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Zebrafish Trap230/Med12 is required as a coactivator for Sox9-dependent neural crest, cartilage and ear development

Marlene J. Rau, Sabine Fischer, Carl J. Neumann *

European Molecular Biology Laboratory (EMBL), Meyerhofstr. 1, 69117 Heidelberg, Germany

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Abstract

The vertebrate Sox9 transcription factor directs the development of neural crest, otic placodes, cartilage and bone. In zebrafish, there are two Sox9 orthologs, Sox9a and Sox9b, which together perform the functions of the single-copy tetrapod Sox9. In a large-scale genetic screen, we have identified a novel zebrafish mutant that strongly resembles the *Sox9a*/*Sox9b* double mutant phenotype. We show that this mutation disrupts the zebrafish Trap230/Med12 ortholog, a member of the Mediator complex. Mediator is a coactivator complex transducing the interaction of DNA-binding transcription factors with RNA polymerase II, and our results reveal a critical function of the Trap230 subunit as a coactivator for Sox9.

Keywords: Zebrafish; Development; Neural crest; Limb; Mediator; TRAP230; MED12; Sox9; Chondrogenesis; Pectoral fins; Trapped

Introduction

The precise control of transcription is of central importance during the development of multicellular animals, as it leads to cell-type-specific gene expression required for differentiation. Sox proteins are a large family of transcription factors implicated in the control of a variety of developmental processes. They are characterized by the presence of an HMG box, a sequence-specific DNA-binding domain, and by their homology to SRY, the mammalian testis-determining factor (reviewed in Pevny and Lovell-Badge, 1997; Wegner, 1999).

One member of the Sox family, Sox9, is known to function in the development of neural crest, epithelial placodes, cartilage and bone. Sox9 is sufficient to induce a number of neural crest-like behaviors in neural tube cells (Cheung and Briscoe, 2003), and tissue-specific inactivation of Sox9 function in neural crest leads to loss of cranial and trunk neural crest derivatives in *Xenopus* and mice (Spokony et al., 2002; Mori-Akiyama et al., 2003; Cheung et al., 2005). At later stages, Sox9 is required in mesenchymal condensations for the morphogenesis and differentiation of cartilage and bone (Bi et al., 1999; Akiyama et al., 2002; Yan et al., 2002). During chondrogenesis, Sox9 is coexpressed with type-II collagen a1 (Col2a1), which encodes the major cartilage matrix protein, and Sox9 has been shown to directly activate transcription of *col2a1* by binding to a chondrocyte-specific enhancer present in its first intron (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998). In contrast to mice, where defects are only observed in homozygous Sox9 mutants, in humans, heterozygous mutations in Sox9 are associated with campomelic dysplasia (CD), which is characterized by skeletal malformations and XY sex reversal (Houston et al., 1983; Wagner et al., 1994).

Sox9 activity is also crucial for inner ear development, as the otic placode fails to form in the absence of Sox9 activity, indicating that Sox9 acts at a very early step in the induction of this placode (Saint-Germain et al., 2004; Yan et al., 2005). Consistent with this proposal, Sox9 is expressed in the otic placode at very early stages, and Pax8 and Tbx2, which are also expressed in the early otic placode, fail to be activated in the absence of Sox9.

Due to the additional genome duplication at the base of the teleost radiation, zebrafish have two Sox9 coorthologs: Sox9a and Sox9b. Functional analysis indicates that these two Sox9 orthologs have partitioned the various functions of the

* Corresponding author. Fax: +49 6221 387 166.
E-mail address: carl.neumann@embl-heidelberg.de (C.J. Neumann).

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ancestral Sox9 gene, leaving each with a subset of these original functions (Yan et al., 2002, 2005). This subfunctionalization appears to be achieved at the level of transcription, as *Sox9a* and *Sox9b* are expressed in largely complementary domains that together approximate the expression of Sox9 in tetrapods.

The Mediator complex is a key coactivator acting as a bridge between DNA-binding transcription factors and RNA polymerase II (pol II), thus conveying regulatory information from enhancer elements to the basal transcription machinery (reviewed in Björklund and Gustafsson, 2005; Kim and Lis, 2005; Malik and Roeder, 2005). It consists of up to 30 subunits that are largely conserved from yeast to mammals. While some Mediator subunits appear to be universally required for the transcription of all genes, a number of subunits are dedicated to the regulation of specific genes. For example, in *Drosophila*, mutations in *Trap80* and *Med6* are cell-lethal, suggesting these subunits are essential for general functions of the Mediator complex (Boube et al., 2000; Gim et al., 2001). Mutations in *Drosophila Trap230/Med12* and *Trap240/Med13*, on the other hand, are cell-viable and show deregulation of specific genes during imaginal disc development (Janody et al., 2003). In *C. elegans*, Trap230/Med12 and Trap240/Med13 are specifically required for the regulation of genes controlling asymmetric cell division (Yoda et al., 2005).

To date, little genetic evidence is available on the vertebrate Mediator complex. Mouse *Med1* mutants are viable, but fibroblasts derived from *Med1* mutants fail to differentiate into adipocytes in response to PPARγ (Ito et al., 2000; Ge et al., 2002), and knock down of Med15 in *Xenopus* leads to defects in activin and nodal signaling through Smad transcription factors (Kato et al., 2002).

Here, we characterize a novel zebrafish mutant strongly resembling the *Sox9a*/*Sox9b* compound phenotype and show that this mutation disrupts zebrafish Trap230. Inactivation of Trap230 leads to dramatically reduced differentiation of bone and cartilage, reduced iridophores and the absence of otic placodes. All these phenotypes are observed in *Sox9a*/*Sox9b* double mutants. Furthermore, injection of *Sox9b* mRNA fails to activate target genes in the absence of Trap230, indicating that Trap230 is required for Sox9 activity. Interestingly, substitution of the Sox9b transactivation domain with a VP16 transactivation domain renders Sox9b independent of Trap230, indicating that Trap230 acts as a coactivator rather than corepressor in this context. Finally, we show that Trap230 is also required during pectoral fin development downstream of Tbx5 and upstream of Fgf24 and therefore also participates in Sox9-independent transcriptional regulation.

Materials and methods

Fish stocks

The *trapped* allele used was *tpd*^{t25870}. Embryos were cultured in E3 medium, with or without the addition of 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to inhibit pigmentation. Embryos were staged according to hours post-fertilization (hpf; Westerfield, 1995).

Linkage analysis and genetic mapping

For fine mapping of *tpd*, SSLPs were generated using the Sanger genome database. The closest SSLP marker to the *tpd* mutation, P1, uses the primer pair GCATCCACCCAAACATGAGG (forward) and GCAGTGCGATTGATGTTGGG (reverse), at a distance of <0.08 cM south of the mutation.

Microinjection of morpholino oligonucleotides and mRNA

Trap230 splice morpholino oligonucleotide (MO) was purchased from GeneTools LLC. The MO, designed to target the exon 26–intron 26 splice junction, has the sequence CAGATCCTCTAAAAATCATCACCTG. A MO stock solution was formed by dilution in water and was stored at −20°C prior to use. Embryos were injected at the single cell stage with 0.25 mM MO.

