

Recombinant *ubb^R* promoter enables highly efficient tamoxifen-inducible Cre recombination in embryonic and adult zebrafish

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The ability to inactivate gene function in an adult organism is essential for studies of biological processes such as regeneration and behavior. This is best achieved by engineering an allele that could be conditionally inactivated using drug-inducible Cre recombinase. Several recent studies clearly demonstrate the feasibility of engineering such conditional alleles in zebrafish. Meanwhile, achieving a sufficient degree of recombination to induce complete loss of function has remained a major limitation. Herein, we address this limitation by engineering a recombinant ubiquitin promoter *ubb^R* consisting of the zebrafish *ubiquitin* promoter supplemented with an intronic enhancer from the carp *beta-actin2* gene. Using phiC31-mediated targeted integration, we demonstrate that *ubb^R* outperforms both parental promoters at all embryonic stages tested. Furthermore, the *ubb^R:CreER^{T2}* driver line we generated ensures a high-level recombination of floxed alleles in adult zebrafish tissues. Finally, we demonstrate that our *ubb^R* promoter-driven construct retains high activity when integrated at other genomic loci, making this promoter a promising new tool for robust expression of transgenes at all stages of zebrafish ontogenesis.

Keywords: conditional mutagenesis; recombinase; transgenesis; zebrafish

Introduction

The Cre/lox recombinase system has been a staple of mouse genetics for more than a quarter century (Zou et al. 1994; Kühn et al. 1995; Rossant and Nagy 1995; Branda and Dymecki 2004). The system consists of 2 key components. The first component is a transgene expressing the recombinase in either native (Cre) or estrogen analog-inducible (CreER and derivatives) form under the control of either ubiquitous, inducible, or tissue-specific promoter. The second component is a cassette containing at least 2 loxP sites. Cre-mediated recombination between the 2 loxP sites leads to either excision or inversion of the intervening DNA element, depending on the nature and orientation of loxP sites.

A wide range of transgenic zebrafish lines expressing Cre and/or CreER derivatives under the control of ubiquitous and tissue-specific promoters have been generated by many different laboratories worldwide (reviewed in Carney and Mosimann 2018). These “driver” lines are most commonly used in lineage-tracing experiments where excision of the intervening DNA element results in permanent labeling of Cre-expressing cells and their progeny by fluorescent protein expression (Carney and Mosimann 2018; Lalonde et al. 2022). While the mouse-optimized CreER^{T2} coding

sequence is most widely used in zebrafish (Hans et al. 2011; Mosimann et al. 2011), some studies suggest that mammalian codon-improved CreER^{T2} (iCre) (Tromp et al. 2023) or zebrafish codon-optimized CreER^{T2} (Kesavan et al. 2018) may result in enhanced recombination efficiency in zebrafish embryos.

The Cre/lox system has also been used to conditionally induce loss-of-function phenotypes in zebrafish. Both cassette excision and cassette inversion paradigms have been successfully deployed for this purpose (Trinh et al. 2011; Ni et al. 2012; Hoshijima et al. 2016; Sugimoto et al. 2017; Burg et al. 2018; Grajevskaja et al. 2018; Gu et al. 2021; Ogawa et al. 2021; Shin et al. 2023). To date, such studies have almost exclusively been carried out at early stages of zebrafish development: only a handful of studies have reported Cre-mediated conditional gene inactivation in adult zebrafish (Sugimoto et al. 2017; Grajevskaja et al. 2018; Ogawa et al. 2021; Angom et al. 2023; Desingu Rajan et al. 2024; Wang et al. 2024).

All transgenes, including those regulated by apparently ubiquitous promoters, are subject to position effects, a phenomenon where the regulatory context of the genomic locus where the transgene is integrated exerts influence on transgene expression pattern and level (Hans et al. 2009, 2011; Mosimann et al. 2011;

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Lalonde et al. 2022; Kalvaityte-Repecke et al. 2026). With the exception of enhancer trapping (Balciunas et al. 2004; Parinov et al. 2004; Scott et al. 2007), position effects are considered to be a detrimental side effect of transgenesis. Attempts to minimize position effects by incorporating border elements or insulators have had variable success (Caldovic et al. 1999; Grajevskaja et al. 2013). Probably the most reliable strategy to mitigate expression variability arising from position effects is to integrate transgenes into a predefined genomic locus (Mosimann et al. 2013; Roberts et al. 2014; Lalonde et al. 2024). This strategy has an additional advantage of generating a stable single-copy harboring zebrafish line already in the F₁ generation, as opposed to Tol2-mediated transgenic zebrafish F₁s that usually have multiple integrations of the transgene (Kawakami et al. 2004; Balciunas et al. 2006; Distel et al. 2009; Zhang et al. 2019; Kalvaityte et al. 2024).

The main goal for this study was to establish a ubiquitous 4-hydroxytamoxifen-inducible Cre driver line that enables efficient mutagenesis at all stages of zebrafish development as well as in adult organisms. We compared the activity of 3 different promoter variants by integrating them into the same locus using phiC31 integrase and found that the recombinant ubiquitin promoter *ubb^R* supplemented with a carp *beta-actin2* enhancer (Liu et al. 1990) significantly outperforms the *ubb* and *actb2* promoters. Removal of bacterial vector backbone further improved activity, resulting in highly efficient recombination in developing and adult zebrafish tissues. Thus, our new ubiquitous CreER^{T2} driver line will be a valuable resource to investigators wishing to completely inactivate genes at various stages of ontogenesis. In addition, we demonstrate broad utility of our recombinant *ubb^R* promoter by showing that *ubb^R*-driven transgenes integrated into other genomic loci display similarly high activity.

Materials and methods

Zebrafish husbandry

Zebrafish (*Danio rerio*) stocks were kept under standard conditions at 27 to 27.5 °C under 14:10 h light:dark cycles, pH (7.4), and salinity-controlled conditions (Vet. Approval No. LT 59-13-002, LT 61-13-007). All experimental procedures were approved by the Lithuanian State Food and Veterinary Service (Approval No. G2-258). Male and female zebrafish at 3 to 18 mo of age were used to obtain embryos and were used for all experiments with adult fish.

Plasmid generation

All plasmids generated in this study were constructed using conventional restriction enzymes and CloneJet PCR Cloning Kit (Thermo Fisher Scientific, K1231).

Plasmids used for targeted integration into Tg(Xla.Crygc:ATTP-GFP)*tp1102* and Tg(Xla.Crygc:ATTP-GFP)*tp1104* zebrafish lines

pEG10 *actb2*:CreER^{T2}*. attB recombination site, mRFP, SV40 late poly(A) signal (Roberts et al. 2014); common carp *beta actin2* promoter (Gibbs and Schmale 2000); zebrafish-optimized CreERT2* sequence (Kesavan et al. 2018); bovine growth hormone poly(A) signal (Goodwin and Rottman 1992) cloned into pUC18 vector (Norlander et al. 1983) with FRT sites flanking the vector backbone. The complete sequence of this vector is provided in Supplementary File 1.

pEG5 *ubb*:CreER^{T2}*. attB recombination site, mRFP, SV40 late poly(A) signal (Roberts et al. 2014); zebrafish ubiquitin promoter (Mosimann et al. 2011) with improved splice acceptor site from AG/AT to AG/GT; zebrafish-optimized CreERT2* sequence (Kesavan et al. 2018); bovine growth hormone poly(A) signal (Goodwin and Rottman 1992) cloned into pUC18 vector (Norlander et al. 1983) with FRT sites flanking the vector backbone (Supplementary File 2).

pEG7 *ubb^R*:CreER^{T2}*. attB recombination site, mRFP, SV40 late poly(A) signal (Roberts et al. 2014); zebrafish ubiquitin promoter (Mosimann et al. 2011) with improved splice acceptor site from AG/AT to AG/GT and with enhancer element from common carp *beta actin2* promoter (Liu et al. 1990) inserted at BsrGI restriction site at forward orientation; zebrafish-optimized CreERT2* sequence (Kesavan et al. 2018); bovine growth hormone poly(A) signal (Goodwin and Rottman 1992) cloned into pUC18 vector (Norlander et al. 1983) with FRT sites flanking the vector backbone (Supplementary File 3).

Plasmid used for Sleeping Beauty-mediated integration of the transgene

pEB19 *ubb^R*:CreER^{T2}*. zebrafish ubiquitin promoter (Mosimann et al. 2011) with enhancer element from common carp *beta actin2* promoter (Liu et al. 1990) inserted at BsrGI restriction site at forward orientation; zebrafish-optimized CreERT2* sequence (Kesavan et al. 2018); SV40 late poly(A) signal, *Xenopus laevis* gamma-crystallin promoter, and mRFP (Grajevskaja et al. 2018), cloned into pT2 HB (Cui et al. 2002) (Supplementary File 4).

Additional plasmids

pJD1 *ubb^R*:MCS. attB recombination site, mRFP, SV40 late poly(A) signal (Roberts et al. 2014); zebrafish ubiquitin promoter (Mosimann et al. 2011) with improved splice acceptor site from AG/AT to AG/GT and with enhancer element from common carp *beta actin2* promoter (Liu et al. 1990) inserted at BsrGI restriction site at forward orientation; polylinker allowing integration of the gene of interest; bovine growth hormone poly(A) signal (Goodwin and Rottman 1992) cloned into pUC18 vector (Norlander et al. 1983) with FRT sites flanking the vector backbone (Supplementary File 5).

pJD2 MCS:CreER^{T2}*. attB recombination site, mRFP, SV40 late poly(A) signal (Roberts et al. 2014); polylinker allowing integration of the promoter of interest; zebrafish-optimized CreERT2* sequence (Kesavan et al. 2018); bovine growth hormone poly(A) signal (Goodwin and Rottman 1992) cloned into pUC18 vector (Norlander et al. 1983) with FRT sites flanking the vector backbone (Supplementary File 6).

Transgenic lines

The following published lines were used for this study

Wild-type lines TL and AB obtained from the European Zebrafish Resource Center (EZRC) (<https://www.ezrc.kit.edu/>), Tg(Xla.Crygc:ATTP-GFP)*tp1102* (Roberts et al. 2014) and Tg(Xla.Crygc:ATTP-GFP)*tp1104* (Roberts et al. 2014), Tg(-3.5*ubb*:CreERT2, myl7:EGFP)*zf2148* (Mosimann et al. 2011; Burg et al. 2018), Tg(-3.5*ubi*:loxP-GFP-loxP-mCherry)*cz1701* (Mosimann et al. 2011), *tbx20*^{tp1455} (Burg et al. 2018), *ift70*^{tp141} (Burg et al. 2018), *tbx5a*^{tp158RGt} (Grajevskaja et al. 2018).

Zebrafish lines generated in this study are listed in Table 1.

Transgenesis

Fertilized zebrafish eggs were obtained by natural crossings. Targeted integration of the transgene into a docking site was performed as described in Roberts et al. (2014). Briefly, 3 nL of solution containing 8.3 ng/μL plasmid DNA and 8 ng/μL of PhiC31-nos1-3' UTR integrase mRNA transcribed from pCS2+PhiC31onos1-3' UTR was injected into the yolks of 1-cell stage *tpl102Tg* or *tpl104Tg* embryos. At 3 dpf, embryos positive for RFP signal in the lens were selected and raised to adulthood. Mature fish were outcrossed to wild-type fish, and their F₁ progeny were screened for the lens RFP. Positive F₁ fish were raised to adulthood and used for further experiments.

Vector backbone removal was performed as described in Grajevskaja et al. (2018). A 3 nL of solution containing 25 ng/μL of Flp^o recombinase mRNA transcribed from pT3TS/Flp^o (pDC50) was injected into the yolks of 1-cell stage embryos obtained from *Tg(tpl102.ubb^R:CreER^{T2})*vln1* outcross. F₀ embryos were screened for RFP signal in the lens, raised to adulthood, outcrossed, and the resulting lens RFP-positive F₁ fish were genotyped by PCR on tail clips to confirm the excision of the vector backbone. Primers used for genotyping are provided in Supplementary Table 1.

Random integration of the transgene was achieved by injecting 3 nL of solution containing 8.3 ng/μL plasmid DNA and 30 ng/μL of *Sleeping Beauty* 100× transposase mRNA transcribed from pT3TS/SB100x (pPS2), as described in Balciunas et al. (2006), into the yolks of 1-cell stage wild-type embryos. At 3 dpf, embryos positive for RFP signal in the lens were selected and raised to adulthood. Mature fish were outcrossed to wild-type fish, and their F₁ progeny were screened for the transgenesis marker. Positive F₁ fish were raised to adulthood, outcrossed to wild-type fish to assess transgene copy number by Mendelian segregation. Lines were considered single-copy if ~50% of progeny were positive for the transgenesis marker. From each founder family, 1 single-copy line was established. Adult single-copy F₂ fish were outcrossed to generate embryos for the experiments.

