CONDITIONAL CONTROL OF GENE EXPRESSION IN THE MOUSE

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One of the most powerful tools that the molecular biology revolution has given us is the ability to turn genes on and off at our discretion. In the mouse, this has been accomplished by using binary systems in which gene expression is dependent on the interaction of two components, resulting in either transcriptional transactivation or DNA recombination. During recent years, these systems have been used to analyse complex and multi-staged biological processes, such as embryogenesis and cancer, with unprecedented precision. Here, I review these systems and discuss certain studies that exemplify the advantages and limitations of each system.

CELL AUTONOMOUS
A genetic trait in which only genotypically mutant cells show the mutant phenotype.

CELL NON-AUTONOMOUS
A cell non-autonomous trait is one in which genotypically mutant cells cause other cells (regardless of their genotype) to show a mutant phenotype.

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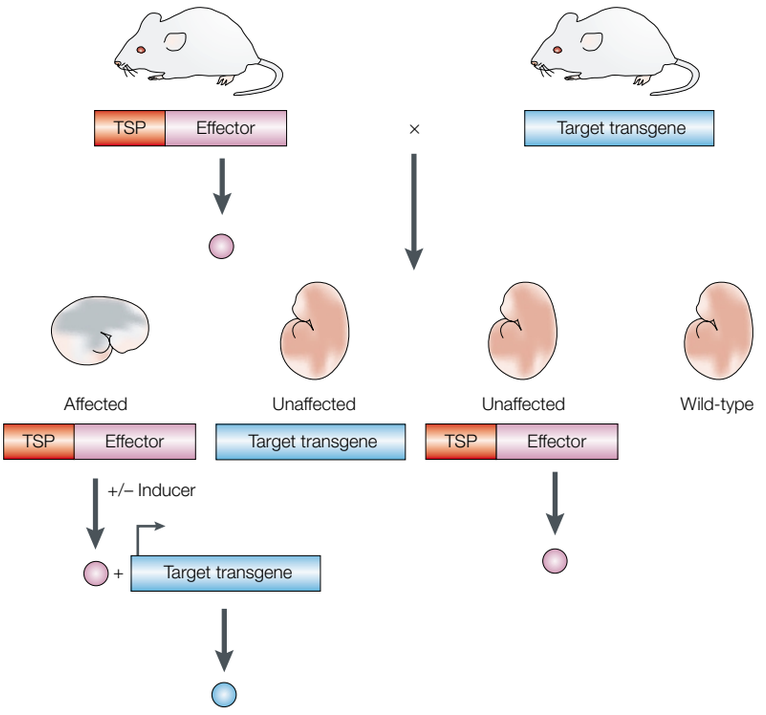
MOUSE GENOMIC TECHNOLOGIES

Our ability to produce mice that carry altered genomic loci or foreign transgenes has facilitated the study of many biological processes, from embryogenesis to animal behaviour. However, not all biological processes can be accessed and studied by gene-inactivation or transgene-expression strategies. For example, many genes have several roles during embryogenesis and adulthood; knocking out these genes can result in an embryonic-lethal phenotype, which reflects the earliest non-redundant role of the gene, and precludes an analysis of its function in later development. Similarly, embryonic lethality due to the expression of a transgene renders it useless for the study of adult biology. Of the phenotypes that are caused by such genetic manipulations, many are difficult to interpret because they might be due to CELL-AUTONOMOUS, CELL-NON-AUTONOMOUS or systemic effects in the whole animal. To circumvent these limitations, mouse geneticists have long searched for experimental 'switches' that allow genes to be activated or silenced where and when the investigator chooses.

The ideal genetic switch results in low or zero basal gene expression when 'off' and high levels of gene expression when 'on'. Furthermore, the switch should be reversible and specific for the target gene, and should not interfere with other cellular components or with general metabolism. The earliest attempts to develop such a genetic switch made use of single transgenes that had promoters that could be induced by heavy metals, heat shock, interferon or steroids. These transgenes fell somewhat short of the ideal because they produced pleiotropic effects or showed high basal activity in the absence of induction[1].

However, the criteria for an ideal genetic switch have largely been met by binary transgenic systems, in which gene expression is controlled by the interaction of two components: an 'effector' transgene, the product of which acts on a 'target' transgene (BOX 1). Binary transgenic systems fall into two categories. In one, the effector transactivates transcription of the target transgene. In the other, the effector is a site-specific DNA recombinase that rearranges the target gene, thereby activating or silencing it. Both systems have their advantages and limitations, as discussed in this review. During recent years, the number of these binary systems has increased, and the more established systems, such as Cre/*loxP* and the TetR-based transactivators (see below), have been refined. Furthermore, these systems have been increasingly used to solve otherwise intractable problems in transgenic mice. Here, I review the current systems for conditional gene expression, focusing on specific examples that illustrate details in experimental design that might be useful to those considering the use of such technologies.

Box 1 | **Binary transgenics in the mouse**



In the ideal conditional system, investigators should be able to activate or silence a transgene where and when they choose. The breakthrough model, on which all the systems in this review are based, is a binary system in which gene expression is controlled through the interaction of two components: the product of an 'effector' transgene acting on a 'target' transgene. The effector transgene alone should not affect the expression of endogenous genes; its expression should therefore be innocuous. Control of target transgene expression should be wholly conditional on the presence of the effector. So, expression of the target transgene should only occur in doubly transgenic F_1 progeny derived from mouse lines that carry either the target transgene or the effector transgene, or in the presence of an exogenous inducer that allows the appropriate interaction of the effector on the target. These criteria have been largely met by developing binary systems with components derived from systems that are too evolutionarily distant from the mouse to have any unintended interactions with the mouse genome. In the example shown (see figure), the effector gene product–target gene interaction results in target activation but, in some cases (such as when using the site-specific recombinases, Cre or Flp), the effector can also inactivate the target transgene. In most of the inducible binary systems, the effector gene product–target gene interaction depends on the presence of an exogenously added inducer, but withdrawal of an inducer can also result in effector action (see text for more details). TSP, tissue-specific promoter.

Transcriptional transactivation

In the first binary systems, transgene transcription was transactivated by viral proteins[2,3], but these systems were not further used because of high basal levels of target-gene expression in the absence of transgene transactivation[2], or because expression of the transactivator caused tumorigenesis[4]. One exception to this has been the VP16 protein of herpes simplex virus, which has been used to control the transcription of target transgenes that contain the VP16 response element[5–7]. However, the use of this system is limited because VP16 is lethal when expressed in pre-implantation embryos, presumably because of the SQUELCHING due to its interaction with mouse transcription factors[8,9]. Squelching might also occur when chimeric transactivators that contain the VP16-activating domain are highly expressed, and *in vitro* solutions to this problem have been reported[9,10]. But, generally, such interactions have not impeded the use of these chimeric transactivators in transgenic mouse studies[11] (described in more detail below) (TABLE 1).

Transcriptional transactivation is used more widely than DNA recombination to bring about transgene activation in mice, mainly because transgene activation by DNA recombination is irreversible, which results in permanent transgene activation in any cell lineage. By contrast, transcriptional transactivation occurs only when the transactivator is present, and, if an inducible system is used, then transgene activation can be reversed at a given time (see below), which provides the investigator with more control over transgene expression.

