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Talking about a Revolution: The Impact of Site-Specific Recombinases on Genetic Analyses in Mice

Review

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Site-specific recombinase systems (Cre-*loxP*, Flp-*FRT*, and ϕC31-*att*) are transforming both forward and reverse genetics in mice. By enabling high-fidelity DNA modifications to be induced in vitro or in vivo, these systems have incited a wave of new biology, advancing our understanding of gene function, genetic relationships, development, and disease.

With the human genome project estimating over 30,000 genes, our task now is to determine the contribution of each gene to development, disease, and disability. A powerful method for inferring the role of human genes is to analyze the expression and function of homologous genes in model organisms, such as the mouse. Nearly every human gene has a counterpart in the mouse, and, among genetic model systems, the mouse is unique in its applicability to human organogenesis, immunology, neurobiology, reproduction, behavior, and epigenetics. Thus, the mouse is an essential model system both for functionally annotating the human genome and for modeling human disease.

The level at which one may use the mouse to explore gene function is dependent, in part, upon the methodologies available to manipulate the genome. By providing a means to delete, insert, invert, or exchange chromosomal DNA with high fidelity, site-specific recombinase (SSR) technology is revolutionizing mouse genetics. SSR technology is being applied to induce null mutations in discrete cells in vivo, bypassing embryonic lethality associated with many germline null alleles. As a result, investigations can now reach beyond the first required function of a gene. Chemical mutagenesis screens and mapping studies are being facilitated by libraries of deletions, duplications, and inversions—reagents historically limited to *Drosophila* but now available in the mouse by the application of SSR technology. Human diseases associated with chromosomal translocations are being modeled in mice using SSR-based genomic engineering. And finally, SSR technology is being applied to map the deployment of cells during embryogenesis in order to reveal their contributions to adult tissues and to provide a framework for analyzing genotype-phenotype relationships uncovered by mutagenesis. In this review, we focus on how mouse genetics is being transformed through SSR technology. We begin by discussing the current contents of the SSR "toolbox" and follow by presenting some new biology made possible through their application.

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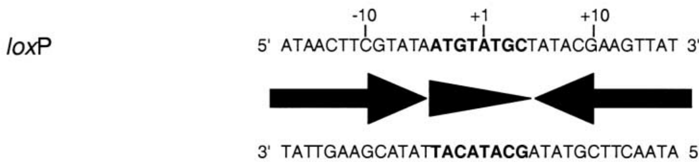
SSRs, Their Target Sites, and Their Function

The SSRs Cre (causes recombination of the bacteriophage P1 genome) and Flp (named for its ability to invert, or "flip," a DNA segment in *S. cerevisiae*) are able to recombine specific sequences of DNA with high fidelity without the need for cofactors (reviewed in Stark et al., 1992; Dymecki, 2000). For this reason, they have been used effectively to create gene deletions, insertions, inversions, and exchanges in exogenous systems such as flies (Golic and Lindquist, 1989; Dang and Perrimon, 1992; Xu and Rubin, 1993), mammalian cell culture (Sauer and Henderson, 1988; O'Gorman et al., 1991), and mice (Lakso et al., 1992; Orban et al., 1992; Dymecki, 1996). Cre and Flp recombine DNA at defined target sites, termed *lox*P (locus of crossover (x) in P1) (Hoess et al., 1982) and *FRT* (Flp recombinase recognition target) (McLeod et al., 1986), respectively, in both actively dividing and postmitotic cells, as well as in most tissue types.

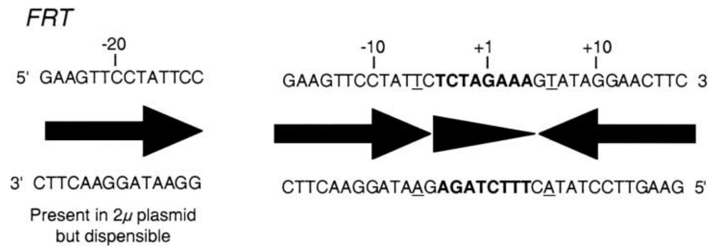
Cre and Flp, both members of the λ integrase superfamily of SSRs, share a common mechanism of DNA recombination that involves strand cleavage, exchange, and ligation (Sadowski, 1995). Although distinct at the nucleotide level, *lox*P (Figure 1A) and *FRT* (Figure 1B) sites share an overall structure which includes two 13 basepair (bp) palindromic sequences, or inverted repeats, separated by an 8 bp asymmetric core, or spacer, sequence. In the presence of two target sites, recombinase monomers bound to the inverted repeats promote DNA synaptic complex formation and recombination between the two sites (Figure 1C) (Hoess et al., 1985). Strand cleavage, exchange, and ligation occur within the spacers (Amin et al., 1991). Because of spacer asymmetry, strand exchange is possible only when target sites are connected by synapsis in one orientation (Hoess et al., 1986). Consequently, the relative orientation of target sites with respect to one another determines the outcome of recombination: Cre and Flp will excise a circular molecule from between two directly repeated target sites (Figure 2A), integrate a circular molecule into a linear molecule each possessing a target site (Figure 2A), invert the DNA between two inverted sites (Figure 2B), and exchange sequences distal to target sites present on two linear molecules, such as a pair of nonhomologous chromosomes (Figure 2C).

The excision reaction is effectively irreversible (due to loss of the circular reaction product) and has been exploited most readily in the mouse. Indeed, it is the basis for both conditional gene inactivation and molecular fate mapping, tools that have allowed gene function and cell deployment to be studied in the late embryo and adult (these applications are discussed further below). By contrast, stable insertion and inversion reactions that permit, for example, a sound comparison of phenotypic consequences of different DNA sequences at a defined locus have been less straightforward to engineer. This is because, in each case, the recombination product harbors two identical target sites in *cis*, which are themselves substrates for recombination (i.e., excision or repeated inversions, respectively). With the goal of being able to further modify an already targeted

A Cre recombinase target site



B Flp recombinase target site



C Mechanism of Cre or Flp-mediated recombination

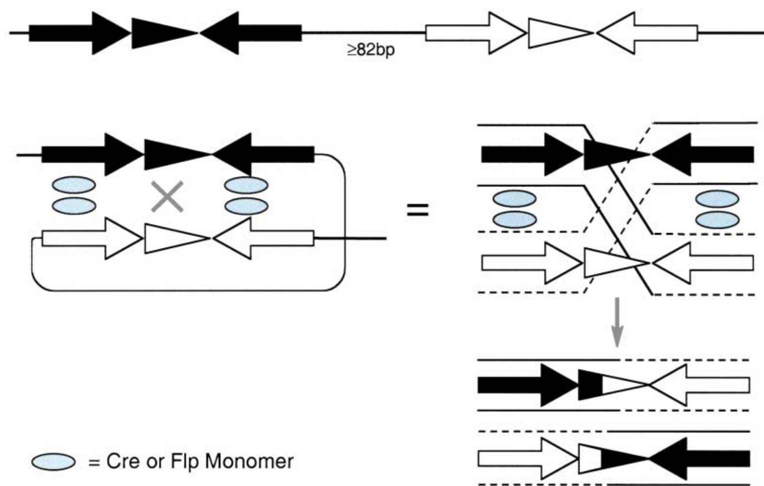


Figure 1. Cre and Flp Recombinase Target Sites and Mechanism of Recombination

The Cre and Flp target sites each contain two 13 bp inverted repeats flanking an 8 bp asymmetrical core, or spacer (nucleotides of the spacer are indicated in bold). Horizontal arrows mark the inverted repeats and a triangle the spacer. The graded sides of the triangle represent the asymmetry of the spacer, which imparts directionality on the target site and which, in turn, imparts directionality on the recombination reaction (see text). By convention, nucleotides are numbered from the center of the site.

(A) The Cre recombinase target site is termed *lox*P. (B) The Flp recombinase target site is termed *FRT*. An additional upstream 13 bp repeat is present in the 2 μ plasmid but is dispensable for Flp-mediated recombination, making the minimal *FRT* site 34 bp. By contrast to a *lox*P site, the inverted repeats of an *FRT* site contain a single bp difference (underlined). (C) Synaptic complex formed between recombinase molecules (Cre or Flp) and two identical target sites (distinguished here as being either black or white). One recombinase monomer binds each 13 bp repeat, while the spacer sequence provides the site of strand cleavage, exchange (via a Holliday intermediate), and ligation. In the final product, there is an exchange of DNA between the two starting sites.

or trapped locus, clever strategies have been devised to make insertions and inversions effectively irreversible. Fundamental to these strategies is the finding that Cre and Flp each tolerate certain variations in their target sequences, but effectively recombine only particular combinations of these alternative sites.

Variant target sites for Cre and Flp fall into two classes: spacer variants and inverted-repeat variants (Table 1). The first class contains nucleotide substitutions within the spacer sequence and exploits the finding that it is spacer length (8 bp), not sequence (Hoess et al., 1986; Senecoff et al., 1988), that is critical for efficient recombination, so long as the sequence between participating sites is identical (Senecoff and Cox, 1986). Recombination is therefore efficiently mediated between pairs of homotypic (e.g., *FRT*/*FRT* or F_3/F_3) but not heterotypic (e.g., *FRT*/F_3) sites. Such exclusivity is the basis of the insertion strategy called "recombinase-mediated cassette exchange" (RMCE) (reviewed in Bode et al., 2000; Baer and Bode, 2001) and the inversion strategy called the "FLEx" switch (Schnutgen et al., 2003).

In RMCE, a selectable marker (such as a fusion gene that permits both positive and negative selection) flanked by heterotypic sites is inserted into a chromosomal site within the mouse embryonic stem (ES) cell genome by homologous recombination (thereby "tagging" the site); a DNA sequence of choice is then inserted into the tagged site replacing the selectable marker by an exchange step (Figure 3A). Cells in which the DNA swap has occurred are enriched by negative selection. The final product is stable because left in *cis* are heterotypic (incompatible) sites. The success of RMCE, therefore, hinges on the inability of heterotypic SSR target sites to recombine with one another. For Flp-mediated RMCE, the spacer variant F_3 and F_5 sites have proven impervious to recombination with a wild-type FRT site (Schlake and Bode, 1994). Likewise, for Cre-mediated RMCE, the spacer variant sites *lox2272* (Lee and Saito, 1998) and *m2* (Langer et al., 2002) appear impervious to recombination with a *lox*P site. By contrast, the alternative Cre target site *lox511* (Bethke and Sauer, 1997) (also termed L1 [Hoess et al., 1986]) has

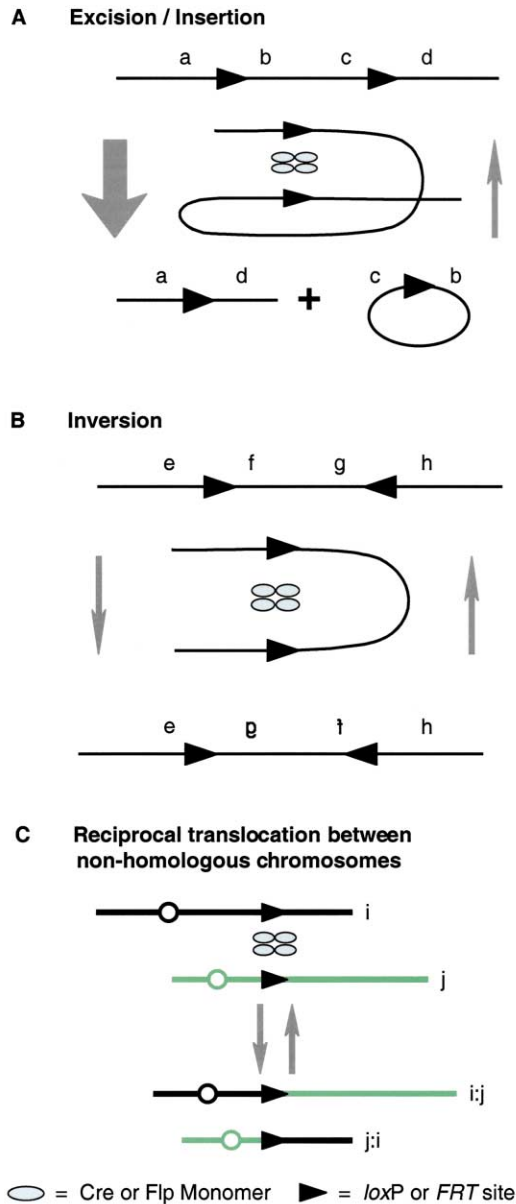


Figure 2. Recombination Reactions Mediated by Cre or Flp

The relative orientation of target sites with respect to one another determines the outcome of the recombination reaction: Cre or Flp will (A) excise a circular molecule from between two directly oriented target sites, integrate a circular molecule into a linear molecule each possessing a target site; (B) invert the DNA between two inverted sites; and (C) exchange sequences distal to target sites present on two linear molecules. This latter type of intermolecular recombination can occur between target sites on nonhomologous chromosomes (indicated by the letters i and j) to produce a balanced translocation. Black and green lines represent chromosomal DNA, with orientation indicated by the letters a–d, or e–h; gray arrows indicate the reversible nature of each reaction.

been found to recombine, albeit at low efficiency (1%–5%), with a wild-type *lox*P site (Feng et al., 1999; Kolb, 2001) and, thus, is less effective in RMCE reactions.

