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# Molecular Neuroanatomy's 'Three Gs': A Primer

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## Abstract

New genetic technologies are transforming nervous system studies in mice, impacting fields from neural development to the neurobiology of disease. Of necessity, alongside these methodological advances, new concepts are taking shape with respect to both vocabulary and form. Here we review aspects of both burgeoning areas. Presented are technologies which, by co-opting site-specific recombinase systems, enable select genes to be turned on or off in specific brain cells of otherwise undisturbed mouse embryos or adults. Manipulated genes can be endogenous loci or inserted transgenes encoding reporter, sensor, or effector molecules, making it now possible to assess not only gene function, but also cell function, origin, fate, connectivity, and behavioral output. From these methodological advances, a new form of molecular neuroscience is emerging that may be said to lean on the concepts of genetic access, genetic lineage, and genetic anatomy – the three 'Gs' – much like a general education rests on the basics of reading, 'riting and 'rithmetic.

## Introduction

One of the enduring mysteries of biology is how the billions of neurons in the developing brain take on specific fates and establish connections in exactly the right place and order. Their deployment into precise functional arrays or circuits—including those that make possible such complex activities as perception, behavior, cognition, and memory—is governed by a vast collection of genes. Over a third of the human genome is thought to be dedicated largely or exclusively to directing the development, maintenance and function of the various cells and circuits of the nervous system. Understanding which neurons depend on which genes is a fundamental challenge of contemporary neuroscience, one made all the more arduous by the extraordinary numbers of genes and cells involved. Though progress has been made in pinpointing gene products responsible for the development and functioning of some brain structures in organisms ranging from fruit flies to chick to mice, efforts in mammalian models such as the mouse, which develop in utero, have presented a thorny set of problems. An obvious obstacle has been access: it has been difficult to manipulate the brains of mammalian embryos in ways that do not interfere with their development. But no longer. Over the past decade, advances have made it almost routine now to turn specific genes on or off in selected subsets of cells either inside an otherwise undisturbed developing mouse embryo or inside a specific organ of a living adult mouse (for reviews see Branda and Dymecki, 2004; Dymecki, 2000; Joyner and Zervas, 2006; Lewandoski, 2001; Miyoshi and Fishell, 2006; Wulff and Wisden, 2005). The manipulated genes can be exogenously engineered transgenes that have been inserted into the mouse genome and which encode various types of reporter, sensor, or effector molecules, or they can be actual endogenous loci.

More recently, neuroscientists have been co-opting this rapidly evolving set of genetic methods to remarkable ends, be it towards expanding our understanding of neural development or adult neurological disease. For example, developmental neurobiologists have fashioned these

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genetic methods to enable tracking molecularly defined populations of cells as they take their place in the developing mouse brain (reviewed in Branda and Dymecki, 2004; Joyner and Zervas, 2006; Pearse and Tabin, 2006; Wingate, 2005). The resulting ‘genetic fate maps’ are yielding surprising information about where in the brain specific cells arise, journey and ultimately take up permanent residence, as well as about what gene products may be involved along the way. The findings, while critical for understanding how the brain develops, could have broader clinical implications for the diagnosis and treatment of a variety of disorders from developmental to degenerative. Knowing which gene products participate in or associate with the formation and functioning of specific brain areas, that is understanding neuroanatomy on molecular terms, could lead to the identification of new disease markers, drug targets, and possibly to cell type-specific gene therapies, as well as to novel approaches for regenerating specific tissue. For studying adult neurological disease, equally exciting approaches have been crafted out of this new mouse genetic toolbox. For example, tools have been fashioned to repair postnatally genes inherited in a silenced form (for example, Gross et al., 2002; Guy et al., 2007). This has made it possible to test whether inherited neurological phenotypes are reversible in postnatal or adult mice – knowledge critical for guiding therapeutic strategies.

Our purpose here is to introduce a series of techniques that are making possible, in many ways, this burgeoning field of molecular neuroanatomy. We do so in the context of a broader discussion of three concepts that are taking shape with respect to both vocabulary and form.

## The ‘Three Gs’

A general education rests on the basics of reading, ‘riting and ‘rithmetic. Similarly, molecular neuroanatomy may be said to lean on three fundamental concepts — *genetic access*, *genetic lineage*, and *genetic anatomy* (reviewed in Branda and Dymecki, 2004; Joyner and Zervas, 2006). Just as reading makes writing and arithmetic possible, so too the ability to construct genetic lineages and genetic anatomies depends on the first G, genetic access. Broadly defined, genetic access is the ability to introduce into selected cells in the brain of living animals a genetically encoded molecule that, when expressed, can visualize, assay, or perturb those neurons' development, connectivity, or function. To achieve such cell-specific effects, the genetically encoded molecule must be paired with promoter and enhancer elements active only in the specific cell types. These transcriptional regulatory elements serve as ‘drivers’ of the system and are incorporated into a transgene along with the coding sequence for the effector molecule, whose only requirement is that it be genetically encoded. Such transgene construction can be accomplished in one of three general ways: by exploiting isolated enhancers known to be transcriptionally active just in the selected cells (among many, examples include Echelard et al., 1994; Palmiter et al., 1991; Zimmerman et al., 1994); by inserting the coding sequence into a bacterial artificial chromosome (BAC) that contains the relevant subset of promoter and enhancer elements (Gong et al., 2002; Heintz, 2001; Lee et al., 2001; Muyrers et al., 2001; Muyrers et al., 1999; Valenzuela et al., 2003; Yang et al., 1997; Zhang et al., 1998); or by introducing the coding sequence into the actual driver gene locus by homologous recombination in embryonic stem cells (reviewed in Hasty et al., 2000; Matise et al., 2000). Note that in this primer, we use the term transgene to refer to any stretch of foreign DNA placed into the mouse genome, whether its site of insertion is random, as occurs following DNA injection into a zygote pronucleus as a means to generate a transgenic mouse, or whether its site of insertion is targeted to a particular locus, as occurs when the avenue to the transgenic first exploits homologous recombination in embryonic stem cells. The latter case is also referred to as a ‘knock-in’ allele.

One of the most exciting advances has involved exploiting selective genetic access to deliver a particular class of molecule, a site-specific recombinase (SSR). The body of this primer will be devoted to describing various SSR-based technologies. The far-reaching impact of SSRs

stems from their capacity to produce very precise DNA rearrangements that, depending on design parameters, can result in deletion, insertion, inversion, or translocation of chromosomal DNA (reviewed in Branda and Dymecki, 2004; Dymecki, 2000; Garcia-Otin and Guillou, 2006; Nagy, 2000). What this means is that, once introduced into a cell, the SSR can modify a second or target gene, effectively turning it on or off (Figure 1A,B).

When coupled with a particular type of target transgene encoding a reporter molecule, the SSR approach can be used to trace a genetic lineage, the second 'G.' A genetic lineage is a population of cells identified by virtue of their arising from a progenitor cell population that is defined through its expression of a particular gene (Chai et al., 2000; Dymecki and Tomasiewicz, 1998; Jiang et al., 2000; Kimmel et al., 2000; Rodriguez and Dymecki, 2000; Zinyk et al., 1998). Genetic lineages are identified by an approach called genetic fate mapping (for reviews see Branda and Dymecki, 2004; Dymecki et al., 2002; Joyner and Zervas, 2006). Briefly, driver elements are used to both define a cell population molecularly and to express an SSR in that cell population; SSR action then results in permanent expression of a reporter molecule in those cells and their descendant cells, even well after SSR expression has ceased. The target reporter has, in effect, been transformed into an indelible cell lineage tracer, marking ancestors and descendants, regardless of cell type (Figure 1C). The resulting genetic fate map owes much of its power to two key features: it depicts a history of gene expression that corresponds to and possibly regulates the development of a particular cell type; second, it does so in a way that makes it possible to visualize, in three dimensions, how such cell lineages assemble, over time, into the various structures that make up the brain (examples include Farago et al., 2006; Landsberg et al., 2005; Machold and Fishell, 2005; Pearse and Tabin, 2006; Rodriguez and Dymecki, 2000; Sgaier et al., 2005; Wingate, 2005; Zervas et al., 2004; Zinyk et al., 1998).

Such molecularly defined structures constitute a genetic anatomy. This third 'G' depends on our ability to link gene expression domains (embryonic or adult) with anatomical structures. In situ detection of mRNA or protein on brain sections can be used to relate adult molecular expression patterns to classical, cytoarchitectonically defined nuclei, layers, regions, and tracts. Exciting progress is being made on this front due to the efforts of various consortia to systematically analyze gene expression in the nervous system using high-throughput RNA in situ hybridization and/or *BAC::reporter* transgenic methods (Gong et al., 2003; Lein et al., 2007; Magdaleno et al., 2006; Visel et al., 2004). Interestingly, many structures, classically defined based on cytoarchitectonic features, are proving to be heterogeneous molecularly, with sets of, rather than single, expressed genes seeming to define individual neuronal cell types.

Understanding genetic anatomy in this way not only highlights genetic programs active in particular neural cell types - crucial information when thinking about how particular cell physiologies and functions are achieved or maintained - but also offers opportunities for gaining genetic access to these cells in the adult organism. The latter is of great impact because, by providing a means to investigate gene, cell and/or circuit function in the adult brain, it offers a critical entrée for examining fundamental disease issues.

Relating embryonic (as opposed to adult) gene expression to adult anatomy is a bit trickier. It involves genetic fate mapping, just as in delineating genetic lineages, but the focus shifts away from simply identifying descendant cell types and instead towards understanding the contributions that specific genetic lineages make to various anatomically-defined structures, such as brain nuclei, layers, or tracts. By establishing genetic anatomies via genetic fate mapping, it becomes possible to classify neuronal assemblies in the adult brain in a new way - based on the molecular programs enacted earlier in their development rather than solely by gross cytoarchitecture. Considering that many antecedent gene expression events are formative with respect to final cell function, these new genetic, developmental classifications are likely to reveal physiologically relevant neuron groupings even if the 'groupings' are highly dispersed

spatially and therefore different from those defined based on shared position and cytoarchitecture.

