

# Novel Tfp2-mediated control of *soxE* expression facilitated the evolutionary emergence of the neural crest

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## SUMMARY

Gene duplication has been proposed to drive the evolution of novel morphologies. After gene duplication, it is unclear whether changes in the resulting paralogs' coding-regions, or in their cis-regulatory elements, contribute most significantly to the assembly of novel gene regulatory networks. The Transcription Factor Activator Protein 2 (Tfp2) was duplicated in the chordate lineage and is essential for development of the neural crest, a tissue that emerged with vertebrates. Using a *tfap2*-depleted zebrafish background, we test the ability of available gnathostome, agnathan, cephalochordate and insect *tfap2* paralogs to drive neural crest development. With the exception of *tfap2d* (lamprey and zebrafish), all are able to do so. Together with expression analyses, these results indicate that sub-functionalization has occurred among Tfp2 paralogs, but that neo-functionalization of the Tfp2 protein did not drive the emergence of the neural crest. We investigate whether acquisition of novel target genes for Tfp2 might have done so. We show that in neural crest cells Tfp2 directly activates expression of *sox10*, which encodes a transcription factor essential for neural crest development. The appearance of this regulatory interaction is likely to have coincided with that of the neural crest, because *AP2* and *SoxE* are not co-expressed in amphioxus, and because neural crest enhancers are not detected proximal to amphioxus *soxE*. We find that *sox10* has limited ability to restore the neural crest in Tfp2-deficient embryos. Together, these results show that mutations resulting in novel Tfp2-mediated regulation of *sox10* and other targets contributed to the evolution of the neural crest.

**KEY WORDS:** Neural crest, Evolution, Tfp2, Transcription factor

## INTRODUCTION

The neural crest (NC) and cranial placodes are present in all vertebrates (including the basal lamprey and hagfish) but are absent from the basal chordate amphioxus, suggesting that these structures were not present in the first chordates. Although recent work suggests that evolutionary precursors of these tissues were probably present in the proximate vertebrate ancestor, bona fide NC and a full complement of cranial placodes are nevertheless considered to be vertebrate synapomorphies (Canestro et al., 2003; Mazet and Shimeld, 2005). Derivatives of the NC and cranial placodes, including jaws and paired sensory structures, contribute to the predatory behavior of vertebrates, which is not exhibited by protochordates (Gans and Northcutt, 1983; Northcutt and Gans, 1983). These NC-derived features were key to the success of vertebrates, which adds interest to identifying the genomic changes that resulted in the emergence of this novel cell type.

The development of the NC is controlled by a gene regulatory network (GRN) composed of transcription factors and other regulatory molecules (Sauka-Spengler and Bronner-Fraser, 2008). Importantly, at least some regulatory interactions within the NC GRN must be evolutionarily novel because many of the transcription factors contributing to the NC GRN were recruited to

the neural-plate border from other tissues (Meulemans and Bronner-Fraser, 2005). The evolution of novel regulatory interactions can result from mutations causing 'protein neo-functionalization', or those causing 'regulatory neo-functionalization'. In the first scenario, mutations in protein-coding sequence would imbue an existing transcription factor with new functionality (e.g. new DNA-binding specificity or affinity for new co-factors), permitting it to make new regulatory connections. A long-standing hypothesis is that protein neo-functionalization is facilitated by gene duplication events, which would relieve constraints on transcription factors imposed by pleiotropy. Indeed, gene duplication followed by protein neo-functionalization has been proposed as a driving force in vertebrate evolution (Ohno, 1970). According to this model, duplicated proteins with initially identical biochemical functions diverge as one copy performs the ancestral functions and the other gains the ability to carry out additional functions (Wagner, 1998). There is support for this model because many examples of paralogous transcription factors with distinct functions have been documented (reviewed by Wagner and Lynch, 2008). However, it remains to be tested whether gene duplication followed by protein neo-functionalization contributed to the emergence of the NC GRN, or to any other vertebrate novelty.

In the alternative scenario, regulatory neo-functionalization, mutations lead to the appearance of a transcription factor's cognate DNA-binding site in cis-regulatory sequences of a gene not previously regulated by that transcription factor. In many cases, a transcription factor from one species can replace the function of its ortholog in another species. This has often been shown in the context of evolutionarily conserved GRNs. For example, Pax6 function is conserved in the photoreceptor GRN across all phyla (Onuma et al., 2002). However, evidence for regulatory neo-

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functionalization requires a transcription factor from one species to replace functionally its ortholog in another species in an evolutionarily novel GRN. The best example to date is that *Drosophila melanogaster* SoxE (Sox100B) can partially replace vertebrate Sox10 in the NC GRN (Cossais et al., 2010); however, whether this example is typical or exceptional remains unclear.

The transcription factor Activator Protein 2 (Tfap2) family, members of which promote growth or differentiation in a variety of cell types (Eckert et al., 2005), presents an excellent opportunity to test whether the formation of one node in the NC GRN required protein neo-functionalization. This family is optimal for such a test because Tfap2 proteins have been duplicated in the chordate lineage, and because they are essential for the early steps of NC development (Hoffman et al., 2007; Li and Cornell, 2007; Luo et al., 2003). Our results support the model that sub-functionalization has contributed to retention of the Tfap2 paralogs in evolution. However, they do not support a role for protein neo-functionalization of Tfap2 in the assembly of the NC GRN. Instead of gaining novel protein function, Tfap2 acquired novel targets, including *sox10* and other genes, and this contributed to the evolution of the NC.

## MATERIALS AND METHODS

### Fish maintenance

Zebrafish embryos and adults were reared as described previously (Westerfield, 1993). Embryos were staged by hours or days post-fertilization (hpf or dpf, respectively) at 28.5°C (Kimmel et al., 1995). Because of the poor effectiveness of *tfap2a* morpholinos (MOs) beyond 48 hpf (our unpublished observations), we used homozygous *tfap2a* mutants, generated from heterozygous adults harboring a presumed null allele of *tfap2a* (*lockjaw*, *tfap2a*<sup>ts213</sup>) (Knight et al., 2003), in analyses of melanophores.

### Generation of cDNAs for in situ hybridization and mis-expression experiments

First-strand cDNA was generated from total RNA extracted from zebrafish embryos at 24 hpf as described (O'Brien et al., 2004) or lamprey and amphioxus embryos/adults as described (Meulemans and Bronner-Fraser, 2002). Partial or full length cDNA clones were amplified (supplementary material Table S1). Digoxigenin (DIG)-labeled probes (Roche Diagnostics, Mannheim, Germany) were synthesized, and whole-mount in situ hybridization was performed following standard methods (Thisse and Thisse, 2008).

For mis-expression experiments, full-length coding regions of each zebrafish gene studied were amplified from a pool of 24 hpf first-strand cDNA. PCR products were cloned into pSCA (Stratagene/Agilent, Santa Clara, CA, USA), pCR4-TOPO (Invitrogen, Carlsbad, CA, USA) or pENTR/D-TOPO (Invitrogen) shuttling vectors, as indicated (supplementary material Table S1). Coding regions were then shuttled into pCS2+ either using standard cloning methods (pSCA and pCR4-TOPO) (Sambrook et al., 1989) or Gateway cloning using LR Clonase II (pENTR-D/TOPO, Invitrogen). All final plasmids were confirmed by sequencing. Plasmids were linearized and capped mRNA synthesized in vitro using the SP6 mMessage mMachine kit (Ambion, Austin, TX, USA) and concentrated using Microcon Spin Columns (Millipore, Billerica, MA, USA).

For forced expression of *sox10*, embryos were injected with *hsp70:sox10* (Elworthy et al., 2003). At 80% epiboly, embryos were transferred to 37°C water and incubated at this temperature for 60 minutes then allowed to develop at room temperature.

### Amphioxus *soxE* enhancer analysis

Regions of amphioxus genomic DNA proximal to *soxE* (see supplementary material Table S2 for genomic coordinates) were amplified and, with the exception of *soxE* promoter region 2, cloned into a *tol-2* based GFP reporter vector described previously (Fisher et al., 2006). Several F0

embryos injected with construct containing the *soxE* region 4 were raised, and two independent stable transgenic strains were isolated that had identical GFP expression to transient transgenic embryos. The amphioxus *soxE* promoter region 2 was amplified and ligated into the *sox10:eGFP* plasmid (Wada et al., 2005) in place of the zebrafish *sox10* element using standard cloning methods (Sambrook et al., 1989).

