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REVIEW

Targeted mutagenesis tools for modelling psychiatric disorders

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Abstract In the 1980s, the basic principles of gene targeting were discovered and forged into sharp tools for efficient and precise engineering of the mouse genome. Since then, genetic mouse models have substantially contributed to our understanding of major neurobiological concepts and are of utmost importance for our comprehension of neuropsychiatric disorders. The "domestication" of site-specific recombinases and the continuous creative technological developments involving the implementation of previously identified biological principles such as transcriptional and posttranslational control now enable conditional mutagenesis with high spatial and temporal resolution. The initiation and successful accomplishment of large-scale efforts to annotate functionally the entire mouse genome and to build strategic resources for the research community have significantly accelerated the rapid proliferation and broad propagation of mouse genetic tools. Addressing neurobiological processes with the assistance of genetic mouse models is a routine procedure in psychiatric research and will be further extended in order to improve our understanding of disease mechanisms. In light of the highly complex nature of psychiatric disorders and the current lack of strong causal genetic variants, a major future challenge is to model of psychiatric disorders more appropriately. Humanized mice, and the recently developed toolbox of site-specific nucleases for more efficient and simplified tailoring of the genome, offer the perspective of significantly improved models. Ultimately, these tools will push the limits of gene targeting beyond the mouse to allow genome engineering in any model organism of interest.

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Introduction

In 2007, Mario Capecchi, Martin Evans and Oliver Smithies received the Noble Prize in Physiology or Medicine "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells" (Mak 2007). The perspicacious combination of the pluripotency of embryonic stem cells with the targeting precision of homologous recombination to produce mice with any desired manipulation of the genome was a major scientific breakthrough accomplished in the 1980s (Evans and Kaufman 1981; Thomas and Capecchi 1987; Koller et al. 1989). In addition to mice offering many practical and economic advantages compared with other mammalian model organisms, this technological quantum leap turned the mouse into the model organism of choice in biomedical research. The preeminence of the mouse is also a consequence of it having been the only mammalian species amenable to this technique until recently (Tong et al. 2010; Li et al. 2008). Its unique position has further been fostered by the sequencing of its genome immediately after that of the human genome (Mouse Genome Sequencing Consortium et al. 2002), ultimately paving the way for the International Knockout Mouse Consortium (IKMC), a high-throughput effort to produce and phenotype knockouts for all mouse genes (Austin et al. 2004; Collins et al. 2007a, 2007b; Bradley et al. 2012; Skarnes et al. 2011). Concomitantly, a wide variety of powerful resources and instrumental infra-structures have been put forward that have developed the mouse into a model organism readily accessible and available to the research community (Eppig and Strivens 1999; Ng et al. 2009; Eppig et al. 2005).

None of today's basic biomedical research disciplines, including neuropsychiatric research, would be imaginable without genetic mouse models based on the principles discovered in the 1980s. Knockout mice are ideally suited to test the function of a specific gene in one of the most thoroughly characterized model organisms. Accordingly, research involving genetic mouse models has significantly contributed to the field of neurobiology in general and to our understanding of psychiatric disorders in particular.

Nevertheless, none of the common psychiatric disorders is the consequence of a single null mutation. As in many other common disease areas, psychiatric research faces the challenge that susceptibility to this type of diseases arises from the complex interaction of many genes or of genetic variants of a relatively small effect size with largely unknown environmental factors rather than from a single gene. These circumstances have to be critically taken into account when trying to model psychiatric disorders or when interrogating the potential underlying disease mechanisms.

In this review, I will give an impression of the way that gene targeting technologies have matured from their infancy into incredibly precise tools for genome engineering. I will touch upon mouse genetic resources available to the research community and provide some practical considerations. Finally, I will highlight the most recent developments that are on the horizon and discuss their potentials for prospective neuropsychiatric research.

Origins of gene targeting procedures

Gene targeting refers to the precise modification of a specific location within the genome via homologous recombination, which takes places between a specifically designed targeting vector and a locus of interest. Today, gene targeting in embryonic stem (ES) cells is a widely used routine method to generate genetically engineered mouse models. The design and construction of a typical replacement type targeting vector has not significantly changed since the early days when these techniques were developed (Thomas and Capecchi 1987) but the fully sequenced mouse genome and technological advancements such as recombineering or direct DNA synthesis have accelerated the process of vector construction tremendously (Skarnes et al. 2011). A targeting vector encompasses two stretches of DNA that are homologous to the target locus (homology arms) and that flank a heterologous DNA sequence, usually a selection marker or a reporter cassette. Homologous recombination results in the insertion of the targeting vector and the replacement of the endogenous sequence by the homology arm-flanked construct sequence (Fig. 1). Homologous recombination is influenced by many factors including the length and the origin of the homology arms with respect to their genetic background (Te Riele et al. 1992; Deng and Capecchi 1992). Homologous recombination events are extremely rare compared with random genome integration events. Therefore, the first gene targeting approaches focused on the hypoxanthine-guanine phosphoribosyl transferase locus (*Hprt*; a deficiency results in Lesch-Nyhan syndrome in humans), because it allowed direct selection via 6-thioguanine. In addition, it was favoured by its X-chromosomal location; thus, only one mutant copy is necessary to yield a phenotype in male cells or mice (Doetschman et al. 1987; Thomas and Capecchi 1987). To enrich for desired homologous recombination events of non-selectable genes, targeting vectors are based on positive–negative selection strategies (Mansour et al. 1988; Yagi et al. 1990). The neomycin resistance gene (*neo*), which confers resistance to the aminoglycoside antibiotic geneticin (G418), is used as a marker that enables the selection of cells that have integrated the construct and the inactivation of the gene of interest. For negative selection, a *Herpes simplex* virus gene, namely the thymidine kinase gene (*HSV-tk*), is placed adjacent to the target homology (Fig. 1). In this way, the *HSV-tk* gene will not be transferred to the endogenous target when homologous recombination occurs. In contrast, random insertion of the targeting vector into the recipient genome will result in the integration of the *HSV-tk* gene. In the presence of *HSV-tk*, the non-toxic guanine analogue gancyclovir will be converted into a toxic metabolite, which will interfere with cellular DNA metabolism and ultimately kill the cell. Another marker, often used but not requiring any external inducer, is the diphtheria toxin A fragment (DTA), which exerts toxicity by inhibiting ADP-ribosylation of elongation factor 2 upon protein synthesis (Yagi et al. 1990). In general, negative selection markers provide a fair enrichment of homologous recombination events but cannot fully avoid random insertions.

Targeting vectors are routinely introduced into ES cells by electroporation. Following positive and negative selection, individual homologous recombination events need to be identified and confirmed by Southern blot or polymerase chain reaction (PCR) methods. ES cells are derived from the inner-cell mass of blastocyst-stage embryos, which retain their pluripotency after in vitro culture, i.e., the ability to contribute to all tissues of the developing embryo proper. However, ES cells do not have the capacity to develop into derivatives of the trophoectoderm (ectoplacental cone and extra-embryonic ectoderm). Therefore, other approaches have to be used to generate mice from targeted ES cells. The injection of ES cells into a blastocyst of donor mice is still the most commonly used method. Alternatively, various injection or aggregation techniques are available (Bradley et al. 1984; Wood et al. 1993; Nagy et al. 1993).

