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Minireview

When reverse genetics meets physiology: the use of site-specific recombinases in mice

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Abstract The use of site-specific recombinases enables the precise introduction of defined genetic mutations into the mouse genome. In theory, any deletion, point mutation, inversion or translocation can be modeled in mice. Because gene targeting is controlled both spatially and temporally, the function of a given gene can be studied in the desired cell types and at a specific time point. This 'genetic dissection' allows to define gene function in development, physiology or behavior. In this review, we focus on the technical possibilities of Cre and other site-specific recombinases but also discuss their limitations.

Key words: Recombinase; Cre-*loxP*; Mutation; Transgenesis; Mouse

1. Introduction

During the last decade, the development of new genetic tools revolutionized reverse genetics in the mammalian organism. The possibility to specifically modify a gene locus by homologous recombination in mouse embryonic stem (ES) cells and to observe the consequences of this modification in living animals provided invaluable information. However, the study of the first generation of mouse mutants was limited by several technical caveats. First, gene targeting in ES cells requires the use of selection markers, usually an open reading frame (ORF) encoding the neomycin resistance gene under the control of a strong promoter. The presence of this promoter has been shown to influence the expression levels of neighboring genes and of the gene of interest itself. The latter can be a problem if subtle genetic modifications such as the introduction of point mutations rather than a total inactivation of the gene of interest is the aim. Second, in many instances the inactivation of a gene can be lethal at early embryonic or postnatal development, preventing the study of its function at later stages. Third, if a gene is expressed in many different cell types, its inactivation may lead to a complex and non-interpretable phenotype that is caused by the accumulation of different alterations in each cell type.

Ten years ago, Lakso et al. and Orban et al. demonstrated the use of a site-specific recombinase in mice [1,2]. This approach has revolutionized reverse genetics and its further development has already solved some of the described caveats.

2. The Cre-*loxP* system

The Cre recombinase (Cyclization recombination) is a member of the integrase family of site-specific recombinases. This protein family groups more than 100 members found in Archae, Eubacteria, mitochondria and yeasts [3]. They are involved in integration and excision of viral and plasmid DNA, transposition, resolution of catenated DNA circles, DNA excision and the control of gene expression.

Cre is a 38 kDa protein encoded by the bacteriophage P1 that recognizes a 34 bp DNA target on the P1 genome called *loxP* (locus of X-over of P1). It catalyzes reciprocal DNA recombination between two *loxP* sites. This mechanism serves to cyclize P1 DNA after infections and during bacterial division, it facilitates the segregation of P1 phages by resolving dimeric plasmids that were before formed by homologous recombination [4]. *LoxP* sites are composed of two inverted DNA segments of 13 bp Cre monomer binding sites and a spacer of 8 bp (Fig. 1A). In vitro and crystallographic studies revealed the molecular mechanisms of Cre-mediated recombination. Two Cre monomers bind cooperatively to the *loxP* site with nanomolar affinity. In the absence of any accessory factors, two *loxP* sites are assembled in an antiparallel fashion by four Cre monomers to form a synaptic structure stabilized by cyclic interactions between neighboring monomers [5] (Fig. 1B). As a first step, two opposite recombinases catalyze single strand breaks in the spacer region by phosphoryl transfer to a tyrosine resulting in the formation of a free hydroxyl group. The hydroxyl group attacks the intact and complementary DNA strand on the other *loxP* site leading to the formation of a Holliday junction. A similar mechanism resolves this structure and leads to the formation of two new *loxP* sites. This event is conservative; it does not cause any nucleotide addition or removal, thus leaving intact the new *loxP* sites that can be reused for further recombination events.

The conservative nature of the reaction makes it reversible.

