A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse

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Functional redundancies, compensatory mechanisms, and lethal phenotypes often prevent the full analysis of gene functions through generation of germline null mutations in the mouse[1]. The use of site-specific recombinases, such as Cre, which catalyzes recombination between *lox*P sites[2], has allowed the engineering of mice harboring targeted somatic mutations, which are both temporally controlled and cell-type restricted[1,3]. Many Cre-expressing mouse lines exist, but only a few transgenic lines are available that harbor a reporter gene whose expression is dependent on a Cre-mediated event[3]. Moreover, their use to monitor gene ablation at the level of individual cells is often limited, as in some tissues the reporter gene may be silenced[1], be affected by position-effect variegation[4], or reside in a chromatin configuration inaccessible for recombination[5]. Thus, one cannot validly extrapolate from the expression of a reporter transgene to an identical ablation pattern for the conditional allele of a given gene. By combining the ability of Cre recombinase to invert or excise a DNA fragment, depending on the orientation of the flanking *lox*P sites[6], and the availability of both wild-type (WT) and mutant *lox*P sites[7], we designed a Cre-dependent genetic switch (FLEx switch) through which the expression of a given gene is turned off, while the expression of another one is concomitantly turned on. We demonstrate the efficiency and reliability of this switch to readily detect, in the mouse, at the single cell level, Cre-mediated gene ablation. We discuss how this strategy can be used to generate genetic modifications in a conditional manner.

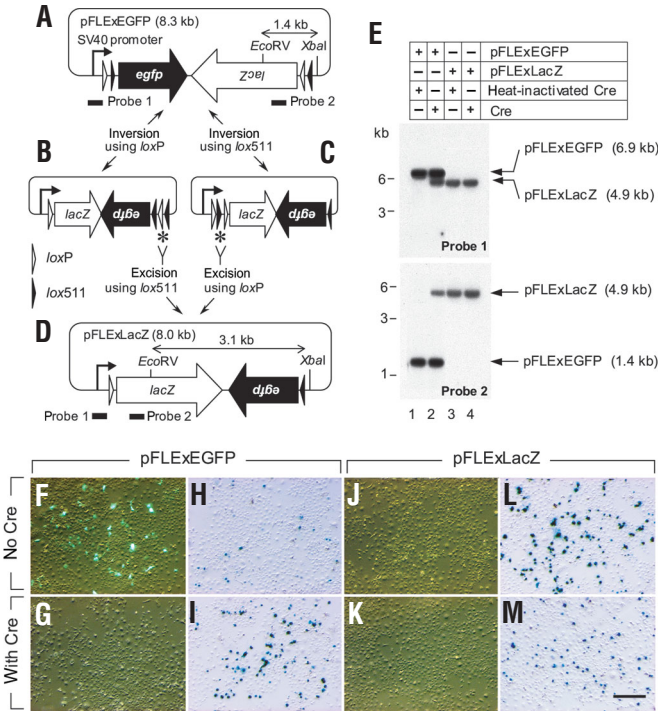

Figure 1. The FLEx switch. (A) pFLExEGFP contains the SV40 promoter (broken arrow), a *lox*P site (open arrowhead), a *lox*511 site (closed arrowhead), the coding sequence for EGFP (*egfp*), the β-galactosidase promoterless minigene (*lacZ*) in the antisense orientation, and *lox*P and *lox*511 sites in the reverse orientation. (B and C) Intermediates after Cre-mediated inversion at the *lox*P and *lox*511 sites, respectively. (D) Final product after Cre-mediated excision between the *lox*511 or the *lox*P sites (asterisks). *Eco*RV and *Xba*I restriction sites, together with location of probes 1 and 2 are indicated. (E) Evidence for *in vitro* Cre-mediated recombination by Southern blot analysis of *Eco*RV- and *Xba*I-digested DNA using probes 1 and 2. Lane 1 and 2, pFLExEGFP; lane 3 and 4, pFLExLacZ (see Experimental Protocol). Cre was added in reactions illustrated in lanes 2 and 4, whereas a heat-inactivated Cre was added in reactions shown in lanes 1 and 3. (F to M) COS-1 cells were transfected with pFLExEGFP (F to I) or pFLExLacZ (J to M) in the absence (F, H, J and L) or in presence (G, I, K and M) of Cre. EGFP was examined by fluorescence microscopy (F, G, J and K) and *lacZ* expression was assessed by X-gal staining (H, I, L and M). Note that the blue staining in H (less than 1% of cells) most probably reflects a low level of *lacZ* transcription initiated from the noncoding strand of pFLExEGFP, as plasmid linearization before transfection reduces it. Scale bar, 250 μm.

