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UDP xylose synthase 1 is required for morphogenesis and histogenesis of the craniofacial skeleton

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ABSTRACT

UDP-xylose synthase (Uxs1) is strongly conserved from bacteria to humans, but because no mutation has been studied in any animal, we do not understand its roles in development. Furthermore, no crystal structure has been published. Uxs1 synthesizes UDP-xylose, which initiates glycosaminoglycan attachment to a protein core during proteoglycan formation. Crystal structure and biochemical analyses revealed that an R233H substitution mutation in zebrafish *uxs1* alters an arginine buried in the dimer interface, thereby destabilizing and, as enzyme assays show, inactivating the enzyme. Homozygous *uxs1* mutants lack Alcian blue-positive, proteoglycan-rich extracellular matrix in cartilages of the neurocranium, pharyngeal arches, and pectoral girdle. Transcripts for *uxs1* localize to skeletal domains at hatching. GFP-labeled neural crest cells revealed defective organization and morphogenesis of chondrocytes, perichondrium, and bone in *uxs1* mutants. Proteoglycans were dramatically reduced and defectively localized in *uxs1* mutants. Although *col2a1a* transcripts over-accumulated in *uxs1* mutants, diminished quantities of Col2a1 protein suggested a role for proteoglycans in collagen secretion or localization. Expression of *col10a1, indian hedgehog*, and *patched* was disrupted in mutants, reflecting improper chondrocyte/perichondrium signaling. Up-regulation of *sox9a, sox9b,* and *runx2b* in mutants suggested a molecular mechanism consistent with a role for proteoglycans in regulating skeletal cell fate. Together, our data reveal time-dependent changes to gene expression in *uxs1* mutants that support a signaling role for proteoglycans during at least two distinct phases of skeletal development. These investigations are the first to examine the effect of mutation on the structure and function of Uxs1 protein in any vertebrate embryos, and reveal that Uxs1 activity is essential for the production and organization of skeletal extracellular matrix, with consequent effects on cartilage, perichondral, and bone morphogenesis.

Introduction

The vertebrate skeleton provides structural support for muscle attachments and a protective casing for vulnerable internal organs. These functions rely on the coordinated secretion of dense extracellular matrix (ECM) by skeletal precursor cells during embryonic development. Proteinaceous components of skeletal ECM include collagens, elastin, and proteoglycans. Collagens anchor and reinforce the ECM; elastin provides flexibility (Velleman, 2000); and proteoglycans impact cell division, cell adhesion, and migration (Holt and Dickson, 2005; Kirn-Safran et al., 2004; Lander and Selleck, 2000; Knudson and Knudson, 2001). Proteoglycans contain repeating disaccharides (glycosaminoglycans, or GAGs) linked to a protein core (Prydz and Dalen, 2000) and include four major classes: dermatan, keratan, chondroitin, and heparan sulfate. In addition to imparting hydrostatic properties to skeletal tissues through GAG sulfation and hydration, proteoglycans can play a role in cell signaling. For example, heparan sulfate proteoglycans (HSPGs) help cell receptors bind growth factors (Izvolsky et al., 2003; Lin et al., 1999) and although the mechanism remains unclear, *Cspg1* (*Aggrecan*)-deficient mutant chickens have dwarfed bones (Velleman and Clark, 1992).

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Not all bones are created equal. Dermal bones differentiate osteoblasts directly via intramembranous ossification, but chondral bones form by endochondral ossification, during which developing chondrocytes and an overlying osteogenic epithelium, the perichondrium, interact (Eames et al., 2003). Understanding how these cell types signal each other and how proteoglycans play structural roles is important because impaired signaling between chondrocytes and osteoprogenitors can lead to osteoarthritis, a disease in which bone spurs replace cartilage in many people over age 65 (Ala-Kokko et al., 1990; Kizawa et al., 2005; Knowlton et al., 1990; Rothschild and Panza, 2007).

