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Initiation of zebrafish hematopoiesis by the TATA-box-binding protein-related factor, Trf3

Daniel O. Hart^{1,2}, Tamal Raha^{1,2}, Nathan D. Lawson², and Michael R. Green^{1,2}

¹ Howard Hughes Medical Institute, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA

² Programs in Gene Function and Expression and Molecular Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA

Abstract

TATA-box-binding protein (TBP)-related factor 3, TRF3 (also called TBP2), is a vertebrate-specific member of the TBP family that has a conserved C-terminal region and DNA binding domain virtually identical to that of TBP¹. TRF3 is highly expressed during embryonic development, and studies in zebrafish and *Xenopus* have shown that TRF3 is required for normal embryogenesis^{2,3}. Here we show that Trf3-depleted zebrafish embryos exhibit multiple developmental defects and, in particular, fail to undergo hematopoiesis. Expression profiling for Trf3-dependent genes identified *mespa*, which encodes a transcription factor whose murine orthologue is required for mesoderm specification⁴, and chromatin immunoprecipitation verified that Trf3 binds to the *mespa* promoter. Depletion of *Mespa* resulted in developmental and hematopoietic defects strikingly similar to those induced by Trf3 depletion. Injection of *mespa* mRNA restored normal development to a Trf3-depleted embryo, indicating *mespa* is the single Trf3 target gene required for zebrafish embryogenesis. Zebrafish embryos depleted of Trf3 or *Mespa* also failed to express *cdx4*, a *caudal*-related gene required for hematopoiesis. *Mespa* binds to the *cdx4* promoter, and epistasis analysis revealed an ordered *trf3-mespa-cdx4* pathway. Thus, in zebrafish commitment of mesoderm to the hematopoietic lineage occurs through a transcription factor pathway initiated by a TBP-related factor.

To analyze the role of TRF3 during embryonic development, we used antisense morpholino oligonucleotides (MOs) to ablate Trf3, and as a control *Tbp*, function in zebrafish embryos. MOs were injected into wild-type one-cell stage fertilized embryos and depletion of Trf3 and *Tbp* was analyzed by immunoblotting at 6 hours post-fertilization (hpf), a time at which expression of both proteins was readily detectable (Supplementary Fig. 1a). Immunoblot analysis confirmed that injection of each MO efficiently and specifically depleted its target gene (Supplementary Fig. 1b). Consistent with previous studies⁵, *Tbp*-depleted embryos appeared to initiate gastrulation but failed to progress past 50% epiboly (Supplementary Fig. 2; n=122/150). By contrast, Trf3-depleted embryos appeared to develop normally until the tailbud stage, but by 14 hpf exhibited delayed development and necrosis compared to siblings injected with a randomized control MO (n=166/177). Inspection of Trf3-depleted embryos at

Correspondence and requests for materials should be addressed to N.D.L. (nathan.lawson@umassmed.edu) or M.R.G. (michael.green@umassmed.edu).

Author Contributions D.H., N.L. and M.G. conceived and designed the experiments. D.H. performed the experiments, with the assistance of N.L., who performed the experiments shown in Supplementary Fig. 2, and T.R., who performed the chromatin immunoprecipitation assays shown in Fig. 1b and Supplementary Fig. 10a. D.H., T.R., N.L. and M.G. analyzed the data. D.H., N.L. and M.G. wrote the paper.

Author Information The microarray data has been deposited in the ArrayExpress database at <http://www.ebi.ac.uk/arrayexpress>. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions.

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21 hpf revealed severe necrosis, although head, trunk and tail rudiments were apparent, suggesting that initial antero-posterior patterning was largely unaffected.

To identify Trf3 target genes, we performed expression profiling in *trf3* MO-treated embryos (at 6 hpf) using a zebrafish oligonucleotide microarray representing ~12,800 genes. As expected, the vast majority of genes were unaffected by Trf3 depletion (Supplementary Data). Three such representative genes are shown as controls in the RT-PCR experiment of Fig. 1a, which confirms that their expression is dependent upon Tbp but not Trf3. Using a candidate-based approach, we selected genes whose expression were significantly decreased by Trf3 knockdown (Supplementary Table 1) and had been previously implicated in embryonic development. These candidates were further analyzed by RT-PCR for Trf3-dependent expression, chromatin immunoprecipitation (ChIP) for selective Trf3 occupancy, and finally for a role in zebrafish development (see below and data not shown). This combined analysis identified *mespa*, whose mouse orthologue, *Mesp1*, encodes a basic-helix-loop-helix (bHLH)-type transcription factor required for proper embryonic development⁴.

