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Cre-inducible Site-specific Recombination in Zebrafish Oligodendrocytes

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Background: The conditional Cre/lox system has recently emerged as a valuable tool for studies on both embryonic and adult Zebrafish. Temporal control and site-specific recombination are achieved by using the ligand-inducible CreER^{T2} and administration of the drug tamoxifen (TAM) or its active metabolite, 4-Hydroxytamoxifen (4-OHT). Results: Here we report the generation of a transgenic Zebrafish line, which expresses an mCherry-tagged variant of CreER^{T2} under the control of the *myelin basic protein a* (*mbpa*) promoter. Our analysis shows that larval and adult expression of the transgene recapitulates the endogenous *mbpa* expression pattern in oligodendrocytes. Furthermore, combination with a Cre-dependent EGFP reporter results in EGFP-expressing oligodendrocytes in the spinal cord, brain, and optic nerve in TAM- or 4-OHT-treated larvae and 4-month-old fish, but not in untreated controls. Conclusions: The transgenic Zebrafish line *Tg(mbpa:mCherry-T2A-CreER^{T2})* elicits *CreER^{T2}* expression specifically in myelinating glia cells. Cre-inducible targeted recombination of genes in oligodendrocytes will be useful to elucidate cellular and molecular mechanisms of myelination in vivo during development (myelination) and regeneration (remyelination) after injury to the central nervous system (CNS). It will also allow targeted expression and overexpression of genes of interest (transgenes) in oligodendrocytes at defined developmental and adult stages. *Developmental Dynamics 246:41–49, 2017*. © 2016 Wiley Periodicals, Inc.

Key words: oligodendrocytes; Cre/lox system; myelin basic protein promoter; glia cell-specific recombination; transgenic Zebrafish; myelination

Background

Myelination of axons is essential for the differentiation, function, and health of the nervous system (Funfschilling et al., 2012; Nave, 2010; Czopka, 2016). Oligodendrocytes and Schwann cells are responsible for the formation of the myelin sheaths in the central and peripheral nervous systems (CNS and PNS), respectively. These sheaths provide electrical insulation and enable fast saltatory propagation of action potentials (Buttermore et al., 2013), but also provide cues that preserve the integrity of the axons (Pan et al., 2005). Axons do, in fact, degenerate when losing oligodendrocyte support, which is—among other factors—a crucial issue in multiple sclerosis (MS) (Franklin, 2002; Compston and Coles, 2008).

Zebrafish has emerged as a cost-efficient model to study vertebrate myelination in vivo (Buckley et al., 2010; Preston and Macklin, 2015; Czopka, 2016) and is particularly interesting and valuable due to its remarkable ability to regenerate injured axons in the CNS (Stuermer et al., 1992; Bernhardt et al., 1996; Welte et al., 2015). Remyelination is one important aspect of axonal regeneration and repair, and elucidating the underlying mechanisms is crucial for understanding regeneration in the CNS and PNS. Expression of genes associated with the formation of the myelin sheath, namely the *myelin basic protein a* (*mbpa*) and *myelin protein zero* (*mpz*, formerly known as P0), starts in Zebrafish at 2 days post-fertilization (dpf) in the ventral hindbrain, as seen by in situ hybridization (Brösamle and Halpern, 2002). Late in embryonic development, the first myelin sheaths around the Mauthner axon define the onset of myelination by 60 hr post-fertilization (hpf) (Almeida et al., 2011). Myelination spreads in the course of the following days into the spinal cord and rostrally to the midbrain and optic nerve. By 3 dpf, loose myelin wraps can also be found around lateral line axons in the PNS, and on ventral axons neighboring the Mauthner axons (Buckley et al.,

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2010). Myelination continues on more dorsally located spinal cord axons (Almeida et al., 2011; Buckley et al., 2010). Myelinating cells were reported to differentiate over a period of 1 month (Park et al., 2007), and myelination continues into adulthood (Jung et al., 2010). Collectively, it is important to label and follow myelinating glial cells in order to better understand the dynamics of myelin formation and remyelination in axonal regeneration in both the CNS and PNS.

New technologies suited to alter gene expression in vivo are making molecular manipulation of target genes and molecules in developing embryos relatively straightforward. For instance, transgenic lines expressing fluorescent proteins under the control of the *oligodendrocyte lineage transcription factor 2* (*olig2*), *SRY (sex determining region Y)-box 10* (*sox10*), or *mbpa* promoters have been used to study oligodendrocyte/Schwann cell differentiation and myelination in the Zebrafish larvae in vivo (Shin et al., 2003; Jung et al., 2010; Gfrerer et al., 2013; Czopka et al., 2013; Almeida et al., 2011). Although these lines have elucidated many aspects of the myelination process, lack of inducible transgenic models is becoming a bottleneck for lineage tracing or remyelination relevant research. Furthermore, methods to conditionally activate transgenes have been adapted to Zebrafish, such as the yeast GAL4/UAS system, where the Gal4 protein is expressed under the control of cell-specific promoters, which in turn activates a UAS cassette driving expression of a desired protein only in a specific population of cells (Halpern et al., 2008). Recently, Cre/lox-mediated systems using heat-shock promoters to drive activation of transgenic constructs permitted a more precise cell-specific and temporal control of gene expression. Temporal control of transgene expression has been successfully achieved by using the ligand-inducible CreER^{T2} (Hans et al., 2009). Specific recombination occurs only upon administration of the drug tamoxifen (TAM) or its active metabolite, 4-Hydroxytamoxifen (4-OHT).

In this study, we generated a transgenic Zebrafish line that expresses a bicistronic mRNA coding for mCherry and CreER^{T2} separated by a viral T2A peptide sequence (Provost et al., 2007) under the control of the *mbpa* promoter. The use of the viral T2A peptide allows equimolar production of mCherry and CreER^{T2} from a single open reading frame in oligodendrocytes in *Tg(mbpa:mCherry-T2A-CreER^{T2})*. Treatment of double-transgenic Zebrafish larvae, carrying the driver *Tg(mbpa:mCherry-T2A-CreER^{T2})* and effector *Tg(hsp70:loxp-DsRed-loxp-EGFP)* alleles, with TAM resulted in selective recombination in cells expressing the mCherry-T2A-CreER^{T2} gene cassette.

