT2 and ERiCreER (Casanova et al., 2002).

With respect to the loxP sites, several issues require attention: First, it is widely assumed that loxP flanked alleles exhibit wild-type expression levels of the floxed gene (Nagy, 2000). Xu and colleagues however reported a 70% reduction of floxed TrkB expression as compared to wildtype mice levels in the absence of cre recombinase (Xu et al., 2000). Furthermore, recombination frequency may be influenced by the position of the floxed locus within the genome (Vooijs et al., 2001). Concerns also arise from the existence of endogenous pseudo-loxP, i.e. degenerate loxP sites, that have been found in various genomes including the mouse genome (Thyagarajan et al., 2000). Consequently, DNA damage through recombination between pseudo-loxP sites has been observed in cultured mouse cells when cre was expressed at high levels (Loonstra et al., 2001). Furthermore, infertility of male mice due to cre expression in spermatids has been demonstrated for the same reason (Schmidt et al., 2000). However, even in the light of the above-mentioned limitations, the general robustness and feasibility of the cre/loxP system for mouse genetics can be clearly deduced from the multitude of reports on its use in a variety of tissues including peripheral organs and the brain (Morozov et al., 2003). How the cre/lox system is applied for use in the mouse adrenal cortex will be described in the following section.

1.5 Applying the Cre/Lox System to the Adrenal Cortex

Thus far, neither a constitutively active nor an inducible cre mouse line have been reported that would specifically be feasible for DNA recombination in the adrenal

cortex. There are, however, several examples of cre lines originally designed for specificity to other tissues that exhibit activity in the adrenal gland such as the αGSU-cre (Cushman et al., 2000), the TH-cre (Lindeberg et al., 2004), the PSA-cre (Ma et al., 2005) and the INHA-iCre (Jorgez et al., 2006). All of these lines though, do not express cre in the adrenal cortex but in the adrenal medulla. Alternatively, expression in the adrenal cortex is extremely low and cursory, while expression in other tissues is high. A truly “adrenocortical” cre mouse line therefore would be complementary to the lines yet in existence and allow for the reliable and independent dissection of adrenal gene function in its two major compartments, cortex and medulla. How cre expression can be restricted to the adrenal cortex will be outlined in the following section.

1.5.1 The Receptor for Adrenocorticotropic Hormone

In the experimental design presented in this work, cre expression is restricted to adrenocortical cells by the endogenous regulatory elements of the receptor for adrenocorticotropic hormone (ACTHR), also termed melanocortin 2 receptor (MC2R). The murine ACTHR coding gene is located on chromosome 18, 37.0 cM in a genomic region that shows high inter-species conservation (Schioth et al., 2003;Schioth et al., 2005). The 296 amino acids of the receptor protein are 89% identical to the human sequence (Kubo et al., 1995). The receptor is a G-protein coupled, seven transmembrane domains receptor and forms part of the melanocortin receptor family. The ACTHR is unique for its exclusive binding of ACTH and its lack of affinity to the other endogenous, agonistic or antagonistic ligands of the melancortic system such as α-melanocyte-stimulating hormone (αMSH), agouti signalling protein and agouti-related peptide (Abdel-Malek, 2001). Of the four other members of the receptor family, MC1R is involved in skin and hair pigmentation, MC3R and MC4R in energy homeostasis and MC5R in sebaceous gland secretion and pheromone release (Butler and Cone, 2002).

The ACTHR is involved in the transduction of the ACTH signal to various second messenger systems including adenylate cyclase, protein kinases A and C and lipooxygenase (Beuschlein et al., 2001). ACTHR stimulation ultimately results in a

variety of effects including increased glucocorticoid release from the adrenal cortex. The ACTHR was also identified as the key molecule in transmitting the lipolytic action of ACTH in mammalian adipose tissue (Abdel-Malek, 2001). A directly proliferative activity in adrenal tumor formation of ACTH through ACTHR has been questioned (Rocha et al., 2003). However, the receptor is thought to play a role in the maintenance of the highly differentiated adrenal phenotype (Beuschlein et al., 2001). In humans, mutations in the ACTHR are causative for the familial glucocorticoid deficiency syndrome type 1, characterized by low levels of plasma cortisol despite high levels of plasma ACTH, which clinically presents as Addison's syndrome (Clark et al., 1993).

The gene encoding the murine ACTHR was first cloned and expressed in 1995 (Cammas et al., 1995;Kubo et al., 1995). It spans approximately 23 kb and is organized in four exons, of which the first three encode the 5' untranslated region (5'-UTR). The fourth exon encodes part of the 5'-UTR and the entire protein coding and 3' untranslated regions. The second exon is alternatively spliced, resulting in two different mRNAs, one with and one without the 57-bp second exon (Shimizu et al., 1997). More recently, an alternative first exon was discovered that encodes part of an alternative 5'-UTR and is solely expressed in adipose tissue (Kubo et al., 2004) (see figure I5).

Figure I5: Genomic organization of the murine ACTH receptor locus. Exon 1a (1a) is expressed in the adrenal, exon 1f (1f) in adipose tissue. Exon 2 (2) is alternatively spliced. Exon 4 (4) encodes the entire open reading frame (depicted in green) and the entire 3' untranslated region. Small numbers indicate bp distances between the exons based on the *ensembl* sequence. For further details, see text.

1.5.2 Properties of the ACTHR Promoter

Cammas and colleagues characterized the ACTHR promoter by showing that a 1,8 kb sequence upstream of the transcriptional start site of the ACTHR restricted luciferase expression to Y1 adrenocortical cells in a cell culture assay. Promoter activity was conserved for increasing deletions of up to position 113 upstream of the transcription start site. However, a fragment of 900 bp upstream of the transcription start site did not effectively restrict reporter expression to adrenal cells any longer but enabled transcription in TM3 Leydig cells. Furthermore, the authors identified transcription factor binding sites for steroidogenic-factor 1 (SF1), one for octamer binding transcription factor (OctB), a glucocorticoid response element (GRE) and one binding site for each activating protein 1 and 2 (AP1 and AP2). By mutating one of the SF1-like binding sites the authors also showed that promoter specificity for the adrenal is at least in part mediated by SF1. A negative control element of so far unknown characteristics precludes ACTHR expression in other SF1 expressing tissues and is situated between 1200 bp and 900 bp upstream of the transcriptional start site. The presence of the GRE is hypothesized by the authors to play a role in glucocorticoid-mediated downregulation of ACTHR expression (Cammas et al., 1997). Zwermann and colleagues were able to show that DAX-1 (“dosage-sensitive sex revearsal, adrenal hypoplasia congenita, critical region on the X-chromosome, gene-1”) is involved in providing adrenal specificity to ACTHR expression (Zwermann et al., 2005). Most recently, the presence and functional relevance of pre-B-cell transcription factor 1 (Pbx1) binding sites has also been demonstrated (Lichtenauer et al., 2007). ACTHR expression in adipose tissue is regulated via a peroxisome proliferator-response element (Noon et al., 2004). A stimulatory effect of the G-protein subunits Gβ and Gγ on ACTHR expression could also be demonstrated (Qiu et al., 1998). Notably, the ACTHR mRNA is upregulated following stimulation by its own ligand ACTH in cultured human and mouse adrenocortical cells (Mountjoy et al., 1994).

In the adult animal, the activity of the murine ACTHR promoter as evaluated by RT-PCR of ACTHR mRNA shows high expression of ACTHR in the adrenal cortex. No expression could be detected in spleen, testis, liver, lung, heart, brain and kidney

(Cammas et al., 1997). Low levels of ACTHR mRNA could however be found in adipose tissue (Boston and Cone, 1996) and more recently also in murine pancreatic islet cells (Al-Majed et al., 2004). Within the adrenal gland, the expression is strongest in the outer layers of the cortex, namely the zona glomerulosa and zona fasciculata with only a few ACTHR mRNA positive cells in the medulla and none in the capsula as demonstrated by in-situ hybridization (Xia and Wikberg, 1996) (see figure I6).

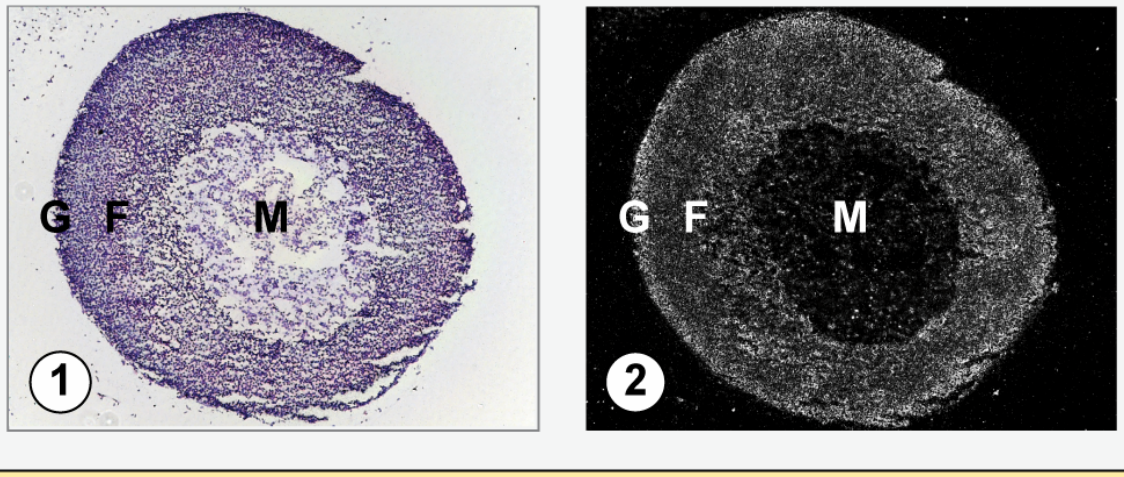

Figure I6: Transversal section of wildtype murine adrenal gland 1) Brightfield image at 50x magnification counterstained with cresyl violet showing the zonal organization of the adrenal (G: glomerular zone, F: fasciculate zone, M: medulla). In contrast to the human adrenal, the mouse lacks a reticular zone as steroid 17α-hydroxylase (CYP17), essential for androgen synthesis, is not expressed. 2) Darkfield image of an ACTH receptor in-situ hybridization on the same slice, revealing the expression pattern of the ACTH receptor in the adrenal. Cursory expression in medullary cells is also detected as described in the literature (image source: own preparation).

In a recent publication, ACTHR expression during developmental days 11.5 to 18.5 (E11.5 to E18.5) and in the adult animal was evaluated by immunohistochemistry. The ACTHR was actively expressed in a variety of tissues including adrenal gland (E13.5 into adulthood), testis (E13.5 to E18.5), genital ridge and ovary (E11.5 to E12.5 and E13.5 to E18.5 respectively), mesonephros (E11.5 to E12.5), metanephros (E12.5 to E18.5), lung (E11.5 to E14.5), brain and spinal cord (E11.5 to E13.5), choroid plexus (E13.5 into adulthood) and the dorsal root and trigeminal ganglia (E13.5 to E15.5). These findings imply a role for the ACTHR in the morphogenesis of tissues that were so far thought to be unaffected by ACTH signaling (Nimura et al., 2006). The ability of ACTH to stimulate testosterone

production in fetal and neonatal testes in the mouse further underlines the importance of ACTHR signaling during embryogenesis (O'Shaughnessy et al., 2003). The implications of this widespread expression of ACTHR during embryogenesis for the strategy of restricting cre activity to the adrenal cortex by the use of the ACTHR gene regulatory sequences will be commented on in the discussion section.

1.6 Aim of the Thesis

In this thesis project, genetic constructs are engineered and applied to the mouse that aim for the generation of novel cre mouse lines with constitutive and inducible cre recombinase activity restricted to the adrenal cortex. Cre mice are versatile tools for studying gene function in a cell-type specific and temporally-controlled manner. Thus far, no cre mouse line has been reported that permits conditional mutagenesis specific for the adrenal cortex.

In order to achieve adrenocortical specificity of cre expression, the “knock-in” of an inducible cre recombinase into the endogenous locus of the adrenocorticotropic hormone receptor (ACTHR) was performed initially. The resulting ACTHR-CE2 mice however did not show the desired cre expression. In a second approach, constructs for ACTHR cre cosmid transgenic mice were designed and generated. Both a constitutively active and a tamoxifen-inducible cre variant are used in this thesis.

The resulting cre mice will allow a vast array of experiments on adrenal gene function, including experiments on adrenal morpho- and tumorigenesis and endocrinology. Interbred with the conditional CRHR 1 knock-out mice created in our group, they will help to elucidate the role of the intra-adrenal CRH system on hormone regulation. Adrenocortical cre mice will therefore reveal insights into the function of the adrenal gland, an organ at the nexus between somatic and psychiatric illness.

2 Materials

2.1 Buffers and Solutions

Buffers and solutions were prepared using Millipore Q purified H_2O. Reagents were purchased from Sigma, Roth or Merck unless indicated otherwise.

2.1.1 Electrophoresis Buffers

2.1.1.1 Buffers for DNA Electrophoresis

TAE buffer:
4.84 g Tris
1.142 ml acetic acid
20 ml 0.5 M EDTA, pH 8.0
800 ml H_2O
adjust pH to 8.3 with acetic acid
adjust volume to 1 l with H_2O

6x Loading buffer Orange:
1 g Orange G
10 ml 2 M Tris/HCl, pH 7.5
150 ml glycerol
adjust volume to 1 l H_2O

6x Loading buffer Blue:
0.25 g bromophenol blue
600 ml glycerol
10 ml 2 M Tris/HCl, pH 7.5
adjust volume to 1 l with H_2O

2.1.1.2 Buffers for RNA Electrophoresis

10x Running buffer:
41.94 g MOPS
4.1025 g sodium acetate
20 ml 0.5 M EDTA, pH 8.0
adjust pH to 7.4 with 2 M NaOH
adjust volume to 1 l with DEPC- H_2O

Loading buffer:
0.0025 g bromophenol blue
4 ml formamide
2 ml formaldehyde
2 ml 10 x Running buffer
adjust volume to 10 ml with DEPC- H_2O

2.1.2 Buffers for Southern Blotting

Denaturation buffer:
100 ml 5 M NaOH
300 ml 5 M NaCl
adjust volume to 1 l with H_2O

Neutralization buffer:
250 ml 2 M Tris/HCl, pH 7.5
300 ml 5 M NaCl
10 ml 0.5 M EDTA, pH 8.0
adjust volume to 1 l with H_2O

20x SSC:
175.3 g NaCl
88.2 g sodium citrate
800 ml H_2O
adjust pH to 7.4 with 1 M HCl
adjust volume to 1 l with H_2O

Washing buffer:
100 ml 20x SSC
10 ml 10% SDS
adjust volume to 1 l with H_2O

Lysis buffer:
5 ml 1 M Tris HCl, pH 8.0
10 ml 0.5 M EDTA, pH 8.0
1 ml 5 M NaCl
12.5 ml 20% Sarcosyl
adjust volume to 500 ml with H_2O
add 5% (20 mg/ml stock) proteinase K prior to use

Precipitation buffer: 0.15 ml 5 M NaCl
10 ml 99% Ethanol

2.1.3 Buffers and Media for Bacterial and Cell Culture

LB medium: 10 g tryptone
5 g yeast extract
10 g NaCl
adjust pH to 7.0 with 5 N NaOH
adjust volume to 1000 ml
sterilize by autoclaving

2x BBS: 1.1 g BES
1.6 g NaCl
0.02 g Na_2HPO_4
adjust pH to 6.95 with 5 N NaOH
adjust volume to 100 ml with H_2O
pass through 0.22 µm filter
store 1 ml aliquots at –20 °C.

Culture medium for ES cells: 500 ml Dulbecco's Modified Eagle Medium, high glucose, plus Na-Pyruvate (DMEM, Gibco)
75 ml fetal calf serum (heat-inactivated, PAN Biotech)
1 ml β-Mercaptoethanol 500 x (Sigma)
5 ml glutamine (Gibco)
90 µl leukaemia inhibitory factor (Chemicon)

Culture medium for feeder cells: 500 ml DMEM (Gibco)
57 ml fetal calf serum (PAA)
5,7 ml glutamine (Gibco)
5,7 ml non-essential amino acids (Gibco)

2x Freezing medium for ES and feeder cells: 5 ml fetal calf serum (Gibco)
3 ml DMEM (Gibco)
2 ml DMSO

Culture medium	500 ml DMEM
for Y1 cells:	55 ml fetal calf serum (PAA)
	0,5 ml penicillin/streptomycin (10 µl/ml)

2.2 Cell Lines

The neomycin resistant embryonic mouse fibroblasts (EMFI) feeder cells and the TBV2 (129S6/SvEv/Tac) ES cells were provided by S. Bourier (GSF). The IDG 3.2 murine hybrid ES cell line (129S6/SvEv/Tac x C57Bl/6) was obtained from R. Kühn (GSF). The Y1 mouse adrenocortical cell line was a gift of F. Beuschlein (University of Freiburg).

2.3 Oligonucleotide Sequences

Oligonucleotides were ordered at MWG Biotech or Metabion and used as PCR or sequencing primers or for linker construction. For sequences consult table 2.

Table 2: Oligonucleotide names and sequences

Oligonucleotide	Sequence
3'vrfyin	ggtaagttacatagcgaaatgc
3'vrfyout	cacttccatgaacagaatggg
3'HAfw	aagcttggcgcgccggatccttaattaaagatctctcgagtgatccctgctttgagtgttg
3'HAinnen1	ttatgacacctggctgttgc
3'HAinnen2	gaaggaacagccaaactacg
3'HAinnen3	ctgttactagagacccaagc
3'HAinnen4	ggactgatttccacaatagc
3'HAinnen5	gatagatatttgcaagtagagc
3'HAinnengap2	atatatcagtggaaaacaggc
3'HAinnnengap1	ctattactacatgtagaatacg
3'HArev	gggcccgtttaaaccttaagtctttagtttgctgc
3'HA-Start	ggctggtaagggatatttgc
3'Probe1	gatgctgcttgtacccacg
3'Probe2	tcaaagctcatggtgcaagc
5'HAss	tgtttcattatcatctcatggc
5'vrfyin	gctccattattctcttgtagc
5'vrfyout	aattctaggtttccacacagc
5'HACregap	actgcatgttacgaattggc
5'HACregap2	aagggccttggaagcagc
5'HAfw	aagcttgtttaaaccttaaggtctcatcggttcc
5'HAinnen1	caggcaggcttgctggc
5'HAinnen2	atttcagagagccccatgc
5'HAinnen3	ctggcaaacatgccaggc
5'HAinnen4	gtagttgagactacaggtgc
5'HAinnen5	ccacattactcataagttggc
5'HArev	ggcgcgccgaattcgtcgacggccggccgacgtcgccatcttccag
5'Probe1	aatctgtaaacaccatgggc
5'Probe2	cgttattctagtaagtgaaagt
ACEvrfyin	gtaacccatatttgcctggc
ACEvrfyout	caccattgtccacttgtccc
AMP-frt-Xho	ctcgaggaagttcctatactttctagagaataggaacttcgagattatcaaaaaggatcttcac
AMP-Pac	ttaattaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagta
BamAs	aactgactaacacagacacg
BamSs	ctagcagaagaagcaagagc
CreAs	acgtaacagggtgttataagc
Cre-BamH1	ggatccgccgcataaccag
Cre-Connect	atgctgtttcactggttatgc
CREE-Aat	gacgtcgagccatctgctggagaca
CREE-Fse	ggccggcctcagactgtggcagggaaa
Cre-End	cctacccggtagaattagc
CREM-c	catggagcagaagctgatctctgaggaggacctgtagggccgg

Table 2 (continued): Oligonucleotide names and sequences	
Oligonucleotide	**Sequence**
CREM-ncneu	ccctacaggtcctcctcagagatcagcttctgctccatgacgt
CreOutw	ctcatcactcgttgcatcg
Crevrfyin	gcaggcaaattttggtgtac
Crevrfyout	cgaacctcatcactcgttg
EcoAs	ctacatctctgggatgaagc
EcoSs	ctttcatcacacaaccaagc
Exon4As	acaatcggagttatttcttgc
FRi-5	agaggtaaaaatggaggaaagg
FRT-BamFs	ggatccgctagcggccggccgagctcgaattcatcg
FRT-mlu	gcagacgcgtgttgatggc
frtOutw	ttcctcgagtgatccctgc
fse-Prom	atcgatggccggccatcgcctgcaggagcttgggcttgaacatcg
HindAs	gctgtaaataggcagtaagc
HindSs	gatccaaatctacaggtagc
IVS-Aat	gacgtcgaattcgctagcggccggccatgattacgccaagctgg
IVS-Notfw	gcggccgccatgattacgccaagctgg
IVS-Notrv	gcggccgcttacttgtacagctcgtcc
IVS-Sal	gtcgacttacttgtacagctcgtcc
IVS-XbaRv	tctagacttacttgtacagctcgtcc
Mar-1	tgatttctgtaagtcaacggc
Mar-2	ctggtcagaagaatcactgg
Mar-Asgr	gggcccaccggtgtcggcttgctgaaactcgta
Mar-Bam	ggatcctgatccctgctttgagtgttg
Mar-Cre	gtgtacggtcagtaaattggacatttttttcctgctggccgtt
Mar-fse	ggccggccagctgccactgctaaccaca
MC2R-Link1	cggccggccgtcgacgaattcggcgcgccggatccttaattaaagatctctcgagat
MC2R-Link2	ctcgagagatctttaattaaggatccggcgcgccgaattcgtcgacggccggccgacgt
PCR-AAH	cgtttaaaccttaaggtttaaaca
PCR-HAA	agcttgtttaaaccttaaggtttaaacgggcc
PmlLinker-1	P-ccgtttaaaccacgtgggccgg
PmlLinker-2	P-cccacgtggtttaaacggccgg
Pneo-Afl-Hind	aagcttcttaaggaagttatcgaagttccta
Pneo-Apa-Afl	gggccccttaagttgtcttcccaatcctccc
Pneo-Asc	ggcgcgccgaagttatcgaagttccta
Pneo-PacI	ttaattaattgtcttcccaatcctccc
pntflprsrsalgap	agatgacaggagatcctgc
pntflprsrsalgap2	cgtctcactagtctcgtgc
post3'HA	tgagtggtttgggcaagtg
prAMP-Pac	ttaattaaaccccctatttgtttatttttct
pre5'HA	agactatacagtcagattgg
Prom-mlu	atcgatacgcgtgttgatggcaggggtacgaagccatattggctgcacggatcctg
Rsr-FRT	tatgtcctgatagcggtccg
SrgSal-FRT	caccggtgtagtgtcgacgaagttatcgaagttcctatt
SV40-Asc	gattaatttcaggcgcgcca
SV40-Sal	gtcgacatgataagatacattga

3 Methods

For detailed protocols on molecular cloning, cell culture techniques etc. refer to Sambrook and Russell, 2001 (Sambrook and Russell, 2001). Updated versions of their protocols are available online at *www.molecularcloning.com*.

3.1 Molecular Cloning Techniques

3.1.1 Transformation of Plasmid DNA

For transformation of plasmids into electrocompetent E. coli, 20 µl aliquots of cells (either MH1, DH5α or XL1-Blue) were thawed on wet ice and 20 ng of plasmid DNA was added. After incubation for 1 minute on wet ice, the mixture was transferred into pre-cooled electroporation cuvettes and an electric pulse was applied by a Gene Pulser Xcell (Biorad) following the manufacturer's recommendations. Immediately after the pulse, 980 µl of 37 °C LB medium were added and the bacteria were shaked for 1 hour at 37 °C.

For transformation of plasmid DNA into chemically competent E. coli, 100 µl aliquots of cells were thawed on wet ice for 15 minutes. Up to 5 µl (100 ng) of plasmid DNA were added and the mixture was incubated on wet ice for 15 minutes, followed by a 45 seconds/42 °C heat shock. After 2 minutes on wet ice, 900 µl of LB medium (37 °C) were added. The bacteria were then allowed to recover for 60 minutes under continuous shaking at 37 °C.

After the recovery phase of both transformation procedures, a suitable volume of the transformation (usually 100 µl) was plated to LB-agar dishes containing either ampicillin (100 µg/ml) or kanamycin (30 µg/ml) and grown overnight at 37°C. Where applicable, LB-agar dishes were additionally plated with 40 µl of a 40 mg/ml X-Gal (Genaxxon) solution for blue-white selection. Subsequent screening of colonies was performed either by restriction digestion, colony lifting or colony PCR. Colony masterplates were stored at 4°C. Storage of clones for longer than 2 months was carried out in glycerol stocks of 250 µl E. coli culture and 750 µl autoclaved 80% glycerol at –80 °C.

3.1.2 Isolation of Nucleic Acids

3.1.2.1 Isolation of Vector DNA

For isolation of plasmid DNA, E. coli cells containing the respective plasmid were grown overnight at 37 °C in autoclaved LB-medium containing antibiotics, usually either ampicillin (100 µg/ml) or kanamycin (30 µg/ml). Plasmid DNA preparation was carried out by means of Qiagen Mini-/Midi-/Maxi-Prep kits according to the manufacturer's protocols. DNA was eluted in sterile water and stored at –20 °C.

For isolation of BAC and cosmid DNA, E. coli cells containing the respective BAC or cosmid were grown overnight at 37 °C in autoclaved LB-medium containing chloramphenicol (20 µg/ml). BAC and cosmid DNA was prepared using the Qiagen Large-Construct kit according to the manufacturer's instructions. DNA was eluted in sterile water and stored at –20 °C.

3.1.2.2 Isolation of Genomic DNA

For preparation of murine tail DNA, tail tips of 0,5 cm length were cut from adult mice. Tails were either stored at –20 °C or used directly for DNA preparation by Promega Wizard Genomic DNA Purification kit following the manufacturer's instructions. Genomic DNA from mouse tails was stored at 4 °C.

To prepare genomic DNA from mouse liver, mouse liver avoiding the gallbladder was preparated and homogenized in a liquid nitrogen cooled mortar. The resulting tissue powder was resuspended in 1 ml NET buffer (100 mM NaCl; 25 mM EDTA; 2 mM Tris pH=7,5) per 0,1 g tissue. 1/10 volume 10% SDS and 1/10 volume 10 mg/ml proteinase k solution were added and incubated overnight at 56°C. The DNA was purified by phenol-chloroform extraction, ethanol-precipitated, solved in 1-2 ml of sterile water and stored at 4 °C.

For extraction of genomic DNA from murine embryonic stem cells, an ES cell pellet of a confluent 9 cm cell culture plate was resuspended in 1 ml NET buffer (100 mM NaCl; 25 mM EDTA; 2 mM Tris pH=7,5). 100 µl of proteinase K solution (10 mg/ml) was added. After mixing and addition of 100 µl of 10% SDS the preparation was

incubated overnight at 56°C. The DNA was purified by phenol-chloroform extraction, ethanol-precipitated, solved in 0,5-1 ml of sterile water and stored at 4 °C.

3.1.2.3 Isolation of Total RNA

For preparation of total RNA from cultured cells, the cells were lysed directly on the culture dish with 1 ml of TRIzol (Invitrogen) per 10 cm^2 of dish surface. After homogenization by pipetting, the lysate was transferred to a 50 ml tube and mixed vigorously. For separation of phases, the samples were incubated 5 minutes at room temperature before adding 0,2 ml of chloroform per 1 ml TRIzol (Invitrogen). The preparation was mixed again and incubated at room temperature and spun down in a table-top centrifuge at maximum speed at 4 °C for 15 minutes. The upper, aqueous phase was transferred into a new tube, mixed with 0,5 ml of isopropanol per 1 ml of TRIzol (Invitrogen) and incubated at room temperature for 10 minutes to precipitate the RNA. By centrifugation in a table-top centrifuge at maximum speed at 4 °C for 10 minutes a RNA pellet was formed and the supernatant discarded. The pellet was washed with 1 ml 70% ethanol per 1 ml TRIzol (Invitrogen) reagent. After vortexing the mixture was centrifuged at 7500 g for 15 minutes at 4 °C. The supernatant was discarded and the RNA pellet dried for approximately 20 minutes at 37 °C and redissolved in a suitable amount of H_2O (usually 40 µl). RNA concentration was measured by spectrophotometry and RNA quality was verified by agarose gel electrophoresis.

3.1.3 Purification of DNA

3.1.3.1 Phenol/Chloroform Extraction

For purification, DNA was extracted from aequous solution by mixing the sample with an equal amount of phenol/chloroform/isoamylalcohol (25/24/1 parts). The resulting upper, aequous phase was carefully separated and mixed with an equal volume of chloroform/isoamylalcohol (24/1 parts). The upper phase was subsequently used for DNA recovery by ethanol precipitation.

3.1.3.2 Ethanol Precipitation

For ethanol precipitation of DNA, the DNA sample was supplemented with 0,1 sample volumes 3 M NaAcetate followed by 2,5 volumes of ice-cold 100% ethanol. After mixing, the DNA was precipitated for 10 minutes at –80 °C. The DNA was pelleted, washed with 70% ethanol, air-dryed and resuspended in the desired amount of H_2O.

3.1.3.3 PCR Purification

Purification of plasmid DNA through binding to silica colums was performed after PCR or restriction digestion by means of the QiaPrep PCR Purification kit (Qiagen).

3.1.4 Restriction Digestion of DNA

Restriction digestion of plasmid DNA was performed as analytical or preparative digest. Analytical digestion was carried out for 1 hour at 37 °C with usually 500 ng of plasmid and 10 U of the respective restriction endonuclease. For preparative purposes, 5 µg of plasmid DNA were digested overnight with usually 50 U restriction endonuclease and restriction buffer, bovine serum albumine (BSA) and H_2O according to the enzyme manufacturer's protocols (New England Biolabs, MBI Fermentas, Roche or Gibco). Digestion of genomic DNA for Southern analysis was equally performed overnight at 37 °C with 10 µg of genomic DNA and 50 U restriction enzyme with supplementation of restriction buffers, BSA, spermidine and H_2O as recommended by the enzyme manufacturer. Special attention was paid to possible star activity, methylation sensitivity and non-classical incubation temperatures of the enzymes. For SgrA I, the relaxation of restriction specifity after successful canonical restriction of its recognition site results in multiple undesired fragments following over-digestion (Bitinaite and Schildkraut, 2002). Therefore, the enzyme kinetics of SgrA I was estimated by stopping the restriction digest at different time points by adding 10-fold loading buffer and heating to 95 °C and the reaction conditions were optimized for the desired digestion.

3.1.5 Isolation of DNA Fragments

For isolation, DNA fragments were routinely separated by agarose gel electrophoresis. The DNA band containing the desired fragment was identified using a UV transillumination table and excised using a disposable scalpel. Gel extraction was performed with the Qiagen QiaQuick Gel Extraction kit according to the manufacturer's protocol. DNA was eluted in H_2O, its concentration was determined and it was stored at –20 °C. In case of formation of single stranded DNA during purification, DNA was supplemented with NEB 10-fold restriction Buffer 3, heated to 95 °C for 3 minutes and cooled down to room temperature. Thereafter DNA integrity was verified by gel electrophoresis.

3.1.6 Ligation of DNA Fragments

Standard ligation of DNA fragments was performed using T4 DNA Ligase (MBI Fermentas). To this end, 10 ng of vector backbone were mixed with a 3- to 10-fold molar excess of insert. H_2O was added up to 17 µl and the mixture was heated to 70 °C for 5 minutes to destroy tertiary structures that could potentially impair ligation efficiency. After 2 minutes on wet ice, 1 µl of T4 DNA Ligase and 2 µl of 10-fold ligation buffer were added. Ligation was routinely performed overnight at 16 °C followed by 15 minutes of heat inactivation at 65 °C. To decrease the formation of undesired plasmids during ligation, 1 µl of a feasible restriction endonuclease was added to ligations where possible. Non-standard ligations, i. e. ligations with fragments of unusual size, more than one insert, blunt-end overhangs etc. were performed as described above or using ligation kits, such as the Alligator (Genaxxon BioScience), Quick Ligation Kit (New England Biolabs) or Rapid DNA Ligation Kit (MBI Fermentas) according to the manufacturer's protocols.