From constitutive to conditional knockout mice

In psychiatric research, as in many other biomedical research disciplines, the generation of constitutive knockout mice by

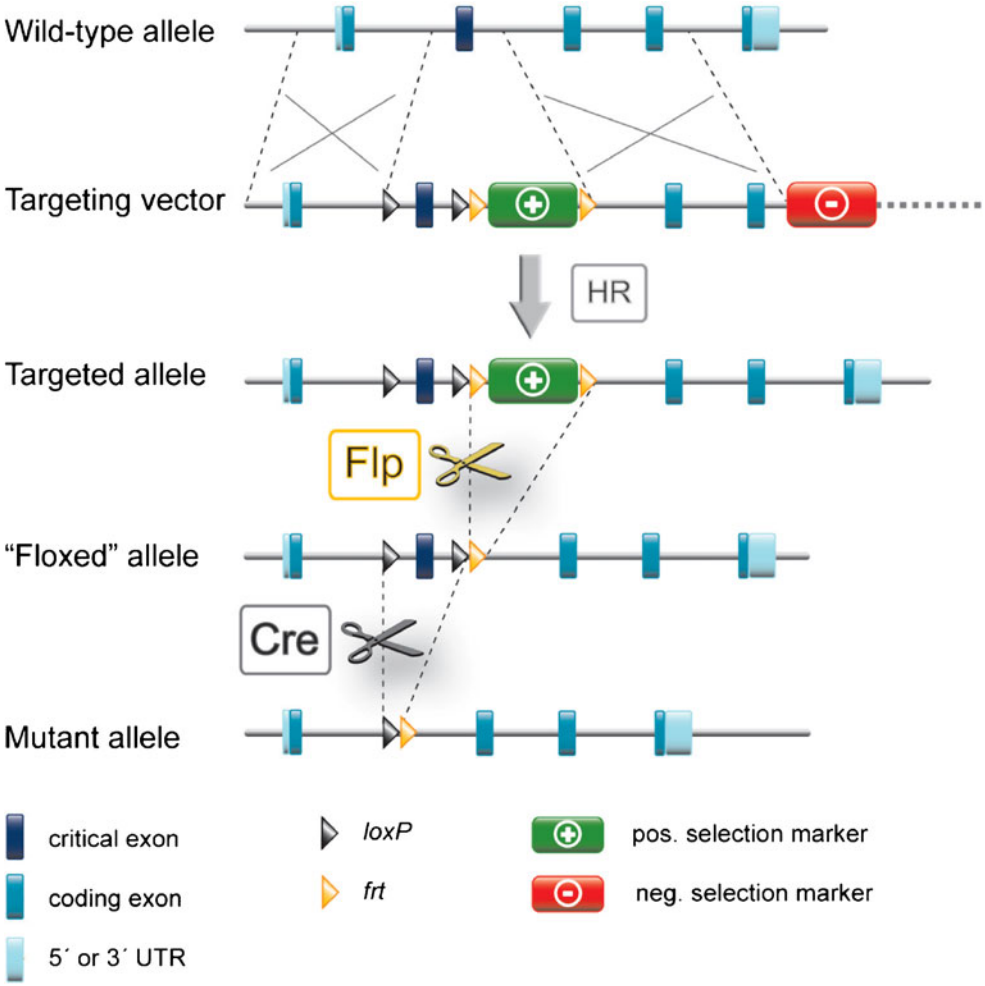


Fig. 1 Generation of a conditional allele by gene targeting. The typical targeting vector harbours a positive (*pos.*) selection marker flanked by *frt* sites located within an intron. Concomitantly, the upstream critical exon is flanked by *loxP* sites. The "flrted" selection marker and "floxed" exon are enframed by stretches of DNA homologous to the gene of interest. In addition, the targeting vector harbours a negative (*neg.*) selection marker for enrichment of homologous recombination events. After successful homologous recombination (*HR*), the positive selection marker can be removed from the targeted allele by Flp-mediated recombination in vitro or in vivo, which will leave the critical exon flanked by *loxP* sites. This conditional allele is now amenable to Cre-mediated inactivation. Recombination between *loxP* sites results in a mutant non-functional allele because of the loss of the critical exon (*UTR* untranslated region)

using gene targeting procedures has been a standard research approach to address the function of a gene of interest. During the last few decades, numerous examples have demonstrated the power of this technology in deciphering a plethora of neurobiological processes (for a review, see Gaveriaux-Ruff and Kieffer 2007). However, this approach comprises inherent problems that might lead to false conclusions. First, the function of a gene essential for embryonic or early postnatal development cannot be studied in adulthood. Second, the disruption of ubiquitously expressed genes might result in rather complex phenotypes with too much noise for an understanding of the specific function of a gene. Particularly in the case of psychiatric disorders, it is mandatory to address the function of a gene of interest in the central nervous system or even more precisely in specific neuronal or glial cell populations. Third, early ablation of a gene might activate compensatory mechanisms that possibly mask or blur the direct effects of the gene of interest. A final intricacy that occurs in conjunction with the original gene targeting strategies is the methodologically unavoidable introduction of positive selection markers, which are used to enrich for homologous recombination events and often for gene disruption (see above, Fig. 1). These selection markers, comprising their own regulatory elements necessary for efficient transcription, have been demonstrated to interfere with the expression of the target gene (even when introduced into intronic regions) and with genes adjacent to the target site (Olson et al. 1996; Meyers et al. 1998).

To overcome these limitations conditional approaches have been developed that allow not only the spatial and temporal control of gene expression but also the more precise engineering of the mouse genome including the introduction of specific mutations, insertions or deletions. Conditional gene targeting has been enabled by the application of site-specific recombinases (SSRs), which are naturally occurring DNA recombinases. A major advantage of these recombinases is that they do not require any accessory co-factors or particular DNA topology to mediate efficient DNA recombination.

Over the past two decades, the Cre/loxP system from the bacteriophage P1 has evolved as the most popular SSR system in the mouse (Hoess et al. 1982). Cre (cyclization recombination; sometimes also: causes recombination) is a 38-kDa site-

specific tyrosine recombinase of the λ integrase family (Argos et al. 1986). This DNA recombinase recognizes a 34-bp site that lies within the P1 genome that is called *loxP* (locus of crossover [x] of P1) and efficiently catalyses the recombination between two pairs of *loxP* sites. A *loxP* site consists in two 13-bp inverted repeats flanking an 8-bp spacer that determines the overall directionality of the *loxP* site. The relative orientation of target sites with respect to each other determines the outcome of the recombination. The DNA fragment between two directly repeated target sites will be excised as a circular molecule leaving a single *loxP* site behind. Because of the rapid loss of the circular reaction product, this reaction is essentially irreversible. In contrast, the reaction between two inverted *loxP* sites produces two identical target sites in cis that are again substrates for further recombination resulting in the continuous inversion of the *loxP* flanked ("floxed") DNA. Cre can also exchange sequences distal to target sites present on two linear molecules such as non-homologous chromosomes. This property has been extensively explored with respect to chromosome engineering (for reviews, see Mills and Bradley 2001; van der Weyden and Bradley 2006). Meanwhile, numerous alternative Cre recognition sites based on sequence substitutions in the spacer or inverted repeat have been established (for reviews, see Branda and Dymecki 2004; Birling et al. 2009). Since Cre mediates efficient recombination between homotypic but not between heterotypic sites, these variants allow novel applications such as insertions by recombinase-mediated cassette exchange (RMCE; for a review, see Turan et al. 2013) or stable inversion by the flip excision switch (FLEX; Schnutgen et al. 2003). The FLEX strategy has been instrumental for developing a conditional gene trap approach (Schnutgen et al. 2005).

With the confirmation of its suitability for genome engineering in mammalian cells (Sauer and Henderson 1988; Gu et al. 1993), the way was cleared for the successful application of the Cre/loxP system in mice (Gu et al. 1994; Lakso et al. 1992; Orban et al. 1992). To generate a conditional allele, *loxP* sites are introduced into the gene of interest by using homologous recombination-based gene targeting. Initially, a strategy comprising three identically oriented *loxP* sites has been utilized in which a critical part of the gene is floxed by two *loxP* sites. These two Cre recognition sites are placed within introns in which they per se should not interfere with gene function. Within the targeting vector, a dual positive/negative selection marker (usually *neo* and *HSV-tk*) is placed adjacent to one of these *loxP* sites and flanked on the opposite site by the third *loxP* site (compare Fig. 1). After successful gene targeting by homologous recombination, the selection marker can be deleted from ES cells by the transient expression of Cre and negatively selected for the desired Cre-mediated recombination, which will leave only two *loxP* sites flanking the essential part of the gene (Gu et al. 1994). Other strategies that allow in vivo disposal of the selection marker have been developed to avoid any additional manipulation of ES cells possibly compromising their pluripotency (Kaartinen and Nagy 2001; Leneuve et al. 2003). With the establishment of additional SSRs (see below), the triple *loxP* site strategy has largely been replaced by the simultaneous use of two SSRs and their respective recognition sites (Meyers et al. 1998; Fig. 1).

