Developmental Biology 349 (2011) 483–493

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Alcama mediates Edn1 signaling during zebrafish cartilage morphogenesis

Priya Choudhry [a], Deepa Joshi [a], Birgit Funke [b], Nikolaus Trede [a,c,*]

[a] Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112, USA
[b] Laboratory for Molecular Medicine (LMM), Harvard Medical School, Cambridge, MA 02139, USA
[c] Department of Pediatrics, University of Utah, Salt Lake City, UT 84112, USA

ARTICLE INFO

Article history:
Received for publication 9 September 2010
Revised 1 November 2010
Accepted 3 November 2010
Available online 10 November 2010

Keywords:
Alcama
Edn1
Cartilage
Endoderm
Neural crest

ABSTRACT

The zebrafish pharyngeal cartilage is derived from the pharyngeal apparatus, a vertebrate-specific structure derived from all three germ layers. Developmental aberrations of the pharyngeal apparatus lead to birth defects such as Treacher-Collins and DiGeorge syndromes. While interactions between endoderm and neural crest (NC) are known to be important for cartilage formation, the full complement of molecular players involved and their roles remain to be elucidated. Activated leukocyte cell adhesion molecule a (*alcama*), a member of the immunoglobulin (Ig) superfamily, is among the prominent markers of pharyngeal pouch endoderm, but to date no role has been assigned to this adhesion molecule in the development of the pharyngeal apparatus. Here we show that *alcama* plays a crucial, non-autonomous role in pharyngeal endoderm during zebrafish cartilage morphogenesis. *alcama* knockdown leads to defects in NC differentiation, without affecting NC specification or migration. These defects are reminiscent of the phenotypes observed when Endothelin 1 (Edn1) signaling, a key regulator of cartilage development is disrupted. Using gene expression analysis and rescue experiments we show that Alcama functions downstream of Edn1 signaling to regulate NC differentiation and cartilage morphogenesis. In addition, we also identify a role for *neural adhesion molecule 1.1* (*nadl1.1*), a known interacting partner of Alcama expressed in neural crest, in NC differentiation. Our data shows that *nadl1.1* is required for *alcama* rescue of NC differentiation in *edn1*$^{-/-}$ mutants and that Alcama interacts with Nadl1.1 during chondrogenesis. Collectively our results support a model by which Alcama on the endoderm interacts with Nadl1.1 on NC to mediate Edn1 signaling and NC differentiation during chondrogenesis.

Introduction

Formation of the pharyngeal apparatus is a crucial part of vertebrate development because it gives rise to the cartilage, connective tissue, sensory neurons, thyroid, parathyroid and thymus. Defects in this process results in human birth defects such as DiGeorge and Treacher-Collins syndromes. Generation of this tissue is highly complex, involving extensive cell migration and signaling between cells derived from all three germ layers. NC cells migrate from the dorsal neural tube in three distinct streams into a series of pharyngeal arches and eventually give rise to cartilages and bones of the head. Each pharyngeal arch is composed of a cylinder of NC surrounding a mesodermal core. The NC is covered by ectoderm on the outside and endoderm on the inside. Between the arches, endoderm meets ectoderm forming the pharyngeal pouches, which later develop into gill clefts and the epithelial lining of the pharynx, thyroid, parathyroid and thymus (Graham, 2003).

Though NC cells carry intrinsic cues for patterning (Noden, 1983), they receive extrinsic cues from the surrounding cells and extracellular matrix as they migrate. Recently, the endoderm has been found to contribute significantly to NC development. Ablation and extirpation experiments in chicken have revealed that the endoderm carries patterning information for the NC in segments along the antero-posterior and medio-lateral axis (Couly et al., 2002; Ruhin et al., 2003). In addition, genetic mutants in zebrafish have also revealed the requirement of endoderm in formation of cartilage. The *sox32*-deficient *casanova* mutant lacks endodermal pouches and cartilages that are rescued by wild-type endodermal transplants (David et al., 2002). Likewise, the *tbx1*-deficient *van gogh* mutant fails to form segmented endodermal pouches resulting in fusion of the pharyngeal cartilages (Piotrowski and Nusslein-Volhard, 2000). Similarly, the zebrafish mutant for *integrinα5* lacks the first endodermal pouch and the anterior part of the hyoid cartilage (Crump et al., 2004). Although these data demonstrate that endoderm is essential for cartilage development, the cellular and molecular interactions between the NC and endodermal cells are not fully understood.

One major signaling factor that provides an extrinsic cue regulating NC differentiation is *endothelin-1* (*edn1*). *edn1* is expressed in the mesodermal cores, ectoderm and endoderm of the pharyngeal arches, but not in NC. Edn1 signals the NC and induces ventralization

* Corresponding author. The Huntsman Cancer Institute, University of Utah, 2000, Circle of Hope, Salt Lake City, UT 84112, USA. Fax: +1 801 581 8547.
E-mail addresses: priya.choudhry@hci.utah.edu (P. Choudhry), nikolaus.trede@hci.utah.edu (N. Trede).

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doi:10.1016/j.ydbio.2010.11.006

of pharyngeal arch cartilage (Miller et al., 2000). Mutations in *edn1*, Edn1 cleaving enzymes and other genes in the Edn1 signaling cascade (*sucker, schmerle, hoover* and *sturgeon*) cause similar cartilage defects, and have been placed in the same class (Kimmel et al., 2001; Piotrowski et al., 1996). Typically, the ventral domains of the first two arches are reduced in size, changed in orientation, and fused with the dorsal domains, while the posterior arches are mostly unaffected. Hence, Edn1 is an important signaling factor that is required non-autonomously for NC differentiation into cartilage.

Other molecular players that may signal from endoderm to NC remain elusive. While Alcama is commonly used as a marker for pharyngeal endoderm in zebrafish (Crump et al., 2004; Piotrowski and Nusslein-Volhard, 2000), its role in this tissue has not been elucidated thus far. In zebrafish, Alcama has been studied primarily for its role in neurogenesis (Diekmann and Stuermer, 2009; Fashena and Westerfield, 1999). Initially identified in chicken for its role in neurite extension (Burns et al., 1991), ALCAMA has now been shown to be involved in axonal pathfinding and axonal fasciculation (Diekmann and Stuermer, 2009; Weiner et al., 2004). Its non-neuronal roles include T-cell activation (Bowen et al., 2000; Fashena and Westerfield, 1999; Ofori-Acquah and King, 2008; Zimmerman et al., 2006), metastasis (Degen et al., 1998; Ofori-Acquah and King, 2008) and cell migration (Heffron and Golden, 2000). Human ALCAM is a transmembrane glycoprotein having five Ig domains, a transmembrane domain and a short cytoplasmic tail. It mediates cell-cell clustering through homophilic (ALCAM-ALCAM) as well as heterophilic (ALCAM-NgCAM and ALCAM-CD6) interactions (DeBernardo and Chang, 1996; Degen et al., 1998). While ALCAM can activate signal transduction pathways in neighbouring cells through heterophilic interactions (Ibanez et al., 2006), a non-autonomous role of ALCAM has not been defined thus far. In this paper we demonstrate that zebrafish Alcama, expressed in the pharyngeal endoderm, is an important non-autonomous molecule for NC differentiation. In addition, we provide evidence that Alcama mediates Edn1 signaling from the endoderm to the NC by interacting with Nadl1.1 (NgCAM in chicken) on the NC cells. These data for the first time link Alcama to Edn1 signaling and identify a role for the molecular interaction between Alcama and Nadl1.1 in cartilage formation.

Materials and methods

Fish stocks and maintenance

Fish were maintained at 28.5 °C under standard conditions (Westerfield, 2000) and were staged as described (Kimmel et al., 1995). The *sucker*^{tf216b} mutant (*edn1*$^{-/-}$) line was obtained from Zebrafish International Resource Center (Miller et al., 2000). Homozygous mutants were obtained by inbreeding of heterozygous carriers. *Tg(fli1:EGFP)* fish have been previously described (Lawson and Weinstein, 2002). Alcian Blue stained larvae of *furina*$^{-/-}$ mutants at 5 days post fertilization (dpf) were a kind gift from Chuck Kimmel (Institute of Neuroscience, University of Oregon).

Identification and genotyping of edn1$^{-/-}$ *mutants*

edn1$^{-/-}$ mutants have an A-to-T missense mutation (Miller et al., 2000). The mutants were identified by Derived Cleaved Amplified Polymorphic Sequences assay (Neff et al., 1998). DNA was extracted from the tails of stained embryos and PCR was conducted using the primers 5'-AGATGCTCCTGCGCAAGTTTTCTAG-3' and 5'-CTGACTTACTCTGGTGTGTTCACCC-3'. The mismatch in the primer which introduces a *XbaI* site in the wild-type (WT) but not in the mutant is underlined. The 93 bp PCR product, when digested with *Xba*I, gives a 68 bp product in WT. The 93 and 68 bp products were resolved on a 4% Metaphor agarose gel (Lonza). All the identified WT and mutants were included in the analysis.