This line provides a novel powerful tool to understand mechanisms underlying myelination or turnover of myelinating cells in vivo, to study the fate of oligodendrocytes after lesion as well as axon-oligodendrocyte interactions and can be used for targeted transgene expression.

Results

The *mbpa* Promoter Drives the Expression of mCherry and CreER^{T2} in Myelinating Cells

In order to achieve spatially controlled expression of the CreER^{T2} recombinase, a 2kb fragment containing the 5'-upstream region of the *mbpa* start codon that has previously been shown to recapitulate the *mbpa* expression (Jung et al., 2010) was cloned into the pTol: mCherry-T2a-CreER^{T2} vector (Hans et al., 2009) (Fig. 1A). The resulting plasmid was injected into one-cell stage Zebrafish embryos along with transposase mRNA (Urasaki et al., 2006). Injected embryos were raised to adulthood, crossed with wild-type fish, and the progeny were analyzed for mCherry expression under a confocal microscope. In total, seven founder fish were identified with offspring expressing mCherry in cells with an oligodendrocyte-like morphology. Animals expressing mCherry were selected, raised to adulthood, and further analyzed in the F2 generation.

Consistent with previously published data on *mbpa* expression in Zebrafish larvae (Brösamle and Halpern, 2002; Jung et al., 2010), mCherry^{+} cells display an oligodendrocyte-like morphology and were predominantly detected in the hindbrain and spinal cord of 4-dpf (Fig. 1B–D) and 6-dpf (Fig. 1F–H) larvae. Analysis of the spinal cord and hindbrain at high resolution showed native mCherry fluorescence in cell bodies as well as processes, e.g., around the hindbrain commissural axons (Fig. 1E,I), as previously described (Brösamle and Halpern, 2002).

Tg(mbpa:mCherry-T2A-CreER^{T2}) Recapitulates the Endogenous *mbpa* Expression at Larval and Adult Stages

To confirm that the observed mCherry expression corresponds to the endogenous *mbpa* expression pattern, 6-dpf *Tg(mbpa:mCherry-T2A-CreER^{T2})* larvae were stained with anti-MBP and anti-mCherry antibodies, showing coexistence of mCherry + cells with an oligodendrocyte-like morphology in MBP-expressing regions of the hindbrain (Fig. 2A,B).

To further corroborate that *Tg(mbpa:mCherry-T2A-CreER^{T2})* faithfully recapitulates *mbpa* expression, we compared mCherry expression with EGFP in *Tg(mbpa:egfp)*, an established reporter line employing the same *mbpa* promoter fragment (Jung et al., 2010), To this aim, we crossed carriers of *Tg(mbpa:mCherry-T2A-CreER^{T2})* and *Tg(mbpa:EGFP)* and analyzed mCherry and EGFP expression in double-transgenic progeny at 6 to 9 dpf. Though *Tg(mbpa:EGFP)* and *Tg(mbpa:mCherry-T2a-CreER^{T2})* lines employ the same promoter fragment, they differ in expression strength, most likely due to different integration sites within the Zebrafish genome. EGFP expression is much stronger, and we found that 94.4% of all EGFP-positive cells co-express mCherry. In the remaining 5.6%, we suspect that mCherry signal was too weak to be detected (n = 26 larvae, 1021 oligodendrocytes were analyzed). It is important to note that we never detected mCherry-positive cells that were not EGFP-positive, indicating that no ectopic mCherry expression is present. Both reporter genes (EGFP and mCherry) were expressed in oligodendrocytes and were abundant in the ventral hindbrain and the ventral spinal cord compared to the dorsal part at 7 dpf (Fig. 2C,D). Similar reporter expression could be found in the myelinated fiber tracts of the ventromedial hindbrain bundle and hindbrain commissural axons.

In addition to the expression in the CNS, *Tg(mbpa:EGFP)* also showed EGFP expression in myelinating Schwann cells. However, Jung et al. observed that the signal was weaker than in oligodendrocytes, and that EGFP-expressing cells could not always be found in the PNS (Jung et al., 2010). Consistent with that, no native mCherry fluorescence was found in the PNS. It is also possible that the established *Tg(mbpa:mCherry-T2a-CreER^{T2})* lines are CNS-specific because founders were selected for mCherry expression at 4 dpf without any selective bias to CNS or PNS.

