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Accessibility
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 transgensics 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:creERt2 transgene, as well as ubi:loxP-EGFPloxP-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 thoroughly characterized transgene using current methodologies controls translation or chromatin maintenance, as well as transgene dependence on the genomic integration context.

Severe 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 CreERt2-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 CreERt2-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 CreERt2 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 CreERt2 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 *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 (Schopp et al., 1996). Notably, *ubi*:EGFP/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 (Schafer et al., 2001).

Based on this precedent, we sought to identify and characterize a *ubi* 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 *ubi* 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, fins, 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*:creER2 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 CreER2/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′-AAAAACTCGAGACGCAAAATCTGCTAGATTT- GTG3′ (forward) and 5′-AAGGATCCCTGTAACACATTCTACA- AGTAAAGATGC3′ (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′* and *3′* BamHI restriction sites (underlined in primer sequence) to facilitate cloning of *ubi*-driven constructs.

pENTR/D creER2 was created by PCR amplification of the creER2 open reading frame (ORF) from pENTR2 (Feil et al., 1997) using the Expand High Fidelity PCR kit (Roche) with primers 5′-CACCATGTCACAT-TAACGCCCCGCTACAGCTCTG3′ (forward, ATG start codon underlined) and 5′-CAGACATGATAAGTACATGATTGAGTGTGGA- CAAACC3′ (reverse) and directional TOPO cloning into pENTR/D (Invitrogen).

*pENTR5_ubi*:loxP-GFP-loxP was created by BsmBI excision of the loxP-GFP-loxP cassette from β-actin-loxP-EGFP-loxP-krasG12D (Le et al., 2007) and subcloning into the *ubi* 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_SV40apolA) and Tg(–3.5ubi:loxP-EGFP) and pCM206, Tol2kit #386 (pME-mCherry), #302, and #394 (ubi:EGFP). Tg(–3.5ubi:creER2;cmkl2-EGFP) derives from vector Tol2Tolp2a2 ubi:creER2;cmkl2-EGFP (pCM283). pCM283 was created by combining pCM206, pENTR/D creER2, and Tol2kit vectors #302 and #395 (Tol2Tol2CG).

Tg(–3.5ubi:loxP-EGFP-loxP-mCherry) (ubi:Switch) based 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/μl of each transgenesis vector were combined with 2.5 ng/μl 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 cmkl2-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/EP-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 CreER2 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 creER2-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.
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 ubiB (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; Schrapp 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 nonintrusively 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. 1F-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.
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 *ef1α, 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...
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:creER<sup>T2</sup> as ubiquitous Cre<sup>ER<sub>T2</sub></sup> source**

Cre/loxP system-based lineage tracing tools are becoming increasingly effective in zebrafish with single-insertion loxP cassettes generated through Tol2-mediated transgenesis (Kawakami, 2007; Yoshikawa et al., 2008). The Cre<sup>ER<sub>T2</sub></sup> 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<sup>ER<sub>T2</sub></sup>;cmlc2-EGFP) (subsequently referred to as ubi:cre<sup>ER<sub>T2</sub></sup>) using Tol2-mediated transgenesis to provide a ubiquitous inducible Cre<sup>ER<sub>T2</sub></sup> source (Fig. 5A). We established three independent zebrafish lines carrying ubi:cre<sup>ER<sub>T2</sub></sup> insertions and subsequently used Tg(−3.5ubi:cre<sup>ER<sub>T2</sub></sup>;cmlc2-EGFP)<sub>V</sub> as a representative line.

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**Fig. 3. ubi-driven transgenes express in all blood cell lineages.**

(A-C) Representative flow cytometry analysis of Tg(−3.5ubi-EGFP)<sub>F</sub> (ubi:EGFP) adult 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.

**Fig. 4. Whole kidney marrow transplantation using ubi:mCherry.**

(A-C) Cells isolated from Tg(−3.5ubi-mCherry)<sub>G</sub> 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<sup>'</sup>) 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).
To characterize *ubi*-driven CreERt2 function, we crossed *ubi:creERt2* males (to circumvent maternal CreERt2 contribution and assure all CreERt2 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 μM 4-OHT starting from shield stage, *ubi:creERt2; 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 CreERt2 activity. These crosses were always single-positive for either EGFP or mCherry (Fig. 5C-D), whereas 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:creERt2* transgene and *Tg(eab2:EGFP-T-mCherry*) (FLEX) reporter cross shown in K and L. Note that FLEX is driven by a compound ef1α-β-actin2 promoter fragment which drives a bi-directional Terminator (T)-separated EGFPtrmCherry cassette that inverts the to enable mCherry expression after Cre-mediated recombination (Boniface et al., 2009). (K,L) Live *ubi:creERt2; FLEX* double-positive zebrafish embryos at approximately 3 dpf. The embryo on top was treated with 5 μM 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 EGFPtrmCherry cassette in FLEX and triggers mCherry expression.

To independently test *ubi:creERt2* functionality, we crossed *ubi:creERt2* 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). CreERt2 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 CreERt2 recombines loxP site-flanked transgenes specifically upon 4-OHT addition in zebrafish embryos.

**Lineage tracing with *ubi:Switch***

We noted in our *ubi:creERt2* 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 CreERt2 is not efficiently excising the tested loxP switch; or (2) the loxP switch transgenes have integrated in suboptimal
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) (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 CreER<sup>T2</sup> responsiveness of ubi:Switch, we crossed ubi:creER<sup>T2</sup> males to ubi:Switch F2 females, again to ensure active CreER<sup>T2</sup> 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 CreER<sup>T2</sup> 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.

**Fig. 6.** ubi:Switch is a ubiquitous lineage-tracing transgene. The asterisks indicate heart-specific cmlc2-EGFP expression marking the ubi:creER<sup>T2</sup> transgene. (A) Schematic of the ubi:creER<sup>T2</sup> transgene and Tg(−3 Subi: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:creER<sup>T2</sup>; 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:creER<sup>T2</sup>; 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 potently 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:creER<sup>T2</sup>; ubi:Switch embryos 5 μM 4-OHT or ethanol control treated at 24 hpf (G, G') 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 cmlc2 marker driver and ubi:creER<sup>T2</sup>. 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.
subsequently grew up in the dark for 3 days before analysis to allow for mCherry accumulation for assessment of resulting switch mosaicism.

ubi:creER2; 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:creER2-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 CreER2 activity.

We next induced ubi:creER2; 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 μM 4-OHT having the strongest effect (Fig. 6C), with a weakened response in the group exposed to 1 μM 4-OHT (Fig. 5D). However, 0.5 μM 4-OHT only inefficiently triggered mCherry expression (Fig. 6E,G).

To assess ubi:creER2 activity during later stages, we induced a cohort of ubi:Switch; ubi:creER2 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 CreER2-mediated loxP excisions required to give homogenous 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:creER2 generates potent CreER2 activity over a broad developmental window to induce tissue-specific loxP switch lines; different loxP switch transgenes have different sensitivities to CreER2 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 CreER2 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 ubiquitous 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 transgensics, 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 (Schafer 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 inloxP:Switch constructs. We found several independent ubi:Switch lines that showed weak and greatly varying Cre-mediated excision efficiencies (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:creER2 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). CreER2-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:creER2) as a recombinase source with eff1a:loxP-dsRed-1-loxP-EGFP, Hans et al. (Hans et al., 2009) reported CreER2 sensitivity to 0.5 μM 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 specific CreER2 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 CreER2 driver paired with a loxP switch will require individual elucidation.

Nonetheless, the use of CreER2 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 CreER2 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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