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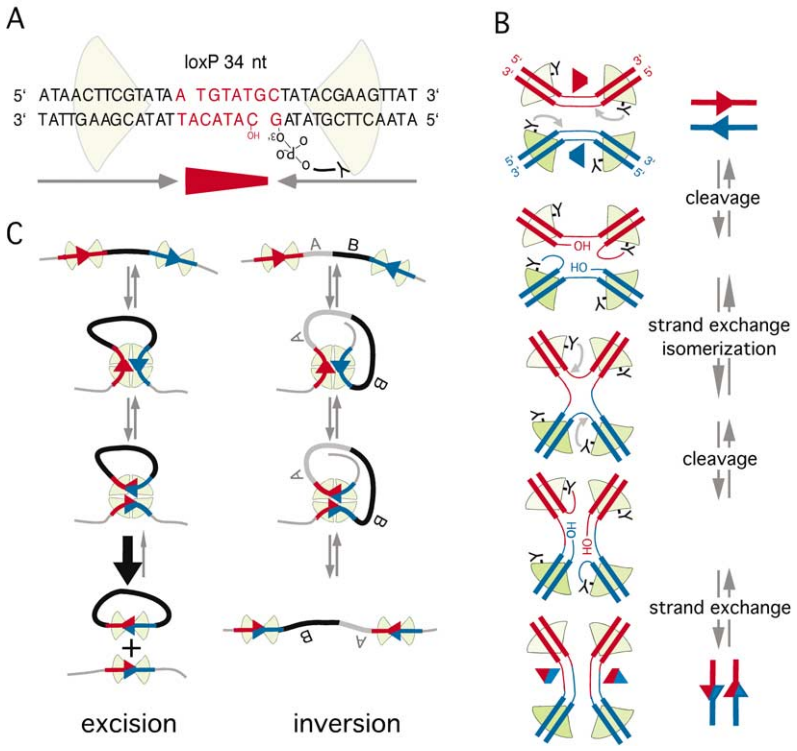

Fig. 1. Mechanism of site-specific Cre recombination. A: The *loxP* site consists of two inverted 13 bp Cre binding sites that surround a central 8 bp spacer. A red arrow indicates the orientation of the *loxP* site. Cre recombinase is pictured in green. The first cleavage reaction is pictured. B: Model of the Cre-*loxP* recombination pathway. Four Cre molecules form a synaptic tetramer. The tyrosines 324 from two of them cleave the DNA backbone. The released 5′ OH ends attack the partner strand to form a Holliday intermediate. A second round of cleavage and strand exchange results in the recombinant products [5,67]. C: If the two recombination sites are in the same orientation the strand exchange leads to excision or integration. If they are in the opposite orientation the outcome is an inversion.

However, if the two *loxP* sites are located on the same DNA molecule, their respective orientation determines the reaction products resulting either in the inversion or the excision of the intervening DNA segment (Fig. 1C).

The simplicity of this system makes it suitable for use in organisms other than bacteria. Indeed, the pioneering work by B. Sauer demonstrated that it efficiently works in organisms with very large genomes [6]. Today it is widely used as a tool for DNA rearrangement in plants, insects and mammals [7–10].

3. Conditional gene activation or inactivation in mice

In mice, the Cre-*loxP* system was initially used to switch on gene expression in a given cell population [1,2]. Two distinct transgenic mouse lines were generated. The first carries a silent transgene, that is spaced from a promoter by a 'stop cassette' (Fig. 2A). The stop cassette prevents transcription of the transgene because it contains either a strong polyadenylation signal and/or a splice donor sequence or it disrupts the ORF of the silent gene [1,2,11]. The second line carries a transgene that drives the expression of Cre recombinase in a cell type-specific way. In every Cre expressing cell, the stop cassette will be excised enabling the expression of the desired transgene exclusively in those cells. This approach has many applications. For example, one can generate transgenic lines that will express toxic proteins, follow the destiny of cells irreversibly marked by the expression of a reporter gene, or determine the recombination pattern of a specific Cre line using β-galactosidase or fluorescent proteins as reporter. The only limitations are those inherent to transgenesis. When small transcriptional regulatory DNA regions are used, the expression might be ectopic, mosaic and sometimes variegated, due to the influence of genomic surroundings at the integration site [12]. In addition, transgenes from prokaryotic origin may be poorly translated and subject to extinction in mice [13]. Recent developments, such as the characterization of a locus that can be targeted in ES cells and allows ubiquitous expression [14], the use of very large DNA segments (YACS and BACs [15]) as vectors for transgenesis, and the generation of 'humanized' ORF, overcome these problems [16].