To avoid religation of single-enzyme cut fragments, 5' phosphate groups were removed when deemed necessary by either calf intestinal phosphatase (New England Biolabs) or shrimp alkaline phosphatase (Roche) according to the manufacturer's recommendations. Advantage of the latter is that it can be heat-inactivated by incubation at 65 °C rendering additional purification of DNA before subsequent ligation unnecessary. Integrity of the dephosphorylated fragment was

checked by T4 polynucleotide kinase (New England Biolabs) mediated re-phosphorylation and subsequent use for ligation.

3.1.7 Recombineering by Red/ET-Cloning

The term recombineering refers to the modification of large DNA vectors like cosmids (or BACs etc.) by homologous recombination (Copeland et al., 2001). The large size of these vectors generally requires their propagation in homologous recombination deficient strains of E. coli to prevent their rearrangement (Shizuya et al., 1992). Recombineering involves the restoration of the homologous recombination potential for a defined period of time while a DNA sequences to be inserted into the cosmid (or BAC etc.) is introduced into the bacteria. After the desired homologous recombination has occured the bacteria will ideally again lose their capacity for homologous recombination in order to preclude subsequent undesired recombination events. This aim can be achieved by the regulated expression of the homologous recombination proteins RecE/RecT from the Rac prophage (Zhang et al., 1998b) and the Redα/Redβ proteins from the lambda phage (Muyrers et al., 1999). In accordance with the utilized recombination pathways, the methods were termed “ET” or “Red” recombination. A combination of these methods called Red/ET-cloning is patented and marketed by Genebridges.

In this thesis, a modified protocol based on the Counter-selection BAC Modification-Kit protocol provided by the manufacturer (Genebridges) was used in order to integrate the targeting vectors pMC2RcreMYC and pMC2RcreERT2 into the cosmid MPMGc121E06653Q2 (purchased at RZPD, Berlin). In a first stage, the cosmid carrying E. coli clone was transformed with the Red/ET expression plasmid pSC101-BAD-gbaA. To this end at least ten colonies carrying the cosmid were picked and they were used to inoculate a 1 ml overnight 37°C-culture in LB medium containing 15 µg/ml kanamycin. The next day, 1.4 ml LB medium culture likewise containing 15 µg/ml kanamycin was started with 30 µl of the overnight culture and grown for 2-3 h at 37 °C under constant shaking. To prepare cells for electroporation with pSC101-BAD-gbaA they were pelleted by centrifugation at 4 °C and washed twice with 1 ml ice-cold H_2O. After the supernatant was decanted, approximately 20 µl of cell suspension remained in the reaction vial to which 1 µl of pSC101-BAD-gbaA was

added. The cell suspension was then transferred to a pre-chilled 1 mm electroporation cuvette and electroporated at 1350 V, 10 µF, 600 Ohms. The cells were resuspended immediately after electroporation with 1 ml LB medium without antibiotics and grown at 30 °C for 70 minutes. The cells were then pelleted and resuspended in 100 µl LB medium and plated onto LB agar plates containing 15 µg/ml kanamycin and 3 µg/ml tetracycline. The tetracycline containing plates were cast just prior to use and were kept light-protected in order to ensure tetracycline integrity. Plates were incubated overnight at 30 °C and subsequently stored at 4 °C.

In a second stage, homologous recombination competent, electrocompetent cells were prepared from the E. coli strain generated in the first stage, that contain both the Red/ET expression plasmid pSC101-BAD-gbaA and the target cosmid MPMGc121E06653Q2. To this end, a 1 ml LB medium culture containing 15 µg/ml kanamycin and 3 µg/ml tetracycline was inoculated with at least ten colonies from the bacterial strain generated as described above. Culture was performed overnight at 30 °C under constant shaking. The following day, a 1.4 ml LB medium culture containing the same antibiotics was started with 30 µl of the overnight culture and grown at 30 °C to an OD_{600} of 0.2. Subsequently, recombination capacity was induced by adding 20 µl L-arabinose 10% to the culture and growing the culture for 1 h at 37 °C up to an OD_{600} of 0.4. For control purposes, parallel cultures were left uninduced. The bacterial cultures were then pelleted by centrifugation and washed twice in ice-cold H_2O. Electroporation was carried out according to the principles described in the first stage, this time with the Pme I- linearized targeting vectors pMC2RcreMYC and pMC2RcreERT2, respectively. After recovery, cells were plated on LB agar plates containing 15 µg/ml kanamycin and 50 µg/ml ampicillin and incubated overnight at 37 °C. Control experiments were performed in parallel as described in the Counter-selection BAC Modification Kit protocol (Genebridges).

3.1.8 Polymerase Chain Reaction

3.1.8.1 Standard PCR

Standard polymerase chain reaction (PCR) amplification of DNA was performed using Taq polymerase. After initial DNA denaturation for 7 minutes at 94 °C, 35 cycles of denaturation (94 °C for 30 seconds), annealing (30 seconds) and

elongation were routinely carried out. The annealing temperature was chosen depending on the primer melting temperature as provided by the primer manufacturer and usually fell into the range of 50-65 °C. Time at 72 °C for elongation was chosen depending on the length of the supposed PCR product, with 1kb elongation estimated to require 1 minute. A final elongation step of 7 minutes at 72 °C followed the 35 cycles. For 100 µl of PCR mix, 20 pmol of each primer were used. In case of plasmids, 20 ng of template DNA were used, in case of genomic or BAC DNA about 100 ng. Taq polymerase, desoxynucleotide triphosphates (dNTPs), PCR buffers, $MgCl_2$ and H_2O were added as described by the Taq polymerase manufacturer (Abgene, Roche).

PCR products were commonly subcloned into pCRII-TOPO vectors (Invitrogen), exploiting the characteristic property of Taq polymerase to add A-overhangs to the 5' end of synthezised strands. To control for the introduction of mutations by the inherently error-prone PCR, subcloned PCR products were sent for commercial sequencing (Sequiserve).

3.1.8.2 PCR Amplification of Long DNA Fragments

PCR using Taq polymerase becomes ineffective and error-prone in case the length of the desired PCR product exceeds 2kb. For generation of longer PCR products the Expand Long Template PCR System (Roche) or Herculase polymerase (Stratagene) that are based on DNA polymerases with proof-reading activity or mixtures of DNA polymerases were used following the manufacturers' protocols.

3.1.8.3 Nested PCR

Nested PCR allows the amplification of templates that are present only in very low amounts. In a first step, a standard PCR is run on the desired template with a specific primer pair. The product of this first PCR is subsequently used as template for a second PCR with "nested" primers, i. e. primers that are located in between the primers of the first PCR.

3.1.8.4 Multiplex PCR

Multiplex PCR utilizes more than two specific primers on a given template. Different primer pairs resulting in different PCR products therefore allow the differentiation between template sequence configurations in a single PCR. Multiplex PCR is typically applied for genotyping.

3.1.8.5 Megaprime PCR

Megaprime PCR allows the ligation of overlapping DNA fragments, i. e. one typically very large “primer” and a “template” strand. The initial denaturation step of the PCR results in single stranded DNAs that bind as PCR primers to the complementary overlap on a strand from the ligation partner. Megaprime PCR efficiency is usually enhanced by combining it with a multiplex PCR approach by adding the 5’ primer of the 5’ fragment and the 3’ primer of the 3’ fragment (compare figure R4).

3.1.8.6 Colony PCR

Colony PCR was used to identify bacterial clones that comprise a desired plasmid. To this end, bacterial colonies are picked and transferred to PCR tubes containing 50 µl of H_2O. These probes are heated to 95 °C for 15 minutes to crack the bacteria. After cooling down, 5 µl of the probes are used as PCR templates with primers that allow the identification of the desired plasmid.

3.1.8.7 Reverse Transcription PCR

To generate cDNA templates for subsequent PCR, reverse transcription was performed using SuperScript II (Invitrogen) reverse transcriptase. 0,5 µg of total RNA were mixed with 1 µl (500 µg/ml) oligo dT primers (Amersham), supplemented with H_2O up to 11 µl, haeted to 70 °C for 5 minutes and put on wet ice for 1 minute. 4 µl of 5x First Strand Buffer (Invitrogen), 2 µl 0,1 M DTT, 1 µl 10 mM dNTP, 1 µl 40U/µl RNasin (Promega) and SuperScript II (Invitrogen) reverse transcriptase were added and the mix was heated first to 42 °C for 60 minutes, then to 70 °C for 15 minutes. After reverse transcription, RNA was destroyed by addition of 2 U/µl RNase H (Invitrogen) and 20 minutes incubation at 37 °C followed by 15 minutes heat inactivation at 70 °C. The resulting first strand cDNA served as template for the following PCR.

3.1.8.8 Primer Design

PCR primers were designed manually according to the following principles: The primer length should be around 20bp and G/C content about 50%. The melting temperature was designed to be around 58 °C with nucleotides A or T calculated to contribute 2 °C and G or C to contribute 4 °C. Additional nucleotides were added to the priming sequences as needed for restriction sites etc. To avoid the positioning of primers into repetitive sequences, oligonucleotides were analyzed in silico using Primer3 and Repeatmasker software when deemed necessary. Primers were ordered at MWG Biotech or Metabion.

3.1.9 Agarose Gel Electrophoresis

Gel electrophoresis for separation of DNA fragments in the range of 100bp to 20kb was performed on agarose gels (Ultra Pure Agarose, Invitrogen) in 1x TAE buffer. Agarose concentration was adjusted within the range of 0,7-2,0% according to the desired separation optimum. Gel running time and voltage were adjusted likewise according to the desired separation characteristics.

For quality control of RNA preparations, 1,5% agarose gels were loaded with a RNA aliquot to be tested that was heated to 65 °C with RNA Loading Buffer.

Staining of nucleic acids was achieved by the intercalating fluorescent dye ethidium bromide which was added to a final concentration of 0,5 µg/ml to the the agarose gel before solidification. For determination of DNA fragment size the 1kb Plus DNA Ladder (Invitrogen) was used. Stained nucleic acids were visualized on a UV-transilluminator and photographed.

3.1.10 Determination of DNA/RNA Concentration

DNA and RNA concentrations between 100 ng/µl and 1 µg/µl were determined by UV-spectrophotometry at 260 nm following the equation $c[\mu g/ml]=OD_{260} \times V \times F$. OD_{260} denominates the optical density measured at 260 nm, V the factor of dilution. Differences in extinction between DNA and RNA are accounted for by F, which is 50 for dsDNA and 40 for RNA. DNA concentrations of less than about 100 ng/µl were

determined by gel electrophoretic comparison to the SmartLadder (Eurogentec) weight standard.

3.2 Blotting Techniques

3.2.1 Southern Blotting of Agarose Gels

Southern blotting was used to transfer DNA from agarose gels onto nylon membranes for subsequent hybridization with specific probes. DNA separation by agarose gel electrophoresis was executed at low voltage overnight (e.g. 40 V for 12-16 h) to ensure sufficient fragment separation. DNA was denatured by bathing the gel twice for 15 min in denaturation buffer followed by twice 15 minutes in neutralization buffer and equilibration for at least 10 min in 20 x SSC.

The nylon blotting membrane (Hybond N, Amersham) and Whatman 3 mm filter paper were pre-wet in 20 x SSC. The agarose gel to be blotted was put onto the Whatman 3 mm filter paper, which had contact to a reservoir containing 20 x SSC. On top of the gel, the nylon membrane was located followed by two additional layers of Whatman paper and a stack of cellulose tissue for absorption of the 20 x SSC transfer liquid and a 1 kg weight. Any air bubbles between the gel, the membrane and Whatman 3 mm papers were carefully removed. The gel was blotted overnight (12-16 h, not more than 24 h) and UV crosslinked (UV Stratalinker® 2400; Stratagene).

25-50 ng of DNA probe were labelled with 50 µCi α-^{32}P-dCTP (Amersham) by the random hexamer-primers based Megaprime DNA Labelling kit (Amersham) according to the manufacturer's instructions. The labelled probe was purified from free nucleotides by MicroSpin S-300 HR columns (Pharmacia). Efficiency of labelling was checked by scintillation counting on a gamma-counter. Pre-hybridization of the blotting membranes was carried out at 65 °C for 30 min in Rapid-Hyb Buffer (Amersham). After denaturation of radiolabelled DNA probes at 95 °C for 5 min, hybridization was carried out for 2-3 h at 65 °C in Rapid-Hyb Buffer (Amersham). The membranes were then washed once in 2 x SSC, 0.1 % SDS for 20 min and twice in 0.2 x SSC, 0.1 % SDS for 15 min at 65 °C. Membranes were exposed for suitable

periods of time (usually 1-2 days) to phospho-imaging screens or to X-ray films (Kodak). For hybridization with additional probes, membranes were stripped of probes by boiling in 0.1 % SDS for 20 minutes followed by rinsing with 2x SSC.

3.2.2 Colony Hybridization

To identify clones with a specific plasmid amongst a large number of E. coli colonies, colony hybridization (also: “colony lifting”) was performed. To this end, Hybond N nylon membranes (Amersham) were put onto agar plates with the E.coli colonies for transfer of colonies. The membranes were subsequently laid twice for 10 minutes facing up in denaturalization buffer and twice in neutralization buffer for 10 minutes, followed by 5 minutes in 2 x SSC for 5 min. After drying, membranes were UV crosslinked (UV Stratalinker® 2400; Stratagene) and used for hybridization. Generation of specific probes and hybridization were carried out as described for Southern blotting of agarose gels. Exposure time to X-ray films (Kodak) however was only 30 minutes to 2 h.

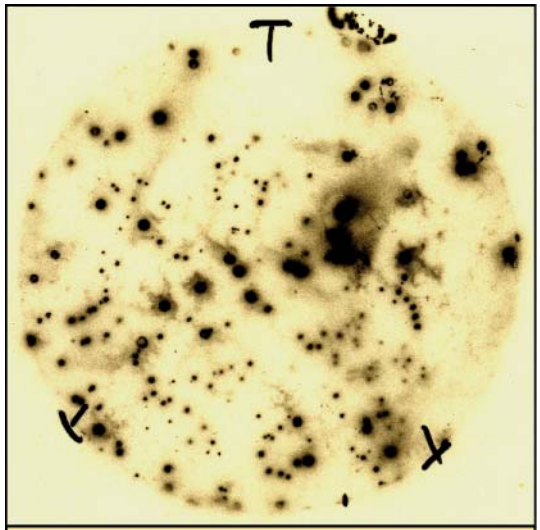

Figure M1: Colony lift. Clones that hybridize with the radioactively labelled probe stain the x-ray film specifically (small black dots) and can subsequently be identified on the culture plates. Larger black areas represent unspecific staining (image source: own preparation).

3.3 Cell Culture Techniques

3.3.1 Manipulation of Embryonic Stem Cells

Murine embryonic stem cell lines (ES cells) were established independently by two groups in 1981 (Evans and Kaufman, 1981;Martin, 1981). They are pluripotential stem cell lines derived from the primitive ectoderm of the mouse blastocyst (Brook and Gardner, 1997). ES cells can be cultured and manipulated in vitro, during which time they remain undifferentiated and maintain their pluripotency. Once reintroduced into the environment of a blastocyst or a morula, the ES cells colonize the inner cell mass and give rise to all the different tissues of the resulting organism, including its

germ cells. A genetic modification introduced into the ES cell genome can thus be transmitted to subsequent generations (Capecchi, 2001). In order to maintain the pluripotency of the ES cells they are cultured on dishes plated with neomycin-resistant embryonic mouse fibroblast (EMFI) feeder cells that secrete differentiation-inhibiting factors into the culture medium. The culture medium is furthermore supplemented with leukaemia inhibitory factor (LIF) to aid maintenance of their undifferentiated state (Chambers, 2004).

3.3.1.1 Culture of Embryonic Mouse Fibroblast Feeder Cells

For preparation of feeder cells plates, a frozen vial of EMFI feeder cells was thawed quickly at 37 °C. Cells were transferred into a plastic tube containing 10 ml feeder cell culture medium and centrifuged. The cell pellet was resuspended gently in 10 ml feeder cell culture medium and cells were seeded onto 3 x 15 cm plates each containing a total of 25 ml feeder cell culture medium. Incubation was performed at 37 °C, 5 % CO_2 for 3 days after which the medium was removed and 15 ml feeder cell culture medium containing 150 µl mitomycin C (MMC) (1 mg/ml) was added in order to mitotically inactive the feeder cells. Plates were swirled to ensure an even distribution of the medium and cells were incubated at 37 °C, 5 % CO_2 for exactly 2.5 h. One culture plate of feeder cells was usually left without MMC treatment in order to maintain a stock culture for subsequent generation of feeder plates.

For trypsinization, the cell monolayer was washed twice with 10 ml phosphate buffered saline (PBS, Gibco) and 7.5 ml trypsin/EDTA (Gibco) were added to each plate. Plates were then incubated for about 5 min at 37 °C and the resulting cell suspension was pipetted up and down for 3 times to break cell aggregates. The resuspended EMFI cells were then transfered to a 50 ml plastic vial, containing at least the same amount of feeder cell culture medium as trypsin/EDTA used for trypsinization. The cells were pelleted by centrifugation and resuspended in feeder cell culture medium to a final concentration of 2.0 x 10^5 cells/ml. Mitotically inactivated feeder cells were immediately plated at a density of 10^4 cells/cm^2 on dishes containing feeder cell culture medium. Feeder cells were allowed to attach overnight or for at least 3 h before medium change to ES cell medium and seeding of ES cells.

3.3.1.2 Culture of Embryonic Stem Cells

A vial of frozen IDG 3.2 ES cells was thawed quickly and transferred to a laboratory tube containing 10 ml ES cell medium without LIF for washing. The cell suspension was spun down by centrifugation and the pellet was resuspended in 5 ml ES cell medium containing LIF. Cells were plated onto a 6 cm- cell culture dish with EMFI cells and grown at 37 °C and 5% CO_2. Medium change was performed daily. For splitting, cells were washed with PBS twice and incubated with 1.5 ml trypsin/EDTA for 10 min at 37 °C, 5 % CO_2, until the cells became detached. The cells were then gently pipetted up and down to obtain a single cell suspension after which 10 ml of ES cell medium without LIF were added. Following centrifugation, the supernatant was aspirated and cells were resuspended in ES cell medium containing LIF. About 2 x 10^6 cells per 10 cm diameter dish were plated to 10 cm feeder plates or, for freezing or electroporation, on plates coated with 0.1 % gelatine.

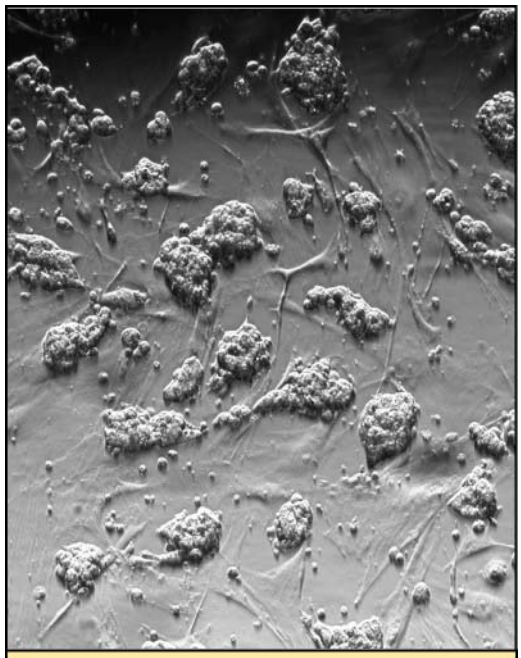

Figure M2: Murine IDG 3.2 embryonic stem cells growing on embryonic fibroblast feeder cells (magnification 100x, image source: own preparation)

3.3.1.3 Electroporation of Embryonic Stem Cells

For electroporation with linearized vector DNA, an ES cell suspension of 7 x 10^6 cells per electroporation was made in 800 µl ice-cold PBS. 20 µg of the linearized targeting vector were added. The cell suspension was then transfered to the electroporation cuvette and electroporation was performed at 0.24 kV, 500 µF for approximately 6 ms on a Bio Rad Gene Pulser. After the pulse, the cells were incubated on wet-ice for 10 to 20 min. The cell suspension from the cuvette was then seeded onto two 10 cm feeder cells plates containing 9 ml ES cell medium each. Medium was changed daily. Two days after electroporation, selection with G418 (geneticin, Life Technologies) was started at 200 µg/ml. Selection with 2 µM

ganciclovir was initiated on the fourth day after electroporation. Drug-resistant colonies were picked on the 12th day after electroporation and transferred into 96-well feeder cell coated plates. Selection was continued and clones were passaged to produce dublicate plates. Two duplicates on gelatine coated 96-well plates were used for screening by Southern blotting. Two duplicates on feeder cell coated 96-well plates were stored with freezing medium in liquid nitrogen for subsequent clone expansion after identifiation of homologously recombined clones.

3.3.1.4 Identification of Homologously Recombined ES Cells

After growing to about 70% confluency, the ES cell clones on the gelatine coated 96-well plates were rinsed twice with PBS and 50 µl lysis buffer per well were added. The plates were then incubated overnight at 50 °C and spun down the next day by centrifugation. To precipitate DNA, 100 µl of NaCl/ethanol (150 µl of 5 M NaCl in 10 ml of Ethanol) were added per well. The 96-well plate was shaked for 30 min at room temperature and spun down again. Supernatants were discarded, carefully leaving the DNA pellets attached to the culture plate. The pellets were rinsed three times with 150 µl of 75% Ethanol per well. After the final washing step, plates were allowed to dry on the bench.

For restriction digestion, 30 µl of restriction digestion mix containing the appropiate restriction enzymes and buffers were added per well and the reaction was incubated overnight at 37 °C. Gel electrophoresis loading buffer was added to the samples and electrophoresis was performed. From the agarose gels, DNA was transferred to Nylon membranes by Southern blotting and hybridized with radioactively labelled probes as described earlier.

3.3.1.5 Preparation of ES Cells for Blastocyst Injection

For expansion of ES cell clones that were identified to exhibit the desired homologous recombination event, 96-well duplicate plates stored in liquid nitrogen were thawed in a waterbath at 37 °C. After centrifugation, the freezing medium was replaced by ES cell medium and the cells were transferred to a fresh 96-well feeder plate. Clones were subsequently expanded by splitting onto 24-well plates, followed by splitting to 6-well plates and 10 cm plates when sufficient confluency was reached.

For storage, expanded clones were trypsinized, medium was changed to freezing medium and clones were stored in liquid nitrogen. For blastocyst injection, vials containing the desired clones were thawed following the principles described earlier and plated onto 6 cm plates. The day before injection, cells were passaged to a gelatine coated 6 cm plate.

3.3.2 Culture of Y1 Adrenocortical Cells

For culture of Y1 adrenocortical cells (Rainey et al., 2004;Schimmer, 1979), a frozen vial of Y1 cells was thawed quickly at 37 °C. Cells were transferred into a plastic tube containing 10 ml Y1 cell culture medium and centrifuged. The cell pellet was resuspended gently in 10 ml Y1 cell culture medium and cells were seeded onto a 10 cm dish. Incubation was performed at 37 °C, 5 % CO_2 until 80 % confluency was reached. For splitting, the cell monolayer was washed twice with 10 ml phosphate buffered saline (PBS, Gibco) and 7.5 ml trypsin/EDTA (Gibco) were added to each plate. Plates were then incubated for about 5 min at 37 °C and the resulting cell suspension was pipetted up and down for 3 times to break cell aggregates. The resuspended Y1 cells were then transfered to a 50 ml plastic vial, containing at least the same amount of Y1 cell culture medium as trypsin/EDTA used for trypsinization. The cells were pelleted by centrifugation, resuspended in Y1 cell culture medium and plated in the desired densities.

For transfection in 6-well plates, 600.000 – 700.000 Y1 cells per well were plated. Transfection was performed at a confluency of approximately 70%, which was typically reached 12 h after plating. Transfections were either performed by the Lipofectamine 2000 (Invitrogen) method according to the manufacturer's protocol or using the calcium phosphate method. For every well to be transfected by the calcium phosphate method, 10 µl 2,5 M $CaCl_2$ were added to 4 µg of DNA and the sample was filled up to 100 µl with H_2O. After adding 100 µl of 2x BBS the sample was vortexed and incubated for 10 minutes at room temperature. The transfection mix was then pipetted dropwise into the well and after gentle rocking, the plate was incubated overnight at 37 °C and 5% CO_2. The following day, medium was changed. In order to confirm transfection success visually, dsRed or eGFP expression plasmids were (co-) transfected and fluorescence was evaluated 24 h after transfection.

4 Results

4.1 Generation of Constructs pPNTflpCremyctagPml and pPNTflpCreERT2Pml

In the following sections, the generation of constructs for gene targeting is described. These targeting constructs are engineered to allow the specific integration of sequences encoding either a constituitively active cre recombinase tagged with the human c-myc epitope (Evan et al., 1985) or the tamoxifen-inducible version CreERT2(Feil et al., 1997) into the murine ACTHR locus. For general strategies in gene targeting, construct design, clone screening etc. see Kwan et al., 2002 (Kwan, 2002) and Joyner et al., 2000 (Joyner, 2000). The targeting constructs are then used for recombination in murine embryonic stem (ES) cells. Of recombinant ES cells, the mouse line ACTHR-CE2 was generated.

4.1.1 Modification of the Universal Gene Targeting Vector pPNTflp

The vector pPNTflp constructed by v. Waldenfels and Deussing in our group is a universal vector for gene targeting and combines the conditional gene targeting vector pPNT4 (Conrad et al., 2003) with a cassette self-excision strategy initially described in vector pRVa3^{ACN} (Bunting et al., 1999). The vector pPNTflp contains the enhanced version of the Flp recombinase (FLPe) (Buchholz et al., 1998) under control of an angiotensin converting-enzyme promoter (ACE). This promoter is only active during spermatogenesis, resulting in expression of the controlled gene early during the formation of male germ-cells. A similar approach was taken by Bunting et al. who generated mice in which ACE promoter driven, cre recombinase mediated self-excision was achieved (Bunting et al., 1999). A neomycin resistance gene (Neo) expressed under the highly active phosphoglycerate kinase I promoter (PGK) is located upstream of the ACE-FLPe cassette. The neomycin resistance serves as positive selection marker for identification of ES cell clones with integration of the targeting construct. As the Neo maker was shown to influence expression of adjacent genes, it needs to be removed after the selection process (Pham et al., 1996). To this end, the vector region containing the Neo and Flp cassettes is flanked with frt sites, the recognition sites for Flp recombinase. Upon ACE promoter driven Flp expression during spermatogenesis, the entire region between the two frt sites will thus excise

itself (see figure R1) abolishing the neomycin selection cassette with its possibly detrimental effect on expression of neighbouring genes.

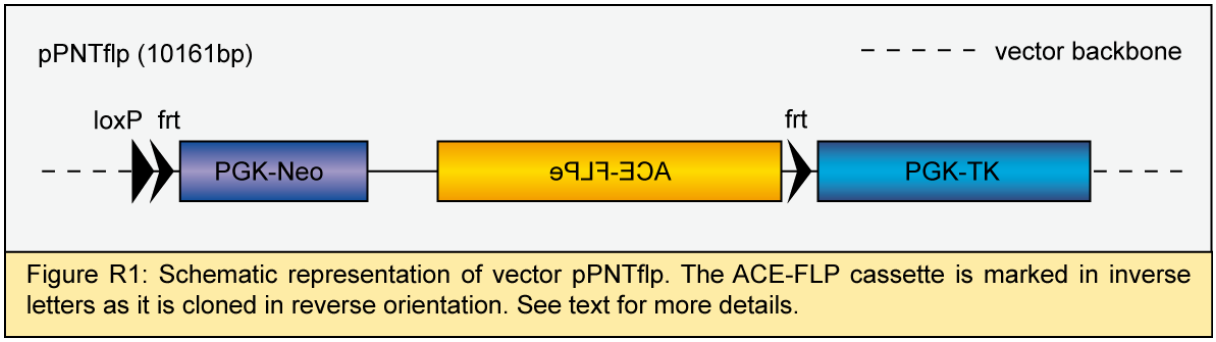

Figure R1: Schematic representation of vector pPNTflp. The ACE-FLP cassette is marked in inverse letters as it is cloned in reverse orientation. See text for more details.

The conditional gene targeting vector pPNT4 (Conrad et al., 2003) contributes a phosphoglycerate kinase I promoter driven thymidine kinase gene to vector pPNTflp. This cassette serves as negative selection marker for construct integration via homologous recombination: Thymidine kinase catalyzes the transformation of the non-toxic substance ganciclovir into a toxic metabolite thereby causing cell death. If integration into the host ES cell genome has occurred through homologous recombination, i.e. non-random, this cassette will be lost as it is designed to be located outside the homologous region in the final constructs. In contrast, ES cell clones with random construct integration will be killed upon ganciclovir administration. The loxP site in pPNTflp likewise originates from vector pPNT4 (Conrad et al., 2003) and has to be eliminated in order to use this vector for generation of a cre knock-in, as it would serve as undesired cre recombinase target (see below).

In order to linearize the final constructs for electroporation into ES cells, an additional Fse I restriction site was introduced into the pPNTflp vector. For this purpose, a PCR using primers FRT-Mlu and FRT-BamFs was performed on pPNTflp creating a 684 bp replacement insert with a Fse I restriction site located 3' of the BamH I restriction site. This fragment was subcloned, sequenced and ligated into pPNTflp using the Bam HI and Mlu I restriction sites. The resulting vector was sequenced for correct ligation and named pPNTflpfse (see figure R2).

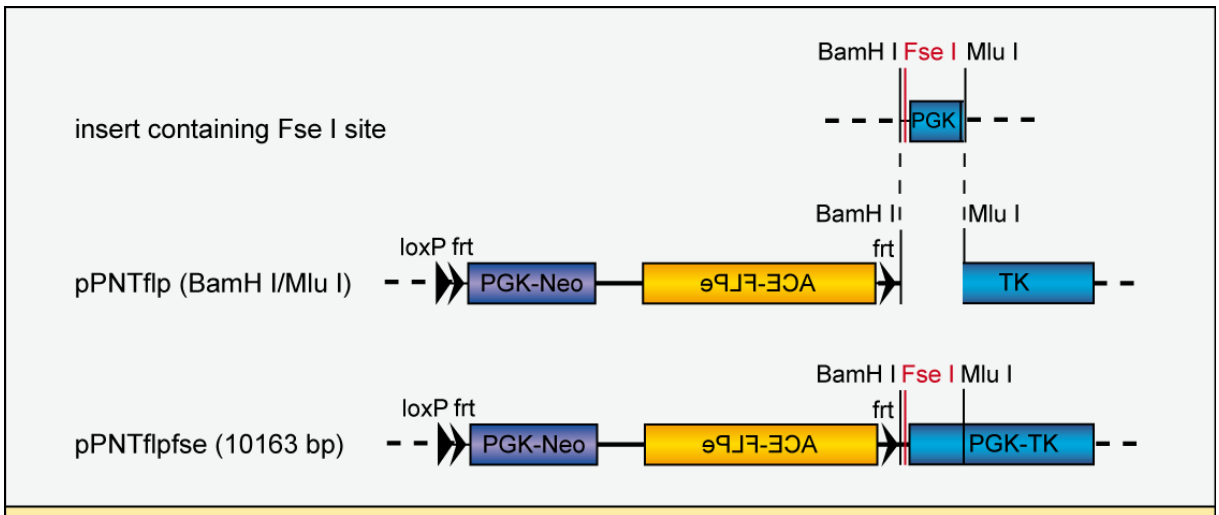

Figure R2: Insertion of a Fse I restriction site into pPNTflp. For details see text.