The N-terminal portion of Sox9b (comprising the HMG-box DNA-binding domain) was fused to the VP16 transactivation domain (amino acids 401–478 of the VP16 protein from human Herpes simplex virus type I). The 5′UTR and the N-terminal coding region of *Sox9b* was PCR amplified and cloned into the *Bam*HI site of a pCS2 + VP16 vector.

prdm1-mRNA, *Sox9b*-mRNA and *Sox9b*-VP16-mRNA were synthesized using the SP6 mMessage mMachine kit (Ambion) from a full-length clone obtained at RZPD and injected at single-cell-stage with a concentration of 90 ng/μl.

Histochemical methods

In situ hybridization and immunohistochemistry were performed as previously described (Hauptmann and Gerster, 1994, Macdonald et al., 1994). The *trap230* in situ probe was synthesized using the following primers: forward primer GGTGGGTGGGATGTTTGAC, reverse primer TTCACAGAACAACGCCAGTATG. All other in situ probes have been previously described. For immunohistochemistry, mouse anti-Hu was used (Marusich et al., 1994). Alcian blue staining of cartilage was performed as described in Grandel and Schulte-Merker (1998). Histological sections were obtained by staining cryosections with methylene blue (Humphrey and Pittman, 1974). TUNEL staining was performed using the in situ cell death TMR red kit (Roche).

Results

Isolation of a mutation in zebrafish Trap230/Med12

In a large-scale genetic screen to identify genes required for zebrafish embryonic development (Habeck et al., 2002), we isolated a novel mutant, *trapped* (*tpd*), with defects in craniofacial development, pigmentation, ear development and pectoral fin outgrowth (Figs. 2A, B, D, E; data not shown). In addition, *tpd* mutants have a heart edema and a curly-down body axis (Fig. 2B).

In order to identify the molecular nature of *tpd*, we used bulked segregant analysis to map it to linkage group 14 (Fig. 1A), between markers Z53264 (0.35 cM away) and Z11725 (0.65 cM away). By making use of the zebrafish genomic sequence (http://vega.sanger.ac.uk/Danio_rerio/) available for this interval, we then constructed new SSLP markers based on CA repeats. For one of these markers, termed P1, we failed to obtain any recombinants with *tpd*, indicating that it must lie very close to the *tpd* mutation (Fig. 1A). We considered several candidate genes in the region close to P1 and compared their sequence between wild type and *tpd* mutants. For one of these, the Trap230/Med12 gene (which we refer to as Trap230 hereafter), we found a difference in cDNA sequence between wild types and mutants (Fig. 1B). Since we found two different

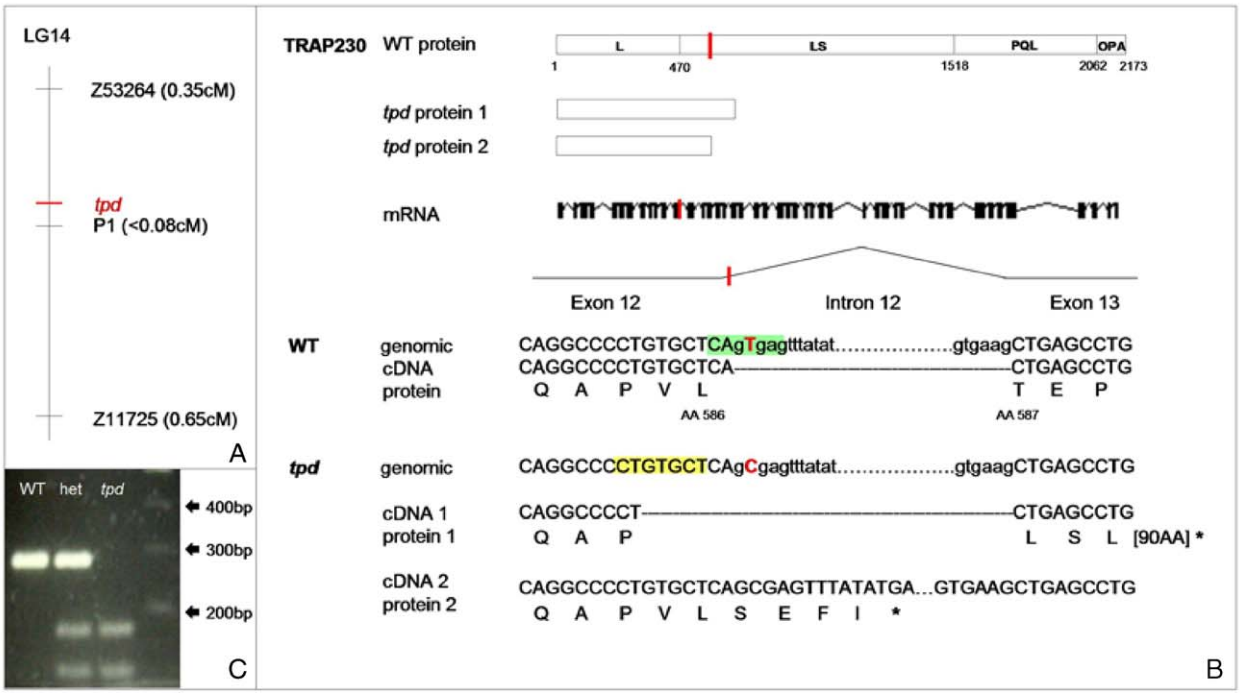

Fig. 1. Molecular characterization of the *tpd* locus. (A) *tpd* maps to linkage group 14 (LG 14), between markers Z53264 and Z11725, close to marker P1. (B) The *tpd* mutation (marked in red) causes an alteration of the splice donor site of intron 12. Two defectively spliced transcripts are generated as a result of this mutation. cDNA1 is spliced at a cryptic splice site (shaded in yellow) within exon 12. This leads to a frame shift and a stop codon after 93 amino acids. cDNA2 contains unspliced intron 12, leading to a stop codon after 4 amino acids. (C) Restriction enzyme Est1 cuts genomic *tpd* but not WT DNA due to the point mutation in its recognition site. het: DNA from a heterozygous embryo. L: leucine-rich domain; LS leucine-and-serine-rich domain; PQL: proline-, glutamine-, and leucine-rich domain; OPA: glutamine-rich domain.