The principle of our Cre-dependent one-way genetic switch is illustrated by plasmid pFLExEGFP (Fig. 1A), which contains one pair of WT *lox*P sites (open arrowheads) and one pair of *lox*511 sites (closed arrowheads), with an alternate organization and a head-to-head orientation within each pair of sites. Both *lox*P and *lox*511 sites are recognized by Cre; however, *lox*511 sites recombine efficiently with themselves but not with *lox*P sites[7]. The plasmid also contains the DNA encoding the enhanced green fluorescent protein (*egfp*) in the sense orientation, and a promoterless *lacZ* gene in the antisense orientation. Initially the SV40 promoter directs expression of *egfp*. Cre-mediated recombination may first induce inversion of the intervening DNA at either the *lox*P (Fig. 1B) or the *lox*511 sites (Fig. 1C), thus yielding a direct repeat (asterisks) of either two *lox*511 (Fig. 1B) or two *lox*P sites (Fig. 1C). A further Cre-mediated excision will then remove the DNA located between the two *lox*P or the two *lox*511 sites. For this, the distance between the compatible *lox* sites (asterisks, Fig. 1B,C) should be at least 82 base pairs (bp)[8]. In the resulting plasmid (pFLExLacZ), single *lox*P and *lox*511 sites are left, making further inversion of the intervening DNA impossible (Fig. 1D). The SV40 promoter now drives expression of *lacZ* instead of *egfp*.

We first established a test system allowing *in vitro* Cre-mediated rearrangements, using a crude Cre preparation that was checked using a plasmid containing a *lacZ* sequence flanked by *lox*P sites (see Experimental Protocol). Plasmid pFLExEGFP (Fig. 1A) was incubated with either Cre (Fig. 1E, lanes 2 and 4) or heat-inactivated Cre (Fig. 1E, lanes 1 and 3), then digested with *Eco*RV and *Xba*I and analyzed by Southern blotting (Fig. 1A,E). The Cre recombinase mediated the rearrangement of pFLExEGFP to pFLExLacZ (Fig. 1E, lower panel), as assessed by the presence of the 4.9 kb *Eco*RV-*Xba*I DNA fragment (Fig. 1E, lane 2). Some unexcised plasmid was left, most probably owing to limiting Cre activity, as increasing amounts of Cre improved the yield of excision (data not shown), and/or to

