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***odd-skipped related 2* is required for fin chondrogenesis in zebrafish**

Pui-ying Lam^{1,2}, Caramai N. Kamei¹, Steve Mangos^{1,3}, Sudha Mudumana¹, Yan Liu¹, and Iain A. Drummond^{1,*}

¹Massachusetts General Hospital, Department of Medicine, Nephrology Division, and Harvard Medical School Department of Genetics, Charlestown MA 02129

Abstract

Background—*odd-skipped related 2* (*osr2*) encodes a vertebrate ortholog of the *Drosophila* odd-skipped zinc-finger transcription factor. *Osr2* in mouse is required for proper palate, eyelid, and bone development. Zebrafish knock down experiments have also suggested a role for *osr2*, along with its paralog *osr1*, in early pectoral fin specification and pronephric development.

Results—We show here that *osr2* has a specific function later in development, independent of *osr1*, in the regulation of *sox9a* expression and promoting fin chondrogenesis. mRNA *in situ* hybridization demonstrated *osr2* expression in the developing floorplate and later during organogenesis in the pronephros and gut epithelium. In the pectoral fin buds, *osr2* was specifically expressed in fin mesenchyme. *osr2* knock down in zebrafish embryos disrupted both three and five zinc finger alternatively spliced *osr2* isoforms and eliminated wild-type *osr2* mRNA. *osr2* morphants exhibited normal pectoral fin bud specification but exhibited defective fin chondrogenesis, with loss of differentiated chondrocytes. Defects in chondrogenesis were paralleled by loss of *sox9a* as well as subsequent *col2a1* expression, linking *osr2* function to essential regulators of chondrogenesis.

Conclusions—The zebrafish *odd-skipped related 2* gene regulates *sox9a* and *col2a1* expression in chondrocyte development and is specifically required for zebrafish fin morphogenesis.

Keywords

odd-skipped related 2; chondrogenesis; pectoral fin; zebrafish

Introduction

The *odd-skipped related 2* (*osr2*) gene encodes a vertebrate ortholog of the *Drosophila* pair-rule gene odd skipped (Lan et al., 2001). In *Drosophila*, the *odd-skipped* family of zinc-finger transcription factors including *odd*, *sob*, *drm* and *bowl*, play an important role in the gut development, leg disk segmentation and initiation of retinal development (Wan et al.,

* Address for correspondence: Iain Drummond, Ph.D., Nephrology Division/MGH 149-8000, 149 13th Street, Charlestown, MA 02129, Tel: 617 726 5647, Fax: 617 726 5669, idrummon@receptor.mgh.harvard.edu.

²Current address: Program in Cellular and Molecular Biology, University of Wisconsin-Madison, Madison, WI 53706

³Current address: Rush University Medical Center, Cohn Research Building, 1735 W. Harrison Street, Ste. 316, Chicago, IL 60612

2000; Iwaki et al., 2001; Johansen et al., 2003; Bras-Pereira et al., 2006; Del Signore et al., 2012). There are two mammalian *odd-skipped related* genes, *Osr1* and *Osr2* (So and Danielian, 1999; Lan et al., 2001). *Osr1* knock-out mice exhibit heart and kidney defects (Wang et al., 2005; James et al., 2006) while kidney development is normal in *Osr2* mutants (Lan et al., 2004). A targeted null mutation in *Osr2* results in cleft palate, open eyelids at birth and supernumerary teeth rows (Lan et al., 2004). *Osr1* and *Osr2* have also been recently shown to be essential for synovial joint formation (Gao et al., 2011). Transgenic mice expressing a dominant-negative chimeric *Osr2* zinc finger transcription factor show decreased osteoblast activity and delayed mineralization in bone tissues (Kawai et al., 2007). In zebrafish, *osr1* is expressed early in germ ring mesendoderm and later in lateral mesoderm (Mudumana et al., 2008). *osr2* expression has been shown to be expressed later in zebrafish development in the proximal pronephros, gut and pectoral fin buds (Tena et al., 2007; Neto et al., 2012). Previous reports have suggested that *osr2* is necessary for the development of the zebrafish pronephros (Tena et al., 2007) and, along with its paralog *osr1*, plays a role in maintaining pectoral fin specification (Neto et al., 2012). However, a detailed characterization of *osr2* expression and its unique functions during zebrafish development have not been reported. We show here that *osr2* is expressed in multiple tissues during organogenesis and in the pectoral fins, is specifically expressed in mesenchyme adjacent to differentiating chondrocytes. *osr2* deficiency prevents expression of the essential chondrogenesis regulator *sox9a* as well as subsequent *col2a1* expression, highlighting a specific role for *osr2* in chondrocyte differentiation.

