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Ubiquitous transgene expression and Cre-based recombination driven by the *ubiquitin* promoter in zebrafish

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SUMMARY

Molecular genetics approaches in zebrafish research are hampered by the lack of a ubiquitous transgene driver element that is active at all developmental stages. Here, we report the isolation and characterization of the zebrafish *ubiquitin* (*ubi*) promoter, which drives constitutive transgene expression during all developmental stages and analyzed adult organs. Notably, *ubi* expresses in all blood cell lineages, and we demonstrate the application of *ubi*-driven fluorophore transgenics in hematopoietic transplantation experiments to assess true multilineage potential of engrafted cells. We further generated transgenic zebrafish that express ubiquitous 4-hydroxytamoxifen-controlled Cre recombinase activity from a *ubi:cre^{ERT2}* transgene, as well as *ubi:loxP-EGFP-loxP-mCherry* (*ubi:Switch*) transgenics and show their use as a constitutive fluorescent lineage tracing reagent. The *ubi* promoter and the transgenic lines presented here thus provide a broad resource and important advancement for transgenic applications in zebrafish.

KEY WORDS: Transgenesis, *ubiquitin* promoter, creER, loxP, Zebrafish

INTRODUCTION

A ubiquitous promoter/enhancer to drive transgenes is a key component of a complete transgenesis toolkit in any model organism. Enhancer trap screening in the mouse previously identified the *Rosa26* locus, which became the current gold standard for ubiquitous transgene expression and for Cre/*loxP*-based lineage tracing at all stages of development and in all adult tissues (Friedrich and Soriano, 1991; Soriano, 1999; Zambrowicz et al., 1997). The lack of a homologous tool in zebrafish has particularly limited the full potential of Cre/*loxP*-based lineage-tracing approaches that complement the powerful imaging possibilities and genetic malleability of the model system. As genomic knock-in methods for zebrafish are currently missing, a broadly applicable ubiquitous transgene control element needs to be accessible in simple cloning vectors. Strong candidates for ubiquitous zebrafish promoters/enhancers are thus control regions of genes encoding factors required in fundamental cellular processes.

Several native zebrafish as well as exogenous control elements have been previously deemed ubiquitous in vivo and successfully applied in genetic experiments; these include the zebrafish *h2afx*, *tbp*, versions of the β -*actin* control elements (Burket et al., 2008; Gillette-Ferguson et al., 2003; Higashijima et al., 1997; Kwan et al., 2007), and the *Xenopus laevis*-derived *elongation factor 1a* promoter (*XIEfla1*) (Johnson and Krieg, 1994; Kawakami et al., 2004). Each of these promoter fragments has shortcomings in different developmental stages and cell

types. A common problem is their progressive inactivation during the course of development, particularly in parts of the nervous system, the fin fold, hematopoietic cell populations, or even ubiquitously in all tissues. For example, the commonly applied *XIEfla1* reportedly only expresses strongly during gastrulation and midsomitogenesis before being restricted to specific cell types, although prolonged expression has been reported for isolated transgenes (Collins et al., 2010; Hans et al., 2009). The β -*actin* promoter fragments do retain expression in adults, yet show no significant activity in erythrocytes or fins, or several other cell types (Burket et al., 2008; Traver et al., 2003). This likely reflects specialization of differentiating cells, potential cell-type-specific requirements for basic molecular mechanisms controlling translation or chromatin maintenance, as well as transgene dependence on the genomic integration context.

Lineage-tracing transgenes using currently available transgene promoters in zebrafish therefore do not allow complete cell-fate tracing to all descendant tissues nor lineage analysis over prolonged time periods. Furthermore, the extensive creation of *loxP* cassette-based transgenes requires a reproducible and reliable driver element with broad cloning compatibility. Seminal work by Hans et al. (Hans et al., 2009) established the functionality of tamoxifen (TAM)-inducible Cre^{ERT2}-mediated *loxP* recombination (Feil et al., 1996; Feil et al., 1997) in zebrafish. The fast uptake and action of the active TAM metabolite 4-hydroxytamoxifen (4-OHT) triggers dose-dependent Cre^{ERT2}-mediated *loxP* excision events within 2–4 hours of administration (Hans et al., 2009). As the drug is easily applied through simple addition to the embryo medium, 4-OHT-inducible Cre^{ERT2} is a desirable tool for studying zebrafish development. However, to reliably and flexibly trace *loxP* excision events in cell-lineage experiments at all stages of development, or to trigger Gal4 and other genetic modifiers using tissue-specific Cre^{ERT2} sources, a ubiquitously expressed *loxP* switch construct driver is eagerly needed (Blackburn and Langenau, 2010; Hans et al., 2009).

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In *Drosophila*, the strongest and most faithful ubiquitous control element currently in use is derived from the 5' region of the *ubiquitin (ubi)* gene, which encodes the evolutionarily conserved multimeric Ubiquitin (Ubi) peptide precursor (Lee et al., 1988). *ubi*-driven green fluorescent protein (*GFP*) transgenes are used widely as reliable markers for various genetic applications at all stages of *Drosophila* development. The evolutionarily conserved *ubi* control region has also been cloned from maize and has found widespread application in plant transgenesis (Christensen et al., 1992). The human *ubi* promoter has been isolated and drives strong transgene expression in a variety of cell culture systems (Schorpp et al., 1996). Notably, *UBI-GFP/BL6* transgenic mice express EGFP in virtually all cells of the hematopoietic system, thus greatly enhancing its utility in bone marrow stem cell transplantation experiments (Schaefer et al., 2001).

Based on this precedent, we sought to identify and characterize a *ubiquitin* locus in zebrafish to generate an endogenously derived ubiquitous transgene driver. Analogous to the reported *Drosophila*, maize, and human loci, we cloned a 3.5 kb 5' region upstream of the zebrafish *ubiquitin B* translational start site spanning the proximal upstream region, the first noncoding exon, and the only intron of the gene. In transient injections into zebrafish embryos, the *ubi* control region drives strong and ubiquitous visible expression of an *EGFP* reporter gene within 4 hours post-injection, likely starting at the onset of zygotic transcription. Stable *ubi:EGFP* lines reveal strong expression in all analyzed external and internal organs, including the retina, fin fold, and across all blood cell types from embryo to adulthood. The blood expression of *ubi*-driven fluorophores enabled us to perform adult zebrafish transplants with whole kidney marrow (WKM), which harbors the adult hematopoietic stem cells, into irradiated recipients and to monitor true multilineage repopulation from the transplanted cell populations. We further created *ubi:cre^{ERt2}* transgenes and showed they confer rapid and ubiquitous 4-OHT-inducible Cre activity at different stages of development. We further established *ubi:loxP-GFP-loxP-mCherry* lineage tracer transgenics that provide strong reporter activity upon Cre exposure. The *ubi* promoter and derived fluorescent reporters, the newly established lineage tracer transgenic lines, and the *Cre^{ERt2}/loxP* procedures reported here therefore provide an effective and valuable addition to the current zebrafish transgenesis tool box.