4-HT treatment for CreER^{T2} induction in zebrafish embryos

4-hydroxytamoxifen (4-HT, H7904; Sigma) was dissolved in ethanol at a final stock concentration of 5 mM and kept in single-use aliquots at -80 °C. To induce recombination in CreER^{T2}-expressing embryos, 30–35 stage-matched embryos were transferred to a Petri dish with egg water that was freshly mixed with 4-HT heated for 10 min at 65 °C to restore its activity (Felker et al. 2016) up to a final concentration of 5 μM. The treated embryos were placed into a closed dark 28.5 °C incubator and remained in 4-HT solution until imaging and sample collection at 3 dpf or 5 dpf.

4-HT treatment for CreER^{T2} induction in adult zebrafish

Adult (3–12-mo-old) fish were incubated in 5 μM 4-HT solution 3 times for 24 h in a dark incubator, with 1-d recovery period between treatments (as described in Grajevskaja et al. (2018); Kalvaityte-Repecke et al. (2026)). One week after the third incubation, the fish were euthanized, and organs (heart ventricle, brain, liver, eye) and caudal fin were collected, submerged in 500 μL of TRI Reagent (Sigma-Aldrich), and stored at -80 °C until further analysis.

Isolation of hematopoietic cells from the adult zebrafish kidney marrow

Adult zebrafish were used to isolate hematopoietic cells from zebrafish kidney marrow. Zebrafish were euthanized by exposure to 0.4% Tricaine (MS-222, Sigma-Aldrich, USA) one at a time. Euthanized fish were placed on their side on a dissection pad. Small scissors were used to cut off the head of the fish. The skin of the abdomen was cut toward the direction of the tail. Internal organs were carefully removed using sharp forceps, leaving the kidney tissue intact. The kidneys were collected carefully using forceps from the dorsal abdominal wall of the fish. Kidneys were placed into a microcentrifuge tube containing 500 μL of 5% BSA in PBS. The cells were dissociated by pipetting 5–10 times. The cells were then poured on a pre-wet 40 μm cell strainer. The strainer was washed 4 times with 500 μL of 5% BSA in PBS. The collected cells were centrifuged at 300 × *g* at 4 °C. The supernatant was discarded, and the cells were suspended in 500 μL of 5% BSA in PBS and used for FACS analysis.

FACS analysis and sorting strategy

Following isolation, the cells were washed and resuspended in 1 mL of 5% BSA in PBS and then stained with the fixable viability dye eFluor 780 (Thermo Fisher Scientific) at a dilution of 1:1,000 for 30 min to exclude nonviable cells. After incubation, the cells were washed and resuspended in 1 mL of 5% BSA in PBS and then analyzed using a FACS Aria III flow cytometer (BD Biosciences, San Jose, USA) according to the manufacturer's guidelines. The initial sorting gate was determined by forward scatter (FSC) and side scatter (SSC) parameters, representing 60% to 80% of the cell population. Doublets were excluded by gating on SSC-W/SSC-H, followed by FSC-W/FSC-H. Dead cells and debris were removed using standard gating procedures. Subsequently, a gate was applied based on the negative staining of the fixable viability dye eFluor 780 to exclude any remaining dead cells. Typical kidney marrow cell populations were distinguished using FSC/SSC parameters, and the selected populations were sorted into 5 mL sample tubes for further analysis. A control tube without staining was consistently analyzed to evaluate autofluorescence. Histograms were generated using 10,000 gated events, with a 2% positive rate threshold applied to the unstained control. Data analysis was performed using packaged BD FACSDiva (BD Biosciences, USA) and FlowJo (Treestar, USA) software.

Imaging and image processing

Three-day-old zebrafish embryos were anesthetized in 0.04% (w/v) Tricaine (Sigma-Aldrich, E10521) solution. After anesthesia, they were mounted in a concave of a glass slide in 1% (w/v) low melting point agarose (Thermo Fisher Scientific, R0801), ensuring their sagittal plane was parallel with the glass slide. Embryos were imaged using Leica DM5500B microscope with a HC PL FLUOTAR 5x/0.15 objective. Leica filter system TXR ET, k (ex: 560/40 nm, Em: 630/75 nm, dichroic: 585 nm) was used to observe mCherry and mRFP signal, while filter system GFP ET, small (Ex: 470/40, Em: 525/50, dichroic: 495 nm) was used to observe eGFP signal. Fiji imaging software version 1.54 h was used to pairwise stitch raw image files (Preibisch et al. 2009). The stitched images were cropped to a size of 2,100 by 550 pixels and colored using "Merge channels" function of the same software. Raw image files are available upon request. A subset of images exhibiting visibly reduced fluorescence due to aging of the metal-halide lamp was adjusted for visualization using a correction factor derived from the

same biological sample imaged on different days. Fluorescent microscopy images are provided as representative examples and were not used for quantitative analysis.

Relative quantification of mRNA and gDNA levels

DNA and RNA were extracted from pools of ten 3-d-postfertilization (dpf) embryos or adult zebrafish organs using TRI Reagent (Sigma-Aldrich, T9424) followed by phenol-chloroform extraction. In brief, pools of embryos or adult fish organ samples were lysed and homogenized in 500 µL of TRI Reagent using syringes with 21G or 23G needles, respectively. Chloroform was added; samples were vortexed and centrifuged. The top aqueous phase (containing RNA) was transferred to a new microcentrifuge tube; RNA was precipitated with isopropanol and ethanol, dissolved in 20 to 40 µL of RNase-free water. At least 400 ng RNA was used for cDNA synthesis (Maxima H Minus cDNA Synthesis Master Mix M1662, Thermo Fisher Scientific) that was used for RT-qPCR. The bottom organic phase (containing DNA) was washed with sodium citrate and subjected to ethanol purification to purify the DNA, which was then dissolved in 8 mM NaOH and used for qPCR.

qPCR was performed in Rotor-Gene Q (QIAGEN) using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, K0253) without ROX passive dye. All reactions were performed in at least technical duplicates. Fold changes were calculated using the 2^{-ΔΔCt} method (Livak and Schmittgen 2001). As a reference gene, *eef1a1/1* was used for mRNA level quantification (McCurley and Callard 2008), and *aldh1a2* was used for gDNA level quantification (Burg et al. 2018). Primers used for qPCR analysis were designed to generate an amplicon only when wild-type mRNA or gDNA is present. Sequences of the primers are provided in Supplementary Table 1. Mean Ct values of technical replicates for each biological sample are listed in Supplementary Table 2.

Statistics

Statistical analysis was performed using GraphPad Prism version 8.4. Ordinary 1-way ANOVA was performed to evaluate differences between control samples and multiple treated samples. Holm-Sidak's multiple comparisons correction test was used, and postcorrection P-values are reported; P ≤ 0.05 was considered significant.

Where multiple comparison was not needed, the 2-tailed unpaired Student's t-test was used to determine statistical significance between control and treated samples.

The P-values are shown in graphs as follows:

*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.

Exact P-values for all statistical tests are provided in Supplementary Table 3.

Determination of the transgene integration site

Genomic DNA was prepared from F₁ fish tail clips. The genomic sequences flanking the integration sites were determined by nested inverse PCR as described in Balciunas et al. (2004) and Hermanson et al. (2004) with a minor modification that the first PCR reaction was diluted 1:100 in water before use as the template in the nested PCR. Briefly, the genomic DNA was digested with *TaqI* or *XbaI*-*NheI*-*AvrII*-*SpeI* and ligated overnight. Amplification of genomic sequences flanking the *SB(ubb^R:CreER^{T2}*, Cry:RFP)* transgene was achieved by nested PCR. The 5' of the end of the construct was amplified using primer pairs LP1 and LP3, followed by second PCR reaction with primers LP2 and LP4. The 3' end of the construct was amplified using nRP1 and RP3, followed by a second PCR reaction with primers RP2 and RP4. Sequences of the primers are provided in Supplementary Table 1.

Use of large language models

ChatGPT (OpenAI) was used for sentence-level language correction and clarity improvement. The authors reviewed and edited the text as needed and take full responsibility for the content of the publication.

The initial sketches of zebrafish in Figs. 1–3 were generated using Google Gemini and manually edited by the authors.

Results

Recombinant ubiquitin promoter *ubb^R* outperforms other promoters

While recombination of 1 allele in a subset of cells is sufficient for lineage-tracing experiments, a very high frequency of biallelic recombination is likely to be required to achieve loss-of-function phenotype for conditional mutants. Notably, using ubiquitous *ubb:CreER^{T2}* driver line *Tg(-3.5ubb:CreERT2, myl7:EGFP)zf2148*, a single-copy subline of *Tg(-3.5ubb:CreERT2, myl7:EGFP)cz1702* (Mosimann et al. 2011), induction of recombination by addition of 4-hydroxytamoxifen (4-HT) at 10 h postfertilization—prior to onset of *tbx20* expression—failed to induce the severe cardiac defect in zebrafish embryos homozygous for the floxed *tbx20^{tp145}* allele (Burg et al. 2018). We speculated that a stronger *CreER^{T2}* driver may yield more robust loss-of-function phenotypes and set out to engineer such line. In our attempts, we decided to use zebrafish codon-optimized *CreER^{T2}* (*CreER^{T2*}*) (Kesavan et al. 2018), as well as 2 widely used promoters: the zebrafish ubiquitin (*ubb*) (Mosimann et al. 2011), with the splice acceptor site modified from AG/AT to a more canonical AG/GT, and the common carp *beta-actin2* (Gibbs and Schmale 2000; Sivasubbu et al. 2006; Clark et al. 2011). We engineered a recombinant zebrafish ubiquitin promoter (subsequently referred to as *ubb^R* for Recombinant) by adding an intronic enhancer element from the carp *beta-actin2* promoter (Liu et al. 1990) into the first intron. Then, we aimed to

Table 1. Zebrafish lines generated in this study.

Abbreviation	Construct	Integration method
<i>vlm4Tg</i>	<i>Tg(Xla.Crygc:ATTR-mRFP, actb2:CreER^{T2*}, Amp, ATTL-GFP)</i>	Targeted integration into <i>Tg(Xla.Crygc:ATTP-GFP)tp1102</i>
<i>vlm3Tg</i>	<i>Tg(Xla.Crygc:ATTR-mRFP, -3.5ubb:CreER^{T2*}, Amp, ATTL-GFP)</i>	Targeted integration into <i>Tg(Xla.Crygc:ATTP-GFP)tp1102</i>
<i>vlm1Tg</i>	<i>Tg(Xla.Crygc:ATTR-mRFP, ubb^R:CreER^{T2*}, Amp, ATTL-GFP)</i>	Targeted integration into <i>Tg(Xla.Crygc:ATTP-GFP)tp1102</i>
<i>vlm2Tg</i>	<i>Tg(Xla.Crygc:ATTR-mRFP, ubb^R:CreER^{T2*}, ATTL-GFP)</i>	Targeted integration into <i>Tg(Xla.Crygc:ATTP-GFP)tp1102</i>
<i>vlm7Tg</i>	<i>Tg(Xla.Crygc:ATTR-mRFP, ubb^R:CreER^{T2*}, Amp, ATTL-GFP)</i>	Targeted integration into <i>Tg(Xla.Crygc:ATTP-GFP)tp1104</i>
<i>vlm10Tg</i>	<i>SB(ubb^R: CreER^{T2*}, Xla.Crygc:mRPP)I</i>	Sleeping Beauty-mediated random integration
<i>vlm12Tg</i>	<i>SB(ubb^R: CreER^{T2*}, Xla.Crygc:mRPP)II</i>	Sleeping Beauty-mediated random integration
<i>vlm13Tg</i>	<i>SB(ubb^R: CreER^{T2*}, Xla.Crygc:mRPP)III</i>	Sleeping Beauty-mediated random integration