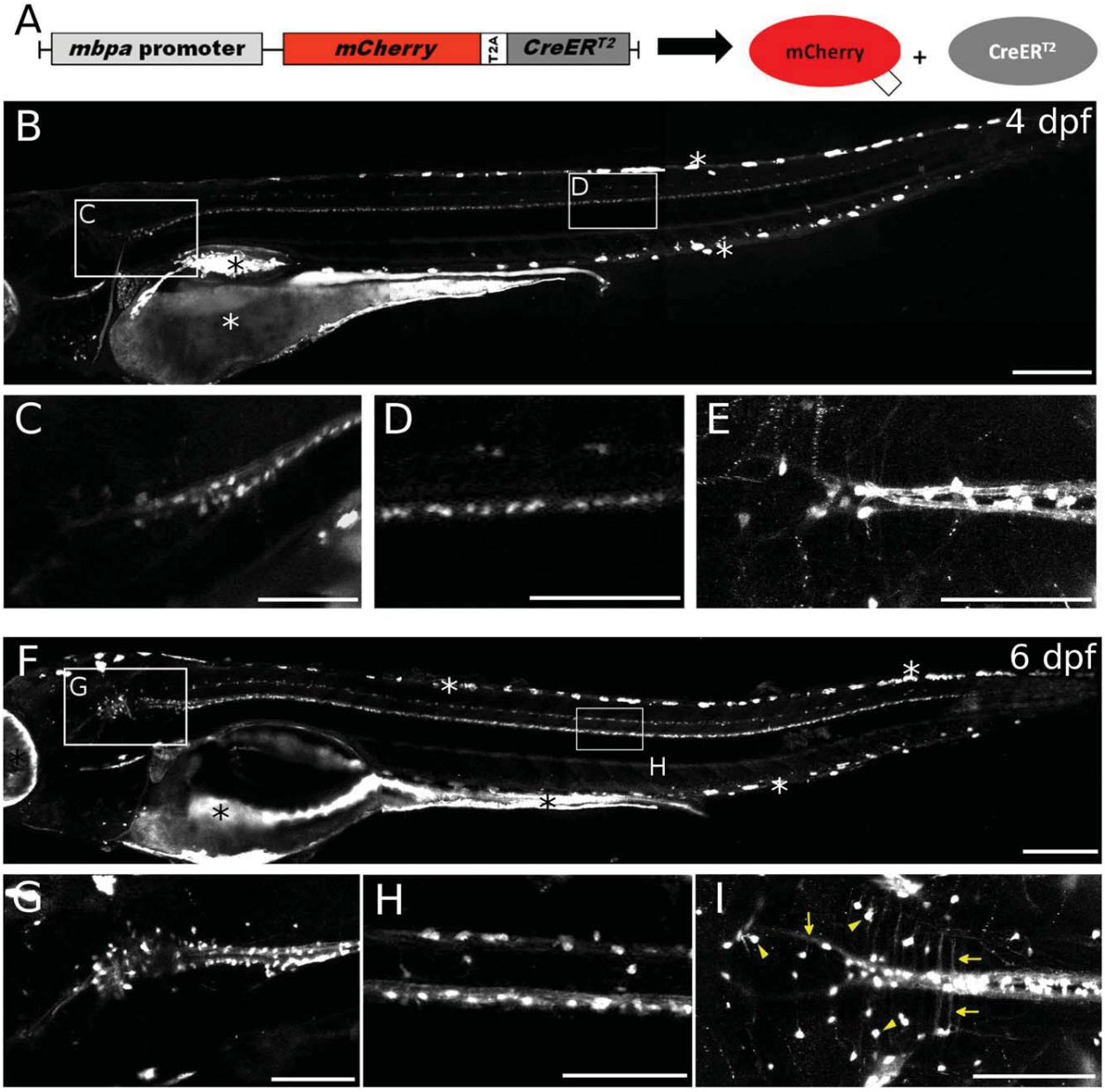

Fig. 1. mCherry expression in 4- and 6-dpf *Tg(mbpa:mCherry-T2A-CreER^{T2})* larvae. **A**: Scheme of the *Tg(mbpa:mCherry-T2A-CreER^{T2})* construct, which expresses a single open reading frame coding for mCherry (red) and CreER^{T2} (dark gray) separated by a viral T2A peptide sequence (white) under the control of the Zebrafish *mbpa* promoter (light gray). **B–I**: Native mCherry fluorescence in 4-dpf (B–E) and 6-dpf (F–I) transgenic Zebrafish larvae. B,F: Lateral view of the larvae, showing mCherry expression in presumptive myelinating cells. Scale bar, 200 μm. C,D,G,H: Higher magnification of the areas indicated by white boxes in B,F. C,G: mCherry expressing oligodendrocytes in the hindbrain and (D,H) the spinal cord. E,I: Dorsal view of the hindbrain with mCherry fluorescence in presumptive oligodendrocyte cell bodies (arrowhead) and processes (arrow). Asterisks mark auto fluorescence from yolk and pigmentation. Scale bar, 100 μm.

However, at 4 dpf, native mCherry expression was predominantly seen in the parts of the CNS.

In cross-sections of adult transgenic fish (Fig. 2E), mCherry + cells were present in the MBP + regions of the optic tract and the optic tectum (Fig. 2F,G), as well as in the optic nerve leaving the retina (Fig. 2H,I), consistent with myelinated areas as previously described (Jung et al., 2010). Remarkably, mCherry expression was always restricted to myelinated areas, indicating that the *mbpa* promoter fragment faithfully recapitulates endogenous *mbp* expression in the adult fish as well. However, MBP staining was much more widespread than mCherry, which was mostly confined to cell somata. This discrepancy is probably due to local translation of *mbpa* mRNA in oligodendrocyte processes where the protein is needed, as previously described (Lyons et al., 2009; Colman et al., 1982; Müller et al., 2013), whereas mCherry, a cytosolic protein, is not specifically transported to the processes and therefore predominantly accumulated in cell body. Taken together, *Tg(mbpa:mCherry-T2A-CreER^{T2})* is selectively expressed by myelinating cells and is well suited to follow myelin-expressing cells over time.

