Development 136, 1987-1994 (2009) doi:10.1242/dev.033209

Genetic ablation of neural crest cell diversification

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The neural crest generates multiple cell types during embryogenesis but the mechanisms regulating neural crest cell diversification are incompletely understood. Previous studies using mutant zebrafish indicated that *foxd3* and *tfap2a* function early and differentially in the development of neural crest sublineages. Here, we show that the simultaneous loss of *foxd3* and *tfap2a* function in zebrafish *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos globally prevents the specification of developmentally distinct neural crest sublineages. By contrast, neural crest induction occurs independently of *foxd3* and *tfap2a* function. We show that the failure of neural crest cell diversification in double mutants is accompanied by the absence of neural crest *sox10* and *sox9a/b* gene expression, and that forced expression of *sox10* and *sox9a/b* differentially rescues neural crest sublineage specification and derivative differentiation. These results demonstrate the functional necessity for *foxd3* and *tfap2a* for neural crest sublineage specification and that this requirement is mediated by the synergistic regulation of the expression of SoxE family genes. Our results identify a genetic regulatory pathway functionally discrete from the process of neural crest induction that is required for the initiation of neural crest cell diversification during embryonic development.

KEY WORDS: Neural crest, Cell fate specification, *foxd3*, *tfap2a*, SoxE, Zebrafish

INTRODUCTION

The neural crest (NC) is an ectoderm-derived embryonic cell population induced at the neural plate border during gastrulation. NC cells subsequently delaminate from the neuroepithelium, migrate and differentiate into a wide variety of derivatives, including peripheral neurons, chromatophores and major elements of the craniofacial skeleton (LeDouarin and Kalcheim, 1999). A number of genes have been identified that are necessary for the development of, and/or that are diagnostic of, different NC sublineages. For example, *mitfa* is essential for melanophore development and is expressed by melanophore precursors well before overt differentiation (Hodgkinson et al., 1993; Hodgkinson et al., 1998; Mochii et al., 1998; Lister et al., 1999; Levy et al., 2006). Genes with analogous functions or expression patterns have been identified for other NC sublineages as well. However, lineage analyses have indicated that the initial specification of distinct sublineages may considerably precede the initial expression of identified genes that are expressed in a sublineage-specific manner (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991; Raible and Eisen, 1994; Schilling and Kimmel, 1994; Henion and Weston, 1997). These observations suggest that an earlier functioning regulatory network initiates neural crest cell (NCC) diversification, resulting in sublineage-specific gene expression and function.

A number of genes that are expressed by NC progenitors upon induction of the neural plate border have been implicated in the early development of the NC and are candidates for mediating the initial specification of NC sublineages (Gammill and Bronner-Fraser, 2003). This group includes the transcription factors *foxd3*, *tfap2a*, *sox10* and *sox9*. Phenotypical analysis of zebrafish presumptive loss-of-function mutants indicates that *foxd3*, *tfap2a*, *sox10* and the *sox9* co-orthologs *sox9a* and *sox9b* are required for the development of both distinct and overlapping NC subpopulations (Dutton et al., 2001; Knight et al., 2003; Barrallo-Gimeno et al., 2004; Yan et al., 2005; Carney et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006), although the genetic interactions among them are incompletely understood. In addition, a large number of studies that primarily used frog, chick and rodent animal models have documented important functions for this same set of transcription factors in NC development (see Gammill and Bronner-Fraser, 2003; Kos et al., 2001; Cheung et al., 2005; Sakai et al., 2006). Although many of these studies indicate crucial roles in NC development for *foxd3*, *tfap2a* and SoxE family genes, inconsistent or conflicting results have been obtained. For example, both gain-of-function and loss-of-function manipulations of *foxd3* expression resulted in the upregulation of NC marker gene expression (Dottori et al., 2001; Kos et al., 2001; Pohl and Knochel, 2001; Sasai et al., 2001). Although some inconsistencies can probably be attributed to differences in experimental paradigms or even species, the lack of consensus on the function(s) of individual transcription factors in the regulation of NC development has hampered efforts to address how these factors interact (cf. Cheung et al., 2005; Sakai et al., 2006). In addition, genetic efforts to this end in mouse have been complicated until recently, and with exceptions (Herbarth et al., 1998; Southard-Smith et al., 1998; Kapur, 1999), by severe early pleiotropic phenotypes or haploinsufficiency issues in knockout models (Schorle et al., 1996; Zhang et al., 1996; Morriss-Kay, 1996; Bi et al., 2001; Hanna et al., 2002; Mori-Akiyama et al., 2003; Sock et al., 2003). Therefore, to investigate potential functional genetic interactions between transcriptional regulators of early NC development, and their molecular and cellular consequences in the process of NCC diversification, we have used a genetic approach in zebrafish.

Using the presumptive null *foxd3*^{zdf10} and *tfap2a*^{low} mutant alleles (Knight et al., 2003; Stewart et al., 2006), we have analyzed NC development in zebrafish *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos and in *tfap2a* morpholino (MO)-injected *foxd3*^{zdf10} embryos. We found that the synergistic functions of *foxd3* and *tfap2a* are required for the specification and differentiation of all major NC sublineages. The absence of NC sublineage specification was preceded by the absence of NC SoxE family gene expression in double mutants. Forced expression of SoxE family genes in *tfap2a*-

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Accepted 9 April 2009

depleted *foxd3*^{zdf10} embryos differentially rescued the specification of all major NC sublineages. Our results define a minimal genetic transcriptional network required for the initiation of NCC diversification.