MATERIALS AND METHODS

Vectors

Zebrafish *ubi* (*ZDB-GENE-050411-10*) promoter sequence was PCR amplified from bacterial artificial chromosome (BAC) *CH211-202A12* using the Expand High Fidelity PCR Kit (Roche, Indianapolis, IN, USA) with primers 5'-AAAACTCCGAGACCAGCAAAGTTCTAGAATTT-GTCC-3' (forward) and 5'-AAAGGATCCCTGTAAACAAATTCAGAGTAAGATTAGC-3' (reverse), and TOPO-cloned into *pENTR5'* (Invitrogen, Carlsbad, CA, USA) to create *pCM206* (*pENTR5' ubi*). *pCM206* thus frames the 3.5 kb *ubi* promoter fragment, including the first non-coding exon and the intron, with *L4* and *R1* sequences for MultiSite Gateway, as well as 5' *XhoI* and 3' *BamHI* restriction sites (underlined in primer sequence) to facilitate cloning of *ubi*-driven constructs.

pENTR/D cre^{ERt2} was created by PCR amplification of the *cre^{ERt2}* open reading frame (ORF) from *pCre^{ERt2}* (Feil et al., 1997) using the Expand High Fidelity PCR kit (Roche) with primers 5'-CACCAIGTCCAATTACTGACCGTACACCAAAATTTGCC-3' (forward, ATG start codon underlined) and 5'-CAGACATGATAAGATACATTGATGAGTTGGACAAACC-3' (reverse) and directional TOPO cloning into *pENTR/D* (Invitrogen).

pENTR5' ubi:loxP-GFP-loxP was created by *BamHI* excision of the *loxP-EGFP-loxP* cassette from β -*actin-loxP-EGFP-loxP-kras^{G12D}* (Le et al., 2007) and subcloning into the *BamHI* site of *pCM206*.

All subsequent MultiSite Gateway assemblies were carried out using LR Clonase II Plus (Invitrogen) according to standard protocols and Tol2kit vectors described previously (Kwan et al., 2007), if not stated otherwise.

Tg(-3.5ubi:EGFP) and *Tg(-3.5ubi:mCherry)* are multisite gateway assemblies of *pCM206*, Tol2kit #383 (*pME-EGFP*), #302 (*p3E_SV40polyA*) and #394 (*pDestTol2A2*) (*ubi:EGFP*), or *pCM206*, Tol2kit #386 (*pME-mCherry*), #302, and #394 (*ubi:mCherry*).

Tg(-3.5ubi:cre^{ERt2};cmlc2-EGFP) derives from vector *pDestTol2pA2 ubi:cre^{ERt2};cmlc2-EGFP* (*pCM283*). *pCM283* was created by combining *pCM206*, *pENTR/D cre^{ERt2}*, and Tol2kit vectors #302 and #395 (*pDestTol2CG*).

Tg(-3.5ubi:loxP-EGFP-loxP-mCherry) (*ubi:Switch*) bases on *pDestTol2A2 ubi:loxP-EGFP-loxP-mCherry*, which was assembled with *pENTR5' ubi:loxP-GFP-loxP* and Tol2kit vectors #383, #302, and #394. Detailed digital plasmid maps of these vectors are available upon request.

Transgenesis

Zebrafish were maintained in accordance with Animal Research Guidelines at Children's Hospital Boston. Zebrafish transgenics were generated using Tol2-mediated transgenesis: 25 ng/ μ l of each transgenesis vector were combined with 2.5 ng/ μ l *Tol2* mRNA as described (Kwan et al., 2007) and injected into one-cell-stage eggs derived from *AB* or *Tu* wild-type crosses, and the injected animals grown up to adulthood. Individual F0 founders were outcrossed to *TL*, and their F1 progeny were screened for the respective transgenesis marker (*ubi:EGFP*, *ubi:mCherry*, or *cmlc2-EGFP*, respectively). On average we achieved efficiencies of F0 transmission of higher than 50%. Positive individual F1 adults were subsequently outcrossed to wild-type zebrafish, and their offspring were then used to establish lines when 50% transgene transmission was observed in the F2 generation, indicating single transgene insertions. If promising clutches had more than 50% positive F2 embryos, individual F2 adults were outcrossed to wild type and their offspring analyzed for 50% transgene positivity before establishing lines. Genotyping of adult *ubi:EGFP* fish was facilitated by the use of UV filter goggles (Modular Fluorescence Head set type: FHS, frame FHS/F-01, lightsource FHS/LS-1B, emission filter FHS/EF-2G2; BLS Ltd, Budapest, Hungary), which reveal EGFP fluorescence (or other fluorophores depending on the filter set) in zebrafish under regular light conditions and do not require any manipulation of the animals.

WKM transplantation

Flow cytometry was performed on a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA, USA) as described previously (Traver et al., 2003). Retro-orbital adult WKM transplantation of *ubi:mCherry* cells into *casper* recipients was performed as described previously (Pugach et al., 2009).

4-OHT treatment for Cre^{ERt2} induction

4-Hydroxytamoxifen (4-OHT, H7904; Sigma, St Louis, MO, USA) was dissolved in ethanol at a final stock concentration of 10 mM and kept in single-use aliquots in the dark at -20°C . 4-OHT is light- and temperature-sensitive, and we found it loses activity after prolonged storage. To induce Cre activity in *cre^{ERt2}*-expressing embryos, 25-30 stage-matched embryos per single well of a six-well culture plate were washed with E3 embryo medium, all medium removed and replaced with E3 medium freshly mixed with 5 μM 4-OHT. The treated embryos were immediately put into a closed and dark 28.5°C incubator to allow for efficient induction and remained in 4-OHT-containing E3 medium for the rest of the experiment. For the 4-OHT pulse experiment, the 4-OHT-containing E3 medium was removed after 15 minutes, the embryos re-suspended in minimal volume of E3 medium, and twice transferred into a fresh glass beaker with 500 ml of fresh E3 medium to remove all traces of 4-OHT. The embryos were subsequently placed in a fresh well of a six-well culture plate in E3 medium and grown as described previously.