The RT-PCR results of Fig. 1a show that Trf3 depletion eliminated *mespa* expression. To determine whether *mespa* was a direct Trf3 target, we analyzed promoter occupancy by ChIP assays using antibodies directed against Trf3, Tbp, RNA polymerase II (Pol II) or, as a negative control, an irrelevant protein (yeast Gal4). As controls, we analyzed two well-characterized housekeeping genes, *actin* and *gapdh*. Figure 1b shows that Pol II was bound to all three promoters, consistent with the transcriptional activity of these genes. Notably, Tbp but not Trf3 was bound to the *actin* promoter, whereas both Tbp and Trf3 were bound to the *gapdh* promoter. By contrast, the *mespa* promoter was selectively bound by Trf3 and not Tbp. Based upon the dependence of *mespa* expression on Trf3 (Fig. 1a) and the selective binding of Trf3 to the *mespa* promoter (Fig. 1b), we conclude that *mespa* is a direct Trf3 target gene.

Phenotypic analysis demonstrated that the *mespa* MO-injected embryo had a developmental defect that was strikingly similar to that of a Trf3-depleted embryo (n=61/89) (Fig. 1c and Supplementary Fig. 3). As a control, injection of a *mespa* mRNA bearing a silent mutation that prevented hybridization with the *mespa* MO restored normal development to the *Mespa*-depleted embryo (Supplementary Fig. 4). To compare the phenotypes of the Trf3- and *Mespa*-depleted embryos in greater detail, we performed whole mount *in situ* hybridization using several developmentally-regulated genes as markers. Figure 1d shows that depletion of either Trf3 or *Mespa* resulted in increased *shh* expression in axial mesoderm, decreased *nkx2.5* expression in cardiac mesoderm, and decreased *gata1* expression in lateral mesoderm. Surprisingly, expression of a second lateral mesoderm marker, *pax2*, was unaffected by loss of Trf3 or *Mespa*. Moreover, expression of the ventral marker *bmp4* was normal in both Trf3- and *Mespa*-depleted embryos, indicating that the developmental defects were not due to loss of proper dorsal-ventral patterning. Thus, the developmental defect observed in *Mespa*-depleted embryos was very similar, if not identical, to that of Trf3-depleted embryos.

We next asked whether ectopic expression of *mespa* could restore normal development to a Trf3-depleted embryo. Figure 1e shows that injection of *mespa* mRNA (n=110/134), but not an unrelated control mRNA (n=0/63; data not shown), restored normal development to the *trf3* MO-injected embryo. Completeness of rescue was verified by differential interference contrast microscopy (Supplementary Fig. 5) and *in situ* hybridization analysis (Supplementary Fig. 6). Collectively, these results indicate that in zebrafish, *mespa* is the single Trf3 target gene required for proper embryonic development.

The results of Fig. 1d suggested a requirement for Trf3 and *Mespa* during development of cell types in the lateral mesoderm, which we elected to analyze in greater detail. We assayed the expression of several hematopoietic, vascular and pronephric markers in *trf3*- or *mespa*-MO

injected embryos. The RT-PCR results of Supplementary Fig. 7 indicate that a number of blood cell-specific genes were significantly down-regulated in *Trf3*- and *Mespa*-depleted embryos, including *hbae1* and *hbae3*, which are terminal markers of erythroid cell fate, as well as *gata1*, which is required for the expression of a variety of genes in the erythroid lineage. In addition, earlier markers of hematopoietic precursors, *scl* and *lmo2*, were similarly reduced although expression of *gata2* was unaffected. Finally, expression of *pu.1*, a marker of myeloid cells that arise in the anterior lateral mesoderm, was also reduced in the absence of *Trf3* or *Mespa*.