## Site-specific recombinase technology figures central to each 'G'

Advances in the three 'Gs' – genetic access, genetic lineage, and genetic anatomy – have depended substantially on the use of SSRs. The most commonly used being Cre (causes recombination of the bacteriophage P1 genome) and Flp (named for its ability to invert, or "flip," a DNA segment in *S. cerevisiae*) (for reviews see (Branda and Dymecki, 2004; Dymecki, 2000; Stark et al., 1992)). They have several advantages. They function well in many species including fruit flies (Dang and Perrimon, 1992; Golic and Lindquist, 1989; Xu and Rubin, 1993) and mice (Dymecki, 1996; Lakso et al., 1992; Orban et al., 1992; Rodriguez et al., 2000); they catalyze recombination between specific DNA target sequences—*lox*P sites, in the case of Cre (Hoess et al., 1982), and *FRT* sites, for Flp (McLeod et al., 1986) – that are not normally found in the fly or mouse genome; and they do so with high fidelity and without the need for cofactors. Creative placement of *lox*P and/or *FRT* sites into the mouse genome can be used to effectively engineer a variety of predetermined modifications, including gene deletions, insertions, inversions, or exchanges. It is the relative orientation of target sites with respect to one another that determines the outcome of SSR-mediated recombination (Hoess et al., 1986) and reviewed in Branda and Dymecki, 2004; Dymecki, 2000). For example, when target sites are arranged in a head-to-tail fashion, the result is the excision of intervening DNA sequences (Figure 1A,B), a reaction that is effectively irreversible due to rapid loss of the excised (circular) product. This simple excision, exploited most readily in the mouse, serves as the basis for most genetic fate mapping and conditional gene activation and inactivation strategies.

In one prototypical SSR strategy, shown in Figure 1A, SSR-mediated excision is used to render active, ie. repair, a functionally silenced transgene. The reporter or effector molecule encoded by the target transgene is expressed upon excision, thus illustrating how SSRs can be used to control gene expression. Typically, such a target transgene contains three elements: (1) a gene encoding the desired reporter or effector molecule; (2) an inserted STOP cassette that functionally silences the gene and is flanked by *lox*P (floxed) or *FRT* sequences (flrted); and (3) promoter/enhancer sequences capable of driving the expression of the reporter or effector gene following SSR-mediated recombination. Thus, expression of the target reporter or effector gene is dependent on two things: excisional recombination, as determined by the cell type-selective expression of the SSR, and on the promoter/enhancer sequences incorporated to drive expression of the recombined target transgene itself. Critical to the conditional aspect of this type of SSR-based strategy is the effectiveness of the STOP cassette. Three are listed here in descending order of efficiency (J. C. Kim and S. Dymecki, unpublished findings): the $lox^2$ cassette (Sauer, 1993), containing SV40 intron and polyadenylation (pA) signal sequences, a gratuitous ATG translation start, and 5′ splice donor signal; a concatemer of SV40 pA sequences (Awatramani et al., 2001; Awatramani et al., 2003; Farago et al., 2006; Lobe et al., 1999; Soriano, 1999; Zinyk et al., 1998); and a concatemer of bovine growth hormone pA sequences (Awatramani et al., 2003; Farago et al., 2006).

A second prototypical strategy (Figure 1B) involves first positioning the *lox*P or *FRT* recombinase target sites within an endogenous gene. Target sites can be positioned to either functionally silence the endogenous gene, which can then be repaired conditionally through SSR-mediated excision of the disrupting cassette (conceptually similar to the transgene activation approach outlined earlier)(Figure 1B, lower scheme)(for example Guy et al., 2007) and reviewed in (Dymecki, 2000); or, as is more common, target sites can be placed so that SSR action results in removal of endogenous gene sequences to create, for example, a

conditional null allele (Figure 1B, upper scheme) (Gu et al., 1994) and reviewed in Branda and Dymecki, 2004).

## Transgene activation begets genetic fate maps that beget genetic lineage information

Fate mapping, by defining relationships between embryonic and adult structures, is, in general, one of the most important tools on hand to developmental neurobiologists and stem cell scientists. Traditionally, it has required unfettered access to the developing embryo for injection of retroviral (Cepko et al., 1990; Galileo et al., 1990; Walsh and Cepko, 1988), fluorescent (Wetts, 1988), or vital dye lineage tracers (Keller, 1975), or the grafting of quail cells into chick embryos (Le Douarin, 1982), thus, making it difficult to carry out in mammals. Furthermore, these methods used for fate mapping in non-mammalian vertebrate systems have been limited by an inability to define, in clear molecular terms, the cell population that was initially labeled. These drawbacks can be circumvented by applying SSR-mediated approaches to activate reporter molecules like ßgalactosidase (ßgal) or green fluorescent protein (GFP) in an indelible, cell-heritable fashion—in effect, turning these reporter molecules into lineage tracers (Figure 1C).

Because the SSR target transgene is integrated into the mouse genome, it is cell heritable. Should recombination occur in a progenitor cell, all of its daughter and granddaughter cells will go on to inherit the target transgene in its recombined form. This heritability feature, coupled with the use of widely active promoter/enhancer sequences to drive target transgene expression once recombination has occurred, means that the recombined transgene, in most cases, will be expressed in the progeny as well as parental progenitor cells, thus turning a simple gene activation strategy (Figure 1A) into a fate mapping strategy (Figure 1C). If the target transgene promoter and enhancer sequences are capable of driving transgene expression in any cell type at any stage of development (ideally), then after a recombination event in a given cell (Figure 1C, lower panel, left) that cell and all its progeny cells should be marked by reporter expression regardless of subsequent cell differentiation (Figure 1C, lower panel, right). It is important to note that genetic fate mapping typically tracks the fate of a molecularly defined *population* of cells, as opposed to delineating the descendants of a single cell. Thus, in most standard genetic fate maps, clonal relationships cannot be strictly discerned; rather, additional features, described later, must be added to the genetic fate mapping technology in order to resolve what are likely to be cell clones. This contrasts the exacting clonal analyses achievable by various retroviral infection methods (Cepko et al., 1990; Galileo et al., 1990; Golden and Cepko, 1996; Walsh and Cepko, 1992) that have been especially useful in, although not limited to, avian systems.

One approach that has proven advantageous in offering the potential to fate map most cell types, has been to drive target transgene expression via a combination of highly active transcriptional regulatory elements (Farago et al., 2006; Zong et al., 2005), for example, collectively using the chicken *ß-actin* promoter, cytomegalovirus enhancer sequence (when paired together, they are referred to as *CAG*, (Niwa et al., 1991)), and regulatory sequences from the endogenous mouse *Gt(ROSA)26Sor* (*R26*) locus (Zambrowicz et al., 1997). The *R26/CAG* partnership may offer improved breadth and levels of expression by comparison to either *R26* or *CAG* alone (Farago et al., 2006; Zong et al., 2005), especially in the postnatal brain. Using broadly active promoter/enhancer elements (like *R26/CAG*) to drive target transgene expression means that the same transgene can be used to study a wide range of cell types, rendering it a fairly universal fate mapping tool. Of course, the actual range of cell types that can be marked by a given target transgene needs to be determined empirically. For example, an approximation of scope can be gained by analyzing tissue from an animal in which the target transgene has been partnered with a broadly expressed SSR transgene, such that target

transgene repair occurs in most, if not all, cell types, each of which then can be sampled for robustness of reporter expression (Awatramani et al., 2001; Rodriguez et al., 2000; Rodriguez and Dymecki, 2000; Soriano, 1999). Another set of regulatory elements shown highly valuable for driving target transgene expression in the nervous system come from the *tau* gene (Kramer et al., 2006). It is important to note that the efficiency of SSR-catalyzed excision events varies depending on where target sites (*lox*P or *FRT*) are placed in the mouse genome, with some loci being more permissive than others – in this regard, both *R26* and *tau* appear to sustain efficient SSR-mediated stop-cassette removal.

In general, we prefer referring to conditional target transgenes for genetic fate mapping as conditional 'indicator' transgenes or alleles, both to distinguish them from more conventional, constitutively driven (non-conditional) *promoter::reporter* transgenes and to emphasize that the target transgene, through reporter expression, serves to 'indicate' or provide a permanent record of all earlier occurring recombination events. These sorts of genetic fate maps have been described as 'cumulative' because such a map will include any cell that has ever in its history expressed the SSR; if the SSR driver is dynamically expressed over space and time, as embryonic development progresses, new populations of expressing cells and their descendants will become successively incorporated in the fate map.

Depending on the type of reporter molecule encoded by the indicator transgene, different features of the mapped cell population may be uncovered in addition to their genetic history. For example, incorporating into the target indicator transgene a nuclear-localized version of the reporter molecule ßgalactosidase (nßgal) allows one to visualize individual cells in a highly sensitive way. Cytoplasmic or membrane-localized reporters, on the other hand, often do not allow for such resolution, especially when cells are tightly clustered. nßgal can further help determine the identity of final progeny cells through colocalization with transcription factors that are capable of serving as cell identity markers. For resolving cell morphology, including axonal projections, it is helpful to employ a reporter molecule capable of revealing cell shape by virtue of either filling or outlining that cell. This can be achieved by many of the standard cytoplasmic reporters such as GFP, or by membrane-tethered reporter molecules such as alkaline phosphatase or farnesylated- (or myristylated-) GFP. Endogenously fluorescing protein reporters such as GFP may offer the further possibility of visualizing dynamic changes in live cell morphology and position, and may permit electrophysiology in cultured brain slices or explants. Indeed, as quickly as new reporter molecules are being developed (Giepmans et al., 2006; Miyawaki, 2003a; Miyawaki, 2003b; Miyawaki, 2005; Shaner et al., 2004), they are being incorporated into Cre- or Flp-responsive indicator transgenes.