The FLEx switch strategy uses both wild-type and spacer variant target sites, and exploits the ability of SSRs to invert a DNA sequence between opposing target sites but delete a DNA sequence between similarly oriented sites (Figure 3B) (as such, FLEx stands for "flip excision"). A DNA fragment to be inverted is flanked by two pairs of matching heterotypic target sites. Recombination between either pair of homotypic sites results in cassette inversion, including inversion of the interior target site. This positions two homotypic sites in direct orientation, resulting in immediate excisional recombination. The end product is an inverted cassette flanked by two heterotypic sites that cannot recombine with one another, thereby preventing further inversions.

The second class of alternative SSR target sites, the inverted-repeat variants, is also being exploited to engineer stable DNA insertions (Figure 4A) and inversions (Figure 4B). Here, variant target sites contain nucleotide substitutions within the inverted repeats that compromise binding of the recombinase to DNA (Albert et al., 1995, and Araki et al., 1997, for Cre; and Senecoff et al., 1988, for Flp). A target site containing a nucleotide substitution in the left inverted repeat (an LE mutant site) can recombine with a site containing an analogous substitution in the right inverted repeat (an RE mutant site), albeit at a slower reaction rate. The recombination product harbors one wild-type and one LE/RE doubly mutant site, the latter being effectively inert. For stable DNA insertions, this class of alternative SSR target sites has typically been used for the incorporation of an incoming plasmid into chromosomal DNA (as depicted in Figure 4A).

Further flexibility in SSR applications has arisen through establishment of the *Streptomyces* phage-derived ϕC31 SSR for use in ES cells (Belteki et al., 2003). The ϕC31 SSR mediates recombination only between the heterotypic sites *attB* (34 bp in length) and *attP* (39 bp in length) (Groth et al., 2000) (see Table 1). *attB* and *attP*, named for the attachment sites for the phage integrase on the bacterial and phage genomes, respectively, both contain imperfect inverted repeats that are likely bound by ϕC31 homodimers (Groth et al., 2000). The product sites, *attL* and *attR*, are effectively inert to further ϕC31-mediated recombination (Belteki et al., 2003), making the reaction irreversible. For catalyzing insertions, it has been found that *attB*-bearing DNA inserts into a genomic *attP* site more readily than an *attP* site into a genomic *attB* site (Thyagarajan et al., 2001; Belteki et al., 2003). Thus, typical strategies position by homologous recombination an *attP*-bearing "docking site" into a defined locus, which is then partnered with an *attB*-bearing incoming sequence for insertion (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/6/1/7/DC1). Importantly, expression of ϕC31 in ES cells (like Cre and Flp) is compatible with germline competence (Belteki et al., 2003).

When designing experiments using ϕC31, an important consideration is the presence of multiple pseudo *attP* sites (that is, sequences that are similar but not identical to the wild-type *attP* site) in the genomes of both human and mouse cell lines (31 and 57 reported [Thyagarajan et al., 2001] thus far, respectively). In certain human and mouse cell lines, some of these endogenous pseudo *attP* sites appear capable of recombining with incoming *attB*-bearing DNA, even in the presence of an engineered wild-type *attP* site (Thyagarajan et al., 2001). Empirical evidence suggests, however, that these pseudo sites do not confound targeted integration

Table 1. Alternative SSR Recognition Sites

Target Site[a]	Inverted repeat 1	Spacer	Inverted Repeat 2	Use of Alternative Site	Reference
*lox*P	ATAACTTCGTATA	ATGTATGC	TATACGAAGTTAT		Hoess, et al., 1982
*lox*511[b]	ATAACTTCGTATA	ATGTATaC	TATACGAAGTTAT	RMCE[c]	Hoess, et al., 1986 Bethke and Sauer, 1997
*lox*5171	ATAACTTCGTATA	ATGTgTaC	TATACGAAGTTAT	RMCE	Lee and Saito, 1998
*lox*2272[d]	ATAACTTCGTATA	AaGTATcC	TATACGAAGTTAT	RMCE	Lee and Saito, 1998
m2	ATAACTTCGTATA	AgaaAcca	TATACGAAGTTAT	RMCE	Langer, et al., 2002
*lox*71 (LE[e] mutant)	taccgTTCGTATA	ATGTATGC	TATACGAAGTTAT	Stable insertion or inversion	Albert, et al., 1995
*lox*66 (RE[f] mutant)	ATAACTTCGTATA	ATGTATGC	TATACGAAcggta	Stable insertion or inversion	Albert, et al., 1995
FRT	GAAGTTCCTATTC	TCTAGAAA	GTATAGGAACTTC		McLeod, et al., 1986
F_3	GAAGTTCCTATTC	TtcAaAtA	GTATAGGAACTTC	RMCE	Schlake and Bode, 1994
F_5	GAAGTTCCTATTC	TtcAaAAg	GTATAGGAACTTC	RMCE	Schlake and Bode, 1994
FRT mutant −10 (LE mutant)	GAAGTTCaTATTC	TCTAGAAA	GTATAGGAACTTC	Stable insertion or inversion	Senecoff, et al., 1988
FRT mutant +10 (RE mutant)	GAAGTTCCTATTC	TCTAGAAA	GTATAtGAACTTC	Stable insertion or inversion	Senecoff, et al., 1988
attB[g]	TCGAGTGAGGTGGAGTACGCGCCCGGGGAGCC CAAGGGCACGCCCTGGCACCCGCA			RMCE or stable inversion	Belteki et al., 2003
attP	CTAGACCCTACGCCCCCAACTGAGAGAACTCAAAGGT TACCCCAGTTGGGGCACG			RMCE or stable inversion	Belteki et al., 2003

[a] Nucleotides that have been altered from the wild-type sites are lowercase.
[b] The alternative Cre target site *lox511* (Bethke and Sauer, 1997) is also referred to as *L1* (Hoess et al., 1986) and mutant 71 (Lee and Saito, 1998). *lox511* can recombine, albeit at low efficiency (1–5%), with a wild-type *lox*P site and therefore may have limited use in RMCE reactions (Kolb, 2001, and see text).
[c] RMCE, recombinase mediated cassette exchange.
[d] *lox2272* is also referred to as *lox2722* (Kolb, 2001).
[e] LE, left-hand element (LE) (inverted repeat 1) of SSR recognition site.
[f] RE, right-hand element (RE) (inverted repeat 2) of SSR recognition site.
[g] Heterotypic target sites of the ϕC31 SSR are included here for reference. Core nucleotide triplets (CAA) where the ϕC31-mediated recombination occurs are underlined.

events into the ES cell genome, at least not when proper strategies are applied (A. Nagy, personal communication). Of note, pseudo *lox*P sites have also been identified in the mouse genome (Thyagarajan et al., 2000), although, given the widespread success of Cre to engineer desired rearrangements in vitro and in vivo, these sites do not appear to be an impediment. In cases where pseudo-*lox*P sites have been involved in unexpected recombination events (resulting in cell growth arrest and chromosomal aberrations), Cre was expressed at very high levels (Schmidt et al., 2000; Loonstra et al., 2001).

To maximize further the utility of SSRs in mice, attention has also been directed at the recombinases themselves. A number of groups have developed protein evolution strategies to manipulate under various conditions the enzymatic properties of Cre and Flp to create new recombinase variants (Buchholz and Stewart, 2001, and Santoro and Schultz, 2002, for Cre; and Buchholz et al., 1998, and Voziyanov et al., 2003, for Flp). For example, an error-prone PCR strategy has been used to develop a Flp variant with increased thermostability (Buchholz et al., 1998). This enhanced form of Flp, called Flpe, exhibits an ~4-fold increase in recombinase activity from wild-type when analyzed in cell culture. In mice, Flpe readily mediates recombination between *FRTs* separated by as little as a few kilobases (kb) (Farley et al., 2000; Rodriguez et al., 2000; Awatramani et al., 2003) or as much as 150 kb (F. Stewart, personal communication). A low-efficiency Flp, Flp-L, contains a single amino acid substitution (F70L) resulting in an ~5-fold decrease in recombinase activity from wild-type (Buchholz et al., 1996). This set of Flp recombinase variants (Flp-wt, Flpe, and Flp-L) collectively span an ~10-fold range in activity in vivo (Dymecki, 1996; Dymecki and Tomasiewicz, 1998; Rodriguez et al., 2000; Rodriguez and Dymecki, 2000; Awatramani et al., 2003), and may be exploited, for example, to achieve a range of cellular resolution in fate mapping studies (the application of SSRs for fate mapping is discussed further below).

With the goal of maximizing Cre activity, the *cre* gene has been modified to be more eukaryotic-like (Shimshek et al., 2002). Changes include a reduced *CpG* content, an improved Kozak translation initiation consensus sequence, and a number of silent mutations reflective of human codon-usage preferences. Indeed, the encoded iCre (for improved Cre) was detected at an ~1.6-fold higher concentration than conventional Cre when expressed from an identical vector; commensurate with this increase in protein levels, iCre was found to be ~1.8-fold more efficient at DNA recombination than conventional Cre.

Ligand-regulated forms of Cre and Flpe have also been developed, with the goal of adding temporal control to SSR activity so as to enable the induction of genetic changes late in embryogenesis and/or in adult tissues (as opposed to at the onset of recombinase expression). One particularly successful strategy for inducing SSR activity has involved fusing a mutant estrogen receptor (ER) ligand binding domain (LBD) to the C terminus of Flp (Logie and Stewart, 1995) or Cre (Metzger et al., 1995; Feil et al., 1996; Kellendonk et al., 1996; Brocard et al., 1997; Schwenk et al., 1998) (Figure 5A).

