Conditional Gene Expression and Lineage Tracing of *tuba1a* Expressing Cells During Zebrafish Development and Retina Regeneration

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ABSTRACT

The *tuba1a* gene encodes a neural-specific α-tubulin isoform whose expression is restricted to the developing and regenerating nervous system. By using zebrafish as a model system for studying CNS regeneration, we recently showed that retinal injury induces *tuba1a* gene expression in Müller glia that reentered the cell cycle. However, because of the transient nature of *tuba1a* gene expression during development and regeneration, it was not possible to trace the lineage of the *tuba1a*-expressing cells with a reporter directly under the control of the *tuba1a* promoter. To overcome this limitation, we generated *tuba1a:CreER^{T2}* and β-*actin2: loxP-mCherrry-loxP-GFP* double transgenic fish that allowed us to label *tuba1a*-expressing cells conditionally and permanently via ligand-induced recombination. During development, recombination revealed transient *tuba1a* expression in not only neural progenitors but also cells that contribute to skeletal muscle, heart, and intestine. In the adult, recombination revealed *tuba1a* expression in brain, olfactory neurons, and sensory cells of the lateral line, but not in the retina. After retinal injury, recombination showed *tuba1a* expression in Müller glia that had reentered the cell cycle, and lineage tracing indicated that these cells are responsible for regenerating retinal neurons and glia. These results suggest that *tuba1a*-expressing progenitors contribute to multiple cell lineages during development and that *tuba1a*-expressing Müller glia are retinal progenitors in the adult. J. Comp. Neurol. 518:4196–4212, 2010.

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Unlike mammals, adult teleost fish such as zebrafish are able to regenerate a damaged central nervous system and therefore provide a good model for investigating mechanisms underlying neuronal repair. The retina is a readily accessible portion of the central nervous system that has served as a model for studying both axonal and neuronal regeneration in fish (Hitchcock et al., 2004; Becker and Becker, 2007, 2008). The robust regenerative power of the teleost retina is demonstrated by the observation that chemically induced whole-retina destruction results in retinal regeneration that is accompanied by restoration of visual function (Sherpa et al., 2007). The cells that contribute to retinal regeneration appear to be resident in the inner nuclear layer, where clusters or columns of proliferating cells are observed (Raymond and Hitchock, 2000; Vihtelic and Hyde, 2000; Wu et al., 2001; Faillace et al., 2002). The observation that inner nuclear layer cell proliferation precedes retina regeneration suggested that retinal stem cells residing in the inner nuclear layer are the predominant source of progenitors for regeneration. Müller glia whose cell bodies reside in the inner nuclear layer were observed to reenter the cell cycle following retinal damage (Braisted et al., 1994; Cameron, 2000;

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Wu et al., 2001). These observations, combined with reports that Müller glia in postnatal chicks and rodents have a limited capacity to regenerate neurons following retinal injury (Fischer and Reh, 2001; Ooto et al., 2004), implicated Müller glia as a source of retinal progenitors that participate in repair of damaged retinas.

Support for Müller glia as a source of retinal progenitors comes from studies with transgenic fish, in which the fate of green fluorescent protein (GFP)-expressing Müller glia can be followed for short periods of time following retinal injury (Fausett and Goldman, 2006; Bernardos et al., 2007; Fimbel et al., 2007; Thummel et al., 2008). By using transgenic fish harboring a 1,016-bp fragment of the *tuba1a* promoter driving GFP expression (*1016 tuba1a:GFP*), we found that *tuba1a*-expressing Müller glia respond to retinal injury by dedifferentiating and proliferating (Fausett and Goldman, 2006). However, because the *tuba1a* promoter was only transiently expressed in these dedifferentiated Müller glia, it was not possible to follow their fate over long periods of time and confirm they were stably integrated into the retinal architecture. To follow the fate of these *tuba1a*-expressing progenitors in the adult retina, we developed a conditional expression system that allowed for permanent labeling of *tuba1a*-expressing Müller glia. Based on previous success in mice, we chose to use the $CreER^{T2}$/LoxP recombination system for this purpose (Branda and Dymecki, 2004).

Cre-mediated recombination offers many advantages for studying gene function by stimulating targeted deletion, insertion, inversion, and exchange of chromosomal DNA (Branda and Dymecki, 2004). Recently, the Cre/LoxP system has been used for conditional gene expression in developing zebrafish in which either a tissue-specific or a ubiquitously expressed heat shock-regulated promoter was used to drive Cre recombinase expression (Feng et al., 2007; Le et al., 2007; Liu et al., 2008; Wang et al., 2008; Hans et al., 2009; Collins et al., 2010). However, these promoters have limitations insofar as they lack temporal control (tissue-specific promoters) or may exhibit leaky expression (heat shock promoters). In addition, stressing fish by a heat shock might have unintended consequences on cell function. For the mouse, these limitations have been surmounted by taking advantage of a ligand-dependent chimeric Cre recombinase where Cre is fused to the mutant ligand-binding domain of the human estrogen receptor ($CreER^{T}$ and $CreER^{T2}$; Feil et al., 1996, 1997; Danielian et al., 1998). These chimeric Cre recombinases are efficiently activated by the synthetic estrogen receptor ligand 4-hydroxytamoxifen (4-OHT) but are insensitive to endogenous 17β-estradiol (Indra et al., 1999).

Recently, transgenic zebrafish were created harboring the *$CreER^{T2}$* transgene under control of different promoters (Boniface et al., 2009; Hans et al., 2009). One study, with fish embryos that harbor the *pax2a:$CreER^{T2}$* transgene and a recombination reporter driven by the *EF1a* promoter, found ligand-dependent $CreER^{T2}$ activation and recombination in developing embryos (Hans et al., 2009). In contrast, another study, using the *her4.1* promoter to drive $CreER^{T2}$ expression, reported ligand-independent $CreER^{T2}$ activation that could be prevented by appending an additional ER ligand binding domain to the $CreER^{T2}$ fusion (Boniface et al., 2009). These studies were restricted to the evaluation of conditional gene expression in early developing embryos by bathing fish embryos in water containing 4-OHT. Although these studies suggest that the $CreER^{T2}$/LoxP system will be useful for conditional gene expression during development, its suitability for conditional gene expression in adults and its use for lineage tracing in developing and adult animals remained untested.

Motivated by the need for a conditional gene expression system that would allow gene recombination at any stage of development, including adulthood, and that was amenable for lineage tracing of Müller glia-derived progenitors in the injured retina, we developed the following transgenic fish: 1) *1016 tuba1a:$CreER^{T2}$*, in which a 1,016-bp fragment of the *tuba1a* promoter directs $CreER^{T2}$ expression to the developing and regenerating CNS; 2) *β-actin2:LCLG*, in which the ubiquitously expressed *β-actin2* promoter drives *mCherry (C)* expression that is flanked by *loxP (L)* sites and followed by an out-of-frame *EGFP (G)* sequence; and 3) *1016 tuba1a:$CreER^{T2}$;β-actin2:LCLG* double transgenic fish, in which the latter transgene serves as a recombination reporter and allows one to label permanently cells that either constitutively or transiently express $CreER^{T2}$ driven by the *1016 tuba1a* promoter. With these fish, we show that transgenic lines expressing $CreER^{T2}$ at low levels do not exhibit basal ligand-independent $CreER^{T2}$ activity. These low-expressing lines allowed us to map the fate of cells expressing the *1016 tuba1a* promoter during development and in the adult injured retina. We found that this recombination system revealed very low and transient *1016 tuba1a* promoter activity that could not be observed with traditional *1016 tuba1a:GFP* transgenes. This improved sensitivity allowed us to identify descendents of *tuba1a*-expressing cells early in development that include neural and nonneural progeny. In addition, we show that this recombination system is suitable for conditional gene expression, which allows one to perform lineage analysis and to assay the function of specific genes at any stage of zebrafish development. With this conditional gene expression system, we mapped the fate of *tuba1a*-expressing Müller glia in the injured retina and found that they regenerate new retinal neurons and glia.

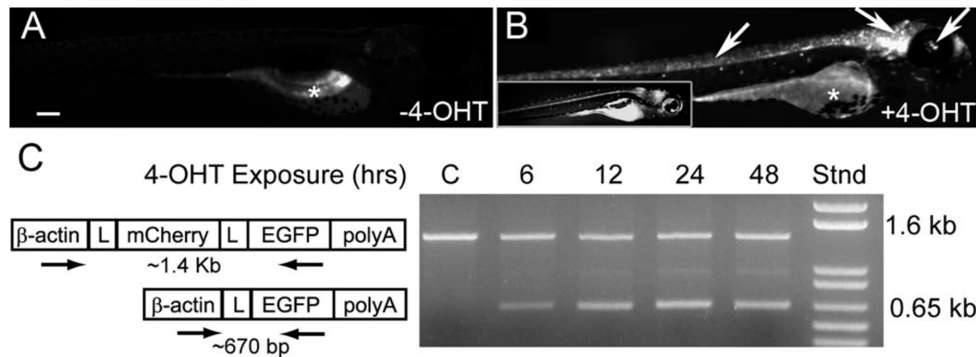

Figure 1. 4-OHT-dependent recombination in developing *CreER^{T2};LCLG* transgenic fish. **A,B:** *CreER^{T2};LCLG* transgenic fish harboring both the *1016 tuba1a:CreER^{T2}* and the β*-act2:LCLG* transgenes were incubated with vehicle (–4-OHT; A) or with 4-hydroxytamoxifen (+4-OHT; B) at 1 dpf and examined under fluorescent microscopy at 4 dpf (days postfertilization). Note the panneuronal induction of GFP expression in fish treated with 4-OHT shown in B, whereas siblings treated with vehicle exhibited undetectable GFP expression shown in A. The **inset** in B shows GFP expression in a *1016 tuba1a:GFP* transgenic fish for comparison with the expression pattern observed in recombined fish (also see Fig. 2B). Asterisk indicates autofluorescence from the yolk. **C:** Agarose gel showing time course of recombination following 4-OHT addition. *CreER^{T2};LCLG* transgenic fish at 24 hpf were immersed in vehicle for 48 hours (C) or 4-OHT for 6, 12, 24, and 48 hours before fish were killed and RNA was harvested for RT-PCR. The illustration at left shows the expected sizes of PCR products using the indicated forward and reverse PCR primers (arrows). The forward primer binds to the untranslated sequence in exon 1 from the β*-actin2* gene, and the reverse primer binds to the *GFP* sequence. Shown on the right is an agarose gel of PCR-amplified products corresponding to the unrecombined (top band) and recombined (bottom band) message. Note that, within 6 hours of 4-OHT application, significant amounts of the recombined mRNA can be detected and that, by 48 hours after 4-OHT application, recombination has leveled off. Stnd are 1 kb DNA molecular weight standards. Scale bar = 100 μm.

