



Published in final edited form as:

*Bone*. 2023 May ; 170: 116719. doi:10.1016/j.bone.2023.116719.

## Perspective: The current state of Cre driver mouse lines in skeletal research: challenges and opportunities

Connor J. Cunningham<sup>1</sup>, Roy B. Choi<sup>1</sup>, Whitney A. Bullock<sup>2</sup>, Alexander G. Robling, Ph.D.<sup>1,3,4,5</sup>

<sup>1</sup>Department of Anatomy, Cell Biology & Physiology, Indiana University School of Medicine, Indianapolis, IN, USA.

<sup>2</sup>Department of Biology, Eckerd College, St. Petersburg, FL, USA.

<sup>3</sup>Richard L. Roudebush Veterans Affairs Medical Center, Indianapolis, IN, USA

<sup>4</sup>Department of Biomedical Engineering, Indiana University–Purdue University at Indianapolis, Indianapolis, IN, USA.

<sup>5</sup>Indiana Center for Musculoskeletal Health, Indianapolis, IN, USA.

### Abstract

The Cre/Lox system has revolutionized the ability of biomedical researchers to ask very specific questions about the function of individual genes in specific cell types at specific times during development and/or disease progression in a variety of animal models. This is true in the skeletal biology field, and numerous Cre driver lines have been created to foster conditional gene manipulation in specific subpopulations of bone cells. However, as our ability to scrutinize these models increases, an increasing number of issues have been identified with most driver lines. All existing skeletal Cre mouse models exhibit problems in one or more of the following three areas: (1) cell type specificity—avoiding Cre expression in unintended cell types; (2) Cre inducibility—improving the dynamic range for Cre in inducible models (negligible Cre activity before induction and high Cre activity after induction); and (3) Cre toxicity—reducing the unwanted biological effects of Cre (beyond loxP recombination) on cellular processes and tissue health. These issues are hampering progress in understanding the biology of skeletal disease and aging, and consequently, identification of reliable therapeutic opportunities. Skeletal Cre models have not advanced technologically in decades despite the availability of improved tools, including multi-promoter-driven expression of permissive or fragmented recombinases, new dimerization systems, and alternative forms of recombinases and DNA sequence targets. We review the current state of skeletal Cre driver lines, and highlight some of the successes, failures, and opportunities

---

Corresponding author: Alexander G. Robling, Ph.D., Department of Anatomy, Cell Biology & Physiology, Indiana University School of Medicine, 635 Barnhill Dr., MS 5035, Indianapolis, IN 46202, Tel: (317) 274-7489, Fax: (317) 278-2040, arobling@iupui.edu.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest

All authors have declared that no conflicts of interest exist.

to improve fidelity in the skeleton, based on successes pioneered in other areas of biomedical science.

## Keywords

Cre; LoxP; recombination; floxed allele; skeletal models

An effective way to understand the function of a particular gene of interest (GOI) on an organ system, physiologic process, developmental checkpoint, or disease initiation/progression, is to disrupt or extinguish the expression of that gene and study the consequences on the relevant biology. For many studies in skeletal biology, an intact postnatal skeleton is required to make meaningful observations regarding the skeletal effects of a particular gene's absence on disease or bone cell behavior, which poses a significant problem if disruption of the GOI causes early lethality. It is not uncommon for such a case to emerge in skeletal biology studies, as many of the genes that are relevant to the skeletal biology community can play significant roles in other tissues and can be critical to the embryonic developmental processes or postnatal survival. Fortunately, tools have been developed to allow selective deletion of GOIs in select cell/tissue types, while other cell/tissue types within the same mouse are spared of gene disruption. This strategy can allow critical processes necessary for life to carry on, while targeted deletion of the GOI can be restricted to the skeleton, where the effects can be studied in an otherwise healthy mouse. Known as the Cre/Lox system, this approach has revolutionized the ability of biomedical researchers to ask very specific questions about the function of individual genes in particular cell types at crucial times during development and/or during disease progression in mammalian models.

The Cre/Lox system has at its core a different strategy for gene disruption than the “global” or “germline” deletion strategy described above. Rather than modifying the GOI sequence in a manner that makes it immediately dysfunctional (e.g., introducing a frameshift mutation, removal of critical exons, introducing stop codons, among others) in every cell, alternatively, the gene can be modified in a manner that takes advantage of the fact that, unlike prokaryotes, eukaryotes have long stretches of non-protein-coding DNA sequence—known as introns—interspersed between the shorter islands of coding sequence (exons). Introns are transcribed in the immature mRNA but they are quickly spliced out by RNA processing machinery, and the exons are joined together, prior to protein translation in the ribosome. The presence of introns provides an opportunity to alter DNA sequence within the GOI without altering the ultimate amino acid sequence of the resulting protein. This might seem pointless, since modifying the introns achieves nothing in terms of altering the function of the gene (i.e., the eventual protein is not changed). But as we discuss below, these intron modifications provide a “trojan horse” that can be selectively manipulated, in both space and time, to dramatically affect gene function. To gain some sense of perspective for intron modification opportunities, in the human genome the average gene has ~9 exons and ~8 introns, but the vast majority of exons are less than 200bp in length, whereas the most introns are several thousand bp in length, and 10% of introns are >10kbp long.<sup>(1)</sup>

What type of intronic modifications can achieve selective deletion/disruption of a GOI, if they do not contribute to coding differences in the protein? Introducing a pair of specific, short DNA sequences (34 bp in length, known as the locus of X-over P1 [LoxP] sequence) into the intronic regions of a gene can provide the “trojan horse” function indicated above, where one LoxP sequence is added to each of two introns (Fig. 1). The addition of loxP sites into the introns that flank crucial exons of a GOI is known as “floxed” a gene, and although the floxed allele is usually only slightly different (i.e., longer by 34 bp X 2 = 64 bp different) than the WT allele, floxed alleles can usually be distinguished from WT alleles by specific PCR reactions on genomic DNA. Transcript expression levels of floxed and WT alleles should be indistinguishable if the recombineering is done properly (LoxP-containing introns are spliced out of the mature mRNA), so gene expression assays (e.g., qPCR) conducted on mature RNA are usually uninformative in differentiating WT from floxed alleles in their unrecombined state. The rationale for choosing which introns to target for LoxP addition, for any particular GOI, is beyond the scope of this review, but very briefly it is based on ensuring that crucial exons for the gene’s function are between the two LoxP sites, and placement of the LoxP sites does not disturb intronic elements such as the branchpoint sequence and polypyrimidine tract. The LoxP sequences have several key properties, when engineered properly into a gene:

1. They do not interfere with the targeted gene’s function. Here, introduction of LoxP sequences into the two introns, if done correctly, has no measurable effect on expression of the gene into which they were introduced (Fig 1). In other words, expression of the “floxed” allele should exhibit the same expression profile as the wild-type allele. Because the LoxP-containing introns are spliced away during mRNA processing, the mature mRNA is identical between the WT and floxed alleles, and therefore the protein product should be identical.
2. They are highly susceptible to recognition by a restriction enzyme know as Cre (Cyclization Recombination Enzyme) derived from the *E. coli* bacteriophage P1. Cre selectively recognizes the 34bp LoxP sequences, and its recombinase activity cuts LoxP near the center of each sequence (at both loxP sites), excises the intervening DNA between the two cuts, and joins the two remaining halves of loxP together. This results in a single “lonely” LoxP site sitting in the newly created hybrid intron, and, more importantly, deletion of the (key) exons that were housed between the loxP sites. Now, transcripts from this “recombined” allele will be missing key coding sequence, rendering the gene dysfunctional.

Because Cre recombinase is a viral gene/protein, it is not contained in the mammalian genome and therefore mice with genes that harbor introduced LoxP sites are not in jeopardy of having those floxed alleles undergo recombination. Consequently mice with floxed alleles represent only half of the system required to achieve time- and tissue-selective gene deletion. The other half of the system requires introducing a (foreign) gene coding for Cre recombinase, into the floxed mice so that the LoxP sites can be cut, recombined, and gene deletion achieved.

If we were to generate mice with homozygous floxed GOI and infect them with an adenovirus that expresses Cre, assuming 100% efficacy of the virus, every cell in the

mouse's body would take up the virus, transcribe and translate the viral DNA for Cre, and the expressed Cre protein would enter the nucleus to recombine the floxed alleles and essentially produce a global knockout. While this strategy is useful for conceptually understanding how to integrate both parts of the Cre/Lox system to achieve LoxP recombination, especially the temporal control aspect of the system mentioned above (we could give the virus at any age of the mouse and achieve deletion), it is not practical experimentally.

A practical and widely used alternative is to generate a separate mouse model, in which a Cre-expressing genetic sequence is added to the mouse genome, so that Cre is made as a normal part of the expression profile of the target cells. It is at this stage that we can begin to understand how the cell/tissue specificity capabilities of the system are possible. If we generate a transgene where Cre is expressed under regulatory sequences that specify expression in, for example, osteoclasts (e.g., use the Cathepsin-K promoter to drive expression of Cre), and we stably introduce that transgene into the genome of a mouse (the strategy for creation of transgenics is beyond the scope of this communication), that mouse will express Cre in any cell that expresses Cathepsin-K. Since osteoclasts strongly express Cathepsin-K but the kidney, for example, does not, we can expect to find Cre production in osteoclasts but not in kidney epithelial cells. If we breed that Ctsk-Cre transgenic mouse to a floxed GOI mouse, Cre will be produced in the osteoclasts, where it will find the LoxP sites in the osteoclast nuclei, recombine them and render the GOI dysfunctional only in those cells that expressed Cre. However, in the kidney epithelial cells (and most other cells) of those mice, we can expect to see no Cre production and therefore, no LoxP recombination and normal expression of the GOI. To further make the point, if we were to cross our floxed GOI mouse with a Cre driver line that uses a promoter expressed in all cells (e.g., CAG-Cre, EIIa-Cre), we would essentially create a global knockout, though we would have arrived at a global knockout by non-traditional means. To put this approach into a historical context, the first published report of tissue-selective gene deletion in a normal mouse appeared in 1994, where loxP sites were introduced into the DNA polymerase  $\beta$  (*Polb*) gene and selective deletion in T-cells was accomplished using the Lck-Cre.<sup>(2)</sup>