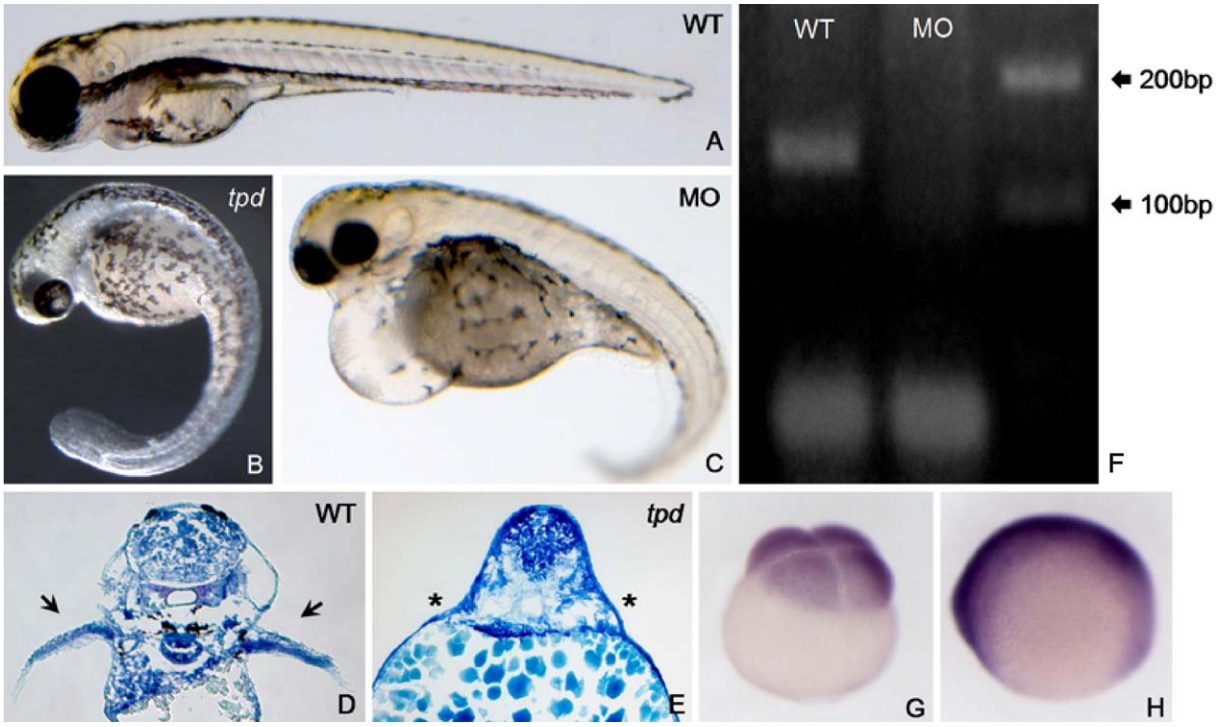

Fig. 2. The overall Trap230 loss-of-function phenotype. (A, D, G, H) WT, (B, E) *tpd*, (C) Trap230 morpholino-injected embryos. (A–E) Embryos at 3 dpf. (D, E) Methylene blue-stained transverse sections at the level of the pectoral fin buds (arrows and asterisks). (F) Trap230 MO-injected embryos lack the RT-PCR product amplified from exons 26 and 27, since intron 26 fails to be spliced out. (G, H) *trap230* mRNA expression. (G) Four-cell stage, animal pole to the top. (H) Three-somite stage, lateral view, dorsal to the top, anterior to the left. MO: Trap230 morpholino injected.

forms of cDNA in *tpd*^{t25870} that could both be explained by aberrant splicing at the exon 12/intron 12 junction, we also sequenced the genomic locus of *tpd*^{t25870}. We find the splice donor site of intron 12 altered from CAgtgag to CAgcgag (capitalized nucleotides belong to exon 12; Fig. 1B). This mutation changes the highly conserved GU found at the intronic 5′ splice site to GC and results in two types of aberrant splicing in *tpd*^{t25870} mutants: In type 1 cDNAs, a cryptic splice site (CTgtgct) located 7 bases 5′ of the correct splice site is used, resulting in a frame shift and a stop codon 90 amino acids downstream of this point (Fig. 1B). In type 2 cDNAs, intron 12 fails to be spliced at all, and its translation generates a stop codon 5 amino acids downstream of this point (Fig. 1B). In both cases, the *tpd*^{t25870} mutation results in a severely truncated Trap230 protein, in which more than half the C-terminus is missing, corresponding to a loss of most functional domains of the protein (Fig. 1B). Nevertheless, this *tpd* allele may be hypomorphic, as it shows some variability in its phenotype (see Figs. 3A–C below; data not shown). This could be due to a small percentage of correctly spliced transcripts. The *trap230* transcript is expressed ubiquitously throughout the embryo at all stages, and is also deposited maternally (Figs. 2G, H; data not shown).

To further investigate the possibility that *tpd* disrupts Trap230, we designed a morpholino oligonucleotide to block splicing of the *trap230* transcript at the exon 26–intron 26 splice junction (Fig. 2F). We find that injection of this morpholino into fertilized one-cell stage zebrafish embryos generates a phenocopy of *tpd* mutants, including absence of pectoral fins, identical ear, pigmentation and craniofacial defects, and a curly-down body axis (Fig. 2C). Although the *tpd* mutation and the morpholino oligonucleotide target different splice sites, both lead to the absence of the C-terminal PQL domain, which is crucial for Trap230 function, and hence, their phenotype is almost indistinguishable.

Taken together, these data show that the *tpd* phenotype is due to disruption of zebrafish Trap230 function.

The Trap230 phenotype resembles loss of Sox9 activity in many different tissues

Since several of the phenotypes we observed in *tpd* mutants affect cell types known to depend on Sox9 function, we systematically compared the characterized phenotypes of zebrafish *Sox9a*/*Sox9b* single and double mutants (Yan et al., 2005) to the phenotypes present in *tpd* embryos. In *Sox9a*/*Sox9b* double mutants, the ear fails to form, while it is only partially reduced in *Sox9a* or *Sox9b* single mutants. We find the ear is variably reduced in *tpd* mutants (Figs. 3A–C), similar to *Sox9b* mutants. In Trap230 morphants, however, the ear is either partially reduced or completely absent (Figs. 3D, E), similar to *Sox9a*/*Sox9b* double mutants, suggesting the morpholino knock down leads to a stronger reduction of Trap230 function than the *tpd* mutation, and further indicating that *tpd*^{t25870} is a hypomorphic mutation.

In *Sox9b* or *Sox9a*/*Sox9b* double mutants, there is an absence of iridophores, which are neural crest-derived pigment cells that can be detected as bright, shiny cells in dark field microscopy (Fig. 3F). As in *Sox9b* single mutants or *Sox9a*/*Sox9b* double mutants, we detect no iridophores in *tpd* mutants (Figs. 3F, G). Melanocytes, on the other hand, are present in *Sox9a*/*Sox9b* mutants but are larger than in wild types, due to dispersed melanosomes even in bright light (Yan et al., 2005). Melanocytes are similarly enlarged in *tpd* mutants and in Trap230 morphants (Figs. 3J–L).

Sox9b mutants fail to straighten the body axis, resulting in a curly-down tail, probably due to Sox9b function in the notochord (Yan et al., 2005). We observe a similar curly-down body axis in *tpd* mutants and in Trap230 morphants (Figs. 2B, C).

Taken together, these results show that Trap230 loss-of-function strongly resembles zebrafish *Sox9a*/*Sox9b* mutants with respect to ear, pigmentation, and body axis development.

Trap230 is required for Sox9-dependent neural crest fates and craniofacial cartilage formation

To further investigate the role of Trap230 in Sox9 signaling, we examined the expression of *col2a1*, which is expressed during the differentiation of craniofacial cartilage elements and is a direct target of Sox9 binding and activation (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998). In *Sox9a*/*Sox9b* double mutants, *col2a1* expression is strongly reduced, although not completely absent, in the pharyngeal arches, the eye capsule and the otic vesicle (Yan et al., 2005). We find a similar reduction of *col2a1* in these expression domains in *tpd* embryos (Figs. 4A, B).

