Targeted knock-in of *CreER^{T2}* in zebrafish using CRISPR/Cas9

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Abstract

New genome-editing approaches, such as the CRISPR/Cas system, have opened up great opportunities to insert or delete genes at targeted loci and have revolutionized genetics in model organisms like the zebrafish. The *Cre-loxp* recombination system is widely used to activate or inactivate genes with high spatial and temporal specificity. Using a CRISPR/Cas9-mediated knock-in strategy, we inserted a zebrafish codon-optimized *CreER^{T2}* transgene at the *otx2* gene locus to generate a conditional *Cre*-driver line. We chose *otx2* as it is a patterning gene of the anterior neural plate that is expressed during early development. By knocking in *CreER^{T2}* upstream of the endogenous ATG of *otx2*, we utilized this gene's native promoter and enhancer elements to perfectly match *CreER^{T2}* and endogenous *otx2* expression patterns. Next, by combining this novel driver line with a *Cre*-dependent reporter line, we show that only in the presence of tamoxifen can efficient *Cre-loxp*-mediated recombination be achieved in the anterior neural plate-derived tissues like the telencephalon, the eye and the optic tectum. Our results imply that the *otx2:CreER^{T2}* transgenic fish will be a valuable tool for lineage tracing and conditional mutant studies in larval and adult zebrafish.

Keywords CRISPR/Cas9 · *CreER^{T2}* · Knock-in · Zebrafish · *Cre-loxp* recombination

Introduction

Recent advances in genome-editing technologies like zinc finger nucleases, transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 have opened up enormous opportunities to knock-in or knock-out genes in several model organisms where gene targeting was previously not possible, such as zebrafish (Hoshijima et al. 2016; Li et al. 2016). The CRISPR/Cas9 system requires two components, namely the endonuclease Cas9 and a target-specific guide RNA (sgRNA), to create a double-strand break (DSB) at the desired locus. These DSBs trigger cell repair mechanisms like the non-

homologous end-joining (NHEJ) pathway that result in site-specific insertion/deletion (indel) mutations in the target sites (Hsu et al. 2014; Sander and Joung 2014). Even though many studies have successfully used this CRISPR/Cas9 technology to generate several loss-of-function mutants (knock-outs) in zebrafish, knock-in strategies to generate reporter lines, fusion proteins, or Gal4- or *Cre*-driver lines are limited. Nonetheless, we and others have described efficient insertion of fluorescent reporters or Gal4-drivers at the targeted locus based on a homology-independent knock-in method (Auer et al. 2014; Kimura et al. 2015; Kesavan et al. 2017). In these methods, the targeting vector contains a “bait” sequence and both the genomic locus and the vector undergo concurrent DSBs. Subsequently, the linearized vector integrates into the targeted genomic locus due to the homology-independent NHEJ mechanism.

Tissue-specific recombination using the *Cre-loxp* system has been extensively used in various model organisms for several applications, including lineage tracing and generation of conditional mutants. This has been made possible largely due to the availability of various tissue/cell type-specific *Cre* or tamoxifen-inducible *CreER^{T2}*-driver lines and floxed alleles in model organisms such as mice (Fuchs and Horsley 2011). However, the *Cre-loxp* system remains poorly explored in zebrafish with limited availability of tissue/cell type-

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specific Cre-drivers and floxed alleles; these are mostly restricted to lineage tracing applications (Kroehne et al. 2011; Ramachandran et al. 2012; Pan et al. 2013; Felker and Mosimann 2016). Notably, there is a significant need for such tools to study tissue-specific gene functions. Although Cre-driver lines can be generated using cell-specific promoters of interest, random integration of the transgene often results in ectopic expression and/or gene silencing (Hans et al. 2009). Thus, new genetic tools that reliably recapitulate endogenous gene expression patterns, even after the insertion of Cre transgenes at their native promoter sites, would be of great interest.