For analysis of conserved transcription factor binding sites the online tool ConSite (Sandelin et al., 2004) was used. Search parameters included a conservation cut-off of 38%, a window size of 50 and a TF score threshold of 80%.

### Morpholinos and microinjection

Plasmids used for in vitro mRNA synthesis are described above. Plasmids and MOs were injected at the one-cell stage and mRNA at the two- to four-cell stage. Approximately 125 pg of *tfap2* mRNAs, 125 pg of all plasmids and 5 ng of MOs were injected. MOs targeting *tfap2a* (*tfap2a* e2i2) and *tfap2c* (*tfap2c* e3i3) used here have been described (Li and Cornell, 2007). Supplementary material Table S1 contains sequences.

### Chromatin immunoprecipitation (ChIP)

A modified ChIP protocol for fish embryos was used (Lindeman et al., 2009). Chromatin was sheared on ice using a probe-tip sonicator (VirTis Virsonic 600) with the following settings: power setting, 5; pulse time, 20 seconds; number of pulses, ten; break between pulses, 2 minutes. Anti-Tfap2a(CT) (Anaspec, Fremont, CA, USA) or Rabbit-IgG (Millipore) were used for immunoprecipitation. ChIP experiments were performed in triplicate, on newly isolated embryos. PCR reactions (10 µl) following ChIP were prepared with immunoprecipitated DNA, using the SYBR Green kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Primers were used at a final concentration of 200 nM in separate PCR reactions. Quantitative real-time PCR in Low 96-well plates (Bio-Rad, Hercules, CA, USA) was conducted using a Bio-Rad thermal cycler (CFX96 Real-Time PCR Detection System) following the default protocol; triplicates of each reaction were carried out simultaneously and mean and standard error were calculated. Melt-curve analysis was also performed to confirm specificity of primers. Figure 5B shows a representative run from one of the three repeats.

### Cycloheximide experiments

Embryos at 90% epiboly were incubated for ~2 hours in 100 µg/ml cycloheximide (diluted in fish water from a 40 mg/ml stock dissolved in DMSO) (Sigma-Aldrich), a dose several times the level previously shown to strongly reduce protein translation in 1.5 hpf zebrafish embryos (Leung et al., 2003), or in diluted DMSO as a negative control. Subsequently, embryos were incubated for an additional 2.5 hours in dexamethasone (100 µM in fish water from a 40 mM stock dissolved in ethanol) (Sigma-Aldrich), or in diluted ethanol as a negative control. Next, RNA was isolated (Trizol, Invitrogen) following the manufacturer's instructions and treated with DNaseI to remove genomic DNA. Quantitative reverse-transcriptase PCR (qRT-PCR) analysis of mRNA levels was conducted, starting with 1 µg of total RNA, as previously described (Van Otterloo et al., 2010). Primer efficiencies were confirmed using a tenfold series dilution of cDNA and generating a standard curve. All primers were designed to span a large intron, preventing amplification of genomic DNA. The 2<sup>ΔΔCt</sup> method was used to calculate fold-enrichment over wild-type samples and all groups were normalized to *β-actin* (Dussault and Pouliot, 2006). Each experiment was repeated in triplicate, starting from injections. Results are shown from a representative experiment.

### Western blotting

Western blotting of zebrafish embryos was carried out essentially as previously described (Link et al., 2006). Antibodies 9E 10 (anti-myc, 1:100) and AA4.3 (alpha-tubulin 1:100) were developed by J. Michael Bishop and Charles Walsh, respectively, and were obtained from the Developmental Studies Hybridoma Bank (Iowa, USA).

## RESULTS

### Sequential sub-functionalization of *tfap2* duplicates in the vertebrate lineage

We first sought to establish the relationships among T<sub>tfap2</sub> paralogs present in amphioxus, lamprey, mammals and zebrafish. Previously, we had identified five *tfap2* homologs in zebrafish (*Danio rerio*, *tfap2a-e*), one in lamprey (*Petromyzon marinus*, *Pm-tfap2*) and one in amphioxus (*Branchiostoma floridae*, *Bf-tfap2*) (Li and Cornell, 2007; Meulemans and Bronner-Fraser, 2002). However, after the pre-assembly lamprey genome became available, we found a second lamprey *tfap2* gene. Phylogenetic analysis of the *Drosophila* (*D. melanogaster*) and deuterostome T<sub>tfap2</sub> proteins grouped this new lamprey T<sub>tfap2</sub> with gnathostome T<sub>tfap2d</sub> genes. The previously described lamprey T<sub>tfap2</sub> fell within a clade that includes the genes encoding gnathostome T<sub>tfap2a</sub>, T<sub>tfap2b</sub>, T<sub>tfap2c</sub> and T<sub>tfap2e</sub> (Fig. 1A). Given the evidence for two genome duplication events in the vertebrate lineage (Holland et al., 1994), these results support a scenario in which whole-genome duplication in the common ancestor of lamprey and gnathostomes generated two paralogs, T<sub>tfap2d</sub> and T<sub>tfap2a/b/c/e</sub>, from an ancestral T<sub>tfap2</sub>. Subsequent duplication events, including a second round of whole-genome duplication, generated all of the gnathostome T<sub>tfap2</sub> paralogs (except T<sub>tfap2d</sub>) from the T<sub>tfap2a/b/c/e</sub> ancestor. By contrast, any duplicates of T<sub>tfap2d</sub> were lost. Although there is evidence for a genome duplication event within the teleost lineage (Postlethwait et al., 2004), given the one-to-one correspondence of tetrapod and zebrafish T<sub>tfap2</sub> homologs, it appears that T<sub>tfap2</sub> duplicates generated in this genome duplication event were lost. In summary, these findings provide an evolutionary scenario for the origin of T<sub>tfap2</sub> paralogs present in vertebrates.