While either *Sox9a* or *Sox9b* single mutants show partial absence of craniofacial cartilages, the *Sox9a*/*Sox9b* double mutant animals lack all traces of pharyngeal cartilages and the neurocranium (Yan et al., 2005). Likewise, *tpd* mutants have a complete absence of both the pharyngeal cartilages and the neurocranium, as well as total absence of the pectoral fin skeleton (Figs. 4C, D).

In *Sox9b* and *Sox9a*/*Sox9b* double mutants, *dlx2a* expression is slightly reduced, with the strongest reduction in the posterior branchial arches (Yan et al., 2005). In *tpd* mutants, there is a similar reduction of *dlx2a*, which is also more pronounced in the posterior arches (Figs. 4C, D).

Sox10 is expressed in a subpopulation of trunk neural crest cells migrating in the medial pathway. In *Sox9b* and *Sox9a*/*Sox9b* double mutants, *Sox10*-expressing neural crest cells fail to migrate as far ventrally as normally observed (Yan et al., 2005). We observed a similar phenotype in the trunk of *tpd* embryos (Figs. 4G, H), indicating that *trap230* and *Sox9b* are required for migration of the same population of *Sox10*-expressing neural crest cells.

An important derivative of trunk neural crest are the neurons and glia found in dorsal root ganglia (DRG), and in mouse *Sox9* mutants, these cell types are strongly reduced (Cheung et al., 2005). Similarly, we failed to detect Hu-positive DRG neurons in Trap230 morphants (Figs. 4I, J). Both in mouse Sox9 mutants and in zebrafish *Sox9b* mutants, neural crest cells are formed but then undergo apoptosis (Cheung et al., 2005; Yan et al., 2005).

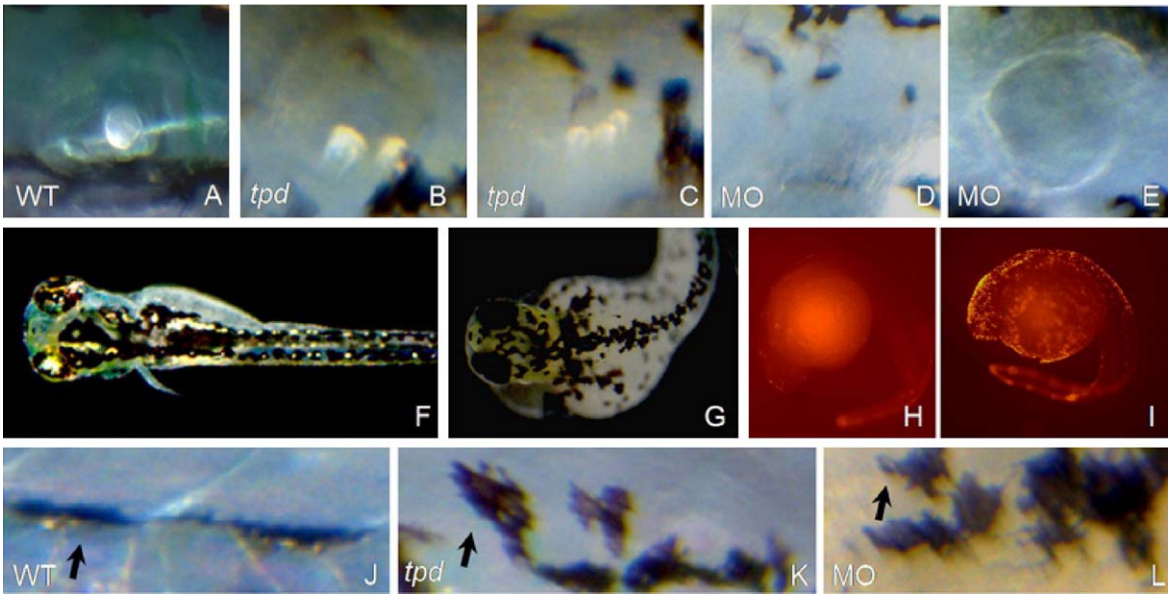

Fig. 3. Ear and pigmentation phenotypes in *tpd* mutants and Trap230 morphants. (A–G, J–L) Embryos at 4 dpf. (H, I) Embryos at 28 hpf. (A–E) Ear phenotypes; note partial reduction in *tpd* mutants (C), and complete absence in some Trap230 morphants (D). (F, G) Dark field microscopy, revealing iridophores (bright shiny cells), present in WT (F), but not in *tpd* mutants (G). (H, I) TUNEL staining, lateral view of embryos. (J–L) Trunk melanocytes (dark cells, arrows). (A, F, H, J) WT; (B, C, G, I, K) *tpd* mutants; (D, E, L) Trap230 MO-injected embryos.

We detect a similar increase of apoptotic cells by TUNEL labeling in *tpd* mutants (Figs. 3H, I).

Altogether, these results show that Trap230 activity is crucial for Sox9-dependent neural crest development and cartilage differentiation, and that many of the defects found in *tpd* embryos are identical to those found in *Sox9* mutants.

Trap230 is required as a coactivator for Sox9

The similarity of the *tpd* phenotype to that of *Sox9a*/*Sox9b* mutants could potentially reflect two different modes of regulatory interaction between Trap230 and Sox9. Since Trap230 is involved in transcriptional regulation, it might be required for expression of *Sox9a* and *Sox9b*. Alternatively, it might be required for the transcriptional regulation of Sox9 targets.

To distinguish between these possibilities, we first examined the expression of *Sox9a* and *Sox9b* mRNA in the absence of Trap230 activity. We find that *Sox9a* expression is largely normal in *tpd* mutants compared to wild type siblings, both at 10 hpf and at 24 hpf (Figs. 5A–D). The exceptions to this are the pectoral fin buds, in which *Sox9a* expression fails to be