Here, we describe the use of a CRISPR/Cas9-mediated knock-in strategy to insert a zebrafish codon-usage-optimized *CreER*^{T2} sequence at the *otx2* locus. *Otx2* belongs to the subfamily of homeodomain-containing transcription factors (Zagozewski et al. 2014). We chose *otx2* as a candidate gene because it is a patterning gene expressed during early development (i.e., from 5-h post fertilization (hpf) onwards) and because structures such as the eye, the telencephalon, the forebrain and the midbrain are derived from *Otx2*-expressing anterior neuroectoderm cells (Martinez-Barbera et al. 2001; Raible and Brand 2004; Rhinn et al. 2006). Further, these structures continue to express *Otx2* in specific cell types like the bipolar cells in the retina and the neuronal and glial cells in the periventricular gray zone of the optic tectum in adult zebrafish (Zagozewski et al. 2014; Kesavan et al. 2017). Critically, the caudal limit of the *Otx2* expression domain marks the midbrain-hindbrain boundary (MHB), an important patterning/organizer center during brain development (Rhinn et al. 2006).

Materials and methods

Zebrafish strains and maintenance

Zebrafish (*Danio rerio*) embryos and adults were obtained, maintained and raised as described previously (Brand et al. 2002; Westerfield 2000). Embryos were staged as hours post fertilization (hpf) (Kimmel et al. 1995); the wild-type strain AB was used to obtain knock-in lines and transgenic fish lines were maintained as outcrosses. None of the larvae or adult fish showed any physiological or behavioral abnormalities. The conditional red-to-green reporter *Tg(hsp70l:loxP-DsRed-loxP-EGFPNLS)tud109* has been described previously (Knopf et al. 2011; Kroehne et al. 2011).

All animal experiments were carried out in accordance with animal welfare laws and local authority requirements (Landesdirektion Sachsen). Protocols for the generation (24-9168.11-1/2013-14) and maintenance of (DD24-5131/346/11 and DD24-5131/346/12) and experimentation with transgenic

animals (24-9168.24-1/2014-4) were also appropriately approved (Landesdirektion Sachsen, Germany).

Zebrafish codon-usage optimization of *CreER*^{T2}

Zebrafish codon-usage optimization was carried out by GenScript USA Inc., using the OptimumGene™ algorithm. Briefly, the algorithm optimizes a variety of parameters that are critical for efficient gene expression. In this case, the native gene contained tandem rare codons, which can negatively affect the efficiency of translation or even disengage the translational machinery. Further, the GC content and methylated CpG sequences were also optimized to prolong the half-life of the mRNA and the stem-loop structures, which control ribosomal binding and stability of mRNA, were broken. Finally, the codon adaptation index was increased from 0.79 to 0.89.

DNA construct and sgRNA

The wild-type strain AB was used to extract genomic DNA and the *otx2* bait was generated by PCR (Phusion Polymerase, Thermo Fischer) using primers listed in supplementary Table S1. The amplified bait fragment was cloned into a pCS2+ vector containing the zebrafish codon-usage-optimized *CreER*^{T2} sequence (Supplementary Fig. S1) generated by GenScript USA Inc., along with a polyA (pA) sequence. The CMV promoter was later removed from the pCS2+ vector and the construct was verified by sequencing. The sgRNA designed for *otx2* had the sequence GGAACCCG GCTAATTGTCTCAGG; the PAM sequence is underlined. The design and efficiency testing of sgRNA sequences have been described elsewhere and we have previously used the same bait and sgRNA to successfully knock-in fluorescent reporters at the *otx2* locus (Kesavan et al. 2017).

Embryo injections

Preparation of sgRNA and Cas9 mRNA and injection into 1-cell stage embryos were done as previously described (Kesavan et al. 2017). The injected embryos were monitored for the next 5 days and approximately 100 embryos were raised to adulthood.

Identification of founders and genotyping

To identify founders, 3–4-month-old F0 fish were outcrossed with wild-type strains (WIK or TL) and 20 embryos from each clutch were used to isolate genomic DNA. Subsequently, PCR was carried out using the primer pair (A + B), where the forward primer was in the 5' sequence of the bait (i.e., upstream of the bait) and the reverse primer was in the *CreER*^{T2}. A positive band indicated a correctly oriented knock-in at the targeted locus and PCR products from

individual embryos were verified by sequencing. The transgenic strain was maintained as an outcross to reduce the general effects of inbreeding.

In situ hybridization

Embryos at specific developmental stages, obtained by crossing *otx2:CreER^{T2}* fish with wild-type fish, were fixed in 4% PFA and stored in 100% methanol at -20°C . Whole-mount in situ hybridization was performed as previously described (Reifers et al. 1998; Kesavan et al. 2017). Briefly, digoxigenin- (DIG) or fluorescein-labeled probes were synthesized from linear DNA using a RNA labeling and detection kit (Roche) and hybridized probes were detected using anti-digoxigenin or anti-fluorescein antibody. Antibody staining was visualized using BM purple (digoxigenin) or fast red (fluorescein).

