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In vivo Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains

Enrico Moro [a,*], Gunes Ozhan-Kizil [b,1], Alessandro Mongera [c,1], Dimitris Beis [d,1], Claudia Wierzbicki [e], Rodrigo M. Young [e], Despina Bournele [d], Alice Domenichini [f], Leonardo E. Valdivia [e], Lawrence Lum [g], Chuo Chen [h], James F. Amatruda [i], Natascia Tiso [f], Gilbert Weidinger [b], Francesco Argenton [f,**]

[a] *Department of Biomedical Sciences, University of Padova, I-35121 Padova, Italy*
[b] *Biotechnology Center, Technische Universität Dresden, 01307 Dresden, Germany*
[c] *Max Planck Institute for Developmental Biology, D-72076 Tübingen, Germany*
[d] *Developmental Biology, Biomedical Research Foundation Academy of Athens, Greece*
[e] *Department of Cell and Developmental Biology, UCL, Gower St, London WC1E 6BT, UK*
[f] *Department of Biology, University of Padova, I-35121 Padova, Italy*
[g] *Department of Cell Biology University of Texas Southwestern Medical Center, Dallas, TX 75390, US*
[h] *Department of Biochemistry University of Texas Southwestern Medical Center, Dallas, TX 75390, US*
[i] *Departments of Pediatrics, Internal Medicine and Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, US*

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ABSTRACT

The creation of molecular tools able to unravel *in vivo* spatiotemporal activation of specific cell signaling events during cell migration, differentiation and morphogenesis is of great relevance to developmental cell biology. Here, we describe the generation, validation and applications of two transgenic reporter lines for Wnt/β-catenin signaling, named *TCFsiam*, and show that they are reliable and sensitive Wnt biosensors for *in vivo* studies. We demonstrate that these lines sensitively detect Wnt/β-catenin pathway activity in several cellular contexts, from sensory organs to cardiac valve patterning. We provide evidence that Wnt/β-catenin activity is involved in the formation and maintenance of the zebrafish CNS blood vessel network, on which sox10 neural crest-derived cells migrate and proliferate. We finally show that these transgenic lines allow for screening of Wnt signaling modifying compounds, tissue regeneration assessment as well as evaluation of potential Wnt/β-catenin genetic modulators.

Introduction

The canonical Wnt/β-catenin signaling is required for a wide range of biological processes during embryonic development, adult organ maintenance and regeneration (Stoick-Cooper et al., 2007; van Amerongen and Nusse, 2009; MacDonald et al., 2009). In the absence of Wnt ligands, the major Wnt signal transducer, β-catenin, is kept at low concentration in the cytoplasm by the activity of a complex of proteins containing Axin, APC and GSK3β, which phosphorylates and targets β-catenin to proteasomal degradation. When Wnt ligands bind to the Frizzled (Fz)/LRP receptor complex, β-catenin's degradation is prevented through incompletely understood mechanisms that involve the recruitment of Axin to the receptor complex by Dishevelled (Dvl) and subsequent inhibition of the β-catenin destruction complex (Clevers, 2006). Stabilized β-catenin translocates into the nucleus to form a transcriptional activator complex with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors (MacDonald et al., 2009). This interaction triggers the transcription of a large number of target genes, through the interaction with transcriptional activators, such as CBP/p300 (Bienz and Clevers, 2003), Pygo (Stadeli and Basler, 2005) and chromatin remodeling factors (Mosimann et al., 2009). The identification and characterization of TCF binding sequences in the promoters of Wnt target genes has led to the development of the TOPFLASH Wnt/β-catenin reporter plasmid, which contains three copies of the optimal TCF binding motif upstream of a minimal c-Fos promoter driving luciferase expression (Korinek et al., 1997). Distinct reporters containing a variable number of tandem TCF/LEF sites, with different minimal promoters and reporter genes have been thereafter generated and successfully

* Correspondence to: Department of Histology, Microbiology and Medical Biotechnology, University of Padova, I-35121 Padova, Italy. Fax: +390498276300.
** Corresponding author. Fax: +390498276300.
E-mail addresses: enrico.moro.1@unipd.it (E. Moro), francesco.argenton@unipd.it (F. Argenton).
[1] These authors contributed equally to the work.

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used in cell culture, invertebrate and vertebrate organisms (Barolo, 2006; Biechele et al., 2009). The TOPGAL and *Tg(TOP:GFP)*^{w25} reporters, which use the regulatory sequence of the TOPFLASH system, have been shown to recapitulate TCF gene expression domains to a certain extent in mouse and zebrafish, respectively (DasGupta and Fuchs, 1999; Dorsky et al., 2002). A different Wnt reporter, BATGal, driving LacZ expression under the control of seven multimerized TCF/LEF binding sites upstream of a minimal promoter of the *Xenopus* direct Wnt target gene *siamois*, has been developed in mice and is consistently expressed in several domains of Wnt/β-catenin activity (Maretto et al., 2003). An additional mouse Wnt reporter, based on a 5.6 kb fragment of the Axin2 promoter upstream of GFP has been shown to recapitulate the activity domains of Wnt signaling (Jho et al., 2002).

Unfortunately, the activity of different transgenic reporter lines does not always overlap in all tissues tested *in vivo* (Al Alam et al., 2011) and reporter activity can also be independent of β-catenin (Ahrens et al., 2011). Thus it is desirable to have several reporters available to assess whether β-catenin signaling is indeed active in a tissue of interest.

In zebrafish, the *Tg(TOP:GFP)*^{w25} transgenic line (also named as TOPdGFP), which expresses a destabilized form of GFP, has been shown to drive reporter expression in known domains of Wnt/β-catenin signaling activity, such as the CNS, migrating neural crest cells and the otic vesicle. Even though *Tg(TOP:GFP)*^{w25} fish have been used in many publications, Wnt signaling activation has been mostly monitored by detecting transgene GFP mRNA expression by *in situ* hybridization (Lewis et al., 2004; Ishitani et al., 2005; Lee et al., 2006; Stoick-Cooper et al., 2007; Veien et al., 2008; Goessling et al., 2009). However, one of the most attractive and powerful aspects of zebrafish is its transparent embryos, which enable *in vivo* tracking of cell fate and movements of all tissues throughout development. This characteristic allows the use of fluorescent transgenic fish lines, and has led to major biological discoveries using live time lapse imaging, as well as the implementation of large scale automated phenotypic analysis (Pardo-Martin et al., 2010; Kissa and Herbomel, 2010; Bertrand et al., 2010). The use of the TOPdGFP reporter line for live imaging is however limited as GFP expression is low due to the use of a destabilized version of GFP.

Here, we describe two novel transgenic lines, the *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} and *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} (designated *TCFsiam*), in which the expression of eGFP or monomeric Cherry protein (mCherry) are under the control of seven multimerized TCF responsive elements upstream of the minimal promoter of the Xenopus direct β-catenin target gene *siamois* (Brannon et al., 1997; Maretto et al., 2003).

We provide a detailed spatial and temporal characterization of the expression pattern of the two Wnt reporter lines. We show that *TCFsiam* transgenic reporter activity is detected *in vivo* in a wide range of tissues, such as brain endothelial cells, cloacal aperture and sensory organs. Most notably, we provide evidence that even in adult fish, *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} and *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} expressing cells are detectable in the endothelial compartment of the brain and in sensory organs. Importantly, we show that in early embryos all expression domains of the reporter are dependent on Wnt/β-catenin signaling and that they respond to enhancement of Wnt pathway activity. We also show that loss of function of *lef1* leads to inhibition of *TCFsiam* expression in specific discrete domains. In addition, we present via live imaging on *TCFsiam* lines that Wnt signaling is also actively transduced in neural crest-derived cells. Finally, we provide a preliminary demonstration that these lines can be easily manipulated to highlight Wnt signaling activation in regeneration studies.

Materials and methods

Generation and microinjection of Tg(7xTCF-Xla.Siam:GFP)^{ia4} *and Tg(7xTCFXla.Siam:nlsmCherry)*^{ia5} *constructs*

A cassette containing seven multimerized TCF/LEF binding sites upstream to a 0.13 kb siamois minimal promoter was amplified from the BAT-GAL construct (Maretto et al., 2003) using the oligonucleotides attb4-TCF (5′–GGGGACAACTTTGTATAGAAAAGTTGGAATTCGATTAAGGACCTCAG3′) and attb1-TCF (5′–GGGGACTGCTTTTTTGTACAAACTTGCAAGCTTATCTCTGTCTCCCAAAATG-3′). The amplified product was recombined with the vector pDONR^{TM}221 (Invitrogen), according to the manufacturer's instructions. Positive clones containing the entry vector were sequenced in both strands and recombined with the eGFP and mCherry-carrying middle vectors and SV40 polyA-containing vector. Entry plasmids were finally recombined into the Tol2 destination vector (pTol2pa) as previously described (Kwan et al., 2007). A total of 25 pg of Tol2 recombinant plasmids with 25–50 pg of *in vitro* synthesized transposase mRNA (Kawakami et al., 2004) were coinjected into zebrafish embryos at one- to two-cell stage.

Microinjected embryos were raised to adulthood and outcrossed to wild type fish. Nearly 15 out of 30 screened fish were identified as founders for both reporter lines *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} and *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} using a M165FC dissecting microscope (Leica).

The following fish lines where crossed with the Wnt reporters: Tg(*hsp70l:wnt8a-GFP*)^{w34} (Weidinger et al., 2005) and Tg(*hsp70l:dkk1-GFP*)^{w32} (Stoick-Cooper et al., 2007) Tg(*fli1a:EGFP*)^{y1} (Lawson and Weinstein, 2002), Tg(*sox10:mRFP*)^{vu234} (Kirby et al., 2006), Tg(*myl7:EGFP*) ^{twu34} (Huang et al., 2003), Tg(*kdrl:EGFP*)^{s843} (Beis et al., 2005) or Tg(*Tie2::GFP*)^{s849} (Motoike et al., 2000). *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} carriers were outcrossed to the apc^{hu745} (Hurlstone et al., 2003), lef1/sinf^{U767} (Valdivia et al., 2011), tcf3a/hdl^{m881} (Kim et al., 2000) and tcf4^{zf55} (Muncan et al., 2007) and raised. Outcross of heterozygote carriers to single mutant carriers were analyzed for the regulation of the transgene.