To abolish the loxP site and to introduce additional Sal I and SgrA I restriction sites subsequently needed for cloning, a PCR was run on pPNTflp with primers Rsr-FRT and SrgSal-FRT resulting in a 1260 bp product that was subcloned, sequenced and ligated into pPNTflpfse using the Xho I/Rsr II and Sal I/Rsr II restriction sites respectively (see figure R3). Sal I and Xho I have compatible overhangs that ligate to form a Taq I recognition site. The resulting vector was named pPNTflpfseSgr as through primer SrgSal-FRT a SgrA I restriction site was introduced upstream of the frt site.

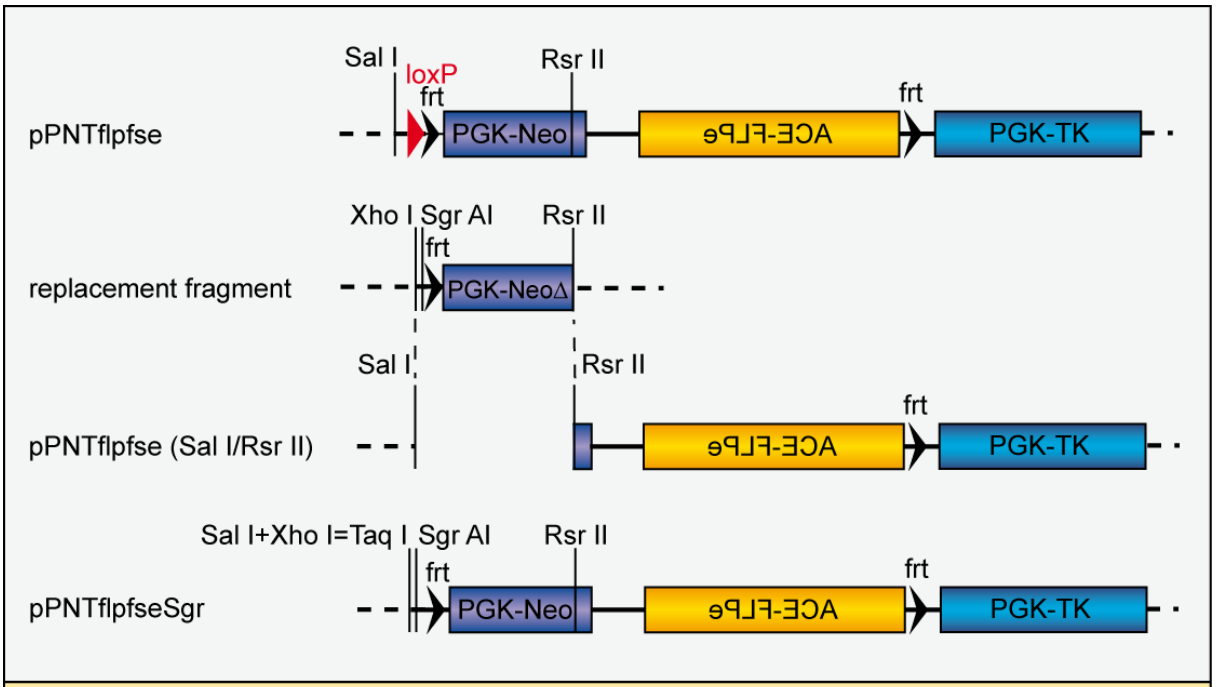

Figure R3: Deletion of loxP site in pPNTflpfse. NeoΔ indicates an incomplete Neo cassette that is complemented by the ligation. For details see text.

4.1.2 Cloning of Homologous Arms

4.1.2.1 Generation of the 5’ Homologous Arm

The 5’ homologous arm (HA) was generated by long range PCR of murine C57BL/6J genomic DNA on the bacterial artificial chromosome pBACe3.6 RP23-179K16 (obtained from F. Beuschlein, genetic strain background C57Bl6/J) using primers Mar-Asgr and Mar-Cre. The PCR product containing 2,8 kb homologous sequence including the ACTH receptor exon 3 as well as the 5’ terminus of the exon 4 up to the endogenous start codon was subcloned and sequenced. Through primer Mar-Asgr, Apa I and SgrA I restriction sites were added. Primer Mar-Cre created a 24 bp overlap to the 5’ terminus of the Cre-ORF allowing the 5’ HA to be used as primer in a megaprime PCR as described below.

4.1.2.2 Fusion of 5’HA to Cre Recombinases

Fusion of the 5’ homologous arm to the 5’ terminus of cre was achieved by combining a megaprime and multiplex PCR with megaprimer 5’HA and primers Cre-BamH1 and Mar-Asgr using pCreERT2 (vector obtained from P. Chambon) as template (see figure R4). The PCR product was subcloned to form vector 5’HACreTOPO and proven to comprise the 5’ HA fused to the first 361 bp of the CreERT2-ORF up to the endogenous BamH I restriction site by sequencing.

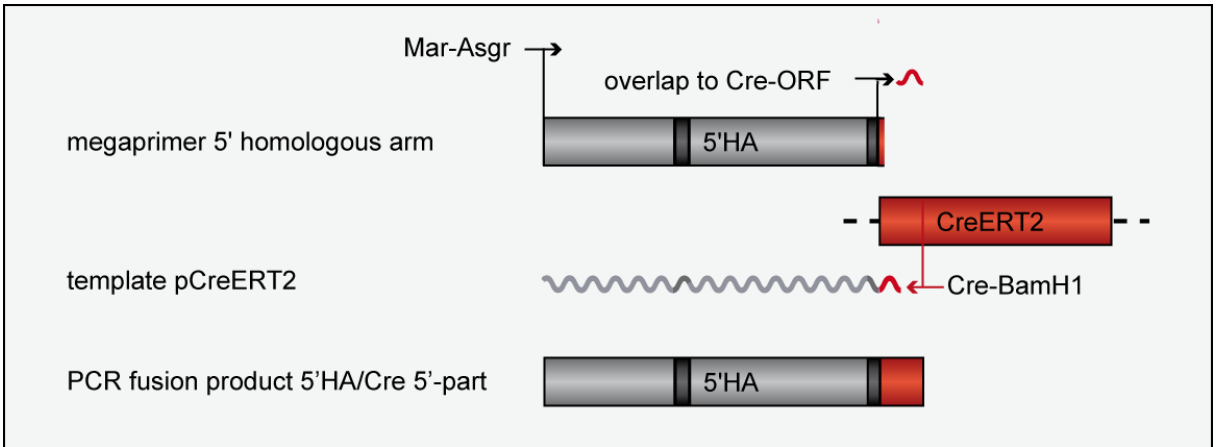

Figure R4: Fusion of 5’ homologous arm to cre 5’ terminus by megaprimed multiplex PCR. The position of ACTHR exons 3 and 4 in the 5’ HA is depicted in dark grey. For details see text.

The resulting fusion product between the 5'HA and the cre 5' terminus was ligated to the remaining 3' terminal part of ORF of the tamoxifen-inducible CreERT2 using restriction sites Apa I and BamH I. The resulting vector 5'HACreERT2 was subcloned and insertion sites were sequenced (see figure R5).

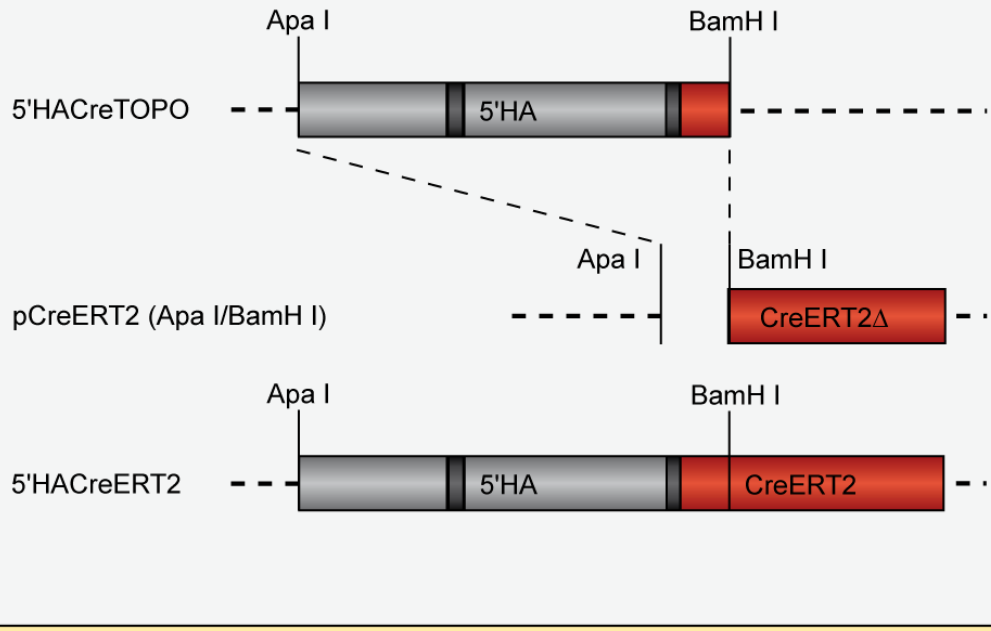

Figure R5: Ligation of 5'HA and 5' terminus of cre to the 3' terminus of CreERT2. CreERT2Δ indicates an incomplete ORF that is complemented by the ligation. For details see text.

In an analogous way, fusion to the 3' terminal part of the constitutively active nlsCremyctag (vector pnlsCremyctag obtained from K. Kobayashi) was achieved by ligation using the restriction sites Xba I/BamH I and Spe I/BamH I respectively. Xba I and Spe I produce compatible cohesive overhangs that after ligation form a Bfa I recognition site. With the fusion to the 5' terminus of the CreERT2, which was used as PCR template to create the fusion to the 5'HA (see above), the 5' terminal nuclear localization signal (nls) of the nlsCremyctag was lost. The vector resulting from the ligation was termed 5'HACremyctag and correct ligation was verified by sequencing.

4.1.2.3 Generation of the 3' Homologous Arm

The 3' homologous arm was generated by long range PCR on pBACe3.6 (RP23-179K16) using primers Mar-Bam und Mar-fse. The Mar-Bam primer adds a BamH I restriction site to the 5' end of the 3' homologous arm. The PCR product (expected

size 5,9 kb) was subcloned and sequenced. Sequencing failed for an adenine/guanine-rich region in the 3' homologous arm which was therefore further characterized by nested PCR using primer pairs 3'HAinnen2/3'HAinnengap2 and FRi-5/3'HAinnengap1. The nested PCR revealed the respective region to be approximately 150 bp shorter in pBACe3.6 (RP23-179K16) as compared to the sequenced published online at *www.ensembl.org*. Nested PCR on pBACe3.6 (RP23-179K16) DNA, genomic TBV2 (derived from strain 129S6/SvEv/Tac) and genomic C57Bl/6 DNA resulted in the same length of product, which therefore likely represents the true wild-type situation in these genetic backgrounds. In silico sequence analysis furthermore revealed an additional BamH I restriction site in the homologous arm, so that the final length of BamH I digest-recovered 3' homologous sequence was 5,0 kb.

4.1.3 Insertion of the 3' Homologous Arm Into the Targeting Vector pPNTflpfseSgr

The 5,0 kb of 3' homologous sequence were recovered from the subcloning vector by BamH I digest and ligated into the BamH I linearized, dephosphorylated pPNTflpfseSgr vector. Clones with insertion of the 3' HA were identified by colony PCR using primers FRi-5 and 3'HAinnengap1. Correct orientation of the 3' HA was demonstrated by restriction digest and the resulting vector was named pPNTflpfseSgr3'HA (see figure R6).

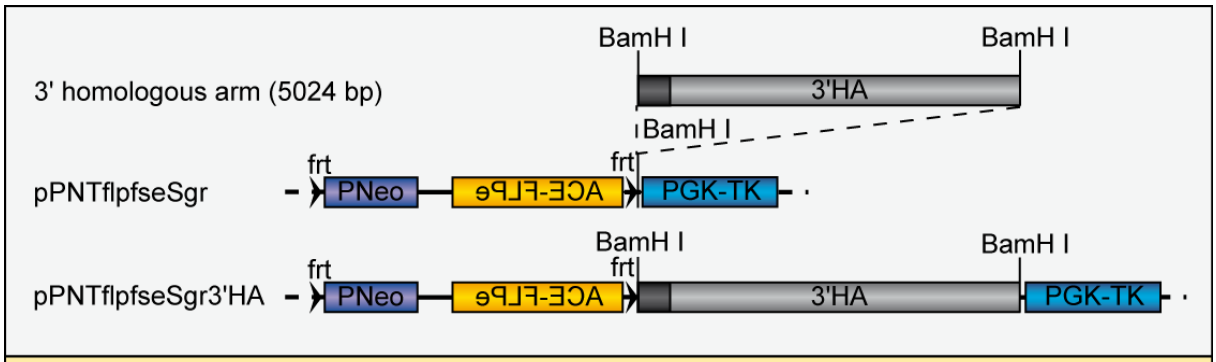

Figure R6: Introduction of the 3' homologous arm into pPNTflpfseSgr. The position of the 3' terminus of the endogenous ACTHR exon 4 within the 3'HA is shown in dark grey. For details see text.

4.1.4 Completion of the Targeting Constructs pPNTflpCremyctagPml and pPNTflpCreERT2Pml

The CreERT2 targeting vector was completed by ligation of the 5'HA/CreERT2 sequence into the SgrA I and Sal I restriction sites of vector pPNTflpfseSgr3'HA. Correct ligation was verified by sequencing of the insertion sites and the resulting vector was named pPNTflpCreERT2 (see figure R7).

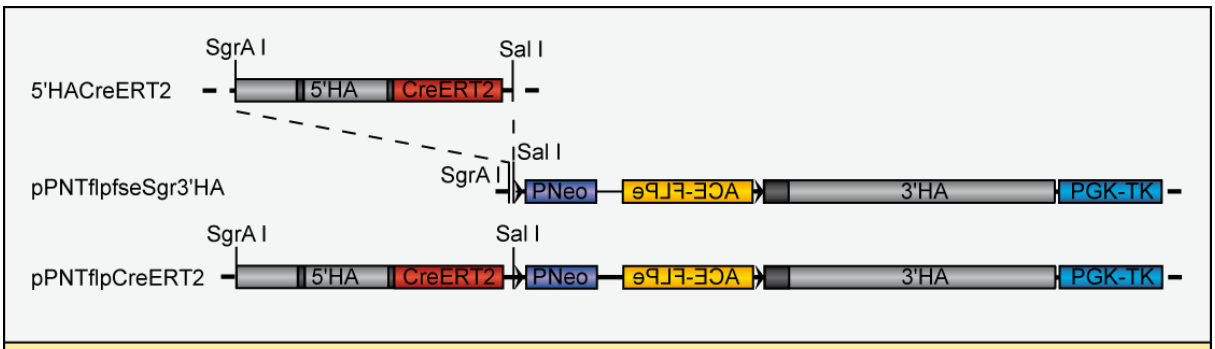

Figure R7: Completion of targeting vector pPNTflpCreERT2. For details see text.

Analogously, the Cremyctag targeting vector was completed by ligation of the 5'HA/Cremyc sequences of vector 5'HACremyctag into the SgrA I and Sal I restriction sites of pPNTflpfseSgr3'HA. The resulting vector was named pPNTflpCremyctag and verified by sequencing (see figure R8).

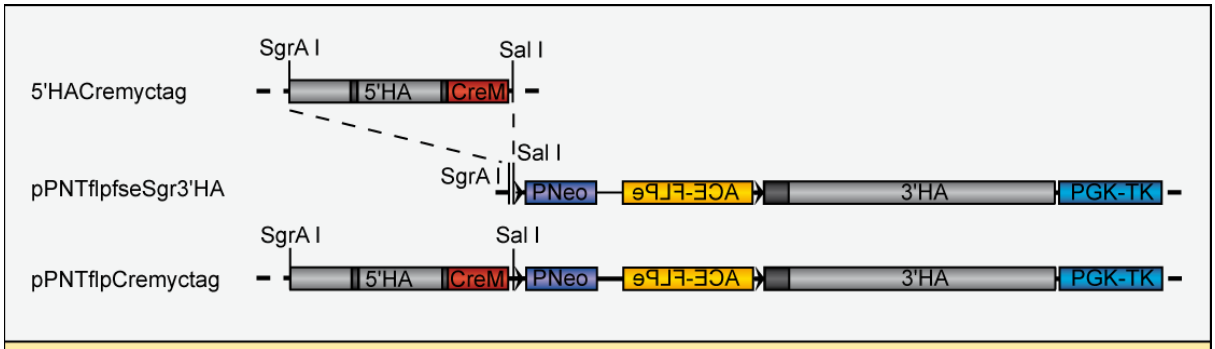

Figure R8: Completion of targeting vector pPNTflpCremyctag. The open reading frame of Cremyctag is depicted as CreM. For details see text.

Because of its low concentration of 2 U/µl, Fse I promoted linearization of the pPNTflpCreERT2 and pPNTflpCremyctag vectors required for electroporation into embryonic stem cells proved to be highly ineffective. To facilitate linearization, Pme I and Pml I restriction sites were inserted into the Fse I linearized, dephosphorylated targeting vectors through ligation with the hybridized linker oligonucleotides

PmlLinker-1 and PmlLinker-2. To anneal, both oligonucleotides were mixed, heated to 95°C and cooled down to room temperature. The resulting vectors were named pPNTflpCreERT2Pml and pPNTflpCremyctagPml (see figure R9), verified by restriction digest (see figure R10) and electroporated into murine IDG3.2 embryonic stem cells after Pml I mediated linearization.

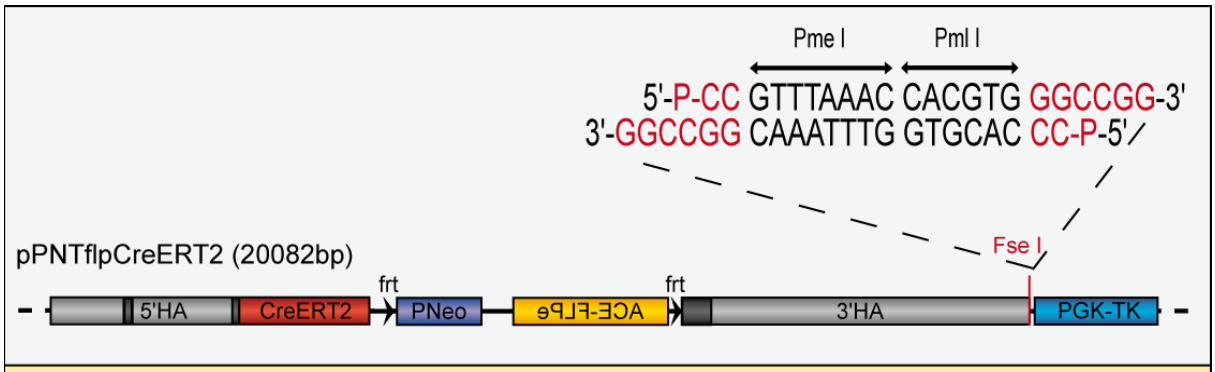

Figure R9: Introduction of Pme I and Pml I restriction sites into targeting vector pPNTflpCreERT2. The ligation was performed with annealed linker oligonucleotides, PmlLinker-1 (upper one) and PmlLinker-2 (lower one). For details see text.

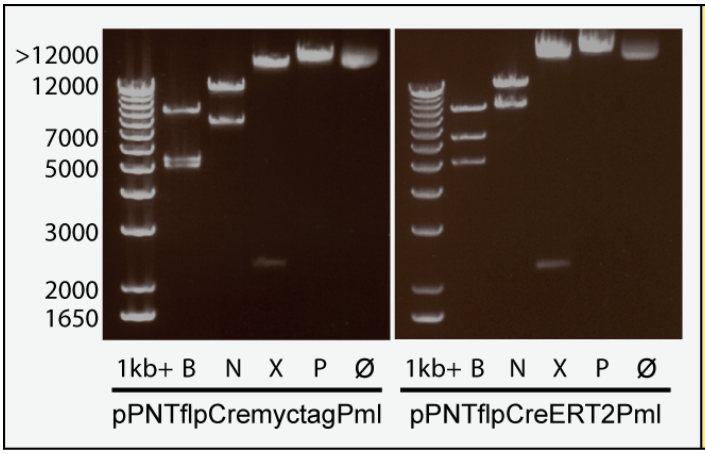

Figure R10: Verification of the targeting constructs by restriction digestion. The 19,0 kb vector pPNTflpCremyctag and the 20,1 kb vector pPNTflpCreERT2 were digested with Bam HI (B), Not I (N), Xma I (X) and Pme I (P) resulting in the desired restriction pattern. Lanes Ø show the uncut vectors, lanes 1kb+ show the 1 KB Plus DNA Ladder (Invitrogen) as size standard. The Pme I linearized constructs as shown in lane P were used for electroporation into ES cells .

4.2 Embryonic Stem Cell Culture

Murine IDG 3.2 hybrid (derived from strains C57Bl/6J and 129S6/SvEv/Tac) embryonic stem (ES) cells (obtained from R. Kühn) were cultivated on mitomycin C inactivated embryonic mouse fibroblast feeder-cells (EMFI). $7x10^6$ ES cells (passage 10) per cuvette were electroporated with 20 µg of Pml I linearized vector pPNTflpCreERT2Pml and pPNTflpCremyctagPml, respectively. Two days after electroporation, positive selection with G418 was initiated at a concentration of 180 µg/ml. Four days after electroporation, G418 concentration was increased to 200

µg/ml. The same day, negative selection with 2 µM ganciclovir was started which was maintained until picking of clones (12 days post electroporation) or for half of the clones till the end of the in vitro manipulations. G418 selection was continued for 2 additional days. Per construct, 192 clones were picked and transferred onto 96-well plates. Colonies were split according to their confluency after 2 days, and again after 4 days, when duplicates of the clones were seeded on 96 well-gelatine coated plates for DNA extraction and subsequent Southern blot analysis.

4.3 Screening of ES Cell Clones for Construct Integration

In order to identify ES cell clones with the desired homologous recombination event, e.g. with knock-in of the CreERT2 coding sequence into the ACTHR locus, screening by Southern blotting was performed.

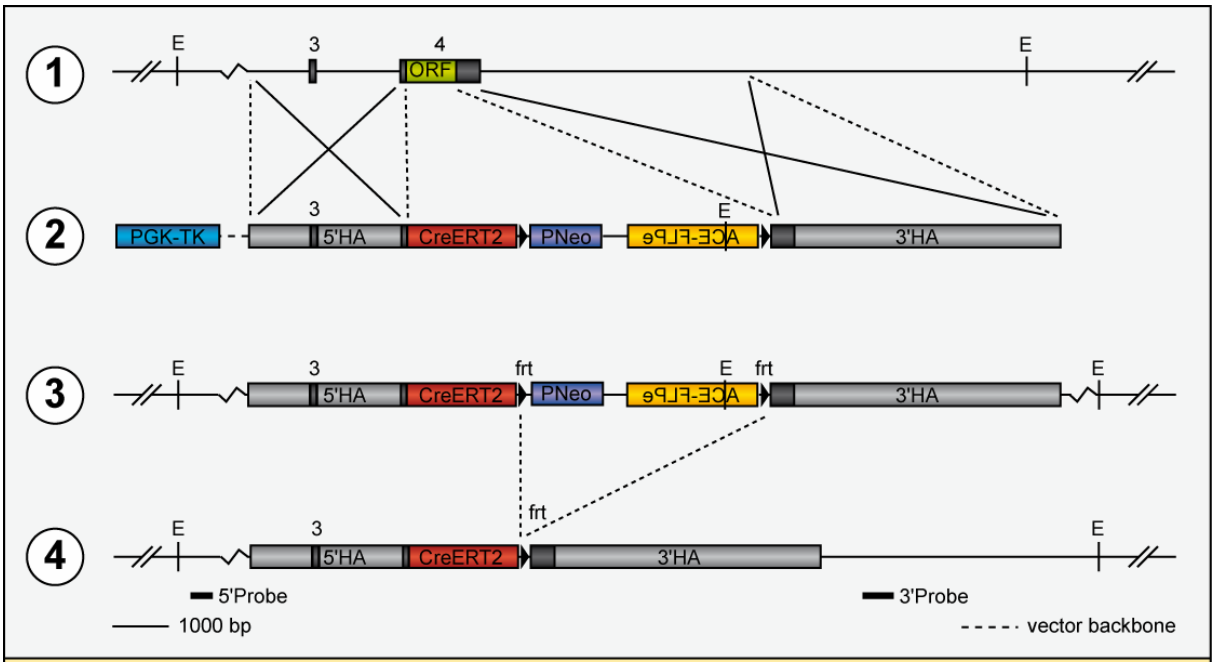

Figure R11: Overview over the desired gene targeting events at the murine ACTHR gene locus. 1) Wild-type genomic organization (only exons 3 and 4 are shown). The distance between the two closest Eco RI restriction sites (E) is 15,3 kb in the wild-type situation according to *ensembl* sequence. 2) The Pml I linearized targeting construct for CreERT2. Homologous recombination between the embryonic stem cell genome and the Pml I linearized targeting construct is indicated by X. As the PGK-TK cassette is located outside the homologous regions, it is lost during homologous recombination allowing for negative selection with ganciclovir. 3) Genomic locus after homologous recombination. The distance between Eco RI restriction sites as detected with the 3' probe is 10,8 kb (as compared to 15,3 kb in wild-type) allowing for clone identification by Southern blotting. 4) Targeted genomic locus after flp mediated excision of the selectable marker cassette. A possible effect of Neo expression on expression of adjacent genes can thereby be circumvented.

To this end, external probes, located outside of the homologous sequences, which detect a modification in restriction fragment length following successful recombination were designed (3' probe and 5' probe). Probes were generated by PCR on bacterial artificial chromosome pBACe3.6 (RP23-179K16) DNA using primers 3'Probe1 and 3'Probe2 and 5'Probe1 and 5'Probe2, subcloned and sequenced. Figure R11 illustrates the screening strategy for the 3' probe-detected change in Eco RI restriction fragment length after recombination with the linearized pPNTflpCreERT2Pml vector.

Southern blotting of ES cell genomic DNA followed by hybridization with the 3' probe revealed three clones with the supposedly desired integration of the pPNTflpCreERT2Pml construct (see figure R12) but no clone with correct integration of pPNTflpCremyctagPml. As no clone with correct integration of the pPNTflpCremyctagPml vector could be retrieved during the first round of ES cell culture, the vector was electroporated into IDG3.2 cells a second time, treated as before and 384 clones were picked.

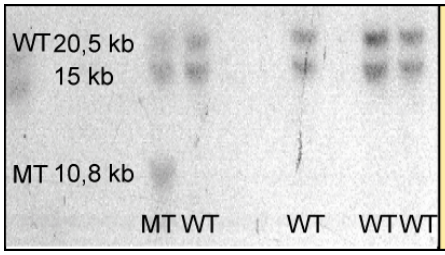

Figure R12: Southern blot of recombinant ES cell DNA carrying the CreERT2 construct (after EcoRI digestion and hybridization with the 3' probe). Wild-type band (WT) at 20,5 kb. The presence of an additional band at 10,8 kb indicates construct integration (mutant=MT). Prior to the extensive characterization of the locus, the 15 kb band was considered unspecific. Refer also to discussion section for further detail.

4.4 Generation of ACTHR-CE2 Mice

One of the three clones showing integration of pPNTflpCreERT2Pml was expanded and used for blastocyst injection in passage 18. Harvesting of the blastocysts and blastocyst injection was performed by the GSF mouse core facility staff, Neuherberg. Following blastocyst transfer into the oviducts of pseudo-pregnant foster mothers, nine chimeric mice were born, of which 3 exhibited germline transmission of the construct as evaluated by genotyping PCR of the offspring. The resulting mouse line was named ACTHR-CE2 and maintained by brother to sister matings (see figure R13).

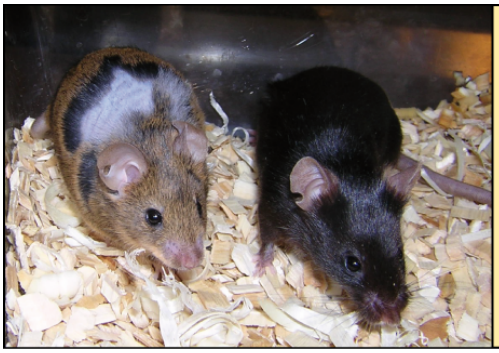

Figure R13: Breeding pair. Male ACTHR-CE2 mouse with 100% coat color chimerism and female breeding partner (black coat color).

4.5 Establishment of a Genotyping PCR for ACTHR-CE2 Mice

A multiplex PCR assay using primers 5'HAss, CreAs and Exon4As was established for genotyping of mutant mice. In the mutant situation a 841 bp PCR product is generated as compared to the 385 bp product of the wild-type situation (see figure R14). Conditions were optimized to the following characteristics: 50 µl samples with 1 µl primer 5'HAss (10 pmol/µl), 1,2 µl primer CreAs (10 pmol/µl) and 0,5 µl primer Exon4As (10 pmol/µl). Cycle number was 35. Primer annealing was carried out at 54°C for 30 s and elongation for 1 min at 72°C. Alternatively, genotyping was performed by PCR using primers detecting cre or flp recombinase, neomycin resistance or the endogenous exon 4 of ACTHR. Of 97 male and female mice genotyped in the F2 generation, 18 (18,6%) were found to be wild-type and 79 (81,4%) to carry the mutant situation. It was however, impossible to distinguish heterozygous from homozygous animals by PCR (please also refer to discussion section).

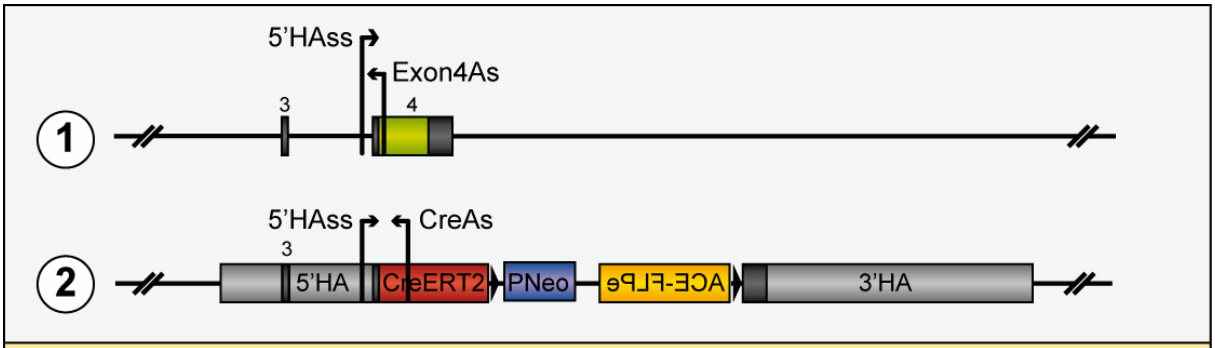

Figure R14: Genotyping PCR. The concurrent use of three PCR primers (5'HAss, Exon4As and Cre As) results in the amplification of a 385 bp product in the wild-type situation as depicted in 1) and a 841 bp product in the recombinant situation as depicted in 2). In case of heterozygosity, both products are generated. For details, see text.

4.6 Characterization of the Targeted ACTHR Locus

Notably, correct integration of CreERT2 from the linearized pPNTflpCreERT2Pml vector could not be verified by hybridization with the 5' probe on Southern blots with ES cell DNA digested with suitable restriction enzymes due to unexpected band sizes and multiple bands. For probe testing, the 3' probe and 5' probe had initially been hybridized with digested pBACe3.6 (RP23-179K16) DNA (derived from strain C57Bl/6). With the tested restriction digests, hybridization had resulted in the restriction fragment lengths predicted from the online mus musculus sequence of *www.ensembl.org* (data not shown). However, when IDG3.2 genomic DNA (hybrid DNA from strains C57Bl/6 and 129Sv) was hybridized with the respective probes, unexpected band numbers and sizes were detected. Therefore extensive efforts were undertaken to characterize the wild-type and the recombinant locus in IDG3.2 cells by Southern blotting with internal and external probes. Figure R 15 shows a representative Southern blot that illustrates the influence of the genetic strain background on restriction length polymorphism. The extent to which restriction length in the murine ACTHR locus is polymorphic depending on the mouse strain can be estimated from figure R16.