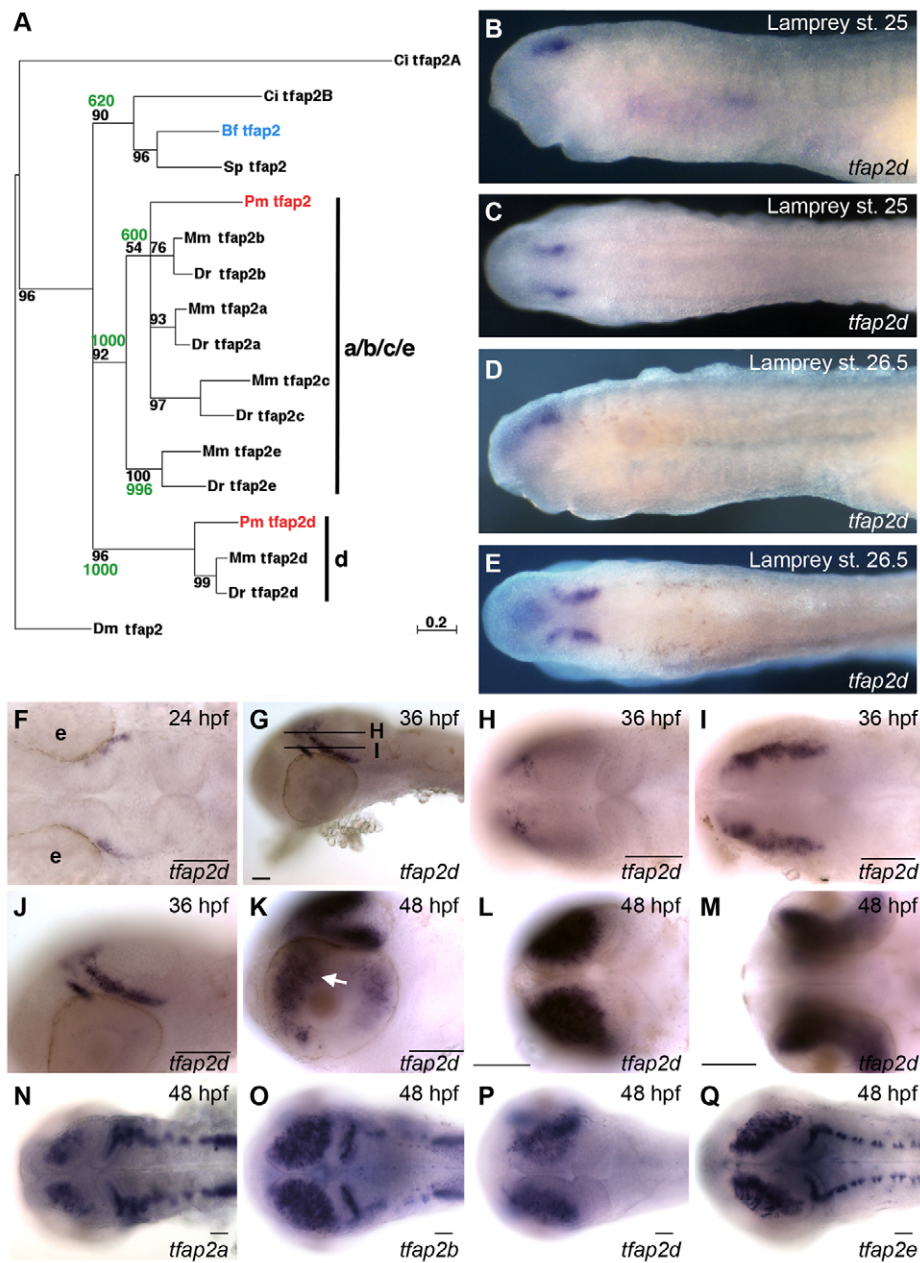
We asked whether the duplication-degeneration-complementation model explains the retention of vertebrate *tfap2d* paralogs (Force et al., 1999). Under this model, sub-functionalization refers to the partitioning of an ancestral gene's expression pattern, or its functional domains, between its duplicates, leading to selective pressure to retain both duplicates (Force et al., 1999). For this analysis, we consider amphioxus to represent the vertebrate ancestor. Amphioxus *tfap2* is expressed in the anterior neural tube and in the non-neural ectoderm (Meulemans and Bronner-Fraser, 2002). Similarly, lamprey *tfap2* is expressed in the brain and the non-neural ectoderm, including the neural plate border/NC (Meulemans and Bronner-Fraser, 2002). However, lamprey *tfap2d* is expressed only in the brain (Fig. 1B-E) [*tfap2d* expression persists from larval stages into adulthood as observed by RT-PCR (data not shown)]. Similarly, mouse *Tfap2d* (Zhao et al., 2003) and zebrafish *tfap2d* are expressed solely within the brain (Fig. 1F-M,P). Together, these data are consistent with sub-functionalization of an ancestral *tfap2* expression domain between two duplicates (*tfap2* and *tfap2d*) pre-dating the split of agnathans and gnathostomes.

We next investigated whether sub-functionalization might also explain the retention of *tfap2a*, *tfap2b*, *tfap2c* and *tfap2e* in gnathostomes. In this analysis, we considered lamprey *tfap2* expression to represent the ancestral condition. Zebrafish *tfap2a*, *tfap2b* and *tfap2c* are also expressed in this tissue but with distinct timing or spatial restrictions (supplementary material Fig. S1). Thus, during the gastrula stage zebrafish *tfap2a* and *tfap2c* are strongly expressed in the ventral ectoderm (presumptive skin) but by 24 hpf *tfap2c* expression remains high in embryonic skin, whereas *tfap2a* expression is relatively low, particularly in the periderm (Hoffman et al., 2007; Li and Cornell, 2007). Expression of *tfap2b* is absent from ventral ectoderm during gastrulation but

is present in discrete regions of anterior skin at 24 hpf (Knight et al., 2005). In addition, lamprey *tfap2* is expressed in premigratory and migratory NC (Meulemans and Bronner-Fraser, 2002). In zebrafish embryos at the gastrula stage, *tfap2a* and *tfap2c* are expressed in lateral neural plate cells, which later give rise to the NC (Li and Cornell, 2007). By the neurula stage and somitogenesis stages, however, only *tfap2a* is expressed at high levels in the premigratory NC. In NC derivatives, *tfap2e* is expressed in melanoblasts, whereas *tfap2b* is expressed in spinal sensory neurons, some of which are derived from the NC (Knight et al., 2005; Knight et al., 2003; O'Brien et al., 2004; Van Otterloo et al., 2010) (supplementary material Fig. S1 and Table S3). Finally, lamprey *tfap2* is expressed in the brain, and zebrafish *tfap2a*, *tfap2b*, *tfap2c* and *tfap2e* are also all expressed in the brain, but in imperfectly overlapping patterns (Fig. 1N-Q) [*tfap2c* is expressed in the brain at 24 hpf (Li and Cornell, 2007)]. In summary, expression of modern *tfap2a*, *tfap2b*, *tfap2c* and *tfap2e* can be viewed as a partitioning of the ancestral pattern. These results are consistent with a model in which multiple rounds of duplication and sub-functionalization have resulted in retention of these four *tfap2* duplicates.

### T<sub>tfap2d</sub> is functionally distinct from other T<sub>tfap2</sub> family members

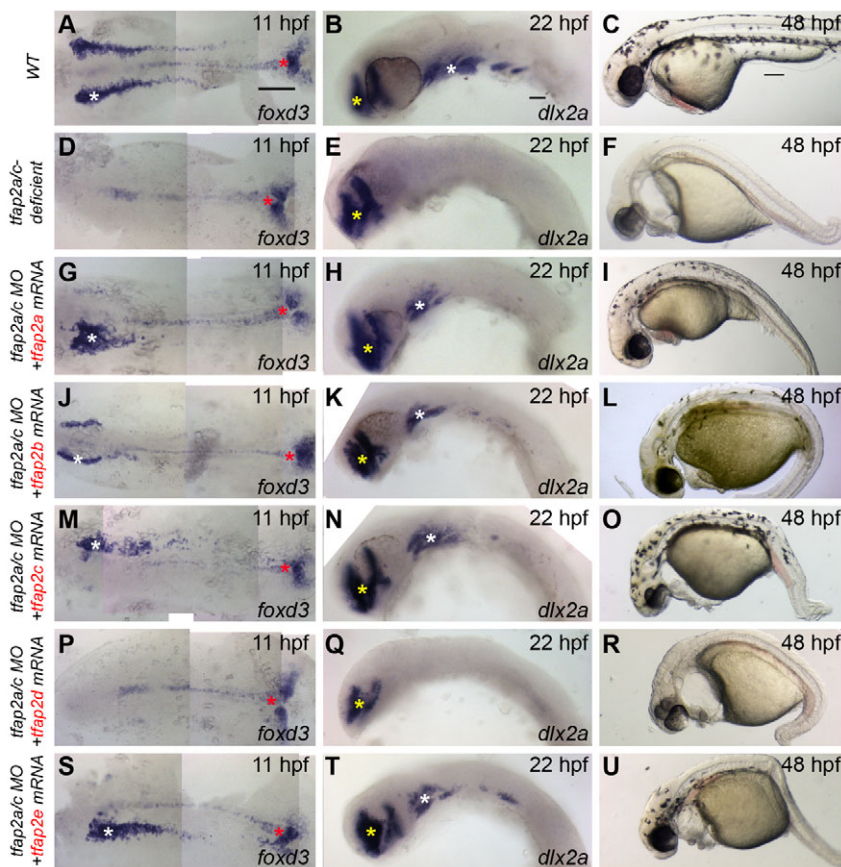
The proteins encoded by paralogs that have undergone sub-functionalization are under relaxed selection. Specifically, they are freed to lose functions appropriate for domains in which they are no longer expressed and to become functionally optimized for expression domains they retain (Conant and Wagner, 2003; Lynch and Wagner, 2008). We tested whether paralogs T<sub>tfap2b</sub>, T<sub>tfap2d</sub> and T<sub>tfap2e</sub>, which have all lost ancestral expression in the lateral neural border, have also lost a function appropriate for this domain, i.e. the ability to generate the NC. Starting with *tfap2a/c*-deficient embryos (which are embryos depleted of both *tfap2a* and *tfap2c* by mutation or by morpholino), we injected mRNAs encoding the various T<sub>tfap2</sub> paralogs. We raised such embryos to appropriate stages then fixed and processed them to reveal a marker of premigratory NC, *foxd3* (Kelsh et al., 2000; Odenthal and Nusslein-Volhard, 1998), or migratory NC, *dlx2a* (Akimenko et al., 1994). As expected, mRNAs encoding T<sub>tfap2a</sub> or T<sub>tfap2c</sub> efficiently rescued *foxd3* expression (Fig. 2G,M), *dlx2a* expression (Fig. 2H,N) and also melanophores (Fig. 2I,O). Interestingly, injection of the *tfap2b* and *tfap2e* mRNAs also rescued *foxd3* expression (Fig. 2J,S), *dlx2a* expression (Fig. 2K,T) and melanophores (Fig. 2L,U) in *tfap2a/c*-deficient embryos. Further tests revealed that overexpression of each of these T<sub>tfap2</sub> paralogs similarly rescued in *tfap2a/c*-deficient embryos the NC derivatives peripheral glia, marked by *foxd3* at 28 hpf (Kelsh et al., 2000), and sensory neuron precursors, marked by expression of *tlxa* at 22 hpf (Andermann and Weinberg, 2001) (supplementary material Fig. S2). By contrast, injection of a similar dose of the *tfap2d* mRNA into *tfap2a/c*-deficient embryos failed to rescue expression of *foxd3*, *dlx2a* or melanophores (Fig. 2P-R). Western blot analysis of epitope-tagged variants of T<sub>tfap2d</sub> and T<sub>tfap2a</sub> revealed similar stability in zebrafish embryos (supplementary material Fig. S3). Finally, we found that injection of lamprey *tfap2* (56% and 55% protein similarity to T<sub>tfap2a</sub> and T<sub>tfap2c</sub>, respectively) also efficiently rescued *foxd3* expression, *dlx2a* expression and melanophores in *tfap2a/c*-deficient embryos (Fig. 3J-L), whereas injection of the lamprey *tfap2d* (45% and 39% protein similarity to T<sub>tfap2a</sub> and T<sub>tfap2c</sub>, respectively) at a similar dose failed to do so (Fig. 3M-O) (all rescue experiments are summarized in Fig. 4).