The promoter sequence chosen to drive Cre expression confers specificity to LoxP recombination activity in space (i.e., in certain cells/tissues but not others) but what about specificity in time (at some predetermined point in time, but not before)? Above, we used an example of temporal control, where Cre-expressing virus was injected to a floxed mouse at any desired time point. Using that approach we could enact an experimental design where we injected virus at 2 days of age, 2 weeks of age, 2 months of age, or 2 years of age (or anything in between), to study GOI deletion at various stages of growth, development, and even senescence. Obviously, systemic administration of Cre-expressing virus, beyond other manifold problems, forfeits any hope of cell/tissue selectivity, and even local tissue injection of virus still has the propensity to spread throughout the body and induce mosaic recombination in unintended tissues. There are, however, better (transgenic) ways to induce GOI recombination at different points in time, that can simultaneously provide spatial control. By far the most widely used method for temporal control of LoxP recombination involves modifying the Cre enzyme to include an additional protein fragment derived from a mutant form of the mouse estrogen receptor (ER), such that the expressed protein is now

a larger chimeric protein containing an active Cre domain and an active ER domain. We are already aware of the function of the Cre domain; the mutant ER domain has several properties that are important. First, the ER domain retains the steroid receptor properties of wild-type ER, which restricts fusion protein localization to the cytosol and outer nuclear envelope (out of the nucleus). Thus the ER fragment, in its unprovoked state, prevents access of the attached Cre domain to the chromatin and LoxP sites contained therein. Second, the mutant ER domain has been intentionally engineered to be highly responsive to the synthetic estrogen analog tamoxifen, with low to no sensitivity to endogenous estrogens (E<sub>2</sub>). Therefore, mice that express the CreER fusion protein in cells that also harbor floxed alleles typically experience no recombination, since the CreER protein is sequestered in the cytosol. However, once the mouse is systemically injected with an appropriate dose of tamoxifen (which can be at any age), cells where CreER is expressed will undergo translocation of the CreER protein from the cytosol to the nucleus, where CreER finds the LoxP sites, ultimately resulting in LoxP recombination and GOI modification. The CreER system has undergone iterative improvement for sensitivity and specificity. Newer versions are known as CreERt2,<sup>(3)</sup> which contains a few additional point mutations that improve selectivity, and the so-called MerCreMer<sup>(4)</sup> protein, which includes an additional (second) mutant ER domain so that Cre is flanked on either side by a mutant ER (Mer = *m*utant *e*strogen *r*eceptor). Whatever the version, the addition of a cell-selective promoter to drive the Cre-ER fusion protein provides spatial control to the temporally controllable protein, and ultimately, LoxP recombination when and where the investigator chooses. For example, returning to our Cstk-Cre model discussed above, there also exists the tamoxifen-inducible Cstk-CreERt2 version in the mouse, which can be treated with tamoxifen to recombine floxed alleles in osteoclasts during a predetermined and defined stage in mouse growth and development.

The CreER approach is not the only system available to achieve temporal control of Cre activity. A second system used for inducible Cre activity is the so-called tet-on/tet-off systems. This model borrows some of the antibiotic (tetracycline, or “tet”) resistance machinery present in certain bacteria. In mice engineered to express the tet-responsive system, two different transgenes are required in order to produce a functional Cre protein that necessary to recombine a floxed allele. The first transgene codes for Cre, and it is driven by a ubiquitously expressed artificial promoter, tetO.CMV. The tetO.CMV-Cre gene is, in its natural state, turned off, because it requires a special transcription factor not present in the mammalian genome, for activity at the tetO.CMV promoter (Fig. 2). Consequently, Cre is not expressed in this transgenic mouse. However, the tetO.CMV promoter can be turned on, and Cre transcribed, only if an engineered transcription factor known as tTA (*t*etracycline-controlled *t*ranscriptional *a*ctivator) is also present and bound to the tetO.CMV promoter. Thus, a second transgene, coding for tTA is required to turn the system on. If the tTA sequence is driven by a tissue selective promoter (e.g., in the context of our osteoclast example, Cstk-tTA), introduced into the mouse genome, and bred to the tetO.CMV-Cre, then we can achieve Cre expression selectively only in Cstk-expressing cells. So essentially, the tetO.CMV-Cre transgene is lying dormant in every cell, waiting to be turned on; but it can only be turned on by co-expression of the permissive tTA. Spatial control of Cre expression is achieved by driving tTA expression using a cell-selective promoter. There is

one crucially important, final detail with this system. tTA is prevented from binding the tetO.CMV promoter if tetracycline is present (usually doxycycline, or “dox” is used). So mice that harbor, for example, both a Ctsk-tTA transgene and a tetO.CMV-Cre transgene will not recombine floxed alleles if they are kept on a diet that includes proper doses of doxycycline in their chow, because the dox prevents tTA-mediated transcription of the tetO.CMV-Cre gene in Ctsk-expressing cells. However, switching these mice to a standard lab diet will remove dox from the circulation, de-inhibit the tTA transcription factor, and Cre will turn on only in Ctsk-expressing cells. The dox removal component provides temporal control of Cre activity (the dox diet can be removed at any age to turn on Cre), and, as mentioned earlier, the promoter used to drive tTA provides spatial control. The system just described constitutes the “tet-off” configuration, because keeping the mice on a tetracycline/doxycycline-supplemented diet keeps Cre activity off. But very clever molecular biologists have discovered a way to reverse tTA activity such that the presence, rather than the absence, of dox turns the system on. This “tet-on” system is identical in principle, but the reverse tTA (rtTA) factor is used instead of tTA, where rtTA requires dox to bind and transcribe tetO.CMV-Cre. The tet-on and tet-off systems are not as widely used as the CreER-based systems in skeletal biology studies, perhaps because an additional transgene is required (one transgene for the tetO.CMV-Cre cassette and another for the tissue-specific tTA or rtTA cassette, in addition to the floxed alleles). To alleviate this additional step, some investigators have combined both constructs into one larger transgene so that a single locus contains both of the tet-on/off elements to turn on Cre. An example of this in the bone field is the *Osx1-GFP::Cre* mouse, which harbors a large, single transgene, where tTA is driven by the Osterix promoter, and immediately downstream of that sequence, within the same donor DNA, is the tetO-CMV-Cre cassette (here, Cre happens to be fused to GFP for visualization of Cre expression).

The list of publicly available floxed mice is growing every year, and it is very likely that floxed mouse models will be available for every gene in the genome by the end of this decade. This endeavor has been fast-tracked because it serves a very large biomedical community, much larger than the skeletal biology community, i.e., researchers from neuroscience, diabetes, cardiovascular disease, renal biology, cancer, and so on. Recall that the floxed models are really only half of the system, and while the number and availability of floxed mouse models has taken off, the number of new skeletal-based Cre driver lines has not. Moreover, the closer we look at the existing skeletal Cre driver lines, the more issues emerge as to their specificity, toxicity, and for inducible models, their induction capacity. Creation of new, next-generation skeletal Cre driver mouse models will be required for improved success and high-fidelity tissue-targeted deletion/recombination studies in the coming years. This effort has languished for bone cell drivers but not for other fields of study, and there is a great deal to learn from the other fields that have successfully implemented newer technologies to improve Cre model fidelity. To further illustrate the depth of the problem, the reader is encouraged to browse the discussion section of the typical paper published (particularly more recent ones) using skeletal Cre/Lox mice, where the authors invariably have to address trying to account for and rectify the off-target, leaky, toxic, or phenotypic effects of the Cre driver they have used in their floxed model. The problem is that there has been no real large-scale movement in the field, in trying to

remedy the state of affairs when it comes to improving our options for Cre models in bone biology. The remainder of this review will focus on problems and solutions to the Cre model shortcomings in skeletal biology. We address the three main issues with skeletal Cre mouse models, namely **(1)** Cre specificity—avoiding Cre expression in unintended cell types; **(2)** Cre toxicity—reducing the unwanted effects of Cre (beyond loxP recombination) on cellular processes and tissue health; and **(3)** Cre inducibility—improving the dynamic range for Cre in inducible models (introduced below), where there is little to no unprovoked Cre activity in the baseline state and high Cre activity after induction.

## Why do we need new Cre driver lines in skeletal biology? The problems of specificity, toxicity, and inducibility

The first step in addressing the “Cre crisis” in the genetic models of bone biology requires identifying how and why the current models fall short. Among the 3 major bone cell types in which investigators seek to recombine floxed alleles—osteocytes, osteoblasts, osteoclasts—there are essentially 2 Cre drivers for osteocytes (Dmp1-Cre and Sost-Cre), 3 drivers for osteoblasts (Ocn-Cre, Col1-Cre, Osx-Cre), and 4 drivers for osteoclasts (Trap-Cre, Ctsk-Cre, Rank-Cre, and LysM-Cre). Tamoxifen-inducible versions (CreERT2) of each of these drivers have also been generated, with the exception of Trap.

### (1) Specificity:

A conceptual example was provided above, regarding the use of an osteoclast Cre driver line (Ctsk-Cre) to recombine a generic floxed GOI allele. We described the system in the context of targeting osteoclasts, while sparing other cell types, including kidney epithelial cells. Although the example served its purpose in highlighting the concept of selectivity of targeted cell types, what was omitted from discussion is that Ctsk-Cre is active not only in osteoclasts, but a variety of other cell types including osteocytes, periosteal cells, marrow cells, and gametes (testes and ovaries). Because of the unwanted/unintended expression in cells other than osteoclasts—a phenomenon known as “off-target expression”—it becomes very difficult to assign a particular phenotype observed in Ctsk-Cre × floxed GOI mice to the osteoclast *per se*, since recombination occurred in a variety of cells and any one of those non-osteoclast cell types (or some combination) could be driving the phenotype. In other words, the phenotype might have nothing to do with GOI loss in the osteoclast. While this phenomenon of off-target expression applies to the Cre driver lines targeting all of the major bone cell types, it should not be that surprising that each promoter has off target effects outside the bone cell of interest. It is extremely rare to find a gene or promoter in the mammalian genome that has exclusive expression to only one cell type, at one stage of differentiation, especially when one considers the very complex and still poorly understood transcriptional events that occur during embryonic development. Below, we briefly review what is known about specificity problems from the published literature and personal observations in a variety of skeletal Cre models. A more thorough treatment of off-target effects can be found in Couasny et al.<sup>(5)</sup>

**Osteocyte lines:** The most widely used osteocyte Cre driver line is Dmp1-Cre, which uses a portion of the Dentin Matrix Protein-1 promoter to drive Cre expression. Dmp1

is an extracellular matrix protein that is highly enriched in mineralized tissues, thus it is intuitive that the *Dmp1* promoter would be active in bone. It is highly expressed by osteocytes and late-stage osteoblasts, and also in dentin-producing odontoblasts (thus, its name). *Dmp1-Cre* produces reliable recombination in the osteocyte population. Two versions of *Dmp1-Cre* have been generated, which differ in their promoter fragment length, and likely, their integration site, though these are not known/reported. The <sup>10kb</sup>*Dmp1-Cre* is more widely used, but the newer <sup>8kb</sup>*Dmp1-Cre* is also used. Despite being primarily known as an osteocyte driver, *Dmp1-Cre* mice exhibit significant Cre activity in osteoblasts. They also exhibit Cre activity in hypertrophic chondrocytes, skeletal muscle, cerebellum, kidney, intestine, and marrow cells.<sup>(6-9)</sup> A thorough characterization of the *Dmp1-CreERT2* expression profile—other than limb tissues—has not been published, so other off target expression, if it exists, is currently unknown.