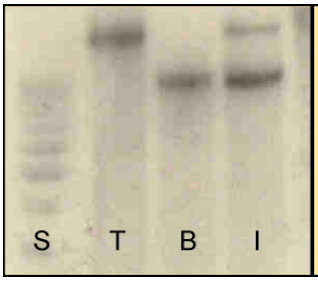

Figure R15: Southern blot of Eco RI digested genomic DNA from different mouse strains (hybridized with the 5' probe). Lane T shows the restriction fragment length in TBV2 (strain 129S6/SvEv/Tac), lane B in C57Bl/6 and line I in IDG3.2 DNA (hybrid ES cells of strains 129S6/SvEv/Tac and C57Bl/6. The two bands in I appear complementary to bands in T and B and therefore likely represent the allelic differences between the two strains that contribute to the hybrid genome of IDG3.2 cells. Lane S shows a size standard. Please also refer to the discussion section.

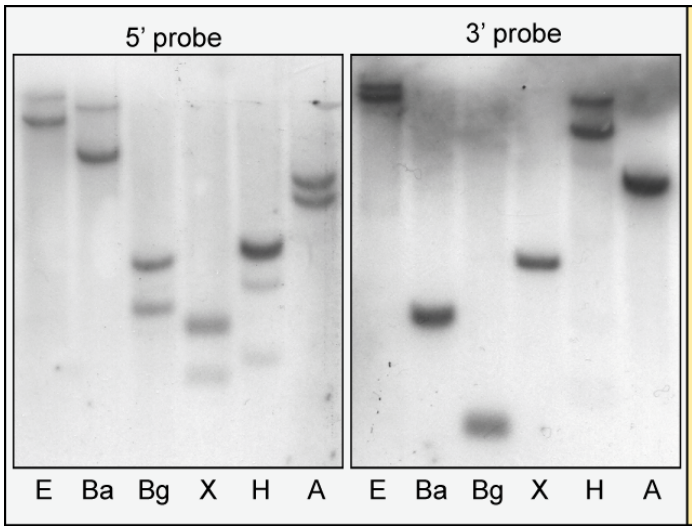

Figure R16: Southern blots of IDG3.2 (hybrid of strains C57Bl/6J and 129S6/SvEv/Tac) ES cell genomic DNA. Hybridization with the 5' probe reveals restriction length polymorphisms (RFLPs) between the two different strains. As shown by hybridization with the 3' probe, this RFLP does not affect all enzymes (E: Eco RI, Ba: Bam HI, Bg: Bgl II, X: Xba I, H: Hind III, A: Acc I)

In order to verify the position of restriction sites as described by the ensembl mus musculus online sequence at *www.ensembl.org*, PCRs were run on the different genomic DNA templates using primer pairs HindSs/As, EcoSs/As and BamSs/As. These primers are located up- and downstream the supposed restriction sites. Lengths of PCR products were compared to predicted lengths and PCR products digested with the respective enzymes (see figure R17).

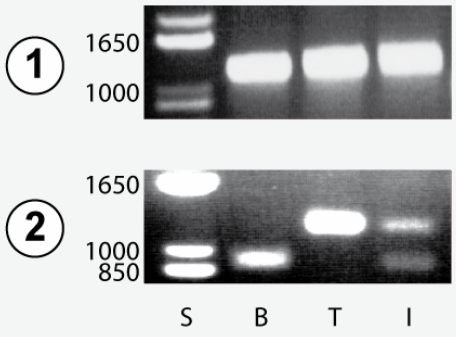

Figure R17: Presence of first Hind III restriction enzyme recognition site downstream of the ACTHR exon 4 is strain dependent. 1) PCR with primers HindSs and HindAs located up- and downstream the supposed restriction site results in the expected fragment length of approx. 1200 bp independent on template DNA mouse strain (B: C57Bl/6J, T: 129S6/SvEv/Tac, I: IDG3.2 hybrid). 2) Following Hind III digestion, the PCR product is cut in B resulting in the expected fragment lengths of 900 bp and 300 bp (300 bp band not shown). However, in strain 129S6 no Hind III restriction site is present. In the case of the hybrid template in I, two different PCR products can be distinguished after Hind III digestion, one with and one without the Hind III site, which corresponds to the two different configurations of the contributing strains (S: 1 Kb Plus DNA Ladder size standard).

As an alternative to detection by Southern blotting, correct integration of the CreERT2 targeting construct into IDG3.2 ES cells was attempted to be verified by long range PCR with primers pairs spanning the entire length of the homologous arms (see figure 18).

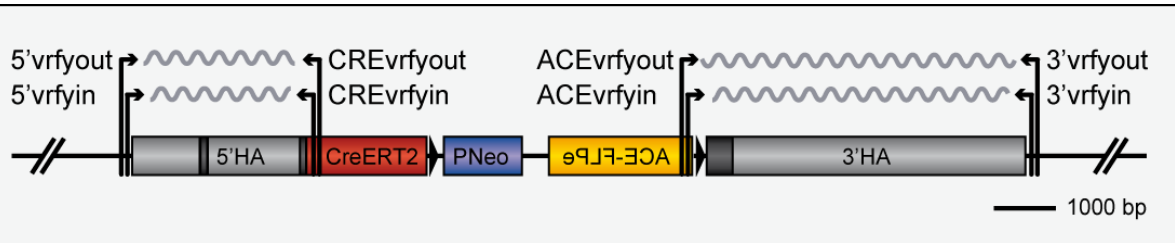

Figure R18: Combined long range/nested PCR strategy to verify correct construct integration. Primers are indicated by arrows. For details see text.

This strategy was pursued in a combined long range/nested PCR approach. PCRs results however were inconclusive, with either not the predicted or multiple bands

(data not shown). In summary, neither Southern blotting nor PCR allowed verification of the desired recombination events.

4.7 Characterization of ACTHR-CE2 Mice

4.7.1 Evaluation for Flp Recombinase Mediated Selection Cassette Excision

As evaluated by flp recombinase detecting PCR, ACE-promoter driven auto-excision of the neomycin selection cassette was unsuccessful. Breeding to universal Flp deleter mice, however, resulted in the abolishment of the frt-site flanked region (data not shown).

4.7.2 Phenotyping of ACTHR-CE2 Mice

On a phenotypic level, ACTHR-CE2 mice were furthermore preliminarily characterized with respect to body weight, adrenal weight, overall adrenal morphology and corticosterone release following ACTH stimulation or restraint stress. No significant differences were found between ACTHR-CE2 and wild-type mice (data not shown).

4.7.3 Evaluation of Cre Expression by RT-PCR and Western Blotting

Total RNA from ACTHR-CE2 mice identified as heterozygous for CreERT2 integration was isolated, cDNA was generated and PCR using primers MAR-1 and Cre3 was performed to detect CreERT2 mRNA. However, CreERT2 mRNA could not be detected despite extensive PCR optimization efforts. Moreover, Western blotting with an antibody directed against the human estrogen receptor ligand binding domain did not show immunoreactivity on proteins of the expected size (see figure R19). As a consequence of the lack of identifiable cre mRNA and protein and the difficulties to characterize the genomic structure at the integration site, breeding of the ACTHR-CE2 mice was discontinued.

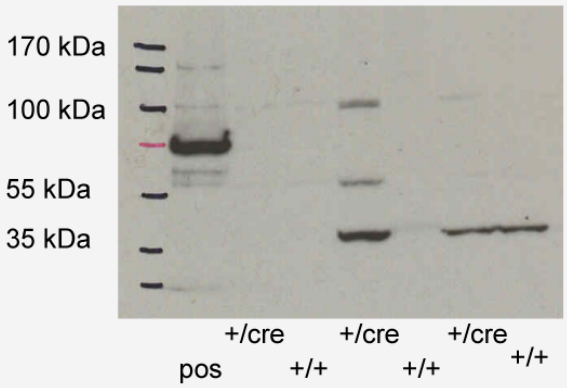

Figure R19: Western Blot of adrenal tissue samples from ACTHR-CE2 mice identified as heterozygous (+/cre) or wild-type (+/cre) labelled with an anti-human estrogen receptor ligand binding domain antibody. This antibody binds the CreERT2 ligand binding domain and exhibits higher specificity than available cre antibodies (Ralf Kühn, personal communication). Sample material (bladder) for positive control (pos) showing the expected protein size of 70 kDa (red mark) was obtained from Robert Feil (TU Munich). In ACTHR-CE2 mice only unspecific immunoreactivity could be detected. Picture courtesy of Constanze Fey.

4.8 Generation of Constructs pMC2RcreMYC and pMC2RcreERT2

In the following sections, the generation of novel constructs targeting the murine ACTHR locus for the introduction of coding sequences of a constituitively active cre variant, CreMYC, and an inducible form, CreERT2, is described. These constructs are designed to overcome the difficulties encountered with the targeting vectors pPNTflpCremyctagPml and pPNTflpCreERT2Pml outlined above as a consequence of which breeding of ACTHR-CE2 mice was discontinued (refer also to discussion). In contrast to these classical knock-in constructs, the novel targeting constructs pMC2RcreMYC and pMC2RcreERT2 were integrated by REd/ET-cloning into a cosmid. This cosmid comprises approximately 43 kb of murine genomic DNA from the ACTHR locus. The recombinant cosmid will be used for homologous recombination in embryonic stem cells. As compared to the prior constructs, the selection strategy with neomycin during ES cell culture is maintained. However, no negative selection marker like thymidine kinase is used. To enable selection during ET cloning, an ampicillin resitance cassette is included. In order to facilitate detection of cre recombinase positive cells in the resulting mouse, an enhanced version of yellow fluorescent protein (Nagai et al., 2002) is additionally cloned into the targeting vectors.

4.8.1 Generation of Homologous Sequences and Cre

4.8.1.1 5’ Homologous Sequence and Cre Open Reading Frame

The 5’ homologous region required for homologous recombination into the targeting cosmid MPMGc121E06653Q2 along with the cre ORF were amplified by PCR from pPNTFlpCremyctagPml using primers 5’HAfw (adding a Hind III and a Pme I restriction site) and 5’HArev (adding a custom made multi cloning site comprising Aat II, Fse I, Sal I and Asc I restriction sites). The introduction of the Aat II restriction site results in a silent mutation in the last amino acid of the cre-ORF (Asp > Asp). The resulting PCR product of 1529 bp was subcloned into pCRII-TOPO vector (Invitrogen), sequenced and checked for homology to the targeting cosmid sequence. To abolish undesired restriction sites from the pCRII-TOPO (Invitrogen) multi cloning site, a Hind III digestion was performed and the vector backbone religated, constituting the new vector p5’HAPCR (see figure R20/1).

4.8.1.2 3’ Homologous Sequence

The 3’ homologous region required for homologous recombination via ET-cloning into the targeting cosmid MPMGc121E06653Q2 was generated likewise by PCR on pPNTFlpCremyctagPml using primers 3’HAfw (adding a multi cloning site with Asc I, Pac I and Xho I restriction sites) and 3’HArev (adding a Pme I and an Apa I restriction site). The 690 bp PCR product was subcloned into a pCRII-TOPO vector (Invitrogen) resulting in vector p3’HAPCR which was sequenced (see figure R20/2) and sequence homology to the targeting cosmid was confirmed.

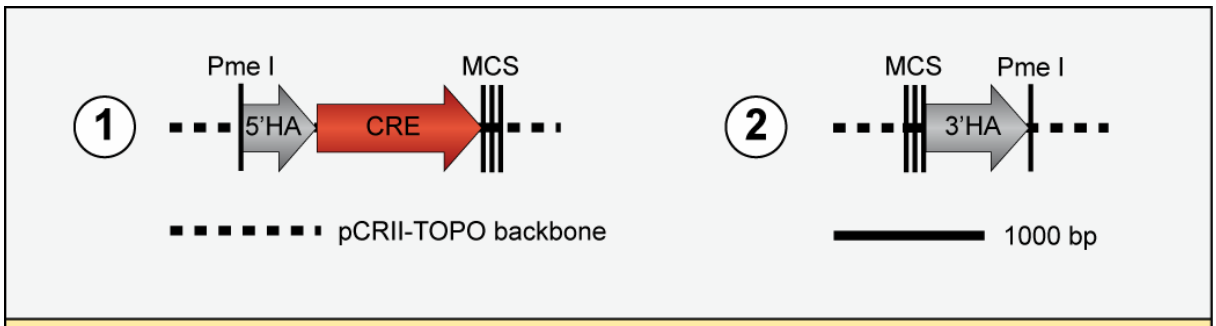

Figure R20: Cloning of homologous arms for pMC2RcreERT2 and pMC2RcreMYC
1) Vector p5'HAPCR which contains the 5' homologous sequence required for the insertion of the construct into the targeting cosmid and the cre recombinase open reading frame. The custom made multi cloning site (MCS) introduced by primer 5'HArev comprises Aat II, Fse I, SalI and Asc I restriction sites. Most of the 5' part of the endogenous pCRII-TOPO MCS was abolished by religation after Hind III digestion (not depicted). 2) Vector p3'HAPCR which contains the 3' homologous sequence required for the insertion of the construct into the targeting cosmid. The custom made multi cloning site (MCS) introduced by primer 3'HAfw comprises Asc I, Pac I and Xho I restriction sites. To facilitate distinction to the previous cloning strategy, cassettes used for the generation of vectors pMC2RcreMYC and pMC2RcreERT2 are depicted as arrows. For further details, see text.

4.8.2 Generation of Cloning Cassettes

4.8.2.1 Improved Yellow Fluorescent Protein (Fragment 1)

Fragment 1 comprises the coding sequence for the improved yellow fluorescence protein (YFP) Venus. It shows faster maturation and higher tolerance to low pH and chloride ions than conventional YFP. Absorption maximum of Venus is at 515 nm, emission maximum at 528 nm (Hadjantonakis et al., 2003;Nagai et al., 2002). Fragment 1 was generated by PCR with primers IVS-Aat and IVS-Sal on template vector 193_IRESVenus (obtained from Claudia Seisenberger, GSF Neuherberg). For cloning purposes, Aat II and Fse I restriction sites were added on the 5' terminus and a Sal I site on the 3' terminus of fragment 1 by the PCR primers. The 1363 bp PCR product was subcloned and sequenced (see figure R21/1). To express the Venus protein, an encephalomyelocarditis virus derived internal ribosomal entry site (IRES) is utilized (Vagner et al., 2001). When coupled to an expressed gene, it allows the cap-independent translation of Venus from a bicistronic mRNA in mammalian cells via entry of the 40S ribosomal subunit at the IRES. Sequencing revealed a guanine to adenine mutation in base pair position 481 which was also detected on the

template vector. Funtionality of the IRESVenus construct was therefore verified by transfection into Y1 murine adrenocortical cells (data not shown).

4.8.2.2 SV40 Polyadenylation Signal (Fragment 2)

Fragment 2 containing a simian virus 40 polyadenylation signal (SV40 pA) was amplified by PCR from vector pPNTFlpCremyctagPml using primers SV40-SalI and SV40-Asc. Through primer SV40-SalI a Sal I restriction site was added. The resulting 311 bp PCR product was subcloned and sequenced (see figure R21/2).

4.8.2.3 Neomycin Resistance (Fragment 3)

Fragment 3, comprising a neomycin resistance gene (Neo) under control of the constitutively active phosphoglycerate kinase-1 promoter (PGK), was amplified from vector pPNT4 (obtained from Jan Deussing, MPI of Psychiatry Munich) using primers PNeo-PacI und PNeo-AscI, adding a Pac I and an Asc I restriction site (see figure R21/3). The resulting 1617 bp PCR product was subcloned and sequenced.

4.8.2.4 Ampicillin Resistance (Fragment 4)

Fragment 4, an ampicillin resistance cassette, was amplified by PCR from vector pcDNA3.1(+) (Invitrogen) using primers prAMP-Pac and AMP-frt-Xho. With these primers, a Pac I restriction site was added at the 5' end and a frt site and a Xho I restriction site at the 3' end of fragment 4. The 1104 bp PCR product was subcloned and sequenced (see figure R21/4). The functionality for selection in bacteria was verified by eliminating the endogenous ampicillin resistance cassette in the vector backbone of the PCRII-TOPO (Invitrogen) subcloning vector and subsequent selection with ampicillin.

4.8.2.5 Mutated Estrogen Receptor Ligand Binding Domain (ERT2)

The ligand binding domain of CreERT2 was generated by PCR on vector pCAG-creER(T2)-bpA SS1 (gift of Ralf Kühn) using primers CREE-Aat and CREE-FseNeu thereby adding a Fse I restriction site to the 3' end of the LBD. The resulting 965 bp PCR product was subcloned to form vector pERT2 which was subsequently sequenced (see figure R21/5).

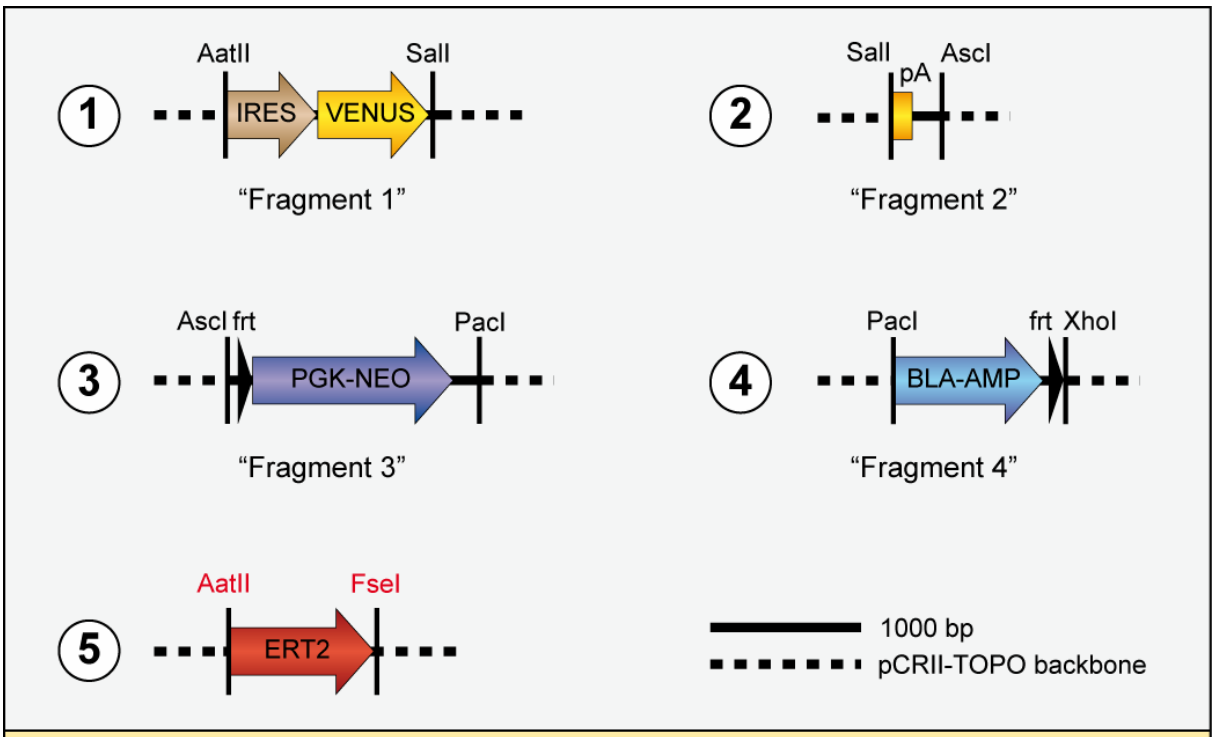

Figure R21: Cloning cassettes for pMC2RcreERT2 and pMC2RcreMYC 1) "Fragment 1", encoding VENUS, a yellow fluorescent protein, translated via ribosomal binding to an internal ribosomal entry site (IRES). 2) "Fragment 2", encoding a Simian Virus 40 polyadenylation signal (pA) required for polyadenylation of the bicistronic CreIRESVENUS mRNA. 3) "Fragment 3", encoding a neomycin resistance required for selection during embryonic stem cell culture. The frt sites in fragments 3 and 4 allow the Flp recombinase mediated excision of the selection markers. 4) "Fragment 4", encoding an ampicillin resistance required for selection during ET cloning. 5) The mutated estrogen receptor ligand binding domain ERT2 providing tamoxifen-inducibility to cre recombinase. To facilitate distinction to the previous cloning strategy, cassettes used for the generation of vectors pMC2RcreMYC and pMC2RcreERT2 are depicted as arrows. For further details, see text.

4.8.3 Assembly of Vectors pMC2RcreMYC and pMC2RcreERT2

4.8.3.1 Ligation of Fragments 1 and 2 into p5'HAPCR

Fragment 2 (SV40 polyadenylation signal) was ligated into the restriction sites Sal I and Asc I of p5'HAPCR. The resulting vector p5'HAF2 was sequenced for correct integration of fragment 2. Ligation of fragment 1 (IRESVenus) into p5'HAF2 was subsequently performed using restriction sites Aat II and Sal I. The resulting vector was named p5'HAF1F2 and sequenced for correct integration (see figure R22).

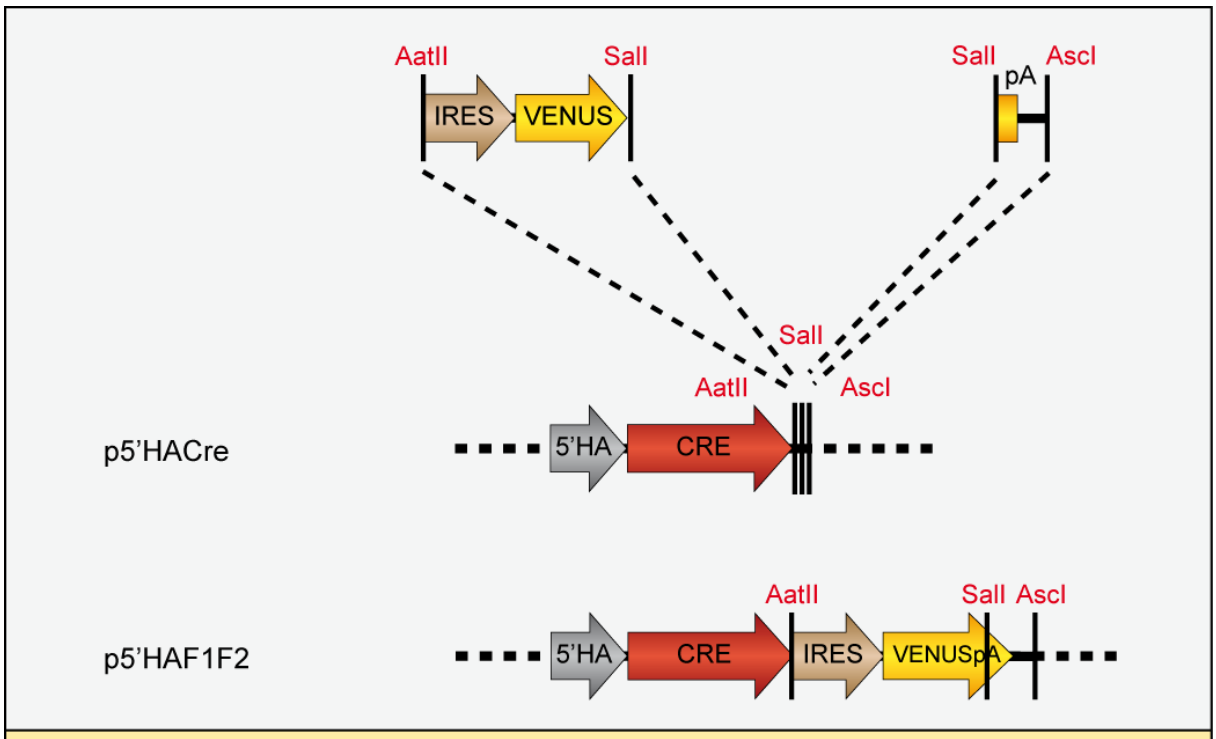

Figure R22: Ligation of fragments 1 (IRESVenus) and 2 (SV40 pA) into vector p5'HACre. The two separate ligations as described in the text are summarized in this picture. The polyadenylation signal ligated to VENUS 3' terminus will not be depicted separately in the following figures anymore. For details, see text.

4.8.3.2 Ligation of CreERT2 c-Terminus and Ligand Binding Domain into p5'HAF1F2

The c-terminal part of CreERT2 and the mutated estrogen receptor ligand binding domain were ligated into the Aat II and Fse I restriction sites of p5'HAF1F2. To this end, p5'HAF1F2 was digested with Bgl II/Aat II and Bgl II/Fse I respectively and the desired backbone fragments 4465 bp and 2622 bp of size were isolated. Triple ligation between these two backbone fragments and the Aat II/Fse I fragment of pERT2 was performed. The resulting vector was named p5'HAF1F2ERT and sequenced (see figure R23). The introduction of an Aat II recognition site for ease of cloning results in an replacement of the first amino acid of the ERT2-LBD (leucine to valine). As both amino acids are of the hydrophobic type, a strong functional effect of this replacement appears unlikely.

4.8.3.3 Ligation of CreMYC c-Terminus into p5'HAF1F2

The c-terminal part of the CreMYC was generated by annealing the linker oligonucleotides CREM-c and CREM-ncneu. As depicted in figure R, the annealed linker shows compatiple cohesive overhangs for ligation into the Aat II and Fse I restriction sites in p5'HAF1F2. As the usual screening for correct integration by restriction digestion was not possible for the size and lack of restriction sites of the inserted linker, colony PCR was performed for clone screening using oligonucleotides CreConnect and CREM-ncneu as primers. Positive clones carrying the desired vector which was named p5'HAF1F2MYC were identified and sequenced (see figure R23).

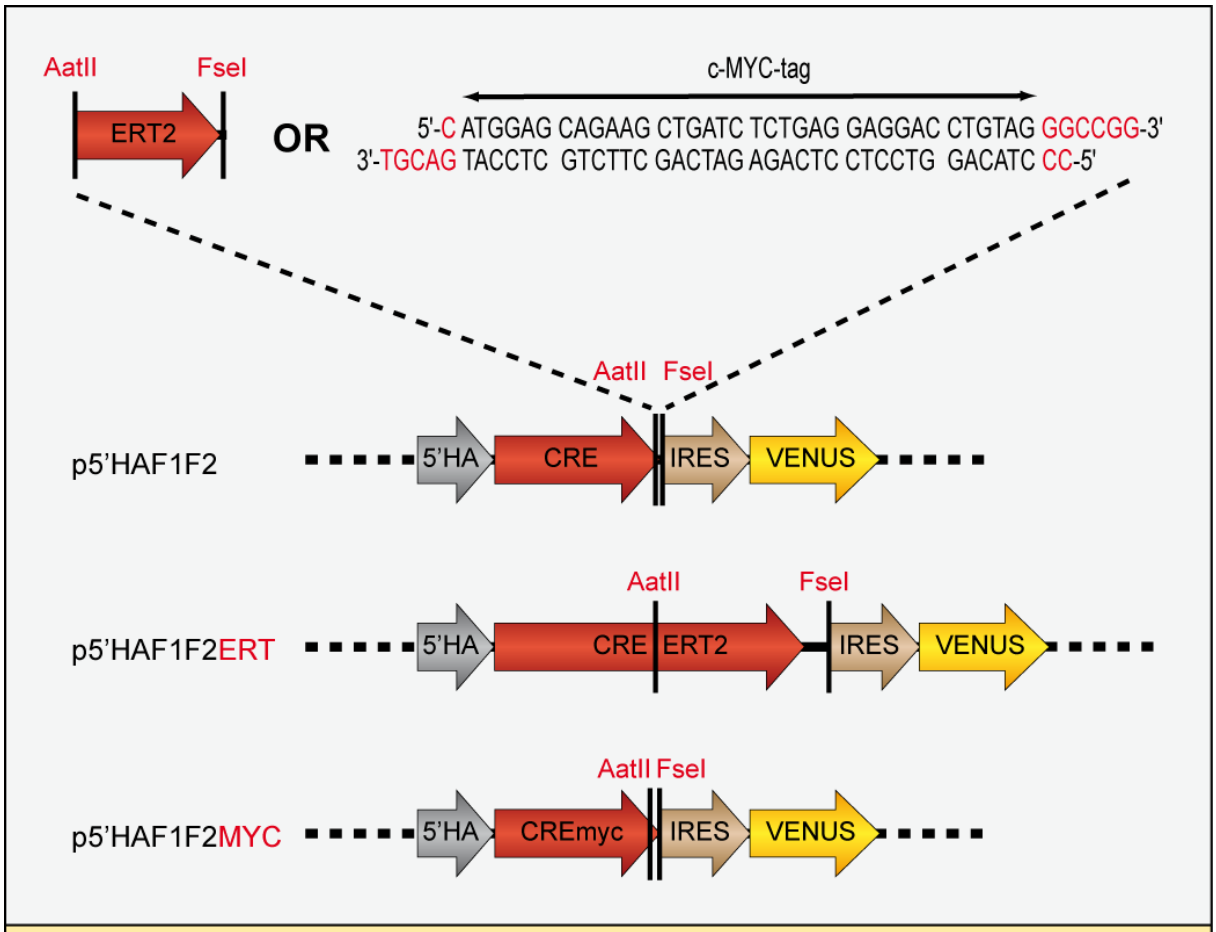

Figure R23: Ligation of alternative c-termini to the cre recombinase. Either the mutated estrogen receptor ligand binding domain ERT2 (resulting in vector p5'HAF1F2ERT) or a c-myc-tag (resulting in vector p5'HAF1F2MYC) is used to complement the cre open reading frame. The c-myc-tag end was generated by annealing the two oligonucleotides CREM-c and CREM-ncneu, thereby also creating the cohesive overhangs (depicted in red letters) required for ligation into the Aat II and Fse I restriction sites. For details, see text.

4.8.3.4 Ligation of Fragments 3 and 4 into p3'HAPCR

Fragment 3 (frt-PGK-Neo) was ligated into p3'HAPCR using restriction sites Asc I and Pac I. The resulting vector was named p3'HAF3 and sequenced for correct integration. For subsequent ligation of fragment 4 (ampicillin resistance cassette) into p3'HAF3, triple ligation between the 4638 bp Xho I digested vector backbone fragment, the 1658 bp Pac I and Xho I digested backbone fragment and fragment 4 digested with Pac I and Xho I was performed. Clones showing the desired ligation event were identified by restriction digestion and sequencing and the resulting vector was named p3'HAF3F4 (see figure R24).