An important strength of this genetic fate mapping approach worth emphasizing is that once an indicator transgene has been 'activated' by SSR-mediated recombination, the encoded reporter molecule is expressed constitutively by that lineage from that point onward. This feature serves to ensure relatively robust marking of descendant cells regardless of cell type or developmental stage; for example, it is typically possible to visualize lineage contributions to adult structures despite the extensive elapse in time between the initial (embryonic) recombination event and the actual (adult) tissue analysis. By contrast, this is not always the case when certain surrogate genetic fate mapping approaches are used, for example, when using perdurance of a reporter molecule that, at an earlier time point, was expressed from a non-conditional, transiently active *promoter::reporter* transgene or knock-in allele. The 'fate maps' resulting from this surrogate type of approach, in the strictest sense, may not be completely accurate because some lineages may be missed due to their more rapid elimination of the residual reporter molecules and/or lower starting level of reporter expression. Further, if (non-conditional) *reporter* knock-in alleles are being exploited that are null for the endogenous gene product, attention must also be given towards understanding whether allele expression is regulated by the endogenously encoded gene product, for example, if it is a transcription factor

that positively regulates/sustains its own expression. In this case, homozygous *reporter* knock-in animals, being devoid of the endogenous gene product, may more rapidly extinguish *reporter* expression than heterozygotes or wild-type animals. The reduction in reporter levels could result in an inability to detect whatever scant reporter molecules remain, resulting in erroneous exclusion of certain cells from a fate map and thus compromising the accuracy of the 'fate map.'

Having reviewed the major parameters surrounding target indicator transgenes, there are considerations to be made with respect to the SSR, especially Flp. There are three variants of Flp that have been employed in mice—Flpe (enhanced Flp), Flp-wt, and FlpL (low-activity Flp). Taken together, the trio collectively spans greater than a ten-fold range in activity in mice (Awatramani et al., 2003; Farago et al., 2006; Landsberg et al., 2005; Rodriguez et al., 2000; Rodriguez and Dymecki, 2000). Enhanced Flp, or Flpe, harbors four point mutations that together confer increased thermostability while maintaining normal target (*FRT*) specificity (Buchholz et al., 1998). To date, Flpe has been shown to function in mice with similar efficacy as Cre. For example, the cell populations fate-mapped using *Wnt1::cre* (Danielian et al., 1998; Jiang et al., 2000) and *Pet1::cre* (Scott et al., 2005) transgenics were mapped with similar efficiencies by *Wnt1::Flpe* and *Pet1::Flpe* transgenics (Awatramani et al., 2003; Farago et al., 2006; Landsberg et al., 2005) (and unpublished findings by A. Farago, N. Hunter, P. Jensen, and S. Dymecki) — the Flpe-based set did not fall short. Still, it is worth noting that Flpe may not perform as well as Cre in cultured mouse embryonic stem cells (Schaft et al., 2001); towards improving Flp activity in ES cells, a mouse codon-optimized form of Flp called Flpo has recently been generated (Raymond and Soriano, 2007).

By contrast to the very robust nature of Flpe in vivo is the modest activity exhibited by the variant FlpL (L for low activity) (Landsberg et al., 2005; Rodriguez and Dymecki, 2000). FlpL contains a single amino acid substitution that renders the recombinase thermolabile (Buchholz et al., 1996), resulting in at least a five-fold reduction in recombinase activity from wild-type (Flp-wt). At first glance, FlpL may appear to have little utility but, it turns out, alongside Flpe it can be exploited to achieve a range of cellular resolution in fate mapping studies. For example, parallel use of FlpL and Flpe has led to the first evidence that the germinal zone called the hindbrain rhombic lip can be fate mapped into two broad dorsoventral domains that give rise to different neurononal cell types (Landsberg et al., 2005; Rodriguez and Dymecki, 2000). These "Flpe/FlpL comparative fate mapping" studies exploited the gradient of *Wnt1* expression that demarcates the hindbrain rhombic lip dorsal-to-ventral. As a consequence of this gradient, a smaller dorsal region of the hindbrain rhombic lip was marked and fate mapped using a *Wnt1::FlpL* transgene (capable of inducing recombination in *Wnt1* mRNA$^{high}$ cells only) than one using a *Wnt1::Flpe* transgene (capable of inducing recombination both in *Wnt1* mRNA$^{low}$ and *Wnt1* mRNA$^{high}$ cells). These experiments led to the finding that the brainstem nuclei that project to different target neurons in the cerebellar cortex actually originate from molecularly and spatially distinct progenitor cell populations in the hindbrain rhombic lip (Landsberg et al., 2005; Rodriguez and Dymecki, 2000). These findings were then substantiated on fate mapping other gene expression domains, like *Math1*, that subdivide dorsoventrally the *Wnt1* domain (Landsberg et al., 2005; Machold and Fishell, 2005; Wang et al., 2005).

As mentioned above and at the beginning of this primer, selective SSR delivery involves exploiting cell type-restricted promoter and enhancer elements as drivers (in the above example, elements from the *Wnt1* gene (Echelard et al., 1994)). This is accomplished either by employing conventional transgenic methods (construct size typically <20kb), BAC transgenic strategies (constructs typically around ~200 kb), or knock-in approaches; choice of method is driven by experimental need and availability of isolated DNA elements. For example, knock-in approaches typically offer the greatest fidelity in matching SSR expression to that of the

endogenous gene of interest. Further, if SSR insertion by knock-in is designed to silence the endogenous gene, then homozygotes can be used in genetic fate mapping strategies to reveal how cell fate may change in the absence of that gene product; a downside, though, would be if heterozgotes exhibit haploinsufficiency thereby precluding generation of the needed wild-type fate map. One way to circumvent haploinsufficiency is to knock-in the SSR-encoding sequence, preceded by an internal ribosome entry sequence (IRES), into the 3′ untranslated region of the gene of interest; a bicistronic transcript is produced encoding the endogenous gene product followed by the SSR. While this approach has been successful (Lee et al., 2000), lower than desirable SSR levels may result in some cases, compromising recombination efficiency and therefore the ability to mark and track all cells arising from the gene expression domain – especially vulnerable would be cells that normally express the endogenous gene at lower levels. Transgenic approaches (conventional and BAC) avoid haploinsuffiency issues as no endogenous gene is typically disrupted. BAC transgenics are quite powerful in that, for most genes, a BAC can be identified that contains all necessary regulatory elements to confer normal gene expression and because they are not subject typically to the strong position effects resulting in transgene silencing or misexpression that can plague conventional constructs (Heintz, 2001). An advantage of conventional transgenics is that they permit use of isolated enhancer elements as drivers, should they be available, such that SSR expression can be delivered to just a subset of an otherwise larger gene-expressing cell population (Zinyk et al., 1998). Regardless of approach taken, it is critical to establish the extent to which SSR expression matches the expected driver gene expression profile, and the extent to which indicator transgene recombination (reporter expression) matches the initial driver gene expression profile - later they will diverge because the reporter expression is cumulatively and permanently tracking all cells that ever in their history expressed the driver gene whereas the driver gene expression is transient (Figure 1C). Further, it is crucial to determine if there is any unexpected ectopic SSR expression, as this would confound subsequent fate mapping studies by switching on the lineage tracer in unrelated cells that would be erroneously interpreted as part of (or lumped into) a given lineage. It is equally critical to assess whether the SSR is capable of activating reporter expression (through indicator transgene recombination) in all driver gene-expressing cells, even low expressors; this is especially important, for example, if the SSR is expressed downstream of an IRES.

Through careful use of Cre- and Flp-mediated techniques, our ability to define genetically related cell populations and lineages is growing exponentially. Resulting fate maps are revealing developmental homologies not only among various brain structures (Chizhikov et al., 2006; Farago et al., 2006; Landsberg et al., 2005; Machold and Fishell, 2005; Nichols and Bruce, 2006; Sgaier et al., 2005; Wang et al., 2005), but also among structures as disparate as the mid/hindbrain and limb (Guo et al., 2003; Kimmel et al., 2000; Zervas et al., 2004). Moreover, possible roles of gene products in development are being suggested and can be tested in gene loss-of-function experiments; how certain gene modifications alter the migratory routes and ultimate fate of specific cell types can now be assayed directly.

Expanding the mouse toolkit further may be two additional recombinase systems, both of which have shown promise in mammalian cell culture (Andreas et al., 2002; Belteki et al., 2003; Sauer and McDermott, 2004). The first is a close relative of Cre, called Dre for D6 site-specific recombinase. Dre is encoded by the bacteriophage D6 genome and recognizes a DNA target site called *rox* (Sauer and McDermott, 2004), The second is ØC31, a recombinase from *Streptomyces lividans*, for which a mouse codon-opitimized version, ØC31o, has recently been synthesized and shown effective in mouse embryonic stem cells (Raymond and Soriano, 2007). It is exciting to ponder all the possible new applications that might be afforded by having additional SSRs for use in mice.

