Improved *Tol2*-mediated enhancer trap identifies weakly expressed genes during liver and β cell development and regeneration in zebrafish

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The liver and pancreas are two major digestive organs, and among the different cell types in them, hepatocytes and the insulin-producing β cells have roles in both health and diseases. Accordingly, clinicians and researchers are very interested in the mechanisms underlying the development and regeneration of liver and pancreatic β cells. Gene and enhancer traps such as the *Tol2* transposon-based system are useful for identifying genes potentially involved in developmental processes in the zebrafish model. Here, we developed a strategy that combines a *Tol2*-mediated enhancer trap and the Cre/loxP system by using loxP-flanked reporters driven by β cell– or hepatocyte-specific promoters and the upstream activating sequence (UAS)-driving Cre. Two double-transgenic reporter lines, *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* and *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)*, were established to label pancreatic β cells and hepatocytes, respectively. These two double-transgenic lines were each crossed with the *Tol2*-enhancer trap founder lines to screen for and identify genes expressed in the β cell and hepatocytes during development. This trap system coupled with application of nitroreductase (NTR)/metronidazole (Mtz)–mediated cell ablation could identify genes expressed during regeneration. Of note, pilot enhancer traps captured transiently and weakly expressed genes such as *rab3da* and *ensab* with higher efficiencies than traditional enhancer trap systems. In conclusion, through permanent genetic labeling by Cre/loxP, this improved *Tol2*-mediated enhancer trap system provides a promising method to identify transiently or weakly expressed, but potentially important, genes during development and regeneration.

Diabetes and liver diseases cause global health problems (1). Loss or dysfunction of insulin-producing β cells and hepatocytes is characteristic of diabetes and liver diseases, respectively (2, 3). Although many genes have been reported to regulate pancreas and liver development (4–6), approaches to identify weakly or transiently expressed genes that are potentially important for organogenesis and regeneration remain to be developed.

69% of zebrafish genes have human orthologs (7). High genetic conservation and larval transparency make zebrafish an ideal model to study development and regeneration of liver and β cells (4, 6, 8–12). In addition to the previous work in other vertebrates (13, 14), genetic screens, including *N*-ethyl-*N*-nitrosourea mutagenesis, in zebrafish have identified a number of factors and signaling molecules that govern differentiation and morphogenesis of pancreas and liver (15–18). However, because many genes reiteratively instruct multiple development processes, early embryonic lethality or malformations caused by gene mutation will conceal its roles in β cell or liver development and regeneration at later stages (19). Thus, considerable work will still be required to achieve a thorough understanding of the temporal sequences of signaling events underlying pancreatic β cell and liver induction during embryonic development and regeneration, which will in turn benefit therapies for diabetes and liver diseases by replenishing damaged cells *in vivo* and generating a new supply of β cells and hepatocytes *in vitro* (20, 21).

Gene and enhancer traps are useful tools to identify genes that potentially regulate developmental processes in zebrafish (22). Previous studies have used *Tol2* transposon-mediated Gal4 to target neural circuits (23, 24) and heart (25). Although gene traps have been widely used to study organ development, this system has little been used to explore organ regeneration. The traditional Gal4-based enhancer trap system requires improvements to overcome two limitations. First, the UAS[2]-driven GFP or other fluorescent proteins can hardly identify weakly or transiently expressed genes involved in organ development and regeneration. Second, traditional enhancer trap lines can hardly be used to trace the cell origins of organogenesis and regeneration.

To improve the traditional gene trap system to overcome the limitations mentioned above, we combined the *Tol2*-mediate enhancer trap with Cre/loxP and nitroreductase (NTR)/metronidazole (Mtz) systems to screen pancreatic β cell– and hep-

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This article contains Figs. S1–S4.

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[2] The abbreviations used are: UAS, upstream activating sequence; hpf, hours postfertilization; hpt, hours post-treatment; Mtz, metronidazole; NTR, nitroreductase; CFP, cyan fluorescent protein; GGFF, green fluorescent protein fused to Gal4FF.

