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FlEx-based transgenic reporter lines for visualization of Cre and Flp activity in live zebrafish

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Summary

Site-specific recombinases such as Cre and Flp are invaluable tools for genetic manipulations, but their usage in zebrafish has been limited. Incorporating recently developed flip-excision (FlEx) design that allows stable inversions, we have established zebrafish reporter lines that express bright and ubiquitous EGFP, but switch to express mCherry in the presence of Cre or Flp. Here, we demonstrate the stable inversion in the reporter lines, both in somatic cells and in the germ line by Cre or Flp, and the subsequent reinversion using the other recombinase. Using the reporter lines, we characterized cardiomyocyte-specific Cre lines and neuronal progenitor-specific and tamoxifen-dependent Cre lines. We also used the reporter lines for screening Cre- and Flp-based enhancer trap lines. Similar to the widely used Cre reporter lines in mice, these FlEx-based reporter lines will facilitate the use of recombinases for genetic manipulations in zebrafish.

Keywords

Cre; Flp; CreERT2; reporter line; enhancer trap; fluorescent proteins; tissue-specific Cre; conditional Cre; zebrafish

Site-specific recombinases Cre and Flp have been extensively used to carry out site-specific recombination in a subset of somatic cells for genetic analysis in mice and flies (Branda and Dymecki, 2004; Feil, 2007; Lee and Luo, 2001; Theodosiou and Xu, 1998). For example, Cre-mediated deletion has been commonly exploited to conditionally inactivate genes and to conditionally activate transgenes in spatial- and temporal-specific fashions in mice (Branda and Dymecki, 2004; Feil, 2007). Similarly, Flp-induced site-specific recombination in flies is used to eliminate gene function by mitotic recombination and to activate transgenes in discrete populations of cells (Lee and Luo, 2001; Theodosiou and Xu, 1998). The use of these recombinases for genetic manipulations in zebrafish is still very limited (Feng *et al.*, 2007; Langenau *et al.*, 2005; Liu *et al.*, 2007; Pan *et al.*, 2005; Thummel *et al.*, 2005).

One prerequisite for genetic analysis using recombinases and conditional mutations is the availability of appropriate Cre or Flp lines. In mice, many recombinase-expressing lines with unique tissue-specificity have been generated and characterized. These lines are mostly

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characterized in two reporter lines that contain a ubiquitous promoter-controlled reporter gene that is interrupted by a transcriptional stop, which is flanked by recombinase target sites (Sato *et al.*, 2000; Soriano, 1999). The presence of Cre removes the stop and allows the expression of the reporter gene. An ingenious new strategy for revealing Cre activity uses a FIEEx switch (Schnutgen *et al.*, 2005; Schnutgen *et al.*, 2003). The switch consists of two tail-to-tail reporter genes that are flanked by opposing dimers of heterotypic target sites. These sites can be recognized by the same recombinase but cannot recombine with each other. Recombination between one pair of homotypic target sites would invert the intervening sequence and bring the other pair in the same orientation for recombinase-mediated excision. After the flip and excision, only two heterotypic target sites remain, one on each side. As a result, the intervening sequence is no longer susceptible to inversion or deletion. Thus, the presence of Cre inverts the switch permanently and changes from expressing one reporter gene to expressing the other. In addition to serving as a recombinase reporter, the FIEEx system also potentially allows subsequent replacement of the reporter genes with any desired DNA fragments by recombinase-mediated cassette exchange (RMCE). This way, one can introduce an allelic series into the same locus, or a recombinase gene into a locus with a desired expression pattern. The FIEEx-based system is thus more advantageous than the commonly used floxed reporter system. Here we report the generation of a FIEEx switch-based reporter line and its use in characterizing several recombinase-expressing transgenic lines obtained by using characterized promoters and by enhancer trap.