## Resolving genetic sublineages—improving the where and when of genetic fate mapping

Despite the molecular precision afforded by genetic fate mapping, many biological questions remain unanswerable because of the broad extent of cell types marked even by a single gene expression domain. For example, spatially, embryonic gene expression domains commonly restrict along one axis of a tissue or germinal zone but extend along the orthogonal axes (Figure 2A). This more expansive dimension will often intersect or overlap with multiple other gene expression domains such that it actually contains multiple uniquely coded molecular subpopulations (Awatramani et al., 2003; Farago et al., 2006; Lumsden and Krumlauf, 1996) (Figure 2A). Resolving these subpopulations and their specific descendant lineages (and thus the relationship between this combination of expressed genes and future cell fate) is not possible using the genetic fate mapping approaches presented so far. Resolution could also be improved along the temporal axis. Genes may be expressed in different cell populations at different times; or they may be constitutively expressed in a given cell population for an extended time period, such as may occur in a germinal zone during a period in which different cell types arise. Resolving the temporal aspects of lineage allocation, for example, from such a molecularly defined germinal zone is not possible by standard genetic fate mapping, as the approach is cumulative in nature.

The need to better distinguish genetic lineages spatially and temporally has driven the development of more sophisticated SSR-based tools. Two general types of advances are presented below: intersectional genetic fate mapping (Awatramani et al., 2003; Farago et al., 2006) and genetic inducible fate mapping (GIFM; reviewed in Joyner and Zervas, 2006). In principle, each has the potential to provide order-of-magnitude improvements in the ability to select cells for fate mapping and to visualize them clearly.

## Improving selectivity using intersectional and subtractive genetic methods

For intersectional genetic fate mapping, two SSRs, Cre and Flpe, are paired in a dual recombinase-mediated transgene activation paradigm (Figure 2B) (Awatramani et al., 2003; Farago et al., 2006) and reviewed in Branda and Dymecki, 2004; Joyner and Zervas, 2006; Miyoshi and Fishell, 2006). Lineage tracer expression is switched-on only in cells that have undergone two genetic events in their history, one mediated by Cre (and therefore driver *gene A*) and the other by Flpe (and therefore driver *gene B*). Only those cells lying at the intersection of the two gene expression domains (A and B) will activate lineage tracer expression (Figure 2B-D,GFP-expressing cells). By mapping cell lineages based on the expression of gene pairs, rather than of a single gene, it is possible to begin defining combinatorial gene expression codes that associate with the development of particular cell types (Figure 2D). It can also lead to the identification of smaller subpopulations of genetically defined cells, thus improving upon the first G, genetic access. The combination of expressed genes (eg., driver *gene A* and driver *gene B*) does not have to coincide temporally. The two genes may be expressed at different times in a cell's developmental history, with activation of the intersectional lineage tracer occurring only after the second recombination event has been completed (Figure 3). This means that temporal, as well as spatial, resolution in lineage allocation can be improved.

In addition to fate mapping intersecting Cre/Flpe cell subpopulations (green cells in Figure 2B-D), the methodology can be engineered to allow simultaneous tracing of Cre/non-Flpe lineages (Figure 2C, left panel, blue nßgal cells). These lineages are referred to as ‘subtractive’ populations because they are what remains when Cre/Flpe intersecting cells are subtracted from the Cre-only expressing domain (Farago et al., 2006). The dual recombinase-responsive target transgene can additionally be engineered such that the subtractive population is the

reverse Flpe/non-Cre population; it simply requires changing the order of floxed and flrted cassettes (Figure 2C).

Using two recombinases simultaneously for intersectional and subtractive genetic fate mapping can be a highly efficient method for marking progenitor cells lying at the intersection of two gene expression domains, as demonstrated in recent developmental studies of the brain stem (Awatramani et al., 2003; Farago et al., 2006). For example, using the PF strategy schematized in Figure 2C, the GFP-positive intersectional domain was found to exhibit few to no nßgal-expressing cells, while nearly all of the cells in the Cre-only domain are ßgal+, indicating nearly 100% efficiency of Flpe-mediated recombination within the intersectional domain and Cre-mediated recombination in both the intersectional and subtractive domains (Farago et al., 2006). Another critical factor in this approach is that the reporter molecule associated with the subtractive population (nßgal in this example) has a relatively short half-life. This ensures that the subtractive reporter (nßgal) will not be detected in intersectional descendants (eg., GFP+ cells), which could be the case even after excision of the coding sequence if the subtractive reporter half-life was long. This would diminish the resolution of the subtractive strategy because it would mean that some of the intersectional cells could be lumped into the subtractive fate map if only the subtractive reporter signal (e.g. nßgal) was analyzed. Importantly, the dual recombinase responsive indicator alleles generated to date show elimination of the subtractive reporter molecule (a particular form of nßgal) in less than 72 hours of coding sequence excision – earlier time points were not examined so the half-life could be even shorter (Farago et al., 2006). Importantly, using a reporter molecule with a relatively short half-life does not compromise one's ability to visualize the desired true subtractive lineages because in those cells the reporter molecule continues to be expressed constitutively, for example from *R26/CAG* sequences.

Using this intersectional and subtractive approach to study brainstem progenitors, we have shown recently that the cochlear nuclear complex, the entry point for all central auditory processing, is assembled from molecularly distinct progenitor cell subpopulations arrayed as rostrocaudal microdomains within and outside the hindbrain (lower) rhombic lip (Farago et al., 2006). This work also uncovered surprising parallels and unexpected distinctions between the development of the brainstem auditory and cerebellar systems.

On a practical level, dual recombinase-responsive indicator transgenes harbor an additional advantage. Three different mouse lines can result from one initial transgene construction and strain generation: one dual recombinase-responsive indicator line and two derivative single recombinase-responsive lines. The latter two are readily generated through germ line deletion of either the *lox*P- or *FRT*-flanked cassette (Farago et al., 2006).

## Genetic Inducible Fate Mapping

A second means by which cell-type selectivity can be enhanced is through GIFM, genetic inducible fate mapping (Ahn and Joyner, 2004; Guo et al., 2003; Harfe et al., 2004; Kimmel et al., 2000; Zervas et al., 2004; Zirlinger et al., 2002) and reviewed in (Branda and Dymecki, 2004; Joyner and Zervas, 2006; Miyoshi and Fishell, 2006). This approach, which depends on ligand-regulated forms of Cre or Flpe, offers a means to temporally control SSR activity. For example, it can allow SSR-mediated recombination to be targeted only to those cells responsible for the late aspects of a dynamic or extended gene expression profile. In this approach, the SSR is fused to an estrogen receptor (ER) ligand-binding domain (LBD) that has been mutated, rendering it insensitive to the natural ligand 17ß-estradiol at physiological concentrations, but responsive to the synthetic ligand 4-hydroxytamoxifen (4-OHT) (Brocard et al., 1997; Danielian et al., 1998; Danielian et al., 1993; Feil et al., 1996; Logie and Stewart, 1995; Metzger et al., 1995; Schwenk et al., 1998). Temporal control in this system occurs as

follows: In the absence of 4-OHT, the ER-LBD domain sequesters the SSR into a cytoplasmic Hsp90 complex. Upon 4-OHT binding, the ER-LBD undergoes a conformational change that frees it, along with the fused SSR, to enter the nucleus, where it can mediate recombination at target sites previously engineered into the genome (Figure 4). At least three different mutant ER-LBDs are available (reviewed in (Branda and Dymecki, 2004; Joyner and Zervas, 2006; Metzger and Chambon, 2001)); the most sensitive one for both nuclear translocation and recombinase activity appears to be a human ER variant harboring three point mutations, referred to as $ER^{T2}$ (Feil et al., 1997; Imai et al., 2001; Indra et al., 1999; Kimmel et al., 2000; Seibler et al., 2003). $CreER^{T2}$ (Imai et al., 2001; Indra et al., 1999; Kimmel et al., 2000; Seibler et al., 2003) and $FlpeER^{T2}$ (Hunter et al., 2005) fusions have been generated and both have been shown effective in vivo. Anecdotally, it appears $CreER^{T2}$ may outperform $FLPeER^{T2}$ when expressed at low levels (N.L. Hunter and S.M. Dymecki, unpublished findings), despite the fact that the constitutive forms, Cre and Flpe, show comparably robust activity in vivo. Also being developed are SSR fusions to mutant forms of the progesterone receptor LBD (eg. *PR) (Kellendonk et al., 1999; Kellendonk et al., 1996). Because *PR activation is regulated by the synthetic steroid RU486 rather than 4-OHT, it, together with $ER^{T2}$, present the possibility of inducing two genetic manipulations in one animal, for example one regulated by RU486/Cre*PR and the other by 4-OHT/$FlpeER^{T2}$.

In this inducible strategy, the window of opportunity for recombination events—and thus the degree to which temporal resolution can be provided—is determined by the half-life of 4-OHT, which in mice appears to be approximately 24 hours. Although 4-OHT is the active inducer, its precursor tamoxifen is both easier to work with (due to its better solubility properties) and less costly and is typically the reagent used. Hepatic conversion of tamoxifen to 4-OHT takes approximately 6 to 12 hours in vivo, resulting in an initial lag between administration of tamoxifen and onset of recombinase-mediated target gene recombination. The ~6-12 hour lag is followed by an ~24 hour window during which recombination is catalyzed (Hayashi and McMahon, 2002; Hunter et al., 2005; Kimmel et al., 2000; Zervas et al., 2004; Zirlinger et al., 2002). This schedule is consistent with well-established tamoxifen pharmacokinetics in rodents (Robinson et al., 1991). Taking this schedule into account, various administration paradigms may be employed depending on the experimental goal. These range from single doses administered to pregnant females (2-14 mg tamoxifen/40 g mouse) in order to induce recombination in embryos within a relatively tight temporal developmental window, to multiple consecutive daily doses in adult animals with the goal of maximizing recombination after bypassing earlier, possibly confounding, aspects of the gene expression profile. Low doses of tamoxifen may even permit the marking of single cells, allowing for clonal analysis (Legue and Nicolas, 2005). A technique called MADM (mosaic analysis with double markers) also permits clonal analysis by relying on rare SSR-mediated translocation events between two homologous chromosomes during the G2 phase of the cell cycle; X segregation of the recombined chromosomes during mitosis then results in two daughter cells each expressing one or the other marker (Zong et al., 2005).