TetR-based systems. The most widely used binary transcription transactivation systems are the tetracycline-dependent regulatory systems developed by Manfred Gossen and Hermann Bujard[12] (see link to Hermann Bujard's trouble-shooting guide). In these systems, the effector is a fusion of sequences that encode the VP16 transactivation domain and the *Escherichia coli* tetracycline repressor (TetR) protein, which specifically binds both tetracycline and the 19-bp operator sequences (*tetO*) of the *tet* operon in the target transgene, which results in its transcription (FIG. 1). There are two versions of this system. In the original system, the tetracycline-controlled transactivator (tTA) cannot bind DNA when the inducer is present ('tet-off') (FIG. 1a), whereas in a modified version, the 'reverse tTA' (rtTA) binds DNA only when the inducer is present ('tet-on')[13] (FIG. 1b). The current inducer of choice is doxycycline (Dox) because of its low cost, commercial availability, and because it efficiently activates rtTA and inactivates tTA at doses that are well below cytotoxic levels[11,13].

The principal difference between tTA and rtTA is the kinetics of target transgene induction. In the tTA system, transgene suppression requires the continuous administration of Dox; induction occurs on Dox removal and is, therefore, dependent on the rate of Dox clearance. In adult transgenic mice, induction can take from 24 hours to 1 week, depending on the tissue of interest[14]. However, in the rtTA system, the addition of Dox can rapidly induce transgene expression, even within an hour[15], whereas subsequent repression depends on the clearance of Dox from the tissue of interest.

Although Dox can cross the placenta and efficiently regulate gene expression during embryogenesis, these systems have been underused to study embryogenesis *per se* and have mostly been used to suppress the embryonic expression of lethal transgenes. In one study by Lee *et al.*[16], which possibly subjected the system to the most stringent test, tTA was used to control the expression of a target transgene that encoded the cell-autonomous, lethal DIPHTHERIA TOXIN A SUBUNIT (DTA). Mouse lines that carried the *tetO*–DTA target transgene were produced at a ten-fold lower frequency compared with this lab's production

SQUELCHING
The titration of interacting molecules that are out of equilibrium by the overexpression of an interacting regulatory partner molecule, which can result in pleiotropic effects.

Table 1 | **Examples of tissue-specific tTA and rtTA mice***

Tissue-specific promoter	Tissue of expression	Transactivator	Target gene	Reference
Lap (*Cebpb*)	Liver	tTA	Luciferase	14
Myh6	Heart	tTA	Diphtheria toxin A	16
Ednrb	Embryonic neural crest	tTA, rtTA	*Ednrb*	17
Camk2a	Hippocampus, striatum	tTA	Mutated calcineurin	19
Camk2a	Hippocampus, cortex, striatum, olfactory bulb, cerebellum	rtTA	Calcineurin inhibitor	18
MMTV-LTR	Submandibular gland	tTA	*SV40*	20
Tyr	Melanocytes	rtTA	*H-ras*^{V12G}	21
Eμ–Sr	Haematopoietic cells	tTA	*Myc*	22
MMTV-LTR	Haematopoietic cells	tTA	*Bcr-Abl1*	23
Camk2a	Hippocampus, cortex, striatum	tTA	*Hdh*	24
Lap	Liver	tTA	*Nfkbia*, luciferase	28
Fabp	Small intestine, colonic epithelium	rtTA	Cre recombinase	34
RB	Retina, cerebellum, monocytes	rtTA	Cre recombinase	116
Wap	Mammary gland	rtTA	Cre recombinase	116

*These are just a few examples of many such mouse lines to illustrate the range of tissues in which tissue-specific transactivator expression can be achieved. (*Bcr-Abl1*, breakpoint cluster region-Abelson protein1; *Camk2a*, Ca^{2+}/calmodulin-dependent protein kinase II, alpha; *Cebpb*, CCAAT/enhancer-binding protein, beta; *Ednrb*, endothelin receptor-B; *Eμ–Sr,* immunoglobulin heavy chain enhancer–SRα promoter; *Fabp*, fatty-acid-binding protein; *Hdh*, huntingtin; *H-ras*, Harvey rat sarcoma virus oncogene; *Lap*, liver activator protein; *Myh6*, myosin heavy chain, cardiac muscle, alpha; *MMTV-LTR*, mouse mammary tumour virus-long terminal repeat; *Myc*, myelocytomatosis oncogene; *Nfkbia*, nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha; *RB*, retinoblastoma; rtTA, reverse tetracycline-controlled transactivator; *SV40*, simian virus 40; tTA, tetracycline-controlled transactivator; *Tyr*, tyrosinase; *Wap*, whey acidic protein.)

of other transgenic lines, presumably because the leaky expression of the transgene caused cell ablation and death[16]. However, the *tetO*–DTA transgene was regulated properly by tTA in the lines that were established, and its specific induction in the hearts of adult mice caused cardiomyopathies[16].

This system has been effectively used during embryogenesis to study endothelin receptor type B (*Ednrb*) signalling[17], which is required for the normal development of neural-crest derivatives. Activation of an *Ednrb* target transgene through rtTA, or its rapid inactivation through tTA, within hours of Dox administration, showed that *Ednrb* is required for neural-crest migration during embryonic days 10–12.5. To achieve this tight regulation and to make all *Ednrb* expression dependent on Dox regulation, gene targeting was used to produce three mouse lines in which the wild-type *Ednrb* allele was replaced with sequences that encoded one of the two transactivators or the *Ednrb*^{tetO} target allele. The SELECTION CASSETTE used to target the *Ednrb* locus, which allowed targeted embryonic stem (ES) cells to be selected, was designed so that it could be removed in transgenic mouse lines by Cre-mediated recombination (as described in more detail below). Its removal was required because the presence of the cassette reduced the expression of the transactivator-encoding transgenes. However, on its removal from the *Ednrb*^{tetO} allele, an unacceptable increase in the basal activity of this allele occurred. As a result, an *Ednrb*^{tetO} allele that still contained the selection cassette was used in experimental crosses. This study exemplifies the importance of having a versatile experimental design that allows targeted alleles to be altered after transgenic mice are produced; the experiment would not have succeeded had the investigators failed to design their selection cassettes for removal, or had they removed them in ES cells before producing transgenic mice.

Theoretically, it is possible to induce and then silence *Ednrb*^{tetO} expression with only tTA or rtTA, but the kinetics of Dox depletion is too slow for either this induction (through tTA) or repression (through rtTA) to be useful in embryological studies. However, in studies of cancer, neurological disease and memory[18,19], such kinetics are not an impediment, and the ability to reverse target-gene expression has produced impressive results. Inducing and then silencing oncogene expression with either tTA or rtTA in both solid tumours[20,21] and leukaemias[22,23] has shown that, in most cases, the oncogene that induces tumorigenesis is also required for tumour maintenance. In a mouse model of Huntington disease that relied on the tTA activation of a mutant *huntingtin* transgene, Dox treatment over 16 weeks resulted in transgene repression, and the reversal of the neuropathology and behavioural abnormalities that characterize the disease[24]. These studies indicate that future cancer or Huntington disease therapies that target the activity of a single molecule might be clinically effective.

The 'tet-on' and 'tet-off' systems continue to be modified with different transactivator domains, trans-repression domains, codon-usage modifications, and variations to the configuration of *tetO* sequences[10,11,25,26] (see also links to Knoll GmbH and ClonTech). One useful variant allows two target transgenes to be co-regulated

DIPHTHERIA TOXIN A SUBUNIT (DTA). Diphtheria toxin consists of two subunits, A and B. The B-subunit binds receptors on the surface of the target cell, facilitating the entry of the A-subunit, which ADP-ribosylates elongation factor 2, thus preventing protein synthesis. The gene that encodes the A-subunit is often used as a cell-autonomous toxin in transgenic ablation experiments.