MATERIALS AND METHODS

Zebrafish husbandry

Zebrafish were obtained from our breeding colony and maintained at 28°C with a 10/14-hour light/dark cycle. Our fish originated from a local pet store. Zebrafish were treated in accordance with the guidelines of the University Committee on Use and Care of Animals at the University of Michigan.

Expression vectors and transgenic fish

The *1016 tuba1a:CreER^{T2}* expression vector (Supporting Information Fig. 1) harbors 1,016 bp of 5′ flanking DNA from the goldfish *tuba1a* gene followed by exon 1 and intron 1 (Heiber et al., 1998), fused in-frame to the *CreER^{T2}* sequence (Feil et al., 1997) and followed by an *SV40 polyA* signal sequence. The *1016 tuba1a* promoter is active throughout the developing nervous system, and in the adult retina this promoter is specifically activated in Müller glia-derived retinal progenitors following injury (Fausett and Goldman, 2006). Just downstream of this expression cassette, we inserted a second expression cassette harboring the same sequences described above except that the *tuba1a* 5′ flanking DNA was truncated to 906 bp and *CFP* was inserted into the noncoding portion of exon 1, which was followed by intron 1 of the goldfish *tuba1a* gene. The reason why we chose a shorter fragment of the *tuba1a* promoter over a heterologous promoter is that 1) we did not want to risk influencing *1016 tuba1a* promoter activity by novel regulatory elements in this second promoter and 2) we already knew that the shorter *tuba1a* promoter fragment was poorly expressed during development and undetectable in the adult nervous system and injured retina (Fausett et al., 2008). The recombination reporter β*-actin2:LCLG* expression vector (Supporting Information Fig. 1) harbors 3,851 bp of 5′ flanking DNA from the zebrafish β*-actin2* promoter followed by exon 1 and intron 1 (Hagashijima et al., 1997) fused in frame with a *LoxP* (Branda and Dymecki, 2004)-flanked *mCherry* sequence that is followed by an out-of-frame *EGFP* sequence and a *SV40 polyA* signal sequence. The plasmid backbone harboring these sequences was the Tol2 vector *pT2AL200R150G* (Urasaki et al., 2006). The *tuba1a* promoter directs gene expression to the developing and regenerating CNS (Hieber et al., 1998; Goldman et al., 2001; Senut et al., 2004).

Transgenic fish were produced by Tol2 transposase-mediated integration of the expression cassette as described by Urasaki et al. (2006). Injected embryos were analyzed for CFP or mCherry expression at 2–5 days postfertilization (dpf) using a Leica MZFLIII fluorescent stereo microscope, and fish expressing the reporter gene were grown to adulthood. F0 fish were bred in groups of six to eight fish, and progeny were examined for reporter gene expression. Groups with fluorescent progeny were then bred individually with wild-type fish to

identify founders with germline integration of the transgene. Progeny were raised to adults and bred to wild-type fish to perpetuate heterozygous lines. Multiple lines of fish harboring either the *1016 tuba1a:CreER*^{T2} or the β-*actin2:LCLG* transgenes were generated. Representative weak and strong expressing lines were saved and maintained for at least four generations, without any noticeable change in transgene expression. *1016 tuba1a:CreER*^{T2} and β-*actin2:LCLG* fish were interbred, and double transgenic fish were identified by CFP and mCherry fluorescence.

The *1016 tuba1a:GFP* transgenic line (formerly referred to as *1016α1TlpEGFP* and *1016α1T*) harbors 1,016 bp of 5′ flanking DNA from the goldfish *tuba1a* gene followed by exon 1 and intron 1 fused in-frame to the GFP sequence and followed by an *SV40 polyA* signal sequence (Heiber et al., 1998; Fausett and Goldman, 2006).

Cell culture transfection

HEK 293 cells were cultured in DMEM with 10% fetal bovine serum and maintained at 5% CO_2 at 37°C. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) using 200 ng recombination reporter and anywhere from 0.1 to 10 ng of *tuba1a:CreER*^{T2} expression vector per 0.5 ml culture media. Twenty-four hours after transfection, 4-hydroxytamoxifen (4-OHT) was added to a final concentration of 1 μM. Control cells received ethanol, which is the vehicle used to dissolve 4-OHT. Cultured cells were observed 48 hours post-4-OHT addition with an inverted DMIL fluorescent microscope (Leica).

Retinal injury

Retinas were injured as previously described (Senut et al., 2004; Fausett and Goldman, 2006; Fausett et al., 2008). Briefly, fish were anesthetized, and under microscopic visualization the right eye was gently pulled from its socket and poked four times, once in each quadrant through, the sclera with a 30-g needle. The needle was inserted to the length of the bevel (∼5mm). The left eye served as an unoperated control.

4-OHT treatment and bromodeoxyuridine labeling

To activate CreER^{T2}, fish were immersed for various lengths of time, up to 3 days, in a small volume of fish water containing 1 μM 4-OHT. After 4-OHT immersion, fish were rinsed extensively in fish water prior to being returned to their tanks. During extended periods of 4-OHT treatment, fish were removed from 4-OHT-containing solution and allowed to feed for a few hours before returning to fish water with 4-OHT. In some experiments, adult fish received 4-OHT simultaneously with retinal injury by injecting ∼ 1 μl of a 50 μM solution of 4-OHT with the same needle used to injure the retina. To identify dividing cells, fish were either immersed in 10 mM bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) for various amounts of time or anesthetized and injected intraperitoneally (IP) with 2–10 μl of a 25 mg/ml BrdU stock solution.

PCR analysis of reporter recombination and tuba1a:CreER^{T2} splicing

Double transgenic embryos at 24 hours postfertilization (hpf) or adult fish whose retinas had been injured 20 hours earlier were immersed in either 1 μM 4-OHT or vehicle and harvested at various times for analysis of recombination by RT-PCR. Embryos or adult injured retinas were lysed in Trizol (Invitrogen) for RNA isolation. RNA was reverse transcribed using oligo(dT) primer and SuperScript II (Invitrogen), and one-twentieth of the resulting cDNA mixture served as template for PCRs. To assay recombination, we designed primers flanking the *mCherry* sequence: therefore, the forward primer (TGCGGAATATCATCTGCTTG) targeted exon 1 of the β-actin2 gene and the reverse primer (GTTGTGGCGGATCTTGAAGT) targeted the *EGFP* sequence. We also assayed whether splicing of the *1016 tuba1a:CreER*^{T2}-derived transcript was accurate by using primers that flanked the *tuba1a* intron; therefore, a forward primer targeted exon 1 of the *tuba1a* gene (CTTACATCGATCCCTTAGTTGTCG) and a reverse primer targeted *CreER*^{T2} (ATGTTTAGCTGGCCCAATG). Cycling conditions were 94°C for 5 minutes, followed by quick cooling on ice and the addition of Taq DNA polymerase and then cycling as follows: 15 seconds at 94°C, 30 seconds at 62°C and 1 min at 68°C, and a final extension at 68°C for 5 min. We used 25 cycles for embryos and 35 cycles for adult retina.

In situ hybridization

In situ hybridization was carried out with antisense digoxigenin-labeled probes as previously described (Gulati-Leekha and Goldman, 2006). Briefly, fixed embryos were dechorionated and stored in methanol at –20°C. Embryos were cleared in methanol-xylene and rehydrated in gradients of methanol (90–50%). Embryos were digested with proteinase K for 5 min at 37°C. Embryos were prehybridized for 2 hours at 56°C and then hybridized with digoxigenin-labeled *CreER*^{T2} (800-bp coding sequence) or *tuba1a* (870-bp 3′ UTR and exon 4) antisense probe overnight at 56°C. Posthybridization procedures were performed as previously described (Gulati-Leekha and Goldman, 2006). The antidigoxigenin antibody conjugated to alkaline phosphatase was used at a 1:5,000 dilution.