To generate the transgene for the Cre and Flp reporter lines, we constructed a synthetic FIEEx switch that can be inverted by both Cre and Flp (Schnutgen *et al.*, 2005). We also constructed a transgene that expresses EGFP but can be switched to express mCherry if inverted. The EGFP and mCherry sequences share a 3xSV40 polyA signal from the pBigT plasmid that works in both orientations (Soriano, 1999; Srinivas *et al.*, 2001). The reporter is controlled by a 2.3 kb regulatory sequence consisting of 0.2 kb *Xenopus EF1* enhancer and 2.1 kb zebrafish *-actin 2* sequence including the 1.5 kb enhancer/promoter sequence, the 1st exon (86 bp) and part of the 1st intron (490bp). To generate a complete intron, the splice acceptor from the rabbit γ -globin gene was placed upstream of the EGFP reporter and a synthetic splice acceptor was placed upstream of the mCherry reporter. The synthetic splice acceptor contains the zebrafish consensus sequence along with intronic and exonic splice enhancers (Yeo *et al.*, 2004)(Fig 1a). The intron in the transgene serves two purposes: increasing the expression of the transgene and housing the FIEEx switch. The switch consists of two fragments, each contains two heterotypic Cre sites (loxP and lox5171) and two heterotypic Flp sites (FRT and F3). One fragment, containing lox5171/loxP/F3/FRT, was placed in the middle of the intron. The other fragment, containing F3/FRT/lox5171/loxP, was placed outside of the synthetic splice acceptor in the opposite orientation (Fig 1a). The transgene was inserted in the mini-Tol2 vector (Urasaki *et al.*, 2006). The original polyadenylation sequence of the bovine growth hormone gene in the vector was kept at the 3' end of the reporters to insure transcriptional termination (Fig 1a). The transgene was denoted as *Tg(eab2:[EGFP-T-mCherry])*. The square brackets indicate the invertible segment. Transgenic lines were made using a standard protocol (Kawakami, 2004). GFP-positive progeny from 3 founders were raised to adulthood. These lines were tentatively named as *Tg(eab2:[EGFP-T-mCherry]) line A, line B, and line C*. All 3 lines exhibited ubiquitous EGFP expression throughout the lifespan (Fig 1c and 1e and data not shown). In fact, these fish could be distinguished with unaided eyes from wild-type fish by their green tone. No mCherry expression was detectable in these lines (Fig 1d, top).

To evaluate the reporter lines, we generated several lines of *Tg(hsp70l:Cre)* and *Tg(hsp70l:Flp)* using the 1.5 kb zebrafish *hsp70l* promoter and standard Tol2 transgenesis protocol (Halloran *et al.*, 2000). To determine whether these *Tg(hsp70l:Cre)* and *Tg(hsp70l:Flp)* lines could support efficient inversion of the FIEEx reporters, we crossed the

germline-transmitting founders of *Tg(hsp70l:Cre)* to the FIE reporters and demonstrated that, when raised at 28°C, double transgenic fish started to express mCherry in the lens by 30 hpf and in the trunk muscles around 60 hpf (data not shown). This is consistent with the basal activity of the *hsp70l* promoter and highlights the potential problem of using this promoter (Blechinger *et al.*, 2002; Scott *et al.*, 2007). This inversion in the trunk muscles is not efficient, however, as the mCherry signal was scattered even at 96 hpf (data not shown). Nevertheless, when 1 day-old embryos from these crosses were treated at 38°C for 1 hour to induce Cre or Flp expression, some embryos expressed mCherry with very faint EGFP signal at 72 hpf (Fig 1d bottom, 1f). No mCherry expression was detected in the absence of Cre (Fig 1d top, 1e). PCR-genotyping confirmed that all mCherry-positive embryos contained the Cre transgene and that most of the reporter transgene was inverted (Fig 1a and 1g). The high efficiency of inversion was further demonstrated at cellular levels. In cross sections of mCherry-positive embryos at 72 hpf, very few EGFP-positive cells were seen (Fig 1f). Therefore, some of the residual EGFP signal observed in live larvae was likely due to expression prior to the inversion. Indeed, at 7 days after heat shock, GFP signal was barely detectable (data not shown). Our unpublished results indicate that EGFP and mCherry have a half-life of more than 24 hours in zebrafish. As expected, no mCherry-positive cells were found in the Cre-negative embryos (Fig 1e). PCR analysis of genomic DNA from a pool of the GFP-positive but mCherry-negative embryos did not detect the Cre transgene. We also found that *Tg(hsp70l:Flp)* yielded similar results (Fig 2a–b). However, both the basal and the induced inversion by Flp seemed to be less efficient. At 72 hpf, only occasional mCherry was detectable in the trunk muscles without heat shock (data not shown). GFP signal remained easily detectable in heat shocked double transgenic embryos even at 7 dpf (Fig 2a). All three reporter lines showed very similar results.