SELECTION CASSETTE
A DNA fragment that contains a transgene, which, when expressed, allows the selection of a subset of cells that have integrated the DNA fragment into their genomes.

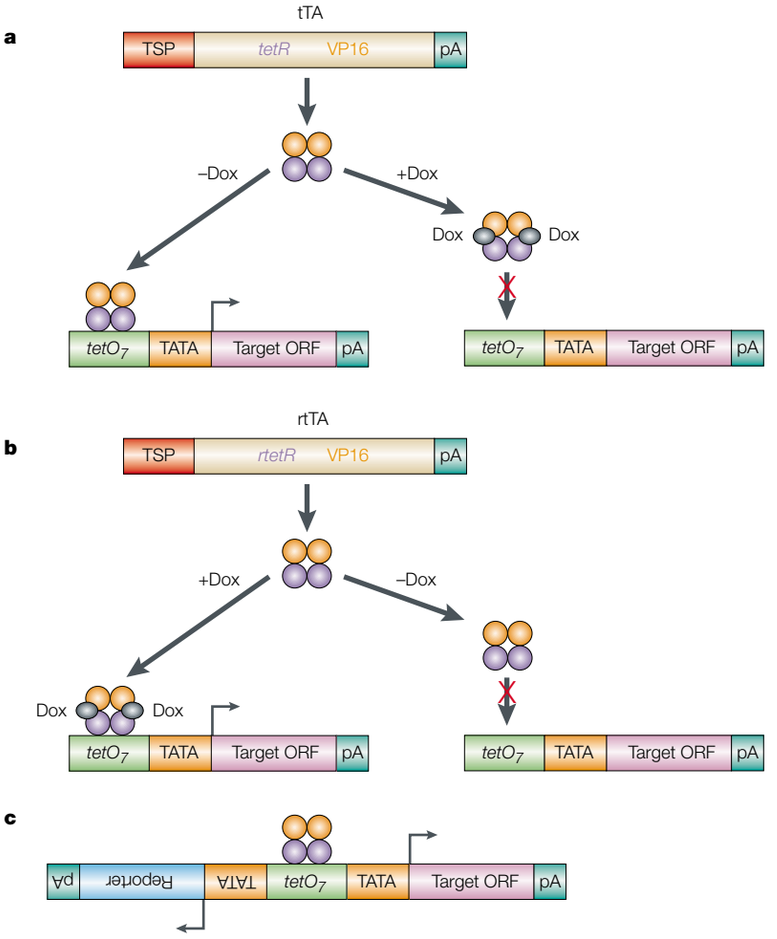


Figure 1 | **The tetracycline-responsive regulatory system for transcriptional transactivation. a** | The effector is a tetracycline-controlled transactivator (tTA) of transcription that consists of a chimeric construct of the *Escherichia coli* Tn10 *tetR* gene (purple) and the VP16 transactivation domain (orange). In the absence of the inducer, doxycycline (Dox), tTA dimers specifically bind to seven tandemly repeated 19-bp *tetO* sequences ($tetO_7$), thereby activating transcription from a minimal promoter (TATA) and driving expression of the target transgene that encodes the gene of interest (target ORF). When bound to Dox, tTA undergoes a conformational change and cannot bind *tetO* sequences. **b** | In the reverse tTA (rtTA) system, the *tetR* gene has been mutated so that it binds *tetO* sequences and activates transcription only in the presence of Dox. **c** | By flanking *tetO* sequences with two minimal promoters, two target transgenes can be activated by the tTA (or rtTA) systems. In the example shown, tTA drives transcription of a reporter gene, as well as the target gene of interest. (bp, base pair; ORF, open reading frame; pA, polyadenylation site; *tetO*, the 19-bp operator sequences of the *tet* operon; *tetR*, tetracycline repressor; TSP, tissue-specific promoter; VP16, transactivation domain of the herpes simplex virus protein VP16.)

by flanking the *tetO* sequences with two minimal promoters[27] (FIG. 1c). This enables transactivation in transgenic mice to be monitored when one promoter drives a reporter gene, such as luciferase[28]. In another variation, the DNA-binding specificity of the transactivator has been modified so that it will discriminate between altered *tetO* sequences, allowing an investigator to switch transactivation between two different target transgenes by altering Dox levels[29]. Finally, a random mutational screen has generated rtTA variants with increased Dox sensitivity. These variants should mediate a greater responsiveness to Dox in those mouse tissues, such as brain, to which Dox has limited access[25]. Although these variants have yet to be used in transgenic mouse studies, the continuing evolution of these systems *in vitro* will soon affect the precision with which we will be able to dissect biological problems *in vivo*.

POSITION EFFECTS
The effect of the local chromosomal environment on the levels or pattern of transgene expression, possibly because of local chromatin configuration or nearby *cis*-acting regulatory elements.

GAL4-*based systems.* In *Saccharomyces cerevisiae*, the transcriptional activator Gal4 directs the transcription of Gal4-responsive genes by binding to upstream activator sequences (UASs). The Gal4/UAS system was used to direct transgene activation in the first non-viral binary system to be used in mice[30] (FIG. 2a). This approach has been used to study the role of Sonic hedgehog (Shh) in the dorsal–ventral patterning of the mouse central nervous system (CNS). In previous studies, Shh, which is normally expressed along the ventral midline of the CNS, had been ectopically expressed along the dorsal midline with a single *Shh* transgene driven by *Wnt1* regulatory sequences (*Wnt1–Shh*)[31]. However, because of an embryonic-lethal phenotype, only a limited number of founder embryos could be analysed. Furthermore, the phenotype varied between these founder embryos presumably because of POSITION EFFECTS. These constraints were bypassed by activating a UAS–*Shh* target transgene with a Gal4 effector transgene that was expressed under the control of a *Wnt1* promoter (*Wnt1–GAL4*) to generate many mutant embryos with a highly reproducible phenotype[32]. It is noteworthy that the initiation of β-galactosidase activity through the transactivation of UAS–*lacZ* by *Wnt1–GAL4* was delayed by ~24 hours relative to that from a single *Wnt1–lacZ* transgene, a time delay which could be significant in some developmental studies.

One example of an inducible Gal4-based system is the GLVP system, which is named after the constituent parts of the chimeric effector protein (FIG. 2). Because of the ligand-binding domain of the GLVP transactivator, the target transgene can be induced by the synthetic steroids, RU486 or ZK98.734 (FIG. 2b). Recently, the topical application of ZK98.734 to mice that express GLVP specifically in the epidermis was used to activate transforming growth factor-β1 (*Tgfb1*) expression in the skin[35]. Previous reports using single transgenes had given contradictory results as to whether Tgf-β1 inhibited or stimulated epidermal growth[36]. By temporally controlling *Tgfb1* expression, locally and in a dose-dependent manner with topical applications of ZK98.734, Tgf-β1 was shown to inhibit epidermal growth[35]. In general, the use of the GLVP system has been limited to controlling gene expression in adult mice[33,35,37], as the abortigenic properties of these synthetic steroids, which act as antiprogestins, limit their use in embryos; although their co-injection with progesterone has been shown to rescue embryonic development (D. R. Roop, personal communication).