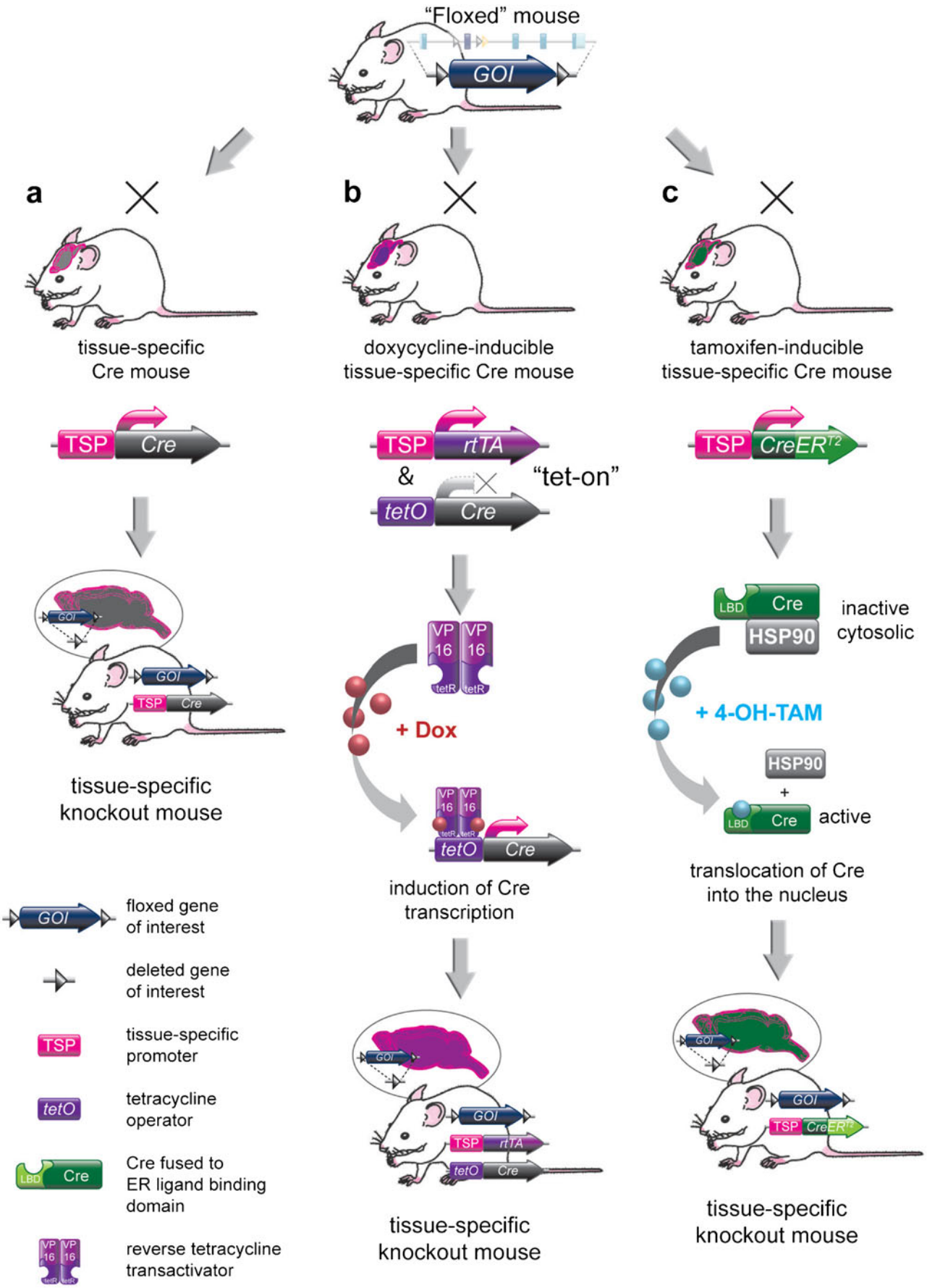
For conditional gene inactivation, mice with a floxed allele of the gene of interest are bred to so-called Cre driver mice, which express the recombinase under the control of a specific promoter or gene. Hence, the spatial and temporal pattern of gene inactivation largely depends on the properties of the Cre driver (Fig. 2).

In addition to the inactivation of a gene of interest, the Cre/loxP system has numerous applications that require the induction of gene expression. For example, the removal of a floxed transcriptional terminator generates a gain-of-function model or allows the restoration of the activity of an inactivated gene. Finally, this genetic switch can be used in vivo to activate specifically various molecular tools (e.g., Madisen et al. 2012; Kim et al. 2009).

Temporal control of Cre-mediated recombination

Unfortunately, most promoters lack the desired tissue- or cell-type specificity and, in particular, the temporal control that would allow gene inactivation at any point in time. Moreover, ectopic expression as a consequence of transient activation of Cre during development and potential toxic effects attributable to continuously high levels of Cre (Schmidt et al. 2000; Loonstra et al. 2001) are further challenges that could be overcome by the control of the temporal onset of Cre recombination. The two main strategies specifically to regulate the temporal onset of Cre-mediated recombination involve transcriptional and posttranscriptional mechanisms (Fig. 2).

Fig. 2 Spatial and temporal control of gene inactivation by using the site-specific recombinase Cre. **a** Tissue-/cell-type-specific inactivation of a gene of interest (*GOI*). Breeding of a mouse with a floxed GOI will result in tissue-specific inactivation of the GOI (e.g., only in the brain as depicted). The spatial and temporal pattern of deletion fully depends on the properties of the tissue-specific promoter (*TSP*) driving Cre expression. **b** Transcriptional control of Cre activity. The combination of a floxed GOI with a Cre recombinase regulated by the tetracycline system provides temporal control over gene inactivation. Exemplified is the reverse tet system (*"tet-on"*). Only upon application of doxycycline (*Dox*) will the reverse transactivator (*rtTA*) bind to the tetracycline operator (*tetO*) and activate Cre expression, which is spatially restricted by the TSP driving rtTA expression. **c** Posttranslational control of Cre activity. The Cre recombinase fused to the ligand-binding domain of the estrogen receptor ($CreER^{T2}$) is sequestered by the heat shock protein 90 (*HSP90*) within the cytoplasm. Only upon binding of 4-hydroxy tamoxifen (*4-OH-TAM*) will Cre be translocated into the nucleus and disrupt the GOI. In addition to the tamoxifen-mediated temporal control, spatial control is provided by the TSP utilized to drive Cre expression


"Floxed" mouse
GOI
a
b
c
tissue-specific
Cre mouse
doxycycline-inducible
tissue-specific Cre mouse
tamoxifen-inducible
tissue-specific Cre mouse
TSP
Cre
rtTA
&
"tet-on"
tetO
CreER^T2
LBD
HSP90
inactive
cytosolic
+ 4-OH-TAM
active
VP
16
tetR
+ Dox
translocation of Cre
into the nucleus
induction of Cre
transcription
tissue-specific
knockout mouse
floxed gene
of interest
deleted gene
of interest
tissue-specific
promoter
tetracycline
operator
Cre fused to
ER ligand binding
domain
reverse tetracycline
transactivator

A first step in this direction was the utilization of a Cre driver expressing the recombinase under the control of the interferon-responsive *Mx1* promoter. In *Mx1-Cre* mice, the application of interferon or the activation of the endogenous interferon response results in the induction of Cre expression and subsequent gene inactivation, albeit with limited efficiency (Kuhn et al. 1995). With the establishment of the *Escherichia coli*-derived tetracycline (tet) system in mammalian cells (Gossen and Bujard 1992), the transcriptional control of Cre has been exploited more extensively (Fig. 2). This two-component system involves, on the one hand, a hybrid transactivator (tTA) consisting in the tetracycline repressor fused to the acidic C-terminal domain of the HSV transcription factor VP16 and, on the other hand, a minimal promoter from the human cytomegalovirus (CMV) immediate early gene fused to tetracycline operator (*tetO*) sequences. A Cre under the control of *tetO* sequences is prevented from being expressed by continuous administration of tetracycline or its analogue doxycycline. Upon removal of the inducer, the transactivator binds to the minimal promoter and activates Cre expression (St-Onge et al. 1996). However, the kinetics of this "tet-off" system are relatively poor because of the slow clearance of the inducer from the organism (Kistner et al. 1996). In contrast, the "tet-on" system allows the induction of Cre expression following inducer administration within 1 h (Hasan et al. 2001; Schonig et al. 2002; Fig. 2). A general problem of the transcriptional control of Cre expression is that a low level of Cre expression will result in undesired recombination contrasting the temporal control of DNA recombination. However, in many cases, the efficient shutdown of Cre expression is disturbed because of endogenous enhancers in the neighbourhood of the transgenic integration site of the *tetO* minimal promoter (Leneuve et al. 2003). Another difficulty of this approach is that it requires the simultaneous presence of (1) tissue-specifically expressed tTA or rtTA, (2) Cre driven by a *tetO* minimal promoter and (3) a floxed gene of interest, making this approach in vivo relatively laborious compared with the more prevalent tamoxifen-inducible Cre system (Fig. 2).