Results

***odd-skipped related2* expression during embryogenesis**

osr2 expression has been reported in the pronephros, gut and pectoral fin buds in developing zebrafish embryos (Tena et al., 2007; Neto et al., 2012). To carefully correlate expression with *osr2* function, we further characterized *osr2* expression domains at multiple developmental stages of zebrafish embryos and in histological sections. *osr2* expression was first detected at 11 hours post fertilization (hpf) in the posterior embryonic midline by whole-mount *in situ* hybridization (Figure 1A). Two-color *in situ* hybridization using *osr2* and *no tail a* (*ntla*) probes demonstrated that *osr2* was expressed in forming floorplate cells overlying the notochord marked by *ntla* expression (Figure 1B) (Schulte-Merker et al., 1994). Floorplate expression was transient and was not detected in the mature floor plate cells. As previously described (Tena et al., 2007), *osr2* was expressed specifically in the proximal pronephros (Figure 1D, E) the gut (Figure 1D, F), and in mesenchyme ventral and lateral to the pronephric duct (Figure 1D, F). *osr2* was first detected in the pectoral fins at 24 hpf and was strongly expressed at 48 hpf (Figure 1G). Cross-sections of the pectoral fins at 48 hpf showed that *osr2* was expressed in fin mesenchyme adjacent to forming cartilage (Figure 1H). We also observed a novel *osr2* expression domain in a specific cluster of anterior endoderm-associated cells at the level of the pectoral fin and on the right side of the embryonic midline (Figure 1G and I). Two-color *in situ* hybridization using *osr2* and *insulin* (Milewski et al., 1998) probes showed that *osr2* expressing cells lie just anterior to the pancreas (Figure 1J). *osr2* positive cells could be distinguished from pancreatic endodermal cells based on two-color *in situ* hybridization using *osr2* and *fgf24* or *osr2* and *agr2* probes

(Manfroid et al., 2007; Shih et al., 2007) (Supplemental figure 1). *osr2* expressing cells instead appeared to be endoderm-associated mesenchymal cells adjacent to the forming pneumatic duct marked by *wnt2* expression (Blader, 1996) (Figure 1K).