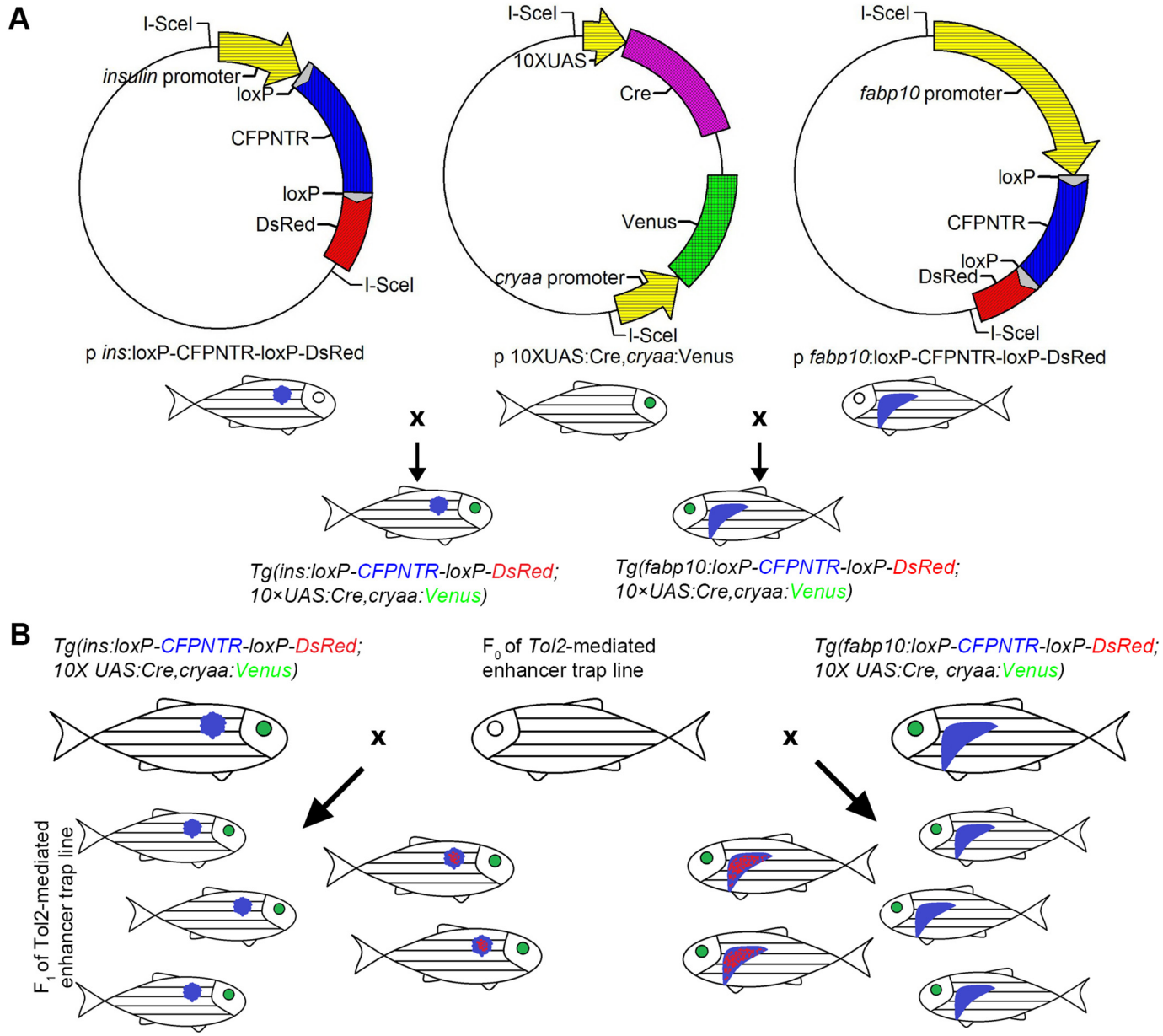


Figure 1. *Tol2*-mediated enhancer trap is combined with Cre/loxP for the screen of pancreatic β cell– and liver-specific genes. *A*, Cre/loxP–based transgenic lines *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* and *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* were constructed as schematically illustrated. The expression of Venus in the eyes indicates the *10×UAS:Cre* transgene in the genome. *B*, schema showing the procedure for the screen. Cre/loxP–based transgenic reporter lines for pancreatic β cells (*left*) and hepatocytes (*right*) were crossed with *Tol2*-mediated F_0 enhancer trap line, respectively. The F_1 larvae with red fluorescence in the β cells or hepatocytes were selected for genomic identification. F_1 larvae without red color in targeted organs were subjected to further regeneration studies.

atocyte-specific genes involved in development or regeneration. Using this strategy, we constructed two transgenic lines, *Tg(ins:loxP-CFPNTR-loxP-DsRed)*^{cq67} and *Tg(fabp10:loxP-CFPNTR-loxP-DsRed)*^{cq66}, which were further crossed with the *Tg(10×UAS:Cre, cryaa:Venus)*^{cq64} line to generate double-transgenic reporter lines for β cells and hepatocytes, respectively (26, 27). Pilot screens by crossing these reporter lines with the *Tol2*-based green fluorescent protein fused to Gal4FF (GGFF)-enhancer trap founders identified six genes with specific expression patterns in β cells or liver during development or regeneration. A traditional enhancer trap strategy using the *Tg(10×UAS:Kaeda, cryaa:Venus)*^{cq65} line was performed for comparison. We conclude that this improved *Tol2*-mediated enhancer trap strategy combining tissue-specific Cre/loxP obtains higher efficiency for identification of weakly or transiently expressed genes.