TABLE 1.
Primary Antibodies

Antibody	Immunogen	Stains	Dilution	Host species	Manufacturer
BrdU monoclonal	BrdU	Proliferating cells	1:400	Rat	Abcam (ab6326)
zpr-1 monoclonal	Whole formaldehyde-fixed retinal cells	Red/green cones	1:250	Mouse	Zebrafish International Resource Center (zpr-1)
GFP polyclonal	Green fluorescent protein from *A. victoria*	Green fluorescent protein	1:1,000	Rabbit	Invitrogen (A-6455)
HuC/D monoclonal	Human HuD peptide, QAQRFRLDNLLN	Amacrine and ganglion cells	1:500	Mouse	Invitrogen (A-21272)
Glutamine synthetase monoclonal	Sheep brain glutamine synthetase	Müller glia	1:500	Mouse	Chemicon/Millipore (MAB302)
PKC-β1 polyclonal	C-terminal peptide fragment of human protein kinase C β1 EFAGFSYTNPEFVINV	Bipolar cells	1:500	Goat	Santa Cruz Biotechnology (sc 209 G)
zn5 Monoclonal	Crude extract of 1–5-day-old zebrafish	Retinal ganglion cells	1:500	Mouse	Zebrafish International Resource Center (zn-5)

Tissue preparation and immunohistochemistry

For analysis of recombination in live embryos and adults, we anesthetized the fish in 0.02% tricaine methane sulfonate (Sigma). Embryos were placed in 3% methylcellulose, whereas adults were placed on a support, and fluorescence was observed with a fluorescent stereo microscope (Leica MZFLIII) with attached digital camera for capturing images. For visualizing retinal sections, fish were overdosed with tricaine methane sulfonate, and eyes from adult fish were enucleated, followed by removal of the lens and fixation by immersion in fresh 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for about 16 hours. After fixation, samples were cryoprotected in phosphate-buffered 20% sucrose before embedding with Tissue-Tek O.C.T. compound (Sakura, Finetek). Embedded samples were kept frozen at –80°C until sectioning to 8 μm on a CM3050S cryostat (Leica). Sections were collected on Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ), dried overnight at room temperature, and stored at –80°C. Immunohistochemistry was performed as previously described (Senut et al., 2004; Fausett and Goldman, 2006). For BrdU staining, sections were pretreated with 2 N HCl for 20 minutes at 37°C. Immunostained slides were washed in PBS and immersed in water containing 10 ng/ml DAPI (Sigma) to visualize nuclei. Slides were rinsed with water and allowed to dry in the dark prior to coverslipping with 2.5% PVA (PVA-polyvinyl alcohol)/DABCO (1,4-diazabicyclo[2.2.2]octane). Slides were examined with a Zeiss Axiophot fluorescent microscope equipped with a digital camera or an Olympus FluoView FV1000 confocal imaging system.

Antibody characterization

See Table 1 for a list of all antibodies used in this study. All antibodies have been previously characterized in zebrafish as described below and gave identical staining patterns in the current study. The BrdU antibody has been used to detect BrdU-labeled proliferating cells and does not detect any cells in animals that have not been treated with BrdU (Adolf et al., 2006; Goldman and Fausett, 2006; Grandel et al., 2006; Pellegrini et al., 2007).

The zpr1 antibody, formerly known as Fret43, identifies the red/green double cone pair in the outer nuclear layer (ONL) of the zebrafish retina as determined by anatomical location (ONL) and morphological features that include labeling of the outer segment, axon, and synaptic terminal and outlining of the inner segment (Larison and Bremiller,1990; Yazulla and Studholme, 2001); an identical staining pattern has been reported when this antibody was used to detect double cones in the adult zebrafish retina (Yazulla and Studholme, 2001; Fausett and Goldman, 2006; Raymond et al., 2006; Bernardos et al., 2007).

The GFP antibody stains only tissue from transgenic fish expressing the GFP transgene, and no signal is detected from wild-type fish that do not harbor the transgene (Fausett and Goldman, 2006; Fausett et al., 2008). The HuC/D antibody identifies amacrine and ganglion cells in the mature rat retina as determined by anatomical localization, morphology, costaining for the ganglion cell marker GAP-43, and lack of costaining with bipolar-specific markers (Ekstrom and Johansson, 2003); an identical staining pattern has been reported when this antibody was used to detect amacrine and ganglion cells in the adult zebrafish retina, where amacrine cells are concentrated in the proximal region of the inner nuclear layer (INL) and extend axons into the inner plexiform layer, whereas ganglion cells, with large round cell bodies, are concentrated in the ganglion cell layer (GCL; Fausett and Goldman, 2006; Bernardos et al., 2007; Fimbel et al., 2007; Kassen et al., 2007).

The glutamine synthetase (GS) antibody recognizes a single 45-kDa protein in adult rat retinal tissue by

Western blot analysis consistent with the size of glutamine synthetase and stains cells with morphological features uniquely characteristic of Müller glia, i.e., cell bodies in the INL along with processes that extend into the GCL and ONL (Chang et al., 2007); an identical staining pattern has been reported when this antibody was used to detect Müller glia in the adult zebrafish retina (Fausett and Goldman, 2006; Kassen et al., 2007, 2008; Thummel et al., 2008). In transgenic fish harboring the Müller glia-specific *gfap* promoter driving EGFP expression, the GS antibody specifically labels EGFP-expressing Müller glia (Kassen et al., 2007).

The PKCβ-1 antibody was made against the C-terminus of human protein kinase C β1 and identifies a 79-kDa protein on Western blots from a variety of mammalian cell types (Korchak and Kilpatrick, 2001; Berdiev et al., 2002). In the zebrafish retina, the PKCβ-1 antibody identifies bipolar cells located in the mid- to distal region of the INL (Kainz et al., 2003; Nevin et al., 2008; Vitorino et al., 2009). These cells have a characteristic bipolar cell morphology that includes a primary dendrite exiting the distal end of the soma that ends in the outer plexiform layer and an axon emanating from the proximal side of the soma that projects into the inner plexiform layer. As with these previous studies (Kainz et al., 2003; Nevin et al., 2008; Vitorino et al., 2009), we also find that the PKCβ-1 antibody identifies retinal cells whose anatomical location and morphology are consistent with bipolar cells.

The zn5 antibody recognizes the activated leukocyte cell adhesion molecule-a (also known as DM-GRASP) that is expressed in zebrafish differentiating motor neurons and retinal ganglion cells (Fashena and Westerfield, 1999; Babb et al., 2005). It does not recognize mature ganglion cells. The characteristic staining of differentiating retinal ganglion cells includes cell bodies located in the GCL and axons extending into the optic nerve layer. This antibody has been used to detect differentiating retinal ganglion cells during zebrafish retina regeneration, and these stained cells exhibit the same features as described above (Fausett and Goldman, 2006; Fimbel et al., 2007).

Primary antibodies were detected by using secondary donkey anti-rat AMCA (1:250; IgG H + L; Jackson Immunoresearch, West Grove, PA; catalog No. 712-156-150), donkey anti-rabbit Alexa Fluor 488 (1:1,000; IgG H + L; Invitrogen; catalog No. A-21206), or donkey anti-mouse Alexa Fluor 555 (1:500; Invitrogen; catalog No. A-31570). Omission of primary antibodies resulted in no specific staining.

Quantification of immunostained cells at the injury site

Quantification of GFP-, BrdU-, HuC/D-, and GS-labeled cells was performed by counting GFP^+ single-positive and GFP^+/GS^+, $GFP^+/HuC/D^+$, or $GFP^+/BrdU^+$ double-positive cells at four injury sites of the retina. Eight retinas were used to quantify cell counts at 2 and 3 days postinjury, whereas two retinas were used to quantify cell counts at 4–7 days postinjury. For this analysis, the retinal sections we analyzed were 8 μm thick and separated by 40 μm. Only cells with well-defined nuclei were counted. We used the Abercrombie formula to correct for counting errors (Abercrombie, 1946). The mean thickness of sections was 8 μm, and the mean nuclear width for GFP^+, GFP^+/GS^+, and $GFP^+/BrdU^+$ cells varied from 5.1 ± 0.34 to 4.9 ± 0.34 over the time course of the experiment. Abercrombie's correction for these samples was 0.6. The mean nuclear width for $GFP^+/HuC/D^+$ cells varied from 3.6 ± 0.5 on day 2 postinjury to 3.1 ± 0.5 on day 3 postinjury, and Abercrombie's correction for these samples was 0.7. Data in Figure 6 are reported as percentage of GFP^+ cells colabeling with GS or BrdU. The raw data and Abercrombie equation-corrected data are presented in Supporting Information Table 1.

Photomicrograph production

Microscopic images were imported into Adobe Photoshop CS2 for processing of figures. Contrast and brightness were changed uniformly across the image when necessary. Graphs were prepared in Adobe Illustrator and then imported into Adobe Photoshop for final figure preparation.

RESULTS

Conditional recombination in transgenic fish

Expression vectors (see Materials and Methods and Supporting Information Fig. 1) used for lineage tracing in transgenic fish were first tested for conditional gene expression in HEK 293 cells. Cotransfection of HEK 293 cells with *1016 tuba1a:CreER^{T2}* and *β-actin2:LCLG* demonstrated 4-OHT-dependent recombination as indicated by GFP expression (Supporting Information Fig. 2A–D). 4-OHT did not cause any detectable GFP expression when HEK 293 cells were transfected with only the *β-actin2:LCLG* recombination reporter (Supporting Information Fig. 2E,F).

After establishing that our vectors were functional, we used the Tol2 transposase system (Urasaki et al., 2006) to introduce vector DNA into the zebrafish germline. *1016 tuba1a:CreER^{T2}* lines were identified by CFP expression, and recombination reporter lines were identified by mCherry expression. We identified both weakly and strongly expressing *1016 tuba1a:CreER^{T2}* lines. Weakly expressing lines showed CFP expression predominantly in neuromasts that make up the lateral line, with weaker expression in the brain (Supporting Information Fig. 3A), whereas strongly expressing lines showed expression in neuromasts, spinal cord, brain, and retina (Supporting Information Fig. 3B).