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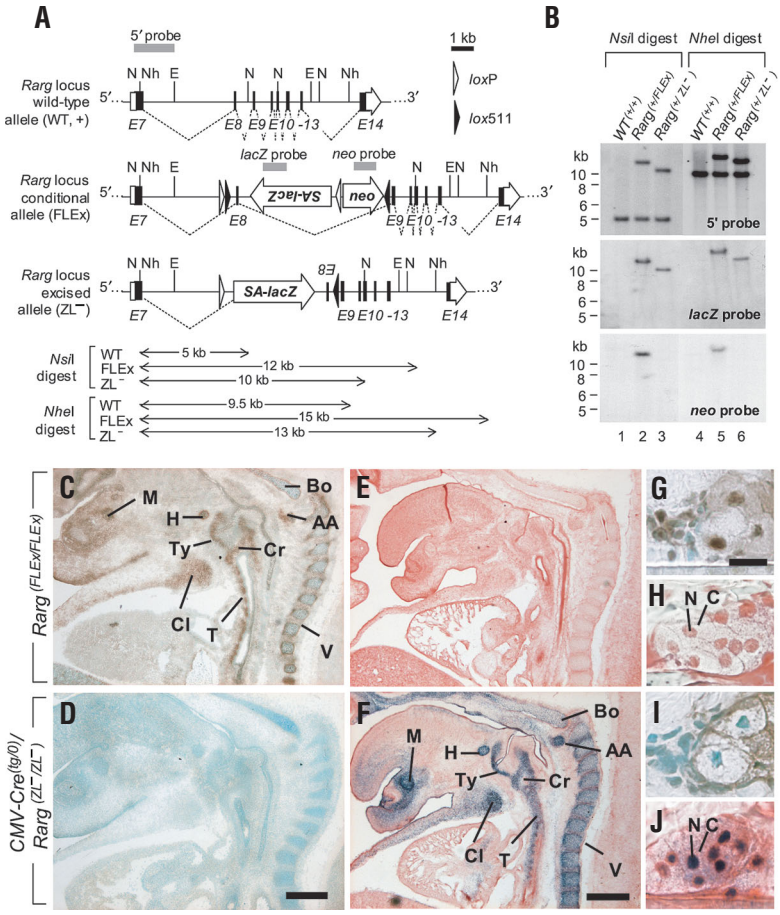

Figure 2. The *Rarg* FLEx allele in the mouse. (A) Scheme of the wild-type (WT, +), conditional (FLEx) and RARγ-null, lacZ-expressing (ZL⁻) alleles. Restriction sites (E, *Eco*RI; N, *Nsi*I; Nh, *Nhe*I) and probe are indicated. Exons 7–14 (E7–E14) are shown. *lox*P and *lox*511 sites are represented by open and closed arrowheads, respectively. Dotted lines represent the expected splicing of the primary transcript. The sizes of the restriction fragments obtained for each allele are in kilobases (kb). (B) Southern blots of *Nsi*I- and *Nhe*I-digested genomic DNA from mice with genotypes as indicated, using the 5′, the *lacZ*, or the *neo* probes. (C and D) Immunohistochemical (IHC) detection of RARγ in E13.5 fetuses: (C) *Rarg*$^{(FLEx/FLEx)}$; (D) *CMV-Cre*$^{(tg/0)}$-*Rarg*$^{(ZL^-/ZL^-)}$. Sections were incubated with antibodies against RARγ, which were revealed using peroxidase-conjugated secondary antibodies and 3,3′-diaminobenzidine as substrate (brown color), then counterstained with 10% (wt/vol) methyl green (green color). (E and F) β-galactosidase activity in the same fetuses as in panels C and D. Sections were incubated with X-gal (blue color) and counterstained with 0.01% (wt/vol) safranine O (red color). (G and I) IHC detection of RARγ in: (G), *Rarg*$^{(FLEx/FLEx)}$; (I), *CMV-Cre*$^{(tg/0)}$-*Rarg*$^{(ZL^-/ZL^-)}$ sections of sebaceous glands of adult mice. (H and J) β-galactosidase activity in the sebaceous glands as in panels G and I. AA, anterior arch of the atlas; Bo, basioccipital bone; C, cytoplasm; Cl, clavicle; Cr, cricoid cartilage; H, hyoid cartilage; M, Meckel's cartilage; N, nucleus; T, trachea; Ty, thyroid cartilage; V, vertebra. Scale bars, 500 μm (C–F) and 20 μm (G–J).