Cloning and RNA transcription

RNA extracted from 48 h post fertilization (hpf) Tü larvae was used for cDNA synthesis. PCR for *alcama* was performed using the forward primer 5'-ggatccgccaccATGCATTCGGTTATCTGCCTTTTCG-3' with a *BamHI* and Kozak overhang and the reverse primer 5'-ctcgagTTAGACATCTGCTTTATGATTGTTCTCCTCC-3' with a *XhoI* overhang. The overhangs are shown in lower case. The PCR product was cloned into pCMV-Script using TOPO TA kit (Invitrogen). The *edn1* cDNA clone in pBK-CMV was obtained from ZIRC.

To make sense RNA for injection, the *edn1* and *alcama* plasmids were cut with *KpnI* and in vitro transcription was driven from the T3 promoter using mMessage Machine kit (Ambion). *ednrb1* was cloned from cDNA into TOPO TA using the primers 5'-ATGCGTTTCCAAATTATTATGGAAACAAGATGCG-3' (forward) and 5'-TCAGTGCCTAATTTGAAGTATACTTGTTGGAGAC-3' (reverse) and this plasmid was used to make ISH probe.

Morpholino anti-sense oligonucleotide and RNA injections

Translation blocking (TB) and splice site blocking (SB) Morpholinos (MOs) were designed to bind 143 bp upstream of the transcription start site and at the donor site of exon 6 *alcama* mRNA, respectively. *alcama* blocking and control MO were purchased from Gene Tools, Inc. The sequences are TB MO: 5'-GTTCTCCTTTATACAGTCCGGCGAC-3'; SB MO: 5'-GCAGTCCCTCACCTTAATGTTAAAG-3'; control MO: 5'-TGATCACCTGCAGATGGACGCTGCG-3'. The optimal doses were determined to be 1.1 ng for the TB MO and 2.2 ng for the SB MO. The control MO was injected at 1.1 ng per embryo. The TB MO for *nadl1.1*: 5-'CAGGCTGACTCTGCACTGGAGGCAT-3' has been previously described (Wolman et al., 2007) and was injected at 4.4 ng per embryo. 26 pg of *alcama* or *edn1* RNA was injected per embryo. MOs and RNA were dissolved in molecular biology grade water and pressure injected into one to four cell zebrafish embryos. For suboptimal doses, the *alcama* TB MO was used at 0.5 ng and the *nadl* MO at 1.1 ng per embryo.

Treatment with proteasome inhibitor MG-132

MG-132 was dissolved in DMSO at a stock concentration of 500 mM. Embryos were dechorionated at 5 hpf and transferred to E2 with 50 μM MG-132 (Bretaud et al., 2007) or with 1% dimethyl sulfoxide (DMSO). MG-132- and DMSO-treated control larvae were fixed at 30 hpf and stained.

Tissue labeling procedures

Alcian Blue cartilage staining and dissection were performed as described (Kimmel et al., 1998). Whole mount RNA in situ hybridization (ISH) with digoxigenin was performed as described (Miller et al., 2000). The plasmids for *dlx2a*, *dlx3b*, *dlx5a* were a kind gift from Gage DeKoeyer Crump (Keck School of Medicine, University of Southern California). The plasmid for nadl1.1 was a gift from Gavin J Wright (Cell Surface Signalling Laboratory, Wellcome Trust Sanger Institute). Alcama protein was stained using Zn-5 antibody from ZIRC at 1:500 dilution. A goat anti-mouse secondary antibody conjugated with Alexa 555 (Invitrogen) was used for fluorescence quantification purposes. DAPI staining was used for counting the number of cells in the pouches.

Imaging and quantification

Skeletons and ISH embryos were photographed on a Nikon Y-IDP microscope at 20× zoom using Spot software. Confocal images of antibody stained embryos were taken on Olympus FV1000 microscope. Images of all larvae from the same experiment were taken

using the same settings and exposure. Cell numbers were counted using Imaris software and fluorescence intensity of each z-section was measured using ImageJ. For immunohistochemistry coupled to ISH, the secondary antibody conjugated to alkaline phosphatase (Bio-Rad) was used with Fast Red for detection (Sigma-Aldrich). Fluorescent ISH was performed following immunohistochemistry as described (Welten et al., 2006).

Results

alcama is necessary for ventral cartilage formation

We tested whether *alcama* plays a role in cartilage morphogenesis by injection of two different MOs: one blocking translation (TB MO), and the other blocking splicing of exon 6 (SB MO; Fig. 1A). Staining with Zn-5 antibody, which recognizes Alcama, revealed effective knockdown of Alcama protein by both MOs (Fig. 1C–E). 5 dpf *alcama* morphants are characterized by a protruding lower jaw, cardiac edema and absence of swim bladder (Fig. 1G). The jaw is shorter in the antero-posterior (A-P) direction and the ventral elements of the anterior arches, Meckels (mc) and ceratohyal (ch), are bent ventrally leading to the protruding lower jaw (Fig. 1I). Injection of the TB MO consistently leads to a more pronounced phenotype than the SB MO. Therefore, in subsequent experiments we use the TB MO exclusively and refer to it as *alcama* MO.

To eliminate the possibility that the jaw defect is caused by non-specific MO-induced p53-activation (Robu et al., 2007), *alcama* MO was injected into the *p53* (I166T) mutant line (Parant et al., 2010). Alcian blue staining of 5 dpf larvae revealed that the percentage of individuals with affected cartilage was equivalent in wild-type (WT) and the $p53^{-/-}$ mutant lines (Fig. S1). The specificity of the phenotype was also tested by co-injection of *alcama* MO and *alcama* RNA. *alcama* RNA can rescue the ventral cartilage defect caused by *alcama* knockdown (Fig. S1). This indicates that the cartilage defect is caused by *alcama* knockdown and not by off-target MO effects and that *alcama* is required for the proper formation of anterior ventral cartilages in zebrafish.