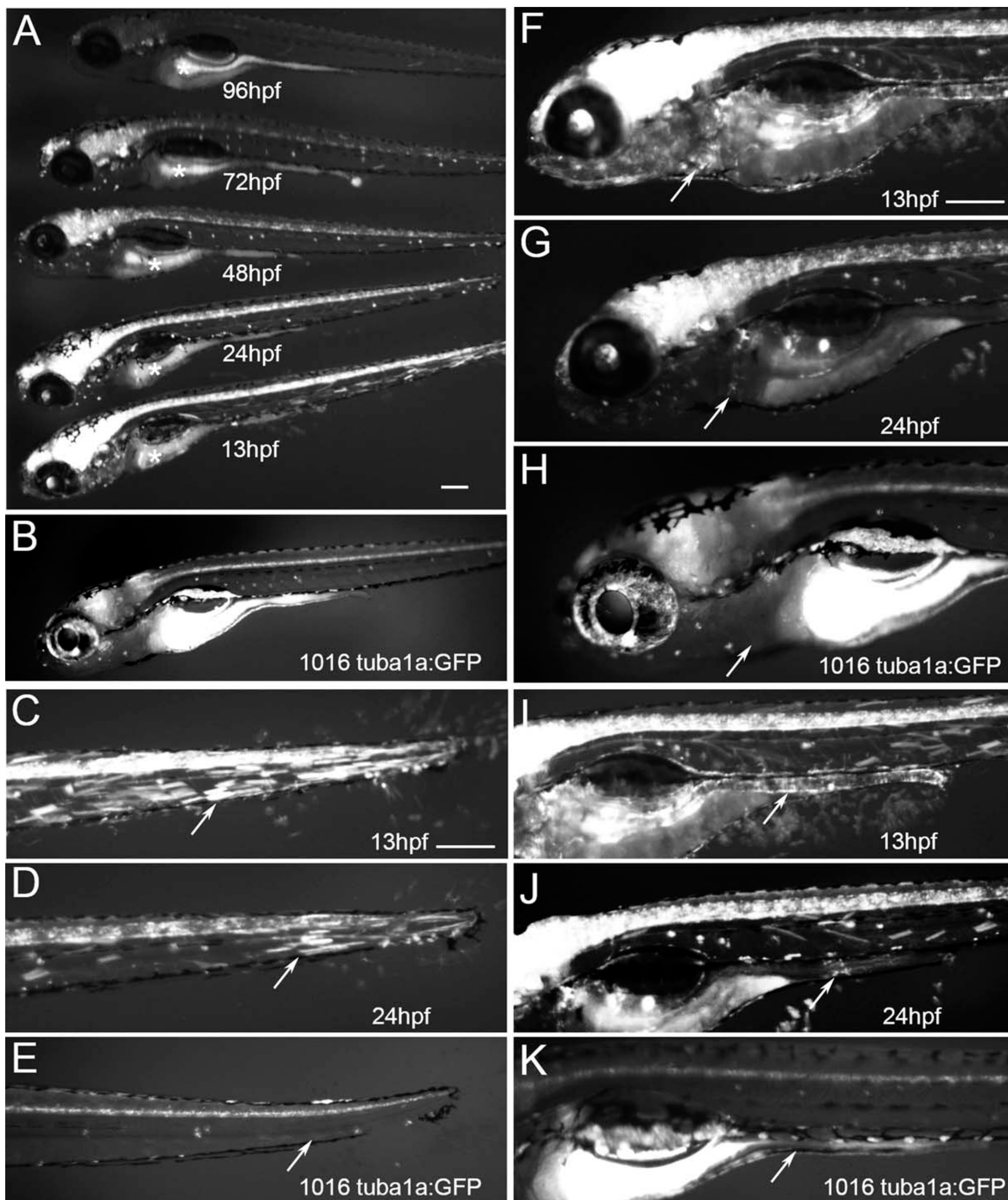

Figure 2. Temporal control of recombination during development reveals transient *1016 tuba1a* promoter activity in neural and nonneural cell populations. **A:** *CreER^{T2};LCLG* transgenic fish were immersed in 4-OHT for 6 hours starting at 13, 24, 48, and 72 hpf and assayed for GFP expression at 7 dpf. Note the robust and panneuronal expression in fish immersed in 4-OHT at 24 hpf or earlier. **B:** GFP expression in *1016 tuba1a:GFP* transgenic fish at 7 dpf for comparison with recombined expression pattern. **C,D:** 4-OHT-dependent recombination in developing muscle (arrow) of *CreER^{T2};LCLG* transgenic fish immersed in 4-OHT at 13 and 24 hpf. **E:** Lack of GFP expression in muscle of *1016 tuba1a:GFP* fish at 7 dpf. **F,G:** 4-OHT-dependent recombination in heart cells is observed only when fish are immersed in 4-OHT at 13 hpf. **H:** Lack of GFP expression in the heart of *1016 tuba1a:GFP* fish. **I,J:** 4-OHT-dependent recombination in the gut of fish immersed in 4-OHT at 13 and 24 hpf. **K:** Absence of GFP expression in the gut of *1016 tuba1a:GFP* fish. Asterisk indicates autofluorescence from the yolk. Scale bars = 100 μm in A (applies to A,B); 100 μm in C (applies to C–E); 100 μm in F (applies to F–K).

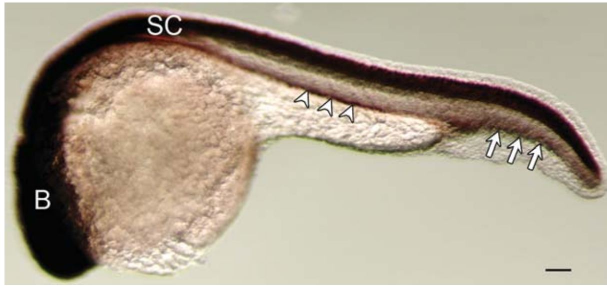

Figure 3. In situ hybridization reveals endogenous *tuba1a* gene expression in gut endoderm and somites. Wild-type fish at 24 hpf were fixed and hybridized with a digoxigenin antisense *tuba1a* RNA probe. Note high level expression of *tuba1a* RNA in the brain (B) and spinal cord (SC). Arrows point to reduced expression in the caudal somites, and arrowheads point to expression in the gut endoderm. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

When we assayed for CreER^{T2} RNA expression in the weakly expressing lines, we detected expression in neuromasts and brain (Supporting Information Fig. 3C,D). In general, mCherry expression in the β-*actin2:LCLG* fish was strong and widespread in developing embryos and adults (Supporting Information Fig. 3E–L).

Conditional gene expression during development identifies novel tuba1a expression domains

Double transgenic lines (*CreER^{T2};LCLG*) harboring *1016 tuba1a:CreER^{T2}* and β-*actin2:LCLG* transgenes were generated and tested for 4-OHT-dependent recombination. We found that *1016 tuba1a:CreER^{T2}* lines, which expressed the CFP reporter at high levels, gave background recombination independent of 4-OHT treatment (data not shown), which suggested that CFP expression reflects CreER^{T2} levels. Therefore, we focused our studies on double transgenic fish made by crossing a weakly expressing *1016 tuba1a:CreER^{T2}* line (L1) with a strongly expressing β-*actin2:LCLG* line (L19). We found that double transgenic fish tolerated 4-OHT concentrations at and below 2 μM, whereas higher concentrations were toxic.

To determine whether *CreER^{T2};LCLG* fish exhibited 4-OHT-dependent recombination, we immersed fish in 4-OHT or vehicle for 3 days beginning at 1 dpf (Fig. 1). Indeed, only fish immersed in 4-OHT exhibited robust panneuronal GFP expression (Fig. 1A,B), which was similar to GFP expression in *1016 tuba1a:GFP* transgenic fish (Figs. 1B, inset, 2B).

To determine whether shorter times of exposure to 4-OHT would also induce recombination, we used RT-PCR to assay recombination at 6, 12, 24, and 48 hours post-4-OHT immersion (Fig. 1C). Sibling control fish incubated in vehicle were also harvested at 48 hours postvehicle immersion and assayed for recombination (Fig. 1C, lane C). Although recombination was not detected in vehicle-treated fish, immersion in 4-OHT for as little as 6 hours was sufficient to induce recombination (Fig. 1C). DNA sequence analysis of the 1.4-kb and 0.67-kb bands confirmed that they represented the original and recombined transcripts, respectively. DNA sequencing showed that the very faint band at about 1 kb represents the recombined product in which the reverse primer bound to an imperfect complementary sequence about 300 nucleotides downstream of its intended target.

We next used *CreER^{T2};LCLG* transgenic fish to investigate when and where the *1016 tuba1a* promoter is activated during development. For these experiments transgenic fish received a 6-hour pulse of 4-OHT at 13, 24, 48, 72 and 96 hpf, and GFP expression was recorded at 7 dpf (Fig. 2A). *1016 tuba1a* Promoter activity, reflected by GFP expression, was most robust in fish pulsed with 4-OHT at 13 and 24 hpf and is consistent with this promoter being activated panneuronally in neural progenitors. This pattern of expression was similar to that of *1016 tuba1a:GFP* fish (Fig. 2B); however, *1016 tuba1a:GFP* fish exhibited a more restricted pattern of neural GFP expression in the brain and spinal cord than did *CreER^{T2};LCLG* fish (Fig. 2A,B, Supporting Information Fig. 4). This difference in expression can be explained largely by the persistent vs. transient nature of the β-*actin2* vs. the *1016 tuba1a* promoters, respectively.

