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Title

Generation of genetically modified mice with conditional alleles of *Dyrk1a*

A minimal animal model of Down syndrome

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Meinem Vater und meiner Familie

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Contents

1 Summary

Down syndrome (trisomy 21) is the major cause of mental retardation. The "Down syndrome critical region" of human chromosome 21 is crucial for both trisomy related mental retardation and monosomy related microcephaly. Among the about two dozen genes identified in this region, DYRK1A is a promising candidate gene for the numerous neurobiological alterations. DYRK1A is highly conserved across species, ubiquitously expressed and encodes for a serine/threonine kinase. Several putative substrates of DYRK1A are identified and transgenic DYRK1A mice suggest that it is involved in learning and memory, but so far most of the underlying molecular mechanisms and the physiological functions of DYRK1A remain unclear. To gain insight into the physiological role of DYRK1A we decided to circumvent the embryonic lethality of the conventional knockout by applying the conditional *Cre*/*loxP* recombination system. The new technique of recombineering allowed for a rapid and efficient generation of mice with a *loxP* flanked *Dyrk1a* allele by gene targeting. Heterozygous Dyrk1a$^{flox/+}$ and homozygous Dyrk1a$^{flox/flox}$ mice are viable and do not show so far any gross anatomic differences compared to wildtype animals. Furthermore RNA analysis revealed no effect of the inserted *loxP* sites on the transcription of the floxed *Dyrk1a* allele. The forebrain-specific conditional knockout of *Dyrk1a* was generated by crossing in CaMKIIa-*Cre* mice. We successfully generated forebrain-specific heterozygous Dyrk1a$^{del/+}$ knockout mice but to this point we could not generate viable homozygous Dyrk1a$^{del/del}$ knockout mice. In a parallel experiment we wanted to mimic the overexpression of *Dyrk1a* in Down syndrome by generating a conditional Tet-inducible *Dyrk1a* transgenic mouse. Thus we further developed a novel transgenic approach. Instead of using random integration transgenesis for the *tetO-Dyrk1a* constructs, we targeted the silent but highly activatable (s/a) locus LC1 identified by K. Schönig in the laboratory of H. Bujard. For gene targeting in ES cells, we successfully constructed the first general targeting vector for the LC1 locus. We subsequently generated mice with a LC1 locus-specific tet-controlled *Dyrk1a* allele, carrying a bidirectional *tetO-Dyrk1a*/*Egfp* transgene in this locus. The conditional expression of *Dyrk1a* and the reporter gene *Egfp* was demonstrated by crossing in CaMKIIa-*tTA* mice. In these double transgenic mice *Dyrk1a* and *Egfp* are expressed in the forebrain according to the expression of the CaMKIIa promoter which drives the expression of tTA. Both genetically modified mouse lines offer a great potential for

further investigation of the pleiotropic effects of Dyrk1a in the complex pathophysiology of Down syndrome and monosomy 21-associated intrauterine microcephaly.

2 Zusammenfassung

Down Syndrom (Trisomie 21) ist die Hauptursache für geistige Behinderung. Die "Down Syndrom kritische Region" auf dem menschlichen Chromosom 21 ist ausschlaggebend für die Trisomie 21-assoziierte geistige Behinderung und für die Monosomie 21-assoziierte Mikrocephalie. Unter den bisher ungefähr zwei dutzend identifizierten Genen in dieser Region, ist DYRK1A ein viel versprechendes Kandidatengen für die zahlreichen neurobiologischen Veränderungen. DYRK1A ist über viele Arten hinweg stark konserviert, ubiquitär exprimiert und kodiert für eine Serin/Threonin Kinase. Einige potentielle Substrate von DYRK1A konnten identifiziert werden und transgene DYRK1A Mäuse deuten darauf hin, dass dieses Gen an Lern- und Gedächtnisprozessen beteiligt ist. Bisher konnten aber die meisten grundlegenden molekularen Mechanismen und die physiologische Funktion von DYRK1A nicht geklärt werden. Um einen Einblick in die physiologische Rolle von DYRK1A zu erhalten, haben wir uns entschlossen die embryonale Letalität des konventionellen Knockouts zu umgehen, indem wir das konditionelle *Cre/loxP* Rekombinationssystem nutzten. Die neue Technik des „Recombineering" ermöglichte eine schnelle und effiziente Herstellung von Mäusen mit einem *loxP* flankierten *Dyrk1a* Allel. Die heterozygoten Dyrk1a$^{flox/+}$ Mäuse und die homozygoten Dyrk1a$^{flox/flox}$ Mäuse sind lebensfähig und sie zeigen bisher keine großen anatomischen Unterschiede im Vergleich zu Wildtyptieren auf. Zudem konnten wir durch eine RNA Analyse zeigen, dass die eingefügten *loxP* Seiten keinen Einfluss auf die Transkription des gefloxten *Dyrk1a* Allels haben. Der spezifische Knockout im Bereich des Vorderhirns wurde durch Verpaarung mit CaMKIIa-*Cre* Mäusen hergestellt. Wir konnten erfolgreich einen heterozygoten Dyrk1a$^{del/+}$ Knockout im Vorderhirn erzeugen aber bisher konnten wir keinen lebensfähigen homozygoten Dyrk1a$^{del/del}$ Knockout im Vorderhirn herstellen. In einem parallelen Experiment wollten wir die Überexpression von *Dyrk1a* im Down Syndrom durch die Herstellung einer konditionalen Tet-induzierbaren *Dyrk1a* Maus nachahmen. Daher haben wir uns für einen neuen transgenen Ansatz entschieden. Anstatt transgene Tiere durch Zufallsintegration des *tetO-Dyrk1a* Transgens herzustellen, wurde der stille aber hochaktivierbare LC1 Lokus angesteuert, der von K. Schönig im Labor von H. Bujard identifiziert wurde. Für das Gene Targeting in ES Zellen konnte der erste generelle Targeting Vektor für den LC1-Lokus erfolgreich hergestellt werden. Anschließend haben wir Mäuse mit einem LC1-Lokus spezifischen, tet-kontrollierten *Dyrk1a* Allel generiert. Das Allel beinhaltete ein bidirek-

tionales *tetO-Dyrk1a/Egfp* Transgen in diesem Lokus. Die konditionale Expression von *Dyrk1a* und dem Reportergen *Egfp* konnte durch Verpaarung mit CaMKIIa-*tTA* Mäusen gezeigt werden. In diesen doppeltransgenen Mäusen wird *Dyrk1a* und *Egfp* im Vorderhirn exprimiert, entsprechend dem Expressionsmuster des CaMKIIa Promotors, der die Expression von tTA steuert.

Beide genetisch veränderten Mauslinien stellen ein großes Potential für die weiteren Untersuchungen der pleiotropen Effekte von *Dyrk1a* in der komplexen Pathophysiologie des Down Syndroms und der Monosomie 21-assoziierten intrauterinen Mikrocephalie dar.

3 Introduction

3.1 The Down syndrome

Down syndrome (DS) is named after the English physician Sir John Langdon H. Down (1828-1896), who published an ethnically incorrect but historically interesting essay in 1866 (Down, 1866). He first described a set of children with common features who were distinct from other children with mental retardation.

Fig. 1 Sir John L. H. Down (1828-1896)

In the first part of the twentieth century, there was much speculation of the cause of Down syndrome. The first researchers who speculated that it might be due to chromosomal abnormalities were Waardenburg and Bleyer in the 1930s (for review see (Carter, 2002)). But it wasn't until 1959 that Jerome Lejeune and Patricia Jacobs, working independently, first determined the cause to be trisomy (triplication) of human chromosome 21 (Jacobs et al., 1959; Lejeune, 1959). Cases of Down syndrome due to translocation and mosaicism were described over the next three years (Carter et al., 1960; Polani et al., 1960; Clarke et al., 1961).

Today, DS is the most frequent genetic cause associated with mild to severe mental retardation in live born children which occurs in 1 out of ~800 live births, independent of races and social status (Hook et al., 1983). Although the complete chromosome 21 is sequenced and putative expressed genes are identified (Hattori et al., 2000), little is known about the mechanism by which trisomy 21 interferes with normal development. So far, the increased dosage of genes from chromosome 21 clearly implicates, as a causative factor, altered levels of gene expression (Wisniewski, 1990; Becker et al., 1993).

3.1.1 Clinical pathology of Down syndrome

Patients with DS show a variety of particular combinations of phenotypes but an invariant concomitant to all is the mental retardation. Additional developmental defects include the characteristic facial malformations (Fig. 2), short stature, hypotonia, congenital heart defects, gastrointestinal abnormalities, hearing loss and thyroid disorders (reviewed in (Epstein, 1989)). Besides the developmental failures, DS is also characterized by Alzheimer's disease-like neuropathology. Characteristic senile beta-amyloid (Ab) plaques, neurofibrillary tangles and neuronal loss are present in the brain of all individuals with DS over the age of 40 (Wisniewski et al., 1985). DS patients are also more frequently affected by leukaemia and leukemoid reactions (Fong and Brodeur, 1987).

Flat facial features, with a small nose	Small, abnormally shaped ears
Asymmetrical or odd-shaped skull (Round head with flat area at the back of the head (occiput), microcephaly)	Single deep crease across the center of the palm
	Hyperflexibility (excessive ability to flex joints)
	Fifth finger has only one flexion furrow instead of two
Short stature, growth retardation	Extra space between the big toe and the second toe
Short neck	Enlarged tongue that tends to stick out
Low muscle tone (babies appear "floppy")	Iris with white spots (Brushfield spots)
Upward slant to the eyes	
Small skin folds on the inner corner of the eyes	

Fig. 2 Characteristic anatomical features of individuals with Down syndrome

3.1.2 Molecular genetics of Down syndrome

The particular combinations of phenotypes in DS are a consequence of the presence of an extra copy of all or part of human chromosome 21. The normal human diploid cell is composed of 46 chromosomes, 44 autosomes and 2 sex chromosomes (XX for female and XY for male individuals) which can be arranged in 23 pairs. DS patients have 47 chromosomes (Fig. 3).

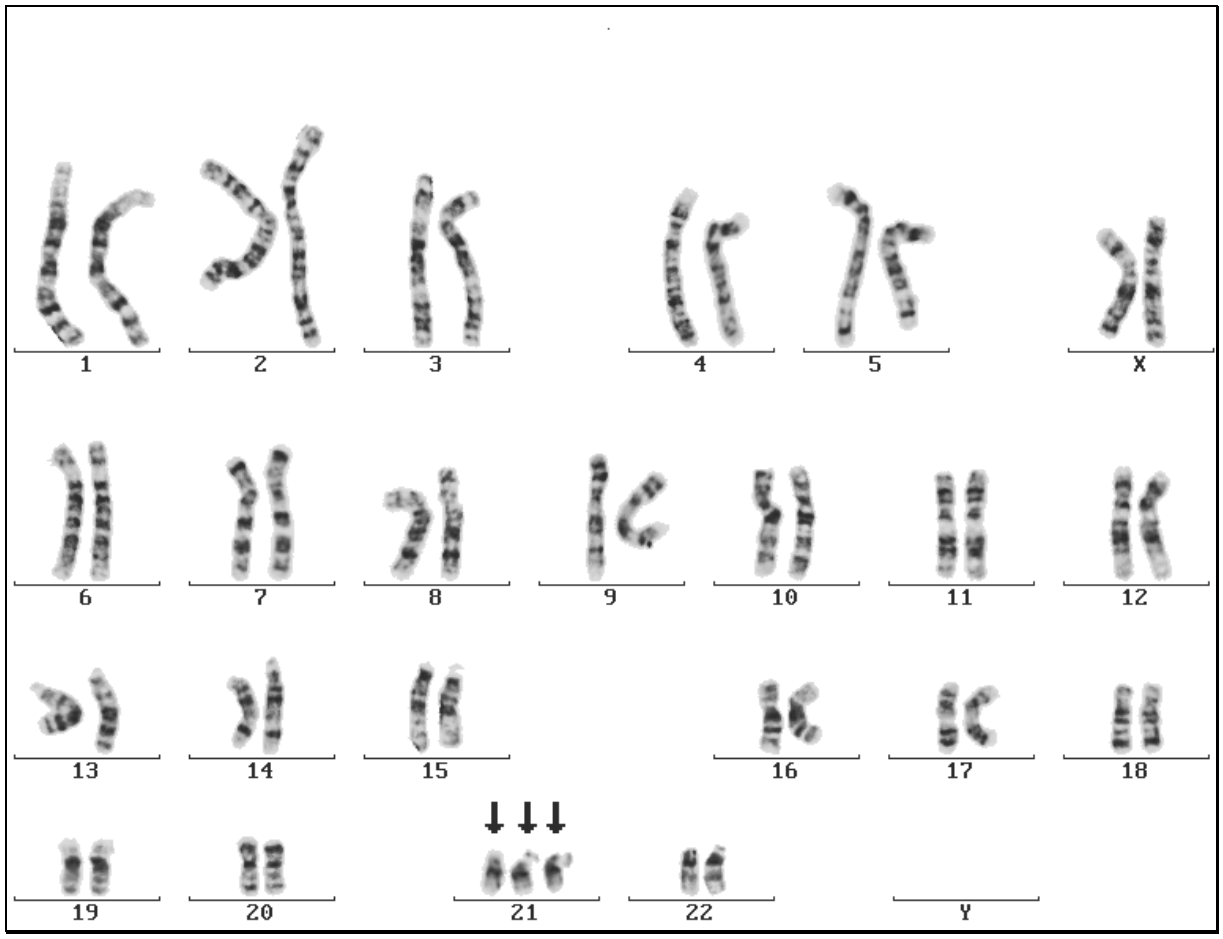


Fig. 3 Karyotype of a female individual with Down syndrome (Trisomy 21)
The metaphase chromosomes are stained with the dye Giemsa to make bands visible on the chromosomes. The chromosomes are arranged in pairs and ordered by size to examine chromosomal aberrations. The karyotype of this individual is 47,XX,+21. Thus the genome consists here of 47 chromosomes including the two female X sex-chromosomes and three copies of chromosome 21 (marked by arrows). The typical human female karyotype is 46,XX (22 pairs of so called autosomes and the 2 X sex chromosomes) and the male karyotype is 46,XY.

Three genetic variations can cause DS:

Free trisomy 21

In most patients, approximately 92% of the time, Down syndrome is caused by the presence of an extra chromosome 21 in all cells of the individual (Antonarakis, 1991). In such cases, the extra chromosome originates in the development of either the egg or the sperm in most cases by meiotic non-disjunction and in just a few cases by mitotic non-disjunction. Consequently, when the egg and sperm unite to form the fertilized egg, three rather than two chromosomes 21 are present. As the embryo develops, the extra chromosome is replicated in every cell. This condition, in which three copies of chromosome 21 are present in all cells of the individual, is called free trisomy 21.

Mosaic trisomy 21

In approximately 2-4% of cases, Down syndrome is due to mosaic trisomy 21 (Mikkelsen, 1977). This situation is similar to simple trisomy 21, but, in this instance, the extra chromosome 21 is present in some, but not all, cells of the individual. For example, the fertilized egg may have the right number of chromosomes, but, due to an error in chromosome division early in embryonic development, some cells acquire an extra chromosome 21. Thus, an individual with Down syndrome due to mosaic trisomy 21 will typically have 46 chromosomes in some cells, but will have 47 chromosomes (including an extra chromosome 21) in others. In this situation, the range of the physical problems may vary, depending on the proportion of cells that carry the additional chromosome 21.

Translocation trisomy

In trisomy 21 and mosaic trisomy 21, Down syndrome occurs because some or all of the cells have 47 chromosomes, including three chromosomes 21 (Hook, 1982). However, approximately 3-4% of individuals with Down syndrome have cells containing 46 chromosomes, but still have the features associated with Down syndrome. In such cases, material from one chromosome 21 gets attached or translocated onto another chromosome, often chromosome 14, either prior to or at conception. In such situations, cells from individuals with Down syndrome have two normal chromosomes 21, but also have additional chromosome 21 material on the translocated chromosome. Thus, there is still too much DNA from chromosome 21, resulting in the features associated with Down syndrome. In such situations, the individual with Down syndrome is said to have translocation trisomy 21.

Extensive research efforts have been undertaken to study the defects in chromosome 21 that cause DS. These studies showed, that in 88% of cases, the extra copy of chromosome 21 is derived from the mother in contrast to ~8% of the cases were the third copy is from the father, both caused by meiotic non-disjunction. In the remaining 2% of cases, DS is due to mitotic cell division errors after fertilization. In addition, incidence of DS rise dramatically with increasing maternal age (Hook, 1982; Hook et al., 1983).

Mothers Age	Incidence of Down syndrome
< 30 years	Less than 1 in 1000
35	1 in 900
36	1 in 400
37	1 in 300
38	1 in 180
39	1 in 135
40	1 in 105
42	1 in 60
44	1 in 35
46	1 in 20
48	1 in 16
49	1 in 12

Fig. 4 Incidence of Down syndrome related to the mothers age (Hook and Lindsjo, 1978)

3.1.3 Neuroanatomy of Down syndrome

The relationship between mental retardation and the trisomic condition of DS is so far unclear and probably very complex. Individuals with DS show in early infancy a typical normal cognitive development but the intelligence quotient decreases in the first decade of life. In adolescent years (age 10-15) cognitive function reaches a plateau that continues into adulthood. Thus DS provides a rare opportunity to explore relationships among genetic, structural, and cognitive or developmental abnormalities.

Early studies of structural brain abnormalities in Down syndrome showed that individuals with DS had consistently a lower brain weight and brachycephaly, with a small cerebellum, frontal and temporal lobes, a simplified appearance of the sulci, and a narrow superior gyrus (Coyle et al., 1986; Wisniewski, 1990).

In contrast fetal DS brains were reported to have the same neuronal morphology and spine counts as brain from normal fetuses. Further microscopical analyses also failed to uncover any difference in development between DS and normal brain in terms of shape, weight, configuration and myelination. Stereological cell counting techniques, however, revealed that the second phase of cortical development and the emergence of lamination are both delayed and disorganized in fetal DS brain (Becker et al.,

1991). In addition, although the emergence and morphology of microglial cells appear not to differ from those in normal fetuses, microglial cells outnumber astroglial cells in fetal DS brain. Modern imaging studies with high-resolution magnetic resonance (MRI) showed that conspicuous morphological abnormalities start to be apparent in brains of newborns and older infants with DS. Subjects with Down syndrome had smaller brain volumes, with disproportionately smaller cerebellar volumes and relatively large subcortical gray matter volumes (Pinter et al., 2001b; Pinter et al., 2001a). They have shortened basilar dendrites, a decreased number of spines with altered morphology and defective cortical layering. In addition, other studies have revealed relatively delayed myelination, fewer neurons, lower neuronal density and distribution, and abnormal synaptic density and length, caused probably by prenatal abnormal neuronal migration and retarded synaptogenesis (Coyle et al., 1986; Becker et al., 1991).
So far the key players and/or modulators of these pathological changes have yet to be identified.

3.1.4 Animal models of Down syndrome

There has been a great effort to generate suitable animal models for DS. In humans, the role of specific genes on chromosome 21 which contribute to the specific symptoms of Down syndrome are difficult to pinpoint because of the large number of genes (~270 protein-coding genes) that likely play a role concertedly in the development of the diverse phenotypes. One important advance was achieved when studies of rare cases with partial trisomy of chromosome 21 suggested, that a considerable portion of the aetiology of Down syndrome and especially the mental retardation is associated with the extra copy of a 2.5 Mb region around locus D21S55 at 21q22.2 located between loci CBR (carbonyl reductase (NADPH) 1) and ERG (transforming protein ERG), called the Down syndrome critical region (DSCR) (Delabar et al., 1993; Korenberg et al., 1994; Takashima, 1997). The DSCR comprises ~ 20 annotated genes and 5 pseudogenes.
Since the distal segment of mouse chromosome 16 is homologous to nearly the entire long arm of human chromosome 21, mice with trisomy (Ts) chromosome 16 were initially thought as useful models for human trisomy 21. However, since the Ts16 mice do not survive the prenatal period, and because segments homologous to human chromosomes other than 21 are present on mouse chromosome 16, Davisson and colleagues have generated a segmental trisomy 16, designated Ts65Dn. The

trisomy is confined to the region of mouse chromosome 16 which is homologous to a large portion of the 4-Mb DSCR on human chromosome 21 (Fig. 5). Unlike Ts16 mice, these mice survive to adulthood.

Among the genes identified within the trisomic region are *Sod1*, *Ets2*, *Gart*, *Dyrk1a* and the amyloid precursor protein (*App*). The genes distal to HMG14 on human chromosome 21 are not present on the trisomic chromosome. The Ts65Dn mice are hyperactive and have deficits in emotional control in response to mildly threatening situations. In addition, they show defects in spatial learning (Reeves et al., 1995; Demas et al., 1996, , 1998). Meanwhile there are additional segmental trisomy mice available (Fig. 5) with even shorter segment of chromosome 16, the Ts1Cje and the Ms1Ts65 mice (Sago et al., 1998; Sago et al., 2000).

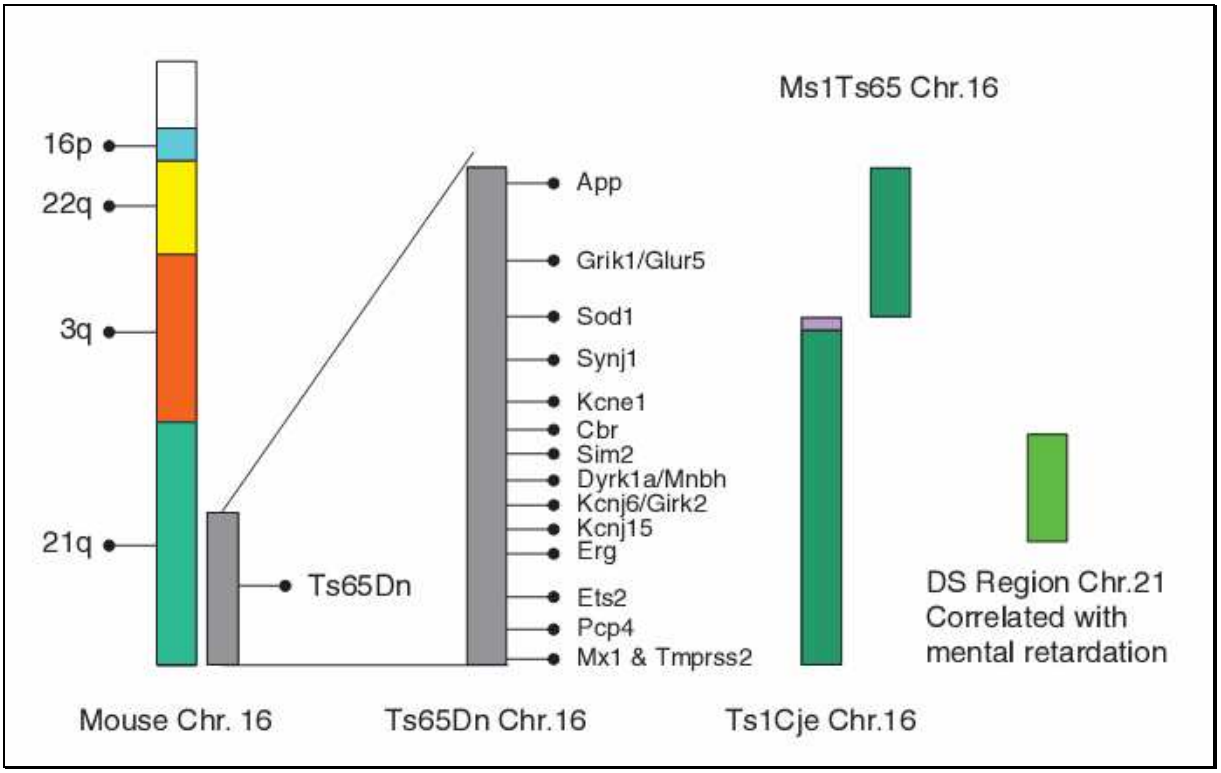


Fig. 5 Schematic representation of mouse chromosome 16 and Ts65Dn fragment

Homology of part of mouse chromosome 16 to respective human chromosome and location of Ts65Dn fragment (left panel). Schematic location of selected genes corresponding to human chromosome 21-homologous segment, in the extra chromosome present in the Ts65Dn mouse genome: *App* (amyloid precursor protein), *Grik1* (glutamate receptor subunit 5, *Sod1* (superoxide dismutase 1), *Cbr* (carbonyl reductase), *Sim2* (single-minded 2), *Dyrk1a* (dual specificity tyrosine-Y-phosphorylation regulated kinase 1a), *Kcnj6* (potassium inwardly rectifying channel, subfamily J, member 6), *Ets2* (E26 avian leukemia oncogene 2), *Pcp4* (purkinje cell protein 4), *Mx1* (myxovirus resistance 1), *Tmprss2* (transmembrane protease, serine 2). (modified from (Galdzicki and Siarey, 2003))

Furthermore Olson and colleagues used chromosome engineering to create mice that are trisomic or monosomic for the mouse chromosome segment orthologous to the DSCR. They assessed the craniofacial skeleton and could show that the DSCR genes were not sufficient and largely not necessary to produce the facial phenotype (Olson et al., 2004). Recently there is also the aneuploid mouse strain Tc1 available, which stably transmits a freely segregating, almost complete human chromosome 21 and shows typically DS phenotypes (O'Doherty et al., 2005).
Several attempts have been made to narrow further the role of genes on the 2.5 Mb critical region of human chromosome 21. Functional screening of 2 megabases of human chromosome 21q22.2 by generation of YAC transgenic mice revealed that one YAC transgenic mouse line with a 180kb fragment displayed defects in spatial learning tests (Smith et al., 1997). This 180 kb region contains *DYRK1A*, the human homologue of a Drosophila gene, *minibrain*, and strongly implicated *DYRK1A* in learning defects associated with Down syndrome.
Thus, although not perfect, the DS animal models provide a powerful tool for the exploration of the effects of gene dosage associated with developmental defects in Down syndrome and associated defects in cognition and learning.

3.2 *Minibrain/Dyrk1a* – a candidate gene for mental retardation

One of the ~20 genes which is located in the DSCR and associated with mental retardation is *DYRK1A*. The *DYRK1A* (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A) encodes for the DYRK1A kinase, which is the vertebrate homologue of the *minibrain* kinase (MNB) of D. melanogaster and *yak1* kinase of S. cerevisiae. The Drosophila *minibrain* mutant was first described in 1979 (Heisenberg and Böhl, 1979) during a larger screen to identify fly mutants which had defects in brain anatomy. The corresponding gene was subsequently cloned in 1995 (Tejedor et al., 1995). The name minibrain is derived form the *mnb* mutants which showed a specific and marked size reduction of the optic lobes and central brain hemispheres due to disturbed neuroblast proliferation during neurogenesis (Fig. 6). The *mnb* mutants also showed defects in learning of olfactory discrimination and hypoactivity.

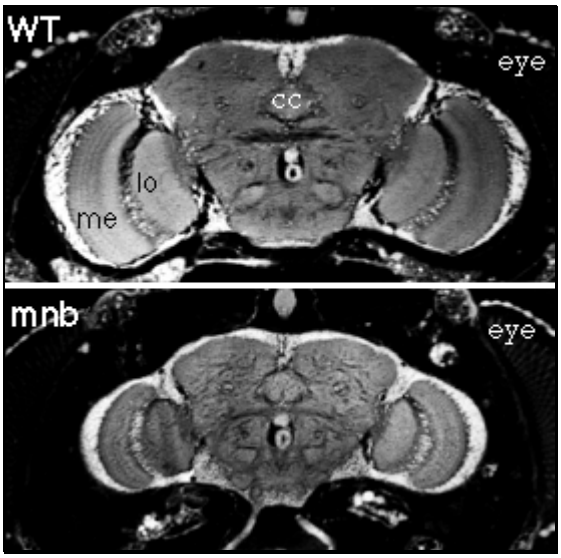


Fig. 6 Reduced adult brain size in mnb *D.melanogaster* mutants
Unstained frontal paraffin sections of male brains viewed under a fluorescence microscope. Having the same size of the head, the minibrain (mnb) mutant has a much smaller brain in contrast to the wildtype (WT) drosophila. cc: central complex; me: medulla; lo: Lobula (from (Tejedor et al., 1995))

3.2.1 Genomic organization of the DYRK1A gene

The human gene for DYRK1A is located between the genes *DSCR3* (Down syndrome critical region protein A) and *KCNJ6* (human G protein coupled inward rectifier potassium channel 2) in the DSCR and in the syntenic region of mouse chromosome 16. The gene comprises 11 coding and 3 non-coding exons and spans over 150kb (Wang et al., 1998; Guimera et al., 1999). The exon-intron structure of *DYRK1A* is highly conserved across species in vertebrates and still comparable in invertebrates like *Drosophila* and *C.elegans*. There are two different promoters in the human gene which drive the expression of transcripts with different 5′-untranslated regions. Exon 1-containing transcript expression is restricted to heart and skeletal muscle and the exon 2-containing transcript is ubiquitously expressed in a broad spectrum of tissues, including 14 tested tissues of the brain – among them cerebellum, cerebral cortex, amygdala and hippocampus (Guimera et al., 1999). The expression of *DYRK1A* was shown to be increased 1.5-fold in the brain of Down syndrome fetuses (Mao et al., 2003) and 2.1-fold in the animal model Ts65Dn (Guimera et al., 1999). Additionally, it has been shown that *DYRK1A* has different isoforms through alternative splicing of the mRNA transcript affecting exon 6. This leads either to the presence or absence of a segment with 9 aa. However no functional differences have been reported so far.

3.2.2 Biochemical properties of DYRK1A

3.2.2.1 The DYRK kinase family

Protein kinases represent one of the largest gene families in the human genome and a large group of protein kinases is the so called CMGC-group (Kostich et al., 2002). The group is named after the protein kinases CDK, MAPK, GSK3 and CLK (Hunter and Plowman, 1997). Protein kinase families are sorted and grouped by similarities of the amino acid sequence and especially by the sequence of their catalytic kinase domains. Thus families and even subfamilies can be defined (Manning et al., 2002). One closely related subfamily of the CMGC group, besides the HIP proteins, is the DYRK family (Miyata and Nishida, 1999). In the human genome, so far 5 genes of the DYRK family have been found with DYRK1A being the first to be identified by several groups in 1996 (Guimera et al., 1996; Kentrup et al., 1996; Shindoh et al., 1996; Chen and Antonarakis, 1997). In the following years DYRK1B, DYRK2, DYRK3 and DYRK4 were discovered and it has been shown that each of this protein has different isoforms through alternative splicing of the mRNA transcript (Becker et al., 1998; Xia et al., 1998; Leder et al., 1999). DYRK proteins are found in every eukaryotic organism and, after intensive studies in yeast (Yak1p), Drosophila (MNB) and the mouse (DYRK1A), are involved in fundamental processes like the regulation of the cell cycle, cell division and the switch between proliferation and differentiation as well as the development of the brain (Bahler and Pringle, 1998; Souza et al., 1998; Becker and Joost, 1999). However DYRK1A is the only DYRK-family member which is located on chromosome 21 and is expressed in the human brain. The members of the DYRK family share a similar kinase-domain and a short N-terminal extension (DYRK homology box or DH-box) which is rich in acidic residues but they have individual N- and C-terminal domains (Becker and Joost, 1999).

3.2.2.2 Structural characteristics of DYRK1A

The 763 amino acids long and ~85 kDa DYRK1A protein harbors several unusual structural features outside the catalytic kinase domain (Fig. 7) in contrast to the other members of the DYRK family. The N-terminus contains a bipartite NLS (nuclear localization signal) which mediates the targeting of DYRK1A to the nucleus (Song et al., 1997; Becker et al., 1998). DYRK1A shows a subnuclear punctuate accumulation inside the nucleus which resembles the subnuclear localization of the splicing machinery (Becker et al., 1998; Marti et al., 2003). Adjacent to the catalytic domain is a

PEST region (rich in proline, glutamic acid, serine and threonine) which is believed to initiate a rapid degradation of the protein but the function in DYRK1A still has to be elucidated (Rogers et al., 1986). Furthermore the C-terminus of DYRK1A harbors a stretch of 13 consecutive histidine residues (His_{13}) and a domain which is rich in serine and threonine repeats (S/T).

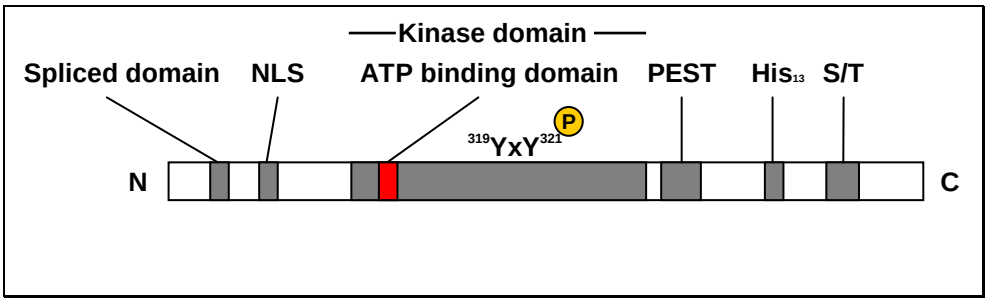


Fig. 7 Schematic presentation of the DYRK1A structure

The sequence of the catalytic kinase domain, the NLS and the DH-box of DYRK1A is largely conserved in orthologous proteins in vertebrates and invertebrates. For example the alignment of the complete human amino acid sequence with rat and mouse DYRK1A showed a similarity of ~99% and the comparison of human against Drosophila MNB kinase domain residues resulted in 85% similarity (Tejedor et al., 1995). A collaboration of our Laboratory with Prof. Eric Kandel showed that even the DYRK1A *C.elegans* homologue MBK-1 shares 78% sequence similarity within the kinase domain (Raich et al., 2003). Thus this suggests that the highly conserved structural elements of DYRK1A might also represent an evolutionary conserved and important function of DYRK1A within the cell and the organism.

3.2.2.3 Enzymatic characteristics of DYRK1A

The name "dual-specificity tyrosine regulated kinase" implicates that the DYRK family kinases are able to phosphorylate tyrosine and also serine and threonine residues. The phosphorylation of the tyrosine-X-tyrosine motif (YxY; x stands for any aa) in the activation loop of the kinase (Fig.7) is essential for the catalytic activity of DYRK proteins.

In DYRK1A (human, mouse and rat) the YxY-motif is located at aa sequence position 319 and 321 (x is the aa glutamine) between the conserved kinase subdomains VII and VIII (Kentrup et al., 1996). This resembles the threonine-X-tyrosine motif in the activation loop of MAP kinases (Canagarajah et al., 1997). Expression of DYRK1A in

E.coli led to the phosphorylation of tyrosine residues. The single mutation Tyr321Phe and also the double mutation Tyr319Phe and Tyr321Phe resulted in the loss of kinase activity. In contrast, the single Tyr319Phe mutation did not influence the kinase activity and the catalytic inactive mutant Lys188Arg showed no tyrosine phosphorylation (Kentrup et al., 1996; Himpel et al., 2001). Furthermore results from mass spectrometry demonstrated that wildtype DYRK1A is only phosphorylated at Tyr321 and not at Tyr319 (Himpel et al., 2001). The activation mechanism of DYRK kinases is similar to MAP kinases. However MAP kinases need the prior phosphorylation of their activation loop by trans-acting superior kinases in the MAP kinase cascade. The DYRK proteins do not require superior kinases for their activation – they can activate themselves by autophosphorylation of their second tyrosine residue (Tyr321 in DYRK1A) in the activation loop (Wiechmann et al., 2003).
Recent data suggest that the activation by autophosphorylation of DYRK1A might be subject to regulation by upstream elements. In hippocampal progenitor cell lines, the extracellular signal molecule FGF (Fibroblast growth factor) induced tyrosine phosphorylation and kinase activity of DYRK1A (Yang et al., 2001). In another study DYRK1A interacts with the adenovirus E1A oncogene and stimulated the kinase activity *in vitro* (Zhang et al., 2001). In the same way the binding of the 14-3-3 protein to the N-terminus of DYRK1A stimulated the kinase activity in a dose-dependent manner *in vitro* and indirectly *in vivo* (Kim et al., 2004). Unlike the activation of Dyrk1a, an *in vitro* kinase assay identified Epigallocatechin-3-gallat, one of the main polyphenolic components of green tea, as a specific inhibitor of the DYRK1A kinase. Additionally Roscovitine and Purvanol A are potent DYRK1A inhibitors (Bain et al., 2003). But the function of this remarkable autoactivation characteristic of the DYRK kinases and upstream signaling pathways of DYRK1A are still a matter of debate and have to be elucidated.

3.2.2.4 Substrates and interaction partners of DYRK1A

The autophosphorylation of tyrosine residues in the activation loop of the kinase is essential for the activity of DYRK proteins. In contrast, exogenous substrates of the kinase are only phosphorylated on serine and threonine residues. First evidence for that phosphorylation pattern came from *in vitro* studies in which DYRK1A phosphorylated Histone H3 and MBP (myelin basic protein) (Kentrup et al., 1996; Becker et al., 1998).

In order to define the consensus phosphorylation sequence for the substrate specificity of DYRK1A, a peptide library was screened (Himpel et al., 2000). The peptide array identified DYRK1A as a proline-directed kinase with a phosphorylation consensus sequence RP*X*(S/T)P similar to that of ERK2 (P*X*(S/T)P). Furthermore DYRK1A showed to be an arginine-dependent serine/threonine kinase. An arginine residue in position -3 relative to the phosphorylated serine or threonine residue in the substrate is essential for the phosphorylation by DYRK1A. Other members of the DYRK family demonstrated to have almost the same substrate recognition sequence (Campbell and Proud, 2002).

After the definition of the consensus sequence, putative *in vitro* and *in vivo* substrates of DYRK1A have been identified in the beginning of this thesis (see also Fig. 8). Four of the first identified substrates belong to the group of transcription factors (FKHR, STAT3, CREB, and GLI1).

STAT3 is a downstream effector of cytokines like IL-6 (Interleukin 6) and LIF (Leukemia inhibitory factor) and they most often control the transition between cell proliferation and differentiation. An *in vitro* screen for peptides that phosphorylate STAT3 on Ser797 identified DYRK1A. DYRK1A stimulates the transcriptional activity of STAT3. It is still unknown if STAT3 is also an in vivo substrate of DYRK1A (Matsuo et al., 2001).

CREB is a transcription factor which is involved in different forms of synaptic plasticity and learning and memory. It was associated with neuronal development and differentiation (Bourtchuladze et al., 1994; Blendy et al., 1995; Bartsch et al., 1998; Kandel, 2001). The CREB activation by phosphorylation triggers the subsequent induction of further genes, most of them being important regulators and effectors. DYRK1A activates CREB by direct phosphorylation of Ser133 during neuronal differentiation in hippocampal progenitor cells (Yang et al., 2001).

FKHR (forkhead in rhabdomyosarcoma) plays a role in the insulin-mediated control of gene expression and in cell survival and proliferation (Brunet et al., 1999; Nakae et al., 1999). DYRK1A phosphorylates *in vitro* Ser329 of FKHR and this leads to the nuclear export of transcription factor FKHR and its inactivation (Woods et al., 2001b). The authors also suggest that FKHR is an *in vivo* substrate of DYRK1A because they could show that the two proteins are both colocalized in discrete regions of the nucleus and coimmunoprecipitated from cell extracts.

GLI1 transcription factor is a key downstream signaling component of the hedgehog pathway in vertebrates. Hedgehog genes encode for secreted signaling proteins that

can function as growth factors and morphogens important for cell proliferation and pattern formation (Ingham and McMahon, 2001). A reporter gene assay demonstrated that DYRK1A can enhance the GLI1-dependent gene transcription, in part by retaining GLI1 in the nucleus. It has been shown that DYRK1A can directly stimulate the transcription of GLI1-dependent genes but the underlying mechanism is still unknown (Mao et al., 2002).

Three additional proteins in the cytoplasm have been identified as *in vitro* DYRK1A substrates. This raises the question wether these proteins are real *in vivo* substrates of DYRK1A since DYRK1A is predominantly localized in the nucleus as described previously. DYRK1A phosphorylates *in vitro* the protein biosynthesis initiation factor EIF2Bε at Ser539 and the microtubule-associated protein Tau at Thr212 (Woods et al., 2001a). In both cases DYRK1A might play the role of a priming kinase for GSK3 phosphorylation of GSK substrates. Efficient phosphorylation of GSK substrates only occurs if another phosphoserine or phosphothreonine is already present four residues C-terminal to the site of GSK3 phosphorylation (Fiol et al., 1987). Thr212 and Thr208 are phosphorylated in fetal tau and hyperphosphorylated in filamentous tau from Alzheimer's-disease brain (Morishima-Kawashima et al., 1995). Dynamin, a GTPase which is a key modulator in clathrin-mediated endocytosis and the recycling of synaptic vesicles, was identified as a substrate with a solid-phase DYRK1A kinase assay in rat brain extracts (Chen-Hwang et al., 2002a). The phosphorylation of Dynamin by DYRK1A strengthens *in vitro* the interaction of Dynamin with components of the endocytosis machinery and recent data showed that DYRK1A and Dynamin colocalize in the growing dendritic tree of differentiating neurons, suggesting a possible role in neuronal differentiation *in vivo* (Hammerle et al., 2003).

Cyclin L2 was identified as a DYRK1A substrate by a new filter array method (de Graaf et al., 2004). Cyclin L2 interacts with several splicing factors and colocalizes with DYRK1A in the nuclear speckle compartment, which is associated with the splicing catalysis and pre-mRNA processing. These data suggest that DYRK1A may regulate splicing by phosphorylation of cyclin L2.

Another yeast-two hybrid screen identified the protein Arip4 (androgen receptor-interacting protein 4), a SNF2-like steroid hormone receptor cofactor, as a DYRK1A-interacting protein (Sitz et al., 2004). DYRK1A or Arip4 alone displays an activating effect on androgen receptor- and glucocorticoid receptor-mediated transactivation, and DYRK1A and Arip4 together act synergistically in the nucleus. These effects are

independent of the kinase activity of DYRK1A. Thus DYRK1A might cause severe changes in the homeostasis of steroid hormone-controlled cellular events.

The cytosolic Huntingtin-interacting protein 1 (Hip-1), a proapoptotic mediator and endocytic adaptor protein, is involved in the pathogenesis of Huntington's disease and a recent report found that DYRK1A selectively binds to and phosphorylates Hip-1 during the neuronal differentiation of embryonic hippocampal neuroprogenitor cells (Kang et al., 2005). Depending on the applied stimuli in cell culture, DYRK1A either activated Hip-1 and blocked neuronal cell death and neurite outgrowth or DYRK1A deactivated Hip-1. The dissociated Hip-1 bound and activated caspase-3 and triggered finally neuronal cell death. Furthermore another study demonstrated that Hip-1 associates with the androgen receptor and modulates the transcriptional activity of nuclear hormone receptors by recruitment to DNA response elements upon androgen stimulation (Mills et al., 2005). In contrast to previous reports, they could also show that Hip-1 is a nucleocytoplasmic protein capable of translocating to the nucleus. Thus this is another hint that DYRK1A and its interaction partners might be involved in the homeostasis of steroid hormone-controlled cellular events.