In summary, the advantages of using transcriptional transactivation to conditionally control gene expression are: the reversibility of the expression of the target gene; the sensitivity of transgene activation levels to

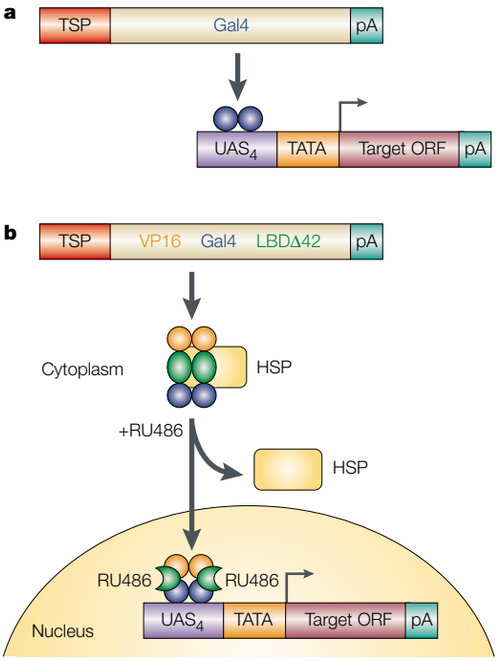


Figure 2 | **Transactivation based on the Gal4/UAS system. a** | The effector is the Gal4 transcriptional transactivator (blue circles) from *Saccharomyces cerevisiae*, which binds as a homodimer to a 17-bp upstream activating sequence (UAS). The target transgene is activated by Gal4 homodimers that bind to four tandemly repeated UASs placed immediately upstream of a minimal promoter (TATA). **b** | GLVP is an inducible Gal4/UAS-based system. In the original version, the transactivator is a chimeric construct (GLVP) that consists of the herpes simplex virus VP16 activation domain (orange), the Gal4 DNA-binding domain (blue) and the ligand-binding domain of the progesterone receptor (LBDΔ42) (green), mutated such that it fails to bind progesterone but can bind antiprogestins, such as RU486. GLVP is believed to be sequestered in the cytoplasm by its binding to heat-shock proteins (HSPs) 70 and 90. In the presence of RU486, GLVP is released, becomes localized to the nucleus and transactivates the UAS-bearing target transgene. Several versions of the transactivator exist owing to the modification of different domains[146] (see link to GeneSwitch™, Valentis, Inc.). (ORF, open reading frame; pA, polyadenylation site; TSP, tissue-specific promoter.)

inducer concentration; and the ability to control the expression of more than one target transgene. These features make this strategy well suited to gain-of-function experiments, in which phenotypes are generated by transgene overexpression or ectopic expression. A potential limitation is that the binary nature of these systems compounds twofold the basic problem of any transgenic experiment: the susceptibility of transgene regulatory elements to position effects. To achieve correct regulation, the transgenes could be flanked with INSULATOR SITES[26,33] or inserted into a known locus that provides low basal and high activated expression[34], but often the best strategy is to test different combinations of effector and target lines.

INSULATOR SITE
DNA sequence that blocks the interaction between *cis*-acting regulatory elements. These sites are sometimes used to protect transgenes from genomic position effects.

CONSERVATIVE DNA RECOMBINATION
A DNA recombination reaction in which there is no net change in base pairs between the products and the reactants.

Site-specific DNA recombination

Two members of the integrase family of site-specific recombinases, Cre from bacteriophage P1 (REF. 38) and Flp from *Saccharomyces cerevisiae*[39], are used at present to conditionally control gene expression by site-specific DNA recombination. Cre and Flp recombinases catalyse a CONSERVATIVE DNA RECOMBINATION event between two 34-bp recognition sites (*loxP* and *FRT*, respectively) that differ in primary sequence but share secondary structure (FIG. 3). Because these recombinases do not require accessory proteins or high-energy cofactors for their activity, they are suitable for use in the foreign milieu of the mouse cell. However, Flp has been modified to alter its temperature optimum for improved activity at 37 °C — the body temperature of mice[40]. Also, many Cre variants now being used have been modified with a heterologous nuclear localization sequence (NLS)[41,42], which has been found to increase Cre-mediated recombination in some *in vitro* assays[41], but not in others[43]. As wild-type Cre contains basic peptides that direct it to the mammalian nucleus[43], the advantages of using such modified Cre variants *in vivo* is questionable.

Originally, the expression of *cre* or *FLP* in mice was expected to be innocuous because it was thought that the mouse genome would not harbour endogenous *loxP* or *FRT* sites. However, these recombinases have been shown *in vitro* to mediate recombination between degenerate *loxP*/*FRT* sequences (pseudo-sites) with reduced efficiency[44,45], and pseudo-*loxP* sites have been identified in the mouse genome[44]. A recent study reported that high levels of *cre* expression in cultured cells leads to growth arrest and DNA damage, indicating that recombination between these pseudo-sites might occur *in vitro*[46]. In one case, *cre* expression in the male germ line resulted in chromosomal aberrations and sterility, indicating that similar interactions might sometimes occur *in vivo*[47]. However, the widespread expression of *cre* in many other transgenic mouse lines (see link to the Cre transgenic database), including both male and female germ lines[48,49], attests to the rare occurrence of such interactions *in vivo*.

The ability to use Cre- or Flp-mediated DNA recombination to invert or delete DNA fragments *in cis*, or to switch DNA fragments *in trans* has many applications beyond conditional gene expression, which include chromosome engineering (see accompanying review by Yu and Bradley on p780 of this issue) or the recycling of selectable markers in cultured cells[50]. Here, I focus on the applications of this system for activating or silencing gene expression by deleting DNA fragments that have been flanked by directly repeated *loxP* or *FRT* sites (so called 'floxed' or 'flrted' alleles, respectively) (FIG. 3). The Cre/*loxP* system is the most frequently used owing to historical contingency, as it was the first well-developed recombination system. Many more effector and target transgenic mouse lines now exist, to which newly generated mouse lines can be crossed; however, the general strategies are the same for the Flp/*FRT* system.

Tissue-specific knockouts. The ability to inactivate an endogenous gene in the mouse in a temporally and spatially controlled manner is not only useful for circumventing early lethal phenotypes, but also allows biological questions to be addressed with exquisite accuracy. In such studies, standard gene-targeting techniques are used to produce a mouse in which an essential region of a gene of interest is floxed, so that tissue-specific *cre* expression results in the inactivation of this allele. Before recombination, the conditional allele should have wild-type activity. In most cases, *loxP* sites are placed in introns, but have sometimes been inserted in 5′- (REFS 51,52) or 3′-flanking regions[53,54] without compromising gene expression. In one published exception[55], a floxed glucokinase allele resulted in a slight reduction (10–15%) in enzyme activity, which might have been due to extra linker sequences adjacent to the *loxP* site (M. Magnuson, personal communication).

By crossing a mouse line with a conditional allele to an effector mouse line that expresses *cre* in a tissue-specific manner, progeny are produced in which the conditional allele is inactivated only in those tissues or cells that express *cre*. Since the first published tissue-specific knockout[56], the use of this strategy has steadily increased (see link to the Cre transgenic database) (TABLE 2), and there are now many instructive examples of its application.