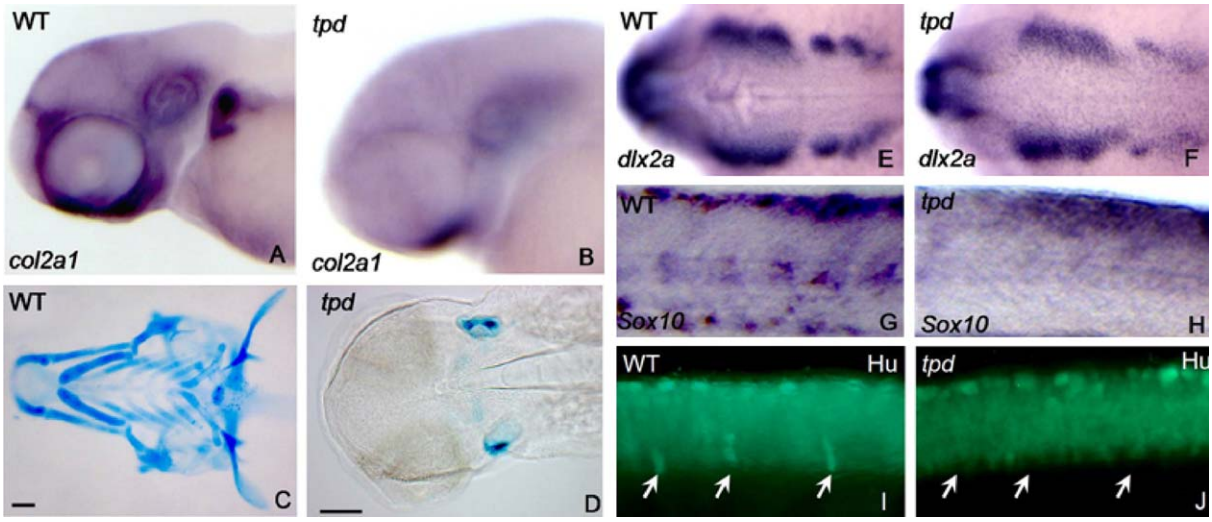

Fig. 4. Neural crest and craniofacial phenotypes of *tpd* mutants. (A, C, E, G) WT; (B, D, F, H) *tpd* mutants. (A, B) Expression of *col2a1* in the head at 68 hpf, lateral view. (C, D) Expression of *dlx2a* in the pharyngeal arches at 24 hpf, dorsal view. (E, F) Alcian blue-stained cartilages at 5 dpf, ventral view. (G, H) *Sox10* Expression in the trunk region of wild type (G) and *tpd* mutants (H). Note the absence of ventrally migrating *Sox10*-expressing cells in *tpd* mutants. (I, J) Anti-Hu immunostaining at 3 dpf, lateral view of the trunk. Arrows indicate the position of DRGs. Note the absence of DRGs in *tpd* mutants.

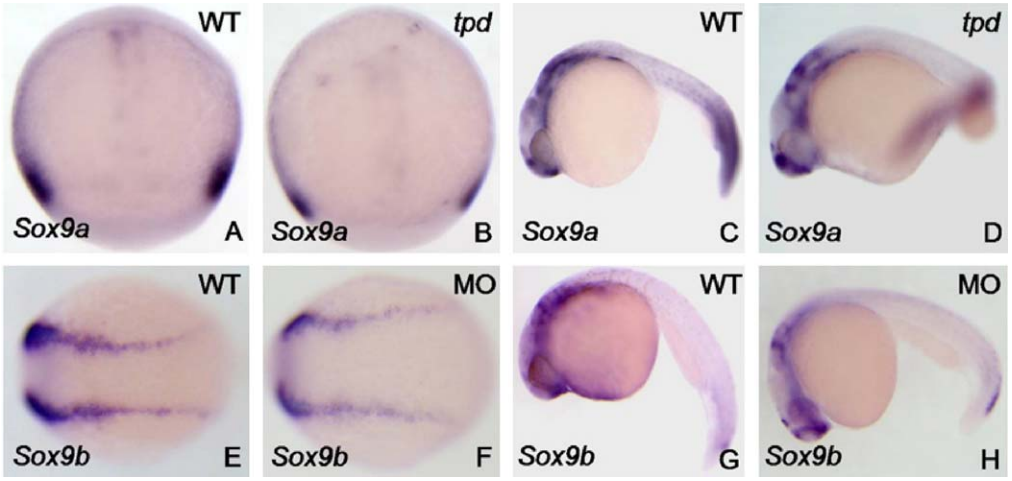

Fig. 5. *Sox9a* and *Sox9b* expression in *tpd* mutants. (A, C, E, G) WT; (B, D) *tpd* mutants; (F, H) Trap230 MO-injected embryos. (A, B) Embryos at 10 hpf, animal pole to the top. (E, F) Embryos at 3-somite stage, dorsal view, anterior to the left. (C, D, G, H) Embryos at 24 hpf, lateral view, anterior to the left. (A–D) Expression of *Sox9a*. (E–H) Expression of *Sox9b*. Note that *Sox9a* and *Sox9b* expression is only weakly reduced in the absence of Trap230 (B, F).

activated (Figs. 5C, D; data not shown), and the otic placodes, in which *Sox9a* expression is activated, but at reduced levels compared to wild type siblings (Figs. 7A, B). *Sox9b* expression is also largely normal in Trap230 morphants (Figs. 5E–H), although there is a weak reduction in the neural crest region at 3 somites (Figs. 5E, F). This reduction of *Sox9b* in neural crest of