Immunohystochemistry

Zebrafish adult tissues were dissected from euthanized fish and fixed with 4% paraformaldehyde/PBS solution overnight. After dehydration with ethanol and paraffin embedding, 8–10 μm slides were collected on SuperFrost slides. Dehydration with chloroform and ethanol was followed by antigen heat retrieval on a steamer with citrate buffer (pH 6.0) and permeabilization with 0.2% Triton X-100, before primary antibody incubation. Primary antisera were anti rabbit GFP-Alexa Fluor 488 conjugated(Invitrogen), zn8 antibody (ZIRC) and Alexa 633 (Molecular Probes) or rhodamine phalloidine (Molecular Probes). Nuclear labeling was performed by 4′-6 diamino-2-phenylindole (DAPI) (Invitrogen).

Heat shock treatments

Wnt/β-catenin signaling was either activated or inhibited by the pairing of heterozygous Tg(*hsp70l:wnt8a-GFP*)^{w34} or Tg(*hsp70l:dkk1-GFP*)^{w32} to homozygous *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish, respectively. Embryos were heat-shocked at three different developmental stages, gastrula (50% epiboly), somitogenesis (3-somite stage) and organogenesis (24 h-post-fertilization, hpf) by replacing the embryo water with water preheated to 40 °C. Following the incubation in an air incubator at 37 °C for 1 h, transgenic embryos were sorted by GFP fluorescence and GFP-negative siblings were used as controls. Embryos were fixed in 4% PFA after an additional incubation period of 2 h at 28 °C.

In situ hybridization

Embryos were fixed in 4% buffered p-formaldehyde in PBS (PFA). RNA *in situ* hybridizations were performed as previously described (Thisse et al., 1993). TOPdGFP probe has been described by Dorsky et al. (2002). For the mCherry probe synthesis, a pCS2 plasmid containing the full mCherry coding sequence was linearized and transcribed with T7 RNA polymerase using DIG-labeled ribonucleotides. For the EGFP probe a linearized pME:EGFP of the Tol2 kit was used to synthesize a DIG-labeled riboprobe.

Zebrafish fin surgeries

Fin amputations of zebrafish at 12 months of age were performed as previously described (Poss et al., 2000). Following amputations, fins were immediately photographed corresponding to 0 h post amputation (hpa) and fish were returned to 28 °C water. Regenerating fins were photographed at 24, 48 and 72 hpa using bright field and epifluorescence for mCherry detection. Regenerating fins were also harvested and fixed at the same stages and RNA *in situ* hybridization was performed for mCherry mRNA detection.

Drug treatments

Zebrafish embryos were incubated in Wnt agonists Alsterpaullone (AP, 5 μM, Sigma) at 3 dpf for 36 h, BIO (5 μM, Sigma) at 24 hpf for 12 h and with LiCl (0.1 M, Sigma) at 24 h for 48 h. Wnt antagonist IWR-1 (10 μM, Sigma) was used at 24 hpf for 24 h. Drugs were directly added to the fish water in 6 well plates. For each treatment performed in triplicates, 10 embryos were used.

Image acquisition

For confocal microscopy PTU-treated larvae were embedded in 0.7% low melting agarose and placed on a Petri capsule filled with fish water. Low laser intensity (20%) was used to minimize induced cell damage as well as photobleaching. Confocal stacks were recorded on different confocal systems. A BioRadiance confocal system using 40× immersion objective (Nikon) was used for most time lapse imaging. However, the Leica SP5 and Nikon A5 confocal systems were also used to record images. Leica TCS SP5 resonant scanner was used for beating heart live imaging. All images were analyzed with ImageJ software (http://rsb.info.nih.gov/ij/).

Results

Generation of Tg(7xTCF-Xla.Siam:GFP)^{ia4} and Tg(7xTCF-Xla.Siam:nlsmCherry)^{ia5} transgenic zebrafish

Regulatory sequences from the previously reported BAT-gal construct (Maretto et al., 2003), containing seven TCF/LEF-binding sites upstream of a 0.13-kb fragment of the minimal promoter-TATA box of the *Xenopus* gene *siamois* were used to create the Gateway destination vectors pDest*Tg(7xTCF-Xla.Siam:GFP)^{ia4}* and *pDestTg(7xTCF-Xla.Siam:nlsmCherry)^{ia5}* in the Tol2 transposon backbone (Fig. 1A and B).

One cell-stage embryos injected with the destination vector DNA displayed strong fluorescence already at the gastrula stage. During somitogenesis transient fluorescence was distributed as a gradient along the trunk, displaying the strongest intensity in the tail (data not shown). Injected fish were raised to adulthood and then outcrossed to two different wild type strains. Almost 50% of the injected fish were found to transmit the transgene to their germline. Of the identified founders, four from the *Tg(7xTCF-Xla.Siam:GFP)^{ia4}* and three from the *Tg(7xTCF-Xla.Siam:nlsmCherry)^{ia5}* lines expressing higher levels of the transgene were raised to adulthood. All the offspring from the different founder fish displayed strong fluorescence in the same body areas, thus ruling out potential genomic positional effects. Moreover, all offspring from the same carrier displayed identical expression domains, therefore, ruling out any potential variegation effect.

In the stable *Tg(7xTCF-Xla.Siam:GFP)^{ia4}* fish line, fluorescence was ubiquitously detectable at dome stage and during epiboly (Fig. 1C and D). Fluorescence in *Tg(7xTCF-Xla.Siam:nlsmCherry)^{ia5}*, was weaker at these stages of development and could only be visualized by epifluorescence by the end of gastrulation (data not shown). This was the only significant difference we found between these two lines and hence from now on we will refer to them in a generic way as *TCFsiam.* During somitogenesis and at 24 hpf, high levels of transgene expression were observed in specific rostral regions of the embryo, specifically in the telencephalon and midbrain–hindbrain boundary (MHB) (Fig. 1E and F). A characteristic graded pattern of Wnt/β-catenin reporter activity was seen in the somites of the trunk (Fig. 1F), corroborating the previously described Wnt/β-catenin-dependent activity of the tail organizer (Agathon et al., 2003). This fluorescent expression domain of Wnt activity in *Tg(7xTCF-Xla.Siam:GFP)^{ia4}* was not detected in the previously reported *Tg(TOP:GFP)^{w25}* (compare Fig. 1H and M).

To assess whether this discrepancy was due to the low fluorescence intensity generated by the destabilized GFP in *Tg(TOP:GFP)^{w25}* embryos, we performed *in situ* hybridization with GFP and mCherry antisense riboprobes in *Tg(TOP:GFP)^{w25}* and *Tg(7xTCF-Xla.Siam:nlsmCherry)^{ia5}* fish, respectively. We found that the posterior mesoderm showed much stronger transgene transcription in *Tg(7xTCF-Xla.Siam:nlsmCherry$^{)ia5}$* compared to *Tg(TOP:GFP)^{w25}* embryos at 20 hpf, and that expression in this domain was exclusively found in our new reporter from 24 hpf onwards (compare Fig. 1J and K with 1O and 1P). Furthermore, additional distinct transgene expression domains in the otic vesicle and lateral line primordium, which are bone fide sites of active Wnt/β-catenin signaling (see below) were labeled in *Tg(7xTCF-Xla.Siam)* fish (green and red arrows in Fig. 1J and K), but not in age matched *Tg(TOP:GFP)^{w25}* fish (Fig. 1O and P). This suggests that the *Tg(7xTCF-Xla.Siam)* lines are a more sensitive Wnt/β-catenin reporters than the *Tg(TOP:GFP)^{w25}* line.

Genetic and pharmacological validation of Tg(7xTCF-Xla.Siam) reporter fish

To first rule out the possible *siamois* dependent regulation of the reporter expression, we generated a Tol2 construct carrying the *Xenopus laevis siamois* minimal promoter, without the TCF repeats, upstream to the eGFP coding sequence and microinjected the vector in one cell-stage embryos. We then compared the pattern of both transiently expressed *siamois-GFP* and *7xTCF-Siam:GFP* by *in situ* hybridization. As shown in Supplemental Fig. 1A, *siamois*-GFP was ubiquitously expressed at early gastrula stage, while *7xTCF-Siam:GFP* injected embryos exhibited a more localized pattern of expression in the blastodermal margin (Supplemental Fig. 1E). We then assayed fish larvae microinjected with either both constructs for Wnt antagonist IWR-1 responsiveness at 24 hpf (Chen et al., 2009). We found that only in *7xTCF-Siam:GFP* injected embryos, was the expression downregulated after drug treatment (compare Supplemental Fig. 1H–D).

To confirm whether *TCFsiam* transgenics reporter expression is regulated by Wnt/β-catenin signaling, we crossed the *Tg(7xTCF-Xla.Siam:nlsmCherry)^{ia5}* reporter fish with the transgenic heat shock inducible lines Tg(*hsp70l:wnt8a-GFP*)^{w34} (Weidinger et al., 2005) and Tg(*hsp70l:dkk1-GFP*) ^{w32} (Stoick-Cooper et al., 2007),