The possibility of cell-specific inactivation of a desired gene within the organism is a unique tool for addressing the function of genes involved in complex physiological networks and for dissecting their respective roles in different tissues. Since its first description in 1994 by Gu et al. [17], a growing number of studies have further developed this strategy [10] (Figs. 2B and 3A). It consists of inserting into the target gene, by homologous recombination in ES cells, two *loxP* sites in the same orientation and at a strategic position, thus ensuring that removal of the intervening DNA segment results in complete inactivation of the gene. The inactivation profile depends on the expression profile of the recombinase (Fig. 3A). It is essential that the insertion of the *loxP* sites does not interfere with the normal expression of the gene. Ideally, they should be placed in introns or non-transcribed regions, avoiding the disruption of regulatory regions. However, in several cases a *loxP* was inserted in transcribed but untranslated regions without negative effects. If the distance between the two

loxP sites was a concern when designing the first conditional alleles, it is now clear that, even if the efficiency of site-specific recombination drops as a function of the distance, very large DNA segments (up to 4 Mb) can be efficiently excised by Cre in vivo [18,19]. It is advisable to remove any DNA cassette used during ES cells targeting since it may interfere with the expression of the targeted or neighboring genes [20]. This was initially done using a targeting construct harboring three *loxP* sites, two of which were flanking the selection cassette. Subsequently, a transient expression of Cre provided ES cells in which a partial recombination event took place, thus excising the cassette but leaving the two *loxP* sites intact [17]. New vectors in which FRT sites flank the selection cassette facilitate this step [21]. The cassette can be removed by expression of the flp recombinase in the ES cells or by mating animals carrying the targeted allele with mice that express the flp in the germ line [22] (Fig. 2E).

A precise determination of the recombination profile is very important for interpreting any phenotype. Unfortunately, the expression pattern of Cre or the recombination pattern of a reporter gene do not necessarily predict the recombination pattern of the targeted gene because the efficiency of Cre-*loxP* recombination also depends on the genomic integration sites of the *loxP* sequences [23]. Ideally, the disappearance of the gene product should be followed using antibodies (Fig. 3A). In some cases, depending on the structure of the gene, an alternative strategy has been used, which couples the excision event with the activation of a silent reporter gene inserted at the targeted locus [24].

Finally, an important consideration is the time course of the disappearance of the gene product that is a function of the stability of mRNAs and proteins present in the cells at the time of the gene deletion. If recombination takes place in dividing cells, the disappearance of the gene product will be facilitated by dilution due to cell division. In non-dividing cells this can be a major problem especially in the case of very stable mRNAs or proteins that are not actively degraded.

4. Chromosomal rearrangements

Cre recombinase proved to be useful for the design of chromosomal aberrations such as deletions, inversions, translocations and duplications [19,25–27]. It can be used as a model for human chromosomal disorders associated with chromosomal deletions and duplications or with translocations as seen in some tumors. Chromosomal rearrangements can be used to establish balanced lethal systems to facilitate stock maintenance [28], large deletions can be used for genetic screening of recessive mutations, and translocations can be used to obtain mosaic animals carrying wild-type, heterozygous and mutant cells after the induction of mitotic sister chromatids exchange [29]. For these purposes, two *loxP* sites have to be introduced into the genome. If they are inserted in the same chromosome, depending on their orientation, Cre will mediate large deletions or inversions. If they are targeted on two heterologous chromosomes, the presence of Cre will lead to a balanced translocation (Fig. 2C). Finally, if they are targeted on two homologous chromosomes but positioned at different locations, the action of Cre will generate a deletion, in one, and a duplication, in the other, homologous chromosome (Fig. 2D). The effect of these rearrangements can be tested directly in chimeric animals generated from the modified ES cells or eventually, when possible through germ line transmission. In ES cells, the rare occurrence of rearrangements makes a selection scheme based on the reconstitution of a selection gene (usually hprt) necessary [27]. However, even without selection, some of these rearrangements can efficiently be induced in animals when Cre is expressed from a transgene and balanced translocations between heterologous chromosomes have been observed at a low efficiency [30]. In addition, translocation between homologous chromosomes could be dramatically increased using an elegant experimental design that takes advantage of chromosomal pairing during meiosis and allowed the generation of balanced deletion and duplication of large DNA segments [31] (Fig. 2D).