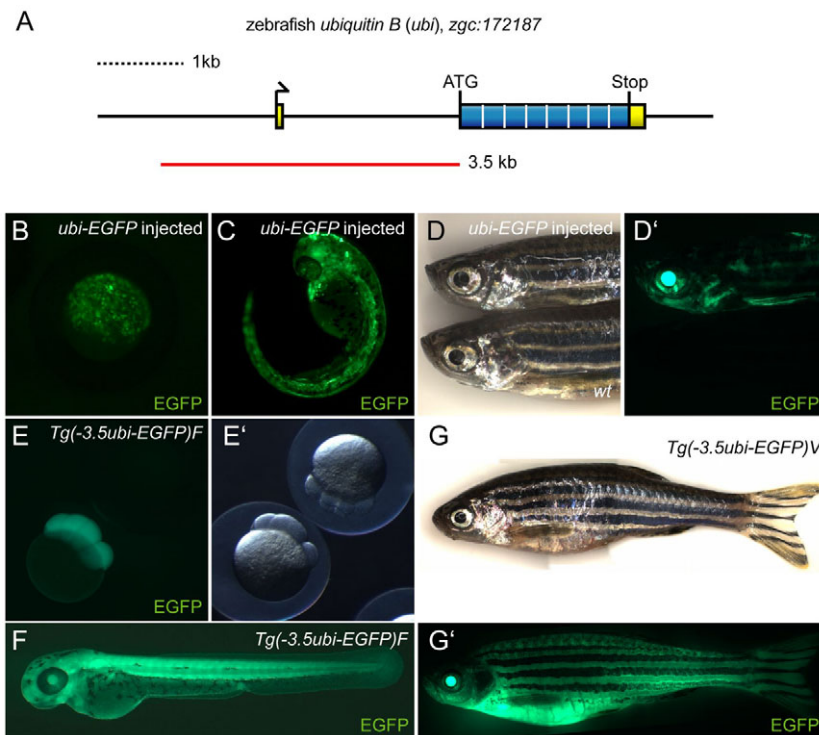


Fig. 1. Cloning and characterization of the zebrafish *ubi* regulatory region. (A) Schematic of the zebrafish *ubiquitin B* locus *zgc:172187* on linkage group 5, featuring a 71 bp non-coding first exon, a single 2 kb intron and a coding exon containing the ORF for a Ubiquitin octamer peptide. The red line indicates the PCR-amplified and subcloned 3.5 kb *ubi* promoter fragment. (B-D') EGFP fluorescence images of zebrafish injected with *Tol2(-3.5ubi:EGFP)* vector at the one-cell stage, (B) oblong to sphere stage (approx. 4 hpf), (C) 36 hpf, and (D, D') adult. Note the transgene injection-derived mosaic EGFP expression that persists through all developmental stages compared with wild type. (E-G') *ubi:EGFP* expression in stable transgenic lines. (E) Maternal EGFP contribution in embryos derived from outcrossing an F2 stable transgenic *Tg(-3.5ubi:EGFP)F* female to wild type compared with a transgene-negative sibling, here shown at the eight-cell stage (1.25 hpf). (F) *ubi:EGFP* expression at 48 hpf in offspring resulting from outcrossing of a F2 *Tg(-3.5ubi:EGFP)F* male to wt. (G, G') Compound image overview of an adult zebrafish female heterozygous for *Tg(-3.5ubi:EGFP)V*, revealing widespread EGFP fluorescence including in the fins. wt, wild type.

Histology

Adult zebrafish were euthanized using approved protocols with tricaine and then fixed in 4% paraformaldehyde (PFA). Paraffin embedding, sectioning, Hematoxylin and Eosin (H&E) staining, and immunohistochemistry (IHC) for enhanced GFP (EGFP) were performed using standard protocols by the Dana Farber/Harvard Cancer Center pathology core facility. EGFP was detected using the JL-8 mouse monoclonal antibody (Clontech, Mountain View, CA, USA).

RESULTS

Isolation and characterization of the zebrafish *ubi* promoter

BLAST searches using the human Ubiquitin peptide ORF as query identified the predicted zebrafish gene locus *zgc:172187* on linkage group five, referred to here as zebrafish *ubiquitin B (ubi)* (Fig. 1A). The zebrafish *ubi* gene features a 71 bp non-coding first exon, a 2 kb spanning intron, followed by a second exon encoding a multimeric (eight repeats) Ubiquitin peptide precursor (Fig. 1A), a gene structure homologous to *ubi* loci in other species.

Analogous to the previously reported *Drosophila* and human *ubi* control region fragments (Lee et al., 1988; Schaefer et al., 2001; Schorpp et al., 1996), we PCR-cloned a 3.5 kb sequence from BAC *CH211-202A12* immediately upstream from the *ATG* start codon at the 5' end of exon 2, including the putative intron/exon splice junctions (Fig. 1A). To test if this 5' *ubi* fragment contains functional transcriptional regulatory sequences, we assembled the *Tol2(-3.5ubi:EGFP)* reporter vector and injected it together with *Tol2* transposase-encoding mRNA into one-cell-stage zebrafish embryos (Kikuta and Kawakami, 2009). Within 4 hours postinjection, we could detect strong mosaic EGFP expression in the injected embryos by fluorescence microscopy (Fig. 1B) confirming that our isolated 5' *ubi* fragment contains sufficient regulatory sequences to drive *EGFP* transgene expression. This early detection of *ubi:EGFP* expression suggests an onset of transgene transcription at the mid-blastula transition (2.75 hours

post fertilization, hpf), in agreement with active histone marks at the *ubi* locus at the mid-blastula transition (Vastenhouw et al., 2010). The injected embryos robustly showed mosaic EGFP-positive cells in a variety of cell types and organ systems over the first days of development (Fig. 1C).

To assess the expression pattern of germline transmitted transgene integrations, we grew mosaic EGFP-positive embryos from the F0 generation to adulthood while periodically monitoring EGFP expression. This was facilitated by using UV filter goggles to noninvasively detect EGFP in vivo (see Materials and methods for details). Throughout development to adulthood, the *ubi:EGFP*-injected animals displayed strong mosaic EGFP in all visible external organs, such as the skin, eyes, and fins (Fig. 1D).

Individual EGFP-positive F0 males were crossed to wild-type females, and their F1 progeny screened for EGFP expression. In several independently derived F1 clutches from different founder parents, EGFP expression became obvious by 4-5 hpf in all blastula cells. These EGFP-positive embryos showed strong ubiquitous expression throughout development and adult stages (Fig. 1E-G'). To establish stable transgenic lines, we selected three independent male F1 founders that give rise to progeny with 50% EGFP-positive F2 embryos, suggesting a single *Tg(-3.5ubi:EGFP)* integration (henceforward referred to as *ubi:EGFP*, results shown for transgenic lines *F* and *V*). Outcrossing of adult F2 females to wild-type males revealed strong maternal deposition of *ubi*-derived EGFP into the egg (Fig. 1E). Incrossing of *ubi:EGFP* F2 parents generated approximately 25% of EGFP-positive embryos emitting notably more intense fluorescence signal than their other positive siblings. These animals were subsequently found to be homozygous for *ubi:EGFP* (data not shown). Our observations thus reveal the strong transgene driving activity of the zebrafish *ubi* promoter and its functional characteristics including maternal contribution, allele dosage effects, and permanent promoter expression throughout zebrafish development.