***osr2* alternative mRNA splicing and knock down of *osr2* transcripts**

We used an antisense translation blocking morpholino as well as an antisense splice donor blocking morpholino targeting *osr2* exon 3 to knock down *osr2* mRNA (Figure 2). In mammals, two different *Osr2* mRNA transcripts, *Osr2A* and *Osr2B*, are generated by alternative splicing of exon 4 (Kawai et al., 2005), generating proteins with either three or five zinc finger domains. Using RTPCR to examine zebrafish *osr2* transcripts we found similar alternate splicing of exon 4 (Figure 2A–D). *osr2* transcript variant 1 (*osr2_tv1*) utilizes an alternate exon 4 splice acceptor and encodes a five zinc finger protein (Figure 2A, B) while *osr2_tv2* utilizes a splice acceptor 82 nucleotides in the 3' direction and encodes a three finger protein (Figure 2A, C). Exon 4 sequence in *osr2_tv1* and *osr2_tv2* is frame-shifted, resulting in alternate protein sequence after exon 3 (Figure 2B,C). The amino acid sequence of the predicted DNA-binding domains of the additional zinc fingers four and five in *osr2_tv1* are 100% conserved between fish and human (Figure 2B,C), suggesting they may specify alternate DNA binding. The predicted DNA contacting amino acids of *osr2* zinc fingers four and five (Wolfe et al., 2000) are also nearly identical to fingers four and five of the five-finger *Drosophila* odd paralogs *bowl* and *sob* (Supplemental figure 2). Targeting the exon 3 splice donor site with antisense morpholino and sequencing RTPCR products demonstrated exon skipping, fusing exon 2 preferentially with the *osr2_tv2* exon 4 splice acceptor out of frame (Figure 2D, E). The resulting frame-shifted transcripts for both *osr2_tv1* and *osr2_tv2* splice forms are predicted to encode Osr2 peptides containing one functional zinc finger followed by part of zinc finger 2 and missense amino acids followed by a stop codon (Figure 2F). No wild-type message was detectable by RTPCR in knock down embryos (Figure 2D). Injection of similar amounts of randomized sequence morpholino had no effects on development. In addition, knock down of *osr2* did not induce phenotypes associated with *osr1*-deficiency (Supplemental figure 4; see below; (Mudumana et al., 2008)), indicating lack of off-target effects and specificity of morpholino knock down.

Chondrogenesis and pectoral fin defects in *osr2* morphants

Previous work has shown that OSR2 regulates osteoblast cell proliferation and bone formation in mice (Kawai et al., 2007). In zebrafish *osr2* morphants, we found that the outgrowth of the pectoral fin was severely affected in almost all morphant embryos compared to controls (59/59 normal length in control morphants, 49/49 *osr2ex3* MO reduced or absent, 58/59 *osr2ATG* MO reduced or absent, results from 2 separate experiments; Figure 3A–D). Cross-sections of wild-type pectoral fins at 72 hpf showed a dense central core of condensing chondrocytes (Figure 3C) that was absent in *osr2* morphant fins (Figure 3D). In extreme cases, *osr2* morphant pectoral fins lacked structure and exhibited what appeared in cross section to be fluid filled bulbous tips (Figure 3D). Expression of *collagen IIa1* (*col2a1*) marks the pectoral fin mesenchyme and apical ectodermal ridge in 48 hpf wild-type embryos (Figure 3E) (Lele and Krone, 1997). In *osr2* morphants, *col2a1* expression in the pectoral fin was either completely absent (13/15 embryos *osr2ex3* MO, 14/14 embryos *osr2ATG* MO; 93%) or markedly reduced (2/29 embryos; 7%) at 48 hpf

(Figure 3F). Since the transcription factor Sox9a is required for proper chondrogenesis in zebrafish fins (Chiang et al., 2001; Yan et al., 2002), we investigated its expression in *osr2* morphants. *sox9a* expression was greatly reduced in *osr2* morphants compared to controls at 24 (1/2 *osr2ex3* MO, 5/9 *osr2ATG* MO; 66%) and 48 hpf (10/10 embryos; 100%, Figure 3G–J). Despite these defects in fin morphogenesis and cell differentiation, fin bud tissue was properly specified as judged by the normal expression of *tbx5a* (Begemann and Ingham, 2000) both at 30 hpf (14/14 *osr2ex3* MO; 100%) and 72 hpf (24/24 *osr2ex3* MO; 100%) (Figure 3K–N). In addition, *fgf24* expression was unchanged in the lateral mesoderm at 24hpf (9/9 *osr2ex3* MO, 12/12 *osr2ATG* MO; 100%) and in the apical ectodermal ridge at 48hpf (10/10 *osr2ex3* MO, 8/8 *osr2ATG* MO; 100%), indicating that the signals that control fin patterning are unaffected by *osr2* deficiency (Figure 3O–R).