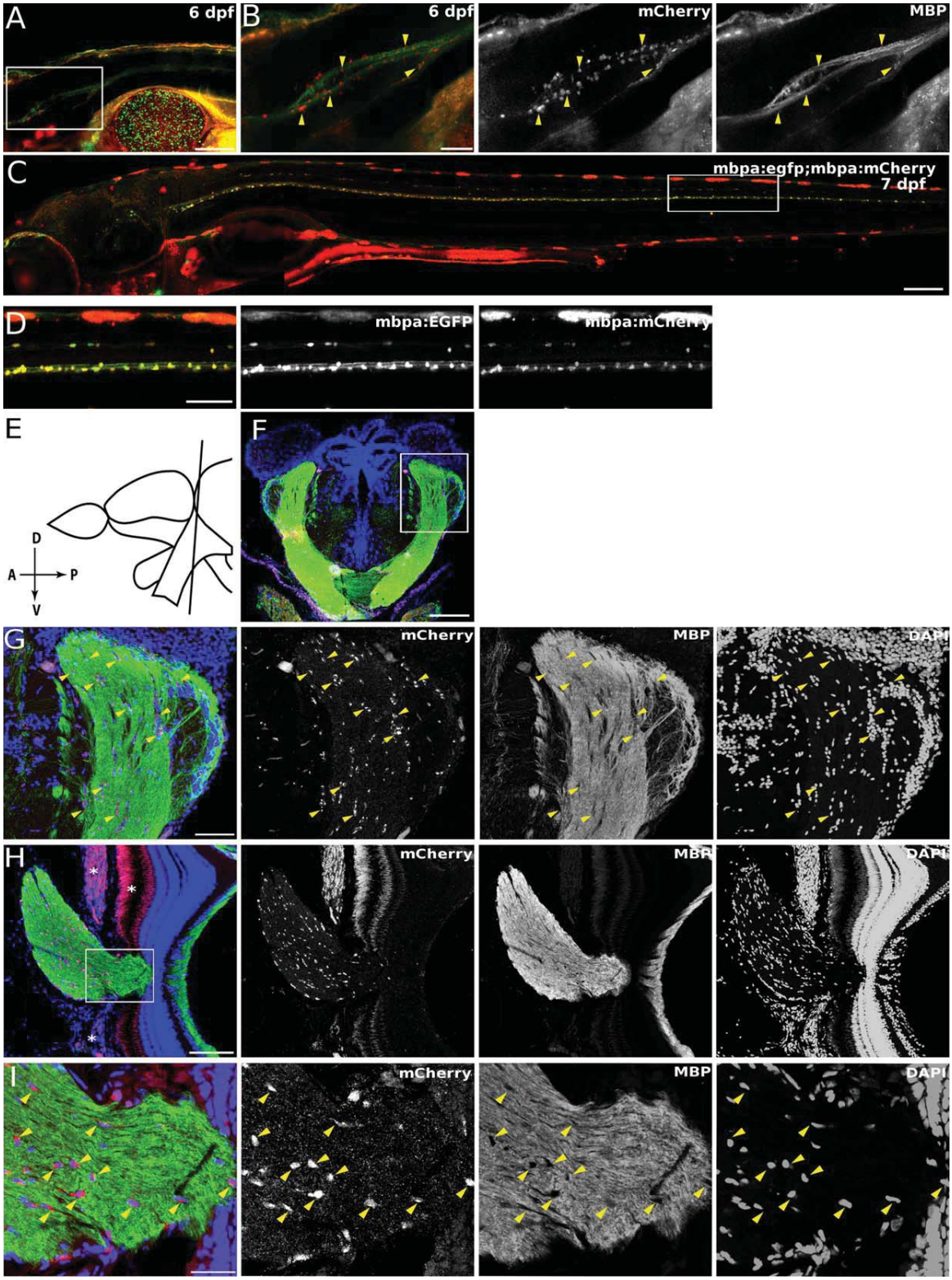

Fig. 2. The *mbpa* promoter recapitulates expression in mature oligodendrocytes in the larval and adult Zebrafish CNS. **A**: 6-dpf larvae from the *Tg(mbpa:mCherry-T2A-CreER^{T2})* line stained with antibodies against mCherry and MBP show coexistence of mCherry + cells (red) in MBP + regions (green), suggesting mature oligodendrocytes. Scale bar, 100 μm. **B**: Higher magnification of the area depicted (white rectangle) in A. Yellow arrowheads mark mCherry-positive oligodendrocytes located in MBP-expressing regions. Scale bar, 50 μm. **C**: Double-transgenic *Tg(mbpa:mCherry-T2A-CreER^{T2}); Tg(mbpa:EGFP)* at 7 dpf show co-localization of EGFP and mCherry expression in oligodendrocytes in the spinal cord and hindbrain. **D**: Higher magnification of the area encircled in C showing EGFP/mCherry-positive oligodendrocytes. **E**: Scheme of cross-sections of the diencephalon with optic nerve/tract in adult Zebrafish brain as shown in F and G. **F**: Expression of mCherry in oligodendrocytes of the optic nerve/tract shown by IHC against mCherry (red) and MBP (green) in *Tg(mbpa:mCherry-T2A-CreER^{T2})* adult fish. Scale bar, 100 μm. **G**: Higher magnification of the area depicted (white rectangle) in F showing mCherry-expressing cells (yellow arrowheads) located within the MBP-expressing optic tract. Yellow arrowheads point to oligodendrocytes. Scale bar, 50 μm. **H**: Cross-section of the retina with the exit point of the optic nerve immunostained for mCherry (red) and MBP (green), showing mCherry-expressing cells within the MBP-expressing processes in the optic nerve as shown in individual panels. Asterisks mark unspecific auto fluorescence of the retinal pigment epithelium, photoreceptors, and extra-retinal tissue. Scale bar, 75 μm. **I**: Higher magnification of the area depicted (white rectangle) in H showing presumptive oligodendrocytes expressing mCherry (yellow arrowheads) and located within the MBP staining along axons of the optic nerve. DAPI stains all nuclei. Scale bar, 25 μm.

Conditional Recombination of Mature Oligodendroglia upon Tamoxifen Treatment in Both Larvae and Adult

In order to test functionality, *Tg(mbpa:mCherry-T2A-CreER^{T2})* was crossed with the Cre-dependent reporter line *Tg(hsp70l:loxP-DsRed-loxP-EGFP)*, which expresses DsRed2 under the control of the ubiquitous, temperature-inducible *hsp70l* promoter, but switches permanently to EGFP after a successful recombination event (Kroehne et al., 2011). In 5-dpf larvae, recombination was induced by a single overnight treatment with 5 μM 4-OHT. Expression of DsRed and EGFP 1 day after heat shock showed that recombination specifically occurred only in oligodendrocyte-like cells. EGFP expression strongly resembled mCherry in oligodendrocyte-like cells (Fig. 3C–F). Although native mCherry fluorescence was not observed in the lateral line of the PNS, recombined EGFP positive cells could be detected in the anterior and posterior lateral line (Fig. 3G,H). This is consistent with *mbpa* expression in Schwann cells of the PNS (Brösamle and Halpern, 2002), indicating that the *mbpa* promoter fragment drives CreER^{T2} expression to some extent in Schwann cells. However, recombination in the PNS was mosaic and occurred only sporadically. Furthermore, no non-conditional recombination was observed in double-transgenic control fish treated with ethanol and heat shock only (Fig. 3I,J).