MATERIALS AND METHODS

Whole-mount in situ hybridization and immunohistochemistry

In situ hybridizations were performed as previously described (Thisse et al., 1993), with minor modifications. Immunohistochemistry was also performed as previously described (An et al., 2002).

Morpholino and expression vectors

A previously described (O'Brien et al., 2004), ap2E2I2 morpholino, which specifically targets splicing of *tfap2a*, was used to phenocopy *tfap2a* mutant phenotypes in both wild-type and *foxd3*^{zdf10} mutant backgrounds. The p53 morpholino was the gift of Dr A. T. Look (Dana-Farber Cancer Institute, Boston, MA, USA). To express SoxE genes, we used *sox9a*-pSP64T and *sox9b*-pSP64T (Yan et al., 2005) kindly provided by Dr John Postlethwait (University of Oregon, Eugene, OR, USA). We tested a wide range of *sox9a*/*b* mRNA concentrations based on Yan et al. (Yan et al., 2005), including concentrations of single *sox9* mRNAs that were greater than the concentrations of the combined mRNAs in double injections. *sox10*-pCSHSP (Elworthy et al., 2003) was a gift from Dr Robert Kelsh (University of Bath, Bath, UK). Heat shock was induced by transferring embryos from 28.5°C to 37°C for 1 hour.

Cartilage staining

Alcian Blue staining was used to detect cartilage, as previously described (Kimmel et al., 1998).

Genotyping

Single and double mutant embryos were genotyped using *foxd3*^{zdf10} and *tfap2a*^{low} allele-specific PCR or sequencing as previously described (Knight et al., 2003; Stewart et al., 2006).

Zebrafish

All zebrafish were maintained in the Ohio State University Zebrafish Facility, raised at 28.5°C, and staged by published criteria (Kimmel et al., 1995). The generation of *foxd3*^{zdf10} and *tfap2a*^{low} mutants has been described previously (Knight et al., 2003; Stewart et al., 2006). The specific nucleotide mutations of both mutants strongly suggest that both represent loss-of-function alleles.

RESULTS

Phenotypical analysis of zebrafish presumptive loss-of-function mutants indicates that *foxd3* and *tfap2a* are required for the development of both distinct and overlapping NC subpopulations (Knight et al., 2003; Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006; Stewart et al., 2006). However, because the induction of *tfap2a* expression is normal in *foxd3*^{zdf10} mutants and *foxd3* expression is only defective in a small number of hindbrain NCCs in *tfap2*^{low} mutants, the reciprocal downregulation of one another's expression in mutant embryos is unlikely to directly contribute to the major phenotypical defects. Consistent with this assertion, we found that misexpression of *tfap2a* mRNA in *foxd3*^{zdf10} mutants failed to rescue any of the NC derivative phenotypes of mutant embryos (n=165; data not shown). Thus, although *foxd3* and *tfap2a* might each directly regulate aspects of NCC diversification, major functions of both transcription factors in NC development are likely to be mediated by other genes. Therefore, to investigate potential functional genetic interactions between *foxd3* and *tfap2a* in the process of NCC diversification, we analyzed NC development in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos. Parallel experiments using *tfap2a* morpholino (MO)-injected *foxd3*^{zdf10} embryos efficiently (90%; n>1000) produced identical results (see Figs S1-S3 in the supplementary material).

Global absence of differentiated NC-derived cells in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos

In both *foxd3*^{zdf10} and *tfap2a* single mutants, NC-derived chromatophore development, after a brief developmental delay, occurs normally (Fig. 1A) suggesting that both of these genes are ultimately dispensable for chromatophore development in zebrafish (Knight et al., 2003; Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006; Stewart et al., 2006). Unexpectedly, *foxd3*^{zdf10};*tfap2a*^{low} double mutants are completely devoid of NC-derived chromatophores, with the exception of occasional xanthophores over the head (Fig. 1A). The normal development of the pigmented retinal epithelium in double mutants demonstrates NC specificity of the genetic requirement for *foxd3* and *tfap2a* function among pigmented cells. This result indicates that a synergistic genetic interaction between *foxd3* and *tfap2a* is required for NC-derived chromatophore development.

Loss of either *foxd3* or *tfap2a* function results in similar but non-identical craniofacial defects involving the reduction and disorganization of upper and lower jaw elements, with more dorsal structures of the neurocranium remaining intact (Knight et al., 2003; Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006; Stewart et al., 2006). By contrast, Alcian Blue staining of *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos revealed the complete absence of all upper and lower jaw structures, and of all but the most posterior portion of the neurocranium (Fig. 1B). The development of pharyngeal mesoderm and endoderm was normal in double mutants (Fig. 2I,J). As was the case for chromatophores, the craniofacial phenotype of double mutants was more severe than would be predicted by an additive effect of the mutations, which further suggests a parallel synergistic interaction between *foxd3* and *tfap2a* that regulates the development of progenitors of the NC-derived craniofacial skeleton.

We also examined the development of NC-derived peripheral neurons of the dorsal root ganglia (DRG) and enteric nervous system by using a neuron-specific antibody, and the development of autonomic sympathetic neurons, identified by expression of *tyrosine hydroxylase* (*th*). DRG neurons are absent in *foxd3*^{zdf10} mutant embryos and are slightly reduced in number in *tfap2a* mutant embryos, enteric neurons are reduced in homozygous mutants for either gene and sympathetic neurons are absent in both *foxd3*^{zdf10} and *tfap2a* single mutants (Fig. 1C,D) (Knight et al., 2003; Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006; Stewart et al., 2006). All three neuronal populations are entirely absent in *foxd3*^{zdf10};*tfap2a*^{low} double mutants at 3 days post-fertilization (dpf) (Fig. 1C,D). Taken together, our analysis of differentiated NC-derived cells indicates a specific, complete failure of the development of major NC derivatives in *foxd3*^{zdf10};*tfap2a*^{low} double mutants. That the NC phenotype of *foxd3*^{zdf10};*tfap2a*^{low} double mutants is more severe than that of either single mutant indicates that *foxd3* and *tfap2a* regulate NC development in parallel.