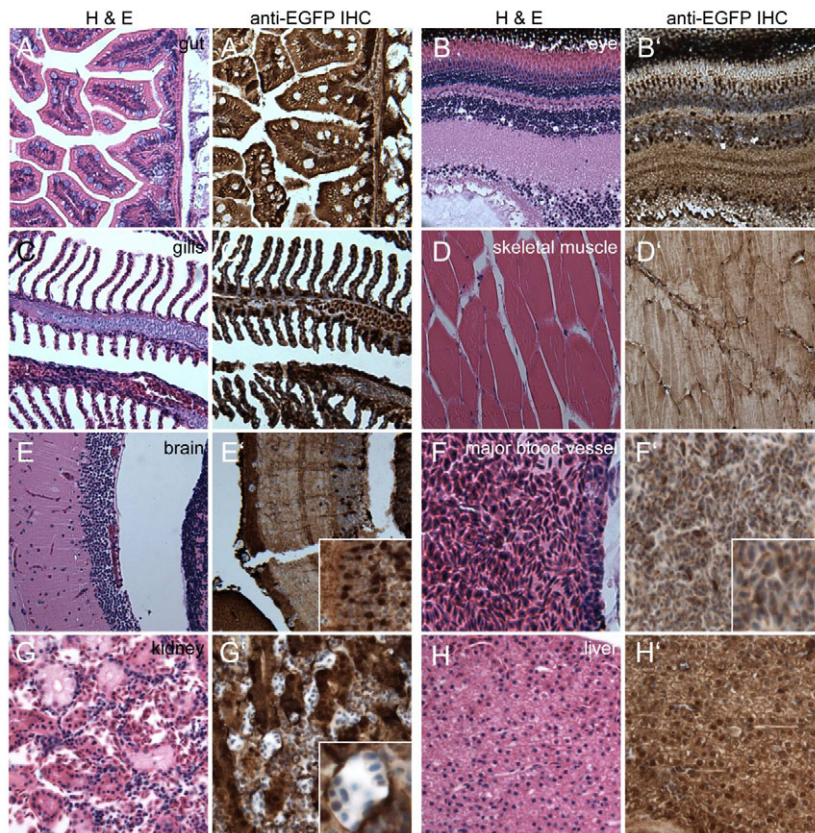


Fig. 2. Sagittal sections through a heterozygous *Tg(-3.5ubi:EGFP)V* adult reveals widespread *ubi* expression in internal organs. (A-H') Sagittal sections of a PFA-fixed heterozygous *Tg(-3.5ubi:EGFP)V* adult zebrafish female stained with H&E. Adjacent sections are stained for EGFP expression through IHC (brown stain). (E',F',G') Insets show magnified areas to illustrate cellular details and variations in EGFP expression levels in cortical neurons (E') and kidney tubule cells (G'). (F') Major blood vessel illustrating EGFP-positive erythrocytes as the only unambiguously identifiable blood cell type.

To determine if *ubi:EGFP* truly expresses ubiquitously, F2 animals that were heterozygous based on Mendelian transgene inheritance were examined and displayed EGFP in a homogenous pattern in all externally visible body structures including the fins (Fig. 1G). These tissues do not show expression with commonly used *efl α* , *tbp* or β -*actin* promoter-driven reporters. Anti-EGFP IHC on sagittal sections of heterozygous F2 *ubi:EGFP* adults further revealed *ubi:EGFP* expression in a multitude of internal organs (Fig. 2). Importantly, tissues that have so far remained untargeted by other putatively ubiquitous promoters, such as retina and erythrocytes, were EGFP-positive (Fig. 2B,C,F). Notably, EGFP IHC revealed that EGFP expression is not homogenous throughout all cell types. We found small regions in internal organs that, despite positive staining, showed substantially lower EGFP levels than adjacent tissues or cell types, such as cell bodies of subsets of CNS neurons (Fig. 2E), or columnar epithelial cells of the kidney tubules (Fig. 2G).

Overall, we therefore conclude that our newly isolated and MultiSite Gateway-compatible zebrafish -3.5 kb *ubi* promoter drives transgene expression ubiquitously in the vast majority of cell types, if not completely ubiquitously. *ubi* expression includes tissues previously untargeted by transgene expression, features maternal deposition, and persists through all stages of zebrafish development, starting at the mid-blastula transition.

***ubi* expresses in all blood cell types**

The β -*actin* promoter that is commonly used as a transgenic marker in zebrafish blood cell transplantation experiments is not active in erythroid cells (Traver et al., 2003). Our sagittal sections and IHC indeed captured erythrocytes as *ubi*-positive

(Fig. 2F), yet these are the only unambiguously identifiable blood cell type using this method. We therefore sought to analyze *ubi* expression in the hematopoietic system and profiled the blood expression of adult *ubi:EGFP* and *ubi:mCherry* heterozygous transgenic zebrafish using flow cytometric analysis of dissected WKM (Traver et al., 2003), the major hematopoietic tissue in adult zebrafish. The analyzed *ubi:EGFP* and *ubi:mCherry* transgenic lines showed robust fluorophore expression in all resolvable blood cell gates (Fig. 3A and Fig. 4A) and in the vast majority of cells in these respective populations, including erythroid, myeloid, and lymphoid lineages, plus precursor cell populations (Fig. 3B,C and Fig. 4B,C). This is in stark contrast to β -*actin* promoter-driven EGFP transgenics, which overall show weaker expression in hematopoietic lineages, particularly in erythrocytes, which barely express β -*actin:EGFP* (Fig. 3D-F).

This broad hematopoietic expression should therefore allow assessment of multilineage engraftment in transplantation experiments using *ubi:EGFP* or *ubi:mCherry*. To test this application, we isolated WKM from either *ubi:mCherry* or *ubi:EGFP* heterozygous adults, and performed retro-orbital WKM transplantation into irradiated *roy^{-/-}*; *nacre^{-/-}* (*casper*) (White et al., 2008) transparent recipients to allow for easy visualization. Three weeks post-transplantation, the transparent *casper* recipients showed strong localized fluorescence marking the kidney territory (Fig. 4D). Flow cytometry analysis of WKM from these *casper* recipients revealed chimeric contribution of fluorophore-expressing donor cells to all light-scatter resolvable blood cell populations, as described above (Fig. 4E-G). We have successfully monitored *ubi:EGFP* and *ubi:mCherry* WKM-transplanted *casper* recipients with long-term transplants over 3 months post transplant, as well