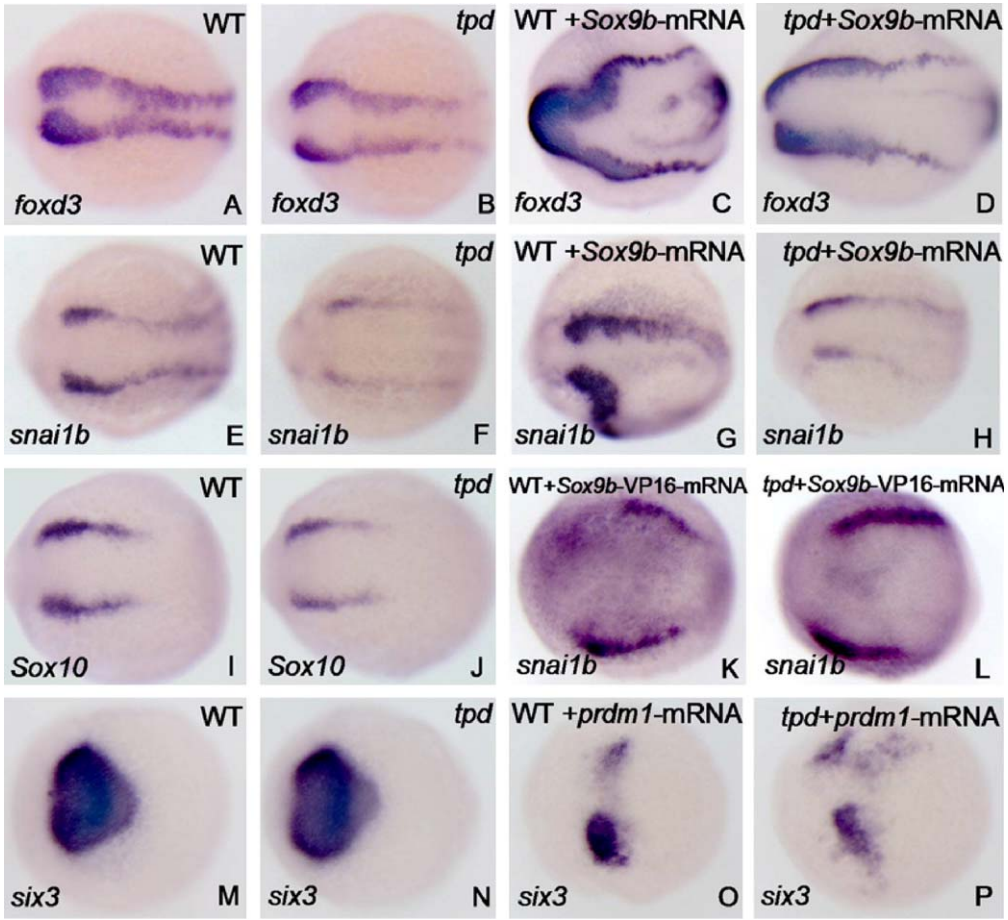

Fig. 6. Trap230 is required as a coactivator for Sox9. (A, C, E, G, I, K, M, O) WT embryos. (B, D, F, H, J, L, N, P) *tpd* mutants. Embryos are at the 3-somite stage, dorsal view, anterior to the left. (A–D) *foxd3* expression. (E–H, K, L) *snai1b* Expression. (I, J) *Sox10* expression. (M–P) *six3* expression. (C, G) WT embryo injected with *Sox9b* mRNA; (D, H) *tpd* mutant injected with *Sox9b* mRNA. (K) WT embryo injected with *Sox9b*-VP16 mRNA; (L) *tpd* mutant injected with *Sox9b*-VP16 mRNA. (O) WT embryo injected with *prdm1* mRNA. (P) *tpd* mutant injected with *prdm1* mRNA.

tpd mutants might be due to an autoregulatory feedback loop of Sox9 on its own expression (Yan et al., 2005).

While the weak reduction of *Sox9a* or *Sox9b* expression may contribute to the *tpd* phenotype, it is not sufficient to explain it entirely, since robust expression of these genes is present in tissues strongly affected by the *tpd* mutation. We therefore examined the ability of *Sox9b* mRNA to trigger downstream target gene activation in the absence of Trap230 activity. Since expression of *foxd3*, *snai1b* and *Sox10* is up-regulated in wild type embryos injected with *Sox9b* mRNA (Yan et al., 2005), we injected *Sox9b* mRNA into one-cell stage wild type and *tpd* embryos, to compare the effect of Sox9b in the presence or absence of Trap230 activity. While *Sox9b* mRNA injection leads to strong up-regulation of *foxd3* and *snai1b* expression in wild type embryos (Figs. 6C, G), we detect only very weak up-regulation in *tpd* embryos (Figs. 6D, H). We obtained similar results with *Sox10*, which also fails to respond to Sox9b in the absence of Trap230 (data not shown).

Our results raise the possibility that Trap230 acts as a coactivator required for expression of Sox9 target genes. To further test this hypothesis, we constructed a fusion protein in which the Sox9b transactivation domain was replaced by the VP16 activator domain. We injected mRNA encoding this construct into wild type and *tpd* embryos and found that *snai1b* expression was equally up-regulated in both cases (Figs. 6K, L), indicating that Sox9b-VP16 activates *snai1b* independently of Trap230. The observation that *Sox9b* mRNA overexpression is phenocopied by a VP16 activator fusion protein shows that Sox9b acts as a transcriptional activator in this context, and that Trap230 is therefore required as a coactivator for Sox9.

As a control, we tested the requirement of Trap230 for activity of Prdm1, an unrelated transcription factor. Overexpression of *prdm1* mRNA leads to downregulation of *six3* expression in the eye field (Wilm and Solnica-Krezel, 2005). Overexpression of *prdm1* mRNA in both wild type and *tpd* embryos was found to similarly reduce expression of *six3* in the eyefield (Figs. 6O, P), indicating that Trap230 is not required for Prdm1 activity.

Taken together, these results show that Trap230 acts as a coactivator for Sox9 and is required for transcription of Sox9 target genes.

The role of Trap230 in Sox9-dependent ear development

Sox9 has been shown to be one of the earliest genes expressed in the otic placode, and knock down of Sox9 activity in *Xenopus* leads to complete absence of the ear (Saint-Germain et al., 2004). *pax8* is also expressed very early in the otic placode, and *pax8* expression fails to be activated following Sox9 knock down. Similarly, we detect no *pax8* expression in the otic placode of Trap230 morphants (Figs. 7E, F), even though *Sox9a* and *Sox9b* expression is present in the otic placode of these morphants, although at partially reduced levels (Figs. 7A–D). In addition, there is also a strong reduction of *pax8* expression in the pronephric anlage of Trap230 morphants (Figs. 7E, F), indicating that Trap230 is also required for Sox9-independent effects during embryogenesis. In the majority of Trap230 morphants, there is a partial recovery of ear development later on, leading to a reduced number of cells expressing *pax2.1* compared to wild-type siblings at 24 hpf (Figs. 7G, H). This partial recovery of morphants is likely due to incomplete blockage of *trap230* splicing, since a small number of morphants do not have any signs of ear development (Fig. 3D).

These results show that Trap230 is required for Sox9-dependent activation of *pax8* in the ear and for Sox9-dependent ear development and further indicate that Trap230 is required for Sox9 activity.

A Sox9-independent role of Trap230 in forelimb development

Since *tpd* mutants show a complete absence of pectoral fins in the most strongly affected mutants (Figs. 2D, E), we were interested to determine how Trap230 fits into the cascade of genes involved in limb induction. It is well established that both *tbx5* mutants (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002) and *fgf24* mutants (Fischer et al., 2003) show a similar absence of pectoral fins. We therefore examined the expression of these two genes, as well as other genes known to be regulated by them, in *tpd* mutants.

We find that *tbx5* expression is activated in *tpd* mutants, but *tbx5*-expressing cells fail to congregate towards the fin bud and stay dispersed in the lateral plate mesoderm (Figs. 8B, F). *fgf24* expression, by contrast, fails to be activated at all in *tpd* mutants (Figs. 8C, G). Consistent with this result, Fgf24-dependent expression of *dlx2a* in the fin bud ectoderm and of *shh* in the fin bud mesenchyme (Fischer et al., 2003) are not detectable in *tpd* mutants (Figs. 8I, M, J, N). We also observed that activation of *erm1*, *pea3* and *msx-c* is strongly reduced in the pectoral fin buds of *tpd* mutants (Figs. 8D, H, K, O, L, P). Since these three genes are activated in the absence of Fgf24 activity (Fischer et al., 2003), these results indicate that Trap230 functions upstream of Fgf24 activation but downstream of Tbx5 during limb development. Consistent with this proposal, *wnt2b*, which functions upstream of Tbx5 (Ng et al., 2002), is expressed normally in *tpd* mutants (Figs. 8A, E).

While Sox9 activity is important for development of the fin cartilage elements, the initial induction, patterning and outgrowth of pectoral fin buds is normal in *Sox9a*/*Sox9b* double mutants (Yan et al., 2005), indicating that the *tpd* fin phenotype cannot be attributed to loss of Sox9 activity, and that Trap230 regulates a Sox9-independent mechanism in forelimb development.