To test if the *CreER^{T2}* insertion affected endogenous *otx2* expression, we performed in situ hybridization for *otx2* in the embryos of incrossed double transgenics (*otx2:CreER^{T2}; hsp70l:loxP-DsRed-loxP-EGFPNLS*) as follows. Embryos were treated with $1\ \mu\text{M}$ 4-OH-tamoxifen at 5 hpf for 12 h, heat shocked at 20 hpf and those positive for EGFP were sorted (wild types excluded) and fixed in PFA at 24 hpf; these were either homozygotes or heterozygotes for *otx2:CreER^{T2}*. Embryos from *otx2:CreER^{T2}* outcrossed with wild-type fish were used as controls.

4-OH-tamoxifen treatment and heat shock

The novel Cre-driver *otx2:CreER^{T2}* fish were crossed with the conditional red-to-green reporter *Tg(hsp70l:loxP-DsRed-loxP-EGFPNLS)tud109*. After 4-OH-tamoxifen treatment, *CreER^{T2}*-positive cells undergo recombination and subsequent heat induction labels recombined cells in green (EGFP) and the non-recombined cells in red (DsRed) (Hans et al. 2009; Chekuru et al. 2017). Embryos were treated with $1\ \mu\text{M}$ 4-OH-tamoxifen from 5 hpf onwards until 19 hpf. Heat shock was administered at 19 hpf for 1 h at 37°C by heating the embryo medium (E3) to 42°C . Embryos were imaged between 24 and 28 hpf.

The *otx2:CreER^{T2}* fish were crossed with the zebrafish responder line *Tg(ubb:lox2272-loxp-RFP-lox2272-CFP-loxp-YFP)* (Pan et al. 2013) and the embryos were treated with $1\ \mu\text{M}$ 4-OH-tamoxifen at 5 hpf or with $15\ \mu\text{M}$ at 24 hpf for 12 h. Control embryos were left untreated. All embryos were imaged at 48 hpf.

Imaging

Live embryos were imaged between 24 and 28 hpf or at 48 hpf and were treated with 1-phenyl 2-thiourea (PTU) to block pigmentation. Embryos were anesthetized using MS-222 in

E2 medium, mounted on a glass bottom dish (MatTek) in 1% low-melting agarose and imaged on a Zeiss LSM 780 with $\times 10$ or $\times 20$ objectives. The following laser lines were used: EFGP (488 nm), DsRed (561 nm), CFP (440 nm), YFP (515 nm) and RFP (561 nm). Images were analyzed using FIJI (open source) or Imaris (ver. 7, Bitplane) software, respective TIFF files generated and figures assembled in Adobe Photoshop (ver. CS5).

Results

CreER^{T2} knock-in into the *otx2* locus

To test if we could knock-in *CreER^{T2}* at the *otx2* locus using the CRISPR/Cas9 genome-editing strategy, a target site was designed at about 500 base pairs (bps) upstream of the ATG start codon (i.e., translational start site). The bait plasmid was constructed by amplifying a sequence that was 1000 bps upstream of the *otx2* translational start site, which included the target site (scheme in Fig. 1a). Embryos were injected with sgRNA, Cas9 mRNA and the bait plasmid at the 1-cell stage so that concurrent double-strand breaks in the genomic target locus and the plasmid DNA could result in plasmid integration at the target locus. We have previously used the same bait and sgRNA to successfully knock-in fluorescent reporters in the *otx2* locus (Kesavan et al. 2017). Founders were identified by PCR, for which the forward primer was located upstream of the bait sequence and the reverse primer was located within the *CreER^{T2}*; this strategy confirmed directionally precise knock-in at the 5' location, i.e., this PCR strategy advantageously identifies insertions that are only correctly oriented (Fig. 1b). The germline transmission rate of successful founders was approximately 3% (1 out of 32 screened) and screening was stopped after identifying one successful knock-in event, as we have never observed differences in gene expression among multiple founders (Kesavan et al. 2017). Sequencing of the PCR amplicon identified a 4-bp insertion at the 5' junction due to the error-prone NHEJ mechanism (Fig. 1c). The *otx2:CreER^{T2}* transgenic strain is being maintained as an outcross to eliminate or dilute any potential off-target mutations and neither the larvae nor the adult fish show any anatomical or behavioral abnormalities.