To confirm the RT-PCR results, we analyzed the expression of these marker genes by whole mount *in situ* hybridization. Figure 2a shows that in *trf3*- or *mespa*-MO injected embryos, *scl* expression was reduced in the posterior lateral mesoderm, although expression was maintained within more anterior cells whose position is consistent with that of endothelial cells^{6,7}. Expression of *lmo2* was moderately reduced in the posterior lateral mesoderm in *Trf3*- and *Mespa*-depleted embryos, whereas *pu.1* expression was absent from anterior lateral mesoderm. Also consistent with the RT-PCR results, expression of the early hematopoietic marker *gata2* was unaffected by loss of *Trf3* or *Mespa*. Similarly, expression of the endothelial cell marker *kdr* was normal in embryos lacking *Trf3* or *Mespa*. As expected, injection of *mespa* mRNA into *Trf3*-depleted embryos fully rescued expression of the hematopoietic markers *gata1* (see below), *hbae3* and *scl* (Supplementary Fig. 6), as well as the cardiac mesoderm marker *nkx2.5* (Supplementary Fig. 6).

Collectively, the results of Fig. 2a suggest a defect in the formation of hematopoietic cells in the posterior lateral mesoderm. To confirm this defect, we simultaneously assayed expression of *fli1*, a marker for both hematopoietic and endothelial cells, and either *gata1* or *pax2*, markers of erythroid or pronephric lateral mesoderm, respectively. Figure 2b shows that expression of a *fli1:EGFP* transgene was maintained in the absence of *Trf3* or *Mespa*, whereas *gata1* expression was absent from the same embryo. By contrast, *pax2* was unaffected by the loss of *Trf3* or *Mespa* and continued to be co-expressed with *fli1* in adjacent cells of the lateral mesoderm. Taken together, these results demonstrate a selective loss of hematopoietic cells within the posterior lateral mesoderm of zebrafish embryos lacking *Trf3* or *Mespa*.

The defects in hematopoietic development described above are reminiscent of those observed in embryos lacking *Cdx4*, a caudal-related transcription factor that is required for hematopoiesis⁶ and functions by activating expression of homeobox genes involved in the commitment of mesoderm to the hematopoietic lineage^{6,8}. RT-PCR and *in situ* hybridization analysis showed that *cdx4* expression was substantially decreased in *Trf3*-depleted embryos, as well as in *Mespa*-depleted embryos (Fig. 3a). As expected, expression of the *Cdx4*-dependent genes *hoxa9a*, *hoxb7a* and *hoxb5a*⁸ was also substantially reduced in *Trf3*- and *Mespa*-depleted embryos (Supplementary Fig. 8). Depletion of *Trf3* or *Mespa* did not affect expression of *cdx1a* (Supplementary Fig. 9), which has been reported to cooperate with *cdx4* in hematopoietic development⁸.

In situ hybridization analysis revealed that at 6 hpf, *cdx4*, *mespa* and *trf3* were co-expressed in the presumptive hematopoietic tissues based on the zebrafish fate map^{9,10} (Fig. 3b), suggesting that the three factors are components of a common pathway. To confirm this idea and determine the order of the pathway, we performed epistasis experiments using *gata1* expression as a phenotypic read-out. Figure 3c shows that injection of *trf3* mRNA restored *gata1* expression in *Trf3*-depleted embryos, but not in *Mespa*- and *Cdx4*-depleted embryos, indicating that *trf3* is upstream of both *mespa* and *cdx4*. Moreover, injection of *mespa* mRNA restored *gata1* expression in *Trf3*- and *Mespa*-depleted embryos, but not in *Cdx4*-depleted embryos, indicating that *mespa* is upstream of *cdx4* and downstream of *trf3*. Finally, injection of *cdx4* mRNA restored *gata1* expression in *Trf3*-, *Mespa*- and *Cdx4*-depleted embryos,

indicating that *cdx4* is downstream of both *trf3* and *mespa*. ChIP analysis supports the possibility that *cdx4* is a direct Mespa target gene (Supplementary Fig. 10a).

Previous studies have shown that ectopic expression of *cdx4* in zebrafish results in increased number of blood cells, as evidenced by expanded expression of hematopoietic markers⁶. Ectopic expression of *mespa* also resulted in expanded expression of *gata1* and *scl* (Fig. 3d) and *cdx4* (Fig. 3e). The collective results of Figs. 2 and 3 reveal that *trf3*, *mespa* and *cdx4* function in a common pathway, which is summarized in Supplementary Fig. 10b.

