Supporting Information

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SI Data

Considerations Regarding the CreER^{T2}/Tamoxifen System. We first sought to test the system in axolotls by using a Cre reporter, pCAG-loxP-mCherry-3xStop-loxP-EGFP (noted as pCLmCSLE in the Fig. S1). We found this construct was a very tight Cre reporter, i.e., in mammalian 293T cells, when transfected with pCLmCSLE alone, there was no EGFP expression, only mCherry (Fig. S1 *A* and *A′*). When cotransfected with pCAG-ERT2-CreERT2 and induced by addition of 1 μM tamoxifen to the media, cells expressed very reduced levels of mCherry, and instead robustly expressed EGFP, indicating that the induction was working (Fig. S1 *C* and *C′*) However, we did see a small amount of leakiness even with this double ER^{T2} construct—even without tamoxifen, a few of the cells recombined out the mCherry and stop cassette and expressed EGFP. Despite a small amount of leakiness without tamoxifen, we tested the pCLmCSLE construct in regenerating axolotl limb blastemas by electroporation to determine if it could function as a Cre reporter there as well. We never detected mCherry expression from this plasmid ($n > 20$ limbs). Upon sequence analysis, we found the mCherry ORF is not preceded by a canonical Kozak sequence (GCCACC), and we postulate that although it is operational in mammalian cells, in axolotls the GCCACC Kozak is necessary for expression.

After encountering the Kozak issue in pCLmCSLE, we decided to construct our own reporter, pCAG-loxP-EGFP-3xStop-loxP-nls-tdTomato (pCLESLT, described in *Results* and *Materials and Methods*). pCLESLT behaved similarly in mammalian 293T cell culture (Fig. S1 *D–G′*) to pCLmCSLE, but we were optimistic that it might work in vivo in blastemas because we engineered GCCACC Kozak sequences immediately upstream of both fluorescent markers. Indeed, the pCLESLT construct is a robust Cre reporter in blastemas (Fig. 1), but we encountered the leakiness problem in animals coelectroporated with the inducible version of Cre, even with two ER^{T2} elements (Fig. 1).

Optimization of the IPTG-Inducible Construct. The first test of the system was performed using reagents described in (1). Briefly, the expression of EGFP in these constructs is controlled by the Rous sarcoma virus promoter separated from EGFP by three Lac operators. The plasmid also encodes a constitutively expressed LacI repressor. Coelectroporation of this construct with CAG-DsRed into blastemas (8 d postamputation) followed by treatment with IPTG (1 mM in water) for up to 5 d starting at two days postelectroporation did not result in detectable EGFP expression, even when DsRed was expressed highly in many cells. We also tried two derivatives of the Stratagene pOPRSVI/MCS construct, pRSV-O-DsRed2 and pRSV-O-EGFP. These constructs should express DsRed2 and EGFP, respectively, from the RSV promoter when LacI is not present, and they contain two operator sequences, one embedded toward the 3′ end of the RSV promoter and one between the RSV promoter and the fluorophore. Each of these constructs expressed the fluorescent marker in mammalian 293T, but even in these mammalian cells the expression was weak compared with the transfection control (CAG driving a different fluorescent protein), but it was repressible by cotransfection of CAG-LacI. When coelectroporated with a control construct, we did not observe any expression of the reporter proteins from the RSV-O promoter/operator constructs in regenerating axolotl limbs ($n > 20$ limbs for pRSV-O-DsRed2, $n > 16$ for pRSV-O-EGFP, Fig. S2). These two experiments led us to believe the RSV promoter might not be active—or active enough—in axolotls to drive significant expression of a reporter fluorophore. Therefore, we decided to build a repressible construct using a promoter we knew drives very strong expression in the axolotl, CAG. For the first experiment, we inserted a single Lac operator into the pCAG-DsRed2 plasmid between CAG and DsRed2, creating pCAG-LacO-DsRed2. We tested pCAG-LacO-DsRed2 in mammalian 293T cells, and we found that this plasmid drove robust and ubiquitous expression of DsRed2, but when cotransfected with CAG-LacI, expression of DsRed2 could not be repressed. We hypothesized that repression did not occur because the single Lac operator sequence in the pCAG-LacO-DsRed2 construct is situated ~1 kb downstream of the transcriptional start site (TATAA), hence bound LacI repressor cannot inhibit transcription. To test this, we constructed pCA$_{mini}$-EGFP, which contains just the CMV enhancer and chicken β-actin promoter element (but not the introns in the full CAG promoter), a minimal promoter, and EGFP. pCA$_{mini}$-EGFP was electroporated into blastemas and found to drive robust expression of EGFP (Fig. S3 *A* and *A′′*). We then modified pCA$_{mini}$-EGFP to contain various numbers and configurations of Lac operators upstream of the TATAA. We tested representative constructs with various numbers of LacO elements by transfection into mammalian 293T cells with and without LacI (Fig. S3 *B–M′*). A construct with >3 LacO sites showed complete repression of EGFP expression in presence of LacI, but expression of EGFP in the absence of LacI was very weak (Fig. S3 *D* and *D′*). Similarly, constructs with three LacO sequences exhibited very low expression of EGFP in the absence of LacI, rendering them essentially useless for further studies (although this low level of EGFP expression was indeed repressible in the presence of LacI) as well (Fig. S3 *F–G′*). In theory, the spacing of the operators, from the central "G" to the central "G" of the next one, can be 28, 45, 50, or 72 bp. We hypothesize operator number, spacing, and intervening sequence might somehow lead to secondary DNA structures that inhibit the processivity of the polymerase and reduce expression of the reporter even in the absence of LacI repressor. Constructs with two operators gave somewhat diminished EGFP expression even in absence of LacI, and in the presence of LacI were not fully repressed (Fig. S3 *H–I′*). All of the constructs tested that contained just a single operator produced the most robust expression, and they all showed very good repression when cotransfected with LacI. Sequencing of these constructs revealed the orientation of the LacO sequence; the reverse inserts gave marginally better repression than the forward insert, but all were very robust (Fig. S3 *J–M′*). We found similar results in blastemas when representative plasmids were electroporated (Fig. S3 *N–O′*).