More recently, a *Sost-Cre* driver has been recently developed to target recombination in osteocytes. *Sost* is the gene coding for sclerostin, a potent secreted inhibitor of the Wnt co-receptors *Lrp5/6*. The *Sost-Cre* is efficient in targeting osteocytes, but ~90% of marrow cells expressed a fluorescent Cre reporter indicating Cre activity in hematopoietic progenitor cells.<sup>(10)</sup> In addition, the fluorescent reporter signal co-localized with Trap staining, indicating *Sost-Cre* activity in osteoclasts. The inducible version, *Sost-CreERT2*, also nicely targets osteocytes and avoids osteoblasts, marrow, and muscle, but this transgenic mouse has a significant muscle mass phenotype that has thus far been poorly understood.<sup>(11)</sup>

**Osteoblast lines:** Many would consider the “gold standard” osteoblast Cre line to be the *Ocn-Cre* (aka *Bglap-Cre*). Like *Dmp1*, *Ocn* is a matrix protein highly enriched in mineralized tissues. Thus, *Ocn-Cre* mice exhibit strong expression of Cre in osteoblasts and osteocytes; however, work from our lab and others indicates that *Ocn-Cre* mice exhibit significant Cre activity in arteriolar pericytes, reticular cells, hippocampus, and cerebellum.<sup>(12)</sup> The off-target effects of *Ocn-Cre* are not trivial, as we found they cause lethality in conditional *Pik3aCA* mice by ~3wks of postnatal life, which could not be explained by hyperactivation of *Pik3a* in bone.<sup>(13)</sup> The inducible version, *Ocn-CreERT2*, has not been as widely used as the non-inducible version, and only a few studies describing its use have been published.<sup>(14-16)</sup> One study looked at off-target effects in liver, fat, and muscle only,<sup>(15)</sup> and reported no discernible recombination in those tissues. It is unclear whether a more thorough evaluation would identify off-target effects for this inducible driver as well (e.g., those Cre-positive tissues identified for *Ocn-Cre*).

The *Col1a1-Cre* (*Col3.6-Cre* *Col2.3-Cre*) lines are perhaps the next most widely used Cre drivers for targeting early- and late-stage osteoblasts, respectively. *Col1a1* is the most abundant protein in bone, so it is an obvious choice to drive Cre expression in bone tissue. But it is also expressed at high levels in most connective tissues, which prompted investigators to begin “chopping up” the *Col1a1* promoter to see if they could find fragments of the promoter that were capable of selective expression in osteoblasts, perhaps at different stages of differentiation.<sup>(17,18)</sup> That work resulted identification of a 3.6kb fragment of the *Col1a1* promoter that could selectively drive expression in preosteoblasts, and a 2.3kb fragment driving expression in more mature, differentiated osteoblasts. Beyond targeting in their intended bone cells, these models shown recombination in brain, tendon, muscle,

skin and fat.<sup>(19–21)</sup> The tamoxifen inducible version—<sup>3.2kb</sup>Col1a1-CreER—exhibits Cre expression (and the IRES-linked transgene *DsRed*) in osteoblasts, but also in ligament, meniscus, and muscle fascia.<sup>(22)</sup>

Finally, the *Osx1-GFP::Cre* driver, which was introduced above, is a tet-off inducible model that has been used to recombine LoxP sites in osteoblasts. Osterix (*Osx*) is an essential transcription factor for osteoblast differentiation. The *Osx1-GFP::Cre* line uses doxycycline supplementation to keep Cre activity in the “off” state. It achieves good osteoblast recombination activity once dox is removed, but careful examination of the expression profile in these mice indicates additional Cre activity in stromal cells, hypertrophic chondrocytes, adipocytes, perivascular cells, olfactory glomerular cells, and gut epithelium.<sup>(23–25)</sup> The tamoxifen inducible version—*Osx-CreERT2*—is expressed osteoblasts, but has off-target activity in the nucleus pulposus and annulus fibrosis of the IV disk.<sup>(26)</sup>

**Osteoclast lines:** Osteoclast Cre driver lines have been equally challenging, in terms of off-target effects, as those described for osteoblasts and osteocytes. The most widely used osteoclast Cre driver has been the *Ctsk-Cre*. Cathepsin-K (*Ctsk*) is a potent protease, secreted by the osteoclast to degrade collagen and other bone matrix proteins during bone resorption. *Ctsk-Cre* driver lines have been developed using both knockin<sup>(27)</sup> and a transgene<sup>(28)</sup> approaches. Both models show Cre activity in mature osteoclasts, but also in inner periosteum.<sup>(29)</sup> We have reported significant Cre activity in the osteocyte population,<sup>(30)</sup> likely due to the expression of this gene during osteocyte perilacunar remodeling.<sup>(31)</sup> Moreover, we found strong evidence for germline recombination in the transgenic,<sup>(30)</sup> and others have reported the same problem with the knockin.<sup>(32,33)</sup> *Ctsk-CreERT2* mice have been created and exhibit Cre expression in osteoclasts upon tamoxifen induction, but these authors only looked at embryos, where gut, lung, and pancreas generated a detectable signal. Further, the authors report that “some leakiness was still observed” the exact extent of which is not well described.<sup>(34)</sup>

The *Trap-Cre* line<sup>(28)</sup> has been much less widely used as compared to the *Ctsk-Cre*. Tartrate resistant acid phosphatase (*Trap*) is an enzyme secreted by osteoclasts that participates in degradation of matrix-bound phosphoproteins during resorption. While *Trap* does exhibit strong activity in osteoclasts, it also (like *Ctsk-Cre*) exhibits significant expression in osteocytes for the same reasons (i.e., perilacunar remodeling). However, this line also exhibits Cre expression in brain, testes, ovary, heart, kidney, colon, lung, liver, stomach, and spleen.

The *Rank-Cre* is the most recently developed osteoclast Cre line.<sup>(35)</sup> The receptor activator for NF- $\kappa$ B (*Rank*, aka *Tnfrsf11a*) is a cytokine receptor for Rank ligand (*RankL*) that is enriched on the osteoclast plasma membrane. This model was created by knocking Cre into the 3'UTR, where an intervening IRES sequence permitted expression of the Cre transcript when and where *Rank* was expressed, without disrupting *Rank* expression. Few off-target reports (either positive or negative) were reported for *Rank-Cre*, but it is expressed in liver macrophages (Kupfer cells),<sup>(36)</sup> and quite possibly in other cell types beyond osteoclasts where *Rank* is expressed, though it simply has not been investigated.

The LysM-Cre is often used for recombination of floxed alleles in osteoclasts, but LysM is expressed so early in the myeloid lineage that it has wide expression in many myeloid-lineage cells including monocytes, granulocytes, and macrophages,<sup>(37)</sup> but also in the earlier hematopoietic stem progenitor cells<sup>(38)</sup> and also in some neurons.<sup>(39)</sup>

## (2) Toxicity:

Cre recombinase is a bacterial enzyme, and its expression in mammalian cells can have toxic effects, usually in a dose dependent fashion.<sup>(40,41)</sup> Eukaryotic cells exposed to Cre can undergo significant DNA damage even in the absence of LoxP sites.<sup>(42-44)</sup> Further, growth arrest, chromosomal abnormalities, and increased cell death have been observed among Cre-expressing cells.<sup>(45-48)</sup> Similar effects have been reported for CreER. In the skeletal Cre models, the most widely known model with overt Cre toxicity issues is the *Osx-tTA,tetO-EGFP/cre*. This mouse has a skeletal phenotype for numerous endpoints, regardless of whether floxed alleles are present or not. Reported effects of the Cre allele system include significant negative effects on long bone growth, cortical bone accrual, calvarial ossification, spontaneous fractures, and reduced body weight,<sup>(49-52)</sup> Beyond the *Osx-tTA,tetO-EGFP/cre* model, other skeletal Cre models have not been thoroughly investigated for toxicity, so it is difficult to clearly define how and why they might introduce confounding factors into *in vivo* Cre/Lox studies. Simple lack of a “Cre-only” gross phenotype sometimes does not preclude toxic effects in the model. In other words, Cre effects need not be as obvious as those for *Osx-Cre* in order to have confounding effects in an experiment. Among fields that have done a more thorough job of evaluating Cre toxicity in their preferred Cre drivers, toxic effects (or at the very least, cell-altering effects) are frequently observed. For example, the most widely used Cre driver line for ocular lens recombination is the *Le-Cre*, which is associated with cataracts and microphthalmia,<sup>(53)</sup> among other abnormalities.<sup>(54)</sup> Significant cellular toxicity has been measured in Cre driver mice designed to target sperm,<sup>(44)</sup> immune cells,<sup>(55,56)</sup> fibroblasts,<sup>(40)</sup> neurons,<sup>(57)</sup> and gastric epithelium,<sup>(58)</sup> among others. Within the musculoskeletal system, the popular muscle driver  $\alpha$ MHC-MerCreMer exhibits heart fibrosis and increased expression of inflammatory cytokines regardless of the presence of floxed alleles.<sup>(59-61)</sup> Numerous studies have revealed that Cre toxicity is related to Cre expression levels,<sup>(40,44,47,48,55)</sup> which is ironic because once a gene promoter is selected to drive Cre, typically the most prominently expressed promoter fragment is chosen from among the various known fragments to drive Cre expression.<sup>(6)</sup>

In summary, many Cre driver lines exhibit toxic effects in the Cre-targeted cell populations. In some cases the toxic effects are overt (e.g., *Osx*), with a gross phenotype clearly discernible. In other models the toxicity might more subtle, affecting gene transcription and/or cell viability. Regarding skeletal Cre models specifically, very few investigators have looked closely enough to determine the presence and/or severity of Cre toxicity for most of the lines; thus we are agnostic as to the toxicity effects for most of the commonly used skeletal drivers.

## (3) Inducibility:

The majority of inducible Cre driver lines, regardless of target tissue, employ the CreER system which was described earlier. The Cre-ER<sup>T</sup> (and later CreER<sup>T2</sup>) system has been

existence in mice since 1996, when the first tamoxifen-inducible mouse line was created using a ubiquitous promoter (CMV-CreER<sup>T</sup>).<sup>(62)</sup> Since then, it has been adopted into virtually every tissue/cell type, to the point where it is difficult to find a promoter used to drive Cre that is not also used to drive CreERt2. As mentioned above, the skeletal biology field has access to Dmp1-CreERt2 and Sost-CreERt2 for inducible osteocyte recombination, to Col1a1-CreERt2 and Osx-CreERt2 for inducible osteoblast recombination, and to the Ctsk-CreERt2 for inducible osteoclast recombination. The chemical induction agent for CreERt2 is tamoxifen. This is an unfortunate requirement since tamoxifen—a clinically used agent—is an active SERM with well-known potent effects on bone metabolism. Tamoxifen is commonly administered to breast cancer patients, who can expect to undergo increases in BMD and reduced fracture risk as a side effect of tamoxifen treatment.<sup>(63)</sup> We have found both anti-catabolic and anabolic effects in the skeletons of mice treated with inductive doses of tamoxifen,<sup>(64,65)</sup> a result that has been confirmed in other labs.<sup>(66,67)</sup> Further, tamoxifen is reported to increase femur length. Thus, ironically, tamoxifen is perhaps one of the least desirable ligands that could be used for an inducible Cre system for bone studies, yet it is the dominant system in bone inducible Cre models. Its strong effects on bone properties, independent of any genetic recombination effects it might have through CreERt2 activation, complicate interpretation of genetic studies, and frequently necessitate large numbers of additional control groups.<sup>(64,65)</sup>

Beyond the complicating effects of CreERt2's active ligand (i.e., tamoxifen) on bone metabolism, another problem with this system is the dynamic range. Here, we define the dynamic range as the range between baseline leakiness and ligand-driven induction of the recombinase. Leakiness is unprovoked, or uninduced, recombination; that is, recombination in the absence of tamoxifen. Conversely, induction is the ability of ligand-provoked cell populations to become active and undergo *Lox* recombination. A large dynamic range is desirable for inducible models: a very low percentage of cells expressing background activity prior to induction, and a very high percentage of target cells exhibiting recombinase activity upon ligand-based induction. Unfortunately, many of the skeletal inducible Cre models have a poor dynamic range. For example, we have found around 15–20% leakiness in the Dmp1-CreERt2 line, and induction with a reasonably strong tamoxifen dose brings the percent of Cre-active osteocytes up to around 45%. The other popular inducible osteocyte driver line—Sost-CreERt2—also has a poor dynamic range (Fig. 3). In 2-mo old female mice, leakiness was around 12% and after tamoxifen induction the active osteocyte population reached 25%, for a net dynamic range of ~13%. In males, 31% baseline recombination and ~63% tamoxifen-induced recombination yielded a dynamic range of ~32%.<sup>(11)</sup> Unfortunately, dynamic ranges for the other bone cell active CreERt2 lines have not been rigorously reported, so it is difficult to estimate whether they have similar dynamic ranges as those reported for inducible osteocyte drivers. Using the mT/mG reporter line, the <sup>2.3kb</sup>Col1a1-CreERt2 has a reported inducibility of 54% in osteoblasts, and low (not specified) leakiness.<sup>(67)</sup>

### **Moving toward solutions for skeletal Cre models:**

While it is apparent that selectivity, toxicity, and inducibility are problems in varying degrees for current skeletal Cre models, solutions do exist for most of these issues. Many of

these solutions have been achieved in other cell types and target tissues, and thus the time is ripe to begin importing those remedies into the skeletal biology world.