We were surprised to find transgene expression in heart, intestine, and skeletal muscle in *CreER^{T2};LCLG* fish pulsed with 4-OHT at 13–24 hpf that was not apparent in *1016 tuba1a:GFP* fish (Fig. 2C–K). This suggested a transient activation of the *1016 tuba1a* promoter in cells giving rise or contributing to these nonneural tissues that is undetectable in *1016 tuba1a:GFP* fish. To determine whether the nonneuronal pattern of *1016 tuba1a* promoter activity reflected that of the endogenous *tuba1a* gene, we assayed expression of the endogenous gene by in situ hybridization at 24 hpf. This analysis showed that the vast majority of the *tuba1a* mRNA is expressed in the developing nervous system (Fig. 3); however, overdevelopment of the in situ signal revealed *tuba1a* mRNA in caudal somites that generate muscle (Fig. 3, arrows; Devoto et al., 1996) and endoderm that gives rise to the gut (Fig. 3, arrowheads; Ober et al., 2003). No in situ hybridization signal was detected, even after long exposures, with a control sense-strand probe (Supporting Information Fig. 5). Thus this conditional recombination system provides a sensitive and robust readout of weak and transient *tuba1a* promoter activity in previously unappreciated expression domains. Although we have not investigated the significance of this expression, the temporal expression pattern suggests that *tuba1a* contributes to progenitor proliferation and differentiation in these nonneural

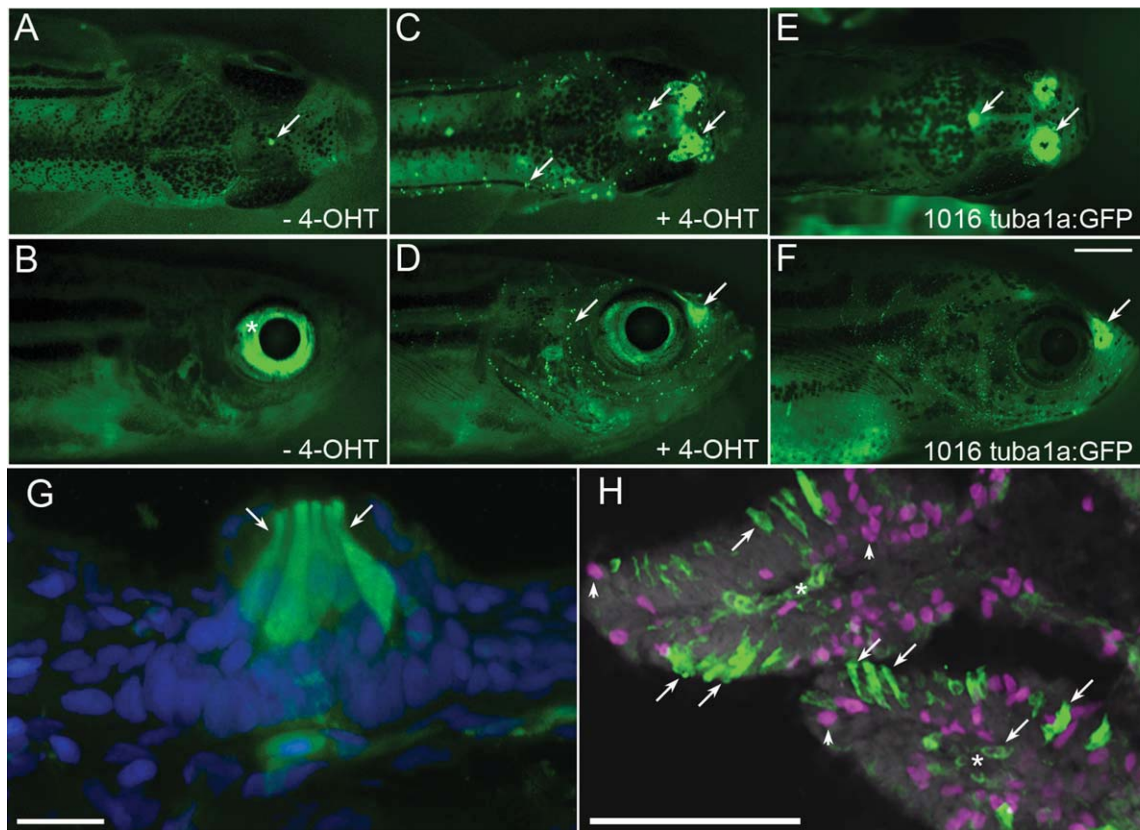

Figure 4. 4-OHT-dependent recombination in adult *CreER^{T2};LCLG* transgenic fish. **A–D:** Adult *CreER^{T2};LCLG* transgenic fish approximately 5 months old were immersed in vehicle (–4-OHT) or 4-OHT (+4-OHT) for 3 days, and GFP expression was visualized by using a stereo fluorescence microscope. **E,F:** GFP expression in *1016 tuba1a:GFP* transgenic fish is shown for comparison. A, C, and E are dorsal views. B, D, and F are lateral views. A: Arrow points to rare GFP$^+$ cell in vehicle-treated fish. C–F: Arrows point to GFP$^+$ cells in olfactory pits, neuromasts, and midbrain. B: Confocal microscopy of cryosections through the lateral line neuromast (**G**) and olfactory pit (**H**) shows GFP expression in putative sensory cells (arrows). The fish shown in H was also exposed to BrdU for 2 days following 4-OHT treatment. BrdU$^+$ nuclei are red/purple and are marked by an arrowhead. Confocal microscopy suggests that GFP$^+$ sensory cells, which are concentrated in the apical olfactory epithelium, do not colabel with BrdU. Asterisk identifies nonspecific fluorescence in the lumen of the olfactory epithelium. Scale bars = 1 mm in F (applies to A–F); 10 μm in G; 50 μm in H. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tissues. These results illustrate the utility of using a conditional recombination system that allows one to probe for weak and transient expression throughout development that would be missed by more traditional approaches and provides a means for labeling these cells or manipulating them by conditional expression of transgenes.

Conditional gene expression in adults and following retina injury allows lineage tracing of Müller glia-derived retinal progenitors

After establishing that recombination and reporter gene expression in *CreER^{T2};LCLG* transgenic embryos and fry were inducible by 4-OHT, we investigated whether recombination could be regulated by 4-OHT in adult fish. The simplest and most reproducible method for inducing widespread recombination with minimal lethality was immersion in 4-OHT. However, for recombination in the eye, immersion in 4-OHT or eye injection of 4-OHT worked equally well.

Control *CreER^{T2};LCLG* fish exhibited very little to no recombination as assayed by GFP expression in live animals (Fig. 4A,B). An example of the type of background that we observed in about 25% of the *CreER^{T2};LCLG* transgenic fish in the absence of 4-OHT is shown in Figure 4A. In contrast, *CreER^{T2};LCLG* fish immersed in 4-OHT for 3 days and examined 2 months later exhibited GFP expression in olfactory pits, brain, and anterior and posterior lateral line (Fig. 4C,D). The pattern of 4-OHT-induced transgene expression in *CreER^{T2};LCLG* fish was similar to that of the *1016 tuba1a:GFP* fish (Fig. 4E,F), although GFP expression in the anterior lateral line was reduced in *1016 tuba1a:GFP* fish. Confocal microscopy of tissue sections from 4-OHT-treated *CreER^{T2};LCLG* fish confirmed that the *1016 tuba1a* promoter was expressed in pear-shaped sensory hair cells of the lateral line neuromast

and putative sensory cells located in the apical portion of the olfactory epithelium (Fig. 4G,H). Consistently with the observation that cells in these anatomical locations do not normally proliferate (Williams and Holder, 2000; Byrd and Brunjes, 2001), we were unable to colabel them with a 3-hour pulse of BrdU (data shown for olfactory epithelium in Fig. 4H).

1016 tuba1a:GFP fish have been instrumental in identifying Müller glia-derived retinal progenitors that facilitate regeneration of an injured retina (Fausett and Goldman, 2006). However, because *1016 tuba1a:GFP* transgene expression does not persist in these putative progenitors after they exit the cell cycle and differentiate, it remained unclear whether newly regenerated cells were derived from the *1016 tuba1a:GFP*-expressing Müller glia. Here we investigated whether the CreER^{T2}/LoxP recombination system could be used to trace the lineage of Müller glia-derived progenitors that reentered the cell cycle.

To visualize injury and 4-OHT-dependent recombination in the retina, we injured retinas of double transgenic fish with a needle poke and immersed fish in 4-OHT for 3 days. Controls received vehicle in place of 4-OHT. Fish then received an IP injection of BrdU on day 4 postinjury. Fish were sacrificed 2 weeks postinjury, and retinas were processed for GFP and BrdU immunohistochemistry. Except for variable GFP expression in a few cells around the optic nerve head (observed in about 50% of the transgenic fish), there was no GFP expression in the uninjured retina regardless of 4-OHT treatment (Fig. 5A–F). A few BrdU-positive rod progenitors were observed in the outer nuclear layer, especially in the retinal periphery (Fig. 5B,E). In contrast, after retinal injury, only fish that received 4-OHT exhibited GFP expression at the injury site (which was visualized by a large increase in BrdU-labeled cells; Fig. 5G–L).

