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Roles of ADAM13-regulated Wnt activity in early *Xenopus* eye development

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ABSTRACT

Pericellular proteolysis by ADAM family metalloproteinases has been widely implicated in cell signaling and development. We recently found that *Xenopus* ADAM13, an ADAM metalloproteinase, is required for activation of canonical Wnt signaling during cranial neural crest (CNC) induction by regulating a novel crosstalk between Wnt and ephrin B (EfnB) signaling pathways (Wei et al., 2010b). In the present study we show that the metalloproteinase activity of ADAM13 also plays important roles in eye development in *Xenopus tropicalis*. Knockdown of ADAM13 results in reduced expression of eye field markers *pax6* and *rx1*, as well as that of the pan-neural marker *sox2*. Activation of canonical Wnt signaling or inhibition of forward EfnB signaling rescues the eye defects caused by loss of ADAM13, suggesting that ADAM13 functions through regulation of the EfnB–Wnt pathway interaction. Downstream of Wnt, the head inducer Cerberus was identified as an effector that mediates ADAM13 function in early eye field formation. Furthermore, ectopic expression of the Wnt target gene *snail2* restores *cerberus* expression and rescues the eye defects caused by ADAM13 knockdown. Together these data suggest an important role of ADAM13-regulated Wnt activity in eye development in *Xenopus*.

Introduction

Members of the ADAM (A Disintegrin And Metalloproteinase Domain) family are type I transmembrane proteins with an extracellular metalloproteinase domain, a disintegrin domain and a cysteine-rich domain. More than half of the known ADAMs contain a conserved zinc-binding motif in the metalloproteinase domain, which is required for protease activity (Edwards et al., 2008; White et al., 2005). These proteolytically active ADAMs, together with other related proteases such as members of the ADAMTS (disintegrin metalloproteinase with thrombospondin type I motifs) and MMP (matrix metalloproteinase) families, form a superfamily of secreted and cell-surface metalloproteinases that regulates turnover of the extracellular matrix, cell–cell and cell–matrix interactions, and cell signaling. Dysregulated metalloproteinase activities often lead to developmental defects and other diseases (Gomis-Ruth, 2009).

Several proteolytically active ADAMs are known to have important roles in cell signaling, mainly through ectodomain cleavage ("shedding") of cell-surface protein substrates. A growing list of substrates has been identified for ADAMs, among which are some key signaling molecules, including receptors and/or ligands of the Notch, EGFR, TNF and Efn signaling pathways (Edwards et al., 2008; White et al., 2005). Ectodomain shedding by ADAMs may have different outcomes for signaling, depending on the cellular and developmental context. For example, cleavage of receptors or ligands has been proposed to be an efficient way to eliminate a functional signaling molecule and, thereby, terminate a signal (Paland et al., 2008; Sapir et al., 2005; Sun et al., 2008). On the other hand, cleavage may also be required to generate a functional fragment of a signaling molecule and activate a pathway. Such a fragment could be the soluble form of a membrane-bound ligand that can signal over a long distance (Black et al., 1997; Blobel, 2005), or the cytoplasmic end of a transmembrane receptor that is further cleaved by γ-secretase and subsequently translocated to the nucleus to regulate target gene expression (Pan and Rubin, 1997; Sardi et al., 2006). For these reasons, several ADAM metalloproteinases have been shown to be indispensable for embryogenesis in mouse and other model animals (Hartmann et al., 2002; Peschon et al., 1998; Rooke et al., 1996).

The diploid amphibian species *Xenopus tropicalis* has emerged as a new and powerful model system to study early vertebrate development (Hellsten et al., 2010). With the aid of bioinformatics tools, we have identified more than a dozen ADAM genes in the *X. tropicalis* genome (Wei et al., 2010a). Loss-of-function studies suggest that ADAM13, one of the proteolytically active ADAMs, is essential for early CNC induction in *X. tropicalis*. We further found that ADAM13

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cleaves Efns B1 and B2, two class B Efns, and that such cleavage alleviates the inhibition of canonical Wnt signaling by these Efns and allows proper induction of CNC (Wei et al., 2010b). Given the coexistence of class B Efns and Wnt components in multiple tissues and the involvement of canonical Wnt signaling in many developmental events, it would be interesting to examine if this ADAM13–EfnB–Wnt cascade also functions in the morphogenesis of other tissues.

Here we show that ADAM13 metalloproteinase activity is required for normal eye development and early eye field formation in *X. tropicalis*. Rescue experiments suggest that ADAM13 functions in this developmental event by regulating EfnB and Wnt signaling. We further identified *snail2* and *cerberus*, two genes that are known to be controlled by the canonical Wnt pathway, as potential downstream effectors that contribute to eye field formation. These data provide new insight into the mechanisms of action for ADAMs and Wnt signaling in vertebrate eye development.

Materials and methods

Embryo manipulation and injection

Wild-type *X. tropicalis* adults were purchased from NASCO, and the *γ1-crys*/GFP3 transgenic line was generated in a previous study (Offield et al., 2000). Embryos were obtained by in vitro fertilization or natural mating, and injected as described (Ogino et al., 2006). Red dextran was co-injected as a lineage tracer. Morpholino (MO) oligos were designed and synthesized by Gene Tools, as described previously (Wei et al., 2010b). The sequences for MOs 13-1 and 13-3 are 5′-TGTGCAGCCAACCCTCCGTCCCCAT-3′ and 5′-CCCCGGCTCAGTCCGCTCT-CAGCC-3′, respectively. Embryos were cultured in 0.1× MBS to desired stages, and in situ hybridization and phenotype scoring were carried out as described below.