To determine whether inversion occurred in the germline and whether Cre-inverted fish can be further inverted by Flp, we raised the Cre-inverted F1 reporter fish from *Tg(eab2:[EGFP-T-mCherry])* line A to maturity. When a Cre-inverted mCherry-expressing fish was crossed to an F1 *Tg(hsp70l:Flp)* fish, about 75% of the progeny (112/150) were positive for fluorescent protein at 24 hpf prior to heat shock, suggesting the reporter fish contains 2 independent copies of the reporter transgene. Among the fluorescent embryos, 85 were mCherry only, 15 were EGFP only, and 12 were positive for both, indicating that 82% of the germline reporter transgene had been inverted by heat shock at 24 hpf (Table 1). We divided the 85 mCherry only embryos into two groups and heat shocked one group at 24 hpf. At 48 hpf, most of heat shocked embryos (30/44) showed strong GFP signal, indicating that the Cre-inverted reporter gene can be re-inverted by Flp, and that there may be two copies of the *Tg(hsp70l:Flp)* transgene in the transgenic F1 fish. None of the embryos in the control group (0/41) showed higher than basal levels of GFP expression. Similar results were found in other Cre-inverted fish tested.

Flp seems to be less active than Cre in inducing germline inversion. To determine whether Flp induces germline inversion, we raised some Flp-inverted mCherry-positive *Tg(eab2:[EGFP-T-mCherry])* line A fish to determine whether they produced mCherry positive progeny. In 150 embryos from an out cross clutch, we found 22 were mCherry only, 19 were positive for both fluorescent proteins, and 57 were EGFP only. That is, only about 31% of the insertions in the germline had been inverted by Flp, much lower than the 82% induced by Cre (Table 1). This could suggest that Flp may not be as active as Cre in zebrafish, although more data are required to compare them more quantitatively. Nevertheless, we also showed that a Flp-inverted transgene can be inverted again by Cre (data not shown). Taken together, our data showed that the reporter lines we generated can detect Cre and Flp activity and the reporter genes can be inverted twice using two different recombinases.

A good Cre/Flp reporter line should only contain one copy of the transgene that does not cause a detectable phenotype. One copy of the transgene should prevent genome rearrangements as a consequence of recombination between multiple transgenes; and an intergenic insertion should minimize the mutagenic effect of the transgene. We therefore determined the insertion sites of the transgene in *Tg(eab2:[EGFP-T-mCherry])* line A using linker-mediated PCR. As expected, we detected 2 insertions, one mapped on chromosome 7, located 2 kb upstream of the *krcp* gene and the other on chromosome 23 in the 1st intron of the *hbsl1* gene. We kept F2 fish with a single insertion upstream of the *krcp* gene and discarded F2 with insertion in the *hbsl1* gene although homozygotes of either of the insertions did not display any overt defects. The *krcp* insertion line is designated as *Tg(eab2:[EGFP-T-mCherry])vu295* and will be deposited in the Zebrafish International Resource Center (ZIRC) stock center. We found that *Tg(eab2:[EGFP-T-mCherry])* line C has only one insertion but it is located on chromosome 7 in an intron of *mGluR6* gene. This may not be a good reporter line given the importance of mGluR6 in vertebrate visual function (Masu *et al.*, 1995).

The primary purpose of developing the reporter lines was to use it for characterization of Cre and Flp lines for spatial- and temporal-specific genetic manipulations. As an example of a tissue-specific Cre line, we generated transgenic Cre lines with Cre under the control of the well-characterized, 1.8 kb cardiomyocyte-specific promoter from the zebrafish *cmhc2* gene (Huang *et al.*, 2003), also known as *myl7* (ZFIN). We identified 10 germline-transmitting *Tg(my17:Cre)* founders. When mated to the reporters, only two of the founders produced offspring with myocardium-specific inversion (Fig 2c). The offspring of the others displayed ectopic inversion, likely due to ectopic Cre expression (data not shown). This clearly indicated the need to screen for multiple candidates for a good tissue-specific Cre line.