While powerful in the enabled science, it is important to note that tamoxifen, when administered at high doses, can kill the developing embryo. This lethality is probably due to some binding of 4-OHT to endogenous estrogen receptors, which may interfere with placental development and/or function, or with the progression of labor and delivery. Such interference can lead to the loss of just those embryos needed for study. Establishing doses of tamoxifen that maximize recombination at the desired embryonic stage while keeping unwanted side affects to a minimum is critical. Inbred strains appear particularly sensitive to tamoxifen dose as compared to outbred Swiss Webster mice, for example (Joyner and Zervas, 2006). Co-administration of progesterone with high tamoxifen doses also appears to improve litter viability (Joyner and Zervas, 2006). Other important variables include gestational age, with later stage embryos better able to tolerate higher tamoxifen doses; and levels of expressed SSR-$ER^{T2}$ protein, with

higher amounts better able to catalyze recombination at lower tamoxifen levels. While higher amounts of SSR-ER$^{T2}$ expression can be helpful, if too high, the capacity for tight inducibility may be compromised – the cell's hsp90 sequestration system may be overwhelmed, which could result in unwanted recombination even in the absence of tamoxifen. The take home lesson is that it may be necessary to screen many mouse lines in order to identify one that expresses the SSR-ER$^{T2}$ at suitable levels.

When applying inducible genetic fate mapping, it is critical to establish the extent to which expression of the reporter molecule (via activation of an indicator transgene) matches the expected driver gene expression profile between 24-48 hours after tamoxifen administration. The degree of matching will determine whether most or only a stochastic subset of the highest expressors can be tracked. Once these parameters are set, it should be possible to visualize the fate of these cells at any later time point. The resulting fate map will mark just those cells that have emerged from a gene expression domain during a particular twenty-four hour window. In other words, it can provide a picture of the various cell types produced successively from a single gene expression domain (Machold and Fishell, 2005; Sgaier et al., 2005; Zervas et al., 2004).

In addition to studying how progeny cells deploy from germinal zones as a function of time during embryogenesis, GIFM has also been used to identify and study adult neural stem cells (Ahn and Joyner, 2005). At late embryonic stages, it appears that quiescent neural stem cells are set aside in the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus and are regulated by Sonic hedgehog. These neural stem cells appear capable of self-renewal for at least a year and of generating multiple cell types over that time.

While intersectional and subtractive genetic fate mapping and genetic inducible fate mapping are important stand-alone tools, it is exciting to ponder the resolution in lineage mapping that might be achieved by incorporating an SSR-ER$^{T2}$ fusion into an intersectional and subtractive genetic fate mapping strategy. Moreover, both of these approaches benefit greatly by the ever-expanding number of genes whose expression has been ascertained within the developing and mature nervous system (Gong et al., 2003; Lein et al., 2007; Magdaleno et al., 2006; Visel et al., 2004), as these genes provide a new source of driver sequences for selective SSR delivery.

While this primer focuses on how SSR technologies are advancing the field of molecular neuroanatomy, other technologies are being developed and having major impact, such as use of inducible enhancers/promoters to gain temporal control of transgene transcription (by contrast to the post-translational induction of recombinase activity described above). For example, an interferon-responsive promoter (Kuhn et al., 1995) or tetracycline (tet) responsive elements (TREs) partnered with their respective transactivators have proven quite powerful, especially because expression of the target transgene is reversible (Furth et al., 1994; Gossen and Bujard, 1992; Hasan et al., 2001; Kistner et al., 1996; Schonig and Bujard, 2003). Incorporating into these approaches an SSR as the induced effector molecule is providing another means by which *lox*P- and/or *FRT*-containing genes can be modified with temporal control (Belteki et al., 2005; Lindeberg et al., 2002; Park et al., 2004; Schonig et al., 2002; Utomo et al., 1999; Yu et al., 2005).

## Going from cell fate to cell function by way of the three ‘Gs’

Once genetic lineage and anatomical maps are defined, a next critical step will be to add to them knowledge of cellular behavior, connectivity, and function. Fortunately, the genetic fate mapping approaches presented (single, dual, and inducible strategies), which have been so instrumental in relating molecular expression to cell fate in the brain, may serve as a template for a new set of tools, capable of revealing additional attributes of the mapped genetic lineages. For example, SSR-based strategies might incorporate into the target transgene (for example,

into an easy to use modular base vector, Figure 5A) various genetically encoded effector molecules (Figure 5B), in addition to or in lieu of reporters. A number of effector molecules hold great promise for their incorporation into this general type of SSR-based strategy because some degree of efficacy has already been established in mice. These include trans-synaptic tracers (Braz et al., 2002; Coen et al., 1997; Farago et al., 2006; Horowitz et al., 1999; Kissa et al., 2002; Maskos et al., 2002; Sakurai et al., 2005; Yoshihara, 2002; Yoshihara et al., 1999) that can be used to map functional afferent and efferent connections of molecularly distinct neuron classes; neuromodulators (Bond et al., 2000; Ehrengruber et al., 1997; Johns et al., 1999; Karpova et al., 2005; Slimko et al., 2002; Sutherland et al., 1999; Tan et al., 2006; Yamamoto et al., 2003; Yu et al., 2004) which allow one to control the activity of discrete circuits as a means to assess their roles in development, perception, behavior, and/or cognition; cell death-inducing molecules which could allow physiological functions to be revealed through targeted cell loss and, in this manner, produce neurodegenerative disease models (Breitman et al., 1990; Buch et al., 2005; Burnett et al., 2004; Chen et al., 2004; Clark et al., 1997; Heyman et al., 1989; Isles et al., 2001; Kobayashi et al., 1995; Leuchtenberger et al., 2001; Mallet et al., 2002; Palmiter et al., 1987; Saito et al., 2001); potential fate specification genes that could shed light on genetic programs that can drive or instruct the development of particular types of neurons. The effector could also be a molecule that acts to reversibly modify the expression of another target transgene. For example the effector could be the transcriptional activator rtTA, enabling tet-regulated (and therefore temporally regulated and reversible) expression of a TRE-driven transgene (Belteki et al., 2005), with the cell-type selectivity enabled through intersectional activation of rtTA expression. Indeed, the possibilities seem without limit.

## Investigating adult neurological disease by way of the three ‘Gs.’

The ability to induce gene modifications at virtually any desired time during the life of an animal is extremely powerful because it permits investigations to reach beyond the first required function of a gene. This can translate into an important capability - gene functions, in many cases, can be assessed specifically in the postnatal or adult brain. Indeed, this presents many potential options for studying adult neurological disease. For example, neurological phenotypes, especially those resulting from inherited, single gene mutations, can be examined for reversibility following gene repair induced postnatally. In other words, it becomes possible to ask whether cellular damage done during development, due to inherited gene inactivation, is irrevocable or whether it can be rectified postnatally following gene repair. This approach was recently applied to the *Mecp2* gene with remarkable results – postnatal reversal of aspects of the autism (Rett)-like neurological phenotype (Guy et al., 2007). An inactive *Mecp2* gene, silenced via insertion of a *lox-stop* cassette, was conditionally repaired postnatally by cassette deletion (similar to the strategy schematized in the lower half of Figure 1b); a *CAG-cre-ER-LBD* transgene along with tamoxifen administration provided the genetic access to mediate gene repair in ~80% of the cells in the brain. Understanding the potential for disease reversibility is of course critical when thinking about possible future therapeutic approaches. This is but one illustration of how SSRs together with the three Gs are impacting fundamental disease issues.

In conclusion, mastering the subjects of reading, writing, and arithmetic opens a world of possibility. Embracing the subjects of genetic access, genetic lineage, and genetic anatomy could have unlimited reward for the field of molecular neuroscience.

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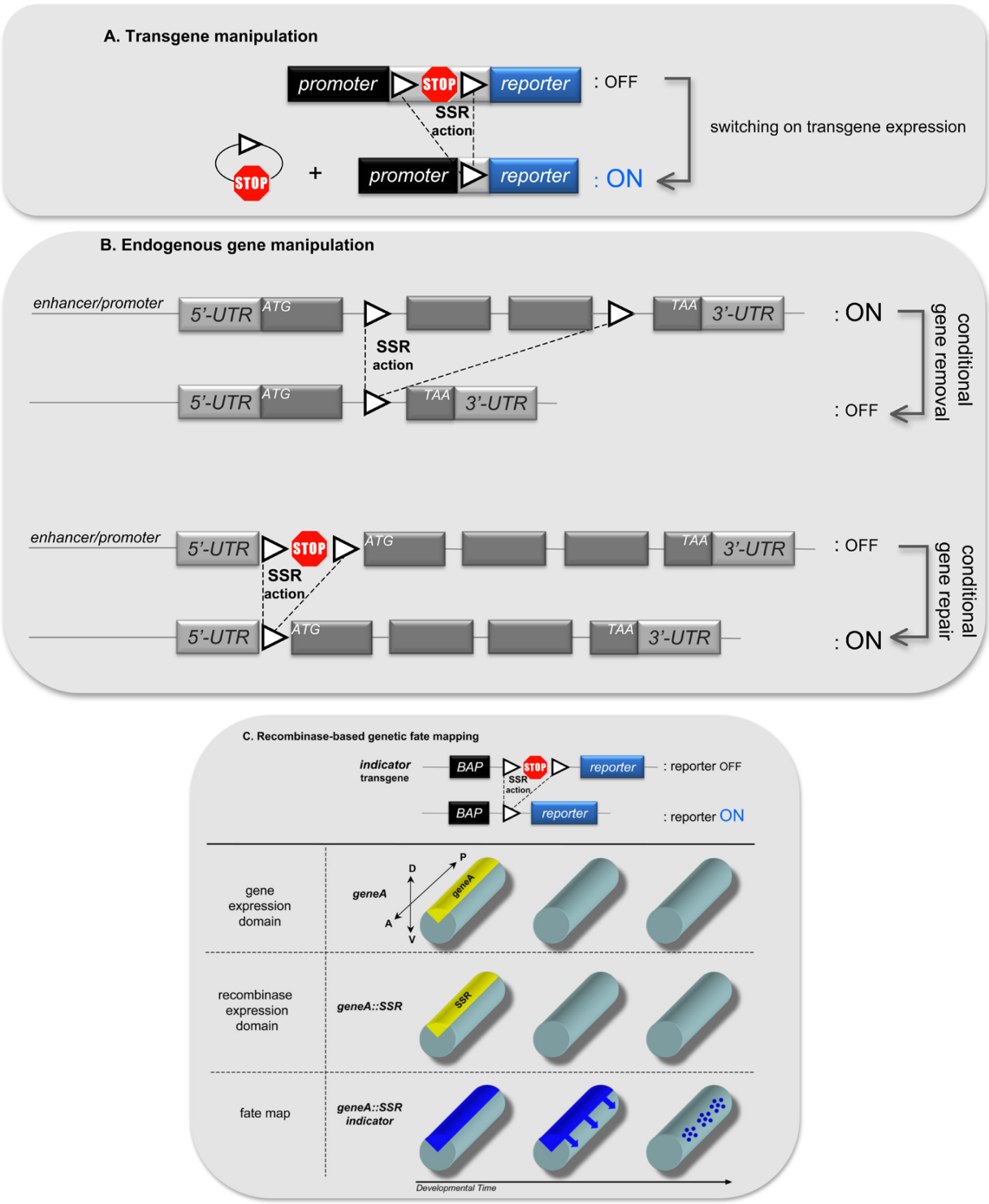