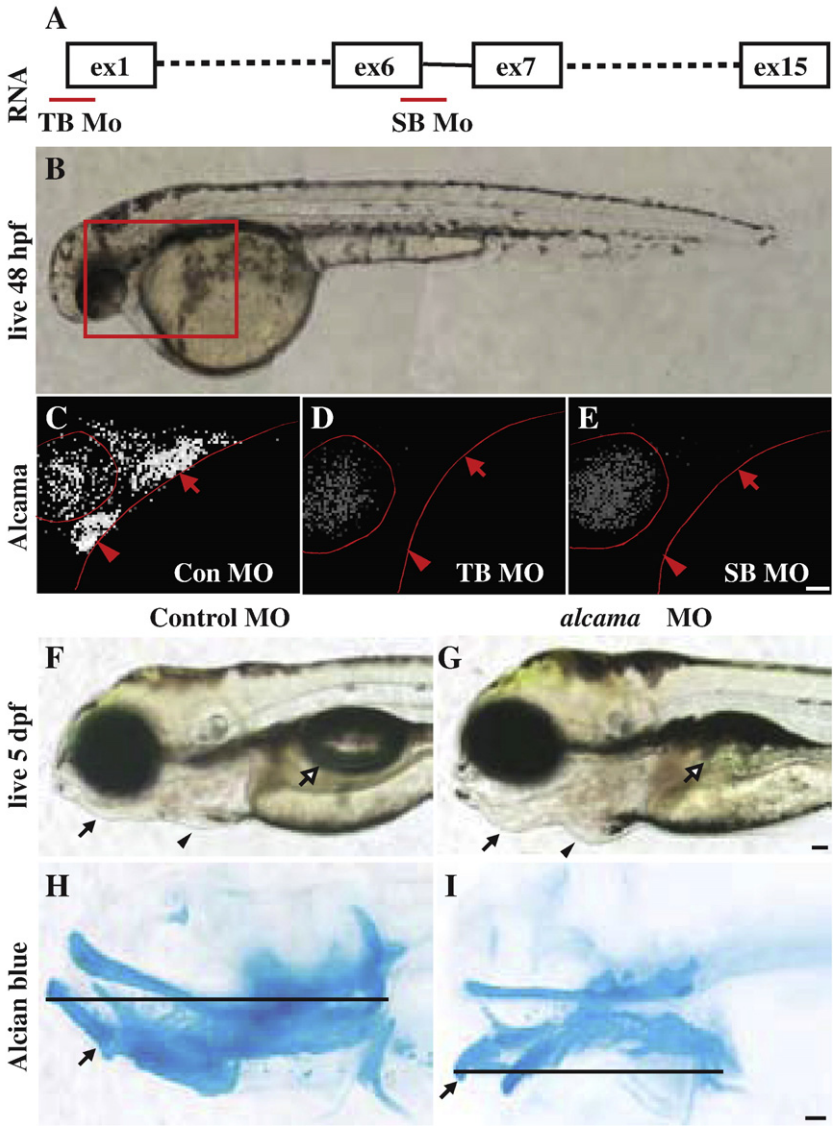

Fig. 1. *alcama* morphants have defects in facial skeletal patterning. (A) Graphic representation of *alcama* RNA illustrating the position of TB and SB MOs. (B) Nomarski image of a 48 hpf WT larva with the box indicating the region shown in (C)–(E). 48 hpf control (C), *alcama* TB (D), and *alcama* SB (E) morphants stained with Zn-5 antibody. Both MOs effectively knock down *alcama* expression in the heart (arrowheads) and pouches (arrows). (F, G) Lateral views of 5 dpf larvae injected with control and *alcama* TB MOs. *alcama* morphants have a protruding jaw (arrows), cardiac edema (arrowheads) and an absent swim bladder (open arrows). Lateral views of Alcian blue-stained control (H) and *alcama* (I) morphants at 5 dpf. The line indicates the length of the pharyngeal cartilage, which is shortened in *alcama* morphants. The arrow points to Meckels cartilage, which is bent ventrally in *alcama* morphants. Scale bars: 50 μm.

Cartilage defects in alcama morphants are reminiscent of the edn1-class of mutants

In *alcama* morphants the pharyngeal skeleton is shorter in the antero-posterior direction and the anterior arch ventral cartilages (mc and ch) point ventrally rather than anteriorly (Fig. 1H, I). In addition, mc and ch are fused to their respective dorsal elements, palatoquadrate (pq) and hyosymplectic (hm) (Fig. 2A, C). The posterior arches are shorter in the antero-posterior direction, but are otherwise unaffected (Fig. 1I). To identify the pathway affected in *alcama* morphants, we compared the jaw phenotype in *alcama* morphants to previously described cartilage mutants. The three characteristic phenotypes of the ventral cartilages in *alcama* morphants; shortening, change of orientation and fusion to dorsal cartilages, are typical of the *edn1*-class of mutants (Piotrowski et al., 1996; Walker et al., 2006, 2007). Particularly striking is the similarity of *alcama* morphants to *furinA*$^{-/-}$ mutants (Fig. 2C, E). This observation suggests that *alcama* and *edn1* may be in the same genetic pathway.

edn1 regulates alcama protein levels in the pharyngeal pouches

In order to test if an epistatic relationship exists between *alcama* and *edn1*, we analyzed *edn1* expression in *alcama* morphants. While *alcama* is also expressed in the heart, retina and the brain (Fig. S2A), expression of *edn1* and *alcama* coincides in the ventral pharyngeal area from 18 to 36 hpf (Thisse and Thisse, 2004, 2005). By 30 hpf *edn1* expression is clearly demarcated in the mesodermal cores and surface ectoderm of pharyngeal arches in addition to the pharyngeal pouches (Miller et al., 2000). At that stage, *alcama* is expressed in pharyngeal pouch endoderm (Kopinke et al., 2006). *edn1* expression is unchanged between control and *alcama* morphants, suggesting that *edn1* may act upstream of *alcama* (Fig. 3A, B).

To test this possibility, we analyzed Alcama protein expression in pharyngeal pouches of *edn1*$^{-/-}$ mutants. 30 hpf larvae were co-stained with Zn-5 Alcama antibody and DAPI (Fig. 3C, D). Confocal images were cropped to individual endodermal pouches and analyzed. Alcama intensity in individual pouches of *edn1*$^{-/-}$ mutants was down-regulated to less than 40% of the WT individuals (Fig. 3E). The total number of Alcama-expressing cells in each pouch (determined by DAPI staining) is comparable between WT and *edn1*$^{-/-}$ mutants (Fig. 3F), indicating that endodermal pouch tissue is present in *edn1*$^{-/-}$ mutants, but it expresses reduced amounts of Alcama. This observation suggests that *edn1* possibly regulates Alcama levels in pharyngeal pouches.

Edn1 is produced as a proprotein, which is cleaved twice to produce a diffusible 21-amino acid peptide. The peptide acts by binding to endothelin receptors (Ednr) coupled to G proteins. In zebrafish, two Ednrs exist; EdnrA and EdnrB. Previous ISH studies

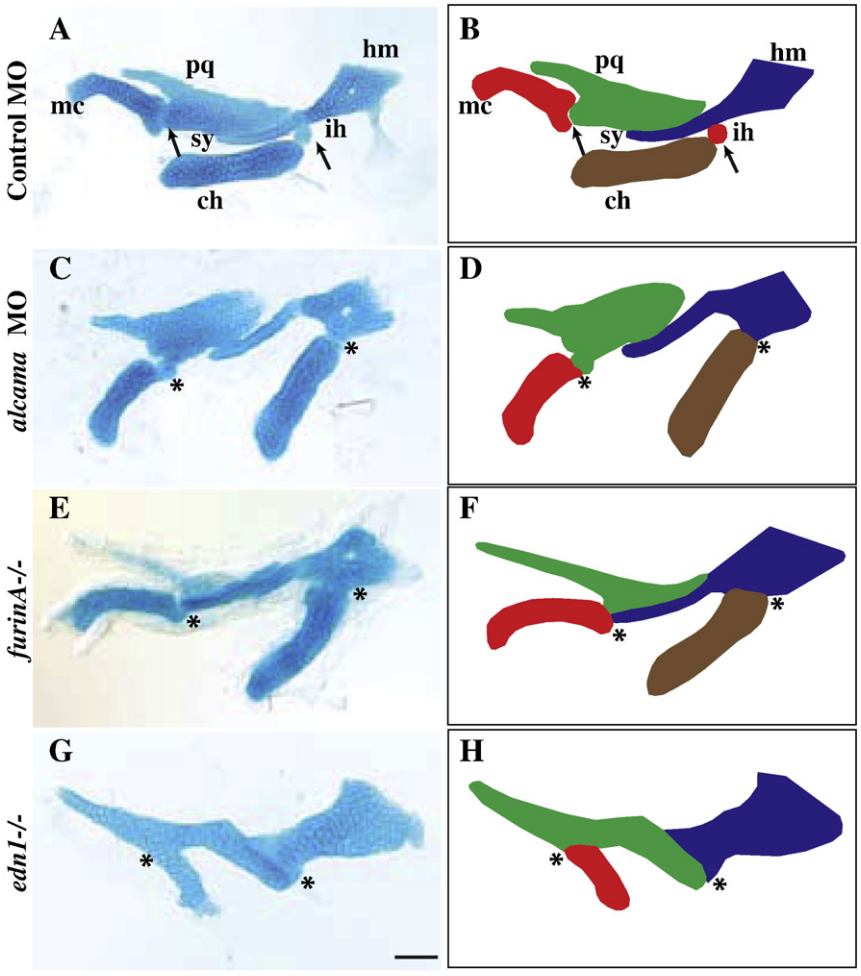

Fig. 2. *alcama* morphants have cartilage defects similar to the *edn1* class of mutants. Flatmounts of mandibular and hyoid cartilage from 5 dpf Alcian Blue-stained larvae (A, C, E, G); corresponding schematics (B, D, F, H). The joint between Meckel's and palatoquadrate is fused in *alcama* morphants and *furinA*$^{-/-}$ and *edn1*$^{-/-}$ mutants. Similarly, the interhyal is absent in *alcama* morphants and *furinA*$^{-/-}$ mutants leading to a fusion of the ceratohyal and hyosymplectic cartilages. *alcama* morphants and *furinA*$^{-/-}$ mutants have misshapen Meckel's cartilage and ceratohyal, but *edn1*$^{-/-}$ mutants have the most severe defect with a lack of ceratohyal and severely misshapen Meckel's cartilage. DV joint regions are indicated with arrows in A and B. Fusions at joints are indicated with asterisks in (C)–(H). Cartilages are labeled as followed: pq (palatoquadrate), mc (Meckel's cartilage), hm (hyomandibula), ch (ceratohyal), sy (symplectic), and ih (interhyal). Scale bar: 50 μm.

