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Nodal signals mediate interactions between the extra-embryonic and embryonic tissues in zebrafish

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Abstract

In many vertebrates, extra-embryonic tissues are important signaling centers that induce and pattern the germ layers. In teleosts, the mechanism by which the extra-embryonic yolk syncytial layer (YSL) patterns the embryo is not understood. Although the Nodal-related protein Squint is expressed in the YSL, its role in this tissue is not known. We generated a series of stable transgenic lines with GFP under the control of *squint* genomic sequences. In all species, *nodal-related* genes induce their own expression through a positive feedback loop. We show that two tissue specific enhancers in the zebrafish *squint* gene mediate the response to Nodal signals. Expression in the blastomeres depends upon a conserved Nodal response element (NRE) in the *squint* first intron, while expression in the extra-embryonic enveloping layer (EVL) is mediated by an element upstream of the transcription start site. Targeted depletion experiments demonstrate that the zebrafish Nodal-related proteins Squint and Cyclops are required in the YSL for endoderm and head mesoderm formation. Thus, Nodal signals mediate interactions between embryonic and extra-embryonic tissues in zebrafish that maintain *nodal-related* gene expression in the margin. Our results demonstrate a high degree of functional conservation between the extra-embryonic tissues of mouse and zebrafish.
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Introduction

In all multicellular organisms, cells differentiate according to their relative position in the embryo generating a highly reproducible pattern of cell fates. The body plan is established at early stages by specialized groups of cells called signaling centers. In many vertebrates, extra-embryonic tissues are the first signaling centers established and act to induce the germ layers and form the major body axes (Beddington and Robertson, 1999; Schier and Talbot, 2005). In the mouse, for example, the first cell fate decision occurs during the cleavage stages and divides the embryo into embryonic and extra-embryonic lineages (Rossant and Tam, 2004). At later stages, signals from the extra-embryonic ectoderm and the extra-embryonic visceral endoderm are required to form the proximo-distal and anterio-posterior axes (Beddington and Robertson, 1999). In teleosts, the enveloping layer (EVL) is an extra-embryonic epithelial covering that forms during the cleavage stages and is sloughed off the embryo at later stages (Bouvet, 1976; Kimmel et al., 1990). Another extra-embryonic tissue is the yolk syncytial layer (YSL), which forms at the onset of zygotic expression (mid-blastula transition; MBT) when the blastomeres juxtaposed to the yolk fuse with each other and release their contents (Kimmel et al., 1995). While a potential signaling role for the EVL has not been tested, the signaling properties of the YSL are well documented (Oppenheimer, 1934; Solnica-Krezel, 1999). In transplant experiments, signals from the yolk can induce ectopic mesoderm (Mizuno et al.,

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1996). Conversely, mesoderm and endoderm fail to form when signals from the yolk are depleted by RNase injection (Chen and Kimelman, 2000). The essential signals produced by the YSL, however, are not known.

Nodal-related proteins form a conserved subclass of the TGF-β superfamily that act in all vertebrates to induce the mesoderm and endoderm, pattern all three germ layers and establish the left–right body axis (Schier, 2003). Consistent with these multiple functions, Nodal-related proteins are dynamically expressed throughout development. In the mouse, for example, *nodal* is expressed across the entire epiblast prior to gastrulation, but rapidly becomes restricted to the primitive streak and the visceral endoderm (Conlon et al., 1994; Zhou et al., 1993). At later stages, *nodal* is expressed in the node and left lateral plate mesoderm (LLPM) (Collignon et al., 1996). Genetic analysis indicates that Nodal signals have different roles in each domain. Conditional mutants showed that *nodal* is required in the node to establish left–right asymmetry (Brennan et al., 2002). By contrast, the primitive streak does not form in null *nodal* mutants, and the resulting embryos lack all mesodermal derivatives (Conlon et al., 1994; Zhou et al., 1993). Analysis of *nodal* mutant chimeras demonstrated that Nodal signals are required in the visceral endoderm for formation of the prechordal plate and anterior neural tissue (Varlet et al., 1997). Other genetic experiments indicate that Nodal signals in the epiblast act to pattern the extra-embryonic tissues (Brennan et al., 2001). Thus in mammalian embryos, Nodal signals mediate reciprocal interactions between the embryonic and extra-embryonic tissues that are essential for embryonic development.