**Figure 1.**

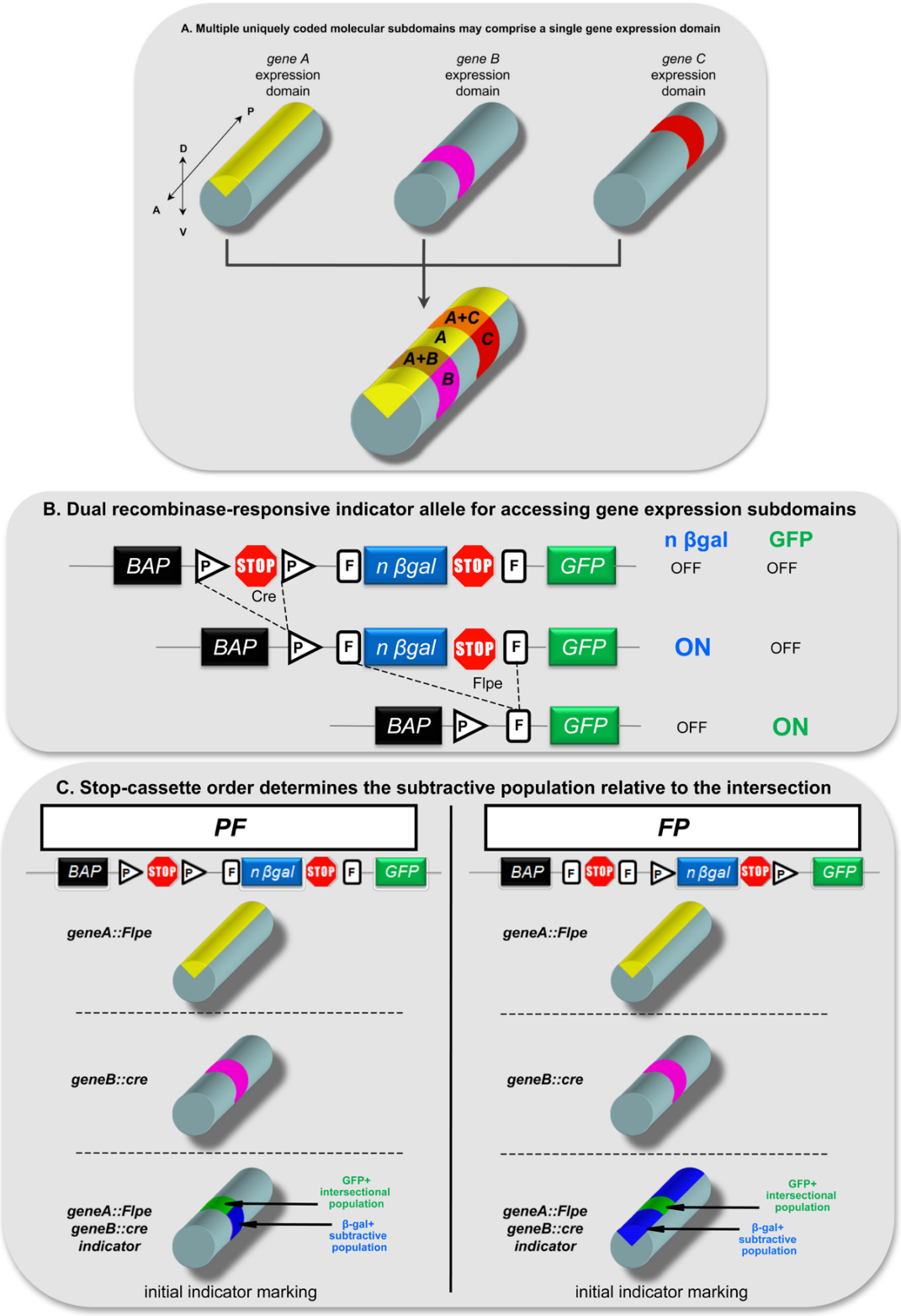
Site-Specific DNA Excisions Serve as "On-Off" Switches for Gene Activity and as the basis for Genetic Fate Mapping.

(A) Structure of a generic SSR-responsive transgene inserted as a single copy into the mouse genome. SSR-mediated recombination between directly repeated SSR recognition sites

(triangles) results in deletion of intervening transcriptional stop sequences (red octagonal stop sign) and consequent expression of a reporter molecule. Depending on the type of promoter incorporated, either constitutive or tissue-specific reporter expression can be achieved. Spatial control of transgene activation is conferred by the regulatory elements used to drive SSR expression.

(B) Two prototypical SSR-based manipulations of an endogenous locus: conditional gene removal versus repair. Depending on recognition site (triangle) placement, SSR-mediated excision can be exploited to remove (B, upper panel) or repair (B, lower panel) endogenous gene sequences. Light gray boxes represent untranslated exon regions (UTRs); dark gray boxes, coding exons; ATG, translation initiation codon; TAA, translation stop codon.

(C) Illustration of how site-specific recombination can be used to study the deployment of progenitor cells and their descendants during development. This method is referred to as genetic fate mapping. The generic SSR-responsive transgene of panel A is modified here (C, upper panel) by incorporation of a broadly active promoter (BAP) ideally capable of driving transgene expression in any cell type at any stage in development, such that after a recombination event in a given cell, that cell and all its progeny cells should be marked by reporter expression regardless of subsequent cell differentiation. We refer to conditional target transgenes for genetic fate mapping as ‘indicator’ transgenes because they indicate or provide a permanent record of all earlier occurring recombination events. Lower panel, strategy for SSR-based genetic fate mapping with development of the neural tube rendered as a simple cylinder and progressing left to right in each row. Top row: hypothetical *gene A* is expressed transiently by progenitor cells located in the dorsal neural tube (yellow domain) at an early developmental stage. Middle row: SSR-expressing transgene utilizes enhancer elements from *gene A*. Bottom row: When *geneA::SSR* is coupled with an indicator transgene, cells expressing the SSR will activate production of the reporter molecule (for example, ßgal). Activation of reporter molecule expression is permanent, and all cells descended from the SSR-expressing (*gene A*-expressing) progenitors will continue expressing the reporter, thereby marking a genetic lineage as it contributes to different brain regions during development. Descendant cells are depicted here as blue circles.