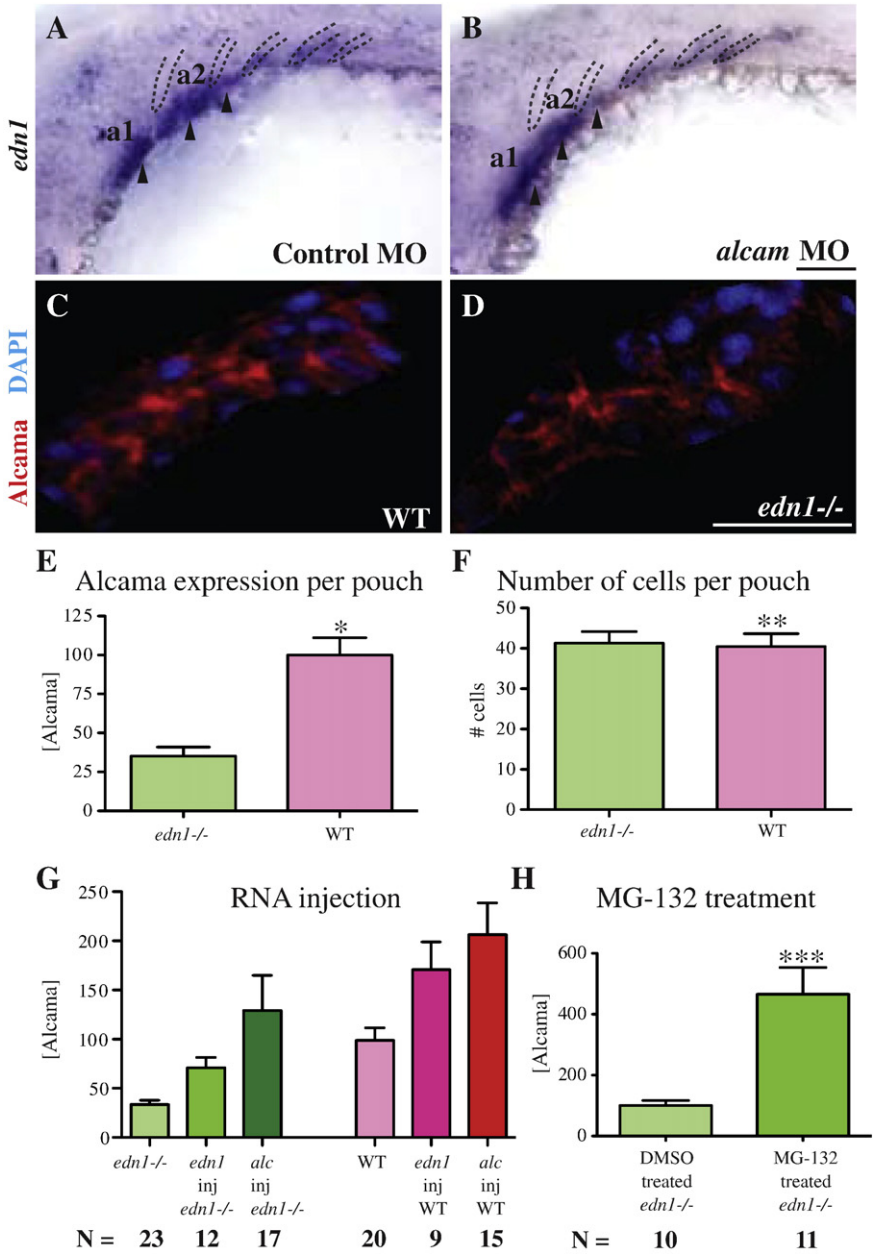

Fig. 3. Edn1 regulates Alcama levels. *edn1* is expressed in the mesodermal cores (arrowheads) of the first three arches and in pharyngeal pouches 2–4 (dotted lines) (A). Its expression is unchanged in *alcama* morphants (B). Cropped image of a single pharyngeal pouch from 30 hpf WT sibling (C) and *edn1*$^{-/-}$ mutant (D) stained with Zn-5 (anti-Alcama) antibody in red and DAPI in blue. (E) Bar graph showing the difference in total Alcama protein in a pouch (second or third pouch), represented as measured fluorescence intensity normalized to WT; *p-value < 0.0001 by a two-tailed t-test. The bar graph in (F) shows the total number of cells per pouch is unchanged in WT and *edn1*$^{-/-}$ mutants; **p-value = 0.08495 by a two-tailed t-test. $n = 14$ for WT; $n = 11$ for *edn1*$^{-/-}$ mutants for this experiment which was repeated with similar results. (G) *edn1* regulates the Alcama levels in pouches. The bar graph shows the sum of Alcama intensity in the first three pouches of 30 hpf *edn1*$^{-/-}$ (green) and WT (red) larvae after the indicated injections (p-value < 0.0001 by one-way analysis of variance). (H) Bar graph showing the sum of Alcama intensity in the first three pouches of 30 hpf *edn1*$^{-/-}$ larvae after treatment with DMSO or the proteasome inhibitor MG-132; ***p-value = 0.0009 by a two-tailed t-test. N is the number of larvae in a single experiment, which was repeated with similar results. a1 and a2 label pharyngeal arches 1 and 2. Scale bars: 50 μm.

have revealed that *ednrA1* and *ednrA2* are expressed almost exclusively in the NC of pharyngeal arches (Nair et al., 2007). We cloned *ednrb1* and validated that this receptor is expressed diffusely throughout the endoderm and NC (Fig. S2B). *ednrb1* expression in the endoderm is compatible with our hypothesis that Edn1 signals to the endoderm to regulate Alcam levels.

To corroborate our hypothesis that *edn1* regulates Alcama levels, we tested whether *edn1* over-expression could rescue Alcama levels in *edn1*$^{-/-}$ mutants. *edn1* RNA was injected into *edn1*$^{-/-}$ embryos at the 1-cell stage and Alcama protein levels were quantified at 30 hpf as discussed previously with one modification. The fluorescence intensity of Zn-5 staining in the pharyngeal area from the first to the third pouch, instead of single pouches, was measured and normalized to uninjected WT (Fig. S3). *alcama* RNA was injected as a positive control. *edn1* RNA injection results in more than 50% increase in Alcama protein in WT and *edn1*$^{-/-}$ mutants (Fig. 3G), suggesting that *edn1* regulates Alcama levels in the endoderm.

Additionally, we investigated the mechanism by which Edn1 regulates Alcama. ISH studies revealed that *alcama* RNA levels, while slightly down-regulated in *edn1*$^{-/-}$ mutants (Fig. S2C, D), do not explain the severe down-regulation observed in Alcama protein levels (Fig. 3D, E). This observation suggests that *edn1* does not regulate Alcama levels via regulating transcription. Previous studies have indicated that *edn1* may lead to Snail and β-catenin protein

stabilization by regulating the proteasome pathway (Rosano et al., 2005; Sato-Jin et al., 2008). To test whether Edn1 stabilizes Alcama protein by interfering with its degradation by the proteasome, we treated *edn1*$^{-/-}$ larvae with the proteasome inhibitor MG132 and measured Alcama levels. The fluorescence intensity was normalized to DMSO-treated *edn1*$^{-/-}$ mutants. Alcama levels in the pharyngeal pouches increased by more than 300% upon treatment with MG-132 (Fig. 3H), indicating that inhibition of proteasomal degradation is involved in Edn1-induced regulation of Alcama. While *edn1* RNA injection increases Alcama levels in the pharyngeal pouches, Alcama levels are unchanged in the heart and retina, where Edn1 signaling is not active (data not shown). These experiments indicate that Edn1 stabilizes Alcama levels specifically in the pharyngeal endodermal pouches by inhibiting some component of the proteasome pathway.

alcama is required for edn1-dependent differentiation of NC

We have determined that *alcama* morphants have defects in ventral cartilages. Since cartilage is formed from NC in the pharyngeal arches, we investigated whether *alcama* knockdown caused a defect in the patterning or specification of NC. We injected *alcama* MO into *Tg(fli:*EGFP*)* line which labels NC cells. Analysis of larvae at 30 hpf, a stage at which the cranial NC has already populated the arches, revealed that NC migration is unaffected in *alcama* morphants (Fig. S4B). This observation was corroborated by ISH with the NC marker *distalless 2a* (*dlx2a*) at the same developmental stage, showing normal patterning of NC (Fig. S4D). In addition *nkx2.3* expression in endoderm (Fig. S4F), and *edn1* expression in mesodermal cores and endodermal pouches (Fig. 3B) is unaffected in *alcama* morphants, suggesting that NC, endoderm and mesoderm are all patterned and specified correctly in the pharyngeal region of *alcama* morphants. This result implies that similarly to genes in the Edn1 pathway, Alcama is not required for NC migration or specification, but is involved in a later stage of cartilage morphogenesis.