In order to address the question of whether *osr2* is acting cell-autonomously to promote *sox9a* expression, we examined the relationship between *osr2* and *sox9a* in the developing fin bud. At 32hpf both *osr2* (Figure 4A,B) and *sox9a* (Figure 4D,E) were expressed in the lateral plate region in and around the newly forming fin bud. *osr2* (Figure 4B) was expressed throughout the fin bud, while *sox9a* (Figure 4E) showed a more restricted expression. Transverse sections through the center of the fin bud revealed that *osr2* was expressed uniformly throughout the fin bud mesenchyme (Figure 4C), while *sox9a* expressing chondrocytes were restricted to the fin bud center (Figure 4F). The results show that *osr2* and *sox9a* are co-expressed in chondrocyte progenitors early in fin bud development before differentiation of cartilage. These results also suggest that *osr2* acts after fin bud specification and prior to or concomitant with the expression of genes regulating fin chondrogenesis (*sox9a*) and morphogenesis (*col2a1*).

Epithelial cell differentiation in *osr2* morphants

Since *osr2* was expressed in posterior gut epithelial cells from as early as 24 hpf (Figure 1D, F), we tested whether it might play a direct role in gut morphogenesis. In histological sections of the trunk of wild-type embryos, the gut tube is a well-formed polarized epithelium at 3 days post fertilization (dpf). However, *osr2* morphants were still able to form a gut tube with a properly polarized lumen indicated by apical localization of Zo-1 staining (Supplemental figure 3). Although a previous study reported edema in zebrafish *osr2* morphants, suggesting kidney defects, we did not observe altered expression of the kidney tubule segment markers *ae2*, *nephrin*, *nbc1*, *trmp7*, *ret1* or Na⁺/K⁺-ATPase in *osr2* morphants (Supplemental Figure 4 G–P). No loss of *osr2*-expressing proximal tubules was observed and the apparent increase in proximal tubule *pax2a* expression (Supplemental Figure 4 A–F) was not reproducible and may be due to differences in RNA probe penetration (Supplemental Figure 5).

Discussion

The *odd-skipped* family of zinc finger transcription factors are now known to have essential functions in a variety of developmental contexts both in *Drosophila* and vertebrate cell differentiation (Hao et al., 2003; Lan et al., 2004; Wang et al., 2005; James et al., 2006; Mudumana et al., 2008; Gao et al., 2011). In addition, recent studies in zebrafish support an

early role for *osr1* and *osr2* acting together to maintain fin bud specification and *tbx5a* expression (Neto et al., 2012). We show here that in addition to a fin patterning role, *osr2* has a specific and essential function independent of *osr1* in fin chondrogenesis upstream of *sox9a* expression. Our results expand our understanding of the genetic hierarchy that is essential for chondrogenesis.

***osr2* and chondrogenesis**

Developmental defects we observed in the pectoral fins of *osr2* morphants reveal a role for *osr2* in zebrafish pectoral fin differentiation and chondrogenesis. Previous studies have shown that fin specification is dependent on retinoic acid and *wnt2ba*-induced expression of *tbx5a* in the fin bud primordium (Mercader, 2007), with a subsequent role for *osr1* and *osr2* together in maintaining *tbx5a* expression (Neto et al., 2012). Consistent with this finding, we observe that *osr2* expression in the pectoral fins begins relatively late at 28 hpf and *tbx5a* expression is normal in *osr2* morphants, suggesting that *osr2* is not required for pectoral fin specification but rather plays a role later in fin morphogenesis and chondrogenesis. In the mouse, *Osr2* is required in palatal mesenchyme for development and elevation of the palatal shelves (Lan et al., 2004). In this context, *Osr2* appears to function independently of other patterning genes, including *Shh*, *BMP2*, and the *Msx1*-*BMP4* pathway, and is specifically required for early mesenchymal cell proliferation (Lan et al., 2004). Similarly, we find no change in expression of the patterning factor *tbx5a* and instead observe failed fin elongation and reduced chondrogenesis. Our results are also consistent with findings from transgenic mice expressing a dominant-negative *Osr2* construct that show reduced chondrogenic tissue, decreased osteoblast activity, and delayed mineralization in bone tissues (Kawai et al., 2007). Since *osr1* and *osr2* share the same zinc finger peptide sequence, this study did not discriminate between their functions in chondrogenesis (Lan et al., 2001). Our results demonstrate that chondrogenesis defects in zebrafish can be specifically ascribed to *osr2* deficiency.