Results

Constructions of Cre/loxP– based double-transgenic reporter lines

To introduce Cre/loxP into the enhancer trap system for pancreatic β cells and liver, we constructed two double-transgenic reporter lines (Fig. 1*A*). Under the control of β cell– and hepatocyte-specific promoters, β cells of the *Tg(ins:loxP-CFPNTR-loxP-DsRed)*^{cq67} and hepatocytes of the *Tg(fabp10:*

loxP-CFPNTR-loxP-DsRed)^{cq66} lines were labeled with CFP fluorescence, respectively. In the *Tg(10×UAS:Cre, cryaa:Venus)^{cq64}* line, the expression of Cre recombinase was under the control of the UAS. *cryaa:Venus* was engineered in the same plasmid with *10×UAS:Cre* to ensure that the existence of Cre recombinase was visible by the Venus fluorescence in the eyes (Fig. 1*A*). Then, the *Tg(10×UAS:Cre, cryaa:Venus)^{cq64}* was crossed with *Tg(ins:loxP-CFPNTR-loxP-DsRed)^{cq67}* and *Tg(fabp10:loxP-CFPNTR-loxP-DsRed)^{cq66}* to generate *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* and *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* double-transgenic reporter lines, respectively. Theoretically, by means of crossing these two transgenic reporter lines with *Tol2*-based enhancer trap founders (F_0), F_1 larvae with red fluorescence appearing in the β cells or liver will be selected as candidates for further genomic identification (Fig. 1*B*). Taking advantage of NTR/Mtz–mediated cell ablation (28), F_1 individuals can be further subjected to screening for genes activated during β cell and liver regeneration.

Validation of the Gal4-UAS system in the double-transgenic reporter lines

In the *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* and *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* lines, β cells and hepatocytes were respectively labeled by CFP. The expression of Cre recombinase is turned on only when UAS is activated by the Gal4 transcriptional activator protein. In this study, we used GGFF, the optimal version of Gal4 with less toxicity to zebrafish cells (24), to evaluate the effectiveness of GGFF-UAS in the double-transgenic reporter lines. After injection of *GGFF* mRNA into one-cell-stage embryos of the reporter lines, the UAS activated the expression of Cre recombinase, which in turn excised the *CFPNTR* cassette flanked by the *loxP* sites specifically in the pancreatic β cells (Fig. 2, *A–E*) and hepatocytes (Fig. 2, *F–J*). Expressions of DsRed were activated in more than 80% of the β cells (Fig. 2, *D* and *E*) and in nearly all of the hepatocytes (Fig. 2, *I* and *J*). The CFP elimination in hepatocytes was more efficient than in β cells (Fig. 2, *D* and *I*). Thus, working efficiencies of GGFF-UAS in these two double-transgenic reporter lines were validated and guaranteed.

Enhancer trap for genes expressed in pancreatic β cells and hepatocytes during development

In a pilot screen, over 600 F_0 enhancer trap lines were generated using the enhancer trap plasmid *T2KhspGGFF*. Then, the F_0 lines were crossed with two double-transgenic reporter lines, *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* and *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)*. F_1 larvae with DsRed expression in the β cells or in the liver were raised to adults. Six and 11 lines were identified with DsRed expression in the β cells and liver, respectively. The expression patterns and mosaicity of DsRed were diverse among the different F_1 enhancer trap lines (Fig. 3, *A–I*). In the F_1 of β cell enhancer trap lines (Fig. 3, *A–D*), some lines exhibited only one or two β cells in red (Fig. 3, *A* and *I*), whereas one line exhibited DsRed over the entire organ and complete elimination of CFP (Fig. 3, *D* and *I*). F_1 of hepatocyte enhancer trap lines exhibited similar phenomena (Fig. 3, *E–I*).

Five of the F_1 trap lines were raised to the F_2 generation, among which two genes with specific expression patterns during development were identified through the reverse PCR method (24). According to the sequencing results of reverse PCR (see Fig. S1A) and BLAST readout from Ensembl (see Fig. S1B), *rab3da* was found to be highly expressed in the endocrine pancreas in addition to spinal cord at 24 hpf (Fig. 4, *A* and *C*). However, its expression in the pancreas was significantly reduced at 48 hpf (Fig. 4, *B* and *D*) and became nondetectable at 96 hpf. Although Rab3d has been reported to play roles in maintaining normal-size secretory granules of pancreatic acini in mammals (29, 30) and be critical for secretory granule maturation in PC12 cells (31), its expression in the developing pancreas has not yet been identified. We found high, but transient, expression of its zebrafish ortholog, *rab3da*, in the pancreas during development. When the F_1 of *rab3da* enhancer trap line was crossed with the traditional reporter line *Tg(10×UAS:Kaede, cryaa:Venus)^{cq65}*, expression of Kaede in the β cells that represents trap of *rab3da* could not be detected at 96 hpf (Fig. 4*E*). By contrast, although expression of *rab3da* was transient, our Cre/loxP– combined enhancer trap strategy could detect the trap more efficiently at 96 hpf (Fig. 4*F*).

The second gene, *rnd2*, identified to be highly expressed in the liver and brain at 34 hpf and 58 hpf (Fig. 5, *A–D*) encodes the Rho family GTPase 2 (see Fig. S2, A and B). In mammals, Rnd2 controls neuron migration in the cerebral cortex (32–34). In addition, the Rnd family is linked to tumorigenesis and metastasis, including lung cancer (35), breast cancer (36), and the most common type of liver cancer, hepatocellular carcinoma (37). We found expression of zebrafish ortholog *rnd2* in the developing liver and brain during embryogenesis. The *rnd2* insertion could also be present under the background of traditional enhancer trap reporter *Tg(10×UAS:Kaede, cryaa:Venus)^{cq65}* (Fig. 5*E*), but its mosaicity of positive cells was obviously less than the Cre/loxP reporter (Fig. 5*F*) and became more difficult to be identified. These data demonstrate that the improved enhancer trap system obtains higher screening efficiencies, thus facilitating identification of genes.