Ultimately, posttranslational control has proven to be superior to the transcriptional control of Cre-mediated recombination. The use of ligand-dependent SSRs that are specifically activated by synthetic compounds has become accepted as the most reliable principle for the control of the temporal onset of Cre recombination. This strategy is based on the observation that the activity of proteins can be controlled by a synthetic ligand when fused to the ligand-binding domain (LBD) of steroid hormone receptors (Picard 1994). This principle has been successfully applied to SSRs (Logie and Stewart 1995; Metzger et al. 1995) and transferred to the mouse, thereby demonstrating the technical feasibility of ligand-induced site-specific recombination in vivo (Fig. 2; Feil et al. 1996; Danielian et al. 1998). LBDs of various steroid hormone receptors have been tested (Kellendonk et al. 1996; Wunderlich et al. 2001; Brocard et al. 1998) but, ultimately, the LBD of the human ($CreER^T$, with a G521R substitution) or mouse ($CreER^{TM}$, with a G525R substitution) estrogen receptor (ER) has turned out to be the most promising tool (Danielian et al. 1998; Feil et al. 1997, 1996). Subsequently, the properties of the chimeric Cre-ER-LBD protein have been further engineered (1) to improve its specificity towards the synthetic ligand tamoxifen ($CreER^T$, named according to its ability to bind the synthetic ER agonist tamoxifen (TAM) and 4-OH-TAM) (2) to reduce its background activity in the absence of the inducer and (3) to increase the sensitivity towards the synthetic ligand. Currently, $CreER^{T2}$ (based on the human ER-LBD with a G400V/M543A/L544A triple mutation) is the most effective variant; it does not bind β-estradiol, has hardly any background activity and is tenfold more sensitive to 4-OH-TAM compared with the former variants (Feil et al. 1997; Indra et al. 1999). The recombination efficacy depends on the expression level of the chimeric recombinase but is also strongly influenced by the route (intraperitoneal or per os) and frequency of TAM administration. Because of the better solubility and lower cost, TAM is usually administered, which is converted to the active inducer 4-OH-TAM by the liver. Therefore, the local induction of Cre-mediated recombination requires the administration of 4-OH-TAM (Vasioukhin et al. 1999; Zadelaar et al. 2006). In general, the efficient induction of $CreER^{T2}$-mediated recombination requires the subchronic administration of relatively high doses of TAM (e.g., intraperitoneal injection of 1 mg/mouse, twice per day for 5 days), which might have adverse effects. However, TAM seems to have only minor effects on general behaviour and does not confound standard behavioural tests (Vogt et al. 2008).

The Cre zoo

The establishment of the Cre/loxP system for genome engineering in the mouse has opened up a plethora of possibilities linked to the flexibility of combining floxed alleles with numerous different Cre drivers (Gaveriaux-Ruff and Kieffer 2007; Refojo et al. 2011; Chen et al. 2010). In the past two decades, many Cre drivers have been generated by individual laboratories, an effort that is now being pushed forward more systematically by large-scale projects such as the GENSAT (gene expression nervous system atlas) Cre project at Rockefeller University (http://www.gensat.org/cre.jsp), the NIH Neuroscience Blueprint Cre Driver Network (http://www.credrivermice.org/), the EUCOMMTOOLS project (http://www.knockoutmouse.org/about/eucommtools) and the CreERT2 zoo of the Institute Clinique de la Souris (ICS) (http://www.ics-mci.fr/mousecre/). The best overview of available Cre lines is provided by the web portal of the CREATE (Coordination of resources for conditional expression of mutated mouse alleles) project (http://www.

creline.org/) and by the MGI (mouse genome informatics) web portal (http://www.creportal.org/).

Numerous different approaches have been pursued to generate Cre drivers including classic random insertion transgenesis by using small promoter fragments, random insertion of large constructs and a variety of knock-in strategies. All of these approaches have their specific pros and cons (for a review, see Murray et al. 2012). Ultimately, the proper characterization of Cre driver lines is crucial for the efficient application of this tool. Usually Cre drivers are characterized by breeding to a respective Cre reporter mouse line. The prerequisite of a suitable reporter mouse line is its capability to express an easy-to-monitor reporter gene (e.g., a fluorescent or histochemical marker) following SSR-mediated recombination in any tissue or cell type of the organism (for a review, see Branda and Dymecki 2004). The most prominent reporters are the ROSA26 (reverse oriented splice acceptor 26) reporter (R26R; Soriano 1999) and the recently generated set of particularly bright fluorescent reporters provided by the Allen Institute for Brain Science (Madisen et al. 2010). Unfortunately, many Cre drivers have only superficially been characterized, as most investigators focus on their favourite target tissues or cell types when generating and characterizing a Cre driver. An awareness of this problem has developed within the research community and has led to initial attempts aimed to introduce a more systematic characterization of Cre drivers (Heffner et al. 2012). Major caveats connected to fragmentary characterization resulting in supposed tissue- or cell-type specificity include unexpected or ectopic expression attributable to transient activation of Cre expression during embryogenesis or early postnatal development and widespread expression because of deletion in the female or male germline (Matthaei 2007; Weng et al. 2008).

Moreover, toxicity of Cre is an issue and has occasionally been reported not only for the original Cre but also for conditional CreERT2 variants (Naiche and Papaioannou 2007; Higashi et al. 2009; Huh et al. 2010; Takebayashi et al. 2008). To some extent, toxicity might be explained by recombination via cryptic *loxP* sites that induce a DNA damage response (Thyagarajan et al. 2000). However, the past 20 years of experience with the Cre/loxP system and the fact that reports of toxicity are rather rare suggest that Cre-induced toxicity is more likely to be an exception. To some extent, the degree of toxicity might depend on the expression level and on the spatial and temporal pattern of Cre expression. The co-expression of reporter genes at high levels might additionally contribute to Cre-mediated toxicity.

Nevertheless, we need controls for potential unspecific and unwanted effects of the utilized Cre driver. This raises the demand for proper controls in SSR-based conditional approaches. Two different strategies are pursued. In the first strategy, the mutant group carries a floxed and a null allele of a gene of interest. In contrast, the control group carries a floxed and a wild-type allele of the gene of interest. Both groups possess the Cre recombinase under the control of a specific promoter. Cre expression will result in a deletion of the floxed allele, leading to heterozygosis in the control group and full gene inactivation in the mutant group. A major shortcoming is that the concomitant haploinsufficiency in the control group might have an effect on the phenotype. Moreover, the breeding to obtain mice with the desired genotypes is less efficient than in the second strategy. In this strategy, both the mutant and control possess a floxed gene of interest on both alleles, but only the mutant group will carry the Cre. However, in this strategy, unwanted side effects of the Cre might interfere with the phenotype. To overcome this difficulty, a parallel characterization of hemizygous or heterozygous Cre mice in comparison with pure wild-type mice of the same genetic background will help to define the unspecific phenotype related to Cre expression.

The Cre/loxP system is clearly superior to the other available SSR systems with regard to the prominence and distribution of driver lines, to some extent because it was the first SSR introduced successfully into the mouse.