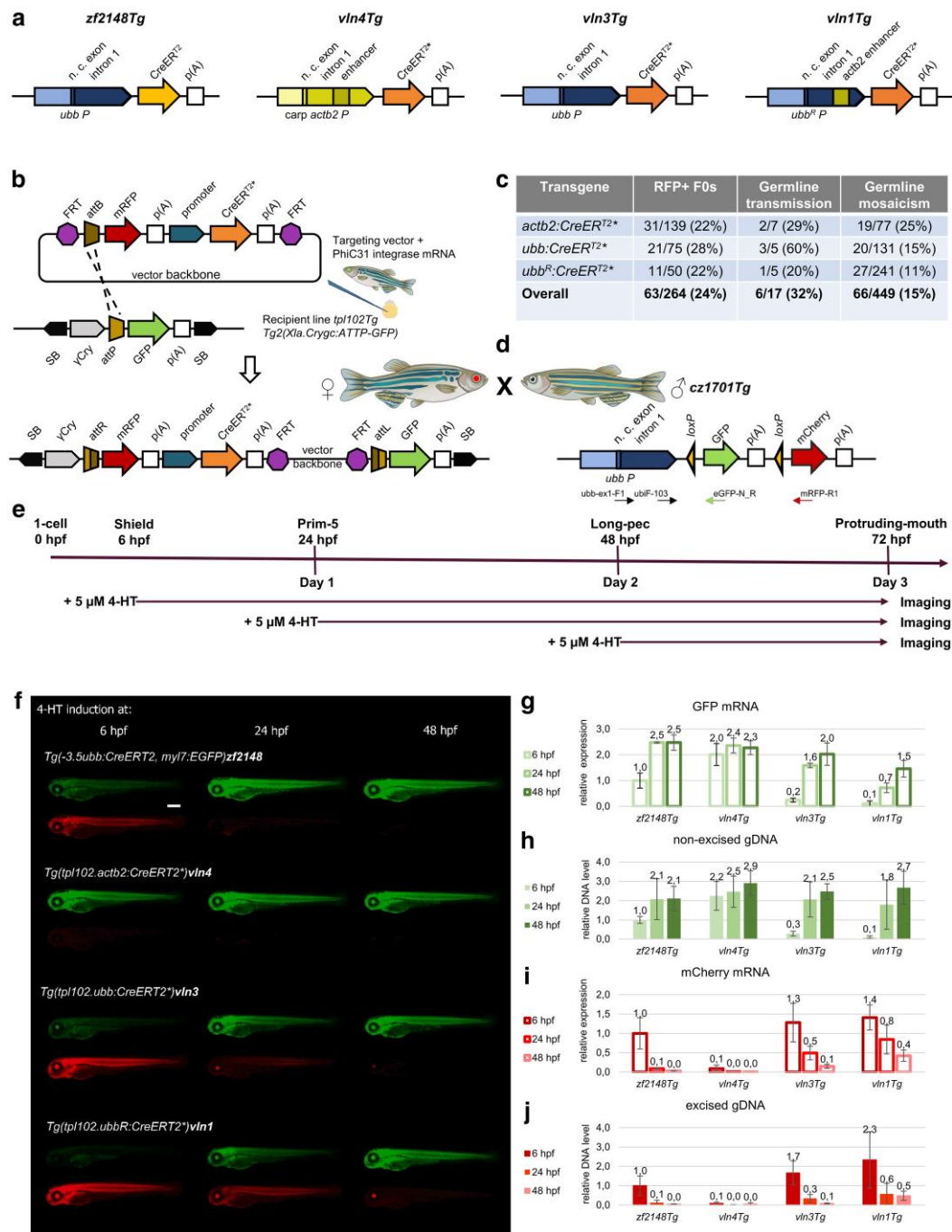


Fig. 1. Construction and comparison of the efficiency of different ubiquitous 4-HT inducible cre-drivers in zebrafish embryos. **a)** Diagrams of CreER^{T2} transgenes used in this experiment. *Tg(-3.5ubb:CreERT², myl7:EGFP)zf2148* is a single-copy derivative of *Tg(-3.5ubb:CreERT², myl7:EGFP)cz1702*, containing a widely used mouse-optimized CreER^{T2} sequence under the control of zebrafish ubiquitin promoter. *Tg(tpl102.actb2:CreERT²)vln4* contains zebrafish-optimized CreER^{T2} sequence under the control of beta-actin 2 promoter from common carp. *Tg(tpl102.ubb:CreERT²)vln3* contains zebrafish-optimized CreER^{T2} sequence under the control of zebrafish ubiquitin promoter. *Tg(tpl102.ubb^R:CreERT²)vln1* contains zebrafish-optimized CreER^{T2} sequence under the control of recombinant *ubb^R* promoter, consisting of zebrafish ubiquitin promoter with carp *actb2* enhancer. **b)** Diagram of phiC31-mediated targeted integration system. *Tg(Xla.Crygc:ATTP-GFP)tpl102* embryos containing an attP docking site have a green lens due to the gamma-crystalline promoter driving a GFP reporter. SB labels recognition sequences of Sleeping Beauty transposase used in generating the recipient transgenic line with the docking site. Injection of a circular plasmid with attB and a red fluorescence reporter (targeting vector) into recipient line embryos leads to eye color change upon phiC31-mediated integration in larvae. Reporter and CreER^{T2} transgenes are flanked with FRT recognition sequences for FLP recombinase, allowing subsequent removal of the vector backbone. **c)** Targeted transgenesis efficiency and germline mosaicism. **d)** Diagram of the ubiquitous lineage-tracing *ubi:switch* transgene used for the evaluation of the recombination efficiency of various transgenic Cre driver lines. Horizontal arrows indicate the primers used for qPCR analysis. **e)** Experimental outline. Zebrafish males harboring *ubi:switch* reporter were crossed to zebrafish females harboring CreER^{T2} driver; embryos were treated with 5 μ M 4-HT solution at 6, 24, or 48 hpf and kept in the dark until 3 dpf. At 3 dpf, embryos positive for the CreER^{T2} driver were imaged, and pools of 10 embryos were collected for quantitative analysis. **f)** Representative images of live 3 dpf embryos treated with 5 μ M 4-HT solution starting at shield stage (6 hpf), prim-5 stage (24 hpf), or protruding mouth stage (48 hpf). After successful recombination, the GFP-encoding DNA fragment is excised and ubiquitin promoter controls the expression of mCherry. Exposure of all red channel microscopy images was increased identically for easier evaluation. Scale bar, 500 μ m. **g)** qPCR analysis of GFP mRNA levels, **h)** unexcised gDNA, **i)** mCherry mRNA levels, and **j)** excised gDNA in 3-d-old larvae harboring different CreER^{T2} transgenes after recombination induction at 6, 24, or 48 hpf.

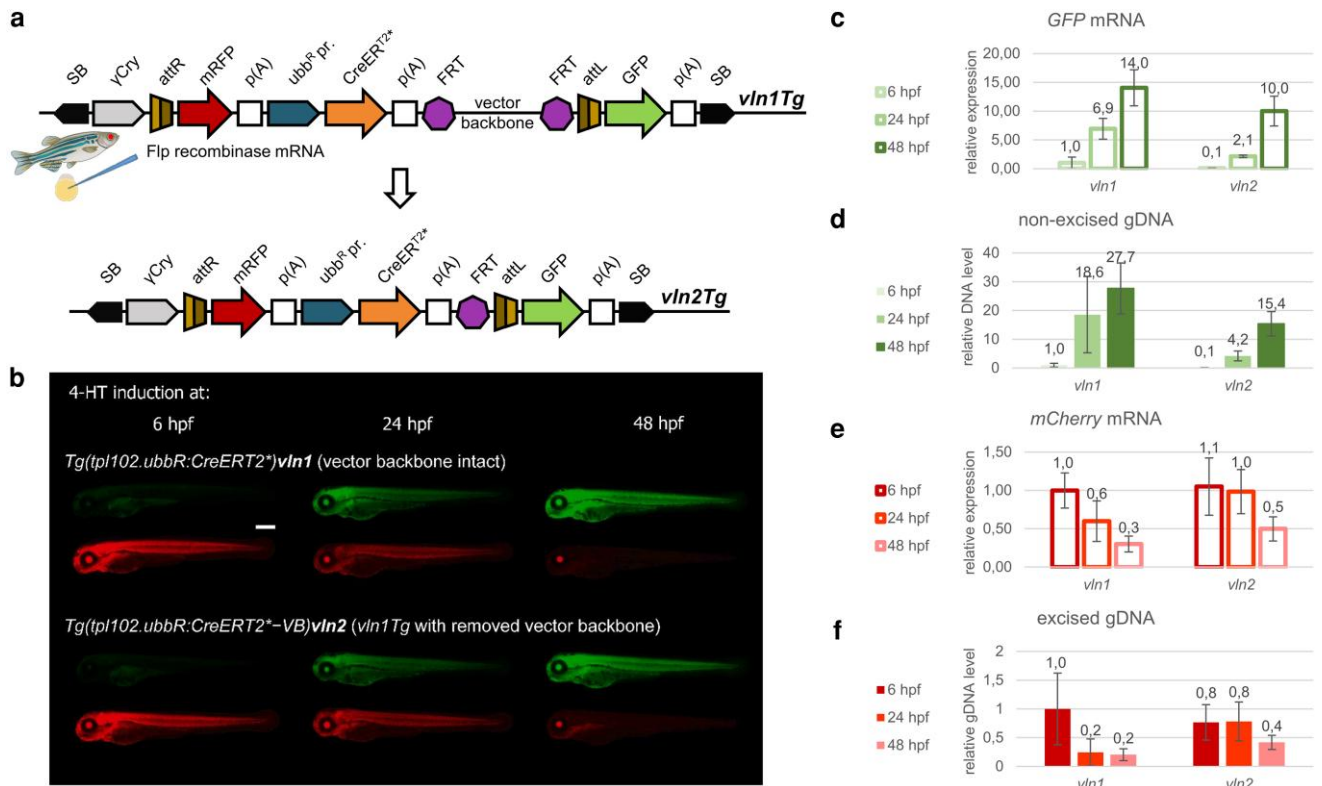


Fig. 2. Vector backbone removal increases recombination efficiency at later stages of development. a) Diagram of vector backbone removal after injection of *Flp* recombinase-encoding mRNA into 1-cell stage transgenic embryos. b) Representative images of live embryos, treated with 5 μ M 4-HT solution at 6, 24, or 48, at 3 dpf. Exposure of all red channel microscope images was increased identically for easier evaluation. Scale bar, 500 μ m. c) qPCR analysis of GFP mRNA levels, d) unexcised gDNA, e) mCherry mRNA levels, and f) excised gDNA in 3-d-old larvae harboring *Tg(tpl102.ubb^R:CreERT^{2*})vln1* or *Tg(tpl102.ubb^R:CreERT^{2*}-VB)vln2* transgenes after recombination induction at 6, 24, or 48 hpf.

compare the activity of both parental promoters and our newly generated *ubb^R* promoter (all constructs are shown in Fig. 1a). It is well-known that the integration locus of the transgene can have an impact on its expression (Hans et al. 2009, 2011; Mosimann et al. 2011; Roberts et al. 2014; Kawakami et al. 2016; Kalvaityte et al. 2024). Therefore, to avoid variability due to position effects, we used a ϕ C31-mediated targeted integration system (Roberts et al. 2014) (Fig. 1b). Thus, we constructed 3 targeting vectors, containing *attB*-mRFP marker for ϕ C31-mediated transgenesis, the promoter being tested, and *CreERT^{2*}*-encoding sequence followed by the bovine growth hormone (*bgh*) poly(A) signal (Fig. 1b). We also added *FRT* sites for subsequent removal of bacterial-derived sequences, as such sequences have been reported to negatively influence transgene expression in other species (Lusky and Botchan 1981; Valera et al. 1994; Chen et al. 2004; Hlavaty et al. 2004; Riu et al. 2005; Etchberger and Hobert 2008; Lu et al. 2012).

For our experiments, we chose the recipient line *Tg(Xla.Crygc:attPGFP)tpl102* with *attP* docking site integrated into the chromosome 3. Fish with the docking site have a green lens due to the *gamma*-crystallin promoter driving a GFP reporter, and the integration of the targeting plasmid leads to eye color change (Roberts et al. 2014). Upon injection of the targeting vectors mixed with ϕ C31 integrase mRNA into 1-cell stage *Tg(Xla.Crygc:attPGFP)tpl102* zebrafish embryos, we observed that 22% (31/139), 28% (21/75), and 22% (11/50) of the embryos injected with *actb2:CreERT^{2*}*, *ubb:CreERT^{2*}*, and *ubb^R:CreERT^{2*}*-encoding targeting vectors, respectively, were positive for lens RFP at 3 dpf. These embryos were raised to adulthood and outcrossed to wild-type

fish to test if the integrated transgene is passed to F₁ embryos. Germline transmission rates varied from 20% to 60% for different constructs. Altogether, 6 out of 17 outcrossed fish transmitted the transgene integrated into the docking site, with germline mosaicism varying between 11% and 25% (Fig. 1c). Single-copy lines were designated *Tg(tpl102.actb2:CreERT^{2*})vln4*, *Tg(tpl102.ubb:CreERT^{2*})vln3*, and *Tg(tpl102.ubb^R:CreERT^{2*})vln1*.