Expression of *CreER^{T2}* recapitulates *otx2* expression

Next, to test if the expression of *CreER^{T2}* matches the expected expression pattern of *otx2*, the *otx2:CreER^{T2}* transgenic fish were outcrossed with wild-type animals and embryos were collected at various developmental stages. Digoxigenin- or fluorescein-labeled probes were synthesized against both zebrafish codon-optimized *CreER^{T2}* and *otx2* and whole-mount in situ hybridization was performed. At the 1-somite

Fig. 1 Targeted knock-in of *CreER*^{T2} into the *otx2* locus. **a** The knock-in strategy is schematized in the cartoon. A target site (Ts) located at about 500 base pairs (bp) upstream of the open reading frame (ORF) was chosen. A bait plasmid was constructed by cloning 1 kb of the upstream sequence from the ORF, including the target site, *CreER*^{T2} and polyA (pA) sequence. The bait plasmid, *sgRNA* against the target site and *Cas9* mRNA were injected at the 1-cell stage. The *Cas9* protein creates double-strand breaks (DSB) at both Ts, i.e., in the genomic locus and the bait plasmid, resulting in integration of the linearized bait

plasmid. Integration in the forward orientation will result in *CreER*^{T2} expression. The primer pair (A + B) can be used to screen and verify precise integration as the forward primer A is located upstream of the bait and the reverse primer B is within the *CreER*^{T2} sequence. **b** Representative gel pictures showing PCR amplicons from F1 embryos using primers A + B. Lanes 1 and 2 show positive amplicons with an expected size of 2 kb, while lanes 3 and 4 are negative for the presence of *CreER*^{T2}. **c** DNA sequence analysis of the 5' junction shows a 4-bp insertion; the PAM sequences are marked in red

stage (10.30 hpf), *CreER*^{T2} expression was observed in the anterior neural plate and a double in situ hybridization with *otx2* showed complete overlap between these two signals, implying that the *CreER*^{T2} expression pattern matches that of *otx2* (Fig. 2a, e, i). At the 6-somites stage (12 hpf), strong *otx2* expression was observed in the prospective midbrain with a sharp boundary abutting the MHB and its expression pattern perfectly matched that of *CreER*^{T2} (Fig. 2b, f, j). Similarly, *otx2* expression at the midbrain matched *CreER*^{T2} expression at later developmental stages, including at 24 and 48 hpf (Fig. 2c, g, k and d, h, i).

To investigate if the endogenous expression of *otx2* is affected by the insertion, heterozygous *otx2:CreER*^{T2} transgenic fish were incrossed and the resulting progeny analyzed for *otx2* expression and morphological defects. At 24 hpf, expression of *otx2* was reduced in approximately one third of the progeny (Fig. 2m–o). However, we did not observe any morphological defects in embryos up to 120 hpf (data not shown), indicating that the lower *otx2* expression in homozygous *otx2:CreER*^{T2} embryos did not affect development. Taken together, these results suggest that the *otx2:CreER*^{T2} knock-in faithfully recapitulates the endogenous expression pattern of *otx2*.

otx2:CreER^{T2} knock-in leads to efficient recombination

To test the functionality of the *otx2:CreER*^{T2} driver line, we crossed it with a *heat shock promoter 70-like* (*hsp70l*)-driven red-to-green responder line (*Tg(hsp70l:loxP-DsRed-loxP-EGFPNLS)*). To avoid potentially unwanted recombination through maternally contributed Cre recombinase, we used males as Cre-drivers and females as responders. In double transgenic progeny, tamoxifen induction resulted in recombination wherein the floxed DsRed sequences were removed and the EGFPNLS (nuclear localized EGFP) sequences became expressed. After the application of heat shock to activate the reporters, recombined and non-recombined cells were labeled in green and red, respectively (see scheme in Fig. 3a).