Expanding the toolbox of SSRs

The establishment of the first SSR, namely Cre, provided convincing evidence that these are efficient tools for manipulating the mouse genome and controlling gene expression. The broad applications of Cre, which allow for excision/insertion, inversion, translocation and cassette exchange, fuelled the search for additional SSRs that would provide increasing flexibility in the engineering of the mouse genome.

With Flp (often called “flippase” because of its property to invert or flip DNA) from the 2-micron plasmid of *Saccharomyces cerevisiae*, another site-specific DNA recombinase of the λ integrase family was established as an SSR applicable to the mouse. Flp recombines DNA at *frt* (Flp recognition target) sites (Sternberg et al. 1981; McLeod et al. 1986; Vetter et al. 1983), which are structurally similar to *loxP* sites and also come in different variants (for a review, see Branda and Dymecki 2004). The Flp/frt system is commonly used for removal of *frt* flanked (“flrted”) selection markers from ES cells by transient expression or by breeding to *Flp-deleter* mice (Fig. 1; Rodriguez et al. 2000; Raymond and Soriano 2010). The original variant of Flp is clearly less active than Cre because of the low temperature optimum of 30 °C, which is perfectly adjusted for efficient recombination in yeast but not in mammalian cells (Buchholz et al. 1996; Andreas et al. 2002). However, molecular evolution (Flpe; Buchholz et al. 1998) and codon optimization (Flpo; Raymond and Soriano 2007) have enhanced the performance of Flp significantly, ultimately resulting in an SSR almost equally as efficient as Cre. Compared with Cre, only a limited number of tissue- or cell-type-specific Flp driver lines has been

established to date (Miyoshi et al. 2010; Awatramani et al. 2003; Farago et al. 2006; Jensen et al. 2008; Rodriguez et al. 2000; Raymond and Soriano 2007). The flp recombinase is also available as a TAM-inducible variant fused to the ER-LBD and has proven its efficacy in vivo (Hunter et al. 2005).

Further flexibility has been gained through the isolation of the SSR Dre from the phage D6 (Sauer and McDermott 2004). This tyrosine recombinase of the λ integrase family mediates recombination between two 32-bp *rox* (region of crossover [X]) sites with similar potency as Cre. The Dre/rox system has been approved as another promising tool for genome engineering in the mouse. However, with the establishment of a first *Dre-deleter* mouse line and Dre reporter mice, the system is still in its infancy (Anastassiadis et al. 2009).

The Streptomycete phage φC31-derived integrase belongs to a different class of SSRs unrelated to Cre, Flp or Dre (Kuhstoss and Rao 1991; Belteki et al. 2003). This serine recombinase of the resolvase family mediates recombination between heterotypic *attB* (34-bp) and *attP* (39-bp) sites, which are original attachment sites for the integrase on the bacterial and phage genomes (Groth et al. 2000). In contrast to Cre and Flp, φC31 promotes unidirectional reactions, i.e., the *attL* and *attR* sites resulting from recombination of *attB* and *attP* sites are inert to further φC31-mediated recombination (Groth et al. 2000). φC31 is more efficient at catalysing intermolecular reactions than intramolecular reactions and therefore is ideally suited for site-specific insertions into the mammalian genome (Monetti et al. 2011). Typically, an *attP*-bearing docking site is introduced into a defined locus, which is subsequently prone to an *attB*-bearing incoming sequence for insertion. The tools for the in vivo application of φC31 integrase are currently limited to a deleter mouse line (Raymond and Soriano 2007).

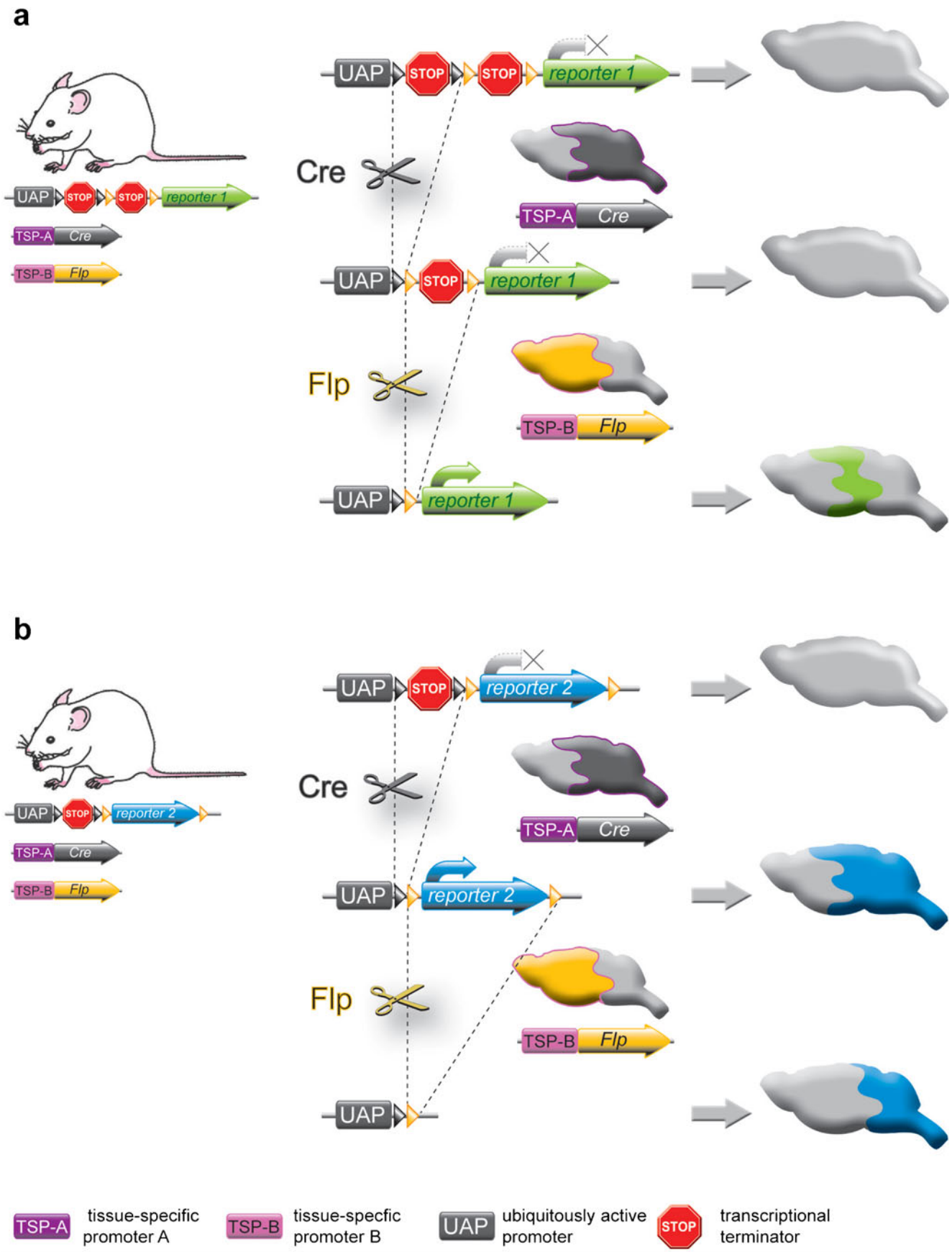
Numerous other SSRs have been identified and investigated in recent years suggesting that many more SSRs are available for genome engineering (e.g., Nern et al. 2011; Karimova et al. 2013). These SSRs just await adaptation for the mammalian system to make them usable for methods that require multiple recombinases such as subtractive and intersectional approaches.