We also tested whether adding back the β-actin and β-globin introns to the CA$_{mini}$ promoter would increase expression of the EGFP in the absence of LacI because EGFP expression from CA$_{mini}$-O-EGFP, although still robust, is not as robust as CAG-EGFP. We added introns 3′ to the operator. There was no improvement in EGFP expression when introns were included; hence, for the remaining optimization they were not included (Fig. S3 *P–Q′*).

We decided to further modify the CAmini-O-EGFP construct in attempts to increase EGFP repression in the presence of LacI. The most obvious means of doing this—based on extensive experimentation with the Lac operator system over the last 50 y—was to add additional operators and vary the spacing between them. Although our LacO oligo concatemers were less successful than a single operator (Fig. S3 *B–M′*), we reasoned that because spacing and intervening sequence is extremely important, testing

a commercially generated plasmid specifically optimized for repressible and inducible expression might help. In one strategy, we replaced the CAT reporter in pOPI3-CAT (Stratagene; 5′-RSV promoter-TATAA-three operators spaced 23 bases apart from each other—TATAA-chloramphenicol acetyl transferase ORF) with EGFP, creating p3xLacO-EGFP. We then replaced the RSV promoter with CAmini (amplified by PCR, does not include TATAA) to make pCAmini-3xLacO-EGFP; we separately amplified the CAmini promoter element to contain the TATAA and similarly created pCAmini-TATAA-OPI3-EGFP. The pCA$_{\mathrm{mini}}$-3xLacO-EGFP was prone to recombination around operators, so we continued with pCA$_{\mathrm{mini}}$-TATAA-3xLacO-EGFP (contains three TATAA sites—one at the end of CA$_{\mathrm{mini}}$, one upstream of the Lac operators, one downstream of the Lac operators). Compared with the original pCA$_{\mathrm{mini}}$-LacO-EGFP, the pCA$_{\mathrm{mini}}$-TATAA-3xLacO-EGFP showed extremely reduced levels of unrepressed EGFP expression. However, we also tested a modified version of this created by ligating an O2 operator sequence upstream of the 3xLacO. When ligated in the forward direction in pCA$_{\mathrm{mini}}$-TATAA-O2-3xLacO-EGFP, we saw extremely robust expression of EGFP in mammalian 293T cells, and this robust expression could be nearly entirely repressed by cotransfection with LacI, and induced in presence of LacI when IPTG was included in the media. The plasmid with the reverse O2 drove extremely weak expression of EGFP when transfected into 293T cells, even in absence of LacI (Fig. S4*A*). Therefore, the pCAmini-TATAA-O2-3xLacO-EGFP construct represented a viable starting point for future studies in the axolotl, and it may indeed still be useful for further studies. However, given that we had sometimes seen recombination in other OPI3 derivatives (likely due to the close spacing of the operators), requiring special attention when amplifying or cloning with these plasmids, we also simultaneously pursued a different strategy for improving upon the pCAmini-O-EGFP reporter configuration. As discussed in the *Results* section, we modified the pCAmini-O-EGFP construct further to contain three operators total, the spacing of which was designed based on the mouse transgene from the Scrable laboratory (2). Ultimately, as described in the *Results* section, we used this alternate plasmid, pCAmini-O1O2O3-EGFP, as the parent plasmid for inducible transgenes. In side-by-side comparisons with pCAmini-O2-3xLacO-EGFP, it performed similarly based on fluorescence of transfected cells and detection of expressed EGFP protein by Western blot (Fig. S4*B*), and displayed no detectable recombination when propagated in bacteria.