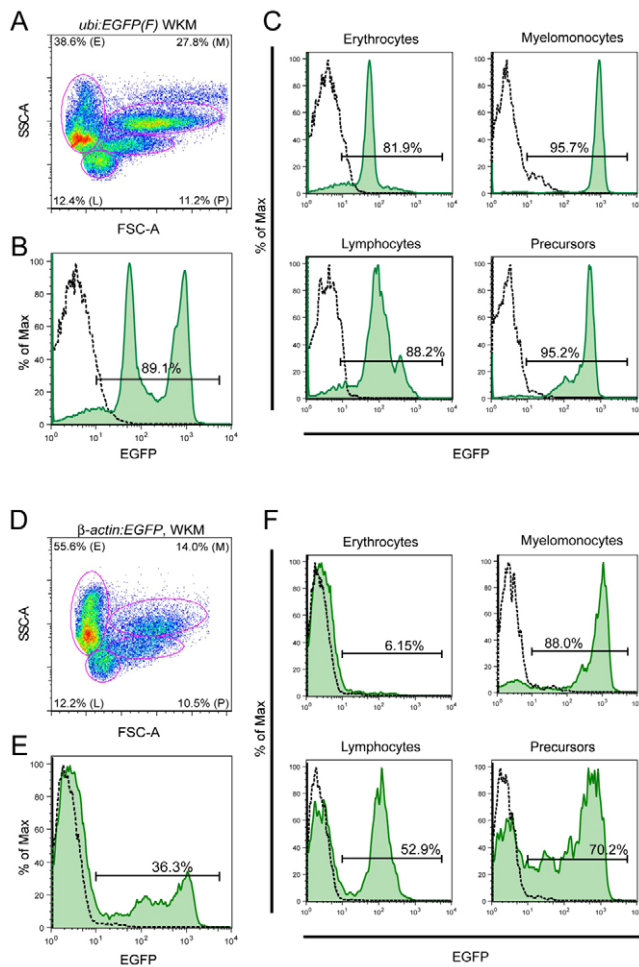


Fig. 3. *ubi*-driven transgenes express in all blood cell lineages. (A-C) Representative flow cytometry analysis of *Tg(-3.5ubi-EGFP)F* (*ubi:EGFP*) adult whole kidney marrow (WKM). (A) EGFP-positive cells fall into all major blood cell populations that are separable by forward scatter and side scatter, and the majority of all WKM and the individual subpopulations express EGFP (B,C) (indicated as percent of total in the respective gate). (D-F) Same comparison run with β -actin:*EGFP* WKM preparation, revealing weak to no expression of the β -actin-driven *EGFP* transgene in erythrocytes (F). E, erythrocytes; FSC, forward scatter; L, lymphocytes; M, myelomonocytes; P, precursors; SSC, side scatter.

as performed transplantation with mixed *ubi:EGFP* and *ubi:mCherry* populations in competitive transplants, without detecting any diminishing fluorophore intensity (data not shown). *ubi*-driven transgenic reporters are therefore suitable reagents for hematopoietic transplantation experiments, and their true multilineage expression offers a clear advantage over previously used transgene reporters.

***ubi:cre^{ERT2}* as ubiquitous Cre^{ERT2} source**

Cre/*loxP* system-based lineage tracing tools are becoming increasingly effective in zebrafish with single-insertion *loxP* cassettes generated through Tol2-mediated transgenesis

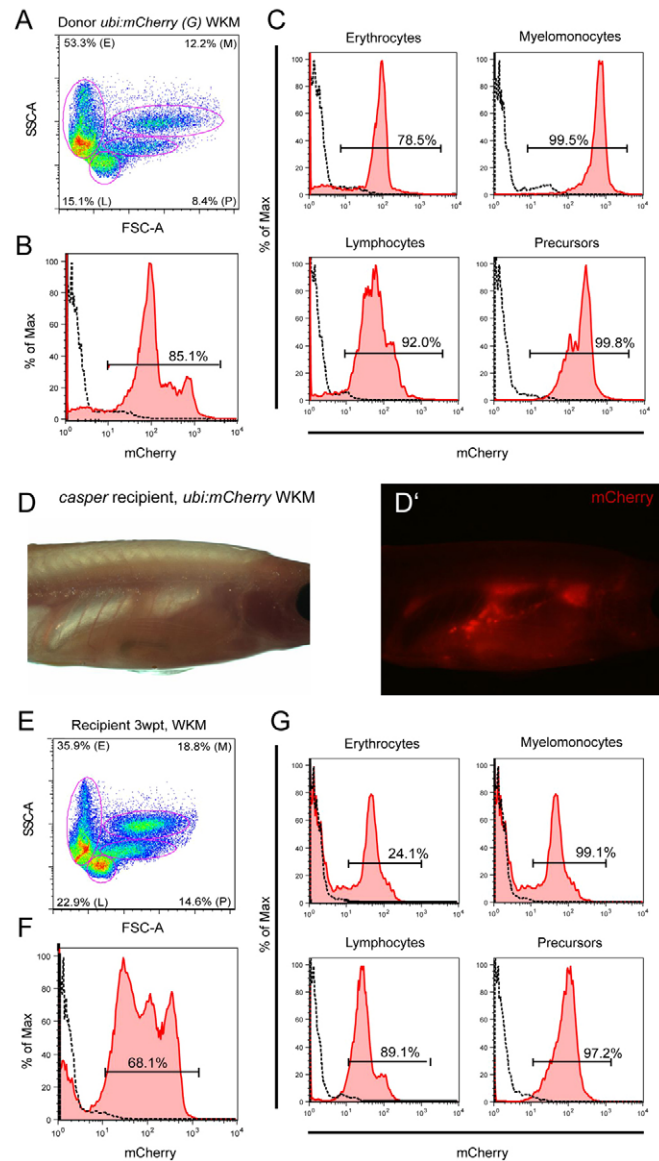


Fig. 4. Whole kidney marrow transplantation using *ubi:mCherry*. (A-C) Cells isolated from *Tg(-3.5ubi-mCherry)G* adult WKM were transplanted via retro-orbital injection into irradiated *casper* recipient adults and read-out 3 weeks post transplant (wpt) for mCherry blood chimerism by fluorescence microscopy (D,D') and flow cytometry (E-G) of isolated recipient WKM. mCherry-positive and thus donor-derived cells efficiently engrafted recipients and contributed to all resolvable blood populations (G).

(Kawakami, 2007; Yoshikawa et al., 2008). The Cre^{ERT2} recombinase fusion protein is inducible by exposure to TAM or its active metabolite 4-OHT (Feil et al., 1996; Feil et al., 1997) and is thus ideal for spatiotemporal tracing of cell types during zebrafish development (Hans et al., 2009). The features of *ubi*-driven reporter transgenes prompted us to create *Tg(-3.5ubi:cre^{ERT2};cmlc2-EGFP)* (subsequently referred to as *ubi:cre^{ERT2}*) using Tol2-mediated transgenesis to provide a ubiquitous inducible Cre^{ERT2} source (Fig. 5A). We established three independent zebrafish lines carrying *ubi:cre^{ERT2}* insertions and subsequently used *Tg(-3.5ubi:cre^{ERT2};cmlc2-EGFP)V* as a representative line.