Our data suggest that Alcama functions downstream of Edn1 in cartilage formation. To assess the functional importance of *alcama* in the Edn1 signaling pathway, we analyzed the expression of genes downstream of *edn1* in *alcama* morphants. Edn1 signaling is required for the expression of Dlx and Hand2 transcription factors in NC (Miller et al., 2000; Walker et al., 2006). Like Edn1, Hand2 is required for ventral cartilage formation and Dlx factors pattern the dorso-ventral axis of pharyngeal arches (Depew et al., 2002; Miller et al., 2003). In *alcama* morphants, the expression of *hand2*, *dlx3b*, *dlx5a* and *dlx6a* is strongly reduced in the first and the posterior arches, and mildly reduced in the second arch at 30 hpf (Fig. 4B, E, H, K). Although *hand2* expression recovers by 48 hpf, *dlx* genes continue to be down-regulated (Fig. S5). At 30 hpf, *hand2* expression is down-regulated in NC, but unaffected in the heart of *alcama* morphants (Fig. 4B) and *edn1*$^{-/-}$ mutants (Fig. 4C), indicating that Alcama is required specifically for *edn1*-dependent expression of NC genes.

edn1-dependent *bapx1* expression in the intermediate region of the precartilage arch is required for cartilage joint formation (Miller et al., 2003). Consistent with the joint fusion observed in *alcama* morphants, *bapx1* expression in the intermediate region of arch1 is down-regulated in *alcama* morphants (Fig. 4N). Down-regulation of Edn1-dependent genes in *alcama* morphants is similar to, but less severe than that observed in *edn1* mutants (Fig. 4C, F, I, L, O). This observation is consistent with the less severe jaw defect observed in *alcama* morphants when compared to *edn1* mutants. Taken together these data suggest that Alcama is required for Edn1-dependent NC differentiation and the proper formation of ventral cartilage and jaw joint.

Our data suggests that NC is patterned and specified correctly in *alcama* morphants (Fig. S3). Hence, the failure of NC to express *edn1*-dependent genes that come on later in development, suggests that *alcama* knockdown causes a defect in NC differentiation. In subsequent experiments, *dlx5a* expression is used as a marker for NC differentiation due to its robust expression. The specificity of MO-induced NC differentiation defect was tested by co-injection of *alcama* MO with *alcama* RNA, and by *alcama* MO injection in *p53*$^{-/-}$ mutants (Robu et al., 2007). *dlx5a* expression was categorized as strong (all arches stained), partial (all arches stained but weaker) and absent (most arches missing staining and some with very weak staining) (Fig. S6A–C). Co-injection of *alcama* RNA with *alcama* MO decreases the number of affected individuals (absent *dlx5a* staining) to WT levels (Fig. S6D), suggesting that the NC differentiation defect is caused specifically by *alcama* knockdown. The percentage of affected larvae is similar in WT and *p53*$^{-/-}$ mutants (Fig. S6D), indicating that similar to the cartilage defect, the NC differentiation defect is caused by *alcama* knockdown and is not dependent on off-target MO effects.

alcama over-expression can rescue NC differentiation defect in edn1$^{-/-}$ *mutants*

Our data suggests that *edn1* regulates Alcama levels and knockdown of either gene results in a NC differentiation defect. We tested our hypothesis that *edn1* affects NC differentiation via Alcama, by over-expressing *alcama* in *edn1*$^{-/-}$ mutants followed by analysis of NC differentiation. We injected *alcama* RNA into *edn1*$^{-/-}$ mutants at the one-cell stage and assessed *dlx5a* expression at 30 hpf as a marker for NC differentiation. *dlx5a* staining was classified as absent, partial and strong (Fig. 5A–C) and the number of larvae in each category were counted. *edn1* over-expression was used as a positive control, resulting in a 300% increase in the number of *edn1*$^{-/-}$ mutants with *dlx5a* staining (sum of strong and partial) (Fig. 5D). *alcama* RNA induces similar increases of rescued *edn1*$^{-/-}$ mutants as *edn1* RNA, although full rescue of *dlx5a* expression was not achieved. This indicates that Alcama functions downstream of Edn1 to regulate NC differentiation. To bolster this hypothesis, we asked if *edn1* rescue of *edn1*$^{-/-}$ mutants could be abrogated by *alcama* knockdown. Indeed, co-injection of *alcama* MO abrogates the ability of *edn1* RNA to rescue *dlx5a* staining in *edn1*$^{-/-}$ mutants (Fig. 5D), supporting our previous finding that *alcama* expression is necessary for Edn1 function. Injection of either *edn1* or *alcama* RNA was unable to rescue the cartilage defect of *edn* mutants at 5 dpf, possibly due to dilution of RNA in rapidly dividing cells.

As opposed to *edn1*$^{-/-}$ mutants, less than 5% of WT larvae have absent *dlx5a* expression. *alcama* or *edn1* RNA injections into WT embryos do not affect *dlx5a* expression (Fig. 5E), demonstrating that these genes do not negatively affect NC differentiation when they are over-expressed. Co-injection of *edn1* RNA with *alcama* MO results in a similar percentage of larvae with absent *dlx5a* staining to that observed in *alcama* morphants (Fig. 5E). However, there are more larvae with strong *dlx5a* expression, suggesting that *edn1* may regulate NC differentiation by another parallel pathway independent of *alcama*. In aggregate, these data provide evidence that Alcama functions downstream of Edn1 in regulation of NC differentiation.

Nadl1.1 mediates Alcama differentiation signal to the NC

Since Alcama is expressed in the endoderm, we investigated the possibility that it interacts with another protein to mediate Edn1-signaling to the adjacent NC. *Nadl1* (Ng-CAM in chick) has been shown to interact with Alcama in the chick brain to promote neurite extension (DeBernardo and Chang, 1996). Zebrafish have two orthologs of Ng-CAM, *nadl1.1* and *nadl1.2*, but only *nadl1.1* is expressed in the NC (Figs. 6A and 7A). To test if *nadl1.1* is involved in cartilage development, we used a previously described TB MO targeting *nadl1.1* (Wolman et al., 2007). Phenotypically, *nadl1.1* morphants are similar to *alcama* morphants: by 5 dpf they have a protruding lower jaw, cardiac edema and absent swim bladder (data not shown). Similar to *alcama* MO, *nadl1.1* MO affects *edn1*-dependent