Enhancer trap for genes activated in the regenerating liver

To evaluate the feasibility of this improved enhancer trap system in the identification of genes activated during regeneration (Fig. 6, *A* and *B*), we crossed the enhancer trap founder lines with the *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* reporter line followed by Mtz treatment to induce liver injury in F_1 larvae (9, 10). DsRed expression in the regenerating liver was found in the F_1 of one line at 48 h post-treatment (hpt) (Fig. 6*C*). The sequencing result of reverse PCR (see Fig. S3A) and BLAST readout from Ensembl (see Fig. S3B) identified the trapped gene *ensab*. Ensa, the ortholog of zebrafish Ensab, inhibits the activity of protein phosphatase 2A and prompts mitosis (38, 39). After liver injury, expression of *ensab* in the regenerating liver was initiated at 8 hpt and became moderately up-regulated at 24 and 48 hpt (Fig. 6*E*, *arrowheads*), validating the gene trap results (Fig. 6*C*). When the F_0

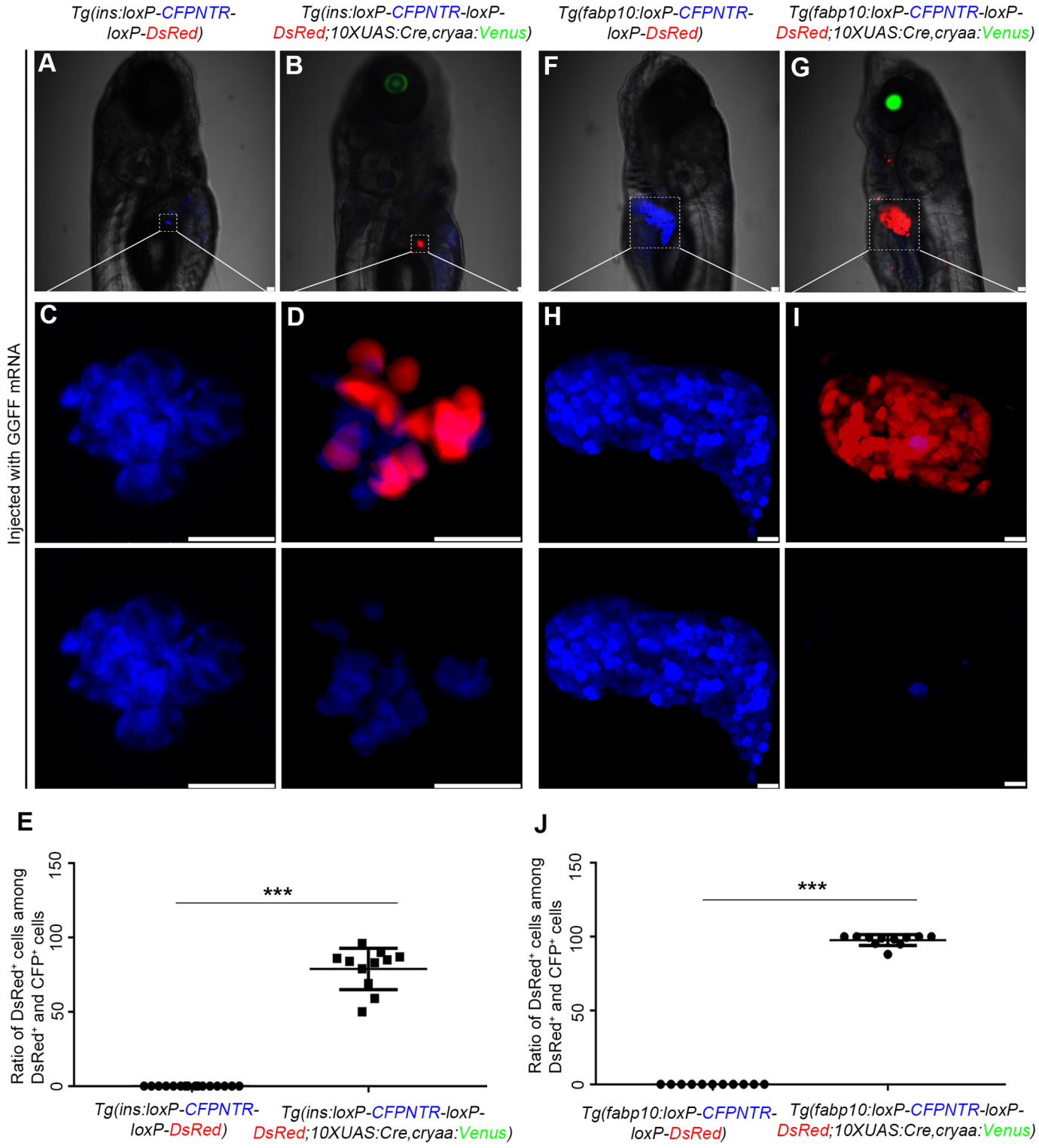
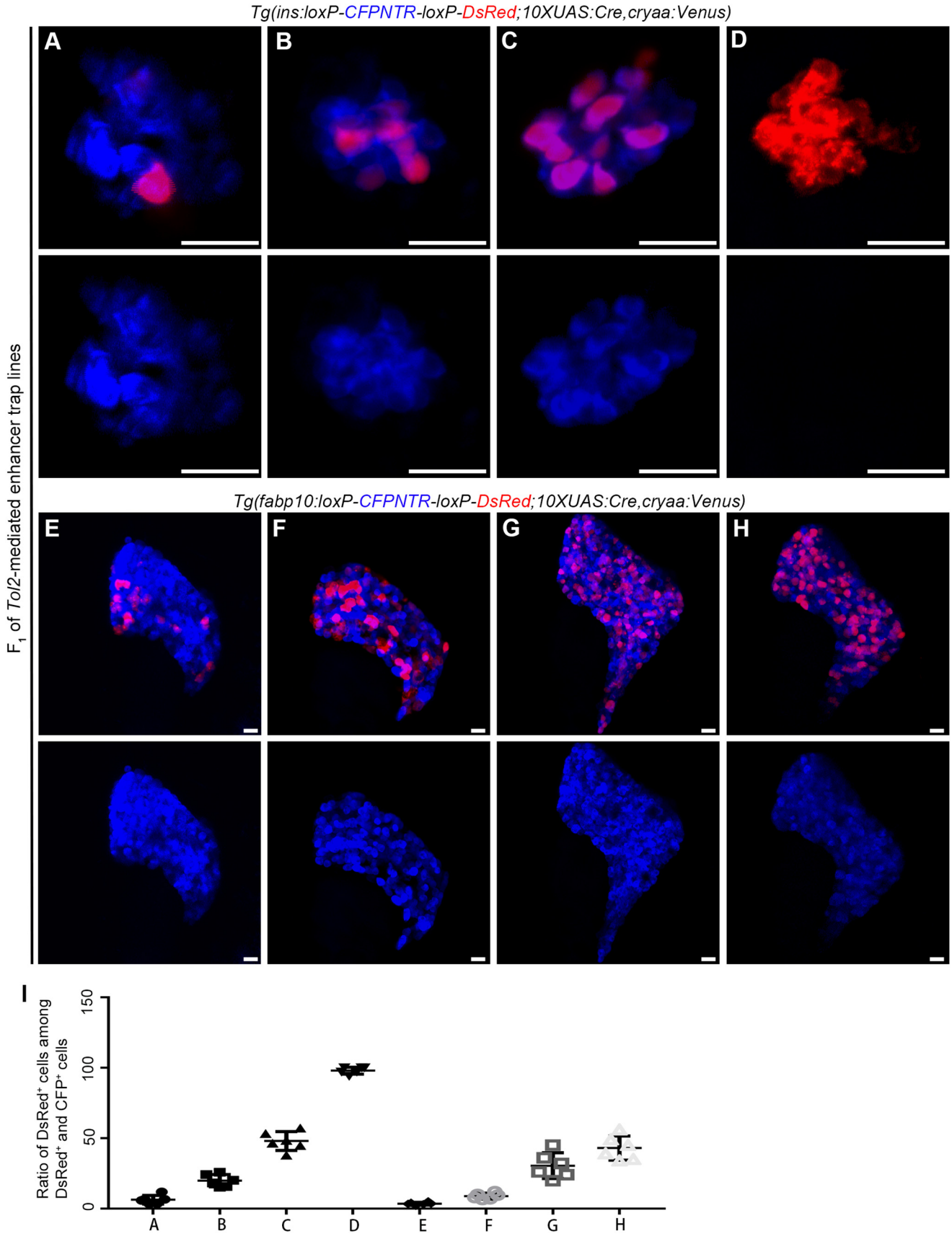