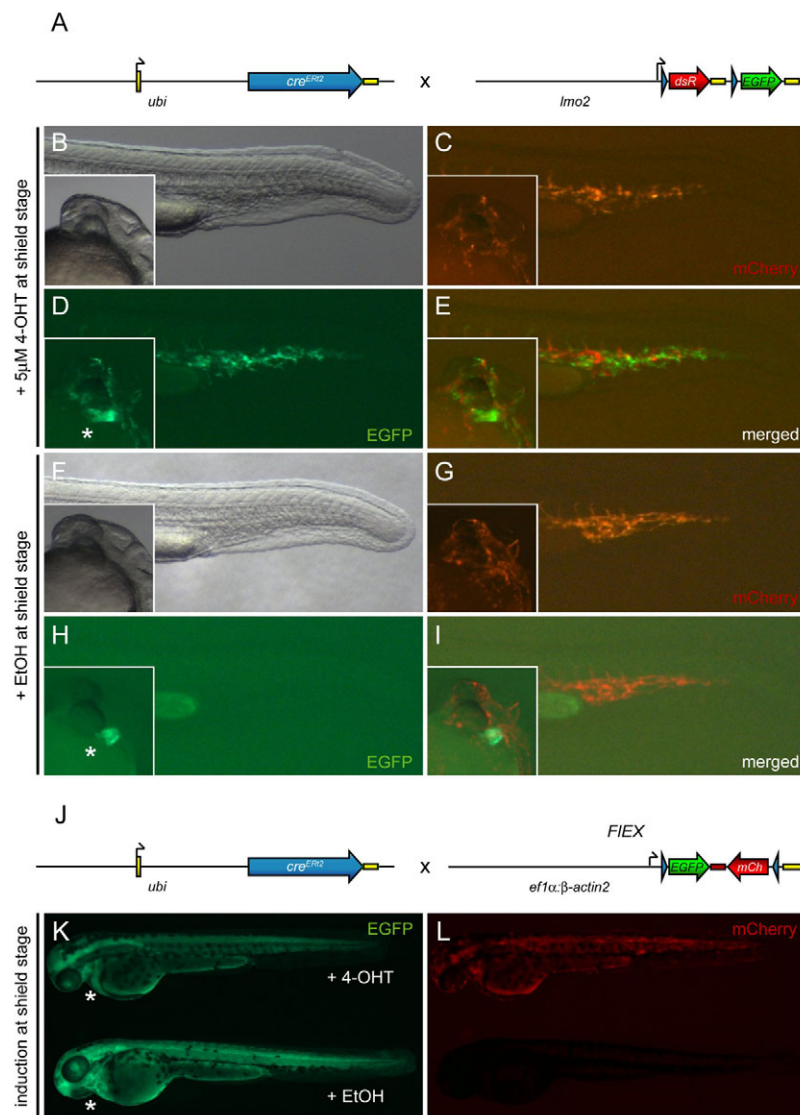


Fig. 5. *Tg(-3.5ubi:cre^{ERT2};cmlc2-EGFP)* provides 4-OHT-inducible ubiquitous Cre activity. Asterisks indicate *cmlc2-EGFP* expression in the heart, which segregates with the *ubi:cre^{ERT2}* transgene. (A) Schematic of the *ubi:cre^{ERT2}* transgene and *lmo2:lox-dsRed-lox-EGFP* (*lmo2:Switch*) reporter cross shown in panels (B-I). Black arrows indicate transcription start positions, yellow boxes are non-coding 5' and 3' UTR/polyA sequences, blue arrow heads indicate *loxP* sites. (B-I) Images depict the posterior region of live *ubi:cre^{ERT2}; lmo2:Switch* double-positive zebrafish embryos at approximately 24 hpf, with insets showing *cmlc2:EGFP* expression to confirm presence of both transgenes. Embryos were induced at shield stage (6 hpf) with 5 μ M 4-OHT (B-E) or ethanol (EtOH) carrier (F-I). mCherry expression marks *lmo2:Switch*-expressing cells in endothelial cell populations of the tail and head (C,G), whereas EGFP reveals specific 4-OHT dependent *loxP* cassette excision from *lmo2:Switch* by Cre (D,E), which did not occur in ethanol controls (H,I). (J) Schematic of the *ubi:cre^{ERT2}* transgene and *Tg(eab2:[EGFP-T-mCherry])* (*FLEX*) reporter cross shown in K and L. Note that *FLEX* is driven by a compound *ef1 α : β -actin2* promoter fragment which drives a bi-directional *Terminator* (*T*)-separated *EGFP/mCherry* cassette that inverts to enable mCherry expression after Cre-mediated recombination (Boniface et al., 2009). (K,L) Live *ubi:cre^{ERT2}; FLEX* double-positive zebrafish embryos at approximately 3 dpf. The embryo on top was treated with 5 μ M 4-OHT at shield stage, whereas the embryo at the bottom was treated with ethanol carrier control. EGFP fluorescence indicates default *FLEX* reporter expression (K), whereas mCherry fluorescence (L) reveals successful Cre-mediated *loxP* recombination, which inverts the *EGFP-T-mCherry* cassette in *FLEX* and triggers mCherry expression.

To characterize *ubi*-driven Cre^{ERT2} function, we crossed *ubi:cre^{ERT2}* males (to circumvent maternal Cre^{ERT2} contribution and assure all Cre^{ERT2} derives from de novo expression) to *lmo2:loxP-dsRed-loxP-EGFP* (*lmo2:Switch*) females (Wang et al., 2008) (Fig. 5A). *lmo2* promoter-driven transgenes start to express from 10–12 hpf in hematopoietic and endothelial precursors (Wang et al., 2008; Zhu et al., 2005). When incubated with 5 μ M 4-OHT starting from shield stage, *ubi:cre^{ERT2}; lmo2:loxP-dsRed-loxP-EGFP* double-positive embryos express strong and mosaic EGFP in the vasculature and hematopoietic system by 24 hpf (Fig. 5B–E), thus revealing 4-OHT dependent Cre^{ERT2} activity. These crosses revealed that *ubi*-derived Cre^{ERT2} is absolutely 4-OHT dependent for its activity, as *ubi:cre^{ERT2}; lmo2:loxP-dsRed-loxP-EGFP* double-positive embryos never showed any detectable GFP expression by fluorescence when treated with ethanol solvent control (Fig. 5F–I). Strikingly, fluorophore-expressing cells were always single-positive for either EGFP or mCherry (Fig. 5C–E), suggesting that 4-OHT activity, and thus the probability of Cre^{ERT2}-mediated *loxP* recombination events, fades during the time window between induction at shield stage and onset of *lmo2* switch expression.

To independently test *ubi:cre^{ERT2}* functionality, we crossed *ubi:cre^{ERT2}* males to females carrying the previously reported fusion promoter-driven *eab2:[EGFP-T-mCherry]* (*FLEX*) (Fig. 5J). Prior to recombination, *FLEX* broadly expresses EGFP and has been proposed to be ubiquitous (Boniface et al., 2009). Cre^{ERT2} activation with 4-OHT at shield stage triggered highly mosaic and strong mCherry expression in double-transgenic embryos as observed 3 days post fertilisation (dpf) (Fig. 5K,L), whereas ethanol solvent control-treated embryos never produced detectable mCherry expression during the observation period (Fig. 5K,L). Together, these experiments establish that *ubi* promoter-driven Cre^{ERT2} recombines *loxP* site-flanked transgenes specifically upon 4-OHT addition in zebrafish embryos.