SI Materials and Methods

The pCAG-loxP-mCherry-3xStop-loxP-EGFP reporter was constructed by modifying the FREPE plasmid (3). The pFREPE configuration is pCAG-FRT-3xStop-FRT-loxP-mCherry-3xStop-loxP-EGFP. We grew this plasmid in Flp-expressing bacteria to recombine out the first stop cassette, resulting in pCAG-loxP-mCherry-3xStop-loxP-EGFP (noted as pCLmCSLE in Fig. S1 *A*–*C*′). pRSV-O-DsRed2, which we generated by cloning DsRed2 into the NotI and KpnI sites in the MCS, and pRSV-O-EGFP, which we generated by cloning EGFP into the NotI site. To generate pCAG-LacO-DsRed2, we inserted a single Lac operator (excised from pOPRSV1-MCS via EcoRI) into the pCAG-DsRed2 plasmid at the EcoRI site between CAG and DsRed2.

To create CA$_{\mathrm{mini}}$-EGFP, we PCR amplified the portion of the promoter/enhancer ("CA$_{\mathrm{mini}}$", contains the CMV enhancer and chicken β-actin promoter missing the β-actin intron) 5′ to the TATAA with SalI on the 5′ end and PacI, XbaI, and SalI on the 3′ end and ligated it into the pStagia3 vector (4) via SalI. We used the PacI site to insert Lac operator sequences. We synthesized two oligos, annealed and polished them, and ligated them into the construct downstream of CA$_{\mathrm{mini}}$ and upstream of the EGFP. The oligos used were: 5′ TAAGAATTGTGAGCGCTCACAATTATTTAAATGCGGCCGCCGATCGTTAAT 3′ and 5′ TAACGATCGGCGGCCGCATTTAAATAATTGTGAGCGCTCACAATTCTTAAT 3′. We analyzed the various configurations by restriction digest. They inserted in both orientations and in varying numbers. Some constructs showed evidence of recombination at/near the LacO sites.

To create pCAmini-TATAA-O2-3xLacO-EGFP, we ligated the oligos: 5′ GATCTATTTAAATAGATCTGTGGAATTGTGAGCGGATAACAATTTCACGGATCCAGATCTA 3′ and 5′ GATCTAGATCTGGATCCGTGAAATTGTTATCCGCTCACAATTCCACAGATCTATTTAAATA 3′; O2 underlined) into the unique BglII site of pCA$_{\mathrm{mini}}$-TATAA-3xLacO–EGFP.

To test the effect of introns, we PCR amplified and cloned the introns into the CA$_{\mathrm{mini}}$-O-EGFP downstream of the operator and TATAA using the AgeI site in the pStagia3 backbone (4), and tested the resulting CA$_{\mathrm{mini}}$-O-introns-EGFP side by side with CA$_{\mathrm{mini}}$-O-EGFP in mammalian 293T cells.

1. Deans TL, Cantor CR, Collins JJ (2007) A tunable genetic switch based on RNAi and repressor proteins for regulating gene expression in mammalian cells. *Cell* 130:363–372.
2. Cronin CA, Gluba W, Scrable H (2001) The lac operator-repressor system is functional in the mouse. *Genes Dev* 15:1506–1517.
3. Dymecki SM, Ray RS, Kim JC (2010) Mapping cell fate and function using recombinase-based intersectional strategies. *Methods Enzymol* 477:183–213.
4. Billings NA, Emerson MM, Cepko CL (2010) Analysis of thyroid response element activity during retinal development. *PLoS ONE* 5:e13739.