The specification of NC sublineages fails to occur in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos

Analysis of the expression of genes required for and/or diagnostic of the specification of major NC sublineages prior to the overt differentiation of derivatives in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos revealed a global failure in the initiation of NCC diversification (Fig. 2, see also Fig. S2 in the supplementary material; data not shown). For example, chromatophore precursor expression of *mitfa* (Lister et al., 1999), *xdh* (Budi et al., 2008), *ltk* (Lopes et al., 2008) and *ednrb1* (Parichy et al., 2000) (Fig. 2A-D), pharyngeal arch progenitor expression of *dlx2* and *dlx3* (Akimenko

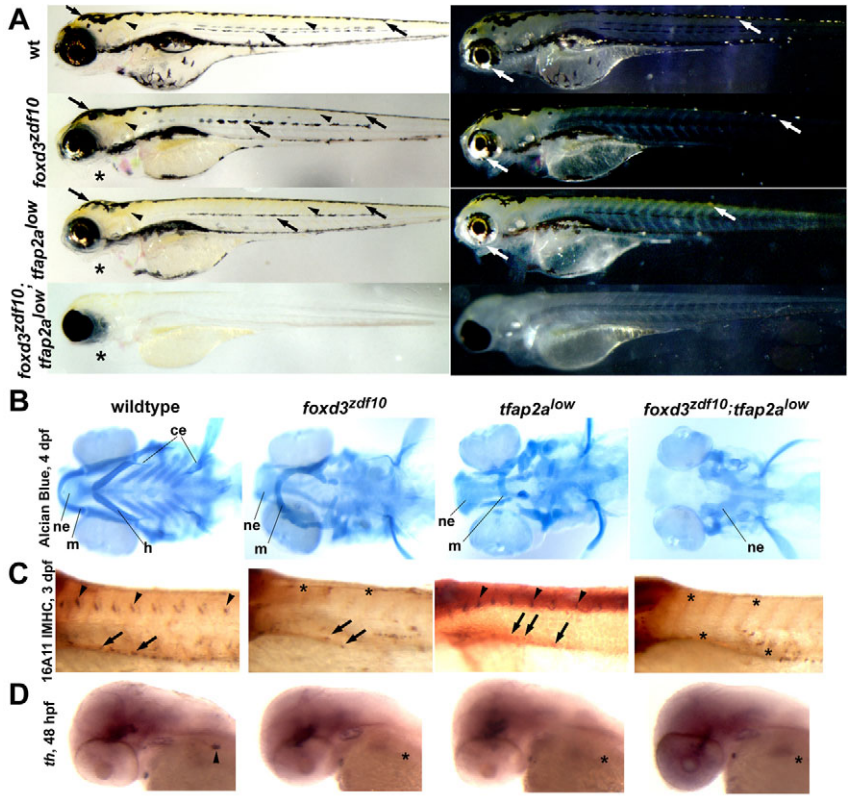

Fig. 1. *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos lack NC derivatives. (**A**) Live larvae at 4 days post-fertilization (dpf), lateral views with either transmitted (left) or reflected (right) light. Wild-type zebrafish exhibit three NC-derived pigment cell types: black melanophores (left, arrows), yellow xanthophores (left, arrowheads) and iridescent iridophores (right, white arrows). *foxd3*^{zdf10} and *tfap2a*^{low} single mutants develop essentially normal pigment patterns by 4 dpf. Jaw structures protrude ventrally in both *foxd3*^{zdf10} and *tfap2a*^{low} single mutants (asterisks). *foxd3*^{zdf10};*tfap2a*^{low} double mutants completely lack NC-derived pigment cells except for occasional head xanthophores. Jaw structures appear to be missing (asterisk). (**B**) Craniofacial cartilage development revealed by Alcian Blue staining at 4 dpf, ventral views with anterior to the left. The wild-type larval head skeleton of zebrafish consists of the dorsal neurocranium (ne), as well as upper (mandibular, m) and lower (hyoid, h, and ceratobranchial, ce) jaw structures. In both *foxd3*^{zdf10} and *tfap2a*^{low} single mutants, mandibular and hyoid structures are disorganized and the ceratobranchial elements are absent, whereas the neurocranium remains intact. Double mutants lack upper and lower jaw structures, and all but the most posterior portion of the neurocranium. (**C**) Immunostaining with 16A11 monoclonal (anti-Hu) antibody at 3 dpf, lateral views, anterior to the left. DRG neurons are found in each trunk segment of wild-type embryos (arrowheads) and enteric neurons populate the gut tube (arrows). DRG neurons are absent in *foxd3*^{zdf10} single mutants (asterisks) and present in reduced numbers in *tfap2a*^{low} single mutants (arrowheads), whereas enteric neurons are reduced in number in both backgrounds (arrows). DRG and enteric neurons are absent in double mutant embryos (asterisks). (**D**) *tyrosine hydroxylase* (*th*) expression in sympathetic neurons at 48 hpf in wild-type embryos (arrowhead). Sympathetic neurons are absent in *foxd3*^{zdf10} and *tfap2a*^{low} single mutants, as well as in double mutants (asterisks).