PAHX-AP1 (phytanoyl-CoA alpha-hydroxylase-associated protein), a Refsum disease gene product which might be involved in brain development by interaction with PAHX (phytanoyl-CoA alpha-hydroxylase), was found to interact with the C-terminus of DYRK1A (Bescond and Rahmani, 2005). Interestingly, in cell culture transfection experiments DYRK1A was translocated from the nucleus to the cytoplasm in the presence of PAHX-AP1 where it colocalizes with PAHX-AP1.

SF3b1/SAP155 is the only spliceosomal protein of the splicing machinery which is phosphorylated concomitantly with the splicing catalysis steps. DYRK1A, which is also localized to the nuclear speckles and is associated with the splicing process, phosphorylates SF3b1 at Thr434 in vitro and in vivo (de Graaf et al., 2006). This further suggests that DYRK1A may be involved in the regulation of pre mRNA-splicing.

The last interaction partner of DYRK1A was identified in a genome-wide Drosophila RNAi screen which showed that DYRK1A is a regulator of NFAT (nuclear factor of activated T cells) (Gwack et al., 2006). Precise regulation of the NFAT family of transcription factors (NFAT1-4) is essential for vertebrate development and function. The localization of the NFAT proteins is cytoplasmic for the heavily phosphorylated form and nuclear after activation by stimuli that raise intracellular free Ca^{2+} levels. The activation of NFATs is controlled by calcineurin-mediated dephosphorylation and they are inactivated by phosphorylation of specific NFAT kinases like CK1 (Casein kinase)

and GSK3 (Glycogen synthase kinase). DYRK1A and DYRK2 counter calcineurin-mediated dephosphorylation of NFAT1 by directly phosphorylating the NFAT regulatory domain. This is another example that DYRK1A can act as a priming kinase for further phosphorylation by downstream kinases like GSK3.
Furthermore mathematical modeling predicted that DYRK1A and DSCR1 (Down syndrome critical region protein 1) act synergistically in Down syndrome to dysregulate the NFAT-mediated gene transcription (Arron et al., 2006). The overexpression of DYRK1A and DSCR1 is speculated to destabilize the NFAT pathway, leading to reduced NFAT activity and subsequently to many features of Down syndrome.

3.2.3 Dyrk1a expression and localization

The localization of a protein is an important indicator for the cellular function within the cell. The DYRK1A nuclear localization has been suppossed to be static and invariant because of the identification of an N-terminal bipartite nuclear localization signal (NLS) in the primary protein sequence, biochemical assays and immunofluorescence studies with DYRK1A-GFP fusion proteins (Kentrup et al., 1996; Song et al., 1997; Becker et al., 1998). But the localization of a protein is dependent on more than only the presence of a NLS. A more detailed subcellular analysis revealed that DYRK1A is also located in the cytoplasm and interacts for example with dynamin in the growing dendritic tree of differentiating neurons (Chen-Hwang et al., 2002b; Hammerle et al., 2003; Marti et al., 2003). Furthermore DYRK1A is located in distinct substructures of the nucleus, the so called nuclear speckles which are associated with the RNA-splicing machinery (Alvarez et al., 2003; de Graaf et al., 2004). The most recent finding showed that the inverse translocation of DYRK1A from the nucleus to the cytoplasm can be induced in the presence of PAHX-AP1 (Bescond and Rahmani, 2005).
Several groups have also investigated the expression pattern of DYRK1A mRNA and protein in several organisms, including *C.elegans* (Raich et al., 2003), *Drosophila* (Tejedor et al., 1995), chicken (Hammerle et al., 2002; Hammerle et al., 2003), mouse (Rahmani et al., 1998), rat (Becker et al., 1998) and human (Guimera et al., 1999). DYRK1A is ubiquitously expressed in all investigated tissues during embryonic development as well as in the adult. In adult tissues, the highest expression is found in brain, skeletal muscle, heart and testis (Guimera et al., 1996; Song et al., 1996; Guimera et al., 1999). In contrast to the rest of the DYRK kinase family members, DYRK1A is the only protein which is expressed in the brain (Becker et al., 1998). In

rodent brain in situ hybridization and immunohistochemistry revealed a high DYRK1A expression in cortex, cerebellum, olfactory bulb, hippocampus, hypothalamus, spinal cord and the brain stem (Guimera et al., 1996; Song et al., 1996; Marti et al., 2003). During rat development the DYRK1A protein expression is high but gradually decreases to very low levels during postnatal stages. The concentrations of DYRK1A mRNA levels remain constant (Okui et al., 1999). The temporal expression pattern of DYRK1A during vertebrate brain development has been extensively studied in chicken with the chicken DYRK1A homologue MNB (Hammerle et al., 2002; Hammerle et al., 2003). In early embryos MNB is transiently expressed during the transition from proliferating to neurogenic division in the three general locations where neural precursors originate: the neural tube, neural crest and cranial placodes. At the cellular level this takes place by asymmetric segregation of MNB mRNA to one of the daughter cells by transient MNB expression during a single cell cycle (Hammerle et al., 2002). There is also a second wave of MNB expression during late embryonic development in which MNB seems to be restricted to certain neuronal populations. MNB expression accompanies the start of dendritic tree formation and is initiated by transient translocation into the nucleus. Thereafter DYRK1A is transported to the growing dendritic tree and colocalizes with the potential substrate Dynamin, implicating a possible role of DYRK1A in dendritic development (Hammerle et al., 2003).

3.2.4 Dyrk1a substrates and interaction partners

Substrate or interacting protein	Substrate evidence	Interaction evidence	Effect
FKHR	In vitro	Co-IP	Stimulation of nuclear export
STAT3	In vivo	Y2H	Stimulation of transcription
CREB	In vitro, in vivo	Y2H, Co-IP	Stimulation of transcription
GLI1	In vitro	Reporter gene assay	Stimulation of transcription
E1A	In vitro	Pulldown assay	E1A stimulates activity DYRK1A
eIF2B-epsilon	In vitro	Y2H	Priming kinase for GSK3
Tau	In vitro	Y2H	Priming kinase for GSK3
Dynamin	In vitro	Pulldown assay	Regulation of protein-protein interaction
SF3b1	In vivo	CoIP, RNAi assay	Stimulation of splicing catalysis
NFAT	In vivo	CoIP, RNAi assay	Priming kinase Modulator of NFAT phosphorylation
HIP-1	In vitro	Pulldown assay	Regulation of proapoptotic activity
PAHX-AP1	In vitro	Y2H, CoIP, IF	Translocation of DYRK1A from the nucleus to the cytoplasm
14-3-3	In vitro, in vivo	Y2H, CoIP	14-3-3 stimulates DYRK1A activity
Cyclin L2	In vitro	Filter array	Regulation of splicing catalysis
Arip4	In vitro, in vivo	Y2H, Transactivation assays, RNAi	Stimulation of steroid hormone-regulated transcription

Fig. 8 Substrates and binding partners of DYRK1A

Y2H: Yeast-two hybrid screen; Co-IP: co-immunoprecipitation; RNAi: RNA interference

3.2.5 Possible functions of DYRK1A in the brain

The physiological function of DYRK1A in the vertebrate brain is still unclear. Considering the existing studies and results of extensive research efforts on the highly conserved DYRK family and especially on DYRK1A, there are several hints for the possible biological function in the brain.

The first evidence for the role of DYRK1A in neurogenesis and neuronal differentiation came from the drosophila mnb mutants (Heisenberg and Böhl, 1979; Tejedor et al., 1995). The mutant flies had specific and marked size reduction of the optic lobes and central brain hemispheres due to disturbed neuroblast proliferation during neurogenesis. They also showed defects in learning of olfactory discrimination and hypoactivity (Tejedor et al., 1995). Further support came from intensive expression studies in chicken which demonstrated that the kinase is transiently expressed in two waves. First, during early development in distinct areas of neural precursors before the onset of neurogenesis and second, in distinct neurons during late embryonic brain development at the onset of dendritic differentiation (Hammerle et al., 2002; Hammerle et al., 2003). This suggests a role for DYRK1A in the transition step from neuronal precursor proliferation to neuronal differentiation. The impairment of neurite outgrowth in hippocampal progenitor cells by blockade of DYRK1A activation might also fit in this idea as well as the localization of DYRK1A in the growing tree of differentiating neurons (Yang et al., 2001; Hammerle et al., 2003). The Dyrk1a$^{+/-}$ haploinsufficient mice showed impairment in the neuromotor development suggesting a possible involvement of DYRK1A in development of the neuromotor system (Fotaki et al., 2004).

These summarized data support that DYRK1A might play an important role in neuronal development of the brain from the early embryo into adulthood. However the underlying molecular mechanisms have to be elucidated.

3.3 Conditional mutagenesis of the *Dyrk1a* gene

The investigation of gene functions in living organisms is one of the prerequisites for the understanding of biological processes. A milestone for the analysis of gene functions in mammals was the targeted inactivation of genes in the mouse. This was made possible by the combination of two methods, which were developed in the late 1980s. First, the isolation of embryonic stem (ES) cells from mouse embryos and their undifferentiated maintenance in cell culture (Evans and Kaufman, 1981; Martin, 1981) and second the targeted integration of artificial genetic information on the chromosomes of eukaryotic cells by homologous recombination (Smithies et al., 1985).

ES cells are isolated from the inner cell mass of mouse blastocyst stage embryos and are undifferentiated and pluripotent cells which have the capacity to differentiate into a mouse embryo including all its tissues. When these ES cells are microinjected

into the blastocyst of a new embryo and subsequently are implanted into a foster mother, mice are born which contain the genetic information of the ES cell and the acceptor embryo (Bradley et al., 1984). These mice with mixed genetic background are called chimeras. With a certain degree of probability, the injected ES cells will also contribute to the development of germ cells in the chimeric mice so that the genetic predisposition of the modified ES cells will be transmitted to the next generation. By specific breeding of these chimeras, mice can be generated which have only the genetic background of the ES cell.
Furthermore the ES cell genome can be manipulated by homologous recombination *in vitro* in cell culture. The first application of this method was the inactivation of the selectable HPRT-locus in ES cells of the mouse and thereof animals were generated which served as a model of the human Leish Nyhan syndrome (Thomas and Capecchi, 1987; Doetschman et al., 1988). Up today about 4000 of the 35000 genes of the mouse are inactivated by the latter methods and more than 500 of these knockout mice serve as animal models of human diseases (Goldstein, 2001).
But not in every case this method proved to be successful. There are several cases where the inactivation of a gene can be compensated by other proteins and consequently no clear phenotype can be seen (Gridley, 1991). Besides, the introduced inactivation or manipulation can act in every cell of the body and more importantly it can act during every step of the ontogenesis of the developing mouse embryo. As a result in about 30% of all cases the inactivation of a gene can cause severe developmental defects which can lead to death of the animals at early embryonic or postnatal stages. Hence the investigation of the phenotype and the function of the gene in the adult animal are impossible. As the genetic modification is seen in every cell of the organism, it is very difficult to decide whether the complex phenotype is caused by a primary or a secondary effect.

3.3.1 Knockout of *Dyrk1a* with the *Cre/loxP* recombination system

3.3.1.1 Sequence specific recombination systems

The problems associated with gene modifications by homologous recombination in ES cells can be circumvented by using sequence specific recombination systems. Sequence specific recombination enzymes made it possible to gain temporal and spatial control over genomic modifications e.g. the deletion of the gene of interest. Two recombinases are almost exclusively used in transgenic animals. The *Cre* recombinase

is derived from the bacteriophage P1 (Sauer and Henderson, 1989) and the *Flp* recombinase from the budding yeast *S. cerevisiae* (O'Gorman et al., 1991).

The *Cre* and *Flp* recombinase belong to the λ integrase family of the sequence specific recombinases. *Cre* catalyses the recombination between two specific recognition sites, called the *loxP* sites. A *loxP* site ("locus of crossing-over P1") is 34 bp long and consists of a directed 8 bp long core sequence which is flanked by two 13 bp long palindromic sequences (Fig. 9). The direction of the central core sequence defines the orientation of the *loxP* site (Rajewsky et al., 1996).

The *Cre* recombinase catalyses without any other cofactors the *intra*molecular excision of the *loxP*-flanked DNA fragments when the two *loxP* sites are oriented in the same way on the same DNA. This reaction is also reversible while one *loxP* site is located on a circular molecule and one is on a linear molecule which directs the *inter*molecular integration of the circular DNA. When the two *loxP* sites on a DNA fragment are oriented towards each other the intervening DNA sequence is inverted and *loxP* sites on two separate linear molecules instruct mutual exchange of regions distal to the target site. These equilibration reactions, the deletion, inversion, exchange and integration of *loxP*-flanked DNA sequences, can be pushed in the desired direction by using suitable selection markers (Nagy, 2000). The molecular mechanisms of the recombination reaction (Voziyanov et al., 1999) in brief are the following: at a time one dimer of the recombinase binds to the palindromic sequence and after the two dimers form a tetramer the two strands are brought together in a complex and are cut in the core region. Thereafter the strands are exchanged and the DNA strands are ligated together in the core sequence. The recombination reaction is carried out with absolute fidelity, such that not a single nucleotide is gained or lost overall.

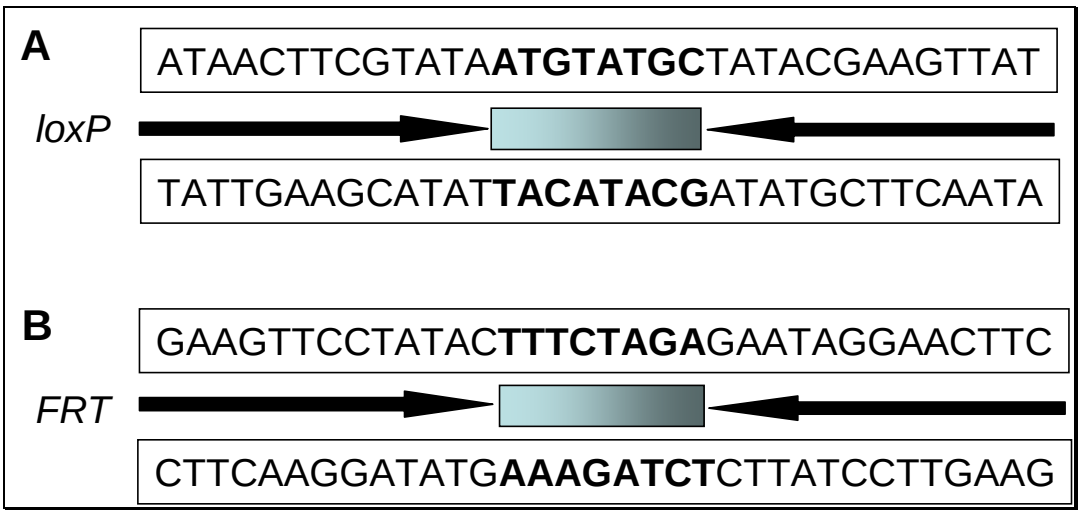


Fig. 9 Recombinase target sites

Target sites have a length of 34 bp and contain inverted 13 bp symmetry elements (horizontal arrows) flanking an 8 bp A:T-rich non-palindromic core (shaded rectangle). **(A)** The target site recognized by the *Cre* recombinase (causes recombination) is called *loxP* site (locus of cross-over P1). **(B)** The *Flp* target recognition site is called *FRT*.

Although the nucleotide sequences are different, the *Flp* recombination system shares the same overall structural elements and molecular mechanisms as the *Cre/loxP* system except the specific 34 bp long recombination sites are called *FRT* sites (Fig. 9). The *FRT* site comprises like the *loxP* site the inverted symmetry elements which act as binding sites for recombinase monomers and the 8 bp asymmetric core element which is the site of strand cleavage, exchange and ligation and directs the manner of the recombination reaction. For the *in vivo* use of the Flp recombinase in mice the recombinase had to be adapted to temperature optimum of 37°C of the mouse (Buchholz et al., 1998). The modified *Flp* recombinase was called *Flpe* and is as efficient as the *Cre* recombinase. It is also noteworthy that both *Cre* and *Flpe* function in the germline and can be used to generate transmissible modifications of *loxP*-flanked ("floxed") or *FRT*-flanked ("flrted") DNA sequences.

A general disadvantage of this recombination-mediated gene modification is that it acts under the "all-or-none" law. If the recombination is induced once, the process can not be reversed. Hence this means that the gene of interest can only be activated once or ablated once. A partial and regulated induction or the tissue-specific overexpression of a transgene is, if at all, complicated.

3.3.2 Inducible Overexpression of *Dyrk1a* with the Tet-system

3.3.2.1 Principles and methods of the tetracycline-regulated transcriptional control

One of the best strategies for the characterization of a gene function *in vivo* is the targeted and reversible control of the corresponding gene activity. Phenotypic changes which can be seen with this method give hints to the function of the gene. Regulation systems are preferable which are controlled by exogenous inductors.
The tetracycline-regulated gene expression system (Tet-system) is based on control elements of the tetracycline resistance operons of the transposon *Tn10* from *E.coli*. The Tet-repressor (TetR) regulates through binding to its operator sequence tetO1 and tetO2 in the central transcriptional control region of the operon its own expression and the expression of *tetA*. *TetA* is a membrane-integrated proton-antiporter for metal-complexed tetracycline which mediates tetracycline resistance through the export of the antibiotic out of the cell (Hillen and Berens, 1994). Stringent control of *tetA* is made possible because of two special binding features of the TetR-dimers. The TetR-dimer has with 10^{-9} M a very high association constant to its operator sequences. Consequently the repression of *tetA* is also feasible at very low Tet repressor concentrations. This is necessary because the antiporter influences the membrane potential of the bacterium and thus leads to a growth drawback (Griffith et al., 1994). In addition the bacterium needs a high expression of *tetA* even at sub-toxic concentrations of antibiotic for survival. This is enabled by the very high complex formation constant of tetracycline to TetR. The binding of tetracycline to the TetR leads to a conformational change and the TetR-dimer can no longer bind its operator sequences and at last the promoters are dereprimed and transcription is activated.

3.3.2.2 The tTA-system

The stringent control of prokaryotic gene expression with the Tet-system was later transferred to eukaryotic cells (Gossen and Bujard, 1992). In the tTA-system the central regulatory component is the tetracycline-dependent transactivator *tTA* (Fig. 10). *tTA* is a fusion protein of the C-terminal transactivation-domain of the virion protein 16 (VP16) of the *Herpes simplex* virus (Triezenberg et al., 1988) with the C-terminal of the TetR. This synthetic transactivator binds to the Tet-operator sequence of the tetracycline-regulated promoter P_{tet} whereby the transcription can be very strongly activated. Ptet itself consists of a minimal promoter which has the elements to bind the

basal transcription-initiation complexes of the RNA-polymerase II (TATA- and the Inr-Box) and 5′-primed a heptamer of Tet-operators. In the case of P_{tet-1} the minimal promoter comprises the -53 to +75 regions, relative to the transcription start, of the IE gene of the human Cytomegalovirus (hCMV) (Boshart et al., 1985). By adding tetracycline (Tet) or some of its derivates (doxycycline (Dox) and anhydrotetracycline (ATC)) the binding of *tTA* to its operators is prevented. The expression is down-regulated to non-measurable levels even at concentrations of the tetracyclines 3 orders of magnitude lower than the toxic limits of the used tetracyclines. Thus the conserved binding- and induction characteristics of TetR and tetracycline in the tTA systems allow for the gradual regulation of gene expression over 5 orders of magnitude in the range of sub-toxic inductor concentrations.

The most important requirement of such a eukaryotic gene regulation system is the specificity of the induced gene expression. In eukaryotic cells the transactivator should not interfere with the expression of endogenous genes. In the Tet-system this is granted by the very high binding-specificity of the TetR to its operators. *In vitro* experiments showed that TetR binds its operators with $4x10^{-11}$ M in contrast to unspecific DNA with $5.5x10^{-3}$ M. As a consequence the discrimination between specific and unspecific binding is in the range of 8 orders of magnitude. Also the operator sequences are of prokaryotic origin and so should not contain any consensus motifs of eukaryotic transcription factors. Another important aspect is the pharmacological and toxicological properties of the inductor. Preferably it should be a synthetic substance which can accumulate in sufficient concentrations in almost every tissue but it should not interfere with the physiology of the cells and cause pleiotropic effects. If possible, a very high and gradual induction at very low basal expression levels in the un-induced state is desirable. This can only be achieved in eukaryotes in a system which is based on the principle of activation.

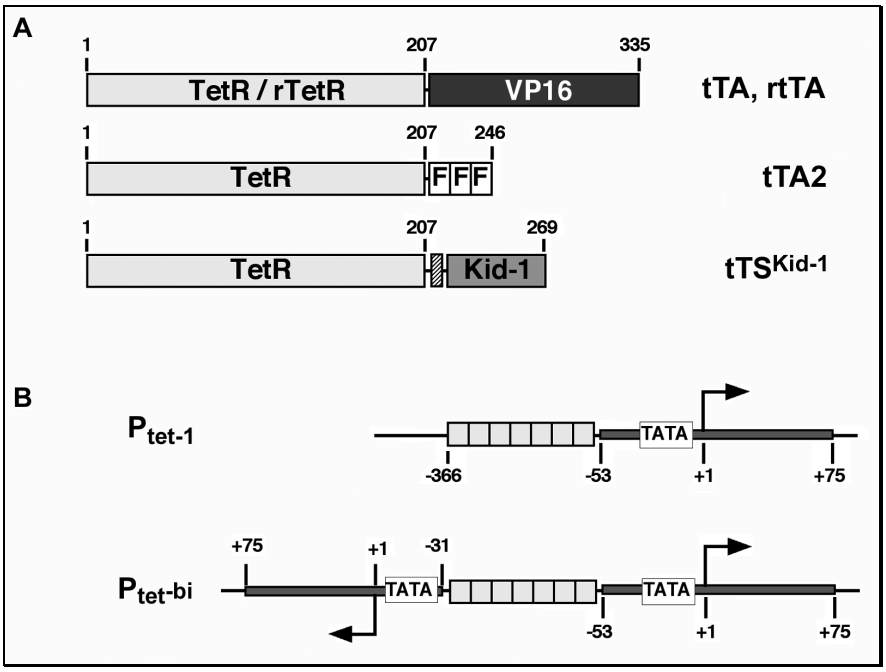


Fig. 10 Schematic overview of the components of the Tet-system

(A) Schematic diagram of different tetracycline-dependent transactivators and repressors. **(B)** Diagram of the tetracycline-inducible promoters (modified from (Gossen and Bujard, 2002)).

3.3.2.3 The rtTA-system

Since its initial characterization, the Tet-system has been permanently extended and improved. The first extension that appeared was the development of the rtTA-system (Gossen et al., 1995). The identification of a TetR-mutant, in which 4 amino acids were substituted, led to a reverted DNA binding behavior of the TetR to its operators. The corresponding reverse transactivator was constructed by fusing the mutant TetR to the VP16 activation domain (Fig. 10). Consequently P_{tet} can now be activated by addition of Dox or ATC. This characteristic is very useful for use in transgenic animals. Due to this additive induction the animals have not to be supplied with Dox all the time to suppress the tet-regulated gene expression (Kistner et al., 1996). This has many advantages concerning the activation- and deactivation kinetics of the gene regulation.

The reverse transactivators itself were also improved by using sensitive yeast-based selection systems. Today there are improved reverse transactivators (Fig. 12) available (rtTA2^s-S2 and rtTA2^s-M2) which differ from the original rtTA in the manner that they have no more binding activity to the Tet-operators in the absence of Dox (Urlinger et al., 2000). Thus they do not cause an elevated basal activity in the non-induced state. The regulation factor is elevated from 3 orders of magnitude to 5 or-

ders of magnitude and the maximal activation of the tet-dependent promoter is achieved with a ten times lower Dox concentration. The constructs which are widely used today consist of synthetic versions of the corresponding transactivators which were optimized for expression in eukaryotic cells. In addition the complete VP16 domain was substituted in most cases with 3 minimal activation domains. These modular domains allow for the application of transactivators with defined activation potentials, thus regulating the toxic effects of such activation domains (Baron and Bujard, 2000).

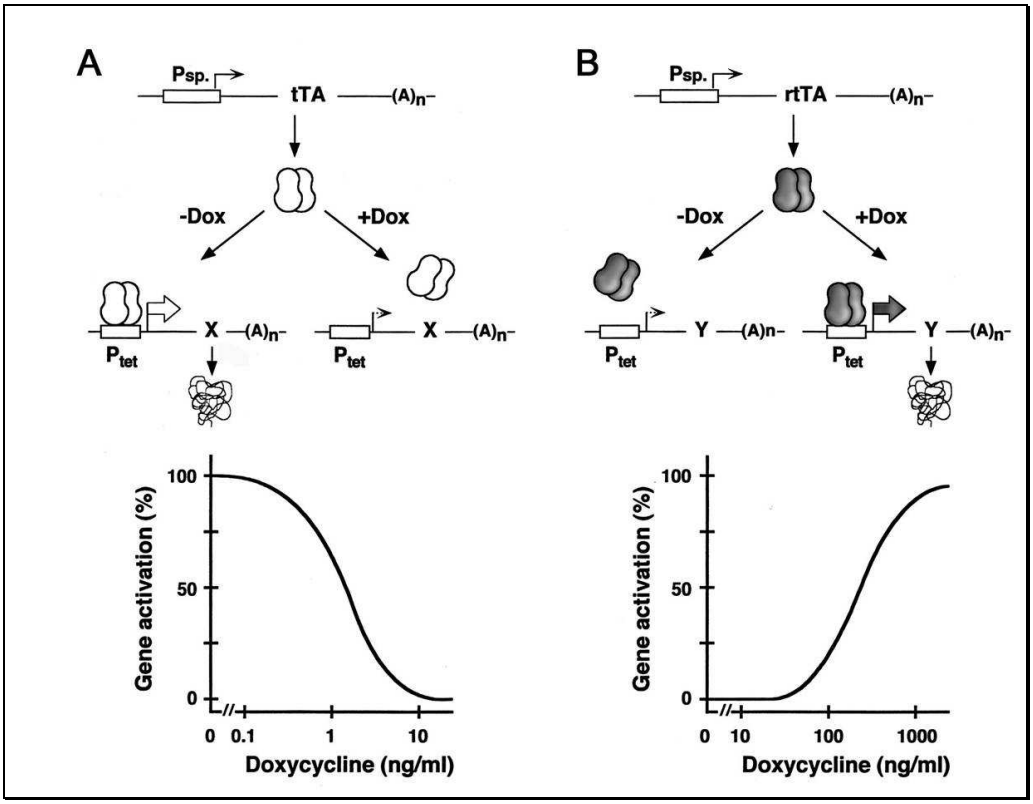


Fig. 11 Schematic diagram of the two different Tet-systems

Top - tTA-system: In the absence of the effector substance doxycycline (Dox), the Tet-regulated transactivator tTA binds to multiple operator sequences within the Tet-inducible minimal promoter P_{tet} and activates the transcription of gene X. When doxycycline is present, the binding and therefore the activation of transcription is inhibited. *Bottom* – Dose-response analysis of Dox on the Tet-dependent gene expression: Gene expression is maximal in the absence of the antibiotic and is down regulated gradually by increasing concentrations of Dox to baseline expression.

Top – rtTA- system: The rtTA differs from the tTA by 4 amino acid substitutions and causes a reverted DNA-binding of the TetR to its operator sequences. In this system, the presence of Dox leads to the Tet-dependent activation of gene expression of gene Y. *Bottom* – Dose-response analysis of Dox on the Tet-dependent gene expression: The inductor Dox can activate gene expression starting at concentrations of about 10ng/ml and increases gradually to a maximum of about 100ng/ml.

The temporal- and spatial regulation specificity of both systems is mediated by the expression profile of the specific promoter $P_{sp.}$, which drives the expression of the tTA/rtTA gene. (courtesy of H.Bujard)

3.3.2.4 Further elements of the Tet-system

In addition a bidirectional tet-regulated promoter (P_{tet}-bi) was developed which consists of the heptameric Tet-operators which are flanked by two minimal promoters (Baron et al., 1995). Thus the transcription is possible in both directions and so allows for the bicistronic simultaneous regulation of two genes. As a result this offers the great opportunity, especially in transgenic mice, to regulate not only your gene of interest but also a reporter gene at the same time, e.g. ß-galactosidase, EGFP or luciferase (Yamamoto et al., 2000; Hess et al., 2001). The development of new live imaging techniques and cameras made it possible to visualize the luciferase and EGFP expression even *in vivo* and so the tet-regulated expression can be studied in living animals (Hasan et al., 2001).

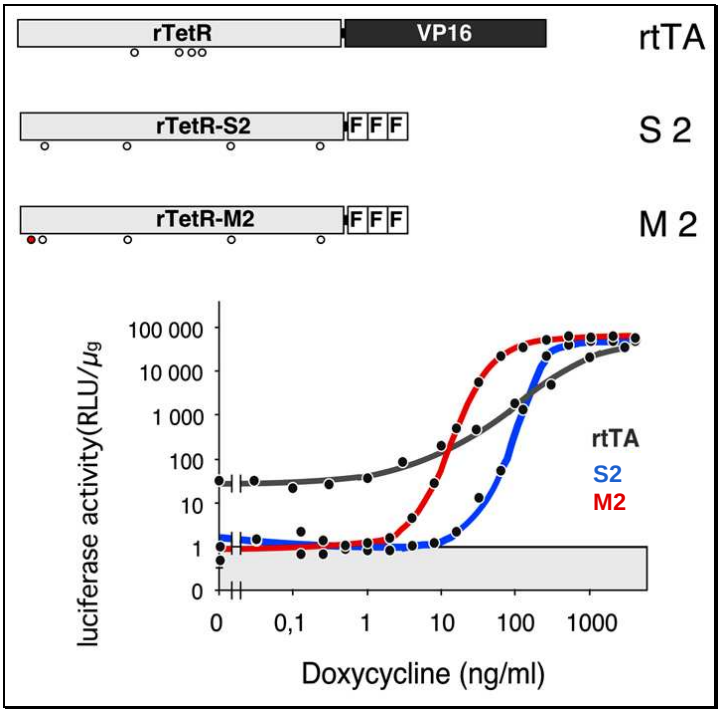


Fig. 12 Comparison of different rtTAs

Schematic diagram of the original reverse transactivators rtTA and the improved rtTA2s-S2 and rtTA2s-M2. In contrast to the original rtTA with a VP16 activation domain, the new rtTAs have 3 minimal activation domains (marked F) and the amino acid substitutions are at different positions within the rTetR (marked by small circles). The dose-response analysis shows the induction of luciferase activity by rtTA, rtTA-S2 and rtTA-M2, dependent on the Dox concentration (modified from (Urlinger et al., 2000).

3.3.2.5 Tetracycline-dependent regulation in mice

The method of choice to establish tet-regulated gene expression in transgenic mice is the breeding of a transactivator-line, in which the tetracycline-dependent transactiva-

tor is expressed under the control of a suitable tissue-specific promoter, and a mouse line wherein the gene of interest is under the control of a tet-regulated promoter. It is very important, that the integration site of the expression cassette in the transactivator line does not influence the tissue-specificity. This can be monitored by breeding the promoter line with a suitable reporter line (Kistner et al., 1996).
Problems could also arise from the genomic integration site of the cassette. The transgenic lines are created by random transgenesis and the number of integrated copies and the integration site itself could lead to positional effects, even if the transgenic inducible cassette is not turned to the "on" state. Depending on the integration site, the expression of endogenous genes or promoter and enhancer elements could be disturbed or, if the cassette is integrated into a heterochromatin region, the tet-induced expression could be silenced. Significant effort has to be taken which mouse line is kept for further analysis and preferably the transgenic line should be selected which shows no complicated basal activity in the un-induced state and has a high activation potential of gene expression in the induced state.
In the resulting double-transgenic mice with the transactivator and the tet-regulated expression cassette the regulation of the gene is controlled by oral application of Dox in the drinking water or the food of the mice (Kistner et al., 1996). This basic scheme was applied up today in several hundred cases for the functional analysis of genes in transgenic animals and so the Tet-system is the best established system for gene regulation in transgenic animals so far. There are more than 100 mouse lines published with tissue-specific transactivators and over 100 mouse lines with tet-regulated expression cassettes (http://www.zmg.uni-mainz.de/tetmouse/index.htm).
It is very important to know, that the application of this strategy is only suitable for scientific questions addressing the analysis of the overexpression of a gene or the overexpression of a dominant active mutant. It has also to be kept in mind that the endogenous gene is still active and accomplishes its normal function in the organism.
Besides the Tet-system there are certain other regulated eukaryotic gene expression systems available. In contrast to the Tet-system they are less widely used (Serguera et al., 1999). These are systems which are regulated by ecdyson (No et al., 1996), mifepristone or RU486 (Wang et al., 1994), rapamycine and its derivates (Ho et al., 1996), streptogramin (Fussenegger et al., 2000) and erythromycin (Weber et al., 2002).

3.3.2.6 The silent but activatable (s/a) locus LC1

To overcome the position effects described before which are associated with the genomic integration site of the tet-regulated expression cassette in random transgenic animals, Kai Schönig characterized a mouse line generated by Frieder Schwenkin the laboratory of H. Bujard called LC1. The LC1 line is a random transgenic mouse line which was generated by DNA-microinjection of a tet-regulated bidirectional expression cassette under the promoter P_{tet}-bi with the *cre* and *luciferase* gene. The basic characterization showed that the LC1 line had the desired regulation properties of an almost "ideal" tet-regulated response line: the LC1 mice showed no basal activity in the un-induced state and showed a high activation potential in the induced state in a wide variety of tissues (Fig. 13). Assuming that the stringent control of expression is due to the genomic integration site of the tet-regulated expression cassette, Kai Schönig further investigated the LC1 line and identified the genomic integration locus by screening of a self-made LC1-BAC library.

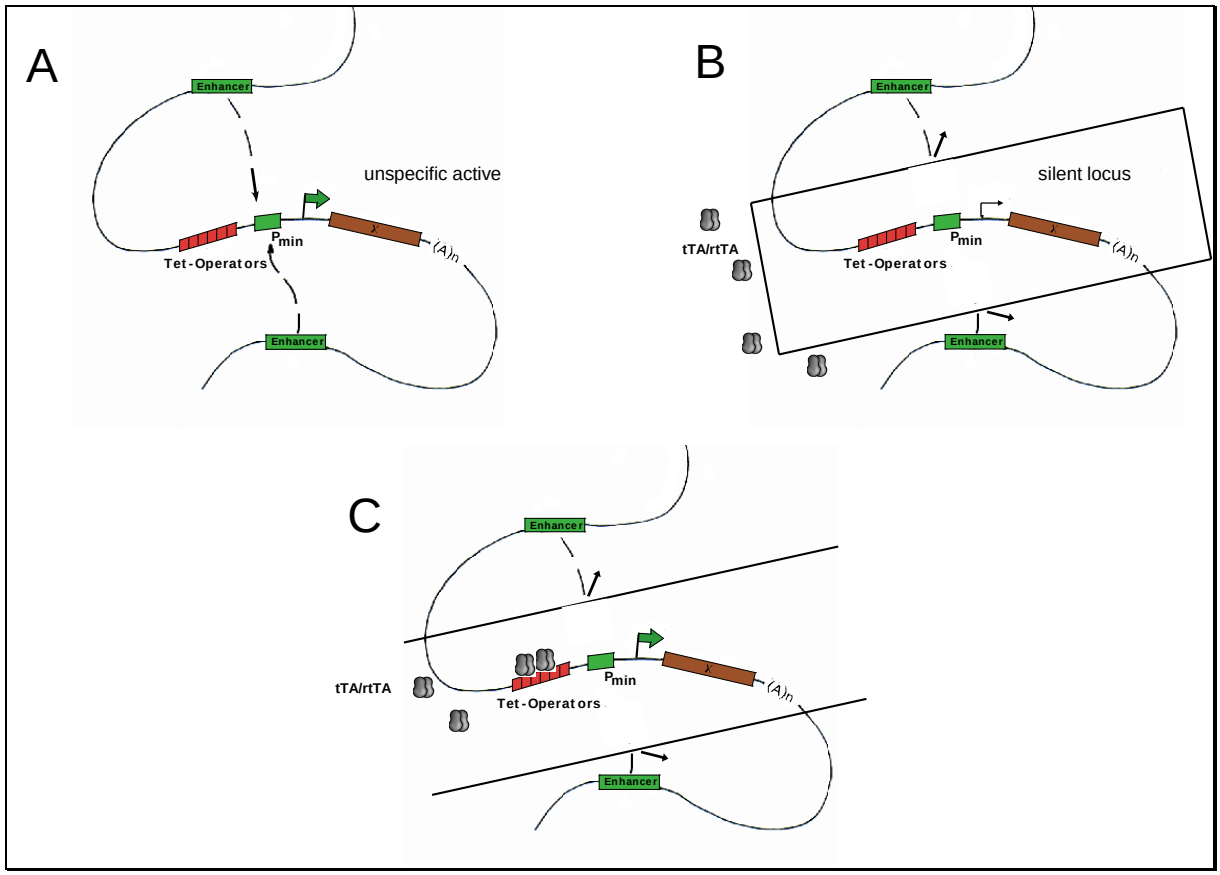


Fig. 13 The silent but activatable (s/a) locus

Dependent on the integration site of the transgene into the genome, position effects can influence the inducible regulation of gene expression via the Tet-system. **(A)** Surrounding genomic elements, mainly enhancer, can induce the unspecific activation of the Tet-regulated transcription cassette even without the presence of the transactivator or the effector Dox. **(B)** The chromatin structure of the integration site can lead to transcriptional silencing of the locus, for example by histone modifications or methylation. There is neither specific Tet-dependent transcription nor unspecific gene expression. **(C)** In the ideal situation, the stringent Tet-dependent gene expression is not influenced by surrounding genomic elements or silenced. Thus there is no basal activity in the un-induced state and a high activation in the induced state in a variety of tissues. A genomic locus with such properties is called a silent but activatable (s/a) locus.

The genomic integration locus LC1 showed to be a "perfect" match to the criterions of a silent but activatable (s/a) locus because:

- Stringent control of P_{tet}-bi, no basal expression detectable
- High induction was detectable in all investigated tissues after breeding with tTA/rtTA-expressing mice
- Both genes are co-expressed at the same levels
- The regulation of gene activity is reproducible and possible over high order of magnitudes and the expression is so far not influenced by position effects

3.4 Aims of the thesis

One of the most elegant ways to study the biological function of a gene or its corresponding protein *in vivo* is the application of reverse genetics. For reverse genetics, the goal is to identify a known gene's phenotype by targeted manipulation of the host genome with molecular genetics techniques, including gene silencing (knockout) and overexpression of a transgene.
The main focus of my thesis was the development and generation of conditional *Dyrk1a* mouse mutants. These minimal models should further deepen the understanding of the general biological function of the DYRK1A kinase and the influence of DYRK1A on the pathophysiology of Down syndrome, especially on the developmental deficits in cognition and learning and memory and mental retardation.

3.4.1 Generation of a conditional *Dyrk1a* Knockout mouse line

The conventional knockout of the *Dyrk1a* gene by targeted disruption showed to be embryonic lethal. Only the heterozygous Dyrk1a$^{+/-}$ knockout mouse mutants were viable and caused developmental delay and abnormal brain morphology in mice (Fotaki et al., 2002).
Consequently, we decided to functionally ablate the gene by using the *Cre/loxP* recombination system to circumvent the embryonic lethality. The strategy included to flank exon 5 of the *Dyrk1a* gene with two *loxP* target recognition sites. Exon 5 codes for the ATP-binding domain of the DYRK1A protein which is essential for the function of every kinase in order to phosphorylate its substrates. Additionally, the "floxed" exon 5 strategy allowed for the later temporal and spatial control of the *Dyrk1a* gene knockout, especially to the brain.

3.4.2 Generation of a tet-regulated *Dyrk1a* mouse line targeted in the genomic s/a-locus LC1

The complex phenotypes of DS and the ubiquitous expression patterns of *Dyrk1a* led us to the conclusion that the temporal and spatial control of the overexpression of *Dyrk1a* would be also advantageous. The Tet-system offered the best and most common strategy for stringent control of transgene expression *in vivo*. Besides the Tet-dependent expression of the *Dyrk1a* gene, we also wanted to simultaneously regulate the *EGFP* reporter gene expression to track and visualize cells with the inducible expression of both genes.

3.4.2.1 Cloning and analysis of a standard targeting vector for the LC1 locus

One limitation of the Tet-system in complex random transgenic organisms is, that dependent on the integration site of the transgenes into the genome, position effects can influence the inducible regulation of gene expression or the phenotype by, for example, disrupting an existing endogenous gene.
To overcome this problem we decided to directly target the Tet-regulated bidirectional expression cassette to the mouse genomic LC1 locus. The LC1 locus has been extensively characterized by Dr. Kai Schönig. He demonstrated in his PhD thesis that the locus has all properties of a silent but activatable (s/a) genomic locus.
However the locus has only been used to generate random transgenic animals with the large E11-BAC with ~75kb of homology to the endogenous genomic locus. So far, the strategy to use the advantages of the LC1 locus was to clone the Tet-regulated expression cassette in the middle of the 75 kb fragment and the modified BAC-DNA was microinjected in the pronucleus to generate random transgenic animals. These animals proved to have a stringent control of expression but they, like any other transgenic line, had to be screened for position effects of the integration site itself and the number of copies of the transgene into the genome. We decided that it would be preferable to target the locus with a targeting vector with short homology arms by homologous recombination in ES cells
Thus another aim of my work was the cloning and analysis of a standard targeting vector to routinely target the s/a – LC1 locus by homologous recombination in ES cells. The strategy included the use of the constructed standard vector for targeting of the LC1 locus with a tet-dependent bidirectional *Dyrk1a* and *EGFP* expression cassette in ES cells and the subsequent generation of single-transgene chimeric offspring. After successful germline transmission of the transgenic allele the strategy allowed for the temporal and spatial control of the transgene expression by using transactivator lines, where the transactivator is under control of specific promoters.
Our strategy for the generation of conditional *Dyrk1a* mutants included the use of the same tissue-specific promoters which drive the expression of the *tTA* or *Cre* gene to investigate the whole bandwidth of *Dyrk1a* gene expression, from the ablation over the wildtype expression to the overexpression *in vivo*.