Embryos were treated with 4-OH-tamoxifen from the onset of *otx2* expression, i.e., at 50% epiboly (5 hpf), to allow recombination in all Otx2-derived cells. To visualize both recombined and non-recombined cells, embryos at the 20-somite stage (19 hpf) were subjected to heat shock at 37 °C and subsequently maintained at 28.5 °C in an incubator. We did not observe any EGFP positive cells in the absence of tamoxifen induction in about 250 embryos, suggesting that

Fig. 2 Expression of *otx2:CreER^{T2}* recapitulates *otx2* expression. Double in situ hybridization for *otx2* (red) and *CreER^{T2}* (blue) was carried out in embryos obtained after crossing *otx2:CreER^{T2}* with wild-type fish; asterisks denote codon-optimized *CreER^{T2}*. **a, e, i**: *otx2* (**a**) and *CreER^{T2}* expression (**e**) were observed in the anterior neural plate ectoderm at 10.5 hpf. Double in situ hybridization shows complete overlap between the two expression patterns (**i**). **b–j** From 12 hpf onwards, *otx2* expression was predominantly observed in the

prospective midbrain (**b, c, d**) and *CreER^{T2}* expression (**f, g, h**) matched *otx2* expression patterns (and **j, k, l**). **m–o** In situ hybridization for *otx2* at 24 hpf. Expression of *otx2* is reduced in approximately one third of the embryos (asterisks) obtained from an *otx2:CreER^{T2}* heterozygote incross (**m**). Higher magnifications of wild-type or heterozygous siblings (**n**) and a homozygous *otx2:CreER^{T2}* embryo (**o**). Scale bar: **a–l, n, o** 60 μ m; **m** 250 μ m

there is no “leakiness” in the *otx2:CreER^{T2}* transgenic line (data not shown). The embryos were imaged between 24 and 28 hpf and recombined cells were predominantly observed in the developing forebrain, eyes and midbrain (Fig. 3b, c); in addition, a small proportion of recombined cells were found in the hindbrain (Fig. 3d, e). In contrast, non-recombined DsRed-positive cells (Fig. 3e, marked in white) were found posterior to the MHB (i.e., hindbrain) as *Otx2* is specific to the anterior neuroepithelium. A few double positive cells (DsRed and EGFP positive) were observed, possibly due to persistence of the DsRed protein even after recombination. Crucially, the *Cre-loxp* recombination pattern matched

the expression pattern obtained by in situ hybridization for both *otx2* and *CreER^{T2}* (Fig. 2), implying that the *otx2:creER^{T2}* knock-in zebrafish line perfectly mirrors *otx2* expression.

Multicolor labeling of forebrain and midbrain cells using the *otx2:CreER^{T2}* knock-in driver line

To test the ability of the *otx2:CreER^{T2}* to flox multiple *loxP* alleles and to visualize its temporal resolution using tamoxifen induction, the *otx2:CreER^{T2}* fish were crossed with the zebrafish responder line (ubiquitin promoter-driven brainbow

Fig. 3 *otx2:CreER^{T2}* knock-in leads to effective recombination. The *Cre-loxp*-mediated recombination strategy is schematized in panel (a). In cells expressing the *CreER^{T2}*, tamoxifen induces recombination between the *loxP* sites. This removes the DsRed cassette and places the EGFPNLS (EGFP with nuclear localization signal) “in-frame,” which is now expressed under the control of the heat shock promoter (*hsp70l*). Upon heat shock, the recombined cells (marked in green) can be visualized. **b–e**: Embryos obtained from *otx2:CreER^{T2}* fish were crossed with the heat shock red-to-green responder line and treated with 4-OH-tamoxifen from 5 hpf onwards and provided heat shock at 19 hpf. Lateral images from live 24 hpf embryos (**b**, **c**) show effective recombination in the forebrain- and midbrain-derived tissues. Dorsal images (**d**, **e**) show clear distribution of the recombined cells abutting the MHB with the exception of a few cells (indicated with red arrows) that are probably derived from cells in the early *Otx-Gbx* overlapping domain crossing the MHB. Images are maximum intensity projections covering about 40 μ m tissue with a Z interval of 1 μ m. MB midbrain, Hb hindbrain. Scale bar 50 μ m