high stability of *lox*P synaptic complexes *in vitro*[9]. All of the plasmids that were recovered after cloning in *Escherichia coli* underwent both inversion and excision (data not shown), indicating that inversion was a transient step that was always resolved by excision, and that illegitimate recombination between *lox*P and *lox*511 sites did not occur. Accordingly, plasmid pFLExLacZ remained unchanged when incubated with Cre (Fig. 1E, lane 4). COS-1 cells were then transiently transfected with pFLExEGFP or pFLExLacZ either alone or together with a fivefold excess of a Cre-expressing vector[10], and the transfected cells (approximately 30%) were analyzed for EGFP fluorescence and β-galactosidase activity. Cells transfected with pFLExEGFP alone clearly showed green fluorescence (Fig. 1F), but only a little β-galactosidase activity (Fig. 1H; see figure legend). In contrast, when expressing Cre, all transfected cells reproducibly did not show green fluorescence (Fig. 1G), but showed prominent β-galactosidase activity (Fig. 1I), indicating that Cre always mediated consecutively both inversion and excision. Regardless of Cre expression, cells transfected with pFLExLacZ did not show green fluorescence (Fig. 1J and K), but showed clear β-galactosidase activity (Fig. 1L and M), indicating that once inversion and excision had occurred, the DNA molecule was stable.

Using homologous recombination in embryonic stem (ES) cells, we integrated the FLEx switch system into the retinoic acid receptor γ (RARγ) gene (*Rarg*), thereby designing a FLEx conditional allele in such a way that β-galactosidase staining should specifically label cells that normally express RARγ and that have lost RARγ expression upon Cre-mediated rearrangement. The *Rarg* locus was chosen because the null mutant phenotype has been characterized[11] and a *lox*P-flanked (L2) allele is available[10] for comparative purposes. *Rarg*$^{(+/FLEx)}$ mice were generated (Fig. 2A) and appeared normal. These mice were crossed with *CMV-Cre* transgenic mice, which express Cre very early in development[1], thus generating *Rarg*$^{(+/ZL^-)}$ mice harboring the Cre-rearranged FLEx allele (ZL⁻) (Fig. 2A,B). In *Rarg*$^{(FLEx/FLEx)}$ mice, RARγ mRNA had the expected size, but its level of expression was nearly fivefold lower than in WT mice, indicating that the presence of the FLEx cassette impaired mRNA production (data not shown). Accordingly, *Rarg*$^{(FLEx/FLEx)}$ males were hypofertile and displayed a less severe form of the RARγ null phenotype[11]—that is, mild prostate keratinization (data not shown). Interestingly, the expression level of the RARγ-LacZ fusion mRNA in *Rarg*$^{(ZL^-/ZL^-)}$ mice was similar to that of the RARγ mRNA in WT mice (data not shown), suggesting that the presence of the *neo* cassette in *Rarg*$^{(FLEx/FLEx)}$ mice may be responsible for the hypomorphic phenotype. Thus, flanking the *neo* cassette with *FRT* and excising it with the Flp recombinase[12] might enable one to restore normal RARγ expression from a FLEx allele. Alternatively, associating one FLEx allele and one L2 allele[10] within a single animal (e.g., *Rarg*$^{(L2/FLEx)}$ mouse) might solve this hypomorphism problem.