Lineage tracing with *ubi:Switch*

We noted in our *ubi:cre^{ERT2}* experiments (Fig. 5 and see above) different degrees of switch construct expression mosaicism despite early 4-OHT induction at shield stage. This suggests that either: (1) *ubi*-driven Cre^{ERT2} is not efficiently excising the tested *loxP* switch; or (2) the *loxP* switch transgenes have integrated in suboptimal

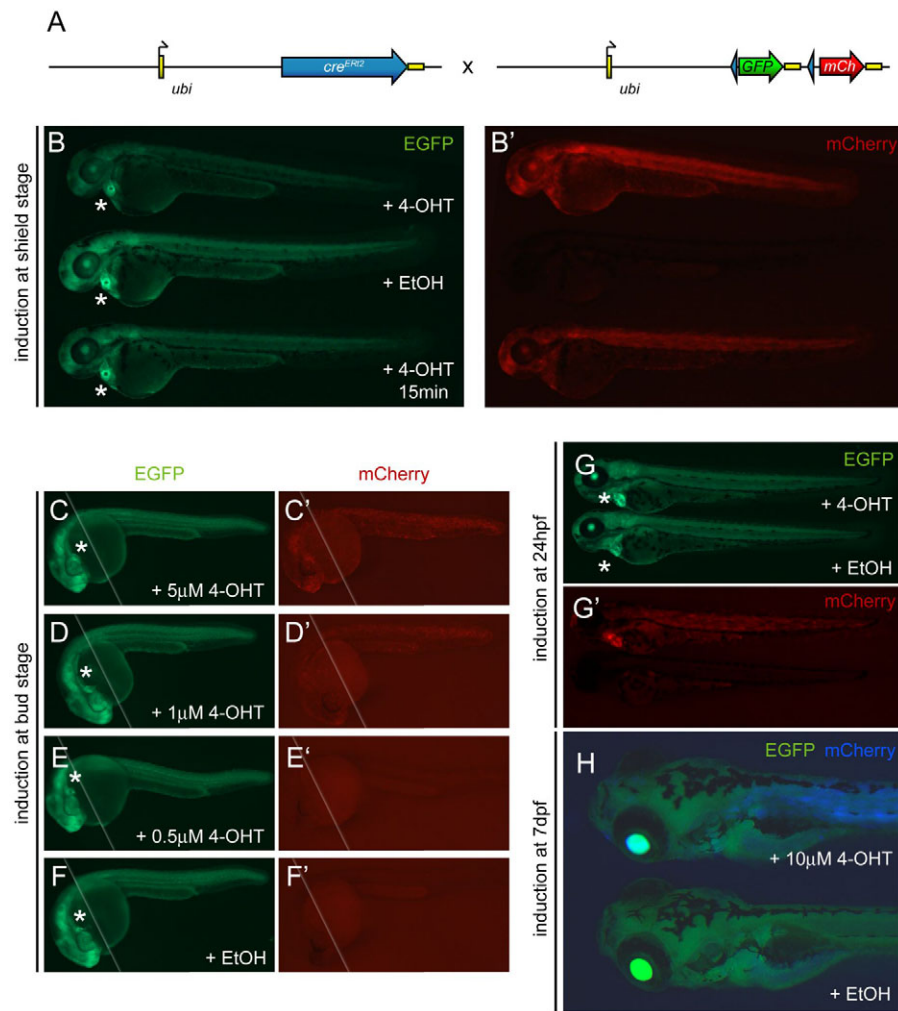


Fig. 6. *ubi:Switch* is a ubiquitous lineage-tracing transgene. The asterisks indicate heart-specific *cmc2*-EGFP expression marking the *ubi:cre^{ERT2}* transgene. (A) Schematic of the *ubi:cre^{ERT2}* transgene and *Tg(-3.5ubi:loxP-GFP-loxP-mCherry)* (*ubi:Switch*) reporter cross. Arrows indicate transcription start positions, yellow boxes are non-coding 5' and 3' UTR/polyA sequences, blue arrowheads indicate *loxP* sites. (B, B') Imaging of live *ubi:cre^{ERT2}*; *ubi:Switch* double-positive zebrafish embryos at approximately 3 dpf for baseline GFP expression (B) and mCherry indicating successful *loxP* excision (B'). Embryos were treated at shield stage (6 hpf) with 5 μM 4-OHT, ethanol (EtOH) carrier, or a short 15 minute pulse of 5 μM 4-OHT (top to bottom). Both 4-OHT treatments induce strong mCherry expression (B'), with apparent GFP fading in the longer 4-OHT treatment (B, top), compared with ethanol control (B, B', center). (C-F') Approximately 30 hpf zebrafish embryos double-positive for *ubi:cre^{ERT2}*; *ubi:Switch* were treated with 4-OHT or ethanol control at bud stage as indicated and compound imaged for GFP fluorescence (C-F) to visualize *ubi:Switch* default expression or mCherry fluorescence (C'-F') to reveal successful *loxP* excision (the white line indicates merge of the anterior and posterior focal planes). Whereas 5 μM 4-OHT potentially induces *loxP* recombination (C'), and 1 μM 4-OHT causes notably reduced mosaicism (D'), 0.5 μM 4-OHT fails to induce a widespread response (E') similar to the ethanol control (F'). (G-H) *ubi:cre^{ERT2}*; *ubi:Switch* embryos 5 μM 4-OHT or ethanol control treated at 24 hpf (G, G') or 7 dpf (H) reveal 4-OHT-dependent *loxP* recombination and *de novo* mCherry expression from the *ubi:Switch* transgene. Note the strong mCherry expression in the heart in the 4-OHT-treated larva (G', top), suggesting promoter cross-talk between the *cmc2* marker driver and *ubi:cre^{ERT2}*. Because of their drastically increased size, the embryos induced at 7 dpf (H) were treated with increased dose (10 μM) of 4-OHT or ethanol control and visualized for GFP (green) and mCherry expression (blue) channel.

genomic locations that decrease the probability of Cre-dependent *loxP* site recombination. To create new *loxP* switch insertions devoid of functionally relevant position effects and to generate a lineage tracing reagent with the temporally stable expression benefits of the *ubi* promoter, we assembled *Tg(-3.5ubi:loxP-GFP-loxP-mCherry)* (subsequently referred to as *ubi:Switch*, Fig. 6A). We established three independent transgenic *ubi:Switch* zebrafish lines and verified their ubiquitous EGFP expression in the F2 generation [the subsequent experiments use *Tg(-3.5ubi:loxP-GFP-loxP-mCherry)III*].

To assess Cre^{ERT2} responsiveness of *ubi:Switch*, we crossed *ubi:cre^{ERT2}* males to *ubi:Switch* F2 females, again to ensure active Cre^{ERT2} *de novo* expression through paternal transmission (Fig. 6A). A first group of the resulting embryos was induced with 5 μM 4-OHT at shield stage, while a second group of siblings was mock treated with ethanol solvent. To also assess Cre^{ERT2} induction dynamics, we performed a pulse treatment experiment by exposing a third group of embryos for 15 minutes to 5 μM 4-OHT, before thoroughly washing the embryos with E3 medium to attempt to remove all 4-OHT in solution. All the embryos

subsequently grew up in the dark for 3 days before analysis to allow for mCherry accumulation for assessment of resulting mosaicism.

ubi:cre^{ERT2}; *ubi:Switch* double-positive embryos induced with 4-OHT at shield stage showed strong and widespread mCherry expression at 3 dpf, which intensified further over time with steadily decreasing EGFP fluorescence (Fig. 6B). Ethanol-treated control, as well as *ubi:cre^{ERT2}*-negative (not shown), embryos remained solely EGFP-positive (Fig. 5B). Strikingly, the 15 minute 4-OHT incubation group revealed strong and widespread mCherry mosaicism that remained less complete than for the first embryo group left in 4-OHT (Fig. 6B). This observation suggests that 4-OHT rapidly enters zebrafish embryos and conveys broad Cre^{ERT2} activity.