Table 2 | **Examples of tissue-specific *cre*-expressing mice***

Tissue-specific promoter	Tissue/cell of expression	Floxed target gene	Reference
Lck	T cells	DNA polymerase-β	56
Cryaa	Eye lens	*SV40* (activated)	82
Ins2	Pancreatic β-cells	*Gck*	55
Alb	Liver	*Gck*	
Myog	Skeletal muscle	Diphtheria toxin A‡	83
KRT5	Epidermis	β1-integrin	54
Nes	Neuronal cells	*Mecp2*	53
Ins2	Pancreatic β-cells	*hGH*‡	79
Gcg	Pancreatic α-cells	*hGH*‡	
Ppy	Pancreatic β-cells	*hGH*‡	
Pdx1	Pancreatic α-cells	*hGH*‡	
En2	Mid/hindbrain	*lacZ*‡	75
Wnt1	Neural crest	*lacZ*‡	78
Camk2a	Forebrain	*Hdh*	51
Nes	Branchial arch	*Fgf8*	65
Msx2	Apical ectodermal ridge of limb bud	*Fgf8*	66
Pax6	Retina	*Pax6*	137

*These are just a few examples of many such mouse lines to illustrate the range of tissues in which tissue-specific *cre* expression can be achieved. ‡In these cases, Cre-mediated recombination resulted in gene activation. (*Alb*, albumin; *Camk2a*, Ca^{2+}/calmodulin-dependent protein kinase II, alpha; *Cryaa*, crystallin alpha A; *En2*, Engrailed 2; *Gcg*, glucagon; *Ins2*, insulin II; *Lck*, lymphocyte-specific tyrosine kinase; *Fgf8*, fibroblast growth factor 8; *hGH*, human growth hormone; *Hdh*, huntingtin; *Mecp2*, methyl-CpG-binding protein 2; *Msx2,* msh-like homeobox gene 2; *Myog*, myogenin; *Nes*, nestin; *Pax6*, paired box gene 6; *Pdx1*, pancreatic and duodenal homeobox factor 1; *Ppy*, pancreatic polypeptide; *SV40*, simian virus 40; *Wnt1*, wingless-related MMTV integration site 1.)

A selectable marker cassette (such as that containing the neomycin resistance gene, *neo*), is routinely used to select for targeted ES-cell clones and is necessarily part of the initial floxed allele. However, the placement of *neo* in introns, or in 5′- or 3′-flanking or untranslated

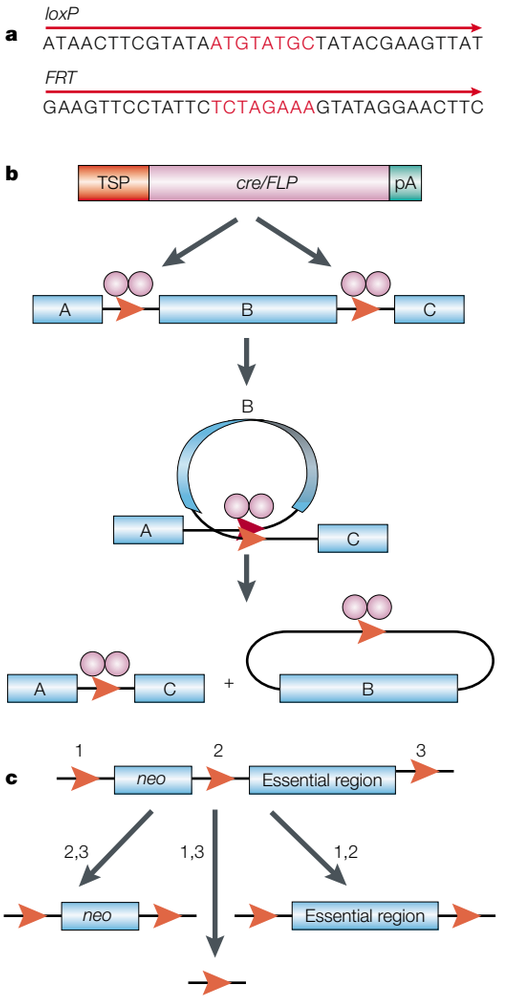


Figure 3 | **Controlling gene expression by DNA recombination. a** | The 34-bp *loxP* and *FRT* sites each consist of two 13-bp inverse repeats (black) that flank an 8-bp core sequence (red). This core sequence confers directionality to these sites (red arrows). **b** | Dimers of Cre or Flp (pink) catalyse *in cis* the conservative recombination between two directly repeated *loxP* or *FRT* sites (red arrowheads), resulting in the formation of a synaptic structure, the excision of region B and the juxtaposition of regions A and C. If region B is an essential region of a gene, then the recombination event results in gene inactivation. Recombination can also activate gene expression. For example, transcription from a promoter in region A could fail to reach protein-coding sequences in region C if polyadenylation sites exist in region B; excising region B would therefore activate transcription of region C. **c** | Use of three *loxP* sites and a 'partial' recombination event (between *loxP* sites labelled 1 and 2) to remove a neomycin resistance (*neo*) selection cassette and leave an essential region floxed. The other two products of recombination between *loxP* sites 2 and 3, and between 1 and 3 are also shown. (pA, polyadenylation site; TSP, tissue-specific promoter.)

HYPOMORPHIC ALLELE
An allele that results in a reduction, but not the elimination, of wild-type levels of gene product or activity, often causing a less severe phenotype than a loss-of-function (or null) allele.

ALLELIC SERIES
An array of possible mutant forms of a gene, which usually cause several phenotypes.

SEMI-DOMINANT ALLELE
An allele that causes an intermediate, incompletely dominant phenotype in heterozygotes.

ROSA26
A genetic locus that was originally identified by gene-trapping technology, which is constitutively transcribed.

regions, can interfere with the expression of the floxed allele, compromising its wild-type activity in the absence of Cre-mediated recombination (TABLE 3). In some cases, it has been determined that cryptic splice sites in *neo* interfere with normal splicing and therefore reduce wild-type mRNA levels[57–59]. A common solution to this problem, carried out in the first tissue-specific knockout experiment, is to use three *loxP* sites, positioned such that *neo*, as well as the essential region of the gene of interest, is floxed[56]. A partial Cre-mediated recombination event then removes *neo*, leaving the essential region floxed (FIG. 3c). This is most often achieved *in vitro* in ES cells by transiently introducing a *cre*-expression cassette[56]. However, it can also be achieved *in vivo* by injecting zygotes with a *cre*-expression cassette[59], or by breeding mice that carry floxed alleles to mice that mosaically express *cre* in the germ line, such that recombination occurs between two of the three *loxP* sites at a reasonable frequency[59,60].

However, because the inclusion of *neo* can generate a HYPOMORPHIC ALLELE, retaining it can often be advantageous, because such an allele, in combination with the null and conditional alleles, can be very informative[57,58,61,62]. One approach, the allelogenic strategy, generates a hypomorphic allele as part of an ALLELIC SERIES from one mouse line by floxing an essential region of the gene of interest and then flirting the *neo* cassette so that it can be removed by Flp-mediated recombination[57]. Three mouse lines can be generated in such an approach. The original line expresses a hypomorphic allele (if *neo* interferes with gene expression); one line expresses a floxed allele, which can be inactivated in a tissue-specific gene manner and is generated by mating the original line to a *FLP*-expressing mouse; and finally, one line carries a null allele, generated by breeding the original line to a *cre*-expressing mouse. Such an allelic series at the fibroblast growth factor 8 (*Fgf8*) locus[57] has been instrumental in studying the role of *Fgf8* in gastrulation[63], left–right axis determination[64], branchial arch development[65] and limb development[66]. An allelic series that includes any desirable genomic alteration can also be generated by introducing specific mutations into the original targeting cassette; the removal of *neo* by Cre-mediated recombination would then leave the specific mutation[58]. Using this approach, an allelic series has been generated at the Fgf receptor 1 (*Fgfr1*) locus, including SEMI-DOMINANT and hypomorphic alleles[61].