Transgenic F₁ females were then crossed to males homozygous for *Tg(-3.5ubi:loxP-GFP-loxP-mCherry)cz1701* (*ubi:switch*) reporter (Mosimann et al. 2011) (Fig. 1d). Obtained embryos were divided into 3 separate groups and treated with 5 μ M 4-HT at shield stage (6 hpf), prim-5 stage (24 hpf), or protruding mouth stage (48 hpf). All the embryos were subsequently incubated in the dark until analysis was performed at 3 dpf. At 3 dpf, embryos positive for the *CreERT^{2*}* driver (RFP signal in the lens) were selected, imaged under the fluorescent microscope to evaluate GFP and mCherry fluorescence (Fig. 1e), and pools of 10 embryos were collected for quantitative analysis of recombination efficiency. For comparison, *Tg(-3.5ubb:Cre-ERT2,myl7:EGFP)zf2148* (Mosimann et al. 2011) females were also crossed to *ubi:switch* males, and the embryos were treated identically.

Our observations made with the *Tg(-3.5ubb:Cre-ERT²,myl7:EGFP)zf2148* driver were entirely consistent with those reported previously (Mosimann et al. 2011; Lalonde et al. 2022). Recombination, as seen by induction of red fluorescence and concomitant reduction in green fluorescence, was strong after the 4-HT induction at 6 hpf and diminished substantially when induced at later stages (Fig. 1f). To our surprise, our transgenic line *Tg(tpl102.actb2:CreERT^{2*})vln4* using the carp *beta-actin2*

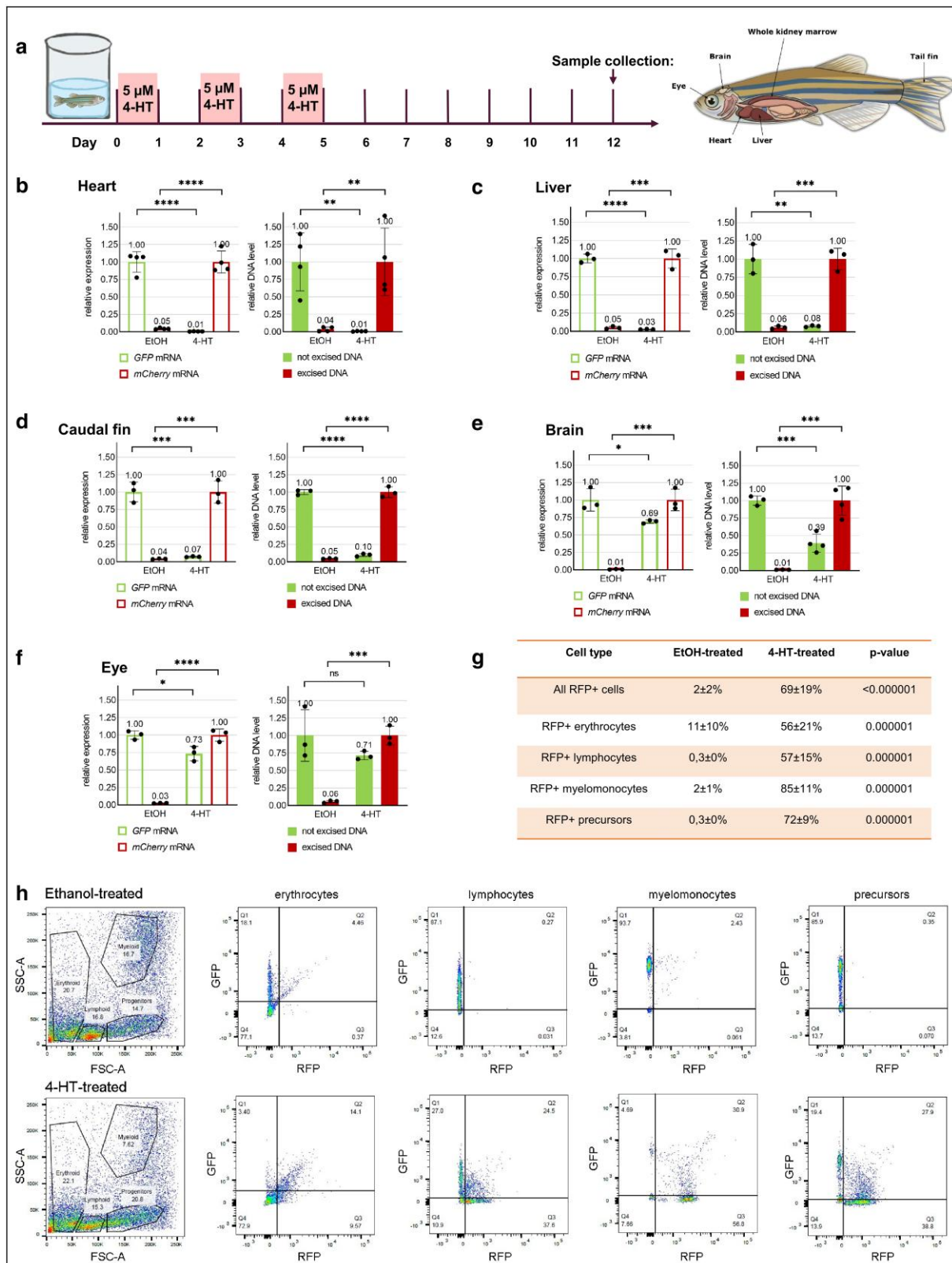


Fig. 3. Recombination efficiency of various tissues of adult zebrafish harboring novel *Tg(tpl102.ubb^R:CreER^{T2}-VB)vlh2* driver. **a**) Experimental outline. Adult (3-mo-old or older) zebrafish females were treated with 5 μ M 4-HT solution 3 times for 24 h with 1-d recovery period between treatments. Control fish were incubated in 0.01% ethanol (solvent for 4-HT) at the same conditions. One week after the last incubation, fish were euthanized and samples of heart ventricles, liver, caudal fin, brain, eye, and whole kidney marrow were collected and used for further analysis. **b**) qPCR analysis of GFP/mCherry mRNA levels and unexcised/excised gDNA in heart ventricles, **c**) liver, **d**) caudal fin, **e**) brain, **f**) eye of 4-HT or ethanol-treated zebrafish with *ubi:switch* reporter. **g**) Analysis of mCherry-positive hematopoietic cells in 4-HT or ethanol-treated adult zebrafish with *ubi:switch* reporter. **h**) Representative flow-cytometry analysis of *Tg(tpl102.ubb^R:CreER^{T2}-VB)vlh2; ubi:switch* adult whole kidney marrow. Cells were separated into major blood cell populations by forward scatter (FSC) and side scatter (SSC), and the percentage of RFP-positive cells in the fluorescence-positive cell population was compared between 4-HT and ethanol-treated fish samples. Error bars, mean, s.d. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

promoter to drive CreER^{T2}* expression exhibited only negligible recombination of *ubi:switch* reporter at all time points tested. Our other 2 new transgenic lines, *Tg(tpl102.ubb:CreER^{T2}*)vln3* and *Tg(tpl102.ubb^R:CreER^{T2}*)vln1*, did not seem to offer a major improvement over *Tg(-3.5ubb:Cre-ERT2,myl7:EGFP)zf2148* when recombination was induced at 6 hpf, perhaps with the exception of some further reduction in green fluorescence. Both lines displayed a consistent increase in mCherry fluorescence when induced at 24 hpf, and this increase was more pronounced for *Tg(tpl102.ubb^R:CreER^{T2}*)vln1*. When induced at 48 hpf (1 d before assessment), we did not observe any decrease in GFP fluorescence. mCherry fluorescence was barely detectable for *Tg(tpl102.ubb:CreER^{T2}*)vln3* and more robustly detectable, but not very strong, for *Tg(tpl102.ubb^R:CreER^{T2}*)vln1*.

We collected pools of 10 embryos from at least 3 different biological replicates of each treatment group for quantitative assessment of recombination efficiency at the molecular level. We extracted total RNA and genomic DNA from these samples, used the RNA to synthesize cDNA, and performed quantitative PCR to assess relative recombination efficiency at the level of mRNA (cDNA) and of genomic DNA (Fig. 1g–j). qPCR results largely confirmed fluorescence-based observations. Relative to *Tg(-3.5ubb:Cre-ERT2,myl7:EGFP)zf2148*, *Tg(tpl102.ubb^R:CreER^{T2}*)vln1* displayed a statistically significant reduction of GFP mRNA levels ($P = 0.01$; primers *ubb-ex1-F1/eGFP-R*; Fig. 1g), however, there were no significant differences in excision efficiency at the gDNA level ($P = 0.0966$; primers *ubi-F103/eGFP-R*; Fig. 1h), when the recombination was induced at 6 hpf. We observed a statistically significant increase in mCherry mRNA levels (primers *ubb-ex1-F1/mRFP-R1*) when recombination was induced at 24 hpf ($P = 0.0101$) and 48 hpf ($P = 0.0012$; Fig. 1i). Similar increase was also observed at gDNA levels (primers *ubi-F103/mRFP-R1*) when we induced the recombination at 48 hpf ($P = 0.0066$; Fig. 1j). P -values of the comparison of mRNA and gDNA levels are provided in Supplementary Table 3.

Removal of the vector backbone increases recombination efficiency at late stages of development

We next tested whether recombination efficiency can be further increased by deleting bacterial vector backbone sequences. This was achieved by injecting the F1p recombinase-coding mRNA into 1-cell stage embryos obtained from *Tg(tpl102.ubb^R:CreER^{T2}*)vln1* outcross (Fig. 2a). Mosaic adults were then outcrossed to wild-type fish, obtained F₁ embryos were raised to adulthood and genotyped for the removal of the vector backbone, and the resulting line was designated *Tg(tpl102.ubb^R:CreER^{T2}*)vln2*. We then crossed *Tg(tpl102.ubb^R:CreER^{T2}*)vln2* females to *ubi:switch* males and exposed the embryos to 4-HT at 6, 24, or 48 hpf to induce recombination.

GFP and mCherry fluorescence intensities of *Tg(tpl102.ubb^R:CreER^{T2}*)vln2* larvae treated with 4-HT at 6 hpf were similar to those of the parental strain *Tg(tpl102.ubb^R:CreER^{T2}*)vln1*, but *Tg(tpl102.ubb^R:CreER^{T2}*)vln2* larvae displayed more intense red fluorescence when treated at 24 and 48 hpf (Fig. 2b). Quantitative PCR analysis confirmed that *Tg(tpl102.ubb^R:CreER^{T2}*)vln2* induced recombination more efficiently than *Tg(tpl102.ubb^R:CreER^{T2}*)vln1*: we observed a reduction of GFP mRNA levels when recombination was induced at 24 hpf ($P = 0.0015$, Fig. 2c); also the amount of nonexcised gDNA was significantly lower after induction at 6, 24, and 48 hpf ($P = 0.0427$, $P = 0.0391$, $P = 0.0115$, respectively; Fig. 2d); and the level of excised gDNA was higher after the induction at 24 and 48 hpf ($P = 0.0331$ and $P = 0.0088$, respectively; Fig. 2e). Therefore, we concluded

that removal of the vector backbone had a positive impact on the expression of the transgene and, subsequently, the efficiency of recombination.

Tg(tpl102.ubb^R:CreER^{T2})vln2* driver highly active in adult zebrafish

We next sought to investigate if *Tg(tpl102.ubb^R:CreER^{T2}*)vln2* can induce recombination in the adult zebrafish. We used adult females of 3 mo of age or older and immersed them in 100 mL of egg water containing 5 μ M 4-HT 3 times for 24 h in the dark, allowing them 24 h of rest at normal husbandry conditions between each treatment. Seven days after the last 4-HT treatment, we euthanized the fish and collected various organs and tissues: heart ventricles, liver, brain, eye, caudal fin, and whole kidney marrow (WKM) (the timeline of the experiment is provided in Fig. 3a).