In order to measure the recombination efficiency, we quantified the number of mCherry + cells before recombination and the number of EGFP + cells after recombination in two different areas of the CNS (300 μm long, see evaluation of recombination efficiency) of the ventral spinal cord and hindbrain of untreated control fish (5 dpf, n = 20) and 4-OHT-treated siblings (7 dpf, n = 20) followed by a heat shock. In controls, on an average, 31.9 mCherry + cells were found in the regions mentioned, while the corresponding areas of 4-OHT-treated fish, showed 29.6 EGFP + cells, indicating high recombination efficiency in approximately 92.8% of cells expressing mCherry and CreER^{T2} at larval stages.

To evaluate the functionality of the line for pulse-chase experiments, we treated 5-dpf double-transgenic larvae with 4-OHT overnight and performed a heat shock the next day. EGFP-positive fish were selected and reexamined at 15 dpf. Without further 4-OHT treatment, a second heat shock was applied to activate reporter expression. Using this paradigm, EGFP-expressing oligodendrocytes could be identified, indicating that cells survived after recombination (data not shown). Cell death, however, was not evaluated.

Finally, we also examined the functionality of *Tg(mbpa:mCherry-T2A-CreER^{T2})* at adult stages. In double-transgenic animals, recombination was induced at the age of ~6 months by overnight treatment with 2.5 μM TAM on three consecutive nights (Fig. 4A). Following two heat shocks with a 1-day interval, animals were sacrificed and analyzed for EGFP fluorescence. Co-staining with anti-MBP antibody showed that recombined cells with strong EGFP expression can be found in myelinated areas of the telencephalon, optic tract, diencephalon, and the optic nerve exiting the retina (Fig. 4B–F). The EGFP signal co-localized with the anti-MBP, especially in cellular processes. Similar to the observations in the larvae no non-conditional recombination was observed in double-transgenic control animals treated with DMSO and subsequent heat shock, indicating tight regulation of CreER^{T2}.

These findings show that the novel transgenic line is functional at larval and adult stages, and allows conditional recombination specifically in myelin-expressing cells.

Discussion

We have successfully generated a transgenic Zebrafish *mbpa* Cre-driver line *Tg(mbpa:mCherry-T2A-CreER^{T2})*, which expresses CreER^{T2} in myelinating cells of the CNS. The expression pattern of mCherry, as well as that of EGFP of a Cre-dependent reporter after successful recombination, faithfully recapitulates the endogenous expression pattern of *mbpa* at larval and adult stages (Brösamle and Halpern, 2002; Almeida et al., 2011), which we confirmed by co-immunostaining. The observed expression also corresponds to the expression pattern in the earlier *Tg(mbpa:egfp)* line, in which EGFP is driven by the same promoter fragment (Jung et al., 2010).

We further demonstrate that recombination in oligodendrocytes can be achieved at all stages examined, even in adults. Finally, we also find that recombination does not impair oligodendrocytes, and that they can survive transgene expression for extended periods of time.

Recombination in our newly generated *Tg(mbpa:mCherry-T2A-CreER^{T2})* line was closely linked to induction by TAM or 4-OHT treatment, and no unconditional recombination was observed in control groups treated with the solvent only. Also, EGFP expression was found only in myelinating cells, even after a 10-day chase.

In larvae, the percentage of recombined oligodendrocytes in the CNS was very high: The number of EGFP-expressing cells reached 92.8% of mCherry-positive cells in untreated siblings. Despite absence of native mCherry fluorescence in the PNS, some recombination was also detected in Schwann cells. However, recombination was only mosaic and sporadic, indicating that our established *Tg(mbpa:mCherry-T2A-CreER^{T2})* lines are a non-reliable tool to recombine in myelinating cells of the PNS.

Moreover, lower or higher recombination rates can potentially be obtained by shorter or longer 4-OHT/TAM treatments, respectively. Control of the recombination rate can be advantageous for the analysis of individual oligodendrocytes, which can be easily controlled by the dose of the ligand administered (Hans et al., 2009). Hence, using our newly established line *Tg(mbpa:mCherry-T2A-CreER^{T2})*, the fate of single recombined oligodendrocytes can be followed, whereas in *Tg(mbpa:EGFP)* transgenic Zebrafish, it is not possible to observe individual oligodendrocytes in heavily myelinated tracts.

Our line can be used to address questions focussing on oligodendrocyte differentiation. In combination with a reporter line, like *Tg(hsp70:loxp-DsRed-loxp-EGFP)*, our line can be used for cell tracing or cell fate determination of myelinating cells in different contexts. It therefore allows elucidating cellular and molecular mechanisms of myelination in vivo during development (myelination) or after CNS injury (remyelination). In our previous study analyzing the fate of oligodendrocytes in the goldfish visual pathway after optic nerve lesion, we used dye injections (Lucifer yellow) into individual oligodendrocytes in situ at different time points after lesion (Ankerhold and Stuermer, 1999). Based on the morphology of injected cells, we concluded that oligodendrocytes dedifferentiate after axonal lesions and redifferentiate to remyelinate the axons after synaptic connections have been restored in the optic tectum. *Tg(mbpa:mCherry-T2A-CreER^{T2})* will allow us to substantiate the earlier findings and will help to decide if indeed “old” cells form new myelin during axon regeneration (Ankerhold and Stuermer, 1999).

This novel line allows temporally controlled myelin-targeted expression of transgenes mainly in oligodendrocytes and