To correctly interpret a phenotype caused by tissue-specific Cre-mediated gene inactivation, it is often essential to know in which cells recombination has taken place. This is usually done by monitoring gene expression at the RNA or protein level, but an alternative is to include a reporter construct (such as one that encodes *lacZ*) downstream of the floxed region, so that recombination both inactivates the target gene and activates the reporter[67]. However, care must be taken to ensure that, like *neo*, the reporter does not compromise the expression of the conditional allele before recombination.

Finally, the success of these experiments depends on the availability of tissue-specific regulatory elements to drive *cre* expression. Controlling *cre* expression is one of the most rigorous tests of the tissue specificity of any regulatory element, as nonspecific expression could render a *cre* transgene useless because of the effective irreversibility of the recombination event. Nevertheless, even if the appropriate Cre line is not available, it is logical for investigators to produce floxed alleles rather than standard 'knockouts'. This is because they can still generate the recombined null allele by using one of the many Cre 'deletor' strains (see link to the Cre transgenic database) and still have mice that carry the floxed allele for a future date when the right *cre*-expressing mouse comes along.

Cell-lineage analysis. Two different approaches have been used to produce mice in which a histological marker can be activated by Cre-mediated recombination in nearly any tissue, thereby reporting Cre activity. Such mice are useful not only for characterizing a newly made *cre*-expressing line, but also for analysing cell lineages, as described below. In one approach, the production of such reporter mice was achieved by using gene targeting to insert Cre-activatable (as described below) reporter constructs that encode β-galactosidase[68,69], or the enhanced fluorescent proteins of the green[70], yellow or cyan varieties[71], into the *ROSA26* locus, which produces constitutive transgene expression. In the other approach, ES clones that contain *cre*-reporter constructs were screened *in vitro* for widespread expression before the production of mice. In this manner, the Z/AP[72] and Z/EG[73] reporter lines were produced, in which Cre-mediated recombination causes a switch from the expression of the *lacZ* gene to

Table 3 | **Loss of target-gene function due to *neo* cassette**

Floxed gene	Location and orientation* of *neo*	Reference
Fgf8	Intron; same orientation	57
N-Myc	Intron; same orientation	58
Fgfr1	Intron; same orientation	61
Gck	Intron; same orientation	55
Pitx2	Intron; same orientation	62
Pck1	Intron; same orientation	147
Krt1-14	Intron; same orientation	90
Krt1-10	Intron; same orientation	89
Fgfr3	Intron; opposite orientation	148
Hnf4	Intron; opposite orientation	149
Brca1	Intron; opposite orientation	59
Slc2a4	3′ UTR	150
Fgf8	3′ UTR	67
Nodal	3′ UTR	151
Scap	5′ UTR	152
Bcl2l	5′ promoter region	153

*Orientation of *neo* cassette relative to transcription of floxed gene. (*Bcl2l*, B-cell lymphoma; *Brca1*, breast cancer gene 1; *Fgf8*, fibroblast growth factor 8; *Fgfr3*, fibroblast growth factor receptor 3; *Gck*, glucokinase; *Hnf4*, hepatocyte nuclear factor 4; *Krt1-x*, keratin complex 1, gene x; *N-Myc*, neuroblastoma myc-related oncogene; *Pck1*, phosphoenolpyruvate carboxykinase; *Pitx2*, pituitary homeobox 2; *Scap*, Srebp cleavage-activating protein; *Slc2a4*, solute carrier family 2, member 4 (also called *Glut4*); Srebp, sterol regulatory element-binding protein; UTR, untranslated region.)

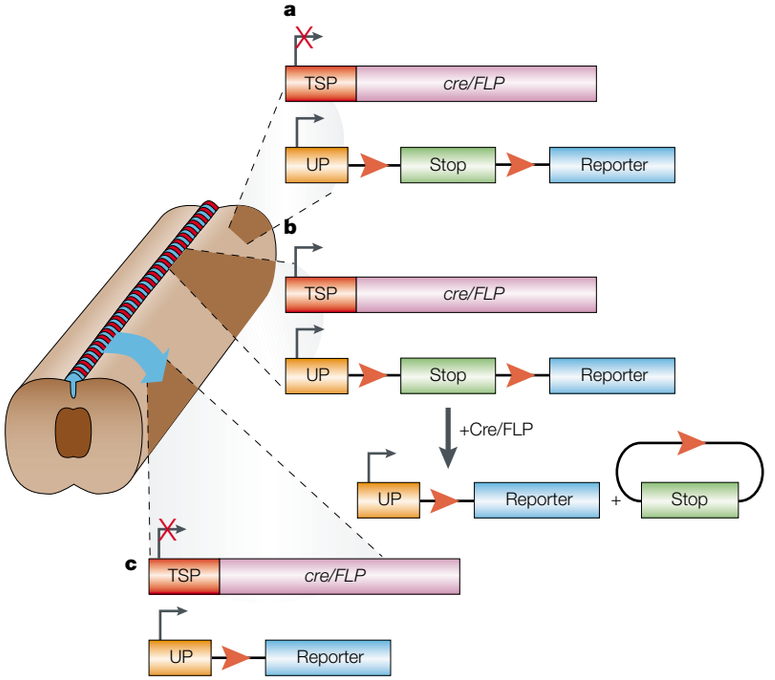


Figure 4 | **Fate mapping the descendants of a gene-expression domain.** In this example, a tissue-specific promoter (TSP) (for example, the *Wnt1* promoter) controls the expression of a *cre* or *FLP* transgene. **a** | The *cre* (or *FLP*) transgene is not expressed outside the dorsal neural tube. A reporter transgene is transcribed from a ubiquitous promoter (UP), but no functional gene product is produced outside the dorsal neural tube because Cre (or Flp)-mediated recombination is required to remove the floxed (or flrted) Stop sequences that separate the promoter from the reporter coding sequences (red arrowheads, *loxP* or *FRT* sites). **b** | Cre activity in the dorsal neural tube (red) results in the removal of the Stop sequence and reporter activation (blue). **c** | Migrating neural-crest cells (blue arrow) no longer express *cre* or *FLP*, but the reporter remains activated (blue).

that for alkaline phosphatase or enhanced green fluorescent protein, respectively. The availability of different reporter lines is useful because recombination can sometimes be locus dependent[74].

These reporter lines are useful for linking gene expression to the fate of specific cell lineages because activation of one of these reporter genes indicates that Cre activity is present in that cell or was present in a progenitor cell (FIG. 4). This strategy has been used to fate map the mid–hindbrain border[75], neural-crest cell derivatives[76–78], pancreatic islet cells[79] and memory T cells[80]. In such experiments, care must be taken to ensure that Cre is not active in a second lineage that is then erroneously assumed to be related to the first lineage. To overcome this problem, it might be useful to insert *cre* into the locus of the gene of interest so that it is free of position effects. As such insertions usually inactivate the gene of interest, one can also compare the fate map of phenotypically wild-type animals that are heterozygous for the *cre* allele, to mutant animals that are homozygous for the *cre* allele. Such an approach has been used to analyse the role of *Hoxb1* on the fate of hindbrain derivatives[81].