We homogenized heart ventricles, liver, brain, eye, and caudal fin, extracted total RNA and gDNA, and evaluated the recombination efficiency by qPCR analysis. Our data show that *Tg(tpl102.ubb^R:CreER^{T2}*)vln2* driver is able to induce recombination in all organs and tissues tested. In adult zebrafish heart, liver, and caudal fin, the level of GFP mRNA dropped to 1%, 3%, and 7% ($P < 0.0001$, $P < 0.0001$, and $P = 0.0004$), respectively (Fig. 3b–d), compared to that observed in the organs of the ethanol-treated fish, indicating a very high recombination efficiency. This was further confirmed by qPCR results using gDNA as a template: the amount of nonexcised DNA in adult zebrafish heart, liver, and caudal fin after recombination induction was reduced to 1%, 8%, and 10% ($P = 0.003$, $P = 0.0014$, and $P < 0.0001$), respectively, compared to the control group. Recombination efficiency in adult zebrafish brain and eye was somewhat lower: GFP mRNA levels dropped to 73% and 69% ($P = 0.0294$ and $P = 0.0179$), respectively (Fig. 3e and f). After treating fish with 4-HT, we observed a reduction to 39% in nonexcised DNA in the brain tissue ($P = 0.0007$), while levels of nonexcised DNA in ethanol-treated and 4-HT-treated zebrafish eye did not change significantly ($P = 0.2542$).

The estrogen receptor domain fused to Cre recombinase is designed to provide precise temporal control of recombination; however, high-level CreER^{T2} expression, resulting from mRNA injection or strong promoter activity, has been reported to cause ligand-independent (“leaky”) recombination (Hans et al. 2009, 2011). This leakiness is thought to arise when endogenous Hsp90 becomes saturated, reducing its capacity to retain the CreER fusion protein in the cytoplasm and thereby permitting the nuclear translocation of unbound CreER (Redeuilh et al. 1987; Picard et al. 1990; Mosimann and Zon 2011). We have also noted a low level of Cre activity on the *ubi:switch* reporter in the absence of induction by 4-HT: with RT-qPCR, we detect transcripts expected to arise only after a successful recombination in ethanol-treated control fish tissues. The levels of these transcripts correlated with 4-HT-induced activity and ranged from 4% to 6% in the heart and liver (Fig. 3b and c) to 1% in the brain (Fig. 3e).

We also evaluated the recombination efficiency in the hematopoietic system of adult zebrafish using flow cytometric analysis of dissected WKM. WKM is the major hematopoietic tissue in adult zebrafish, and previous studies have indicated that it may be challenging to achieve robust transgene expression in this tissue, making it relevant to assess quasi-ubiquitous promoter activity in WKM (Traver et al. 2003; Burket et al. 2008; Mosimann et al. 2011). Our results show that our driver *Tg(tpl102.ubb^R:CreER^{T2}*)vln2* was able to induce successful recombination in hematopoietic cells. Specifically, we observed that 85% of fluorescence-positive myelomonocytes, 72% of

fluorescence-positive precursors, 66% of fluorescence-positive erythrocytes, and 57% of fluorescence-positive lymphocytes expressed mCherry, indicating a successful recombination event in the cell. Overall, mCherry fluorescence was observed in 69% of all the cells in which the *ubi:switch* reporter was active after the fish were treated with 4-HT. Same as in the aforementioned tissues, we observed some nonspecific recombination: 2.4% WKM cells of the fish that were treated with ethanol displayed a low level of nonspecific induction of recombination.

***Tg(tpl102.ubb^R:CreER^{T2}*-VB)vlm2* zebrafish line is able to induce loss-of-function phenotypes in developing embryos**

To further evaluate the efficiency of our new *Tg(tpl102.ubb^R:CreER^{T2}*-VB)vlm2* driver, we tested it on 3 different conditional mutants. Firstly, we wanted to see if this new driver could induce loss-of-function phenotypes of floxed *tbx20*^{tpl145} zebrafish line (Fig. 4a) (Burg et al. 2018). *Tbx20* is an essential regulator of heart development, and the loss of *tbx20* function leads to significantly reduced cardiomyocyte numbers by impaired proliferation of heart muscle cells, resulting in cardiac hypoplasia and weak cardiac contraction (Just et al. 2016). The expression of *tbx20* in developing zebrafish is not detected until 1–4 somite stage (ZFIN Publication: Thisse et al. 2001). We crossed *Tg(tpl102.ubb^R:CreER^{T2}*-VB)vlm2*; *tbx20*^{tpl145/tpl145} females to *tbx20*^{tpl14/tpl1455} males, then treated the resulting embryos with 5 μM 4-HT solution at 6, 10, 12, or 24 hpf. At 3 dpf, we collected the larvae with RFP signal in the lens (selection marker for *Tg(tpl102.ubb^R:CreER^{T2}*-VB)vlm2*), evaluated the development of the heart, and collected pools of 10 larvae for quantitative analysis of recombination efficiency.

qPCR analysis of gDNA revealed that excision of the floxed allele was almost complete at all time points tested. Compared to untreated larvae, the relative quantity of unexcised (exon 2-containing) gDNA levels dropped to 0%, 0%, 1%, and 2% after recombination was induced at 6, 10, 12, or 24 hpf, respectively (in all cases, $P < 0.0001$; Fig. 4b). RT-qPCR analysis of *tbx20* mRNA in the larvae indicates that the relative quantity of mRNA that has an intact exon 2 was equal to 0% after the 4-HT induction at 6 and 10 hpf (in both cases, $P < 0.0001$). Even when we treated the embryos at 12 hpf (2 h after the start of the *tbx20* gene expression), we detected only 1% of full-length mRNA relative to untreated embryos ($P < 0.0001$) at 3 dpf. While qPCR analysis of gDNA showed that the floxed exon was excised almost completely when the embryos were treated with 5 μM 4-HT solution at 24 hpf, we still detected around 32% of wild-type *tbx20* mRNA in Cre-positive larvae ($P < 0.0001$). We speculate that this mRNA was transcribed before the recombination was completed. The structure of the floxed allele and the position of stop codon after successful recombination is provided in Supplementary Fig. 1a and b.

Tbx20 loss-of-function has previously been shown to cause severe defects in heart development. While early heart tube formation proceeds normally, from the looping stage onward, the heart fails to undergo proper chamber morphogenesis, causing defects in blood flow and resulting in pronounced pericardial edema (Szeto et al. 2002; Just et al. 2016). Our results are consistent with these observations: almost complete excision of exon 2 of the *tbx20* gene had a strong impact on cardiac development when recombination was induced at 6, 10, and 12 hpf (Fig. 4c). All (141/141) or nearly all (91/92) Cre-positive larvae displayed severe cardiac edema when induced at 6 and 10 hpf, respectively. However, when the recombination was induced at 12 hpf, the

observed phenotypes were different: all the embryos had noticeable circulation defects, but severe or mild heart edema was evident only in a small subset of the embryos (11/130 and 15/130, respectively). When the recombination was induced at 24 hpf, all the embryos ($N = 43$) had a functioning heart, and no edemas were observed. Representative images of the phenotypic groups are provided in Fig. 4d–f; the representative movie of circulation defects observed in embryos treated with 4-HT at 12 hpf is presented as Supplementary Video 1.

We next tested our *Tg(tpl102.ubb^R:CreER^{T2}*-VB)vlm2* driver on floxed *ift70*^{tpl141} zebrafish line (Burg et al. 2018). The *ift70* (intraflagellar transport 70, previously known as *fleer* or *fir* [Pathak et al. 2007]) gene encodes an essential regulator of cilia tubulin polyglutamylation, and mutant embryos exhibit ventral axis curvature, hydrocephalus, and kidney cysts (Pathak et al. 2007). Expression of the gene is first detected in Kupffer's vesicle of 7 somite embryos and the lateral mesoderm of 11 somite embryos (Pathak et al. 2007). This conditional allele also has 2 unidirectional loxP sites integrated into introns; however, the 2 loxP sites are ~5 kb apart. (Fig. 4g). *Tg(tpl102.ubb^R:CreER^{T2}*-VB)vlm2* zebrafish females homozygous for *ift70*^{tpl141} were crossed to *ift70*^{tpl141/tpl141} males, and the resulting embryos were treated with 5 μM 4-HT solution at 6, 10, 12, and 24 hpf. Cre-positive larvae were selected for phenotypical analysis, and pools of 10 larvae were taken for quantitative evaluation of excision of the floxed part at 3 dpf.

The phenotypical analysis revealed the absence of gross developmental defects. The qPCR analysis of gDNA showed that relative quantities of unexcised *ift70* sequence were significantly reduced in samples taken after recombination induction at 6 hpf (unexcised gDNA level dropped to 47%, $P = 0.0007$), 10 hpf (51%, $P = 0.0009$), and 12 hpf (71%, $P = 0.0203$). However, even though we observed a significant recombination efficiency, RT-qPCR analysis indicated that the expression of *ift70* mRNA was even higher in Cre-positive embryos. Compared to untreated embryos, mRNA levels after 4-HT treatment increased and reached 258% ($P = 0.0021$) after treatment at 6 hpf and 396% ($P < 0.0001$) after treatment at 24 hpf (Fig. 4h). The structure of the floxed allele and the position of stop codon after successful recombination is provided in Supplementary Fig. 1c and d.

The third zebrafish line used for the analysis of the efficiency of conditional mutagenesis induced by our Cre driver was the *tbx5a*^{tpl58t} that has an invertible gene trap cassette flanked by LE/RE mutant loxP sites in the exon 2 of the *tbx5a* gene (Gräjevskaja et al. 2018) (Fig. 4f). *Tbx5* is required for heart and upper limb development (Ahn et al. 2002; Garrity et al. 2002), with expression of the gene starting at around 12 hpf, at the 6–7 somite stage (Begemann and Ingham 2000). *Tg(tpl102.ubb^R:CreER^{T2}*-VB)vlm2* zebrafish females homozygous for *tbx5a*^{tpl58R} allele were crossed to *tbx5a*^{tpl58R/tpl58R} males, and the resulting embryos were treated with 5 μM 4-HT solution at 6, 10, 12, and 24 hpf. Heart development was evaluated at 3 dpf, and development of pectoral fins was assessed at 5 dpf. Pools of 10 larvae sorted for RFP signal in the lens, indicating the presence of the driver, were collected for quantitative analysis of gene trap inversion and *tbx5a* mRNA expression at 3 dpf.

Inversion of the gene trap results in exon 2 of *tbx5a* gene mRNA being spliced into to AUG-less Gal4-VP16 followed by zebrafish beta actin 3' UTR and transcriptional termination sequences. This allows for quantification of both the conditional mutant allele with inverted gene trap cassette and unrecombined *tbx5a* allele that ensures the expression of wild-type *tbx5a*. qPCR analysis revealed that after the induction of recombination at different developmental stages the relative quantity