Figure 2. The validation of the Cre/loxP–based transgenic reporter lines. *A–D*, the fluorescence of the pancreatic β cells in *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* was converted from *blue* to *red* by *GGFF* mRNA. *E*, quantification of the percentage of the DsRed$^+$ cells among the DsRed$^+$ and CFP$^+$ cells in *C* and *D*. *F–I*, the color of the hepatocytes of the *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* shifts from *blue* to *red* when injected with *GGFF* mRNA at one-cell stage. *J*, quantification of the percentage of the DsRed$^+$ cells among the DsRed$^+$ and CFP$^+$ cells in *H* and *I*. *Asterisks* indicate statistical significance: ***, $p < 0.001$. *Scale bars*, 20 μm. *Error bars*, ±S.D.

was crossed with the traditional gene trap reporter line *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Kaede, cryaa: Venus)*, only a few Kaede-positive cells were present at 48 hpt (Fig. 6*D*), making it more difficult to be identified from the screen compared with the improved gene trap strategy (Fig. 6*C*). These data demonstrate that our improved gene trap system provides a useful tool to identify genes activated during regeneration.


Tg(ins:loxP-CFPNTR-loxP-DsRed;10XUAS:Cre,cryaa:Venus)
A
B
C
D
Tg(fabp10:loxP-CFPNTR-loxP-DsRed;10XUAS:Cre,cryaa:Venus)
E
F
G
H
F1 of Tol2-mediated enhancer trap lines
I
Ratio of DsRed+ cells among DsRed+ and CFP+ cells
0
50
100
150
A
B
C
D
E
F
G
H

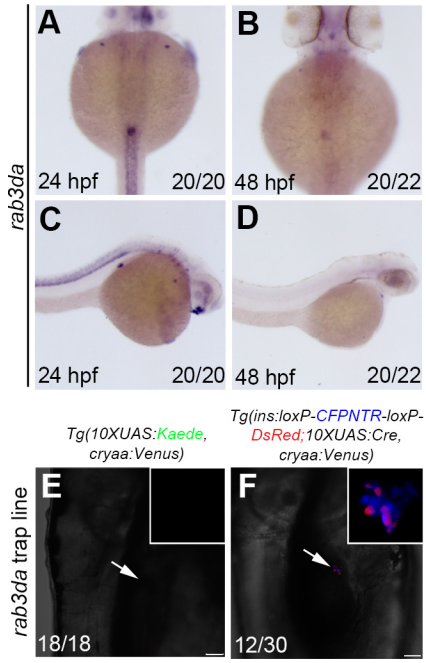


Figure 4. Identification of *rab3da* expressed in the pancreatic β cells using the improved enhancer trap system. *A–D, in situ* results show *rab3da* expressed in pancreatic endocrine cells. *E* and *F*, the double-transgenic reporter line *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* expresses the *Tol2*-mediated GGFF insertion in pancreatic β cells with higher efficiency. *Numbers* indicate the proportion of larvae exhibiting the expression shown. *Arrows* indicate the region of pancreatic β cells. *Scale bars*, 50 μm.

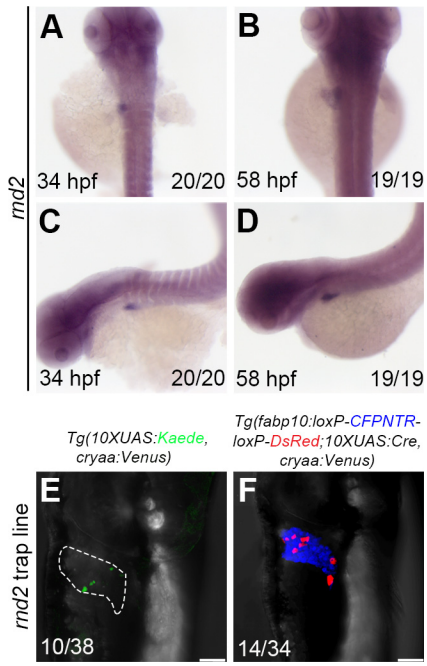


Figure 5. Identification of *rnd2* expressed in the liver using the improved enhancer trap system. *A–D, in situ* results show *rnd2* expressed in the liver. *E* and *F*, the double-transgenic reporter line *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* expresses the *Tol2*-mediated GGFF insertion in hepatocytes with higher efficiency. *Numbers* indicate the proportion of larvae exhibiting the expression shown. *Scale bars*, 100 μm.

Discussion

Although the process of organ regeneration shares many common molecular pathways with organogenesis, it cannot be ruled out that some molecules play roles only in organ regeneration (40). Moreover, other cell types could convert to regenerating cells through trans-differentiation under certain injury circumstances (9, 10, 41, 42). In addition to the well-established NTR/Mtz injury models in zebrafish (28, 43), this improved enhancer trap system should also be applicable to other injury models to identify transient and weak genes. For example, a previous study has revealed that macrophages repair ruptures of brain blood vessels through direct physical adhesion and mechanical traction forces (44). Generation of double-transgenic reporter lines to label macrophages or blood vessel endothelial cells will enable identifications of genes important for this repair process.

A study of genome-wide enhancer–promoter interactions revealed that the interaction between the enhancer and promoter decreases with increasing distances (45). The transcriptional efficiency of *Cre* depends on the distance from the insertion site to the candidate enhancer. It accounts in part for the mosaic patterns of DsRed fluorescence embedded on the CFP background in pancreatic β cells and hepatocytes (Fig. 3, *A–H*). However, this does not overshadow the power of the Cre/loxP–combined enhancer trap to screen the gene of interest. A portion of DsRed-positive pancreatic β cells and hepatocytes retained the CFP fluorescence (Figs. 2 and 3), which could be caused by the activation of Cre at different time points dependent on the insertion sites, and therefore the residual CFP protein has not been degraded yet.

Benefiting from the characteristics of permanent labeling, introduction of the Cre/loxP into the enhancer trap system improves the efficiency to screen genes of interest, in particular those weakly or transiently expressed. For genes with strong expression, this improved enhancer trap system shows no significant difference compared with the traditional reporter. For example, insulin was trapped using both Cre/loxP–combined and traditional reporter lines (see Fig. S4, *A–C*). Taken together, the Cre/loxP–combined, improved enhancer trap provides an approach to study gene expression in the organ of interest and could be genetically engineered to match the organ injury model for regeneration studies.