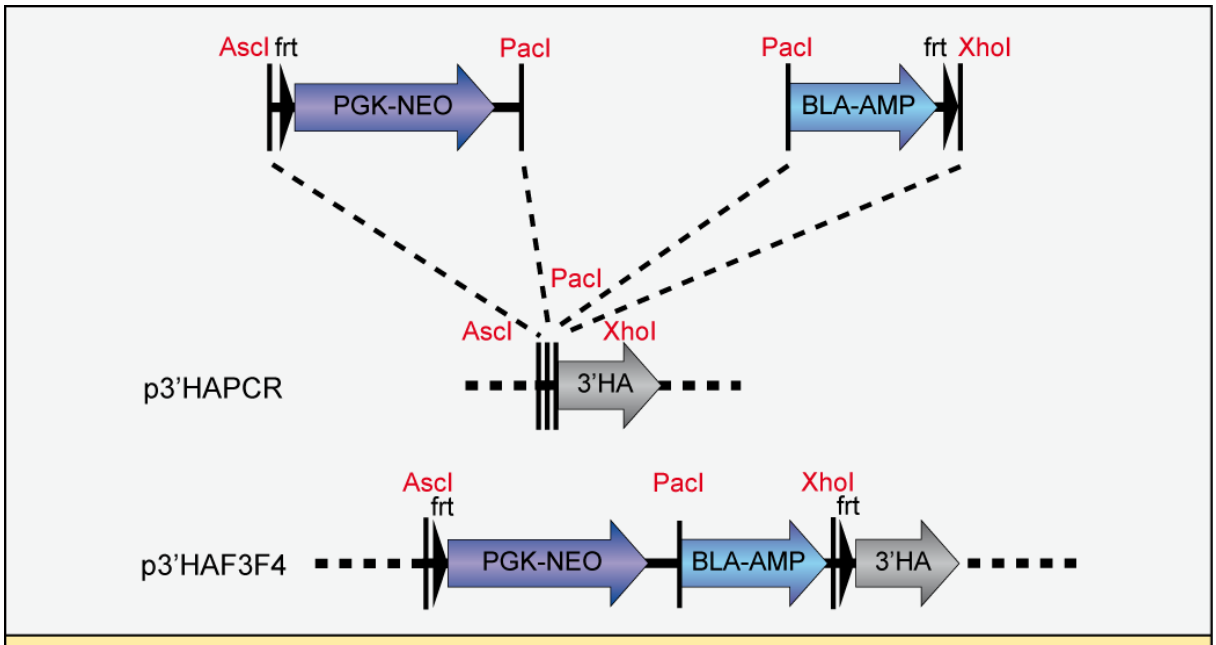

Figure R24: Ligation of fragments 3 (neomycin resistance cassette) and 4 (ampicillin resistance cassette) into p3'HAPCR forming vector p3'HAF3F4. The two independent ligations are described in more detail in the text.

4.8.3.5 Completion of the Targeting Constructs pMC2RcreERT2 and pMC2RcreMYC

The targeting construct pMC2RcreERT was completed by triple ligation of the 3406 bp Asc I/Apa I fragment of p3'HAF3F4 with the 2809 bp Asc I/Cla I and the 5168 bp Apa I/Cla I vector backbone fragments of p5'HAF1F2ERT2 (see figure R25). Escherichia coli clones carrying the desired vector were identified by restriction digestion (see figure R26) and the targeting vector pMC2RcreERT2 was sequenced

entirely. Mutations were detected in the CreERT2 ligand binding domain which was therefore replaced by ligation of a correct ERT2 ligand binding domain into the Aat II and Fse I sites of pMC2RcreERT2.

For completion of the targeting construct pMC2RcreMYC the 3406 bp Asc I/Apa I fragment of p3'HAF3F4 was ligated with the 1894 bp Asc I/Cla I and the 5168 bp Apa I/Cla I vector backbone fragments of p5'HAF1F2MYC in a triple ligation. E. coli clones carrying the desired vector were identified by restriction digestion (see figure R26) and vector pMC2RcreMYC was sequenced for correct ligation. Complete sequencing of the vector was not performed as only one cloning step differs between pMC2RcreMYC and pMC2RcreERT2 which was entirely sequenced as described above.

In order to electroporate the targeting constructs into E. coli containing cosmid MPMGc121E06653Q2, vectors pMC2RcreMYC and pMC2RcreERT2 were digested with Pme I and the 6607 bp and 7522 bp inserts were isolated by gel extraction.

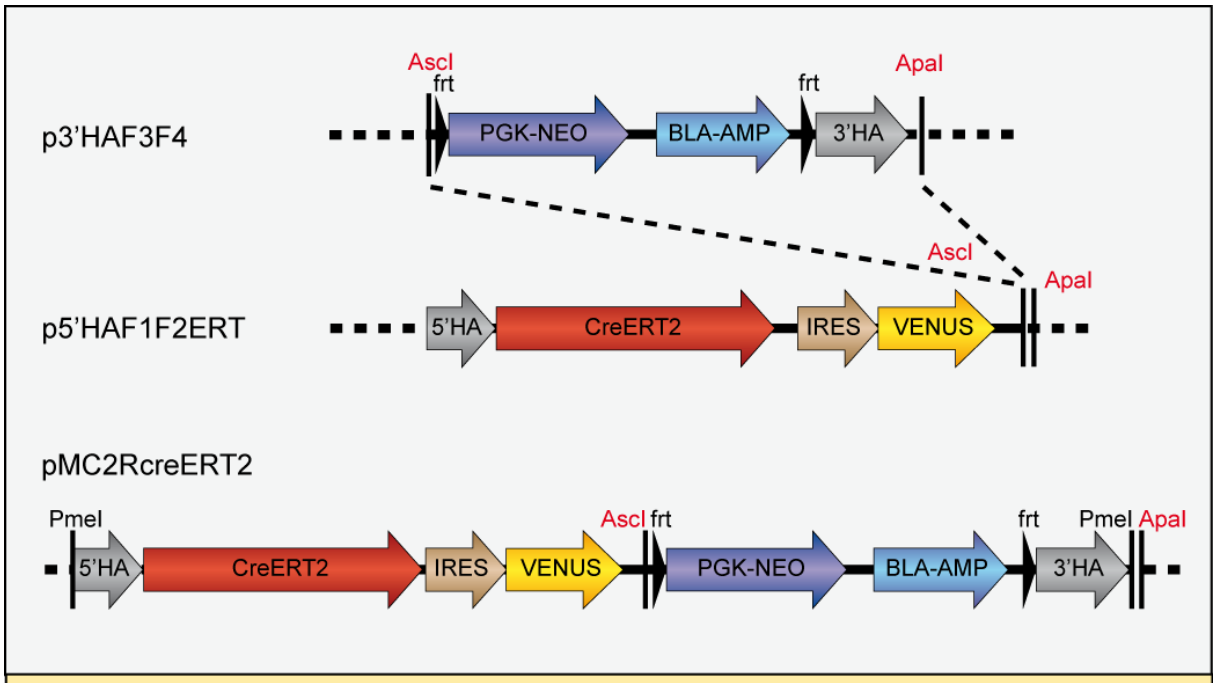

Figure R25: Completion of the targeting construct pMC2RcreERT2 by ligation of the Asc I/Apa I fragment of p3'HAF3F4 into p5'HAF1F2ERT. The targeting vector pMC2RcreMYC was generated analogously (not depicted). To extract the linearized targeting constructs without vector backbone sequences as needed for ET cloning, Pme I digestion was performed. For further details, see text.

In summary, the described targeting vectors both contain a cre coding sequence, either constituitively active or inducible by tamoxifen. An improved yellow fluorescent protein sequence (Venus) to be translated by ribosomal binding to an IRES site is coupled to the cre sequences. Antibiotic resistances that serve as selection marker during ET cloning or ES cell culture are flanked by frt sites and can thus be removed by flp recombinase mediated excision.

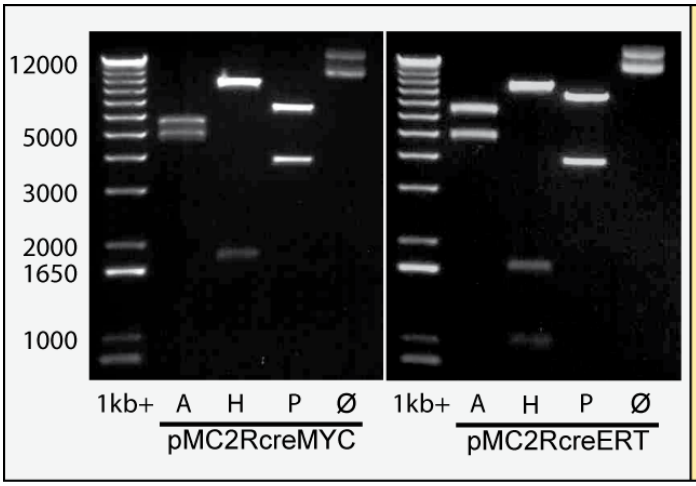

Figure R26: Verification of the new targeting constructs by restriction digestion. The 10,5 kb vector pMC2RcreMYC and the 11,4 kb bp vector pMC2RcreERT were digested with Apa I (A), Hind III (H), Pme I (P) resulting in the desired restriction pattern. Lanes Ø show the uncut vectors, lanes 1kb+ show the 1 KB Plus DNA Ladder (Invitrogen) as size standard. The larger fragments of the Pme I digests, 6607 bp for pMC2RcreMYC and 7522 bp for pMC2RcreERT, were used for homologous recombination into the targeting cosmid.

4.9 Recombineering of pMC2RcreMYC and pMC2RcreERT2 into the Targeting cosmid

In order to complete the targeting cosmids CosCremyc and CosCreERT2, homologous recombination between cosmid MPMGc121E06653Q2 and the Pme I-digested inserts of vectors pMC2RcreMYC and pMC2RcreERT2 was performed by Red/ET-cloning (see figure R27). Bacterial clones carrying the desired, homologously recombined cosmids were identified by colony PCR using primers pre5'HA and CreOutw. Homologous recombination was then verified by sequencing using primers pre5'HA, CreOutw, post3'HA and frtOutw.

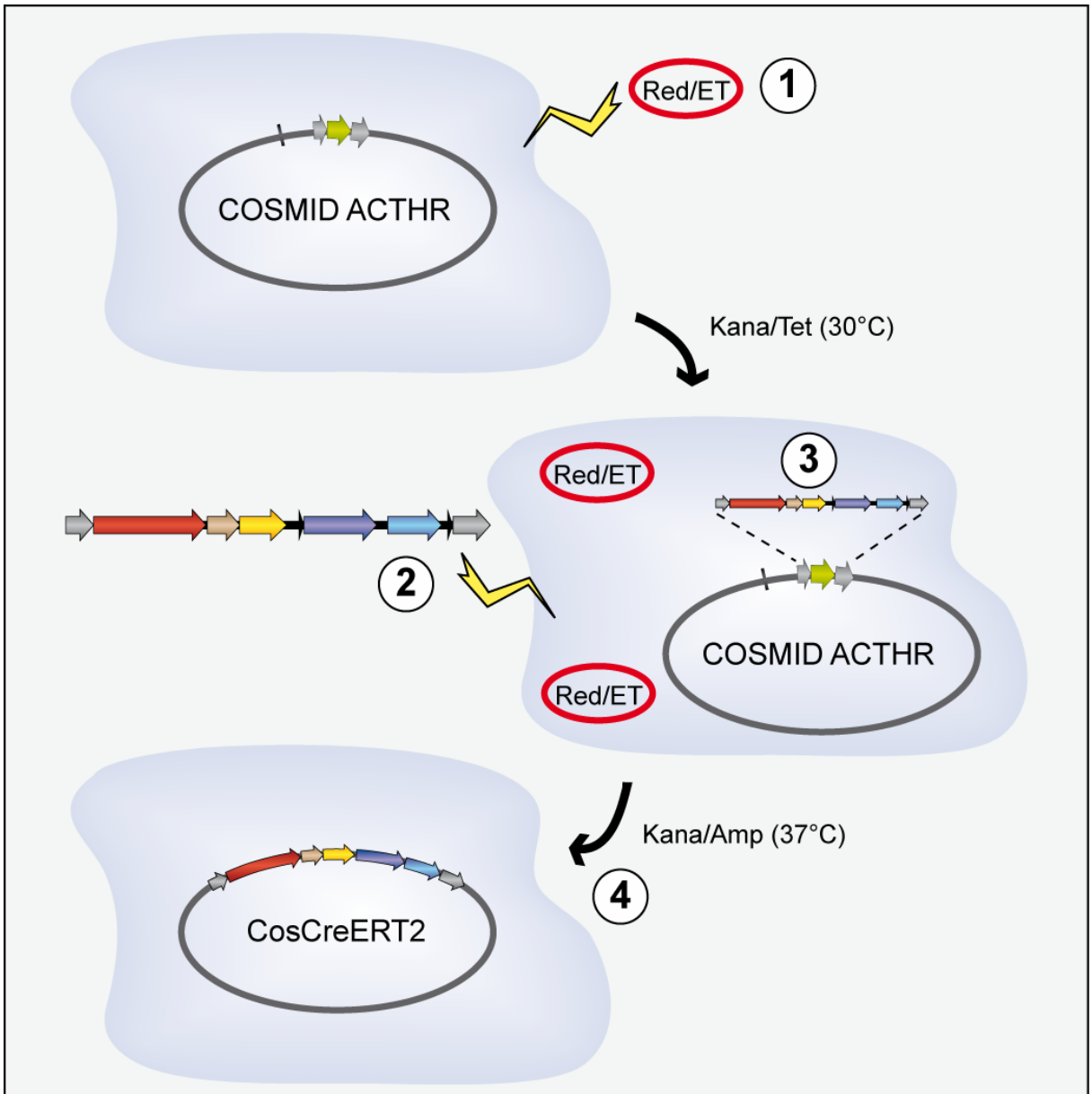

Figure R27: ET-Cloning of construct pMC2RcreERT2 into the targeting cosmid 1) In a first transformation step, the host E. coli strain carrying the cosmid to be recombined, in our case cosmid MPMGc121E06653Q2 (Cosmid ACTHR) with murine genomic DNA from the ACTH receptor locus of mouse strain 129/ola, is electroporated with a Red/ET expression plasmid, in our case pSC101-BAD-gbaA (Genebridges). As the cosmid comprises a kanamycin resistance (Kana) and the Red/ET plasmid a tetracyclin resistance (Tet), selection is carried out with both antibiotics. The vector pSC101-BAD-gbaA (Genebridges) is engineered to be lost at 37°C, therefore culture is performed at 30°C. 2) In a second step, the Pme I linearized construct pMC2RcreERT2 is transformed into E. coli clones that contain both cosmid ACTHR and the Red/ET plasmid. 3) After L-arabinose administration, expression of recombination proteins from the Red/ET plasmid is induced (not depicted), so that homologous recombination between pMC2RcreERT2 and the cosmid ACTHR occurs (homologous sequences shown as grey arrows). 4) As the integrated construct provides ampicillin (Amp) resistance, the desired cre recombinase carrying cosmids (CosCreERT2) can be selected for. By growing the bacteria at 37°C the Red/ET plasmid pSC101-BAD-gbaA is eliminated. For further details, consult the Genebridges BAC modification kit protocols and the methods section.

5 Discussion

5.1 Overview

In the first part of the discussion, the rationale for the generation of cre mouse lines that allow DNA recombination specifically in the adrenal cortex is given. In the second part, the two strategies chosen in this thesis to generate mice with cre expression under the control of the ACTHR promoter are discussed. The initial strategy pursued, a classical knock-in strategy lead to the generation of the ACTHR-CE2 mouse line. Possible reasons for the lack of cre expression in the adrenals of these mice are discussed with a special emphasis on the effects of genetic heterogenity between inbred mouse strains. Subsequently, the generation of constructs for cosmid transgenic mice in order to overcome the problems encountered with the first strategy is discussed. In an outlook, the necessary steps for generating mouse lines on the basis of these novel cosmid targeting constructs are described. Finally, future applications of adrenocortical cre mice are outlined. Exemplarily, CRHR1 is chosen, a target gene with well-known involvement in psychiatric pathology. How targeted gene disruption may be applied to generate a mouse model for adrenocortical carcinoma serves as a second example.

5.2 Rationale

A powerful way to infer the role of human genes is to analyze the expression and function of homologous genes in model organisms, such as the mouse. Traditionally, this was achieved by generating classical gene knock-out or overexpressing mice although this broad approach has obvious drawbacks. As the gene is disrupted (or overexpressed depending on the experimental strategy) in every cell of the organism, the specific function of the gene in a certain tissue may be obscured by its function in another tissue. Likewise, as the gene of interest is disrupted from conception onwards, its function during embryogenesis may preclude the interpretation of its function in adult animals, e.g. by an embryonic lethal phenotype in the most drastic case. Conditional and inducible gene targeting, i.e. activation of targeting constructs in a cell-type-specific and/or time dependent manner, are methods to overcome these disadvantages (see also introduction to this thesis). Primary aim of this project was to generate tools that allow conditional and inducible control of gene expression

in the mouse adrenal cortex in order to specifically study gene function in this organ compartment (see figure D1).

Figure D1: Differences between classical and conditional knock-out mice. In the wild-type situation, the expression of a gene of interest is unaltered (indicated by colors). In a classical knock-out, the gene of interest is disrupted in every cell of the organism from conception onwards (indicated in grey). In a conditional, adrenocortical knock-out mouse, the gene of interest is likewise disrupted from conception onwards but exclusively in adrenocortical cells (depicted as grey adrenal cortex). Adrenal medulla and all other tissues of the mouse remain unaffected (indicated by colors). The generation of inducible, conditional knock-out mice (not depicted) additionally allows the determination of the onset of the knock-out phenotype through the administration of an inducing agent. In other words, inducible knock-out mice before induction represent a quasi-wild-type state which greatly enhances their experimental utility.

Several systems, which are based on two fundamental principles, transcriptional transactivation and site-directed DNA recombination, are available to achieve conditional control of gene expression in the mouse (Branda and Dymecki, 2004;Sorrell and Kolb, 2005). Although characterized by reversibility of activation, transcriptional transactivation systems have not found as extensive use as the DNA recombination based approaches. Both transcriptional transactivation methods, the lac (Scrable, 2002) and the tet (Corbel and Rossi, 2002) system are amongst other reasons impaired by the necessity to generate operator responsive promoters for the genes of interest which may require extensive cloning and testing procedures. Of the site-directed DNA recombination systems, flp recombinase is usually reserved for working tasks such as the removal of selection markers as in this thesis (Glaser et al., 2005). The ΦC31 recombinase (Thyagarajan et al., 2001) and the recent discovery of the cre related, heterospecific dre recombinase (Sauer and McDermott, 2004) will further extend the tool set for site-directed mutagenesis, but as Glaser et al. point out, cre-mediated DNA recombination is today “the sharpest tool in the box” (Glaser et al., 2005). Cre recombinase mediates DNA recombination between two of its 34 bp target sites, the so called loxP sites, which allows a multitude of genetic

experiments amongst them gene activation and inactivation experiments (see also introduction to this thesis).

Although numerous cre mouse lines exist that cover many tissues and developmental stages, no cre mouse line was yet established with reliable expression of cre in the adrenal cortex, although recombinase activity in the adrenal gland can be found in several cre lines originally designed for specificity to other tissues including the αGSU-cre (Cushman et al., 2000), the TH-cre (Lindeberg et al., 2004), the PSA-cre (Ma et al., 2005) and the INHA-iCre (Jorgez et al., 2006). All of these lines however, do not express cre in the adrenal cortex but in the adrenal medulla or alternatively, expression in the adrenal cortex is low and heterogenous, while expression in other tissues is high. A novel, truly adrenocortical cre mouse line therefore would be complementary to the already existing lines.

5.3 Driving Cre Expression by the ACTHR Promoter

As discussed, the ultimate aim of this thesis consists in generating a mouse line with reliable expression of cre recombinase restricted to the adrenal cortex. In order to drive cre expression in a tissue-specific manner, the endogenous ACTHR promoter receptor was chosen. This choice offers the additional benefit, that homozygous ACTHR cre knock-in mice constitute classical ACTHR knock-out mice as the ACTHR coding sequence is replaced by a cre recombinase open reading frame. Up to now, no ACTHR knock-out mouse has been published which adds further interest to the generation of ACTHR cre knock-in mice. The evaluation of suitability of this promoter choice to drive cre expression requires discussion of two variables, first, strength of expression, second, specificity of expression. Of these two, strength of expression appears less of an issue, as even very low expression will eventually lead to a considerable recombination efficiency because cre-mediated DNA recombination is an irreversible and therefore accumulating event (Nagy, 2000). If cre expression can be limited effectively to the adrenal cortex or at least to a subset of tissues including the adrenal cortex is more difficult to answer.

In theory, the use of an endogenous promoter to drive a deliberately introduced exogenous gene will restrict its expression to the domains of expression of the

naturally occuring product of the endogenous gene (Rickert et al., 1997). This holds true with some exception such as e. g. in cases that expression is additionally influenced by genomic position effects like in some classical transgenes (Branda and Dymecki, 2004). At the initiation of the project, ACTHR mRNA had been detected in the adrenal cortex of adult mice (Xia and Wikberg, 1996), namely in the the zona glomerulosa and zona fasciculata and in only a few scattered cells in the adrenal medulla. The latter could correspond to scattered cortical cells which are known to be present in the medulla (Ehrhart-Bornstein et al., 1998). ACTHR mRNA could also be found in adipose tissue where ACTHR activation exerts a potent lipolytic effect (Boston and Cone, 1996). In contrast, there was no detectable ACTHR mRNA in spleen, testis, liver, lung, heart, brain and kidney (Cammas et al., 1997). ACTHR promoter driven cre expression consequently was expected to mimic this pattern, resulting in cre activity mainly in the steroid producing cells of the adrenal cortex and to a lesser extent in fat cells.

More recent publications however, have revealed that the ACTHR can additionally be found in murine pancreatic islet cells of adult mice (Al-Majed et al., 2004), on certain leukocyte populations (Johnson et al., 2001) and most importantly, in a variety of tissues during prenatal development (Nimura et al., 2006). Expression was confirmed in adrenal gland (developmental day 13.5 (E13.5) into adulthood), testis (E13.5 to E18.5), genital ridge and ovary (E11.5 to E12.5 and E13.5 to E18.5 respectively), mesonephros (E11.5 to E12.5), metanephros (E12.5 to E18.5), lung (E11.5 to E14.5), brain and spinal cord (E11.5 to E13.5), choroid plexus (E13.5 into adulthood) and the dorsal root and trigeminal ganglia (E13.5 to E15.5). This widespread expression during embryogenesis has important consequences for ACTHR promoter controlled cre expression. As pointed out earlier, cre mediated DNA recombination is a cumulative event. With the use of a constituitively active cre, all cells from first cre expression in these cells onwards – and likewise all cells derived from them – will show a recombinant genotype. In the light of the extensive prenatal ACTHR expression in the mouse, cre-mediated DNA recombination by an ACTHR promoter driven constitutively active cre may thus not be limited to the adrenal cortex.

This potential drawback requires some attention: First of all, it depends on the experimental questions in how far cre expression in other tissues will confound results that were primarily intended to represent the adrenal cortex only. Such confounding influence will supposedly be high in cases like secretory molecules with tissue non-autonomous effects and low in genes with expression that is a priori restricted to the adrenal cortex. Secondly, the apparently broad promoter activity during embryogenesis can be entirely counteracted by using an inducible cre which can be activated later in life. The ACTHR promoter controlled CreERT2 in our case, may be expressed during embryogenesis, recombinase activity however is induced only after tamoxifen administration. The developmental state and the extent of expression can thus be chosen depending on experimental interest. Thirdly, the widespread expression of the ACTHR in prenatal development can be considered an unexpected, but highly welcome research opportunity as the developmental functions of the ACTHR in tissues other than the adrenal cortex is entirely unclear. Homozygous ACTHR cre knock-in mice, i. e. classical ACTHR knock-out mice, will be valuable tools to investigate them. The knock-out phenotype that was originally expected from the expression and function in the adult animal would likely have been mainly caused by glucocorticoid deficiency due to the lack of ACTH signalling. A possible role of the ACTHR during embryogenesis in contrast renders the knock-out phenotype virtually unpredictable and therefore greatly enhances its interest.

Still, the question remains, if there are alternative promoters available that would more readily restrict gene expression to the adrenal cortex. Besides gene promoters of steroidogenic enzymes, especially the 21-hydroxylase promoter, (Morley et al., 1996) and a 0.5 kb promoter fragment in combination with a 3.5 kb intronic region of the mouse vas deferens protein (MVDP) gene (also termed akr1-b7) (Martinez et al., 1999) (bi-Dargham et al., 2000) have been used with some success for adrenocortical-specific transgene expression. Notably, like with the ACTHR (Cammas et al., 1997), restriction to adrenocortical cells seems to be at least partly conferred by SF-1 response elements in all these cases, which put possible advantages of one promoter over the other into question. The determination of the exact expression pattern is however not determined by SF-1 alone but rather by

specific transcription factor combinations or yet unidentified cell-specific factors (Val et al., 2004) which can therefore so far not be used for mouse transgenesis.

In summary, controlling cre recombinase by the endogenous ACTHR promoter may not perfectly restrict expression to the adrenal cortex of adult mice. However, this promoter choice is certainly justified by the lack of better alternatives and the possibility to avoid cre activation during embryogenesis by means of an inducible cre. The supposed widespread expression furthermore opens additional research opportunities besides the elucidation of gene function in the adrenal cortex. The characteristics of the strategies that were chosen for targeting of the ACTHR locus will be discussed in the following section.

5.4 Strategy One: Classical Knock-In Vectors

The fundamental structure of knock-in vectors with homologous arms, selection markers and the gene-to-be-introduced has been discussed extensively elsewhere (Joyner, 2000). Discussion will therefore focus on the choice of the cre variants and the use of a self-excizing selection cassette which are issues specific to pPNTflpCremyctagPml and pPNTflpCreERT2Pml, the classical knock-in vectors constructed and utilized in this thesis project initially (see figure D2).

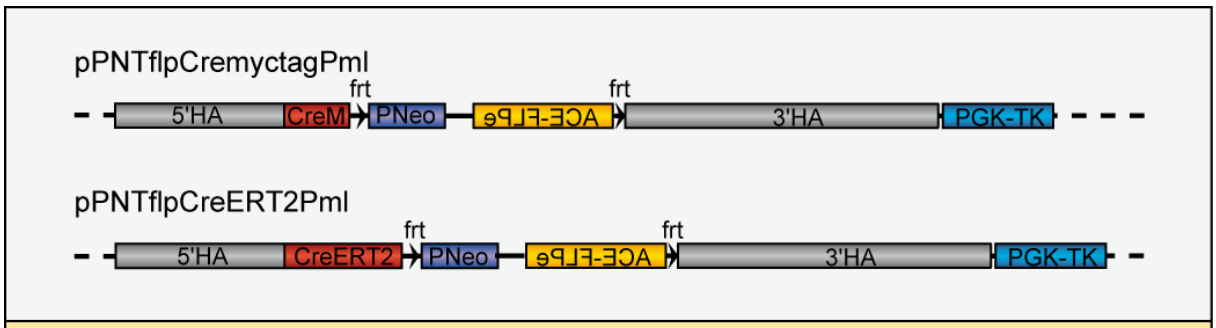

Figure D2: Targeting vectors pPNTflpCremyctagPml and pPNTflpCreERT2Pml. Homologous arms required for homologous recombination in ES cells depicted in grey, vector backbone indicated by dotted line. For details see text.

5.4.1 Choice of Constituitively Active Cre Variant

As a constituitively active cre variant, a cre tagged with an epitope from the human c-myc gene, was chosen for the generation of the targeting vector pPNTflpCremyctagPml. The tagging of proteins with small epitopes improves their immuno-detectability which facilitates expression studies. In the case of cre, tagging appears useful as the commercially available and published cre-antibodies (Schwenk et al., 1997) show only limited specificity (Ralf Kühn, personal communication). In contrast, the monoclonal antibody (Ab) against the human c-myc epitope is well characterized (Evan et al., 1985). In the first report on immunological tagging of a cre recombinase the authors used an epitope from the herpes simplex virus (HSV). In transgenic mice generated with this HSV-tagged cre, recombinase activity was fully retained and the cre protein was readily detectable by a monoclonal anti-HSV Ab (Stricklett et al., 1998). Furthermore, Watanabe et al. recently published the generation of a myc- and his-tagged cre variant that exerted full cre activity and detectability in cell culture and in an in-vivo intraoviductal injection assay (Watanabe et al., 2006). However, to my knowledge, no mouse line expressing a solely myc-tagged cre recombinase has been published so far. Generation of a Cre-myc-tag line therefore will provide the proof-of-principle for the feasibility of this approach in mice along with the generation of mice with constitutive cre activity in the adrenal cortex.

5.4.2 Choice of Inducible Cre Variant

The ideal genetic switch was defined as enabling low or zero basal gene activity when switched “off” and high levels of gene activity when switched “on” (Lewandoski, 2001). Only under these stringent conditions reliable conclusions can be derived from conditional gene expression experiments as high background activity and low inducibility both lead to diminished differences between experimental and control animals. When compared to other inducible cre variants available today, CreERT2 comes closest to these requirements and is consequently used in this thesis. CreERT2 is a cre recombinase fused to a mutated human estrogen receptor LBD that comprises three point mutations (G400V/M543A/L544A) (Feil et al., 1997). These mutations render the ERT2-LBD insensitive to estrogen binding, but allow a

very high inducibility following tamoxifen administration even at comparatively low doses. In an attempt to further improve the characteristics of CreERT2, modified versions of it have been generated. The codon usage improved version iCreERT2 was designed to achieve higher cre activities after induction. The ERiCreER variant on the other hand with a codon-usage improved cre fused to ERT2-LBD on both carboxy- and amino-terminus was intended to allow an even tighter control in the absence of tamoxifen while maintaining sufficient inducibility. However, none of these modified cre recombinases showed clear advantages over CreERT2 in cell culture (Casanova et al., 2002) and they have consequently not been widely used for the generation of transgenic mice.

In contrast to its two variants, CreERT2 itself has been successfully used in a variety of tissues including mouse skin (Indra et al., 1999), smooth muscle (Kuhbandner et al., 2000), adipose tissue (Imai et al., 2001), bone (Kim et al., 2004), glia cells (Leone et al., 2003) (Mori et al., 2006) (Hirrlinger et al., 2006) and melanocytes (Yajima et al., 2006). The most recently published CreERT2 mouse line with expression in neurons of the dorsal root ganglia is characterized by complete absence of cre activity without tamoxifen. Activity after tamoxifen administration was found to be only about 10% of the activity of a constituitively active cre expressed under the identical promoter. After experimental exclusion of cytosolic CreERT2 sequestration by high levels of Hsp90, the authors favor low tissue penetrance of tamoxifen as possible explanation (Zhao et al., 2006). They argue, that increased doses of tamoxifen would ultimately lead to a higher induction efficiency. Unfortunately, mice display symptoms of toxicity for orally administered tamoxifen amounts of more than 2 mg/day.

As in all other cases, the minimum dosage and time of application of tamoxifen for an optimal induction of cre activity will also need to be established in ACTHR promoter controlled cre mice. It appears plausible to assume that the adrenal gland as a highly perfused endocrine organ will show sufficient penetration of tamoxifen. The amounts required for induction will therefore likely be considerably lower than for example in the neuronal or glial CreERT2 variants and will thus not reach the toxic range. In the case of ACTHR promoter controlled cre mice, the differential tissue penetrance may

even be of benefit in the light of a possible expression of cre in adipose and other tissue: Tissue specificity might be titrable.

As a further point of note, the transcription of ACTHR mRNA is upregulated following ACTH stimulation in cultured human and mouse cells (Mountjoy et al., 1994). Consequently, cre recombinase expression might be increased by ACTH administration in cre lines as well in which expression is controlled by the endogenous ACTHR promoter. ACTH administration could possibly modify the recombination kinetics by accelerating recombination or increasing the maximum recombination efficiency. Conversely, adrenostatic compounds such as aminoglutethimide and metyrapone suppress ACTHR expression (Fassnacht et al., 1998) and might be used to slow down recombination.

With respect to detectability as discussed earlier for the myc-tagged cre variant, CreERT2 offers the advantage of immuno-reactivity with antibodies directed against the human estrogen receptor (ER) ligand binding domain. These are more specific than the available cre-antibodies (Ralf Kühn, personal communication) and were successfully used in this thesis for detection of the CreERT2 protein by Western blotting.