et al., 1994) (Fig. 2F,G), and *dhand* (Yelon et al., 2000) (Fig. 2H), and sympathetic and enteric neuron progenitor expression of *dhand* (Lucas et al., 2006) (Fig. 2E) and *zash1a* (Allende and Weinberg, 1994; Lucas et al., 2006) (data not shown) fail to occur. By contrast, the development of mesendodermal components of the early craniofacial skeleton appears to occur normally in double mutants (Fig. 2I,J). Taken together, the absence of NC derivatives in double mutants appears to be a consequence of the failure of NC sublineage specification mediated directly or indirectly by the functions of *foxd3* and *tfap2a* during early stages of NC development.

foxd3 and *tfap2a* are synergistically required for NC SoxE family gene expression

Because members of the SoxE family of genes in zebrafish have been implicated in the specification and development of some NCC sublineages that generate all chromatophore types, DRG, sympathetic and enteric neurons (*sox10*) (Dutton et al., 2001; Elworthy et al., 2005; Carney et al., 2006), and craniofacial skeleton and melanophores and iridophores (*sox9a and sox9b*) (Yan et al., 2005), we examined the expression of these genes in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos. In both *foxd3*^{zdf10} and *tfap2a*^{low} mutants, NC expression of *sox9a*, *sox9b* and *sox10* was reduced but still readily detectable by in situ hybridization in the majority of the NCC population (Fig. 3A-E) (Knight et al., 2003; Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006; Stewart et al., 2006). By stark contrast, NC expression of *sox9b* and *sox10* was induced at strikingly reduced levels (Fig. 3A,D; see also Fig. S3 in the supplementary material), was then rapidly and completely extinguished (Fig. 3B,E) in *foxd3*^{zdf10};*tfap2a*^{low} double mutants, and remained undetectable at all axial levels through 27 hours post-fertilization (hpf), the latest stage examined (not shown). NC *sox9a* expression was absent at all stages in double mutants (Fig. 3C; data not shown; see Fig. S3 in the supplementary material). Failure in the expression of these SoxE genes in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos was NC specific, as the induction and maintenance of expression in non-NC-derived tissues was similar to that in wild-type embryos (Fig. 3). Lastly, we also examined the expression of another transcription factor, *snai1b*, that is normally expressed by premigratory NC during early somitogenesis (Thisse et al., 1993). Consistent with previous findings (Knight et al., 2003; Barrallo-Gimeno et al., 2004; Stewart et al., 2006), we found that NC *snai1b* expression in *tfap2a*^{low} mutants was indistinguishable from that in wild-type embryos, whereas NC expression was significantly reduced but still detectable in *foxd3*^{zdf10} mutants. NC *snai1b* expression in *foxd3*^{zdf10};*tfap2a*^{low} double mutants was qualitatively indistinguishable from that in *foxd3*^{zdf10} single mutants (data not shown) (see Stewart et al., 2006).

NC induction is independent of *foxd3* and *tfap2a* function

Importantly, NCCs are present at stages when SoxE family genes are expressed in wild-type embryos based on the normal pattern of *foxd3* expression in *foxd3*^{zdf10};*tfap2a*^{low} double mutants (Fig. 3F; data not shown), indicating that NC induction is qualitatively normal, although these NCCs are molecularly abnormal. In addition, the level of NCC death over time (6 somites-24 hpf), assessed

