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

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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

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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