5.4.3 Selection Cassettes

Two antibiotic resistance cassettes are included in the classical knock-in constructs for selection of clones during embryonic stem cell culture: Firstly, a phosphoglycerate kinase I promoter driven thymidine kinase for negative selection (also refer to results chapter) which follows well established priniciples and will not be discussed further, secondly, the phosphoglycerate kinase I promoter driven neomycin resistance cassette for positive selection. In contrast to cassettes for negative selection, positive selection cassettes are located in between the homologous arms, so that they are not lost during homologous recombination. However, as the neomycin gene was shown to influence expression of adjacent genes, it needs to be removed after the selection process (Pham et al., 1996). To this end, the marker is usually flanked with frt sites that allow flp-mediated DNA recombination and thereby cassette excision (compare also design of the recombineering vectors depicted on figure D3). This

approach requires time-consuming manipulations, either flp expression in ES cells or breeding to flp expressing mice. The latter approach furthermore will likely lead to mosaic cassette removal only as flp activity is not uniform in all cells (Rodriguez et al., 2000).

To circumvent these common problems, v. Waldenfels and Deussing in our group generated a novel positive selection cassette which was tested in vectors pPNTflpCremyctagPml and pPNTflpCreERT2Pml in a proof-of-principle experiment. This cassette is flanked with frt sites and carries a neomycin resistance gene expressed under a phosphoglycerate kinase I promoter (PGK) and a flp recombinase gene which is driven by an ACE promoter. The ACE promoter is active during spermatogenesis and was therefore thought to a activate flp expression during sperm cell formation effectively leading to self-excision of the cassette. A similar approach was taken by Bunting et al. who generated mice with an ACE promoter driven, cre-mediated self-excision cassette (Bunting et al., 1999). In contrast to these mice, the ACTHR-CE2 mice generated from construct pPNTflpCreERT2Pml failed to self-excise, while deletion by breeding to flp expressing mice did result in cassette deletion. In conjunction with the failure of self excision in another mouse line generated in our laboratory (unpublished data), the self-excising property of the selection cassette unfortunately has to be considered non-functional. As flp recombinase activity in mammalian cells is much lower than cre recombinase activity (Andreas et al., 2002) it is apparently not sufficient to cause recombination when flp expression is driven by the ACE promoter as in the vectors presented here. The self-excision strategy was therefore not further pursued in subsequent constructs such as the cosmid targeting vectors discussed later.

5.4.4 ACTHR-CE2 Mice

As described in the results chapter of this thesis, the targeting vector pPNTflpCreERT2Pml was used to generate the mouse line ACTHR-CE2 by homologous recombination in ES cells. As desired, characterization of heterozygous ACTHR-CE2 revealed no significant differences to wild-type mice with respect to body weight, adrenal weight, gross adrenal morphology and corticosterone release following ACTH administration or restraint stress. Unfortunately, the mouse line also

showed no detectable cre expression as evaluated by RT-PCR and Western blotting. The functional activity of cre was consequently not tested anymore by breeding to cre reporter mice such as R26R (Soriano, 1999), also in the light of the required tamoxifen induction. Possible reasons for the lack of expression will be discussed in this section.

Absence of expression may be caused by one or more of several factors. Firstly, the targeting construct itself may be non-functional. This, however, appears little plausible in our case as the fundamental construct design follows well established principles. Technical issues that could result in lack of expression such as frame shift mutations and absence of polyadenylation signals were excluded by analysis of the vector sequencing results. Secondly, the activity of the endogenous promoter at the target locus may be insufficient to drive expression of the exogenous gene. The knock-in strategy as pursued here replaces the open reading frame of the ACTHR. All putative regulatory elements situated up- or downstream as well as the regions forming the 5' and 3' UTRs are unaffected. Consequently, as ACTHR expression itself is detectable by RT-PCR and Western blotting, the exogenous gene should behave equally. Thirdly, the integration of the targeting construct via homologous recombination exactly into the desired locus failed, which is the explanation with most evidence in our case.

Success of gene targeting in ES cells is commonly verified by one of two methods, Southern blotting and long range PCR of ES cell DNA. By means of Southern blotting, three ES cell clones were identified in this thesis that supposedly had undergone homologous recombination with the targeting vector pPNTflpCreERT2Pml (also see results chapter). One of these clones was used to generate the ACTHR-CE2 mouse line. In an isogenic background as normally the case, a wild-type situation is represented on a Southern blot by a single band of a certain length expected from prior in silico sequence analysis. This band is constituted by the hybridization of the probe to both wild-type alleles that are located on the two homologous chromosomes. However, in our case the wild-type situation was characterized by two bands of different sizes in almost all digests with different restriction enzymes. One of the bands usually matched the expected wild-type size,

while the other did not. The unexpected band was therefore initially thought to be caused by unspecific cross-hybridization of the probe to another genomic region. Restriction length polymorphism by mutation of one of the alleles as alternative explanation was considered improbable, because a second band consistently appeared in different digests. At the time of ES cell clone screening, the detection of a third fragment showing the size expected for the recombinant band was thus taken as indicative for successful gene targeting by homologous recombination. Retrospectively however, the amount of restriction length polymorphism, i.e. sequence polymorphism, between the two wild-type alleles was underestimated. It was caused by the utilized ES cell line as will be discussed in the following section.

Traditionally, almost all ES cell lines used for gene targeting are generated from epiblast cells of mouse strain 129 (Brook and Gardner, 1997). For unknown reasons, this strain has proven particularly permissive for the derivation of ES cell lines and the colonization of blastocysts usually gives rise to chimeric animals with high likelihood of germline transmission of an introduced mutation. Acceptor blastocysts in contrast are usually derived from strain C57Bl/6 which allows for coat color selection and provides better breeding efficiencies. To control for strain influence on the phenotype, the mutant offspring are backcrossed continously to C57Bl/6 mice in order to re-instate an inbred background. This procedure usually delays reliable phenotype analysis of mutant mice for more than one year and still does not abolish the influences of loci that are inherited linked to the targeted locus (Crusio, 2004). Backcrossing can be shortened by one generation of breedings by means of hybrid ES cells derived from two different strain backgrounds, so called F_1 (first filial generation) ES cells. Their genome is constituted by homologous chromosomes that originate from either of the parental strains, which are substrains of 129 and C57Bl/6, i. e. half of their genome is already congenic to the C57Bl/6 acceptor blastocyst DNA. A further advantage of F_1 ES cells besides speeding the backcrossing procedure seems to be their “hybrid vigor” which allows extended culture periods and manipulations while their capacity to give rise to fertile offspring is maintained (Eggan et al., 2001).

In order to generate ACTHR-CE2 mice, IDG3.2 cells (gift of Ralf Kühn) were used which are F_1 ES cells created from breedings between strains 129S6/SvEv/Tac and C57Bl/6 (Schwenk et al., 2003). The possible advantages of robustness and shortening of breeding were however in our case outweighed by the fact that the use of the hybrid ES cells led to the misinterpretation of the Southern blotting results. The detected two bands were considered one wild-type and one unspecific band as the amount of genetic heterogenity between the two strains was underestimated. However, as later demonstrated by comparative Southern blotting, the wild-type situation in IDG3.2 genomic DNA is indeed characterized by extensive restriction length polymorphisms between the two ACTHR loci derived from either parental strain.

The extent to which sequence disparities prevail between different mouse strains is only beginning to be fully recognized since the publication of the mouse genome data in 2002. Commercial mouse sequencing by Celera Genomics was carried out on four mouse strains (129X1/SvJ, 129S1/SvImJ, A/J and DBA/2J) with a total 5.3-fold genome coverage. The publicly funded Mouse Genome Sequencing Consortium in contrast was sequencing a single strain, C57Bl6/J, covering the genome 6.5-fold and three additional strains (129S1/SvImJ, BALB/cByJ and C3H/Hej) for only minimal coverage (Wade and Daly, 2005). As of August 2006, TranscriptSNPView has been added to the genome browser (*www.ensembl.org*) that allows convenient comparison of interstrain sequence differences identified by these sequencing projects. Alltogether, this genome-wide catalog displays coding single nucleotide polymorphism (SNP) variations from 48 mouse strains (Cunningham et al., 2006). Mouse haplotype information on inbred strains can also be found on www.broad.mit.edu/personal/claire/MouseHapMap/Inbred.htm, however up to now only in form of SNP lists. Another third comprehensive online source for comparative mouse genomics is *www.informatics.jax.org*, which besides extensive phenotypic data on multiple mouse strains since recently also offers information on inter-strain sequence differences.

Interestingly, genetic variation between mouse strains is not homogenously distributed throughout the genome, which reflects the evolutionary and breeding

history of the inbreed laboratory mouse strains (Wade and Daly, 2005). When the C57Bl/6J Mouse Genome Sequencing Consortium sequence was compared to sample sequences from ancestral mouse strains and common inbred strains, genome segments were identified showing SNP frequecies of up to one SNP per 250 bp, while other regions only comprised about one SNP per 20 kb (Wade et al., 2002). Between mouse strains 129S5 and C57Bl/6J, which only diverged by breeding within the last century (Beck et al., 2000), SNPs lead to an estimated > 100 premature transcriptional termination codons and > 62.000 coding changes and splice-site alterations (Adams et al., 2005). Yalcin et al. showed exemplarily by inter-strain comparison of a 4.8 Mb region on chromosome 1 which contains a quantitative trait locus (QTL) influencing anxiety that the unexpected haplotype complexity they encountered could be represented by “strain distribution patterns” (Yalcin et al., 2004). These common patterns of alleles suggest that there is both extensive linkage disequilibrium and limited diversity potentially facilitating phenotypic mapping experiments. Besides SNPs, other types of inter-strain genetic variations include sequence insertions, deletions and copy number polymorphisms likewise offer interesting insights (Wade and Daly, 2005) but will not be discussed further.

Despite extensive efforts to characterize the wild-type and the targeted locus it still remains enigmatic what exact genetic rearrangements occurred in the ES cell clone used to generate ACTHR-CE2 mice. Integration did take place as PCR testing with internal primers showed. Correct integration of the construct via homologous recombination into the exact position at the ACTHR locus however did likely not occur. In this case, one of the wild-type bands would have been shifted to the length of the recombinant band when screened with e. g. the external 3’ probe in Southern blotting. The appearance of an additional band points to the duplication of the binding site for the probe. Karyotyping of the ES cell clone used for generation of the ACTHR-CE2 mice however showed no obvious abnormalities (data not shown) so that the size of duplication must be low. Although a more detailed characterization of the events at the targeted locus could have been undertaken, this line of investigation was not pursued further for the lack of practical consequences. After all, the insert was unable to produce CreERT2 protein.

Comparability of this case to the existing literature is limited, since reports on aberrant gene targeting are scarce due to publication bias: Negative results are not published. One extremely rare exception from this rule is the publication of an aberrantly targeted p53 allele (Tyner et al., 2002). For unknown reasons, targeting in this case resulted in the unexpected deletion of p53 exons 1-6 and an approximately 20 kb region upstream of the gene. Mice derived from these ES cells exhibited enhanced tumor resistance and an increased lifespan. Interestingly, like with the ACTHR-CE2 mice, the authors were unable to detect a truncated (p53) protein. Even this publication does not offer mechanistic explanations for the aberrant gene targeting event but benefits from the otherwise interesting phenotype of the mice. In conclusion, as no specific data on aberrant gene targeting is available, general parameters that influence gene targeting success were considered in order to modify the targeting strategy and to improve the targeting efficiency. Besides the length and design of the targeting vector and the target genetic locus (Zhou et al., 2001), two major factors determine the efficiency of homologous recombination: The length (Hasty et al., 1991) and the grade of sequence identity (te Riele H. et al., 1992) of the homologous regions. In the second strategy employed in this thesis for the generation of targeting vectors, both factors are accounted for, as will be discussed in the following chapter.

5.5 Strategy Two: Recombineering Vectors

In comparison with the classical knock-in vectors pPNTflpCremyctagPml and pPNTflpCreERT2Pml described earlier, the same cre variants were used to generate the targeting cosmids CosCremyc and CosCreERT2 (see figure D3). In their design, two points require further attention, the major being the change to a recombineering strategy, the minor the introduction of the Venus fluorescence marker which is translated by means of an internal ribosomal entry site (IRES). The choice of resistance cassettes for selection during recombineering and ES cell culture is largely determined by the cassettes present in the carrier cosmid and is therefore not discussed. Notably, no negative selection marker was included into the targeting cosmids CosCremyc and CosCreERT2. Its inclusion would have required difficult cloning steps and is largely unnecessary as the expected overall rate of correct

targeting with a cosmid is high and the usual enrichment achieved by negative selection low (Joyner, 2000).

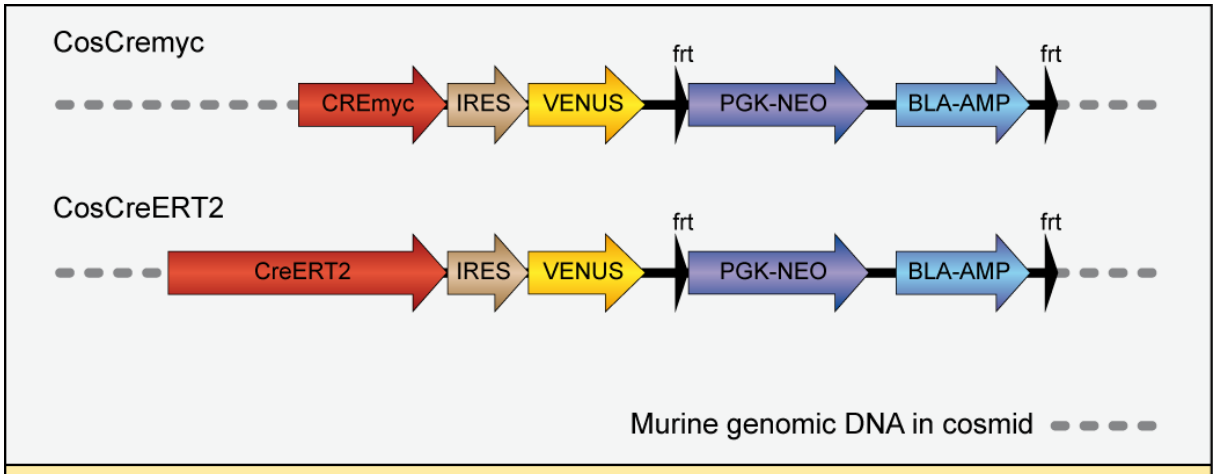

Figure D3: Targeting cosmids CosCremyc and CosCreERT2 which were generated by homologous recombination between vectors pMC2RcreMYC and pMC2RcreERT2 and cosmid MPMGc121E06653Q2. In the cosmids, the targeting constructs are embedded in approximately 40 kb of strain 129/ola genomic DNA from the ACTHR locus which will serve as homologous sequence for homologous recombination into ES cells. Likewise, the surrounding genomic DNA contains all essential regulatory elements that are required for ACTHR expression.

5.5.1 Cosmid Recombineering

“Recombineering” refers to the cloning of DNA in E. coli by homologous recombination (Copeland et al., 2001). It is usually applied to large DNA vectors such as bacterial artifical chromosomes (BACs) and cosmids that are otherwise not easily modifiable. In this thesis, recombineering was used to integrate a 6.6 kb fragment of plasmid vector pMC2RcreMYC and a 7.5 kb fragment of plasmid vector pMC2RcreERT2, respectively, into the cosmid MPMGc121E06653Q2. This cosmid carries approximately 40 kb of strain 129/ola genomic DNA from the ACTHR locus which provides more than sufficient homologous DNA for subsequent gene targeting in ES cells. To account for sequence disparities in the homologous regions of the plasmid (derived from strain C57Bl/6) and cosmid (129/ola) that would decrease recombineering efficiency (Liu et al., 2003), longer homologies (approximately 500 and 700 bp) than the 60 bp of the original publication were used (Zhang et al., 1998a).

For the production of gene targeting vectors, recombineering has been applied most commonly to integrate targeting constructs into BACs (Yang and Seed, 2003) (Valenzuela et al., 2003). BACs carrying genomic DNA from a region of interest are readily available and even annotated in the genome browsers as C57Bl/6 BACs were used for mouse genome sequencing (Branda and Dymecki, 2004). For their size of around 300 kb, BACs can be identified that contain all necessary regulatory elements to confer normal gene expression to a transgene (Heintz, 2001). Moreover, even BAC transgenes are typically not subject to the strong position effects resulting in transgene silencing or misexpression that affect conventional constructs (Giraldo and Montoliu, 2001). Besides BACs, other large DNA vectors like cosmids may be used for gene targeting. Although to my knowledge no mouse line has been reported so far generated on the basis of cosmid recombineering and homologous recombination making our strategy the proof-of-principle for this approach, the general feasibility of cosmid targeting has been demonstrated in fungi (Chaveroche et al., 2000). When used for this purpose in mice, cosmids offer, although not characterized by the advantages of BACs to full extent due to their lower size of about 40 kb, the benefit of easier handling, especially with respect to screening of ES cell clones by Southern blotting or long range PCR. These assays are based on finding ES cell clones in which sequences within the native locus (but outside of the flanking regions shared by the targeting vector) have been linked to sequences unique to the targeting vector. Such linking of sequences can only occur in correctly targeted ES cells. In order to be able to use these techniques, the length of homology arms used in targeting vector is effectively limited to about 10 kb. This condition is granted on the 3’ homologous region of the targeting cosmids presented here. BAC targeting on the other hand requires fluorescence in situ hybridization (FISH) (Yang and Seed, 2003) or indirect screening techniques such as the real-time PCR based “loss-of-native-allele” approach (Valenzuela et al., 2003).

The novel cosmid targeting vectors generated by recombineering are suitable to overcome the problem of inefficient and aberrant gene targeting encountered with the classical knock-in constructs pPNTflpCremyctagPml and pPNTflpCreERT2Pml described earlier. The homologous arms of these constructs had a combined length

of approximately 8 kb while the cosmid vectors comprise homologies of about 40 kb. Deng and Capecchi could show in extensive experiments that efficiency of targeting at the Hprt locus increased exponentially with the length of homologous sequence up to at least 14 kb (Deng and Capecchi, 1992). In how far longer homologies further improve recombination efficiency has not been systematically tested. It is however known, that longer homologies diminish the influence of disparities in their genetic sequence, which is the second major factor influencing targeting efficiency (Valenzuela et al., 2003). Te Riele et al. first showed that targeting the retinoblastoma gene locus with isogenic DNA was 10- to 20-fold more efficient than with non-isogenic DNA (te Riele H. et al., 1992). With the μ-opioid receptor locus, targeting between isogenic DNA was 15-fold more efficient than between non-isogenic DNA, although the sequences between the two strains used in this experiments (129/Sv and C57BL/6) varied only about 2% at this locus (Zhou et al., 2001). In fact, isogeny is such an important factor that sequence differences even in between substrains of 129 reduce targeting efficiency and even let some loci appear untargetable. For example, attempts to target a region tightly linked to Tyr on chromosome 7 were unsuccessful when a 129/SvJ-derived construct was electroporated into a 129/Sv ES cell line; however, the same construct underwent homologous recombination very efficiently in a 129/SvJ derived ES cell line (Joyner, 2000).

Notably, the genetic differences between different mouse strains do not only affect gene targeting efficiencies but also the phenotypes of the resulting offspring. For example, when targeting the epidermal growth factor receptor (Egfr) locus on a CF-1 or 129/Sv background animals died prenatally, while on a CD-1 they lived for up to 3 weeks post partum (Threadgill et al., 1995). Even between certain substrains of 129 there are differences in behavioral paradigms (Montkowski et al., 1997) and reciprocal skin grafts are rejected (Simpson et al., 1997). Unfortunately, it is frequently impossible to tell which exact mouse strain was used in an experiment or to generate a genomic library as denominations are outdated or incomplete. Strain names such as 129/SvJ as opposed to 129/SvJae do not facilitate precision in this respect (Festing et al., 1999).

In conclusion, the DNA for construction of targeting vectors should be ideally isogenic with the utilized ES cells. Alternatively, length of homology may compensate for sequence disparities. The cosmid targeting vectors presented here, will therefore likely show considerably higher targeting efficiency than the classical targeting vectors used before.

5.5.2 Fluorescent Marker

In order to facilitate identification of cre expressing cells, the cosmid vectors CosCremyc and CosCreERT2 were designed for expression of a bicistronic mRNA which contains the coding information for both cre recombinase and a fluorescent marker (Venus). Translation of the Venus fluorescent protein is granted by means of an IRES (see figure D3). IRESs, originally identified in picorna viruses (Pelletier and Sonenberg, 1988), have been found in a variety of viruses. They allow the cap-independent translation of a bicistronic mRNA in eukaryotes by internal binding of the ribosomal 40S subunit to the mRNA (Vagner et al., 2001). The encephalomyocarditis IRES site was first used in mice by Kim et al., 1992 (Kim et al., 1992). In contrast to a fusion protein, the use of the IRES offers the advantage of leaving the biological properties of the cre recombinase unaffected as two independent proteins are generated from one mRNA. A possible disadvantage is the unpredictable strength of IRES-mediated translation. For example, in CamKIIα iCre-IRES-eGFP BAC transgenic mice fluorescence was found to be weak and was not detected uniformely in all hippocampal neurons (Casanova et al., 2001). The IRES-translated fluorescent marker Venus used in this thesis is a variant of the yellow fluorescent protein (YFP) with faster maturation and increased fluorescence yield (Nagai et al., 2002). Its properties may therefore compensate possible low IRES-driven expression. A Venus fusion protein construct has been used to visualize endoplasmatic reticulum stress (Iwawaki et al., 2004), but to my knowledge no IRES-Venus construct has been applied to the mouse so far. In summary, the inclusion of the IRES-dependent Venus fluorescent marker into the cosmid targeting vectors allows an indirect but rapid and – if needed – even in vivo detection (Hadjantonakis et al., 2003) of cells that express cre.

5.6 Outlook

5.6.1 Towards The Generation of ACTHR Cre Cosmid Mice

Ultimately, four mouse lines can be generated with the cosmid targeting vectors CosCremyc and CosCreERT2 presented in this thesis. Of these four lines, two lines express the constituitively active Cremyctag, one of them as transgenic, one of them as knock-in mouse. The two remaining lines are one transgenic and one knock-in line for the ACTHR promoter driven inducible CreERT2. Every one of these lines is characterized by inherent strengths and limitations. The widespread expression of the ACTHR during embryogenesis may result in too little restriction of cre activity in the case of the constituitively active cre. The inducible CreERT2 mouse lines on the other hand require tamoxifen administration for cre activation which precludes a rapid evaluation. The transgenic lines might be prone to position effects necessitating the characterization of several lines. Indeed, Lee et al. generated Eno2-Cre mice from both full-size BACs and from 25 kb BAC fragments. Expression domains varied between the two lines created from the fragment and were also different from the expression pattern of the full-size BAC derived line (Lee et al., 2001). The cosmid knock-in lines for their part might be hampered by the loss of homozygosity at the ACTHR locus which might influence hormonal parameters. Homozygous knock-in animals in contrast are of special interest as they will reveal the ACTHR knock-out phenotype. Thus the different lines will be complementary in the sense that drawbacks of one line are overcome by the other lines. In addition, the four lines allow addressing different sets of questions, with a functioning transgenic CreERT2 line being most useful for the analysis of gene function in the adrenal cortex.

Technically, the cosmid vectors CosCremyc and CosCreERT2 can be used in ES cells at the same time to generate transgenic mice, i.e. with random construct integration, and knock-in mice, i.e. with integration by homologous recombination. To facilitate clone screening by Southern blotting, no F_1 hybrid but isogenic 129 derived ES cells will be used in order to avoid the interstrain restriction length polymorphisms encountered with the first targeting strategy. After identification of clones with either random or targeted construct integration, by Southern screening with internal and 3' probes on e. g. Xho I digests, blastocyst injection will be performed as for the generation of ACTHR-CE2 mice. In the resulting offspring, the presence of cre and

Venus mRNA will be verified by RT-PCR or in situ hybridization and the presence of the respective proteins by Western blotting or immunohistochemistry and fluorescence microscopy. Subsequently, the neomycin/ampicillin selection cassette will be removed by breeding to mice with ubiquitous flp recombinase expression (Dymecki, 1996). The mouse stock will then be maintained by continuous breeding to C57Bl/6 mice.

The characterization of cre activity will cover two main parameters, efficiency and spatial distribution of recombination. For the inducible CreERT2 recombinase leakyness, i.e. activity without tamoxifen induction, and efficiency after tamoxifen administration as compared to the constituitively active cre will be evaluated additionally. To this end, cre mice will be crossed to R26R LacZ reporter mice (Soriano, 1999). In these mice, a lacZ reporter gene is activated after cre mediated excision of a transcription termination signal. As expression from the R26 locus is ubiquitous, all cells with cre recombinase activity are stained by lacZ thus revealing the cre expression pattern. Alternatively, other cre reporter strains can be used, e.g. with activation of a fluorescent marker by cre recombination (Branda and Dymecki, 2004). LacZ is however more sensitive than fluorescence detection as staining intensity accumulates.

The characterization of ACHTR cre mice will furthermore include the demonstration that key biological parameters do not differ between cre and wild-type mice as this might obscure phenotypes in subsequent conditional gene targeting experiments with these mice. Phenotyping of cre mice will therefore cover adrenal hormone release under basal and stress conditions and adrenal morphology. Moreover, parameters reflecting other functions of tissues with expression of ACTH will be measured e.g. body weight and total fat mass as markers for adipose tissue. In the case of homozygous cre knock-in mice, i.e. ACTHR knock-out mice, phenotyping might lead to insights into yet unknown functions of the ACTHR. Which conditional gene targeting experiments exploiting the specific cre activity might ultimately be performed with the ACTHR cre mice is illustrated in the next section.

5.6.2 Targets for ACTHR Cre Mice

As the cre/lox system is binary, cre mice alone are of only limited use unless they may serve a second function e. g. as classical knock-out mice like in this thesis. Cre mice however become an exceptional tool when combined with the other component of the system, mice with floxed target alleles. The system then does not only allow targeted gene disruption but also other experiments such as targeted gene overexpression and knock-down. In this last part of the discussion, possible fields of interest for gene function experiments in the adrenal cortex are presented (see figure D4).

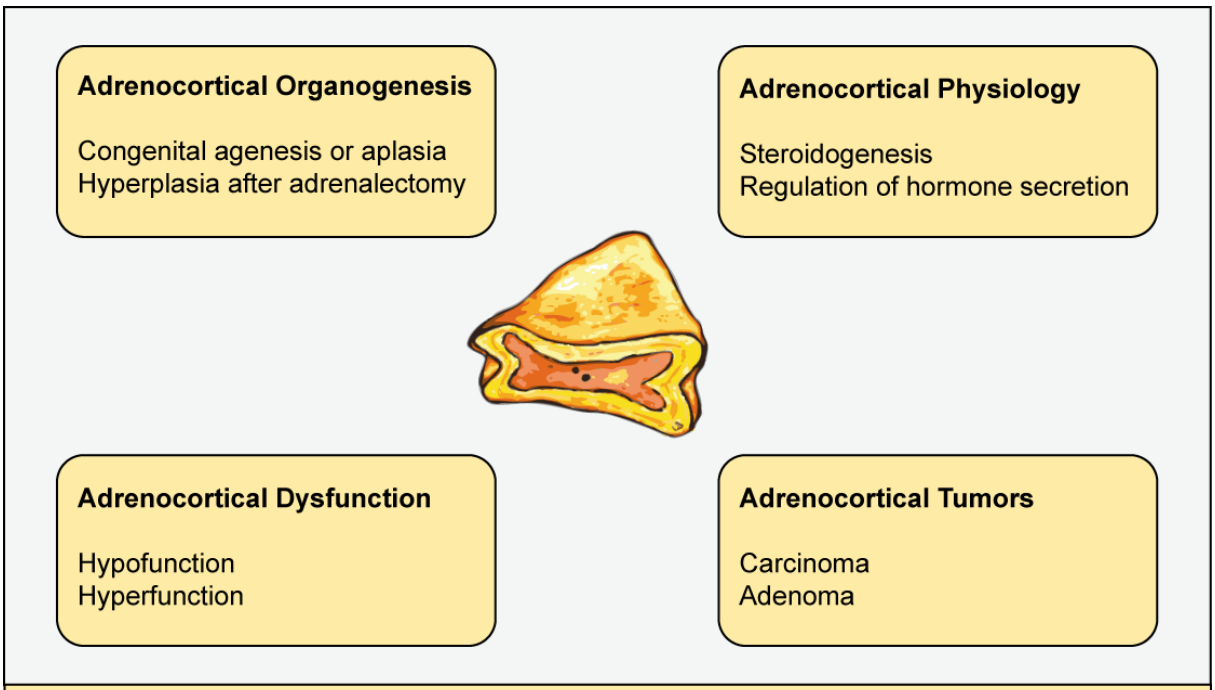

Figure D4: Possible fields of interest for targeted mutagenesis in the adrenal cortex. For further details see text.

In the field of adrenocortical organogenesis, questions include the developmental origins of the adrenal cortex and the molecular regulation of its formation (Hammer et al., 2005). Targeted mutagenesis may help to better understand these processes and to develop models for congenital syndromes of aplasia or agenesis (Else and Hammer, 2005). Even in adulthood, the adrenal cortex displays constant renewal of steroidogenic cells and is able to compensate contralateral loss of an adrenal cortex by ipsilateral hyperplasia. Its underlying mechanisms and the provenience of cells

remain largely unknown, although a subcapsular stem cell zone is hypothesized to be involved (Else and Hammer, 2005).

Within the human adrenal cortex, steroid hormone synthesis depends on the coordinated action of several enzymes. It is the differential expression of these enzymes within the adrenocortical zones that allows the synthesis of the wide array of steroid hormones secreted by the gland. However, the factors causing the coordinated differential expression are only incompletely understood (Hsu et al., 2006). Targeted gene disruption of steroidogenic enzymes may improve this understanding and provide models for adrenocortical dysfunction. It may also be applied to circumvent the necessity for surgical adrenalectomy in mice as sometimes needed to experimentally control for endogenous glucocorticoid release.

Hormone secretion from the adrenal cortex is subdued to much more regulatory influences than assumed in the past. Humoral and neuronal factors but also direct intercellular interactions play important roles besides the classical secretagogues angiotensin and ACTH (Ehrhart-Bornstein et al., 1998). In how far structures like gap junctions or molecules like serotonin modulate adrenocortical function may be addressed by gene targeting. One of the newly identified regulators of hormonal secretion from the adrenal cortex is CRHR1, whose targeted disruption will be discussed exemplarily in the following section.

5.6.2.1 CRHR1 as Regulator of Glucocorticoid Secretion

CRH was first purified and characterized in 1981 (Spiess et al., 1981;Vale et al., 1981). Since then, data from patients and model organism have revealed the important role of CRH and its two receptors, CRHR1 and CRHR2, in mood and anxiety disorders (Deussing and Wurst, 2005) (see also introduction to this thesis). For the elucidation of these disorders, the obvious focus of research has been CRH function in the central nervous system (CNS). Our research group made important contributions to this field e. g. by generating both classical (Timpl et al., 1998) and conditional (Muller et al., 2003) CRHR1 knock-out mice.

Nonetheless, peripheral organs, especially the adrenal gland, may be crucial for a better understanding of affective disorders. Several lines of evidence point into this direction: The importance of adrenocortical steroid hormones in the brain to modulate behavior is well known (de Kloet et al., 2005;Holsboer, 2000). Circulating interleukin-18 is released from the adrenal after stress, indicating a possible neuropsychoimmunological function of the adrenal (Sekiyama et al., 2006;Sugama et al., 2006). Even on the level of gross morphology, an antidepressant treatment reversible, increased adrenal volume determined by computed tomography has been reported in depressed patients (Nemeroff et al., 1992;Rubin et al., 1995). Indeed, to investigate adrenal function in affective disorders seems to be of utmost interest as evidence about the connection between major depressive disorder and somatic illnesses such as coronary heart disease is accumulating (Joynt et al., 2003). As adrenocortical glucocorticoids exert their effects on multiple central as well as peripheral tissues and cell types, they could represent the missing link between the different pathologies (Brown et al., 2004).