Conditional Cre lines have been instrumental for analysis of gene function during post-embryonic development. To determine if the commonly used tamoxifen-dependent Cre, CreERT2 (Indra *et al.*, 1999), can be used for conditional induction of recombination, we generated lines in which CreERT2 was under the control of the 3 kb neuronal progenitor-specific promoter of the zebrafish *her4.1* gene (Yeo *et al.*, 2007). Of the 3 *Tg(her4.1:CreERT2)* lines we analyzed by crossing to the reporter, all displayed markedly increased Cre activity after incubation in embryo medium containing 5 µg/ml tamoxifen from 28 to 48 hpf (Fig 3b and 3c, bottom). However, all three also exhibited detectable basal activity without induction (Fig 3b and 3c, top), indicating that the CreERT2 may not be as tightly regulated in zebrafish as in mice. We therefore tested whether ERT2CreERT2, which has two tamoxifen binding domains, is more tightly regulated (Matsuda and Cepko, 2007). We analyzed 3 *Tg(her4.1:ERT2CreERT2)* lines in the reporter and found all had tamoxifen-induced Cre activity and undetectable basal activity (Fig 3d-f). Nevertheless, the tamoxifen-induced inversion by ERT2CreERT2 was not as extensive as that by CreERT2. Thus, ERT2CreERT2 may be a better choice for generating conditional Cre lines in zebrafish if the application requires no basal activity, such as lineage tracing. However, if maximal induced Cre activity is essential, such as in switching conditional mutations, CreERT2 may be better suited. Of course, it is possible to obtain a line with little basal activity and very strong induced activity by screening a large number of lines.

A battery of Cre- and Flp-expressing zebrafish lines with various expression patterns will be valuable for conditional and tissue-specific expression of transgenes, and potentially for conditional and tissue-specific gene inactivation when conditional alleles become available. The advent of an efficient enhancer trap in zebrafish provides another route to generate lines with suitable recombinase expression patterns (Davison *et al.*, 2007; Ellingsen *et al.*, 2005; Kawakami *et al.*, 2004; Scott *et al.*, 2007). Such a screen, however, requires an easily

detectable indicator of expression. In the case of recombinases, for example, EGFP-Cre may be used (Thummel *et al.*, 2005). However, fusion proteins are generally less active than the original proteins. Furthermore, a fluorescently-tagged Cre may interfere with fluorescence-based phenotype analysis. Our reporter line presents an excellent opportunity to screen these lines. To test the feasibility of this approach, we generated Tol2-based enhancer trap constructs using the 600 bp *hsp70l*/basal promoter (*hsp70lb*) to direct the codon-improved Cre (iCre) or Flp (Flpo) (Halloran *et al.*, 2000; Raymond and Soriano, 2007; Shimshek *et al.*, 2002). We crossed the Cre-injected male founders to the reporter fish to look for embryos with mCherry expression at 24, 48, and 120 hpf. After screening 64 *Et(hsp70lb:iCre)* founders, we found 12 lines with Cre activity, as measured by mCherry expression. We also identified 1 line with Flpo activity after screening 5 *Et(hsp70lb:Flpo)* founders (Table 2). Unfortunately, mCherry expression in all 13 lines was very broad (Fig 4a, 4b) and therefore they were not propagated. Although the broad expression could partially result from the basal activity of the *hsp70l* promoter (Scott *et al.*, 2007), it most likely reflected the fact that most genes are widely expressed. Nevertheless, these lines did exhibit some specificity. Histological analysis indicated that Cre activity was only present in a subset of cells. For example, histological analysis of two iCre enhancer trap lines showed different inversion patterns at the forebrain/retina levels (Fig 4c, 4d). In the brain, much more inversion was seen in line 6 than in line 10. In the retina, although both lines had extensive inversion in the photoreceptor and the retinal ganglion cell layers, the inversion in the inner nuclear layer (ONL) is different. In line 6, INL inversion was nonspecific, while in line 10, it seemed to be more restricted to the Müller cells. In the future, a better basal promoter and a larger scale screen may help generate a battery of interesting Cre and Flp lines.