Zebrafish *sox9a* is essential for normal chondrogenesis (Yan et al., 2002) and its ortholog *Sox9* is required in mammals during chondrocyte mesenchymal condensation to repress *wnt* signaling and promote the chondrocyte differentiation pathway (Akiyama et al., 2002; Akiyama et al., 2004). *Sox9* is subsequently down-regulated in proliferating chondrocytes where continued, ectopic *Sox9* expression results in chondrodysplasia (Akiyama et al., 2004). *Sox9* also directly induces expression of the *col2a1* gene in chondrocytes (Lefebvre et al., 1997). We find that *osr2* is required for *sox9a* and *col2a1* expression in fins, placing *osr2* function downstream of patterning genes (*tbx5a*, *fgf24*) and upstream of fin morphogenesis genes (*sox9a*, *col2a1*). Also, similar to *sox9a*, *osr2* is broadly expressed in fin mesenchyme but is down-regulated in mature, *col2a1*-expressing chondrocytes. The simplest interpretation of our results is that *osr2* is required in the mesenchyme for *sox9a* expression and mesenchymal condensation and then, like *sox9a*, must be down-regulated for progression of the chondrogenesis pathway (Akiyama et al., 2004). This interpretation would be consistent with recent observations on the role of *Osr* proteins in synovial joint formation where prechondrogenic mesenchymal condensations are segmented into distinct bone elements by zones of persistent *Osr1* and *Osr2* expression, blocking further chondrocyte differentiation (Gao et al., 2011). It remains possible that in the zebrafish

pectoral fins, *osr2* could have non-cell autonomous roles in chondrogenesis. Further experiments will be required to determine how *osr2* regulates *sox9a* expression and subsequent chondrogenesis.

***osr2* and epithelial cell differentiation**

A previous report suggested that *osr2* is necessary for the development of the zebrafish pronephros, based in part on the expression of *osr2* in the pronephric kidney and on the appearance of edema in *osr2* knock down embryos (Tena et al., 2007). While we did confirm that *osr2* was expressed in the proximal pronephric nephron, we did not observe edema in *osr2*-deficient embryos. Using a panel of pronephric kidney markers we found instead that pronephric development was essentially normal in *osr2* morphants. Our results are consistent with mouse *Osr2* knockout studies demonstrating that a targeted null mutation in *Osr2* does not affect kidney development (Lan et al., 2004).

Alternative splicing of *osr2* mRNA

We found that zebrafish *osr2* mRNA is alternatively spliced to generate transcripts predicted to encode proteins with either three or five zinc fingers, similar to alternative splicing of the mammalian *Osr2* mRNA (Kawai et al., 2005). Sequence comparisons have revealed the high degree of conservation in *osr2* fingers 1–3 between all vertebrates (Buckley et al., 2004). We found that the alternatively spliced fingers 4 and 5 are also highly conserved between fish *osr2_tv1* and human *Osr2A*. Interestingly, the alternatively spliced zinc fingers four and five are also highly conserved between vertebrate *Osr2* and fingers four and five of the *Drosophila* odd skipped family members *bowl* and *sob*. The function of the five finger odd skipped family isoforms remains unclear however. While in vitro studies have suggested that *Osr2* splice variants may have opposite transcriptional activity (Kawai et al., 2005), *in vivo*, targeted knock-in studies have shown that expression of the five finger *Osr2A* protein (as well as the three finger *Osr1* protein) can rescue developmental defects in *Osr2*^{−/−} mice, suggesting that the two *Osr2* isoforms are functionally equivalent. On the other hand, *Drosophila* hypomorphic *bowl* alleles with mutations in the fourth zinc finger have been isolated (Wang and Coulter, 1996), although the presence of additional mutations confounds interpretation of possible functional deficits. Further studies will be required to determine whether alternate DNA recognition by five finger odd skipped family proteins can specify a unique set of *Osr2* target genes.