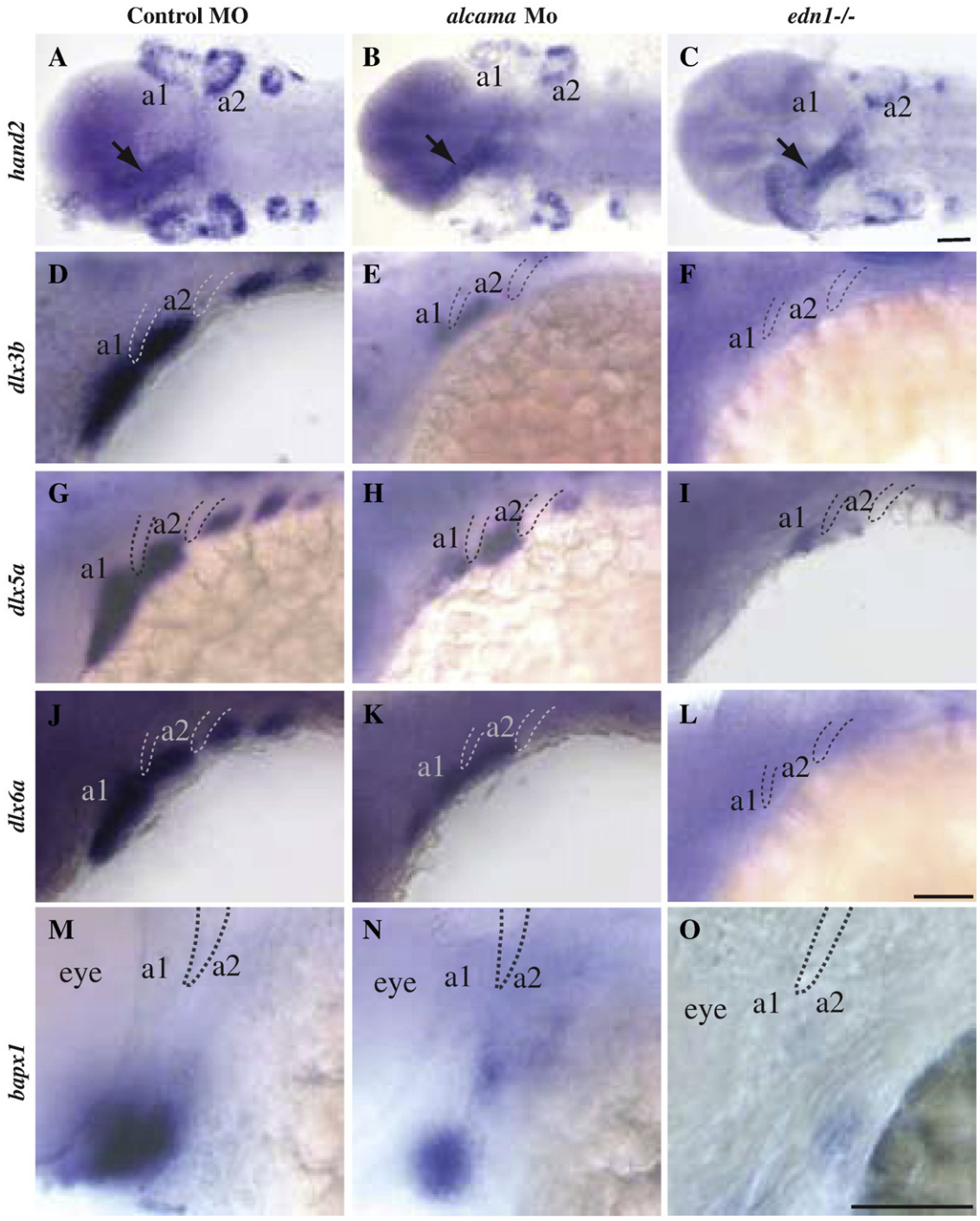

Fig. 4. *alcama* is required for *edn1*-dependent gene expression. Ventral views of larvae at 30 hpf (A–C), and lateral views at 30 hpf (D–L) and at 48 hpf (M–O) respectively. ISH for *hand2* (A–C), *dlx3b* (D–F), *dlx5a* (G–I), *dlx6a* (J–L), and *bapx1* (M–O) in control and *alcama* morphants and in *edn1*$^{-/-}$ mutants. *hand2, dlx3b, dlx5a* and *dlx6a* expression is strongly reduced in the first and branchial arches and moderately reduced in the second arch in *alcama* morphants. Their expression is strongly reduced in all arches in the *edn1*$^{-/-}$ mutants. *bapx1* expression in the first arch intermediate domain is moderately reduced in *alcama* morphants and strongly reduced in *edn1*$^{-/-}$ mutants. a1 and a2 label pharyngeal arches 1 and 2. Dotted lines highlight pharyngeal pouches 1 and 2. Scale bars: 50 μm.

gene expression: *hand2* and *dlx5a* expression is strongly reduced in the arches with a more pronounced effect on the posterior arches (Fig. 6B–E). Alcian blue staining reveals cartilage defects reminiscent of *alcama* morphants: mc and ch are changed in orientation and are fused to pq and hm, respectively (Fig. 6G–H'). Injection of *nadl1.1* MO into *p53*$^{-/-}$ mutants yields similar percentages of larvae with *dlx5a* down-regulation and jaw abnormalities (Fig. 6F, I), indicating that the observed effect is specific to *nadl1.1* knockdown. These data suggest that *nadl1.1* may be another player in the Edn1 signaling pathway that regulates ventral cartilage formation.

We investigated the possibility that *nadl1.1* is another downstream effector of Edn1-mediated NC differentiation and cartilage morphogenesis that functions independently of Alcama. *nadl1.1* expression is not down-regulated in *edn1*$^{-/-}$ mutants as compared to WT at 30 hpf (Fig. S2E, F), indicating that *edn1* does not regulate *nadl1.1* at the transcriptional level. Hence, it is unlikely that *nadl1.1* functions in the

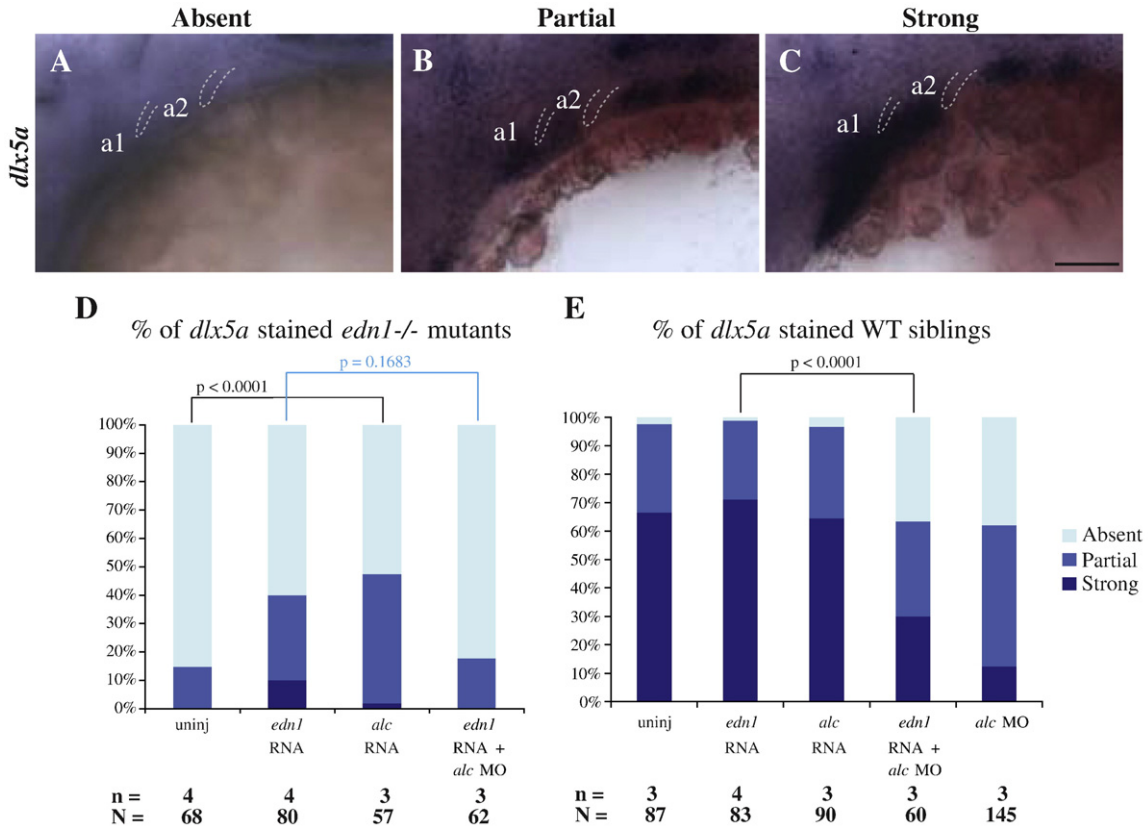

Fig. 5. *alcama* rescues NC differentiation defect in *edn1*$^{-/-}$ mutants. Lateral views of 30 hpf larvae with representative absent (A), partial (B) or strong (C) staining for *dlx5a*. Bar graphs show the percentage *dlx5a* expressing larvae in *edn1*$^{-/-}$ mutants (D) and WT (E) following the stated injections. *edn1* or *alcama* RNA was injected with or without *alcama* MO. *alcama* RNA decreases the percentage of affected individuals (absent *dlx5a* staining) by nearly 30% in *edn1*$^{-/-}$ mutants and co-injection of *alcam* Mo abrogates the ability of *edn1* RNA to rescue *dlx5a* expression in *edn1*$^{-/-}$ mutants. Indicated *p*-values are calculated by Fishers exact test. n depicts the number of experiments and N depicts the total number of larvae represented in the columns of the plot (*p*-value<0.0001 for both D and E by χ^2 analysis). a1 and a2 label pharyngeal arches 1 and 2. Dotted lines highlight pharyngeal pouches 1 and 2. Scale bar: 50 μm.

edn1-pathway independently of Alcama. These data show that loss of *alcama* and *nadl1.1* causes very similar defects in cartilage morphogenesis and NC differentiation, supporting our hypothesis that the two adhesion molecules might interact.

alcama and *nadl1.1* are expressed adjacent to each other in the endoderm and NC respectively (Fig. 7A), supporting our hypothesis that Nadl1.1 may propagate Alcama differentiation signal from the endoderm to NC. To bolster this hypothesis, we first asked if *alcama*-mediated rescue of *dlx5a* expression in *edn1*$^{-/-}$ mutants (Fig. 5) is abrogated by *nadl1.1* knockdown. Co-injection of *nadl1.1* MO with *alcama* RNA indeed blocked rescue of *dlx5a* expression in *edn1*$^{-/-}$ mutants (Fig. 7B), suggesting that Nadl1.1 expression is necessary for transmission of Edn1 signal through Alcama to NC.

To corroborate this observation we next tested possible interaction of Alcama and Nadl1.1 in synergy experiments by co-injecting suboptimal doses of *alcama* MO and *nadl1.1* MO into WT embryos. The read-out consisted of assessment of NC differentiation (*dlx5a* expression) and cartilage morphogenesis. While suboptimal doses of *alcama* and *nadl1.1* MOs alone do not cause significant changes in *dlx5a* expression or cartilage shape, co-injection resulted in synergistic increase in the number of larvae with down-regulated *dlx5a* expression and cartilage defects (Fig. 7C, D). This indicates that Alcama and Nadl1.1 interact during cartilage morphogenesis. Taken together with the ability of *nadl1.1* MO to block rescue of NC differentiation by Alcama over-expression in *edn1*$^{-/-}$ mutants, these data suggest that Alcama affects cartilage and NC differentiation by interacting with Nadl1.1.