1.0 cassette) where a combination of three fluorescent proteins (RFP, CFP, or YFP) is expressed in cells based on stochastic recombination events (Pan et al. 2013). In a cell with multiple copies of RFP, CFP and YFP, *CreER^{T2}*-mediated stochastic recombination events lead to cell clones being labeled by different colors. The combinatorial nature of fluorescent protein expression gives a unique barcode to each cell and marks their progenies in the same color (Schematized in Fig. 4a). The ubi:zebrabow; *otx2:CreER^{T2}* embryos were treated with 4-OH-tamoxifen either at 5 hpf (1 μ M) or at 24 hpf (15 μ M) for 12 h and live embryos were imaged at 48 hpf. Untreated embryos (No Tam) showed only the default RFP expression (non-recombined cells) (Fig. 4b). In contrast, treated embryos showed effective recombination in both the fore- and midbrain tissue with combinatorial expression of CFP, YFP and RFP, depending on the number of recombined and non-recombined alleles. In embryos treated at the onset of *Otx2* expression (5 hpf), broad labeling could be observed in the eye and other forebrain- and midbrain-derived cells (Figs. 4c and 5a). A few labeled cells could also be detected in the hindbrain, similar to that observed with the heat shock responder line (Figs. 4c and 5a). In comparison to the 5 hpf-treated embryos, sparse labeling of recombined cells was observed in the eye of embryos treated at 24 hpf as *Otx2* is predominantly expressed in the midbrain and in specific cell types in the eye (Figs. 4d and 5b). Further, no recombined cells could be observed in the hindbrain of 24 hpf-treated embryos (Figs. 4d and 5b). These results imply that the *otx2:CreER^{T2}* driver line can be used for effective recombination with high spatial and temporal resolution.

Discussion

We demonstrated that *CreER^{T2}* can be efficiently knocked-in at a specific locus for a gene of interest, *otx2* in this case, while advantageously utilizing the native promoter and enhancer elements to control expression of the knocked-in gene. Thus, *CreER^{T2}* expression pattern recapitulated the native gene expression pattern of *otx2* at all developmental stages tested and resulted in effective recombination when combined with responder lines containing floxed alleles. Although *otx2* expression was reduced in homozygous *otx2:CreER^{T2}* embryos, we found that diminished expression did not affect development. Furthermore, as effective recombination could be achieved using heterozygous carriers, we believe that deriving and raising homozygous animals is unnecessary. Recently, we also described the knock-in of two different fluorescent reporters at the *otx2* locus using the same strategy where reporter expression perfectly recapitulated both embryonic and adult gene expression patterns (Kesavan et al. 2017). Together, these results imply that this knock-in strategy is a robust method for generating fluorescent reporters and

Cre-driver lines. An important aspect of this strategy is that knocking in reporters or Cre-drivers at the non-coding region just upstream of the ATG renders screening for in-frame insertions unnecessary. The exact mechanisms mediating plasmid integration at the targeted locus have not been explored in this study but based on available literature on repair mechanisms in zebrafish (Hagmann et al. 1998; Dai et al. 2010) and recent studies on CRISPR/Cas9-mediated knock-in (Auer et al. 2014; Kimura et al. 2014; Ota et al. 2016), we think that plasmid integration occurs most likely due to homology-independent integration mediated by the NHEJ mechanism.

Synthesis of heterologous proteins like *CreER^{T2}* in zebrafish may be inhibited by the usage of rare codons that result in translational errors such as stalling, termination, or amino acid substitution (Kudla et al. 2009; Zhou et al. 2016). Hence, the coding sequence of *CreER^{T2}* was re-synthesized using codon sequences preferentially found in zebrafish. In our experience with Cre-mediated recombination, we have observed that the codon-optimized *CreER^{T2}* exhibits enhanced recombination efficiency (unpublished results).

Although *otx2* is exclusively expressed in and is a determining factor for the anterior neuroepithelium, a small proportion of *otx2*-derived recombined cells were also observed in the hindbrain. The zebrafish MHB forms at the interface of two transcription factor domains in the neural plate epithelium, namely the anterior *Otx* domain and the posterior *Gbx* domain (Rhinn et al. 2003; Rhinn et al. 2006). At 60% epiboly (6 hpf), the anterior border of the *gbx1* expression domain directly abuts the *otx2* expression domain with an overlapping domain spanning 3–4 cell layers. This overlap in expression domains disappears at around 80% epiboly (8 hpf) and results in two sharply defined, mutually exclusive, adjacent domains (Rhinn et al. 2003). The induction of recombination using tamoxifen at around 50% epiboly onwards would have resulted in recombination in cells located at this *Otx*-*Gbx* overlapping domain. Thus, the small proportion of recombined cells observed later in the hindbrain and in close proximity to the MHB may have been derived from cells in this overlapping domain. This theory is further supported by similar observations in the zebrafish responder, where tamoxifen induction at 5 hpf showed recombined cells both in the midbrain and the hindbrain while induction at 24 hpf showed no recombined cells in the hindbrain. Similar observations of *Otx2*-positive cells in the hindbrain domain have been reported in mice (Sunmonu et al. 2011). Additionally, fluorescent proteins are known to have a half-life of about 24 h and the presence of double positive cells (EGFP+ and DsRed+) suggests that, in these cells, recombination probably occurred after the onset of DsRed expression.