SSRs in intersectional and subtractive strategies

The combination of independent SSR systems allows further temporal and spatial refinement of conditional approaches. For instance, the simultaneous operation of two SSRs, driven by different promoters, can be used to define a specific cell type or tissue domain tagged by overlapping expression of SSRs (Fig. 3). In addition, such an approach can also be utilized in a subtractive manner. If, for example, the expression of a gene of interest is induced by SSR-mediated recombination in a given domain or cell type, then the expression can be turned off by another SSR wherever both SSRs are co-expressed (Fig. 3). Several examples with Cre and Flp have demonstrated the utility of this approach, in particular in the context of fate mapping and cell lineage tracing (Awatramani et al. 2003; Farago et al. 2006; Hunter and Dymecki 2007; Jensen et al. 2008; Miyoshi et al. 2010; Taniguchi et al. 2011). In general, the combined usage of two or more independent SSRs offers a versatile tool for intersectional or subtractive gain- or loss-of-function approaches in vivo (for a review, see Dymecki and Kim 2007).

In addition to the simultaneous application of different SSRs, the Cre recombinase itself has been subjected to intersectional strategies based on a split-protein approach similar to the widely used α complementation of the *E. coli* β-galactosidase (Ullmann et al. 1967). The Cre recombinase can be split into two independent polypeptides: an N-terminal α part and a C-terminal β part. The individual halves show no Cre activity by themselves but, when both fragments are co-expressed, they are able to re-associate spontaneously to reconstitute enzyme activity and to execute Cre-mediated recombination (Casanova et al. 2003). However, since the spontaneous reconstitution is relatively ineffective (in vitro up to 30 % of wild-type Cre), more efficient complementation strategies had to be developed to convert this approach into a feasible method. To enhance the reconstitution of Cre, the complementation-competent fragments coding for amino acids 19–59 and 60–343 of Cre were fused to the dimerizing coiled-coil leucine zipper domain of the yeast transcriptional activator GCN4 (Hope and Struhl 1987). When the two modified fragments are expressed under the control of two different promoters, the leucine zippers effectively support the complementation of the Cre fragments. Only within cells with overlapping expression of the two promoters did this result in an active Cre that mediated efficient recombination (Hirrlinger et al. 2009b). This strategy was further developed and successfully applied to the TAM-inducible variant CreERT2

Fig. 3 Intersectional and subtractive strategies to manipulate gene expression. **a** For intersectional expression, e.g., of a reporter gene, a strategy is used that involves two transcriptional terminator sequences, which interrupt the expression of a reporter from a ubiquitously active promoter (*UAP*). The first transcriptional terminator sequence is floxed, whereas the second terminator is flrted. Expression of the reporter will only be activated in cells in which both terminator sequences are deleted. Combining this reporter allele to a Cre driver expressing Cre under the control of the tissue-specific promoter A (*TSP-A*) and to a Flp driver expressing Flp under the control of the tissue-specific promoter B (*TSP-B*) will result in the expression of the reporter only in regions/cells of overlapping Cre and Flp expression. **b** For subtractive expression, the activity of a reporter gene driven by a UAP is blocked by a floxed transcriptional terminator. In addition, the reporter is flrted. Cre will activate reporter gene expression based on the spatial and temporal properties of TSP-A. Simultaneously, Flp will delete the reporter from all regions/cells in which TSP-B is active. This combination of Cre and Flp will result in an expression of the reporter in cells expressing Cre but not Flp


a
UAP
STOP
STOP
reporter 1
TSP-A
Cre
TSP-B
Flp
Cre
Flp
b
UAP
STOP
reporter 2
TSP-A
Cre
TSP-B
Flp
TSP-A tissue-specific promoter A
TSP-B tissue-specfic promoter B
UAP ubiquitously active promoter
STOP transcriptional terminator

providing temporal control in addition to increased spatial precision (Hirrlinger et al. 2009a).

Another variant of the temporally controlled split-Cre system is the dimerizable Cre (DiCre) system (Jullien et al. 2003). In this case, an exogenous ligand (rapamycin) triggers the dimerization and restoration of Cre activity. The binary DiCre system consists in (1) a fusion of the FK506-binding protein FKBP12 to the N-terminal portion of Cre and (2) a second chimeric protein comprising the binding domain of the FKBP12-rapamycin-associated protein (FRB) fused to the C-terminal portion of Cre. The efficient rapamycin-induced heteromerization of the two chimeric proteins, which results in the reconstitution of an active Cre recombinase, has been demonstrated in vitro and in vivo (Jullien et al. 2007, 2003).

Recently, a significant advancement of the split-Cre system has been demonstrated by the utilization of intein-mediated protein splicing (Wang et al. 2012). Inteins are peptides capable of protein splicing, i.e., of excising themselves from a precursor protein while rejoining the remaining portions of the protein (exteins) with a peptide bond (Liu 2000). The combination of the N-terminal (amino acids 19–59) and C-terminal (amino acids 60–343) moieties of Cre with the two parts of a split-intein from the cyanobacterium *Synechocystis sp* allows the formation of a peptide bond between the two Cre fragments in a process of protein trans-splicing when they are present in the same cell. The fact that the peptide bond prevents dissociation of the two domains of Cre makes this a highly effective way of reconstituting Cre activity and thus an ideal mehtod for generating Cre drivers with previously unmet specificity (Wang et al. 2012).

Humanized mouse models

Humanized mice often refer to immunodeficient animals that allow the engraftment and study of a functional human immune system (Shultz et al. 2012). In contrast, genetically or genomically humanized mouse models, as designated in this review, refer to mice that have been generated by genome engineering and that harbour human coding or non-coding DNA sequences, respectively (Devoy et al. 2012). In a broader sense, humanized mice carry human DNA sequences added to the mouse genome, for instance, by insertional transgenesis. In a narrower sense, the term "humanized" refers to the substitution of a mouse gene or a genomic sequence by its human orthologue. Human proteins have repeatedly been reported as differing in their biochemical properties from their murine counterparts. Therefore, transgenic mice have often been generated by using human cDNAs. Genetic humanization via gene targeting strategies ideally places human coding sequences into the regulatory context of the orthologous mouse gene. The conclusive functional investigation of human disease-associated protein variants in vivo should be based on a direct comparison with the human wild-type protein and not on a comparison with murine variants of the protein. In general, this type of approach is able to model the human disease situation more accurately than approaches addressing the function of orthologous mouse proteins.

For obvious reasons, study of the vast majority of current mouse models has focused on the changes in the coding genome. For various psychiatric disorders, numerous robust and replicable genetic findings have been reported in recent years, although non-coding or structural variants such as copy number variations clearly outnumber coding variants (for a review, see Sullivan et al. 2012). With the upsurge of genome-wide association studies (GWASs) and the prospect of the increasing availability of human whole genome sequencing data, a growing number of non-coding and regulatory variants, which confer susceptibility or resistance to psychiatric diseases, is to be expected. Therefore, demand is increasing for the creation of "genomically" humanized mouse models. This is a relatively young research field that is just beginning to evolve with various methodological strategies being under development to address this challenging task. Current approaches are based on the transfer of entire human genomic loci into the mouse genome either by the addition of genomic sequences or by the replacement of regions of the mouse genome with equivalent human genome sequences.

As a straight-forward method, yeast artificial chromosomes (YACs; comprise up to 1 Mb) or bacterial artificial chromosomes (BACs; comprise up to 300 kb) have been used to transfer large segments of the human genome into the mouse genome usually by pronuclear injection of zygotes. These large constructs have the potential to include all cis-regulatory elements required to recapitulate endogenous expression and thus provide a higher likelihood of position-independent expression (Giraldo and Montoliu 2001; Heaney and Bronson 2006). As an add-on, a genomically humanized mouse line might be bred onto a null background for the gene of interest so that the human variant is the only version of the gene that is expressed (Johnson and Wade-Martins 2011).

Human artificial chromosomes (HACs) offer an alternative way of gene delivery. Micro-cell-mediated chromosome transfer into ES cells has been used to generate mouse models carrying a freely segregating partial or complete human chromosome (O'Doherty et al. 2005; Tomizuka et al. 1997). HACs exhibit several prerequisites for an ideal gene delivery vector, including stable episomal maintenance and the capacity to carry large genomic loci with their regulatory elements, thus allowing the physiological regulation of the introduced gene in a manner similar to that within native chromosomes (Kazuki and Oshimura 2011).