We next used RT-PCR to assay recombination in the injured retina of *CreER^{T2};LCLG* fish (Fig. 6A). For this analysis, retinas were injured by a needle poke, and 1 day later fish were immersed in 4-OHT for up to 3 days. Retinas were harvested beginning at the time when fish were first immersed in 4-OHT (0 days post-4-OHT immersion) and then at daily intervals for up to 14 days (three retinas per time point). Retinal RNA was isolated and used in RT-PCRs with a β-*actin2* exon 1 forward primer and a *GFP* reverse primer (arrows in Fig. 6A). This analysis showed that expression of the recombined message began at about 2 days post-4-OHT immersion. In general, recombination in the uninjured retina was undetectable, even after exposure to 4-OHT, or in the injured retina of fish that received vehicle devoid of 4-OHT (Fig. 6A). However, periodically we observed a very faint band corresponding to recombined message in the uninjured retina (for example, see Fig. 6A, uninjured 4 days post-4-OHT immersion), which we suspect represents the variable and small amount of recombination observed near the optic nerve head. In comparing 7 with 14 days post-4-OHT immersion, it appears that there is a slight reduction in the recombined 670-bp band, which likely reflects a less severely injured retina used for that time point. DNA sequence analysis of the unrecombined 1.4-kb band and the recombined 670-bp band indicated that RNA processing and recombination occurred without any errors (data not shown). The 1.4-kb band is present in all samples, because the RNA used for RT-PCR was from whole retina, in which most cells express unrecombined reporter (1.4-kb band) but do not express CreER^{T2} and hence exhibit no recombination. These experiments were repeated three times with similar results.

We had previously shown that the *1016 tuba1a* promoter is activated in Müller glia at early times following retinal injury (Fausett and Goldman, 2006). To determine whether recombination is also restricted to these cells, we costained injured retinas with anti-GFP and anti-GS antibodies (Fig. 6C–Z). Consistent with the RT-PCR analysis, immunohistochemical detection of GFP was first observed on day 2 postretinal injury, and the vast majority of these cells costained with the Müller glia marker GS (Fig. 6C–F, Supporting Information Table 1). Examination of eight injured retinas indicated that, among approximately 3,725 GFP$^+$ cells identified at 2 and 3 days postinjury, 3,501 (94%) of these were also GS$^+$ (Fig. 6B, Supporting Information Table 1). By using cell-type-specific antibodies, we found that the remaining GFP$^+$/GS$^-$ cells expressed the HuC/D antigen, exhibited extensive dendritic branching, and were localized to the proximal portion of the INL (Supporting Information Fig. 6), which suggests that they are amacrine cells (Godinho et al., 2005). In addition, these cells did not reenter the cell cycle, inasmuch as they could not be labeled with BrdU. Therefore, to show unambiguously that *1016 tuba1a*-expressing Müller glia-derived progenitors give rise to amacrine cells, additional methods must be employed, such as conditional gene expression in combination with BrdU labeling (see below and Fig. 7).

At later times postinjury, we noticed a gradual decrease in the percentages of GFP$^+$ cells that were also GS$^+$ (Fig. 6B, Supporting Information Table 1) and an increase in the number of GFP$^+$ cells found in all retinal layers (Fig. 6W–Z, arrowheads). This is compatible with the idea that GFP$^+$ Müller glia give rise to other retinal cell types that contribute to repair of the damaged retina.

Müller glia-derived progenitors reenter the cell cycle around day 2 postretinal injury, exhibit peak proliferation at day 4 postinjury, and return to baseline levels by day 7 postinjury (Fausett and Goldman, 2006). To investigate whether GFP$^+$ cells were proliferating Müller glia-derived progenitors, *CreER^{T2};LCLG* fish retinas were injured and

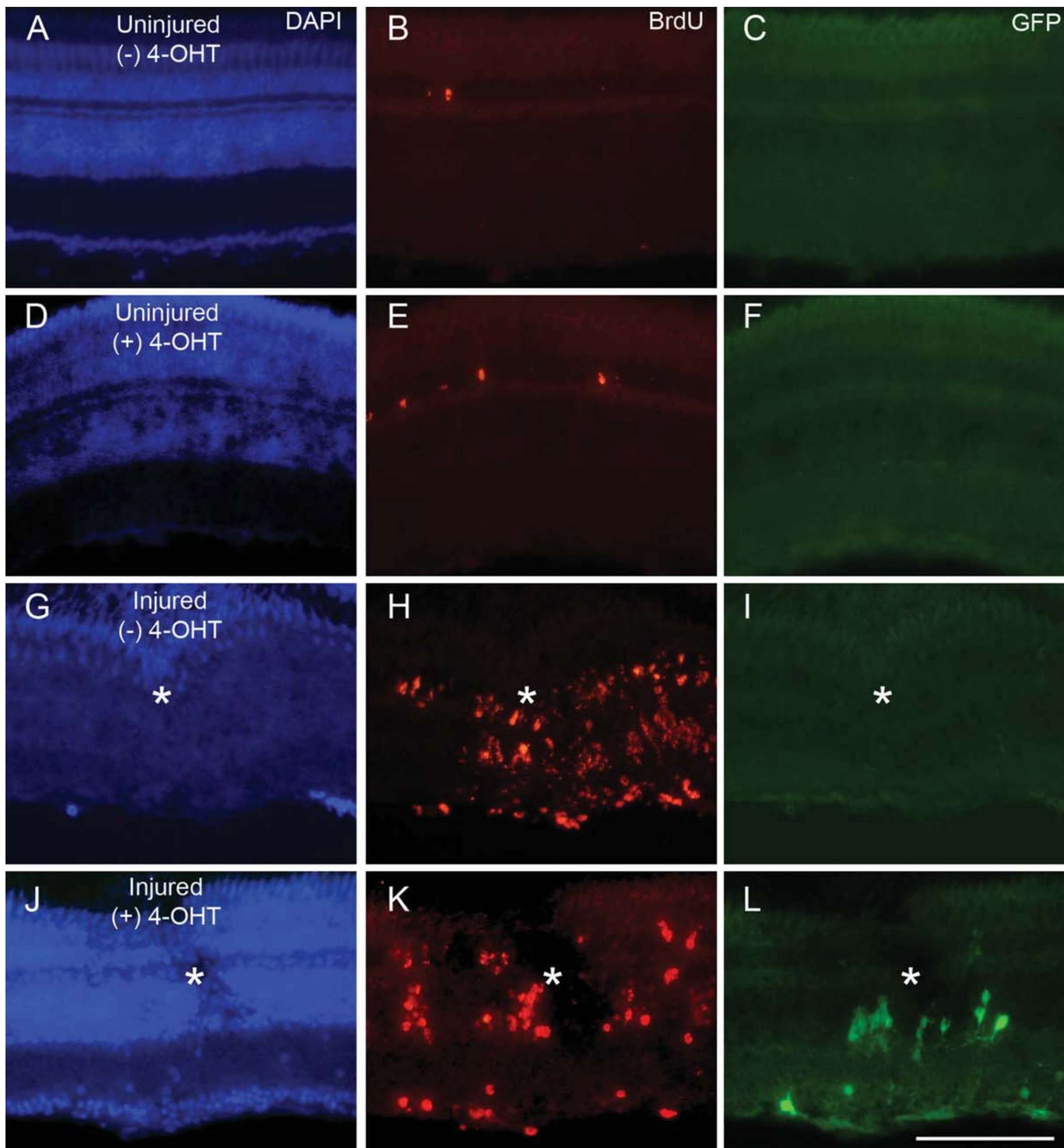

Figure 5. 4-OHT-dependent recombination in the injured retina is restricted to the injury site. Adult *CreER^{T2};LCLG* transgenic fish were anesthetized, and retinas were left uninjured (**A–F**) or injured by a needle poke (**G–L**). Twenty hours later, fish were immersed in vehicle (–4-OHT, A–C,G–I) or 4-OHT (+4-OHT, D–F,J–L) for 3 days and then received an intraperitoneal injection of BrdU on day 4 postinjury and returned to their tank for 2 weeks before harvesting retinas. Retinas were sectioned and stained with DAPI (A,D,G,J), anti-BrdU (B,E,H,K), and anti-GFP (C,F,I,L) antibodies, to identify proliferating and recombined cells, respectively. A–C: Uninjured retina shows a single BrdU-positive rod progenitor but no GFP expression. D–F: Uninjured retina treated with 4-OHT shows a few BrdU-expressing rod progenitors but no GFP expression. G–I: Injured retina shows a large induction of BrdU-expressing cells at the site of injury (asterisk) but no GFP expression. J–L: Injured retina treated with 4-OHT shows a large induction of BrdU expressing cells at the site of injury (asterisk) and an increase in GFP-expressing cells. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

exposed to 4-OHT, and then, 3 hours prior to harvesting, fish received an IP injection of BrdU. At 3 days postinjury, we found that approximately 5% of the GFP$^+$ cells were also BrdU$^+$, which increased to approximately 15% on day 4 postinjury (Fig. 6B,G–N). Some of the fish that received BrdU on day 4 postinjury were allowed to develop further (up to 7 days postinjury), and the percentage of GFP$^+$ cells that were also BrdU$^+$ was quantified. This percentage remained relatively constant (Fig. 6B) and suggested that the *1016 tuba1a* promoter may be