Experimental procedures

Ethics statement

All experimental protocols were approved by the School of Life Sciences, Southwest University (Chongqing, China), and the methods were carried out in accordance with the approved guidelines. The zebrafish facility and study were approved by the Institutional Review Board of Southwest University

Figure 3. Improved enhancer trap combined with Cre/loxP is used to screen genes expressed in the β cells and hepatocytes during development. *A–D*, F_1 *Tol2*-mediated enhancer trap larvae of *Tg(ins:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* possess β cells marked by red fluorescence in various degrees. *E–H*, F_1 *Tol2*-mediated enhancer trap larvae of *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* possess hepatocytes marked by red fluorescence in various degrees. *I*, quantification of the percentage of the $DsRed^+$ cells among the $DsRed^+$ and CFP^+ cells in *A–H*. *Scale bars*, 20 μm. *Error bars*, ±S.D.

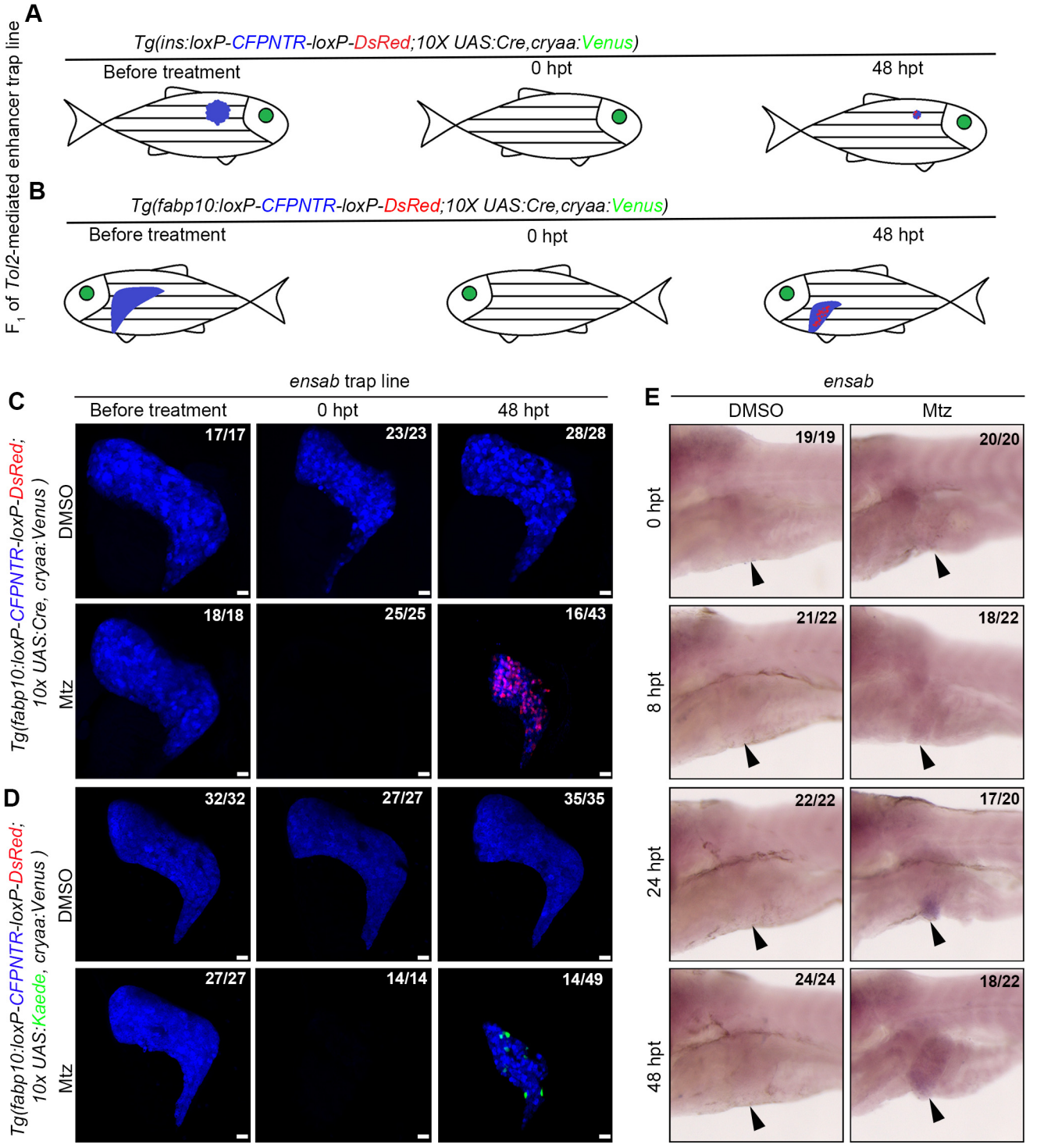


Figure 6. Identification of *ensab* expressed in the regenerating livers using the improved enhancer trap system. *A*, the pancreatic β cells marked by red fluorescence would appear exclusively during the regeneration when regeneration-specific genes or genes regulating trans-differentiation have been captured with the improved enhancer trap system. *B*, the red colored hepatocytes would appear only during the recovery of liver when regeneration-specific genes or genes regulating trans-differentiation have been captured with the improved enhancer trap system. *C* and *D*, the double-transgenic reporter line *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus)* expresses the *Tol2*-mediated GGFF insertion in regenerating livers with higher efficiency. *E*, *in situ* results show *ensab* expressed in the regenerating livers after Mtz treatment at 24 and 48 hpt (*arrowheads*). *Numbers* indicate the proportion of larvae exhibiting the expression shown. *Scale bars*, 20 μm.