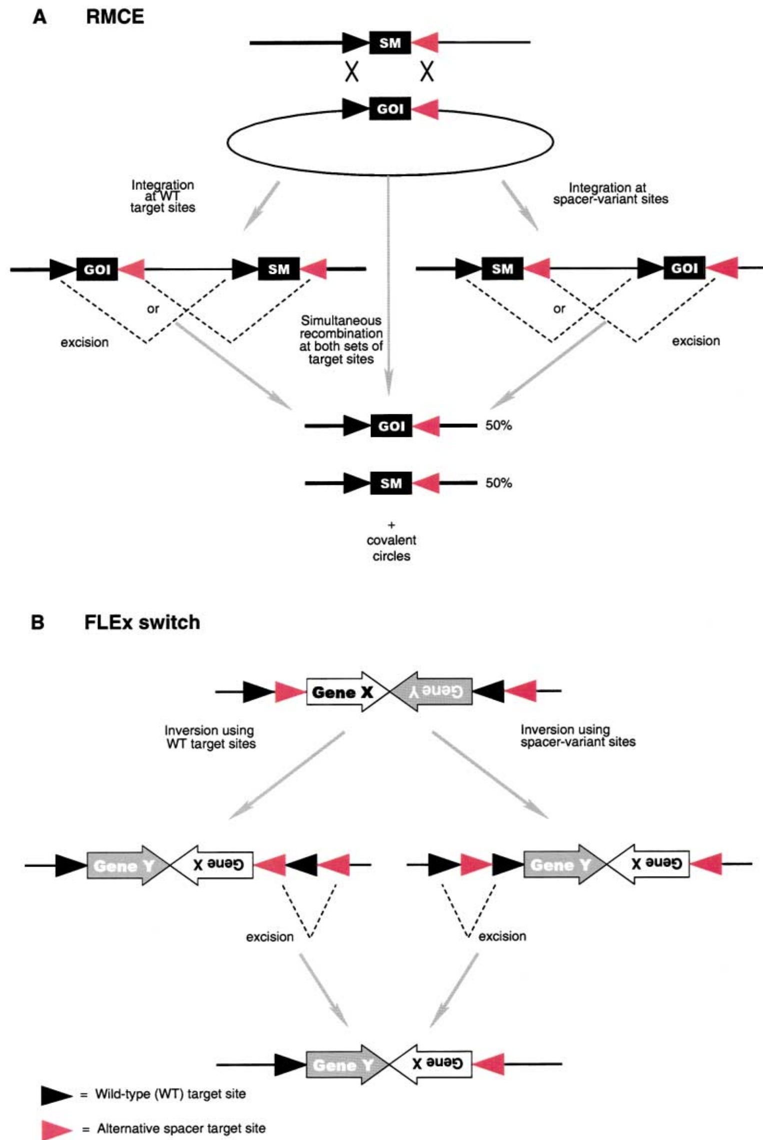


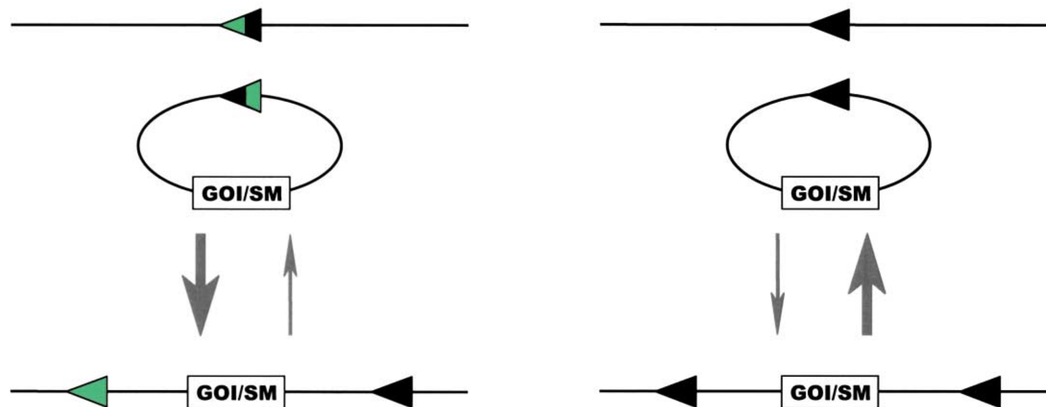
Figure 3. Use of Cre or Flp Spacer-Variant Target Sites to Achieve Stable DNA Cassette Exchange or Inversion

(A) Recombinase-mediated cassette exchange (RMCE). This insertion reaction exploits the fact that recombination is efficiently mediated only between pairs of homotypic, but not heterotypic target sites that vary in their spacer sequence. A selectable marker gene (SM) flanked by a pair of heterotypic spacer-variant *lox* or *FRT* sites may be stably replaced by a gene-of-interest (GOI) flanked by similar sites. This reaction involves two recombinase-mediated events: an integration step, followed by an excision step, leaving heterotypic target sites (e.g., an *FRT* and an F_3 site) flanking the exchanged cassette that may be used in additional rounds of RMCE. In RMCE reactions, the heterotypic target sites may be directly oriented or inverted with respect to one another; an advantage of using inverted sites (as shown) is that cassette excision is prevented should promiscuous recombination be possible between heterotypic target sites.

(B) The FLEx switch. Stable DNA inversion using the FLEx principle, applied here to invert the orientation of gene X and gene Y. Such a strategy could be employed to swap a coding sequence downstream of a promoter/enhancer element. The FLEx switch involves two recombination events: an inversion step, followed by an excision step. Following inversion at either wild-type or mutant target sites, pairs of homotypic sites flank a heterotypic target site. Recombination between these directly oriented homotypic sites (separated by at least 82 bp) results in the excision of the heterotypic site, and the inverted cassette is locked into place. Although not subject to further inversion, the recombinant product, flanked by heterotypic spacer-variant target sites, could serve as a substrate for subsequent rounds of RMCE.

Currently, there are three different ERs available, all of which are insensitive to endogenous β-estradiol, but responsive to the synthetic estrogen antagonist 4-OH tamoxifen (4-OH-TAM): (1) mouse ER^{TM} with a G525R mutation (Danielian et al., 1998; Guo et al., 2002; Hayashi and McMahon, 2002; Zirlinger et al., 2002), (2) human ER^{T} with a G521R mutation (Logie and Stewart, 1995; Metzger et al., 1995; Feil et al., 1996; Brocard et al., 1997; Schwenk et al., 1998; Li et al., 2000; Vooijs et al., 2001), and (3) human ER^{T2} containing three mutations G400V/M543A/L544A (Feil et al., 1997; Indra et al., 1999; Kimmel et al., 2000; Imai et al., 2001; Seibler et al., 2003). (Note that TAM, which is converted by the liver to the active inducer 4-OH-TAM, is often administered rather than 4-OH-TAM because it is more soluble in solution and is less costly.) Using the broadly active *CAG* promoter (chicken β-*actin* promoter coupled with cytomegalovirus enhancer sequences) (Niwa et al., 1991) to drive $CreER^{TM}$ expression in embryos, recombination was detectable in many cell types by 15 hr post-TAM injection, peaking by 48 hr (Hayashi and McMahon, 2002). (Methods of detecting recombinase activity within cells are discussed below.) Assays comparing the activity of $CreER^{T}$ with $CreER^{T2}$ indicate that $CreER^{T2}$ is ~10-fold more sensitive than $CreER^{T}$ for both nuclear translocation and recombinase activity (Indra et al., 1999). A $FlpeER^{T2}$ system has been developed recently; preliminary studies show inducible recombinase activity in vitro and in vivo comparable to that observed with CreERs (N. Lu, R. Awatramani, and S.M.D., unpublished data).

This ligand-regulated "switch" for turning on recombinase activity is now being exploited in two prototypal ways: (1) broad expression of recombinase::ER fusions to enable temporal control of recombinase activity in most tissues (Guo et al., 2002; Hayashi and McMahon, 2002; Seibler et al., 2003; and N. Lu, R. Awatramani, and S.M.D., unpublished data); and (2) tissue-restricted expression of these fusions using specific enhancer elements (Logie and Stewart, 1995; Danielian et al., 1998; Schwenk et al., 1998; and N. Lu, R. Awatramani, and S.M.D., unpublished data)—these mice permit both temporal and spatial control of recombinase activity. While the doses, routes, and frequency of TAM or OH-TAM administration are still being optimized, initial reports



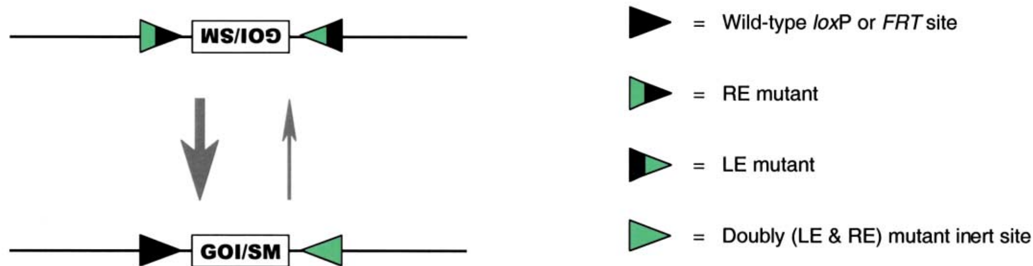


Figure 4. Use of Cre or Flp Inverted Repeat-Variant Target Sites to Achieve Stable DNA Insertion or Inversion

Stable DNA insertions (A) or inversions (B) may also be achieved exploiting *lox* or *FRT* sites that contain established substitutions in either the left or right inverted repeat, also referred to as the left element (LE) or right element (RE), respectively. While two wild-type (wt) target sites can recombine with each other repeatedly, an LE and RE mutant site will recombine efficiently only once, creating two different sites, a wild-type and a doubly (LE & RE) mutant inert site, that cannot recombine efficiently with each other. SM, selectable marker; GOI, gene-of-interest.

have uncovered interesting biology, including the timing of specification of sensory and autonomic neural crest sublineages (Zirlinger et al., 2002).

The fusion of a mutant progesterone receptor (PR) LBD linked to the C terminus of Cre has also been explored. For example, the fusion Cre-PR1 has been found to be largely insensitive to endogenous progesterone, but responsive to the synthetic steroid RU 486 (Kellendonk et al., 1996). For certain applications, Cre-PR1 has been effective in vivo, such as in adult epidermis to model human skin disorders (Arin et al., 2001; Cao et al., 2001). Evidence suggests that it may be effective in utero as well (Zhou et al., 2002). A drawback to Cre-PR1, however, is the leakiness of this system, with some degree of recombinase activity detectable even in the absence of inducer. An improved Cre-PR, however, has been developed, termed Cre*PR, which confers lower background activity and increased sensitivity to RU 486 (Wunderlich et al., 2001). Through application of both Cre*PR and FlpeER^{T2}, it should be possible to induce two genetic events at unique time points in the same animal. This capability offers tremendous potential to the types of experiments possible with SSR technology.

An alternative approach to temporally regulate SSR activity is to regulate SSR transcription. The first such report described use of the interferon-α/β inducible promoter of the *Mx1* gene to drive *cre* expression in vivo (Kuhn et al., 1995). The *Mx1* gene, normally involved in defense to viral infection, is silent in healthy mice, but can be induced to high levels of expression upon application of interferon-α or -β. While an important proof of concept, use of this system has been limited by the inability to induce recombination in certain cell types, slow induction kinetics, and the cost of the inducing agent.

A second transcriptional mechanism to achieve temporal control of *cre* or *Flp* expression involves the tetracycline (tet) responsive system (Gossen and Bujard, 1992) (Figure 5B). This system involves two components: (1) a transactivator gene, encoding a fusion protein that specifically binds tet (or the tet analog, doxycycline [dox]) as well as operator sequences of the tet operon (*tetO*), and (2) a *tetO*::*recombinase* transgene (St-Onge et al., 1996; Strathdee et al., 1999). The fusion protein encoded by the transactivator gene is termed tTA and is composed of the *E. coli* tetracycline repressor fused to the acidic domain of the herpes simplex viral protein 16 (VP16) transactivation domain. In the absence of tet (or dox), tTA binds *tetO* DNA to activate transcription from a minimal promoter located immediately upstream