3.5 Abbreviations

aa	amino acids
AA	acryl amide
Amp	ampicillin
AP	alkaline phosphatise
APS	ammonium persulfate
ATC	anhydrotetracycline
Bis-AA	Bis-acryl amide (N,N´-methylen-bis-acryl amide)
bla	gene for the enzyme ß-Lactamase (ampicillin resistance)
ß-Gal	ß-galactosidase
bp	base pairs
BSA	bovine serum albumin
CaMKIIa	Ca^{2+}/Calmodulin-dependent protein kinase II α
Carb	carbenicillin
C-terminal	Carboxy-terminal of a protein
cDNA	copy DNA
cko	conditional knockout
Cm	chloramphenicol
cre	gene for the Cre-recombinase
DAB	diaminobenzidine
DSCR	Down syndrome critical region
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	2´-deoxyribonucleotide
ddNTP	2´,3´-deoxyribonucleotide
Dox	doxycycline
dsDNA	double stranded DNA
DTT	dithiothreitol
DYRK1a	dual-specificity tyrosine-Y-phosphorylation regulated kinase 1a
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EtBr	ethidium bromide
EtOH	ethanol
f.c.	Final concentration

FBS	fetal bovine serum
FCS	fetal calf serum
Fig.	figure
G418	Geneticin
hCMV	human cytomegalovirus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horse radish peroxidise
HS	horse serum
Hyg	hygromycin
IgG	immunoglobulin G
i.p.	intraperitoneal
KAc	potassium acetate
Kan	kanamycin
kb	kilo base pairs
KO	knockout
NLS	nuclear localisation signal
lacZ	gene for the ß-galactosidase of E.coli
LB	Luria broth
LTD	long term depression
LTP	long term potentiation
luc	gene for the enzyme luciferase
MCS	multiple cloning site
MeOH	methanol
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
N-terminal	Amino-terminal of a protein
NaAc	sodium acetate
nt	nucleotides
NTP	nucleoside triphosphate
OD	optical density
ori	origin of replication
ORF	open reading frame
P	promoter
p	plasmid
pA	polyadenylation signal
p.A.	pro analysis
PAA	polyacryl amide
PAGE	PAA-gel electrophoresis
PBS	phosphate buffered saline

PCR	polymerase chain reaction
pH	negative base-10 logarithm of H^+-ion concentration
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PNK	polynucleotide kinase
PVDF	polyvinylidenfluoride
Pwo	pyrococcus woesei
r	resistant
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	ribonuclease inhibitor
RT	room temperature
rtTA	reverse tetracycline-inducible transactivator
s/a	silent but activatable
SAP	shrimp alkaline phosphatase
SDS	sodium dodecylsulfate
ssDNA	single stranded DNA
SV40	simian virus 40
tab.	table
TAE	Tris-acetate-EDTA
Taq	thermos aquaticus
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tm	melting point
tetO	tetracycline operator sequence
tetR	gene for the repressor of the Tn10 resistanceoperon of E.coli
TetR	repressor of the Tn10 resistanceoperon of E.coli
TEMED	N,N,N',N'-Tetramethylethylenediamine
tet/Tet	tetracycline
Tris	tris-hydroxymethylaminomethane
tTA	tetracycline-inducible transactivator
tRNA	transfer RNA
UTR	untranslated region
UV	ultraviolet light
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
x g	times gravity

3.5.1 System of Units

Symbol	Name
A	Ampere
Ci	Curie
cpm	counts per minute
Da	Dalton
F	Farad
g	gram(s)/Force of gravity
h	hour(s)
l	liter(s)
m	meter(s)
M	molar (mol/l)
min	minute(s)
mio.	million(s)
mol	Avogadro constant (6.022×10^{23})
Rlu	relative light units
s	second(s)
U	enzyme activity (units)
rpm	rounds per minute
V	Volt

3.5.2 Prefixes

Symbol	Name	Factor
k	kilo	10^{3}
c	centi	10^{-2}
m	milli	10^{-3}
µ	micro	10^{-6}
n	nano	10^{-9}
p	pico	10^{-12}
f	femto	10^{-15}

3.5.3 Bases/nucleosides

Symbol	Name
A	Adenine/Adenosine
C	Cytosine/Cytidine
G	Guanine/Guanosine
T	Thymine/Thymidine
U	Uracil/Uridine
X	C,U and A

3.5.4 Amino acids

Three-Letter symbol	One-Letter symbol	Name
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophane
Tyr	Y	Tyrosine
Val	V	Valine

4 Materials

4.1 Laboratory equipment

4.1.1 Microscopy

Microscopes	Wilovert S, Hund
	Axiovert, Zeiss
	DM IRB, Leica

4.1.2 Agarose-gelectrophoresis

Electrophoresis chamber	B1A, B2 ;B3, Owl Scientific
Power supply	Poer Pac 300, BioRad
Gel photo imager	Gene Flash, Syngene

4.1.3 Cell culture

Hood	Nuair class II
Cell culture incubator	Nuaire US autoflow
Vacuum pump	IBS Integra Biosciences
Cell culture water bath	GFL

4.1.4 Data processing

Computer	AMD Athlon 1,2Ghz, 512MB RAM
Software	Windows XP SP2, Microsoft
	Office XP Professional edition, Microsoft
	Photoshop 6.01, Adobe
	Endnote 9.0, ISI ResearchSoft
	pDRAW32, freeware
	Vector NTI 10, Invitrogen
	AanalySIS_B, Soft Imaging Systems Olympus

4.1.5 Other equipment

Accurate scales	BP210S, Satorius
Autoclave	Fritz Gössner
Incubator	Heraeus
Centrifuge Tubes	Beckman-Coulter
Electroporator	ECM630, BTX
Magnetic stirrer	MR3001, Heidolph

Thermal Cycler	I-cycler, BioRad
	Primus 96, MWG Biotech
	UNO II Biometra
Pipetting controller	Pipetus akku, Hirschmann Laborgeräte
pH-meter	pH537, WTW
Refrigerator	Liebherr
-80°C freezer	Sanyo
Shaker	Duomax 1030, Pharmacia Biotech
Spectrophotometer	Ultrospec 200, Pharmacia Biotech
	NanoDrop ND-1000, MWG Biotech
Vacuum concentrator	Speed Vac SC110, Savant
Tabletop centrifuge	Centrifuge 5415D, Eppendorff
	Centrifuge 5415R, Eppendorff
	Multifuge 3 S-R, Heraeus
	CS-6, Beckman-Coulter
Thermo block	Thermomixer comfort, Eppendorff
Ultracentrifuge	Optima L-70, Beckman-Coulter
	J2 MC, Beckman-Coulter
Vortexer	MS1 Minishaker, IKA
Vacuum pump	Mini-Vac E1, Axon Labs
Water purifier	Milli-Q RG, Millipore

4.2 Consumables

Electroporation cuvettes	*E.coli* Pulser Cuvette, Bio-Rad, Munich
Laboratory film	Parafilm M, American Can Company
Pasteur-Capillary- Pipettes	WU, Mainz
Plastic syringes	Discardit II, Becton Dickinson GmbH
Petri dishes	Greiner-Bio-One GmbH
Plastic material for cell culture	Sarstedt GmbH, Nümbrecht
Plastic material for cell culture	Falcon, Becton Dickinson GmbH, Heidelberg
Pipette Tips	Easy load, Greiner-Bio-One GmbH, Frickenhausen
Reaction tubes 1,5ml, 2ml, 15ml, 50ml	Sarstedt GmbH, Nümbrecht
PCR tubes	Sarstedt GmbH, Nümbrecht

4.3 Chemicals

The commonly used chemicals had analysis quality (p.A.) and have been sourced by the companies listed below:

Acros Organics, Geel (Belgien)
AppliChem GmbH, Darmstadt
Biomol Feinchemikalien GmbH, Hamburg
Bio Rad GmbH, Munich
Carl Roth KG, Karlsruhe
Difco Laboratories, Detroit Michigan (USA)
Fluka Feinchemikalien GmbH, Neu-Ulm
Gerbu Biotechnik GmbH, Gaiberg
Invitrogen (Gibco) GmbH, Karlsruhe
J.T. Baker, Deventer (Holland)
Merck AG, Darmstadt
Pharmacia Chemikalien GmbH, Dübendorf
Promega, Mannheim
Riedel de Haen Laborchemikalien AG, Seelze
Roche Diagnostics GmbH, Mannheim
Roth Chemikalien, Karlsruhe
Serva Feinbiochemika GmbH & Co., Heidelberg
Sigma-Aldrich GmbH, Seelze

4.4 Enzymes and recommended buffers

4.4.1 Enzymes

ß-Agarase	Fermentas, St. Leon-Roth
DNA Ligase T4	Fermentas, St. Leon-Roth 10 x T4 DNA Ligation buffer: 400 mM Tris-HCl (pH 7,8 at 25°C), 100 mM $MgCl_2$, 100 mM DTT, 5 mM dATP
DNA Polymerase I E.coli (Klenow fragment)	Fermentas, St. Leon-Roth 10 x Klenow reaction buffer: 500mM Tris-HCl (pH 8.0 at 25°C), 50mM $MgCl_2$, 10mM DTT.
DNA Polymerase T4	Fermentas, St. Leon-Roth 5 x Reaction Buffer: 335mM Tris-HCl (pH 8.8 at 25°C), 33mM $MgCl_2$ 5mM DTT, 84mM $(NH_4)_2SO_4$
DNA Polymerase *Taq*	Bioron, Ludwigshafen PCR-Puffer (10x): 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20, 25 mM $MgCl_2$ dNTP-Mix: 10 mM dATP, 10 mM dCTP , 10 mM dGTP, 10 mM dTTP in ddH_2O
DNA Polymerase *Pwo* (proofreading Taq)	Roche Diagnostics, Mannheim PCR-Puffer (10x) with $MgSO_4$: 100 mM Tris-HCl (pH 8.85, 20°C), 250 mM KCl, 50 mM $(NH_4)_2SO_4$,20 mM $MgSO_4$
Luciferase	Sigma, Seelze
Mung-Bean Nuclease	New England Biolabs, Frankfurt 10 x Mung bean buffer: 50 mM Sodiumacetate, 30 mM NaC1, 1 mM $ZnSO_4$ (pH 5.0)
Polynucleotide Kinase T4	Fermentas, St. Leon-Roth 10 x PNK buffer A: 500 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine and 1 mM EDTA
Proteinase K	Qiagen, Hilden
Reverse Transcriptase	Qiagen, Hilden Invitrogen, Karlsruhe

Restriction enzymes	Fermentas, St. Leon-Roth
	New England Biolabs, Frankfurt
RNase A	Roche Diagnostics, Mannheim
Shrimp alkaline phosphatise	Promega, Mannheim
	10X Reaction Buffer:
	100mM $MgCl_2$, 0.5M Tris-HCl (pH 9.0)

4.4.2 Reaction buffers for restriction endonucleases

4.4.2.1 Fermentas buffers

1X Buffer B (blue)
10mM Tris-HCl (pH 7.5 at 37°C)
10mM MgCl2
0.1mg/ml BSA

1X Buffer R (red)
Tris-HCl (pH 8.5 at 37°C
10mM MgCl2
100mM KCl
0,1mg/ml BSA

1X Buffer G (green)
10mM Tris-HCl (pH 7.5 at 37°C)
10mM MgCl2
50mM NaCl
0.1mg/ml BSA

1X Buffer Tango™ (yellow)
33mM Tris-acetate (pH 7.9 at 37°C)
10mM magnesium acetate
66mM potassium acetate
0.1mg/ml BSA

1X Buffer O (orange)
50mM Tris-HCl (pH 7.5 at 37°C)
10mM MgCl2
100mM NaCl
0.1mg/ml BSA

4.4.2.2 New England Biolabs buffers

1X NEBuffer 1
10 mM Bis-Tris-Propane-HCl
10mM MgCl2
1 mM DTT
pH 7.0 at 25°C

1X NEBuffer 3
100 mM NaCl
50 mM Tris-HCl
10mM MgCl2
1 mM DTT
pH 7.9 at 25°C

1X NEBuffer 2
50 mM NaCl
10mM MgCl2
10 mM Tris-HCl
1 mM DTT
pH 7.9 at 25°C

1X NEBuffer 4
50 mM potassium acetate
20 mM Tris-acetate
10 mM magnesium acetate
1 mM DTT
pH 7.9 at 25°C

4.5 Antibodies

Antibody	Source	Supplier
Anti-EGFP	Rabbit	Clontech
Anti-Flag	Mouse	Sigma Aldrich
Anti-Dyrk1a	Rabbit	Dusan Bartsch

4.6 Nucleic acids

4.6.1.1 Oligonucleotide- and sequencing primers

4.6.1.2 Plasmids

Name	Origin	Resistance
pBi-EGFP	Clontech, Heidelberg	Amp
pBelo BAC12 SB2	Tina Baldinger, ZMBH Heidelberg	Cm
pL253	N. Copeland, Frederick, USA	Amp
pL451	N. Copeland, Frederick, USA	Amp/Kan
pL452	N. Copleand, Frederick, USA	Amp/Kan
pUC 18	Stratagene, Heidelberg	Amp
pGemT	Promega, Mannheim	Amp
gWiz	GTS, San Diego, USA	Kan
pMCS5	Molecular Probes	Amp
pBlueskript II SK (+)	Stratagene, USA	Amp
pMC1-Cre	D. Bartsch	Amp
pCaggsFlpE	F. Stewart, TU Dresden	Amp/Puro

4.6.1.3 DNA marker

PFGE Low Range	New England Biolabs, Frankfurt
PFGE Mid Range I & II	New England Biolabs, Frankfurt
λ/2 HindIII ladder	Fermentas, St. Leon-Roth
1 Kb DNA-ladder	Fermentas, St. Leon-Roth
100bp ladder	Fermentas, St. Leon-Roth

4.7 Bacteria strains

DH5a	Invitrogen, Karlsruhe	Δ(lac)U169, endA1, gyrA46, hsdR17(r_K-m_K+), phi80, Δ(lacZ)M15, recA1, relA1, supE44, thi-1.
DH10B	Invitrogen, Karlsruhe	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15; Δ*lac*X74; *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G

EL250	Neil G. Copeland, National Cancer Institute, Frederick, USA	DH10B [_cl857 (*cro-bioA* < > *araC-P*$_{BAD}$*flpe*]
EL350	Neil G. Copeland, National Cancer Institute, Frederick, USA	DH10B [_cl857 (*cro-bioA* < > *araC-P*$_{BAD}$*cre*]

4.8 Cell lines

HeLa	Human cervix carcinoma cells	ATCC-LGCPromochem
HtTa	Helas with stabely integrated tTA construct	From Gossen, ZMBH, Heidelberg
PC12	rat adrenal pheochromocytoma cells	ATCC-LGCPromochem
R1	Mouse embryonic stem cells	From A. Nagy, Toronto, Canada

4.9 Mouse lines

TgN(EIIa-Cre)C5379Lmgd	Adenovirus EIIa promoter directs expression of Cre recombinase in preimplantation mouse embryos. This transgene expresses Cre recombinase in nearly all tissues	H.Westphal, NIH Bethesda, USA
Tg(ACTFLPe)9205Dym/J	Human ACTB promoter drives the expression of FLPe also in the germline	S.Dymecki, Harvard, USA
TA^{CAMK}-1/TgN(CamK2a-tTA)1Mmay)	αCamKII-promoter directs expression of the Tet-systsem Tansactivator tTA	M.Mayford, San Diego, USA
Camk2a-Cre	αCamKII-promoter directs expression of the Cre recombinase	K.Schoenig, ZI Mannheim
B6D2F1/Crl	C57BL6/N and DBA2 F1-hybrids for ES cell injection foster mothers and vasectomised males	Charles River, Sulzfeld
C57BL6/NCrl	Standard mouse line for backcross	Charles River, Sulzfeld

4.10 Buffers, media and solutions

Acrylamide stock solution	30% Acrylamide, 0.8% Bis
Antibiotics stock solutions:	
Ampicillin (1000x)	100mg/ml in H_2O
Carbenicillin (1000x)	100mg/ml in EtOH
Doxycyclin-hydrochloride (1000x)	1mg/ml in H_2O
Kanamycin (2000x)	25mg/ml in H_2O
Chloramphenicol (2000x)	25mg/ml in H_2O
D-MEM	Dulbecco´s modified Eagle Medium, Invitrogen GmbH, Karlsruhe with GlutaMAX I4500 mg/L D-Glucose Sodium-pyruvate
10xDNA-loading buffer	30 % (w/v) Glycerol 1 % (w/v) SDS 0,3 % (w/v) bromine phenol blue 50 mM EDTA 10 mM Tris-HCl; pH 7.5
Ethidium bromide	10 mg/ml in H_2O
FCS	Fetal calw serum, Invitrogen GmbH, Karlsruhe
In situ ß-Gal-staining solution	1 mg/ml X-Gal 2 mM $MgCl_2$ 5 mM $K4(Fe(CN)_6)$ 5 mM $K3(Fe(CN)_6)$
IPTG stock	400mM in H_2O
LB-agar	1.5 % (w/v) Bacto-Agar (Difco-Laboratories, Detroit, USA) in LB-medium
LB-medium	10 g Bacto-Trypton (Difco-Laboratories) 5 g yeast-extract (Difco-Laboratories) 10 g NaCl adjusted with NaOH to pH 7.5 ddH_2O ad 1000 ml
Mini-prep solutions	solution I: 50 mM glucose 25 mM tris-HCl, pH 8.0 10 mM EDTA

	solution II:
	0.2 M NaOH
	1% SDS
	solution III:
	3 M potassium acetate, pH 4.8
	(adjusted with glacial acetic acid)
Opti-MEM	serum reduced medium, Invitrogen GmbH, Karlsruhe
	with GlutaMAX I
	2400 mg/L sodium carbonat, HEPES, Sodium pyruvate, Hypoxathine, Thymidin, Growthfactors, 1.1 mg/L Phenolred
PBS(1x), pH 7.4	140 mM NaCl
	2.7 mM KCl
	1.5 mM K_2PO_4
	8.1 mM Na_2HPO_4 x $2H_2O$
TAE(1x)	Tris acetate EDTA:
	40 mM Tris-HCl, pH 8.3
	20 mM Sodium acetate
	2 mM EDTA
TBE(1x)	Tris borate/EDTA:
	90 mM Tris-HCl, pH7.8
	90 mM Boric acid
	3 mM EDTA
TE	Tris/EDTA:
	10 mM Tris-HCl pH 7.5
	1 mM EDTA
Trypsin-EDTA	Invitrogen GmbH, Karlsruhe
	0.05% Trypsin
	0.53 mM EDTA x 4Na
YENB	0.75% Bacto yeast extract
	0.8% Bacto nutrient broth
APS	10% (w/v) Ammoniumpersulfat in water
SDS separating Tris buffer (1x)	1.5 M Tris-HCl, pH 8.8
	0.4% (w/v) SDS
SDS stacking Tris buffer (1x)	0.5 M Tris-HCl, pH 6.8
	0.4% (w/v) SDS
SDS electrophoresis buffer (10x)	23 mM Tris-HCl, pH8.8
	0.19 M Glycine
	0.2% (w/v) SDS

Western Blot buffer (10x)	23 mM Tris-HCl, pH8.8 0.19 M Glycine
TBS (10x)	20 mM Tris-HCl, pH 7.4 150 mM NaCl
TBST (1x)	1x TBS + 0.1% Triton X-100
Blocking solution	1x TBST + 5% (w/v) BSA
SG/PBS	0.01% saponin, 0.2% gelatine, 0.02% sodium azide in PBS

5 Methods

5.1 Cultivation of Bacteria

E.coli was routinely cultivated in an aerobic environment at 37°C. The liquid cultures were cultivated in autoclaved small glass tubes or in Erlenmeyer flasks at 37°C by shaking at 180rpm. For short-term storage or the retrieval of single colonies, bacterial suspensions were spread on LB Agar plates containing the appropriate antibiotic and were incubated over night at 37°C. The plates were stored for up to 2 month at 4°C. For long-term storage, one volume of a log-phase bacterial culture was mixed with one volume of autoclaved 50% Glycerin in water and stored at -20°C in a freezer.

5.2 Cell densitometry

The cell density was measured with a spectrophotometer at a wavelength of 600nm. LB-medium served as the reference solution.

5.3 Transformation of Bacteria

5.3.1 Generation of competent *E.coli* cells for electroporation

E.coli cells naturally do not efficiently absorb surrounding nucleic acid. The uptake of DNA can be increased, making the *E.coli* cells "electrocompetent" by the following method:

To convert *E.coli* in electrocompetent cells 5 ml YENB-Medium are inoculated with a -70°C single colony stock solution and incubated overnight at 37°C. 2 ml of this overnight culture are used to inoculate 1 liter of YENB-Medium which is subsequently incubated overnight at 37°C. At an OD_{600} value of 0.9 the bacteria culture is incubated for 5 min on ice and centrifuged for 10 min at 4.000 x g at 4°C. The supernatant is discarded. The bacteria pellet is washed twice with ice cold ddH_2O and centrifuged as described above. Afterwards the pellet is resuspended in 20 ml H_2O containing 10% glycerol and centrifuged. The supernatant is discarded. Finally bacteria pellet is resuspended in 2-3ml cold H_2O containing 10% glycerol.

The cell count of the final solution is supposed to be $2\text{-}4x10^{10}$ cells / ml. These electrocompetent cells are aliquoted in pre chilled tubes, frozen in liquid nitrogen and stored at – 80°C. The transformation efficiency ought to be $10^8\text{-}10^9$ colonies/µg plasmid used for the transformation reaction.

5.3.2 Electroporation of competent E.coli cells

When electrocompetent bacteria and plasmids are mixed together, the plasmid can be transferred into the cell by using an electric pulse to open the cell wall and membrane. Several hundred volts are typically used in this process. Afterwards, the cells have to be handled carefully as cell walls have been perforated by the electric pulse. Following a general electro-transformation protocol is described:
50µl of the electrocompetent *E.coli* cells are thawed on ice and mixed with 1-2 µl DNA (ligation-) solution. This mixture is transferred into a pre-chilled electroporation cuvette (0.1 cm electrode gap). The electroporation is carried out at 1250 V, 125 Ω, 50 µF. Immediately after the electroporation 1 ml LB-medium (without antibiotic) is added and the mixture is incubated for 1 hour at 37°C to ensure that the resistance gene expression can take place. Following a certain volume of the bacteria solution is plated on LB-agar plates containing the corresponding antibiotic.

5.4 Isolation and purification of DNA

5.4.1 Isolation of plasmid DNA

Transformed bacteria can be analyzed for the uptake of the correct plasmid by amplification of individual colonies and extraction of the DNA. In the following a quick method to isolate plasmid DNA is illustrated:

- 5 ml LB-medium containing 100 µg/ml ampicillin is inoculated by a single colony. Incubate the mixture at 37°C overnight.
- 1.5 ml overnight culture is transferred into a 1.5 ml reaction tube and centrifuged for 5 min in a table centrifuge at 9000 rpm. The supernatant is carefully discarded.
- The bacteria pellet is resuspended in 100 µl miniprep. solution I (containing RNase) and incubated at room temperature for 5 min.
- 200µl miniprep. solution II is added, mixed gently by inverting the tube five times and incubated for 2-3 min.
- 150 µl ice cold miniprep. solution III is added, mixed gently by inverting the five times and incubated for 5 min on ice.
- The sample is centrifuged in a table centrifuge for 15 min at maximum speed.
- The supernatant is transferred in a clean microcentrifuge tube and the nucleic acid is precipitated by adding 1 ml 100% ethanol for 30 min at – 20°C.

- The precipitated DNA is centrifuged for 10 min at maximum speed at 4°C.
- The precipitated DNA is washed with 0.5 ml 70%ethanol and centrifuged 15 min at maximum speed.
- The supernatant is carefully discarded.
- The dried DNA pellet is solved in 50 µl TE buffer

The detergent solution (miniprep. solution II) given to the bacteria cells causes a cell lysis followed by an alkaline denaturation of the nucleic acid. Subsequent the pH-value is decreased (as a result of miniprep. solution III) to renaturate the nucleic acid. Within this process the chromosomal E.coli DNA renaturates intermolecular by forming a highly complex structure while the plasmid DNA renaturates intramolecular. Proteins are denaturated via SDS (miniprep. solution II). Membrane particles, denaturated proteins and chromosomal DNA can be separated from plasmid DNA and RNA by centrifugation.

5.4.2 Isolation of plasmid DNA on a large scale

Within this thesis the preparative purification of plasmid DNA from bacteria cultures was carried out with the Plasmid DNA Purification Kit NucleoBond PC 500 (MACHEREY-NAGEL GmbH), which employs a modified alkaline/SDS lysis procedure to prepare the bacterial cell pellet for plasmid purification. Both chromosomal and plasmid DNA are denatured under these alkaline conditions. Potassium acetate is then added to the denatured lysate, which causes the formation of a precipitate containing chromosomal DNA and other cellular compounds. The potassium acetate buffer also neutralizes the lysate. Plasmid DNA, which remains in solution, can revert to its native supercoiled structure. After equilibrating the NucleoBond column with equilibration buffer, plasmid DNA is bound to the anion-exchange resin and finally eluted after efficient washing of the column. After precipitation of the eluted DNA it can easily be dissolved in TE buffer for further use.
The general procedure has been fulfilled as follows:

- Centrifuge up to 500 ml overnight culture at 4,500 - 6,000 x g for 15 min at 4°C.
- Carefully resuspend the pellet of bacterial cells in 12 ml buffer S1 + RNase A.
- Add 12 ml buffer S2 to the suspension. Mix gently by inverting the tube 6-8 times. Incubate the mixture at room-temperature (20-25°C) for 2–3 min (max. 5 min). Do not vortex, as this will release contaminating chromosomal DNA from cellular debris into the suspension.

- Add 12 ml pre-cooled buffer S3 (4°C) to the suspension. Immediately mix the lysate gently by inverting the flask 6-8 times until a homogeneous suspension containing an off-white flocculate is formed. Incubate the suspension on ice for 5 min.
- Place a NucleoBond folded filter in a small funnel for support, and prewet the filter with a few drops of buffer N2 or sterile deionized H2O. Load the lysate onto the wet filter and collect the flow-through.
- Equilibrate a NucleoBond column with 6 ml buffer N2. Allow the column to empty by gravity flow. Discard flow-through.
- Load the cleared lysate from onto the column. Allow the column to empty by gravity flow.
- Wash the column with 32 ml buffer N3. Discard flow-through
- Elute the plasmid DNA with 15 ml buffer N5.
- Add 11 ml room-temperature isopropanol to precipitate the eluted plasmid DNA. Mix carefully and centrifuge at ≥ 15,000 x g for 30 min at 12°C. Carefully discard the supernatant.
- Add 5ml room-temperature 70% ethanol to the pellet. Vortex briefly and centrifuge at ≥ 15,000 x g for 10 min at room-temperature (20-25°C).
- Carefully remove ethanol from the tube with a pipette tip. Allow the pellet to dry at room-temperature (20-25°C), at least for the indicated time.
- Redissolve pellet in an appropriate volume of buffer TE.

5.4.3 Isolation of genomic DNA from tail tissue of the mouse

The Qiagen DNeasy 96 Tissue Kit provides the fastest and easiest procedure for high-throughput purification of total genomic DNA from rodent tails and animal tissues. The DNeasy 96 procedure is ideal for simultaneous processing of 96 samples. The eluted DNA is ready for use in any downstream application, including PCR, Southern blotting, and other enzymatic reactions. DNA can be safely stored at –20°C for later use. The purified DNA is free of proteins, nucleases, and other contaminants and enzyme inhibitors. Routinely a 0.5cm long tail biopsy of 2-3 weeks old mice is cut by a sterile scalpel to purify the whole genomic DNA.
The general procedure has been fulfilled as follows:

- A 0.5 cm mouse tail sample was cut and placed into a collection microtube.
- 180µl Buffer ATL and 20µl Proteinase K solution were added and mixed by vortexing

- The sample was incubated at 55°C overnight or until the sample was completely lysed
- The collection tube was shaken vigorously and spun shortly down in a microfuge
- 400µl buffer AL/E was added, mixed well and spun shortly down
- The supernatant was carefully transferred to the DNeasy 96 Plate and centrifuged for 10min at 6000rpm.
- 500µl Buffer AW1 was added to each well and centrifuged for 5min at 6000rpm.
- 500µl buffer AW2 was added to each well and centrifuged for 5min at 6000rpm.
- The DNeasy 96 plate was dried at 70°C for 15 min
- The genomic DNA was eluted with 250µl of pre-warmed elution buffer AE
- The eluted DNA was stored at -20°C

5.4.4 Phenol extraction of DNA

To eliminate proteins in a liquid nucleic acid sample the same volume of equilibrated phenol is added to the solution. The two solutions are mixed consistently for 2-3 min and afterwards separated via centrifugation (table centrifuge, 5 min, 13000 rpm). Following centrifugation, the mixture separates into a lower phenol-chloroform phase, an organic interphase and a colorless upper aqueous phase. DNA remains exclusively in the aqueous phase. . If required the phenol extraction of the upper phase can be repeated. For the following use of the DNA solution it is necessary to eliminate phenol contamination. For this purpose the same volume of a Chloroform/Isoamyl alcohol (24:1) mixture is added to the contaminated DNA solution, mixed and centrifuged (as indicated above). The upper aqueous solution contains the DNA which might be precipitated with ethanol (see below).
A faster way to remove the proteins from a DNA solution (if there is little protein contamination or less demand for a high quality standard of the DNA solution) is carried out by adding one volume of Phenol / Chloroform / Isoamylalcohol (at a ratio of 25:24:1; equilibrated with 100 mM Tris-HCl to pH 8) to the aqueous DNA solution. Subsequently the solution is mixed thoroughly for 1 min and centrifuged at 13.000 rpm; 10 min; 4°C. The upper aqueous phase contains the purified DNA.

5.4.5 Photometric analysis of concentration and purity of nucleic acids

The nucleic acid concentration of an aqueous solution is due to the Beer-Lambert law directly proportional to its extinction and absorption, respectively (indicated as the optical density OD value). This relation is utilized to quantify the nucleic acid con-

centration spectrophotometrically. The maximum absorption of nucleic acid is at 260 nm whereas proteins have their maximum absorption at 280 nm. The absorption/concentration relations are:

- 1 OD_{260} = 50 µg/µl ds DNA
- 1 OD_{260} = 40 µg/µl ss RNA
- 1 OD_{260} = 30 µg/µl ss oligo nucleotides

Therefore the DNA concentration in µg/µl of an unknown DNA sample equals its OD_{260}, multiplied by 50 and any dilution factors. The purity of a DNA solution is given by the OD_{260}/OD_{280} ratio, which is supposed to be between 1.8 and 2.0 for a pure DNA solution without protein-contamination. All DNA solutions used in the experiments of this thesis had a value between 1.8 and 2.0.
By using the NanoDrop ND-1000 Spectrophotometer technology there is no need for cuvettes and other sample containing devices. To quantify the nucleic acid concentration, the 1µl sample is pipetted directly onto the measurement pedestal. The measurement is initiated using the NanoDrop software on the PC. A concentration from 2 to 3700 ng/µl is measurable without dilution

5.4.6 DNA extraction from agarose gels

Following Agarose gel electrophoretic separation of a DNA sample the particular DNA band of interest is excised from the agarose.
For DNA extraction of 40-bp to 50-kb DNA fragments from 0.3-2% standard agarose gels in TAE buffers the Qiaex II Kit (Qiagen), was used according to the manufacturer's instructions listed below. Purification of DNA fragments with the QIAEX II system is based on solubilization of agarose and selective adsorption of nucleic acids onto QIAEX II silica-gel particles in the presence of chaotropic salt. QIAEX II separates DNA from salts, agarose, PAA, dyes, proteins, and nucleotides.
User manual:

- The DNA band from the agarose gel was excised with a clean, sharp scalpel. The size of the gel was minimized by removing excess agarose. A 1.5-ml microfuge tube was used for processing up to 250 mg agarose.
- The gel slice was weighed in a colorless tube. 3 volumes of Buffer QX1 were added to 1 volume of gel for DNA fragments 100 bp - 4 kb; otherwise:

DNA fragments < 100 bp	Add 6 volumes of Buffer QX1
DNA fragments > 4 kb	Add 3 volumes of Buffer QX1 plus 2 volumes of H_2O
> 2% or Metaphor agarose gels	Add 6 volumes of Buffer QX1

- QIAEX II was resuspended by vortexing for 30 sec. QIAEX II was added to the sample according to the table below and mixed.

< 2 µg DNA	Add 10 µl of QIAEX II
2-10 µg DNA	Add 30 µl of QIAEX II
Each additional 10 µg DNA	Add additional 30 µl of QIAEX II

- The sample was incubated at 50°C for 10 min to solubilize the agarose and bind the DNA. It was mixed by vortexing* every 2 min to keep QIAEX II in suspension. The color of the mixture was checked that it is yellow.
- If the color of the mixture was orange or purple, 10 µl 3M sodium acetate, pH 5.0 was added, and mixed. The color should turn to yellow. The incubation was then continued for an additional 5 min at least.
- The adsorption of DNA to QIAEX II particles is only efficient at pH 7.5. Buffer QX1 contains a pH indicator which is yellow at pH 7.5, and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.
- The sample was centrifuged for 30 sec and the supernatant was carefully removed with a pipette.
- The pellet was washed with 500 µl of Buffer QX1 and the pellet was resuspended by vortexing*. The sample was centrifuged for 30 sec and all traces of supernatant were removed with a pipette. This wash step removed residual agarose contaminants.
- The pellet was washed twice with 500 µl of Buffer PE and was resuspended by vortexing*. The sample was centrifuged for 30 sec and carefully all traces of supernatant were removed with a pipette. These washing steps removed residual salt contaminants.
- The pellet was air-dried for 10-15 min or until the pellet became white. If 30 µl of QIAEX II suspension was used, the pellet was air-dried for approximately 30 min. Do not vacuum dry, as this may cause overdrying. Overdrying the QIAEX II pellet may result in decreased elution efficiency.
- To elute DNA, 20 µl of 10 mM Tris-HCl, pH 8.5 or H_2O were added and the pellet was resuspended by vortexing*. The sample was incubated according to the table below.

DNA fragments < 4 kb	Incubate at room temp. for 5 min
DNA fragments 4-10 kb	Incubate at 50°C for 5 min
DNA fragments > 10 kb	Incubate at 50°C for 10 min

- Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, the pH should also be within this range and the DNA was stored at -20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) but the EDTA may inhibit subsequent enzymatic reactions.
- The sample was centrifuged for 30 sec and the supernatant was carefully pipetted into a clean tube. The supernatant now contained the purified DNA.
- For fragments larger than 10 kb, the pellet was resuspended by inverting and flicking the tube because vortexing can cause shearing of large DNA fragments.

5.4.7 DNA purification

The cleanup of DNA fragments from primers, nucleotides, enzymes or salts of PCR and other enzymatic reactions might be crucial for subsequent experiments. For this purpose the PeqLab E.Z.N.A. Cycle-Pure kit was used. The corresponding protocol is listed below:

- Add 4-5 volumes of CP-buffer to the reaction batch and mix (for DNA fragments < 200 bp add 6 volumes CP-buffer, for DNA fragments > 4 kbp add 3 volumes CP-buffer plus 1 volume of ddH_2O).
- Place a HiBind-centrifuge column in a 2 ml collection tube and apply the mixture (reaction batch plus CP-buffer) to the column.
- Centrifuge collection tube with the column for 1 min at 10.000 x g and room temperature. Discard the flow-through and place the column back in the same collection tube.
- To wash, add 750 µl of completed DNA-wash buffer (buffer concentrate plus 1.5 volumes absolute ethanol) to the column and centrifuge 1 min at 10.000 x g and room temperature. Discard the flow-through and place the column back in the same collection tube. Repeat the washing step once.
- To dry the column completely centrifuge the column for an additional 1 min at maximum speed

- To elute DNA place the column in a clean 1.5 ml microcentrifuge tube, add 50-100 µl ddH_2O or TE- buffer to the center of the matrix and centrifuge the column for 1 min at 10.000 x g and room temperature.

5.5 Analysis and modification of DNA

5.5.1 Fragmentation of DNA with restriction enzymes

Restriction endonucleases recognize specific DNA sequences. These sequences usually cover four to eight base pairs, which are characteristic for each enzyme. Once a restriction enzyme recognizes its specific DNA sequence, the phosphodiester bond is hydrolyzed whereby the DNA fragment is cleaved at this particular position. Depending on the restriction endonuclease, this enzymatic reaction generates blunt end or cohesive end termini. The enzymatic activity is indicated in units (u), whereupon 1 u corresponds to the amount of enzyme needed for the digestion of 1 µg DNA (depending on the assay: λ phage DNA or adenovirus DNA) in one hour. In general these enzymes are used to cut circular DNA molecules, e.g. to drop out defined parts of a plasmid. The restriction reactions were conducted in the recommended buffer and incubated for at least two hours at 37°C. In addition it is recommended, that the volumetric content of enzyme does not exceed 10% of the final volume since glycerin is major component of the enzyme solution and inhibits the enzymatic activity if its concentration is higher then 5%. More over if restriction endonucleases are used under extreme non-standard conditions (such as to many units, to long incubation time or eventually the use of a not recommended buffer), they might be capable of cleaving sequences which are similar but not identical to their defined recognition sequence, termed “star activity”.

A typical analytical restriction digest reaction was composed as follows:

1 µg DNA in x µl TE
2 µl 10x restriction buffer
1-10 U restriction endonuclease
ddH_20 ad 20 µl

After an appropriate incubation-time the generated DNA fragments can be separated via agarose-gel-electrophoresis.

5.5.2 Gelelectrophoretic analysis of nucleic acids

Agarose gel electrophoresis can be used to separate DNA strands by their size. By comparison of fragments of known length it is possible to determine the size of the separated strands. The principle of this method is similar to sifting molecules through a sieve; here an electric field is used to drag the negatively charged DNA molecules through the gel matrix. Shorter DNA molecules move faster than the longer ones since they are able to slip through the gel more easily. The DNA fragments in the gel can be visualized by staining with ethidium bromide (EtBr). EtBr is a fluorescent dye which intercalates between bases of the DNA and is excited by light of a wavelength around 300 nm. It is often incorporated into the gel but the gel can also be stained after electrophoresis by soaking in a solution of ethidium bromide.

5.5.2.1 Separation of DNA on Agarose gels

To make an agarose gel the agarose (~1-2%) is dissolved in 1x TAE buffer and boiled in a microwave oven until the agarose completely dissolves. After the solution cools down to about 60°C ethidium bromide is added to a final concentration of 0.5 µg/ml. The agarose solution is poured into a horizontal gel-electrophoresis device containing a gel-comb with pockets of appropriated size. Upon the gel becomes solid the comb is removed and the gel chamber is filled with TAE (1X) buffer. Subsequently the lanes are loaded with the DNA sample together with loading buffer. Alternatively 1x TBE buffer can also be used.
The negatively charged DNA is separated according to its size in an electric field (~100 V; 45 min). The agarose gel electrophoresis allows DNA separation from about 100 bp to several 1000 bp depending on the agarose concentration of the gel. In order to determine weight and size of the DNA sample it is necessary to apply a molecular DNA marker (100 bp / 1 kb DNA ladder, Fermentas GmbH) on a separate lane in the same gel. The ethidium bromide in the gel is used to visualize the DNA fragments via ultraviolet irradiation (wavelength 302nm).

5.5.2.2 Separation of high-molecular DNA by PFGE (Pulsed Field Gel Electrophoresis)

Pulsed field electrophoresis is a technique for resolving chromosome size DNAs. Conventional electrophoresis does not permit resolution of DNA fragments larger than 50,000 base pairs. By alternating the electric field between spatially distinct pairs

of electrodes, DNAs on the order of 10 megabases are able to reorient and move differentially through the pores in an agarose gel. The BioRad CHEF-DR III system separates large and small DNA fragments with better resolution, speed, and accuracy, than initial pulsed field methods. The system uses two leading technologies, CHEF (Clamped Homogeneous Electric Fields) and PACE (Programmable Autonomously Controlled Electrodes). The system provides highly uniform, or homogeneous, electric fields within the gel, using an array of 24 electrodes, which are "clamped" or held to intermediate potentials to eliminate lane distortion. Like in normal gelelectrophoretic separation of DNA different buffers can be used. Routinely 0.5 x TBE is used. The migration rate of DNA molecules through an agarose gel is dependent on pulse time, voltage (field strength), pulse angle and run time.

Switch interval:

In pulsed field electrophoresis, DNA molecules are subjected to alternating electric fields imposed for a period of time called the switch time. Each time the field is switched, the DNA molecules must change direction or reorient in the gel matrix. Larger molecules take longer to reorient and have less time to move during each pulse, so they migrate slower than smaller molecules. Resolution will be optimal for DNA molecules with reorientation times comparable to the pulse time. So, as the DNA size increases, the pulse time needs to increase to resolve the molecules. Under some conditions, larger molecules sometimes run ahead of smaller ones.

Voltage (Field Strength):

DNA migration increases with increases in voltage or field strength. However, greater migration is accompanied by decreased band sharpness. In general, as the size of the DNA molecules increases, the field strength should decrease. At high field strengths (6 V/cm) some very large DNA (>3 Mb) cannot be resolved on the gel and the field strength must be reduced. Moreover, some large DNA molecules will not enter the gels at high field strengths. Therefore, in selecting the field strength for an experiment, a compromise between run time and resolution has to be made.

Field Angle:

The system allows separations to be carried out with electric field vectors oriented in any direction in the plane of the gel (90°–120°). With two field vectors, resolution of DNA molecules up to 1 mb is independent of the angle between them. It has been shown that decreasing the included angle from120° to 94° increases the velocity of the DNA, with the mobilities of large DNAs (>1 mb) affected to a greater degree by the change in angle than are smaller DNAs (<1 mb). Decreasing the included angle

will decrease the resolution of smaller DNAs by causing them to pile up on each other. This same effect on small DNA can be seen with long switch times. It is recommended that the included angle be decreased (<120°) when separating large DNA molecules greater than 2 mb.