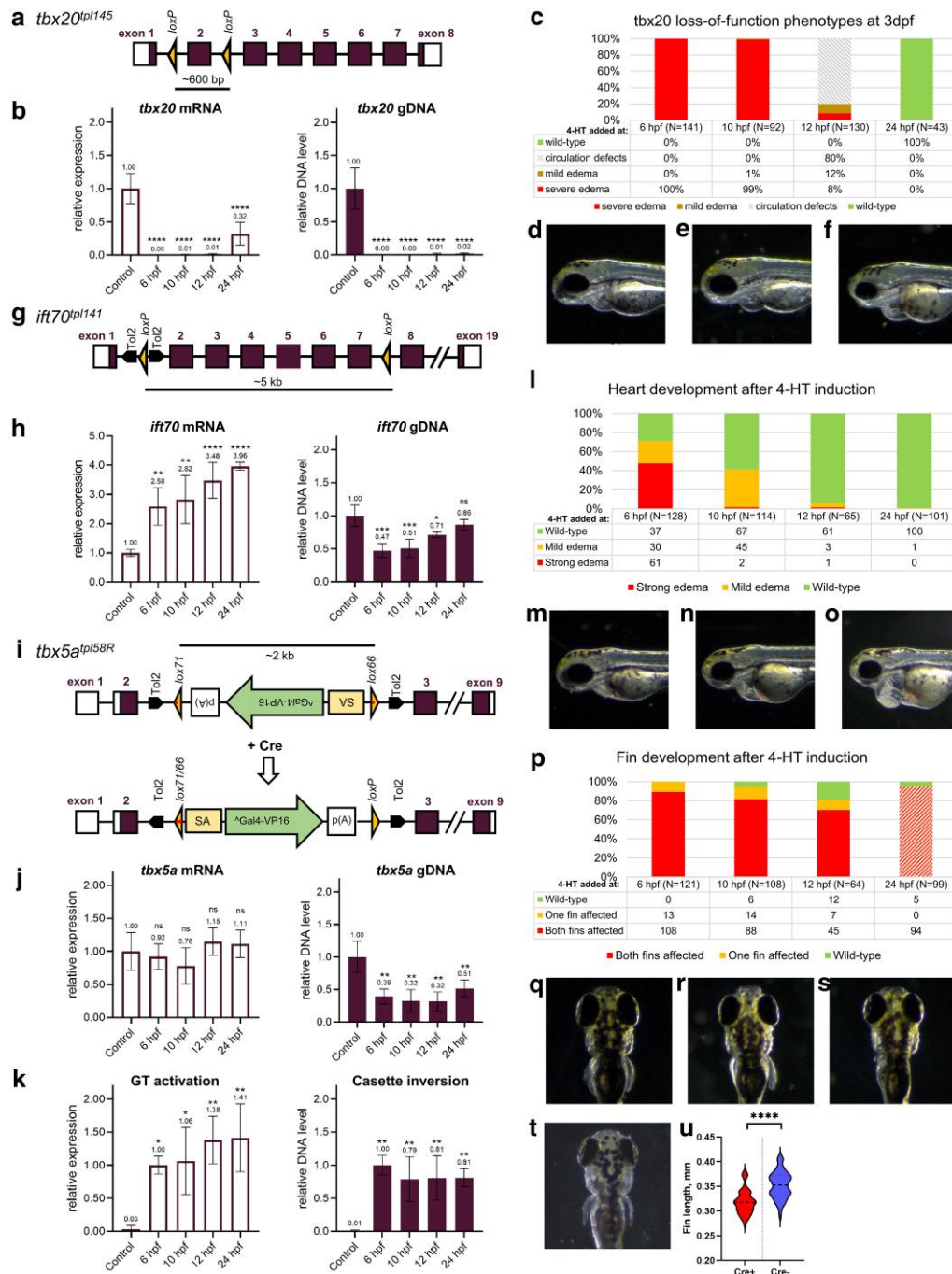


Fig. 4. Recombination efficiency of 3 different conditional zebrafish mutants harboring a novel *Tg(tp102.ubb^R:CreER^{T2}-VB)u1n2* driver. a) Diagram of the floxed *tbx20* allele *tbx20*^{tp145}. b) qPCR analysis of the relative quantities of unexcised *tbx20* mRNA and *tbx20* gDNA levels after the induction of recombination at different time points during early development of the zebrafish embryos homozygous for *tbx20*^{tp145} allele. c) Phenotypic analysis of 3-d-old embryos homozygous for *tbx20*^{tp145} allele after the induction of recombination at 6, 10, 12 or 24 hpf. d) Representative images of wild-type, e) mild edema, and f) severe edema phenotypes observed after conditional inactivation of the *tbx20*^{tp145} allele. g) Diagram of the floxed *ift70* allele *ift70*^{tp141}. h) qPCR analysis of the relative quantities of unexcised *ift70* mRNA and *ift70* gDNA levels after the induction of recombination at different time points during early development of the zebrafish embryos homozygous for *ift70*^{tp141} allele. i) Diagram of the *tbx5a* conditional gene trap allele. SA, carp beta actin splice acceptor; Δ Gal-VP16, AUG-less Gal4-VP16; zp(A), zebrafish beta actin 3' UTR and transcriptional termination sequences; cry, *X. laevis* gamma crystalline promoter; p(A), SV40 poly(A). The gene trap cassette is identical to that used in GBT-B1 gene trap vector (Balciuniene et al. 2013). j) qPCR analysis of the relative quantities of *tbx5a* mRNA and *tbx5a* gDNA levels and k) gene trap activation at the mRNA level and cassette inversion at the gDNA level after the induction of recombination at different time points during early development of the zebrafish embryos homozygous for *tbx5a*^{tp158R} allele. l) Heart development of the zebrafish embryos homozygous for *tbx5a*^{tp158R} allele after 4-HT induction at different developmental points. m) Wild-type zebrafish heart, n) mild heart edema, and o) severe heart edema of a 3-d-old zebrafish embryo observed after the inversion of the gene trap that turns off the expression of the *tbx5a* gene. p) Pectoral fin development of the zebrafish embryos homozygous for *tbx5a*^{tp158R} allele after 4-HT induction at different developmental points. q) Pectoral fins of a 5-d-old zebrafish embryo with wild-type phenotype. r) Five-day-old zebrafish embryo with 1 shortened pectoral fin or o) both shortened pectoral fins observed after the inversion of the gene trap that turns off the expression of the *tbx5a* gene. t) Representative image of a 5-d-old zebrafish embryo that was treated with 5 μ M 4-HT solution at 24 hpf. The length of both fins is unaffected, although the shape of the fins differs from the embryo with the wild-type phenotype. u) Violin plot showing the distribution of pectoral fin lengths of 5-d-old zebrafish embryos homozygous for *tbx5a*^{tp158R} allele with and without the driver.

of wild-type *tbx5a*-expressing alleles was very similar and decreased to 39% ($P = 0.0024$), 32% ($P = 0.002$), 32% ($P = 0.002$), and 51% ($P = 0.005$), when embryos were treated with 4-HT at 6, 10, 12, and 24 hpf, respectively. However, RT-qPCR analysis did not detect a corresponding decrease in wild-type *tbx5a* mRNA levels (Fig. 4j): no significant differences in the relative amount of wild-type mRNA in untreated embryos vs 4-HT-treated embryos were observed.

Nonetheless, phenotypic analysis of 4-HT-treated zebrafish larvae performed at 3 dpf revealed that gene trap activation has a time-dependent impact on cardiac development (Fig. 4l). After 4-HT treatment at 6 hpf, only 29% (37/128) of the driver-positive larvae had normally developed hearts (Fig. 4m), while 23% (30/128) and 48% (61/128) of larvae had mild (Fig. 4n) or severe (Fig. 4o) cardiac edemas, respectively. The percentage of larvae with normal heart increased to 59% (67/114) and 99% (100/101) after recombination induction at 10 and 24 hpf; meanwhile only 2% (2/114) of larvae treated with 4-HT at 10 hpf had severe heart edemas, and none of the larvae treated at 24 hpf displayed severe heart defects ($N = 101$) (Fig. 4l).

Tbx5a is also required for pectoral fin development. *tbx5a* knockdown leads to hypoplasia or aplasia of pectoral fins (Ahn et al. 2002; Lu et al. 2019; Boyle-Anderson et al. 2022). *tbx5a*^{hst} mutants lack pectoral fins (Garritty et al. 2002). Heterozygous zebrafish embryos with 1 gene trap allele also display fully penetrant variable fin defects (Grajevskaja et al. 2018). We observed impaired development of pectoral fins after the gene trap inversion (Fig. 4p). When the recombination was induced at 6 hpf, none of the 5-d-old larvae displayed wild-type pectoral fins (Fig. 4q): they all had defects of one (11%, example in Fig. 4r) or both (89%, example in Fig. 4s) fins ($N = 121$). Proportion of the impaired individuals was similar after the 4-HT treatment at 10 hpf: only 6% (6/108) of larvae had normally developed fins, while 13% (14/108) and 81% (88/108) of larvae had one or both underdeveloped fins, respectively. When the recombination was induced at later stages of development, the results were similar and most of the larvae had disturbed pectoral fin development; however, the fins of these larvae were less severely impaired. When 4-HT induction was performed at 24 hpf, the majority of the fins of Cre-positive larvae resembled wild-type fish (Fig. 4t), but they had different postures and were slightly shorter (Fig. 4u) than those of their siblings: the average length of the pectoral fins in larvae without the driver was 0.3523 mm, while larvae with the Cre driver had fins averaging 0.3185 mm in length ($P < 0.0001$). This suggests that *tbx5a* may be important not only for specification and development but also for the outgrowth of the pectoral fins. It must be stressed, however, that the gene trap cassette contains the Gal4-VP16 fusion protein that is expressed following successful recombination. Therefore, these phenotypes may reflect not only altered *tbx5a* dosage but also potential toxicity of Gal4-VP16 (Scott et al. 2007; Distel et al. 2009).

We also evaluated the recombination efficiency ensured by *Tg(tpl102.ubb^R:CreER^{T2}–VB)vlm2* driver in adult zebrafish heart ventricles of all 3 aforementioned conditional mutants that have different functions and expression levels in the adult zebrafish heart. *Tbx20* is expressed in adult zebrafish heart ventricle and atrium (Fang et al. 2020). In the heart ventricle, the expression level is 41.40 TPM (Zuppo et al. 2023). Its expression is induced in regenerating hearts after the injury, and the overexpression of this transcription factor promotes cardiomyocyte proliferation (Fang et al. 2020); however, the function of this gene in the uninjured heart is not described, because no conditional loss-of-function studies have been performed to date.

The expression of *tbx5a* in adult zebrafish hearts is restricted to the trabeculae (Sánchez-Iranzo et al. 2018). Bulk RNAseq data using the whole ventricle show that the expression level is 62.22 TPM (GEO accession GSE201139, Zuppo et al. 2023). During heart regeneration, trabecular cardiomyocytes can switch their fate and differentiate into cortical myocardium (Sánchez-Iranzo et al. 2018), and the loss of *tbx5a* leads to severe regeneration defects (Grajevskaja et al. 2018). The *Irf70* role in adult heart function, homeostasis, or regeneration has not been studied; the expression level of this gene in adult zebrafish heart is very low (2.52 TPM) (GEO accession GSE201139, Zuppo et al. 2023).

Similar to the assessment of recombination efficiency during zebrafish development, the most efficient recombination is observed in floxed allele that has the loxP sites close to each other: according to RT-qPCR, after 4-HT induction, the relative quantity of exon 2 of *tbx20*^{tpl145} mRNA drops to 0%, compared to that observed in ethanol-treated hearts ($P < 0.0001$, Fig. 5a), even though approximately one-fourth of the *tbx20*^{tpl145} remained refractive to excision at the gDNA level. The recombination efficiency of floxed *itf70*^{tpl141} and gene trap of *tbx5a*^{tpl158R} was noticeably lower, but the differences between ethanol-treated and 4-HT-treated fish were still statistically significant. We were able to induce the excision of two-thirds of the copies of *itf70*^{tpl141} allele ($P = 0.002$) and inversion of half of the copies of *tbx5a*^{tpl158R} allele ($P < 0.0001$), thus reducing the mRNA level of wild-type-like transcripts of these genes to 38% ($P = 0.0025$) and 50% ($P = 0.0082$), respectively (Fig. 5b–d).

***ubb^R:CreER^{T2}** transgene displays high activity at other genomic loci**

High recombination efficiency observed using the *Tg(tpl102.ubb^R:CreER^{T2})*vln1* and subsequent *Tg(tpl102.ubb^R:CreER^{T2}–VB)vln2* lines could be attributed to inherent qualities of the transgene, serendipitously fortunate selection of the integration site (“safe harbor”, (Kotin et al. 1992; Vooijs et al. 2001; Lalonde et al. 2022, 2024)), or a combination of both factors. We therefore sought to test the activity of the *ubb^R:CreER^{T2}** transgene at other loci by integrating this transgene into a different docking site line and by performing a random integration using *Sleeping Beauty* transposon (Davidson et al. 2003; Balciunas et al. 2004).