Collectively, our results indicate that binding of Trf3 to the *mespa* promoter is the earliest documented step in committing mesoderm to the hematopoietic lineage in the developing zebrafish embryo. A recent study has provided definitive evidence for the existence of hemangioblasts, bipotential progenitors that can give rise to both endothelial and hematopoietic cells, within the zebrafish embryo¹⁰. Significantly, in zebrafish embryos, hemangioblasts are present at the same time and place in which *trf3*, *mespa* and *cdx4* are expressed. Thus, the timing and location of *trf3*, *mespa* and *cdx4* co-expression suggests that this transcription factor pathway may function within hemangioblasts to specify hematopoietic progenitor cells (i.e., commitment of mesoderm to the hematopoietic lineage). Although the defects associated with loss of Trf3 or Mespa are restricted to hematopoietic cell types in the lateral mesoderm, the effects on other tissues appear to be more widespread. We predict that there will be additional Mespa target genes that function analogous to but independent of *cdx4* in mediating other developmental pathways, such as specification of cardiac mesoderm, in the early embryo.

METHODS SUMMARY

Zebrafish maintenance, embryo production and microinjection

Zebrafish (*Danio rerio*) were maintained under standard conditions¹¹, and embryos were produced and staged as described¹². Antisense morpholino oligonucleotides (MOs) were designed against the start codon/5'UTR to block translation. MO sequences are as follows: control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'; *trf3* MO, 5'-GATGCCTCCTCATCCATGTTCA T-3'; *mespa* MO, 5'-GAAGAGAAAACGTGGAGGCGTCCAT -3'; and *zfz* MO, 5'-GCCCAACTTCCCGTACCGCCATCAT-3'. The *cdx4* MO (5'-CTCCAAAAGGTATCCAACGTACATG-3') was purchased from Open Biosystems, and the *thp* MO has been previously described⁵. MOs were injected at a concentration of 5 mg ml⁻¹, except the *cdx4* MO, which was injected at 0.2 mg ml⁻¹. Embryos were analyzed morphologically up to 24 h post injection.

To generate mRNAs for phenotypic rescue and epistasis experiments, full-length *trf3*, *mespa* and *cdx4* genes were amplified from shield-stage cDNA by RT-PCR (primer sequences are listed in Supplementary Table 2), and subcloned into pCS2 (ref. 13) for mRNA synthesis. The mRNAs were then injected into MO-treated or wild-type embryos at the 1–2 cell stage at concentrations of 100 ng µl⁻¹, 200 ng µl⁻¹, and 30 ng µl⁻¹ for *trf3*, *mespa* and *cdx4*, respectively.

Immunoblot analysis

Protein extracts were prepared from zebrafish embryos as described¹¹. Blots were probed with either an α -zebrafish Trf3 polyclonal antibody raised to the unique N-terminal region of zebrafish Trf3 (CPQKESTQADIDTSNS; amino acids 90–105), or an α -human TRF3 polyclonal¹, α -TBP monoclonal (3G3; Eurogentec), or α -RNA Pol II monoclonal (8WG16; Covance Research Products) antibody.

METHODS

Microarray analysis

Total RNA was prepared from 50 control MO- or *trf3* MO-injected zebrafish embryos 6 h after MO treatment using Trizol reagent. The KCC 17K Zebrafish Microarray was constructed using a commercially available 17K Zebrafish OligoLibrary™ consisting of 16,399 oligos (XEBLIB96; Compugen/Sigma-Genosys), corresponding to ~12,800 genes, based on UniGene clusters. Microarray construction, hybridization, and scanning were performed at the Kimmel Cancer Center Microarray Core Facility at Thomas Jefferson University. Chips were scanned using a Perkin Elmer ScanArray XL5000 Scanner, software version 3.1 and images were quantified by PerkinElmer QuantArray Software 3.0. The raw data for all genes induced or repressed by *trf3* MO treatment is provided in Supplementary Data. Background threshold values were determined for both microarrays and calculated as the mean plus two (for the *trf3* MO microarray) or three (for the control MO microarray) standard deviations of the channel intensities for the five bacterial probes present on each array. The background threshold values were then subtracted from the channel intensity value of each probe set (representing a single gene). The resulting data from the two microarrays were compared to identify Trf3-affected genes, which were classified as those whose probe values satisfied the following condition: >0 in the control microarray (i.e., the gene is significantly expressed over background) and ≤ 0 in the *trf3* MO microarray (i.e., the gene is significantly down-regulated, essentially “off”, upon Trf3 depletion); these genes are listed in the “Analyzed data” tab in Supplementary Data. To determine transcript identity and gene descriptions, we first annotated the gene list by identifying Unigene numbers (Unigene build #104) that corresponded with the Genbank accession number for each oligo on the array. We subsequently annotated gene names and descriptions through downloadable datasets obtained from the Zebrafish Information Network (ZFIN) and ENSEMBL (Zv7). In each case, we used FileMaker Pro to generate relational databases to annotate gene sets.