### **Solutions for specificity:**

The most high-yield improvement to tissue/cell selectivity is the two-promoter approach. As mentioned earlier, there is no “silver bullet” single promoter that achieves selectivity in only one cell type through all stages of embryonic development, growth, and maturity. Thus it is unreasonable to expect that a single promoter can yield the specificity requires by many experimental hypotheses and designs. The two promoter approach is based on the principle of overlapping expression in the cell type of interest. For example, osteocytes strongly express both *Mepe* and *Sost*. However, neither of these genes are exclusive to the osteocyte. *Sost* expression is found in coronary arteries and hypertrophic chondrocytes, and *Mepe* expression is found in the amygdala and cerebral cortex. The key point here is that the non-osteocytic expression of *Sost* does not overlap with the non-osteocytic expression of *Mepe*, an observation that is best represented by the Venn diagram in Fig. 4A. If the expression of Cre is restricted to only those cells where both *Mepe* and *Sost* are expressed (the overlapping green area in 2A), true osteocyte selectivity could be achieved. This can be accomplished at least two different ways. The first is a “sequential release” approach, where expression of one promoter is required to turn on the Cre, driven by a different promoter. If we take the *Mepe/Sost* example, we could design a simple *Mepe-Dre* transgenic mouse, where the recombinase *Dre* is driven by the *Mepe* promoter. *Dre* is an alternate form of Cre that recognizes its own target sequence known as *Rox*. Much like Cre recognizes and recombines *LoxP*, *Dre* recognizes and recombines *Rox* sites.<sup>(68)</sup> Fortunately, Cre does not cut *Rox* and *Dre* does not cut *LoxP* (i.e., there is no detectable “crossover”), making these two systems independently functional within the same cell. If we designed a second transgenic mouse, *Sost-RSR.Cre*, where *Sost* drives expression of Cre but the Cre is inactive due to a “roxed” stop sequence (i.e., *Rox-Stop-Rox-Cre*), then only when both transgenes are present will the *Mepe*-driven *Dre* unlock expression of the *Sost*-driven Cre. If only *Sost* is expressed in a cell (and not *Mepe*), there will be no Cre expression since *Dre* is required to turn on Cre. If only *Mepe* is expressed, (and not *Sost*) there will be no Cre expressed since the promoter for Cre is not expressed. It is only among cells where both are expressed that we will get Cre recombinase expression. The sequential release requires an understanding of expression profiles during differentiation in order to fully capitalize on the system. The system has been used successfully to improve Cre selectivity in the mouse heart, where  $\alpha$ MCH-*Dre* and *Ki67-RSR.Cre* were used sequentially to selectively recombine cardiomyocytes.<sup>(69)</sup>

Another successful use of the two-promoter approach is to split the coding sequence for Cre into two pieces, and have each portion driven by a different promoter (Fig. 5). Of course, there must be a mechanism for post-translationally rejoining the two Cre protein portions back together so that the ligated Cre protein is functional and capable of inducing recombination. Rejoining the two portions can be accomplished by fusing each Cre fragment with a dimerization fragment, which either induces self-assembly to the other Cre fragment without chemical prompting (e.g., inteins<sup>(70)</sup>) or reassembles Cre in the presence of a dimerization molecule.<sup>(71)</sup> Choices for dimerization fragments are

broad, and include peptides that respond to the rapamycin analog rapalog (AP29167), and the plant hormones gibberellic acid or abscisic acid. Moreover, dimerization fragments are available that respond to other co-expressed proteins like GFP,<sup>(72)</sup> and light-inducible systems for Cre reassembly are available,<sup>(73)</sup> but these are unlikely to gain much traction in bone Cre models given the density of bone tissue and refraction properties of packed mineral. To provide an example of how the split Cre system could be used in bone models, we will use the Mepe/Sost dual promoter example. We can create a transgenic mouse that harbors a Mepe-<sup>N</sup>Cre.GAI allele. Here, the Mepe promoter drives expression of an N-terminal Cre fragment, which is fused to the dimerization molecule GAI. A second transgenic mouse could be designed to harbor a Sost-<sup>C</sup>Cre.GID1 allele. Here, the Sost promoter drives expression of the remaining C-terminal portion of Cre, fused to the complementary dimerization molecule GID1. In a mouse that harbors both transgenes, both Cre fragments are made simultaneously only in cells that express both Mepe and Sost (i.e., mature osteocytes) but the two fragments do not associate and therefore there is no recombination activity. Injection of gibberellin, a synthetic phytohormone that is not present in the mammalian genome, induces the dimerization of GID1 and GAI, bringing the two Cre fragments into alignment and ultimately, inducing recombination. Note some differences between the sequential Cre approach and the split Cre approach, both of which use two promoters for specificity. The sequential Cre approach does not require that both promoters to be active in the same cell at the same time. It does require that the Dre expresser be active sometime prior to the RSR.Cre expresser. For example, if we were to use Dmp1 and Sost promoters for our two-promoters in a sequential Cre approach, we would need to drive Dre with Dmp1 and drive RSR.Cre with Sost, and not vice versa, since Dmp1 comes on earlier in the osteocyte (i.e., in the transitional osteocyte) than does Sost. By the time Sost is expressed, there is very little Dmp1 expression. Conversely, the disadvantage of the split Cre approach is the requirement of simultaneous expression of both promoters, but the advantage is that it represents an inducible model once both constructs are expressed (though RSR.CreErt2 could be used instead of RSR.Cre, making the sequential Cre approach inducible). Another advantage of the split Cre approach over current (CreErt2) approaches for induction is that the ligands used for dimerization and Cre activation in the split Cre model likely come with milder side effects than tamoxifen, though this needs to be demonstrated experimentally. The split-Cre approach has been used successfully in neuroscience applications to target parenchymal microglia (Sall1-<sup>N</sup>Cre + Cx3cr1-<sup>C</sup>Cre) and vasculature associated macrophages (Lyve-<sup>N</sup>Cre + Cxcr1-<sup>C</sup>Cre). Partial models have also been developed where one component is a transgene in the mouse genome and the other Cre component is injected as a virus.<sup>(74)</sup> The successful use of two promoter approaches in neuroscience suggests that similar advances can be made in the skeletal biology field to improve specificity.

### **Solutions for toxicity:**

One of the main problems with Cre expression, whether constitutive or activated inducible, is the persistence of Cre after LoxP sites are recombined. LoxP recombination can occur relatively quickly after Cre is translated or activated with ligand, and once the LoxP sites are recombined there is no need for Cre to be present in the cell. Once LoxP is recombined by Cre, the DNA is permanently altered in that cell and all daughter cells that arise, so

the persistence of Cre expression after LoxP recombination has no utility and is only problematic. To provide some context, an Ocn-Cre driver will express Cre in the osteoblast population as these cells mature and begin producing matrix; but some of those cells will go on to become osteocytes and potentially live in the mineralized matrix for a year or more, all the while producing Cre (since osteocytes express Ocn, albeit at lower levels than the osteoblast). Self-limiting Cre constructs have been developed and validated in vitro for mammalian cells, but to our knowledge they have not been adopted to in vivo models. The approach basically works by flanking the Cre coding sequence with LoxP sites, so that once Cre is transcribed and translated, it will find and recombine the LoxP sites with the GOI but also those that flank the Cre gene itself (Fig. 6). That way, the GOI is inactivated simultaneously with Cre gene inactivation, and only a very brief window of Cre expression occurs in order to achieve the genomic modification to the target gene. These self-excising modifications have not made their way into Cre mouse drivers but the successful proof-of-principle experiments done in cultured cells,<sup>(48,75)</sup> as well as in plants<sup>(76)</sup> (including in the context of split Cres), suggest that the time is ripe to begin engineering Cre drivers in mice with self-limiting capacity to reduce toxicity.

### Solutions for inducibility:

As discussed earlier, all of the inducible skeletal Cre models currently in existence suboptimal dynamic ranges, driven either by high baseline (unprovoked) Cre activity, relatively low induction activity/penetrance after stimulation, or both. Moreover, the tamoxifen/CreERT2 system for induction was never designed for bone—it was simply adopted into skeletal models “after-the-fact” because it worked in other tissues where tamoxifen effects were of little concern. However, other approaches have been developed that could replace the tamoxifen/CreERT2 system. The first is a next-generation induction strategy known as ddCre (Fig. 5). This system similar to CreER in that it creates a Cre fusion protein where Cre is joined to a ligand-sensitive protein. In this case, the protein domain is a mutant (destabilizing) form of bacterial dihydrofolate reductase (ecDHFR), which causes the entire fusion protein to be rapidly and consistently degraded by the proteasome. Injecting the mice with small doses of antibiotic trimethoprim (TMP) blocks proteasome access to the ddCre protein, and Cre can survive to recombine LoxP sites. TMP-induced protein stabilization is particularly attractive for in vivo applications because TMP can be easily delivered to laboratory animals, exhibits a high rate of diffusion in peripheral tissues and does not have known endogenous targets in mammals. The ddCre model was successfully adopted in the neuroscience field to achieve recombination in forebrain by generating a Camk2 $\alpha$ -ddCre transgenic mouse, and in the ventromedial hypothalamus by generating a cFos-ddCre mouse line. This approach is reported to achieve a greater dynamic range than the tamoxifen-based CreERT2 system.