Using the methodology described above for *Tg(Xla.Crygc:attP-GFP)tpl102*, we performed targeted integration of our cassette into *Tg2(Xla.Crygc:attP-GFP)tpl104* (Roberts et al. 2014) and named the established line *Tg(tpl104.ubb^R:CreER^{T2})*vln7*. We have also subcloned the *ubb^R:CreER^{T2}** into a *Sleeping Beauty* (SB) transposon containing a lens-specific RFP transgenesis marker (Davidson et al. 2003; Balciunas et al. 2004) and generated 3 independent single-copy lines: *SB(ubb^R:CreER^{T2}*, Cry:RFP)vln10*, *SB(ubb^R:CreER^{T2}*, Cry:RFP)vln12*, and *SB(ubb^R:CreER^{T2}*, Cry:RFP)vln13*. We then assessed the activity of these transgenes by crossing heterozygous transgenic female fish harboring the driver to the *ubi:switch* reporter line males; exposing embryos to 4-HT at 6, 24, and 48 hpf; and assessing fluorescence under a microscope at 3 dpf (Fig. 6a). We noted that GFP fluorescence in line *Tg(tpl104.ubb^R:CreER^{T2})*vln7* was stronger after recombination induction at 6 and 24 hpf, and mCherry fluorescence was not as evident as in line *Tg(tpl102.ubb^R:CreER^{T2})*vln1*, indicating that integration of the same transgene into a different docking site results in slightly lower activity. In the random-integration line *SB(ubb^R:CreER^{T2}*, Cry:RFP)vln10*, the fluorescence intensity looked similar to that of the *Tg(tpl102.ubb^R:CreER^{T2})*vln1* line, and in lines *SB(ubb^R:CreER^{T2})*vln12* and *SB(ubb^R:CreER^{T2})*vln13*, recombination efficiency appears even stronger than in the targeted integration

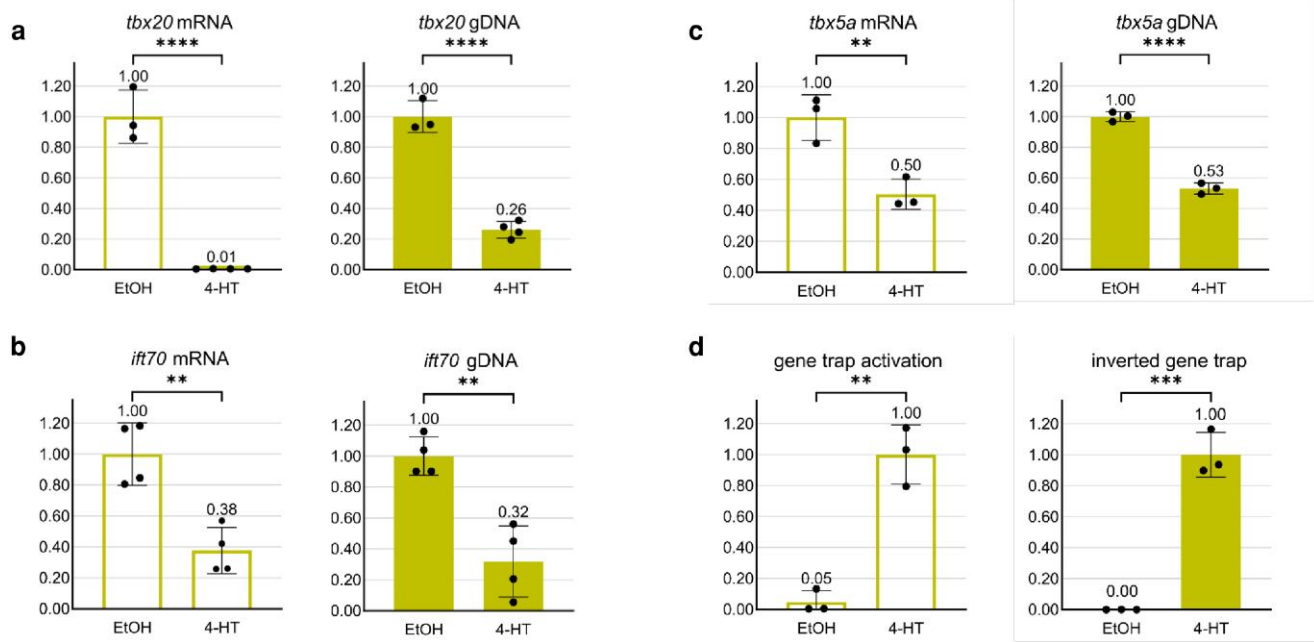


Fig. 5. Recombination efficiency of different conditional mutants in adult zebrafish heart ventricles. a) qPCR analysis of *tbx20* mRNA and gDNA levels in heart ventricles of 4-HT or ethanol-treated adult *tbx20*^{tp1145} zebrafish harboring *Tg(tp102.ubb^R:CreER^{T2}–VB)vlm2* driver. b) qPCR analysis of *ift70* mRNA and gDNA levels in heart ventricles of 4-HT or ethanol-treated *ift70*^{tp1141} zebrafish harboring *Tg(tp102.ubb^R:CreER^{T2}–VB)vlm2* driver. c) qPCR analysis of *tbx5a* mRNA/gDNA levels and d) gene trap activation/inversion in heart ventricles of 4-HT or ethanol-treated adult *tbx5a*^{tp158R} zebrafish harboring *Tg(tp102.ubb^R:CreER^{T2}–VB)vlm2* driver. a–d) $n = 3–4$ biologically independent samples. Error bars, mean, s.d. Two-tailed Student's *t*-test used to assess *P*-values. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

line *Tg(tp102.ubb^R:CreER^{T2})vlm1*: no green fluorescence was observed in either line after induction at 6 hpf, and the GFP intensity after recombination induction at 24 hpf was also clearly diminished. Similarly, mCherry expression was stronger in these 2 random-integration lines after 4-HT treatment at 48 hpf.

In zebrafish, the early events of embryogenesis are primarily regulated by maternal factors synthesized during oogenesis under the control of maternal-effect genes. These maternal factors regulate the processes of embryonic development until midblastula transition and may even contribute to later development of the organism (Dosch et al. 2004; Kishimoto et al. 2004). Maternal contribution of Cre recombinase mRNA and/or protein can cause recombination even when the embryo itself does not contain this transgene and might have a substantial impact on recombination efficiency, especially at early stages of development, when the parent providing the driver is a female. To assess the level of maternal contribution in the driver lines we generated, we imaged larvae from different zebrafish lines, in which the driver was transmitted exclusively through the female parent, that were treated with 4-HT at shield stage but lacked the Cre driver. Our results show that only the line *SB(ubb^R:CreER^{T2})vlm13* displays a significant maternal contribution of Cre as Cre-negative larvae display quite strong mCherry fluorescence at 3 dpf. Targeted integration lines *Tg(tp102.ubb^R:CreER^{T2})vlm1*, *Tg(tp102.ubb^R:CreER^{T2}–VB)vlm2*, and *Tg(tp104.ubb^R:CreER^{T2})vlm7* displayed almost no maternal contribution of Cre mRNA and/or protein as almost no recombination is observed in Cre-negative larvae, and 2 other random integration lines *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm10* and *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm12* displayed low recombination efficiency in the embryos that did not express the recombinase (Supplementary Fig. 2).

We collected pools of 10 embryos exposed to 4-HT at 48 hpf and performed quantitative analysis of GFP excision and resulting

mCherry expression at the mRNA and gDNA levels (Fig. 6b–e). Quantitative analysis revealed that *Tg(tp104.ubb^R:CreER^{T2})vlm7* and *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm10* display activity highly reminiscent of that of *Tg(tp102.ubb^R:CreER^{T2})vlm1* (with the only significant difference being higher mRNA level of mCherry in the *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm10* line, compared to *Tg(tp102.ubb^R:CreER^{T2})vlm1*; $P = 0.0193$), and 2 other random-integration lines *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm12* and *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm13* have even higher mCherry expression ($P < 0.0001$ and $P = 0.0007$, respectively) and lower levels of unexcised ($P = 0.005$ and $P = 0.0273$, respectively) and higher levels of excised ($P = 0.0087$ and $P = 0.0414$, respectively) gDNA, compared to line *Tg(tp102.ubb^R:CreER^{T2})vlm1*.

We also performed inverse PCR to determine the integration loci of *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm10*, *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm12*, and *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm13* lines. We were able to design genotyping primers allowing to distinguish these 3 lines (Supplementary Table 1, Supplementary Fig. 3). However, only one integration locus, *SB(ubb^R:CreER^{T2}, Cry:RFP)vlm12*, mapped onto the zebrafish genome (Supplementary Fig. 4).

Altogether, our data clearly demonstrate that the newly engineered *ubb^R* promoter enables a high level of CreER^{T2} activity when integrated into several different loci of the zebrafish genome.

Discussion

This study describes generation and validation of a strong ubiquitous CreER^{T2} driver line that enables highly efficient 4-HT inducible recombination at all tested stages of zebrafish ontogenesis, thus providing a critical resource needed for studies of biological processes that occur late in development or in adults.

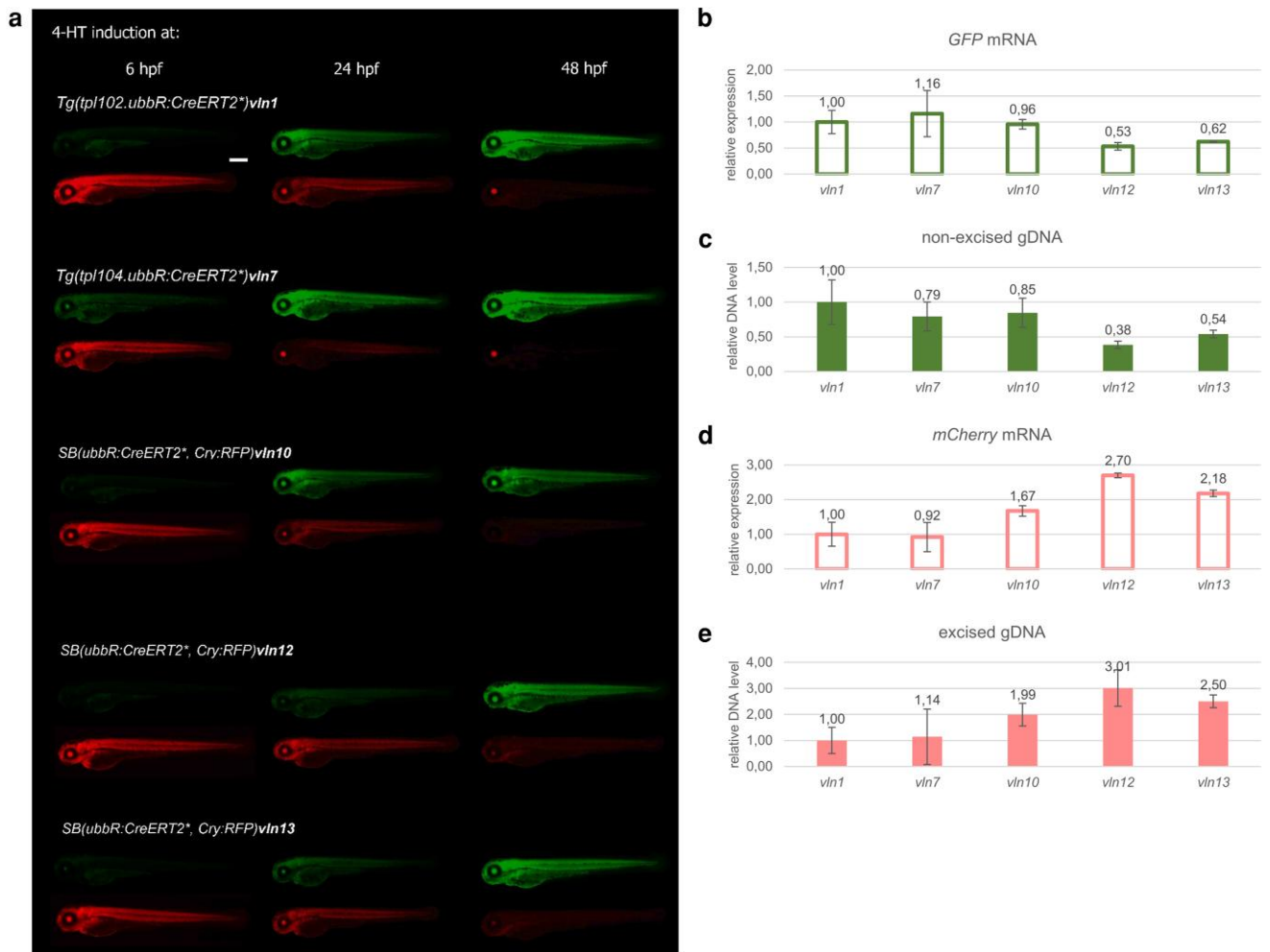


Fig. 6. *ubb^R:CreERT²** transgene displays high activity at other genomic loci. a) Representative images of live 3 dpf embryos treated with 5 μ M 4-HT solution at 6, 24, or 48 hpf. *Tg(tpl102.ubb^R:CreERT²*)vln1*, *ubb^R:CreERT²** transgene with vector backbone at *Tg(Xla.Crygc:ATTP-GFP)tpl102* docking site; *Tg(tpl104.ubb^R:CreERT²*)vln7*, *ubb^R:CreERT²** transgene with vector backbone at *tpl104Tg* docking site; *vln8Tg, ubb^R:CreERT²** transgene without vector backbone at *Tg(Xla.Crygc:ATTP-GFP)tpl104* docking site; *SB(ubbR:CreERT2*, Cry:RFP)vln10*, *SB(ubbR:CreERT2*, Cry:RFP)vln12*, and *SB(ubbR:CreERT2*, Cry:RFP)vln13*, 3 independent random integrations of *ubb^R:CreERT²** transgene using SB100x transposase. Exposure of all red channel microscope images was increased identically for easier evaluation. Scale bar, 500 μ m. b) qPCR analysis of GFP mRNA levels, c) unexcised gDNA, d) mCherry mRNA levels, and e) excised gDNA in 3-d-old larvae in zebrafish lines harboring *ubb^R:CreERT²** in different genomic loci following 4-HT treatment at 48 hpf.