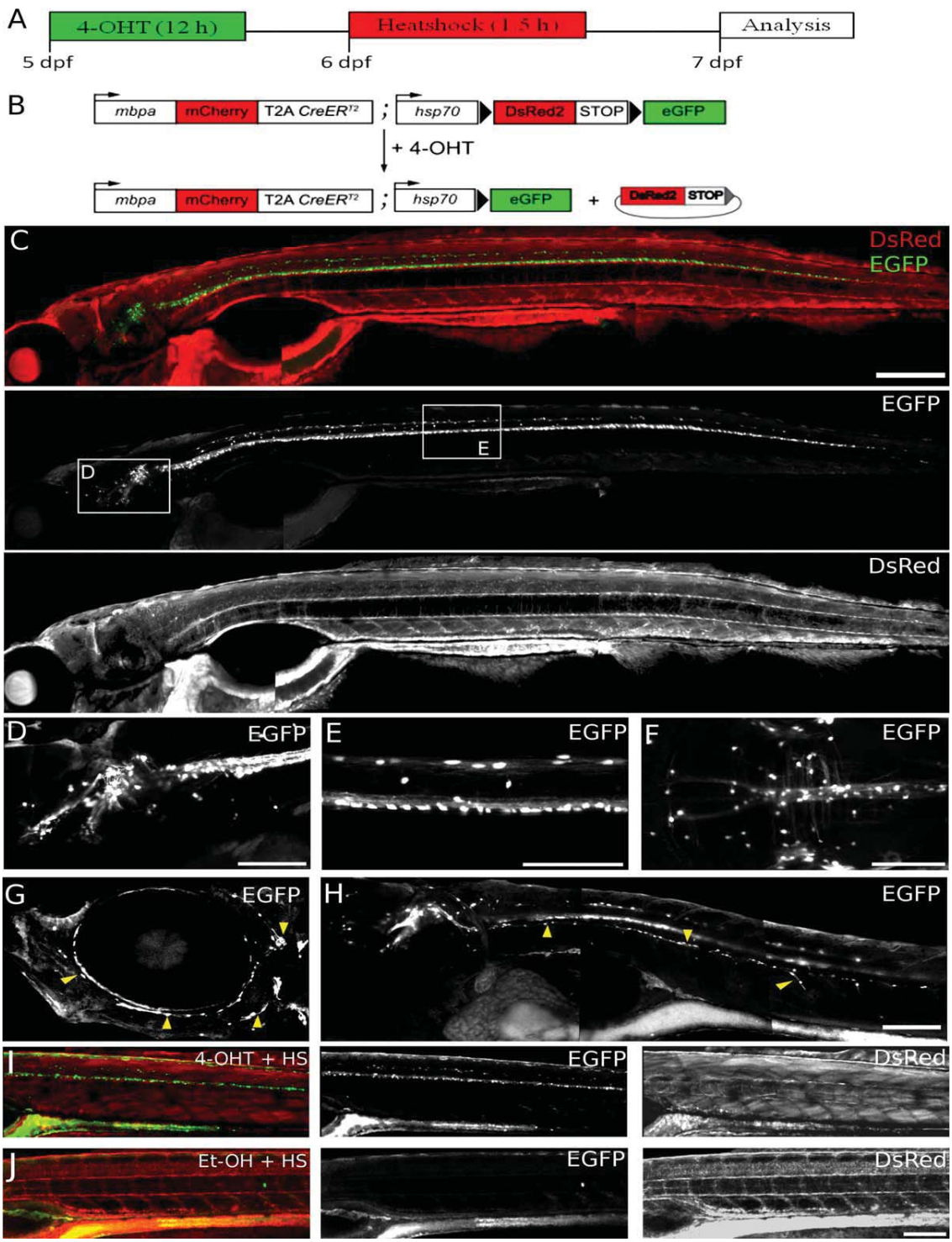

Fig. 3. Conditional CreER^{T2}-mediated recombination in *Tg(mbpa:mCherry-T2A-CreER^{T2})* in 5-dpf larvae. **A**: Schematic representation of treatments applied to achieve conditional CreER^{T2}-mediated recombination with the temperature-inducible, Cre-dependent reporter line *Tg(hsp70l:loxP-DsRed-loxP-EGFP)*. **B**: Scheme of the ligand-dependent recombination event in double-transgenic *Tg(mbpa:mCherry-T2A-CreER^{T2})*, Tg(*hsp70:loxP*-DsRed-*stop-loxP*-EGFP) larvae. Application of TAM results in Tg(*hsp70: loxP*-EGFP) in *mbpa*-expressing cells. **C**: Lateral view of a 7-dpf larva after 4-OHT treatment and heat shock. Recombination is indicated by a switch from red to green fluorescence in cells in the hindbrain and spinal cord. Scale bar, 200 μm. **D,E**: Higher magnification of the areas indicated by white boxes in C showing EGFP-expressing oligodendrocytes. **F**: Dorsal view of the hindbrain with EGFP fluorescence in presumptive oligodendrocyte cell bodies (arrowhead) and processes (arrow). Scale bar, 100 μm. **G,H**: Recombination can also be observed in Schwann cells myelinating the anterior and posterior lateral lines (arrowheads) Scale bar, 200 μm. **I**: Recombination only ever occurred in larvae treated with 4-OHT and HS, but never in control siblings treated with ethanol and HS. Scale bar, 200 μm. **J**: Asterisks mark auto fluorescence from yolk and pigmentation. Scale bar, 200 μm.