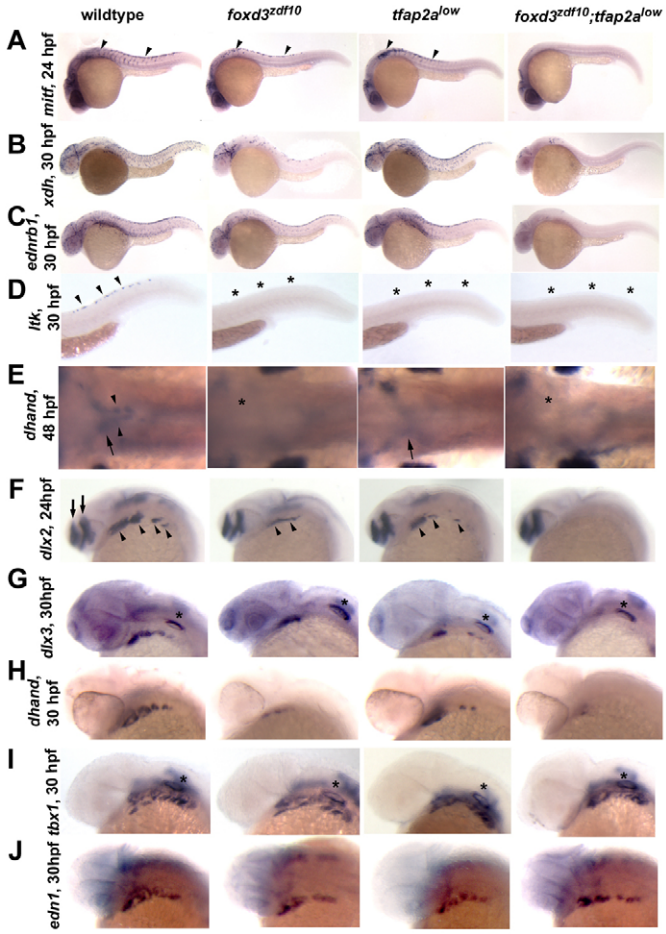

Fig. 2. Failure of NC sublineage specification in *foxd3*^{zdf10};*tfap2a*^{low} double mutants. (A-D) Lateral views with anterior to the left. (**A**) *mitf* expression at 24 hpf (arrowheads). *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos completely lack NC-derived melanoblast *mitf* expression. (**B**) *xanthine dehydrogenase* (*xdh*), diagnostic of xanthophore precursors, at 30 hpf. (**C**) *endothelin receptor B* (*ednrb1*), presumed to be expressed by all chromatophore precursors, at 30 hpf and (**D**) *leukocyte tyrosine kinase* (*ltk*), reported to be expressed by iridophore precursors, at 30 hpf. (**E**) Dorsal views of *dhand* expression, anterior to left, ventral of the caudal hindbrain, at 48 hpf. Sympathetic neuron precursors are labeled in wild-type embryos (arrowheads), as are the more ventral and laterally localized enteric neuron precursors (arrows). In the majority of *foxd3*^{zdf10} embryos, both sympathetic and enteric neuron progenitors are absent (asterisk) (Stewart et al., 2006). In *tfap2a*^{low} mutants, sympathetic neuron precursors are absent whereas a small number of enteric precursors are present (arrow) (Knight et al., 2003). In *foxd3*^{zdf10};*tfap2a*^{low} double mutants, both sympathetic and enteric neuron precursors are absent (asterisk). (F-J) Lateral (F-I) and dorsolateral views (J), anterior to the left. (**F**) *dlx2* expression at 24 hpf. *dlx2* expression (arrowheads) is absent in the branchial arches of double mutant embryos, but is retained in the forebrain (arrows). (**G**) *dlx3* expression at 30 hpf; (**H**) *dhand* expression at 30 hpf. NC expression of both genes is absent in double mutant embryos with *dlx3* expression maintained in non-NC otic vesicle (asterisks). (**I**) *tbx1* expression in the branchial arches (asterisk) at 30 hpf is diagnostic of the endodermal component of the arches. *tbx1* expression is normal in all experimental embryos. (**J**) *endothelin 1* (*edn1*) expression in the mesodermal component of the branchial arches at 30 hpf. Branchial mesoderm appears to develop normally in *foxd3*^{zdf10} and *tfap2a*^{low} single mutants, as well as in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos.

qualitatively by TUNEL and Acridine Orange staining, in *foxd3*^{zdf10};*tfap2a*^{low} double mutants was modest and not more pronounced than the levels observed in *tfap2a*^{low} or *foxd3*^{zdf10} single mutants nor than the theoretical sum of both (data not shown). Nevertheless, NCC death might play a role in the development of aspects of the single as well as the double mutant phenotypes. Consistent with this possibility, we found that p53 morpholino injection into *tfap2a*-depleted *foxd3*^{zdf10} embryos resulted in limited and variable melanophore and xanthophore rescue in 100% of mutant morphant embryos (n=50), and iridophore rescue in 30% of these embryos when examined at 4 dpf and 7 dpf (Fig. 4C). Qualitatively, chromatophore rescue by p53 morpholino was markedly less robust than that by *sox10* misexpression (not shown, see below). No rescue of craniofacial cartilage development was found based on Alcian Blue staining (data not shown). However, we also found that activation of *sox10* cDNA expression driven by a heat-shock promoter (Halloran et al., 2000) at 24 hpf in *tfap2a* MO-injected *foxd3*^{zdf10} mutant embryos resulted in robust melanophore rescue (50%, n=17; Fig. 4K-M). Because *sox10* function is not necessary for NC induction in zebrafish (not shown) (Kelsh and Eisen, 2000) and has a demonstrable NCC survival function only after 35 hpf (Dutton et al., 2001), these results indicate the persistence of an NCC population in *tfap2a*-depleted *foxd3*^{zdf10} embryos at least as late as 24 hpf. Interestingly, the persistence and/or *sox10* responsiveness of these cells is limited, as chromatophore rescue was not observed when *sox10* expression was induced at 48 hpf (not shown). Taken together, these results strongly suggest that *p53*-mediated NCC death is unlikely to fully account for the observed defects in NC sublineage specification and development in double mutants. Finally, the induction and patterning of the neural plate border occurs normally in double mutants, as does the development of Rohon-Beard sensory neurons, identified by *huC* (*elevl3* – Zebrafish Information Network) and *isl1* expression (see Fig. S4 in the supplementary material; data not shown). Thus, *foxd3* and *tfap2a* are dispensable for NC induction but are genetically required for NCCs to express SoxE genes and diversify.

Coordinate regulation of SoxE family gene expression by *foxd3* and *tfap2a* initiates NCC diversification

To test whether the absence of NC SoxE family gene expression in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos can functionally account for the failure of NC sublineage specification and/or the differentiation of NC derivatives, we misexpressed SoxE family genes using mRNA constructs in *tfap2a* MO-injected *foxd3*^{zdf10} mutants. Again, these mutant morphants precisely and efficiently phenocopy double mutants, including NC SoxE family gene induction deficiencies (see Figs S1-S3 in the supplementary material). Misexpression of *sox10* mRNA resulted in the efficient rescue of melanophores (50%, n=69), xanthophores (50%, n=69), and DRG and sympathetic neurons (50%, n=38), but had no effect on the failure of craniofacial NC development (0%, n=75; Fig. 4B,F,G,I,J). By contrast, misexpression of combined *sox9a* and *sox9b* mRNAs globally rescued the sublineage specification (76%, n=28) and differentiation of craniofacial cartilages (68%, n=44), as well as melanophores and xanthophores (76%, n=29; Fig. 4A,E,H). No phenotype rescue was detected after misexpression of either *sox9a* (n=44) or *sox9b* (n=33) alone (data not shown), even when concentrations of single species were in excess of that of the concentration of the combined mRNAs that resulted in phenotype rescue (see Materials and methods). These results