**Fig. 1. Characterization of embryonic *tfap2d* expression in zebrafish and lamprey.** (A) Phylogenetic tree of deuterostome and *Drosophila melanogaster* *tfap2* genes, constructed using the Maximum Likelihood method (Schmidt et al., 2002). A similar tree topology was obtained using the Neighbor-Joining method (not shown) (Saitou and Nei, 1987). Confidence values for both methods are shown at branch points with quartet-puzzling reliability scores in black and Neighbor-Joining bootstrap values in green. Each gene name includes a prefix with the initials of the corresponding species name. Ci, *Ciona intestinalis*; Pm, *Petromyzon marinus*; Mm, *Mus musculus*; Dr, *Danio rerio*; Bf, *Branchiostoma floridae*; Sp, *Strongylocentrotus purpuratus*; Dm, *Drosophila melanogaster*. (B-E) Lamprey embryos, fixed at the stage indicated and processed to reveal *tfap2d* expression. (B,C) Expression of *tfap2d* is first observed in bilateral spots in the presumed forebrain after neurulation (Tahara st. 25) in lateral (B) and dorsal (C) views. (D,E) Expression in the brain at late larval stage (st. 26.5) in (D) lateral and (E) dorsal views. (F-Q) Wild-type zebrafish embryos, fixed at the stages indicated and processed to reveal expression of *tfap2d* (F-M) or the indicated *tfap2* family member (N-Q) by RNA in situ hybridization. (F) Dorsal view of the head showing *tfap2d* expression in a distinct structure within the presumptive mesencephalon. (G) Lateral view showing *tfap2d* expression within two distinct domains of the tegmentum; lines represent field of view for optical sections shown in H and I. (H,I) Dorsal views of the head, expression is in presumed tegmentum. (J) Higher magnification of embryo shown in G. (K) Lateral view of the head of a 48 hpf embryo, showing expression in tegmentum and optic tectum and retina (arrow). (L,M) Dorsal (L) and ventral (M) views of embryo shown in K, focusing on *tfap2d* expression within the optic tectum and retina, respectively. (N-Q) Embryos at 48 hpf, revealing shared and distinct expression domains of *tfap2* family members in the brain. Embryos shown with anterior to the left, unless otherwise indicated. e, eye. Scale bars: 50 μm.

In summary, Tfap2b and Tfap2e, retain the ability to promote NC development, while Tfap2d virtually lacks this ability. As discussed above, it appears that *tfap2d* lost enhancers possessed by the *tfap2*

ancestor that drive expression in the non-neural ectoderm, and did so prior to the split of agnathans and gnathostomes. However, the functional experiments just described do not resolve whether



**Fig. 2. Assessment of the ability of gnathostome Tfap2 paralogs to restore neural crest in *tfap2a/c*-deficient zebrafish embryos.** (A,D,G,J,M,P,S) Dorsal views of flat-mounted wild-type zebrafish embryos (A) or embryos injected with the indicated mRNA and/or MO (D,G,J,M,P,S), fixed at 11 hpf and processed to reveal *foxd3* expression. Restored *foxd3* expression was found on left, right, or both sides as a result of mosaic injection of mRNA. White asterisks indicate premigratory neural crest. Red asterisks indicate non-neural crest-derived tailbud. (B,E,H,K,N,Q,T) Lateral views of wild-type zebrafish embryos (B) or embryos injected with the indicated mRNA and/or MO (E,H,K,N,Q,T), fixed at 22 hpf and processed to reveal *dlx2a* expression. With the exception of *tfap2d*, no trend was seen in the spatial extent of *dlx2a* expression and the *tfap2* paralogs used. White asterisks indicate migratory neural crest. Yellow asterisks indicate brain. (C,F,I,L,O,R,U) Lateral views of live embryos at 48 hpf that are wild type (C) or *tfap2a* mutants injected with *tfap2c* MO (F,I,L,O,R,U), and injected with the indicated mRNA. Embryos shown with anterior to the left, unless otherwise indicated. Scale bars: in A, 100  $\mu$ m for A,D,G,J,M,P,S; in B, 50  $\mu$ m for B,E,H,K,N,Q,T; in C, 100  $\mu$ m for C,F,I,L,O,R,U.

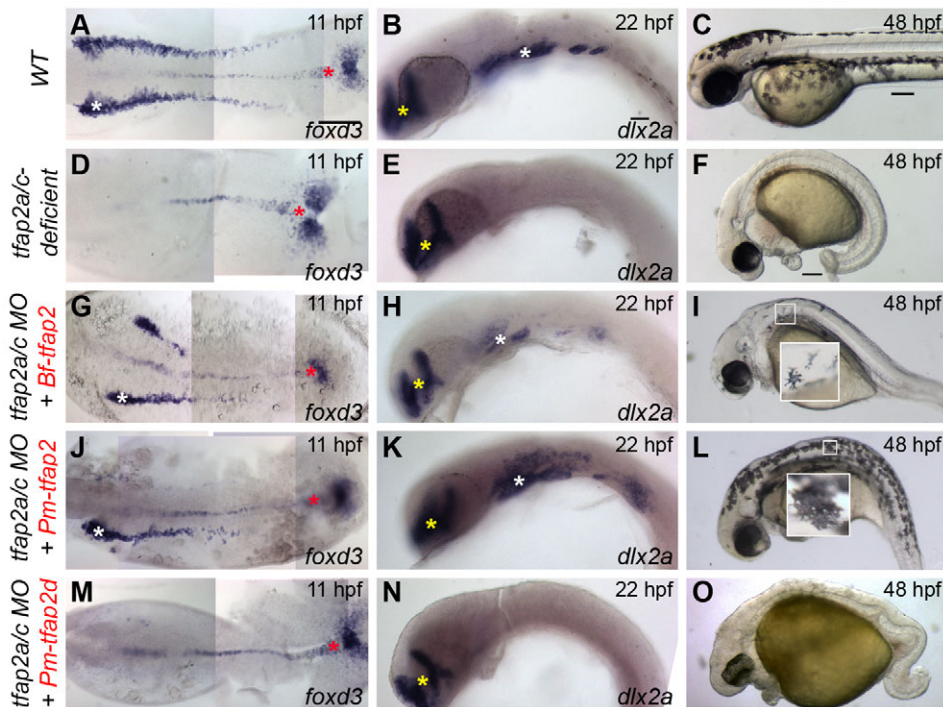
Tfap2d subsequently lost the ability to induce NC, i.e. because of the absence of selective pressure to retain this function, or alternatively, whether the other Tfap2 paralogs, which retained expression in non-neural ectoderm, gained this ability, i.e. via protein neo-functionalization.