(Chongqing, China). Zebrafish were maintained in accordance with the Guidelines of Experimental Animal Welfare from Ministry of Science and Technology of People's Republic of China (2006) and the Institutional Animal Care and Use Committee protocols from Southwest University (2007).

Plasmid constructs

The *10×UAS* fragment was amplified from P5EUAS with PCR and then cloned upstream of Cre coding sequence in the backbone of modified pBluescript, which harbors the meganuclease I-SceI site. On this base, the whole *cryaa:Venus* was also

cloned in the *10×UAS:Cre* construct flanked by the I-SceI site. *10×UAS:Kaede* was constructed by replacing Cre coding sequence with Kaede coding sequence. *fabp10:loxP-CFPNTR-loxP-DsRed* was constructed by insertion of the *CFPNTR* fused sequence into the previously reported *fabp10:loxP-stop-loxP-DsRed* (9). *ins:loxP-CFPNTR-loxP-DsRed* was made by replacing the *fabp10* promoter of *fabp10:loxP-CFPNTR-loxP-DsRed* with the insulin promoter. Enhancer trap vector pT2KhspGGFF was a kind gift from the Kawakami Lab.

Zebrafish strains

Transgenic lines *Tg(10×UAS:Cre, cryaa:Venus)*^{cq64}, *Tg(10×UAS:Kaede, cryaa:Venus)*^{cq65}, *Tg(fabp10:loxP-CFPNTR-loxP-DsRed)*^{cq66}, and *Tg(ins:loxP-CFPNTR-loxP-DsRed)*^{cq67} were all generated based on the standard I-SceI meganuclease transgenesis technique from the AB genetic background. The *Tg(fabp10:loxP-CFPNTR-loxP-DsRed; 10×UAS:Cre, cryaa:Venus*) double-transgenic line was generated from the cross of *Tg(fabp10:loxP-CFPNTR-loxP-DsRed)*^{cq66} with *Tg(10×UAS:Cre, cryaa:Venus)*^{cq64}, and the *Tg(ins:loxP-CFPNTR-loxp-DsRed; 10×UAS:Cre, cryaa:Venus*) double-transgenic line was generated from the cross of *Tg(10×UAS:Cre, cryaa:Venus)*^{cq64} with *Tg(ins:loxP-CFPNTR-loxP-DsRed)*^{cq67}. Enhancer trap F_0 was made by injecting *Tol2*-mediated enhancer trap vector pT2KhspGGFF with transposase mRNA into zebrafish embryos at one-cell stage. All zebrafish lines were brought up and maintained under standard laboratory conditions according to institutional animal care and use committee protocols.

Mtz treatment

The *Tg(fabp10:loxP-CFPNTR-loxP-DsRed)*^{cq66} transgenic larvae at 5 days postfertilization was incubated with 10 mM Mtz (Sigma-Aldrich) in 0.2% DMSO for 24 h. Then, larvae were washed three times and recovered in egg water, marking the regeneration 0 hpt.

Microinjection of mRNA

Transposase mRNA was synthesized from the linearized pCS-zTP according to the protocol in the mMESSAGE mMACHINE SP6 kit (Ambion Inc., Austin, TX). GGFF coding sequence was cloned into pCS2(+) plasmid and linearized by XbaI to use as a template to synthesize the *GGFF* mRNA according to the protocol in the mMESSAGE SP6 kit.

Microscopic analysis

A fluorescence stereomicroscope (M165FC, Leica) was used to observe and screen embryos that express DsRed in their hepatocytes or pancreatic β cells. The selected embryos were mounted with 1.2% low-melting-point agarose and subjected to confocal microscopy using a Zeiss LSM 780 META laser confocal microscope. Images of embryo were acquired as serial sections along the z axis at 1.0-μm intervals and processed using Zeiss LSM 780 Image Browser and Adobe Photoshop CS2.

Whole-mount in situ hybridization

In situ hybridization was performed as described previously (36). The primers used for synthetic probes were as follows: *rab3da* primers, 5′-AGAGCCGGATAAGATGGCGT-3′ and 5′-ATCAGGGGGCGTGTCTTGAA-3′; *rnd2* primers, 5′-CCGTCCACTCACAGTCACAG-3′ and 5′-GTCCCGTAGGCCTCAGTATG-3′; and *ensab* primers, 5′-CACCGTGGGTGGATCAGATCGG-3′ and 5′-ACCAGTCCTGGTGAAGCTGG-3′.

Quantification and statistical analysis

All statistical tests were performed with GraphPad Prism version 7.0 for Windows (GraphPad Software). Data were analyzed with Student's t test, and multiple comparisons performed with analysis of variance tests were used to determine statistical significance. Statistical significance was defined as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Author contributions—L. L. writing-review and editing; Y. Z. and L. L. designed the experimental strategy, analyzed data, and wrote the manuscript; W. H. performed plasmid construction; J. D. and Z. W. joined the screen process; J. H. analyzed data and wrote the manuscript.

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