There are three *nodal-related* genes in zebrafish but only two, *squint* (sqt/ndr1) and *cyclops* (cyc/ndr2), are required for mesoderm and endoderm formation (Feldman et al., 1998). The third *nodal-related* gene, *southpaw* (*spaw/ndr3*), is only expressed after gastrulation and is required to establish left–right asymmetry (Long et al., 2003). In the absence of *sqt* function, the zebrafish organizer, known as the embryonic shield, does not form (Feldman et al., 1998). These embryos subsequently recover, however, because Cyc signals induce mesoderm and endoderm during gastrulation (Dougan et al., 2003; Hagos and Dougan, 2007). At 24 hours post-fertilization (hpf), most *sqt* mutants are indistinguishable from wild type, but a variable minority have reduced prechordal plates and display mild cyclopia (Dougan et al., 2003; Heisenberg and Nusslein-Volhard, 1997). In contrast, all *cyc* mutants have reduced prechordal plate, resulting in cyclopia, and lack the floorplate (Hatta et al., 1991; Rebagliati et al., 1998b; Sampath et al., 1998). The defects in *sqt;cyc* double mutants are much more severe than either single mutant. These embryos lack all derivatives of the mesoderm and endoderm in the head and trunk, including the notochord, prechordal plate, trunk somites, pronephros, heart, blood and gut (Feldman et al., 1998). Thus, *sqt* and *cyc* have partially overlapping functions in germ layer formation.

Nodal signaling is mediated by a bipartite receptor complex containing the TGF-β Type I receptor, ALK4 and the Type II receptor, ActR–IIB (Reissmann et al., 2001). In order to bind and activate the ALK4/ActR–IIB receptor complex, Nodal-related proteins require the function of the Cripto/One-Eyed-pinhead (Oep) co-receptor (Cheng et al., 2003; Gritsman et al., 1999; Yeo and Whitman, 2001). ALK4 is a Ser/Thr kinase that phosphorylates cytoplasmic Smad2 and Smad3. PSmad2 or PSmad3 then dimerizes with Smad4 and the complex translocates to the nucleus, and activates transcription of target genes (Massague and Chen, 2000). The Smad heterodimers associate with any of several nuclear co-factors to stimulate gene expression, the most prominent of which are the winged-helix transcription factor FoxH1 and the paired-like homeodomain protein, Mixer (Kunwar et al., 2003). A few direct transcriptional targets of this pathway have been identified, including the *nodal-related* genes themselves (Meno et al., 1999). Conserved elements in the introns of *Xenopus xnr1* and mouse *nodal* mediate the autoregulatory response (Brennan et al., 2001; Hyde and Old, 2000; Osada et al., 2000). In both species, this element drives expression in the LLPM after gastrulation (Hyde and Old, 2000; Osada et al., 2000; Saijoh et al., 2000). At earlier stages, transcription factors acting on this element boost expression levels in the margin in frog embryos, and mediate expression in the epiblast of mouse embryos (Brennan et al., 2001; Hyde and Old, 2000; Osada et al., 2000).

sqt is initially expressed during oogenesis, but its function during these stages is controversial (Gore et al., 2005; Gore and Sampath, 2002; Hagos et al., 2007; Schier, 2005). In the zygote, *sqt* and *cyc* are expressed in three independent phases (Rebagliati et al., 1998a). *sqt* expression initiates in dorsal blastomeres soon after MBT (3 hpf), under control of the dorsal determinant β-catenin (Bellipanni et al., 2006; Dougan et al., 2003). After initiation, *sqt* expression extends into the YSL and the EVL (Erter et al., 1998; Feldman et al., 1998). Although overexpression experiments demonstrated that Sqt signals in the YSL could induce overlying blastomeres to become dorsal mesoderm, it is not known if *sqt* is required in the YSL (Erter et al., 1998; Feldman et al., 1998). During the late blastula stages, *sqt* and *cyc* are co-expressed in all marginal blastomeres. Two lines of evidence indicate that expression in the marginal ring is independent of the earlier expression of *sqt* in the dorsal blastomeres. First, overexpressing β-catenin induces ectopic expression of *sqt* at 3.5 hpf, but has no effect on expression at the margin (Dougan et al., 2003). Second, depletion of β-catenin eliminates the early dorsal expression of *sqt*, but does not effect *sqt* expression in the marginal ring (Bellipanni et al., 2006; Kelly et al., 2000). Although the T-box transcription factor VegT induces marginal expression of the *nodal-related* genes in *Xenopus*, the factors that induce this phase of *nodal-related* gene expression in zebrafish are not known (Stennard, 1998; White and Heasman, 2007). Expression of both *sqt* and *cyc* at this stage is maintained by an autoregulatory loop (Meno et al., 1999). In the third phase, *sqt* expression during gastrulation is maintained in a few blastomeres at the dorsal midline, called dorsal forerunners (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998a). By contrast, *cyc* transcripts accumulate in the axial mesoderm (Rebagliati et al., 1998b; Sampath et al., 1998).

We have undertaken an analysis of *sqt* genomic sequences in order to understand the regulatory networks that control *sqt*

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