Turning to the two-promoter system (which also improved specificity), the split-Cre approach is quite amenable to inducibility since there are two protein fragments that can be brought together by a ligand. As discussed earlier, this involves splitting the Cre protein into two portions, and attaching a dimerization peptide that can allow the two fragments to rejoin. Considerable work has been performed in how to best split Cre into two (and even three) pieces. Cre contains 334 amino acids, and there is some controversy in the

literature as to where the best split site is located, though several good sites have emerged. Splitting between aa270/aa271 or aa229/aa230 appears to be highly successful in multiple models.<sup>(70,71,77–79)</sup> There are now numerous dimerization domain pairs to choose from (FKBP/FRB joined by rapalog, PYL/ABI joined by abscisic acid, GID1/GAI joined by gibberellin, GBP2/GBP7 joined by GFP).<sup>(71,72,77,80–82)</sup> The most efficient, dynamic, and least leaky system at this stage appears to be the gibberellin (GIB) -induced dimerization of GID1 and GAI.<sup>(71)</sup> In cultured mammalian cells, the GID1/GAI system exhibited several fold increase in signal to noise ratio compared to the CreERT2 system. These next-generation dimerization systems for Cre have not made their way into mouse driver lines but the successful proof-of-principle experiments done in cultured cells suggest that the skeletal biology field can engineer Cre drivers with new dimerization tools to improve the dynamic range of recombination in bone cells.

## Summary

Cre/lox mouse models have revolutionized our ability to conduct *in vivo* research, to better understand the molecular biology musculoskeletal diseases and disorders. As with any model system, they are imperfect and have their shortcomings. However, new discoveries in genetic engineering, dimerization kinetics, recombineering, and cell identity provide new opportunities to improve *in vivo* experimental design in genetic studies. Some of these improvements have already made their way into mouse models in other fields of science, while others show great promise *in vitro* but have yet to be validated for *in vivo* model use. If skeletal research is to advance at a pace needed to combat the increasing burden of musculoskeletal diseases, the time is upon us to begin improving skeletal Cre models to more precisely understand the *in vivo* biology of different skeletal cells, and ultimately, the origins of disease.

## REFERENCES CITED

1. Sakharkar MK, Chow VT & Kanguane P (2004) Distributions of exons and introns in the human genome. *In Silico Biol* 4(4):387–393 [PubMed: 15217358]
2. Gu H, Marth JD, Orban PC, Mossmann H & Rajewsky K (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* 265(5168):103–106 [PubMed: 8016642]
3. Feil R, Wagner J, Metzger D & Chambon P (1997) Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun* 237(3):752–757 [PubMed: 9299439]
4. Zhang Y, Riesterer C, Ayrall AM, Sablitzky F, Littlewood TD & Reth M (1996) Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res* 24(4):543–548. [PubMed: 8604292]
5. Couasnay G, Madel MB, Lim J, Lee B & Elefteriou F (2021) Sites of Cre-recombinase activity in mouse lines targeting skeletal cells. *J Bone Miner Res* 36(9):1661–1679 [PubMed: 34278610]
6. Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF & Feng JQ (2007) DMP1-targeted Cre expression in odontoblasts and osteocytes. *J Dent Res* 86(4):320–325 [PubMed: 17384025]
7. Lim J, Burclaff J, He G, Mills JC & Long F (2017) Unintended targeting of Dmp1-Cre reveals a critical role for Bmpr1a signaling in the gastrointestinal mesenchyme of adult mice. *Bone Res* 5:16049. [PubMed: 28163952]
8. Kalajzic I, Matthews BG, Torreggiani E, Harris MA, Divieti Pajevic P & Harris SE (2013) *In vitro* and *in vivo* approaches to study osteocyte biology. *Bone* 54(2):296–306. [PubMed: 23072918]

9. Gorski JP, Huffman NT, Vallejo J, Brotto L, Chittur SV, Breggia A, Stern A, Huang J, Mo C, Seidah NG, Bonewald L & Brotto M (2016) Deletion of *Mbtps1* (*Pcsk8*, *S1p*, *Ski-1*) Gene in Osteocytes Stimulates Soleus Muscle Regeneration and Increased Size and Contractile Force with Age. *J Biol Chem* 291(9):4308–4322. [PubMed: 26719336]
10. Xiong J, Piemontese M, Onal M, Campbell J, Goellner JJ, Dusevich V, Bonewald L, Manolagas SC & O'Brien CA (2015) Osteocytes, not Osteoblasts or Lining Cells, are the Main Source of the RANKL Required for Osteoclast Formation in Remodeling Bone. *PLoS One* 10(9):e0138189. [PubMed: 26393791]
11. Maurel DB, Matsumoto T, Vallejo JA, Johnson ML, Dallas SL, Kitase Y, Brotto M, Wacker MJ, Harris MA, Harris SE & Bonewald LF (2019) Characterization of a novel murine *Sost* ER(T2) Cre model targeting osteocytes. *Bone Res* 7:6. [PubMed: 30820362]
12. Zhang J & Link DC (2016) Targeting of Mesenchymal Stromal Cells by Cre-Recombinase Transgenes Commonly Used to Target Osteoblast Lineage Cells. *J Bone Miner Res* 31(11):2001–2007. [PubMed: 27237054]
13. Dasgupta K, Lessard S, Hann S, Robling AG & Warman ML (2021) Sensitive detection of Cre-mediated recombination using droplet digital PCR reveals Tg(BGLAP-Cre) and Tg(DMP1-Cre) are active in multiple non-skeletal tissues. *Bone* in press
14. Ono N, Ono W, Mizoguchi T, Nagasawa T, Frenette PS & Kronenberg HM (2014) Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. *Dev Cell* 29(3):330–339. [PubMed: 24823376]
15. Frey JL, Kim SP, Li Z, Wolfgang MJ & Riddle RC (2018) beta-Catenin Directs Long-Chain Fatty Acid Catabolism in the Osteoblasts of Male Mice. *Endocrinology* 159(1):272–284. [PubMed: 29077850]
16. Yoshikawa Y, Kode A, Xu L, Mosialou I, Silva BC, Ferron M, Clemens TL, Economides AN & Kousteni S (2011) Genetic evidence points to an osteocalcin-independent influence of osteoblasts on energy metabolism. *J Bone Miner Res* 26(9):2012–2025. [PubMed: 21557308]
17. Kalajzic Z, Liu P, Kalajzic I, Du Z, Braut A, Mina M, Canalis E & Rowe DW (2002) Directing the expression of a green fluorescent protein transgene in differentiated osteoblasts: comparison between rat type I collagen and rat osteocalcin promoters. *Bone* 31(6):654–660 [PubMed: 12531558]
18. Krebsbach PH, Harrison JR, Lichtler AC, Woody CO, Rowe DW & Kream BE (1993) Transgenic expression of COL1A1-chloramphenicol acetyltransferase fusion genes in bone: differential utilization of promoter elements in vivo and in cultured cells. *Mol Cell Biol* 13(9):5168–5174. [PubMed: 8355676]
19. Liu F, Woitge HW, Braut A, Kronenberg MS, Lichtler AC, Mina M & Kream BE (2004) Expression and activity of osteoblast-targeted Cre recombinase transgenes in murine skeletal tissues. *Int J Dev Biol* 48(7):645–653 [PubMed: 15470637]
20. Scheller EL, Song J, Dishowitz MI, Soki FN, Hankenson KD & Krebsbach PH (2010) Leptin functions peripherally to regulate differentiation of mesenchymal progenitor cells. *Stem Cells* 28(6):1071–1080. [PubMed: 20506495]
21. Scheller EL, Leininger GM, Hankenson KD, Myers MG Jr. & Krebsbach PH (2011) Ectopic expression of *Col2.3* and *Col3.6* promoters in the brain and association with leptin signaling. *Cells Tissues Organs* 194(2–4):268–273. [PubMed: 21555864]
22. Ouyang Z, Chen Z, Ishikawa M, Yue X, Kawanami A, Leahy P, Greenfield EM & Murakami S (2014) *Prx1* and 3.2kb *Col1a1* promoters target distinct bone cell populations in transgenic mice. *Bone* 58:136–145. [PubMed: 24513582]
23. Rodda SJ & McMahon AP (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 133(16):3231–3244 [PubMed: 16854976]
24. Rankin EB, Wu C, Khatri R, Wilson TL, Andersen R, Araldi E, Rankin AL, Yuan J, Kuo CJ, Schipani E & Giaccia AJ (2012) The HIF signaling pathway in osteoblasts directly modulates erythropoiesis through the production of EPO. *Cell* 149(1):63–74. [PubMed: 22464323]
25. Chen J, Shi Y, Regan J, Karuppaiah K, Ornitz DM & Long F (2014) *Osx*-Cre targets multiple cell types besides osteoblast lineage in postnatal mice. *PLoS One* 9(1):e85161. [PubMed: 24454809]

26. Silva MJ & Holguin N (2020) Aging aggravates intervertebral disc degeneration by regulating transcription factors toward chondrogenesis. *FASEB J* 34(2):1970–1982. [PubMed: 31909538]
27. Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, Harada Y, Azuma Y, Krust A, Yamamoto Y, Nishina H, Takeda S, Takayanagi H, Metzger D, Kanno J, Takaoka K, Martin TJ, Chambon P, & Kato S (2007) Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell* 130(5):811–823 [PubMed: 17803905]
28. Chiu WS, McManus JF, Notini AJ, Cassady AI, Zajac JD & Davey RA (2004) Transgenic mice that express Cre recombinase in osteoclasts. *Genesis* 39(3):178–185 [PubMed: 15282744]
29. Ruiz P, Martin-Millan M, Gonzalez-Martin MC, Almeida M, Gonzalez-Macias J & Ros MA (2016) CathepsinKCre mediated deletion of betacatenin results in dramatic loss of bone mass by targeting both osteoclasts and osteoblastic cells. *Sci Rep* 6:36201. [PubMed: 27804995]
30. Kang KS, Hong JM, Horan DJ, Lim KE, Bullock WA, Bruzzaniti A, Hann S, Warman ML & Robling AG (2019) Induction of Lrp5 HBM-causing mutations in Cathepsin-K expressing cells alters bone metabolism. *Bone* 120:166–175. [PubMed: 30409757]
31. Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, Wysolmerski J & Bonewald LF (2012) Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. *J Bone Miner Res* 27(5):1018–1029. [PubMed: 22308018]
32. Winkler CL, Kladney RD, Maggi LB Jr. & Weber JD (2012) Cathepsin K-Cre causes unexpected germline deletion of genes in mice. *PLoS One* 7(7):e42005. [PubMed: 22860046]
33. Dallas SL, Xie Y, Shiflett LA & Ueki Y (2018) Mouse Cre Models for the Study of Bone Diseases. *Curr Osteoporos Rep* 16(4):466–477. [PubMed: 29934753]
34. Sanchez-Fernandez MA, Sbacchi S, Correa-Tapia M, Naumann R, Klemm J, Chambon P, Al-Robaiy S, Blessing M & Hoflack B (2012) Transgenic mice for a tamoxifen-induced, conditional expression of the Cre recombinase in osteoclasts. *PLoS One* 7(5):e37592. [PubMed: 22624050]
35. Maeda K, Kobayashi Y, Udagawa N, Uehara S, Ishihara A, Mizoguchi T, Kikuchi Y, Takada I, Kato S, Kani S, Nishita M, Marumo K, Martin TJ, Minami Y, & Takahashi N (2012) Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. *Nat Med* 18(3):405–412 [PubMed: 22344299]
36. Mass E, Ballesteros I, Farlik M, Halbritter F, Gunther P, Crozet L, Jacome-Galarza CE, Handler K, Klughammer J, Kobayashi Y, Gomez-Perdiguerro E, Schultze JL, Beyer M, Bock C, & Geissmann F (2016) Specification of tissue-resident macrophages during organogenesis. *Science* 353(6304).
37. Clausen BE, Burkhardt C, Reith W, Renkawitz R & Forster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 8(4):265–277 [PubMed: 10621974]
38. Ye M, Iwasaki H, Laiosa CV, Stadtfeld M, Xie H, Heck S, Clausen B, Akashi K & Graf T (2003) Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity* 19(5):689–699 [PubMed: 14614856]
39. Orthgiess J, Gericke M, Immig K, Schulz A, Hirrlinger J, Bechmann I & Eilers J (2016) Neurons exhibit Lyz2 promoter activity in vivo: Implications for using LysM-Cre mice in myeloid cell research. *Eur J Immunol* 46(6):1529–1532 [PubMed: 27062494]
40. Loonstra A, Vooijs M, Beverloo HB, Allak BA, van Drunen E, Kanaar R, Berns A & Jonkers J (2001) Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc Natl Acad Sci U S A* 98(16):9209–9214. [PubMed: 11481484]
41. Anonymous (2007) Toxic alert. *Nature* 449(7161):378 [PubMed: 17898721]
42. Janbandhu VC, Moik D & Fassler R (2014) Cre recombinase induces DNA damage and tetraploidy in the absence of loxP sites. *Cell Cycle* 13(3):462–470. [PubMed: 24280829]
43. Schmidt-Supprian M & Rajewsky K (2007) Vagaries of conditional gene targeting. *Nat Immunol* 8(7):665–668 [PubMed: 17579640]
44. Schmidt EE, Taylor DS, Prigge JR, Barnett S & Capecchi MR (2000) Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc Natl Acad Sci U S A* 97(25):13702–13707. [PubMed: 11087830]
45. Higashi AY, Ikawa T, Muramatsu M, Economides AN, Niwa A, Okuda T, Murphy AJ, Rojas J, Heike T, Nakahata T, Kawamoto H, Kita T & Yanagita M (2009) Direct hematological toxicity