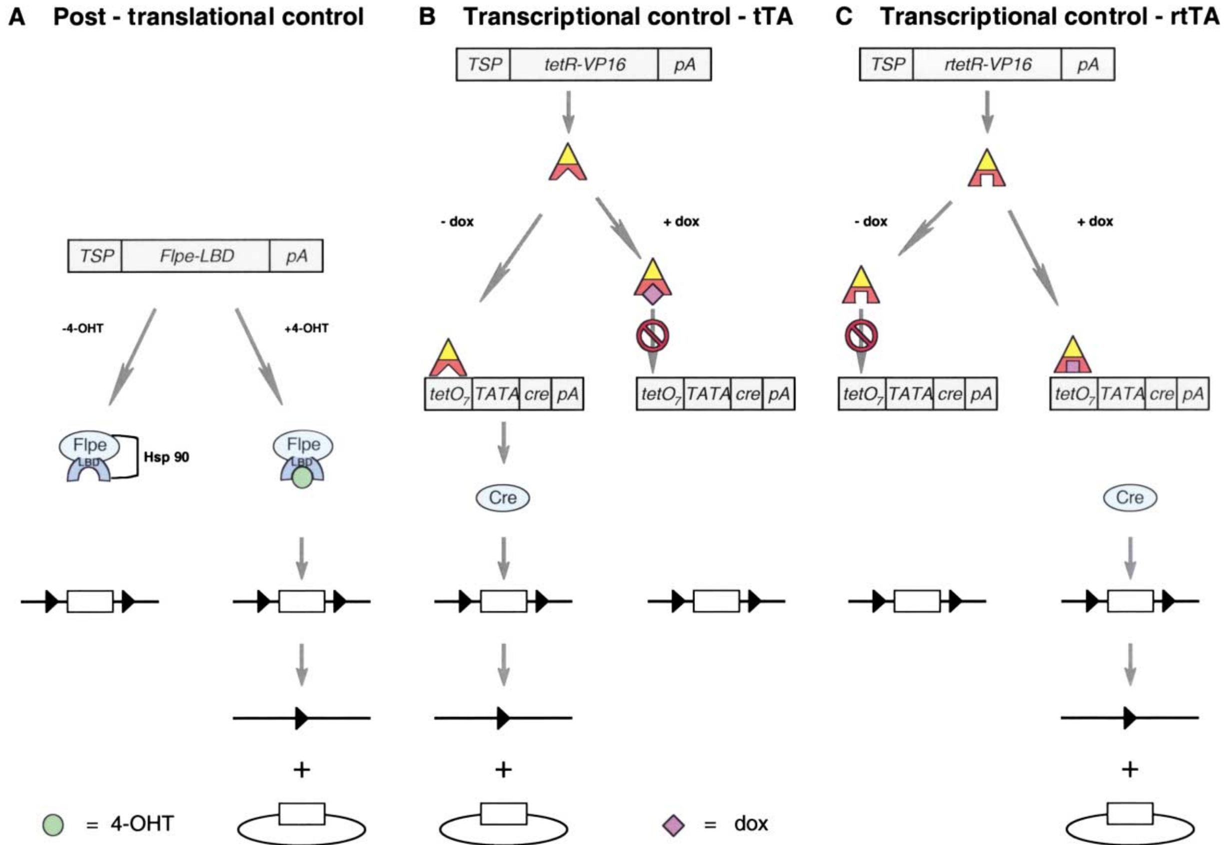


Figure 5. Mechanisms for Temporal Control of SSR Activity

(A) Posttranslational control of SSR activity. Recombinase-steroid fusion proteins are inactive in the absence of cognate steroid hormone due to sequestration of the fusion protein into an Hsp90 complex. Binding of cognate steroid hormone to the recombinase-steroid fusion protein results in disruption of the Hsp90 interaction, freeing the recombinase to enter the nucleus and mediate recombination at its target sites (black triangles). As an example of this system, Flpe is shown fused to the ligand binding domain (LBD) of a mutant estrogen receptor. Activation of the Flpe-ER fusion protein is achieved by administration of 4-hydroxy tamoxifen (4-OHT).

(B) Transcriptional control of SSR expression using the tTA tetracycline-responsive regulatory system. In the absence of the inducer in this system, doxycycline (dox), the tTA (tetR-VP16) fusion protein is capable of binding to seven tandemly repeated 19 bp *tetO* sequences, resulting in transcriptional activation of the recombinase and, in turn, SSR-mediated recombination at target sites. When bound to dox, tTA undergoes a conformational change and cannot bind to *tetO* sequences.

(C) Transcriptional control of SSR expression using the reverse tTA (rtTA) tetracycline-responsive regulatory system. In the rtTA system, the mutated rtetR-VP16 fusion protein is only capable of binding to *tetO* sequences in the presence of dox, resulting in transcriptional activation of the recombinase and, in turn, SSR-mediated recombination at target sites. As an example in (B) and (C), *cre* expression is regulated by the tTA and rtTA systems, respectively. TSP, tissue-specific promoter; TATA, representing a minimal promoter required for transcription; pA, polyA signal sequence.

of the recombinase-encoding transgene. Thus, continuous tet administration is required to prevent tTA binding to *tetO* and for the suppression of recombinase expression. In order to induce recombinase expression, tet administration is suspended, enabling tTA to bind to *tetO*. By contrast, a "reverse" tTA (termed rtTA) has been engineered that only binds *tetO* in the presence of tet (Figure 5C). Using rtTA, recombinase expression is induced by the administration of tet, thereby enabling rtTA to bind to *tetO*.

In addition to their converse mechanisms of *tetO* binding, rtTA and tTA also differ significantly in their kinetics of transcriptional activation. Using rtTA, transgene expression has been detected within 1 hr of dox administration in adults (Hasan et al., 2001; Schonig et al., 2002) and within 13 hr in embryos (Shin et al., 1999). By contrast, with tTA, target induction, which depends on the rate of dox clearance, was reported to take from 24 hr to a week, depending on the tissue examined (Kistner et al., 1996). Target silencing using tTA, on the other hand, was achieved in embryos after 6 hr, and possibly sooner as earlier time points were not examined (Shin et al., 1999). Taking advantage of the kinetics achieved for gene activation and silencing using the rtTA and tTA systems, respectively, it was determined that *endothelin receptor-B* expression is required for a restricted period of neural crest development, between embryonic days 10 and 12.5 (Shin et al., 1999).

An important point to consider when using the tet system is that tight regulation of recombinase expression depends on the integration site of the *tetO::recombinase* transgene. Indeed, a number of *tetO::cre* strains have been generated that express *cre* in a manner that is neither dependent on nor affected by dox treatment

(Leneuve et al., 2003), presumably due to neighboring endogenous enhancers acting on the *tetO*-associated minimal promoter. Importantly, a *tetO*::*cre* strain has been described, termed LC-1, in which *cre* expression is indeed tightly controlled in the absence of dox, but efficiently induced following rtTA expression and dox administration (Schonig et al., 2002). Coupled with tissue-restricted tTA or rtTA transgenes and *lox*P-flanked target sequences, the LC-1 locus should have broad use.

Driven by experimental need, the sophistication and versatility of SSR-based tools has grown tremendously over the last decade through the efforts of numerous investigators. Equally impressive has been the application of these tools to further our understanding of development and disease.

Reverse Genetics Takes a Big Step Forward: Partnering Homologous Recombination with SSRs to Achieve Conditional Gene (In)Activation

Gene-targeting in ES cells and the production of knockout (KO) mutant mice substantiated the utility of the mouse as a genetic model system for elucidating mammalian gene function (Kuehn et al., 1987; Thomas and Capecchi, 1987, 1990; Koller and Smithies, 1989; McMahon and Bradley, 1990). Fundamental to these achievements were the successful isolation and culture of mouse ES cells (Evans and Kaufman, 1981; Martin, 1981), the demonstration that they could be used to recolonize the germline (Bradley et al., 1984), even following genetic manipulation (Gossler et al., 1986; Robertson et al., 1986), and the development of gene-targeting, the introduction of DNA sequences into a chromosome by homologous recombination (Smithies et al., 1985; Thomas and Capecchi, 1986).

Toward creating a KO mouse, the traditional gene-targeting strategy is to replace the target gene, or at least an essential part of the gene (up to ~20 kilobases [kb] [Zou et al., 1994]), with a positive selection marker, usually the neomycin phosphotransferase gene (*neo*), permitting isolation of recombinant ES cells in culture. A standard replacement vector contains the positive selection marker flanked by genomic DNA homologous to the target, with a negative selection gene, usually the *HSV-tk* or *diphtheria toxin A subunit* (*DTA*) gene, on one end. The negative selection marker simply enriches for the desired homologous recombination event over random integrations by killing cells that have retained the negative selection gene.

Although a powerful approach, this traditional method of gene-targeting has two potential pitfalls that have been resolved with SSR technology. The first and major pitfall is that many genes serve an essential function, and so the elimination of gene activity throughout the entire animal can result either in early embryonic lethality, precluding the analysis of gene function at later stages, or, alternatively, a masking of the full mutant phenotype due to genetic compensatory mechanisms. In these cases, site-specific recombination may be used to inactivate a gene in discrete cells and/or at discrete times during development within the context of an otherwise normal mouse (a method termed conditional gene modification). The second pitfall in traditional gene-targeting strategies is the maintenance of the positive selection cassette within the targeted gene. Its presence can cause a number of problems, such as the disruption of neighboring gene expression due to strong transcriptional regulatory elements frequently present in selection cassettes (Lerner et al., 1993; Ohno et al., 1994). While removal of a positive selection cassette to avoid the associated deleterious effects can be achieved with a second gene-targeting step leaving a "clean" mutation (using, for example, the "hit-and-run" [Askew et al., 1993] or "double-replacement" [Stacey et al., 1994; Wu et al., 1994] approach), a relatively simpler strategy is to use Cre or Flp either in vitro or in vivo to excise a selection cassette that has been either *lox*P-flanked ("floxed") or *FRT*-flanked ("flrted"), respectively.

The first report of conditional gene modification examined the effect in T cells of a null mutation in the essential *DNA polymerase* β (*pol*β) gene (Gu et al., 1994). In this type of experiment, a mouse line is constructed that carries two components: (1) a recombinase transgene for the expression of Cre or Flpe in a restricted domain, and (2) a target gene in which a given segment has been either floxed or flrted, respectively (note that mice should be either homozygous or heterozygous over a null allele for the floxed or flrted locus). In mice, the targeted gene segment will be deleted specifically in cells expressing the recombinase and their descendants, while the target gene will remain intact in other cells of the mouse, where the recombinase is not expressed. This binary design offers great versatility to gene function studies: the role of one gene may be assessed in a variety of lineages, by crossing different recombinase lines (with differing recombinase expression patterns) to one strain bearing a conditional allele of a single gene; alternatively, the function of different genes may be assessed in a single lineage, by crossing one recombinase line to multiple strains each carrying a conditional allele of a different gene.

Regarding the second pitfall of traditional gene-targeting, removal of a floxed or flrted positive selection cassette can be achieved either in vitro or in vivo, with advantages inherent to both strategies. A benefit of removing the positive selection marker in ES cells is that the second allele can be subsequently modified with the same targeting vector to create ES cells homozygous for the desired alteration. Removal of a floxed cassette in cell culture has been aided by the fusion of Cre to a basic peptide from HIV-TAT, which has been demonstrated to promote the cellular uptake of Cre (Peitz et al., 2002), eliminating the need to electroporate recombinant ES cells with a Cre-encoding plasmid. By contrast, a benefit of removing the positive selection cassette in vivo is the opportunity to create an allelic series for a given gene, by taking advantage of the fact that the presence of the *neo*^{r} cassette often partially disrupts gene expression. In this case, removal of the selection cassette can be achieved by crossing mice carrying the targeted allele to a "deleter" strain, a transgenic strain that expresses the relevant recombinase either early in embryonic development, or in the developing germline (Lewandoski et al., 1997, O'Gorman et al., 1997, and Leneuve et al., 2003 for Cre, and Rodriguez et al., 2000, and Farley et al., 2000, for Flpe).

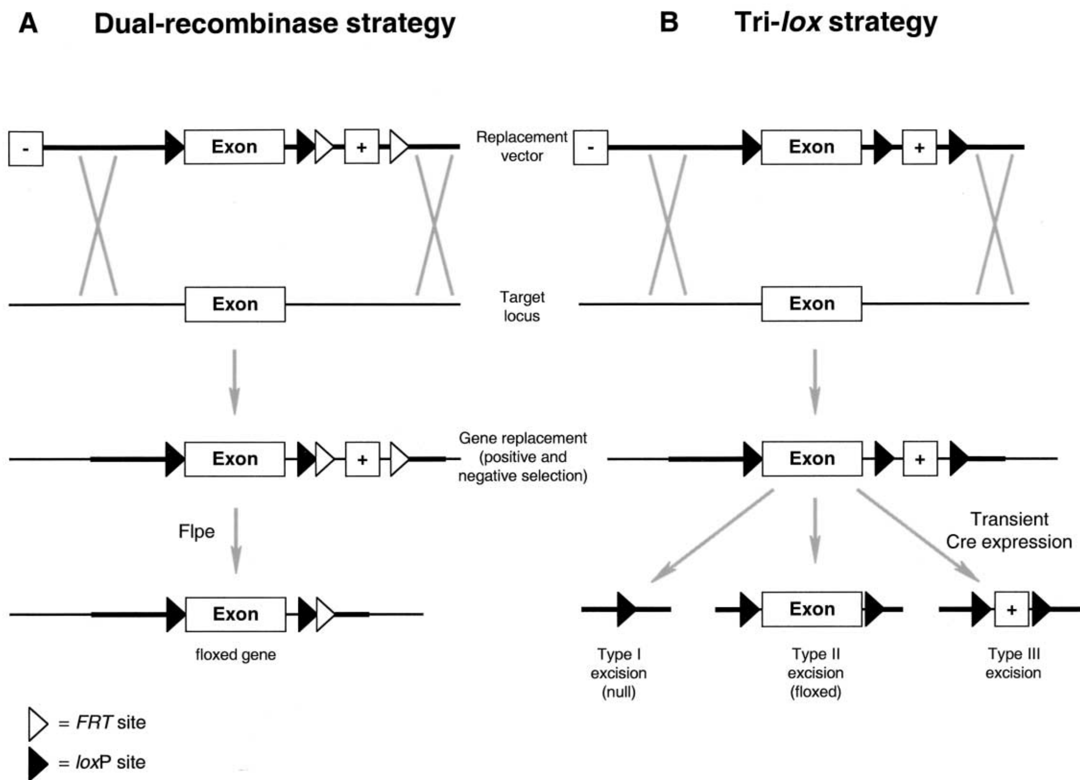


Figure 6. Strategies for Removing the Selection Cassette from a Conditional Allele

The thick line represents sequences within a targeting replacement vector homologous to a target locus in ES cells. + represents a positive selection marker (such as *neo*^{r}) and −, a negative selection marker (such as *HSVtk*). Both positive and negative selection marker genes contain a promoter and polyA sequences. The gray Xs represent homologous recombination crossover points. Positive and negative selection are applied to select for the desired gene replacement event.