Experimental Procedures

Plasmid Constructs

The full-length zebrafish *osr2* gene cloned into pME18sFL3 was obtained from open Biosystems (Catalogue #7406070). The full-length insert was isolated and recloned into pBluescriptII-SK+ to enable transcription of sense and antisense RNA. The additional plasmid constructs used in this work (*ntla* (Schulte-Merker et al., 1994), *insulin* (Milewski et al., 1998), *wnt2* (Blader, 1996), *collagen IIa1* (*col2a1*) (Lele and Krone, 1997), *tbx5a* (Ruvinsky et al., 2000) (gift from Chi-Bin Chien), *sox9a* (Chiang et al., 2001), *pax2a* (Krauss et al., 1991), *ae2* (Shmukler et al., 2005), *nephrin* (Kramer-Zucker et al., 2005), *nbc1* (Nichane et al., 2006), *trpm7* (Elizondo et al., 2005), *ret1* (Bisgrove et al., 1997), *agr2*

(Shih et al., 2007), *fgf24* (Manfroid et al., 2007) and *foxa2* (previously named as *axial*) (Strahle et al., 1993) have been previously described.

Zebrafish embryos

Ekk and TUAB wild-type zebrafish were maintained as described (Westerfield, 1995). Embryos were kept at 28.5°C in E3 with or without 0.003% PTU (1-phenyl-2-thiourea, Sigma) to suppress pigmentation. For histology or *in situ* hybridization, embryos were fixed in 4% paraformaldehyde (PFA) at the indicated developmental stages then processed as described. Homozygous *p53* mutant embryos (*tp53*^{M214K} (Berghmans et al., 2005)) were used in morpholino injection experiments to confirm the reported phenotypes were specific effects of the morpholino and not due to non-specific morpholino toxicity.

Morpholino design, microinjections and molecular analysis

Morpholino oligonucleotide was designed to target the splice donor site of exon3 of *osr2* gene, which resulted in skipping exon3 in morphant embryo *osr2* mRNA. A second morpholino designed to block translation was also used. Randomized sequence morpholinos were used as negative controls and showed no effect on development.

Morpholino oligonucleotides used in this study were

osr2ex3d: 5' GTGTGTTGATGGAGATTCACCTGCA 3'

osr2ATG: 5' AGCCGGGAGAGTCTTACTGCCCATT 3'

Negative control MO:

5'-CCTCTTACCTCAGTTACAATTTATA-3'

Morpholino oligonucleotides were diluted in 100 mM KCl, 10 mM HEPES, 0.1% phenol red (Sigma-Aldrich) and 4.6 nl was injected to each embryo using a nanoject2000 microinjector (World Precision Instruments). Initial fin morphology and expression of kidney markers were assessed using an injection concentration of 0.125 mM and subsequent *in situ* hybridization experiments for expression in the fin were performed at 0.0625mM which generated equivalent *osr2* knock down efficiencies. Zo-1 staining was performed at both doses with equivalent results. Efficiency of splicing was confirmed by nested RT-PCR. Total RNA from single *osr2ex3d* morpholino-injected embryos at 24 hours post fertilization (hpf) was extracted using TRI Reagent® (Molecular Research Center, Inc.) according to the manufacturer's instructions. Sequences of the primers flanking exon 3 are listed here, with the letters F and R denote forward and reverse, respectively:

Out-L 5'AGGAGTTCATCTGCCGGTTCTGC 3',

Out-R 5'CAGGAGTGTGTCCGTGATCGTGA 3',

In-F 5'CCTTCCGGAGACAAGACCACCTG 3',

In-R 5'GCGGTGTGTGTGTCAGACTGTGG 3'.

PCR products were gel purified (QIAquick Gel Extraction Kit) for sequencing.