DNA constructs and in vitro transcription

The expression construct for dominant-negative *X. laevis* EphB1 receptor (pCS107-*ephB1ΔC*) was kindly provided by Dr. Ira Daar, and constructs encoding full-length and mutant forms (ΔDix and ΔDep) of Xdsh were provided by Dr. Mungo Marsden. *X. tropicalis* cDNA clones for *snail2* (in pCS107; clone ID: Ttba075D05; from Geneservice) and *cerberus* (in pCS108; clone ID: 7579004; from OpenBiosystems) were identified by bioinformatics and used directly for expression (sequences of all clones were confirmed by DNA sequencing). Cloning of *X. tropicalis adam13* has been described previously (Wei et al., 2010a). The coding sequence for ADAM13 was then subcloned into a pCS2+ expression vector modified to append a C-terminal myc_6-tag. To generate rescue constructs, site-directed mutagenesis was carried out using a QuickChange mutagenesis kit (Stratagene). Sense mutations were introduced by using the primers 5′-TATGCGGCCACATGGGCACC-GAAGGCTGGTTACATACATGGCTGG-3′ (forward) and 5′-CCAGCCATG-TATGTAACCAGCCTTCGGTGCCCATGTGGCCGCATA-3′ (reverse). The protease-inactive E340A mutation was introduced by using the primers 5′-GCTGCTGCAACAATGGCCCATGCAATTGGACACAAT-3′ (forward) and 5′-ATTGTGTCCAATTGCATGGGCCATTGTTGCAGCAGC-3′ (reverse). Capped mRNA with poly(A) tail was generated by in vitro transcription as described in Sive et al. (2000).

In situ hybridization

Clones of *X. tropicalis pax6* and *rx1*, as well as *X. laevis sox2*, *chordin* and *xbra*, were used to produce digoxigenin-UTP labeled antisense RNA probes. To generate *cerberus* probe, the coding sequence of *X. tropicalis cerberus* gene was amplified by PCR using the Sp6 promoter primer and a reverse primer that introduced a NotI site (5′-ATATGCGGCCGCTTTAATTGTGCAGGGTGG-3′). The PCR product was digested with SalI and NotI, and subcloned into pCS108. Antisense RNA probes were generated by in vitro transcription using T3 RNA polymerase (Promega). Whole-mount in situ hybridization was carried out as described (Sive et al., 2000).

Cell fate analyses

Embryos were injected with different MOs and red dextran as a lineage tracer and cultured to stage 37/38. Whole and sectioned embryos were analyzed for the presence of red fluorescence in the eye field as described (Lee et al., 2006; Moody, 1987).

Phenotype scoring and statistics

Embryos were cultured to stage ~35 and scored for defects in eye morphology (see Fig. 1A for examples of phenotypes). Cartilage phenotypes of the same embryos as those shown in Figs. 1A, 2B, 3B and 4B have been reported elsewhere (Wei et al., 2010b). The percentage of normal, moderate and severe phenotypes was calculated for each experiment, and averaged for multiple independent experiments. For statistics, the percentage of normal embryos in each experiment was used for comparison, and Student's *t* tests were performed for a series of independent experiments.

Results

Knockdown of ADAM13 in X. tropicalis leads to defects in eye morphology

Two antisense MOs (MOs 13-1 and 13-3) with no overlap between their target sequences, as designed in earlier studies (Wei et al., 2010b), were used to block ADAM13 translation. Western blot analyses demonstrated that both MOs effectively knock down endogenous ADAM13 protein, as well as translation of exogenous ADAM13 expressed by a co-injected transcript that contains the MO binding sites (Wei et al., 2010b). When either MO 13-1 or 13-3 was injected into a dorsal-animal blastomere of 8-cell stage embryos, we observed defects in eye morphology with high penetrance at tadpole stages (Fig. 1A), in addition to the aberrant head cartilage structures reported previously (Wei et al., 2010b). More than 90% of such morphants developed abnormal eye phenotypes on the injected side, ranging from reduced eye pigment (moderate) to little or no visible eye structure (severe). Both 13-1 and 13-3 morphants displayed the same eye phenotypes with no significant difference in penetrance (Fig. 1A). The uninjected side (not shown), as well as embryos injected with the same dose of the standard control MO, appeared to have normal eye morphology (Fig. 1A), indicating that the eye phenotypes were caused specifically by loss of ADAM13 function.

Function of ADAM13 in early eye field formation

In *Xenopus*, the presumptive eye tissue (eye field) is specified at the anterior neural plate as early as stage 12.5, when the embryos are transitioning from gastrula to early neural plate stages (Li et al., 1997). A number of eye field transcription factors (EFTFs) are expressed before stage 15, with the homeobox genes *pax6* and *rx1* among the earliest to appear in the forming eye field (Zuber et al., 2003). The expression domain of *pax6* is slightly larger than that of *rx1* in the eye field, and also includes two lateral stripes (Casarosa et al., 1997; Hirsch and Harris, 1997; Zuber et al., 2003; Figs. 1B and C). Injection of either MO 13-1 or 13-3 resulted in a significant decrease in *pax6* and *rx1* mRNA in the anterior neural plate at stage 12.5, as shown by in situ hybridization (Figs. 1B and C), suggesting that early formation of the eye field was perturbed. We also observed