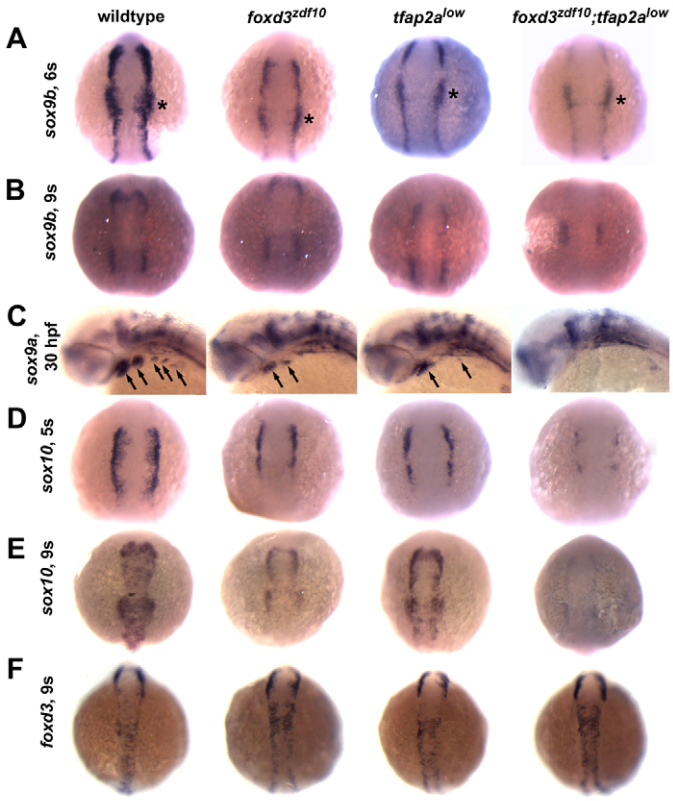

Fig. 3. Defective expression of NC SoxE family genes in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos. (A,B,D-F) Dorsal views with anterior to the top of 6, 5 and 9 somite stage (s) embryos. (C) Lateral views with anterior to the left of 30 hpf embryos. (**A**) NC *sox9b* expression is severely depleted in *foxd3*^{zdf10};*tfap2a*^{low} double mutants at the 6 somite stage, but is retained in the non-NC-derived otic placode (asterisks). (**B**) NC *sox9b* expression remains reduced in both single mutants, whereas NC expression is undetectable in double mutants by the 9 somite stage. (**C**) NC *sox9a* expression in the branchial arches (arrows) is absent in double mutant embryos. (**D**) There are slight reductions in NC *sox10* expression in *foxd3*^{zdf10} and *tfap2a*^{low} single mutants at the 5 somite stage, whereas in double mutant embryos NC *sox10* expression is much more reduced. (**E**) By the 9 somite stage, NC *sox10* expression is undetectable in double mutants. (**F**) In contrast to SoxE gene expression, NC *foxd3* expression is maintained in *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos, demonstrating that NC induction occurs.

indicate that the *foxd3*;*tfap2a*-dependent initiation of NCC diversification is mediated in part by SoxE family genes, which in turn differentially specify subpopulations of all of the major NC sublineages.

DISCUSSION

We have shown that *foxd3*^{zdf10};*tfap2a*^{low} double mutant embryos completely lack differentiated NC-derived cells. Furthermore, our results indicate that *foxd3* and *tfap2a* are required in parallel for the initiation of NCC diversification in zebrafish embryos, as demonstrated by the absence of NC expression of genes required for and/or diagnostic of specified NC sublineages. This phenotype is preceded or accompanied by the complete loss of NC expression of the SoxE family genes *sox10*, *sox9b* and *sox9a*. We show that restoration of NC expression of *sox10* and both *sox9a* and *sox9b* differentially rescues NCC diversification. Together, based on the results of our genetic manipulations, we conclude that the requirement for *foxd3* and *tfap2a* function for the initiation of NCC diversification is mediated to a significant extent through the synergistic regulation of SoxE family gene expression. We also show that *p53*-mediated NCC death also plays at least a limited role in the double mutant phenotype, although whether this results directly or indirectly from the absence of *foxd3*, *tfap2a* or SoxE function, or a combination thereof, is unclear. Our results also provide further evidence for the decoupling of the genetic regulation of the processes of NC induction and NCC diversification, as the generation of the premigratory NC population is largely normal and significant numbers of NCCs persist for an extended period of embryogenesis in *foxd3*^{zdf10};*tfap2a*^{low} double mutants. Our results suggest that in *foxd3*^{zdf10};*tfap2a*^{low} double mutants substantial numbers of NCCs persist in an unspecified state and do not appear to adopt an alternative ectodermal fate.