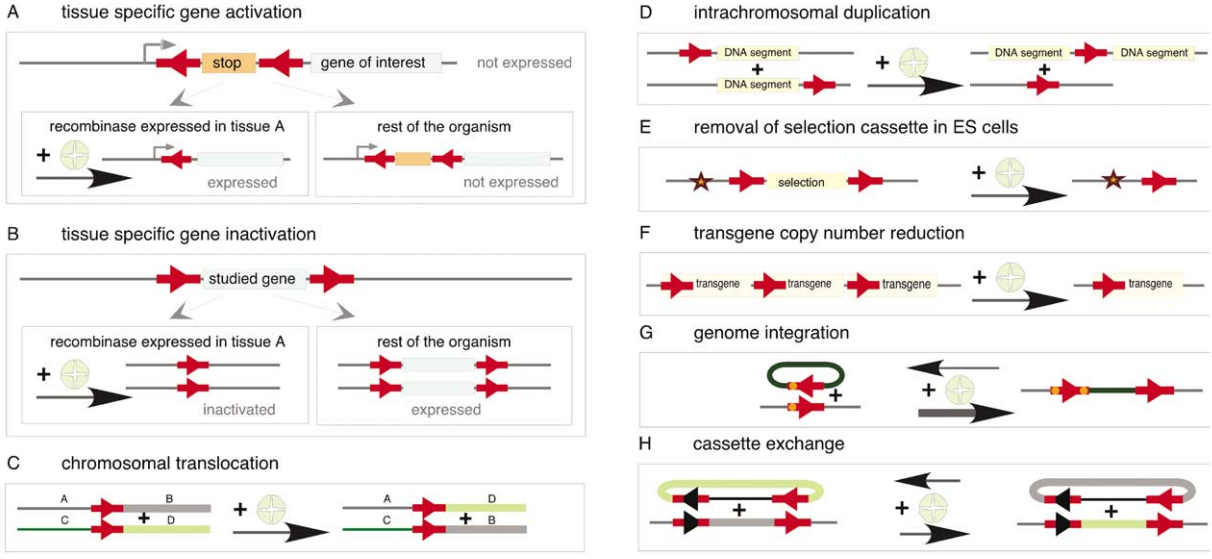

Fig. 2. Application of site-specific recombination as a genetic tool in mammals. For A–H details are described in the text. *LoxP* sites are indicated by red arrows and Cre recombinase in green. If the *loxP* site is located in the 5′ untranslated region (A), an inverted orientation is advisable as it prevents the introduction of ATG codons in front of the expressed ORF [8]. In E, instead of *loxP* sites also FRT sites could be used. The star indicates as an example an introduced point mutation. The Cre-*loxP* system can also be used to resolve transgene silencing due to concatemerization of transgene copies (F) [68]. In G, the mutation of the *loxP* site in the Cre binding site is represented by a dot. In H, the mutation in the *loxP* spacer is indicated by a black arrow.