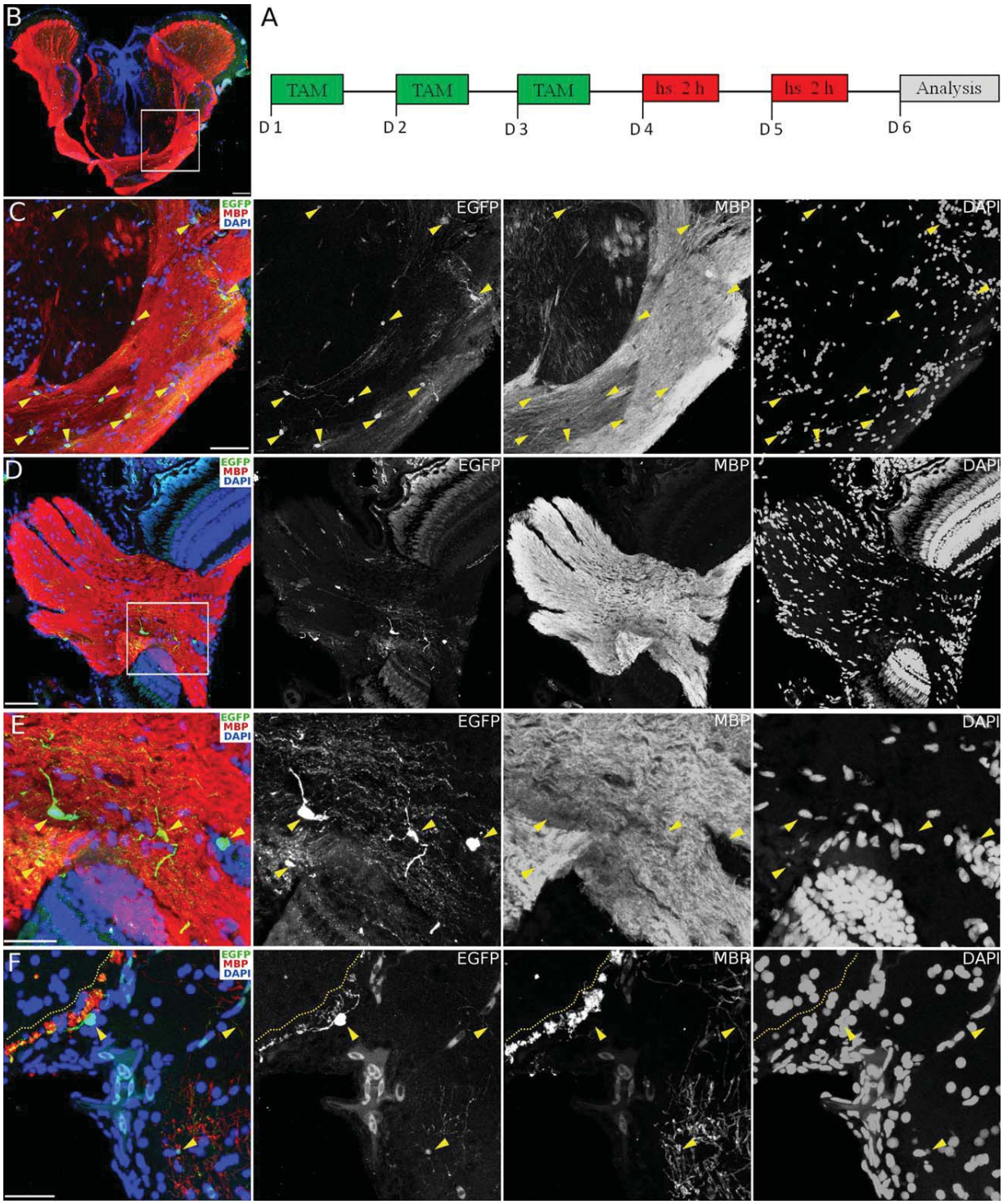

Fig. 4. Conditional CreER^{T2}-mediated recombination in *Tg(mbpa:mCherry-T2A-CreER^{T2})* in the adult Zebrafish CNS. **A**: Schematic representation of treatments applied to achieve conditional CreER^{T2}-mediated recombination with the temperature-inducible, Cre-dependent reporter line *Tg(hsp70l:loxP-DsRed-loxP-EGFP)*. **B**: Cross-section of the diencephalon with optic tracts and the rostral optic tectum immunostained for MBP (red) and EGFP (green). Scale bar, 100 μm. **C**: Higher magnification of the area depicted (white rectangle) in B showing presumptive oligodendrocytes (marked by arrowheads) after recombination expressing EGFP and co-stained with MBP (red). Scale bar, 50 μm. **D**: Cross-section of the retina with the exit point of the optic nerve immunostained for EGFP (green) and MBP (red), showing recombined cells (EGFP) within the MBP-expressing optic nerve. Scale bar, 50 μm. **E**: Higher magnification of the area depicted (white rectangle) in C showing EGFP-expressing cells (yellow arrowheads) co-expressing MBP. Scale bar, 25 μm. **F**: Cross-section of the dorsal telencephalon showing presumptive mature oligodendrocytes after recombination expressing EGFP (yellow arrowheads), co-immunostained with MBP (red) and located along the MBP-positive lateral olfactory tract (yellow dotted line) and the dorsal part of the entopeduncular nucleus. DAPI stains nuclei. Scale bar, 25 μm.

potentially in some Schwann cells as well by crossing it to effector lines containing loxP constructs with genes of interest. Moreover, TAM-dependent recombination allows different recombination rates, sparse or robust, and subsequent labeling of few or many oligodendrocytes and Schwann cells. Furthermore, the fact that the expression of these genes can be induced at a specific time point circumvents potential negative effects that permanent expression of non-endogenous genes during embryonic or larval development might cause. The line could additionally be used for oligodendrocyte-specific cell ablation.

Conclusions

Taken together, cell type–specific recombination in Zebrafish using inducible Cre is a novel technical methodology. We report here for the first time functional CreER^{T2} expression specifically in myelinating glia of Zebrafish. Our newly established *Tg(mbpa:mCherry-T2A-CreER^{T2})* line will be a useful tool in future studies aiming at a better understanding of myelination during development, under homeostatic conditions as well as during axon regeneration after injury. Furthermore, it allows targeted expression of transgenes in larvae and adults without interfering with earlier developmental stages.

Experimental Procedures

Zebrafish Husbandry

Zebrafish (*Danio rerio*) were maintained at 28 degC under a 14-hr light, 10-hr dark cycle (Brand et al., 2002). Developmental stages are indicated according to Kimmel et al. (Kimmel et al., 1995) and in hours, days, and months post-fertilization (hpf, dpf and mpf respectively). Some embryos were raised in fish water containing 0.003% 1-phenyl 2-thiourea to prevent pigmentation (Karlsson et al., 2001). *Tg(hsp70:loxp-DsRed-loxp-EGFP)^{tud107}* and *Tg(mbpa:EGFP)^{ck1}* have been previously described (Kroehne et al., 2011; Jung et al., 2010). All experiments were performed in compliance with animal welfare legislation. Procedures were approved by the ethical approval committee of the Regierungspräsidium Dresden, Germany: AKZ: 24-9168.11-1/2013-29 and the Regierungspräsidium Freiburg, Germany: AKZ: 35–9185.81/G-13/103. All efforts were made to minimize animal suffering and the number of animals used.