### Tfap2 from amphioxus can induce neural crest

To determine whether the ability of Tfap2 to induce NC resulted from protein neo-functionalization, we generated a full-length amphioxus *tfap2* cDNA and generated mRNA from it in vitro. Interestingly, injection of amphioxus *tfap2* mRNA (48% and 46% protein similarity to Tfap2a and Tfap2c, respectively) into *tfap2a/c*-deficient embryos efficiently rescued expression of *foxd3* (in NC and in peripheral glia), expression of *dlx2a*, expression of *tlxa* and melanophores (Fig. 3G-I; supplementary material Fig. S2). Testing an even more ancient AP2 homolog, we injected *Drosophila tfap2* mRNA (40% and 39% protein similarity to Tfap2a and Tfap2c, respectively) into *tfap2a/c*-deficient zebrafish embryos, and observed robust *foxd3* expression in the lateral neural plate at 11 hpf (supplementary material Fig. S4G); however, in such embryos, expression of *dlx2a* at 22 hpf and melanophores at 48 hpf were rarely present (supplementary material Fig. S4H,I, legend). The less robust rescue of NC by *Drosophila tfap2* might reflect instability of *Drosophila* Tfap2 relative to other Tfap2 proteins tested, or possibly the absence in *Drosophila* Tfap2 of a function essential for NC maintenance or other later events in NC development. Nonetheless, because amphioxus efficiently rescued NC in *tfap2a/c*-deficient embryos, we conclude that recruitment of Tfap2 into a GRN that controls induction of NC at the neural plate border did not require Tfap2 to gain new protein function.

### *sox10* is targeted directly by Tfap2a and forced expression of *sox10* partially restores trunk neural crest in *tfap2a/c*-deficient embryos

We sought to identify the novel regulatory connections gained by Tfap2 in the context of the NC GRN. In amphioxus, the orthologs of multiple NC regulatory proteins (e.g. SoxE, FoxD, Twist, ID) are expressed in the mesoderm but not in the neural plate border (Sauka-Spengler et al., 2007). Mutations in the regulatory regions of the genes encoding such regulatory proteins might have introduced Tfap2 binding sites, thereby recruiting their expression to the neural plate border. We investigated this possibility for *sox10*, a gene necessary for early steps of NC induction (Dutton et al., 2001; Honore et al., 2003) and expression of which in NC is lost in *tfap2a/c*-deficient embryos (Hoffman et al., 2007; Li and Cornell, 2007). We conducted anti-Tfap2a chromatin immunoprecipitation (ChIP) on 4.9 kb upstream of the start codon of *sox10*, a region previously shown to drive reporter expression in the NC (Wada et al., 2005). We started with lysates of zebrafish embryos at 12 hpf, a stage at which both *tfap2a* and *sox10* are expressed at high levels in the NC, using an antibody generated against zebrafish Tfap2a, specificity of which was confirmed in *tfap2a* mutants (supplementary material Fig. S5). A pair of predicted Tfap2 binding sites are present proximal to the transcription start site (Fig. 5A). On the precipitated chromatin we performed quantitative PCR (qPCR) with primer pairs positioned near these predicted Tfap2a binding sites, or with primer pairs positioned 1.2 kb downstream, as a negative control (off-target site) (Fig. 5A). We observed 12- to 22-fold enrichment at the target site in anti-Tfap2a-precipitated chromatin versus IgG-precipitated



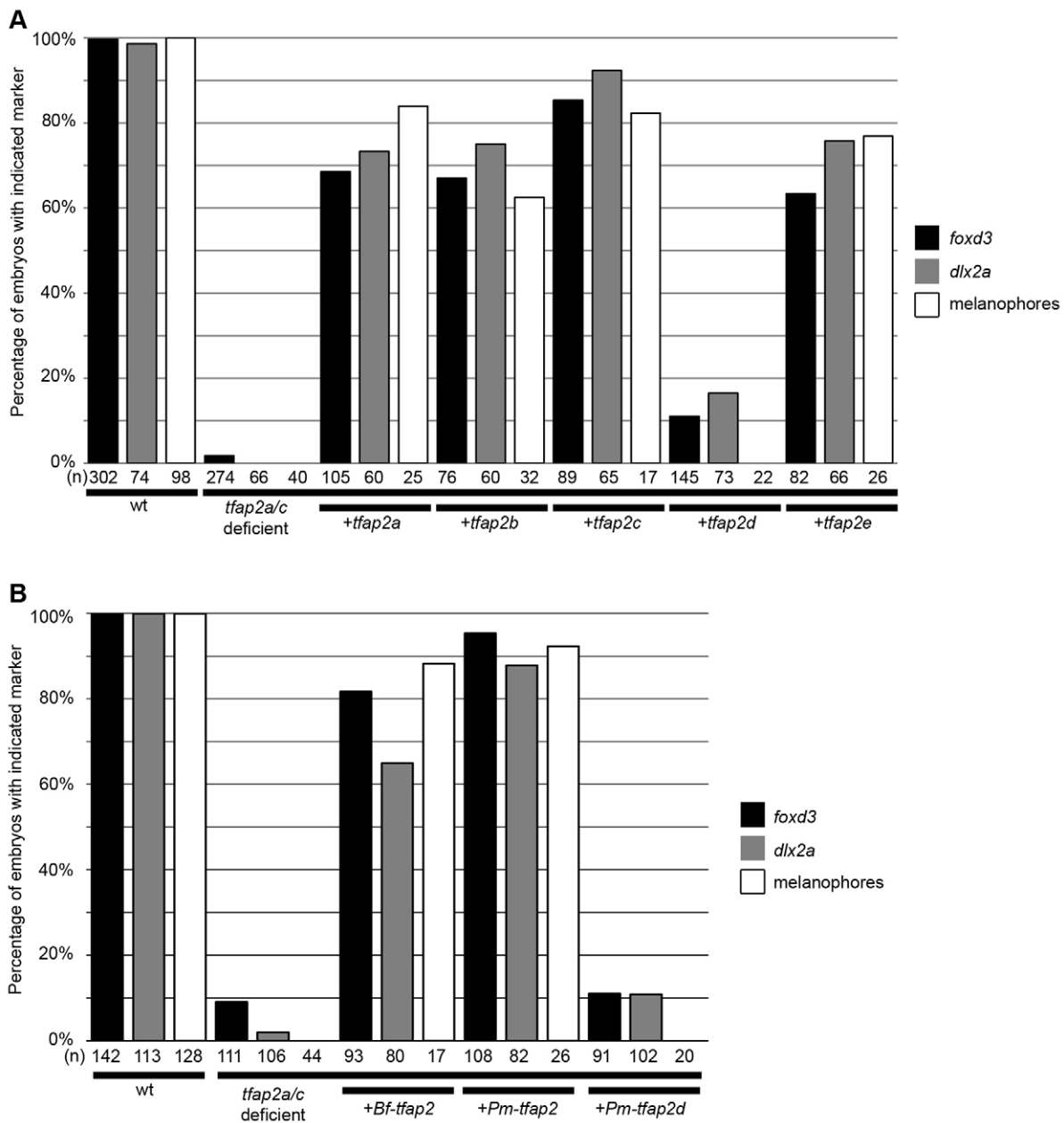
**Fig. 3. Assessment of the ability of lamprey and amphioxus Tfap2 paralogs to restore neural crest in *tfap2a/c*-deficient zebrafish embryos.** (A,D,G,J,M) Dorsal views of flat-mounted wild-type zebrafish embryos (A) or embryos injected with the indicated mRNA and/or MO (D,G,J,M), fixed at 11 hpf and processed to reveal *foxd3* expression. White asterisks indicate premigratory neural crest. Red asterisks indicate non-neural crest-derived tailbud. (B,E,H,K,N) Lateral views of wild-type zebrafish embryos (B) or embryos injected with the indicated mRNA and/or MO (E,H,K,N), fixed at 22 hpf and processed to reveal *dlx2a* expression. White asterisks indicate migratory neural crest. Yellow asterisks indicate brain. (C,F,I,L,O) Lateral views of live embryos at 48 hpf that are wild type (C) or *tfap2a* mutants injected with *tfap2c* MO (F,I,L,O), and injected with the indicated mRNA. No trend was seen in the ability of *Bf-tfap2* versus *Pm-tfap2* to rescue more melanophores per embryos. Insets in figure I and L are higher magnification images of boxed melanophores. Embryos shown with anterior to the left, unless otherwise indicated. Scale bars: in A, 100  $\mu$ m for A,D,G,J,M; in B, 50  $\mu$ m for B,E,H,K,N; in C, 100  $\mu$ m for C,F,I,L,O.

chromatin, and minimal enrichment at the off-target site (Fig. 5B). These findings indicate that Tfap2a binds the zebrafish *sox10* promoter at a time when *sox10* is expressed in the NC.