For routine separation of BAC mini-preps the following settings are used:

- Run time: 15h
- Voltage: 6V/cm
- Field angle: 120°
- Switch interval: 1-15 sec
- Temperature: 14°C

5.5.3 Staining of nucleic acid agarose gels with ethidium bromide

Nucleic acids can be visualized in agarose- and PAA-gels by staining with ethidium bromide (EtBr). EtBr intercalates in nucleic acids and can emit light after excitation with ultraviolet light (optimal excitation wavelength is 254nm). The gel is stained for 10-30min in an EtBr solution (1µg/ml). Unspecific bound EtBr should be removed by washing the gel 2 times for 10min in water. The nucleic acids can then be visualized on a UV gel documentation system

5.5.4 Oligonucleotide annealing

Single stranded DNA oligonucleotides with complementary DNA sequences can hybridize to double stranded DNA. For this purpose a 2 µl aliquot of each oligonucleotide (100 mM) is added to 10 µl 1x TE, 2 µl KCl (3M), and 24 µl dH_2O. The sample is vortexed and incubated at 95°C in a thermo block for 3 minutes. The thermo block is then switched off, so that the block cools slowly down to room temperature (takes about 1 hour), allowing the oligonucleotides to anneal. The sample can be used immediately for a ligation reaction (see below), or stored at -20°C for future use. It has to be considered that in general the 5´termini of synthesized oligonucleotides are not phosphorylated; consequently the 5´ termini of a vector used in a ligation reaction together with the synthetic oligonucleotide should not be dephosphorylated since otherwise ligation can not occur.

5.5.5 Ligation of DNA fragments

The formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA is catalyzed by DNA ligases. The commonly used enzyme is the T4 DNA ligase, which is able to join blunt end and cohesive end termini. The enzymatic reaction only takes place in the presence of sufficient ATP, and a free 5´phosphate group. The enzymatic activity is indicated in Weiss units (u),

whereupon 1 u is equivalent to approximately 200 cohesive-end ligation units. One cohesive–end ligation unit corresponds to the amount of enzyme required to ligate 50% of *Hind*III fragments of λ DNA in 30 min at 16°C in 20 µl of the assay mixture and a 5′-DNA termini concentration of 0.12 µM. For a general standard cloning ligation purified vector and insert are combined in a molar ratio of 1 : 3, using the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \quad X \text{ insert:vector molar ratio} = \text{ng of insert}$$

A typical ligation reaction was composed as follows:

60 ng of a 1 kb insert DNA-fragment
60 ng of a 3 kb vector-DNA fragment (backbone)
1 µl 10 x ligation buffer
1 µl T4-DNA-Ligase (1 Weiss unit / µl)
ddH_2O ad 10 µl

The reaction is incubated either for one hour at 22°C or overnight at 16°C. Subsequently the enzymatic reaction is stopped by 10 min incubation at 65°C.

5.5.6 Blunting of dsDNA molecules with 5′- or 3′-protruding ends

If vector and insert used for a ligation reaction have not been digested with corresponding restriction endonucleases it might be necessary to blunt the 5′- or 3′- protruding termini. The T4 DNA polymerase catalyzes 5′→3′synthesis of a primed single-stranded DNA template and has a 3′→5′ exonuclease activity. It can therefore be used to fill 5′ protruding ends with dNTPs; alternatively due to its exonuclease activity the T4 DNA polymerase can be used for the generation of blunt ends from DNA molecules with 3′ overhangs. One unit of enzyme catalyzes the incorporation of 10 nmol of dNTPs into a into a polynucleotide fraction in 30 min at 37°C. In general the reaction mixture is incubated for 20 min at 11°C (alternatively 5 min. at room temperature) whereupon the reaction is stopped by a 10 min taking incubation at 65°C.

To degrade a single-stranded 3′overhang, the reaction mix was prepared as follows:

4µl 5xT4 DNA polymerase reaction buffer
1µg of digested DNA
1U of T4 DNA polymerse
ddH_2O ad 20 µl

To catalyze 5′→3′synthesis of a primed single-stranded DNA template, 1 µl dNTP (2 mM) has to be added to the reaction mix.

5.5.7 Dephosphorylation of 5′phosphates from DNA

By removing phosphate groups from both 5′-termini recircularization and religation of linearized cloning DNA fragments is prevented. For this purpose the shrimp alkaline phosphatase (SAP) is used. One unit of enzyme is the amount required to catalyze the hydrolysis of 1 µmol 4-nitrophenyl phosphate per minute at 37°C. SAP is completely and irreversibly inactivated by heating at 65°C for 15 minutes.
A typical "dephosphorylation reaction" was composed as follows:

1µg of DNA
3µl of 10xSAP buffer
1U SAP
ddH_2O ad 30 µl

The reaction mixture is incubated for 15 min at 37°C, subsequently the reaction is stopped by a 15 min incubation at 65°C. Alternatively, after a restriction digest of e.g. a plasmid one can just add 1µl of SAP to the restriction mixture because SAP is also active in almost all 1x restriction buffers.

5.5.8 Polymerase chain reaction – PCR

The Polymerase Chain Reaction (PCR) is a method for amplifying DNA without using a living organism, such as *E.coli* or yeast. The DNA fragment to be amplified is specified by selecting primers. Primers are short, synthesized single stranded oligonucleotides that are complement (at the least 12-15 nucleotides) to the 5′and 3′ end of the DNA fragment to be amplified. They *anneal* to the DNA template by hydrogen bonds and thus define the starting and ending points of the amplification. DNA-Polymerase binds to the primed single-stranded DNA template and catalyzes the 5′→3′ synthesis of the new DNA strand. The length of the primers and their melting

temperature is depended on various conditions (such as the C/G content within the sequence of the primer). The melting temperature of a primer is defined as the temperature below which the primer will anneal to the DNA template and above which the primer will *dissociate* (break apart) from the DNA template. The melting temperature increases with the length of the primer and its C/G content, since these bases generate three hydrogen bonds whereas A/T bases only build up two. Primers that are too short would anneal at unspecific positions which would result in non-specific copies. On the other hand, the length of a primer is limited by the temperature required to melt it. Melting temperatures that are too high, i.e., above 80°C, can also cause problems since the DNA-Polymerase is less active at such temperatures. The optimum length of a primer is generally from thirty to forty nucleotides with a melting temperature between 60°C and 75°C.

The PCR process consists of a series of twenty to thirty five cycles. Each cycle comprises three steps. First, the double-stranded DNA has to be heated to 94°C in order to separate the strands. This step called *melting* breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often melted for an extended time (3 to 7 min) to ensure that both the template DNA and the primers have completely separated and are now single-stranded only. Afterwards, the temperature is lowered so the primers can attach themselves to the single DNA strands. The temperature of this step called *annealing* is usually 5°C below the melting temperature of the primers. Finally, the DNA-Polymerase has to fill in the missing strands. It starts at the 3´-OH-end of the annealed primer and works its way along the DNA strand. This step is called *elongation.* The elongation temperature depends on the DNA-Polymerase (68-72°C). The time for this step depends both on the DNA-Polymerase itself and on the length of the DNA fragment to be amplified. The PCR run is completed by a single 3-7 min taking incubation at elongation temperature.

A typical PCR reaction was composed as follows:

~50-200ng	Template DNA
5µl	10 x PCR buffer complete (+25mM MgCl2)
0,5µl	dNTP-mix (10mM each)
0,25µl	5´ Primer forward (100pM)
0,25µl	3´ Primer reverse (100pM)
0,15µl	Taq-Polymerase (5U/µl)
Ad 50µl with ddH20	

The reaction was mixed together on ice in special PCR reaction tubes. Following the "PCR run" an aliquot of the reaction was analyzed in respect of size and amount via agarose gel electrophoresis.

5.5.9 Colony PCR

Purified DNA is most often used as a template in the PCR reaction. However, it is possible to amplify specific DNA sequences without DNA purification by starting with a single living *E.coli* colony. This technique is known as colony PCR and provides a powerful and reliable method for the rapid detection of transformation success when primers are available which allow determination of correct ligation products by size or hybridization.
Apart from the DNA template, the PCR reaction mix (PCR-buffer, dNTPs, primers and *Taq*-DNA polymerase) is similar to the standard protocol. To inoculate the PCR reaction a colony is picked with a sterile pipette tip and dabbed into the PCR tube and then placed in a correspondingly labeled culture tube. Subsequently the PCR reaction mix is added to the reaction tube and the PCR is carried out with appropriate conditions for the used primers and expected product. It is required to begin the PCR protocol with an extended time at 95°C, (e.g. 5 minutes) to ensure that complete lysis of the bacterial cells has occurred.

5.5.10 Sequencing of DNA

With the methods of sequencing which were developed in the late seventies, sequences of cleaned DNA-fragments could be quickly and easily specified. During the last 20 years, sequencing had been continuously automated. During the enzymatic sequencing, the DNA to be sequenced is multiplied with a synthesis-reaction. This method takes part in two steps. First the **labeling-reaction** in order to produce a labeled source sample and second the **termination-reaction** where the synthesis of the fragments produced in the first reaction is ended by incorporation of fluorescence-labeled dideoyxnucleotides (ddNTPs). The sequencing reaction products were ethanol precipitated and the sequencing analysis was carried out with an ABI Prism 3130 xl Sequencer (Applied Biosystems). The laser detects the sequence of the fluorescence-labeled DNA fragments which were separated by capillary gel electrophoresis.
A typical sequencing reaction was composed as follows:

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems):

Terminator Ready Reaction mix	8µl
Template	150ng Plasmid or BAC DNA; 10-50ng PCR product
Primer	3.2 pmol
ddH2O	Ad 20µl

5.5.11 Southern Blot

Southern blotting was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s. DNA fragments are separated through gel electrophoresis and are then transferred onto a membrane and immobilized. By hybridizing the membrane with a 32P-labelled DNA probe, DNA fragments can be localized which contain a complementary region to the probe. In my experiments I used the PosiBlot 30-30 pressure blotter from Stratagene. The PosiBlot 30-30 pressure blotter is part of an integrated system designed to transfer DNA or RNA from agarose gel quickly and efficiently. The DNA was digested with suitable restriction enzymes and then separated by agarose gel electrophoresis (0.8% agarose in 1xTAE buffer). Following electrophoresis the agarose gel was stained with ethidium bromide (5µg/ml) and photographed with a ruler on the side. For depurination, the gel was treated with 0.25N HCl for 30 minutes with gentle shaking (the bromphenol blue dye should turn green at the end of the HCl treatment). Then the DNA was denatured by pouring off the HCl and by addition of the 0.5 N NaOH and 1.5 M NaCl denaturation solution. The gel was treated for ~30 minutes with gentle shaking. Subsequently the denaturation solution was discarded and the gel was neutralized with 1M Tris-HCl (PH 7.5) and 1.5 M NaCl between 30 minutes and 1 hour with gentle shaking. The transfer of the gel onto the membrane was made according to the protocol of Stratagenes PosiBlot manual. In brief the:
The cellulose sponge serves as a buffer reservoir and has to be cut to a final size 1.5-2 cm larger than gel on all four sides. It has to be rehydrated with transfer buffer before use. The plastic mask was cut at least 0.3 cm smaller than the rectangle of the gel to be blotted. The transfer membrane was cut 1-2 cm larger than the window in the mask. The membrane was prewetted in distilled water and than soaked 5 minutes in transfer buffer. The PosiBlot was assembled according to the manual and connected to the Pressure control station. The pressure was adjusted to 75 mm Hg and the gel

was blotted for 1 hour. Subsequently the membrane was UV-crosslinked for 30 seconds in the Stratalinker UV crosslinker and dried for 1 hour at 80°C.

Prehybridization:

The membrane with the immobilized DNA was incubated for 3 hours with prewarmed 60°C hybridization solution in hybridization roles in the hybridization oven.

Probe labeling:

The DNA probe (100-1000bp) was generated through digest of a plasmid or PCR and was purified through gel electrophoresis and gel extraction. According to the protocol of the manufacturer for radioactive labeling with ^{32}P-dATP (20µCi/µl, Amersham Pharmacia Biotech Europe), 100ng of purified DNA served as a template for the Fermentas HexaLabel Plus DNA labeling Kit. The unincorporated Nucleotides where separated with the Roche Nucleotide removal Kit.

Hybridization:

The ^{32}P-labelled DNA probe was boiled for 5 minutes and immediately added to the prehybridized membrane (~8x10^5 cpm/ml hybridization solution) and incubated over night at 60°C. The next day the membrane was washed several times with washing buffer until the membrane was only weakly radioactive (measured by hand counter below 50 counts per second). The membrane was then dried on a whatman paper and wrapped into plastic foil. The detection of radioactive signals was subsequently performed through autoradiography with an X-ray film (Hyperfilm, Amersham Pharmacia Biotech) in a -80°C freezer for 1-14 days.

5.6 *In vitro* modification and recombination in bacteria

Modification of large genomic DNA fragments e.g. in BACs or PACs is normally very time-consuming and difficult because the strategies for cloning a suitable targeting vector with conventional DNA modification enzymes like restriction enzymes and ligases is very limited. A newer and more convenient method is the use of homologous recombination in bacteria to construct the targeting vector. This new form of DNA modifications is called "recombineering". Efficient homologous recombination in *E.coli* is made possible by the so called *Red* genes of bacteriophage λ, which permits linear double-strand DNA (dsDNA) fragments (e.g. those carrying loxP sites and selection markers) to be inserted into DNA cloned on plasmids, BACs, or PACs via homologous recombination. Only two Red genes are required for the recombination: *exo*, which encodes a 5′–3′ exonuclease (Exo) that acts on the 5′ ends of the linear dsDNA fragment to produce 3′ single-strand DNA (ssDNA) overhangs, and *bet*,

which encodes a pairing protein (Beta) that binds to the 3´ssDNA overhangs created by Exo and promotes annealing to its complementary DNA strand on the cloned DNA. The recombination functions of Exo and Beta are further assisted by λ-encoded Gam protein, which inhibits the RecBCD exonuclease activity of *E.coli*. Unlike in yeast, linear dsDNA is unstable in *E.coli* because of the activity of RecBCD. In our system the recombination functions are expressed from a defective prophage integrated into the E.coli chromosome (strains EL250 and EL 350). The expression of the recombination genes are under control a temperature-sensitive repressor. At 32°C, expression of *exo*, *bet*, and *gam* is undetectable. However, by shifting the culture temperature to 42°C for 15 minutes, expression of *exo*, *bet*, and *gam* can be induced to very high levels.

5.6.1 Transformation of BAC DNA into recombinogenic strains

The EL250 or EL350 *E.coli* strains were grown in a 5 ml YENB broth overnight at 32°C to suppress the expression of the *Red* genes. The next day the cells were centrifuged for 5 minutes at 5000 rpm and 4°C. Now the pellet was resuspended in 1 ml of ice-cold sterile water and transferred to a 1.5 ml microfuge tube. The cells were washed 3 times with ice-cold water by centrifugation in a 4°C benchtop centrifuge for 20 seconds at 13000xg. Finally the bacterial pellet was resuspended in ~50 µl ice-cold water and transferred to a pre-cooled 1 mm gap electroporation cuvette. Subsequently ~100 ng of BAC DNA or 1-10 ng of plasmid DNA was added and mixed by pipetting. The electroporation conditions were: 1800 V, 25 µF and 200 Ω. After the electroporation 1ml of LB medium was added, the cells were transferred to an Eppendorf tube and incubated for 1 hour at 32°C. The bacteria were spread on LB plates which contained the appropriate antibiotics.

5.6.2 GAP repair

To circumvent the problems which are associated with BAC modifications it is possible to subclone the desired 5-10 kb genomic DNA fragment to be modified into a linearized plasmid backbone by homologous recombination. The DNA was subcloned before the recombination sites and the selection marker were introduced.
The EL350 cells containing the BAC with the desired genomic DNA were grown overnight in 5 ml YENB medium at 32°C. The next day 1 ml of the overnight culture was used to inoculate a new 20 ml YENB flask and incubated at 32°C until the OD_{600} reached 0.5 (~2-3 hours). To activate the expression of the *Red* genes 10ml of the cells

were transferred into a new flask and incubated for 15 minutes at 42°C in a shaking water bath. The flask was put on wet ice and steadily shaken for 2 minutes and left for another 5 minutes on ice. The cells were centrifuged for 5 minutes at 5000 rpm and 4°C. The pellet was resuspended in 1 ml of ice-cold sterile water and transferred to a 1.5 ml microfuge tube. The cells were washed 3 times with ice-cold water as described above. Finally the bacterial cells were resuspended in ~50 µl ice-cold water, transferred to a pre-cooled 1 mm gap electroporation cuvette and ~10 ng of digested and purified retrieval vector was added. The cells were mixed by pipetting and electroporated as mentioned above. After the electroporation 1ml of LB medium was added, the cells were transferred to an Eppendorf tube and incubated for 1 hour at 32°C. The bacteria were spread on LB plates which contained the appropriate antibiotics.

5.6.3 Targeting through temperature control

Targeting of the subcloned genomic locus in the plasmid backbone was performed by coelectroporation. In brief 5 ml EL250 or EL350 overnight cultures in YENB were regrown in a larger volume (500ml YENB) until OD_{600} 0.5, temperature-induced and made electrocompetent as mentioned above. Finally the cells were resuspended in 4 ml ice-cold 15% glycerol in water. Then 50µl of cells were aliquoted in pre-cooled Eppendorf tubes and stored at -80°C. For targeting the cells were thawed on ice and 100ng of the purified targeting cassette and 10ng of template plasmid DNA were coelectroporated as mentioned above. After the electroporation 1ml of LB medium was added, the cells were transferred to an Eppendorf tube and incubated for 1 hour at 32°C. The bacteria were spread on LB plates which contained the appropriate antibiotics.

5.6.4 Excision through arabinose induction

In the recombinogenic bacteria strains the expression of the Cre recombinase (EL350) or the FlpE recombinase (EL250) are under the control of an arabinose-inducible promoter. After arabinose induction, the expression of the single site specific recombinases could be used to excise loxP or FRT site flanked selection markers.
In brief, a 10ml overnight culture of e.g. EL350 cells was added to 500ml of YENB broth in a 2 l flask. The culture was incubated at 32°C until the OD_{600} reached 0.4. Then 5 ml of 10% L-arabinose (Sigma Aldrich) in water was added to the culture to a final concentration of 0.1% and shaken at 32°C for another hour. Then cells were pel-

leted, washed and frozen as described above. Next, ~1ng of plasmid DNA with the recombination site flanked selection marker was electroporated into 50µl of frozen competent cells. After the electroporation 1ml of LB medium was added, the cells were transferred to an Eppendorf tube and incubated for 1 hour at 32°C. Then 10-100µl of the cells were plated on LB plates with and without the antibiotic of the selection marker and grown overnight at 32°C to control for the correct excision of the marker.

5.7 RNA analysis

5.7.1 Isolation of RNA from tissue or cultured cells

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA isolation procedures. Disruption and homogenization was done by using the Mixer Mill MM 300 from Qiagen. In bead-milling, cells and tissues can be disrupted by rapid agitation in the presence of beads and lysis buffer. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Therefore approximately 1-3x10^7 animal cells or 100mg tissue were put in a 2ml RNase-free reaction tube with 2-4 0.5mm diameter stainless steel beads and 1ml of QIAzol Lysis Reagent. The sample tube was transferred to the Mixer mill and disrupted for 2min at a frequency of 30 Hertz. For the isolation the total RNA the RNeasy Lipid Tissue Mini Kit from Qiagen was used and performed according to manual of the manufacturer. In brief:

- The sample tube with the homogenate was placed on the benchtop at RT for 5 min
- 200µl chloroform were added and the tube was shaken vigorously for 15 s
- The sample tube with the homogenate was placed on the benchtop at RT for 3 min
- The tube was centrifuged at 12000xg for 15min at 4°C
- The upper aqueous phase was transferred to a new collection tube and 1 volume of 70%
- EtOH/DEPC was added and mixed by vortexing
- Up to 700µl of the sample were transferred into a RNeasy Mini spin column and centrifuged at
- 8000xg for 15s at RT. The flow-through was discarded and the remainder of the sample was processed as described before

- 700µl of buffer RW1 were added to the sample, centrifuged for 15s at 8000xg and the flow-through was discarded
- The RNeasy column was transferred to a new collection tube, 500µl of buffer RPE were pipetted onto the column and centrifuged. The flow-through was discarded
- To dry the column, another 500µl of RPE were added and the column was centrifuged at 8000xg for 2min. Next, the column was transferred to new RNase-free tube.
- For elution 30-50µl of RNase-free water were pipetted directly onto the column and centrifuged at 8000xg for 1min. The elution step was repeated

5.7.2 RT-PCR

The Superscript II Reverse Transcriptase (Invitrogen) was used according to the user's manual to generate cDNA from a complementary total RNA or mRNA. The cDNA can then be used for amplification in PCR. In Brief:

Oligo(dT)$_{18}$ or random primer (~500ng/ml)	1 µl
1 ng- 5 µg total RNA or	x µl
1-500 ng mRNA	
1 µl dNTP mix (10 mM each)	1µl
RNase-free water	to 12 µl
5x First-strand buffer	4 µl
0.1 M DTT	2 µl
RNasin (40U/µl)	1µl
Superscript II RT	1 µl

The RT-mix was incubated for 50 min at 42°C and finally inactivated by heating at 70°C for 10 min.

5.8 Protein biochemistry

5.8.1 Protein precipitation

5.8.1.1 Acetone precipitation

A mixture of proteins can be precipitated by adding 4 times the sample volume of cold (-20°C) acetone in an acetone-compatible tube. The tube was vortexed and incubated for 60 min at -20°C. Then the tube was centrifuged for 10 min at 13000xg. The supernatant was decanted, being careful not to dislodge the protein pellet and the acetone was allowed to evaporate for ~ 30 min from the uncapped tube. Buffer appropriate for the downstream process was added and the tube was vortexed thoroughly to dissolve the protein pellet.

5.8.1.2 TCA precipitation

Another method to precipitate proteins is the tri-chlor acetic acid method. 100% TCA was added to a protein sample to a final concentration of 15% TCA and mixed thoroughly. The mixture was incubated for 60 min or alternatively overnight at 4°C. Then the sample was centrifuged for 10min at 13000xg and the pellet was washed first with 90% cold acetone (-20°C) and then with 100% cold acetone. The supernatant was decanted, being careful not to dislodge the protein pellet and the acetone was allowed to evaporate for ~ 30 min from the uncapped tube. Buffer appropriate for the downstream process was added and the tube was vortexed thoroughly to dissolve the protein pellet.

5.8.2 Determination of protein concentration

For the determination of the protein concentration in samples the Pierce BCA Protein assay Kit was used. The assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 μg/ml). After the assay was set

up in a 96 well plate according to the manual of the manufacturer the assay was automatically measured in the Victor Wallac Workstation at 562nm wavelength.

5.8.3 SDS-Polyacrylamide gel electrophoresis

Gel electrophoresis is a useful method to separate and identify proteins and nucleic acids. In SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated largely on the basis of polypeptide length, and so their molecular weight can also be estimated. SDS does however denature the protein, so activity stains cannot be used to identify particular enzymes. Described below is the protocol for preparing and using Laemmli discontinuous gels. In this system, two sequential gels are actually used; the top gel, called the stacking gel, is slightly acidic (pH 6.8) and has a lower acrylamide concentration to make a porous gel. Under these conditions, proteins separate poorly but form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is more basic (pH 8.8), and has a higher polyacrylamide content, which causes the gel to have narrower channels or pores. As a protein, concentrated into sharp bands by the stacking gel, travels through the separating gel, the narrower pores have a sieving effect, allowing smaller proteins to travel more easily and hence rapidly, than larger proteins.
The BioRad MiniProtean II gel chamber system was used to cast and run the PAGE gels according to the manual of the manufacturer.

Stock solution	Stacking gel	8% Separating Gel
30%AA/0.8%BisAA	0,65 ml	4.0 ml
SDS Separating Buffer pH 8.8	-	3.75 ml
SDS Stacking Buffer pH 6.8	1.25 ml	-
Water	3 ml	7.25 ml
10% APS	20µl	50 µl
TEMED	10µl	10 µl

5.8.4 Coomassie Blue staining of protein gels

Polypeptides separated by SDS-PAGE can be fixed with methanol and glacial acetic acid and stained simultaneously with Coomassie Brilliant Blue R250. Therefore 0.25g Coomassie Brilliant Blue R250 were dissolved in 90ml of a 1:1 dilution (v/v) of methanol and water and 10ml of glacial acetic acid were added. The mixture was filtered to remove any particulate matter. The PAA gel was immersed for at least 4

hours in 5 volumes of staining solution at room temperature by slowly shaking. Afterwards the gel was destained in the methanol/acetic acid solution without the dye for at least 4 hours. The destaining solution was changed several times.

5.8.5 Western Blot

5.8.5.1 Electro transfer of proteins onto nitrocellulose

After the separation of the proteins in a PAA gel, the proteins were transferred with a constant current of 400mA for 1 hour onto a nitrocellulose filter membrane (Hybond) with the vertical BioRad Mini Trans-Blot system (Tank Transfer system). The vertical chamber was filled with 1x Western Blot buffer.
Setup of the Electro transfer:

- Trans-Blot pad, equilibrated in Western Blot buffer (+ side)
- 3 layers of Whatman paper, equilibrated in Western Blot buffer
- Nitrocellulose membrane, equilibrated in Western Blot buffer
- SDS-PAA gel
- 3 layers of Whatman paper, equilibrated in Western Blot buffer
- Trans-Blot pad, equilibrated in Western Blot buffer (- side)

After the transfer the proteins on the nitrocellulose membrane were briefly stained with 1:10 diluted Ponceau S solution (Sigma) for 5 min at RT by shaking. Excess staining solution was removed by washing several times with distilled water and the bands of the protein size marker and the position of the other lanes were marked with a pencil. The PAA gel was stained with Coomassie blue to control for the complete transfer of the proteins onto the nitrocellulose membrane.

5.8.5.2 Immunoblot analysis

Unspecific protein binding sites on the nitrocellulose membrane, with the transferred proteins, were blocked with blocking solution (1x TBST/ 5% BSA) for at least 1 hour at room temperature with gentle agitation on a platform shaker. The blocking solution was discarded and the membrane was washed 5 min with 1x TBST and afterwards the membrane was directly incubated with the primary antibody diluted in 1xTBST for 2 hours at RT or over night at 4°C with gentle agitation on a platform shaker. To remove unspecific bound antibodies the membrane was washed 3 times with 1x TBST for 5 min and then incubated with the secondary antibody for 30 min at

RT. Finally the membrane was washed at least 6 times with 1xTBST for 5 min each time. The signal detection was carried out by enzymatically catalyzed chemiluminescence (ECL) reaction.
Once the secondary antibody was coupled to horseradish peroxidase (HRP), the washed membrane was immersed with 2 ml of ECL reaction solution for 1 min. Then the membrane was briefly and gently dried with a paper towel, placed into a sealable clear plastic bag and exposed to an x-ray film (Kodak X-Omat XLS) in an x-ray cassette. After a suitable incubation time the film was developed in a Kodak x-ray developer.

5.8.6 Immunofluorescence

The cultured cells were grown in 6 well plates on covers lips. They were fixed with 4% PFA for 15 min at RT and washed 3 times with PBS. The fixed cells were permeabilized and blocked for 10 min with 0.1% saponin and 0.2% gelatin in PBS. A 20µl droplet with the diluted primary antibody in SG/PBS was pipetted on a clean stripe of parafilm and the cover slip was put on the droplet upside down (the cells faced into the droplet). It is important to remove the PBS of the backside of the cover slip before placing on the droplet. The cells were incubated in the dark for 30 to 60 minutes. Next they were transferred into a 24 well plate and washed for 3 times with SG/PBS. The incubation with the secondary antibody was carried out for 40 minutes in the dark in a 200µl droplet containing the diluted antibody in SG/PBS as described before. Afterwards the cells were washed 4 times with PBS and 1 time in distilled water. To mount the cells on a microscope slide, the cells were finally placed on a drop of mowiol and stored in the dark.

5.8.7 *In situ* hybridization (ISH)

In situ hybridization is currently one of the essential methods for gene expression studies. ISH traces the regional and cellular sites of gene expression (mRNA distribution) within a tissue. A nucleic acid probe tagged with either radiolabeled nucleotides or molecules allowing colorimetric/light detection is applied to a tissue section. The probe hybridizes to its corresponding mRNA in the cells and forms a probe-mRNA double helix. After excess probe has been washed away, the section is either exposed for autoradiography (radiolabeled probes) or processed histochemically (non-radioactive probes) to reveal sites on the section targeted by the probe.

5.8.7.1 Labeling of RNA probes with Digoxigenin (DIG)

The DIG molecule is a unique steroid isolated from the plant *Digitalis purpurea* (foxglove). Linked to UTP, DIG can be incorporated as a marker into cRNA probes by *i*n *vitro* transcription. Specific antibodies against DIG then allow the detection of the DIG-labeled hybrid RNAs and the expressing cells are stained with a colorimetric reaction product.
We routinely use the Roche DIG RNA labeling Kit and follow the protocol of the manufacturer. In brief:

Linearized transcription vector	1-2µg
10x Transcription buffer	2µl
DIG RNA labeling mix	2µl
RNase inhibitor (40U/µl)	1µl
RNA polymerase (Sp6, T7 or T3) (40U/µl)	1µl
DEPC-H_2O	ad 20µl

Incubation was performed for 2h at 37°C. After RNA labeling, the DNA was degraded by adding 20U of RNase-free-DNase I for 30min at 37°C and the reaction was stopped with SDS 0.04% and EDTA 1.6mM final concentration. Riboprobes were precipitated with 0.1 volumes 3M sodium acetate and 2.5 volumes 100% ethanol and stored at -20°C until use.

5.8.7.2 Non-radioactive DIG in situ-hybridization

Pre-hybridization
The freshly prepared tissue was fixed with 4%PFA and then sliced with a cryostat in 30µm thick slices. 2 slices were put in one well of a 12 well culture plate and processed at a time with ~1 ml of solution. The slices were consecutively hybridized in the following order:

Solution	Time
PBS-DEPC	10 min
0.2M HCl-DEPC	10 min
PBS-DEPC	5 min
PBS-DEPC + Triton 0.1%	5 min
PBS-DEPC	5 min
PBS-DEPC	5 min

Subsequently the slices were incubated for 1h at 55°C in the hybridization oven, the culture plate sealed with Parafilm.

Hybridization

The probe was denatured in hybridization buffer for 10min at 95°C and put on ice. The denatured probe was diluted 1:100 in hybridization buffer (generally 1ng probe per ml hybridization buffer) and incubated over night or at least for 16h at 55°C in the hybridization oven.

The next day the hybridized slices were washed in the following order:

Solution	Time	temperature
4x SSC	10 min	RT
RNase A (50µg/ml)	20 min	37°C
2x SSC	2 h	RT
0.1x SSC	30 min	55°C
DIG 1 buffer	10 min	RT
DIG 1 buffer	10 min	RT
DIG 2 buffer	10 min	RT

Finally the slices were incubated over night at 4°C with the alkaline phosphatase-coupled anti-Digoxigenin antibody, diluted 1:750 in DIG 3 buffer.

The following day the slices were washed again and incubated with the substrate:

Solution	Time
DIG 1	15 min
DIG 1	15 min
DIG 1	15 min
TBS	5 min
TBS	5 min
TBS-Mg pH 9.5	5 min
BCIP	Variable
TE	10 min

The stained slices were quickly washed in water and then mounted on gelatin coated slides, air dried and cover slipped with Mowiol.

5.9 Histology

5.9.1 Mouse perfusion and fixation

For RNA *in situ* hybridization of brain tissue the mouse has to be fixed by perfusion fixation with PFA-intracardial infusion after anaesthesia.
This procedure must be performed under a hood and has been fulfilled as follows:
The butterfly apparatus was setup by attaching a two-way connecting valve with two 20ml syringes (one containing saline (4.25mg NaCl/500ml ddH_2O) and the other containing cold 4% PFA fixative). It was made sure that the valve for the PFA is shut off to ensure that the mouse is initially perfused with saline. Then the mouse was anesthetized with Avertin (0.3 ml/10 g/body weight) by using a 26 gauge needle. The eyes were removed using scissors and placed in a cassette in PFA and the mouse was pinned to an angled board (head angled down) which is inside a container, large enough to collect the drain-off of saline and PFA. The skin was cut at thoracic area just below the rib cage with small scissors and forceps and the stenum was removed to expose the heart. Next, the right atrium was snipped with scissors and the butterfly needle was inserted into the left ventricle (snip approximately 2mm of plastic off tip of butterfly to expose the needle and act as a gauge for entering the heart). The mouse was perfused with saline at a slow and steady pace. Afterwards the shut off valve was switched to the saline syringe and the mouse was perfused with 4% PFA for about 10 min. After perfusion the top of the skull was opened and the brain was removed. The brain was transferred to a 50ml falcon tube and further fixed over night in 4% PFA at 4°C. The next morning the PFA was substituted by 1x PBS and the brain was stored at 4°C for further analysis

5.10 Cultivation of mammalian cells

All cell lines were incubated at 37°C in humidified cell culture incubators with 5% $C0_2$. Additionally all used materials, solutions and medias were sterilized for cell culture work and the work was performed under sterile cell culture hoods.
Hela cells were grown in DMEM medium (Gibco) substituted with 10% FCS and 100µg/ml (1x) Penicillin/Streptomycin (Gibco) to prevent bacterial contamination. PC12 cells were grown in DMEM with 5% FCS, 10% Horse serum and 1x Penicillin/Streptomycin (Gibco). ES cell media and solutions are described below.
The cell lines used in my experiments grow in a monolayer on the bottom of the cell culture plate. Only the PC12 cells need a Poly-L Lysine coated cell culture dish for

attachment to the bottom. The PC12 culture dishes were incubated with a 100µg/ml Poly-L Lysine solution for 10 min at RT and were subsequently washed two times with sterile water or PBS.

5.10.1 Hela cells

Passaging of the Hela cells was done by aspirating of the culture medium and by washing the cells with PBS. PBS-EDTA without Magnesium and Calcium was added and the solution was incubated for ~10 min until the cells started to detach from the bottom of the culture dish. The cell suspension was pipetted gently up and down and was transferred to a 15 ml Falcon tube. The cells were pelleted by centrifugation at 270xg for 5 minutes. The supernatant was aspirated and the cells were resuspended in an appropriate volume of Hela medium to dilute the cells.

5.10.2 PC12 cells

Passaging of PC 12 cells was done by trypsinization. Thus the medium was aspirated and the cells were washed twice with PBS. To trypsinize the cells 3 ml of Trypsin/EDTA was added and the dish was incubated for ~5 minutes in the incubator at 37°C. Trypsinization was stopped by addition of 7 ml of medium and the suspension was gently pipetted up and down to break the cell clumps. The cell suspension was transferred to a 15 ml falcon tube and pelleted by centrifugation at 270xg for 5 minutes. The supernatant was aspirated and the cells were resuspended in an appropriate volume of PC12 medium to dilute the cells.

5.11 DNA transfer in mammalian cells

Foreign DNA can be transferred into eukaryotic cells by several chemical or physical methods for the analysis of gene regulation and expression.

5.11.1 Transient Transfection

Transient transfection of cells was done by the lipid transfection method. In my experiments we routinely used the Lipofectamine 2000 (Invitrogen) transfection reagent according to the cell-type specific protocol of the manufacturer.

5.11.2 Electroporation

Electroporation, or electropermeabilization, is a significant increase in the electrical conductivity and permeability of the cell membrane caused by externally applied electrical field. The pulsed electrical field causes pore formation in the cell membrane and the subsequent entry of the foreign DNA into the cell.
Hela cells and PC12 were electroporated with the appropriate amount of DNA in the Electroporation generator BTX ECM 630 in suitable 4mm gap electroporation cuvettes (BioRad) with 250V and 960µF in 300µl medium.

5.12 Generation of genetically modified mice

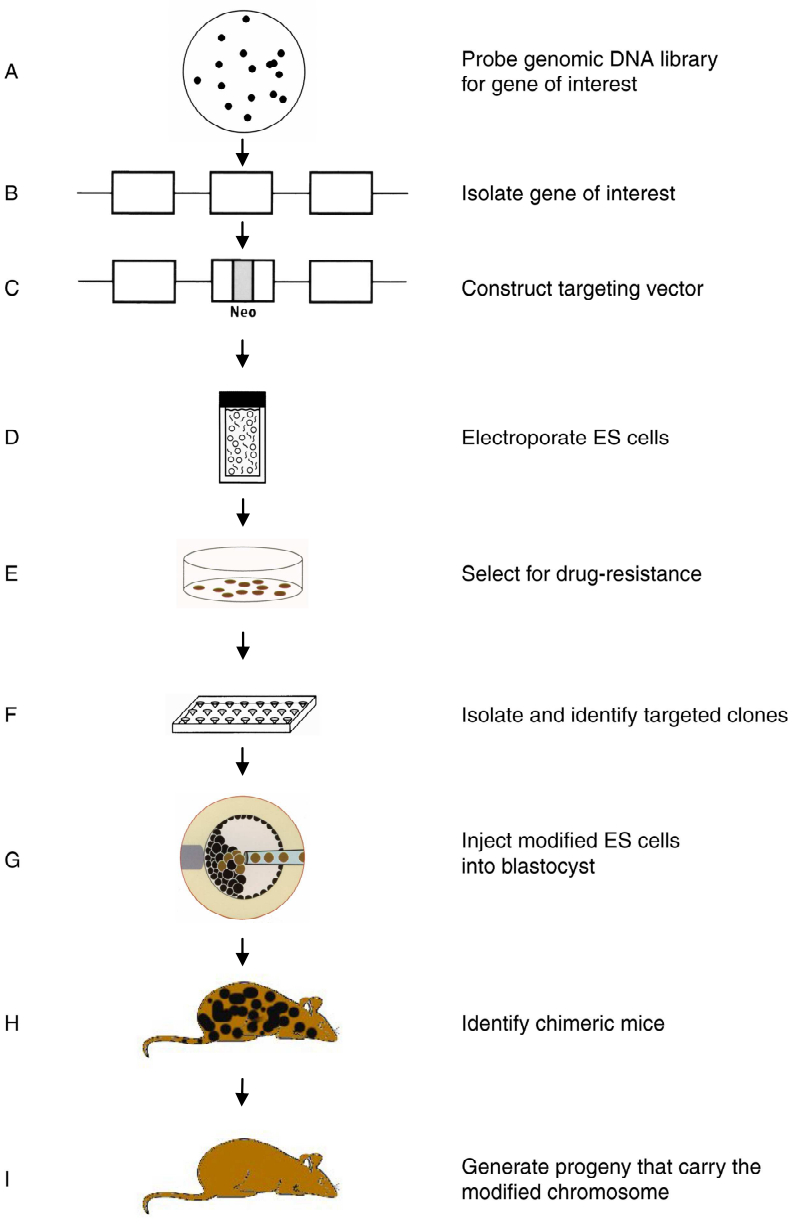


Fig. 14 General strategy for gene targeting in mice

(A) An isogenic DNA library is screened for the genomic DNA of the gene of interest by Southern blot hybridization. (**B)** The corresponding BAC with the genomic DNA is isolated. (**C)** The targeting vector is designed and constructed with a positive selectable marker gene (Neo) and commonly also a negative selectable marker gene (TK) outside of the homology arms. (**D)** The targeting vector DNA is linearized an electroporated into the ES cells. (**E)** The electroporated ES cells are spread out and selected for drug-resistance in cell culture until single ES colonies are present. (**F)** The drug-resistant ES clones are isolated and molecularly characterized. (**G)** The selected ES cells are injected into mouse blastocysts and the embryos are transferred into the uteri of pseudo-pregnant foster mothers. (**H)** Chimeras that are generated from blastocyst injection are mated with black wild-type mice to establish germline transmission of the modified genome. (**I)** The agouti-colored progeny derived from the chimeras are characterized and a mutant mouse line that carries an engineered chromosome is established

5.12.1 Cultivation of embryonic stem cells

Mouse Embryonic stem (ES) cells are pluripotent cells which were derived from the inner cell mass (ICM) of mouse blastocyst stage embryos. ES cells resemble ICM cells in many respects, including their ability to contribute to all embryonic tissues in chimeric mice. By using stringent culture conditions, the embryonic development potential of ES cells can be maintained following genetic manipulation and after many passages in vitro. Furthermore, permanent mouse lines carrying genetic alterations introduced into ES cells can be obtained by transmitting the mutation through the germline by generating ES cell chimeras.

5.12.2 Media and solutions

ES Medium	KnockOut DMEM (Invitrogen)
	0.1 mM non-essential amino acids (100x stock, Invitrogen)
	0.1 mM ß-mercaptoethanol (Sigma Aldrich)
	2 mM Glutamax (100x stock, Invitrogen)
	1x Penicillin/Streptomycin (100xstock, Invitrogen)
	15 % Fetal bovine serum (FBS), ES qualified (Invitrogen)
	1000U/ml RESGRO LIF (Chemicon)
Trypsin/EDTA	0.05 % trypsin in saline/EDTA (Invitrogen)
1x Freezing Medium	25 % FBS
	10 % DMSO
	In DMEM
Feeder Medium	DMEM high glucose
	10 % Fetal calf serum (FCS)
	1x Penicillin/streptomycin
	2 mM Glutamax
Gelatine solution	0.1 % Gelatine in water, autoclaved (Sigma)
Gancyclovir	2 µM (5000x stock , 10mM in PBS, sterile filtered)
G418	200µg/ml (1000x stock, 200mg/ml in PBS, sterile filtered)
ES Lysis buffer	10 mM Tris-HCl, pH 7.5
	10 mM EDTA
	10 mM NaCl
	1 mg/ml Proteinase K (Qiagen)
	0.5 % SDS

Mineral oil	Sigma Aldrich
NaCl/EtOH mix	15µl of 5M NaCl per 1ml of cold 100 % EtOH(.made fresh)
Mitomycin C	1 mg/ml stock

5.12.3 Embryonic Fibroblasts

For long-term culture and maintenance, pluripotent ES cells should be grown on monolayers of mitotically inactivated fibroblast cells. For the preparation of Feeder cell stocks 15 days post coitum pregnant mice were sacrificed and the uterus was transferred into a Petri dish containing PBS. The embryos were dissected away from the uterus and transferred to a new dish with PBS. Next, heads and all internal organs were removed. The remaining was washed 2-3 times in PBS to remove the blood. Then about 10 carcasses were minced together into ~1mm cubes with sterile small scissors and incubated in 20 ml Trypsin/EDTA in a 50 ml falcon tube. 200µl DNase I (10 mg/ml stock) was added and a sterile magnetic stirring bar was put inside the tube. The tube was placed on a magnetic stirrer in a 37°C incubator for 30 minutes. Then 10 ml of Trypsin/EDTA were added to the tube and the tube was incubated for another 30 minutes. To stop the Trypsin activity, the cell suspension was decanted into a new 50 ml falcon containing 3 ml of FBS. The cells were pelleted by centrifugation at 270 x g for 5 minutes and the pellet was resuspended in 10 ml of Feeder medium. The cells were counted in a Neubauer chamber and $5x10^6$ cells were plated on a 15 cm culture dish in 25 ml Feeder medium. The cells were grown to confluence and all the cells from one plate were frozen in 1 ml of 1x freezing medium and stored in liquid nitrogen.