***In situ* hybridization and immunohistochemistry**

Whole-mount single and double *in situ* hybridization was performed as described (Thisse et al., 2004). Probes were synthesized using DIG-UTP (Boehringer Mannheim, Germany) for *in situ* hybridization or a combination of DIG-UTP and FITC-UTP for two-color *in situ* applications. Antisense mRNA probes for *osr2* was made by linearizing and transcribing with EcoR1/T7. Additional probes used in this work have been described before (see section on plasmid constructs) and were synthesized as indicated: *ntla* (XhoI/T7), *insulin* (XbaI/T7), *wnt2* (NotI/T3), *col2a1* (HindIII/T3), *tbx5a* (NotI/T3), *pax2a* (BamHI/T7), *ae2* (NotI/Sp6), *nephrin* (NotI/Sp6), *nbc1* (NotI/Sp6), *trpm7* (EcoRV/T7), *ret1* (EcoRI/T7), *agr2* (BamHI/T7), *fgf24* (NotI/T7), *foxa2* (SacI/T3), *twhh* (HindIII/T7), and *sox9a* (EcoRV/T7). Stained embryos were fixed with 4% PFA, cleared with dimethylformamide, transferred into PBS:glycerol (1:1) and imaged on a Leica MZ12 microscope equipped with a Spot Image digital camera. Whole-mount immunohistochemistry for Na⁺/K⁺-ATPase (α6F antibody Developmental Studies Hybridoma Bank) and ZO-1 (Mouse monoclonal anti-rat ZO-1; a kind gift from S. Tsukita (Itoh et al., 1991)) was performed on embryos fixed in Dent's fixative (80% methanol, 20% dimethyl sulfoxide) as previously described (Drummond et al., 1998). Images were processed using Adobe Photoshop 7.0 software.

Histology

For embryos analyzed by *in situ* hybridization and for general histological examination, processing involved fixation with 4% PFA in phosphate-buffered saline at 4°C overnight followed by dehydration in an ethanol:PBS series. Dehydrated embryos were embedded in JB-4 plastic resin (Polysciences) and then sectioned to a thickness of 5 μm using a LEICA RM 2165 rotary microtome. Histological sections were stained in methylene blue/Azure II and coverslips were mounted using Permount (Fisher Scientific). Following double *in situ* hybridization, embryos were infiltrated with 20% sucrose at 4°C overnight followed by embedding in 1.5% agarose in 20% sucrose and cryosectioned to a thickness of 10 μm LEICA CM 1900 Cryostat. Sections were imaged on a Nikon E800 microscope equipped with a Spot Insight CCD digital camera. Image handling was performed using Adobe Photoshop 7.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Bullet points

- zebrafish *odd-skipped related 2* is required specifically for pectoral fin chondrogenesis.
- zebrafish *odd-skipped related 2* is alternatively spliced, generating three and five zinc finger isoforms.
- *odd-skipped related 2* is required for fin *sox9a* expression and subsequent expression of *col2a1*.
- *odd-skipped related 2* is not required for fin specification or for kidney or gut morphogenesis.

Figure 1. *osr2* expression during zebrafish development

(A) At 11 hpf, *osr2* is expressed in the posterior embryonic midline (tilted lateral view, anterior to the left). (B) Cross-section of an 11 hpf embryo at the level of the dotted line indicated in A showing *osr2* (blue) expression in floorplate (fp) cells overlying the notochord marked by red *ntl* expression (black arrowheads). (C) Expression of *osr2* is biased to cells on the right side of the forming floorplate. Black arrowheads mark the boundaries of the notochord. L, left; R, right. (D) At 24 hpf, *osr2* is expressed in the pronephric proximal tubule and gut. (E and F) Cross-section of 24 hpf embryo indicated in Figure 1D showing *osr2* expression in the proximal pronephros (E) and gut (F; red arrow). Distal tubules of the pronephros do not express *osr2* (dotted circles). *osr2* expression is also observed in mesenchyme cells (black arrowheads) ventral and lateral to the pronephros. (G) At 48 hpf, *osr2* is expressed in fin (red arrow), pronephric proximal tubule (white arrowhead), posterior gut (black arrowhead) and in gut mesenchyme (red arrowhead) at the level for the pectoral fin. (H and I) Cross-section of the embryo in F at the level indicated showing expression of *osr2* in pectoral fin mesenchyme (H) pronephric tubules, and gut mesenchyme (I; red arrowhead). (J) Double *in situ* hybridization showing gut mesenchyme (red arrowhead) expressing *osr2* (blue) anterior to the exocrine pancreas as indicated by *insulin* (red) expression at 48 hpf. (K) Gut mesenchyme (red arrowhead) expressing *osr2* (blue) was adjacent to the pneumatic duct as indicated by *wnt2* (red) expression at 55 hpf.