We engineered a recombinant *ubb^R* promoter consisting of the zebrafish ubiquitin promoter (Mosimann et al. 2011) and an intronic enhancer element from the carp *beta-actin2* (Liu et al. 1990). The activity of both parental promoters, zebrafish ubiquitin and common carp *beta-actin2*, that are widely used for quasi-ubiquitous transgene expression in zebrafish, and the derived *ubb^R* promoter, was compared by performing phiC31-mediated targeted integration of otherwise identical transgenes driving zebrafish-optimized CreERT²* (Kesavan et al. 2018) into the *Tg(Xla.Crygc:attP-GFP)tpl102* docking site line (Roberts et al. 2014) to minimize expression variability due to position effects and to quickly derive single-insertion lines and compared to a widely used ubiquitous zebrafish driver line *Tg(-3.5ubb:Cre-ERT2,myl7:EGFP)zf2148* (Mosimann et al. 2011). To assess Cre recombinase activity, we used the ubiquitous green-to-red switch line *ubi:switch* (Mosimann et al. 2011). Females heterozygous for different Cre-drivers were crossed to *ubi:switch* males, and recombination was induced by adding 4-HT at 6, 24, or 48 h postfertilization. Recombination efficiency was assessed by fluorescence microscopy and quantitative PCR at the mRNA and gDNA levels at 3 dpf.

To our surprise, activity of the carp *beta-actin2* promoter was barely detectable in our assay. Our findings contrast several previous observations noting strong expression in developing larvae as well as in adult zebrafish (Gibbs and Schmale 2000; Sivasubbu et al. 2006). We also noted reduced expression of the transgenesis marker, lens-specific RFP (confirmed in independently derived targeted integration lines, data not shown). It remains to be determined if a specific component of our transgene or the genomic context of the *Tg(Xla.Crygc:attP-GFP)tpl102* docking site is responsible for silencing of transgenes containing the carp *beta-actin2* promoter.

Our observations made with the *Tg(tpl102.ubb:CreERT²*)vln3* line, in which the expression of CreERT²* is regulated by the ubiquitin promoter, are entirely consistent with the recombination efficiency induced by the *Tg(-3.5ubb:Cre-ERT2,myl7:EGFP)zf2148* line in the original study: recombination efficiency is very high when induced at 6 hpf but diminishes significantly at later stages of development (Mosimann et al. 2011; Lalonde et al. 2022).

Meanwhile, the recombinant *ubb^R* promoter demonstrated improved recombination efficiency at all time points tested, including improvement by over an order of magnitude when induced at

48 hpf. Thus, although the carp *beta-actin2* promoter itself does not seem to work well under our experimental conditions, its enhancer element is able to increase the activity of the zebrafish ubiquitin promoter.

The ability of the *Tg(tpl102.ubb^R:CreER^{T2}*-VB)uIn2* driver to induce a high level of recombination of the *ubi:switch* transgene at embryonic and larval stages prompted us to test its activity in adults heterozygous for *Tg(tpl102.ubb^R:CreER^{T2}*-VB)uIn2* and *ubi:switch*. We tested it on several organs and tissues: heart ventricle, liver, caudal fin, brain, eye, and WKM cells. Our results confirm that our driver is active and efficient in adult zebrafish: we observed a high level of recombination in the heart ventricle, liver, and caudal fin. In these tissues, after 4-HT induction, the expression of the floxed GFP drops to less than 10% of that observed in ethanol-treated control fish. However, the recombination efficiency was lower in tissues with lower mitotic activity, such as the brain and the eye. We also observed low levels of recombination in tissues from fish treated with EtOH only, indicating background Cre activity in the absence of 4-HT. Levels of background activity ranged between 1% and 6% of tamoxifen-induced activity. This leakiness is an important limitation of this driver that should be taken in account when designing experiments and selecting appropriate controls. Ligand-independent recombination can be particularly problematic in lineage-tracing approaches, where even rare recombination events may lead to persistent false-positive labeling. In contrast, for conditional gene knock-out applications, the impact is expected to be limited, since efficient gene inactivation requires high recombination levels across the target cell population.

To reveal loss-of-function phenotypes in gene knock-out experiments, a high level of biallelic recombination is likely to be needed, in contrast to lineage-tracing experiments, where 1 recombination event per cell is sufficient. We used different conditional mutants to evaluate if *Tg(tpl102.ubb^R:CreER^{T2}*-VB)uIn2* driver is strong enough to achieve this. The results obtained with the *tbx20^{tp145}* line show that the addition of 4-HT at 10 hpf leads to heart defects in 100% of the treated *tbx20^{tp145/tp145}* embryos heterozygous for the driver, and even when we induced the recombination 2 h after the start of the gene transcription (12 hpf), all larvae have abnormal heart shape, indicating of incomplete looping, and strong circulation defects. qPCR results additionally confirm extremely high efficiency of recombination: even after recombination induction at 24 hpf, the relative level of unexcised allele in the whole organism was around 2%, compared to untreated embryos. We also observed near-complete loss of *tbx20^{tp145/tp145}* expression in 4-HT-treated adult zebrafish heart ventricles, further confirming that *Tg(tpl102.ubb^R:CreER^{T2}*-VB)uIn2* driver is suitable for temporally regulated inactivation of floxed alleles in adult zebrafish. Very high degree of excision of *tbx20^{tp145/tp145}* at the level of genomic DNA—74%—is particularly noteworthy. Only about 39% of all the cells in the adult zebrafish heart ventricle are cardiomyocytes (Patra et al. 2017), and not all cardiomyocytes express *tbx20*. Thus, our data clearly demonstrate that our driver is capable of inducing recombination at loci that are not transcriptionally active. Furthermore, the *Tg(tpl102.ubb^R:CreER^{T2}*-VB)uIn2* driver line was already used for highly efficient conditional *tcf21* inactivation in both zebrafish larvae and adult zebrafish hearts (Kalvaityte-Repecke et al. 2026), further demonstrating the strong potential for widespread application of this driver line.

Another conditional mutant that we tested was floxed *ift70^{tp141}* allele. In this conditional mutant, we observe lower recombination efficiency that might be at least partially caused by a longer

distance between the loxP sites: in this line, the distance between them is ~5 kb, compared to ~600 bp in the *tbx20^{tp145}* line. However, although we were able to achieve a noticeable reduction in intact gene-coding sequence, we observed no loss-of-function phenotypes even when we induced the recombination at early stages of development. The analysis of *ift70* mRNA expression revealed an interesting phenomenon: when we induce recombination in *ift70^{tp141}* embryos, instead of the reduction, we detect a significant increase in wild-type mRNA levels, explaining the wild-type resembling embryos. The most likely explanation for this increase is genetic compensation induced by the nonsense-mediated RNA decay of mRNAs containing premature termination codon (El-Brolosy and Stainier 2017; El-Brolosy et al. 2019). The clearing of defective transcripts can trigger self-transcriptional adaptation (Falcucci et al. 2025), resulting in elevated transcription of the impaired gene and a wild-type-like phenotype. This phenomenon is not evident in the hearts of the adult zebrafish, probably because while this gene is highly important in zebrafish development (Pathak et al. 2007), the expression of it in the adult zebrafish heart is low, and there is not enough defective mRNA to induce the transcriptional adaptation (El-Brolosy et al. 2019).

Finally, we evaluated whether our driver could elicit the characteristic developmental phenotypes associated with *tbx5a* LoF by using a conditional gene trap cassette containing inverted lox66/lox72 sites separated by ~2 kb in *tbx5a^{tp158R}* (Grajevskaja et al. 2018). Recombination induction produced pronounced defects in cardiac and pectoral fin development, consistent to those in previous studies (Ahn et al. 2002; Garrity et al. 2002; Burket et al. 2008; Grajevskaja et al. 2018).

In this study, we used homozygous floxed alleles rather than compound heterozygotes carrying 1 floxed allele and 1 preexisting null allele, an approach that is frequently used in other model systems. Our strategy offers several advantages, including the need to validate only a single allele, wild-type-like phenotypes in cases of haploinsufficiency, simplified line maintenance, and more reliable genotyping. However, complete gene inactivation in this context requires 2 recombination events within the same cell to achieve full gene knock-out. Consequently, in situations where recombination efficiency is limiting, combining a floxed allele with a null allele may represent a more sensitive strategy.

Although variability in recombination efficiency may partly reflect locus-specific genomic context, our data support the notion that the design of the conditional alleles also plays a major role. Recent work in mice shows that Cre-mediated recombination is most efficient when loxP sites are spaced within 1 to 4 kb (or ≤3 kb for mutant sites [Erhardt et al. 2025]). Consistent with this, our results support the conclusion that conditional alleles with closely spaced, standard loxP sites are likely to outperform gene trap-based or more distantly spaced designs.

Finally, we demonstrate that *ubb^R:CreER^{T2}** transgene is highly active when integrated at 4 additional genomic loci. Compared to *Tg(tpl102.ubb^R:CreER^{T2}*-VB)uIn2*, *SB(ubb^R:CreER^{T2}*, Cry:RFP)uIn12* and *SB(ubb^R:CreER^{T2}*, Cry:RFP)uIn13* facilitated a higher degree of *ubi:switch* recombination when the larvae were treated with 4-HT at 48 hpf. Line *SB(ubb^R:CreER^{T2}*, Cry:RFP)uIn12* is further notable for strong maternal contribution of CreER^{T2} activity. This data indicate that the recombinant *ubb^R* promoter could be tremendously useful for strong ubiquitous expression of other transgene as well. However, given the maternal contribution observed in some of the generated lines, using males as the Cre-positive parent represents a practical strategy to avoid unintended recombination and secondary effects on the experimental outcome.

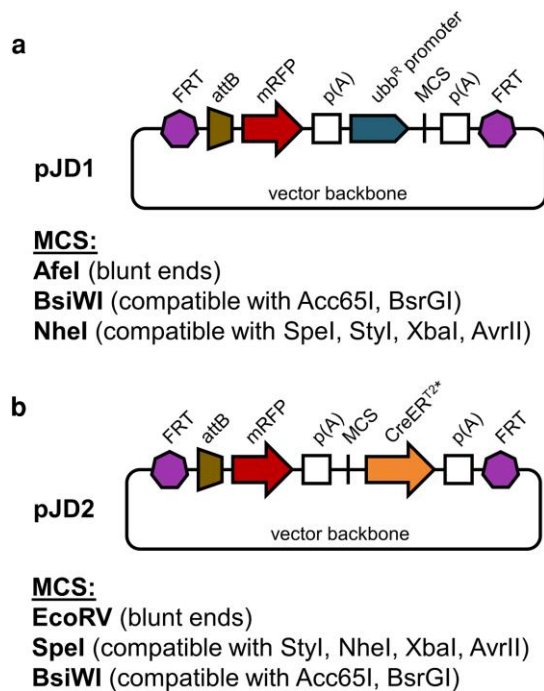


Fig. 7. Vectors for targeted integration of transgenes. a) Plasmid pJD1, containing attB-mRFP marker for phiC31-mediated transgenesis, the *ubb^R* promoter, and a multiple cloning site (MCS) for cloning a transgene of interest followed by the bovine growth hormone (*bgh*) poly(A) signal. b) Plasmid pJD2, containing attB-mRFP marker for phiC31-mediated transgenesis, a MCS for cloning the promoter of interest, and CreER^{T2}-coding sequence followed by the bovine growth hormone (*bgh*) poly(A) signal.

The integration locus of SB(*ubb^R:CreER^{T2}*, *Cry:RFP*)*vlh12* has been determined and may contribute to future efforts aimed at identifying potential “safe harbor” loci in zebrafish.

Overall, the results presented here will offer valuable tools for the zebrafish community, enabling more efficient experiments with conditional mutants in developing embryos and in adult organisms. To facilitate the adoption of the *ubb^R* promoter and the targeted transgenesis system, we constructed 2 plasmids suitable for classical cloning and Gibson assembly that can be used in all attP-harboring zebrafish lines: one for cloning any other transgene of interest under the *ubb^R* promoter (pJD1) and one for cloning any promoter of interest in front of the zebrafish-optimized CreER^{T2} (pJD2) (Fig. 7). The FRT sites integrated in these plasmids allow for subsequent removal of the vector backbone, minimizing the possibility of its negative impact on transgene expression.

Data availability

The data underlying this article are available in the article and in its online [Supplementary material](#). Plasmids and zebrafish lines described in this article will be shared upon request to the corresponding author.

Supplemental material available at [GENETICS](#) online.

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Conflicts of interest

None declared.

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