Intriguingly, CRH, classicaly known only as ACTH secretagogue and regulator of brain function as pointed out already, seems to be directly implicated in the regulation of adrenocortical steroid hormone secretion. Strong evidence for this hypothesis is provided by the failure of CRHR1 knock-out mice to release corticosterone after ACTH stimulation (Muller et al., 2001). CRH levels in the general bloodstream are low, so that the existence of an intra-adrenal regulatory CRH system has been postulated (further evidence reviewed in Ehrhart-Bornstein et al., 1998). The involvement of the CRH receptor family in the production and release of catecholamines from the adrenal medulla has furthermore been demonstrated in a recent paper. To date, the physiological significance of these findings is entirely unclear (Dermitzaki et al., 2007). It is however tempting to speculate, that CRH might be involved in the differentiation of adrenal response to either somatic of psychological stressors. Ultimately, targeted disruption of CRHR1 in the adrenal cortex will help answering these questions and may also shed light on the morphological abnormalities encountered in the adrenals of classical CRH receptor knock-out mice (Preil et al., 2001). How targeted gene disruption may furthermore be used to generate a model for adrenocortical carcinoma, will be discussed next.

5.6.2.2 A Mouse Model for Adrenocortical Carcinoma

Adrenocortical carcinoma (ACC) is a rare neoplasm with poor prognosis. Its incidence is approximately 1-2 per year per million population. R0 resection is the treatment of choice for stage I-III ACC. However most patients enventually develop either local recurrence or distant metastases so that overall 5-year survival ranged only between 23% and 60% depending on the study population. First line treatment for metastatic disease (stage IV) is mitotane, whose clinical efficacy remains disputed (Allolio et al., 2004). Unfortunately, progress in elucidating the molecular pathology and improving the pharmacological treatment of ACC is slowed by the absence of a suitable, orthotopic mouse model. The traditional in vivo models rely on the subcutaneous injection of Y1 cells and, more recently, of modified mouse myeloma cells (Ortmann et al., 2004). In an attempt to generate novel stable tumor cell lines, targeted SV40 T antigen transgene expression in the mouse adrenal cortex lead to tumor formation (Mellon et al., 1994) (Sahut-Barnola et al., 2000). Homozygous Inhibin α (Inha) knock-out mice develop adrenal tumors, in case prior death due to gonadal tumors is prevented by gonadectomy. However, this rodent model appears of only limited value as the contribution of Inha in human tumorigenesis is unclear (Stratakis, 2003).

Mice with targeted, adrenocortical disruption of gatekeeper genes such as p53 or PTEN may be able to provide a more reliable ACC model. These molecules are known to be involved in the formation of a least a subset of adrenocortical tumors (Stratakis, 2003) and the generation of tumor models by cre-mediated conditional mutagenesis of p53 (Attardi and Donehower, 2005) and PTEN (Kishimoto et al., 2003) has already been successful in a variety of organs and tissues. Mice with floxed p53 and PTEN alleles are therefore already available. The development of tumors in other tissues that confer early-onset lethality as in the classical knock-outs of these genes can be elegantly avoided by the tissue specificity of cre expression. One example for this approach is the PSA-cre mediated disruption of PTEN in the prostatic gland. With this model, the embryonic lethal phenotype of homozygous conventional PTEN knock-out mice was bypassed and the role of PTEN in the development of prostate neoplasias could be demonstrated (Ma et al., 2005). The

use of the inducible adrenocortical CreERT2 mouse line would furthermore enable the determination of the onset of tumor formation by tamoxifen administration. The possibility to generate conditional double mutants, e. g. with targeted disruption of p53 and the telomerase RNA component mTERC (Attardi and Donehower, 2005) gene, might even enhance the value of adrenocortical cre mice for providing ACC models.

5.7 Conclusion

In conclusion, the number of targets for cre-mediated genetic manipulation in the adrenal cortex is extensive and will further increase with the number of available floxed genes. Large scale mouse mutagenesis consortia such as the European conditional mouse mutagenesis project EUCOMM (Schnutgen et al., 2005) (Glaser et al., 2005) have recently been established with the aim to generate conditional alleles for every known gene (Grimm, 2006). The number of target strains will therefore steadily increase eventually leading to comprehensive insight into the differential function of genes depending on the site of expression. Whole genome expression profiling data for the adrenal is already available, revealing the entire set of candidate genes for targeted mutagenesis in the adrenal (Zhang et al., 2004). As Else and Hammer state in their 2005 review (Else and Hammer, 2005): “The founder of modern anatomy, Vesalius, overlooked the adrenal glands, and, even after the first description by Eustachius in 1564, it took almost another 300 years until Addison’s description of adrenal insufficiency and Brown-Sequard’s first scientific investigations of the organ elucidated the vital functions of the gland. The future looks bright for many years of discovery, with basic and clinical investigations beginning to unravel the genetic and molecular underpinnings of adrenocortical development and disease.” Despite all the difficulties with their generation, adrenocortical cre mice will contribute their share to this bright future of discoveries.

6 Summary

The stress reaction of mammals is controlled by the hormones Corticotropin-Releasing Hormone (CRH), Adrenocorticotropic Hormone (ACTH) and Cortisol, which are released by the constituents of the Hypothalamus-Pituitary-Adrenal Cortex axis (HPA axis). An exact regulation of this system is of essential importance and is mainly achieved by negative feedback loops. A dysregulation of the HPA axis is a prominent feature e. g. in affective disorders. To investigate HPA axis functioning in vivo, mouse models may be used that either do not express ("knock-out" mouse) or overexpress key molecules of the system. Observing the consequences of these manipulations allows to infer the function of the modified component. However, the inference of a molecule function in a certain tissue may be hampered by its function in another tissue.

The cre/lox-system offers a solution to this problem. The DNA-recombinase cre catalyzes the recombination between two short DNA-sequences, termed lox-sites. This property is used in mouse genetics to obtain spatial control over DNA-recombination. To this end, a mouse expressing cre in a certain tissue or celltype of interest is bred with a mouse, in which the genetic region of interest is flanked with lox-sites. In cells where cre-protein is expressed and has access to the lox-sequences, recombination occurs, while the genetic sequence in all other cells remains unaltered. In addition to spatial control, temporal control over DNA-recombination can be achieved by means of inducible cre-recombinases allowing e.g. the indepent evaluation of molecule function in different stages of organismic development. However, there are currently no mice available that selectively express cre-recombinase in the adrenal cortex. Aim of this thesis therefore was the engineering of genetical constructs that allow the generation of mouse that selectively express cre-recombinase in the adrenal cortex.

In order to restrict expression of cre-recombinase to adrenocortical cells, the classical strategy of "knocking in" the cre-coding sequence into the open reading frame of an endogenous gene, in our case the receptor for ACTH, was chosen initially. This

approach permits gene expression according to the properties of the promoter of the endogenous gene. Corresponding constructs were generated for a constitutively active variant of cre and a tamoxifen inducible form (CreERT2). These constructs were used for homologous recombination in murine embryonic stem cells (ES-cells). A neomycin selection marker was integrated via an self-excising cassette designed in our laboratory. One ES-cell clone with supposedly correct integration of CreERT2 was selected to generate mouse line ACTHR-CE2. Unfortunately, the characterization of this mouse line revealed no presence of cre neither on RNA level nor on protein level, so that breeding was discontinued.

In a second, entirely novel approach targeting constructs on the basis of the abovementioned cre-variants integrated into cosmids carrying genomic DNA from the ACTH receptor locus were generated by means of homologous recombination in E. coli. In these constructs, the gene for the Venus fluorescent marker was introduced in order to improve visualization of cre expression domains. The fluorescent marker will be translated from cre/Venus bicistronic RNA by means of an internal ribosomal entry site. With the cosmid-based constructs mice can be generated either by classical “knock-in” through homologous recombination in ES-cells or as transgenic mice by means of pronuclear injection.

In the discussion section of this thesis, the justification for the use of the endogenous promoter of the ACTH receptor to restrict cre-expression to the adrenal cortex is given. Secondly, the choice of cre-variants used in this thesis is discussed. Why the self-excising cassette employed in the initial strategy did not function as expected is considered next. How an aberrant gene-targeting event lead to the generation of the ACTHR-CE2 mouse line is subsequently discussed with a special focus on the use of F1-hybrid ES-cells and the effect of non-isogenic DNA in homologous recombination. It is outlined, how the novel cosmid-based constructs serve to increase recombination efficiency. In addition, the rationale for the integration of the fluorescent marker is given. Which steps have to be taken towards the generation of mice from the constructs generated in this thesis is delineated next. In an outlook, the discussion section points out several possible applications of adrenocortical cre mice.

7 Zusammenfassung

Die Stressreaktion von Säugetieren wird maßgeblich durch die Hormone Corticoliberin (CRH), Adrenocorticotropes Hormon (ACTH) und Cortisol beeinflusst, welche von den Strukturen der Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HHN-Achse) ausgeschüttet werden. Eine genaue Regulation dieses Systems ist dabei von entscheidender Bedeutung und erfolgt nach klassischem Modell vor allem über negative Rückkopplungen. Eine Dysregulation dieses Systems ist ein bekanntes Phänomen u. a. bei affektiven Erkrankungen. Zur Untersuchung der HHN-Achse in vivo werden Mausmodelle verwendet, bei denen zentrale Moleküle des Systems entweder überhaupt nicht („knock-out"-Maus) oder aber vermehrt („überexprimierende" Maus) gebildet werden. Aus den Auswirkungen einer derartigen, selektiven Veränderung kann auf die Funktion der veränderten Komponente geschlossen werden. Dabei gilt jedoch die Einschränkung, dass die Auswirkungen der Veränderung in einem Gewebe die Interpretation der davon unabhängigen Auswirkungen in einem anderen Gewebe verunmöglichen kann.

Eine Lösung dieses Problems bietet das cre/lox-System. Die DNA-Rekombinase cre katalysiert die Rekombination zwischen zwei kurzen DNA-Regionen, die als lox-Sequenzen bezeichnet werden. In der Mausgenetik wird dies u. a. genutzt, um räumliche Kontrolle über DNA-Rekombination zu erreichen. Eine Maus mit cre-Expression in einem bestimmten Zelltyp oder einer bestimmten Region wird mit einer Maus gekreuzt, in der ein bestimmtes Gen von Interesse mit lox-Sequenzen flankiert worden ist. Nur in den Zellen, in denen cre-Protein gebildet wird und Zugang zur lox-markierten DNA hat, kommt es danach zur Rekombination. Die genetische Sequenz in allen anderen Zellen bleibt unverändert. Neben dieser Möglichkeit zur räumlichen Kontrolle lässt sich mit Hilfe von sogenannten induzierbaren cre-Rekombinasen auch die zeitliche Kontrolle erlangen, was die Differenzierung der Funktion von Genprodukten z. B. in verschiedenen Entwicklungsstadien erlaubt. Z. Zt. gibt es jedoch noch keine Mäuse, die cre-Rekombinase selektiv in der Nebennierenrinde exprimieren, mithin also die Untersuchung der Genfunktion in diesem integralen Bestandteil der HHN-Achse erlauben würden. Ziel dieser Arbeit war deswegen die

Erzeugung genetischer Konstrukte zur Herstellung von Mäusen, in deren Nebennierenrinde cre-Rekombinase exprimiert wird.

Zur Restriktion der Expression der cre-Rekombinase auf die Zellen der Nebennierenrinde wurde zunächst eine klassische Strategie des „knock-in“ der cre-kodierenden Sequenz in den offenen Leserahmen des ACTH-Rezeptors verfolgt, um dadurch die Eigenschaften des endogenen Promoters dieses Gens auszunutzen. Entsprechende Konstrukte wurden sowohl mit einer dauerhaft aktiven cre-Variante als auch mit einer Tamoxifen-induzierbaren Variante (CreERT2) erstellt. Diese Konstrukte wurden für den Austausch der Wildtyp- durch die rekombinante Genkonfiguration durch homologe Rekombination in embryonalen Stammzellen (ES-Zellen) der Maus eingesetzt. Die für die Selektion als Marker notwendige Neomycinresistenz wurde dabei in einer in unserem Labor entworfenen, selbst-ausschneidenden Kassette integriert. Ein Stammzellklon mit vermeintlich korrekter Rekombination mit dem CreERT2-tragenden Vektor wurde zur Erzeugung der ACTHR-CE2-Mauslinie verwendet. Die Charakterisierung dieser Mauslinie ergab jedoch sowohl auf RNA- als auch auf Protein-Ebene kein Vorliegen von cre, so dass die Zucht der Linie eingestellt wurde.

In einer zweiten Strategie wurden mit Hilfe homologer Rekombination in E. coli mit den bereits zuvor verwendeten cre-Varianten neuartige Konstrukte auf Grundlage eines ACTH-Rezeptor-Genlocus tragenden Cosmids generiert. Zur Verbesserung der Darstellbarkeit der cre-Expressionsdomänen wurde in diese Konstrukte zudem das Gen für den Venus-Fluoreszenzmarker eingeführt, dessen Translation mittels einer „internen ribosomalen Eintrittstelle“ (IRES) von bicistronischer cre/Venus-mRNA erfolgen soll. Die Cosmid-basierten Konstrukte eignen sich sowohl zur Erzeugung von Nebennierenrinden-cre-Mäusen durch homologe Rekombination in ES-Zellen im Sinne eines „knock-in“ wie im zuvor verwendeten Ansatz als auch zur Generierung von transgenen Mäusen durch pronukleäre Injektion. Der Einsatz eines Cosmids als Vektor zur Generierung von Mäusen ist dabei ein neuartiger Ansatz.

Im Rahmen der Diskussion wird zunächst dargestellt, dass die Verwendung des endogenen Promoters des ACTH-Rezeptors geeignet für die Restriktion der cre-

Expression auf Nebennierenrindenzellen ist. Des Weiteren wird die Wahl der benutzten cre-Varianten diskutiert. Mit Bezug auf die initial erstellten, klassischen „knock-in“-Konstrukte wird erörtert, warum die verwendete Selektionskassette sich nicht wie gewünscht selbst exzidierte. Im Anschluss wird diskutiert, warum auf Grundlage von erst post hoc erkannter, aberranter Rekombination die Generation der nicht-funktionalen ACTHR-CE2-Mauslinie erfolgte. Besonderes Augenmerk wird dabei auf die Verwendung von F1-Hybrid-ES-Zellen und nicht-isogener-DNA für homologe Rekombination in ES-Zellen gelegt. Sodann wird der Frage nachgegangen, wie mit Hilfe der neuartigen Cosmid-Konstrukte die Effizienz des Rekombinationsprozesses erhöht werden kann. Ergänzend wird die Verwendung des zusätzlich eingeführten Fluoreszenzmarkers erläutert. Es wird dargestellt, welche Mauslinien mit den vorliegenden Konstrukten generierbar sind und welche weiteren Schritte dafür notwendig sind. Abschliessend erfolgt ein Ausblick auf mögliche Anwendungsbereiche.

8 References

1. Abdel-Malek,Z.A. (2001). Melanocortin receptors: their functions and regulation by physiological agonists and antagonists. Cell Mol. Life Sci. *58*, 434-441.

2. Adams,D.J., Dermitzakis,E.T., Cox,T., Smith,J., Davies,R., Banerjee,R., Bonfield,J., Mullikin,J.C., Chung,Y.J., Rogers,J., and Bradley,A. (2005). Complex haplotypes, copy number polymorphisms and coding variation in two recently divergent mouse strains. Nat. Genet. *37*, 532-536.

3. Al-Majed,H.T., Jones,P.M., Persaud,S.J., Sugden,D., Huang,G.C., Amiel,S., and Whitehouse,B.J. (2004). ACTH stimulates insulin secretion from MIN6 cells and primary mouse and human islets of Langerhans. J. Endocrinol. *180*, 155-166.

4. al-Shawi,R., Kinnaird,J., Burke,J., and Bishop,J.O. (1990). Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. Mol. Cell Biol. *10*, 1192-1198.

5. Allolio,B., Hahner,S., Weismann,D., and Fassnacht,M. (2004). Management of adrenocortical carcinoma. Clin. Endocrinol. (Oxf) *60*, 273-287.

6. Andreas,S., Schwenk,F., Kuter-Luks,B., Faust,N., and Kuhn,R. (2002). Enhanced efficiency through nuclear localization signal fusion on phage PhiC31-integrase: activity comparison with Cre and FLPe recombinase in mammalian cells. Nucleic Acids Res. *30*, 2299-2306.

7. Attardi,L.D., and Donehower,L.A. (2005). Probing p53 biological functions through the use of genetically engineered mouse models. Mutat. Res. *576*, 4-21.

8. Bear,M., Connors,B., and Paradiso,M. (1996). Neuroscience: Exploring the Brain (Baltimore: Wiliams&Wilkins).

9. Beck,J.A., Lloyd,S., Hafezparast,M., Lennon-Pierce,M., Eppig,J.T., Festing,M.F., and Fisher,E.M. (2000). Genealogies of mouse inbred strains. Nat. Genet. *24*, 23-25.

10. Berton,O., and Nestler,E.J. (2006). New approaches to antidepressant drug discovery: beyond monoamines. Nat. Rev. Neurosci. *7*, 137-151.

11. Beuschlein,F., Fassnacht,M., Klink,A., Allolio,B., and Reincke,M. (2001). ACTH-receptor expression, regulation and role in adrenocortial tumor formation. Eur. J. Endocrinol. *144*, 199-206.

12. bi-Dargham,A., Rodenhiser,J., Printz,D., Zea-Ponce,Y., Gil,R., Kegeles,L.S., Weiss,R., Cooper,T.B., Mann,J.J., Van Heertum,R.L., Gorman,J.M., and

Laruelle,M. (2000). Increased baseline occupancy of D2 receptors by dopamine in schizophrenia. Proc. Natl. Acad. Sci. U. S. A *97*, 8104-8109.

13. Bitinaite,J., and Schildkraut,I. (2002). Self-generated DNA termini relax the specificity of SgrAI restriction endonuclease. Proc. Natl. Acad. Sci. U. S. A *99*, 1164-1169.

14. Boston,B.A., and Cone,R.D. (1996). Characterization of melanocortin receptor subtype expression in murine adipose tissues and in the 3T3-L1 cell line. Endocrinology *137*, 2043-2050.

15. Boyle,M.P., Brewer,J.A., Funatsu,M., Wozniak,D.F., Tsien,J.Z., Izumi,Y., and Muglia,L.J. (2005). Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proc. Natl. Acad. Sci. U. S. A *102*, 473-478.

16. Branda,C.S., and Dymecki,S.M. (2004). Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev. Cell *6*, 7-28.

17. Brocard,J., Warot,X., Wendling,O., Messaddeq,N., Vonesch,J.L., Chambon,P., and Metzger,D. (1997). Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. Proc. Natl. Acad. Sci. U. S. A *94*, 14559-14563.

18. Brook,F.A., and Gardner,R.L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. Proc. Natl. Acad. Sci. U. S. A *94*, 5709-5712.

19. Brown,E.S., Varghese,F.P., and McEwen,B.S. (2004). Association of depression with medical illness: does cortisol play a role? Biol. Psychiatry *55*, 1-9.

20. Brunner,H.G., Nelen,M., Breakefield,X.O., Ropers,H.H., and van Oost,B.A. (1993). Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. Science *262*, 578-580.

21. Bucan,M., and Abel,T. (2002). The mouse: genetics meets behaviour. Nat. Rev. Genet. *3*, 114-123.

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

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

24. Butler,A.A., and Cone,R.D. (2002). The melanocortin receptors: lessons from knockout models. Neuropeptides *36*, 77-84.

25. Cammas,F.M., Kapas,S., Barker,S., and Clark,A.J.L. (1995). Cloning, Characterization and Expression of A Functional-Mouse Acth Receptor. Biochemical and Biophysical Research Communications *212*, 912-918.

26. Cammas,F.M., Pullinger,G.D., Barker,S., and Clark,A.J.L. (1997). The mouse adrenocorticotropin receptor gene: Cloning and characterization of its promoter and evidence for a role for the orphan nuclear receptor steroidogenic factor 1. Molecular Endocrinology *11*, 867-876.

27. Capecchi,M.R. (2001). Generating mice with targeted mutations. Nature Medicine *7*, 1086-1090.

28. Casanova,E., Fehsenfeld,S., Lemberger,T., Shimshek,D.R., Sprengel,R., and Mantamadiotis,T. (2002). ER-based double icre fusion protein allows partial recombination in forebrain. Genesis *34*, 208-214.

29. Casanova,E., Fehsenfeld,S., Mantamadiotis,T., Lemberger,T., Greiner,E., Stewart,A.F., and Schutz,G. (2001). A CamKII alpha iCre BAC allows brain-specific gene inactivation. Genesis *31*, 37-42.

30. Cases,O., Seif,I., Grimsby,J., Gaspar,P., Chen,K., Pournin,S., Muller,U., Aguet,M., Babinet,C., Shih,J.C., and . (1995). Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science *268*, 1763-1766.

31. Chambers,I. (2004). The molecular basis of pluripotency in mouse embryonic stem cells. Cloning Stem Cells *6*, 386-391.

32. Chaveroche,M.K., Ghigo,J.M., and d'Enfert,C. (2000). A rapid method for efficient gene replacement in the filamentous fungus Aspergillus nidulans. Nucleic Acids Res. *28*, E97.

33. Chemelli,R.M., Willie,J.T., Sinton,C.M., Elmquist,J.K., Scammell,T., Lee,C., Richardson,J.A., Williams,S.C., Xiong,Y., Kisanuki,Y., Fitch,T.E., Nakazato,M., Hammer,R.E., Saper,C.B., and Yanagisawa,M. (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell *98*, 437-451.

34. Clark,A.J., McLoughlin,L., and Grossman,A. (1993). Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. Lancet *341*, 461-462.

35. Conrad,M., Brielmeier,M., Wurst,W., and Bornkamm,G.W. (2003). Optimized vector for conditional gene targeting in mouse embryonic stem cells. Biotechniques *34*, 1136-8, 1140.

36. Copeland,N.G., Jenkins,N.A., and Court DL (2001). Recombineering: a powerful new tool for mouse functional genomics. Nat. Rev. Genet. *2*, 769-779.

37. Corbel,S.Y., and 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. *13*, 448-452.

38. Crusio,W.E. (2004). Flanking gene and genetic background problems in genetically manipulated mice. Biol. Psychiatry *56*, 381-385.

39. Cryan,J.F., and Holmes,A. (2005). The ascent of mouse: advances in modelling human depression and anxiety. Nat. Rev. Drug Discov. *4*, 775-790.

40. Cryan,J.F., and Mombereau,C. (2004). In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. Mol. Psychiatry *9*, 326-357.

41. Cunningham,F., Rios,D., Griffiths,M., Smith,J., Ning,Z., Cox,T., Flicek,P., Marin-Garcin,P., Herrero,J., Rogers,J., van der,W.L., Bradley,A., Birney,E., and Adams,D.J. (2006). TranscriptSNPView: a genome-wide catalog of mouse coding variation. Nat. Genet. *38*, 853.

42. Cushman,L.J., Burrows,H.L., Seasholtz,A.F., Lewandoski,M., Muzyczka,N., and Camper,S.A. (2000). Cre-mediated recombination in the pituitary gland. Genesis. *28*, 167-174.

43. Dallman,M.F. (2005). Fast glucocorticoid actions on brain: back to the future. Front Neuroendocrinol. *26*, 103-108.

44. Danielian,P.S., Muccino,D., Rowitch,D.H., Michael,S.K., and McMahon,A.P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr. Biol. *8*, 1323-1326.

45. de Kloet,E.R., Joels,M., and Holsboer,F. (2005). Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. *6*, 463-475.

46. Debruyne,J.P., Noton,E., Lambert,C.M., Maywood,E.S., Weaver,D.R., and Reppert,S.M. (2006). A Clock Shock: Mouse CLOCK Is Not Required for Circadian Oscillator Function. Neuron *50*, 465-477.

47. Deng,C., and 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.

48. Dermitzaki,E., Tsatsanis,C., Minas,V., Chatzaki,E., Charalampopoulos,I., Venihaki,M., Androulidaki,A., Lambropoulou,M., Spiess,J., Michalodimitrakis,E., Gravanis,A., and Margioris,A.N. (2007). Corticotropin-releasing factor (CRF) and the urocortins differentially regulate catecholamine secretion in human and rat adrenals, in a CRF receptor type-specific manner. Endocrinology *148*, 1524-1538.

49. Deussing,J.M., and Wurst,W. (2005). Dissecting the genetic effect of the CRH system on anxiety and stress-related behaviour. C. R. Biol. *328*, 199-212.

50. 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.

51. Easton,A., Arbuzova,J., and Turek,F.W. (2003). The circadian Clock mutation increases exploratory activity and escape-seeking behavior. Genes Brain Behav. *2*, 11-19.

52. Ebmeier,K.P., Donaghey,C., and Steele,J.D. (2006). Recent developments and current controversies in depression. Lancet *367*, 153-167.

53. Eggan,K., Akutsu,H., Loring,J., Jackson-Grusby,L., Klemm,M., Rideout,W.M., III, Yanagimachi,R., and Jaenisch,R. (2001). Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc. Natl. Acad. Sci. U. S. A *98*, 6209-6214.

54. Ehrhart-Bornstein,M., Hinson,J.P., Bornstein,S.R., Scherbaum,W.A., and Vinson,G.P. (1998). Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. Endocr. Rev. *19*, 101-143.

55. Ellenbroek,B.A. (2003). Animal models in the genomic era: possibilities and limitations with special emphasis on schizophrenia. Behav. Pharmacol. *14*, 409-417.

56. Else,T., and Hammer,G.D. (2005). Genetic analysis of adrenal absence: agenesis and aplasia. Trends Endocrinol. Metab *16*, 458-468.

57. Evan,G.I., Lewis,G.K., Ramsay,G., and Bishop,J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell Biol. *5*, 3610-3616.

58. Evans,M.J., and Kaufman,M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.

59. Fassnacht,M., Beuschlein,F., Vay,S., Mora,P., Allolio,B., and Reincke,M. (1998). Aminoglutethimide suppresses adrenocorticotropin receptor expression in the NCI-h295 adrenocortical tumor cell line. J. Endocrinol. *159*, 35-42.

60. Feil,R., Brocard,J., Mascrez,B., LeMeur,M., Metzger,D., and Chambon,P. (1996). Ligand-activated site-specific recombination in mice. Proceedings of the National Academy of Sciences of the United States of America *93*, 10887-10890.

61. Feil,R., Wagner,J., Metzger,D., and Chambon,P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochemical and Biophysical Research Communications *237*, 752-757.

62. Feltus,F.A., Cote,S., Simard,J., Gingras,S., Kovacs,W.J., Nicholson,W.E., Clark,B.J., and Melner,M.H. (2002). Glucocorticoids enhance activation of the

human type II 3beta-hydroxysteroid dehydrogenase/Delta5-Delta4 isomerase gene. J. Steroid Biochem. Mol. Biol. *82*, 55-63.

63. Festing,M.F., Simpson,E.M., Davisson,M.T., and Mobraaten,L.E. (1999). Revised nomenclature for strain 129 mice. Mamm. Genome *10*, 836.

64. Flores,B.H., Kenna,H., Keller,J., Solvason,H.B., and Schatzberg,A.F. (2006). Clinical and biological effects of mifepristone treatment for psychotic depression. Neuropsychopharmacology *31*, 628-636.

65. Fukaya,M., Kato,A., Lovett,C., Tonegawa,S., and Watanabe,M. (2003). Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 knockout mice. Proc. Natl. Acad. Sci. U. S. A *100*, 4855-4860.

66. Giraldo,P., and Montoliu,L. (2001). Size matters: use of YACs, BACs and PACs in transgenic animals. Transgenic Res. *10*, 83-103.

67. Glaser,S., Anastassiadis,K., and Stewart,A.F. (2005). Current issues in mouse genome engineering. Nat. Genet. *37*, 1187-1193.

68. Gossen,M., Freundlieb,S., Bender,G., Muller,G., Hillen,W., and Bujard,H. (1995). Transcriptional Activation by Tetracyclines in Mammalian-Cells. Science *268*, 1766-1769.

69. Greer,J.M., and Capecchi,M.R. (2002). Hoxb8 is required for normal grooming behavior in mice. Neuron *33*, 23-34.

70. Grimm,D. (2006). Mouse genetics. A mouse for every gene. Science *312*, 1862-1866.

71. Gu,H., Marth,J.D., Orban,P.C., Mossmann,H., and Rajewsky,K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science *265*, 103-106.

72. Hadjantonakis,A.K., Dickinson,M.E., Fraser,S.E., and Papaioannou,V.E. (2003). Technicolour transgenics: imaging tools for functional genomics in the mouse. Nat. Rev. Genet. *4*, 613-625.

73. Hamilton,D.L., and Abremski,K. (1984). Site-specific recombination by the bacteriophage P1 lox-Cre system. Cre-mediated synapsis of two lox sites. J. Mol. Biol. *178*, 481-486.

74. Hammer,G.D., Parker,K.L., and Schimmer,B.P. (2005). Minireview: transcriptional regulation of adrenocortical development. Endocrinology *146*, 1018-1024.

75. Hasler,G., Drevets,W.C., Manji,H.K., and Charney,D.S. (2004). Discovering endophenotypes for major depression. Neuropsychopharmacology *29*, 1765-1781.

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

77. Heintz,N. (2001). BAC to the future: the use of bac transgenic mice for neuroscience research. Nat. Rev. Neurosci. *2*, 861-870.

78. Hirrlinger,P.G., Scheller,A., Braun,C., Hirrlinger,J., and Kirchhoff,F. (2006). Temporal control of gene recombination in astrocytes by transgenic expression of the tamoxifen-inducible DNA recombinase variant CreERT2. Glia *54*, 11-20.

79. Holsboer,F. (2000). The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology *23*, 477-501.

80. Holsboer,F. (2001). Prospects for antidepressant drug discovery. Biol. Psychol. *57*, 47-65.

81. Hsu,H.J., Hsu,N.C., Hu,M.C., and Chung,B.C. (2006). Steroidogenesis in zebrafish and mouse models. Mol. Cell Endocrinol. *248*, 160-163.

82. Huang,H., Winter,E.E., Wang,H., Weinstock,K.G., Xing,H., Goodstadt,L., Stenson,P.D., Cooper,D.N., Smith,D., Alba,M.M., Ponting,C.P., and Fechtel,K. (2004). Evolutionary conservation and selection of human disease gene orthologs in the rat and mouse genomes. Genome Biol. *5*, R47.

83. Imai,T., Jiang,M., Chambon,P., and Metzger,D. (2001). Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinoid X receptor alpha mediated by a tamoxifen-inducible chimeric Cre recombinase (Cre-ERT2) in adipocytes. Proceedings of the National Academy of Sciences of the United States of America *98*, 224-228.

84. Indra,A.K., Warot,X., Brocard,J., Bornert,J.M., Xiao,J.H., Chambon,P., and Metzger,D. (1999). Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. Nucleic Acids Res. *27*, 4324-4327.

85. Inoue,K., and Lupski,J.R. (2003). Genetics and genomics of behavioral and psychiatric disorders. Curr. Opin. Genet. Dev. *13*, 303-309.

86. Iwawaki,T., Akai,R., Kohno,K., and Miura,M. (2004). A transgenic mouse model for monitoring endoplasmic reticulum stress. Nat. Med. *10*, 98-102.

87. Johansson,C., Willeit,M., Smedh,C., Ekholm,J., Paunio,T., Kieseppa,T., Lichtermann,D., Praschak-Rieder,N., Neumeister,A., Nilsson,L.G., Kasper,S., Peltonen,L., Adolfsson,R., Schalling,M., and Partonen,T. (2003). Circadian clock-related polymorphisms in seasonal affective disorder and their relevance to diurnal preference. Neuropsychopharmacology *28*, 734-739.

88. Johnson,E.W., Hughes,T.K., Jr., and Smith,E.M. (2001). ACTH receptor distribution and modulation among murine mononuclear leukocyte populations. J. Biol. Regul. Homeost. Agents *15*, 156-162.