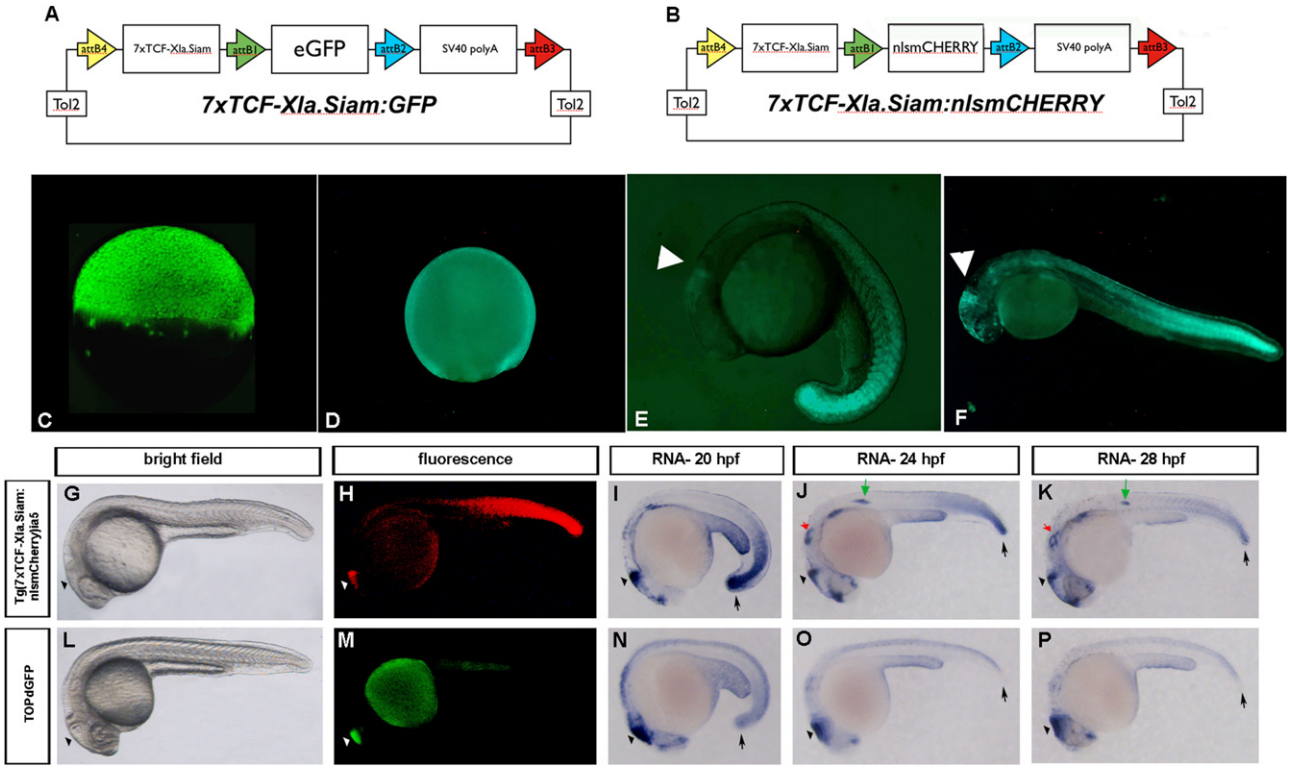

Fig. 1. Generation of the *TCFsiam* lines and comparison with the *Tg(TOP:GFP)*^{w25} fish. (A, B) Schematic rapresentations of the Tol-2 based vectors used to generate the *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} line (vector shown in A) and the *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} (vector shown in B). (C and D) Strong fluorescence is detectable in *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} early staged embryos. Lateral views with anterior to the left are shown in C-P. At dome (C) and 80% epiboly (D) *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} transgene expression is evident in all blastodermal cells. (E) During somitogenesis (19 hpf), eGFP is detected in the midbrain-hindbrain boundary (MHB) and in somites with a gradient distribution pattern. (F) 24-hpf embryo. Strong reporter expression is seen in the tail, while the gradient distribution is more clearly visible along the trunk. Positive cells are visible also in the fronto-nasal ectoderm and midbrain-hindbrain boundary (arrowhead). (G–P) Comparison of Wnt reporter activity under epifluorescence and by *in situ* hybridization in different embryonic stages reveals a stronger and broader reporter activity in *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} embryos than in *Tg(TOP:GFP)*^{w25} embryos. (H and M) Reporter fluorescence in *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} embryos is detectable anteriorly in the brain (arrowhead) and posteriorly in tail mesoderm, while in *Tg(TOP:GFP)*^{w25} embryos reporter activity is evident only in the brain. (I and N) Reporter transcription in both transgenic lines is detectable in the brain, while *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} additionally display a much stronger activity in the posterior mesoderm at 20 hpf (arrows). (J,K and O,P) After 24 hpf, Wnt reporter transcription is additionally detectable in otic vesicles (red arrow in J and K) and lateral line primordium (green arrow in J and K) in *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}, but not in *Tg(TOP:GFP)*^{w25} embryos. I-K and N-P are 20, 24 and 28 hpf embryos stained for mCherry and TOPdGFP RNAs, respectively. G and L are brightfield images of the same embryos represented in H and M, respectively.

which activate or inhibit Wnt/β-catenin signaling, respectively. Reporter gene expression was analyzed in double transgenic embryos after activation of Wnt8 or Dkk1 expression at gastrula, somitogenesis and organogenesis. All *TCFsiam* expression domains were strongly reduced 3 h after global overexpression of Dkk1 at all developmental stages tested, compared to age-matched heat-shocked *TCFsiam* single transgenic control embryos (Fig. 2A–F). Conversely, after global induction of Wnt8, GFP intensity in all expression domains was significantly enhanced at all three tested stages (compare Fig. 2G–I with Fig. 2J–L). Thus, reporter expression levels are fully responsive on Wnt/β-catenin signaling, suggesting that reporter activity reliably reflects endogenous regions of active Wnt/β-catenin signaling.

We next tested whether the *TCFsiam* reporter activity was modified in response to mutations in known Wnt/β-catenin signaling components. In agreement with the functional role of mutations in *apc*, which constitutively activate Wnt/β-catenin signaling, *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} reporter expression in the *apc*^{hu745} mutant background was significantly upregulated in the brain, heart, spinal cord and pectoral fins (arrows in Fig. 2N and P).

To further assess whether the *TCFsiam* lines were more sensitive compared to the *Tg(TOP:GFP)*^{w25}, we tested transgene expression level recovery by *in situ* hybridization after global Dkk1 overexpression using Tg(*hsp70l:dkk1-GFP*)^{w32}. *Tg(TOP:GFP)*^{w25} fish reporter gene transcription was first detected twelve hours after the heat shock (Panel F of Supplemental Fig. 2), while in *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} the recovery of reporter expression started approximately eight hours earlier (Panel D' of Supplemental Fig. 2). Therefore, the *TCFsiam* lines display a faster recovery after inhibition of Wnt signaling probably due to a higher sensitivity.

The zebrafish embryo has emerged as a valuable *in vivo* platform for large scale screening of small molecules (Barros et al., 2008; Rihel et al., 2010; Kokel et al. 2010). We therefore explored whether *TCFsiam* reporter fish could represent a suitable *in vivo* tool to screen for molecules that modulate Wnt signaling. To enhance Wnt activity we used BIO (Polychronopoulos et al., 2004), alsterpaullone (Lengfeld et al., 2009) and lithium chloride (LiCl) (Klein and Melton, 1996), three small molecules known to inhibit glycogen-synthase kinase (GSK-3β).

TCFsiam embryos treated with alsterpaullone at four dpf for 36 h displayed a notable increase in the reporter activity in the branchial arches, pectoral fin and heart (Fig. 2R). Similarly, the number of fluorescent cells in the branchial arches increased in 3 dpf *TCFsiam* reporter fish treated with LiCl (Fig. 2T). We also noticed that LiCl slightly enhanced the reporter fluorescence intensity in the otic vesicle. Exposure of *TCFsiam* embryos to BIO from 24 to 48 hpf resembles several aspects of *apc* mutants, which display overactive Wnt signaling, strongly enhancing *TCFsiam* expression in the lateral line primordium, otic vesicle and developing fins pharyngeal arches, telencephalon and tail (Fig. 2V and Valdivia et al., 2011). Importantly, 48 hpf BIO treated embryos showed a lateral line phenotype