To test for concomitant Cre-mediated RARγ ablation and β-galactosidase expression, we compared the distribution of RARγ protein with X-gal staining in *Rarg*$^{(FLEx/FLEx)}$ mice bearing, or not bearing, the *CMV-Cre* transgene[1]. RARγ protein was detected in the precartilaginous condensations of *Rarg*$^{(FLEx/FLEx)}$ embryonic day 13.5 (E13.5) fetuses (Fig. 2C), and in the forming epidermis (surface ectoderm, data not shown). Importantly, no β-galactosidase activity could be detected in these fetuses (Fig. 2E), indicating that the *lacZ* cassette was fully silent before Cre-mediated rearrangement. As the *CMV-Cre* transgene directs recombination in one-cell-stage embryos[1], each cell of the transgenic fetuses should be RARγ-null. Accordingly, the RARγ protein was not detectable in E13.5 transgenic *CMV-Cre*$^{(tg/0)}$-*Rarg*$^{(ZL^-/ZL^-)}$ fetuses (Fig. 2D), indicating efficient *Rarg* gene disruption. It is noteworthy that these fetuses displayed a β-galactosidase activity restricted to precartilaginous condensations (Fig. 2F) and surface ectoderm (data not shown), indicating that the ZL⁻ allele faithfully reproduced the normal expression pattern of RARγ. As shown in the adult sebaceous gland (Fig. 2G–J), identification of RARγ-null, lacZ-expressing cells was facilitated by the presence of a nuclear targeting sequence