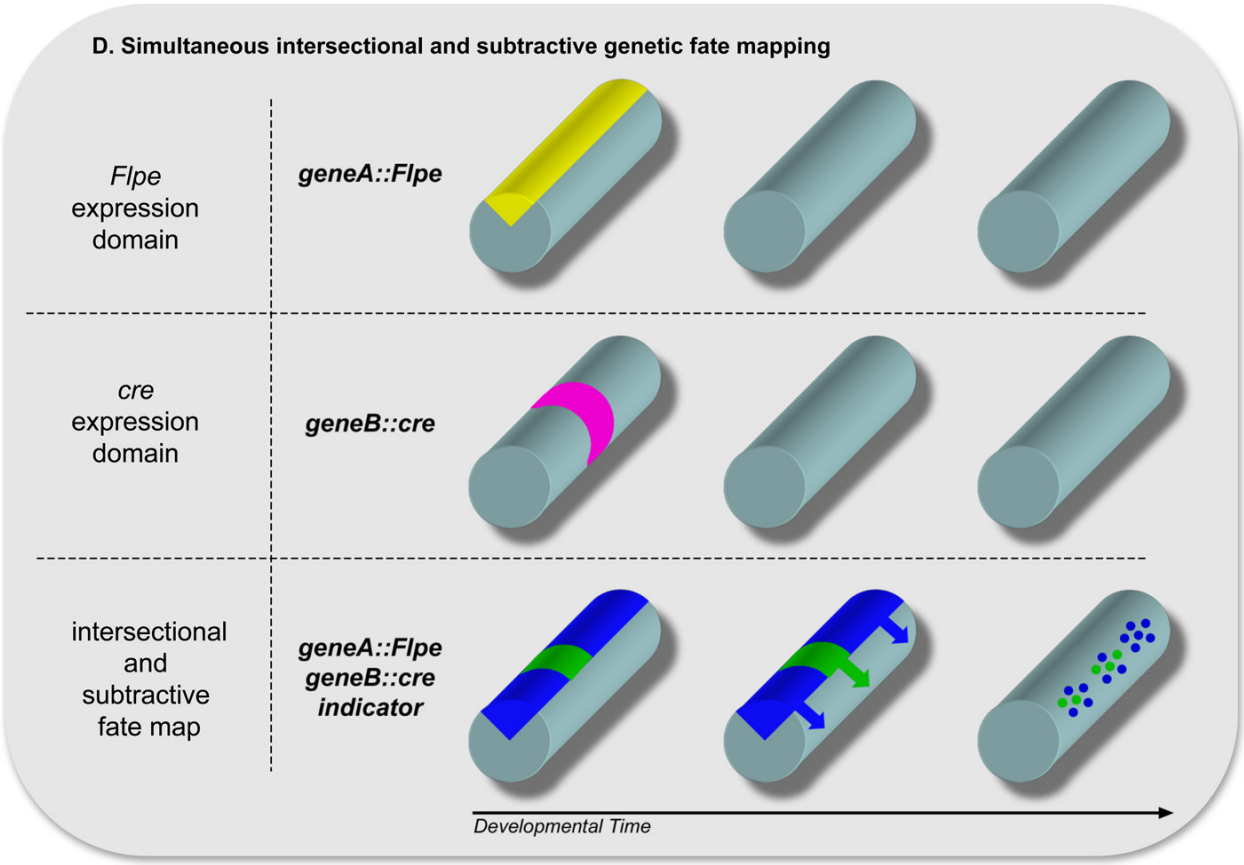



**Figure2.**

Intersectional and Subtractive Genetic Fate Mapping Strategy and an Enabling Prototypical Dual Recombinase-Responsive Indicator Allele.

(A) Multiple uniquely coded molecular subdomains may comprise a single gene expression domain. Shown are schematics of the neural tube (gray cylinder), with different gene expression domains depicted in different colors. The expression domain for hypothetical *gene A* (yellow) restricts along the dorsoventral (DV) axis but extends along the anteroposterior (AP) axis; by contrast, the expression domains for *genes B* (pink) and *C* (red) restrict along the AP axis but extend along the DV axis. Thus, the *gene A* expression domain (yellow) is subdivided into three molecularly distinct subdomains: one in which *genes A* and *B* are co-expressed (tan domain); another in which *genes A* and *C* are co-expressed (orange domain), and finally, that territory (yellow) marked by *gene A* expression, but not *B* or *C*. Similarly, both the *gene B* and *C* expression domains are each subdivided.

(B) Structure of a prototypical dual recombinase (Cre and Flpe)-responsive indicator allele. By contrast to a single recombinase-responsive indicator allele (Figure 1C), a dual recombinase-responsive indicator allele has two stop cassettes, one flanked by directly oriented *lox*P sites (triangles) and the other, by *FRT* sites (vertically oriented rectangles). Cre-mediated stop cassette removal results in expression of nßgal, while the remaining *FRT*-flanked stop cassette prevents GFP expression. Following removal of both stop cassettes, requiring Cre- and Flpe-mediated excisions, GFP expression is turned on and nßgal expression off.

(C) Illustration of intersectional and subtractive populations and the latter dependency on stop-cassette order. In the ‘PF’ configured allele, the *lox*P-flanked stop cassette precedes the *FRT*-flanked cassette (left panel), while the reciprocal order characterizes the ‘FP’ configuration (right panel). Shown are schematics of the neural tube (gray cylinder), with the expression

domain for hypothetical *gene A* and *Flpe* recombinase (yellow) restricting along the dorsoventral (DV) axis but extending along the anteroposterior (AP) axis (top row); by contrast, the expression domain for *gene B* (pink) restricts along the AP axis but extends along the DV axis (middle row). When *geneA::Flpe* and *geneB::cre* are coupled with a PF dual recombinase-responsive indicator allele (bottom row, left), cells expressing *cre* and *Flpe* activate production of GFP (green domain, intersectional population) while cells expressing only *cre* activate production of nßgal (blue domain, subtractive population). When *geneA::Flpe* and *geneB::cre* are coupled with an FP configured allele (bottom row, right), cells expressing *cre* and *Flpe* still activate production of GFP in the same intersectional population (green domain) but now cells expressing only *Flpe* (rather than *cre*) activate production of nßgal (blue domain, new subtractive population).

(D) Illustration of the selective fate mapping achievable using an intersectional and subtractive approach. Development of the neural tube is again rendered as a simple cylinder progressing left to right in each row. Top row: *gene A* drives transient *Flpe* expression in progenitor cells located in the dorsal neural tube (yellow domain) at an early developmental stage. Middle row: *gene B* drives transient *cre* expression in progenitor cells located at a particular AP level of the neural tube at an early developmental stage (pink domain). Bottom row: when *geneA::Flpe* and *geneB::cre* are coupled with a dual recombinase-responsive indicator allele (FP configuration), cells expressing *Flpe* and *cre* activate production of GFP, while cells expressing only Flpe activate production of nßgal. Activation of reporter molecule expression is permanent, and all cells descended from *Flpe*-expressing or *Flpe*- and *cre*-expressing progenitors will continue expressing the blue or green marker, respectively. Descendant cells from the intersectional domain are denoted by green circles, those from the subtractive population by blue circles.

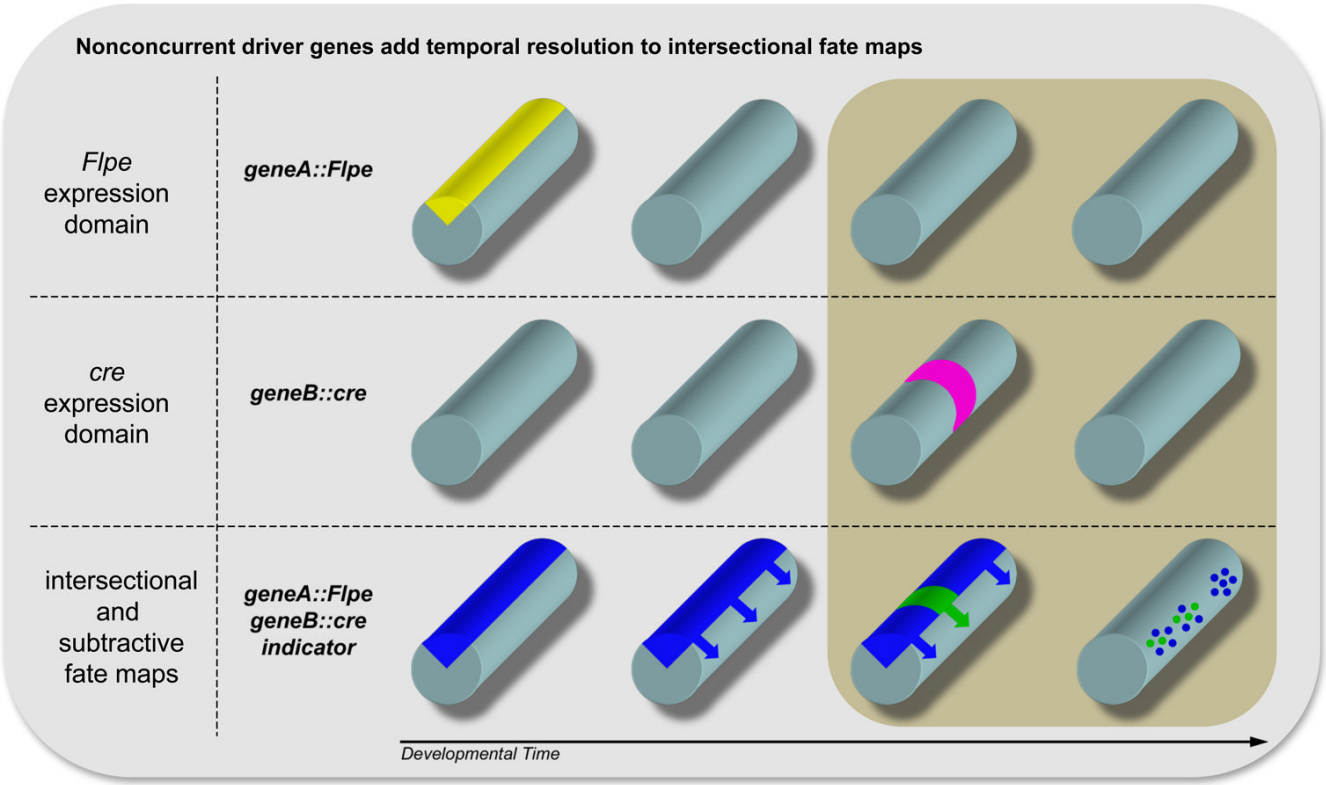



**Figure 3.**

Nonconcurrent driver genes add temporal resolution to intersectional fate maps. Development of the neural tube is again rendered as a simple cylinder progressing left to right in each row. Top row: *gene A* drives transient *Flpe* expression in progenitor cells located in the dorsal neural tube (yellow domain) at an early developmental stage. Middle row: *gene B* drives transient *cre* expression in a population of later-stage progenitor cells located at a particular AP level of the neural tube (pink domain). Bottom row: when *geneA::Flpe* and *geneB::cre* are coupled with a dual recombinase-responsive indicator allele (FP configuration), expression of nßgal, as a lineage tracer of *geneA*-expressing progenitor cells, is activated first. Cells with a history of *gene A* expression that go on to express *gene B*, and therefore *cre*, will, following Cre-mediated recombination, turn-off nßgal expression and turn on GFP expression (the intersectional population).