(A) A dual-recombinase strategy for selection marker removal. Following gene replacement with the targeting vector, a flrted positive selection cassette is excised by Flpe. The reciprocal target site arrangement can be used to generate a flrted allele for conditional mutagenesis, with *lox*P sites flanking the positive selection cassette.

(B) A tri-*lox* strategy for selection marker removal. Here, following gene replacement with the targeting vector, the floxed positive selection cassette may be excised by transient expression of Cre, leaving the floxed exon in place (type II excision). Cre recombination may also result in a null allele (type I excision) or excision of the floxed exon, leaving a floxed selection marker in place (type III excision).

The availability of two highly efficient site-specific recombinase systems (Cre-*lox*P and Flpe-*FRT*) provides a convenient means to create a conditional allele while maintaining the potential to remove the positive selection cassette from the gene-targeting construct (Figure 6A). Such a dual-recombinase strategy was used to create an allelic series for *Fgf8*: two essential exons of *Fgf8* (exons 2 and 3) were floxed, and a flrted *neo*^{r} cassette placed in an intron just upstream of the 5′ *lox*P site (Meyers et al., 1998). Mice homozygous for this targeted allele displayed perturbed brain development, consistent with a hypomorphic phenotype for *Fgf8*; this mutant phenotype was due to the presence of the *neo*^{r} cassette, as its removal by crossing to Flp deleter mice (Dymecki, 1996) resulted in reversion to a wild-type phenotype. Positioning the *neo*^{r} cassette within the first intron apparently disrupted *Fgf8* mRNA splicing, effectively reducing mature *Fgf8* transcripts and protein. By contrast, crossing to a Cre deleter strain, creating a null allele in nearly all cells of the early embryo, produced an embryonic lethal phenotype, demonstrating an essential role for *Fgf8* in gastrulation. Thus, three alleles, including a null, hypomorphic, and reversion-to-wild-type allele, were generated in a single gene-targeting experiment using SSR technology. Most notably, by crossing mice harboring the reversion-to-wild-type allele (i.e., the cleaned-up floxed allele) to a strain that expresses Cre in early limb ectoderm, it was determined that *Fgf8* is absolutely required for multiple aspects of normal limb development (Lewandoski et al., 2000).

An alternative strategy to create a conditional allele in which the positive selection cassette may be removed is to position three *lox*P sites such that the selection cassette is flanked as is the gene segment to be conditionally deleted (a "tri-*lox*" approach) (Gu et al., 1993) (Figure 6B). A major caveat of this strategy, however, has been the difficulty in obtaining a partially deleted allele in which the gene segment remains floxed but the positive selection cassette has been removed (Kaartinen and Nagy, 2001). However, using a new Cre recombinase mouse termed MeuCre40 (Leneuve et al., 2003), the desired partial deletion can be generated with relative ease. MeuCre40 is one of several *tetO*::*cre* strains found to produce mosaic, early embryonic, ubiquitous (Meu) Cre expression neither dependent on nor affected by dox treatment (Leneuve et al., 2003), presumably

due to positional effects associated with the *tetO*::*cre* chromosomal integration site. Upon intercrossing Meu-Cre40- and tri-*lox* allele-carrying mice, it is estimated that approximately two F2 litters (~30 F2 mice) need to be screened in order to isolate one animal carrying a deletion specifically of the selection cassette. An advantage of using the Cre-*lox*P system both to create a conditional allele and to remove the positive selection cassette is that it permits application of the Flpe-*FRT* system to achieve other conditional manipulations in the animal.

Conditional gene modification provides the potential for careful analysis of gene inactivation in specific cell lineages. In many cases, however, gene inactivation is not complete due to a failure of the recombinase to modify the target gene in all cells expressing the recombinase. Such mosaicism of recombinase-mediated excision precludes an analysis of a null phenotype in the target lineage, due to the existence of wild-type cells that may mask a potential defect. In fact, this issue confounded the analysis of *pol*β in T cell lineages in the first conditional KO experiment (Gu et al., 1994). To overcome this caveat, it is possible to use conditional gene modification to achieve lineage-specific gene repair in an otherwise null background. This method could also be used to determine where in the animal a gene is required for viability.

By combining a gene-targeting strategy with a reporter for recombinase activity, it is also possible to mark cells directly that have undergone Cre-dependent gene inactivation, enabling the precise distinction of mutant versus wild-type cells in the target gene expression domain (Theis et al., 2001; Schnutgen et al., 2003). For example, the FLEx switch (Schnutgen et al., 2003) (described above) was used to activate β-galactosidase (β-gal) activity in cells in which the retinoic acid receptor γ (RARγ) gene had been inactivated by Cre. *LacZ* in this case is expressed under the regulatory control of the target *RAR*γ gene and is therefore expressed in mutant cells only within the window of time that *RAR*γ is normally expressed; notably, it does not serve as a lineage tracer (application of SSRs for tracing cell lineages is discussed below). The FLEx switch strategy of replacing one open reading frame with another using site-specific recombination could be applied for a number of applications, such as conditional rescue of a gene KO, conditional point mutations, and conditional gene replacement.

While essential for functional analyses, conditional gene modification strategies are encumbered by the time required to generate mice that are homozygous for the floxed or flrted allele and carry the recombinase transgene—three breeding steps, requiring up to ~14 months. An alternative approach requiring less than half this amount of time as well as far fewer animals is to generate ES cells harboring the desired genotype and to then generate mice derived entirely from these cells. Use of F1 hybrid ES cells permits the necessary consecutive rounds of gene-targeting while maintaining ES cell totipotency; use of tetraploid blastocyst complementation methods enable the production of entirely ES cell-derived mice. Tetraploid blastocysts are developmentally compromised, but when complemented by diploid ES cells will complete fetal development with the resultant embryos being completely ES cell derived (Nagy et al., 1990, 1993; Seibler et al., 2003). Diploid F1 hybrid ES cells (C57Bl/6 X 129S4Sv/Jae) generate live pups with high efficiency, by contrast to most diploid inbred ES cells (Eggan et al., 2001).

Recombineering BACs to Generate Conditional Gene-Targeting Vectors and Recombinase Transgenes

An important consideration for conditional gene modification experiments is the time required for vector construction, be it a gene-targeting vector or a recombinase transgene. This process has been expedited through development of "recombineering" strategies that exploit *E. coli* homologous recombination systems to modify introduced exogenous DNA. Indeed, recombineering represents a flexible and efficient means with which to construct insertions, deletions, and point mutations in a given DNA sequence without any other residual changes in the recombinant product and without the need for suitable restriction enzyme recognition sites.

For the production of gene-targeting vectors and reporter constructs, recombineering strategies have been applied most commonly to bacterial artificial chromosomes (BACs). This is because (1) BACs are readily available for most genes having represented the primary source of archived DNA for the mouse genome sequencing project; (2) BACs can be used directly in ES cells for high-fidelity gene replacement (Liu et al., 2003; Valenzuela et al., 2003; Yang and Seed, 2003); and (3) for most genes, BACs can be identified that contain all necessary regulatory elements to confer normal gene expression (Heintz, 2001). Moreover, BAC transgenes are not subject typically to the strong position effects resulting in transgene silencing or misexpression that plague conventional constructs (<20 kb). It is important to note that BAC vector sequences contain *lox*P sites. If left in the mouse genome, as is often the case when generating a BAC transgenic by zygote injection (but not by homologous recombination), these sites may participate in undesired recombination events, complicating use of Cre-*lox*P technology in these animals. These unwanted sites may be removed using additional recombineering strategies (Yang et al., 1997; Lee et al., 2001; Gong et al., 2002) or by isolating the BAC insert away from vector sequences.

In general, the large size of BACs requires their maintenance and propagation in a homologous recombination-deficient strain of *E. coli* (DH10B) to prevent their rearrangement (Shizuya et al., 1992). Recombineering strategies involve the restoration of homologous recombination potential to bacteria for a brief window of time concurrent with the introduction of DNA sequences to be inserted into or exchanged with the BAC. Ideally, the window of homologous recombination potential is just wide enough to permit the desired alteration, following which the *E. coli* return to a recombination-deficient state suitable for maintenance of the modified BAC. The first reported recombineering methodology involved restoration of the RecA homologous recombination pathway (Yang et al., 1997), followed soon after by use of the RecE/RecT pathway for this purpose (Zhang et al., 1998). Notably, both strategies have been used in

high-throughput BAC modification projects for the production of transgenic mice: RecA-mediated recombineering (Gong et al., 2002) has been used for the insertion of reporter sequences into hundreds of BACs with the aim of reporting gene expression patterns in the brain and of gaining genetic access to every cell type in the CNS (as part of the GENSAT BAC Transgenic Project [http://www.gensat.org/index.html]) (Gong et al., 2003), and a modified RecE/RecT-based strategy has been applied in VelociGene, a high-throughput and largely automated method for the genetic modification of endogenous genes, applied recently to generate reporter expression patterns for hundreds of genes in mice (Valenzuela et al., 2003). Using the gene expression data derived from these projects, *BAC::recombinase* transgenics can be generated to achieve SSR activity in specific cell populations in vivo.

The RecA strategy (Yang et al., 1997; Gong et al., 2002) involves the introduction of a single plasmid, referred to as a "shuttle vector," into the BAC *E. coli* host. Contained within the shuttle vector are *RecA* and DNA sequences to be incorporated into the BAC, such as a reporter or recombinase gene, flanked by 500 bp homology arms corresponding to sequences in the BAC. The desired modification of the BAC is achieved in two consecutive homologous recombination steps, termed "cointegration" and "resolution" (mechanistically similar to the RMCE reaction, depicted in Figure 3A). Cointegration describes the insertion of the entire shuttle vector into the BAC by homologous recombination between the homology arms of the vector and the BAC, while unwanted BAC vector sequences are then "resolved," or eliminated, through a second homologous recombination event. In the course of modifying an initial set of BACs, average efficiencies of 75% for cointegration and 63% for resolution were reported (Gong et al., 2002).

The alternative method of BAC modification involves regulated expression of phage homologous recombination proteins in BAC host cells: either RecE/RecT from Rac prophage (Zhang et al., 1998) or Redα/Redβ from the Red operon of lambda phage (Muyrers et al., 1999; Yu et al., 2003). Thus, this method is referred to as either "ET" or "Red" recombination. Upon expression of the phage proteins in a BAC host, a linear double-stranded DNA fragment, flanked by relatively short homology arms (50 bp), will be homologously recombined into the identical sequences in the BAC at high efficiency. This method was devised following an observation that homologous recombination between linear and circular DNA molecules can be achieved in *sbcA* strains in which RecE and RecT are expressed (due to the *sbcA* mutation that activates expression of an integrated prophage encoding the *recE/recT* operon [Clark, 1973; Lloyd, 1974; Winans et al., 1985]). The method was then developed using the analogous *red* operon from lambda phage. To enable ET/Red recombination in any recombination-deficient *E. coli* cell line, several ways to regulate the expression of RecE and RecT, or Redα and Redβ, have been developed. These usually include coexpression of Redγ, which inhibits the major *E. coli* exonuclease, RecBCD, preventing degradation of linear DNA fragments (Elledge and Walker, 1985; Murphy, 1998; Poteete et al., 1999). Notably, both RecA and ET/Red cloning strategies deliver high ratios of correct (homologous) to incorrect (illegitimate) recombinations.