Fig. S1. Transfections of two versions of a Cre reporter in mammalian 293T cells. (*A*–*C*′) pCAG-loxP-mCherry-3xSTOP-loxP-EGFP (pCLmCSLE) was transfected into 293T cells and imaged 48 h posttransfection. (*A and A*′) When a Cre-encoding plasmid is not cotransfected, cells express mCherry and no EGFP. (*B* and *B*′) When cotransfected with pCAG-$ER^{T2}CreER^{T2}$ and treated with 1 μM tamoxifen for 24 h, lower levels of mCherry are observed, and most cells robustly express EGFP, indicating that tamoxifen induced $ER^{T2}CreER^{T2}$ entered the nucleus and catalyzed recombination. (*C* and *C*′) When cotransfected with pCAG-ER^{T2}-$CreER^{T2}$, nearly all cells express mCherry alone, but a few express EGFP (arrowheads), indicating a low level of leakiness in the absence of tamoxifen. (*D*–*G*′) pCAG-loxP-EGFP-3xStop-loxP-nls-tdTomato (pCLESLT) was transfected into 293T cells and imaged 48 h posttransfection. (*D* and *D*′) Cells transfected with only

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pCLESLT are green and never red. (*E* and *E*′) In the presence of 1 μM tamoxifen for 24 h, cells transfected with only pCLESLT are still green and never red. (*F* and *F*′) Cells transfected with both pCLESLT and pCAG-$ER^{T2}CreER^{T2}$ and cultured in 1 μM tamoxifen for 24 h show robust tdTomato expression, demonstrating that tamoxifen can potently induce recombination at loxP sites in the pCLESLT plasmid. (*G* and *G*′) Some leakiness is seen in the absence of tamoxifen. When cells are cotransfected with pCLESLT and pCAG-$ER^{T2}CreER^{T2}$, a few cells express tdTomato after 48 h in culture with media alone and no tamoxifen (arrowheads).

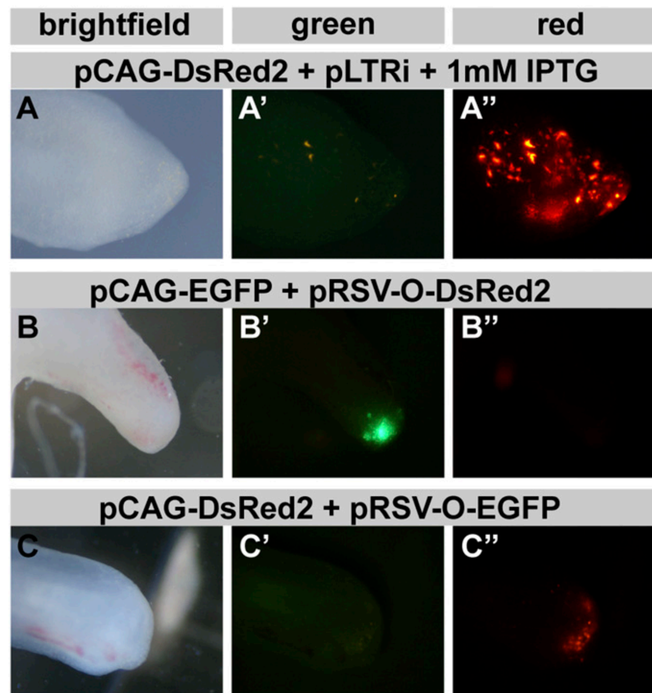

Fig. S2. The RSV promoter does not drive appreciable expression of fluorescent reporters in blastemas. (*A*–*A*′′) The LTRi construct (1) does not drive expression of EGFP in blastemas in the presence of IPTG. Blastemas were electroporated with pLTRi and pCAG-DsRed2 (electroporation control). Animals were housed in 1 mM IPTG. (*A*) Brightfield image of blastema. (*A*′) No EGFP expression was detectable. (*A*′′) Intense DsRed2 expression indicates the electroporation was successful. (*B*–*B*′′) RSV does not drive detectable expression of DsRed2. Blastemas were electroporated with pCAG-EGFP (electroporation control) and pRSV-O-DsRed2. (*C*–*C*′′) RSV does not drive detectable expression of EGFP. Blastemas were electroporated with pRSV-O-EGFP and pCAG-DsRed2 (electroporation control).