- and illegitimate chromosomal recombination caused by the systemic activation of CreERT2. *J Immunol* 182(9):5633–5640 [PubMed: 19380810]
46. de Alboran IM, O'Hagan RC, Gartner F, Malynn B, Davidson L, Rickert R, Rajewsky K, DePinho RA & Alt FW (2001) Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. *Immunity* 14(1):45–55 [PubMed: 11163229]
  47. Pfeifer A, Brandon EP, Kootstra N, Gage FH & Verma IM (2001) Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. *Proc Natl Acad Sci U S A* 98(20):11450–11455. [PubMed: 11553794]
  48. Silver DP & Livingston DM (2001) Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. *Mol Cell* 8(1):233–243 [PubMed: 11511376]
  49. Davey RA, Clarke MV, Sastra S, Skinner JP, Chiang C, Anderson PH & Zajac JD (2012) Decreased body weight in young Osterix-Cre transgenic mice results in delayed cortical bone expansion and accrual. *Transgenic Res* 21(4):885–893 [PubMed: 22160436]
  50. Chen Z, Yue SX, Zhou G, Greenfield EM & Murakami S (2015) ERK1 and ERK2 regulate chondrocyte terminal differentiation during endochondral bone formation. *J Bone Miner Res* 30(5):765–774. [PubMed: 25401279]
  51. Huang W & Olsen BR (2015) Skeletal defects in Osterix-Cre transgenic mice. *Transgenic Res* 24(1):167–172. [PubMed: 25139670]
  52. Wang L, Mishina Y & Liu F (2015) Osterix-Cre transgene causes craniofacial bone development defect. *Calcif Tissue Int* 96(2):129–137. [PubMed: 25550101]
  53. Lam PT, Padula SL, Hoang TV, Poth JE, Liu L, Liang C, LeFever AS, Wallace LM, Ashery-Padan R, Riggs PK, Shields JE, Shaham O, Rowan S, Brown NL, Glaser T, & Robinson ML (2019) Considerations for the use of Cre recombinase for conditional gene deletion in the mouse lens. *Hum Genomics* 13(1):10. [PubMed: 30770771]
  54. Dora NJ, Collinson JM, Hill RE & West JD (2014) Hemizygous Le-Cre transgenic mice have severe eye abnormalities on some genetic backgrounds in the absence of LoxP sites. *PLoS One* 9(10):e109193. [PubMed: 25272013]
  55. Zeitrag J, Alterauge D, Dahlstrom F & Baumjohann D (2020) Gene dose matters: Considerations for the use of inducible CD4-CreER(T2) mouse lines. *Eur J Immunol* 50(4):603–605 [PubMed: 32087088]
  56. Becher B, Waisman A & Lu LF (2018) Conditional Gene-Targeting in Mice: Problems and Solutions. *Immunity* 48(5):835–836 [PubMed: 29768166]
  57. Forni PE, Scuoppo C, Imayoshi I, Tauli R, Dastru W, Sala V, Betz UA, Muzzi P, Martinuzzi D, Vercelli AE, Kageyama R & Ponzetto C (2006) High levels of Cre expression in neuronal progenitors cause defects in brain development leading to microencephaly and hydrocephaly. *J Neurosci* 26(37):9593–9602. [PubMed: 16971543]
  58. Huh WJ, Mysorekar IU & Mills JC (2010) Inducible activation of Cre recombinase in adult mice causes gastric epithelial atrophy, metaplasia, and regenerative changes in the absence of “floxed” alleles. *Am J Physiol Gastrointest Liver Physiol* 299(2):G368–380. [PubMed: 20413717]
  59. Hall ME, Smith G, Hall JE & Stec DE (2011) Systolic dysfunction in cardiac-specific ligand-inducible MerCreMer transgenic mice. *Am J Physiol Heart Circ Physiol* 301(1):H253–260. [PubMed: 21536850]
  60. Hougen K, Aronsen JM, Stokke MK, Enger U, Nygard S, Andersson KB, Christensen G, Sejersted OM & Sjaastad I (2010) Cre-loxP DNA recombination is possible with only minimal unspecific transcriptional changes and without cardiomyopathy in Tg(alphaMHC-MerCreMer) mice. *Am J Physiol Heart Circ Physiol* 299(5):H1671–1678 [PubMed: 20802136]
  61. Lexow J, Poggioli T, Sarathchandra P, Santini MP & Rosenthal N (2013) Cardiac fibrosis in mice expressing an inducible myocardial-specific Cre driver. *Dis Model Mech* 6(6):1470–1476. [PubMed: 23929940]
  62. Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D & Chambon P (1996) Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A* 93(20):10887–10890. [PubMed: 8855277]

63. Tzeng HE, Muo CH, Chen HT, Hwang WL, Hsu HC & Tsai CH (2015) Tamoxifen use reduces the risk of osteoporotic fractures in women with breast cancer in Asia: a nationwide population-based cohort study. *BMC Musculoskelet Disord* 16:123. [PubMed: 25989902]
64. Kedlaya R, Kang KS, Hong JM, Bettagere V, Lim KE, Horan D, Divieti-Pajevic P & Robling AG (2016) Adult-Onset Deletion of beta-Catenin in (10kb)Dmp1-Expressing Cells Prevents Intermittent PTH-Induced Bone Gain. *Endocrinology* 157(8):3047–3057. [PubMed: 27253995]
65. Bullock WA, Hoggatt AM, Horan DJ, Lewis KJ, Yokota H, Hann S, Warman ML, Sebastian A, Loots GG, Pavalko FM & Robling AG (2019) Expression of a Degradation-Resistant beta-Catenin Mutant in Osteocytes Protects the Skeleton From Mechanodeprivation-Induced Bone Wasting. *J Bone Miner Res* 34(10):1964–1975. [PubMed: 31173667]
66. Perry MJ, Gujra S, Whitworth T & Tobias JH (2005) Tamoxifen stimulates cancellous bone formation in long bones of female mice. *Endocrinology* 146(3):1060–1065 [PubMed: 15576459]
67. Zhong ZA, Sun W, Chen H, Zhang H, Lay YE, Lane NE & Yao W (2015) Optimizing tamoxifen-inducible Cre/loxP system to reduce tamoxifen effect on bone turnover in long bones of young mice. *Bone* 81:614–619. [PubMed: 26232373]
68. Chuang K, Nguyen E, Sergeev Y & Badea TC (2015) Novel Heterotypic Rox Sites for Combinatorial Dre Recombination Strategies. *G3 (Bethesda)* 6(3):559–571. [PubMed: 26715092]
69. Bradley LA, Young A, Li H, Billcheck HO & Wolf MJ (2021) Loss of Endogenously Cycling Adult Cardiomyocytes Worsens Myocardial Function. *Circ Res* 128(2):155–168 [PubMed: 33146578]
70. Wang P, Chen T, Sakurai K, Han BX, He Z, Feng G & Wang F (2012) Intersectional Cre driver lines generated using split-intein mediated split-Cre reconstitution. *Sci Rep* 2:497. [PubMed: 22773946]
71. Weinberg BH, Cho JH, Agarwal Y, Pham NTH, Caraballo LD, Walkosz M, Ortega C, Trexler M, Tague N, Law B, Benman WKJ, Letendre J, Beal J & Wong WW (2019) High-performance chemical- and light-inducible recombinases in mammalian cells and mice. *Nat Commun* 10(1):4845. [PubMed: 31649244]
72. Tang JC, Rudolph S, Dhande OS, Abairra VE, Choi S, Lapan SW, Drew IR, Drokhyansky E, Huberman AD, Regehr WG & Cepko CL (2015) Cell type-specific manipulation with GFP-dependent Cre recombinase. *Nat Neurosci* 18(9):1334–1341. [PubMed: 26258682]
73. Link KH, Shi Y & Koh JT (2005) Light activated recombination. *J Am Chem Soc* 127(38):13088–13089. [PubMed: 16173704]
74. Khoo ATT, Kim PJ, Kim HM & Je HS (2020) Neural circuit analysis using a novel intersectional split intein-mediated split-Cre recombinase system. *Mol Brain* 13(1):101. [PubMed: 32616061]
75. Mahonen AJ, Airene KJ, Lind MM, Lesch HP & Yla-Herttuala S (2004) Optimized self-excising Cre-expression cassette for mammalian cells. *Biochem Biophys Res Commun* 320(2):366–371 [PubMed: 15219836]
76. Yin Q, Li R & Ow DW (2022) Split-Cre mediated deletion of DNA no longer needed after site-specific integration in rice. *Theor Appl Genet* 135(7):2333–2340 [PubMed: 35596798]
77. Jullien N, Sampieri F, Enjalbert A & Herman JP (2003) Regulation of Cre recombinase by ligand-induced complementation of inactive fragments. *Nucleic Acids Res* 31(21):e131. [PubMed: 14576331]
78. Hirrlinger J, Scheller A, Hirrlinger PG, Kellert B, Tang W, Wehr MC, Goebbels S, Reichenbach A, Sprengel R, Rossner MJ & Kirchhoff F (2009) Split-cre complementation indicates coincident activity of different genes in vivo. *PLoS One* 4(1):e4286. [PubMed: 19172189]
79. Wen M, Gao Y, Wang L, Ran L, Li J & Luo K (2014) Split-Cre complementation restores combination activity on transgene excision in hair roots of transgenic tobacco. *PLoS One* 9(10):e110290. [PubMed: 25329460]
80. Liang FS, Ho WQ & Crabtree GR (2011) Engineering the ABA plant stress pathway for regulation of induced proximity. *Sci Signal* 4(164):rs2. [PubMed: 21406691]
81. Razavi S, Su S & Inoue T (2014) Cellular signaling circuits interfaced with synthetic, post-translational, negating Boolean logic devices. *ACS Synth Biol* 3(9):676–685. [PubMed: 25000210]