The homology arms required for ET/Red recombination are short. Consequently, they can be generated by oligonucleotide synthesis, which can be attached to a selectable marker by PCR amplification. *FRT* or *lox*P sites can also be included in the synthetic oligonucleotides, and hence the PCR product that is then recombined into the mouse genomic DNA clone. These experimental simplicities permit (1) the rapid, one step, construction of targeting constructs for homologous recombination in ES cells (Angrand et al., 1999; Lee et al., 2001; Muyrers et al., 2000; Zhang et al., 2002; Yang and Seed, 2003; Valenzuela et al., 2003; Liu et al., 2003), and (2) rapid insertion of *lox*P or *FRT* sites into any chosen position in targeting constructs or BAC transgenes (Zhang et al., 1998, 2000; Angrand et al., 1999; Liu et al., 2003; Testa et al., 2003). This cloning strategy greatly facilitates the introduction of site-specific recombinase target sites into the mouse genome, which has been a limiting step for the application of SSR technology. Through further implementation of recombineering, it is now possible to simultaneously alter two or more sites separated by distances up to the cloning capacity of BACs (250 kb). For example, Red recombination has been employed to produce gene-targeting constructs to create conditional alleles in which recombinase target sites are positioned at significant distances from each other (Liu et al., 2003; Testa et al., 2003). More specifically, a 64 kb targeting construct was used to direct the simultaneous insertion of two cassettes, one flanked by *FRT*, and the other by *lox*P, sites into the *Mll* gene in ES cells. The dually modified *Mll* allele has four possible configurations depending upon recombination or not by Flp, Cre, or both, thus permitting the production of three *Mll* alleles in vivo including a null allele, a truncation allele typically observed in *Mll*-associated childhood leukemia, and a reversion-to-wild-type allele (Testa et al., 2003).

Trap and Release: A New Generation of Gene-Trap Vectors Enable Postinsertional Modifications

A significant step toward functionally annotating the human genome will be the determination of loss-of-function phenotypes for every gene in the mouse. As an alternative approach to systematically knocking out genes by gene-targeting, a number of groups have launched large-scale, random mutagenesis screens using either gene-trap vectors in ES cells (Hansen et al., 2003; Stryke et al., 2003) or the chemical agent N-ethyl-N-nitrosourea (ENU) in vivo (Hrabe de Angelis et al., 2000; Nolan et al., 2000; Coghill et al., 2002; Herron et al., 2002). Both strategies make no a priori assumptions regarding gene function but differ in the types of mutations produced. A standard gene-trap vector contains a promotorless reporter gene flanked by an upstream splice acceptor and a downstream polyadenylation (polyA) signal sequence (Gossler et al., 1989). When inserted into an intron, transcriptional activation of the "trapped" gene will result in a fusion transcript composed of upstream coding sequences and the reporter.

(β-geo, a β-gal-Neo fusion, tolerates N-terminal extensions and so is well suited for this purpose.) This gene-trap design generally produces a null allele while simultaneously revealing the expression of the endogenous gene. Variations in the design of gene-trap vectors have increased the efficiency and selectivity of gene trapping in ES cells (Stanford et al., 2001). Building on the basic vector design described above, some gene-trap vectors not only contain a promotorless reporter gene, but also include either a start ATG or an internal ribosomal entry site (IRES), and, as such, are able to trap both the coding and the 5′ untranslated region (5′ UTR) of genes, with the potential to mutate specific isoforms as well as to produce null alleles. Still other gene-trap vectors permit the identification of particular classes of genes, such as those that are transiently or actively transcribed (Thorey et al., 1998), that are induced following steroid hormone stimulation (Wan and Nordeen, 2002), or that encode secreted or transmembrane proteins (Skarnes et al., 1995; Mitchell et al., 2001). By contrast, ENU alkylates DNA to produce point mutations or small deletions, the majority of isolated alleles predicted to be missense mutations (Justice et al., 1999). As a result, ENU-derived alleles can reveal critical amino acids for protein function, complementing loss-of-function gene studies. ENU-derived mutations must be mapped to determine the affected gene and then molecularly characterized. By contrast, gene-trapping offers a comparatively quick means of gene identification by 5′ rapid amplification of cDNA ends (RACE), for which the fusion transcript serves as template. Alternatively, some gene-trap vectors permit gene identification by plasmid rescue (Araki et al., 1999). Currently, BayGenomics (Stryke et al., 2003) (http://baygenomics.ucsf.edu) and the German Gene Trap Consortium (Hansen et al., 2003) (http://genetrap.de) are the largest libraries of gene-trap ES cell lines available to the public. Combined, these resources contain insertions in ~14% of genes currently annotated in Ensembl (http://ensembl.org) (W. Skarnes and H. von Melchner, personal communication).

To increase the versatility of the gene-trap approach, several groups have modified gene-trap vectors to include wild-type and variant SSR recognition sites enabling postinsertional modifications of the trapped gene (Araki et al., 1999; Hardouin and Nagy, 2000). Postinsertional modification could involve SSR-mediated manipulation of the integrated vector to achieve an allelic series of the trapped gene (for example, a null, hypomorphic, reversion-to-wild-type, and neomorphic allele), or, alternatively, to achieve ectopic expression of another gene under the regulatory control of the trapped *cis*-acting promoter or enhancer elements. Notably, *FRT* and heterotypic *lox* sites have been incorporated into the vectors used to generate most of the ES cells lines in the BayGenomics resource, enabling the application of Cre-*lox* and Flp-*FRT* technology to transform gene-trap events into virtually any desired allele (W. Skarnes, personal communication). Furthermore, a FLEx switch strategy (see Figure 3B) has been applied in a new generation of gene-trap vectors, permitting these vectors to be inverted twice following their insertion into the ES cell genome by the successive activation of two different SSR systems (H. von Melchner, personal communication). The result of this clever design is that a gene-trap-induced mutation may be repaired in ES cells, facilitating passage of the modified locus through the germline in the production of transgenic mice, followed by the regeneration of the gene-trap-induced mutation in a conditional manner in vivo.

Chromosomal Engineering with the Cre-*lox*P System to Model Human Disease

Chromosomal rearrangements are a major cause of inherited human disease and fetal loss, and have been associated with the progression and maintenance of cancer (Ramirez-Solis et al., 1995; Rabbitts et al., 2001). For example, a number of the contiguous gene syndromes, associated with disruptions in gene dosage, such as Smith-Magenis and DiGeorge, have been attributed to specific chromosomal rearrangements (Walz et al., 2003). In addition, many cancers are associated with specific translocations, such as the coincidence of B lymphoid cell tumors with the translocation t(4;11), affecting the MLL and AF-4 genes (Rabbitts et al., 2001). Chromosomal translocations often result in abnormal gene fusions and, consequently, tumor-specific mRNAs and proteins that represent attractive targets for therapy. Thus, a capacity to engineer chromosomal rearrangements with specific breakpoints is critical to our ability to model human disease in the mouse, and could facilitate the development of disease therapies.

Four groups independently pioneered use of the Cre-*lox*P system for chromosome engineering to generate either a specific translocation (Smith et al., 1995; Van Deursen et al., 1995), a large deletion (Li et al., 1996), or a set of deletions, duplications, and inversions (Ramirez-Solis et al., 1995). Which of these modifications is generated depends on the relative orientation of *lox*P sites positioned within the genome. If two *lox*P sites are positioned in the same orientation on a single chromosome or homologous chromosomes, then a deletion and/or duplication can be made. If instead, two *lox*P sites are positioned in the opposite orientation, an inversion can be isolated; dicentric and acentric chromosomes may also be produced in this case but cause cell lethality. Finally, if two *lox*P sites are positioned on different chromosomes, a balanced translocation can be generated by mutual exchange of DNA distal to the *lox*P sites. Using the publicly available mouse genome sequence, the relative orientation of genes with respect to each other and the centromere can be determined with few exceptions, enabling the correct orientation of *lox*P sites in the ES cell genome depending on the rearrangement desired. This also abrogates the need to target *lox*P sites in both directions within target genes. Following the sequential targeting of two *lox*P sites into the ES cell genome, Cre-mediated recombination to produce the rearrangement may be achieved either in vitro or in vivo.

For the generation of substantial genomic rearrangements involving the placement of *lox*P sites on different chromosomes or on the same chromosome but at distances exceeding ten megabases (Mb) (Mills and Bradley, 2001, and discussed below), the efficiency of Cre recombination is low enough that a selection

system for the desired rearrangement is recommended. To address this need, two groups independently developed strategies in which a positive selection marker is reconstituted following the Cre-mediated genomic alteration (Ramirez-Solis et al., 1995; Smith et al., 1995). For example, Cre-mediated recombination between *lox*P sites has been used to juxtapose the 5′ and 3′ halves of an *Hprt* gene, thereby reconstituting this selectable marker and rendering ES cells bearing this rearrangement resistant to hypoxanthine, aminopterin, and thymidine (HAT) (Ramirez-Solis et al., 1995). (Note that when using this type of selection, the ES cells must be initially *Hprt* deficient.)

To aid in the production of chromosomal rearrangements using this selection system, two different genomic libraries of pre-made gene-targeting insertion vectors have been made. They are referred to as the 5′ *Hprt* and 3′ *Hprt* libraries (Zheng et al., 1999). The vector used to create the 5′ *Hprt* library contains the 5′ *Hprt* cassette (containing the 5′ half of the *Hprt* gene which itself is nonfunctional) with an embedded *lox*P site, the *PGKneopbA* (*neo*) gene as a positive selection marker, and the *Tyrosinase* (*Ty*) minigene. The vector used to create the complementary 3′ *Hprt* library contains the 3′ *Hprt* cassette (containing the 3′ half of the *Hprt* gene which itself is nonfunctional) with an embedded *lox*P site, the *PGKpuropbA* (*puro*) gene as a positive selection marker, and the *K-14 Agouti* (*Ag*) minigene. The vectors were designed such that following recombination at *lox*P sites, the reconstituted *Hprt* gene is flanked by the *Ty* and *Ag* coat-color markers, enabling the visible identification of mice carrying the rearrangement. In addition, the *neo* and *puro* genes were designed to segregate away from the reconstituted *Hprt* minigene in a characteristic fashion depending on the rearrangement made, enabling the identification of ES cell clones bearing the desired chromosomal rearrangement by sib selection. Sib selection is performed by duplicate plating individual clones in order to assess their viability in the presence of various drug combinations (HAT, G418, puromycin) (Yu and Bradley, 2001), and is particularly useful given the range of recombination products that can occur, depending, for example, on whether *lox*P sites are localized to one chromosome (recombination occurs in *cis*) or homologous chromosomes (recombination occurs in *trans*), as well as on when during the cell cycle recombination occurs, that is, in either G1 or in G2 following DNA synthesis (see Supplemental Figure S2 at http://www/developmentalcell.com/cgi/content/full/6/1/7/DC1).

To assess the efficiency of Cre-mediated recombination as *lox*P sites become separated by increasing distance, a study was performed in ES cells in which the frequency of isolating Cre-mediated inversions was determined (Zheng et al., 2000). The focus was on mouse chromosome 11 as it is extensively conserved with human chromosome 17 and contains several potential tumor suppressor genes (Zheng et al., 2001). Inversions, as opposed to deletions, were analyzed in order to bypass any confounding issues of haploinsufficiency. As predicted, HAT-resistant colonies were isolated at decreasing frequencies as the distance between *lox*P sites increased. In addition, recombination in *cis* occurred roughly 200 times more frequently than in *trans*. Thus while the efficiency of Cre-mediated recombination decreases with increasing genetic distance between *lox*P sites on the same chromosome (in *cis*), it does not appear to be limiting, with the extent of the rearrangement that can be made apparently more a function of ES cell viability than a limitation of the Cre-*lox*P system. By contrast, the efficiency by which Cre mediates recombination in *trans* is fairly low, in the range of in 10^{-3} (Van Deursen et al., 1995) to 10^{-5} (Smith et al., 1995; Zheng et al., 2000), and may reflect chromosomal position within the cell during interphase and mitotic metaphase (Testa and Stewart, 2000).