Next, we investigated whether Tfap2a overexpression will elevate *sox10* expression when protein translation is inhibited. We co-injected embryos with *tfap2a* MO, *tfap2c* MO, and mRNA encoding a dexamethasone-inducible variant of Tfap2a (Tfap2a fused to glucocorticoid-receptor, *tfap2a-GR*) (Luo et al., 2002). At 10 hpf, we treated the embryos with cycloheximide to block translation (Leung et al., 2003). Two hours later, we added dexamethasone to induce nuclear transport of Tfap2a-GR. Finally, 2.5 hours later (~14 hpf), we harvested RNA from these embryos, generated first-strand cDNA and used qPCR to measure the levels of *sox10* and *zgc:85942* (*ednra* – Zebrafish Information Network), an *ednra* homolog which, like *sox10*, is expressed at high levels in the NC at 13 hpf (Thisse and Thisse, 2004). As expected, in *tfap2a/c*-deficient embryos the levels of both *sox10* and *zgc:85942* mRNA were reduced relative to those in control embryos (Fig. 5C,D). In *tfap2a/c*-deficient embryos injected with *tfap2a-GR* and not treated with dexamethasone, *sox10* and *zgc:85942* expression were barely elevated above the levels detected in *tfap2a/c*-deficient embryos (Fig. 5C,D), confirming that Tfap2a-GR is only minimally active in the absence of dexamethasone. By contrast, in embryos treated with dexamethasone but not cycloheximide, the expression of both NC markers was significantly elevated (Fig. 5C,D).

Interestingly, in embryos treated with dexamethasone in the presence of cycloheximide, the expression of *sox10*, but not that of *zgc:85942*, was raised to levels significantly higher than those in *tfap2a/c*-deficient embryos (Fig. 5C,D). Together, these findings strongly suggest that Tfap2 activates *sox10* expression directly.

To assess whether activation of *sox10* is the sole requirement of Tfap2a in NC development, we re-introduced *sox10* into *tfap2a/c*-deficient embryos. To do so, we used the *hsp70:sox10* plasmid, which contains a heat-shock-inducible promoter upstream of the *sox10* coding region (Elworthy et al., 2003). In *hsp70:sox10*-injected, *tfap2a/c*-deficient embryos exposed to heat-shock at 9 hpf and fixed at 12 hpf, *snai1b* and *sox9b* were detected in a scattered pattern around the embryo (mosaic expression is typical of transient transgenesis) (Thisse et al., 1995; Yan et al., 2005) (Fig. 5M,N). However, *foxd3* expression was absent in these embryos, and *crestin* expression was absent from similarly treated embryos fixed at 22 hpf (Rubinstein et al., 2000) (Fig. 5O; data not shown). Interestingly, in similarly treated embryos melanophores were present at 48 hpf, but only in the trunk, not in the head (Fig. 5P). Control experiments revealed the requirement for both the *hsp70:sox10* plasmid and the heat-shock for the results described in this section. Because forced expression of *sox10* was insufficient to restore a full NC program in *tfap2a/c*-deficient embryos, it is very likely that there are Tfap2 regulatory targets other than *sox10* that are essential in the NC GRN.

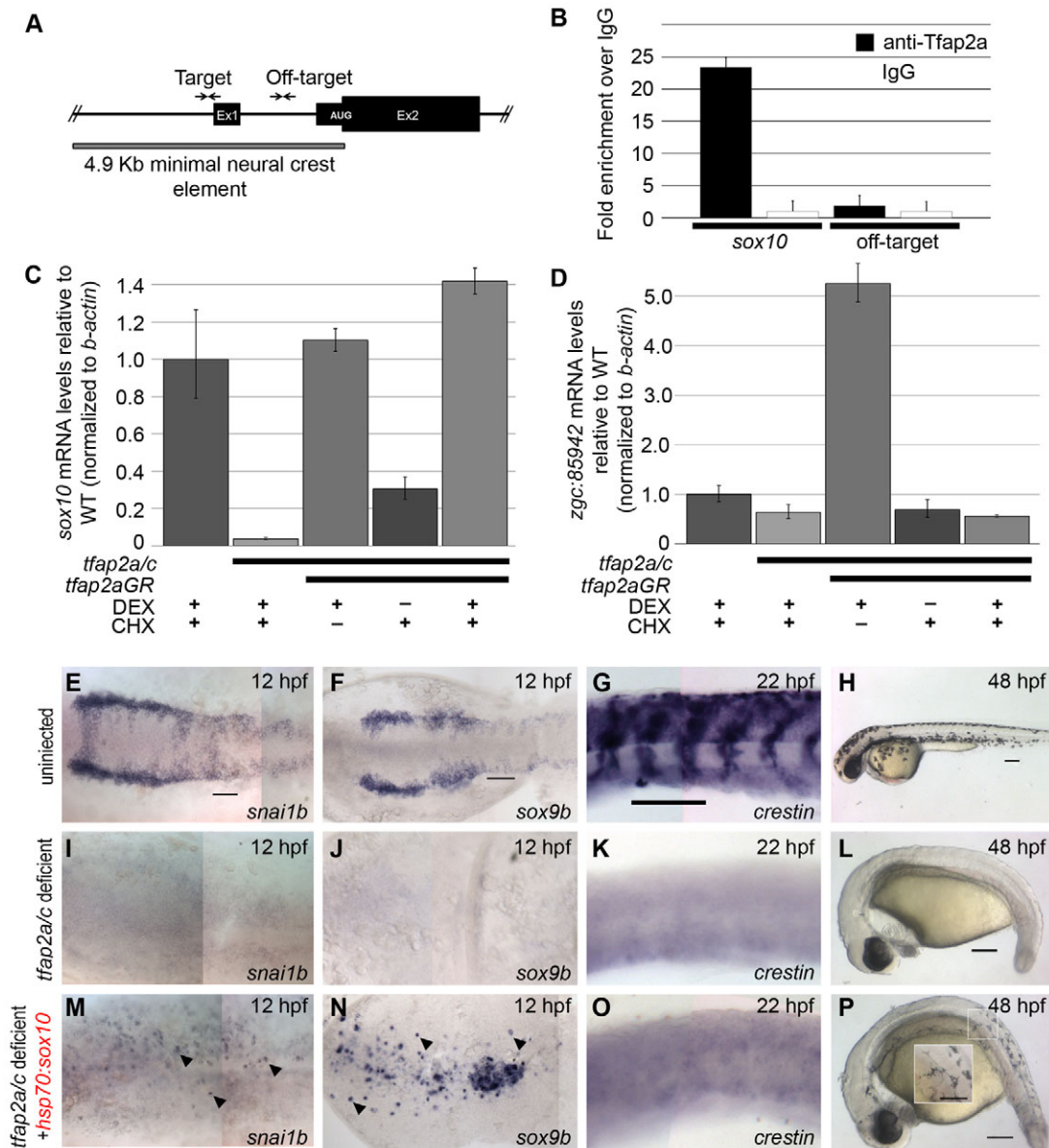


**Fig. 4. Summary of Tfp2 rescue results.** (A,B) Histograms summarizing neural crest rescue results in *tfap2a/c*-deficient embryos injected with zebrafish *tfap2* paralogs (A), or amphioxus (Bf) or lamprey (Pm) *tfap2* homologs (B), as indicated. Number of embryos (*n*) per group is displayed below bars.