Discussion

The trapped mutation shares many phenotypes with Sox9 mutants

Although the Mediator complex functions as a general coactivator for most pol II-driven transcription in eukaryotes, it seems that different parts of Mediator regulate distinct sets of genes by interacting with specific DNA-binding transcriptional activators. In this study, we have shown that zebrafish Trap230

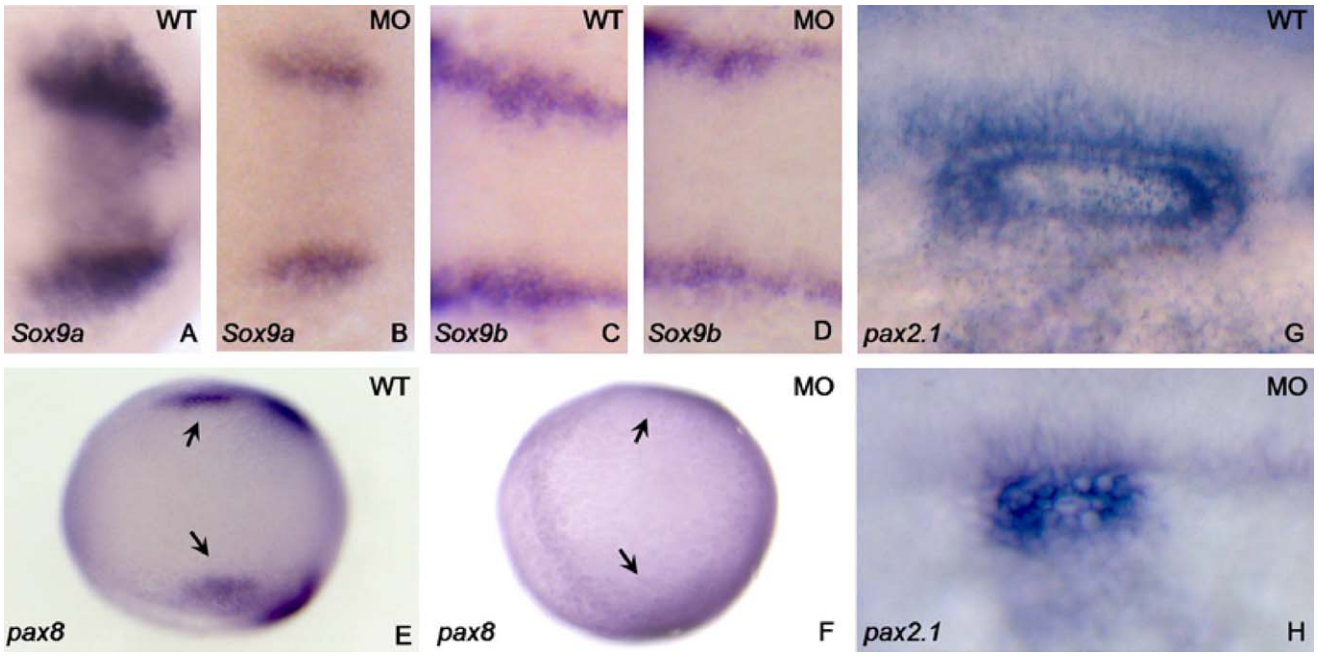

Fig. 7. Ear development in the absence of Trap230. (A, C, E, G) WT; (B, D, F, H) Trap230 MO-injected embryos. (A–D) Embryos at 3-somite stage, dorsal view, anterior to the left. (E, F) Tailbud stage embryos, dorsal view, anterior to the left. (G, H) Lateral views of the ear at 24 hpf. (A, B) *Sox9b* expression in the otic placodes; (C, D) *Sox9b* expression in the otic placodes. (E, F) *pax8* expression in the otic placodes (arrows). (G, H) *pax2.1* expression in the otic vesicle.

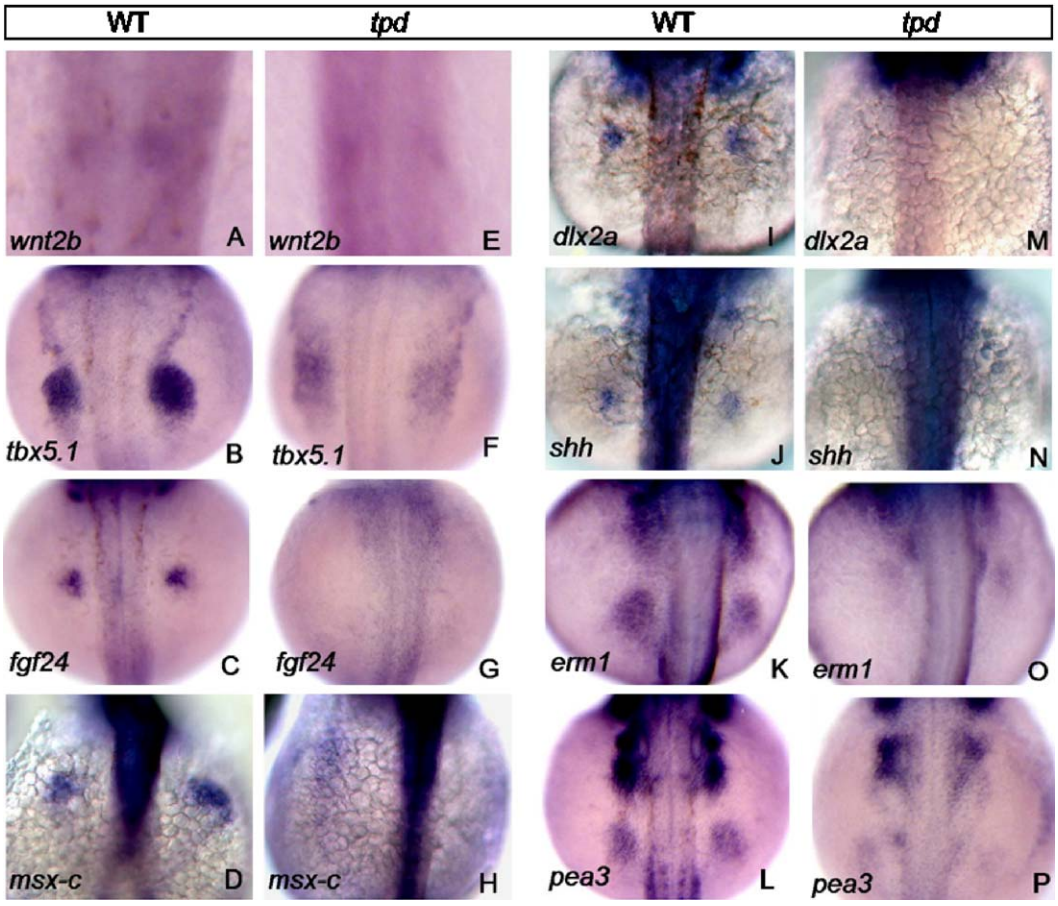

Fig. 8. The pectoral fin phenotype of *tpd* mutants. All embryos shown are 24 hpf, dorsal views, anterior to the top. (A–D, I–L) WT; (E–H, M–P) *tpd* mutant embryos. (A, E) *wnt2b* expression. (B, F) *tbx5.1* expression. (C, G) *fgf24* expression. (D, H) *msx-c* expression. (I, M) *dlx2a* expression. (J, N) *shh* expression. (K, O) *erm1* expression. (L, P) *pea3* expression.

is required for Sox9 activity. We have isolated *trapped*, a zebrafish Trap230 mutant, and our characterization of this mutant shows that it strongly resembles the *Sox9a*/*Sox9b* double mutant in many different tissues.