Our results demonstrate that the coordinated regulation of the NC expression of SoxE family genes by *foxd3* and *tfap2a* underlies the specification of multiple NC sublineages, although the rescue experiments we performed do not address whether all cells within a given sublineage are specified by SoxE genes. For example, the fact that in zebrafish *sox10* null mutants subsets of DRG, sympathetic and enteric neurons develop successfully (Kelsh and Eisen, 2000; Dutton et al., 2001) indicates that additional regulators of sublineage specification, dependent upon *foxd3* and *tfap2a* function, are required for the specification of these sublineages in their entirety. Likewise, although our results show that forced *sox9a/b* expression in *tfap2a*-depleted *foxd3*^{zdf10} mutants is sufficient for the specification of craniofacial skeleton progenitors and their subsequent differentiation, the fact that craniofacial NC precursors are specified in *sox9a*;*sox9b* double mutants (Yan et al., 2005) might indicate that one or more different genes, whose expression is dependent on *foxd3* and *tfap2a* function, are normally required as well for the initial specification of the NC craniofacial skeleton sublineage. In addition, the chromatophore rescue activity of *sox9a/b* is somewhat surprising given the phenotype of *sox9a*;*sox9b* double mutants (Yan et al., 2005). We did not detect rescue of NC *sox10* expression in *sox9a/b*-injected, double deficient, 9-somite-stage embryos at the level of in situ hybridization, although this does not preclude low-level *sox10* induction sufficient to drive chromatophore development in these embryos. It could also be possible that forced *sox9a/b* expression is sufficient to drive chromatophore development in the absence of *sox10* even though the *sox9a*;*sox9b* double mutant phenotype indicates that they are not normally necessary (Yan et al., 2005). Lastly, unlike zebrafish *sox10*, which is entirely dispensable for NC induction (not shown) (Kelsh and Eisen, 2000), *sox9a* and *sox9b* have been shown to induce ectopic NC-like cells in zebrafish upon forced misexpression (Yan et al., 2005). However, this activity appears to be qualitatively slight, and although it possibly contributes to the robust craniofacial phenotype rescue observed, it would seem unlikely to entirely account for it. The *foxd3*^{zdf10};*tfap2a*^{low} double mutant model provides an excellent experimental template for the identification of these additional regulators of NCC diversification. Taken together, however, based on the phenotypes of zebrafish SoxE family gene mutants and the robust phenotype rescue observed upon their forced expression in *tfap2a*-depleted *foxd3*^{zdf10} mutants, SoxE family genes are clearly principal regulators of NC sublineage specification genetically downstream of *foxd3* and *tfap2a*. It will be important to determine in the future whether the genetic interactions between *foxd3*, *tfap2a* and SoxE genes are direct or indirect by using biochemical analyses.