Proteoglycan biosynthesis initiates with the addition of a common tetrasaccharide linker to a core protein. Vertebrates use UDP-xylose, the first sugar in the linker, almost exclusively for proteoglycan synthesis (xylose is also added to EGF-repeat domains of some proteins (Bakker et al., 2009; Ishimizu et al., 2007). UDP-xylose biosynthesis begins with the conversion of UDP-glucose into UDP-glucuronic acid by UDP-glucose dehydrogenase (Ugdh). Zebrafish with diminished Ugdh activity have defective craniofacial and coronary development (Neuhauss et al., 1996; Walsh and Stainier, 2001). Next, UDP-xylose synthase (Uxs1, also called UDP-glucuronic acid decarboxylase or UGD, EC 4.1.1.35) converts UDP-glucuronic acid into UDP-xylose (Kearns et al., 1993; Vertel et al., 1993). The GAG tetrasaccharide linker of proteoglycans is initiated by a xylosyltransferase, which adds UDP-xylose to a serine residue of the core protein. Galactose and glucuronic acid transferases then add two galactoses and one glucuronic acid, completing the tetrasaccharide linker. GAG synthesis continues as exostosins (Ext1a, Ext1b, Ext1c, Ext2, Extl2, and Extl3 in zebrafish) add disaccharide constituents (Kjellen and Lindahl, 1991; Knudson and Knudson, 2001; Lin, 2004). Zebrafish mutations in *ext2* (*dackel* (*dak*)), *extl3* (*boxer* (*box*)), and *solute carrier family 35, member b2* (*pinscher* (*pic*); previously termed *3'-phosphoadenosine 5'-phosphosulfate transporter 1*, or *papst1*) show that GAG synthesis and sulfation are important for axon sorting and cartilage morphogenesis (Clement et al., 2008; Lee et al., 2004; Schilling et al., 1996). The developmental roles of Uxs1, however, are poorly understood because vertebrate models that lack Uxs1 activity have not yet been investigated.

In a mutation screen for neural crest defects, we identified *man o'war* (*mow*), which, like the *sox9a* mutation *jellyfish* (Yan et al., 2002), fails to form craniofacial cartilages. Our molecular genetic analyses showed that the *mow*^{w60} mutation causes an amino acid replacement in the zebrafish *uxs1* gene and is allelic to the viral insert *hi3357* (Amsterdam et al., 2004; Golling et al., 2002; Nissen et al., 2006). Sequence alignments reveal Uxs1 to be one of the most highly conserved non-mitochondrial proteins, preserving 57% amino acid identity between the bacterium *Rhodospirillum rubrum* and human. Using the crystal structure of human UXS1, we modeled the *mow*^{w60} substitution and found it to disrupt interactions at the enzyme's dimer interface, which should reduce or eliminate enzymatic activity. Indeed, our biochemical analyses revealed that an amino acid replacement homologous to the *mow*^{w60} allele destroys human UXS1 activity. Transcripts of *uxs1* are deposited maternally, and then *uxs1* is expressed zygotically in regions of the developing craniofacial skeleton. Histochemical and immunohistochemical investigations showed that wild-type *uxs1* is essential for the production and organization of many components of the ECM, including both proteoglycans and collagens. Additionally, confocal microscopy of GFP-labeled cranial neural crest cells revealed a critical role for *uxs1* in directing the morphology of chondrocytes, perichondrium, and bone during craniofacial development. Our molecular analyses demonstrated that chondrocyte maturation and Hedgehog signaling is dependent upon *uxs1*. Finally, our observation that the early chondrogenic markers *sox9a, sox9b,* and *runx2b* were up-regulated in *uxs1* mutants provide a mechanistic explanation for many of the defects in endochondral ossification, and furthermore suggest a novel feedback role for proteoglycans as skeletal progenitor cells undergo differentiation. Specifically, our data show proteoglycans to modify signaling pathways in early chondrogenic condensations and in later interactions between chondrocytes and perichondrium.