For the preparation of Feeder cell layers one vial of feeders were thawed and split onto five 15cm plates. After ~3 days the cells formed a confluent monolayer and the cells were directly treated with 10µg/ml Mitomycin C for 2.5 hours in the incubator. The Mitomycin C treated cells were washed with PBS, trypsinized, resuspended in Feeder medium and counted. The cells were diluted to $2x10^5$ cells/ml, frozen and stored in liquid nitrogen.

5.12.4 Stem cell culture

5.12.4.1 Thawing of ES cells

Frozen ES cells were quickly thawed by warming at 37°C. Before all the ice has disappeared the cells were transferred into a 15 ml falcon tube containing 10 ml auf prewarmed ES medium. The cells were centrifuged at 270 x g for 5 minutes. The supernatant was aspirated and the pellet was resuspended in 10 ml of ES medium and plated on a 10 cm dish with a feeder layer or gelatin-coated. The medium was changed the next day to remove any debris.

5.12.4.2 Growth of ES cells

After the cells were thawed and plated on a 10 cm dish and grown to sub-confluence (~ after 2 days) the medium was aspirated and the cells were washed twice with PBS. To trypsinize the cells 3 ml of Trypsin/EDTA was added and the dish was incubated for ~5 minutes in the incubator at 37°C. Trypsinization was completed when the cells detached from the plate as small clumps, not as a single sheet or single cells. The cells were observed under a microscope. To stop trypsinization, 7 ml of ES medium was added and the suspension was gently pipetted up and down to break the cell clumps. The cell suspension was transferred to a 15 ml falcon tube and pelleted by centrifugation at 270xg for 5 minutes. The supernatant was aspirated and the cells were resuspended in an appropriate volume of ES medium to dilute the cells.

5.12.4.3 Freezing of ES cells

The cells from a 10 cm dish were trypsinized as described above (~2-3x10^7 cells). They were centrifuged at 270xg for 5 minutes to pellet the cells. The pellet was resuspended in 3 ml of pre-cooled 1 x freezing medium. Immediately 1 ml of the cell suspension was aliquoted into freezing vials on ice. Next, the vials were quickly transferred into a pre-cooled isopropanol slow-cool container and put into the -80°C freezer. After 24 hours the vials were transferred to the liquid nitrogen freezer for long-term storage.

5.12.5 Homologous recombination in ES cells

5.12.5.1 Preparation of DNA

A large scale preparation of the gene targeting vector DNA was prepared. After restriction digest to linearize the gene targeting vector, the DNA was extracted twice with phenol/chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in sterile ddH_20 at a concentration of ~1μg/μl.

5.12.5.2 Preparation of ES cells

The R1 ES cells (Passage #14 from A. Nagy, Toronto, Canada) were freshly thawed and were cultured for at least two passages on Feeder cells as described previously. Then a sub-confluent 10 cm dish (60-80 % confluence) was trypsinized and pre-plated as described above to remove the feeder cells. The ES cells were collected, re-suspended, counted in a Neubauer chamber and were kept on ice. The cell concentration was adjusted to $1x10^7$ cells/ml with sterile PBS.

5.12.5.3 Electroporation of ES cells

For electroporation 800 μl of ES cells ($1x10^7$/ml) and 25-50μg of digested and purified gene targeting DNA was mixed and transferred to a pre-cooled 4 mm gap electroporation cuvette. The electroporation was carried out at 240 V, 500 μF and 0 Ω in the BTX ECM 630 electroporator. The cuvettes were removed from the cuvette holder and put on ice for 20 minutes. The cells from the cuvette were removed and diluted in an appropriate volume of ES medium (normally 30 ml of medium for three gelatinized 10 cm dishes). The cells were incubated overnight and the next morning the medium was changed.

5.12.5.4 Selection of ES cells

Two days after electroporation the drug selection begun by adding 200 μg/ml G418 and 2 μM gancyclovir to the medium. The medium was changed every day since gancyclovir can brake down and become toxic. After 3 days of selection, widespread cell death appeared. After about 8 to 10 days of selection individual drug-resistant colonies appeared and were large enough for picking and sub-cloning.

5.12.5.5 Isolation of drug-resistant ES cell clones

The drug-resistant ES cell containing plates were washed twice with PBS and left in PBS during picking. The colonies were marked on the bottom of the plate by holding the plate up to the light. Under a dissection microscope drug-resistant colonies of approximately the same size and shape were picked up with a Gilson P20 pipette adjusted to a volume of 3 µl with yellow tips. Each colony in PBS was transferred into one well of a 96 well V-shaped plate containing 35 µl of Trypsin/EDTA at room temperature. Usually 48 colonies were picked at one time. Next, the plate was incubated for ~ 10 minutes in the 37°C incubator to break up the cell clumps. The reaction was stopped by adding 100 µl of medium containing G418 to each well with a multichannel pipettor and the cells were mixed by pipetting up and down. The cells were transferred to a new gelatinized flat-bottom 96 well plate. Each V-well was washed again with 100µl of medium and added to the same well of the 96 well gelatinized plate. The plate was incubated at 37°C in the incubator and the medium was changed daily. In two to three days the cells should have reached ~80% confluence and were passaged, frozen and replicated for analysis. If the colonies have not grown to confluence in 2-3 days the cells were tryplated as followed: The medium was aspirated and the cells were washed twice with PBS. 35 µl of Trypsin/EDTA was added and incubated for 5 minutes at 37°C. Then 200 µl of medium was added and the cell clumps were broken up by pipetting up and down. 12 hours later the medium was changed.

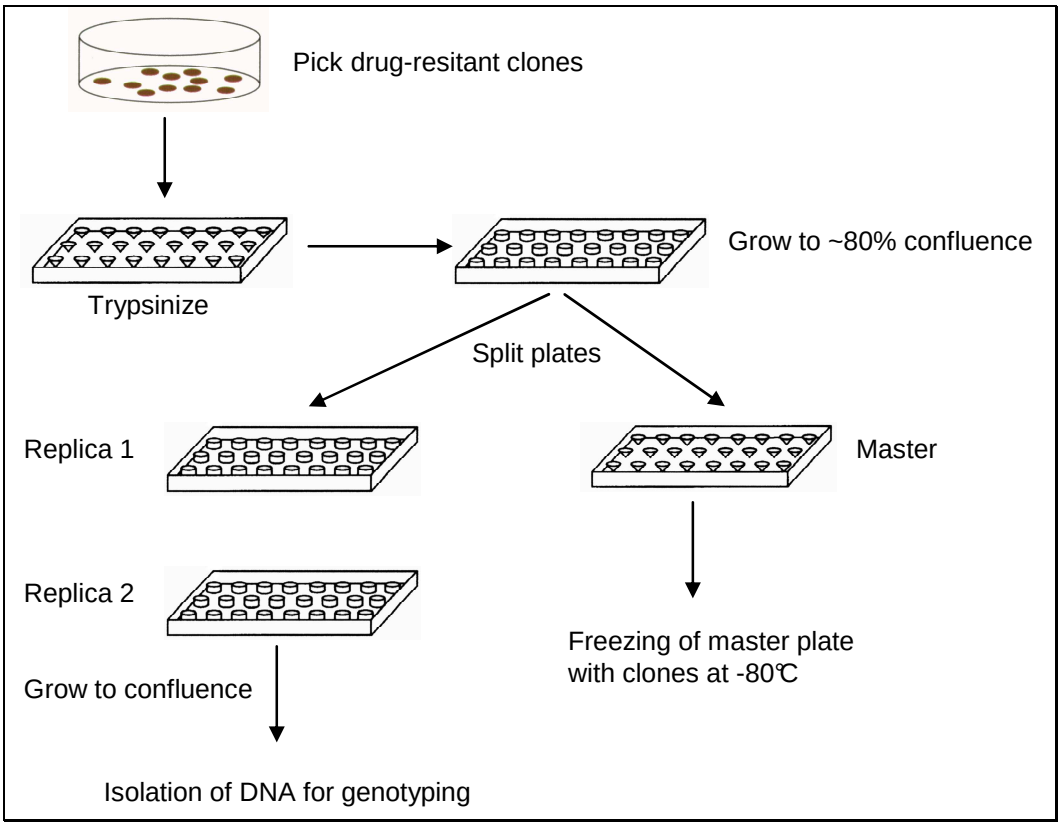


Fig. 15 Isolation, expansion and freezing of drug-resistant ES clones

5.12.5.6 Freezing and preparation of replica plates

Two sets of flat-bottom 96 well tissue culture plates were gelatinized for two replica plates and one 96 V-shaped masterplate was prepared containing 50µl of cold 2 x freezing medium and kept on ice. The medium of the growing ES cells was aspirated and washed twice with 200 µl PBS. The cells were trypsinized with 50 µl of Trypsin/EDTA and were incubated for 10 minutes at 37°C in the incubator. Trypsinization was stopped by adding 50 µl of ES medium and the cells were mixed by pipetting. Immediately 50 µl of the suspension was transferred to the V-shaped masterplate containing the 2x freezing medium and mixed well. Then 100µl of mineral oil was layered on top of each well containing ES cells in freezing medium. The plate was completely sealed with Parafilm, wrapped into aluminum foil and was transferred into a pre-cooled Styrofoam box to a -80°C freezer.

The remaining cell suspension was mixed and 25 µl was transferred into each of the two gelatinized replica plates containing 200µl of medium per well. The replica plates were incubated at 37°C in the incubator and the medium was changed daily.

5.12.6 Identification of genetically modified ES cells

5.12.6.1 Isolation of DNA from 96 well plates

The genomic DNA of the drug-resistant ES colonies was extracted from over-confluent cells of the replica plates. The medium of the confluent cells was aspirated and washed twice with PBS. Per well, 50 µl of ES Lysis buffer was added. The Plate was sealed with Tesa tape and incubated overnight at 55°C to perform the Proteinase K digest. The next day, the sealing tape was removed and 100µl of freshly prepared ice-cold NaCl/EtOH mix was added to each well and mixed slowly by pipetting up and down to precipitate the DNA. The mixture was incubated for at least 2 hours at room temperature avoiding vibration. The lid was removed and several layers of paper towel were put over the open wells. The plate was turned over and the liquid was blot away. The DNA attached to the bottom of each well. Each well was washed three times with 200µl 70 % EtOH by blotting the alcohol by inversion on paper towels. Finally the plate was dried for ~ 5 minutes at room temperature and the DNA was usually resuspended in 50 µl of TE buffer. The DNA was stored at -20°C until proceeding with the analysis.

5.12.6.2 PCR genotyping of ES cell clones

After the selection of single resistant clones and DNA isolation, as described before, the clones were genotyped by PCR. To demonstrate the correct site-directed integration by homologous recombination into the genomic locus, two primers were designed; one which is specific for the inserted DNA sequence of the targeting vector and the other outside of the homology arm.

5.12.6.3 Expansion and freezing targeted ES cell clones

The 96 well plate with the positive targeted ES cell clones was removed from the -80°C freezer and unwrapped. The plate was quickly warmed at 37°C in the incubator until the ice crystals disappeared (~ 5 minutes) and the outside of the plate was cleaned with 70% EtOH. When the mineral oil was thawed, 100 µl of pre-warmed ES medium was added under the oil to each well containing a targeted positive clone and the cells were transferred to a 3.5 cm plate with feeders in 2 ml of ES medium. The original 96 well was rinsed again with 200µl of ES medium and added to the same 3.5 cm dish. The positive cells were incubated at 37°C in the incubator and the medium was changed after 6 hours and then daily. When the cells reached semi-

confluence after two days the cells were passaged to a 10 cm plate with feeders. If the cells didn't reach semi-confluence they were tryplated as described previously. The cells were split 1:5 or 1:6 every two days and the remainder of the cells was frozen at each passage and stored at -80°C. The passage number was noted for future reference.

5.12.7 Cre-mediated recombination in ES cells

The *LoxP*- or *FRT*-flanked heterozygous positive targeted ES cell clones can be analyzed for their functionality by *in vitro* recombinase-mediated deletion. Therefore the ES cells were transiently transfected with a *Cre* or *FlpE* expressing plasmid in cell culture. The gene targeted G418-resistant ES cells were grown for at least 2 passages in medium containing G418 to eliminate any cell clones that have lost the antibiotic resistance gene. Then $1x10^7$ cells were electroporated with 30µg of supercoiled recombinase vector (pMC1-Cre or pCaggsFlpE) and plated at a density of $0.5x10^7$ cells per 10cm dish in ES medium without G418. After two days, when the colonies were established, the cells were washed twice with PBS, trypsinized and resuspended to a single cell suspension. The cells were counted and 10^3 cells were plated onto a 10 cm dish. Several dishes (3-5) were plated out so that there were enough colonies to be picked and the remainder was frozen for later plating. After five days incubation after re-plating the established colonies were washed with PBS and picked as described previously. Following trypsinization half the cells were transferred to a master 48 well plate containing feeders and ES medium and the other half was transferred to a gelatinized 48 well plate with ES medium containing G418. After 3-5 days the desired G418-sensitive clones were identified and the sibling G418-sensitive clones from the master plate were expanded, frozen and genomic DNA was made for later analysis.

5.12.8 Injection of genetically modified ES cells into blastocysts

The R1 mouse ES cell line is derived from the 129/Sv mouse strain. The coat color of this mouse strain is agouti (brown-yellow). These agouti-colored ES cells were injected into blastocysts derived from black C57/BL6 mice. The blastocysts were collected on day 3.5 post coitum from the pregnant female donor mice.

5.12.8.1 Preparation of the ES cells for injection

To guarantee the optimal quality and performance of the ES cells a strict protocol has to be applied for treating the cells before the ES cell injection (after F. Zimmermann, ZTL Heidelberg):

- 3.5 to 3 days before the ES cell injection $4x10^6$ Mitomycin C-treated Feeder cells were plated onto three 6 cm dishes
- 2.5 days before the injection 1 vial of frozen ES cells (~$5x10^6$ cells) was plated on one 6 cm Feeder plate
- 1.5 days before injection the subconfluent ES-Feeder plate was split onto two 6 cm dishes with a ratio of 1:3 and 2:3
- 7 am on the injection day, the best plate was chosen (when the colonies are grown to dense the quality of the ES cells is affected; when there are too few ES cells on the plate the enrichment for injection is difficult). The medium was changed 2 hours before collecting the cells.
- 9 am on the injection day, the plate was washed twice with PBS and trypsinized with 1ml of Trypsin/EDTA. After ~5 minutes the cells were resuspended by gently pipetting up and down with a polished Pasteur pipette. The reaction was stopped by adding 2 ml of medium and pipetted again to get a single cell suspension. 8 ml of medium was added and the cells were centrifuged at 270xg for 5 minutes. The supernatant was aspirated and the cells were resuspended in 1ml of medium.
- Afterwards the cells were pre-plated in 6 ml of medium on a new gelatinized 6 cm plates and incubated at 37°C to separate the ES cells from the Feeder cells. After 20-30 minutes of incubation the cells were checked under the microscope. In theory the feeder cells should reattach to the bottom of the plate and the ES cells should float in the supernatant. The supernatant was collected and plated onto a new gelatinized 6 cm plate. The incubation was repeated and if there were still too much Feeder cells present, the cells were pre-plated for a third time. Then the supernatant was centrifuged at 270xg for 5 minutes and the pellet was resuspended with a polished Pasteur pipette with 1ml of ES medium plus 20µl/ml 1 M HEPES buffer (f.c. 20 mM). The cells were transported in 15 ml falcon tube on wet ice.
- 10.30 am the prepared ES cells were injected into blastocysts at the ZTL Heidelberg under supervision of Frank Zimmermann.

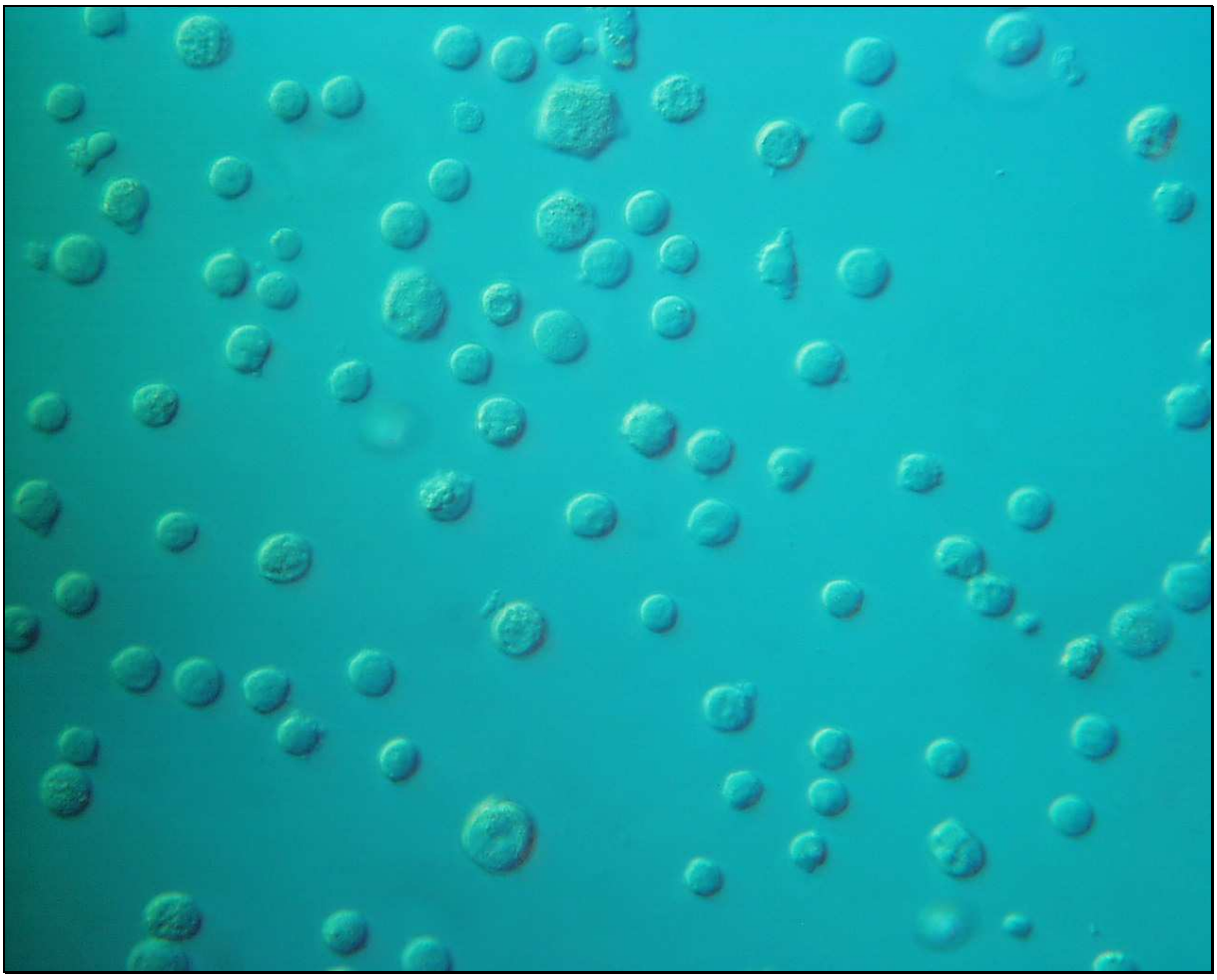


Fig. 16 Single mutated ES cell suspension before blastocyst injection

5.12.9 Germ line transmission from chimeric mice

The chimeras which were generated through ES cell injection are animals which have two different populations of cells, which are genetically distinct. They are genetic mosaics of the blastocyst donor, the black-colored C57/BL6 mouse strain, and the agouti-colored R1 ES cells from the 129/SvJ mouse strain. Thus the contribution of the agouti-colored ES cells to the chimera's coat color can be easily seen and they usually have a more or less speckled coat color of black and agouti.

However the mutant allele of the chimera has to be transmitted through the germ line in order to get a genetically modified mouse line. Thus the genetically modified ES cells have to contribute to the development of germ cells of the chimera. Therefore male chimeras with a high percentage of agouti-colored coats (> 60%) were mated with female C57/BL6 mice. The agouti color is genetically dominant over the black coat color and so the genetically modified gene can not be transmitted to the off-spring with black coats. In contrast the agouti-colored offspring has a genome that is

heterozygous (129/Sv//C57/BL6), meaning that one allele was derived from the 129/Sv and one from the C57/BL6 genome respectively. As a consequence every agouti offspring mouse has to be analyzed for their genomic content because the wildtype allele or the mutant allele of the chimera can be transmitted.
For the analysis of the agouti colored-offspring DNA was prepared from tail biopsies and analyzed by PCR or Southern Blot. The animals which were PCR positive for the genetic modification became the founders of the mutant mouse line and where back-crossed on the C57/BL6 strain to get a pure genetic background of C57/BL6 for the behavioral analysis of the mutant animals.

5.12.10 Animal husbandry and breeding

The animals were housed in the animal facility of the Central Institute for Mental Health, approved by the Regierungspräsidium Karlsruhe for animal husbandry and breeding. All animal experiments were permitted according to the German animal welfare law by the ethics committee and the Regierungspräsidium Karlsruhe (AZ 35-9185.81/G-157/03). The Animals were maintained under standard housing conditions in line with the guidelines of the FELASA (Federation of European Laboratory Animal Science Association) and the GV-SOLAS (Society of Laboratory Animal Science). In brief, the animal rooms are constantly air-conditioned with a temperature range of 20-22°C and a humidity of ~50%. A constant biorhythm for the animals is guaranteed by fixed bright and dark phases of light (Day from 6 am to 6 pm). The animals were maintained according to their size and the experiment in clear Makrolon cages of type II and III and food and water was provided ad libidum.

6 Results

6.1 Generation of mice with a *loxP* flanked *Dyrk1a* allele

During this thesis genetic modifications were introduced into the genome of ES cells and mouse respectively. To construct a suitable gene targeting vector for the conditional knockout of the *Dyrk1a* gene by using the phage P1 derived *Cre/loxP* site-specific recombination system murine genomic sequences containing parts of the *Dyrk1a* gene had to be obtained and characterized. Thus a genomic BAC library had to be screened for the complete or partial genomic DNA sequence of *Dyrk1a*. The minimal components of such a vector are sequences which are homologous with the desired chromosomal integration site, in my case exon 5 of the *Dyrk1a* gene.

6.1.1 Screening of a genomic mouse library for *Dyrk1a*

The mouse genomic BAC libraries were obtained from ResGen, USA and are created from the 129/SVJ mouse strain. This is very important because the targeting frequencies during homologous recombination are elevated when the DNA of the targeting vector is isogenic compared to the genome of the ES cells. The R1 ES cells (for genotype see material section) we use are also derived from a 129/SVJ substrain and are therefore isogenic. The screening of the BAC library was performed by Southern Blot hybridization. 36 potential BAC clones were identified with a radioactively-labeled Dyrk1a cDNA probe from the whole library. To further narrow down the number of possible clones a bacteria filter hybridization of these 36 clones was carried out (Fig. 17). Of these first 36 clones only 6 could be verified in the filter hybridization experiment. The exact genomic composition of the identified BACs had to be screened by PCR. For the PCR screening strategy PCR primer pairs were designed which were specific for every one of the 11 exons of the Dyrk1a gene (Fig. 18). In all of the PCR screened BAC clones the first exon was not present. In terms of the exon content clone #1, # 29 and #30 are identical and comprise exons 2 to exon 11. Clone #3 and 24# are also identical and include exon 2 to exon 8. The BAC clone #33 contains exons 2 to 9.

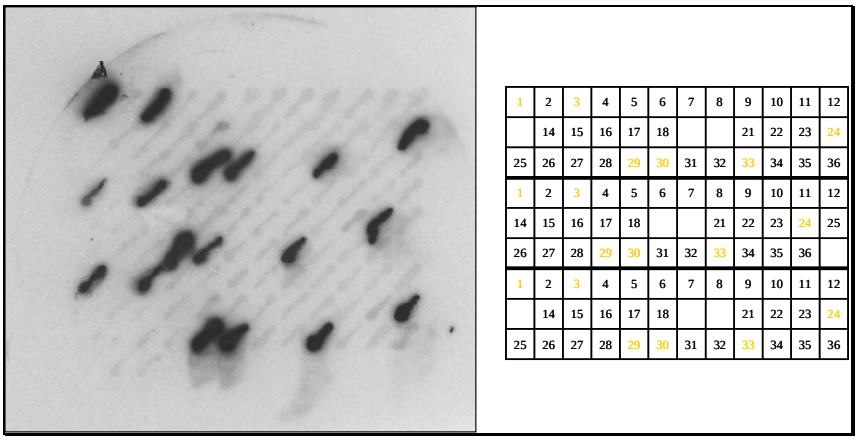
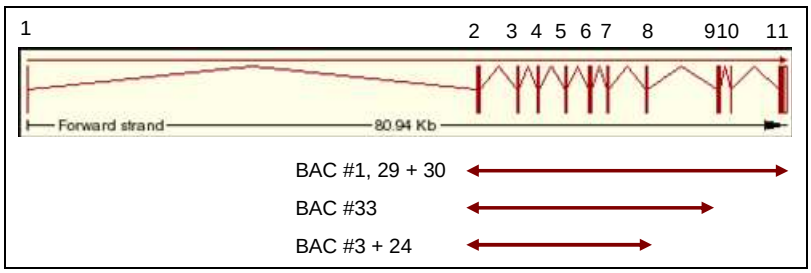


Fig. 17 Radioactive Filterhybridization of genomic Dyrk1a BAC clones

The 36 preliminary *Dyrk1a*-positive BAC clones from the mouse whole genome BAC library were streaked out in triplicates (BAC clone number grid on the right side) on a LB agar plate containing chloramphenicol, incubated over night at 37°C and transferred onto a nylon filter membrane. The membrane was probed with a radioactive-labeled *Dyrk1a* cDNA probe and finally visualized on an X-ray film (left side). 6 clones (#1, 3, 24, 29, 30 and 33; in yellow) were verified in the filterhybridization experiment.



Dyrk1a exon	Forward primer	Reverse primer	Amplicon length [bp]
1	Two21	Two22	45
2	Two1	Two2	197
3	Two7	Two8	108
4	Two9	Two10	125
5	Two3	Two4	102
6	Two11	Two12	288
7	Two13	Two14	147
8	Two15	Two16	138
9	Two17	Two18	224
10	Two19	Two20	108
11	Two5	Two6	588

Fig. 18 Exon/Intron structure of *Dyrk1a* and genotyping primers

The murine *Dyrk1a* gene spans over 80.94 kb and consists of 11 exons. Exon 1, containing the ATG start codon, is ~48 kb upstream of the next exon 2. The identified 6 positive *Dyrk1a* genomic BAC clones were characterized by PCR with the shown Two primer sets for the presence of the *Dyrk1a* exons. (See appendix for primer sequences and exon/intron information)

6.1.2 Targeting strategy for the murine Dyrk1a gene

The fundamental elements of a targeting vector are the homology to the target chromosomal locus, a positive selection marker, bacterial plasmid sequences and linearization sites flanking the homologous sequence of the vector (Fig. 19). A negative selection marker can also be used to enrich the transfected cells against random integration events.

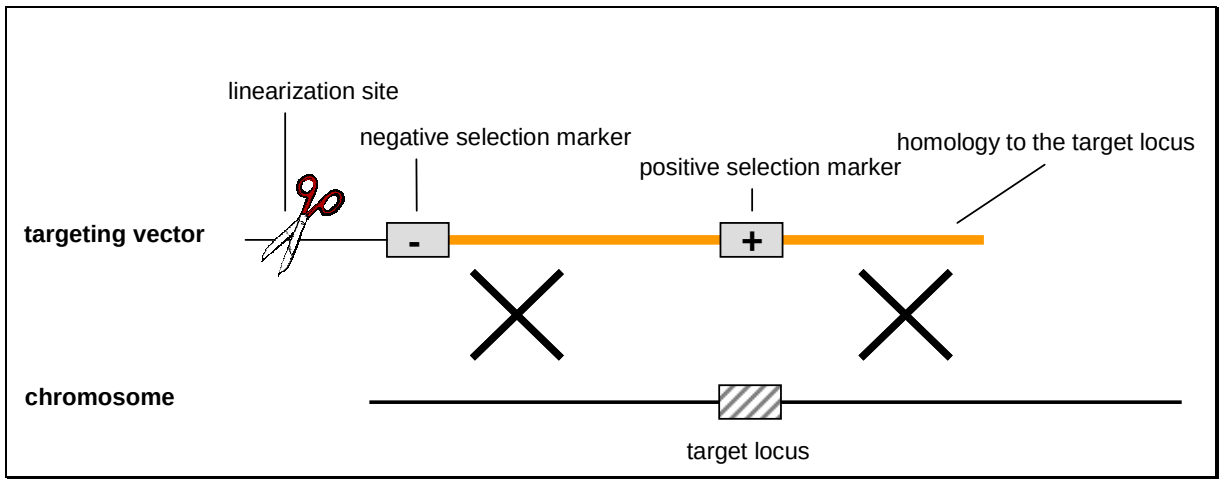


Fig. 19 Fundamental elements of a targeting vector

The yellow line represents the vector homology to the target locus, thin line represents bacterial plasmid, and line of intermediate thickness represents the target locus in the chromosome. The positive selection marker (+) is integrated into the vector homology in contrast to the negative selection marker (-) that is adjacent to the homology. The targeting vector is cut with a unique restriction enzyme prior to transfection.

Based on the determined exon-intron structure of the genomic subclone, a *Cre/loxP* based gene targeting strategy specific for the murine *Dyrk1a* gene was designed (Fig. 20). To enable conditional inactivation of the *Dyrk1a* gene, a gene targeting construct was generated in which a *FRT* flanked G418 resistance cassette (*Neo*) allowing positive selection in ES cells was introduced downstream of putative exon 5. One *loxP* sites was introduced upstream of exon 5 and the other *loxP* site was introduced in the same orientation downstream of exon 5 and of the *FRT*-flanked *Neo* cassette. A 3.5 kb long genomic fragment was included 5′of the first *loxP* site and 5 kb 3′of the second *loxP* site in order to allow the endogenous *Dyrk1a* allele to be replaced with the targeting construct by homologous recombination in ES cells. Adjacent to the homology arm, a negative selection cassette thymidine kinase (*TK*) from Herpes simplex virus was included.

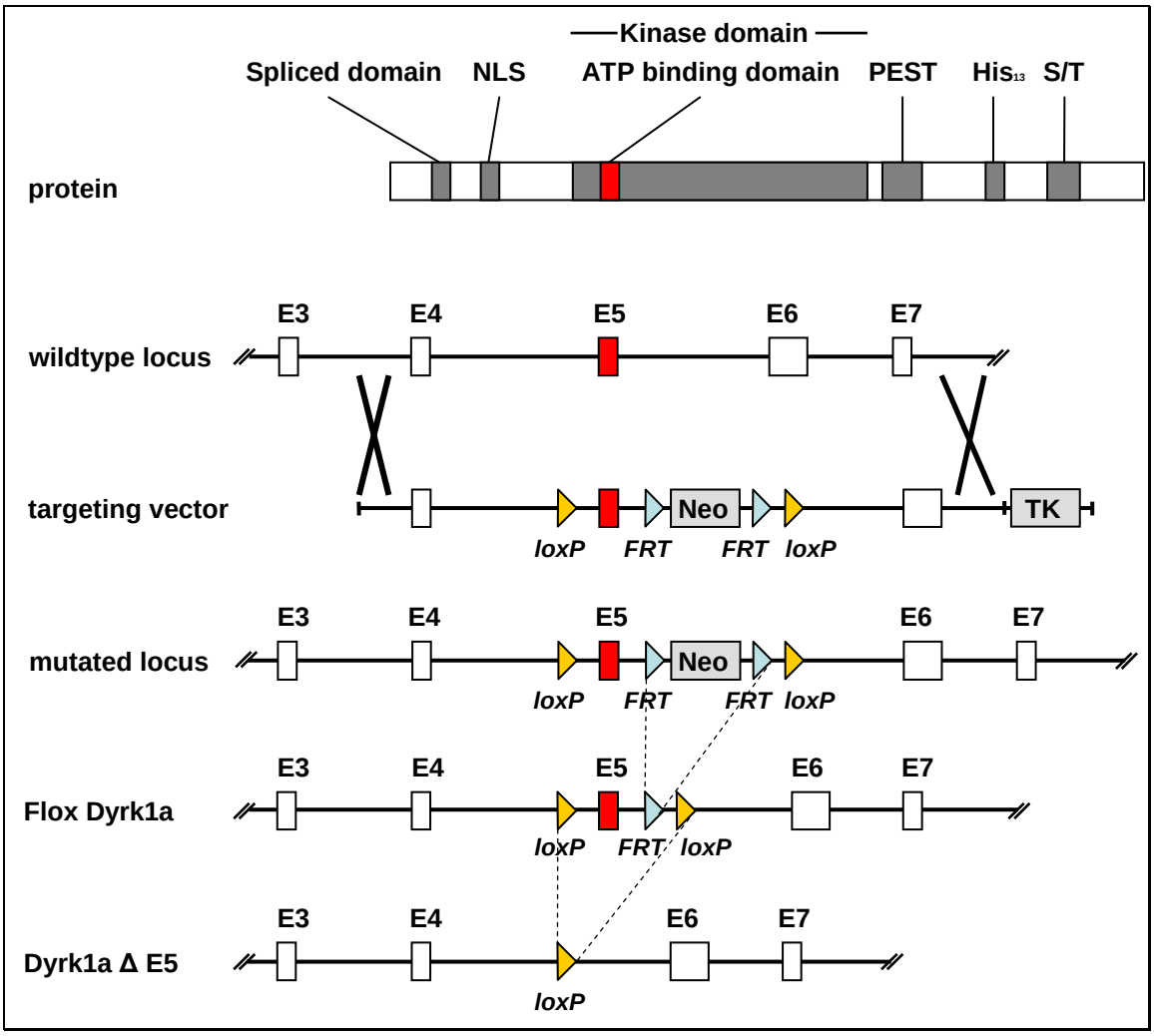


Fig. 20 Targeting strategy for the murine Dyrk1a gene

Primary structure of the DYRK1A kinase with the alternatively spliced domain, the bipartite nuclear localization signal (NLS), the kinase domain including the ATP-binding domain, the PEST sequence, the Poly-Histidine (His_{13}) repeat and the serine/threonine repeat domain (S/T). The ATP-binding domain that includes the conserved amino acid sequence AIK is encoded by exon 5 of the Dyrk1a gene. The targeting vector contains a *FRT*-flanked neomycin gene for positive selection in ES-cell culture, the *loxP* flanked exon 5 of *Dyrk1a*, ~4kb homology arms on each side of exon 5 and a Herpes simplex virus type 1 thymidine kinase (TK) gene for negative selection in cell culture. The mutated locus in genetically modified ES-cells still comprises a neomycin resistance gene which can then be removed by *FlpE*-mediated excision of *FRT*-flanked sequences (Flox Dyrk1a). The final step for the generation of the *Dyrk1a* conditional knockout allele is then the excision of *Dyrk1a* exon 5 by *Cre*-mediated recombination, leaving behind a single *loxP* site (Dyrk1a Δ E5).

6.1.3 Construction of the targeting vector by recombineering

For the construction of the vector we established a modified highly efficient recombination-based method in our laboratory called recombineering, adopted from the laboratory of N. Copeland. The standard procedure so far for generating a cko targeting vector was to identify a genomic BAC vector with the desired homology to the

target locus and then to modify the BAC to introduce the *loxP* sites, and the positive and negative selection markers. But the introduction and modification of large BAC constructs is very difficult. Therefore we subcloned just a 7-10 kb fragment of the desired genomic DNA into a normal plasmid backbone to eliminate the problems associated with BAC modification.

6.1.3.1 Subcloning DNA via GAP repair

Homologous recombination via a process called GAP repair provides a convenient method for subcloning DNA from a BAC into a linear plasmid vector backbone without restriction enzymes or DNA ligase (Fig. 21). The BAC must first be electroporated from its strain of origin (DH10B) into a modified *E.coli* strain (EL250 or EL350) containing the necessary enzymes *exo*, *bet* and *gam* for homologues recombination and the Cre (EL350) or FlpE (EL250) recombinase. In the EL350 cells, the homologous recombination functions encoded by the *red* genes can be controlled by temperature, whereas the *Cre* gene can be controlled by arabinose.
When BAC DNA is electroporated into stationary electro-competent EL350 cells and the BAC containing cells are selected using the chloramphenicol resistance (Cam^r) gene that is carried in the BAC vector backbone, we routinely obtained 100 to 1000 Cam^r colonies from 50 ng of BAC DNA, and virtually all of the colonies contained unrearranged BACs. We used the GAP repair method that makes use of longer homology arms (250–500 bp). These larger homology arms significantly increase the frequency of subcloning by GAP repair, and because of this, unwanted recombination products were rare. In this method, two sets of PCR primers were produced and used to amplify two 250–500-bp regions of the BAC (primers A_Mnb and B2_Mnb and Y_Mnb and Z2_Mnb). Ultimately these two regions will mark the ends of the fragment to be subcloned by GAP repair. The PCR products were purified using spin columns and digested with either *Not*I and *Hin*dIII or *Hin*dIII and *Spe*I. Restriction sites for these enzymes were included in the amplification primers to permit directional cloning of the PCR products into pL253. The digested fragments were again purified and ligated to *Not*I- and *Spe*I-cut pL253 DNA that also has a *TK* (*MC1TK*) gene for use in negative selection in ES cells. The retrieval vector was subsequently linearized with *Hin*dIII to create a DNA double-strand break for GAP repair. When we electroporated the linear retrieval plasmid into electro-competent EL350 cells, which contained Dyrk1a BAC 30, and which had been induced for *exo*, *bet*, and *gam* expression by prior growth at 42°C for 15 min, we found that we were able to rou-

tinely generate several thousand Amp^r colonies in a single electroporation experiment. About 15% of these Amp^r colonies were background colonies derived either from self-ligation of the linearized GAP repair plasmid or from uncut DNA. The other 85% of the colonies contained GAP-repaired plasmids with the expected 9.5 kb genomic insert, including exon 5 of Dyrk1a.

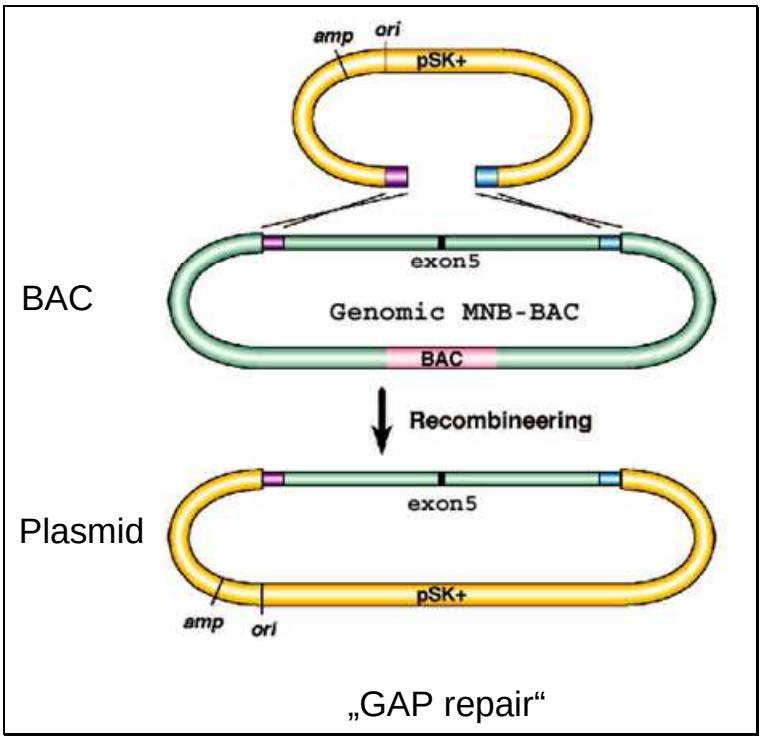


Fig. 21 GAP repair of the genomic Dyrk1a homology arms into a plasmid

6.1.3.2 Targeting the first loxP site of Dyrk1a Exon 5

After the GAP repair the first *lox*P site was integrated 5′ of Dyrk1a exon 5 into the subcloned DNA (Fig. 22). This was achieved by introducing a modified floxed neomycin resistance (*Neo*) cassette from plasmid pL452 via homologous recombination into the subcloned plasmid DNA, and by subsequent removal of the *Neo* gene via *Cre* recombinase. The floxed *Neo* gene in pL452 is expressed from a hybrid PGK–EM7 promoter. PGK permits efficient *Neo* expression in mammalian cells, whereas EM7 allows *Neo* to be expressed in bacterial cells. Subsequent removal of the floxed *Neo* gene via *Cre* recombinase leaves behind a single *lox*P site at the targeted locus. To introduce a floxed *Neo* gene at the correct location the Minitargeting vector 1 was cloned. The Neo cassette was first flanked with 250–500-bp arms that are homologous to the targeting site. These homology arms, as described above, were generated

by PCR amplification of the Dyrk1a BAC 30 DNA. The PCR primer pairs were engineered to contain *Not*I and *Eco*RI (primers C_Mnb and D_Mnb) or *Bam*HI and *Sal*I (primers E_Mnb and F_Mnb) restriction sites. These restriction sites allowed for the directional cloning of the homology arms, and the floxed *Neo* gene, into pBluescript. Following PCR amplification, the products were purified, restriction digested, and ligated to the floxed *Neo* cassette excised from pL452 with *Eco*RI and *Bam*HI, and to pBluescript that was linearized by *Not*I and *Sal*I digestion to generate Minitargeting vector 1. Four to six colonies selected by their kanamycin resistance, conferred by *Neo*, were picked and checked by restriction enzyme digestion to ensure that they were properly constructed. The floxed *Neo* gene, together with the homology arms, was excised from Minitargeting vector 1 by *Not*I and *Sal*I digestion, and gel-purified. The purified *Neo* cassette (150ng) was coelectroporated along with the GAP-repaired subcloned DNA (pL253-Mnb, 10 ng) into EL350 cells, which had been induced for *Red* recombination functions by prior growth at 42°C for 15 min, and frozen at -80°C. Transformants were selected on kanamycin plates.

Excision of the *Neo* cassette from the recombined DNA was achieved by electroporating the targeted plasmid DNA into EL350 cells, which had been induced for *Cre* expression by prior growth in arabinose-containing media for 1 h. The electroporated cells were spread on either ampicillin or kanamycin plates. *Cre*-mediated recombination is highly efficient; therefore, the kanamycin plates usually did not have any colonies. Colonies from the ampicillin plates were checked for their kanamycin sensitivity and restriction digestion patterns to make sure that the floxed *Neo* cassette was properly excised. All Amp^r colonies picked for analysis in this experiment were kanamycin-sensitive, and contained a single *lox*P site at the targeted locus.