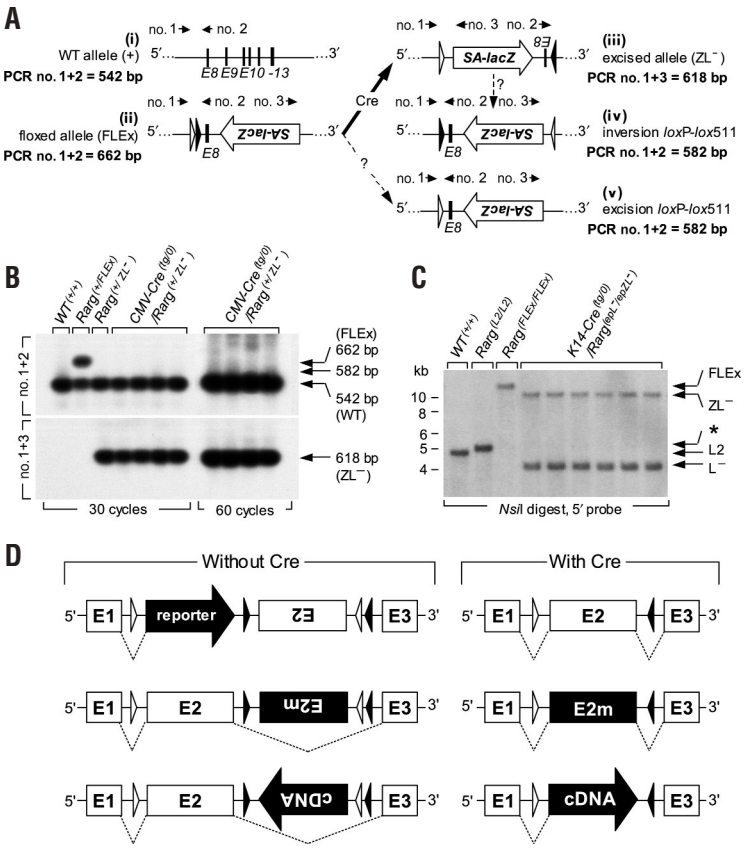

Figure 3. The FLEx switch, which is reliable and efficient in the mouse, makes possible novel applications. (A) Strategy to detect illegitimate recombination between *lox*P and *lox*511 sites. The structure of the *Rarg* wild-type (WT, i) and FLEx (ii) alleles is schematized (see also Fig. 2A). Arrows (nos. 1–3) indicate PCR primers. The *lox*P and *lox*511 sites are represented by open and closed arrowheads, respectively. Primers no. 1 and no. 2 allow amplification of 542 bp-long and 662 bp-long DNA fragments from WT (i) and FLEx (ii) alleles, respectively. Cre-mediated recombination generates the excised *Rarg* ZL^{-} allele (iii; see also Fig. 2A), from which primers no. 1 and no. 3 amplify a 618 bp–long DNA fragment. Illegitimate inversion or excision between *lox*P and *lox*511 sites would yield alleles iv and v, respectively, and in both cases primers no. 1 and no. 2 would amplify a 582 bp–long DNA fragment. (B) PCR analysis of DNA from fetuses with the indicated genotypes. Primers, size, and identity of the amplified fragments are indicated. The 582 bp–long fragment corresponding to illegitimate recombination (iv and v alleles) was not detected. (C) Southern blot analysis of *Nsi*I-digested genomic DNA from tail epidermis of mice with the indicated genotypes (see Fig. 2A for details). Neither L2 nor FLEx alleles could be detected in *K14-Cre*$^{(tg/0)}$-*Rarg*$^{(epL^{-}/epZL^{-})}$ mice, indicating similar efficacy for Cre-mediated recombination of L2 and FLEx alleles. The asterisk points to the position of the fragment expected after complete deletion between *lox*P and *lox*511 sites. (D) Additional possible applications of the FLEx switch. Top: conditional rescue. The scheme represents a knock-in reporter allele (left). After Cre-mediated rearrangement (right), the reporter cassette is removed, whereas the WT exon is restored in sense orientation. Middle: conditional point mutation. The scheme represents a conditional allele expressing the WT protein (left). Upon Cre-mediated rearrangement (right), exon 2 is removed and replaced by the mutated exon 2 (E2m), resulting in the synthesis of a mutated protein. Bottom: conditional gene replacement. The scheme represents a conditional allele expressing the WT protein (left). After Cre-mediated rearrangement (right), exon 2 is removed and replaced by a cassette containing an internal ribosomal entry site (IRES) followed by a given cDNA and a polyadenylation signal. Synthesis of the WT protein is abrogated whereas the cDNA-encoded protein is expressed. Dotted lines represent the expected splicing of the primary transcript, and E1–E3 stands for exons. Open and closed arrowheads represent *lox*P and *lox*511 sites, respectively.

in the *lacZ* gene[13]. Importantly, *CMV-Cre*$^{(tg/0)}$-*Rarg*$^{(ZL^{-}/ZL^{-})}$ mice displayed the characteristic features of the RARγ-null phenotype[11], namely Harderian gland agenesis, tracheal rings malformations, and keratinization of the prostate and seminal vesicles (data not shown). Thus, Cre-mediated rearrangement of the *Rarg* FLEx allele indeed produces a *Rarg*-null allele.

To further illustrate the usefulness of the FLEx allele, *Rarg*$^{(FLEx/FLEx)}$ mice were crossed with *K14-Cre* transgenic mice[14]. In these mice, every keratinocyte expressing Cre also expressed β-galactosidase, but not RARγ. In contrast, dermal fibroblasts in which Cre was not expressed still expressed RARγ, but not β-galactosidase (see Supplementary Fig. 1 online; other data not shown). Thus, *lacZ* expression was observed exclusively in domains in which the *Cre* transgene and RARγ expression patterns overlap.