Generation of the *Tg(mbpa:mCherry-T2A-CreER^{T2})*

To create the pTol mbpa:mCherry-T2A-CreER^{T2} plasmid, ~2.0 kb of the *mbpa* [ENSDARG00000036186] regulatory region were amplified by polymerase chain reaction (PCR) with restriction sites EcoNI at the 5' end and FseI at the 3' end. PCR products were sequentially subcloned into the EcoNI-FseI site of the pTol:-mCherry-T2a-CreER^{T2} (Hans et al., 2009). For germ line transformation, plasmid DNA and Tol2-transposase mRNA were injected into fertilized eggs (F0), raised to adulthood, and crossed to wild-type Zebrafish from the AB line, which is the primary background of all transgenic and mutant fish that come from the Zebrafish International Resource Stock Center (ZIRC) in Eugene, Oregon.

F1 embryos were screened by PCR using mbp (ttgccaacgttgtaggctactacc) and Cre (tagagcctgttttgcacgttcacc)-specific primers that result in an 867 base pair fragment. Positive embryos were examined under a fluorescence microscope and mCherry + embryos were raised. Out of 16 PCR-positive F0 fish, seven lines showed a distinctive CreER^{T2} expression pattern. Two lines, *Tg(mbpa:mCherry-T2A-CreER^{T2})^{kn4}* and *Tg(mbpa:mCherry-T2A-CreER^{T2})^{kn5}*, with weak and strong mCherry expression, respectively, were established.

Pharmacological Treatments and Heat Induction

Larvae

For pharmacological treatments, the following stock solutions were made and stored at -20 degC: 10 mM 4-OHT (Sigma, H7904) in ethanol. Larvae (5 dpf onward) were incubated overnight in 5 μM 4-OHT. For control treatments, sibling embryos were incubated in corresponding dilutions of ethanol. All incubations were conducted in the dark. For heat-shock experiments, larvae were transferred into fresh petri dishes. After removal of excess embryo medium, pre-warmed 40 degC embryo medium was added and the petri dishes were kept for 1.5 h in a 38 degC incubator. Afterward, embryos were returned to 28.5 degC. The analysis of the fluorescent proteins occurred 1 day after heat shock.

Adults

Adult fish of ~6 months of age were used in the study. Adult Zebrafish were subjected to three consecutive overnight treatments with 2.5 μM TAM with a 12-hr resting period in between. Subsequently, fish were treated on two consecutive days with 2-hr heat shocks. This was achieved by slowly warming the water in which the fish were located from 28 degC to 38 degC over the course of 1 hr, and fish were kept at this temperature for an additional hour.

Fluorescence expression in larvae

mCherry, DsRed, and EGFP expression in larvae was analyzed by using a confocal laser-scanning microscope (LSM700 META; Carl Zeiss).

Evaluation of recombination efficiency

Twenty untreated and 4-OHT- and heat stock (HS)-treated sibling larvae (treatments applied as described in Fig. 3A) were photographed at 5 and 7 dpf, and the number of fluorescent cells (mCherry in untreated control fish, EGFP in treated larvae) was counted in 300 μm-long segments in the hindbrain and the ventral spinal cord anterior to the pronephric duct. Altogether, 848 cells were counted in controls, and 787 EGFP-positive cells in recombined fish.

Tissue Preparation

Fish heads were fixed in freshly prepared 4% PFA (paraformaldehyde) in 0.1 M phosphate buffer (PB), pH 7.4, overnight followed by a decalcification treatment with 0.5 M EDTA and 20% sucrose in PB solution overnight prior to embedding in 7.5% gelatin and 20% sucrose in PB. Fish heads were instantly frozen on dry ice and cryosectioned at 10 μm thickness using Microm HM 560 cryostat. Cryopreserved heads were stored at -80 degC, and the cryosectioned slides were stored at -20 degC prior to immunohistochemistry (IHC).

Immunohistochemistry on Larvae

Anesthetized larvae were fixed in 4% PFA in phosphate-buffered saline (PBS) for 30 min at room temperature (RT), washed in

PBS-Tween 20 (PBST), and permeabilized in acetone for 7 min at -20 degC. MBP and mCherry antibodies were used for whole-mount immunostainings: polyclonal anti-MBP, 1:50 (kindly provided by William S. Talbot and David Lyons, Stanford University School of Medicine, Stanford, California); and monoclonal anti-mCherry, 1:200 (Clontech, Cat.No: 632543). The MBP antibody was raised against a peptide that is present only in Mbpa (CSRSRSPPKRWSTIF), but not in Mbpb, and therefore recognizes only this isoform (Lyons et al., 2005).

Immunohistochemistry on Cryosections

For immunohistochemistry, the slides were thawed and air-dried prior to the washes with 1x PBS with 0.3% Triton-X100 (PBS-TX). Primary and secondary antibodies were incubated in PBS-TX. Primary antibodies were incubated overnight at 4 degC and secondary antibodies for 2 hr at room temperature. The slides were washed in PBS-TX and mounted with 80% glycerol. Primary antibodies used were polyclonal rabbit anti-MBP (1:100), monoclonal mouse anti-mCherry (1:200, Clontech, Cat.No: 632543), and polyclonal chicken anti-GFP (1:2000, Abcam, Cat.No: ab13970). Alexa conjugated (488, 555, and 633) highly cross-adsorbed secondary antibodies raised in an animal corresponding to the primary antibody were used for detection (1:750, Invitrogen). Immunostained samples were imaged at a laser-scanning confocal microscope (Leica-SP5).

Abbreviations

4-OHT: 4-Hydroxytamoxifen, CNS: central nervous system, dpf: days post fertilization, hpf: hours post fertilization, IHC: Immunohistochemistry, mbp: myelin basic protein, MS: multiple sclerosis; Olig2: oligodendrocyte lineage transcription factor, P0: myelin protein zero, PB: 0.1 M phosphate buffer, PBS: phosphate buffered saline, PBST: phosphate buffered saline with Tween20, PBSTx: phosphate buffered saline with Triton-X100, PFA: Paraformaldehyde, PNS: peripheral nervous system, Sox10: SRY-box 10, SRY: sex determining region Y, TAM: Tamoxifen.

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