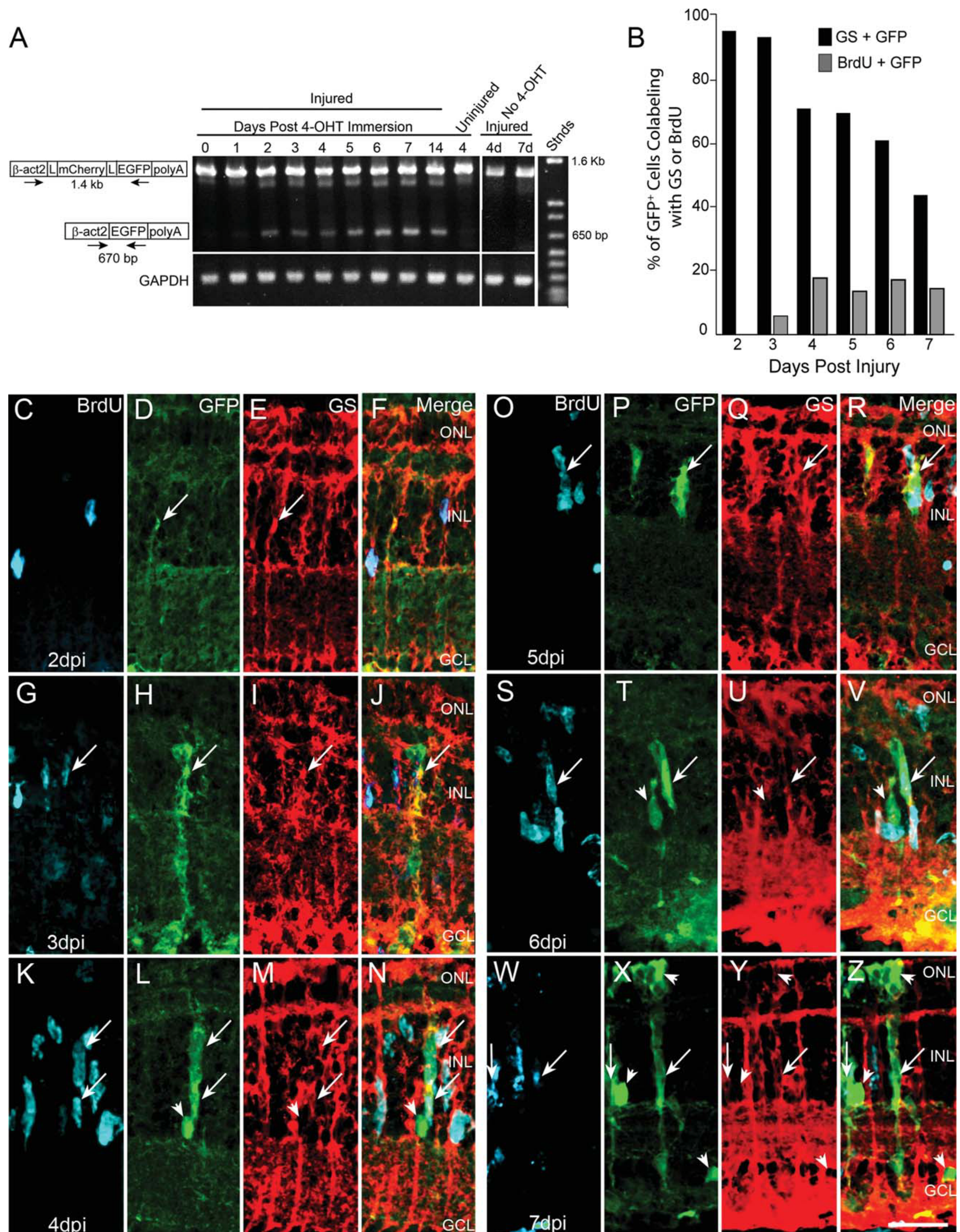

Figure 6

most active in Müller glia-derived progenitors that are entering their last cell division and preparing to differentiate. Because we are selecting very low-expressing *1016 tuba1a:CreER^{T2}* lines to prevent ligand-independent recombination, we may be able to observe recombination only in these late-stage progenitors. Consistent with this idea is our observation that only 5–10% of the BrdU$^+$ cells in an injured retina express GFP at 4 days postinjury. In contrast, most BrdU$^+$ cells express GFP in injured retinas of *1016 tuba1a:GFP* fish (Fausett and Goldman, 2006), which likely reflects selection for high promoter activity that results in GFP expression at early and late progenitor stages.

To determine whether *tuba1a* expressing progenitors contribute to regeneration of mature retinal neurons and glia following retinal injury, we took advantage of our *CreER^{T2};LCLG* fish to label the *tuba1a*-expressing Müller glia permanently with GFP. We also labeled dividing cells by injecting BrdU IP into fish on day 4 postinjury, when cell proliferation is maximal (Fausett and Goldman, 2006). At 10 days to 2.5 months postretinal injury, fish were killed and retinas were sectioned and stained for GFP, cell-type specific markers, and BrdU and analyzed by confocal microscopy. GFP expression serves as a lineage tracer for descendents of *tuba1a*-expressing progenitors; cell type-specific antibodies allow for identification of individual retinal cell types, and BrdU-labeling confirms that cells were derived from cycling Müller glia-derived progenitors. We found that *tuba1a*-expressing progenitors gave rise to photoreceptors in the ONL that expressed the Zpr1marker of red/green cones (Fig. 7A–D) and also gave rise to Müller glia (Fig. 7I–L), bipolar cells (Fig. 7E–H), and amacrine cells (Fig. 7M–P) that reside in the INL and were identified by the coexpression of cell-specific markers GS, PKC, and HuC/D, respectively. At 10 days postinjury, when newly differentiating cells can still be identified, we found that *tuba1a*-expressing progenitors gave rise to retinal ganglion cells expressing the developmental marker Zn5 (Fig. 7Q–T).

DISCUSSION

The *tuba1a* promoter has traditionally been viewed as a neural-specific promoter that is induced during nervous system development and differentiation (Heiber et al., 1998; Goldman et al., 2001). However, this promoter is also active in adult neural progenitors lining brain ventricles, the central canal of the spinal cord, and the periphery of the retina (Goldman et al., 2001). In the adult retina, the *tuba1a* promoter is activated, following injury, in Müller glia-derived retinal progenitors (Fausett and Goldman, 2006). These progenitors are thought to be responsible for regenerating a damaged retina in zebrafish. In mammals, Müller glia are much more restricted in their response to retinal injury; regenerating only a very limited number of retinal cell types with very low frequency even when stimulated by growth or differentiation factors (Ooto et al., 2004; Karl et al., 2009).

To visualize these *tuba1a*-expressing cells better during CNS development and regeneration, we previously created transgenic fish harboring the *1016 tuba1a:GFP* transgene (Fausett and Goldman, 2006). This transgene contains a fragment of the *tuba1a* promoter that appears to restrict transgene expression to the nervous system during development and to Müller glia-derived progenitors in the adult injured retina. Although *1016 tuba1a:GFP* transgenic fish have facilitated our studies of CNS development and regeneration by marking Müller glia-derived retinal progenitors in the injured retina, they do not allow us to follow the fate of these progenitors, because transgene expression is extinguished as they differentiate.

To facilitate visualization of *tuba1a* progenitors and their fate, we generated double transgenic fish harboring *1016 tuba1a:CreER^{T2}* and *β-actin2:LCLG* transgenes (Fig. 1). These fish allow us to take advantage of the power of conditional gene expression and recombination to label, specifically and permanently, *tuba1a*-expressing cells in the developing and adult CNS. Recombination during early stages of development showed that *tuba1a*-expressing progenitors contribute not only to the nervous system

Figure 6. Time course of 4-OHT-dependent recombination in the adult injured retina. **A:** Adult *CreER^{T2};LCLG* transgenic fish were anesthetized, and the right retina was injured by a needle poke. Twenty hours later, fish were immersed in 4-OHT for the indicated times prior to harvesting retina for RT-PCR. The illustration at left shows the expected sizes of PCR products using the indicated forward and reverse primers (arrows). Shown at right is an agarose gel of PCR-amplified products corresponding to the unrecombined (top band) and recombined (bottom band) message. **B:** Quantification of the percentage of GFP$^+$ cells that is also GS$^+$ or BrdU$^+$ at various times following retinal injury. Adult *CreER^{T2};LCLG* transgenic fish retinas were injured and harvested at the indicated days postinjury (dpi). Fish harvested at 2–4 days postinjury received an IP injection of BrdU 3 hours before being killed, whereas fish harvested at 5–7 days postinjury received an IP injection of BrdU at 4 days postinjury. Retinas were isolated, fixed, and sectioned for GFP, glutamine synthetase (GS), and BrdU immunofluorescence detection. **C–Z:** Representative confocal images used for the quantification reported in B. Recombination was restricted to Müller glia (arrows) at early times following retinal injury, and at later times recombination was detected in Müller glia (arrows) and other retinal cell types (arrowheads) found in all three retinal cell layers. BrdU, 5-bromo-2′-deoxyuridine; GFP, green fluorescent protein; GS, glutamine synthetase; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