89. Jordan,V.C. (2003). Tamoxifen: a most unlikely pioneering medicine. Nat. Rev. Drug Discov. *2*, 205-213.

90. Jorgez,C.J., De Mayo,F.J., and Matzuk,M.M. (2006). Inhibin alpha-iCre mice: Cre deleter lines for the gonads, pituitary, and adrenal. Genesis. *44*, 183-188.

91. Joyner,A. (2000). Gene Targeting (Oxford: Oxford University Press).

92. Joynt,K.E., Whellan,D.J., and O'Connor,C.M. (2003). Depression and cardiovascular disease: mechanisms of interaction. Biol. Psychiatry *54*, 248-261.

93. Keays,D.A., and Nolan,P.M. (2003). N-ethyl-N-nitrosourea mouse mutants in the dissection of behavioural and psychiatric disorders. Eur. J. Pharmacol. *480*, 205-217.

94. Keck,M.E., Ohl,F., Holsboer,F., and Muller,M.B. (2005). Listening to mutant mice: a spotlight on the role of CRF/CRF receptor systems in affective disorders. Neurosci. Biobehav. Rev. *29*, 867-889.

95. Kellendonk,C., Simpson,E.H., Polan,H.J., Malleret,G., Vronskaya,S., Winiger,V., Moore,H., and Kandel,E.R. (2006). Transient and selective overexpression of dopamine D2 receptors in the striatum causes persistent abnormalities in prefrontal cortex functioning. Neuron *49*, 603-615.

96. Kim,D.G., Kang,H.M., Jang,S.K., and 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.

97. Kim,J.E., Nakashima,K., and de,C.B. (2004). Transgenic mice expressing a ligand-inducible cre recombinase in osteoblasts and odontoblasts: a new tool to examine physiology and disease of postnatal bone and tooth. Am. J. Pathol. *165*, 1875-1882.

98. Kishimoto,H., Hamada,K., Saunders,M., Backman,S., Sasaki,T., Nakano,T., Mak,T.W., and Suzuki,A. (2003). Physiological functions of Pten in mouse tissues. Cell Struct. Funct. *28*, 11-21.

99. Kromer,S.A., Kessler,M.S., Milfay,D., Birg,I.N., Bunck,M., Czibere,L., Panhuysen,M., Putz,B., Deussing,J.M., Holsboer,F., Landgraf,R., and Turck,C.W. (2005). Identification of glyoxalase-I as a protein marker in a mouse model of extremes in trait anxiety. J. Neurosci. *25*, 4375-4384.

100. Kubo,M., Ishizuka,T., Kijima,H., Kakinuma,M., and Koike,T. (1995). Cloning of A Mouse Adrenocorticotropin Receptor-Encoding Gene. Gene *153*, 279-280.

101. Kubo,M., Shimizu,C., Kijima,H., Nagai,S., and Koike,T. (2004). Alternate promoter and 5'-untranslated exon usage of the mouse adrenocorticotropin receptor gene in adipose tissue. Endocr. J. *51*, 25-30.

102. Kuhbandner,S., Brummer,S., Metzger,D., Chambon,P., Hofmann,F., and Feil,R. (2000). Temporally controlled somatic mutagenesis in smooth muscle. Genesis *28*, 15-22.

103. Kuhn,R., Schwenk,F., Aguet,M., and Rajewsky,K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

104. Kwan,K.M. (2002). Conditional alleles in mice: Practical considerations for tissue-specific knockouts. Genesis *32*, 49-62.

105. Lai,C.S., Fisher,S.E., Hurst,J.A., Vargha-Khadem,F., and Monaco,A.P. (2001). A forkhead-domain gene is mutated in a severe speech and language disorder. Nature *413*, 519-523.

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

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

108. Lee,E.C., Yu,D., Martinez,d., V, Tessarollo,L., Swing,D.A., Court DL, Jenkins,N.A., and Copeland,N.G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics *73*, 56-65.

109. Leone,D.P., Genoud,S., Atanasoski,S., Grausenburger,R., Berger,P., Metzger,D., Macklin,W.B., Chambon,P., and Suter,U. (2003). Tamoxifen-inducible glia-specific Cre mice for somatic mutagenesis in oligodendrocytes and Schwann cells. Mol. Cell Neurosci. *22*, 430-440.

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

111. Lichtenauer,U.D., Duchniewicz,M., Kolanczyk,M., Hoeflich,A., Hahner,S., Else,T., Bicknell,A.B., Zemojtel,T., Stallings,N.R., Schulte,D.M., Kamps,M.P., Hammer,G.D., Scheele,J.S., and Beuschlein,F. (2007). Pre-B-cell transcription factor 1 and steroidogenic factor 1 synergistically regulate adrenocortical growth and steroidogenesis. Endocrinology *148*, 693-704.

112. Lindeberg,J., Usoskin,D., Bengtsson,H., Gustafsson,A., Kylberg,A., Soderstrom,S., and Ebendal,T. (2004). Transgenic expression of Cre recombinase from the tyrosine hydroxylase locus. Genesis. *40*, 67-73.

113. Liu,P., Jenkins,N.A., and Copeland,N.G. (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res. *13*, 476-484.

114. Loonstra,A., Vooijs,M., Beverloo,H.B., Al Allak,B., van Drunen,E., Kanaar,R., Berns,A., and Jonkers,J. (2001). Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America *98*, 9209-9214.

115. Ma,X., Ziel-van der Made AC, Autar,B., van der Korput,H.A., Vermeij,M., van,D.P., Cleutjens,K.B., de,K.R., Krimpenfort,P., Berns,A., van der Kwast,T.H., and Trapman,J. (2005). Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis. Cancer Res. *65*, 5730-5739.

116. Mangiarini,L., Sathasivam,K., Seller,M., Cozens,B., Harper,A., Hetherington,C., Lawton,M., Trottier,Y., Lehrach,H., Davies,S.W., and Bates,G.P. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell *87*, 493-506.

117. Manji,H.K., Drevets,W.C., and Charney,D.S. (2001). The cellular neurobiology of depression. Nat. Med. *7*, 541-547.

118. Mann,J.J. (2005). The medical management of depression. N. Engl. J. Med. *353*, 1819-1834.

119. Martin,G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U. S. A *78*, 7634-7638.

120. Martinez,A., Lefrancois-Martinez,A.M., Manin,M., Guyot,S., Jean-Faucher,C., Veyssiere,G., Kahn,A., and Jean,C. (1999). 5'-flanking and intragenic sequences confer androgenic and developmental regulation of mouse aldose reductase-like gene in vas deferens and adrenal in transgenic mice. Endocrinology *140*, 1338-1348.

121. McKernan,R.M., Rosahl,T.W., Reynolds,D.S., Sur,C., Wafford,K.A., Atack,J.R., Farrar,S., Myers,J., Cook,G., Ferris,P., Garrett,L., Bristow,L., Marshall,G., Macaulay,A., Brown,N., Howell,O., Moore,K.W., Carling,R.W., Street,L.J., Castro,J.L., Ragan,C.I., Dawson,G.R., and Whiting,P.J. (2000). Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. Nat. Neurosci. *3*, 587-592.

122. Mellon,S.H., Miller,W.L., Bair,S.R., Moore,C.C., Vigne,J.L., and Weiner,R.I. (1994). Steroidogenic adrenocortical cell lines produced by genetically targeted tumorigenesis in transgenic mice. Mol. Endocrinol. *8*, 97-108.

123. Metzger,D., Clifford,J., Chiba,H., and Chambon,P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc. Natl. Acad. Sci. U. S. A *92*, 6991-6995.

124. Montkowski,A., Poettig,M., Mederer,A., and Holsboer,F. (1997). Behavioural performance in three substrains of mouse strain 129. Brain Res. *762*, 12-18.

125. Mori,T., Tanaka,K., Buffo,A., Wurst,W., Kuhn,R., and Gotz,M. (2006). Inducible gene deletion in astroglia and radial glia--a valuable tool for functional and lineage analysis. Glia *54*, 21-34.

126. Morley,S.D., Viard,I., Parker,K.L., and Mullins,J.J. (1996). Adrenocortical-specific transgene expression directed by steroid hydroxylase gene promoters. Endocr. Res. *22*, 631-639.

127. Morozov,A., Kellendonk,C., Simpson,E., and Tronche,F. (2003). Using conditional mutagenesis to study the brain. Biol. Psychiatry *54*, 1125-1133.

128. Mountjoy,K.G., Bird,I.M., Rainey,W.E., and Cone,R.D. (1994). ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. Mol. Cell Endocrinol. *99*, R17-R20.

129. Muller,M.B., Preil,J., Renner,U., Zimmermann,S., Kresse,A.E., Stalla,G.K., Keck,M.E., Holsboer,F., and Wurst,W. (2001). Expression of CRHR1 and CRHR2 in mouse pituitary and adrenal gland: implications for HPA system regulation. Endocrinology *142*, 4150-4153.

130. Muller,M.B., Zimmermann,S., Sillaber,I., Hagemeyer,T.P., Deussing,J.M., Timpl,P., Kormann,M.S., Droste,S.K., Kuhn,R., Reul,J.M., Holsboer,F., and Wurst,W. (2003). Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. Nat. Neurosci. *6*, 1100-1107.

131. Muyrers,J.P., Zhang,Y., Testa,G., and Stewart,A.F. (1999). Rapid modification of bacterial artificial chromosomes by ET-recombination. Nucleic Acids Res. *27*, 1555-1557.

132. Nagai,T., Ibata,K., Park,E.S., Kubota,M., Mikoshiba,K., and Miyawaki,A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat. Biotechnol. *20*, 87-90.

133. Nagy,A. (2000). Cre recombinase: The universal reagent for genome tailoring. Genesis *26*, 99-109.

134. Nagy,A., and Mar,L. (2001). Creation and use of a Cre recombinase transgenic database. Methods Mol. Biol. *158*, 95-106.

135. Nemeroff,C.B., Krishnan,K.R., Reed,D., Leder,R., Beam,C., and Dunnick,N.R. (1992). Adrenal gland enlargement in major depression. A computed tomographic study. Arch. Gen. Psychiatry *49*, 384-387.

136. Nestler,E.J. (2000). Genes and addiction. Nat. Genet. *26*, 277-281.

137. Nimura,M., Udagawa,J., Hatta,T., Hashimoto,R., and Otani,H. (2006). Spatial and temporal patterns of expression of melanocortin type 2 and 5 receptors in the fetal mouse tissues and organs. Anat. Embryol. (Berl) *211*, 109-117.

138. Nolden,L., Edenhofer,F., Haupt,S., Koch,P., Wunderlich,F.T., Siemen,H., and Brustle,O. (2006). Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase. Nat. Methods *3*, 461-467.

139. Noon,L.A., Clark,A.J., and King,P.J. (2004). A peroxisome proliferator-response element in the murine mc2-r promoter regulates its transcriptional activation during differentiation of 3T3-L1 adipocytes. J. Biol. Chem. *279*, 22803-22808.

140. Norman,A.W., Mizwicki,M.T., and Norman,D.P. (2004). Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat. Rev. Drug Discov. *3*, 27-41.

141. O'Shaughnessy,P.J., Fleming,L.M., Jackson,G., Hochgeschwender,U., Reed,P., and Baker,P.J. (2003). Adrenocorticotropic hormone directly stimulates testosterone production by the fetal and neonatal mouse testis. Endocrinology *144*, 3279-3284.

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

143. Ortmann,D., Hausmann,J., Beuschlein,F., Schmenger,K., Stahl,M., Geissler,M., and Reincke,M. (2004). Steroidogenic acute regulatory (StAR)-directed immunotherapy protects against tumor growth of StAR-expressing Sp2-0 cells in a rodent adrenocortical carcinoma model. Endocrinology *145*, 1760-1766.

144. Palmiter,R.D., Brinster,R.L., Hammer,R.E., Trumbauer,M.E., Rosenfeld,M.G., Birnberg,N.C., and Evans,R.M. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature *300*, 611-615.

145. Parker,K.L., and Schimmer,B.P. (2001). Genetics of the development and function of the adrenal cortex. Rev. Endocr. Metab Disord. *2*, 245-252.

146. Pelletier,J., and Sonenberg,N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature *334*, 320-325.

147. Pham,C.T., MacIvor,D.M., Hug,B.A., Heusel,J.W., and Ley,T.J. (1996). Long-range disruption of gene expression by a selectable marker cassette. Proc. Natl. Acad. Sci. U. S. A *93*, 13090-13095.

148. Preil,J., Muller,M.B., Gesing,A., Reul,J.M., Sillaber,I., van Gaalen,M.M., Landgrebe,J., Holsboer,F., Stenzel-Poore,M., and Wurst,W. (2001). Regulation of the hypothalamic-pituitary-adrenocortical system in mice deficient for CRH receptors 1 and 2. Endocrinology *142*, 4946-4955.

149. Qiu,R., Frigeri,C., and Schimmer,B.P. (1998). A role for guanyl nucleotide-binding regulatory protein beta- and gamma-subunits in the expression of the adrenocorticotropin receptor. Mol. Endocrinol. *12*, 1879-1887.

150. Rainey,W.E., Saner,K., and Schimmer,B.P. (2004). Adrenocortical cell lines. Mol. Cell Endocrinol. *228*, 23-38.

151. Rickert,R.C., Roes,J., and Rajewsky,K. (1997). B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res. *25*, 1317-1318.

152. Rocha,K.M., Forti,F.L., Lepique,A.P., and Armelin,H.A. (2003). Deconstructing the molecular mechanisms of cell cycle control in a mouse adrenocortical cell line: roles of ACTH. Microsc. Res. Tech. *61*, 268-274.

153. Rodriguez,C.I., Buchholz,F., Galloway,J., Sequerra,R., Kasper,J., Ayala,R., Stewart,A.F., and Dymecki,S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. *25*, 139-140.

154. Rubin,R.T., Phillips,J.J., Sadow,T.F., and McCracken,J.T. (1995). Adrenal gland volume in major depression. Increase during the depressive episode and decrease with successful treatment. Arch. Gen. Psychiatry *52*, 213-218.

155. Sahut-Barnola,I., Lefrancois-Martinez,A.M., Jean,C., Veyssiere,G., and Martinez,A. (2000). Adrenal tumorigenesis targeted by the corticotropin-regulated promoter of the aldo-keto reductase AKR1B7 gene in transgenic mice. Endocr. Res. *26*, 885-898.

156. Sambrook,J., and Russell,D. (2001). Molecular Cloning - A Laboratory Manual (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

157. Sauer,B., and McDermott,J. (2004). DNA recombination with a heterospecific Cre homolog identified from comparison of the pac-c1 regions of P1-related phages. Nucleic Acids Res. *32*, 6086-6095.

158. Schimmer,B.P. (1979). Adrenocortical Y1 cells. Methods Enzymol. *58*, 570-574.

159. Schioth,H.B., Haitina,T., Ling,M.K., Ringholm,A., Fredriksson,R., Cerda-Reverter,J.M., and Klovins,J. (2005). Evolutionary conservation of the structural, pharmacological, and genomic characteristics of the melanocortin receptor subtypes. Peptides *26*, 1886-1900.

160. Schioth,H.B., Raudsepp,T., Ringholm,A., Fredriksson,R., Takeuchi,S., Larhammar,D., and Chowdhary,B.P. (2003). Remarkable synteny conservation of melanocortin receptors in chicken, human, and other vertebrates. Genomics *81*, 504-509.

161. Schmidt,E.E., Taylor,D.S., Prigge,J.R., Barnett,S., and Capecchi,M.R. (2000). Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proceedings of the National Academy of Sciences of the United States of America *97*, 13702-13707.

162. Schnutgen,F., De-Zolt,S., Van,S.P., Hollatz,M., Floss,T., Hansen,J., Altschmied,J., Seisenberger,C., Ghyselinck,N.B., Ruiz,P., Chambon,P., Wurst,W., and von,M.H. (2005). Genomewide production of multipurpose alleles for the functional analysis of the mouse genome. Proc. Natl. Acad. Sci. U. S. A *102*, 7221-7226.

163. Schwenk,F., Kuhn,R., Angrand,P.O., Rajewsky,K., and Stewart,A.F. (1998). Temporally and spatially regulated somatic mutagenesis in mice. Nucleic Acids Res. *26*, 1427-1432.

164. Schwenk,F., Sauer,B., Kukoc,N., Hoess,R., Muller,W., Kocks,C., Kuhn,R., and Rajewsky,K. (1997). Generation of Cre recombinase-specific monoclonal antibodies, able to characterize the pattern of Cre expression in cre-transgenic mouse strains. J. Immunol. Methods *207*, 203-212.

165. Schwenk,F., Zevnik,B., Bruning,J., Rohl,M., Willuweit,A., Rode,A., Hennek,T., Kauselmann,G., Jaenisch,R., and Kuhn,R. (2003). Hybrid embryonic stem cell-derived tetraploid mice show apparently normal morphological, physiological, and neurological characteristics. Mol. Cell Biol. *23*, 3982-3989.

166. Scrable,H. (2002). Say when: reversible control of gene expression in the mouse by lac. Semin. Cell Dev. Biol. *13*, 109-119.

167. Sekiyama,A., Ueda,H., Kashiwamura,S., Nishida,K., Yamaguchi,S., Sasaki,H., Kuwano,Y., Kawai,K., Teshima-kondo,S., Rokutan,K., and Okamura,H. (2006). A role of the adrenal gland in stress-induced up-regulation of cytokines in plasma. J. Neuroimmunol. *171*, 38-44.

168. Seong,E., Seasholtz,A.F., and Burmeister,M. (2002). Mouse models for psychiatric disorders. Trends Genet. *18*, 643-650.

169. Shen,J., Bronson,R.T., Chen,D.F., Xia,W., Selkoe,D.J., and Tonegawa,S. (1997). Skeletal and CNS defects in Presenilin-1-deficient mice. Cell *89*, 629-639.

170. Shimizu,C., Kubo,M., Saeki,T., Matsumura,T., Ishizuka,T., Kijima,H., Kakinuma,M., and Koike,T. (1997). Genomic organization of the mouse adrenocorticotropin receptor. Gene *188*, 17-21.

171. Shizuya,H., Birren,B., Kim,U.J., Mancino,V., Slepak,T., Tachiiri,Y., and Simon,M. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc. Natl. Acad. Sci. U. S. A *89*, 8794-8797.

172. Shu,W., Cho,J.Y., Jiang,Y., Zhang,M., Weisz,D., Elder,G.A., Schmeidler,J., De,G.R., Sosa,M.A., Rabidou,D., Santucci,A.C., Perl,D., Morrisey,E., and

Buxbaum,J.D. (2005). Altered ultrasonic vocalization in mice with a disruption in the Foxp2 gene. Proc. Natl. Acad. Sci. U. S. A *102*, 9643-9648.

173. Simpson,E.M., Linder,C.C., Sargent,E.E., Davisson,M.T., Mobraaten,L.E., and Sharp,J.J. (1997). Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat. Genet. *16*, 19-27.

174. Smith,G.W., Aubry,J.M., Dellu,F., Contarino,A., Bilezikjian,L.M., Gold,L.H., Chen,R., Marchuk,Y., Hauser,C., Bentley,C.A., Sawchenko,P.E., Koob,G.F., Vale,W., and Lee,K.F. (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron *20*, 1093-1102.

175. Soriano,P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nature Genetics *21*, 70-71.

176. Sorrell,D.A., and Kolb,A.F. (2005). Targeted modification of mammalian genomes. Biotechnol. Adv. *23*, 431-469.

177. Soukharev,S., Miller,J.L., and Sauer,B. (1999). Segmental genomic replacement in embryonic stem cells by double lox targeting. Nucleic Acids Res. *27*, e21.

178. Spiess,J., Rivier,J., Rivier,C., and Vale,W. (1981). Primary structure of corticotropin-releasing factor from ovine hypothalamus. Proc. Natl. Acad. Sci. U. S. A *78*, 6517-6521.

179. Stratakis,C.A. (2003). Genetics of adrenocortical tumors: gatekeepers, landscapers and conductors in symphony. Trends Endocrinol. Metab *14*, 404-410.

180. Stricklett,P.K., Nelson,R.D., and Kohan,D.E. (1998). Site-specific recombination using an epitope tagged bacteriophage P1 Cre recombinase. Gene *215*, 415-423.

181. Sugama,S., Wang,N., Shimokawa,N., Koibuchi,N., Fujita,M., Hashimoto,M., Dhabhar,F.S., and Conti,B. (2006). The adrenal gland is a source of stress-induced circulating IL-18. J. Neuroimmunol. *172*, 59-65.

182. Sung,Y.H., Song,J., and Lee,H.W. (2004). Functional genomics approach using mice. J. Biochem. Mol. Biol. *37*, 122-132.

183. Tan,S.S. (1991). Liver-specific and position-effect expression of a retinol-binding protein-lacZ fusion gene (RBP-lacZ) in transgenic mice. Dev. Biol. *146*, 24-37.

184. te Riele H., Maandag,E.R., and 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.

185. Tecott,L.H. (2003). The genes and brains of mice and men. Am. J. Psychiatry *160*, 646-656.

186. Threadgill,D.W., Dlugosz,A.A., Hansen,L.A., Tennenbaum,T., Lichti,U., Yee,D., LaMantia,C., Mourton,T., Herrup,K., Harris,R.C., and . (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science *269*, 230-234.

187. Thyagarajan,B., Guimaraes,M.J., Groth,A.C., and Calos,M.P. (2000). Mammalian genomes contain active recombinase recognition sites. Gene *244*, 47-54.

188. Thyagarajan,B., Olivares,E.C., Hollis,R.P., Ginsburg,D.S., and Calos,M.P. (2001). Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. Mol. Cell Biol. *21*, 3926-3934.

189. Timpl,P., Spanagel,R., Sillaber,I., Kresse,A., Reul,J.M., Stalla,G.K., Blanquet,V., Steckler,T., Holsboer,F., and Wurst,W. (1998). Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat. Genet. *19*, 162-166.

190. Tsien,J.Z., Huerta,P.T., and Tonegawa,S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell *87*, 1327-1338.

191. Tyner,S.D., Venkatachalam,S., Choi,J., Jones,S., Ghebranious,N., Igelmann,H., Lu,X., Soron,G., Cooper,B., Brayton,C., Hee,P.S., Thompson,T., Karsenty,G., Bradley,A., and Donehower,L.A. (2002). p53 mutant mice that display early ageing-associated phenotypes. Nature *415*, 45-53.

192. Vagner,S., Galy,B., and Pyronnet,S. (2001). Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep. *2*, 893-898.

193. Val,P., Aigueperse,C., Ragazzon,B., Veyssiere,G., Lefrancois-Martinez,A.M., and Martinez,A. (2004). Adrenocorticotropin/3',5'-cyclic AMP-mediated transcription of the scavenger akr1-b7 gene in adrenocortical cells is dependent on three functionally distinct steroidogenic factor-1-responsive elements. Endocrinology *145*, 508-518.

194. Vale,W., Spiess,J., Rivier,C., and Rivier,J. (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science *213*, 1394-1397.

195. Valenzuela,D.M., Murphy,A.J., Frendewey,D., Gale,N.W., Economides,A.N., Auerbach,W., Poueymirou,W.T., Adams,N.C., Rojas,J., Yasenchak,J., Chernomorsky,R., Boucher,M., Elsasser,A.L., Esau,L., Zheng,J., Griffiths,J.A., Wang,X., Su,H., Xue,Y., Dominguez,M.G., Noguera,I., Torres,R., Macdonald,L.E., Stewart,A.F., DeChiara,T.M., and Yancopoulos,G.D. (2003). High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nat. Biotechnol. *21*, 652-659.

196. Vasioukhin,V., Degenstein,L., Wise,B., and Fuchs,E. (1999). The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc. Natl. Acad. Sci. U. S. A *96*, 8551-8556.

197. Vitaterna,M.H., King,D.P., Chang,A.M., Kornhauser,J.M., Lowrey,P.L., McDonald,J.D., Dove,W.F., Pinto,L.H., Turek,F.W., and Takahashi,J.S. (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science *264*, 719-725.

198. Vooijs,M., Jonkers,J., and Berns,A. (2001). A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. Embo Reports *2*, 292-297.

199. Wade,C.M., and Daly,M.J. (2005). Genetic variation in laboratory mice. Nat. Genet. *37*, 1175-1180.

200. Wade,C.M., Kulbokas,E.J., III, Kirby,A.W., Zody,M.C., Mullikin,J.C., Lander,E.S., Lindblad-Toh,K., and Daly,M.J. (2002). The mosaic structure of variation in the laboratory mouse genome. Nature *420*, 574-578.

201. Watanabe,S., Honma,D., Furusawa,T., Sakurai,T., and Sato,M. (2006). Preparation of enzymatically active human Myc-tagged-NCre recombinase exhibiting immunoreactivity with anti-Myc antibody. Mol. Reprod. Dev.

202. Wei,Q., Lu,X.Y., Liu,L., Schafer,G., Shieh,K.R., Burke,S., Robinson,T.E., Watson,S.J., Seasholtz,A.F., and Akil,H. (2004). Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability. Proc. Natl. Acad. Sci. U. S. A *101*, 11851-11856.

203. Wells,S., and Murphy,D. (2003). Transgenic studies on the regulation of the anterior pituitary gland function by the hypothalamus. Front Neuroendocrinol. *24*, 11-26.

204. Wunderlich,F.T., Wildner,H., Rajewsky,K., and Edenhofer,F. (2001). New variants of inducible Cre recombinase: a novel mutant of Cre-PR fusion protein exhibits enhanced sensitivity and an expanded range of inducibility. Nucleic Acids Res. *29*, E47.

205. Xia,Y., and Wikberg,J.E.S. (1996). Localization of ACTH receptor mRNA by in situ hybridization in mouse adrenal gland. Cell and Tissue Research *286*, 63-68.

206. Xu,B., Gottschalk,W., Chow,A., Wilson,R.I., Schnell,E., Zang,K., Wang,D., Nicoll,R.A., Lu,B., and Reichardt,L.F. (2000). The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. J. Neurosci. *20*, 6888-6897.

207. Yajima,I., Belloir,E., Bourgeois,Y., Kumasaka,M., Delmas,V., and Larue,L. (2006). Spatiotemporal gene control by the Cre-ERT2 system in melanocytes. Genesis. *44*, 34-43.

208. Yalcin,B., Fullerton,J., Miller,S., Keays,D.A., Brady,S., Bhomra,A., Jefferson,A., Volpi,E., Copley,R.R., Flint,J., and Mott,R. (2004). Unexpected complexity in the haplotypes of commonly used inbred strains of laboratory mice. Proc. Natl. Acad. Sci. U. S. A *101*, 9734-9739.

209. Yang,Y., and Seed,B. (2003). Site-specific gene targeting in mouse embryonic stem cells with intact bacterial artificial chromosomes. Nat. Biotechnol. *21*, 447-451.

210. Yu,J., and McMahon,A.P. (2006). Reproducible and inducible knockdown of gene expression in mice. Genesis. *44*, 252-261.

211. Yu,Y., and Bradley,A. (2001). Engineering chromosomal rearrangements in mice. Nat. Rev. Genet. *2*, 780-790.

212. Zhang,W., Morris,Q.D., Chang,R., Shai,O., Bakowski,M.A., Mitsakakis,N., Mohammad,N., Robinson,M.D., Zirngibl,R., Somogyi,E., Laurin,N., Eftekharpour,E., Sat,E., Grigull,J., Pan,Q., Peng,W.T., Krogan,N., Greenblatt,J., Fehlings,M., van der,K.D., Aubin,J., Bruneau,B.G., Rossant,J., Blencowe,B.J., Frey,B.J., and Hughes,T.R. (2004). The functional landscape of mouse gene expression. J. Biol. *3*, 21.

213. Zhang,Y., Buchholz,F., Muyrers,J.P., and Stewart,A.F. (1998a). A new logic for DNA engineering using recombination in Escherichia coli. Nat. Genet. *20*, 123-128.

214. Zhang,Y., Riesterer,C., Ayrall,A.M., Sablitzky,F., Littlewood,T.D., and Reth,M. (1996). Inducible site-directed recombination in mouse embryonic stem cells. Nucleic Acids Research *24*, 543-548.

215. Zhang,Y.M., Buchholz,F., Muyrers,J.P.P., and Stewart,A.F. (1998b). A new logic for DNA engineering using recombination in Escherichia coli. Nature Genetics *20*, 123-128.

216. Zhao,J., Nassar,M.A., Gavazzi,I., and Wood,J.N. (2006). Tamoxifen-inducible Na(V)1.8-CreERT2 recombinase activity in nociceptive neurons of dorsal root ganglia. Genesis. *44*, 364-371.

217. Zhou,L., Rowley,D.L., Mi,Q.S., Sefcovic,N., Matthes,H.W., Kieffer,B.L., and Donovan,D.M. (2001). Murine inter-strain polymorphisms alter gene targeting frequencies at the mu opioid receptor locus in embryonic stem cells. Mamm. Genome *12*, 772-778.

218. Zobel,A.W., Yassouridis,A., Frieboes,R.M., and Holsboer,F. (1999). Prediction of medium-term outcome by cortisol response to the combined dexamethasone-CRH test in patients with remitted depression. Am. J. Psychiatry *156*, 949-951.

219. Zwermann,O., Beuschlein,F., Lalli,E., Klink,A., Sassone-Corsi,P., and Reincke,M. (2005). Clinical and molecular evidence for DAX-1 inhibition of

steroidogenic factor-1-dependent ACTH receptor gene expression. Eur. J. Endocrinol. *152*, 769-776.

9 Acknowledgments

I am much obliged to Prof. Florian Holsboer and Prof. Wolfgang Wurst for giving me the opportunity to work and learn in their great institutes. I furthermore thank Prof. Florian Holsboer, Prof. Rainer Rupprecht and PD Dr. Christoph Auernhammer for their appraisal of this thesis.

Above all, I thank my supervisor, Dr. Jan Deussing, for introducing me into molecular biology and supporting me in infinite ways.

I am greatly indebted to the following members of our laboratory: Martin Ableitner, Dr. Johannes Breu, Dr. Maria Brenz-Verca, Dr. Constanze Fey, Dr. Thomas Fischer, Nele Graf, Dr. Ralf Kühn, Claudia Kühne, Dr. Judit Oldekamp, Dr. Nilima Prakash, Dr. Damian Refojo, Dr. Juliane Rhode, Dr. Daniela Vogt-Weissenhorn, Dr. Juliane Wunsch, Yoh-Ra Yi. They were my teachers both in professional and personal matters.

For performing technical procedures I thank Dr. Jan Deussing (for breeding and phenotyping of mice), Dr. Constanze Fey (for Western blotting), Dr. Ralf Kühn (for mouse karyotyping) and the IDG mouse facility staff (for blastocyst injection).

Finally, I thank my parents, Ingrid & Godlib Riese, and Elisabetta Fiorentino for everything that ultimately matters.

10 Curriculum Vitae

Personal Information	
Full Name:	Florian Erasmus Simon Hagen Riese
Date of Birth:	01/09/1977
Place of Birth:	Hannover
Marital State:	Single
School	
06/1997	Abitur Kaiser-Wilhelm- und Ratsgymnasium Hannover
Civil Service	
09/1997 – 09/1998	Caring for severely disabled, Hannover
Medical School	
10/1998 – 09/2001	Dresden Technical University
10/2001 – 09/2002	Universidad Autonoma de Madrid
10/2003 – 10/2004	Dresden Technical University
11/2004 – 03/2005	Harvard Medical School Boston
03/2005 – 10/2005	Dresden Technical University
02/2006	Medical licensure
Laboratory	
11/2002 – 10/2003	Max-Planck-Institute of Psychiatry, Munich
01/2006 – 09/2006	Max-Planck-Institute of Psychiatry, Munich AG Molecular Neurogenetics (Prof. Wurst, Dr. Deussing)
Residency	
10/2006 – ongoing	Psychiatric University Hospital Zurich Division for Psychiatric Research and Geriatric Psychiatry (Prof. Nitsch, Prof. Hock)