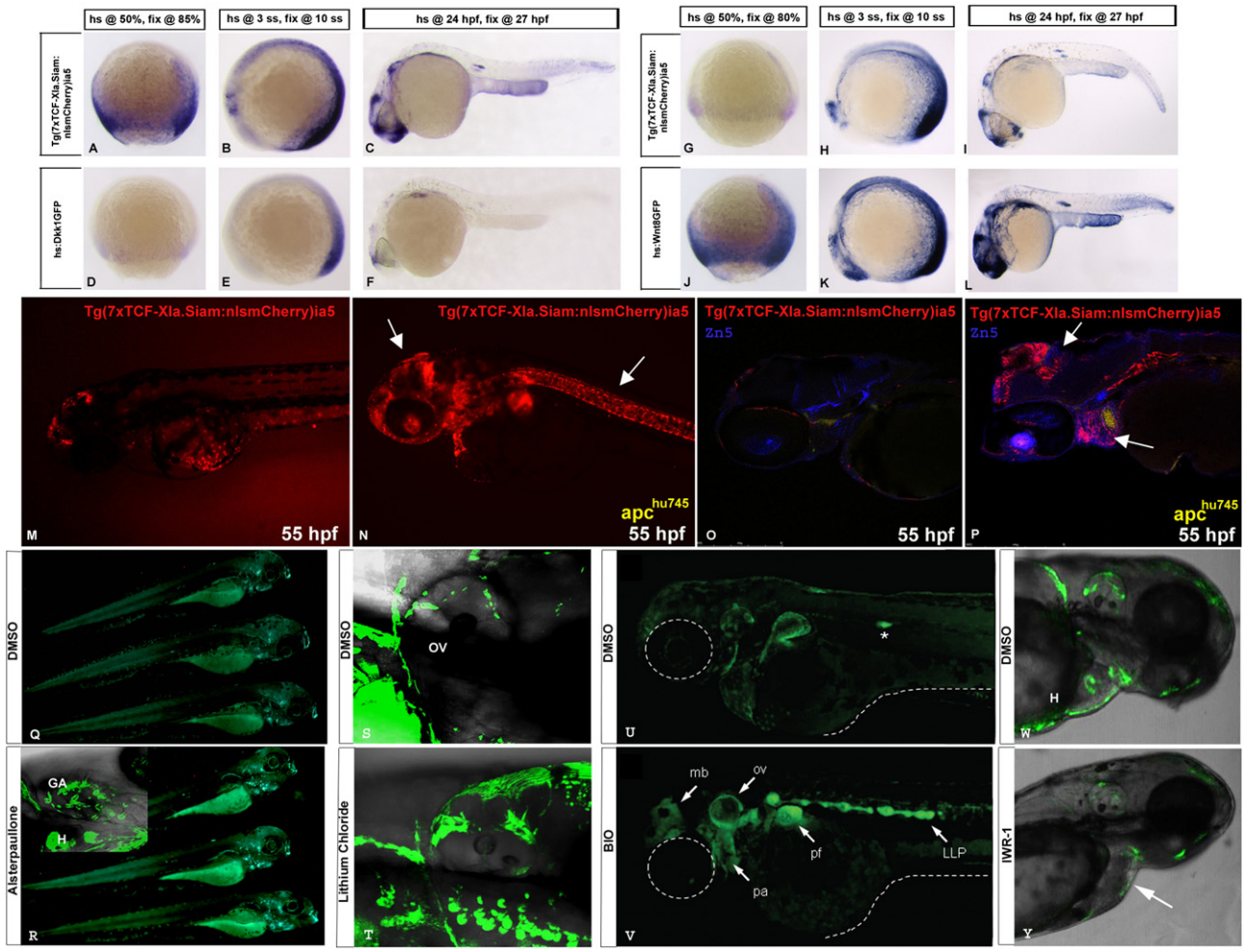

Fig. 2. *TCFSiam* lines represent *bona fide* Wnt/β-catenin reporters. (A–F) *In situ* hybridization with an antisense *mCherry* probe on *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}/Tg(*hsp70l:dkk1-GFP*)^{w32} embryos. In embryos heat-shocked at 50% epiboly, 3 somite-stage and 24 hpf, mCherry RNA is detected at 85% epiboly, 10 somite-stage and 27 hpf, respectively. (A–C) Heat-shocked *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} single transgenic control embryos stained for *mCherry* RNA. (A) During late gastrula, reporter mRNA is expressed in a broad marginal domain. (B) During somitogenesis, Wnt/β-catenin activity is detectable in the midbrain-hindbrain boundary, and the posterior neuroectoderm and mesoderm. (C) During organogenesis, the reporter is expressed in multiple domains of the CNS, the lateral line primordium, the epidermis of the yolk extension and weakly in posterior tail. (D–F) In heterozygous *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}/Tg(*hsp70l:dkk1-GFP)*^{w32}, *mCherry* signal is strongly reduced in all expression domains. (G–L) *In situ* hybridization with an antisense *mCherry* probe on Tg(*hsp70l:wnt8a-GFP)* ^{w34}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} embryos. (G–I) Heat-shocked *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} single transgenic control embryos stained for *mCherry* RNA. Note that the signal intensities in A-C and G-I are different due to variable duration of staining. (J–L) In heterozygous Tg(*hsp70l:wnt8a-GFP*) ^{w34}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}, a much stronger signal is detected. (M–P) Reporter activity in 55 hpf *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} in apc^{hu745} background (N and P). Wild type siblings (sib) are shown in M and O. Immunostaining for zn8 antibody is visible in blue. *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} expression is upregulated ectopically in the heart, brain region and lateral region (arrows) according to a constitutive activation of the Wnt signaling pathway in *apc* mutants. (Q,R) Treatment for 36 h with 5 μM Alsterpaullone (R) increases Wnt reporter activity in the pharyngeal arches of 3 dpf *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} larvae. Parallel treatment with control DMSO (Q) does not alter reporter activity. (S,T) Administration of 0.1 M LiCl for 36 h increases GFP fluorescence in the otic vesicle and pharyngeal arches (white arrowheads) of 3 dpf *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} (T) when compared to age-matched transgenic larvae treated with DMSO (S). (U,V). Incubation of transgenic embryos in BIO from 24 hpf to 48 hpf, induces a robust tissue specific activation of the reporter (arrows in V). Notice that BIO treated animals resemble *apc* phenotype in lateral line, with an hypertrophic, stalled and strongly GFP expressing primordium, while in 2% DMSO control embryos the secondary lateral line primordium is already migrating (asterisk in U). (W,Y) Treatment of 24 hpf *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} embryos with 10 μM IWR-1 for 24 h (Y) reduced reporter fluorescence particularly at the heart level (white arrow) when compared with DMSO treated embryos (W). ga: gill arches; h:heart, ov:otic vesicle.

comparable to that of *apc* mutants with a hypertrophic and stalled lateral line primordium, which is evidence of constitutive activation of Wnt/β-catenin signaling (Aman and Piotrowski, 2008; Valdivia et al., 2011).

To test whether the *TCFsiam* reporters respond to Wnt-inhibiting compounds, we treated 24 hpf embryos with the Wnt inhibitor IWR-1 (Chen et al., 2009). After 24 h IWR-1 was able to strongly decrease reporter activity particularly in the tectum, pectoral fins and heart, whereas in the tail residual EGFP was still present (Fig. 2Y and not shown).

These experiments show that the *TCFsiam* reporter is sensitive to well known Wnt agonists and antagonists *in vivo,* and that the changes in levels of fluorescence are coherent with its function as readout to image centers of Wnt signaling activity.

Mutation in lef1 but not tcfs modulates the expression of the TCFsiam reporter in several tissues

The transcriptional output of Wnt/β-catenin signaling is mediated by TCF/LEF transcription factors (Behrens et al., 1996). The zebrafish genome codes for *lef1* and five *tcfs*. Zygotic loss of *lef1* causes defects in lateral line development, *tcf7* mutation weakly affects pectoral fin development, while loss of the zygotic function of *tcf3a* (*tcf7la1*) and *tcf4* (*tcf7l2*) causes no defects during early development (Kim et al., 2000; Muncan et al., 2007; Nagayoshi et al., 2008; Valdivia et al., 2011). This is probably because of the great level of functional redundancy among *tcfs*. To test whether expression of *TCFsiam* reporters is dependent on TCF/LEF transcription factors and to assess whether more subtle

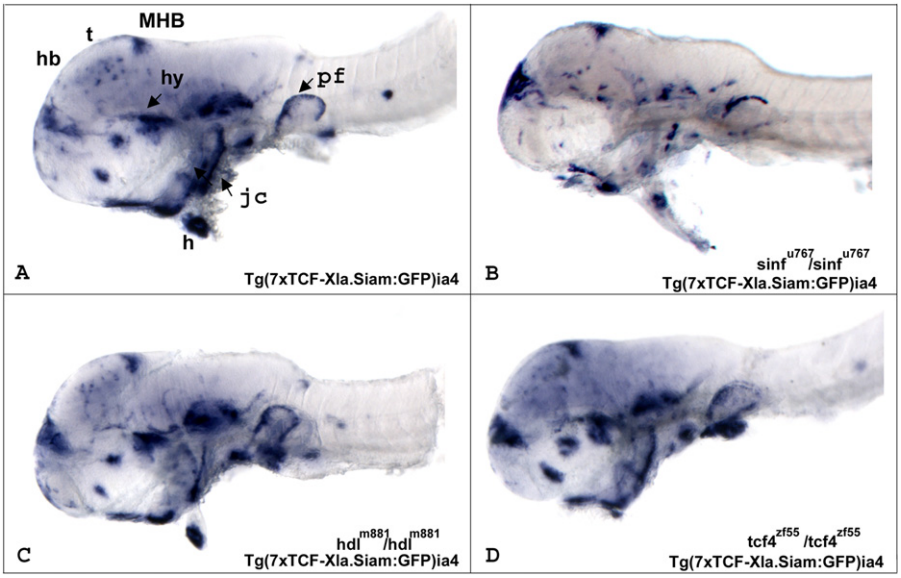

Fig. 3. Transgene expression in *TCFsiam* transgenics is highly regulated by *lef1*. All images (A–D) show a representative 48 hpf larva. Expression analysis of the *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} reporter in lef1/sinf ^{U767} homozygous mutants (B) is significantly reduced in several tissues, when compared to age matched *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} fish (A). In *tcf7l1a*$^{m881/m881}$ (*hdl*) (C) and tcf7l2^{zf55}/^{zf55} (D) no significant differences can be observed in respect to A. Anterior is shown to the left. Hb: habenula; hy:hypothalamus; t.tectum; pf: pectoral fin; jc: jaw cartilages; MHB: midbrain-hindbrain boundary; h: heart.

requirements of *tcf3a* and *tcf4* during early development could be revealed by the reporters, we generated transgenic *TCFsiam* lines in *lef1*^{U767} (Valdivia et al., 2011), *tcf3a/hdl* (*tcf7l1a*$^{m881/m881}$) (Kim et al., 2000) and *tcf4* (tcf7l2^{zf55}/^{zf55}) (Muncan et al., 2007) mutant backgrounds. *TCFsiam* reporter expression was only absent in the eyes, tectum, hypothalamus, lateral line primordium and tail somites in *lef1*^{U767} mutant (Fig. 3B). *tcf3a/hdl* and *tcf4* mutants (Fig. 3C and D) showed no difference compared to sibling embryos. This suggests that even though *tcf*s are expressed in many tissues, the overlapping expression in many of them could probably be enough to avoid overt reduction in the *TCFsiam* reporter expression during embryogenesis and larval stages.

Dynamic expression of fluorescent proteins in transgenic TCFsiam fish highlights novel domains of Wnt signaling activity

Wnt reporter expression is persistent in the 48 hpf *TCFsiam* larvae brain (Fig. 4A) and also detected in the notochord (Fig. 4B), otic vesicle (Fig. 4C), somites (Fig. 4D), fin buds (Fig. 4E), eye (Fig. 4F), and posterior lateral line (PLL) (Fig. 3G).

The migrating primordium of the PLL, which gives rise to primary neuromasts, was strongly labeled (Fig. 4G) and transgene expression colocalised with the *claudin b* primordium expression domain at 36 hpf labeled by the *-8.0cldnb:lynEGFP* transgene (Fig. 4H; Haas and Gilmour, 2006). From juvenile stages to adulthood we observed *TCFsiam* expression in the retina pigmented epithelium (Fig. 4I), brain endothelial cells (see below) and novel expression domains, such as the hypothalamus (Fig. 4K), gill arches (Fig. 4J and Supplementary Figure 4C), more caudally in the pronephric duct terminus and cloacal aperture (Supplemental movie 1) and finally in major vessels of the liver (Supplementary Figure 4E).

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Later in adult fish the olphactory bulb (Fig. 4L) and barbles (Supplementary Fig. 4D) were marked by the transgene expression. Furthermore, we found that *TCFsiam* transgenics displayed a fluorescent signal in the bifurcation of primary lamellae, at the tip of small gill rakers and along the bodies and on the tips of larger gill rakers (Fig. 4J and Supplementary Fig. 4C). Therefore, we were able to detect *TCFsiam* transgene expression both during larval stages and in adult fish. Moreover, novel tissues, such as the hypothalamus, gill arches and rakers, olphactory bulb and cloacal aperture were shown to actively express the Wnt reporter in distinct domains.