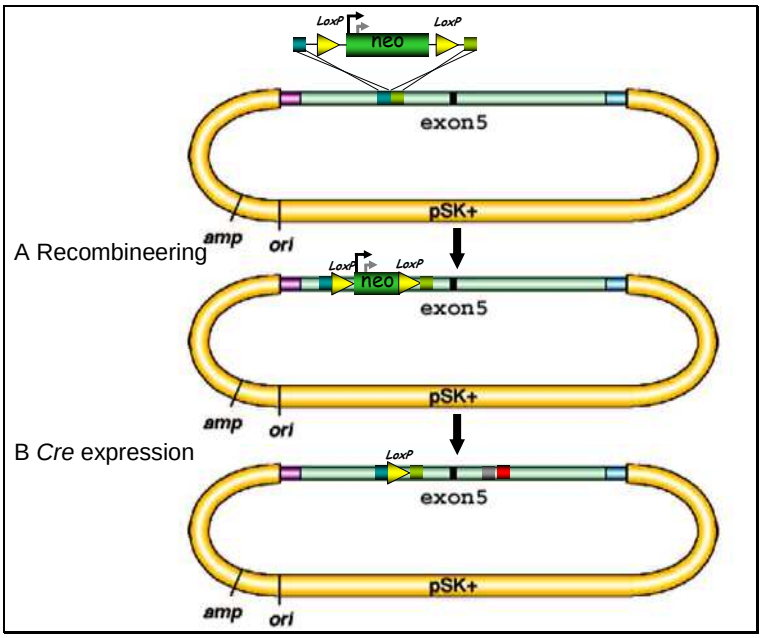


Fig. 22 Targeting the first loxP site 5′ of Dyrk1a exon 5

6.1.3.3 Targeting a second loxP site downstream of Dyrk1a exon 5

The final step in the construction of the cko-targeting vector is the introduction of a second *loxP* into the subcloned DNA downstream of Dyrk1a exon 5 (Fig. 23). This was accomplished by using a new selection cassette. The plasmid pL451 shares the same mammalian and bacterial neomycin expression cassette as described above with pL452 but in this case the Neo cassette is flanked 5′ with a *FRT* site and downstream of Neo with a *FRT* and a *loxP* site. Since *FRT* is the DNA recognition site for *Flp* recombinase, DNA located between two *FRT* sites can be excised by expression of the genetically enhanced *Flp* recombinase *Flpe* leaving behind a single *FRT* and a single *loxP* site at the targeted locus.

The PL451 selection cassette was introduced into the subcloned DNA in the same manner used to introduce the floxed *Neo* gene upstream of Dyrk1a exon 5. The *Neo* cassette was also flanked with homology arms that are homologous to the targeting site which were PCR amplified using the Dyrk1a BAC 30 as a template. The PCR primer pairs were engineered to contain *Not*I and *Eco*RI (primers G_Mnb and H_Mnb) or *Bam*HI and *Sal*I (primers I_Mnb and J_Mnb) restriction sites. Following PCR amplification, the products were purified, restriction digested, and ligated to the floxed *Neo* cassette excised from pL451 with *Eco*RI and *Bam*HI, and to pBluescript that was linearized by *Not*I and *Sal*I digestion to generate Minitargeting vector 2. Four to six colonies selected by their kanamycin resistance, conferred by *Neo*, were

selected and checked by restriction enzyme digestion. The floxed *Neo* gene, together with the homology arms, was excised from Minitargeting vector 2 by *Not*I and *Sal*I digestion. The purified *Neo* cassette (150ng) was coelectroporated along with the plasmid with the first *loxP* site (pL253-Mnb1.lox, 10 ng) into EL350 cells, which had been temperature-induced for *Red* recombination functions and transformants were selected on kanamycin plates. The resulting cko-targeting vector was referred to as pL253-MnbKO.

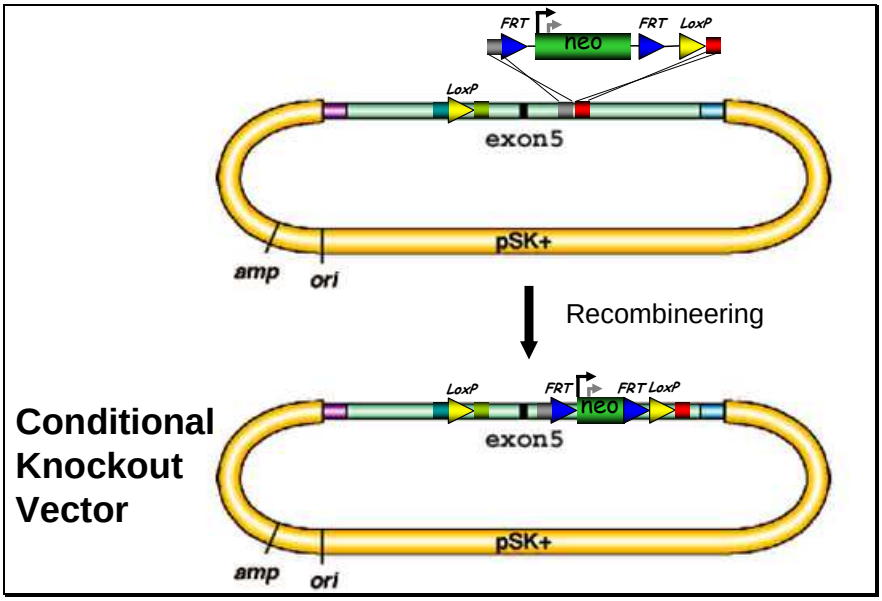


Fig. 23 Targeting the second loxP site 3′ of Dyrk1a exon 5

All *Dyrk1a* exons, which are present in the subcloned DNA (exon 4-7), including both targeted regions, were sequenced to make sure that no mutations in coding sequences occurred during the recombination process. To functionally test the *lox*P and *FRT* sites in the targeting vector, the cko-targeting vector plasmid DNA was transformed into arabinose-induced EL350 and EL250 cells. The cells were plated on ampicillin plates to select for the plasmid. Plasmid DNA was prepared and digested to confirm the expected recombination patterns.

The negative selection cassette can be removed by transient transfection with *Flpe* or, in my case, the PL451 selection cassette can be removed after the conditional allele is introduced into the mouse germ line by breeding the mice to one of the mouse strains that expresses *Flpe* in the mouse germ line, the so called *Flp*-deleter lines.

Subsequent expression of *Cre* recombinase will excise the entire DNA between the *lox*P sites located on either side of *Dyrk1a* exon 5, and create a *Dyrk1a* null allele. *Cre*

can be expressed in the mouse germ line to create a germ-line null allele, or in somatic cells.

6.1.4 Genetic modifications of ES cells

The discovery that a technique termed "gene targeting" allows cloned DNA, introduced into tissue culture cells, to undergo homologous recombination at specific chromosomal loci revolutionized the ability to study gene function in cultured cells and *in vivo*. Gene targeting includes the transfer of the targeting vector into the ES cells by electroporation and the locus specific integration into the ES cell genome. However, stringent cell culture conditions have to be maintained during transfection and selection. ES cells are pluripotent which means that they are not differentiated and that they are able to contribute to all embryonic tissues in chimeric mice. It is a prerequisite for the embryonic development potential and the later germline transmission of the ES cells to maintain the undifferentiated state during the whole gene targeting process. This is achieved by the addition of Leukemia-inhibitory factor (LIF) to the culture medium and the growth of the ES cells on feeder cell layers.

6.1.4.1 Homologous recombination KO vector

R1 ES cells (passage 14) from A. Nagy were used for gene targeting in our experiments. The R1 cells were grown for at least two passages on confluent feeder cell layers under stringent cell culture conditions. The targeting vector pL253-Mnb KO was digested with *NotI* to linearize the gene targeting vector before electroporation. The digested DNA was extracted twice with phenol/chloroform, precipitated with ethanol and resuspended in sterile PBS (at a concentration of 1µg/µl) in a laminar flow hood. Two days after the electroporation the positive drug selection began by supplementing the ES culture medium with 200µg/ml G418. The negative selection with 2µM gancyclovir was delayed for 24 h. The medium was changed every day since gancyclovir can break down and become toxic to the cells. After ~4 days after electroporation the selection started to become detectable and the G418/gancyclovir-sensitive ES cells started to die. After ~8-10 days of drug selection, individual drug-resistant colonies appeared and were large enough to pick into gelatin-coated 96 well plates, to subclone for PCR analysis and to freeze for storage. In total, about 250 G418 and gancyclovir-resistant ES cell clones were obtained after selection and 192 clones were selected and analyzed.

6.1.4.2 PCR-analysis of the KO ES cell clones

To confirm that the mutation is inserted in the correct locus in the genome of the ES cells we used PCR screening (Fig. 24). The basic strategy included the design of PCR primer pairs that will amplify a novel junction fragment created by the correct homologous recombination event, but not in wildtype DNA. The primers for the PCR were designed in such a way that one primer binds to the inserted first *loxP* site, upstream of exon 5, and the second to a region just past the short homology arm of the targeting vector within the endogenous locus. Aliquots of the PCR reactions were subsequently separated by agarose gel-electrophoresis and visualized under UV-light.

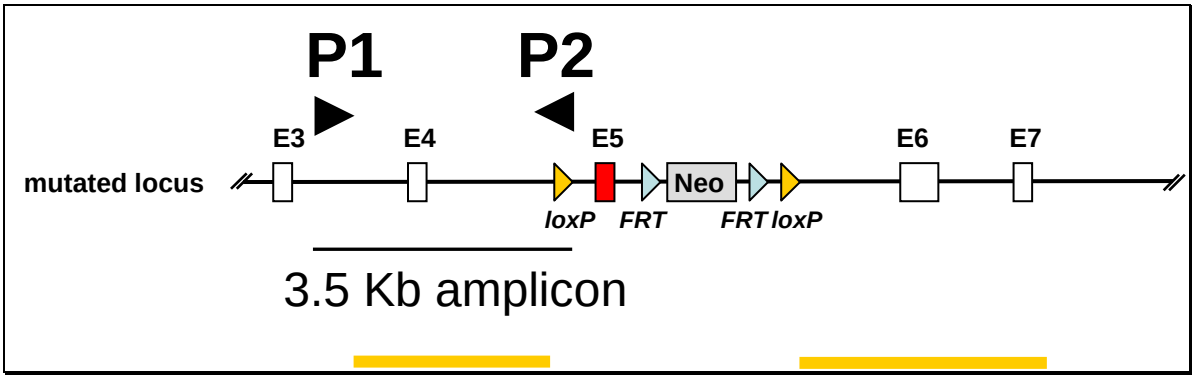


Fig. 24 PCR genotyping strategy for the mutated Dyrk1a locus after homologous recombination

The reverse primer P2 (MnbIntRev1) binds within the first *loxP* site and the forward primer P1 (MnbIntRev1) binds outside of the homology arm (yellow line) within the endogenous locus. Only the ES cells in which the gene targeting by homologous recombination was successful will generate a 3.5 kb PCR amplicon.

We could show that from the total 192 picked drug-resistant ES cell clones 6 were positive for the designed PCR genotyping reaction. The clones #51.1, 54.1, 60.1, 65.1, 66.2 and 84.2 generated the theoretical 3524 bp long amplicon after PCR using the primers MnbExtRev1 and MnbIntFor1 (Fig. 25).

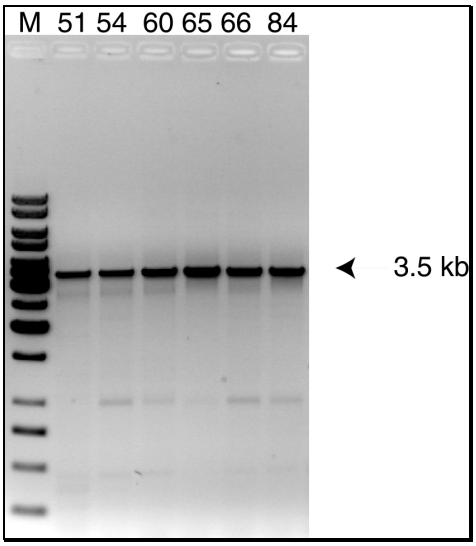


Fig. 25 PCR screen for the mutated Dyrk1a locus in ES cells

DNA was prepared after Proteinase K digest and submitted to PCR with primer MnbExtRev1 and MnbIntFor1. A 3.5 kb amplification product was detected for ES cell clones that had undergone correct homologous recombination in the short arm of homology. (M: 1 kb DNA marker)

After thawing and expansion of the positive ES cell clones all 6 clones were prepared according to the stringent protocol for blastocyst injection. At the day of the injection ES clones 60.1 and 66.2 were chosen to be injected into blastocysts because they were grown to the needed confluence and correct ratio between feeder cells and ES cells. The single ES cells had mainly a round shape and a smooth surface.

6.1.5 Injection of ES cell clones into blastocysts

The ES cell clones which were positive after PCR-analysis and thus heterozygous for the mutated locus were prepared for injection into blastocyst following a stringent protocol from Frank Zimmermann of the ZTL Heidelberg. For this purpose the positive ES cell clones were thawed and expanded in cell culture to maintain the embryonic development potential of the genetically modified ES clones.

The microinjection of the genetically modified ES cells into blastocysts was done by Arianna Frömmig at the ZTL in Heidelberg. Blastocysts with genetically modified ES cells were transferred into the uterus of pseudo-pregnant foster mothers. Consequently these implanted blastocysts produced chimeric offspring. In general the degree of coat color chimerism of a particular animal correlates with the degree of germline contribution. In our experiments only male offspring, which were 70-90% chimeric (i.e. high percentage of agouti-colored coat) were selected for breeding with C57BL/6 females to demonstrate the germline transmission of the genetic modifica-

tion. In contrast, chimeras which only produced black offspring did not contribute to the germline because the agouti-color coat of the ES cell phenotype was not inherited. Fig. 26 shows a summary of all chimeras and their contribution to the germline.

ES cell clone	*Reimplanted blastocysts*	*Offspring*	*Chimeras (male)*	*GLT*	*TierBase Code*	*Mouse line*
# 66.2 (KO)	32	21	15 (5)	2	Line 21	CB2
# 60.1 (KO)	24	14	6 (5)	1	Line 21	CB2

Fig. 26 Efficiency of injection and germline transmission (GLT) of different ES cell lines

6.1.6 Genotyping of agouti-colored litters

The resulting agouti-colored litters from the backcross of the male chimeras on black-colored females (C57BL/6) ascertain the germline transmission of the genetically modified R1 ES cells (129X1/SvJ x 129S1/Sv). Since the ES cells were in general heterozygous for the introduced mutation after gene targeting, the litters were genotyped by PCR reaction because both wildtype and heterozygous mice can be obtained from the agouti-colored offspring. For PCR genotyping, the DNA was isolated from tail tissue of the mice.

6.1.6.1 Genotyping of Dyrk1a$^{flox\ (FRT\text{-}Neo)/+}$ mice (CB2)

The CB2 mouse line is derived from the backcross of the male chimeras from the genetically manipulated ES cells to C57BL/6 mice. To demonstrate that the cko allele is transmitted through the germline and not the wildtype allele, the agouti-colored offspring was PCR genotyped with the same primers used for checking the correct integration during homologous recombination in ES cells (primers MnbIntFor1 and MnbExtRev1) and additionally with *Neo* genotyping primers (Neo 5′and Neo 3′) (Fig. 27). From 53 agouti-colored offspring from 3 different chimeric fathers only 10 were considered as germline transmitters because they were PCR genotyping positive (TierBase codes 21.26-27, 21.33, 21.59-62, 21.66-67 and 21.70) for both primer pairs. In summary we had 10 founders of the mouse line CB2 which transmitted the conditional knockout allele through the germline.

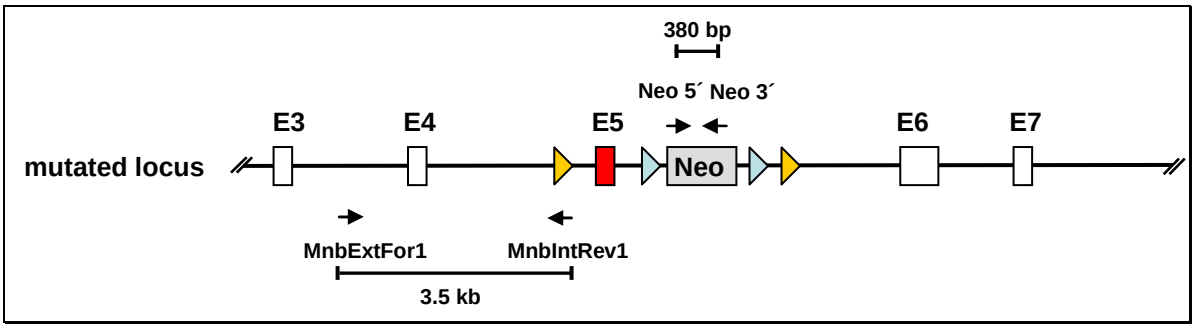


Fig. 27 PCR genotyping strategy for the CB2 mice

The agouti-colored offspring of the chimeric mice were first PCR genotyped with the primers used for the ES cells after homologous recombination (MnbIntFor1 and MnbIntRev1), generating a 3.5 kb fragment and then with the internal neomycin primers (Neo 5´and Neo 3´) which amplify a 380 bp fragment.

6.1.6.2 Genotyping of Dyrk1a$^{flox/+}$mice (CB4)

After the mutated Dyrk1a cko allele was transmitted through the germline in the CB2 mouse line we had to remove the positive selection marker n*eo*. It has been shown that the presence of neomycin resistance gene in the genome might influence the phenotype of the genetically modified mice. To prevent the silencing effect of *neo* we crossed the CB2 mice with *Flpe*-deleter mice. The *Flpe*-deleter mice express the *Flpe* recombinase under the control of the human ACTB promoter in a variety of tissues, especially in the germ cells. By *Flpe*-mediated recombination the *FRT* flanked *Neo* is removed from the genome by excision. To test whether the excision of *Neo* was successful we analyzed the offspring of the mating between CB2 and the *Flpe*-deleter mice by PCR (Fig. 28).

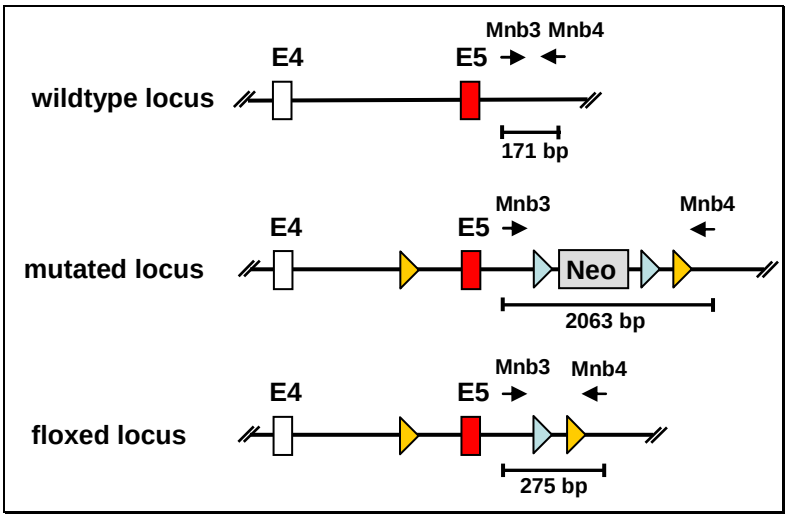


Fig. 28 PCR genotyping strategy for the detection of the Flpe-mediated recombination

The offspring of the mating of the CB2 with the *Flpe*-deleter mouse line were analyzed with the primers Mnb3 and Mnb4. The wildtype locus generates a 171 bp amplicon, whereas the mutated locus with the neomycin cassette generates a 2063 bp fragment. In case the neomycin cassette is successfully deleted by *Flpe*-mediated recombination, the amplicon is 275 bp long leaving behind the floxed exon 5 of *Dyrk1a* and a single *FRT* site.

We designed primer pairs that could not only detect the excision of *Neo* but could also detect if the resulting CB4 offspring is wildtype (+/+), heterozygous (Flox/+) or homozygous (Flox/Flox) for the floxed Dyrk1a conditional knockout allele. The primer Mnb3 is located 5′ before the first *FRT* site of *Neo* and primer Mnb4 is located 3′ after the second *FRT* site and the second *loxP* site in the endogenous sequence of the genomic *Dyrk1a* locus. When *Neo* is present there is a long PCR fragment of 2063bp and when *Neo* is *Flpe*-excised it results in a short 275 bp fragment. The wildtype locus generates an even shorter 171 bp fragment without the *FRT* and *loxP* site sequence downstream of *Dyrk1a* exon 5. The mating between CB2 and the *Flpe*-deleter mice resulted in 27 offspring mice (now referred to as line CB4 or TierBase line #24) from which 5 mice were PCR positive for the excision of the FRT-flanked *Neo* cassette (24.12, 24.19, 24.20, 24.23 and 24.27). The *Flpe*-excision of the *Neo* gene leaves behind a single *FRT* and *loxP* site 3′of *Dyrk1a* exon 5. To preclude the possibility that the *Neo* gene is also located anywhere else in the genome by random integration we checked the 5 positive mice in a negative PCR using the *Neo* genotyping primers (primers Neo5′and Neo3′; data not shown). None of the 5 positive mice did amplify the *Neo* gene but did amplify an internal 200bp genomic control (primers Fabpi 5′and Fabpi 3′) in the same PCR reaction.

Taken together, these results show that we succeeded in deleting the negative selection marker gene *Neo* by *Flpe*-mediated recombination from the mouse genome of the CB2 mice and generated the desired floxed *Dyrk1a* exon 5 conditional allele in the new mouse line CB4.

6.2 Conditional Dyrk1a knockouts generated by crossing in Cre transgenes

6.2.1 Genotyping of Dyrk1a$^{flox/+}$ x CaMKIIa-Cre mice (CB4 x E11Cre)

The generated floxed *Dyrk1a* exon 5 allele in the CB4 mouse line can now be excised by *Cre*-mediated recombination. To confirm that we can succeed in generating a functional knockout of Dyrk1a we mated the heterozygous CB4 mouse line, containing the floxed allele, with the CaMKIIa-*Cre* mouse line of Dr. Kai Schönig (E11-*Cre*). These transgenic mice express the *Cre* recombinase under the control of the mouse calcium/calmodulin-dependent protein kinase II alpha promoter. *Cre* recombinase expression is detected postnatally in the forebrain, specifically to the CA1 pyramidal cell layer in the hippocampus. Our breeding strategy included the mating of heterozygous Dyrk1a$^{flox/+}$ mice with CaMKIIa-*Cre* mice to get heterozygous forebrain-specific Dyrk1a$^{del/+}$ knockout mice. In the next step heterozygous knockout mice were breed together to obtain homozygous forebrain-specific knockout mice.

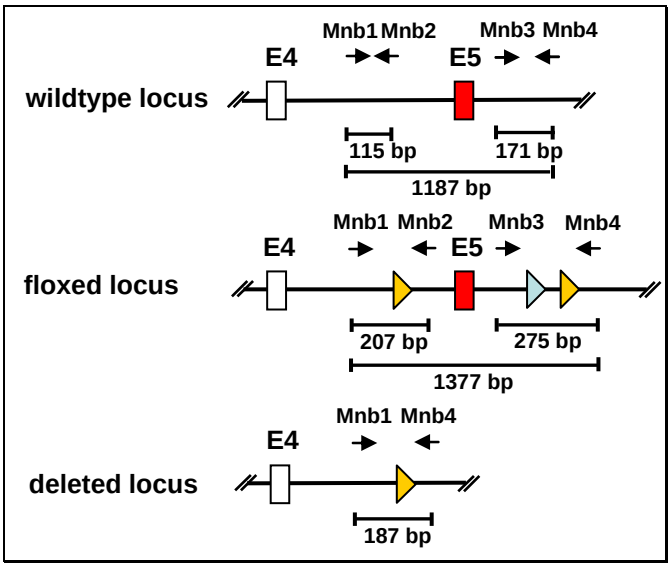


Fig. 29 PCR genotyping strategy for the detection of Cre-mediated recombination

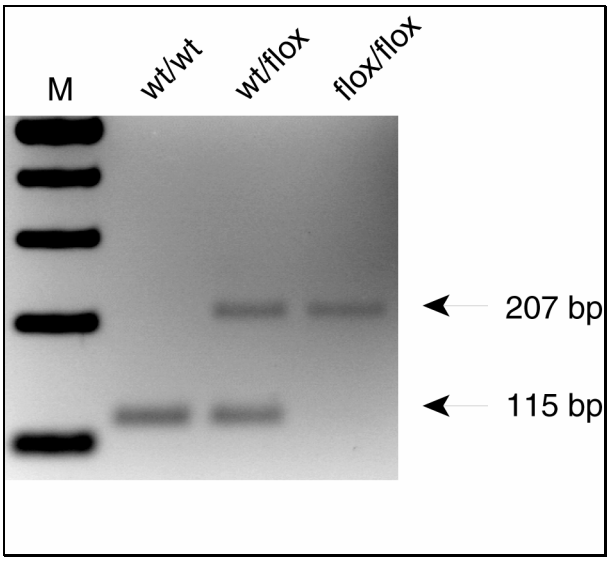


Fig. 30 Genotyping PCR for detection of floxed Dyrk1a mice
Tail DNA was prepared from the CB4 offspring and submitted to PCR using the primers Mnb1 and Mnb2.

Primer pairs	*Wildtype Locus*	*Floxed Locus*	*Deleted Locus*
Mnb1/Mnb2	115 bp	207 bp	-
Mnb3/Mnb4	171 bp	275 bp	-
Mnb1/Mnb4	1187 bp	1377 bp	187 bp

Fig. 31 PCR Genotyping amplicons of different cko loci

6.2.2 RNA analysis of conditional Dyrk1a mice

To determine whether the modified genetic loci had an effect on the transcription of the *Dyrk1a* gene the RNA expression of the heterozygous cko mice CB4$^{flox/CaMKIIa\text{-}Cre}$ was analyzed. Therefore tissues were the *Cre* recombinase under the control of the CaMKIIa promoter is known to be active and tissues were there is no *Cre* expression were prepared. Heart, cortex and hippocampus of the different mouse genotypes were extracted and the total RNA was isolated. We reverse transcribed the total RNAs into single-stranded cDNAs and performed PCRs (Fig. 32). The resulting PCR

products were separated by agarose gel-electrophoresis, gel-purified and subsequently directly sequenced. The computational alignment of the sequenced DNA fragments shows that we deleted *Dyrk1a* exon 5 in a tissue-specific manner in the hippocampus (Fig. 33) but, as expected, not in the heart were there is no Cre expression due to the CaMKIIa promoter. Further computational analysis demonstrates that the *Cre*-mediated excision of exon 5 causes a frameshift in the ORF of *Dyrk1a* (Fig. 34). The frameshift leads to a translation termination stop codon TGA in the beginning of Dyrk1a exon 6 and thus encodes for a truncated Dyrk1a protein of only 174 aa length. The truncated Dyrk1a protein represents a functional knockout of Dyrk1a because it lacks the ATP-binding domain (exon 5) and only contains the nuclear localization signal (NLS) and small parts of the first kinase domain (exon 4).

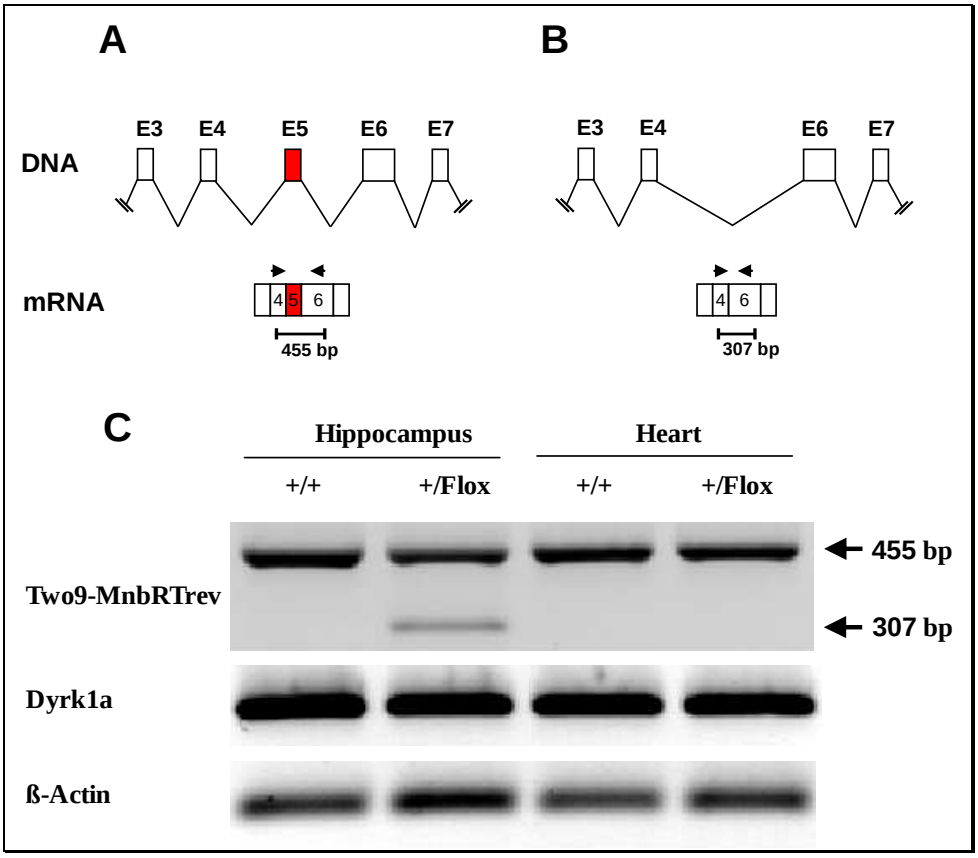


Fig. 32 RT-PCR analysis of the CB4 x CaMKIIa-Cre mice

(A) Schematic diagram of the wildtype DNA and mRNA structure of *Dyrk1a* and **(B)** the structure of *Dyrk1a* with the deleted exon 5 by Cre-mediate excision. The arrows indicate the binding site of primer Two9 (left) and Mnb_RT_rev (right) **(C)** RT-PCR of mRNA prepared from hippocampus and heart of wildtype (+/+) and heterozygous floxed (+/Flox) mice with primers flanking *Dyrk1a* exon 5 (Two9 and Mnb_RT_rev). *Dyrk1a* and *ß-Actin* served as an internal control. Since *Cre* is expressed in the hippocampus of CaMKIIa-*Cre* mice and not in the heart, the resulting mRNA of heterozygous floxed mice generates the wildtype 455 bp fragment and an additional 307 bp fragment in hippocampus due to the excision of exon 5 by *Cre* recombination.

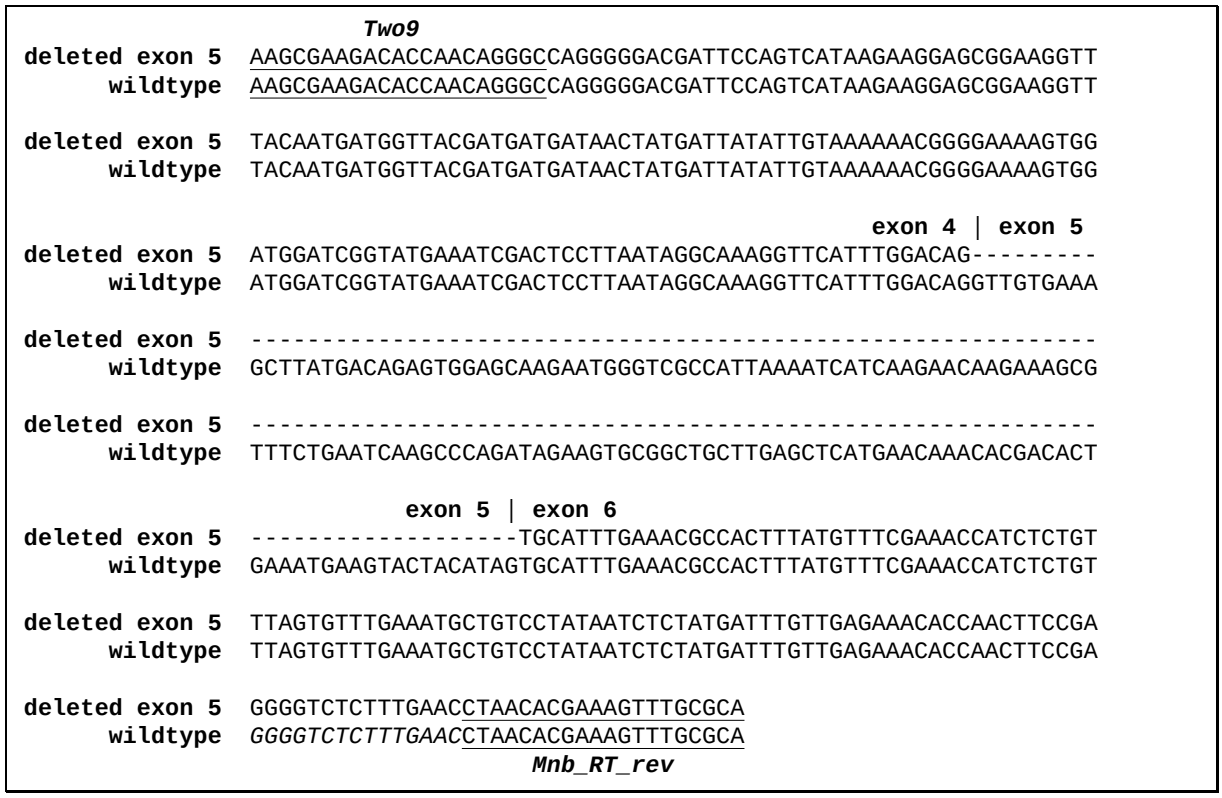


Fig. 33 Sequence alignment of the wildtype and deleted exon 5 cDNAs

The RT-PCR fragments of the hippocampus from CB4 x CaMKIIa-*Cre* mice (455 bp for the wildtype allele and 307 bp for the deleted exon 5 allele) were gel-purified and directly sequenced with the Two9 and Mnb_RT_rev primer (underlined). Sequence alignment was performed with the Vector NTI 10 alignment tool.

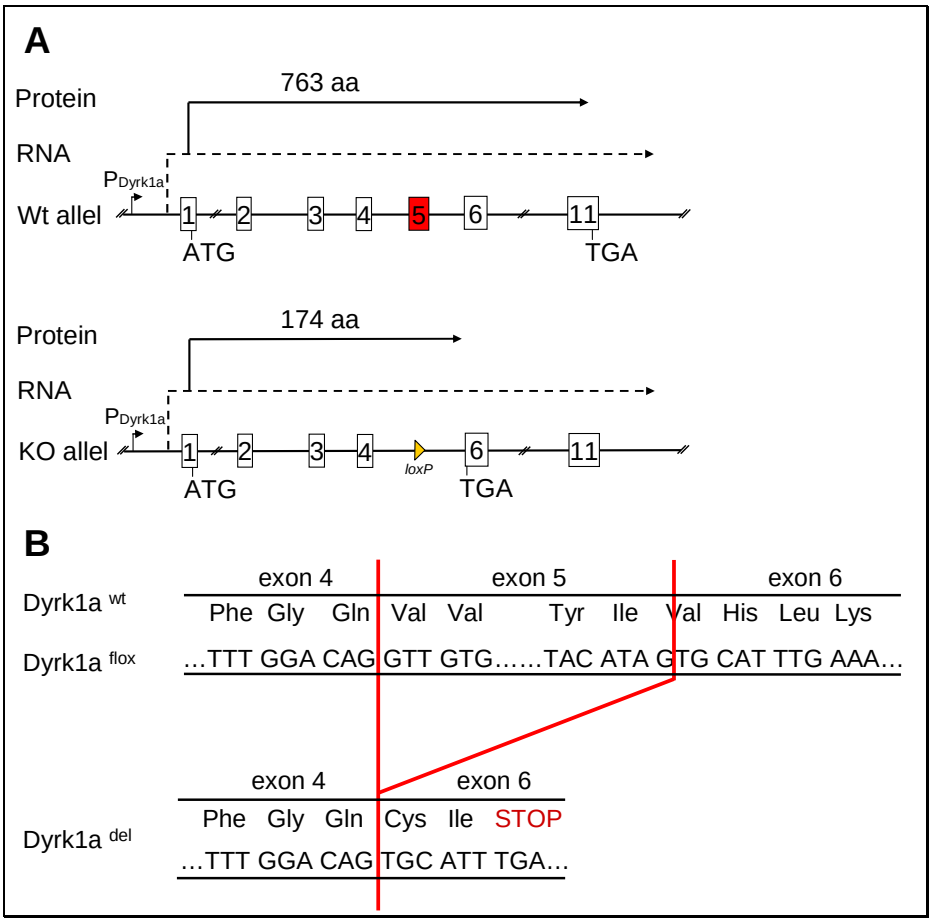


Fig. 34 Schematic overview of the wildtype and knockout *Dyrk1a* alleles

(A) The wildtype *Dyrk1a* allele codes for the 763 aa long Dyrk1a protein. **(B)** In contrast the knockout allele with the deleted exon 5 of *Dyrk1a* leads to a frameshift in the mRNA sequence within exon 6 (new translation termination stop codon TGA) and results in a truncated Dyrk1a protein of 174 aa (splice junctions are represented by red lines). The truncated protein only contains the nuclear localization signal (NLS) and a small part of the first kinase domain. It represents a functional knockout of the Dyrk1a kinase.

6.3 Generation of mice with a LC1 locus-specific tet-controlled *Dyrk1a* allele

The complex phenotypes of DS and the ubiquitous expression patterns of Dyrk1a led us to the conclusion that the temporal and spatial control of the overexpression of Dyrk1a would be advantageous. The Tet-system offered the best and most common strategy for stringent control of transgene expression *in vivo*. Besides the Tet-dependent expression of the *Dyrk1a* gene, we also wanted to simultaneously regulate the *Egfp* reporter gene expression to track and visualize the inducible expression of both genes. For the conditional overexpression of the *Dyrk1a* gene we decided to develop a novel transgenic approach. Instead of using random integration transgenesis for the *tetO-Dyrk1a*/*Egfp* constructs, we have decided to target the silent but highly activatable (s/a) locus LC1 identified by Kai Schönig to overcome the position effects associated with random transgenesis.

6.3.1 Targeting strategy for the locus-specific tet-controlled Dyrk1a targeting vector

To target the endogenous LC1 locus, located on mouse chromosome 6, by homologous recombination with a general targeting vector we decided to use about 7.5 kb of homology to the LC1 locus. The targeting vector also contained a Herpes simplex virus type 1 thymidine kinase (*TK*) cassette for negative selection in ES cells. After construction of the general targeting vector we cloned the tet-controlled bidirectional expression cassette with *Dyrk1a* and the *FRT* flanked *Egfp*. Subsequently we introduced a *loxP* flanked G418 resistance cassette (*Neo*) for positive selection in ES cells. The gene targeting vector was completed by cloning the bidirectional expression cassette into the asymmetric homology arms of the general LC1 targeting vector. The targeting strategy is depicted in Fig. 35.

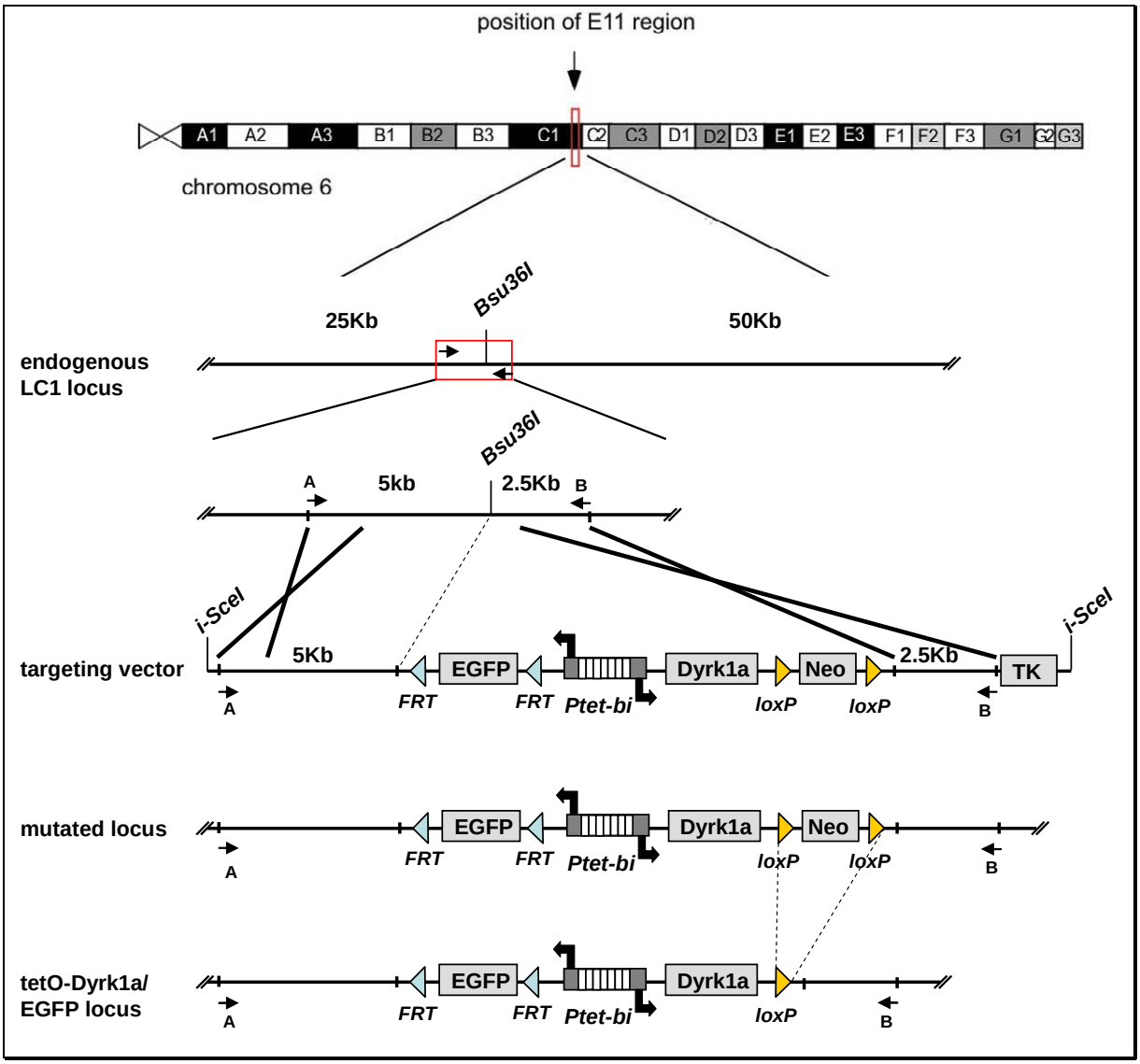


Fig. 35 Targeting strategy for the locus-specific tet-controlled Dyrk1a gene

The endogenous LC1-locus with the integration site of the ~75 kb E11 BAC analyzed by Kai Schönig is located on mouse chromosome 6. For the generation of a versatile general targeting vector for homologous recombination in ES cells a~7.5 kb fragment of the LC1-locus was subcloned into a BAC with a Herpes simplex virus type 1 thymidine kinase (*TK*) gene for negative selection in cell culture (5′end and 3′end of homology to the endogenous locus marked by arrows A and B). The *tetO* transcription unit, with the bidirectional *tetO*-promoter cassette which regulates the simultaneous expression of *Dyrk1a* and *Egfp*, and a *loxP* flanked *Neo* cassette for positive selection was integrated in the *Bsu36I* restriction site of the 7.5 kb LC1-homolgy. The *Egfp* gene was *FRT*-flanked for *Flpe*-mediated excision in later control experiments. The mutated locus in genetically modified ES-cells still comprises a neomycin resistance gene which can then be removed by *Cre*-mediated excision of *loxP*-flanked sequences *in vivo.*

6.3.2 Construction of the tet-inducible targeting vector

For the construction of the general targeting vector for the silent but highly activatable (s/a) locus LC1 we used the similar modified recombineering method described before for the cko of *Dyrk1a*. Thus we subcloned a 7.5 kb fragment of the E11 BAC genomic DNA into a plasmid backbone to eliminate the problems associated with BAC modifications. The cloning of the tet-controlled expression cassette was done by conventional methods.