Discussion

Our data shows for the first time that endodermally expressed Alcama is required for differentiation of NC and ventral pharyngeal cartilage morphogenesis. The *alcama*-deficient cartilage defect resembles that seen in *edn1*-class of mutants. In addition, *edn1* regulates Alcama protein levels in the pharyngeal endoderm. Moreover, similar to genes involved in Edn1 signaling, *alcama* is dispensible for NC patterning and migration, but is required for NC differentiation. Additionally, *alcama* over-expression partially rescues the NC differentiation defect in *edn1*$^{-/-}$ mutants, indicating that Alcama mediates Edn1 signaling to NC. Finally, we identify that Nadl1.1, an interacting partner of Alcama, is crucial for transmitting Alcama differentiation signal to NC and promoting ventral cartilage morphogenesis. These data lead us to propose a model (Fig. 8), whereby Edn1 signaling turns on *dlx* genes in the NC by two independent pathways. Edn1 binds to its receptor on NC to activate NC genes directly. In parallel, Edn1 binds to its receptor on the endoderm to stabilize Alcama protein, which in turn binds to Nadl1.1 on NC and further activates NC genes. Activation of both pathways is required for normal differentiation of NC and cartilage morphogenesis.

edn1 signals to NC by two parallel pathways

Edn1 is synthesized as a proprotein, which is cleaved twice by Furin and endothelin-converting enzyme resulting in a short, active Edn1 peptide. This short, active Edn1 peptide binds to the G protein-

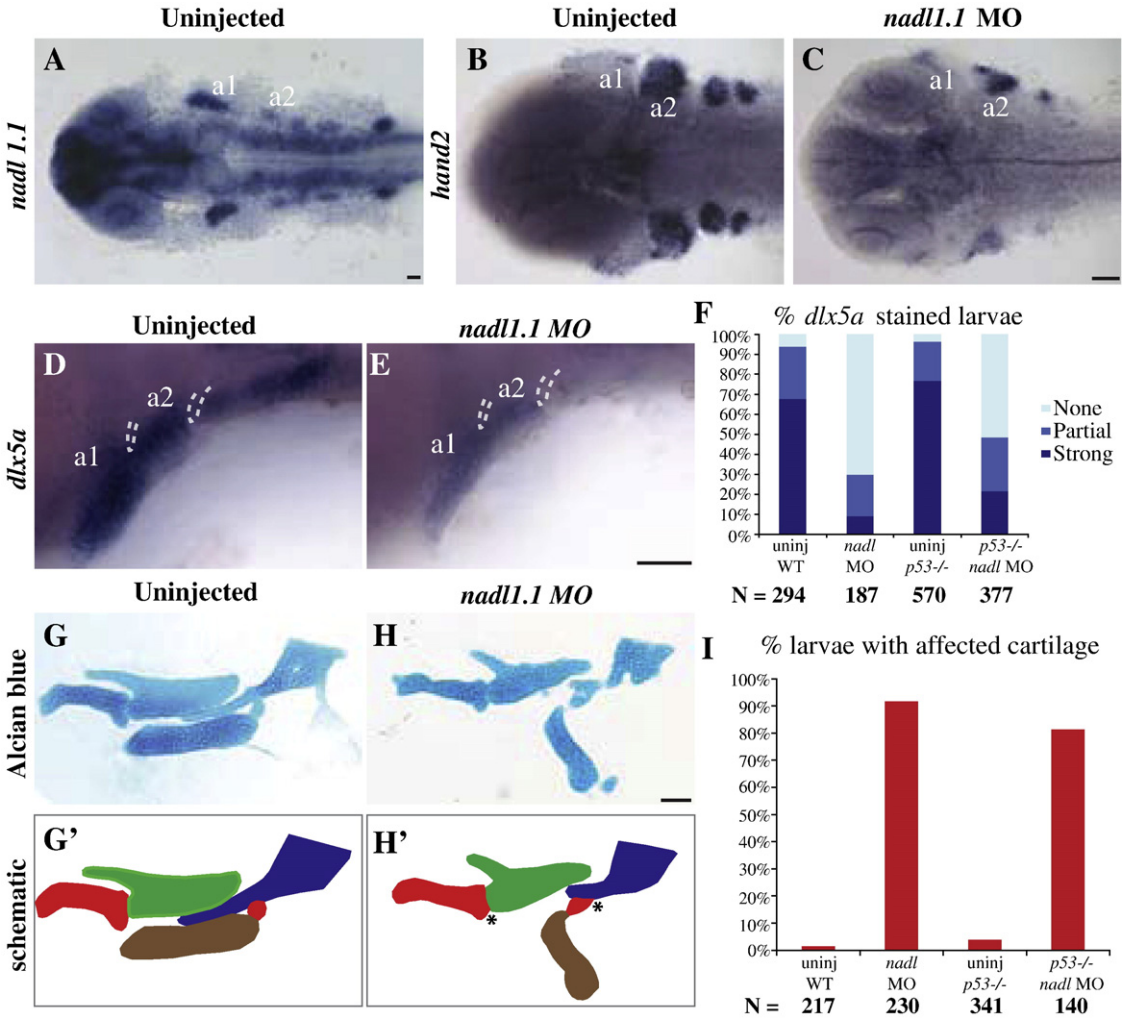

Fig. 6. *nadl1.1* morphants phenocopy *alcama* morphant cartilage defects. Dorsal views (A–C) and lateral views (D, E) of 30 hpf larvae. *nadl1.1* is expressed in the pharyngeal arches, diencephalon, telencephalon, hindbrain neurons, neural tube and pectoral fin in 30 hpf WT larvae (A). 30 hpf *nadl 1.1* morphants have down-regulated *hand2* (C) and *dlx5a* (E) expression as compared to uninjected WT (B, D). (F) Bar graph showing that the percentage of larvae with down-regulated (absent) *dlx5a* expression remains unchanged in the $p53^{-/-}$ background (p-value = 1.000 by Fishers exact test, indicating that WT and $p53^{-/-}$ mutants are not significantly different). Dissected mandibular and hyoid elements from 5 dpf WT and *nadl1.1* morphant larvae stained with Alcian blue (G, H) and their corresponding schematics (G', H'). The joint fusions in *nadl1.1* morphants (H, H') marked by * resemble those seen in *alcama* morphants. (I) Bar graph comparing the percentage of larvae with affected cartilage in *alcama* morphants in WT versus $p53^{-/-}$ mutants (p-value = 0.1941 by Fishers exact test, indicating that WT and $p53^{-/-}$ mutants are not significantly different). *N* is the total number of larvae from three experiments. Scale bars: 50 μm.

coupled Edn1 receptor. Phospholipase C enzyme further transmits the signal by producing inositol trisphosphate and diacylglycerol. Mouse mutants for Edn1, Ednra and the G-protein share skeletal defects with zebrafish mutants for *edn1*, *furinA* and *phospholipase C beta 3 (plcβ3)*; the ventral domains of the lower jaw are reduced in size and fused to the dorsal domains in both species (Clouthier et al., 1998, 2000; Dettlaff-Swiercz et al., 2005; Kurihara et al., 1994; Miller et al., 2000; Walker et al., 2006, 2007). Thus Edn1 signaling has been conserved during evolution to regulate ventral cartilage morphology.

Zebrafish $edn1^{-/-}$ and $plcβ3^{-/-}$ mutants have more severe ventral cartilage reductions and joint fusions than *alcama* morphants. The milder cartilage defect in *alcama* morphants correlates with the less pronounced reduction of *dlx*, *hand2* and *bapx1* gene expression in NC. We propose that this milder defect is due to two concomitant, additive pathways for *edn1* signaling; the first acts directly in the NC through the Edn1 receptor and the second activates Alcama signaling from the endoderm to the NC (Fig. 8). While both pathways are affected in *edn1* and *plcβ3* mutants, in *alcama* morphants direct signaling from the Edn1 receptor is still intact and can partially activate *dlx* gene expression in the NC, resulting in a milder phenotype.

Edn1 regulates alcama protein levels in pharyngeal endoderm

Previous data has implicated Edn1 as a regulator of the proteasome pathway (Rosano et al., 2005; Sato-Jin et al., 2008). Our data suggest that *edn1* stabilizes Alcama protein by inhibiting the proteasome pathway because inhibiting the proteasome pathway in $edn1^{-/-}$ mutants results in increased Alcama levels. It is still unknown whether Edn1 stabilizes Alcama by inducing a structural change that prevents polyubiquitination, or indirectly by regulating transcription of proteasome subunits or by activation of de-ubiquitinases. Alternatively, Alcama may not be a direct target of the proteasome pathway; instead a chaperone protein that stabilizes Alcama might be the target of proteasome pathway. An alternative possibility is that Edn1 signals to NC and thus induces Alcama stabilization indirectly. Further investigation into this mechanism will broaden our understanding of the process of NC differentiation.