In TCFsiam transgenic fish fluorescent cells are found in atrioventricular valves and the outflow tract.

In agreement with the expression pattern of the BAT-GAL transgene in mouse (Maretto et al., 2003), we observed cardiac-specific expression in the *TCFsiam* reporters from 36 hpf onwards. To better characterize transgene expressing cell populations, we crossed *TCFsiam* reporter to transgenic lines labeling specific tissues of the developing heart. The myocardial specific line, *Tg(myl7:EGFP)*^{twu34}, shows that *TCFsiam* expression in the heart is initially found in atrial myocardial cells adjacent to the atrioventricular canal (AV) (Fig. 5A). Endocardial cells at the AV form a multilayered structure, which functions as a leaflet to prevent retrograde blood flow after 72 hpf. *Tg(kdrl:EGFP)*^{s843} positive cells at the AV canal endocardium were observed to express the *TCFsiam* transgene at 72 hpf (Fig. 5B). At 96 hpf, *TCFsiam* positive cells were found at the boundary between the ventricle and bulbus arteriosus (BA) (arrowheads in Fig. 5C, D, and G). In addition, *TCFsiam* positive cells were also found at the AV boundary (Fig. 5E and Supplemental Movie 2). Two morphologically different types of endocardial cells were observed in developing cardiac valves from 72 hpf to 100 hpf. The ones, adjacent to the flowing blood remain cuboidal and maintain expression of the adhesion molecule Alcam (zn8) and the *Tg(tie2:GFP)*^{s849} transgene (Fig. 5 D–I). Internal endocardial cells show less Alcam expression and appear mesenchymal rather than epithelial (arrows in Fig. 5F and I). These more internal AV canal endocardial cells predominantly express the *TCFsiam* transgene, indicating that they maintain Wnt/β-catenin signaling activity.

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Therefore, mesenchymal looking endocardial cells at the atrioventricular canal and ventricular/bulbus arteriosus boundary express the *TCFsiam* transgene during zebrafish heart development.

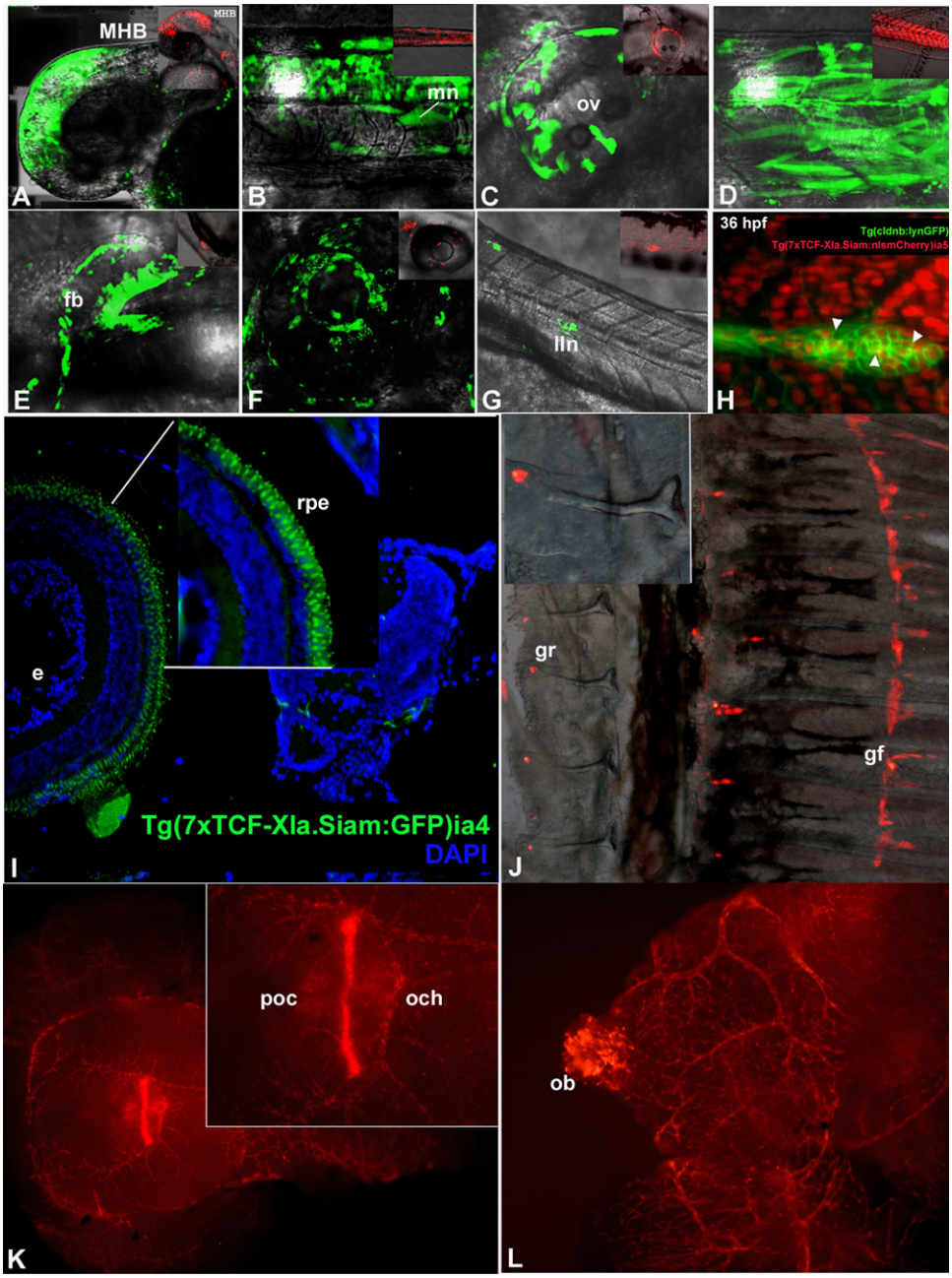

Fig. 4. *TCFsiam* expression domains resemble characteristic regions of Wnt/β-catenin signaling activity. (A–H) are reconstructed confocal projections images. (I–L) are images taken with conventional fluorescent microscope. In A–G a comparison between *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} and *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} (small inset) show an identical pattern of expression. (A-H) 48-hpf larva. (A) *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} is highly expressed in the brain, particularly in the MHB. More caudally, strong *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} expression is seen in the spinal cord (B), in the cristae of the otic vesicle (C), in the somitic mesoderm (D), in fin buds (E) and eye(F). (G–H) Posterior lateral line neusromasts actively express the *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} transgene. Confirmation of the neuromasts identity of the cell cluster seen in H (arrowheads) was obtained by Tg(-8.0cldnb:lynEGFP)^{zf106} transgene coexpression in *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} expressing cells of the neuromasts primordium at 36 hpf of *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}*/Tg(-8.0cldnb:lynEGFP)*^{zf106} double transgenics. (I) Longitudinal paraffin section of a representative 30 dpf *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} larva showing GFP-expressing cells in the retinal pigmented epithelium (rpe). Counterstaining was performed with the nuclear dye DAPI. (J) Representative image of the branchial arch of a one year old *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish demonstrating transgene expression in the tip of gill rackers (gr) and in gill filaments. In the small inset, a magnification of a single gill raker with its fluorescently labeled tip is depicted. (K) Ventral view of a representative forebrain from a one year old *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish, showing strong fluorescence in the hypothalamic region and optic commissure together with labeled endothelial cells of the vascular newtork. (L) Magnification of a representative olphactory bulb labeled in a representative one year old *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish. MHB: midbrain-hindbrain boundary; e: eye; fb: fin bud; e:eye; gf: gill filaments; gr:gill rackers; lln:lateral line neuromasts; ob: olphactory bulb; och: optic commissure; ov: otic vesicle. poc: post-optic commissure; rpe: retinal pigmented epithelium.

Persistent transgene expression in the central nervous system (CNS) endothelial compartment of Wnt reporter lines during early and late life stages

In mice Wnt ligands have been demonstrated to act as migrating factors for CNS endothelial cells and to trigger blood brain barrier (BBB) formation (Liebner et al., 2008; Daneman et al., 2009). To test whether CNS endothelial cells express the *TCFsiam* transgene, we crossed *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia} reporter fish to the *fli1a:EGFP* transgenic line (Lawson and Weinstein, 2002). No cells were co-labeled before 3dpf. However, after 3dpf few *TCFsiam* reporter expressing-cells colocalised with *fli1a:EGFP*-