In summary, we have developed a FIEEx-based reporter line for both Cre and Flp activity in zebrafish. We demonstrated the utilities of the reporter in characterizing tissue-specific, as well as conditional Cre lines. Furthermore, we demonstrated that it is feasible to use the reporter to screen for interesting Cre and Flp enhancer trap lines. The reporter line, as well as the Cre and conditional Cre lines should facilitate genetic manipulations in zebrafish.

Materials and Methods

Fish

Zebrafish were raised in Aquatic Habitats systems (Apopka, FL) on a 14 to 10 light dark cycle as described previously (Chen and Casey Corliss, 2004). Tubingen fish were acquired from the Zebrafish International Resource Center (ZIRC). Embryos were reared in 0.3x Danieau's solution at 28°C.

Transgenes and transgenesis

The two arms of the Cre/Flp target were made by annealing and filling-in. Arm 1 consists of lox5171/loxP-F3/FRT, and was made from 2 pairs of primers in two steps. First, the 2 primer pairs were annealed and filled-in using the Klenow fragment of DNA Polymerase I separately. Then, the reactions were mixed in equal molarity and treated with Exonuclease I to eliminate free primers. After inactivating Exonuclease I, the mix was annealed and extended by Taq DNA polymerase. The resultant DNA was analyzed in a 2% agarose gel, purified and cloned into pCR4-TOPO (Invitrogen). The sequence of the primers is: pair 1: 5' GGGCCCACTTTCGTATAGTACACATTATACGAAGTTATGGGTCGATGGTGA GATCTGGAC TAGAGGGTCGATGGTG3' and 5' ATTACCCTGTTATCCCTAATAACTTCGTATAATGTATGCTATACGAAGTTATCC AAGCATCAC CATCGACCCTCTAG3'; pair 2: 5' CATTATACGAAGTTATTAGGGATAACAGGGTAATGAAGTTCCTATTCCGAAGT TCCTATTCT TCAAATAGTATAGGAACCTTCGTGTGTGTGTG3' and

5 CTCGAGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTC
 ACACACACA CACACACACACACACGAAGTTC3 . Arm 2 consists of loxP/lox5171-
 FRT/F3, and was made in 2 steps. First, a primer pair,
 CTTGGGGGAGGGGGAGGCCAGAATGAGAAGTTCCTATACTTTCTAGAGAA
 TAGGAACTTCTAACTATAACGGTCCTAAGGTAGCGAATA and
 CATCGACCCGAATTGCCAAGCATCACCATCGACCCATAACTTCGTATAGTACAC
 ATTATACGA AGTTATTCGCTACCTTAGGACCGTTA, was annealed and filled-in with
 Klenow, and free primers eliminated with Exonuclease I. After heat inactivation of
 Exonuclease I, the product was used as a template for PCR using a forward primer
 5 GGATCCGAAGTTCCTATACTATTTGAAGAATAGGAACTTCGGAATAGGAA
 CTTCTGGCCTTGGGGGAGGGGGAGGCCAG3 and a reverse primer 5
 GCTAGCATAACTTCGTATAGCATACATTATACGAAGTTATCCAAGCTTCACCATC
 GACCCGAATTGCCAAGCATC. The product was cloned into pCR4-TOPO. The synthetic
 splice acceptor, consisting of branchpoint, intronic splice enhancer, splice acceptor, and
 exonic splice enhancer identified by bioinformatical analysis (Yeo *et al.*, 2004), was made
 by annealing and filling-in two primers:
 CTCGAGGACTATCCGGAGTGTGTGTGTGTTTCTGACGTGTCTCTCTCTCCCTTTT
 TTTTTTCAG GTCGACAACTCTTCG and
 CCATGGTGGTTCTTCTTCTTCTCCGACCGCGAAGAGTTTGTCGACCTG. The
 product was cloned into PCR4-TOPO. The cDNA for mCherry was obtained from the
 laboratory of Roger Tsien (Shaner *et al.*, 2004) and the 3xpolyA sequence was from pBigT
 (Srinivas *et al.*, 2001). The zebrafish promoters were obtained by PCR using TAB14
 genomic DNA as template, primers designed based on Ensembl database, and a Pfu/Taq
 polymerase mixture (1:2). The plasmids for Flpo, Cre, iCre, CreERT2 and ERT2CreERT2
 were obtained from Addgene (Cambridge, MA). To generate transgenic lines, about 1 nl
 sterile isotonic saline solution containing a mixture of the plasmid DNA of a miniTol2-based
 transgene (30 ng/μl) and transposase RNA (30 ng/μl) was injected into fertilized eggs, and
 injected embryos raised to maturity. The founders of reporter transgenes were screened
 based on GFP expression to establish 3 lines, *Tg(eab2:[EGFP-T-mCherry])* line A, B and C.
 All Cre lines were either screened by mCherry expression after crossing to a reporter line, or
 by PCR using primers 5 CCATGTCCAATTTACTGACCGTAC3 and
 5 CATCTTCAGGTTCTGCGGAAAC3 . All Flp, CreERT2 and ERT2CreERT2 lines
 were similarly screened. The PCR genotyping primers for Flp were 5
 GCTTCCACCATGCCACAATTTG3 and 5 CATGAATGTGGCTCTCTTGATTGCTG3 ,
 and for ERT2 containing lines were 5 GACCTCCATGATCAGGTCCACC3 and
 5 GACCGTGGCAGGAAACCCTCTG3 . To screen for enhancer trap lines, male
 founders injected with *Et(hsp70lb:iCre)* or *Et(hsp70lb:Flpo)* were crossed to females of the
 Cre/Flp reporter and mCherry expression was examined under a Zeiss M2Bio microscope at
 days 1, 2, and 5.