As the *lox*P and *lox*511 sites are not strictly incompatible[7], the question of how often they could recombine in our FLEx switch is important because illegitimate excision between *lox*P and *lox*511 sites would generate a RARγ-expressing allele containing the *lacZ* gene locked in the antisense (silent) orientation (Fig. 3A,v), whereas illegitimate inversion between the *lox*P and *lox*511 sites left in the ZL^{-} allele could cause the RARγ-null, lacZ-expressing allele to revert to a silenced lacZ, RARγ-expressing allele (Fig. 3A,iv). Importantly, the 582 bp–long DNA fragment corresponding to such illegitimate rearrangements could not be amplified by PCR from mice bearing the FLEx allele and expressing Cre (Fig. 3B). Similarly, no illegitimate recombination between the *lox*P and *lox*511 sites flanking the *neo* cassette was detected (data not shown). Because recombination is less efficient between *lox*511 sites than between *lox*P sites, and because *lox*P can form highly stable[9] 'dead-end' synaptic complexes with *lox*511 that compete for productive recombination[15], the presence of both *lox*P and *lox*511 sites within the FLEx allele may significantly reduce its capability to recombine. We therefore generated mice bearing both a *Rarg* allele flanked by *lox*P(L2) and a FLEX allele (*Rarg*$^{(L2/FLEx)}$ mice), and bred them with *K14-Cre* transgenic mice[14] to yield transgenic *K14-Cre*$^{(tg/0)}$-*Rarg*$^{(L2/FLEx)}$ mice, in which Cre-mediated recombination was analyzed on tail epidermis DNA (Fig. 3C). Both *Rarg* L2 and FLEx alleles were fully converted into L^{-} and ZL^{-} alleles in the epidermis, generating *K14-Cre*$^{(tg/0)}$-*Rarg*$^{(epL^{-}/epZL^{-})}$ mice. Thus, rearrangement of the FLEx allele appears to be as efficient as excision of the *lox*P-flanked allele. Finally, we did not observe inversion or complete excision of the entire *lox*P/*lox*511-flanked FLEx cassette (Fig. 3C, asterisk; data not shown), ruling out the possibility that the altered directionality of the Cre recombination pathway previously observed in bacteria[16] also occurred in the mouse. Altogether, these data show that the FLEx switch is both reliable and efficient in the mouse, at least at the *Rarg* locus. However, we suggest that the functionality of any FLEx allele should be checked in ES cells before proceeding to blastocyst injections. Should an allele be less successful than a *lox*P-flanked L2 allele, it may be modified by using *lox*5171 or *lox*2272 sites, which are less permissive[7] and more efficient[17] than *lox*511 sites.

Conditional null alleles containing a *lacZ* reporter have recently been described[18]. Although operational, with β-galactosidase expression selectively replacing the normal gene product in Cre-expressing cells, such alleles cannot be used for further purposes. In contrast, the *lox*P and *lox*511 sites left in the rearranged *Rarg* FLEx (ZL^{-}) allele can be used as a substrate for Cre-mediated cassette exchange (CMCE[19]), allowing a different sequence to be inserted between the *lox*P and *lox*511 sites. Applying CMCE to the

ES cell clone FK177.4 harboring the *Rarg* ZL^{-} allele (see Experimental Protocol), we successfully replaced the *SA-lacZ* cassette by a *lacZ-neo* minigene (see Supplementary Fig. 2 online). Thus, through CMCE, any DNA sequence can be targeted into the *Rarg* locus. The most attractive features of this method are that (i) the *lox*P/*lox*511-flanked exchanged fragment will stay in position, even in the presence of an active Cre recombinase, and (ii) its expression will be controlled by the *Rarg* promoter. Therefore, it becomes possible to integrate the *cre* gene itself in the rearranged FLEx locus. Finally, the FLEx switch strategy paves the way for the engineering of more sophisticated genetic modifications in the mouse, including those previously considered 'impossible' to perform[3]: accurate conditional rescue of a gene knockout (Fig. 3D, top), conditional point mutations (Fig. 3D, middle), and conditional replacement of a given gene product by another one (Fig. 3D, bottom).

Experimental protocol

DNA constructs. For pFLExEGFP, a *lox*P site followed by a 21 bp spacer was introduced into the *Eco*RI site of pSG5. A *lox*511 site[15] followed by a 21 bp spacer was introduced 3′ to the *lox*P site. A reverse *lox*P site followed by a 21 bp spacer was introduced 3′ to the first *lox*511 site. A second *lox*511 site, also in the reverse orientation, followed by a 21 bp spacer was introduced 3′ to the reverse *lox*P site. The coding sequence of EGFP (*egfp*) and a *lacZ* cassette were introduced between the two sets of *lox*P sites, in the sense and the antisense orientation, respectively. To obtain pFLExLacZ, pFLExEGFP was incubated with a crude Cre preparation and the recombined DNA was cloned in *E. coli*.