Cre-mediated gene activation. The first demonstration of Cre activity in mice, in which a transgene was activated by Cre-mediated recombination[82], provides a model for this general strategy. Activation occurs by Cre removing a 'Stop' DNA fragment, which prevents transcription and translation because it contains polyadenylation sequences, false translation initiation codons and/or splice donor sequences. Alternatively, recombination can restore an open reading frame that is interrupted by floxed sequences. In this case, the *loxP* site that remains after recombination must be tolerated as part of the opening reading frame[83]. Probably the best experimental approach to using this strategy is to design the target transgene for near-ubiquitous expression and then make use of the growing battery of mice that express *cre* in a tissue-specific manner (see link to the Cre transgenic database) to activate the target transgene. The creation of the *Rosa26* knock-in and Z/AP (or Z/EG) reporter mice, described above, exemplify how to ensure the otherwise difficult goal of achieving widespread transgene promoter activity in mice.

Investigators pursuing the Z/AP (or Z/EG) strategy should characterize the final ES clones *in vitro* before producing mice to ensure that ubiquitous expression of the target transgene is retained after Cre-mediated recombination. This will screen out the ~30% of ES clones in which the transgene is silenced after recombination for reasons that are at present unclear (C. Lobe, personal communication). Another advantage to using ES-cell technology to produce these target mice is that one can ensure that the transgene is present in a single copy, thereby avoiding a multiple array that occurs with the production of transgenic mice through PRONUCLEAR INJECTION. Cre-mediated recombination of such multiple arrays can result in chromosome loss[84], although it should be noted that this does not always occur[85].

As with the TetR-based system, the most rigorous demonstration of gene expression control has come from studies in which the activated target transgene expresses DTA driven by a tissue-specific promoter[83,86,87]. Several labs are now producing universal 'ablator' mice in which the DTA target is driven by a ubiquitous promoter, such that different Cre effector mice can be used for tissue-specific ablation experiments.

Endogenous genes can also be altered by gene targeting to render their expression conditional on Cre activity. In this case, Cre-mediated recombination results in the excision of a floxed fragment, such as *neo*, targeted to the intron of a specific locus to prevent expression[58,88]. So, in a design that is complementary to a tissue-specific knockout experiment, gene function can be studied by using Cre-mediated recombination to achieve tissue-specific gene repair. This approach has been used to model the human dominant skin-blistering disorders — epidermolytic hyperkeratosis and epidermolysis bullosa simplex. In these studies[89,90], mouse keratin genes that contain point mutations identical to human disease alleles were silenced with a floxed *neo*. Cre activation in the skin of these mice that was produced by the topical application of an inducer (described below), activated the mutant allele, resulting in a phenotype that models the human syndromes[89,90].

PRONUCLEAR INJECTION
One of two methods for producing a transgenic mouse line (the other method being through germ-line transmission of transgenic embryonic stem cells). In this approach, DNA is microinjected into the nucleus of a mouse zygote. The DNA integrates randomly, usually into one genomic locus, as a multiple array.

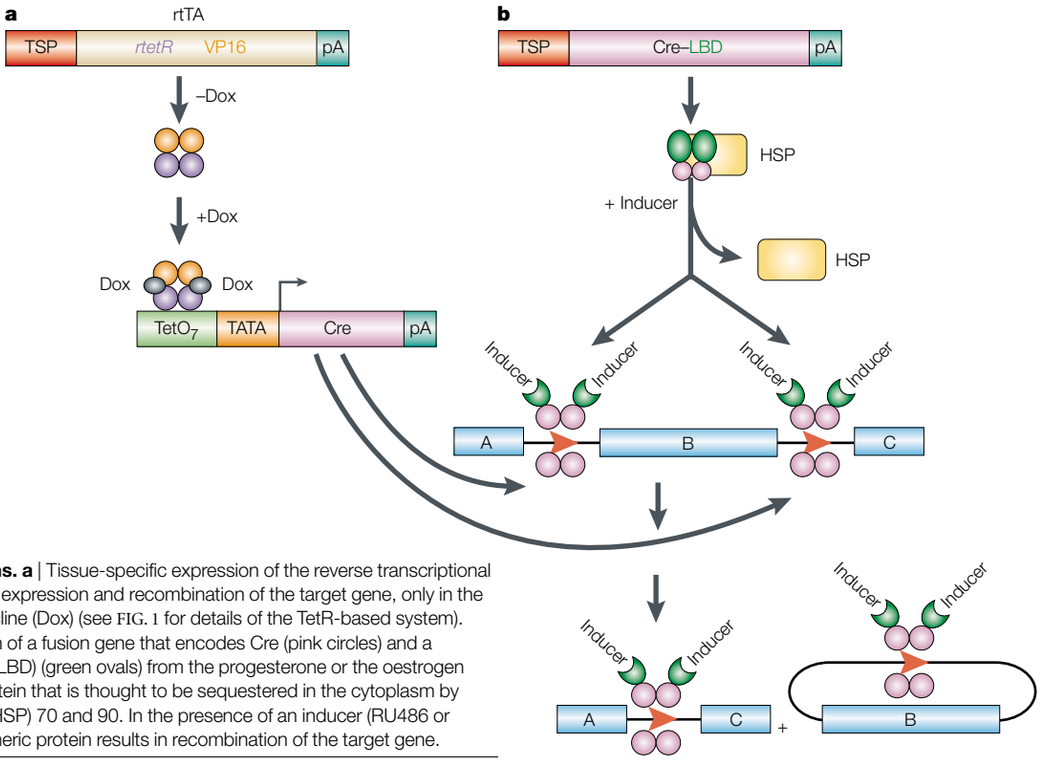


Figure 5 | **Inducible Cre systems. a** | Tissue-specific expression of the reverse transcriptional transactivator (rtTA) results in *cre* expression and recombination of the target gene, only in the presence of the inducer, doxycycline (Dox) (see FIG. 1 for details of the TetR-based system). **b** | The tissue-specific expression of a fusion gene that encodes Cre (pink circles) and a mutated ligand-binding domain (LBD) (green ovals) from the progesterone or the oestrogen receptor results in a chimeric protein that is thought to be sequestered in the cytoplasm by binding to heat-shock proteins (HSP) 70 and 90. In the presence of an inducer (RU486 or tamoxifen, respectively), the chimeric protein results in recombination of the target gene.

Animated online

Temporal control of Cre expression. The first inducible Cre mouse line used the Mx1 promoter, which can be activated by injecting mice with interferon-α or interferon-β or with synthetic double-stranded RNA (polyinosinic–polycytidylic acid)[91]. The use of these mice has been limited to regulating gene expression in the liver[92–95] and immune system[96–98] where recombination efficiency is high[91,94].