Linker-mediated PCR

To identify the insertion site of the transgene, tail DNA from transgenic fish was digested
 with TaqI or NlaIII. A linker with compatible overhang was ligated to the digested DNA.
 The linker consisted of a long and a short primer. The sequence for the primers excluding
 the overhang are:
 CTAGGATTAGCTGCTGGAGTACACGATCGCTTAATAGAGGCACGTGGAACGCGG
 GC (long) and pGCCCCGTTCCACGTGZZOG (short). The genomic sequence flanking
 the 5' end of the transgene was amplified by nested PCR. The first round PCR consisted of
 25 cycles of 94°C 20 sec and 65°C 3 min, in the presence of primers
 CTAGGATTAGCTGCTGGAGTACACG and
 CATCTGATAGTGGACCTTAAGCCGACAC. The product was diluted 25x and 1μl of the
 diluted product was used in the 2nd round PCR. The 2nd round of PCR consisted of 35

cycles of 94°C 20 sec and 65°C 3 min, in the presence of primers ATCGCTTAATAGAGGCACGTGGAAC and CATTGGAAAGGGTCGCTGGCTT. The PCR product from the 2nd PCR was sequenced directly to map the insertion sites onto the zv7 of Ensembl zebrafish genome assembly.

Transgene induction

Expression of *hsp70l* promoter-driven transgenes was induced by placing a 50 ml conical tube containing transgenic embryos in embryo medium in a 38°C water bath for 1 hour. Heat shocked embryos were returned to Petri dishes in the 28.5°C incubator. To induce the activity of conditional Cre, tamoxifen stock solution (10 mg/ml in DMSO) was added to embryo medium to a final concentration of 5 µg/ml. This concentration was found to be tolerated by embryos, with occasionally mild but recoverable tail deformation.

Histology and image processing

Embryos were fixed in 4% paraformaldehyde at room temperature for 2 hours or 4°C overnight and processed as described previously (Chen and Casey Corliss, 2004). Images were acquired using a Zeiss M2Bio or an Axiovert 200 microscope equipped with a color CCD camera controlled by AxioVison (Zeiss). Images were processed in Photoshop (Adobe).

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Fig 1.