The phenotypes shared between *tpd* and *Sox9a*/*Sox9b* mutants include the complete absence of craniofacial cartilages, the absence or strong reduction of otic placodes, the absence of iridophores, the presence of expanded melanophores and the presence of a curly-down body axis and heart edema. The absence of craniofacial cartilages correlates with strongly reduced *col2a1* expression in this region. *col2a1* is a direct target of transcriptional activation by Sox9 (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998) and is strongly reduced in *Sox9a*/*Sox9b* mutants (Yan et al., 2005), therefore raising the possibility that Trap230 functions as a transcriptional coactivator participating in the regulation of Sox9 targets such as *col2a1*. In regulating chondrogenesis, Sox9 has an important interaction with beta-catenin (Akiyama et al., 2004), and it will be interesting to determine whether Trap230 plays a role in this interaction.

The absence of DRG neurons in *tpd* mutants has also been described in mouse Sox9 mutants (Cheung et al., 2005), and both zebrafish and mouse Sox9 mutants show elevated apoptosis in pre- and post-migratory neural crest cells, similar to *tpd* mutants.

Taken together, these results indicate that Trap230 is crucial for Sox9 activity and is required for both Sox9a and Sox9b in zebrafish, suggesting that the split of an ancestral Sox9 gene into two teleost copies did not affect their interaction with Trap230. Our results suggest that the interaction of Trap230 with Sox9 is relatively specific, since most *tpd* phenotypes can be explained by disrupted Sox9 activity. However, Trap230 clearly also functions in Sox9-independent events, such as limb induction and kidney development. It therefore remains to be seen how many different vertebrate transcription factors depend on Trap230. In the present study, we have focused on the role of zygotically expressed Trap230, and it could be that maternally expressed Trap230 is required for distinct transcription factors.

The role of Trap230 in Sox9 activity

Since transcription of *Sox9a* and *Sox9b* is activated normally in most tissues in the absence of Trap230 activity, this suggests that Sox9 activity is disrupted in these mutants. Consistent with this possibility, *trap230* transcript is expressed ubiquitously throughout the embryo, and Trap230 protein is therefore likely to be present in the same cells as Sox9 protein, and the two proteins could function together in the same gene regulatory events. In further support of this proposal, injection of *Sox9b* mRNA can trigger target gene activation in wild-type embryos but not in *tpd* mutants.

These results are in strong agreement with the finding that human SOX9 protein binds directly to TRAP230 in vitro, both in a yeast two-hybrid assay, and in a GST pull-down assay (Zhou et al., 2002). The PQL domain of TRAP230 is both necessary and sufficient for this interaction with the SOX9 transactivation domain. The PQL domain is located near the C-terminus, which is deleted in both *tpd* mutants and in Trap230 morphants, suggesting these truncated forms are unable to bind Sox9. Our in vivo data support this hypothesis, since substitution of the Sox9b transactivation domain by a VP16 transactivation domain abolishes the requirement of Sox9b for Trap230 activity. This result also shows that Trap230 acts as a coactivator in this context, which is in contrast to its role as a corepressor in other contexts. In addition, our results show that Trap230 is required for Sox9 activity not only during chondrogenesis, since other functions of Sox9 are also impaired in *tpd* mutants.

Trap230 as a transcriptional coregulator during vertebrate development

The Trap230/Med12 and Trap240/Med13 subunits are part of a Mediator subcomplex that also contains Cdk8 and CycC (reviewed in Björklund and Gustafsson, 2005; Malik and Roeder, 2005). This module is variably present in the Mediator complex, and its presence correlates with transcriptional repression in yeast (Holstege et al., 1998; Samuelsen et al., 2003; Spahr et al., 2003) and in mammalian cells (Naar et al., 2002; Wang et al., 2001). These observations have led to a model in which recruitment of Mediator containing this module represses transcription, whereas Mediator devoid of this module activates transcription. The role of this module may be more complex, however, since Cdk8 is linked to the positive regulatory effect of Mediator on pol II in *Drosophila* (Park et al., 2001), and activation of the Notch signaling pathway leads to the recruitment of Cdk8 and CycC to a Notch target promoter (Fryer et al., 2004). Mediator containing this module may thus be involved both in transcriptional activation and repression. In *C. elegans*, Trap230 appears to be required for transcriptional repression (Yoda et al., 2005), while in *Drosophila* it is not yet clear if Trap230 participates in transcriptional repression or activation (Janody et al., 2003).

Our results indicate that Trap230 is required for transcriptional activation by Sox9. Thus, *col2a1*, a direct transcriptional target of Sox9 activation (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998), shows strongly reduced expression in *tpd* mutants. Furthermore, injection of *Sox9b* mRNA can up-regulate *foxd3*, *snai1b* and *Sox10* expression in wild type embryos but not in *tpd* mutant embryos. In further support of the proposal that Trap230 functions as a coactivator for Sox9, replacement of the Sox9b transactivation domain by a constitutive VP16 activation domain leads to activation of *snai1b* by Sox9b independently of Trap230 activity. This result is also consistent with the observation that over-expression of a Trap230 fragment only containing the Sox9-binding domain acts as a dominant negative, blocking the ability of Sox9 to activate target gene expression in vitro, thus suggesting that binding of full-length Trap230 is crucial for transcriptional activation by Sox9 (Zhou et al., 2002).

The role of Trap230 in forelimb initiation

We have shown that *tpd* mutants fail to form pectoral fin buds and lack all pectoral fin structures. Since Sox9 signaling is not involved in the early steps of limb development, this is a clear example of Trap230 involvement in a Sox9-independent pathway. In addition, our data indicate that Trap230 is also required for Sox9-independent kidney development, since *pax8* expression in the pronephric anlage is strongly reduced in *tpd* mutants.

Our results show that Trap230 functions upstream of Fgf24 during limb development, as *fgf24* expression fails to be activated in *tpd* mutants. Also, several genes that are activated independently of Fgf24 in the limb bud mesenchyme, including *msx-c*, *erm1* and *pea3* (Fischer et al., 2003), are strongly reduced or absent in *tpd* embryos, thus further indicating that an event upstream of Fgf24 is blocked in the absence of Trap230 activity.

Since *tbx5* expression is activated in *tpd* mutants, our data suggest that Trap230 is required for an event between Tbx5 and Fgf24 activation. In the simplest scenario, Trap230 might bind directly to Tbx5 and function as a coregulator, similar to its interaction with Sox9. Alternatively, it could bind to a transcription factor acting downstream of Tbx5. Future experiments will help to distinguish between these possibilities. The failure of *tbx5*-expressing cells to migrate towards the limb bud is likely due to the absence of Fgf24, as the same effect is observed in *fgf24* mutants (Fischer et al., 2003).

In summary, we have shown that the zebrafish Trap230 gene, a member of the Mediator complex, functions as a coactivator necessary for activation of Sox9 target genes. In addition, it is also required for several Sox9-independent events during embryogenesis, including limb induction and kidney development. It remains to be seen in which other regulatory events during vertebrate development Trap230 participates.

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