An advantage of constructing large-scale chromosomal rearrangements in vitro is the option of using a positive selection system to facilitate isolation of the rearrangement. In certain cases, however, it must be accomplished in vivo. This is achieved by generating mice with appropriately positioned *lox*P sites, followed by restricted *cre* expression within the animal, thereby producing the desired rearrangement within a small number of cells, much like a conditional gene-targeting experiment. An in vivo approach may be required due to ES cell haploinsufficiency or infertility associated with the transmission of translocations through the germline, or might be preferred in order to better model cancers that arise in somatic lineages due to a loss of heterozygosity. For example, translocations corresponding to the human rearrangements t(8;21)(q22;q22) and t(9;11)(p22;q23) have been induced successfully in the mouse (Buchholz et al., 2000, and Collins et al., 2000, respectively) in order to model acute leukemias. In addition, in vivo chromosomal engineering may be used for the production of genetic mosaics, defined as individuals that are heterozygous for a particular mutation but that contain patches of homozygous mutant cells (not to be confused with chimeras, defined as individuals derived from cells from different sources) (Liu et al., 2002). Genetic mosaics enable an assessment of the requirement of gene function on clonal cell lineages, and can be extremely informative in studying cell fate determination and imprinting. Genetic mosaics are generated when mitotic recombination occurs after DNA synthesis in the cell cycle (in G2) and the recombinant chromatids segregate to different daughter cells (referred to as X segregation), as opposed to the same daughter cells (referred to as Z segregation) (Liu et al., 2002). In *Drosophila*, the Flp-*FRT* site-specific recombination system has facilitated the production of genetic mosaics, yet because *Drosophila* chromosomes are unique in that homologs align during mitosis (Metz, 1916), it was unclear if genetic mosaics could be identified among mice. However, Cre-*lox*P site-specific recombination has been used successfully to induce mitotic recombination with X segregation in ES cells, with a frequency of 7.0×10^{-3} to 4.2×10^{-5} depending on genomic location (Liu et al., 2002).

Mouse Forward Genetics on the Fly Using SSR Technology

Using chromosome engineering, an arsenal of reagents previously limited to *Drosophila* is being assembled for the mouse, including marked deletions, duplications, and inversions that collectively span the genome. These

reagents will greatly increase the efficiency and flexibility of genetic screens and mapping studies conducted in mice (for example, see Kile et al., 2003). Deletions, often referred to as deficiencies, render a segment of the genome haploid and so can be used in focused F1 or F2 screens for recessive mutations. Deletions are also useful for mapping studies if their endpoints are known, and those generated with the Cre-*lox*P system have the advantage that their endpoints are predetermined at the nucleotide level, unlike those produced by ionizing radiation, for example. Cre-*lox*P site-specific recombination has also enabled the construction of nested deletions series (Su et al., 2000), useful for mapping recessive mutations to progressively smaller intervals. Such deletions have been isolated by embedding the first of two *lox*P sites at a fixed position in the genome by conventional gene-targeting, followed by random integration of the second site using a recombinant retrovirus. Deletions and their complementary duplications, attainable using the Cre-*lox*P system, are extremely useful for classifying uncharacterized mutations, such as those isolated in ENU mutagenesis screens. For example, a mutation can be classified as a bona fide loss-of-function allele if (1) it fails to complement an overlying deletion for a given mutant phenotype and (2) if the phenotype associated with the homozygosed mutation is rescued by an overlying duplication. Finally, Cre-*lox*P site-specific recombination has permitted the generation of balancer chromosomes, which are dominantly marked inversions rendered recessive lethal by placing at least one of the endpoints within an essential gene. Balancers are used to maintain recessive lethal mutations and the integrity of mutagenized chromosomes by suppressing meiotic crossovers within the inverted region (Zheng et al., 2001). They can also be instrumental to the isolation of ENU-induced recessive mutations (Kile et al., 2003, and discussed further below). Each balancer chromosome can span about one-third of a chromosome without issues of haploinsufficiency that plague large deletions. An effort to make a genome-wide balancer series using SSRs is currently underway at the Sanger Center (UK), each balancer tagged with dominant coat-color markers (Nishijima et al., 2003, and A. Bradley, personal communication).

To increase the efficiency and flexibility of genetic screens using deletions and balancer chromosomes, a system using SSRs has been developed that permits generating two distinct dominant fluorescent markers at any location within the genome (Frank et al., 2003). Dominant visible markers, such as those described here, are extremely useful for genetic analysis because they enable a chromosome to be followed without selection or genotyping, and, used in conjunction with an overlying deletion or balancer chromosome, can be used to distinguish all classes of progeny in genetic screens. In this case, a plasmid, *pCX-YNC*, was devised that drives broad expression of two different color variants of enhanced green fluorescent protein (eGFP), eCFP (cyan), and eYFP (yellow) (derived from *peYFP-C1* and *peCFP-1*, respectively, from Clonetech). The eYFP cassette is flanked by *lox*P sites, and the eCFP cassette by *FRT* sites, such that following the production of transgenic mice, one or the other cassette may be removed by intercrossing with the appropriate deleter strain, resulting in a chromosome tagged at the same genetic location with either eCFP or eYFP. Two key attributes of this marker system are (1) that they may be used to identify individuals carrying a marked chromosome at any developmental stage, including embryos, unlike coat-color markers that are restricted to postnatal animals, and (2) that they may be used to dominantly mark, in theory, any position within the genome with either of two fluorescent tags, and thus could be used to facilitate targeted genetic screens at any region within the genome. To assess the utility of the transgene *CX-YNC*, it was randomly inserted into ES cells and the insertion sites mapped by plasmid rescue. ES cells in which the transgene had integrated as a single copy were readily isolated and found to strongly and uniformly express both markers. One ES cell line was used to generate mice in which dual color fluorescence was easily visualized throughout development, including in pre- and postimplantation embryos, newborn pups, and adult mice. Widespread cyan and yellow fluorescence was observed in all organs and tissues examined, including the brain, heart, lung, skin, and skeletal muscles. This transgene could be homozygosed with no effect on viability or fertility. Ideally, a bank of ES cells will be generated harboring the *CX-YNC* dual reporter in locations throughout the genome. Figure 7 depicts use of these markers to distinguish the classes of progeny in F1, F2, or F3 focused screens: for recessive mutations in vivo accompanying a deletion (Figure 7A), in ES cells accompanying a deletion (Figure 7B), or in vivo accompanying a balancer chromosome (Figure 7C). Importantly, these markers can also be used to flag a missing class of offspring due to the generation of a recessive lethal allele, and provide a mechanism for its recovery by facilitating the identification of carriers.

Molecular Fate Mapping in the Mouse: Switching on Lineage Tracers Using SSRs

Analyses of cell fate greatly inform our understanding of normal development and provide an important framework for analyzing genotype-phenotype relationships uncovered by mutagenesis. With rare exception (Tam and Beddington, 1987; Turner and Cepko, 1987; Lawson and Pedersen, 1992; Walsh and Cepko, 1992), vertebrate fate maps have been plotted in avian and amphibian systems because of the ease of manipulating tissue in ovo. Employed methods include the injection of retroviral (Galileo et al., 1990; Cepko et al., 1993), fluorescent (Wetts and Fraser, 1991), or vital dye lineage tracers (Keller, 1975, 1976), or the grafting of quail cells into chick embryos (Le Douarin, 1982). Because mouse embryos develop in utero, they are less accessible, making these established tracing methods more difficult. To circumvent this obstacle, SSR-based strategies have been developed to genetically (and noninvasively) activate lineage tracers in mice.

Initial reports of SSR-based fate mapping included use of the Cre-*lox*P system to determine the adult fates of *Engrailed-2*-expressing cells originating in the mid-brain-hindbrain constriction (Zinyk et al., 1998), and use of the Flp-*FRT* system to analyze the adult expansion of neural progenitors which transiently express *Wnt-1*

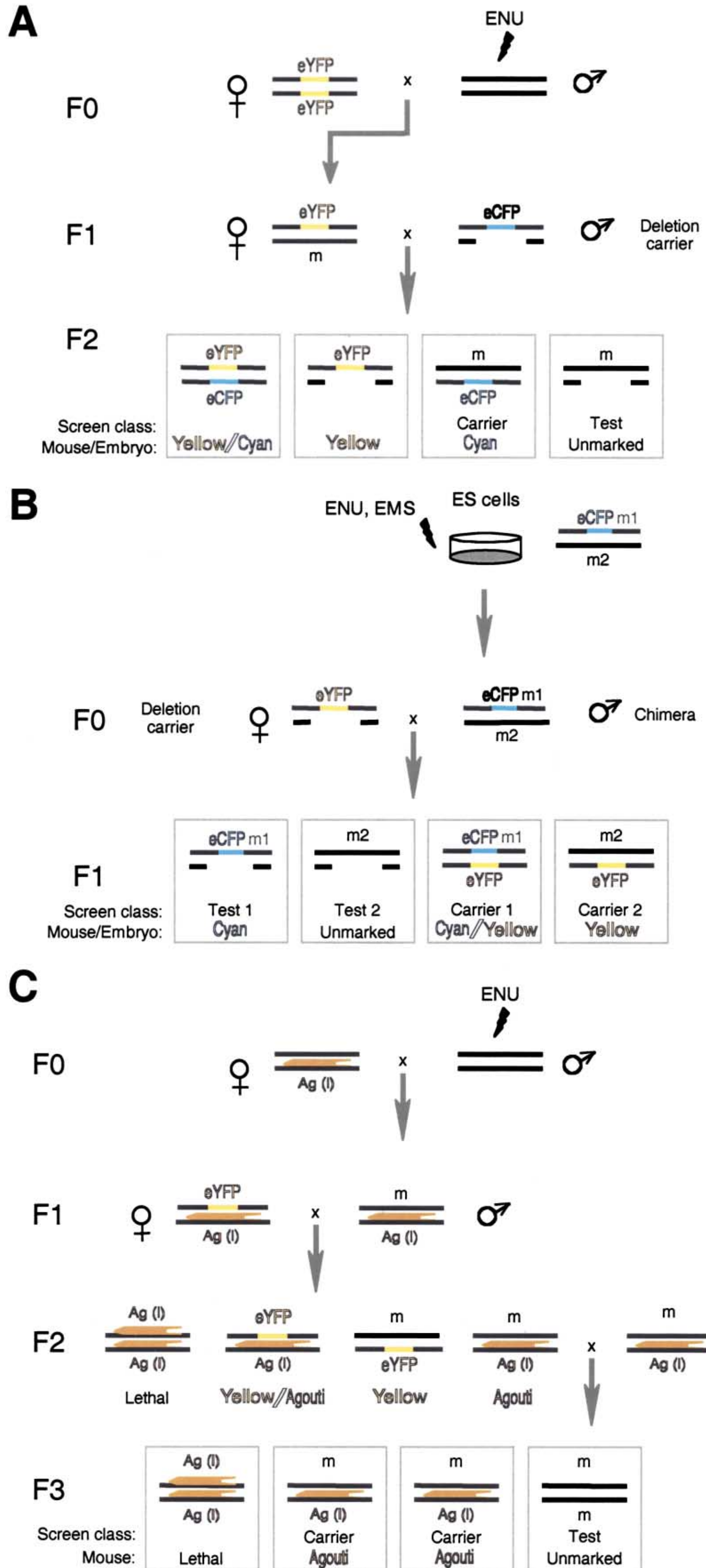


Figure 7. Marking Focused Mutagenesis Screens Using the pCX-YNC Reporter

(A) A traditional hemizygosity screen using visibly marked chromosomes and deletions. In this example, an ENU-mutagenized male (F0) is mated with an eYFP homozygous female to generate an eYFP-marked F1 female heterozygous for an ENU-induced mutation (m). Mutations in the region are uncovered in a test cross with an eCFP-marked deletion carrier male. The F2 test class progeny are color-coded to visually distinguish the test class (unmarked), mutation carrier (cyan), and two discard classes (yellow/cyan and yellow).

(B) A hemizygosity screen combining visibly marked chromosomes, deletions, and ES cell mutagenesis. ENU- or EMS-mutagenized ES cell clones are used to produce germline competent chimeric mice. Mutations in the region produced in the diploid ES cells residing on the eCFP-marked chromosome (m1) and on the unmarked chromosome (m2) are uncovered in a single-generation test cross with an eYFP-marked deletion carrier female. The F1 test class progeny are color-coded to visually distinguish the m1 test class (cyan), the m1 carrier (cyan/yellow), the m2 test class (unmarked), and the m2 carrier (yellow).