### Genomic elements proximal to amphioxus *SoxE* lack neural crest enhancer activity

If regulation of the *soxE* gene by Tfp2 emerged in the context of the novel NC GRN then amphioxus *soxE* should lack regulatory elements sufficient to drive expression in the NC. To test this notion, we amplified 4.9 kb upstream of the amphioxus *SoxE* coding region and engineered it into the same GFP reporter vector used to show that the analogous 4.9 kb element upstream of zebrafish *sox10* drives NC-specific expression (Wada et al., 2005). In addition, because canonical Wnt signaling promotes NC development in zebrafish (Lewis et al., 2004), we amplified several regions proximal to amphioxus *SoxE* that contain TCF/LEF sites, and cloned them into a reporter vector with a minimal promoter (Fisher et al., 2006) (supplementary material Table S2). We

injected each of these constructs, or, as a positive control, a reporter construct containing 4.9 kb upstream of zebrafish *sox10*, into zebrafish embryos (Wada et al., 2005). However, only in embryos injected with the zebrafish construct did we detect GFP expression concentrated in the NC, as recognized by anti-Sox10 immunoreactivity (Fig. 6H,I). Interestingly, although amphioxus embryos are believed to lack ears, zebrafish embryos injected with a construct containing an element downstream of amphioxus *soxE* (Fig. 6A, *soxE* region 4) exhibited GFP expression in the ear and presumed midbrain-hindbrain boundary; embryos from two independent stable transgenic lines made from this reporter revealed the same expression (Fig. 6B-G). Similar to endogenous *sox10* expression in the ear, expression of GFP in these lines was insensitive to knockdown of Tfp2a and Tfp2c (Fig. 6F,G).

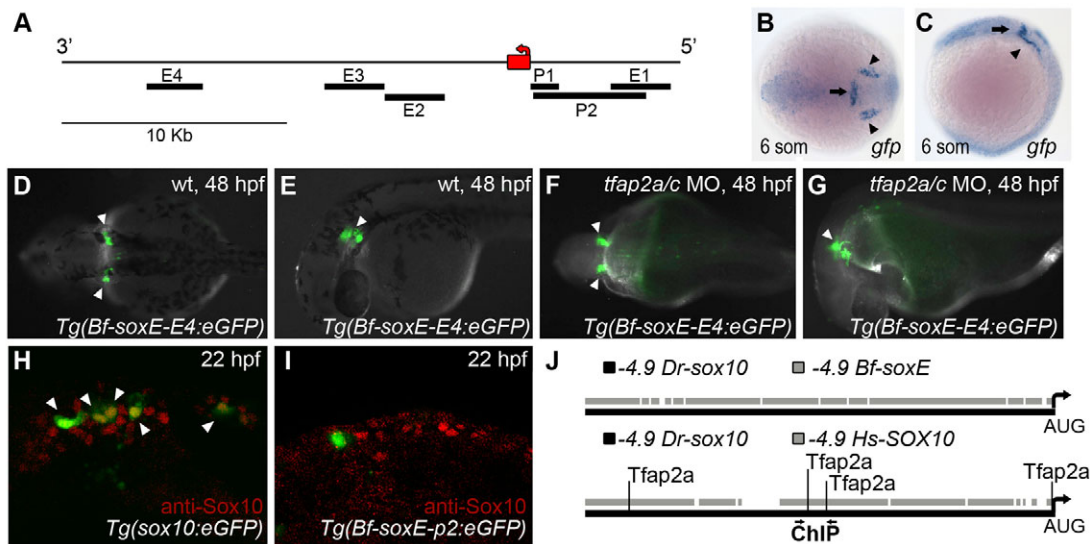


**Fig. 5. *sox10* is directly regulated by Tfap2a in zebrafish embryos, and forced expression of *sox10* can restore melanophores in *tfap2a/c*-deficient embryos.** (A) Schematic of *sox10* upstream element sufficient to drive NC-specific expression (Wada et al., 2005). Ex1, exon 1; Ex2, exon 2. Arrows indicate primers used to quantify results of ChIP experiments. (B) Representative quantitative real-time PCR (qPCR) results from anti-Tfap2a antibody ChIP conducted on lysates of 12 hpf zebrafish embryos (mean  $\pm$  s.e.m. in technical replicates from a single representative experiment). In two additional biological replicates, qPCR revealed  $\sim$ 12-fold enrichment at the target site with anti-Tfap2a relative to IgG, and minimal enrichment at the off-target site (data not shown). (C,D) Representative results of qRT-PCR analysis of *sox10* (C) or the *ednra* homolog *zgc:85942* (D) mRNA expression in lysates of embryos that were uninjected or injected with MOs targeting *tfap2a* and *tfap2c* and with *tfap2a-GR* mRNA, as indicated, and treated with cycloheximide (CHX) to inhibit protein translation, and with dexamethasone (DEX) to trigger nuclear localization of Tfap2a-GR, as indicated. The results are shown as fold-enrichment relative to levels in uninjected embryos treated with CHX/DEX, after normalization to *b-actin*. These experiments were performed three times with similar results observed (graphs represent mean  $\pm$  s.e.m. of one repeat). (E-P) Dorsal (E,F,I,J,M,N) or lateral (G,H,K,L,O,P) views of embryos at the times indicated stage processed with the indicated marker. Embryos are either uninjected or injected with *tfap2a* and *tfap2c* MOs (or *tfap2c* MO injected into *tfap2a* mutant; K,L,O,P) and *hsp70:sox10* plasmid, as indicated. All embryos exposed to heat-shock for 60 minutes at 90% epiboly. Rescue numbers: *snai1b*, uninjected, 20/23; *tfap2a/c*-deficient, 0/10; *tfap2a/c*-deficient plus *hsp70:sox10*, 19/27; *sox9b*, uninjected, 24/25; *tfap2a/c*-deficient, 0/10; *tfap2a/c*-deficient plus *hsp70:sox10*, 26/27; *crestin*, uninjected, 20/20; *tfap2a/c*-deficient, 0/5; *tfap2a/c*-deficient plus *hsp70:sox10*, 0/17; melanophores, uninjected, 59/59; *tfap2a/c*-deficient, 0/21; *tfap2a/c*-deficient plus *hsp70:sox10*, 22/30. Embryos shown with anterior to the left, unless otherwise indicated. Scale bars: 100  $\mu$ m.

Finally, we examined sequences upstream of the Sox10 or SoxE start codon in human, fish and amphioxus for conserved Tfap2 binding sites using the online analysis software ConSite (Sandelin et al., 2004). This analysis revealed multiple conserved predicted

Tfap2 binding sites between human and zebrafish sequences (Fig. 6J). Importantly, two of these conserved binding sites were located precisely within the region we showed by ChIP analysis to be enriched with Tfap2a. Using identical parameters, no conserved





**Fig. 6. Amphioxus *soxE* elements fail to drive neural crest specific expression in zebrafish embryos.** (A) Schematic of amphioxus *soxE* locus showing location of DNA elements tested in zebrafish reporter analysis. Red box, coding region of *soxE*; red arrow, initial AUG. Genomic coordinates are shown in supplementary material Table S2. (B, C) Anterior dorsal (B) and lateral (C) views of a *Tg(Bf-soxE-E4:eGFP)* zebrafish embryo fixed at 12 hpf and processed for *gfp* expression. *gfp* expression in midbrain-hindbrain (arrows) and otic placodes (arrowheads) is apparent. (D-G) Dorsal (D, F) and lateral (E, G) views of live, 48 hpf *Tg(Bf-soxE-E4:eGFP)* embryos. Arrowheads in D and E indicate GFP expression in otic vesicle (observed in five of 20 injected embryos). (F, G) A *Tg(Bf-soxE-E4:eGFP)* embryo injected with MOs targeting *tfap2a* and *tfap2c*, revealing eGFP expression in otic vesicle (arrowheads) despite an absence of melanophores (otic vesicle expression seen in three of 14 similarly injected transgenic embryos). (H, I) Lateral views of 22 hpf fixed embryos injected with (H) *sox10:eGFP* plasmid (Wada et al., 2005), showing many GFP-positive, anti-Sox10-positive NC cells (arrowheads) in the dorsum (18/22 injected embryos showed dorsal GFP-positive cells), or (I) an analogous plasmid harboring 4.9 kb upstream of amphioxus *soxE* (*Bf-soxE-p2:eGFP*), with very few such cells (4/22 embryos showed few dorsal GFP-positive cells all of which were negative for anti-Sox10 immunoreactivity). (J) Modified output of ConSite software showing conservation of sequence and Tfp2 transcription factor binding sites in 4.9 kb of genomic DNA upstream of the start of the initiator AUG in human *SOX10* (-4.9 *Hs-SOX10*) and zebrafish *sox10* (-4.9 *Dr-sox10*). Arrows, region tested in ChIP analysis. An identical comparison of amphioxus *soxE* (-4.9 *Bf-soxE*) and zebrafish *sox10* using identical parameters fails to detect conserved Tfp2 binding sites. Embryos shown with anterior to the left, unless otherwise indicated.