Further inquiry is also needed into other proteins/signals regulating *alcama* expression. Although *edn1* RNA injections and MG132 treatment led to higher Alcama expression, in both cases expression was mostly restricted to the endoderm and not the NC. This data

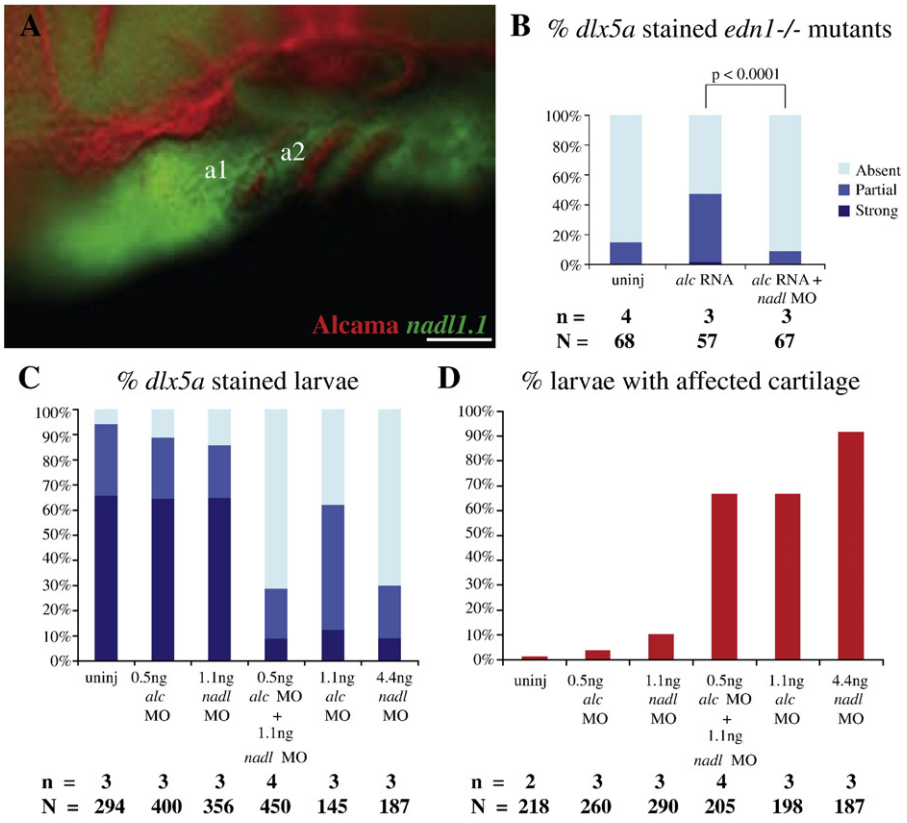

Fig. 7. *alcama* and *nadl1.1* interact during cartilage formation. (A) Lateral view of a 30 hpf WT larva stained with Zn-5 antibody (Fast Red) and anti-sense *nadl1.1* RNA (Fluorescein), revealing that *nadl1.1* is expressed in the NC adjacent to *alcama* in the endoderm. The bar graphs display the percentage of 30 hpf *edn1*$^{-/-}$ (B) and WT (C) larvae with *dlx5a* staining after the indicated injections. (B) shows that *nadl1.1* is required for *alcama* rescue of *dlx5a* expression in *edn1*$^{-/-}$ mutants (p-value<0.0001 by Fishers exact test). (C) and (D) show that *alcama* and *nadl1.1* interact during *dlx5a* expression and cartilage morphogenesis. (D) Bar graph displaying the percentage of 5 dpf larvae with affected cartilage after the indicated injections (p-value<0.0001 by χ^2 analysis). *n* indicates the number of experiments and *N* the total number of larvae. Scale bar: 50 μm.

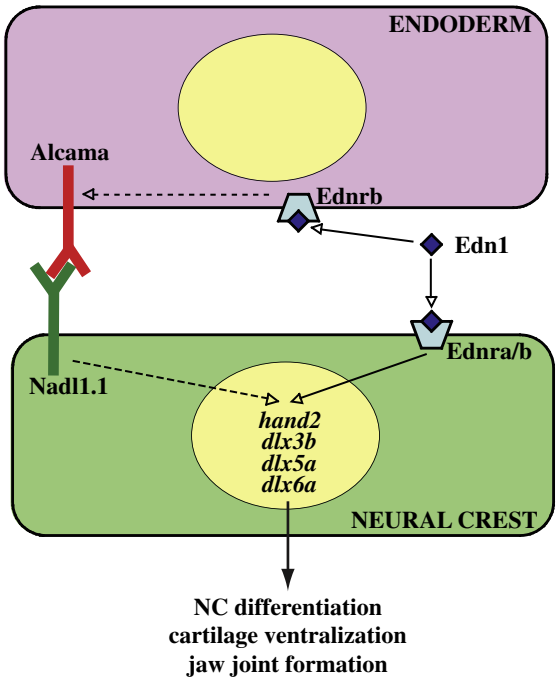

Fig. 8. Model demonstrating how Alcama mediates Edn1 signaling and NC differentiation. Previous data has supported the model that Edn1 peptide binds to its G-protein-coupled receptor on NC to turn on transcription of NC genes. We propose a parallel pathway by which Edn1 signaling stabilizes Alcama protein in endodermal cells. Alcama, in turn, binds to Nadl1.1 in NC and regulates NC differentiation.

points to an endoderm-specific role of Alcama in cartilage formation. Similarly, *edn1* and *alcama* RNA injections into the one-cell stage induced rescue of *dlx5a* expression, specifically in NC. Further investigation of these mechanisms, that restrict Alcama expression to endoderm and *dlx* genes to NC, is needed to gain a more complete understanding of the process of chondrogenesis.

Alcama interacts with Nadl1.1 on the NC to mediate differentiation signals

We established that Nadl1.1, a known interacting partner of Alcama expressed in NC, is important for cartilage morphogenesis and its knockdown results in similar cartilage defects to *alcama* knockdown. We also demonstrated that *nadl1.1* is necessary for Alcama-mediated rescue of NC differentiation in *edn1*$^{-/-}$ mutants and that Alcama and Nadl1.1 interact during cartilage formation, either directly or indirectly. Hence we concluded that Nadl1.1 possibly transmits Alcama-mediated Edn1 signaling to the NC. Future studies will focus on how Nadl1.1 activates *dlx* genes in the NC.

One possible function for Alcama and Nadl1.1 interaction during NC differentiation and cartilage morphogenesis may be to simply maintain cell–cell contacts between the endoderm and NC. Support for this hypothesis is provided by the *integrinα5* mutant and the Eph-ephrin system. Integrins are required for cell adhesion and migration in many tissues (Benoit et al., 2009; Brakebusch and Fassler, 2005). The zebrafish *integrinα5* mutant has defects in formation of the first pouch, resulting in defective compaction and survival of NC cells adjacent to the first pouch, giving rise to a deformed hyoid cartilage (Crump et al., 2004). Eph genes encode tyrosine kinase receptors for ephrin ligands. Eph-ephrin mediated repulsion establishes polarity and boundaries during development (Holder and Klein, 1999; Robinson et al., 1997). Since EphA3 is expressed

in ventral NC, it is also postulated that Eph-ephrin repulsion may play a role in separating the dorsal and ventral cartilages, giving rise to the joint between them (Kimmel et al., 2001). Hence, both adhesive (integrins) and repulsive forces (ephrins) play a role in NC differentiation. By analogy it is conceivable that Alcama-Nadl1.1 mediated adhesion between the endoderm and NC may play a similar role in chondrogenesis.

Conclusion

In summary, this is the first demonstration that Alcama, a commonly used marker for endoderm, plays a critical role in NC differentiation and cartilage morphogenesis. We show that Edn1 regulates Alcama levels in vivo and that Alcama functions downstream of Edn1 during cartilage formation. In addition, we provide support for a mechanism by which Alcama mediates Edn1-signaling by interacting with Nadl1.1 on NC cells.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.11.006.

Acknowledgments

The authors wish to thank Raju Kucherlapati and Len Zon for material and technical support, Sarah Hutchinson for intellectual contributions, and Gavin Wright (and his student) for technical expertise. We wish to thank Chuck Kimmel for Alcian Blue-stained *furina*$^{-/-}$ mutant zebrafish, and Gage Crump for *dlx* plasmids. This work was supported in part by R01 HD047863-01 and by the Huntsman Cancer Foundation. Huntsman Cancer Institute core facilities, supported by grant P30 CA042014, also facilitated this work.

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