Materials and methods

Mapping and cloning of mow^{w60}

Adult male AB zebrafish (*Danio rerio*) were treated with ethylnitrosourea to induce point mutations and out-crossed to wild-type females. F2 families were produced and F3 larvae were screened for mutant phenotypes. The *mow*^{w60} allele showed reduced pharyngeal cartilages. For mapping, heterozygous *mow*^{w60} fish on an AB background were mated to WIK wild-type fish. F2 individuals were genotyped for 311 well-distributed simple sequence repeats (Knapik et al., 1998; Shimoda et al., 1999), identifying linked zmarker z3124. The zebrafish genome sequence nearby was screened for candidate genes involved in skeletal differentiation and mapping primers were designed in candidate genes. For *uxs1,* primers were designed to amplify a simple sequence repeat (SSR) in intron-7 (scaff346.117 + GCAGCGTGAAAAAGCAAAGAC and scaff346.524-ACCGCCGC-CTGTGACGA). cDNA for *uxs1* was amplified and isolated for sequencing using overlapping fragments amplified by primer sets designed from NM_173242 (Uxs1.114 + TGACCGTTGGACAAGGGAGGATTTA, Uxs1.421-CTATTTGAAGAGCGGCTGCACGACTAT; Uxs1.309 + AGCCGAAAA-TAAACTGCCCAGACTACTT, Uxs1.594-CATCCGCATCATCCTCCAGCACAC; Uxs1.394 + CATAGTCGTGCAGCCGCTCTTCAAAT, Uxs1.757-GTCCCACTGCCTCATCTATCCTCTGCTC; Uxs1.854 + TCACCGGTGGGGCAG-GATTC, Uxs1.1397-ACCACTCGCCCGTCGTTCAT; Uxs1.945 + CGGCCGCAAGCGCAATGTAGA, Uxs1.1340-ACTCGCACCTCCA-CTCCTTCCTGTTTC; Uxs1.1251 + TGGTCCCCGGGCCTGTTATGATG, Uxs1.1685-AGTTTGGCCCTGCGGATGTCG). To genotype *mow*^{w60} fish, we used primers MOW.928 + CACCCCCAAAATGAGGACTACTG and MOW.1277-AGAGCTCGCAACGGCATAAGAT, which amplify a 349 bp fragment that yields 274 and 75 bp fragments from the *mow*^{w60} mutant amplicon but leaves the wild-type amplicon intact after digestion by *NspI*. To detect the *hi3357* pro-viral insertion, we used primers that flank the insertion site (Uxs1.e1.398 + GTCGTGCAGCCGCTCTTCAAAT and Uxs1.e1.597-GCTCATCCGCATCATCCTCCAG) and yield a 199 bp fragment from wild type but no fragment from homozygous *hi3357* mutants; a *wnt5a* amplicon verified DNA quality (Golling et al., 2002). All work with animals was approved by the appropriate Institutional Animal Care and Use Committee.

Sequence alignments

Uxs1 sequences: human, *Homo sapiens* NP_079352 (179/311, 57% identity to the bacterial protein); mouse, *Mus musculus* NP_080706 (179/311, 57%); chicken *Gallus gallus* XP_416926 (180/311, 57%); frog *Xenopus tropicalis* NP_001006849 (178/311, 57%); pufferfish *Tetraodon nigroviridis* CAG05807 (145/252, 57%); zebrafish *Danio rerio* NP_775349 (178/311, 57%); beetle *Tribolium castaneum* XP_969232 (180/305, 59%); fruitfly *Drosophila melanogaster* NP_648182 (186/311, 59%); fungus *Cryptococcus neoformans* XP_572003 (176/316, 55%); rice *Oryza sativa* EAY89464 (192/304, 63%); mustard *Arabidopsis thaliana* NP_180443 (192/305, 62%); bacterium *R. rubrum* YP_428334 (311/311, 100%). Tgds sequences: human *H. sapiens* NP_055120; mouse *M. musculus* EDL00567; chicken *G. gallus* XP_416988; frog *Xenopus laevis* NP_001088301; zebrafish *Danio rerio* NP_956111; mustard *A. thaliana* NP_564633; rice *O. sativa* NP_001049724; bacterium *R. rubrum* YP_425086. Clustal-X alignment is available on request; sequences were trimmed to include only unambiguously aligned sequences.