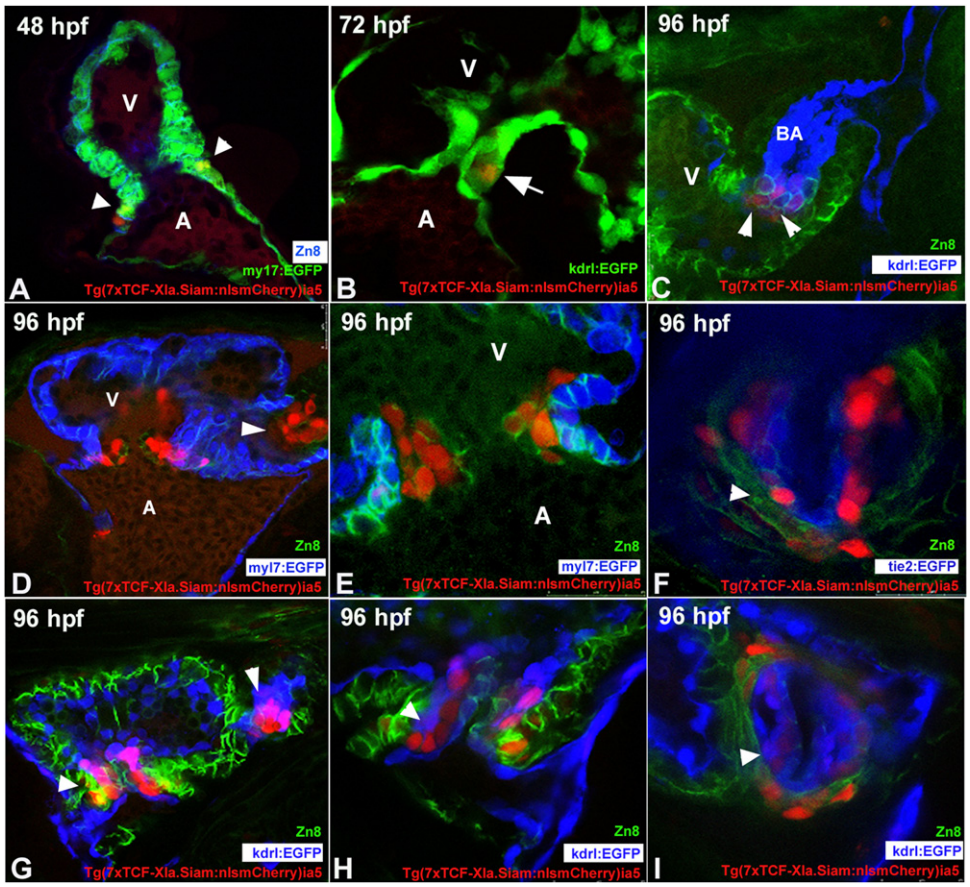

Fig. 5. Expression analysis of the *TCFsiam* transgene in wild-type AV canal between 48 and 96 hpf. (A, B, D, E) Confocal images of the AV canal from Tg(*myl7:EGFP*)^{twu34}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish (A, D, E) and from Tg(*kdrl::EGFP*)^{s843}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} double transgenics (B,C,G,H,I). (F,I) are cross section from the AV canal of Tg(*Tie2:EGFP*)^{s849}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} (F) and Tg(*kdrl:EGFP*)^{s843}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish (I). (C) Cross section of the bulbus arteriosus (BA) of a 96 hpf Tg(*kdrl:EGFP*) ^{s843}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish. Green fluorescence has been pseudocolored in blue for Tg(*myl7:EGFP*) ^{twu34} in D, E, for Tg(*kdrl::EGFP*) ^{s843} in C,G,H,I and for Tg(*Tie2:EGFP*) ^{s849} in F. (A–I). Immunostaining with zn8 (blue in A, pseudocolored green in C–I). (A) Few atrial myocardial cells adjacent to the atrioventricular canal (arrowhead) express the *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}. (B) Endocardial cells forming the multilayered structure of cardiac valves at 72 hpf express the transgene. (C) Endocardial cells at the boundary between the ventricle and bulbus arteriosus express the transgene (arrowheads). (D, E, G, H, I) round shaped cells at the AV canal adjacent to the cuboidal epithelial cells express the transgene. (D–I) *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} positive cells downregulate alcam/zn8 at the AV boundary and the AV marker Tg(*Tie2:EGFP*) ^{s849} (F). A:atrium, V:ventricle; AV: atrio-ventricular.

expressing trunk vessels (Fig. 6A). Detailed analyses of blood vessels after 5dpf, showed *TCFsiam* expression in individual endothelial cells located along the same vessels in the head (Fig. 6B and C), while none were labeled in the trunk region. Lack of expression of the *TCFsiam* transgene in the trunk vessels persisted in one month old double transgenics fish (Supplemental Fig. 6). As shown by coexpression with Tg(*kdrl:EGFP*) ^{s843} at 7 dpf, *TCFsiam* reporters labeled the endothelial compartment of the brain (Fig. 6E and F). At early larval (at 14 and 20 dpf) as well as juvenile stages (30 and 60 dpf) *TCFsiam* reporter was found to be still expressed in the CNS endothelial cells (Fig. 6G–M, and Supplemental Movie 3). Since the blood brain barrier (BBB) is essential for the control of the brain fluid milieu in adult mammals, we asked whether transgene expression persisted in late life stages of the reporter fish CNS. Surprisingly, we found that in one year old fish, eGFP labeled the entire brain vascular network (Fig. 6N); and using computer assisted reconstruction we were able to confirm that even the endothelial cells of the small capillary network were labeled by *TCFsiam* transgene with strong fluorescence intensity (data not shown).

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These observations led us to conclude that the *TCFsiam* transgene is persistently expressed in endothelial cells within the CNS.

Wnt/β-catenin reporter is expressed in both cephalic and trunk neural crest derived-cells

Because Wnt/β-catenin signaling has been involved in neural crest cell induction and proliferation (Dorsky et al., 1998; Lewis et al., 2004), we assessed *TCFsiam* coexpression in neural crest lineage expressing *Tg(sox10:mRFP)* fish (Kirby et al., 2006). At 36 hpf, we observed *TCFsiam* expression in several *sox10* positive otic vesicle (Fig. 7A) and neural crest derived pharyngeal arch cells (Fig. 7B C). From 72 hpf *TCFsiam* was expressed in dorsal root ganglia (DRG) cells along the trunk (Fig. 7D), and after 5 dpf we identified double co-labeled cephalic Schwann cells (Supplemental Fig. 4A).

We next generated time-lapse movies to assess the reporter activation in neural crest derived-cells. As shown in Fig. 7E and Supplemental Movie 4A B, we observed that *sox10:mRFP* positive cells migrate over *TCFsiam*-expressing endothelial cells at 3 dpf. Notably, after three independent experiments in 3 dpf larvae we observed a single TCFsiam/sox10-doublet of cells generated after a presumptive cell division. The absence of eGFP signal in the single migrating Sox10 positive cell (indicated by the arrow) rules out the possibility that colabeling occurred before cell division. Indeed, the orange-labeled doublet of cells suggests that TCF expression was induced during cell division.

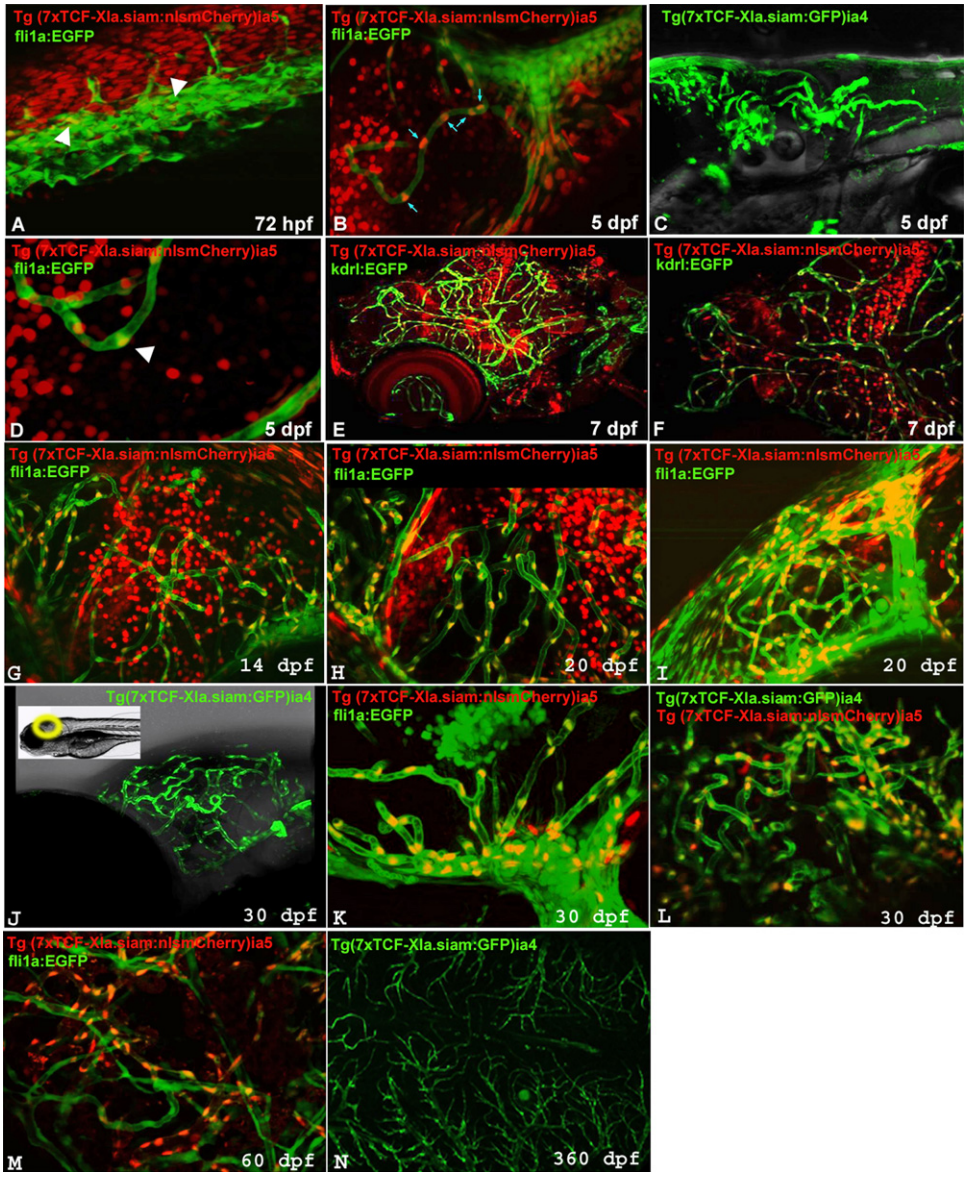

Fig. 6. *TCFsiam* transgene expression in the CNS endothelial compartment during larval, juvenile and adult stages. (A) Confocal image of the trunk vessels in a representative 72 hpf Tg(*fli1a:EGFP*)^{y1}/ *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish. Few endothelial cells are positive for the transgene (arrowheads in A). (B) Confocal image of the CNS vessels in a 5 dpf representative Tg(*fli1a:EGFP*)^{y1}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish. Several endothelial *fli1a:EGP* expressing cells are positive for the *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} transgene (highlighted by light blue arrows). (C) Confocal Z-stack projection of a 5 dpf *Tg(7xTCF-Xla.Siam:GFP)*^{ia4}, showing the targeted labeling of the cerebral vascular network. (D) Magnification of a CNS blood vessel of a representative 5 dpf Tg(*fli1a:EGFP*)^{y1}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} larva. A double positive endothelial cell is marked by a arrowhead. (E,F) Confocal Z-stack projection of a representative 7 dpf Tg(*kdrl:EGFP*)^{s843}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}, showing a perfect localization of the *TCFsiam* signal in the CNS endothelial compartment. In F a higher magnification of (E) is shown. (G) Confocal Z-stack projection in the midbrain area of a representative 14 dpf Tg(*fli1a:EGFP*)^{y1}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish. (H,I) Confocal Z-stack projection of a representative 20 dpf Tg(*fli1a:EGFP*)^{y1}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish in the midbrain area (H) and in the forebrain area (I), showing strong labeling of the brain vessels in the transgenic reporter fish. (J) Confocal projection a 30 dpf fish brain showing localized expression of the *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} transgene in the vascular compartment of the brain. A small inset illustrates the area analyzed by confocal microscopy in the living fish. (K, L, M) Confocal Z-stack projection of a representative 30 dpf (K) and 60 dpf (M) Tg(*fli1a:EGFP*)^{y1}/ *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} and 30 dpf (L) *Tg(7xTCF-Xla.Siam:GFP)*^{ia4}/*Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish showing a persistent expression of the *TCFsiam* transgene in the brain vascular network. (N) Confocal acquisition of a whole fixed brain from a one year old fish demonstrating long-term expression of the *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} transgene in CNS vascular network.