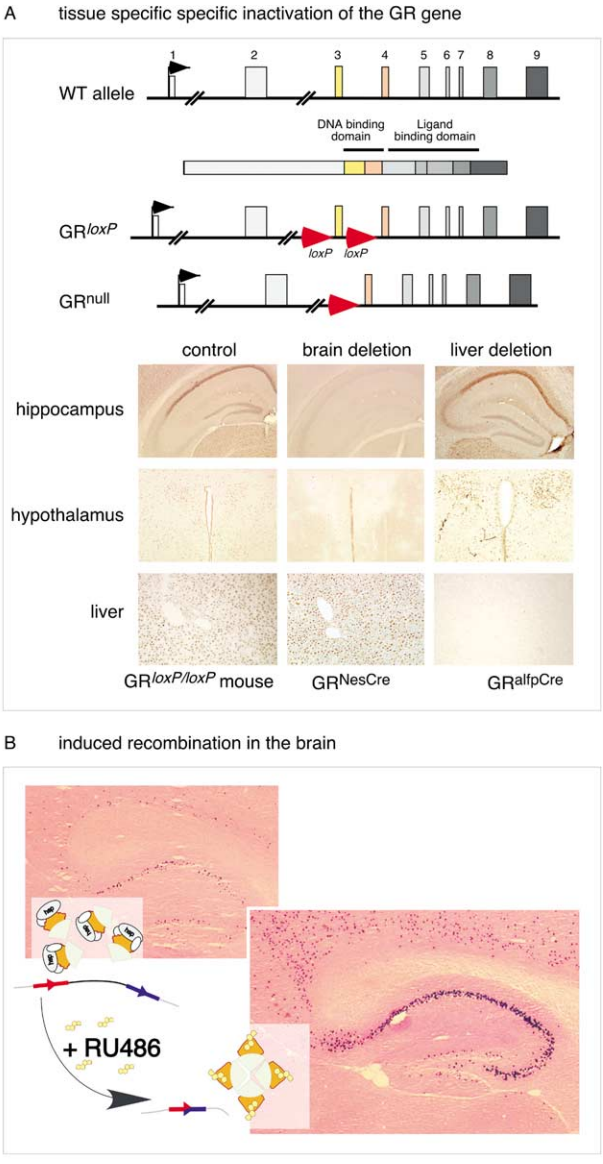

Fig. 3. Example of somatic mutagenesis of the glucocorticoid receptor using site-specific recombination in mice. A: Conditional inactivation of the glucocorticoid receptor (GR) gene. The gene and protein structures of this transcription factor are indicated. The presence of *loxP* sites (red arrows) in the GR^{loxP} allele makes it sensitive to Cre-mediated inactivation. Cre activity deletes the third exon including the DNA-binding domain. In addition, it introduces a frameshift after the second and fourth exons are fused. The expression of Cre in the brain or in the liver specifically depletes GR protein levels in the corresponding tissues, as shown by immunohistochemistry using an antibody directed against GR [69,70]. B: Inducible site-specific recombination in the brain. See details in the text. The CrePR fusion protein is expressed in the brain under the control of the CamKIIα promoter. Injection of RU486 activates the recombinase and results in the expression of a Cre dependent LacZ transgene [32].

5. Inducible somatic mutations in mice

Adding temporal control over the recombinase activity would not only allow the generation of tissue-specific mutations that would be otherwise lethal, but also the study of the physiology and behavior in the same animal before and after the mutation. This avoids variations between individual organisms and limits the time for compensatory mechanisms. Several strategies have been developed. One is to control the recombinase activity. This requires the fusion of Cre ORF to a mutated form of the ligand-binding domain (LBD) of a nuclear receptor (progesterone (PR), estrogen (ER) or glucocorticoid (GR) receptors [32–34]). The mutated LBDs bind synthetic steroid analogs (RU486, tamoxifen and dexamethasone, respectively) but do not bind endogenous steroids, thus preventing the induction of recombination by endogenous hormones. If Cre is fused to a PR-LBD, the activity of the CrePR fusion protein is strongly reduced due to binding of chaperones. Injection of RU486 to an adult animal releases the fusion protein and results in Cre-mediated recombination (Fig. 3B). When tested in cell culture, comparable CreER and CrePR fusion proteins displayed similar induced and background activities [32]. Both systems can regulate site-specific recombination in different organs, such as the blood, the brain, the heart, the liver and the skin [32,33,35–39]. It is difficult to compare the two systems in vivo since the fusion proteins, their expression levels, their targets and the cell types were different. However, the tightest control was observed with CreER expressed in the skin. Recently, for both systems, new fusion proteins that are more tightly regulated and more sensitive to the inducer have been developed ([33,40] and E.C., unpublished).