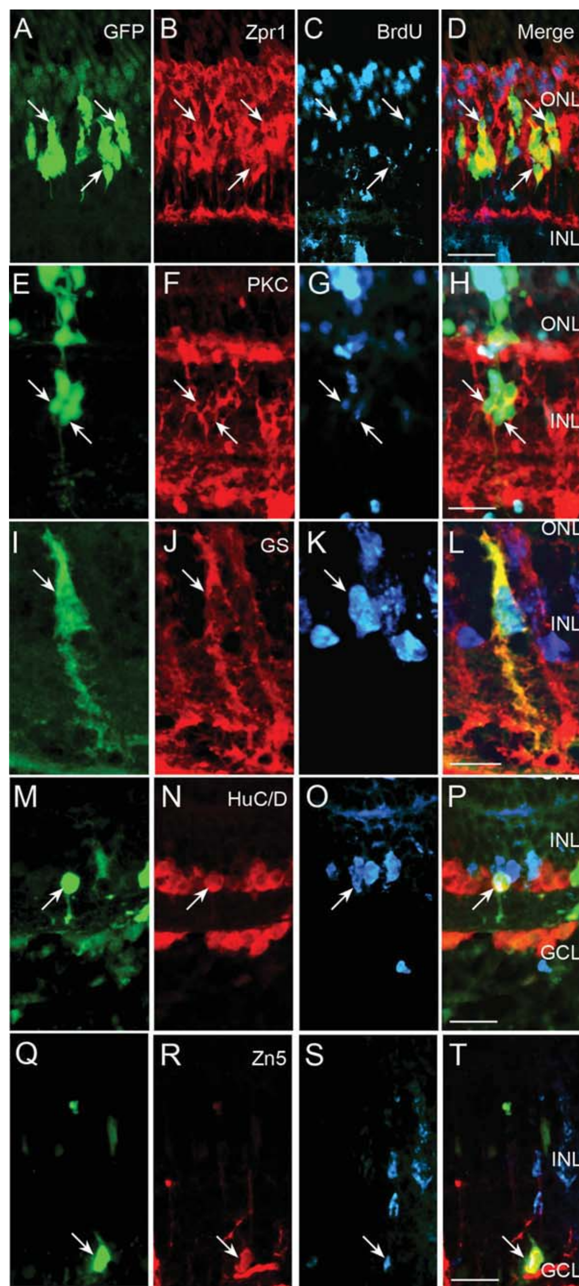

Figure 7. A–T: *tuba1a*-Expressing progenitors regenerate retinal neurons and glia. Adult double transgenic fish retinas were injured by a needle poke, and then fish were exposed to 4-OHT either by immersion or by direct injection into the eye. On day 4 postinjury, fish were injected intraperitoneally with BrdU and allowed to survive for 1–2.5 months. Retinas were then harvested, sectioned, and stained with anti-GFP, anti-cell-type-specific (zpr1 for photoreceptors in the ONL, GS for Müller glia, PKC for bipolar cells, HuC/D for amacrine cells in the INL, and zn5 for ganglion cells in the GCL), and anti-BrdU antibodies. Sections were analyzed by using confocal microscopy. Arrows point to triple-labeled cells. BrdU, bromodeoxyuridine; GFP, green fluorescent protein; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars = 20 μm in D (applies to A–D); 20 μm in H (applies to E–H); 15 μm in L (applies to I–L); 20 μm in P (applies to M–P); 20 μm in T (applies to Q–T). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

but also to heart, skeletal muscle, and intestine (Fig. 2). This was a surprising result, in that previous analysis of *1016 tuba1a:GFP* transgenic fish suggested that this promoter was restricted to neural tissue (Fausett and Goldman, 2006). We reason that this nonneural expression escaped detection in previous studies because the *tuba1a* promoter is expressed transiently and at a low level in cells that contribute to development of heart, skeletal muscle, and intestine. In contrast, even very low levels of CreER^{T2} expression in *1016 CreER*^{T2}*;LCLG* fish that were selected for weak expression would stimulate recombination and maintain strong GFP expression driven by the β-actin promoter and thus facilitate visualization of these nonneural cells.

By immersing embryos in 4-OHT at different times during development, we were able to identify a developmental window (13–24 hpf) when this nonneural expression was occurring (Fig. 2). In situ hybridization assays confirmed our lineage tracing studies and demonstrated very low, but detectable, expression of the endogenous *tuba1a* gene in these nonneural tissues at 24 hpf (Fig. 3). It is interesting that an α-tubulin isoform, like *tuba1a*, that is predominantly expressed in the developing nervous system is also expressed in a limited number of nonneural tissues during early development. Although the reason for the expession of different tubulin isoforms in different tissues is not known, our data suggest that the unique function it imparts to the developing nervous system might also be shared with cells contributing to heart, skeletal muscle, and intestine. These studies also indicate that the conditional recombination system used here is a very sensitive readout of gene expression that allows one not only to trace cell lineages but also to define developmental windows of gene expression that can be visualized in live animals. Finally, our studies suggest that the *1016 tuba1a* promoter can be used to label or manipulate these nonneuronal progenitors and their progeny by using conditional gene expression.

Adult *1016 tuba1a:GFP* fish express GFP in neuromasts of the lateral line, olfactory pits, and restricted brain regions. As expected, a similar pattern of expression is observed following immersion of *CreER*^{T2}*;LCLG* fish in 4-OHT (Fig. 4). BrdU labeling and immunohistochemistry of sectioned material showed that, in the lateral line and olfactory epithelium, recombination is restricted to nondividing sensory cells (Fig. 4G,H).

We previously showed that, in *1016 tuba1a:GFP* fish, retinal injury transiently induces GFP expression in a cycling population of Müller glia-derived retinal progenitors (Fausett and Goldman, 2006). However, because GFP expression is transient in these fish, we were not able to demonstrate directly that Müller glia expressing the *1016 tuba1a* promoter were the same cells that regenerated

the retina following injury. This is also true for other transgenic models using promoters that are active either in Müller glia or retinal progenitors but shut off in differentiated retinal neurons (Fausett and Goldman, 2006; Bernardos et al., 2007; Fimbel et al., 2007; Thummel et al., 2008). Thus, there were no lineage tracing studies demonstrating that Müller glia-derived retinal progenitors gave rise to retinal neurons or glia that integrate into the retinal architecture and were stably maintained. To address this issue, we created *1016 CreER^{T2};LCLG* fish to label Müller glia-derived retinal progenitors in the injured retina permanently.

Analysis of recombination at 2–3 days postretinal injury indicated that 94% of the recombined GFP^{+} cells could be identified as Müller glia, whereas the remainder were identified as amacrine cells (Supporting Information Table 1). We never detected GFP expression in amacrine cells following retinal injury in our *1016 tuba1a:GFP* transgenic fish, suggesting that *1016 tuba1a* promoter activity may be too low and/or transient to drive enough GFP expression for detection. However, this low and transient promoter activity may be sufficient to induce recombination in *1016 CreER^{T2};LCLG* fish, which, after recombination, would express GFP constitutively from the strong β-*actin* promoter and therefore allow easy detection. Thus, similarly to what we reported in our lineage tracing experiments during development, the recombination system allows for a more sensitive detection of low and transient *1016 tuba1a* promoter activity that may be missed by using more traditional transgenic approaches. Although this low level of recombination in amacrine cells precluded us from using solely recombination to trace the amacrine lineage, we were able to combine BrdU labeling with lineage tracing to show clearly that *tuba1a*-expressing Müller glia-derived progenitors regenerate amacrine cells, along with most other retinal cell types (Fig. 7).

Quantification of recombination and cell proliferation at 4 days postinjury suggested that only 5–10% of the BrdU^{+} cells underwent recombination in *CreER^{T2};LCLG* fish, whereas the majority of BrdU^{+} cells express GFP in *1016 tuba1a:GFP* fish (Fausett and Goldman, 2006). This latter observation suggests that the *1016 tuba1a* promoter is normally active in the proliferating cell population, but the former data suggest that its highest activity is restricted to a subpopulation of proliferating cells. Indeed, a BrdU pulse/chase experiment suggested that these cells are late-stage retinal progenitors that are undergoing their final divisions (Fig. 6B). Thus, by selecting a very weakly expressing *1016 tuba1a:CreER^{T2}* line to prevent basal ligand-independent recombination, we might have restricted recombination to these late-stage retinal progenitors. In addition, recombination itself may be reduced by low-level CreER^{T2} expression as reported for mice (Feil et al., 1996).

With regard to the transgenic lines that we generated, our highly and ubiquitously expressing β-*actin2:LCLG* line should provide investigators with a universal reporter for Cre activity, and our analysis of different *1016 tuba1a:-CreER^{T2}* lines suggests that only low CreER^{T2}-expressing lines will be useful for conditional expression experiments. This latter observation, which is similar to what we found in transfected tissue culture cells, suggests that CreER^{T2} may escape sequestration when expressed at high levels. Indeed, CreER sequestration may be enhanced and ligand-independent recombination reduced by appending an additional ER^{T2} domain (Matsuda and Cepko, 2007; Boniface et al., 2009).

One goal of generating a conditional gene expression system using CreER^{T2}/LoxP technology, in addition to lineage tracing, is to be able to induce genes in a specific cell type at any time during an animal's life cycle and examining the consequence of that gene on cell function. The conditional recombination system described here is ideally suited for this purpose. By choosing appropriate promoters to drive CreER^{T2} expression and tagging the conditionally expressed protein (for example, generating fusions with fluorescent proteins), one can direct expression to any cell type at any time of development and follow the cell's fate. Because of the mosaic nature of the conditional recombination system, one should be able to follow these cells and compare them with their normal, genetically unmodified neighbors for changes in behavior and function.

In conclusion, we have shown that the CreER^{T2}/LoxP system applied to zebrafish allows conditional gene expression and lineage tracing in developing and adult zebrafish. We used this system to identify the fate of *tuba1a*-expressing cells during development and during retinal regeneration. Our data suggest that, during development, *tuba1a*-expressing progenitors contribute not only to neural tissue but also to heart, muscle, and intestine. For the adult injured retina, our data suggest that *tuba1a*-expressing Müller glia-derived progenitors are responsible for regenerating most retinal neurons and glia and that these cells are stably integrated into the retinal architecture. Conditional gene expression and lineage tracing in zebrafish will open up new avenues for studying development and regeneration.

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