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To further evaluate the potential use of the reporter in tracking neural crest cell-derived cell fate, we performed time-lapse imaging of double transgenic 5dpf larvae. We were able to track the migration of individual *TCFsiam/sox10* expressing cells in the trunk, crossing the somitic mesoderm to probably differentiate into DRG or sympathetic ganglia (Fig. 7F and Supplemental Movie 5). Although only two larvae were evaluated, this observation suggests that neural crest derived migrating cells can be followed due to the long half-life of the eGFP. Thus, the *TCFsiam* transgene is highly expressed in the neural crest lineage, and allows for observation of its dynamic behavior during fish development.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2012.03.023.

Wnt signaling upregulation during epimorphic fin regeneration can be recapitulated in reporters

We and others have previously shown that Wnt/β−catenin signaling is active and necessary for zebrafish tail fin regeneration (Kawakami et al., 2006; Stoick-Cooper et al., 2007). However, using

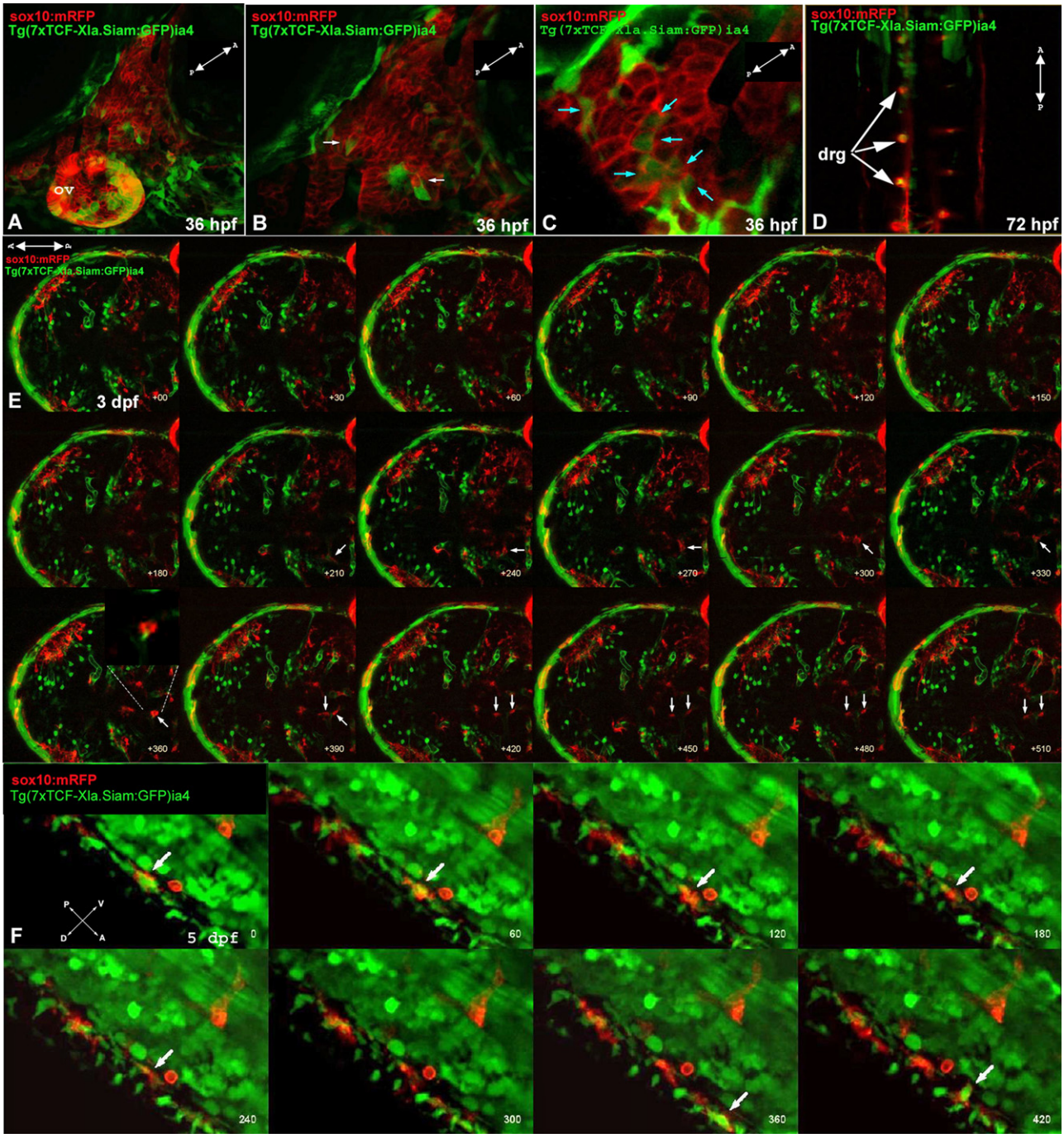

Fig. 7. Persistent *TCFsiam* expression allows fine tracking of neural crest-derived cells. (A) *sox10:mRFP* expressing cells in otic vesicle of a representative 36 hpf double transgenic larva Tg(*sox10:mRFP*)^{vu234}/(*7xTCF-Xla.Siam:GFP*)^{ia4} coexpress the *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} transgene. (B,C) Neural crest-derived cells in the pharyngeal arches coexpress the transgene *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} at a high level at 36 hpf. (D) At 72 hpf, Tg(*sox10:mRFP*)^{vu234}/*Tg(7xTCF-Xla.Siam:GFP)*^{ia4} coexpressing cells are dorsal root ganglia (DRG) scattered along the trunk. (E) Montage of a time lapse movie, showing neural crest-derived cells dynamics in the midbrain of a representative 56 hpf Tg(*sox10:mRFP*)^{vu234}/*Tg(7xTCF-Xla.Siam:GFP)*^{ia4} larva. One to two small white arrows in the bottom left indicate migration and neural crest derived cell division along a brain vessel. Note at the 13th frame a small inset showing a doublet of sox10 positive cells coexpressing the *TCFsiam* transgene. Each frame was recorded every 30 min. (F) Sequential images of a time lapse movie in a representative 5 dpf Tg(*sox10:mRFP*)^{vu234}/*Tg(7xTCF-Xla.Siam:GFP)*^{ia4} larva showing the migration of double positive cells across the somitic mesoderm. Copositive migrating cells are labeled by a white arrow. Each frame was recorded every 1 h.

the TopdGFP transgenic line, pathway activation in regenerating fins is only weakly detectable by RNA *in situ* hybridization (Stoick-Cooper et al., 2007). We thus asked whether Wnt signaling activity during regeneration is more efficiently reported in the *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} line. We found no reporter activity either by detecting Cherry RNA or Cherry fluorescence in unamputated tail fins or in fins immediately after amputation (Fig. 8B F). However, already at 24 h post amputation (hpa), robust Cherry RNA expression could be detected (Fig. 8I), and was followed by Cherry fluorescence at 48 hpa (Fig. 8K). Cherry activity increased and was strongly detected both by RNA and fluorescence at 72 hpa (Fig. 8N, O). We conclude that the *TCFsiam* reporter is a sensitive tool to monitor Wnt/β-catenin signaling during fin regeneration. Importantly, these results show that β-catenin signaling is activated at early stages of regeneration.

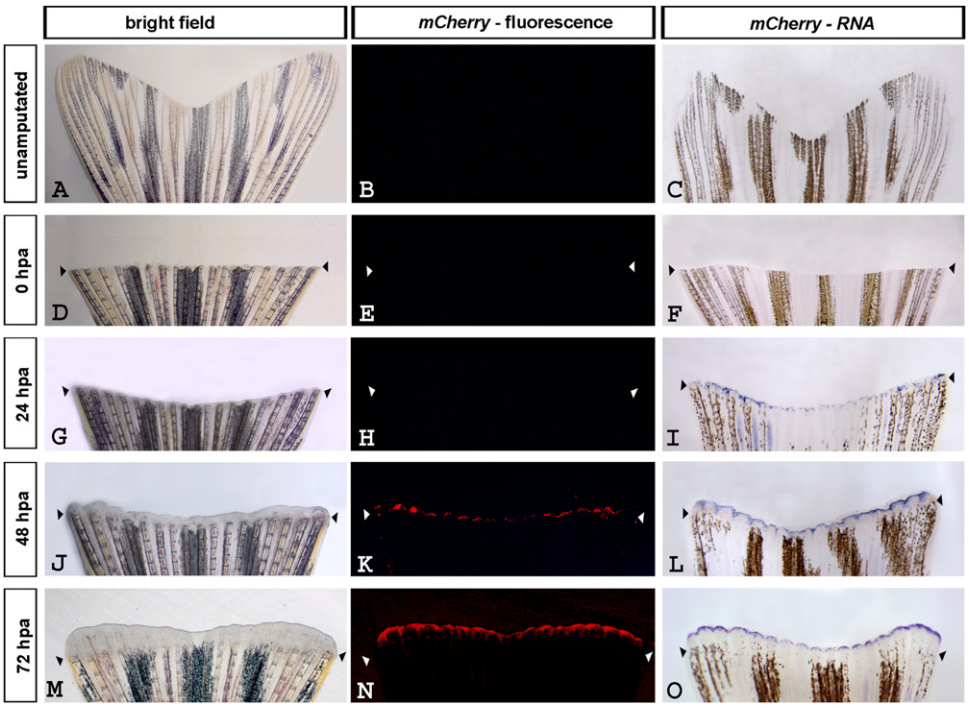

Fig. 8. Wnt/β-catenin signaling is detectable in regenerating tail fins of *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} fish. (Left column) Bright field live images of unamputated and regenerating fins. (Middle column) mCherry fluorescence in live unamputated and regenerating fins. (Right column) *mCherry* expression detected by RNA *in situ* hybridization in unamputated and regenerating fins. (A–C and D–F) Wnt/β-catenin signaling is not detectable in unamputated and newly amputated fish fins, respectively. (G–I) β-catenin activity is detected by strong *mCherry* RNA expression at 24 hpa while mCherry fluorescence is not visible yet. (J–L) mCherry expression is detectable by both RNA and fluorescence at 48 hpa. (M–O) Wnt/β-catenin signaling is robustly evident by both *mCherry* RNA and fluorescence at 72 hpa.