Another strategy is to control the expression of the recombinase using inducible promoters. The Mx1 promoter that can be induced by application of interferon or poly dIdC to mice, and tetracycline dependent artificial promoters that have the advantage to allow tissue specificity have both been used [41,42]. Finally, to induce a mutation at a particular time, the recombinase can also be introduced using viral transduction with vectors derived from viruses [43], or it can be fused to membrane translocation sequences that allow a protein to enter a cell simply by crossing the cell membrane [44].

6. Targeted insertions

Targeting of transgenes into a defined genomic location abolishes the variability of transgene expression due to positional effects. Cre catalyzes both integration and excision but the monomolecular nature of the excision favors the excision reaction. Therefore, although integration events can be selected in cell culture, their low occurrence does not allow the use of this approach in vivo. Several attempts have been performed on this system to reduce the efficiency of the excision step and thus favor integration. Recombination between a pair of *loxP* sites, one carrying a mutation on the left 13 bp repeat and the other one on the right repeat, generates an intact and a doubly mutated *loxP* site ([45], Fig. 2G). This reduces, but unfortunately does not abolish, the reverse excision. An alternative approach would be the use of an unidirectional recombinase that in contrast to Cre performs only one reaction (the integration in this case). The phiC31 integrase is such a recombinase and is active in mammalian cells [46]. Another approach is the so-called 'recombination-mediated exchange of cassette' (RMCE) that has the advantage of not integrating vector sequences. It relies on the use of *loxP* sites mutated in the 8 bp spacer region. Cre catalyzes recombination between two mutant sites but only poorly between a mutant and an intact site. As picture in Fig. 2H, a double recombination between two mutant and two wild-type *loxP* sites in *trans* leads to the cassette exchange [47,48]. In one study, RMCE has been demonstrated in the genome of

injected mouse fertilized oocytes with a frequency of 15%. However, since the mutation did not completely abolish the recombination between heterospecific *loxP* sites, excision subsequently occurred [49]. Further improvements, such as the use of inverted *loxP* sites (the cassette is inverted but never excised [50]) and more exclusive *loxP* mutations [51], are required to improve this approach in animals.

7. Some problems with the Cre-*loxP* system

Although the large number of publications using the Cre-*loxP* system in mammals suggests that the recombinase is not toxic, recent reports indicated that this assumption might not be true. The attempt to express high levels of Cre in spermatids resulted in sterility of the transgenic males, due to a very high occurrence of chromosomal aberrations [52]. Several other reports observed a decrease in cell proliferation as well as an increase in apoptosis in cells expressing high levels of Cre [43,53,54], which is associated with the accumulation of Cre expressing cells in the G2/M phase of the cell cycle, chromosomal rearrangement and the appearance of micronuclei. These aberrations could be due to the action of Cre on cryptic target sites that exist in the genome [55]. Alternatively, Cre may induce nicks, which are then converted into double strand breaks and repaired via non-homologous recombination resulting in the accumulation of fragmented DNA. To prevent such deleterious effects, special care has probably to be taken on the level of Cre expression. Self-excising Cre vectors may minimize the time window for the toxicity of Cre, as long as it ensures recombination of the targeted locus before disappearance of the recombinase [43,53].

Another concern is the fact that sensitivity of the *loxP* target to recombination may vary from locus to locus [23]. In addition, a given target might be recombined at early stages of development but not in adult tissues [32] or can become insensitive to recombination, due to methylation [56]. Therefore, a given Cre line may recombine different targets with different kinetics and cellular specificities. This can intuitively be explained by differential accessibility of the target site due to differences in the chromatin state. These facts illustrate that the recombination pattern obtained with one conditional allele is not sufficient to predict the profile of recombination of another conditional allele, even if exactly the same Cre line was used.