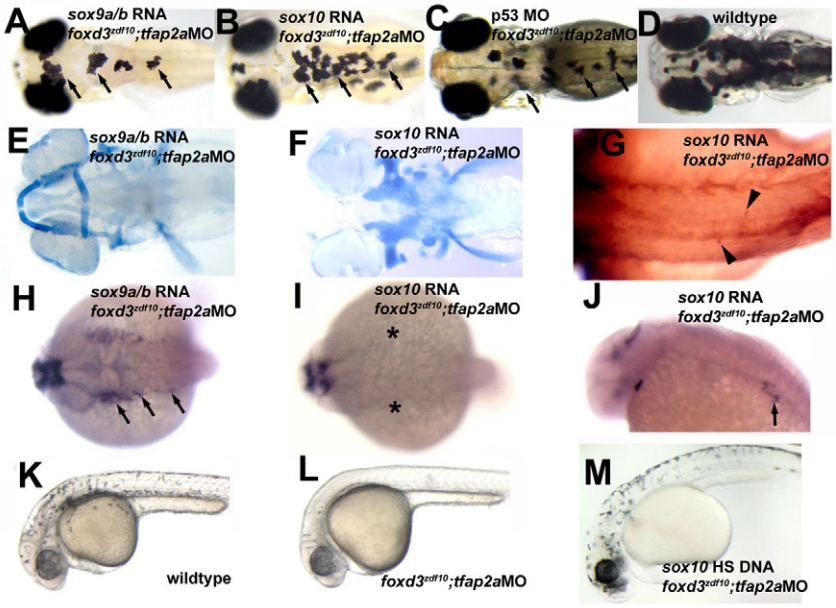

Fig. 4. Misexpression of SoxE genes differentially rescues NC sublineage specification and differentiation in *foxd3^{zdf10};tfap2a*MO embryos. (A,E,H) *sox9a* and *sox9b* (*sox9a/b*) RNA co-injection with *tfap2a* morpholino (*tfap2a*MO) into *foxd3^{zdf10}* mutant embryos (*foxd3^{zdf10}-tfap2a*MO). (**A**) Live 4 dpf larva, dorsal view, anterior to the left. Melanophore and xanthophore development is rescued in *foxd3^{zdf10}-tfap2a*MO embryos co-injected with *sox9a/b* RNA (arrows; compare with wild-type embryo, **D**). (**E**) Alcian Blue staining of a 4 dpf larva, ventral views with anterior to the left. Misexpression of *sox9a/b* in *foxd3^{zdf10}-tfap2a*MO embryos results in rescue of the neurocranium, as well as of the mandibular and hyoid jaw structures. (**H**) *dlx2* expression at 24 hpf, dorsal views, anterior to the left. Specification of NC craniofacial progenitors occurs in *foxd3^{zdf10}-tfap2a*MO embryos co-injected with *sox9a/b* RNA, based on *dlx2* expression (arrows). (B,F,G,I,J) *foxd3^{zdf10}-tfap2a*MO embryos co-injected with *sox10* RNA. (**B**) Live 4 dpf larva, dorsal view, anterior to the left. Consistent with a role in development of non-ectomesenchymal NC derivatives, *sox10* misexpression rescues melanophore and xanthophore development in *foxd3^{zdf10}-tfap2a*MO embryos (arrows; compare with wild-type embryo, D). (**F**) Alcian Blue staining of 4 dpf larva, ventral view, anterior to the left. *sox10* misexpression does not rescue cranial cartilage structures in *foxd3^{zdf10}-tfap2a*MO embryos. (**G**) 16A11 immunoreactivity, 3 dpf, dorsal view. Dorsal root ganglion neuron development is rescued by *sox10* misexpression (arrowheads). (**I**) *dlx2* expression at 24 hpf, dorsal view with anterior to the left. *sox10* RNA injection also does not rescue NC *dlx2* expression in *foxd3^{zdf10}-tfap2a*MO embryos (asterisks), indicating that *sox10* function cannot rescue the specification of NC precursors for craniofacial cartilages. (**J**) *th* expression at 48 hpf, lateral view, anterior to the left. *sox10* misexpression rescues sympathetic neuron development (arrow). (**C**) Live 4 dpf larva, dorsal view, anterior to the left. Morpholino-mediated knockdown of p53 in *foxd3^{zdf10}-tfap2a*MO embryos rescues melanophore, xanthophore (arrows; compare with wild-type embryo, D) and iridophore (not visible) development. (K-M) *sox10*-responsive NCCs persist in *foxd3^{zdf10}-tfap2a*MO embryos. 33 hpf embryos, lateral view, anterior to left. (**K**) Wild-type embryo with abundant melanophores. (**L**) In *foxd3^{zdf10}-tfap2a*MO embryos injected with a heat shock-inducible *sox10* construct but not exposed to heat shock, melanophores fail to develop. (**M**) In *foxd3^{zdf10}-tfap2a*MO embryos injected with a heat shock-inducible *sox10* construct and heat shocked at 24 hpf, robust melanogenesis occurs.

We have shown that a *foxd3-tfap2a*-SoxE transcriptional network regulates the fate specification of NC sublineages. In addition, we (Stewart et al., 2006) and others (Kelsh and Eisen, 2000; Dutton et al., 2001; Knight et al., 2003; Barrallo-Gimeno et al., 2004; Yan et al., 2005; Montero-Balaguer et al., 2006) have documented additional consequences of the loss- or gain-of-function of these transcription factors during NC development. Specifically, zebrafish *foxd3* (Montero-Balaguer et al., 2006; Stewart et al., 2006), *tfap2a* (Knight et al., 2003; Barrallo-Gimeno et al., 2004), *sox10* (Kelsh and Eisen, 2000; Dutton et al., 2001), *sox9a*;*sox9b* (Yan et al., 2005) and *foxd3*;*tfap2a* (this study) mutants all display NC phenotypes that include limited cell death that is likely to contribute in a limited way to the mutant phenotypes, although these phenotypes vary widely in both their extent and spatiotemporal pattern between different mutants. Importantly, a similar range of defects has been demonstrated in experiments in other vertebrate models as well (see Gammill and Bronner-Fraser, 2003; Kos et al., 2001; Cheung et al., 2005; Sakai et al., 2006), although differences exist between these models and zebrafish, perhaps most noticeably regarding roles in NC induction (see, for example, Kelsh and Eisen, 2000). Thus, whereas our conclusion that the coordinate regulation of SoxE family gene expression by *foxd3* and *tfap2a* is essential for the initial specification of NC sublineages, operationally defined by the absence of the expression of genes diagnostic of NC sublineages, the mechanism(s) underlying this developmental defect is currently not well defined. Conceptually, NCC death could deplete the numbers of NCCs available to be specified and develop, although in all of the zebrafish mutants relevant to this study, with the exception of *foxd3^{zdf10};tfap2a^{low}* double mutants, significant numbers and types of derivatives successfully develop, and we have shown that significant numbers of NCCs persist in *foxd3^{zdf10};tfap2a^{low}* double mutants. This suggests that NCC death does not entirely account for the *foxd3^{zdf10};tfap2a^{low}* double mutant phenotype. Indeed, it seems just as likely that the failure in NC sublineage specification as a result of simultaneous *foxd3*;*tfap2a* loss of function might result in some cells that would normally be specified to adopt a specific sublineage fate adopting an alternative fate, cell death. Our results demonstrating limited phenotype rescue upon p53 morpholino injection do not distinguish between the alternative mechanisms described. Taken together, although cell death might underlie aspects of the *foxd3^{zdf10};tfap2a^{low}* double mutant phenotype, it is unlikely to entirely account for it. Ultimately, it will be important to determine whether and to what extent defects in NC sublineage cell fate specification are the cause or consequence of NCC death observed in zebrafish mutant for the transcription factors investigated in this study.

In summary, using a genetic approach, we have demonstrated that the transcription factors *foxd3* and *tfap2a* are required for the initiation of NCC diversification in zebrafish embryos through the synergistic regulation of the expression of SoxE family genes that function to differentially specify NC sublineages. These results also provide further evidence that the processes of NC induction and NC sublineage specification can be regulated independently. Our results

identify a genetic network of transcriptional regulators that initiate NCC diversification. This network represents a minimal genetic scaffold to which additional regulators of NCC diversification can be appended to ultimately construct a comprehensive genetic network regulating the generation of NCC diversity.

We thank T. Schilling for sharing *tfap2a*^{low} fish, J. Postlethwait and R. Kelsh for expression vectors, R. Stewart for technical advice and numerous colleagues for reagents used in this study. This research was supported by NIH GM076505 to P.D.H. with additional support from NIH P30-NS045758. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/12/1987/DC1

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