(C) A regional homozygosity screen using a visibly marked, recessive lethal inversion. An ENU-treated F0 male is mated with a female heterozygous for a marked inversion chromosome. Cre-*lox*P-mediated chromosome engineering has been used to produce recessive lethal inversion chromosomes dominantly marked with the K-14-Agouti transgene (Ag) (see text). F1 offspring carrying a mutation (m) and the marked inversion are mated with an animal carrying both the marked inversion and eYFP- (or eCFP-) to produce F2 progeny. F2 mice carrying the mutation balanced by the marked inversion are selected based on the absence of yellow (or cyan) fluorescence. These F2 offspring can be intercrossed to produce the F3 test class progeny that are marked to distinguish the homozygous mutation test class (unmarked) and carrier (Ag). Homozygosity for the marked inversion is lethal (l), typically owing to the disruption of an essential locus at an inversion breakpoint. This figure is a modified recreation of Figure 4 from Frank et al., 2003.

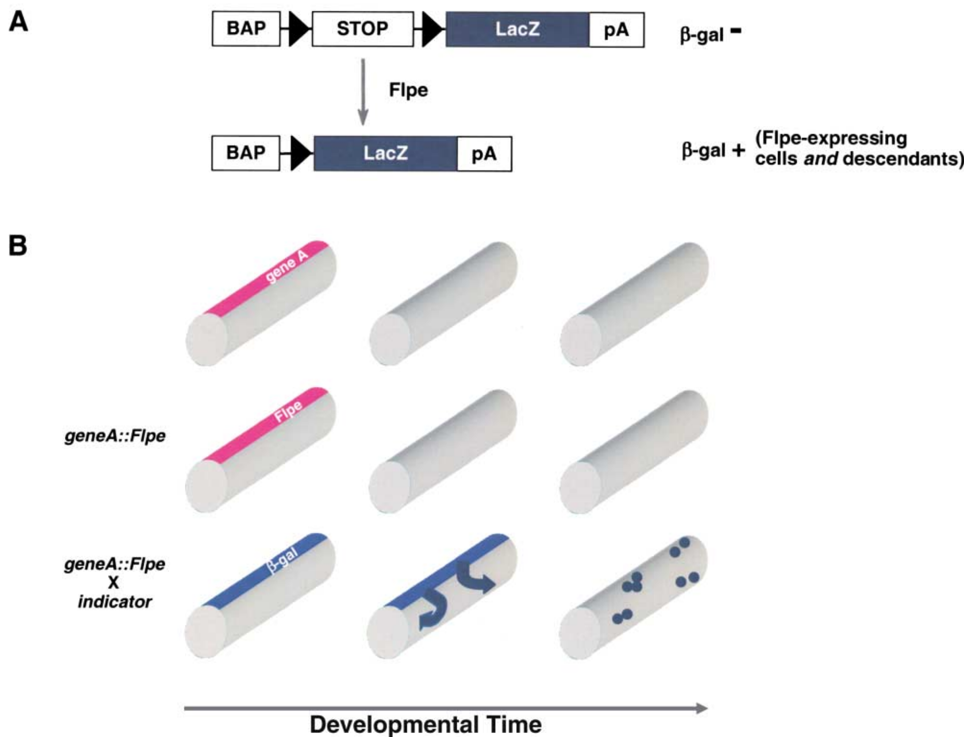


Figure 8. Molecular Fate Mapping

Illustration of how site-specific recombination can be used to study the deployment of progenitor cells and their descendants during development.

(A) Structure of a Flp-responsive indicator transgene. Flpe-mediated recombination between direct *FRT* repeats results in deletion of intervening DNA and restoration of the *lacZ* open reading frame (rendering cells β-gal$^+$). Permanent expression of the recombined indicator transgene requires use of a constitutive, broadly active promoter (BAP).

(B) Development of the neural tube is represented as a simple cylinder. Top row, transient expression profile of hypothetical gene A (purple). Middle row, transient expression profile of Flpe recombinase (purple) driven by regulatory elements from gene A. Bottom row, switching on a heritable *lacZ* (dark blue) lineage tracer by Flpe-mediated excisions. Descendant cells (β-gal$^+$, depicted here as dark blue circles) are visualized throughout development.

during embryonic development (Dymecki and Tomasiewicz, 1998). Both systems involve two mouse strains that are intercrossed: (1) a recombinase mouse, expressing Cre or Flp in a gene-specific fashion, and (2) an indicator mouse, harboring a transgene that "indicates" that a recombination event has occurred and provides a permanent record of this event by transforming it into a heritable lineage marker (Figure 8). The basic elements contained within an indicator transgene are (1) a reporter gene, functionally silenced by insertion of a STOP cassette (containing, for instance, a head-to-tail array of four *simian virus 40* (*SV40*) polyA sequences coupled with translational stop codons in all reading frames [Maxwell et al., 1989; Soriano, 1999]) that has been either floxed or flrted; and (2) a widely active promoter capable of driving reporter expression ideally in all cell types and at all stages of development, such that following a recombination event in any given cell, that cell and all progeny cells will be marked regardless of subsequent fate specification. In *recombinase; indicator* doubly transgenic animals, excision of the STOP cassette in the indicator transgene will occur only in the recombinase-expressing cells, thereby activating (i.e., repairing) the reporter gene. Because the excision event activating the reporter is at the level of chromosomal DNA, it is stably inherited to progeny cells, thereby marking these cells and revealing their contribution to embryonic and adult tissues. By delineating the adult structures that arise from a gene expression domain within the embryo, such molecular fate maps not only inform developmental processes, but also guide an investigator where to look for subtle phenotypes in the adult animal, potentially long after the time of normal expression of the mutated gene. (For further review on molecular fate mapping in the mouse, see Dymecki et al., 2002.)

A number of useful indicator mice are available and are listed in Table 2. Each reporter molecule contained within the various indicators has a unique advantage. For example, nuclear-localized β-gal facilitates the identification of individual cells, human placental alkaline phosphatase (PLAP) has the potential to provide information on cell morphology (Fields-Berry et al., 1992), and green fluorescent protein (GFP) and other color variant fluorescent proteins can be used to visualize live cells. Of the listed indicator mice, *R26::FLAP* (*ROSA26*::*FRT*- and *lox*P-disrupted PLAP) represents the first

Table 2. Cre- and Flp-Responsive Indicator Mice

Indicator	Responsive to:	Promoter/ Enhancer Sequences	Transgenic vs. Knock In	Reporter(s)	Condition for Reporter Expression	Reference
cAct-XstopXlacZ	Cre	*chicken β-actin*	T	nuclear β-gal	ELC	(Tsien et al., 1996)
floxLacZ	Cre	*chicken β-actin*	T	β-gal	ELC	(Akagi et al., 1997)
cβ-STOP-lacZ	Cre	*chicken β-actin*	T	nuclear β-gal	ELC	(Zinyk et al., 1998)
R26R	Cre	*ROSA26*	KI	β-gal	ELC	(Soriano, 1999)
ROSA26^{flox}	Cre	*proviral ROSA26*	KI	β-gal	ELC	(Mao et al., 1999)
Z/AP	Cre	*CAG*	T	β-gal	--	(Lobe et al., 1999)
				PLAP	ELC	
UCR	Cre	*G3BP (BT5)*	KI	β-gal	ELC	(Michael et al., 1999)
Z/EG	Cre	*CAG*	T	β-gal	--	(Novak et al., 2000)
				eGFP	ELC	
CAG-CAT-EGFP	Cre	*CAG*	T	eGFP	ELC	(Kawamoto et al., 2000)
R26R-EYFP	Cre	*ROSA26*	KI	eYFP	ELC	(Srinivas et al., 2001)
R26R-ECFP	Cre	*ROSA26*	KI	eCFP	ELC	(Srinivas et al., 2001)
ROSA26-EGFP^{f}	Cre	*ROSA26*	KI	eGFP	ELC	(Mao et al., 2001)
LSLMAP2GFP	Cre	*Chicken β-actin*	T	MAP2-GFP	ELC	(Huang et al., 2002)
Hmgcr::FRTZ	Flp	*Hmgcr* sequences	T	nuclear β-gal	EFC	(Rodriguez and Dymecki, 2000)
R26::FRAP	Flp	*ROSA26*	KI	PLAP	EFC	(Awatramani et al., 2001)
R26FR	Flp	*ROSA26*	KI	β-gal	EFC	(Possemato et al., 2002)
R26::FLAP	Flp + Cre	*ROSA26*	KI	eGFP(F)	EFC	(Awatramani et al., 2003)
				PLAP	EFC + ELC	

Hmgcr, hydroxymethylglutaryl-coenzyme A reductase; β-gal, beta-galactosidase; ELC, excision of *lox*P-flanked STOP cassette; *CAG*, *chick β-actin* promoter coupled with cytomegalovirus enhancer sequences; PLAP, human placental alkaline phosphatase; UCR, universal conditional *lacZ* reporter; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; EFC, excision of *FRT*-flanked STOP cassette; eGFP(F), enhanced green fluorescent protein, farnistylated. Dashed lines indicate constitutive expression of the reporter prior to recombinase activity.

dual-recombinase-responsive indicator strain (Awatramani et al., 2003). Coupled with two recombinase-expressing alleles, *R26::FLAP* allows the tracking of cells that have undergone two genetic events in their history, and therefore is useful for studying many multistep processes relevant to development and/or disease. For example, through its application, progenitor-specific expression of gene combinations can be mapped onto specific cell fates, helping to define combinatorial gene expression codes important in the development of diverse cell types. Moreover, it represents a general strategy for intersectional gene activation (IGA), which could be employed to ectopically express any gene of interest solely at the intersection (in space or time) of two gene expression domains. In most cases, this provides great improvement in selectivity, allowing more precisely defined cell populations to be studied.

One of the first demonstrations of the utility of molecular fate mapping has been the tracking of cells arising from the *Wnt-1* expression domain. *Wnt-1* mRNA is detectable largely within dorsal neuroepithelium of the midgestation embryo (Wilkinson et al., 1987), a tissue that gives rise to a wide variety of migratory cell populations over the course of development. These migratory cell populations include the neural crest and derivatives of the rhombic lip, the latter referring specifically to neuroepithelium adjacent to the roof plate of the fourth ventricle. Although these *Wnt-1*-expressing cell populations have been the subject of many studies, a comprehensive analysis of their terminal fates had not been possible in mice due to the cells' extensive migration patterns and lack of a marker to follow them throughout development and within the adult. By expressing Cre within the *Wnt1* domain and permanently marking these Cre-expressing cells and their descendants with β-gal, the contribution of marked neural crest cells to the development of craniofacial and cardiac structures could be explored carefully in mice for the first time (Chai et al., 2000; Jiang et al., 2000). Extending this analysis, the fate of derivatives of cardiac neural crest cells in mice null for the α1 and β retinoic acid receptors was traced, revealing that the number, migration, and terminal fate of these cells is normal in the double mutants (Jiang et al., 2002). This study serves to highlight the added utility of SSR-based molecular fate mapping to track cells in mutant backgrounds to determine, for example, if they assume abnormal cell fates, or migrate to abnormal positions. In addition, by expressing Flp within the *Wnt1* expression domain and permanently marking these Flp-expressing cells and their descendants, it was demonstrated definitively for the first time that dorsal germinal zones give rise to neurons of the precerebellar afferent system, a group of ventral brainstem nuclei that provide major input to the cerebellum (Rodriguez and Dymecki, 2000; R. Awatramani, C. Rodriguez, and S.M.D., unpublished data).

Conclusion

The completion of the human (Lander et al., 2001; Venter et al., 2001) and mouse (Lindblad-Toh et al., 2001; Waterston et al., 2002) genome sequencing projects together with advances in SSR technology have heralded a new era of genetic analysis in the mouse. The genetic similarity of mice and humans, the availability of sophisticated SSR-based tools with which to explore gene function, and the genetic tractability of ES cells, make the mouse a powerful model system. Indeed, with the tools of SSR technology in hand, the field of mouse genetics is primed to determine the contribution of every human gene to development, disease, and disability.

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