8. Other site-specific recombinases

The parallel development of alternatives to the Cre/LoxP system is necessary. The utilization of other recombinases than Cre may allow studying sequential biological events by introducing more than one somatic mutation into the same animal (Fig. 4A). As depicted in Fig. 4B,C it also enhances the potency of site-specific recombination. In the first example recombinase A inverts a DNA segment and this inversion is stabilized by the presence of recombinase B, allowing one to swap the expression of an intact exon with that of a mutated one. In the second example, a cell type-specific recombination is achieved by combining the expression patterns of two different gene promoters. Recombination would occur only in cells that express both recombinases.

Two strategies have been followed to develop further recombinases that are active in mammalian cells. The first is to take advantage of the diversity of existing recombinases

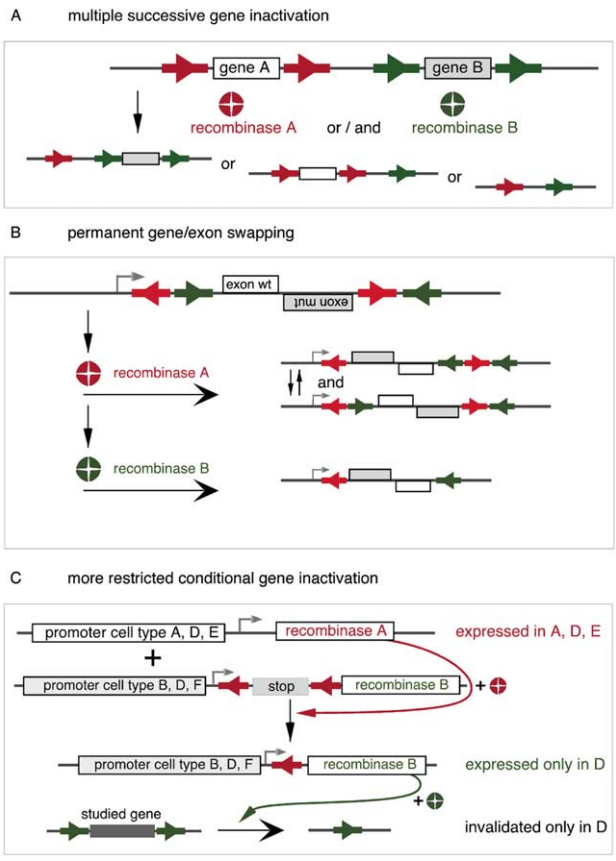

Fig. 4. Possible applications of using two distinct site-specific recombinases. See the text for comments.

in nature, the second to modify the existing ones. The yeast Flp recombinase and its target, FRT, do work in mice, in particular the mutated version that was selected for higher efficiency at 37°C [57] and has been widely used recently to remove selection cassettes after homologous recombination in mice [22]. Recently, other recombinases from yeast [58] or bacteriophages (R4, lambda, phi31, HK022 and TP901-1 [46,59,60]) have been also demonstrated to catalyze recombination events in mammalian cells. In parallel, new recombinases were obtained by mutating the Cre, generating chimeric proteins generated from Cre and flp enzymes or by accelerated protein evolution [61–63]. In the latter case, the authors were able to select for a Cre variant that recognizes a DNA target naturally present in the human genome, opening up the possibility of specifically targeting existing DNA sequences in the genome for genetic modifications.

The use of site-specific recombinases allows us to examine the consequences of sophisticated genetic modifications at the level of an entire mammalian organism. Conditional mutagenesis is however tedious and requires long breeding schemes. It will provide answers to specific questions with an exquisite precision, but the parallel development of other approaches, including the targeting of gene products by small molecules, RNA interference and systematic mouse mutagenesis [64–66], is essential to get an insight into genetic networks, by targeting simultaneously several partners, at a genome scale.

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