A commonly used strategy to achieve more reproducible expression is based on site-directed BAC transgenic approaches. BACs have been designed to integrate themselves

at a specific and ubiquitously expressed chromosomal locus such as the *Hprt* gene on the X chromosome, which warrants reliable expression from tissue-specific promoters, including expression from human BAC clones (Bronson et al. 1996; Guillot et al. 2000; Heaney et al. 2004). ES-cell-based BAC integration can either occur by homologous recombination (Heaney and Bronson 2006; Schmouth et al. 2012; Yang et al. 2012) or by SSR-mediated recombination (Prosser et al. 2008). Homologous recombination-based integration uses a modified BAC with homology to the mouse *Hprt* locus. Successful integration of the BAC and of the concomitant human genomic fragment results in the restoration of the *Hprt* gene, an event that can be selected by using hypoxanthine-aminopterin-thymidine (HAT) medium (Schmouth et al. 2012; Yang et al. 2012). In the case of the SSR-based RMCE approach, *loxP* and *lox511* sites have been introduced by homologous recombination into the *Hprt* gene to act as docking sites for a genomic DNA fragment containing similar lox sites. Many BAC vectors used for creating genomic libraries contain such an arrangement of *loxP* and *lox511* sites greatly simplifying the SSR-mediated integration of a BAC of interest (Frengen et al. 1999).

The most precise means for humanizing the mouse genome is to replace the mouse genomic sequence by its syntenic human equivalent. The "VelociGene" method has demonstrated that the substitution of larger fragments is possible via homologous recombination by using a hybrid BAC vector in which a human DNA segment is placed, by recombineering, between long regions of mouse genomic sequence together with a positive selection marker. Homologous recombination in ES cells will integrate the human genomic sequence into the mouse genome at the desired site (Valenzuela et al. 2003). For replacement of large fragments of mouse genomic sequences with a human sequence from a BAC clone, a variant of RMCE, the so-called recombinase-mediated genomic replacement (RMGR) has been developed. For RMGR, two heterospecific SSR sites and connected positive selection markers are integrated into a desired genomic location by sequential gene targeting to delineate the region for replacement. The human BAC containing the genomic sequence of interest is accordingly modified to carry respective heterospecific SSR sites. The BAC is then stably integrated by SSR-mediated integration (Wallace et al. 2007; Hasegawa et al. 2011). RMGR offers a selectable, precise and reproducible strategy for the substitution of large pieces of mouse genomic DNA (Redon et al. 2006).

Finally, structural variations such as copy number variations (CNVs) are a major class of genetic determinants identified by GWASs in psychiatric disorders. CNVs are highly polymorphic in the human population and either inherited or caused by *de novo* mutations. CNVs include translocations, inversions, duplications, triplications and deletions that can range in size from a few kilobases to several megabases. In particular, the Cre/loxP system seems to be ideally suited to model CNVs by chromosome engineering. Numerous induced chromosome rearrangements based on the Cre/loxP-system have demonstrated the feasibility of this strategy (Nomura and Takumi 2012; van der Weyden and Bradley 2006).

Gene targeting procedures 2.0

The frequency of homologous recombination events is extremely low and strongly depends on DNA double-strand breaks (DSBs). Thus, the generation of genomic DSBs at desired genomic sites would be advantageous in the employment of homology-directed repair mechanisms for targeted gene modification. Cells have evolved two major repair pathways in order to effectively overcome DSBs, which otherwise would be lethal: (1) the error-prone non-homologous end joining mehtod (NHEJ) and (2) the high fidelity homology-directed repair method (HDR; Fig. 4). Interestingly, homologous recombination is the predominant DSB repair pathway in murine ES cells with a minimal contribution of NHEJ; this has serendipitously assisted the breakthrough of gene targeting technologies (Tichy et al. 2010). However, the generally low frequency of HDR events in mammalian cells precludes their application in systems other than murine ES cells. Only recently, custom-designed nucleases have been implemented to introduce site-specific DSBs into the genome of any type of mammalian cell. As a consequence of DSBs, NHEJ can disrupt a gene by introducing frame-shift mutations, whereas HDR provides the opportunity for the deletion, insertion or correction of genes (Fig. 4). The site-specific nucleases (SSNs) under development for efficient genome engineering include homing endonucleases (HEs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the recently developed RNA-guided endonucleases (RGNs; Sun et al. 2012; Gaj et al. 2013).

HEs, also known as meganucleases, are naturally occurring rare-cutting endonucleases cleaving recognition sites of 14–40 bp (for a review, see Marcaida et al. 2010). A recent report demonstrated their successful application in zygotes to generate knockout mice and rats (Menoret et al. 2013). Despite the identification of several hundred HEs, the number of sites that these endonucleases are able to recognize is relatively limited. Therefore, the directing of HEs to specific target sites requires laborious engineering, which clearly limits their practical application.

ZFNs are artificial DNA nucleases combining the non-specific cleavage domain of the *Fok*I endonuclease from *Flavobacterium okeanokoites* with zinc finger proteins (Kim et al. 1996). With their DNA-binding motifs, zinc finger proteins provide a modular structure for designing ZFNs with customized sequence specificities. Zinc finger proteins