Tfp2 binding sites were detected when comparing human with amphioxus sequences or zebrafish with amphioxus sequences (Fig. 6J; data not shown). In combination with the enhancer analysis of *soxE* discussed above, and the absence of co-expression of *soxE* and *tfap2* in amphioxus (and *Drosophila*), these findings support the hypothesis that *sox10* became a regulatory target of Tfp2 after the split of the cephalochordates from the lineage leading to vertebrates.

## DISCUSSION

### Selective pressure to retain Tfp2 paralogs resulted from sub-functionalization

Here, we began by showing that fish and mammalian Tfp2d paralogs resulted from an ancient duplication of an ancestral Tfp2 prior to the split of agnathans and gnathostomes, potentially during a genome-duplication event. Interestingly, both Maximum Likelihood and Neighbor Joining (not shown) analyses grouped amphioxus Tfp2, sea urchin Tfp2 and ascidian Tfp2B together in one clade, and placed ascidian Tfp2A as an outgroup to all other chordate Tfp2s with moderate to high support. This grouping contradicts recent phylogenomic analyses showing that urochordates are the vertebrate sister group (Delsuc et al., 2006). These results probably reflect the rapid evolution of ascidian *tfap2* genes, as has been seen with many other urochordate developmental regulators (Holland and Gibson-Brown, 2003). However, regardless of the phylogenetic position of the ascidian genes, a comparison of the expression pattern of the single *tfap2*

homolog in amphioxus (a basal chordate) and the two homologs in lamprey (a basal vertebrate) supports the duplication-degeneration-complementation model (Force et al., 1999). This model posits that selective pressure to retain duplicated, otherwise redundant, genes emerges through a complementary loss of gene-regulatory elements inherited from the ancestral gene (a process termed sub-functionalization) (Force et al., 1999). Sub-functionalization also appears to have occurred among the *tfap2a/b/c/e* genes in gnathostomes. Like lamprey *tfap2*, the collective expression pattern of zebrafish *tfap2a/b/c/e* paralogs includes the presumptive skin, the neural plate border, NC and NC derivatives. However, as described here and elsewhere (Hoffman et al., 2007; Knight et al., 2005; Knight et al., 2003; Li and Cornell, 2007; Meulemans and Bronner-Fraser, 2002; O'Brien et al., 2004; Van Otterloo et al., 2010), the individual patterns of these paralogs are not identical. Thus, it appears that through the loss of tissue-specific enhancers associated with an ancestral *tfap2a/b/c/e*, initially redundant *tfap2* duplicates have acquired distinct expression territories, resulting in selective pressure to retain all four paralogs.

### Relaxed selective pressure on Tfp2d permitted the loss of its neural crest-promoting function

We have also tested the notion that expression-domain partitioning relaxes selective pressure on duplicated proteins. We showed that Tfp2d has lost, to a great degree, the ability to generate NC. Whether Tfp2d has also become optimized for its brain function, relative to the ancestral Tfp2, will require further tests. However,

the DNA-binding preference of TFAP2D is distinct from that of the other homologs, supporting this possibility (Zhao et al., 2001). Of note, sub-functionalization usually refers to complementary loss of regulatory elements possessed by an ancestral gene, but it can also apply to complementary loss of subfunctions possessed by the ancestral protein (Force et al., 1999). Indeed, the loss of NC-inducing function by Tfap2d might have preceded the loss of its expression in the non-neural ectoderm and neural border. This loss of functionality would have released selective pressure to retain expression of *tfap2d* in these tissues. Although not expressed broadly in surface ectoderm, Tfap2b and Tfap2e retain NC-inducing ability, in contrast to Tfap2d. These proteins have a more recent origin than Tfap2d and might have not had time to lose this ability; alternatively, the functions for which they have been selected in their current expression domains are not easily separated from the surface ectoderm function [for a general discussion, see Force et al. (Force et al., 1999)].

### Changes in the cis-regulatory elements of Tfap2 targets contributed to the origins of the neural crest

Our results favor the importance of regulatory versus protein neo-functionalization in the insertion of Tfap2 into the evolving NC GRN. Protein neo-functionalization, facilitated by gene duplication, has been proposed to drive new morphologies over the course of evolution (Ohno, 1970). However, although the Tfap2 family has been duplicated and is required for NC development, we found no evidence that protein neo-functionalization conferred a novel 'NC-inducing' function to a Tfap2 paralog. Specifically, we found that Tfap2 from basal animals that lack NC nonetheless harbored NC-promoting function. By contrast, we did find evidence for regulatory neo-functionalization of the *sox10* gene. Thus, here we show that Tfap2a directly activates *sox10* in zebrafish, a vertebrate. The evidence that this regulatory interaction emerged after the split of the lineage leading to vertebrates from cephalochordates is that *soxE* and *tfap2* are not co-expressed in amphioxus nor is *AP2* co-expressed with *sox100B* in flies (Hui Yong Loh and Russell, 2000; Meulemans and Bronner-Fraser, 2002; Meulemans and Bronner-Fraser, 2007; Monge and Mitchell, 1998; Yu et al., 2008). Moreover, multiple DNA elements proximal to amphioxus *soxE*, including the 4.9 kb upstream, lack NC enhancer function in zebrafish. The discovery of NC-like features in tunicates is consistent with the possibility that the novel regulation of *sox10* by Tfap2 appeared prior to the split of urochordates and vertebrates (Jeffery et al., 2004).

In the evolution of the NC GRN, Tfap2 probably acquired targets in addition to *sox10*, as judged by the failure of forced expression of *sox10* in *tfap2a/c* deficient embryos to fully restore the gene expression profile of premigratory NC. The absence of *foxd3* expression in this paradigm is consistent with earlier findings that Sox10 is downstream of Foxd3 in the NC GRN (Arduini et al., 2009). The presence of *sox9b* and *snailb* expression in this paradigm is noteworthy because although *sox10*, *sox9b* and *snailb* are all expressed in the NC, they are also co-expressed in mesoderm (Chimal-Monroy et al., 2003; Thisse et al., 1995; Yan et al., 2005). NC shares qualities with the mesoderm, such as migratory properties and the ability to make bone. The novel activation of *sox10* by Tfap2 might have served to recruit part of the mesoderm GRN to the NC, conferring mesoderm-like qualities to the NC. Interestingly, forcing *sox10* expression in *tfap2a/c*-deficient embryos restored trunk melanophores. It has recently been shown that dynamic regulation of Sox10 is essential for

differentiation of melanophores, and it remains to be seen whether trunk melanophores rescued by *sox10* in this study are fully normal (Greenhill et al., 2011). Clearly, there are many details of the NC GRN and the melanophore GRN that remain to be discovered. However, the failure of *sox10* to fully restore NC in this paradigm strongly suggests that other Tfap2 targets, whether novel or ancient, contribute to the NC GRN.

Finally, the presence of an apparent otic placode enhancer adjacent to amphioxus *soxE* serves to demonstrate that regulatory neo-functionalization is not the only mechanism by which novel GRNs are assembled. Amphioxus lack cranial placodes, indicating that at least some regulatory connections within the otic placode GRN are novel. Our findings reveal that cis-regulatory information sufficient to drive *soxE* expression in the ear was present prior to the split of cephalochordates and the lineage leading to vertebrates. Therefore regulatory neo-functionalization did not necessarily play a role in recruitment of *SoxE* into the otic placode GRN. Instead, *soxE* might have been recruited to the otic placode GRN via protein neo-functionalization, or as part of a conserved sub-network co-opted from another GRN. This unexpected finding supports other evidence that both protein neo-functionalization and regulatory neo-functionalization contribute to assembly of novel GRNs (Wagner and Lynch, 2008).

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### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071308/-/DC1>

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