Genetics-based lineage tracing using inducible *CreER^{T2}*-drivers, in combination with zebrafish responders, allows stochastic recombination and the resultant fluorescent protein combinations provide an opportunity to understand lineage decisions and clonal origins with high temporal resolution

Fig. 4 Multicolor labeling of forebrain and midbrain cells using the *otx2:CreER^{T2}* knock-in driver line. The *otx2:CreER^{T2}*-mediated recombination strategy using the zebrafish transgenic responder fish is schematized in (a). In cells expressing *CreER^{T2}*, tamoxifen induces recombination between either the two *lox2272* sites (marked by dotted triangles) or the two *loxP* sites (marked by a triangle), resulting in the stochastic labeling of cells due to the expression of both CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) or YFP alone in the recombined cells, and RFP (red fluorescent protein) in the non-recombined cells. In a cell with multiple copies of RFP, CFP and YFP, *CreER^{T2}*-mediated stochastic recombination events lead to clones marked by different colors. b–d Embryos obtained from *otx2:CreER^{T2}* fish

crossed with the zebrafish responder line were treated with 4-OH-tamoxifen either at 5 hpf (1 μ M) or at 24 hpf (15 μ M) for 12 h and live embryos were imaged at 48 hpf. (b, b', b'') Embryos not treated with 4-OH-tamoxifen (No Tam) showed only RFP expression. (c, d) Embryos treated with tamoxifen at 5 hpf (c, c', c'') and 24 hpf (d, d', d'') show effective recombination in both forebrain- and midbrain-derived tissues and are marked by clones expressing a combination of CFP and YFP. A few recombined cells were found in the hindbrain region (arrowhead) but only in samples treated at 5 hpf (c, c', c'') and not at 24 hpf (d, d', d''). Lateral images are shown. Images are maximum intensity projections covering about 100–120 μ m of tissue with a Z interval of 4 μ m. MB midbrain, Hb hindbrain. Scale bar 100 μ m

(Pan et al. 2013). Further, the transparency and optical clarity of zebrafish embryos provide a unique opportunity to use photoactivatable substrates like the caged cyclofen to activate *CreER^{T2}*, which permits spatially and temporally controlled recombination using UV laser illumination of a single cell or a particular clone of cells (Sinha et al. 2010). Similarly, laser-induced gene expression in the *otx2:CreER^{T2}* line described

here, in combination with a heat shock responder line, can be used to activate or suppress molecules in single cells or cell clones in the developing forebrain and midbrain. Interestingly, laser-induced gene expression by activation of a Cre recombinase has been recently used to lineage trace hematopoietic cells in zebrafish embryos (Henninger et al. 2017). Besides a role in embryonic development, *Otx2* continues to

Fig. 5 Multicolor labeling using the *otx2:CreER^{T2}* knock-in driver line. Dorsal images of embryos obtained from the *otx2:CreER^{T2}*; zebrafish cross. (a, a', a'') Embryos treated with tamoxifen at 5 hpf show effective recombination in the midbrain while a few recombined cells could be observed in the hindbrain region (arrowheads). (b, b', b'') Embryos treated with 4-OH-tamoxifen at 24 hpf show recombined cells only in the midbrain. Images are maximum intensity projections covering about 40 μm of tissue with a Z interval of 2 μm . MB midbrain, Hb hindbrain. Scale bar 50 μm

be expressed in various neuronal and glial cells in the adult optic tectum and in the bipolar cells of adult retina, which make the *otx2:CreER^{T2}* transgenic fish a valuable tool for regeneration studies.

Until genome-editing technologies like zinc finger nucleases, TALENs and CRISPR/Cas became available, loss-of-function mutants in zebrafish were largely obtained from mutagenesis screens. While these have been fundamental in identifying the core genetic network essential for early embryonic development, most of these mutants survive only a few days after birth, making them unsuitable for gene function studies in the late larval and adult stages (Brand et al. 1996; Schier et al. 1996). Generating conditional Cre-driver lines using this knock-in strategy and combining them with methods to generate floxed alleles can overcome this limitation. Importantly, this strategy will be useful for understanding gene functions under homeostatic and regenerative conditions in both larval and adult zebrafish.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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