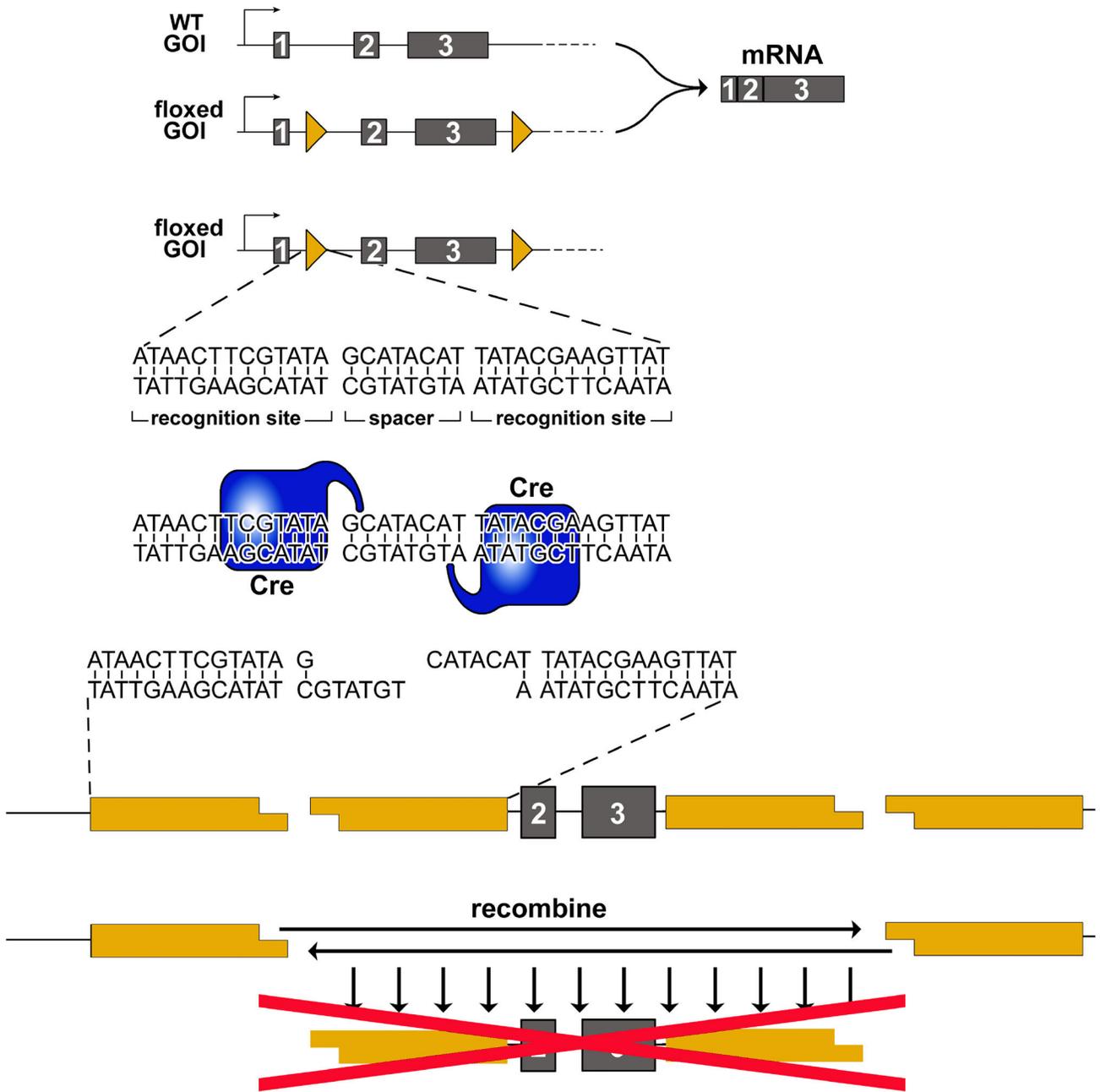
82. Miyamoto T, DeRose R, Suarez A, Ueno T, Chen M, Sun TP, Wolfgang MJ, Mukherjee C, Meyers DJ & Inoue T (2012) Rapid and orthogonal logic gating with a gibberellin-induced dimerization system. *Nat Chem Biol* 8(5):465–470. [PubMed: 22446836]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 1:** Schematic overview of the Cre/Lox system for recombining genomic DNA. A typical gene of interest (GOI) is depicted, with hypothetical exons shown in grey boxes and the intervening introns shown as black lines. 34bp LoxP sequences introduced into the 1<sup>st</sup> and 3<sup>rd</sup> introns are indicated by orange triangles, but their presence does not affect the messenger RNA molecule, as indicated by the identical transcript shown for WT and floxed GOI. (B) Nucleotide-level view of the 5' loxP site, indicating the 13bp inverted palindromic Cre recognition sites, as well as the intervening spacer which provides directionality and is the site for restriction activity. The lower panel shows a pair of Cre molecules binding to the

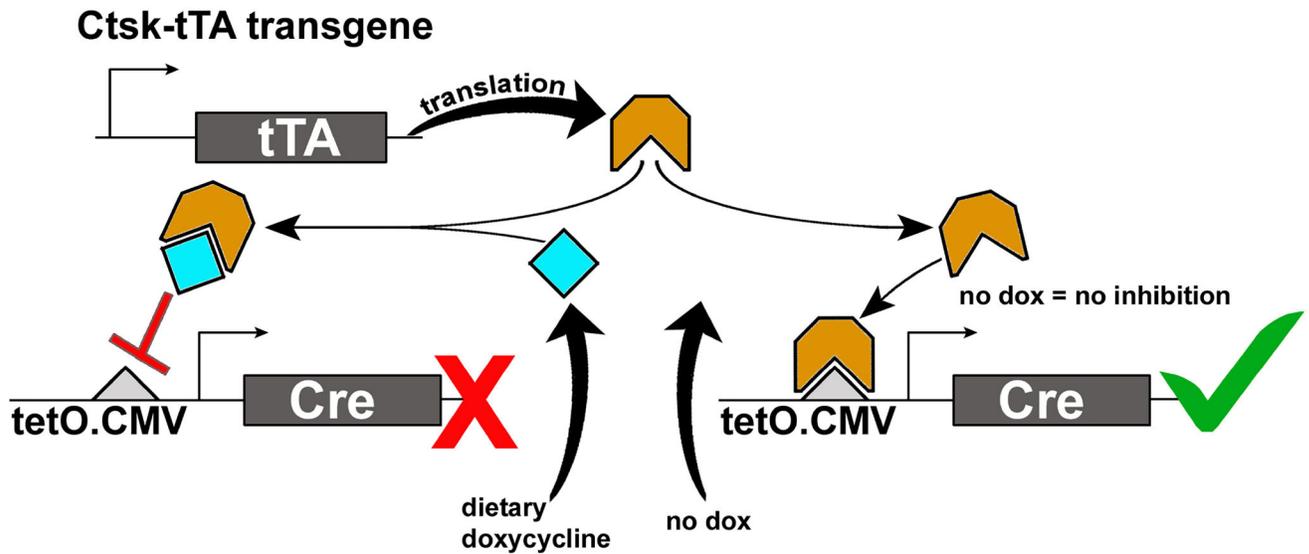
recognition sites, with idealized extensions cutting after the first base (in each direction) of the spacer region. (C) Enzymatically cleaved LoxP site, opened up to show the 6bp overhang created, which will ultimately ligate with the complementary overhang from the 3' loxP site after the intervening piece is removed.