Figure 2. Alternative splicing of *osr2* mRNA and morpholino knock down

(A) *osr2* gene structure depicting use of alternative splice acceptors in exon 4. Morpholino oligo (red line) targeting exon 3 is indicated by a red bar. RTPCR primers used for analysis are depicted as black arrows in exons 2 and 4. (B) Protein sequence alignment of *osr2* transcript variant 1 (Osr2_tv1) vs. human Osr2a (five finger isoform). Zinc finger sequence is shaded yellow. (C) Protein sequence alignment of *osr2* transcript variant 2 (Osr2_tv2) vs. human Osr2b (three finger isoform). Zinc finger sequence is shaded yellow. (D) Gel analysis of *osr2* RTPCR products from control embryo RNA (WT) and *osr2* ex3d morpholino (ex3d MO) injected embryo RNA. No wildtype mRNA was detected in morphants; misplicing caused exon skipping and fusion of exons 4 and 2 primarily at the *osr2_tv2* exon 4 splice acceptor. (E) Diagram of predicted protein products of wildtype and morphant mRNAs. Both mis-spliced mRNA products are predicted to lack the final histidine in the zinc coordination pocket of zinc finger 2 (F; asterisk) and produce proteins with only one functional zinc finger.

Figure 3. *osr2* knock down results in abnormal pectoral fin

Brightfield images of control fins at 48 hpf show the pectoral fin extending toward the posterior (A) while *osr2* morphants display only a fin stump (B). Embryos are oriented with anterior to the left and dorsal to the bottom in (A, B). (C) Methylene blue stained transverse section of normal pectoral fin at 72 hpf. (D) *osr2* morphants showed abnormal pectoral fin morphology and lacked differentiated chondrocytes. *col2a1* *in situ* hybridization analysis at 48 hpf of control embryos (E) and *osr2* morphants (F). *osr2* morphants showed reduction or disappearance of *col2a1* expression in the pectoral fin. (G–J) *sox9a* expression was reduced in *osr2* morphants at both 24 hpf (H) and 48 hpf (J) compared to controls (G, I). (K–N) *tbx5a* expression in lateral mesoderm at 30 hpf (K, L) and in the pectoral fin buds at 72 hpf (M, N) was not affected in *osr2* morphants compared to controls. (O–R) *fgf24* expression is unaffected by *osr2* knock down and is unchanged in the lateral mesoderm at 24 hpf (O, P) and is restricted to the apical ectodermal ridge in both control and *osr2* morphants at 48 hpf (Q, R). Embryos in (E, F, G–R) are displayed from a dorsal view and anterior to the top.

Figure 4. *osr2* and *sox9a* are coexpressed in the developing fin bud

Wholemount *in situ* hybridization at 32hpf shows that both *osr2* (A) and *sox9a* (D) are expressed in the lateral plate in the region of the newly forming fin bud. *osr2* (B) appears to be expressed throughout the fin bud, while *sox9a* (E) shows a more restricted expression. Transverse sections through the center of the fin bud show that *osr2* is expressed uniformly throughout the fin bud mesenchyme (C), while *sox9a* expressing chondrocytes are restricted to the center (F). Embryos in (A, D) are displayed from a dorsal view with anterior to the top. In (B, E) arrowhead indicates fin bud with proximal to the left.