Generation and characterization of the Cre/Flp reporter line. (a) schematic map of the original reporter transgene and the resultant transgenes after Cre and Flp-mediated flip and excision. Bgh pA, polyA signal from the bovine growth hormone gene; SA, splice acceptor; SD, splice donor; T, 3xSV40 polyA; Dark blue triangles, loxP; light blue triangles, lox5171; red triangles, FRT; pink triangles, F3. (b–g) Analysis of embryos produced by a *Tg(eab2:[EGFP-T-mCherry])* F1 and a *Tg(hsp70l:Cre)* founder. The embryos were heat shocked at 24 hpf and sampled at 72 hpf for analysis. Two representative embryos, one GFP-positive and one GFP- and mCherry- double positive, were imaged in bright field (b), GFP channel (c), and mCherry channel (d). Embryos from the two groups were cross-sectioned and stained with Hoechst 33342 to determine the extent of inversion. No mCherry-positive cell was found in the GFP-positive embryos (e) and very sparse EGFP-positive cells were found in the GFP-positive/mCherry-positive embryos (f). Images are at the level of hindbrain. Genotype analysis of these GFP-positive and GFP-positive/mCherry-positive embryos indicates that embryos in the latter group were double transgenic (g). The location and orientation of primers (p1, p2, p3) for genotyping the reporter transgene are indicated in (a). g, green; r, red.

Fig 2.

Characterization of *Tg(hsp70l:Flp)* and *Tg(myf7:Cre)* transgenic lines. (a-b) Images of two embryos at 7 dpf in GFP channel (a) and mCherry channel (b) indicating efficient inversion by Flp. Both embryos carry the reporter transgene but only the bottom one also carries the *Tg(hsp70l:Flp)* transgene. The embryos were heat shocked at 24 hpf. (c) Merged GFP and mCherry image of two embryos at 72 hpf. Both embryos harbor the reporter transgene, but only the bottom one also carries the *Tg(myf7:Cre)* transgene. Expression of mCherry indicates heart-specific inversion of the reporter transgene. Insert, closer view of the cardiac region indicating mCherry expression in the myocardium, but not in the endocardium or blood cells in the heart.

Fig 3.

Characterization of tamoxifen-dependent Cre lines. (a) GFP channel image and (b) mCherry channel image of a *Tg(her4.1:CreERT2); Tg(eab2:[EGFP-T-mCherry])* double transgenic embryo at 72 hpf incubated with DMSO (top) or tamoxifen (bottom) from 28 to 48 hpf. (c) cryosections of the retina in DMSO- (top) and tamoxifen- (bottom) treated double transgenic embryos at 72 hpf. (d) a GFP channel image and (e) an mCherry channel image of *Tg(her4.1:ERT2CreERT2); Tg(eab2:[EGFP-T-mCherry])* double transgenic embryos at 72 hpf treated with DMSO (top) or tamoxifen (bottom) from 28 to 48 hpf. (f) Cryosections of the retina in DMSO (top) or tamoxifen-treated double transgenic embryos at 72 hpf.

Fig 4.

Examples of the *Et(hsp70lb:iCre)* enhancer trap lines. (a–b) mCherry channel image of a *Et(hsp70lb:iCre); Tg(eab2:[EGFP-T-mCherry])* embryo at 96 hpf from line 6 (a) and line 10 (b). (c–d) EGFP and mCherry expression in cross cryosections at the level of the retina and forebrain. Differential mCherry expression indicates distinct iCre expression patterns. Br, brain; Ret, retina; RGC, retinal ganglion cell layer; INL, inner nuclear cell layer; ONL, photoreceptor layer. Bar, 50 μm.

Table 1

Comparison of germline inversion efficiency of Cre and Flp

	mCherry-EGFP+ ¹	mCherry+/EGFP+ ¹	mCherry+/EGFP- ¹	Total ²	Efficiency ³
Cre	15	12	85	150	82%
Flp	57	19	22	130	31%

Heat shocked double transgenic fish with mCherry expression were raised and out crossed.

¹The number of fluorescent progeny in the group

²The number of progeny analyzed

³Calculated percentage assuming 2 copies of independently segregating reporter genes

Table 2

Summary of pilot enhancer trap screens for Cre/Flp expression

Construct	Founders Screened	Founders with mCherry+ progeny	Detection rate
<i>Et(hsp70lb:iCre)</i>	64	12	18.75%
<i>Et(hsp70lb:Flpo)</i>	5	1	20%