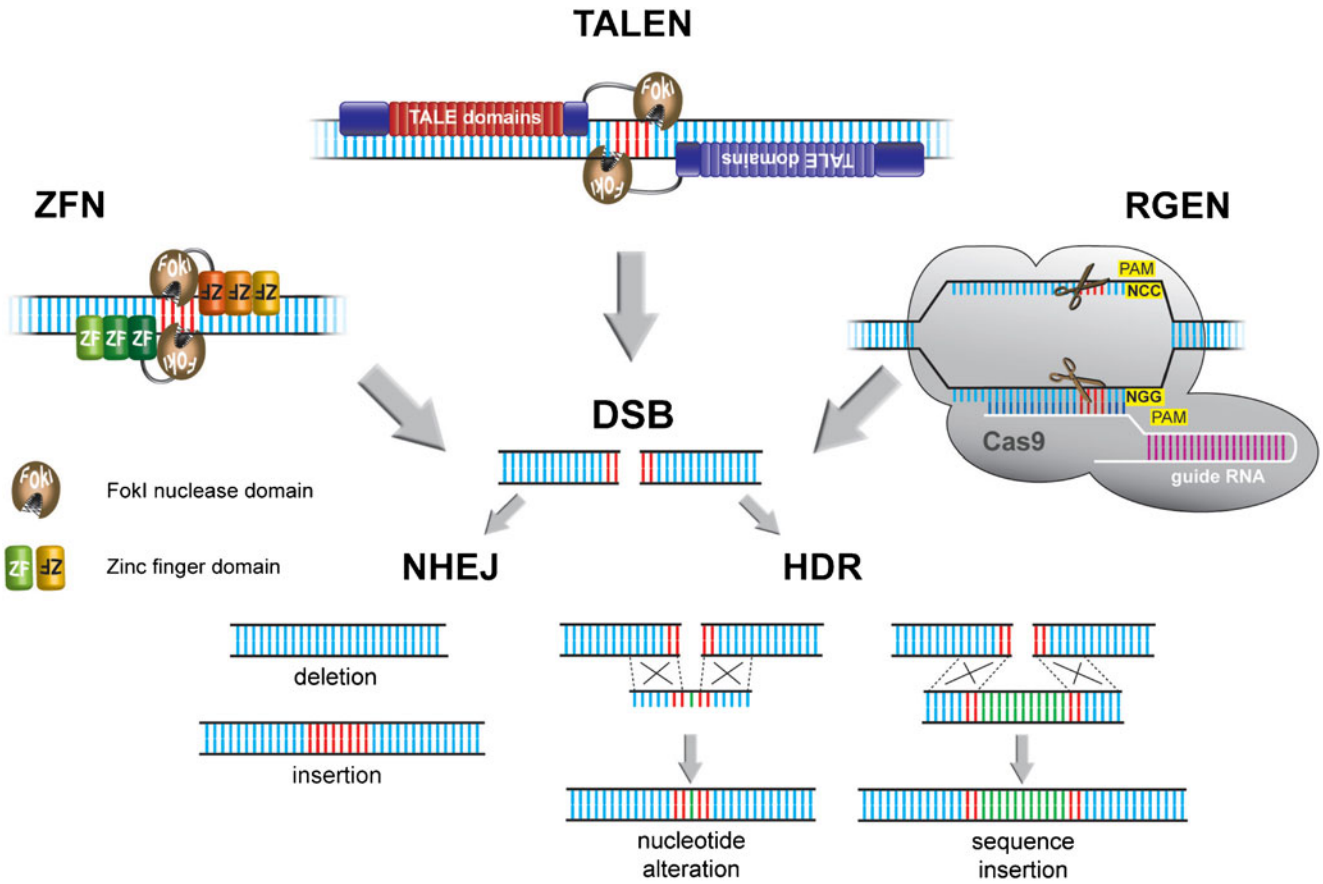


Fig. 4 Targeted manipulation of the genome by site-specific nucleases. Zinc finger nucleases (*ZFN*), transcription activator-like effector nucleases (*TALEN*) and RNA-guided nucleases (*RGEN*) such as the CRISPR/Cas system efficiently cause site-directed double-strand breaks (*DSB*) activating two main DNA repair mechanisms, i.e., non-homologous end joining (*NHEJ*) and homology directed repair (*HDR*). The error-prone NHEJ produces insertions and deletions and thus can be utilized to generate mutant alleles in a random fashion. High-fidelity HDR allows targeted and specific modifications such as single nucleotide alterations or larger insertions. HDR can use various templates such as single-stranded oligonucleotides or targeting vectors with longer stretches of homologous DNA (*PAM* protospacer adjacent motif)

usually contain three to four zinc finger domains that recognize a 9- or 12-bp DNA target site. Since the non-specific DNA cleavage domain of *Fok*I is active as a dimer, the typical tailor-made ZFN functions as a heterodimer recognizing an 18-bp to 24-bp DNA sequence with a 4-bp to 6-bp spacer between each half site (Fig. 4). Applications include gene disruption, gene insertion, gene correction and chromosomal rearrangement. Despite improved design algorithms, ZFNs are still comparatively difficult to generate or expensive when purchased from commercial suppliers.

Similar to ZFNs, TALENs consist in a specific DNA-binding domain fused to the cleavage domain of *Fok*I. TAL effector proteins were first discovered in the plant pathogens of the genus *Xanthomonas*, which uses them to activate transcription of plant genes that are important for pathogen multiplication and spread (Bogdanove et al. 2010; Scholze and Boch 2011, 2010). TAL effector proteins are typically composed of an N-terminal translocation domain, a central repeat domain that mediates binding to the DNA and a C-terminal transcriptional activation domain (Mussolino and Cathomen 2012). Since the deciphering of the TAL effector recognition code (Boch et al. 2009; Moscou and Bogdanove 2009), the utilization of TALEN technology has developed with stunning speed. TALENs with any desired specificity can be easily designed by assembling individual repeats in a modular fashion or by direct synthesis (Fig. 4). Because of the simplicity of the TALEN code, these SSNs are far more amenable to custom design and thus superior to ZFNs.

Recently, another SSN with genome-engineering capacity has been discovered. The bacterial CRISPR/Cas system (clustered regulatory interspaced short palindromic repeats/CRISPR-associated protein) naturally provides adaptive immunity against viruses and plasmids (Horvath and Barrangou 2010; Bhaya et al. 2011). It comprises short sequences of foreign DNA that are integrated within the CRISPR locus. These sequences are transcribed and processed into short CRISPR RNA (crRNA). The crRNAs anneal to a so-called trans-activating crRNA (tracrRNA), which ultimately directs

sequence-specific cleavage of pathogen-derived foreign DNA by Cas proteins. Target recognition by the Cas protein requires a 20-bp seed sequence within the crRNA and a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence upstream of the crRNA binding region (Fig. 4). In particular, the Cas9 protein has been proven capable of introducing DSBs. Cas9 can be retargeted to cleave virtually any DNA sequence by redesigning the crRNA. Further streamlining of the system by using a chimeric crRNA-tracrRNA fusion transcript called a guide RNA (gRNA) has turned this RNA-guided nuclease into an efficient tool for genome engineering (Fig. 4). The CRISPR/Cas system can be easily transferred to any cell type by co-expression of the Cas9 endonuclease and the necessary crRNA components (Cong et al. 2013; Mali et al. 2013). The future will demonstrate the efficacy of the CRISPR/Cas system and whether it can compete with the currently favoured TALENs (Barrangou 2012).

The reliability of these custom-designed SSNs to generate DSBs has been demonstrated in various cell types (for a review, see Perez-Pinera et al. 2012). Moreover, the successful application of ZFNs, TALENs and RGENs to directly target the genome of one-cell-stage embryos of mice, rats, pigs and zebrafish inevitably pushes the limits for genome engineering into a new era beyond murine or rat ES cells (Wefers et al. 2013; Meyer et al. 2010, 2012; Bedell et al. 2012; Carlson et al. 2012).

Conclusions and future perspectives

In the past two decades, gene targeting technologies have matured in a previously unimagined manner. Numerous novel strategies and tools have evolved rapidly. Whereas some of these tools are extremely successful and dominate the scenery, others have turned out to be dead ends (e.g., in vivo knockdown based on transgenic RNA interference) depending on whether they have withstood in vivo approval. The establishment and acceptance of these tools as gold standards has further been backed up by trend-setting decisions of large-scale approaches and involved consortia. Along these lines, the usage of conditional mouse mutants based on the Cre/loxP system to interrogate the role of genes or pathways in psychiatric disorders is the most appropriate strategy at the moment. Similarly, the CreER^{T2} system appears to be unbeatable when it comes to the temporal control of gene regulation. Nevertheless, additional and increasingly sophisticated tools are evidently needed in order to make significant progress with respect to psychiatric disorders. For instance, we still lack a regulatory system that would allow the reliable switching on and off of endogenous genes in a repeatedly reversible manner. Approaches with multiple SSRs remain the exception but have proven extremely powerful. In the future, an increase in the number and availability of Flp and Dre drivers will obviously be demanded. The use of Flp and Dre drivers as alternative tools for major cell types or neuronal populations, in addition to the existing Cre drivers, will mean a significant extension of flexibility and fidelity based on intersectional and subtractive methods. The split-Cre, particularly the intein-based system, has high potential in this direction.

The current efforts to generate large numbers of floxed alleles, Cre drivers and other tools require stringent coordination to achieve maximum synergistic effects. These large-scale approaches also point to the need for efficient and accessible repositories that maintain or preserve these tools for the scientific community. In general, the research community will strongly benefit from the availability and accessibility of mouse tools. However, this will also increase the standard criteria for high impact publications, criteria that might be difficult to be met by smaller laboratories, since experiments involving several generations of mouse breeding are time- and cost-intensive. One way to bypass this problem is the efficient combination of SSR drivers with SSR-inducible viruses, thus avoiding laborious breeding.

Targeted mutagenesis with all of the above-discussed possibilities offers intriguing entry points into the study of genes or pathways underlying psychiatric disorders; however, we are far from having mouse models that resemble human psychiatric diseases in a narrower sense. The polygenicity of psychiatric disorders poses two major questions. (1) Can these polygenic disorders be approached from the single gene perspective? (2) How can these many genes be coherently modelled in the mouse? In addition, GWASs and recent meta-analyses indicate that the effects of identified disease-associated common variants are rather small, which is a major challenge for the phenotypic analyses of respective genetic mouse models. Along these lines, the generation of genomically humanized mice is an important step in understanding the effect of non-coding potentially regulatory variants. In addition to gene-gene interactions, gene-environment interactions are of importance and need to be addressed more carefully in genetic mouse models. Finally, increasing genome sequencing efforts might well identify rare disease-associated variants of larger effect size and even coding variants, both of which would provide better accessibility in the mouse.

With the advent of SSNs, a powerful novel class of tools has entered the scene with the potential to redefine the boundaries of biomedical research. It provides, for the first time, the possibility of genome engineering in any model organism of interest. The current enthusiasm is reasonable, despite the many open questions, such as potential off-target effects that await clarification. The recent establishment that this technology can allow the simultaneous targeting of several independent genomic loci in the mouse is a spectacular demonstration of its power (Wang et al. 2013).

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