Author Manuscript

Author Manuscript

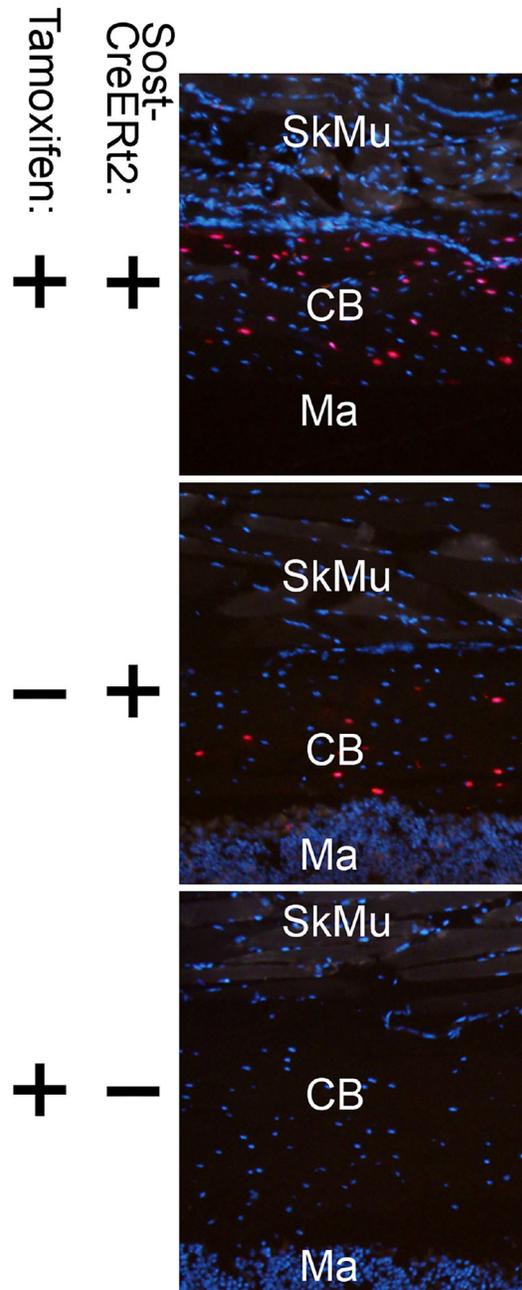
Author Manuscript

Author Manuscript

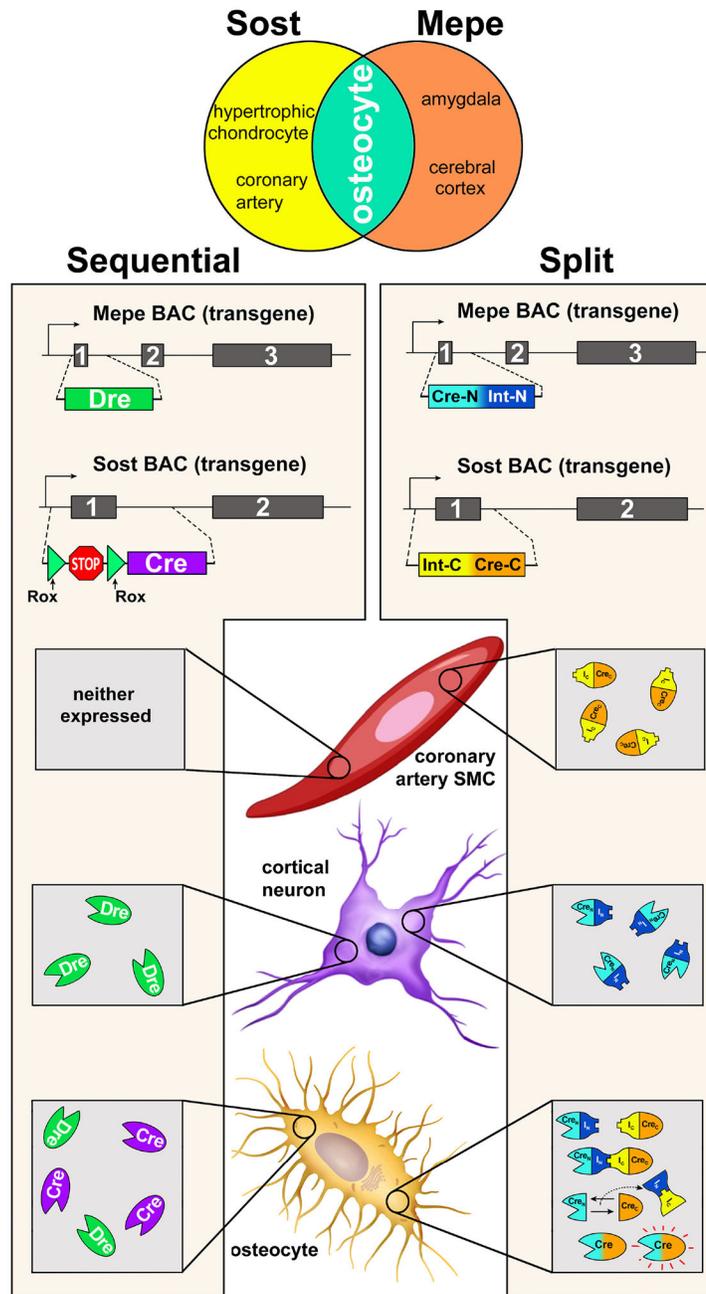


**Figure 2:**

Idealized depiction of the tet-off system for driving Cre expression in osteoclasts. The lower left image shows the Cre transgene, which is driven by the tetO.CMV promoter. The tetO.CMV promoter requires 2 components in order to transcribe Cre. The first is the presence of a specialized transcription factor known as tTA, and the second is the absence of the antibiotic doxycycline in order to allow tTA to be active. The upper panel shows tTA expression being driven by the cathepsin-K promoter, which gives cell selectivity (osteoclast) to the system. The lower right panel shows the presence of tTA, and the absence of dox, which meets the permissive requirements for tetO.CMV activation and Cre transcription. The Ctsk promoter driving tTA expression provides spatial (cell type) control of Cre, while the removal of dox from the diet provides temporal control of Cre. As mentioned in the text, reverse tet systems are also used (tet-on), where dox activates rather than inhibits tTA.

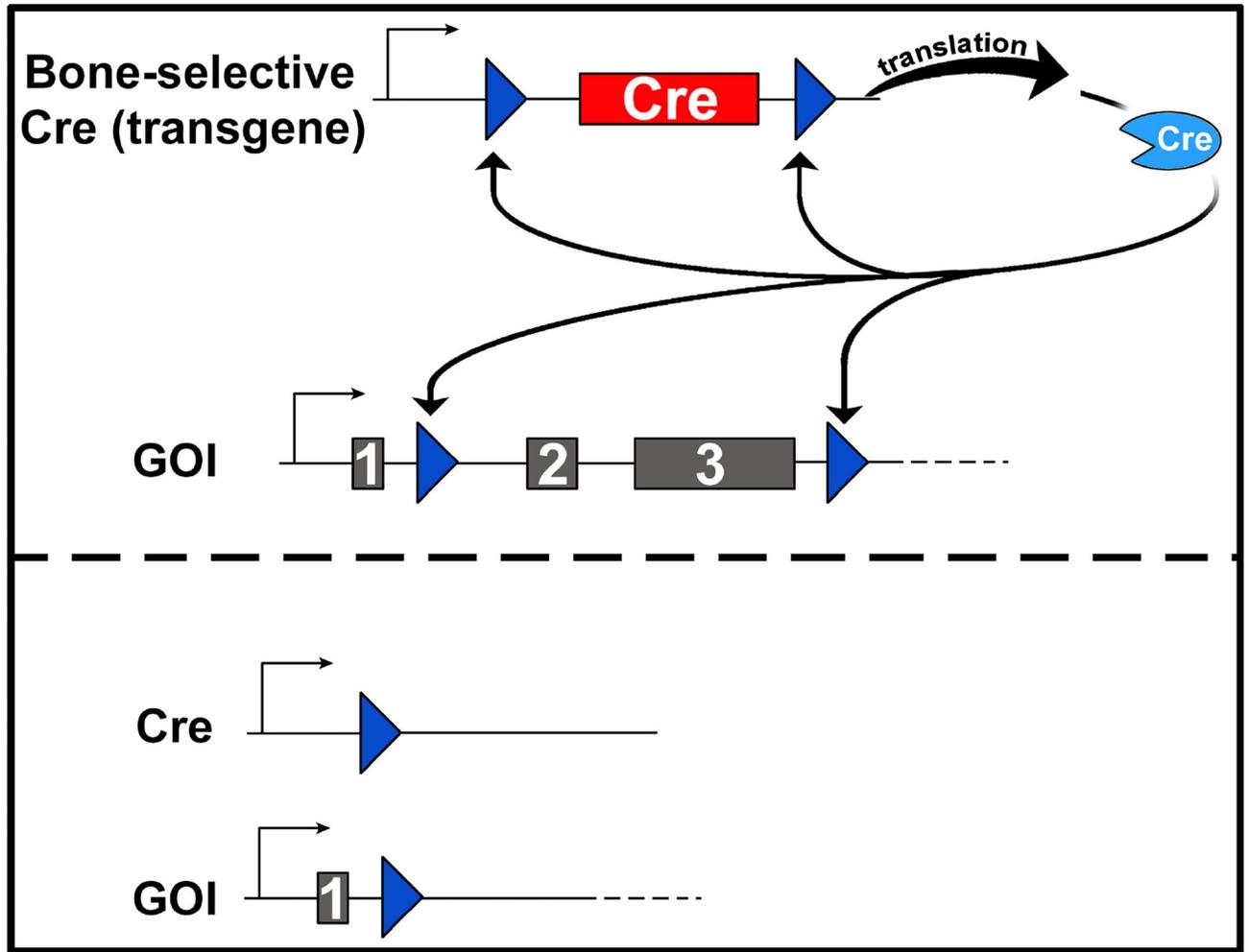


**Figure 3:** Three doses of tamoxifen in *Sost-CreERT2* mice crossed onto the Ai9-tdTomato reporter reveals a poor dynamic range (compare top induced panel with middle uninduced panel). The tdTomato reporter has no detectable activity in Cre-negative mice (lower panel).

**Figure 4:**

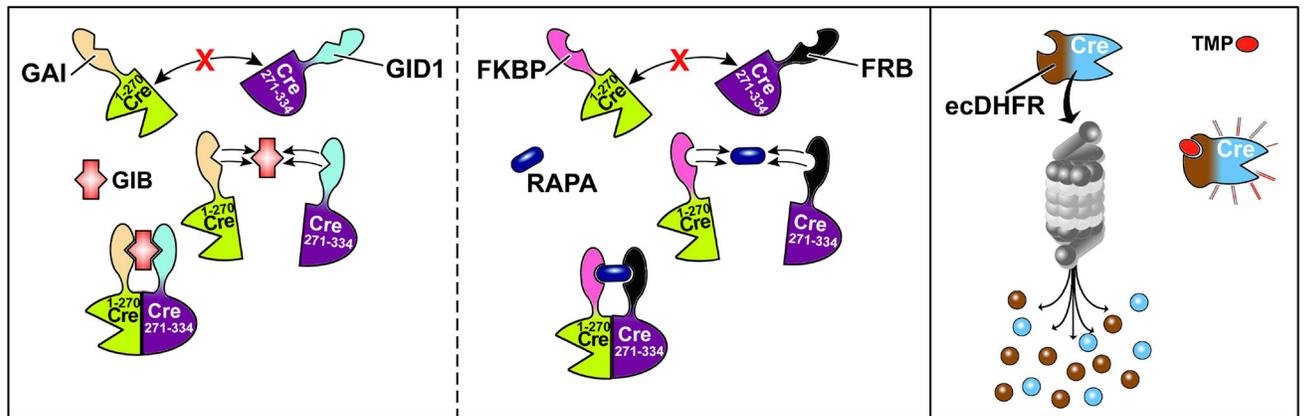
(A) Venn diagram indicating the expression characteristics for two well-known osteocyte genes—*Sost* and *Mepe*. Both exhibit expression outside of the osteocyte population, but their extra-osteocytic expression profiles do not overlap. (B) Splitting the Cre molecule into two components and driving each component by different promoters (in this case, using *Sost* and *Mepe* as hypothetical drivers) allows expression of both Cre components only in cells where *Mepe* and *Sost* expression overlap—i.e., in the osteocytes. The components are able to reassemble and gain activity by designing the Cre sequences such that the two resulting protein fragments are attached to protein splicing elements known as inteins.

Complementary inteins attract to one another and enact a series of autocatalytic peptide bond rearrangements that induces self-excision of the intein components and ligation of the flanking polypeptides. (C) In cells that express Sost but not Mepe (for example, coronary artery smooth muscle cells), the c-terminal fragment of Cre alone is expressed, but without the complementary n-terminal fragment, no active Cre is produced. In cells that express Mepe but not Sost (for example, cerebral cortex neurons), the n-terminal fragment of Cre alone is expressed, but without the complementary c-terminal fragment, no active Cre is produced. In cells that express both Sost and Mepe (i.e., osteocytes), both n- and c-terminal Cre fragments are expressed, and the associated inteins self-assemble the Cre fragments and auto-excise the intein, rendering active Cre protein only in osteocytes.



**Figure 5:**

Next-generation inducible Cre systems to achieve greater inducibility, lower leakiness, and reduced off-target effects (e.g., tamoxifen effects on bone) in the cell type of interest. (A) The two-promoter approach is explained in Fig 3, but here it is assumed to drive Cre fragments that are fused to dimerization elements. For sake of example, the Cre protein is split between amino acids 270 and 271 such that the n-terminal contains aa1-270 and the c-terminal fragment contains aa271-334. Two dimerization systems are depicted; on the left is the GAI/GID1 system which dimerizes via exposure to gibberellin (GIB) and brings the two Cre fragments into a configuration where they can ligate and induce recombination activity. On the right is the rapamycin (RAPA)-sensitive FKBP/FRB system, which functions similarly to the GAI/GID1 system. (B) Inducible system using degraded Cre-ecDHFR fusion protein (ddCre). ddCre is rapidly degraded by the proteasome, rendering it useless to recombine LoxP sites. ddCre is stabilized by the addition of the antibiotic analog trimethoprim (TMP), which induces ddCre survival and results in Cre activity.



**Figure 6:** Self-inactivating Cre systems have been designed and implemented in other model organisms, but not in mice. Self-inactivation can be achieved by flanking the Cre-producing sequence with LoxP sites, such that both the gene of interest (GOI) and the Cre sequence are recombined away soon after Cre turns on, leaving a lonely LoxP site at each locus. This self-limiting approach is likely to reduce the toxic effects of sustained Cre expression, which serves no purpose once LoxP is recombined.