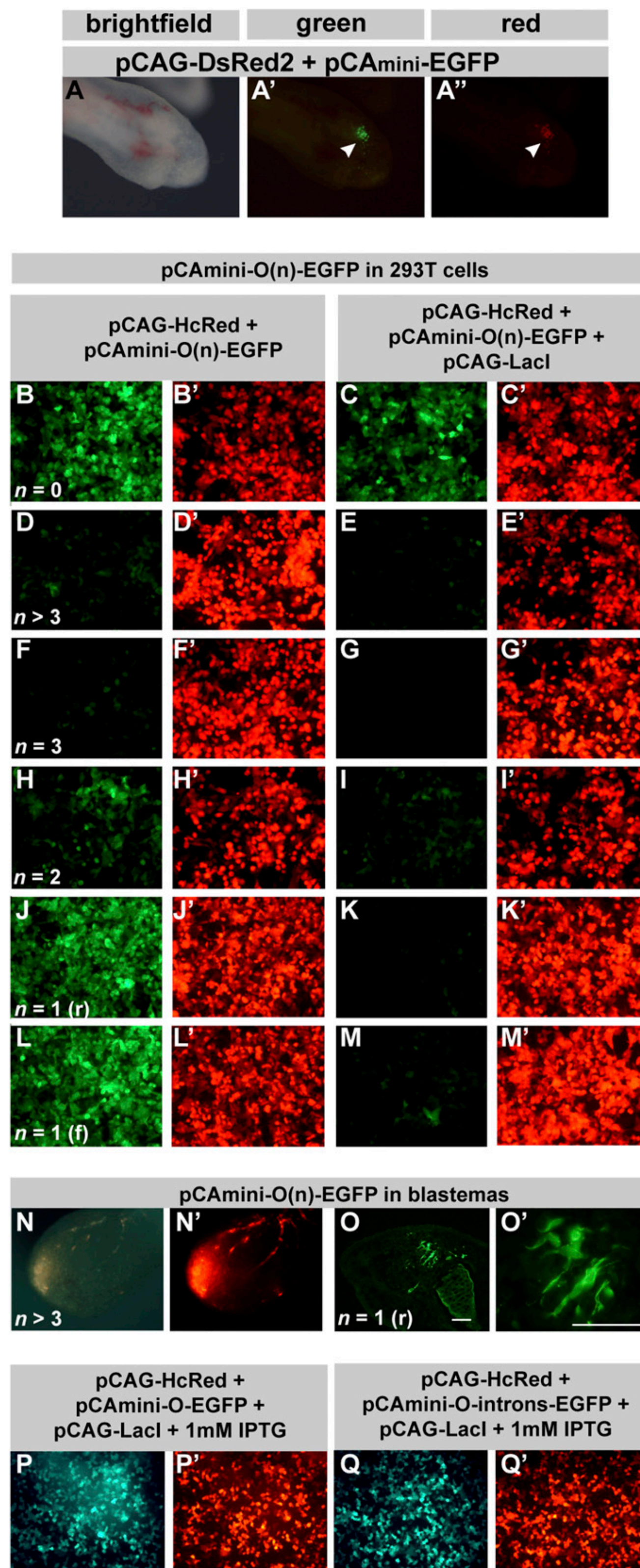

Fig. S3. Early versions of the reporter constructs. (*A*–*A*′′) The CAmini element cloned into a plasmid with a minimal promoter can drive EGFP expression in blastemas (electroporated cells indicated by arrowhead). Coelectroporation marker was pCAG-DsRed2. *B*–*M*′ are modifications of the CAmini-EGFP including various numbers (denoted O_n) and orientations [forward (f) or reverse (r)] of Lac operator elements cloned into a PacI site in the pCAmini-EGFP plasmid produced various effects in EGFP expression (*B* and *B*′, *D* and *D*′, *F* and *F*′, *H* and *H*′, *J* and *J*′, and *L* and *L*′) in the absence of LacI and in the presence of LacI (*C* and *C*′, *E* and *E*′, *G* and *G*′, *I* and *I*′, *K* and *K*′, and *M* and *M*′) in mammalian 293T cells. Cotransfection control was pCAG-HcRed for all. (*N* and *N*′) With greater than three operators at this site, EGFP expression was not detectable in blastemas (N), even when the coelectroporation marker was very strong (*N*′)

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(pCAG-DsRed2). (*O* and *O*′) Low- and high-magnification views of limbs sectioned following electroporation with a plasmid with only one operator showing robust EGFP expression. Scale bar is 100 microns. (*P*–*Q*′) Adding back the introns to reconstitute the entire CAG promoter/enhancer/intron configuration (with introns after the Lac operator) does not appreciable enhance EGFP expression. (*P* and *P*′) Mammalian 293T cells transfected with a construct without introns. (*Q* and *Q*′) Mammalian 293T cells transfected with a construct including introns. Note that *P* and *Q* are similar.

Fig. S4. Optimization of the reporter. Western blots detecting expression, repression, and induction of EGFP (with GAPDH as loading control). Comparison of the original pCAmini-O-EGFP (one operator) to pCAmini-3xO-EGFP (derived from Stratagene's pOPI3-CAT, described in text, contains three LacO1 elements spaced 23 bases apart), pCAmini-O2-3xO-EGFP (addition of LacO2), and pCAmini-O2(r)-OPI3-EGFP (addition of a reverse LacO2) and pCA_{mini}-O1O2O30EGFP (construct with configuration ultimately used).