We next induced *ubi:cre^{ERT2}*; *ubi:Switch* double-positive embryos with varying concentrations of 4-OHT starting at bud stage. These experiments revealed concentration-dependent *loxP* recombination efficiency when read out at 30 hpf, with 5 μ M 4-OHT having the strongest effect (Fig. 6C), with a weakened response in the group exposed to 1 μ M 4-OHT (Fig. 5D). However, 0.5 μ M 4-OHT only inefficiently triggered mCherry expression (Fig. 6E,G).

To assess *ubi:cre^{ERT2}* activity during later stages, we induced a cohort of *ubi:Switch*; *ubi:cre^{ERT2}* double-positive embryos with 4-OHT at 24 hpf, and 6 dpf. Compared with their siblings induced at shield stage, the embryos exposed to 4-OHT at 24 hpf showed slightly lower mCherry mosaicism with weaker mCherry levels when observed at 3 dpf (Fig. 6H), which again intensified over developmental time with decreasing EGFP fluorescence. The 6 dpf cohort, when observed at 9 dpf, showed widespread mCherry expression, with faint expression in the head region (Fig. 6I). These effects are expected because: (1) more cells exist in the embryo at 24 hpf and 6 dpf, increasing the number of successful Cre^{ERT2}-mediated *loxP* excisions required to give homogeneous mCherry expression; (2) mCherry has less time to accumulate and mature starting from 24 hpf than after *loxP* excision events at shield stage; and (3) tissue penetration in the growing zebrafish larvae may limit 4-OHT access, particularly in the head region.

In summary, we conclude that: *ubi:cre^{ERT2}* generates potent Cre^{ERT2} over a broad developmental window to induce tissue-specific *loxP* switch lines; different *loxP* switch transgenes have different sensitivities to Cre^{ERT2} activity, potentially depending on their genomic integration site and *loxP* sequence accessibility; and 4-OHT is rapidly taken up by zebrafish embryos and is able to trigger transient Cre^{ERT2} activity within minutes of application, in agreement with previously reported findings (Hans et al., 2009). This last observation suggests the use of temporal pulses of 4-OHT induction during zebrafish development to trace temporally distinct cell populations, an application that is technically challenging in mouse development.

DISCUSSION

The number of potent zebrafish tools to manipulate genetic events and trace cell lineages through development has steadily increased in recent years; however, a common obstacle has been the lack of a ubiquitous transgene driver (Blackburn and Langenau, 2010; Collins et al., 2010; Hans et al., 2009). Our identification and characterization of the zebrafish *ubi* promoter described here adds a promising new candidate to fill this gap. *ubi* expresses from early development throughout the zebrafish life cycle (Figs 1-3). We have further monitored stable *ubi*-driven transgene expression through multiple generations (F5 with *ubi:EGFP*), suggesting no

particular susceptibility to transgene silencing. The *ubi* promoter fragments practical size (3.5 kb) plus compatibility with both MultiSite Gateway and traditional restriction enzyme cloning allows for simple use in applications that depend on persistent ubiquitous transgene expression, as illustrated by our adult WKM transplantation experiments with *ubi*-driven reporters (Fig. 4). Although our analysis cannot rule out cell types that fail to express *ubi*-driven transgenes, *ubi* provides altogether a substantial and significant improvement compared with existing supposedly ubiquitous promoters in zebrafish.

Still, a 'ubiquitous' promoter should not be confused with a 'homogenous' promoter: *ubi* does not express at the same level in all cell types. We noted markedly different expression levels by EGFP expression in subsets of cell types within various tissues, such as the brain, retina, kidney (Fig. 2) and blood (Fig. 3). When comparing independent *ubi:EGFP* or *ubi:mCherry* transgenics, we also found different fluorophore intensity distributions in our fluorescence-activated cell sorting analysis for WKM (compare Fig. 3B and Fig. 4B; and data not shown), as also described for *UBI-GFP/BL6* and *ROSA26* mice (Schaefer et al., 2001). Such effects are anticipated for randomly integrated transgene constructs, which are susceptible to environmental influences of the genomic integration locus.

Position effect influences are especially pronounced in *loxP:Switch* constructs. We found several independent *ubi:Switch* lines that showed weak and greatly varying Cre-mediated excision potencies (data not shown). Although significantly increasing the required workload, these observations suggest that *loxP* switch transgenes need to be functionally screened for efficient integration lines, by using *ubi:cre^{ERT2}* for example. Screening with *Tg(hsp70:cre)*, particularly when this transgene is maternally contributed and thus pre-loads the egg cell with mRNA or possibly protein, probably greatly overestimates the *loxP* recombination efficiencies of switch lines (Hans et al., 2009). Cre^{ERT2}-mediated recombination induced at different developmental time points exposes *loxP* transgene dynamics more reliably through de novo transcription events after recombination (see also Fig. 6).

By combining the tissue-specific *Tg(pax2.1:cre^{ERT2})* as a recombinase source with *ef1 α :loxP-dsRed-loxP-GFP*, Hans et al. (Hans et al., 2009) reported Cre^{ERT2} sensitivity to 0.5 μ M 4-OHT, which at the time points we tested triggered minimal recombination with our *ubi*-driven transgenes (Fig. 6). This is probably owing to stronger cell-type-specific Cre^{ERT2} expression in the diencephalon and hindbrain through the *pax2.1* driver. These observations combined with our findings suggest that the optimal 4-OHT dose for a given Cre^{ERT2} driver paired with a *loxP* switch will require individual elucidation.

Nonetheless, the use of Cre^{ERT2} in zebrafish reveals a striking simplicity and efficiency for drug-inducible *loxP* cassette recombination, thus crucially extending existing genetic manipulation methods. Furthermore, the possibility of pulsed Cre^{ERT2} induction in zebrafish allows for control of the onset of cell lineage labeling and misexpression in restricted developmental time windows. Similar experiments in mouse embryos are technically challenging and ambiguous in their readouts, as TAM needs to be injected or administered in the drinking water of the mother, which does not allow for uniformly controlled temporal induction. Zebrafish molecular genetics have received a major boost with the dawn of transposon-mediated transgenesis (Kawakami, 2005; Kwan et al., 2007; Villefranc et al., 2007); yet much work remains to establish reliable cloning standards and transgenesis markers. The *ubi* promoter now provides a solid basis

to the construction of elaborate recombinase-dependent or drug-inducible transgene systems for all developmental stages in zebrafish.

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Competing interests statement

L.I.Z. is a founder and stock holder of Fate, and a scientific advisor for Stemgent.

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