Discussion

Addressing the time and position of Wnt/β-catenin signaling pathway activity during vertebrate development is important to further understand its functional role in human pathogenesis. Despite previous efforts in creating a Wnt/β-catenin fish reporter, a model which allows for robust imaging of Wnt/β-catenin reporter activity in the living organism is lacking.

We describe here the generation and characterization of two novel transgenic Wnt reporter lines, *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} and *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5}, which we propose as tools for live imaging and drug screening. We consider *TCFsiam* lines as *bona fide* Wnt reporters for several reasons.

First of all, we have shown how the expression of the reporter can be positively and negatively modulated by using the heat-shock inducible expression of Wnt8 and Dkk1, respectively. In particular, using the Wnt8 heat-shock inducible line, it is possible to observe an ectopic Wnt reporter activity of *TCFsiam* transgenic domains only during early stages of development such as gastrulation and somitogenesis (compare Fig. 2G–H with J–K, respectively), while at later stages, (after 24 hpf) increased levels of transgene expression are observed only within tissues already restricted in competence, such as posterior somites, main sensory organs and specific regions of the brain (compare Fig. 2I and L). This expression can raise the question of how restriction of canonical Wnt responsiveness is acquired during fish development. However, results from intracellular manipulation of Wnt signaling by agonists such as alsterpaullone, BIO and LiCl treatments, suggest that intracellular mechanisms are limiting factors in restriction of Wnt responsiveness.

By testing Wnt pathway modifiers (LiCl, BIO, IWR and alsterpaullone), we have demonstrated that TCFsiam lines can be used as a platform to screen for compounds that modulate Wnt/β-catenin pathway activity in a tissue specific manner. Interestingly, we have detected a specific activation of the *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} transgene in the otic vesicles after lithium chloride treatment. This is in agreement with previous findings, showing that the activation of the TOPGAL reporter with lithium in the otic vesicle was responsible for the expansion of Wnt-responsive genes in mouse (Riccomagno et al., 2005). On the other hand, we have shown that the expression of the reporter in the same otic vesicle region is less sensitive to the treatment of IWR-1 at the transcriptional level (see Fig. 2Y and Supplemental Fig. 3). This finding can be explained by an Axin-independent regulation of TCF/LEF mediated transcription, as previously seen in an *in vitro* model (Seo and Jho, 2007).

The pharmacological test of Wnt pathway activity modifiers by means of the *TCFsiam* lines allows to identify novel Wnt responsive domains in fish tissues. To this purpose, we have demonstrated increased reporter activity in gill filaments after both LiCl, alsterpaullone and BIO treatments. Gill filaments are known to bud from the ectodermal epithelium of the 3–6 pharyngeal arches (Shadrin and Ozerniuk, 2002). This drug-dependent upregulation of Wnt reporter activity expression in the gill arches represents a novel finding, which underscores the role of Wnt pathway during differentiation and maintenance of this tissue.

Given the long half-life of eGFP or mCherry (more than 24 h), we are aware that, in specific domains, fluorescence detection simply reflects Wnt signaling activity already ceased in the tissue. However, *in situ* hybridization mRNA detection performed with antisense eGFP/mCherry riboprobes on *TCFsiam* reporters demonstrated that the pattern of expression was included within the fluorescently labeled domains (Fig. 1, Supplemental Fig. 3 and not shown).

Here, we have also shown that zygotic loss of *lef1* but not of *tcf3 and tcf4* reduces the *TCFsiam* reporter activity. One possible interpretation of the *lef1*-specific transcriptional control is that the *tcfs* have redundant roles and, therefore, the effect of only

combinatorial loss of *tcf3* and *tcf4* function on Wnt/β-catenin might be detectable. The hypothesis has indeed been supported by previous studies showing that individual *tcfa* and *tcf4* zygotic homozygous mutants display no obvious abnormalities during early development; moreover, the expression profile of some *tcfs* is overlapping in some embryonic regions (Kim et al., 2000; Dorsky et al., 2003).

A second major reason supporting *TCFsiam* as bona fide Wnt/β-catenin reporters is the spatiotemporal expression profile of the reporter transgene. This latter resembles the characteristic domains of the Wnt/β-catenin signaling pathway, previously described using the *Tg(TOP:GFP)*^{w25} line, such as the CNS, spinal cord, otic vesicles, heart valve cushions, lateral line ganglia, neural crest cells and retinal pigmented epithelium (Hurlstone et al., 2003; Lewis et al., 2004; Lee et al., 2006; Stoick-Cooper et al., 2007).

As regards the expression profile in the heart, we have shown a particular subset of mesenchymal-like endocardial cells expressing the *TCFsiam* transgene. Intriguingly, we have observed that prolonged administration of lithium chloride expands the expression domain in cardiac valves (data not shown). This observation could be associated with a well known human congenital condition, called Ebstein's anomaly, characterized by an enlargement of the anterior leaflet of the heart, which occurs after lithium exposure in infants during pregnancy (Zalzstein et al., 1990).

In *TCFsiam* lines, however, we have been able to further show reporter expressing-cells in the endothelial compartment, especially in CNS endothelial cells, which have not yet been reported as active domains of β-catenin signaling in zebrafish. The involvement of the Wnt/β-catenin signaling pathway in the CNS angiogenesis and blood brain barrier (BBB) formation has been clearly demonstrated in rodents, through the use of the TOPGAL transgene (Daneman et al., 2009). Different ligands of this pathway, such as Wnt3a, Wnt7a and Wnt7b have been shown to play a fundamental role in both CNS endothelial migration and interaction with neural progenitors (Stenman et al., 2008; Daneman et al., 2009). Moreover, through the use of conditional knockout mouse models, it has been demonstrated that Wnt/β-catenin signaling modulates BBB permeability by acting on the tight junction-specific claudin 3 (Cldn3) protein (Liebner et al., 2008). Notably, the expression of *TCFsiam* reporters persisted until adulthood in the vascular compartment, suggesting a long-term role of the Wnt/β-catenin in the maintenance of the endothelial compartment of the BBB. This reporter shows that the Wnt/β-catenin signaling acts in a delimited portion of the whole body vascular network. The CNS-endothelial specific signaling detected by this reporter suggests a tissue specific mechanism of endothelial differentiation limited to the CNS (Supplemental Fig. 6).

Here, we have also shown how the *TCFsiam* reporters combined with the *Tg(sox10:mRFP)* line allow to visualize the integration between CNS vessel network and migration of neural crest derived-cells. The cephalic neural crest-derived cells are known to give rise to pericytes and smooth muscle cells of the brain vessels (Etchevers et al., 2001). However, while a close relationship between enteric neural crest cells (ENCC) and endothelial cells has been previously shown in the zebrafish gut (Nagy et al., 2009), we provide *in vivo* evidence that this tight interaction exists also in the brain vascular network.

Among other tissues, we have also found that the olphactory bulb and gill rackers of the branchial arches are active sites of *TCFsiam* activity. Since taste buds in zebrafish are known to be at the tip of gill rakers (Hansen et al., 2002) and in mammals Wnt signaling has been recently reported to be essential for taste bud formation and olphactory bulb (Liu et al., 2007; Gaillard and Barlow, 2011), it is very likely that fluorescent cells at the tip and along the body of gill rakers are indeed taste receptors. The fact that taste buds, olphactory bulb, barbels, and lateral line organs all express the transgene suggests an essential role for the Wnt/β-catenin pathway in sensory organs formation and maintenance.

Among novel tissues where Wnt/β-catenin pathway appears to be active in *TCFsiam* transgenics we detected reporter activity also in the cloacal aperture. Recently, a negative interaction between nephrocystin4 and Wnt/β-catenin pathway has been reported during fish pronephric duct and cloacal aperture formation (Burcklé et al., 2011), suggesting a critical role of this pathway in either physiological or pathogenetic conditions.

In this paper, we have also addressed the use of *TCFsiam* reporter lines in regeneration studies. Our finding that active Wnt/β-catenin signaling in regenerating fin is detectable earlier and stronger than reported (Stoick-Cooper et al., 2007), indicates that *TCFsiam* reporter provides a more sensitive assessment of the pathway activation in regenerative response. Considering that the identification of new soluble factors or small molecules modulating regenerative capacity is one of the major goals in regenerative biology, we propose the use of these reporters as readout to screens aimed to identify mitogens and drugs that enhance regeneration. This could as well be applied to the discovery of molecules capable of blocking Wnt-dependent cell stemness especially related to the field of familial and sporadic colorectal cancers (Fodde and Brabletz, 2007). In this context, the tumor suppressor protein APC (adenomatous polyposis coli) is a candidate target known to be frequently mutated in a high number of colorectal cancers (Segditsas and Tomlinson, 2006). We, therefore, suggest the use of *TCFsiam* reporters in the apc^{hu745} mutant background as a tool to screen new molecules able to counteract Wnt activation.

In conclusion, we have shown that *TCFsiam* reporters represent a step forward in analyzing Wnt/β-catenin activity *in vivo*, which can be applied in a wide context, such as analysis of mutants that modulate Wnt signaling, detailed examination of Wnt signaling in specific tissues and stages, and identification and characterization of small molecules or drugs that could be useful for regenerative medicine or cancer biology.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.03.023.

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