Overlaying temporal regulation with promoter specificity has been achieved in two ways: by combining the Cre/*loxP* system with the TetR-based systems or by using Cre variants that function only in the presence of an exogenous inducer (FIG. 5). In the latter approach, the Cre-coding region is fused with a mutated ligand-binding domain (LBD) from either the progesterone[99,100] or the oestrogen receptor[101–106]. These mutated LBDs will bind and become localized to the nucleus only in the presence of the synthetic steroids — RU486 or tamoxifen, respectively — but not in the presence of endogenous steroids. High recombination efficiencies with these Cre–LBD fusion proteins have been obtained in mouse skin[107,108], but, in general, recombination mediated by such fusion proteins has been mosaic and dependent on tissue type[99,103–104] despite nearly toxic doses of inducer[102,103,108]. Furthermore, the doses required for induction *in utero* are perilously close to abortigenic levels[102]. Nevertheless, these systems have been successfully applied to addressing several biological questions[89,109,110]. In one elegant study, boundaries that define specific structures in the developing limb were fate mapped at different embryological time points by tamoxifen-induced, Cre-mediated activation of a *lacZ* reporter gene[111]. New progesterone and oestrogen receptor-based Cre–LBD fusion proteins with enhanced sensitivities to their respective inducers are likely to increase the usefulness of this approach[112,113]. One of these — Cre–ERT2 — has been used to inactivate the mouse retinoid X-receptor-α in mouse adipocytes[114] and epidermis[115].

Several groups have combined the TetR-based system with Cre/*loxP* so that Dox controls *cre* expression. As recombination results in a permanent genomic change, monitoring Cre-mediated excisions in such transgenic mice is a sensitive assay for the basal, uninduced, expression of the *tetO*-containing promoter from which *cre* is expressed (FIG. 5a). Inducing *cre* expression with Dox in the rtTA system results in tighter regulation of *cre* expression (although not without basal expression)[34,116,117] in comparison with the tTA system[118]. Using this essentially tertiary transgenic system complicates mouse husbandry and increases the risk that position effects might occur, although this can be remedied by using a single Cre–rtTA construct[116,117]. It is also helpful to prescreen *tetO*/Cre floxed target mice to exclude mouse lines in which leaky, uninduced recombination occurs[34]. A *tetO–cre* transgene that responds well to induction and has low basal activity has been flrted so that Flp-mediated recombination might be used to replace *cre* at this locus with other potential target genes[34].

An approach that obviates the need for producing Cre effector mice and that achieves temporal control of Cre-mediated recombination is the use of viral vectors,

such as adenovirus[119–121] or herpes simplex[122], to deliver *cre*. This approach is most useful in adult animals, although it is possible to deliver adenoviral vectors to embryos *in utero*[123]. Limitations to this approach include complications due to possible host–virus interactions, mosaic recombination in the targeted tissue and variable infectivity between different tissues. Nevertheless, the viral delivery of *cre* to both activate transgenes and inactivate endogenous genes has been used in studies of liver function, cancer and the neurological basis of learning[124–126]. Limitations can also be turned into advantages depending on the question being posed. For example, the adenoviral delivery of *cre* has been used to generate pancreatic β-cells in which the glucokinase gene is mosaically inactivated, showing that intercellular coupling has little to no function in glucose-stimulated insulin secretion[127].

The Flp/FRT system. Initial attempts to develop the Flp/*FRT* system in transgenic mice resulted in either no recombination[128] or mosaic recombination[129] because wild-type Flp and an Flp variant (Flp-F70L) have decreased enzyme stability at 37 °C (REF. 130). However, a themostable variant — Flpe — has been generated[40] that mediates recombination efficiencies *in vivo* resembling those of Cre[131]. All three Flp variants have their uses depending on whether an experiment calls for complete or mosaic recombination levels[40,131].

Although use of the Flp/*FRT* system is unlikely to be as widespread as that of the Cre/*loxP* system, it continues to be developed as a genetic tool and will be useful as a supplementary system. For example, Flp has been used in tissue-specific gene-inactivation studies[132], *FLP*–LBD fusions have been generated[133,134] and a *Rosa26*-based Flp reporter mouse has been produced[135]. Recently, fate mapping through Flp-mediated recombination showed that *Wnt1* expression in the neural crest might define a subset of progenitor cells that eventually gives rise to specific types of precerebellar neurons[136]. As the authors point out, Flp-based lineage mapping can now be used to analyse phenotypes caused by Cre-based, tissue-specific gene knockouts.

In summary, the effective irreversibility of site-specific DNA recombination makes systems such as Cre/*loxP* and Flp/*FRT* uniquely suited for tissue-specific gene-inactivation and cell-lineage studies during embryogensis and in the adult. However, as the list of tissue-specific *cre* mice grows, we might find that in some cases high recombinase activity leads to chromosomal aberrations[46,47]. Another limitation is shown by the recent demonstration that recombination efficiencies can be locus dependent[74], although other studies have shown the efficient recombination of three floxed alleles in the same cell[137].

The future

To ask increasingly more sophisticated biological questions, we will eventually need to individually control the expression of two or more genes in the same mouse line. For example, inactivating a ligand in a specific tissue, rescue of the resulting phenotype by transactivating a constitutively active receptor in an adjacent tissue, and then characterizing the final phenotype by DNA-recombination-based fate mapping might be required. Therefore, systems such as the ecdysone-regulated gene switch[138] or the *lac* operator–repressor[139], might be increasingly used to address biological questions in transgenic mice. The ecdysone system is a transactivating system, based on a modified *Drosophila* steroid receptor that has been used to achieve high levels of target-gene induction in cultured cells. However, stocks of the ecdysteroid inducer — muristerone A — which is derived from the seeds of the Himalayan kaladana plant, have become depleted, limiting the use of this system in mice, although this barrier might be overcome with a recent report of alternative inducers[138]. The *E. coli lac* operator–repressor system has been recently modified for use in mice by altering the *lacI* codon usage, eliminating cryptic splice sites and removing methylation-prone CpG islands[139]. Alternative DNA recombination-based systems, such as β-recombinase[140] and ϕC31 integrase[141] are also being developed as genetic tools. The generation of Cre and Flp variants (or hybrids such as 'Clp' and 'Fre'[142]) that recognize different recognition sequences might also serve as future recombination systems[143].

All of these systems rely on appropriate promoters and/or enhancers to control the expression of effector transgenes whether they encode either DNA recombinases or transactivators. So, we are often still dependent on the most basic level of transgenesis: the ability to control the tissue-specific transcription of a transgene, ideally free of position effects. Approaches such as gene targeting, 'promoter traps' (for more on insertional mutagenesis, see accompanying review by Stanford *et al.* on p756 of this issue), BAC engineering (for more on this strategy, see accompanying review by Copeland *et al.* on p769 of this issue) and traditional transgenics, each with its particular advantages and disadvantages, expand our ability to achieve tissue specificity with simple transgenesis and therefore our control over these binary systems.

Finally, the molecular domains of the two broad categories of conditional transgenesis described here are DNA (site-specific recombination) and RNA (transcriptional transactivation). What about altering activity at the protein level? Technology that allows enzyme stability and activity to be regulated might soon be used in mouse transgenics as well. For example, chemical inducers of dimerization have been used to regulate protein activity[144], and small molecules that regulate specific enzyme activities or biological processes have been identified in chemical genetic screens[145], which can be considered to be a type of 'conditional epigenetics' because only protein and not DNA is affected. So, we are approaching an era in which we will not only be able to conditionally control gene expression in mice where and when we choose, but also at the desired level of gene expression. We might be asymptotically approaching this era, because there are always some limitations and unforeseen technical problems to be solved, but with every step the limiting reagent becomes not our tools but, increasingly, our imaginations.

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