The vector (pγ6.0FLEx), used for homologous recombination in ES cells[10], contained a 6 kb genomic fragment[11] encompassing exons E8–E13, in which were inserted: a *lox*P and a *lox*511 upstream of E8; a DNA cassette (*SA-lacZ*) containing the splice acceptor site and the first 4 codons of *Rarg* E8 (*SA*) in frame with *lacZ*[13] and followed by an SV40 polyadenylation signal, all in the antisense orientation; a *lox*P site in the reverse orientation; a neomycin resistance (*neo*) cassette[11]; and a *lox*511 site in the reverse orientation. The *Rarg SA* was chosen to minimize, as far as possible, the risk of aberrant mRNA splicing once the FLEx allele is recombined. Two ES cell clones (out of 234) exhibited targeted gene replacement (FK39 and FK177). To verify the functionality of the FLEx allele, clone FK177 was transfected with pSG5-Cre[10]; 2 of the resulting subclones (out of 25) showed recombination as expected (FK177.4 and FK177.18). Clone FK177 was injected into blastocysts and 2 (out of 11) of the chimeras obtained transmitted the *Rarg* FLEx allele to their germ line. The detailed procedure for constructing pγ6.0FLEx and the sequences of the *lox* sites or spacers are available upon request.

***In vitro* Cre reactions.** A crude Cre extract was prepared from a culture of *E. coli* 294-Cre[20] resuspended in 50 mM Tris/HCl, pH 7.5, 33 mM NaCl, 10 mM $MgCl_2$, 5% glycerol, and 0.02% (wt/vol) NaN_3, and lysed by sonication. The extract was cleared by centrifugation (14,000*g*, 15 min, 4 °C). For control reactions, Cre was inactivated by 10 min at 70 °C. The plasmids (3 μg) were incubated with 100 μl of Cre for 1 h at 37 °C, then isolated and transformed into XL1-Blue cells (Stratagene, La Jolla, CA). Recombined plasmids were isolated, digested by *Eco*RV and *Xba*I and analyzed by Southern blotting using the radiolabeled oligonucleotides 5′-AATACGACTCACTATAG-3′ (probe 1) or 5′-GTGCATCTGCCAGTTTGAGG-3′ (probe 2).

EGFP detection, LacZ staining and immunohistochemistry. COS-1 cells were cultured and transfected according to standard methods. Each transfection was carried out at least five times. Three days after transfection, cells were fixed for 5 min with 2% (wt/vol) formaldehyde in phosphate-buffered saline. EGFP activity was examined with a Leica MS FL-III microscope. For β-galactosidase activity detection, samples (cell plates or 10 μm–thick frozen sections) were fixed with 4% (wt/vol) paraformaldehyde, then incubated overnight at 30 °C in 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ containing 1 mg/ml X-gal. Immunodetection of Cre[1] and RARγ[10] was performed on frozen sections. Digital images were generated using a CCD camera.

Genotyping and PCR reactions. Tail DNA was genotyped by PCR[1,10]. Illegitimate recombination (Fig. 3) was tested using primers no. 1 (5′-TTTTTGTGCCTGGTGCCCATGGAAG-3′) and no. 2 (5′-ATGGCTTATAGACCCGAGGAGGTGG-3′) to amplify WT (542 bp long) and FLEx alleles (662 bp long), or primer no. 1 and no. 3 (5′-AGTGCCAAGCTTGGACTCAAAAAAC-3′) to amplify the ZL^{-} allele (618 bp long). Conditions were denaturation at 92 °C, annealing at 60 °C, and elongation at 72 °C in a standard buffer using 100 ng of genomic DNA. Amplified fragments were resolved on 2.5% (wt/vol) agarose gels, transferred onto HybondN+ (Amersham, Orsay, France) and detected using the 5′-CCTGGTCCTTGGGAGAGCAGCCAGT-3′ ^{32}P-radiolabeled probe.

Note: Supplementary information is available on the Nature Biotechnology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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