6.3.2.1 Cloning of the general LC1-targeting vector

The first step in generating a targeting vector for the LC1 locus was the decision which homology sequence should be used for the homology arms of the targeting vector. After intense computational analysis of the genomic elements within the original BAC E11 by Dr. Kai Schönig, we decided to clone a ~7.5kb long homology DNA sequence into the plasmid vector pL253 that also has a *TK* (MC1-*TK*) gene for use in negative selection in ES cells. The computational analysis showed that the endogenous 75 kb LC1 locus has 3 peaks of so called S/MAR or scaffold matrix attachment regions at position 12100, 40000 and 67000. S/MARs play a crucial role within the structural organisation chromatin domains because they mediate the anchoring of DNA to the matrix of the nucleus (Schoenig, 2003). These elements might flank independent sequence areas and so we decided to integrate our response cassette between the first to S/MAR peaks from position ~16870 to 24380.
We subcloned the 7.5 kb long genomic DNA fragment from the E11 BAC, using the similar GAP repair method as described before for the construction of the Dyrk1a cko-targeting vector. Therefore two sets of PCR primers were produced and used to amplify two 250–500-bp regions of the E11 BAC (primers A_LC1 and B_LC1 and Y_LC1 and Z_LC1). The PCR products were purified using spin columns and digested with either *Not*I and *Hin*dIII or *Hin*dIII and *Spe*I. Restriction sites for these enzymes were included in the amplification primers to permit directional cloning of the PCR products into pL253. The digested fragments were purified and ligated to *Not*I- and *Spe*I-cut pL253 DNA that also has a *TK* (MC1-*TK*) gene for use in negative selection in ES cells. The retrieval vector pL253-ABYZ-LC1 was subsequently linearized with *Hin*dIII to create a DNA double-strand break for GAP repair. Following GAP repair plasmid DNA was prepared and digested to confirm the expected patterns and later sequenced. The generated plasmid was referred to as pL253-LC1 (Fig. 36). In the further process we recognized that this plasmid vector is not suitable for the

integration of larger DNA fragments, in my case 9.5 kb of the tet-response cassette and so we decided to subclone the 7.5 kb LC1 homology fragment plus the MC1-*TK* selection cassette in the BAC vector pBeloHD. Thus the desired fragment was excised from pL253-LC1 by *NotI* and *SfoI* digestion and blunt-ended using T4 DNA polymerase. Finally it was cloned into pBeloHD that was digested before with *EcoRI* and blunted. The final general targeting vector for the LC1-Locus was called pLC1-BeloHD (Fig. 36).

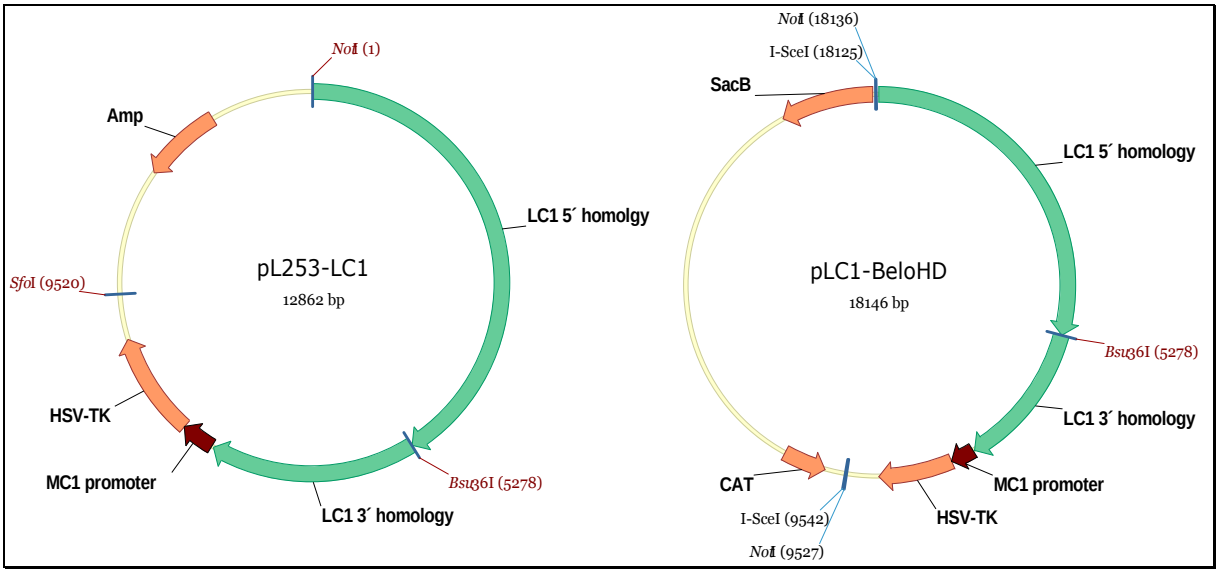


Fig. 36 Maps of the general targeting plasmid and BAC vector for the LC1 locus

Both LC1 locus general targeting contain the same 7.5 kb homology arms to the endogenous locus (including the *Bsu36I* restriction site for asymmetric homology arms) and a Herpes simplex virus thymidine kinase (HSV-TK) cassette for negative selection in ES cells. The plasmid vector is linearized with *NotI* and the BAC is cut with either *i-SceI* or *NotI* before transfection.

6.3.2.2 Cloning of the Tet-inducible bidirectional expression cassette

The bidirectional *tetO*-regulated vector pBi-EGFP (Clontech) served as a starting point for the construction of the expression cassette. The plasmid pBi-EGFP allows the simultaneous regulation of both a gene of interest and EGFP by one central tetracycline responsive element (TRE). We modified pBi-EGFP in the way that the open reading frame of the reporter gene *Egfp* is flanked by two *FRT* recombination sites. Thus we are able to excise the reporter gene *Egfp* in the *in vivo* situation by crossing the transgenic mice with *Flp*-deleter mice. This is especially important in later behavioral analysis of the mutated mice to demonstrate that the expression of the reporter *EGFP* has no effect on the phenotypes of the mutated mice.

The *FRT*-sites were generated by PCR-cloning of *Egfp*. The forward PCR primer FRT-NotI_rev was engineered to contain at the 5′-end a *NotI* restriction site, the 34bp *FRT*-site and 20bp *Egfp* homology sequence and at the 3′-end the reverse PCR primer AatII-FRT_for contained an *AatII* site, the 34bp *FRT*-site and 20bp SV40pA sequence. The PCR product was cut with *AatII* and *NotI*, gel-purified and ligated into the pBi-EGFP fragment digested before with *AatII* and *NotI*. The correct orientation of the *FRT* sites was checked by sequencing and the functionality of the *Egfp* expression cassette was ascertained in cell culture experiments.

In the next step, the open reading frame of my gene of interest, *Dyrk1a*, was cloned into pBi-Frt.EGFP. Therefore the 2292 bp long ORF of *Dyrk1a* was excised from gWiz-Mnb by NotI digestion. After purification and blunt-ending of the *NotI-Dyrk1a* fragment by T4 DNA-polymerase the DNA was ligated into the pBi-Frt.EGFP MCS, cut by *MluI* and blunt-ended. The resulting plasmid was referred to as pBi-Frt.EGFP-Mnb. The sequence of *Dyrk1a* and the orientation was furthermore checked by sequencing.

Finally we integrated a positive selection marker for the selection in mouse ES cells. Thus we used the floxed *Neo* gene in pL452 which is expressed from a hybrid PGK–EM7 promoter. PGK permits efficient *Neo* expression in mammalian cells, whereas EM7 allows *Neo* to be expressed in bacterial cells. pL452 was digested with *NotI* and *SalI* and blunt-ended with T4 DNA Polymerase. pBi-Frt.EGFP-Mnb was digested with *SapI* and also blunt-ended. Both fragments were ligated together and the electroporated cells were double-selected on LB-plates containing both ampicillin and kanamycin. The orientation of the *loxP*-flanked *Neo* cassette was checked by restriction digests.

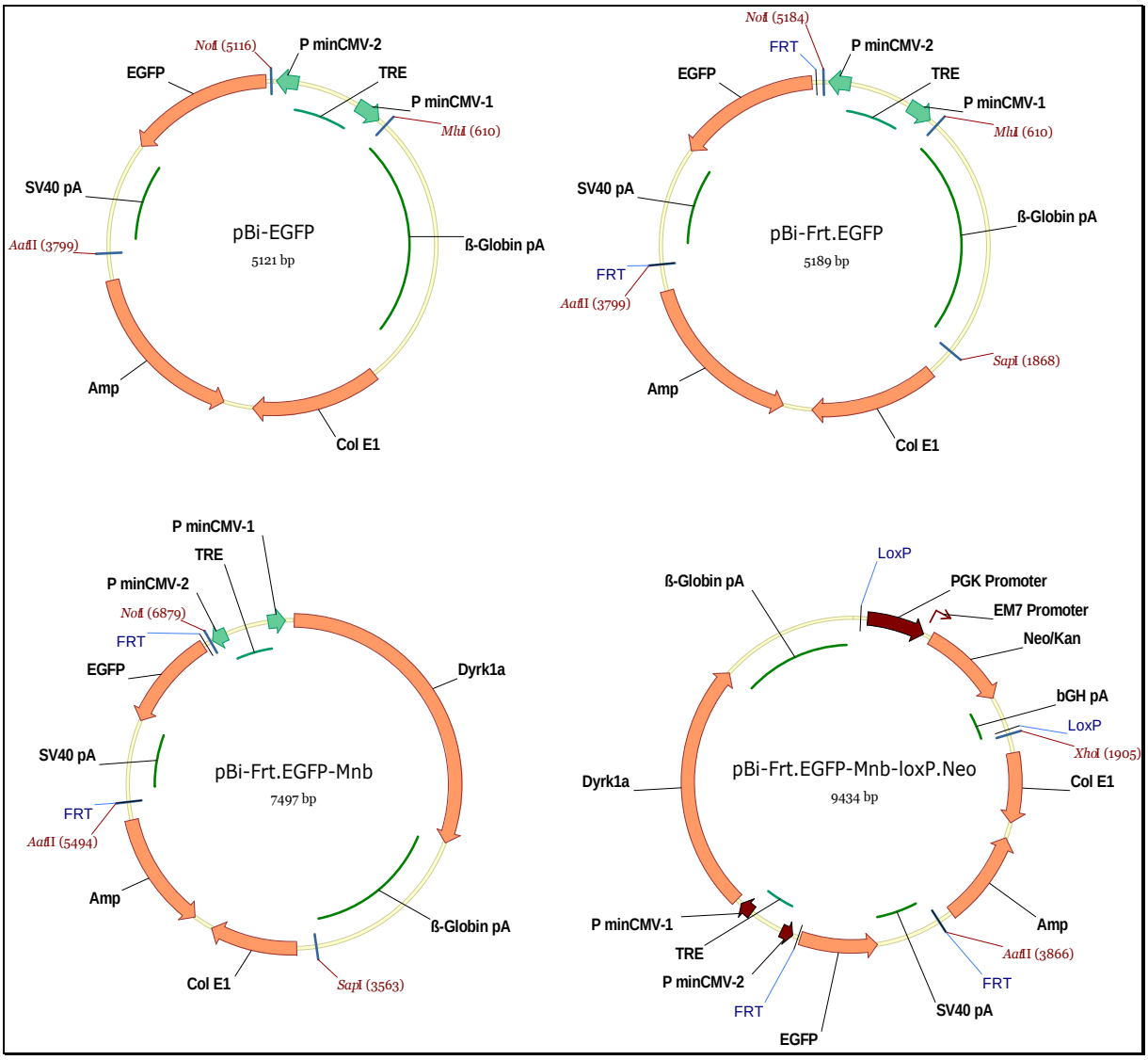


Fig. 37 Cloning of the Tet-inducible bidirectional expression cassette – vector maps

The plasmid pBi-EGFP served as a base for cloning of the conditional expression cassette. The *Egfp* gene was first *FRT* flanked by PCR-cloning in pBi-Frt.EGFP and then the mouse *Dyrk1a* cDNA was cloned into the multiple cloning site of pBi-FRT.EGFP, resulting in the plasmid pBi-FRT.EGFP-Mnb. Finally the *loxP* flanked PGK-*Neo* cassette (including a prokaryotic EM7 promoter for kanamycin selection in bacteria) was cloned downstream of the bovine growth hormone poly-A signal (bGH pA). For cloning in the general LC1 targeting vector, the expression cassette was cut out from pBi-Frt.EGFP-Mnb-loxP.Neo by digestion with *AatII* and *XhoI*.

6.3.2.3 Cloning of the final targeting vector

The final bidirectional *tetO*-expression cassette with the positive selection cassette was then cloned blunt into the general LC1-Locus targeting vector pLC1-BeloHD in the blunted *Bsu36I* site of the BAC. The *Bsu36I* digest produced asymmetric homol-

ogy arms. The 5´ arm is ~5 kb and the 3´ arm is ~ 2.5 kb long which helps detecting positive ES-cell clones after homologues recombination by PCR-genotyping. The bidirectional cassette plus the floxed *Neo* cassette was excised by digestion of pBi-Frt.EGFP-Mnb-loxPNeo with *AatII* and *XhoI.* The fragment was blunt-ended with T4 DNA polymerase, gel-purified and ligated to pLC1-BeloHD. The electroporated cells were now double-selected for chloramphenicol and kanamycin resistance. BAC DNA was prepared and digested to confirm the expected restriction patterns.

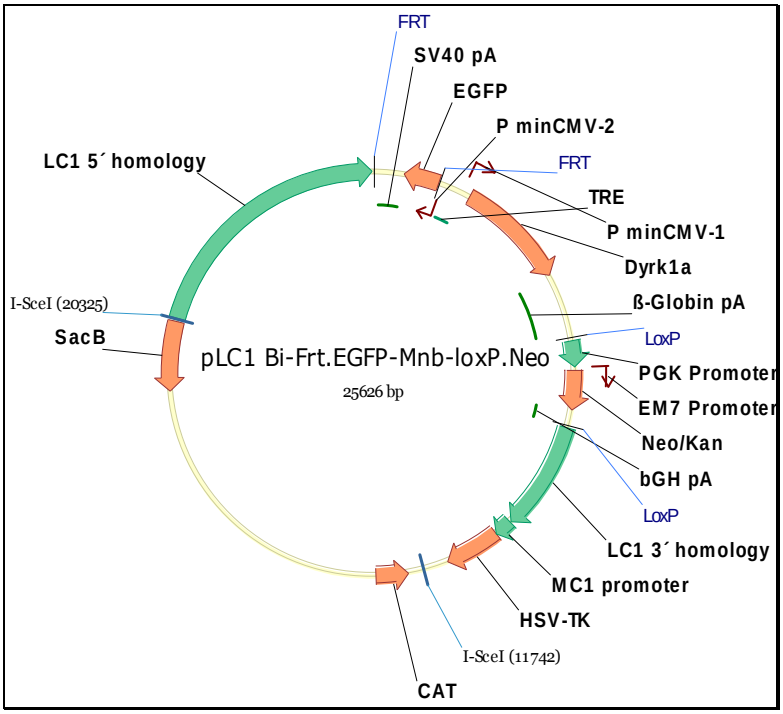


Fig. 38 LC1 locus specific targeting vector for tet-controlled Dyrk1a expression

The bidirectional Dyrk1a/Egfp tet-response cassette with the loxP flanked Neo cassette was cloned blunt into the *Bsu36I* site of the general LC1 locus targeting vector pLC1-BeloHD. For gene targeting in ES cells the vector was digested with *i-SceI,* the vector backbone was removed and the targeting fragment was purified.

6.3.3 Transient transfection in Hela and ES cells

The functional test of the whole targeting construct was accomplished by electroporation of the final BAC into modified Hela cells which constitutively express the rtTA-M2 reverse-transactivator. Without doxycycline in the culture medium there is virtually no expression of *tetO*-regulated genes. By adding doxycycline to the medium the expression is intiated. In our experiment we could clearly show that EGFP

and Dyrk1a are coexpressed after the medium was supplemented with doxycycline (Fig. 39).

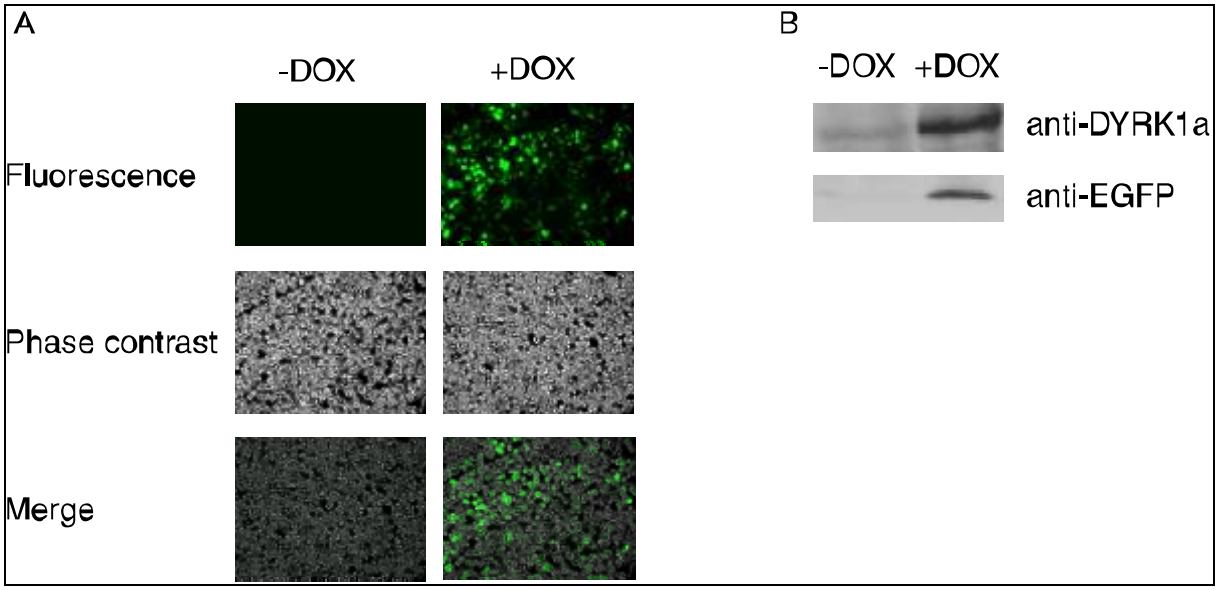


Fig. 39 Transient transfection of the LC1-targeting vector in Hela-M2 cells
Hela-M2 cells which constantly express the rtTA-M2 transactivator were transfected with 4µg DNA of the final LC1-targeting vector and were grown in the presence or absence of 1µg/ml doxycycline (DOX) in the culture medium for 24h. **(A)** Fluorescence microscopy of the transfected Hela-M2 cells and **(B)** Western Blot analysis of the transfected cells using anti-DYRK1a and anti-EGFP antibody

6.3.4 Genetic modifications of ES cells

The gene targeting was done as described before for the conditional knockout of *Dyrk1a* by homologous recombination. In brief, gene targeting includes transfer targeting vector DNA into the ES cells by electroporation and the locus specific integration into the ES cell genome under culture conditions that maintain the undifferentiated state during the whole gene targeting process.

6.3.4.1 Homologous recombination LC1 vector

The targeting vector pLC1-Bi-Frt.EGFP-Mnb-loxPNeo was linearized with *i-SceI*, purified and electroporated into R1 mouse ES cells as described before for the cko vector. The electroporated ES cells were also selected for their G418 and gancyclovir resistance. After selection, about 80 G418 and gancyclovir-resistant ES cells clones were obtained and 44 colonies were selected and analyzed.

6.3.4.2 PCR-analysis of the LC1 ES cell clones

To ascertain that the P_{tet}-bi cassette is correctly integrated into the endogenous LC1 locus on mouse chromosome 6 we also decided to use a similar PCR screening strategy (Fig. 40) as described before for the cko of *Dyrk1a*. Aliquots of the PCR reactions were subsequently separated by agarose gel-electrophoresis and visualized under UV-light.

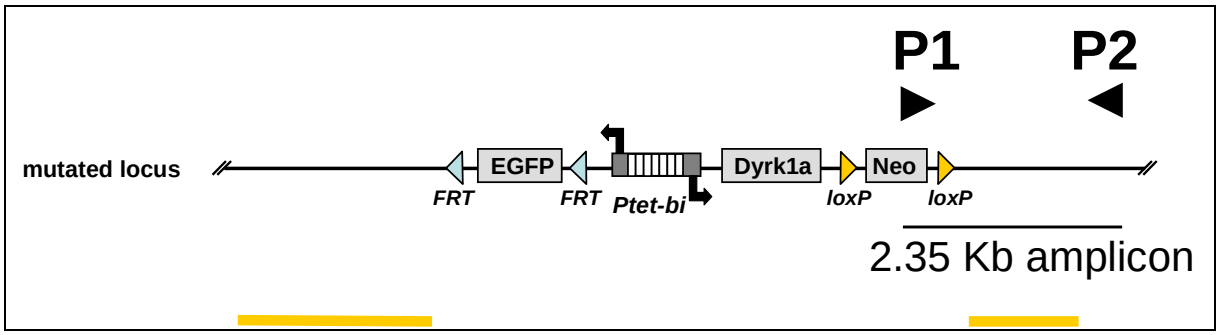


Fig. 40 PCR genotyping strategy for the mutated LC1 locus after homologous recombination

The forward primer P1 (pBi_NeoIntFor2) binds within the Neo cassette and the reverse primer P2 (HygTK_IntRev2) binds outside of the homology arm (yellow line) within the endogenous locus. Only the ES cells in which the gene targeting by homologous recombination was successful will generate a 2.35 kb PCR amplicon.

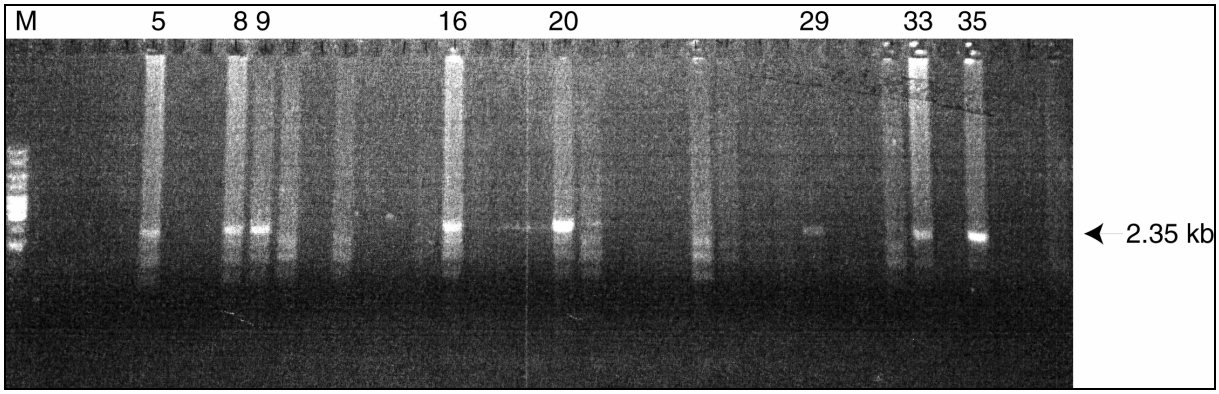


Fig. 41 PCR screen for the mutated LC1 locus in ES cells

DNA was prepared after Proteinase K digest and submitted to PCR with primer pBi_NeoIntFor2 and HygTK_IntRev2. A 2.35 kb amplification product was detected for ES cell clones that had undergone correct homologous recombination in the short arm of homology. (M: 1 kb DNA marker)

We could verify that 9 of the 44 selected neomycin and gancyclovir resistant ES cell clones PCR-amplified the theoretical 2349 bp fragment using the *Neo*-internal primer pBi_NeoIntFor2 and the external primer HygTK_IntRev2 (Fig. 41). To ensure that the

identified clones # 5, 8, 9, 16, 20, 29, 33, 35 and 39 were not truncated we also checked the ES cell clones for the presence of the genes for *Dyrk1a* (primers Mnb_RT_for and Mnb_RT_rev) and *Egfp* (primers GFP5´and GFP3´) by PCR genotyping. For blastocyst injection only clones # 8 and 20 were thawed, expanded and prepared according to the protocol of the ZTL.

6.3.5 Injection of ES cell clones into blastocysts

The ES cell clones which were positive after PCR analysis and thus heterozygous for the mutated locus were prepared for injection into blastocyst following a stringent protocol from Frank Zimmermann of the ZTL Heidelberg. Thus the positive ES cell clones were thawed and expanded in cell culture to maintain the embryonic development potential of the genetically modified ES clones.
The microinjection of the genetically modified ES cells into blastocysts was done by Sascha Dlugosz at the ZTL in Heidelberg. Blastocysts with genetically modified ES cells were transferred into the uterus of pseudo-pregnant foster mothers. Consequently these implanted blastocysts produced chimeric offspring. In general the degree of coat color chimerism of a particular animal correlates with the degree of germline contribution. In our experiments only male offspring, which were 70-90% chimeric (i.e. high percentage of agouti-colored coat) were selected for breeding with C57BL/6 females to demonstrate the germline transmission of the genetic modification. In contrast, chimeras which only produced black offspring did not contribute to the germline because the agouti-color coat of the ES cell phenotype was not inherited. Fig. 42 shows a summary of all chimeras and their contribution to the germline.

ES cell clone	*Reimplanted blastocysts*	*Offspring*	*Chimeras (male)*	*GLT*	*TierBase Code*	*Mouse line*
# 20 (LC1)	44	22	11 (8)	0	-	-
# 8 (LC1)	23	15	2 (2)	2	Line 20	CB1

Fig. 42 Efficiency of injection and germline transmission (GLT) of different ES cell lines

6.3.6 Genotyping of agouti-colored litters

The resulting agouti-colored litters from the backcross of the male chimeras on black-colored females (C57BL/6) ascertain the germline transmission of the genetically

modified R1 ES cells (129X1/SvJ x 129S1/Sv). Since the ES cells were in general heterozygous for the introduced mutation after gene targeting, the litters were genotyped by PCR reaction because wildtype and heterozygous mice can be obtained from the agouti-colored offspring. For PCR genotyping, the DNA was isolated from tail tissue of the mice.

6.3.7 Genotyping of LC1-*tetO-Dyrk1a/Egfp-loxPNeoloxP* mice (CB1)

The CB1 mouse line is derived from the backcross of the male chimeras from the ES cell injection of the targeted ES cells with the *Dyrk1a*/*Egfp tetO*-transcription unit in the silent but activatable (s/a) locus LC1 on C57BL/6 mice. To demonstrate that the mutated allele is transmitted through the germline and not the wildtype allele, the agouti-colored offspring was PCR genotyped with the same primers used for checking the correct integration during homologous recombination in ES cells (primers pBiIntFor2 and HygTKIntRev2) and additionally with *Neo* genotyping primers (Neo 5′and Neo 3′) (Fig. 43). From the first injection of ES clone #20 we got 8 male chimeras but only two of them had agouti-colored offspring. Their backcross on C57Bl6 gave rise to more than 60 offspring but none of them did transmit the mutated allele. Therefore we injected another mutated ES cell clone (clone # 8). This time we got 2 male chimeras and their backcross on C57BL/6 resulted in 35 agouti-colored offspring. Only 4 were considered as germline transmitters because they were PCR genotyping positive (TierBase codes 20.134, 20.142, 20.147 and 20.148) for both primer pairs. In summary we had 4 founders of the mouse line CB1 which transmitted the allele with *tetO-Dyrk1a*/*Egfp* transcription unit in the silent but activatable (s/a) locus LC1 through the germline.

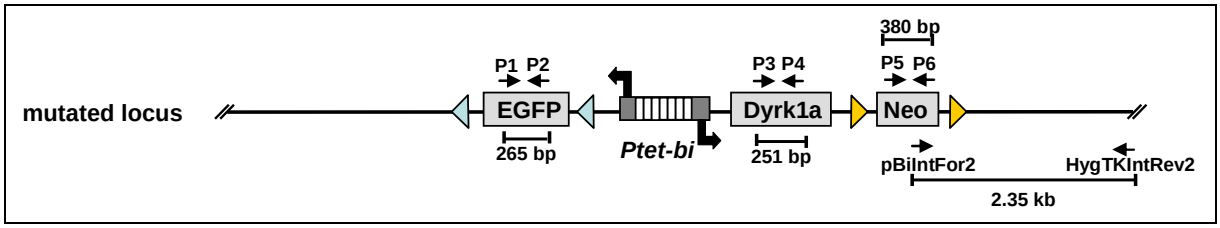


Fig. 43 PCR genotyping strategy for the CB1 mice

The agouti-colored offspring of the chimeric mice were first PCR genotyped with the primers used for the ES cells after homologous recombination (pBiIntFor2 and HygTKintRev2), generating a 2.35 kb fragment and then with the internal neomycin primers (Neo 5′and Neo 3′) amplifying a 380 bp fragment and with the internal *Egfp* primers (GFP5′ and GFP3′) amplifying a 280 bp fragment.

6.3.8 Genotyping of LC1-*Dyrk1a/EGFP* delta *Neo* mice (CB3)

After the mutated *tetO-Dyrk1a/Egfp* allele in the LC1 locus was transmitted through the germline in the CB1 mouse line we had to remove the positive selection marker *Neo*. It has been shown that the presence of neomycin in the genome might influence the phenotype of the genetically modified mice and to prevent the latter we crossed the CB1 mice with *Cre*-deleter mice. The *Cre*-deleter mice express the *Cre* recombinase under the control of the adenovirus EIIa promoter that targets expression of *Cre* recombinase to the early mouse embryo. By *Cre*-mediated recombination, the *loxP*-flanked *Neo* is removed from the genome by excision. To test whether the excision of *Neo* was successful we analyzed the offspring of the mating between CB1 and the *Cre*-deleter mice by PCR (Fig. 44).

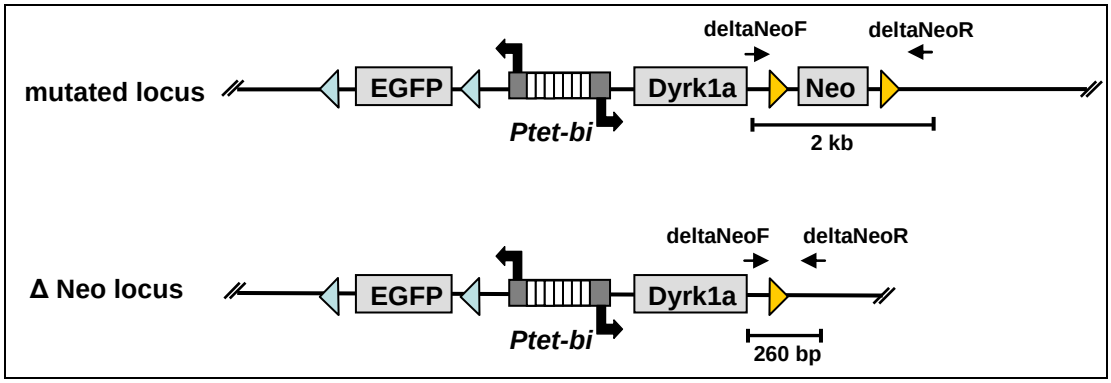


Fig. 44 PCR genotyping strategy for the detection of *Cre*-mediated recombination
The offspring of the CB1 mating with the *Cre*-deleter mouse line EIIa-*Cre* were analyzed with the primers deltaNeoF and deltaNeoR. The mutated locus with the neomycin cassette generates a 2057 bp fragment and when Neo cassette is successfully deleted by *Cre*-mediated recombination (ΔNeo), the amplicon is 260 bp long leaving behind a single *loxP* site downstream of the *Dyrk1a* cDNA.

Similar to the CB4 mouse line described before, we designed primer pairs that detect the excision of *Neo* in the *Dyrk1a/Egfp tetO* allele in the LC1 locus. The primer deltaNeoF is located 5′ before the first *loxP* site of *Neo* and primer deltaNeoR binds 3′ after the second *loxP* site in the endogenous sequence of the genomic *Dyrk1a* locus. When *Neo* is present there is a long PCR fragment of 2057bp and when *Neo* is *Cre*-excised it results in a short 260 bp fragment. The mating between CB1 and the *Cre*-deleter mice resulted in 9 offspring mice (now referred to as line CB3 or TierBase line #25) from which 2 mice were PCR positive for the excision of the *loxP*-flanked *Neo* cassette (25.2 and 25.3). The *Cre*-excision of the *Neo* gene leaves behind a single *loxP*-site 3′ of *Dyrk1a* cDNA. To preclude the possibility that the *Neo* gene is also located anywhere else in the genome by random integration we checked the 2 positive mice in a nega-

tive PCR using the *Neo* genotyping primers (primers Neo5′and Neo3′; data not shown). None of the 2 positive mice did amplify the Neo gene but did amplify an internal 200bp genomic control (primers Fabpi5′and Fabpi 3′) in the same PCR reaction.

Taken together, these results show that we succeeded in deleting the negative selection marker gene *Neo* by *Cre*-mediated recombination from the mouse genome of the CB1 mice and generated the desired *tetO*-response allele controlling the expression of *Dyrk1a* and *Egfp* in the new mouse line CB3.

6.4 Conditional expression of *Dyrk1a/Egfp* by crossing in *tTA* mouse lines

6.4.1 Genotyping of LC1-*tetO-Dyrk1a/EGFP* x CaMKIIa-*tTA* mice (CB1 x KT1LZR)

The method of choice to establish tet-regulated gene expression in transgenic mice is the breeding of a transactivator-line, in which the tetracycline-dependent transactivator is expressed under the control of a suitable tissue-specific promoter, and a mouse line wherein the gene of interest is under the control of a tet-regulated promoter. To test whether our *Dyrk1a*/*Egfp tetO*-response allele can be regulated, we decided to breed the CB1 mice with the CaMKIIa-*tTA* transactivator mouse line KT1LZR. In this mouse line the Tet-transactivator *tTA* is under the control of the same 8.5 kb forebrain-specific CaMKIIa promoter we also used for the *Cre*-mediated excision of *Dyrk1a* as described before with the floxed *Dyrk1a* mouse line CB4. The KT1LZR mouse line expresses tTA in sufficient amounts and shows its maximal expression in absence of Dox. We mated heterozygous CB1$^{tetO/+}$ mice with KT1LZR$^{CaMKIIa\text{-}tTA/+}$ and analyzed the offspring with genotyping PCR for the cDNA of *Egfp*, *Dyrk1a* and *tTA* (Fig. 45).

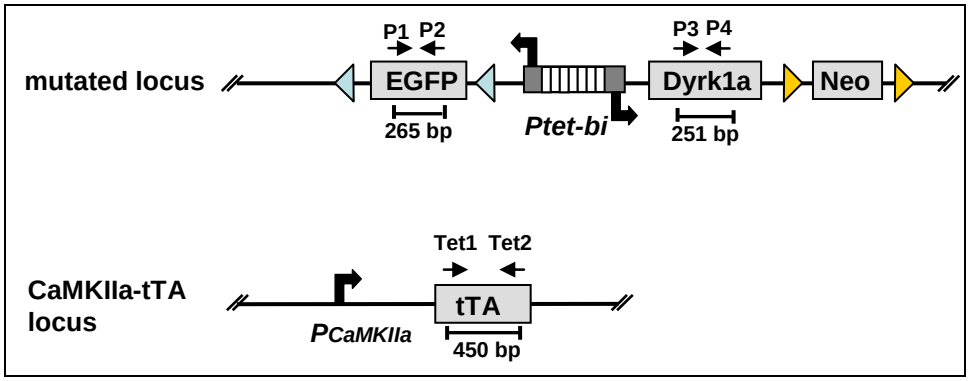


Fig. 45 PCR genotyping strategy for CaMKIIa-*tTA* and CB1 mice

The CB1 mice were genotyped as described before with the primers for *Egfp* (P1=GFP 5′ and P2=Gfp3′) and for the *Dyrk1a* cDNA (P3=Mnb_RT_for and P4=Mnb_RT_rev). The CaMKIIa-*tTA* allele was genotyped with the primers Tet1 and Tet2 which amplify a 450bp fragment of the original tTA transactivator.

6.4.2 Expression analysis of genetically modified mice

To determine wether the *tetO*-response cassette in the CB1 mouse line is functional, we analyzed the mice for conditional forebrain specific gene expression of *Dyrk1a* and the reporter *Egfp*, after the genetic characterization of the intercross offspring between the CB1 mouse line and the KT1LZR mouse line. Therefore we explanted the whole brain of single *tetO* transgenic (+ Dox) and double transgenic *tetO*/CaMKIIa-*tTA* (- Dox) mice and divided the brain sagittaly in two parts. One part of the brain was sagittaly sectioned with a vibratome and brain slices were analyzed by *in-situ* hybridization for gene expression of *Dyrk1a* and *Egfp* (Fig. 46). We could show, that the overall expression of *Dyrk1a*, using a *Dyrk1a* cDNA antisense DIG-probe, is just slightly enhanced in the induced state compared to the uninduced state. The ubiquitous and strong endogenous expression of *Dyrk1a* led us think of another strategy to determine wether the induced expression pattern that we see is overlayed by the the strong wildtype expression of *Dyrk1a*. Toward that goal we generated another DIG-probe with the antisense sequence of the rat ß-globin polyA tail that is expressed at the 3′end of *Dyrk1a* cDNA in the *tetO* expression cassette (see also vector map of pBi-EGFP). This ß-globin polyA probe allowed us to determine the gene expression of the transgenic Dyrk1a cDNA, since the rat ß-globin polyA sequence is not present or expressed in the mouse (BLAST analysis, data not shown).

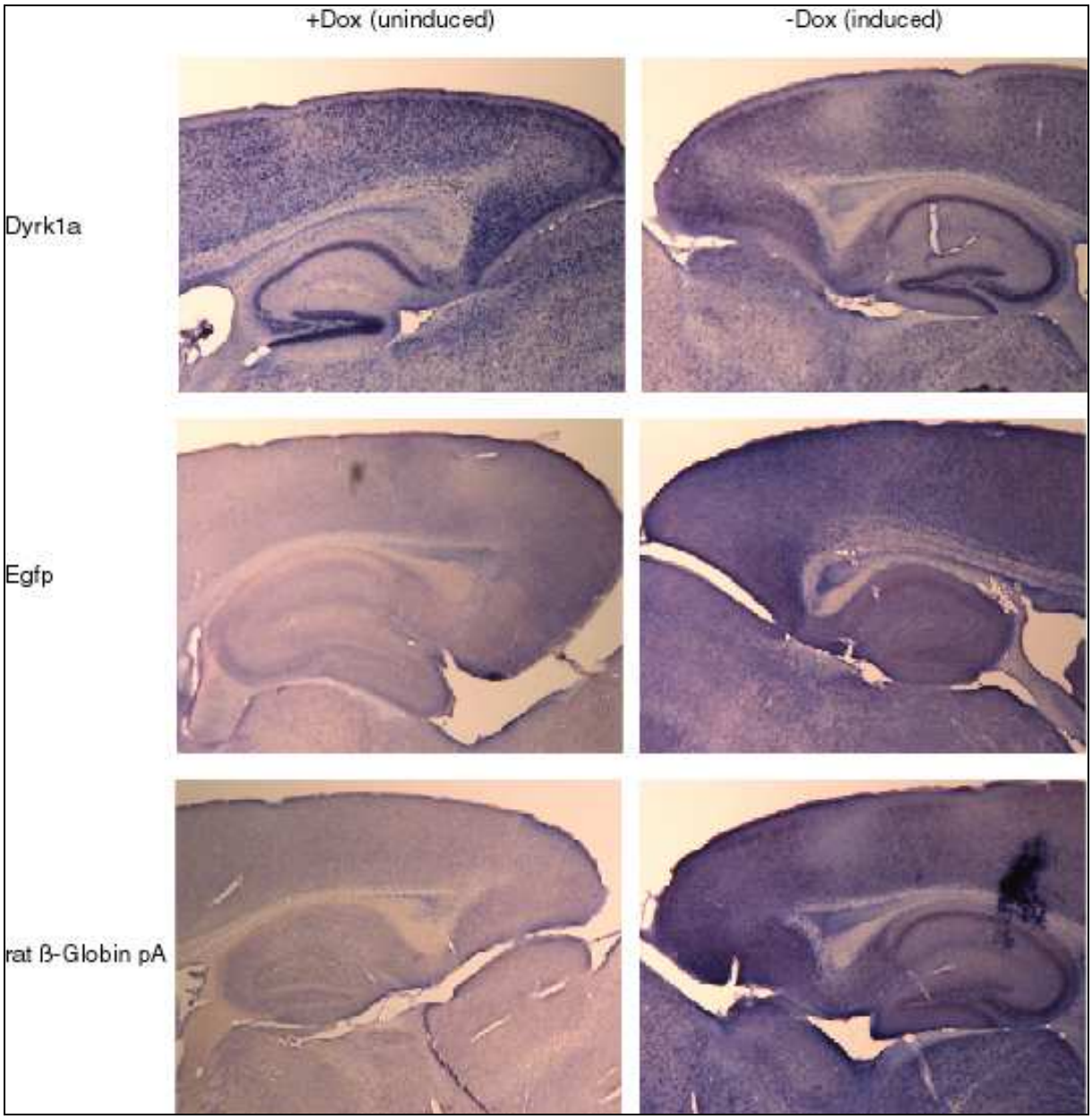


Fig. 46 Expression profile of the LC1-*tetO-Dyrk1a*/*Egfp* / CaMKIIa-*tTA* mice

Brains of single transgenic LC1-*tetO-Dyrk1a*/*Egfp* mice (+Dox: uninduced) and double transgenic LC1-*tetO-Dyrk1a*/*Egfp* / CaMKIIa-*tTA* mice (-Dox: induced) were sagittaly sectioned and were analyzed for mRNA expression by *in situ* hybridization with DIG-labelled RNA antisense probes for *Dyrk1a, Egfp* and the rat ß-Globin pA sequence. The rat ß-Globin pA probe detects the transgenic *Dyrk1a* expression because it is expressed at 3´end of the *Dyrk1a* cDNA. Hybridization was visualized by alkaline phosphatase/BCIP staining (blue color).