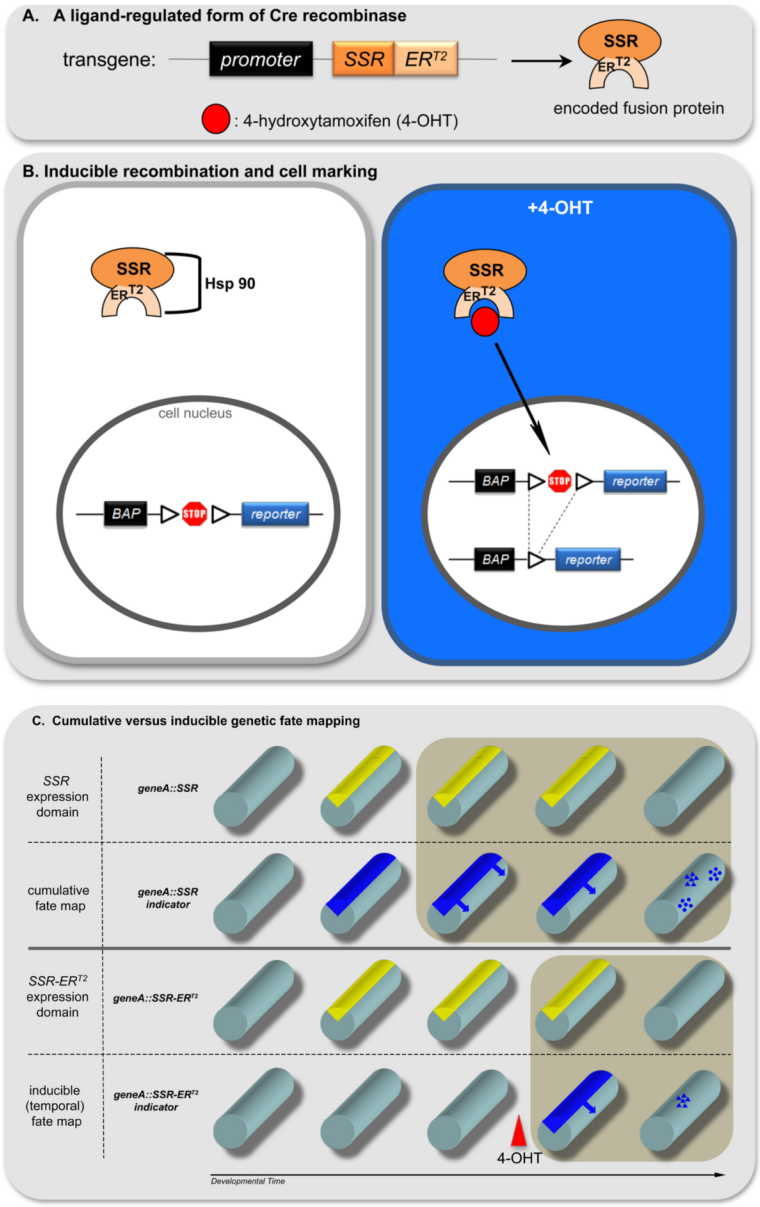



**Figure 4.**

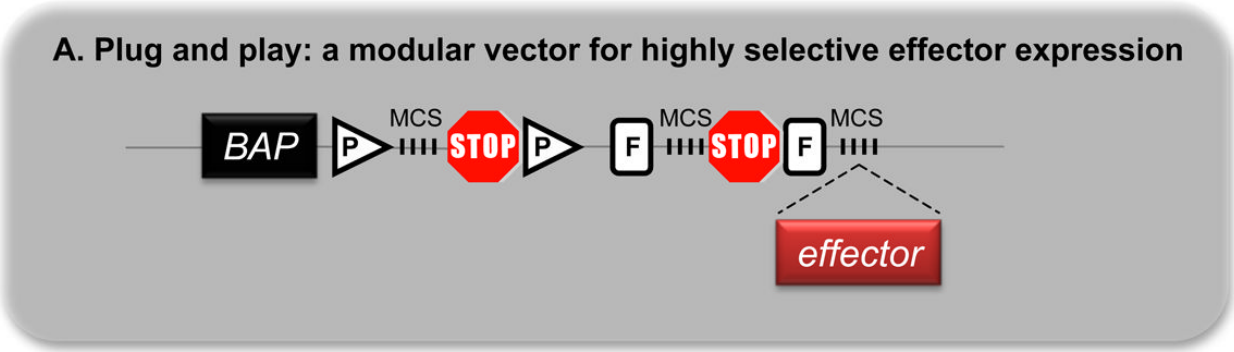
Addressing temporal aspects of lineage allocation by controlling SSR activity.

(A) Schematic of a transgene encoding the recombinase-steroid fusion protein, SSR-ER$^{T2}$, whose activity is regulated posttranslationally by the ligand 4-hydroxy tamoxifen (4-OHT, red circle).

(B) Inducible recombination and cell marking using SSR-ER$^{T2}$. In the absence of 4-OHT, SSR-ER$^{T2}$ is inactive due to sequestration of the fusion protein into an Hsp90 complex. Binding of 4-OHT to SSR-ER$^{T2}$ results in a conformational change that disrupts the Hsp90 interaction, freeing the recombinase to enter the cell nucleus and mediate recombination at its target sites (triangles) positioned within an indicator transgene. Excisional recombination renders cells positive for reporter expression (for example, cytoplasmic ßgal as indicated in dark blue).

(C) Cumulative versus inducible genetic fate mapping. Development of the neural tube is again rendered as simple cylinders progressing left to right in each row. Cumulative genetic fate mapping is schematized in the top two rows, much as done previously in Figure 1C. Top row:

transient, midgestation expression of *cre* recombinase in progenitor cells of the dorsal neural tube defined by their expression of *gene A*. Second row: activation of nßgal, for example, as a lineage tracer in all cells that ever in their history expressed *gene A::cre*. Inducible genetic fate mapping is schematized in the bottom two rows. Third row: transient, midgestation expression of *SSR-ER*$^{T2}$ in progenitor cells of the dorsal neural tube defined by expression of *gene A*. Bottom row: induction of recombinase activity and consequent indicator transgene expression following administration of 4-OHT permits selective tracking of late-emerging cohorts in virtual isolation.




**B. A sample of plug-in genetically encoded effector molecules**

| Effector category | Name | Reference |
|---|---|---|
| Reporters | Fluorescent proteins | (Giepmans et al., 2006; Miyawaki, 2005; Shaner et al., 2004) |
| Cell activity indicator | Fluorescent protein based indicators | (Miyawaki, 2003a; Miyawaki, 2003b; Miyawaki, 2005) |
| Neuronal Circuit tracer | Wheat germ agglutinin (WGA) | (Yoshihara et al., 1999) |
| | Barley lectin (BL) | (Horowitz et al., 1999) |
| | Tetanus toxin C-terminal fragment (TTC) | (Maskos et al., 2002; Sakurai et al., 2005) |
| Neuronal activity modulator | Tetanus toxin light chain | (Yamamoto et al., 2003; Yu et al., 2004), |
| | VAMP2-FK506 binding protein (FKBP) fusion protein (VAMP/Syb MIST) | (Karpova et al., 2005) |
| | Shaker type $K^+$ channel | (Sutherland et al., 1999) |
| | Inwardly rectifying $K^+$ channel (Kir) | (Johns et al., 1999; Yu et al., 2004) |
| | G-protein coupled inwardly rectifying $K^+$ channel (GIRK) | (Ehrengruber et al., 1997) |
| | Allatostatin receptor (AlstR) | (Tan et al., 2006) |
| | Small-conductance $Ca^{2+}$-activated $K^+$ channel (SK channel) | (Bond et al., 2000) |
| | Glutamate-gated chloride channel (GluCl) | (Slimko et al., 2002) |
| Cell ablator | Diphtheria toxin fragment A (DTA) | (Palmiter et al., 1987) |
| | Diphtheria toxin fragment A, attenuated form (DTA176) | (Breitman et al., 1990) |
| | Diphtheria toxin receptor (DTR) | (Buch et al., 2005; Saito et al., 2001), |
| | Interleukin 2 receptor alpha subunit (IL-2R alpha) | (Kobayashi et al., 1995) |
| | Caspase 3 | (Mallet et al., 2002) |
| | Herpes simplex virus 1 thymidine kinase (HSV-TK) | (Heyman et al., 1989) |
| | Puromycin *N*-acetyltransferase-HSV1 *tk* fusion (Pu$\Delta$tk) | (Chen et al., 2004) |
| | *E. coli* nitroreductase (NTR) | (Clark et al., 1997; Isles et al., 2001), |
| | RNase from *Bacillus amyloliquefaciens* (Barnase) | (Leuchtenberger et al., 2001) |
| | Low-affinity nerve growth factor receptor ($\Delta$LNGFR)-FKBP-Fas fusion (Mafia) | (Burnett et al., 2004) |
| Transactivator | tTA and rtTA | (Belteki et al., 2005; Furth et al., 1994; Kistner et al., 1996) |
| Recombinase | Flp and Cre | (Dymecki, 1996; Lakso et al., 1992) |

**Figure 5.**

Turning genetic fate maps into functional connectivity maps using SSR technology as template. (A) Schematic of a modular, 'plug and play' vector designed for assembly of dual recombinase responsive transgenes that offer highly selective, conditional expression of effector molecules of choice (J. Kim and S. Dymecki, unpublished reagent). *BAP*, broadly active promoter (for example, *CAG/R26*); MCS, multiple cloning sites; STOP, transcriptional stop cassette; *lox*P site, triangle; *FRT* site vertical rectangle; sequence encoding an effector molecule and pA, red rectangle.

(B) A sample of 'plug-in' genetically encoded effector molecules that have shown some degree of efficacy in mice.