RT-PCR

RT-PCR analysis was performed according to standard protocols¹⁴. Total RNA was prepared using Trizol reagent (Invitrogen) and RT-PCR products were separated by agarose gel electrophoresis and visualized by autoradiography. Primer sequences are listed in Supplementary Table 2.

Chromatin immunoprecipitation

Embryos (~3000 at 6 hpf) were washed in 1X PBS three times, suspended in 1X PBS containing 1% formaldehyde (final concentration), transferred to a 10 ml dounce homogenizer and then dounced in one stroke. The reaction mixture was incubated at room temperature for 15 min and quenched by addition of glycine (0.125 M final concentration) for 10 min. The cells were centrifuged at 14K for 10 min at 4°C, and the pellets were washed twice with ice-cold 1X PBS containing protease inhibitors, and resuspended in 5 ml lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton-X 100, 0.1% SDS, 1 mM PMSF, and protease inhibitor cocktail). The suspension was then sonicated eight times on ice to generate ~500 bp fragments. The lysates were centrifuged, precleared with protein-A agarose beads (Upstate Biotech), and then divided into 1.5 ml aliquots per immunoprecipitation. Antibodies for immunoprecipitation were as follows: Trf3, Tbp and Pol II antibodies were described above; the Mespa antibody was raised to the C-terminal portion of the protein (CYQTQNPVQGDFHS; amino acids 191–204); yeast Gal4 (sc577; Santa Cruz). Following addition of the antibody, lysates were incubated for 12 h at 4°C, and then incubated with protein-A agarose beads (50 µl) for 2 h at 4°C (5% of the lysate was kept as “input” prior to the addition of the antibody). The beads were washed twice with lysis buffer without protease inhibitor, twice with the lysis buffer containing 1 M NaCl, and once with LiCl immune-

complex wash buffer (50 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% N-P40, 0.5% sodium deoxycholate). Finally the beads were washed thrice in TE and pelleted, and chromatin was eluted from the beads by adding 500 μ l freshly prepared 0.1 M NaHCO₃ and 1% SDS and incubated with rotation for 15 min at room temperature. Following addition of 2 μ l proteinase K (18.6 ml ml⁻¹), reverse cross-linking was performed for all samples for 6 h at 65°C. Chromatin was purified from the beads by phenol extraction followed by alcohol precipitation. The input sample was dissolved in 200 μ l and the immunoprecipitated samples were dissolved in 40 μ l, 2 μ l of which was used in the PCR reaction. ChIP PCR primers are listed in Supplementary Table 2.

***In situ* hybridization**

In situ hybridization was performed on whole-mount zebrafish embryos as described¹⁵, or on embryos flat-mounted in glycerol. Digoxigenin-labeled antisense probes were synthesized *in vitro* and obtained as follows: *shh*, *pax2*, *cdx4*, *hoxa9a*, *hoxb5a* and *hoxb7a* (Charles Sagerstrom, UMMS), *bmp4* (Michael Tsang, NIH), *gata1*, *ephrinb2a* and *kdr* (Nathan Lawson, UMMS), *nkx2.5* (The Zebrafish International Resource Center¹⁶), *scl* (NIH Zebrafish Gene Collection), *lmo2* (IMAGE clone 7433557), *pu.1* (IMAGE clone 6960940), *gata2* (IMAGE clone 6789690). For the *trf3* and *mespa* probes, N-terminal fragments of either *trf3* and *mespa* were first subcloned into the vector pGEM-T (Promega), and riboprobes were synthesized from the corresponding constructs as T7 or SP6 transcripts using the DIG RNA labelling mix (Roche). Riboprobe primers are listed in Supplementary Table 2.