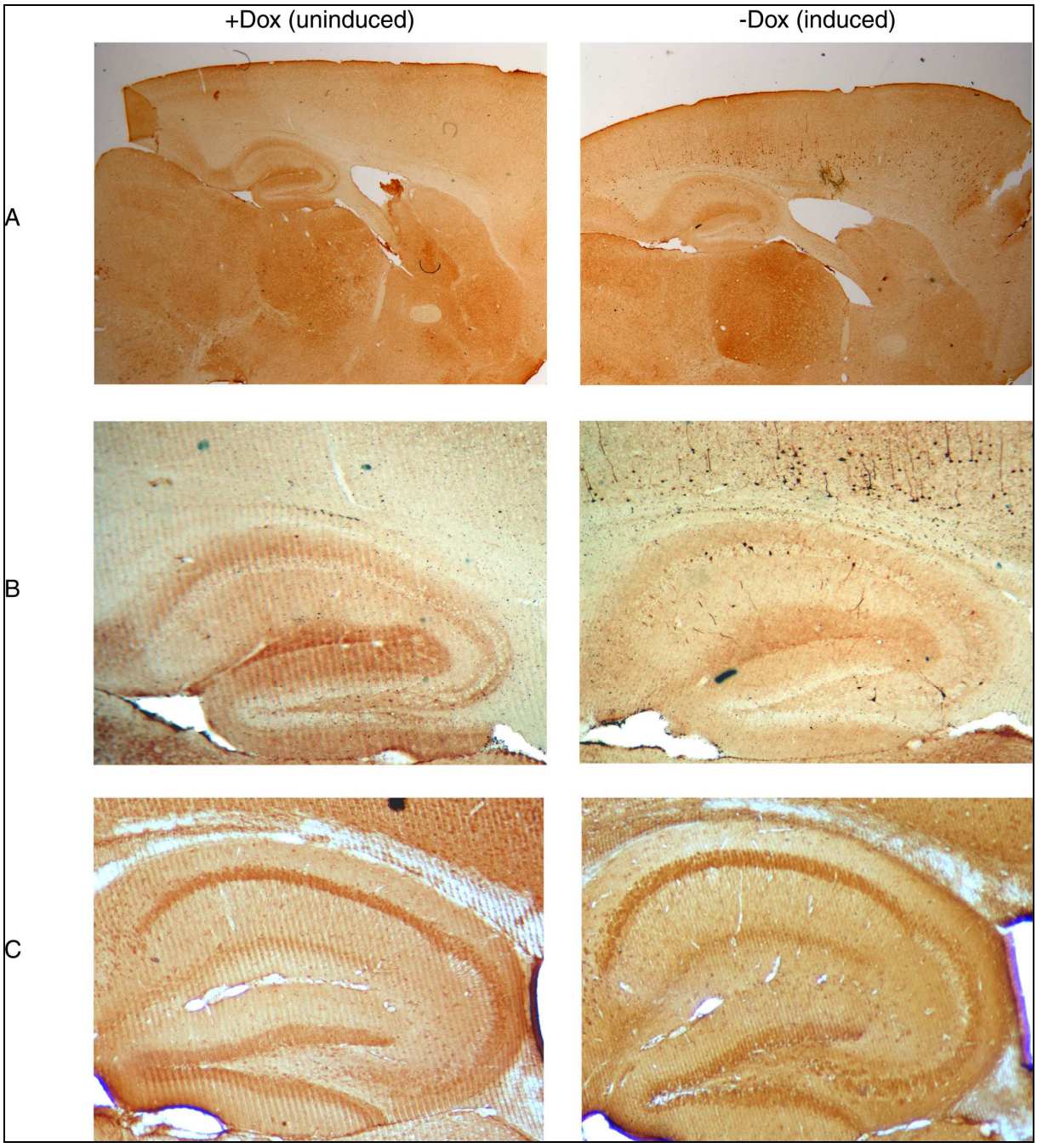


Fig. 47 Protein expression in LC1-*tetO-Dyrk1a*/*Egfp* / CaMKIIa-*tTA* mice

Brains of single transgenic LC1-*tetO-Dyrk1a*/*Egfp* mice (+Dox: uninduced) and double transgenic LC1-*tetO-Dyrk1a*/*Egfp* / CaMKIIa-*tTA* mice (-Dox: induced) were sagiatally sectioned and were analyzed for EGFP expression by immunocytochemistry with an anti-EGFP antibody and for Dyrk1a expression with an anti-Dyrk1a antibody. **(A)** EGFP expression in the forebrain and hippocampus **(B)** magnified section of EGFP in the hippocampus **(C)** Dyrk1a expression in the hippocampus. Antigen expression was detected by HRP/DAB staining (brown color).

As predicted, we could see no expression of transgenic *Dyrk1a* mRNA in the uninduced state, but we could see transgenic tet-regulated expression of *Dyrk1a* cDNA in the induced state (-Dox). *Dyrk1a* gene expression was detected according to the CaMKIIa-*tTA* expression pattern in the brain, with highest expression in the hippocampus and the cortex. The simultanious expression of the reporter *Egfp* in the induced state could also be detected but the overall expression of *Egfp*, especially in the hippocampus was decreased compared to transgenic *Dyrk1a* expression. To test if the mice also express the corresponding Dyrk1a and EGFP protein in a conditional way, we sliced the other part of the explanted brains to analyze protein expression by using antibodies against Dyrk1a and EGFP (Fig 47). The immunocytochemistry with the Dyrk1a antibody showed no obvious differences in the induced and uninduced state (Fig. 47C), as predicted by the results of the *in situ* hybridization experiments. In contrast to the mRNA expression experiments we were unable to use an alternative way to detect the transgenic Dyrk1a expression. The antibody only detects the N-terminal part of Dyrk1a, independent of transcription from endogenous mRNA or transgenic cDNA, and the transgenic Dyrk1a does not contain a protein tag like FLAG or His. Therefore the only way to detect the inducible protein expression of either genes was done by EGFP staining since EGFP is not endogenously expressed but is expressed upon tTA induction. We could clearly show that EGFP is expressed by tTA induction in the absence of Dox (Fig. 47A). But in contrast to the mRNA expression results, EGFP could only be detected in a few single cells in the hippocampus and several defined single cells in the cortical layers of the cortex (Fig. 47B).

7 Discussion

The ability to introduce virtually any mutation into the mouse genome following gene targeting in mouse embryonic stem (ES) cells provides a powerful approach for elucidating the physiological function of genes and their corresponding proteins in the whole animal.

In the course of this work, we established two mutagenesis systems that allow conditional control of *Dyrk1a* gene expression, including gene silencing (knockout) and overexpression. With the help of homologous recombination in ES cells and the *Cre*/*loxP* recombination system, we generated mouse lines with floxed *Dyrk1a* alleles to ablate *Dyrk1a* by *Cre*-mediated recombination. Furthermore we generated with the help of the Tet-system a tet-response mouse line to overexpress *Dyrk1a* by Tet-induction. Both genetically modified mouse lines should lay the foundation for further elucidating the *in vivo* function of *Dyrk1a* in Down syndrome and its role in associated deficits in cognition and learning and memory.

7.1 Targeting strategy for cko of *Dyrk1a* exon 5

To generate conditional gene targeted mice, gene targeting by homologous recombination in ES cells and a combination of the *Cre*/*loxP*- and the *Flp*/*FRT*-recombination system were employed. Genomic BAC clones containing sequences of the murine *Dyrk1a* gene were obtained from a mouse genomic BAC library and characterized to comprise putative exons 4-7 of the murine *Dyrk1a* gene.

Whereas conventional knockouts are usually designed so that a selection marker disrupts the transcription/translation of the target gene, in conditional gene knockouts, the gene must function normally until recombined by *Cre* recombinase like the endogenous gene. Thus any modification must be placed outside coding regions and not interfere with regulatory intronic regions like splice acceptor- or donor sites. For conditional gene targeting, *loxP* sites are thus either positioned to block transcription/translation by *Cre*-mediated deletion of promoter elements including transcription or translation start sites, or alternatively to remove exons encoding important functional domains. The removal of exons might result in a frameshift if alternative splicing occurred after recombination.

Our strategy was designed on the principal of removal of functional domains, especially the ATP-binding domain of the kinase encoded by exon 5 of the *Dyrk1a* gene. The binding of ATP to the kinase is essential for its function because the bound ATP

is needed to transfer a phosphate moiety from the cleavage of ATP in ADP+P to its substrate, thus generating a functional knockout of Dyrk1a kinase.

As shown in the targeting strategy in Fig. 20, exon 5 of *Dyrk1a* was flanked by two *loxP* sites and a *FRT*-flanked positive selection *Neo* cassette in-between exon 5 and the second *loxP* site downstream of exon 5. The *loxP* sites were positioned more than 250bp away from the exon on both sites to remove the complete exon 5 and not interfere with intronic regulatory elements surrounding *Dyrk1a* exon 5. Furthermore according to putative determined exon-intron boundaries all exons downstream of exon 5 will not be expressed upon *Cre*-mediated deletion, since alternative splicing of the remaining exon 4 to exon 6 would introduce a frameshift and result in a premature termination signal (TGA) at the third amino acid codon in exon 6, which turned out to be the case (Fig. 34).

This left a possible transcript encoded by exon 1 to exon 4 with a premature termination signal introduced in case of alternative splicing to exon 6. Thus the new transcript potentially was translated into a truncated Dyrk1a protein comprising the putative spliced domain, the nuclear localization signal (NLS) and the first subdomain of the kinase before the ATP-binding domain.

In many cases the introduction of a premature stop signal had been shown to degrade the mutated transcript, a process termed nonsense mediated decay (Maquat, 2005). Hence it was possible that the truncated Dyrk1a protein encoded by exon 1 to 4 would not be translated at all or not very efficiently after *Cre*-mediated excision of *Dyrk1a* exon 5. Therefore we analyzed the expression of Dyrk1a in the forebrain of mice carrying a *Cre*-mediated heterozygous knockout of *Dyrk1a* and could not detect any truncated form of Dyrk1a (data not shown). The results we obtained resemble what was found in the conventional knockout were no expression of the truncated Dyrk1a protein could be identified, despite the fact that our Dyrk1a antibody was generated against the N-terminus of Dyrk1a. It is still noteworthy that our putative truncated form of Dyrk1a is longer than the truncated Dyrk1a of the conventional knockout described previously. Through the strategy we used, the truncated form includes exon 1-4, lacking exon 5 with a stop codon in exon 6 in contrast to the conventional knockout which comprises only exon 1-3 with exons 4-5 deleted and a new stop codon in exon 6. We do not know wether the nonsense mediated decay and the degradation of the mutated transcript is due to degradation of the protein or due to low level expression after *Cre*-mediated deletion.

Taken together our targeting strategy and the new technique of recombineering, allowed for a rapid and efficient generation of a conditional *Dyrk1a* knockout gene targeting vector.

7.2 Targeting of the *Dyrk1a* gene in ES cells

The targeting strategy was unusual in respect to the cloning method used for the generation of the targeting vector. A major limitation for generating conditional knockout mice is the difficulty and time it takes to make a targeting vector. The conventional approach is to find appropriate restriction enzyme sites that are located in or near the gene. These sites are then used to ligate together *lox*P sites and various other DNA fragments such as homology arms, a positive selection marker such as *PGK-Neo*, and a negative selection marker such as *MC1-TK*. The problem with this approach is that restriction sites are not always located in convenient places, and this can severely limit where *lox*P sites are placed.

For the generation of our conditional knockout mouse allele we modified the phage-based *E.coli* homologous recombination system described by Copeland et al (Liu et al., 2003). With this advanced system we were able to subclone or modify large pieces of DNA cloned into BACs or plasmids without the need for restriction enzymes or ligases. This new form of DNA engineering, termed recombineering, has many different uses for functional genomic studies.

Homologous recombination is mediated by the lambda-phage Red proteins and is used to subclone DNA from BACs into high-copy plasmids by GAP repair, and together with *Cre* or *Flp* recombinases, to introduce *FRT* or *loxP* sites into the subcloned DNA. The first *loxP* site upstream of *Dyrk1a* exon 5 was introduced via homologous recombination in bacteria by using a modified *loxP* flanked *Neo* cassette with ~250bp of homology to the target locus on each side. The *loxP* flanked Neomycin cassette has a eukaryotic promoter for G418 resistance and a prokaryotic EM7 promoter for kanamycin selection in bacteria. Thus the successful homologous recombination event could be detected in bacteria by double selection with chloramphenicol (resistance of the genomic BACs) and kanamycin. Arabinose-induced *Cre* expression in the EL350 led to the subsequent recombination between the *loxP* sites and left behind the first *loxP* site 5′of exon 5. In a similar way, the second *loxP* site downstream of *Dyrk1a* exon 5 and the *PGK-Neo* cassette for positive selection in ES cells was introduced in only one step. Thus targeting was carried out with another modified *FRT* flanked *Neo* cassette. This time the prokaryotic and eukaryotic *Neo* expression cassette was

flanked with *FRT* sites and an additional *loxP* site downstream of the second *FRT* site. After homologous recombination the resulting vector represented the final targeting vector for generation of the floxed allele in ES cells (Fig. 23). Despite the fact that we also used a negative selection marker (*MC1-TK*) in our targeting vector to select against random integration events into the genome, the targeting of the *Dyrk1a* gene turned out to be reasonably efficient and 6 ES cell clones (3.13% of total isolated clones) were identified and had undergone correct homologous recombination, replacing the endogenous locus.

Two independently derived ES cell clones that carried the *loxP* flanked *Dyrk1a* and the *FRT* flanked *Neo* cassette were injected into the blastocysts to generate chimeric mutated Dyrk1a mice. They did not show any differences in lifespan, reproductive vigor, as well as embryonic lethality. Both lines were intercrossed and maintained on the C57BL/6 background, being the standard strain for genetic and behavioral analysis since the R1 ES cells are derived from a 129/SvJ substrain. Effects exerted by mixed backgrounds may influence the results obtained from conditional mutagenesis and may lead to false positives (for further information visit www.jax.org). Therefore all *Cre* or *Flp* expressing transgenic mouse lines in this study had been derived from pure C57BL/6 background or from advanced backcrosses to C57BL/6 in order to minimize the differences in the genetic background of the mice. Particularly for future behavioral analysis of the mutated mice, it is essential to backcross the germline transmitting offspring of chimeric mice on a pure genetic background for at least 10 generations to get at least 99.9% genetic identity. This extremely time consuming step usually takes about 3 years but can now be shortened to ~2 years of backcrossing by applying a novel technique, called "speed congenics" (Wong, 2002). Instead of mating just mutation positive mice to wildtype C57BL/6 mice in every generation, speed congenics selects in every backcross generation via SNP PCR screening those mice, which show the highest percentage of genetic identity to the C57BL/6 background. This shortens the time needed for a pure genetic background to ~5-6 generations. The most recent and sophisticated approach, that unfortunately has not been available before this thesis, is the application of pure inbred strains like C57BL/6 derived ES cells which totally abolish the need for backcrossing of the germline transmitting offspring since the ES cells are isogenic to the background strain. The problem so far, was the poor performance of the C57BL/6-derived ES cells during blastocyst injection, germline transmission and especially the loss of coat color selection for chimeric offspring since these ES cells are derived from the same black-colored mice as the

blastocysts in which they are injected. This problem has now been solved by genetic manipulation of just the locus responsible for the coat-color in C57BL/6 mice, resulting in "white" colored C57BL/6 ES cells (e.g. Chemicon´s Pluristem B6-White™ murine stem cells).
In summary, our targeting strategy and the new technique of recombineering, allowed for a rapid and efficient generation of conditional floxed *Dyrk1a* mice.

7.3 Knockout of *Dyrk1a* through Cre recombination

Although the cellular and neurobiological role of Dyrk1a is still more or less an enigma, its vital importance, however, has been strikingly demonstrated by the embryonic lethal phenotype caused by the ubiquitous knockout of the *Dyrk1a* gene in mice (Fotaki et al., 2002). Heterozygous Dyrk1a$^{+/-}$ mice showed decreased neonatal viability and a significant body size reduction from birth to adulthood. In contrast homozygous Dyrk1a$^{-/-}$ mice presented a general growth delay and died during midgestation between embryonic day E10.5 and E13.5. The broad developmental abnormalities of Dyrk1a$^{+/-}$ and Dyrk1a$^{-/-}$ mice are consistent with the ubiquitous expression of *Dyrk1a* and suggest that its function is fundamental to many different cell types and tissues.
In this study we wanted to circumvent the embryonic lethality of the conventional knockout by applying the conditional *Cre/loxP* recombination system. After the floxed allele of *Dyrk1a* was successfully transmitted through the germline we excised the *FRT*-flanked *Neo* cassette by breeding the CB2 mouse line to *Flp*-deleter mice resulting in a pure heterozygous floxed *Dyrk1a* exon 5 allele without a positive selection cassette (Dyrk1a$^{flox/+}$). These heterozygous floxed mice were then intercrossed to obtain homozygous *Dyrk1a* floxed mice (Dyrk1a$^{flox/flox}$). The heterozygous and homozygous floxed mice showed to be viable and are comparable to wildtype mice.
For *Cre*-mediated recombination we mated Dyrk1a$^{flox/+}$ mice with the CaMKIIa-*Cre* mice to get double transgenic mice with a heterozygous forebrain-specific deletion of *Dyrk1a* exon 5. Finally we intercrossed these heterozygous mice to generate a forebrain-specific homozygous knockout of *Dyrk1a*. Strikingly we could not get viable homozygous forebrain-specific knockout offspring from these matings in contrast to the heterozygous knockout mice. A possible explanation for this maybe the embryonic lethality of the homozygous cell type-specific disruption of the *Dyrka1* gene due to *Cre* expression during embryogenesis. This interpretation contrasts with the current view that the CaMKIIa promoter is activated postnatally in a forebrain-specific

manner (Tsien et al., 1996). Since *Cre* expression should not occur during embryogenesis there should also be no embryonic *Cre* recombination consistent with our findings that the heterozygous and homozygous floxed mice did not show any obvious differences compared to wildtype mice. With the help of RT-PCR and DNA sequencing, we could confirm that the floxed allele and so the introduction of *loxP* sites into the intronic sequences encompassing *Dyrk1a* exon 5 did influence neither the transcription nor the splicing of exons and introns of the *Dyrk1a* gene. The amplification is consistent with the amplicon size and the nucleic acid sequence of the wildtype gene locus. On the other hand we could clearly show on the transcriptional level that the mRNA of the *Cre*-recombined locus lacks exactly the nucleic acid sequence of *Dyrk1a* exon 5 thus leading to a putative truncated protein (Fig. 33 and 34).
A complete picture thus requires an alternative approach using other tissue-specific Cre mice (in particular other available CaMKIIa-Cre mouse lines) and especially the use of tamoxifen drug-inducible *Cre* mice to avoid embryonic lethality of homozygous conditional Dyrk1a knockout mice. The floxed Dyrk1a mice offer a great potential and widen the repertoire of tools to investigate the physiological role of Dyrk1a *in vivo* and *in vitro*.

7.4 Targeting strategy for Tet-inducible *Dyrk1a* mice

The need for silent but activatable (s/a) loci in the genome of the mouse for stringent tetracycline-inducible regulation convinced us to generate a general targeting vector for the LC1 locus identified by Frieder Schwenk and characterized by Kai Schönig. We made use of the same advanced technique of recombineering as described before for the cko of *Dyrk1a*. As shown in the targeting strategy (Fig. 35) we decided to subclone a 7.5 kb genomic fragment from the original E11 BAC, homologous to the endogenous LC1 locus on mouse chromosome 6 into a plasmid with a *TK* cassette for negative selection during homologous recombination in ES cells (pL253-LC1). But in contrast to the cko *Dyrk1a* targeting vector which was generated and modified only in a plasmid backbone, we had to generate a second general targeting vector for the LC1 locus in a BAC backbone (pLC1-BeloHD) because we did not succeed in cloning the large 9.5 kb fragment of the bidirectional *tetO*-response cassette into the LC1 homology arms of pL253-LC1. The bidirectional *tetO*-response construct was designed to simultaneously regulate the expression of the reporter gene *Egfp* and *Dyrk1a*. In addition the reporter gene *Egfp* was flanked with two *FRT* sites to excise the reporter gene *in vivo* for phenotypic control experiment reasons and we introduced a *loxP*

flanked *Neo* cassette for positive selection in ES cells. The final targeting vector was tested in cell culture and proofed to be functional by activation with a transactivator plasmid in the presence and absence of the inductor doxycycline (Fig. 39).
In summary we successfully generated a plasmid and BAC based general targeting vector for gene targeting of the (s/a) LC1 locus in ES cells by using recombineering and we effectively cloned and integrated a bidirectional *tetO*-response cassette for tet-inducible expression of *Dyrk1a* and the reporter gene *Egfp* into the LC1 homology arms.

7.5 Targeting of the LC1 locus in ES cells

Gene targeting was done in ES cells after digestion of the targeting vector, releasing the target fragment for electroporation. The efficiency of homologous recombination in ES cells was interestingly high compared to the cko of *Dyrk1a*, despite the fact that we used approximately the same homology length and the same negative and positive selection markers. The reason for this may be the different accessibility of the genomic loci for recombination events. We could identify 9 ES cell clones (20.45% of total isolated clones) which had undergone correct integration into the endogenous target locus. Two independently derived ES cell clones that carried the LC1-targeted *tetO*-response cassette were injected into blastocysts to generate chimeric mutated *tetO*-response mice. To test for germline transmission we backcrossed both chimeric offspring on C57BL/6 but only the chimeric offspring from ES cell clone #8 successfully transmitted the mutated allele through the germline. As discussed previously for the *Dyrk1a* cko mice we maintained the germline transmitting offspring on the C57BL/6 background to generate a pure genetic background for analysis.

7.6 *Dyrk1a/Egfp* expression in the brain

To test the functionality of the *tetO-Dyrk1a/Egfp* response cassette in the LC1 locus *in vivo* and to mimic the effects of *Dyrk1a* in the pathogenesis of Down syndrome, we overexpressed *Dyrk1a* in a conditional way in the brain. Previous studies only showed that the transgenic overexpression of *Dyrk1a* in all cells of the body leads to impairment of cognition and learning and memory but due to the ubiquitous expression pattern of *Dyrk1a* the underlying defects could not be elucidated. Therefore we breed the heterozygous CB1 mouse line (Dyrk1a$^{tetO\text{-}Dyrk1a/Egfp}$), with the mutated *tetO*-response allele, to transgenic mice expressing the *tTA* transactivator under the control of the same CaMKIIa promoter used in the previously described *Dyrk1a* cko ex-

periments (CaMKIIa-*tTA*). The obtained double transgenic offspring were analyzed for *Dyrk1a* expression and additionally for expression of the reporter gene *Egfp*. On the transcriptional level we could show by in-situ hybridization that the overall expression of *Dyrk1a* mRNA was slightly enhanced in the hippocampus, in particular in the CA1 region, and the cortex (Fig. 46) of induced mice consistent with the expression pattern of the CaMKIIa-*tTA* mouse line (Tsien et al., 1996). In contrast the immunocytochemistry of these mice showed a slightly different expression pattern (Fig. 47). In the hippocampus, the *EGFP* reporter protein expression could only be detected in just a few single cells and in the cortex *EGFP* was clearly present in some cells. This may be due to lower levels of protein expression or due to detection problems associated with the *EGFP* antibody. The expression of *Dyrk1a* ORF was not detectably altered in *tetO*/*tTA* double transgenic mice since the endogenous *Dyrk1a* is already ubiquitously expressed at high levels in the brain, even in tissues were the CaMKIIa-promoter drives the expression of *tTA*. The use of quantitative RT-PCR may significantly improve the results and may detect even slight and desired changes in *Dyrk1a* expression by tet-induction since the expression in individuals with Down syndrome is changed by only the factor 1.5. It also has to be kept in mind that we used the CB1 mouse line, including the positive selection *Neo* cassette, for our preliminary functionality tests of the *tetO*-response cassette *in vivo*. Since the presence of the *Neo* cassette can influence the phenotype of the mouse, best results might be obtained by crossing the CB1 mouse line to *Cre*-deleter mice to excise the *Neo* cassette from the genome. This has now been done and in subsequent experiments we will cross those CB3 mice again to the CaMKIIa-*tTA* mice and also to other *tTA* and *rtTA* mouse lines for further analysis.

In summary, we generated a *tetO-Dyrk1a*/*Egfp* response mouse line which offers a great potential for Down syndrome research and basic research elucidating the underlying mechanisms of learning and memory.

7.7 Future perspectives

With the steadily increasing number of available *Cre* and *tTA*/*rtTA* transgenic mice, that express *Cre* and *tTA*/*rtTA* in a tissue-specific or drug-inducible manner or in combination, and their collection in freely accessible internet databases, floxed *Dyrk1a* mice and tet-inducible *Dyrk1a* mice represent a versatile tool for the analysis of the physiological function of *DYRK1A in* vivo. They may help to verify the function of upstream and downstream targets of DYRK1A which have been identified so far *in vitro*. As a result both conditional *Dyrk1a* mice also represent a minimal animal model for Down syndrome and monosomy 21-associated intrauterine microcephaly, especially for the associated developmental deficits in cognition and learning and memory of individuals with Down syndrome. However due to the ubiquitous expression of DYRK1A these conditional models also permit to dissect the possible pleiotropic effects of DYRK1A in other Down syndrome associated clinical pathologies. So far the role of DYRK1A in heart development, hypotonia, leukemia, Alzheimer disease, Parkinson disease, infertility and anatomical changes of individuals with Down syndrome still has to be investigated.

Furthermore, the animal models can serve as a source to derive conditional floxed or tet-inducible *Dyrk1a* cell lines of various cell types. Corresponding cell lines can be obtained from these by transient *Cre* or *tTA*/*rtTA* expression in cell culture *in vitro*.

Thus various analyses of mechanisms in which *Dyrk1a* is involved become feasible in the future as indicated throughout the discussion. In summary, this includes the general molecular, biochemical and cellular function of the *DYRK1A* kinase and the analysis of loss-of-function and gain-of-function effects of *Dyrk1a* on the physiology *in vivo*, in particular effects involved in learning and memory e.g. electrophysiology and behavior.

8 Appendix

8.1 Primer sequences

8.1.1 PCR and sequencing primers

Primer name	*Sequence in 5´-3´ direction*	*Localization*
Two1	GAGGAGAGACTTCAGCATGCA	Exon 2 for
Two2	CTGGTTAGTTAGAGGTTG	Exon 2 rev
Two3	CAAGAATGGGTCGCCATTAA	Exon 5 for
Two4	AGTGTCATGTTTGTTCATGAG	Exon 5 rev
Two5	TGGTCAGGCACTGAAGCTCCT	Exon 11 for
Two6	CGAGCTAGCTACAGGACTCTG	Exon 11 rev
Two7	ATTGTCATGTTACAGAGGCGG	Exon 3 for
Two8	CTCATTAATATGCTTGTAT	Exon 3 rev
Two9	AAGCGAAGACACCAACAGGGC	Exon 4 for
Two10	TCCATCCACTTTTCTCCGT	Exon 4 rev
Two11	AGTGCATTTGAAACGCCAC	Exon 6 for
Two12	CCTCTGCCCCAACTGACAAGA	Exon 6 rev
Two13	ATATACCAGTATATTCAGAGT	Exon 7 for
Two14	CTCATTGGCACCACTGAACAG	Exon 7 rev
Two15	GATCAGATGAATAAAATAGTG	Exon 8 for
Two16	CCGTTTTCCATCTTTGGTCTT	Exon 8 rev
Two17	GAGTACAAACCACCAGGAACCC	Exon 9 for
Two18	TTTGTACCTTCATCAGCTGT	Exon 9 rev
Two19	AGCAACAGTGGGAGAGCC	Exon 10 for
Two20	CTGGGGACTATGTGTCTC	Exon 10 rev
Two21	CCCCATCAGGATGATATGA	Exon 1 for
Two22	CTGTATGCATCGTCTTCTTT	Exon 2 rev
M13F	TGTAAAACGACGGCCAGT	
M13R	CAGGAAACAGCTATGACC	
T7	TAATACGACTCACTATAGGG	
T3	ATTAACCCTCACTAAAGGGA	
Sp6	ATTTAGGTGACACTATAGAA	

8.1.2 Primers for the construction of targeting vectors

Primer name	*Sequence in 5´-3´ direction*	*Localization*
A_Mnb	GAATGCGGCCGCGGCATTTGCTTCTGGTCA	5´ Dyrk1a homology arm for
B2_Mnb	GAATAAGCTTTGGGGTGACAACAATAACAT	5´ Dyrk1a homology arm rev
C_Mnb	ATAAGCGGCCGCCGTGTTTAAAGGCGTAGC	5´ of 1.loxP for
D_Mnb	GTCGAATTCTCTGGGTTTACTGCTATCTA	5´ of 1.loxP rev
E_Mnb	ATAGGATCCGAGACAAAGGCAGGAAGATA	3´ of 1.loxP for
F_Mnb	GTCGTCGACCCTGATGAAGGCTGAGTTGAAC	3´ of 1.loxP rev
G_Mnb	ATAAGCGGCCGCGCTCTCATGTGTCTTTGTCC	5´ of 2.loxP for
H_Mnb	GTCGAATTCGGTACCGACCATTTATGTTTACCACTG	5´ of 2.loxP rev
I_Mnb	ATAGGATCCCTCTGTAGCTGGCATCTGC	3´ of 2.loxP for
J_Mnb	GTCGTCGACGCAGAACTCTGTCTCTACCCTG	3´ of 2.loxP rev
Y_Mnb	GATAAGCTTGATTTAGTCCTTCAAAAGC	3´ Dyrk1a homology arm for
Z2_Mnb	GAATACTAGTAAGAAGATGGTGACTGGAGAT	3´ Dyrk1a homology arm rev
A_LC1	GAATGCGGCCGCACACAAATACAAGGCTGATG	5´ LC1 homology arm for
B_LC1	GAATAAGCTTGGTGTTCTTTTCCTGTGTTTTA	5´ LC1 homology arm rev
Y_LC1	GATAAGCTTTTGAAAATTTCCCATGCATC	3´ LC1 homology arm for
Z_LC1	GAATACTAGTTTGCCCGTGAGTTAGAAT	3´ LC1 homology arm rev
AatII-FRT_for	ATAAGAATGACGTCGAAGTTCCTATACTTTC TAGAGAATAGGAACTTCTAAGAAACCATTATTATC	5´ of EGFP in pBi-EGFP
FRT-NotI_rev	TAAAGCGGCCGCGAAGTTCCTATTCTCTAGA AAGTATAGGAACTTCCTAGAACTAGTGGATCCCCG	3´ of SV40pA in pBi-EGFP

8.1.3 Primers for genotyping

Primer name	*Sequence in 5´-3´ direction*	*Localization*
Mnb1F	GCAGTAGATAGCAGTAAACCC	5´ of 1.loxP
Mnb2R	TCTCTCTGTTTGACCCTGG	3´ of 1.loxP
Mnb3F	CCCTGCTGCTTTACTTCTGG	5´ of 2.loxP/FRT
Mnb4R	GGAAGATGGCATGACAATGGA	3´ of 2.loxP/FRT
Mnb_RT_for	TGGGTCGCCATTAAAATCAT	For in Dyrk1a Exon5
Mnb_RT_rev	TGCGCAAACTTTCGTGTTAG	Rev in Dyrk1a Exon 6
MnbExtFor1	ATTTTCTGTTGCAGTGTTTG	For in Neomycin
MnbIntRev1	TATGATCGGAATTGGGCT	Rev 3´ external of Dyrk1a homology
pBi_NeoIntFor2	CTTGCGGAACCCTTAATATA	For in Neomycin
HygTK_IntRev2	CCTTCATTTGGTTGATTCTT	Rev 3´ external of LC1 homology
Neo5´	TGCTCCTGCCGAGAAAGTATCCATCATGGC	Neomycin for
Neo3´	CGCCAAGCTCTTCAGCAATATCACGGGTAG	Neomycin rev

Cre#3	TGCCTGCATTACCGGTCGATGC	Cre recombinase for
Cre#3	CCATGAGTGAACGAACCTGGTCG	Cre recombinase rev
Tet1	CGCTGTGGGGCATTTTACTTTAG	Tet Transactivator for
Tet2	CATGTCCAGATCGAAATCGTC	Tet Transactivator rev
GFP 5´	GCGGATCTTGAAGTTCACCTTGATGCC	EGFP for
GFP 3´	GCACGACTTCTTCAAGTCCGCCATGCC	EGFP rev
FLP1	CACTGATATTGTAAGTAGTTT	Flpe recombinase for
FLP2	CTAGTGCGAAGTAGTGATCAGG	Flpe recombinase rev
Fabpi 5´	TGGACAGGACTGGACCTCTGCTTTCCTAGA	200bp genomic control for
Fabpi 3´	TAGAGCTTTGCCACATCACAGGTCATTCAG	200bp genomic control rev
deltaNeoF	AGGCGGTTTGCGTATTGG	5´ before Neo in LC1
deltaNeoR	TGCTGGGTTCTGGTGATG	3´ after Neo in LC1

8.2 Murine *Dyrk1a* gene information

Exon Information

No.	Exon / Intron	Chr	Strand	Start	End	Start Phase	End Phase	Length	Sequence
	5' upstream sequence								..........ttcacacagtgttatagttttgccgctggactcttccctccctccccca
1	ENSMUSE00000494598	16	1	94,720,258	94,720,302	-	1	45	CCCCATCAGGATGATATGAGACTTGAAAGAAGACGATGCATACAG
	Intron 1-2	16	1	94,720,303	94,768,294			47,992	gtgactcaagtttgaaatacagta..........atctgaacttgctgtgtgttcccag
2	ENSMUSE00000263429	16	1	94,768,295	94,768,491	1	0	197	GAGGAGAGACTTCAGCATGCAAACCTTCATCTGTCCGGCTTGCACCGTCGTTCTCATTCC ATGCTGCTGGCCTTCAGATGGCTGCACAGATGCCCCACTCACACCAGTACAGTGACCGTC GCCAGCCGAGCATAAGTGACCAGCAGGTGTCTGCCTTACCATATTCTGACCAGATTCAGC AACCTCTAACTAACCAG
	Intron 2-3	16	1	94,768,492	94,772,539			4,048	gtaatttcattattatcagaaatga..........ctgctagtctctctgtctatttaag
3	ENSMUSE00000131724	16	1	94,772,540	94,772,659	0	0	120	GTGATGCCTGACATTGTCATGTTACAGAGGCGGATGCCCCAAACCTTCCGTGATCCAGCA ACTGCTCCTCTGAGAAAACTCTCTGTGGACTTGATCAAAACATACAAGCATATTAATGAG
	Intron 3-4	16	1	94,772,660	94,774,713			2,054	gtaagagtattgatttgctatagta..........ttaactttgtatatatttattacag
4	ENSMUSE00000131728	16	1	94,774,714	94,774,902	0	0	189	GTTTACTATGCAAAAAAGAAGCGAAGACACCAACAGGGCCAGGGGGACGATTCCAGTCAT AAGAAGGAGCGGAAGGTTTACAATGATGGTTACGATGATGATAACTATGATTATATTGTA AAAAACGGGGAAAAGTGGATGGATCGGTATGAAATCGACTCCTTAATAGGCAAAGGTTCA TTTGGACAG
	Intron 4-5	16	1	94,774,903	94,777,602			2,700	gtaagtctatcagttgttcattgtt..........gatgataacttgttttgttttgtag
5	ENSMUSE00000131727	16	1	94,777,603	94,777,750	0	1	148	GTTGTGAAAGCTTATGACAGAGTGGAGCAAGAATGGGTCGCCATTAAAATCATCAAGAAC AAGAAAGCGTTTCTGAATCAAGCCCAGATAGAAGTGCGGCTGCTTGAGCTCATGAACAAA CACGACACTGAAATGAAGTACTACATAG
	Intron 5-6	16	1	94,777,751	94,780,100			2,350	gtaagggctcatcacgatgccttga..........ggtgaaattttcttttctctttcag
6	ENSMUSE00000555140	16	1	94,780,101	94,780,387	1	0	287	TGCATTTGAAACGCCACTTTATGTTTCGAAACCATCTCTGTTTAGTGTTTGAAATGCTGT CCTATAATCTCTATGATTTGTTGAGAAACACCAACTTCCGAGGGGTCTCTTTGAACCTAA CACGAAAGTTTGCGCAACAGATGTGCACAGCATTGCTTTTTCTTGCGACTCCAGAACTTA GTATCATTCACTGTGACTTAAAGCCTGAGAACATCCTTCTGTGTAACCCCAAACGGAGTG CAATCAAGATTGTTGATTTTGGCAGCTCTTGTCAGTTGGGGCAGAGG
	Intron 6-7	16	1	94,780,388	94,782,134			1,747	gtaagtattcctttaggacttgtga..........aatactactttgaaatatgtttcag
7	ENSMUSE00000555136	16	1	94,782,135	94,782,281	0	0	147	ATATACCAGTATATTCAGAGTCGCTTTTATCGGTCTCCAGAGGTGCTACTGGGAATGCCT TATGACCTTGCTATCGACATGTGGTCCCTTGGATGTATCTTGGTTGAAATGCACACTGGA GAGCCTCTGTTCAGTGGTGCCAATGAG
	Intron 7-8	16	1	94,782,282	94,786,249			3,968	gtaggtaaagggtactctgtagtga..........ttttgtttgtttgtttttaatacag
8	ENSMUSE00000263402	16	1	94,786,250	94,786,390	0	0	141	GTCGATCAGATGAATAAAATAGTGGAAGTCTTGGGCATCCCACCTGCTCATATTCTTGAC CAAGCACCGAAAGCAAGAAAGTTCTTTGAGAAGTTGCCCGATGGCACTTGGAGCTTAAAG AAGACCAAAGATGGAAAACGG
	Intron 8-9	16	1	94,786,391	94,793,832			7,442	gtaaaatgggggtgcacttattctt..........atttctgttctctcttctttaatag
9	ENSMUSE00000263397	16	1	94,793,833	94,794,139	0	1	307	GAGTACAAACCACCAGGAACCCGTAAACTTCATAATATTCTTGGAGTAGAAACAGGAGGA CCTGGCGGGCGGCGTGCTGGGGAATCGGGTCATACTGTAGCTGACTACTTGAAGTTCAAA GACCTCATTTTAAGGATGCTTGATTATGACCCCAAAACTCGGATTCAACCTTATTATGCC CTGCAGCACAGTTTTTTCAAGAAAACAGCTGATGAAGGTACCAACACAAGTAACAGTGTG TCTACCAGCCCTGCGATGGAGCAGTCTCAGTCTTCAGGCACCACCTCCAGCACCTCCTCC AGCTCAG
	Intron 9-10	16	1	94,794,140	94,795,276			1,137	gtccgccacggcgcagcgcggcccg..........gtttttccatgtcctgatattaaag
10	ENSMUSE00000131722	16	1	94,795,277	94,795,401	1	0	125	GTGGATCCTCGGGAACGAGTAACAGTGGGAGAGCCAGGTCGGATCCGACGCACCAGCATC GACACAGCGGTGGACACTTCGCTGCTGCTGTCCAGGCCATGGACTGTGAGACACACAGTC CCCAG
	Intron 10-11	16	1	94,795,402	94,800,378			4,977	gtgagtgttcctagtgctcctgcca..........taacctttcacaatgcccccacag
11	ENSMUSE00000434351	16	1	94,800,379	94,801,197	0	-	819	GTGCGCCAGCAGTTTCCGGCTCCTCTGGGATGGTCAGGCACTGAAGCTCCTACACAAGTC ACTGTTGAAACTCATCCTGTTCAAGAGACAACCTTTCATGTAGCCCCCCAGCAGAACGCA TTGCATCATCACCATGGAAACAGTTCCCATCACCACCACCACCACCATCACCACCACCAC CACCATGGACAGCAAGCCTTGGGTAACCGGACCAGGCCAAGGGTCTACAATTCTCCAACA AATAGCTCCTCTACCCAGGATTCTATGGAGGTTGGCCACAGTCACCACTCCATGACATCC CTGTCTTCCTCAACAACTTCTTCCTCGACATCTTCCTCCTCTACTGGTAATCAAGGCAAT CAGGCCTATCAGAACCGCCCAGTGGCTGCTAACACCTTGGACTTTGGACAGAATGGAGCT ATGGACGTTAATTTGACCGTCTACTCCAATCCCCGCCAAGAGACTGGCATAGCTGGACAT CCAACATACCAATTTTCTGCTAATACAGGTCCTGCACATTACATGACTGAAGGACATCTG GCGATGAGACAAGGGGCTGATAGAGAAGAGTCTCCCATGACAGGAGTTTGTGTGCAACAG AGTCCTGTAGCTAGCTCGTGACTACATTGAAACTTGAGTTTGTTTCTTGTGTGTTTTTAT AGAAGTGGTGTTTTTTTCCCAAAAAACAAAAGTGCAAAGCTGCTTGAATCAGAAGGAGAT TAACACACTGAACCGCTACAAGAGGGCAAAGCTGACTTTTTTTTTTAACTTGAAAAGATT GCAAAGGGACAATGAAGTTTTTAAGAGCCATGTCCACCC
	3' downstream sequence								atcttcacggagagctcagagtgtcctctttctgcctccccatttaact..........

Fig. 48 Mouse Dyrk1a exon information from Ensembl database (www.ensembl.org)

Schematic representation of the exon/intron structure of the murine *Dyrk1a* gene. Mouse *Dyrk1a* consists of 11 exons and the transcript length is 2525 bp including the 5′ and 3′ untranslated region (pink colored sequence). The coding sequence of 2289 bp is translated into the 763 aa Dyrk1a protein (black colored sequence).

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