Differential interference contrast (DIC) microscopy

High-resolution DIC microscopy was carried out on a Zeiss Axiophot microscope equipped with a Zeiss AxioCam HRC digital camera.

Fluorescence microscopy

Embryos (14 hpf) expressing the *fli1:EGFP* transgene [*TG(fli1:EGFP)^{y1}*; ref. ¹⁷] were hybridized with a riboprobe to either *gata1* or *pax2* (described above) for 16 h at 70°C. Samples were then washed twice in 2X SSC, 0.1% Tween 20 and 50% formamide for 30 min, once in 2X SSC and 0.1% Tween 20 for 15 min, and twice in 0.2X SSC and 0.1% Tween 20 for 30 min, all at 65°C, and blocked with Western Blocking Reagent (Roche) diluted in PBS for at least 1 h at room temperature. Primary antibody staining was performed in 100 μ l of blocking buffer consisting of a 1:200 dilution of anti-GFP rabbit IgG (A11122; Molecular Probes) and a 1:200 dilution of pre-absorbed anti-DIG Fab fragments (Roche) for 2 h at room temperature with gentle shaking. Embryos were washed six times in blocking buffer for 20 min at room temperature. Secondary antibody staining was performed using goat anti-rabbit Alexa Fluor 488 (A11008; Molecular Probes) at a 1:200 dilution in blocking solution for 2 h at room temperature. Embryos were washed six times in blocking buffer for 20 min at room temperature. Fast Red tablets (Roche) were dissolved (1 tablet per 2 ml of buffer) in 0.1 M Tris-HCl, pH 8.3 and 0.1% Tween 20 by shaking, and embryos were stained in 1 ml per sample well for 4 h at room temperature. After staining was assessed, embryos were washed six times in PBST for 20 min and stored in 90% glycerol at 4°C. Embryos were flat-mounted in glycerol and fluorescence microscopy was carried out using a Leica TCS SP2 confocal microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

mespa is the single Trf3 target gene required for proper embryonic development. **a**, RT-PCR analysis. **b**, ChIP analysis. **c**, Phenotypic analysis at 24 hpf. **c**, Whole-mount *in situ* hybridization with riboprobes to *shh* (14 hpf), *nkx2.5* (10 hpf; arrowheads denote decreased expression in cardiac mesoderm in *trf3* MO- and *mespa* MO-treated embryos), *gata1* (12 hpf), *pax2* (10 hpf; showing dorsal posterior (top) and anterior (bottom) views) and *bmp4* (6 hpf). **d**, Phenotypic analysis of embryos injected with a control MO or *trf3* MO, or *trf3* MO-treated embryos injected with *mespa* mRNA at 28 hpf.

Figure 2.

Trf3- and Mespa-depleted embryos fail to undergo hematopoiesis. **a**, Whole-mount *in situ* hybridization with riboprobes to *scl* (12 hpf), *lmo2* (14 hpf), *pu.1* (12 hpf), *gata2* (14 hpf) and *kdr* (12 hpf). **b**, Fluorescence microscopy monitoring expression of an *fli1:EGFP* transgene (top panels), and either *gata1* or *pax2* (middle panels). The merged signal is shown in the bottom panels.

Figure 3.

Trf3 initiates a transcription factor pathway required for *cdx4* expression and hematopoiesis. **a**, (Top) RT-PCR analysis (9 hpf). (Bottom) *In situ* hybridization (6 hpf). **b**, *In situ* hybridization monitoring *cdx4*, *trf3* and *mespa* expression in wild-type embryos (6 hpf). **c**, Epistasis analysis; *gata1* expression was monitored by *in situ* hybridization at 14 hpf. **d**, *In situ* hybridization monitoring *gata1* and *scl* induction (14 hpf). Arrowheads show a "third stripe" indicative of expanded *scl* expression. **e**, *In situ* hybridization monitoring *cdx4* induction (14 hpf). Lateral (top) and dorsal (bottom) views are shown. Arrowheads indicate normal (left) and expanded (right) *cdx4* expression.