Emilin3 is required for notochord sheath integrity and interacts with Scube2 to regulate notochord-derived Hedgehog signals

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ABSTRACT
The notochord is a transient and essential structure that provides both mechanical and signaling cues to the developing vertebrate embryo. In teleosts, the notochord is composed of a core of large vacuolated cells and an outer layer of cells that secrete the notochord sheath. In this work, we have identified the extracellular matrix glycoprotein Emilin3 as a novel essential component of the zebrafish notochord sheath. The development of the notochord sheath is impaired in Emilin3 knockdown embryos. The patterning activity of the notochord is also affected by Emilin3, as revealed by the increase of Hedgehog (Hh) signaling in Emilin3-depleted embryos and the decreased Hh signaling in embryos overexpressing Emilin3 in the notochord. In vitro and in vivo experiments indicate that Emilin3 modulates the availability of Hh ligands by interacting with the permissive factor Scube2 in the notochord sheath. Overall, this study reveals a new role for an EMILIN protein and reinforces the concept that structure and function of the notochord are strictly linked.

KEY WORDS: Extracellular matrix, EMILIN, Notochord, Hedgehog, Scube2, Zebrafish

INTRODUCTION
EMILINs (elastin microfibril interface located proteins) are extracellular matrix (ECM) glycoproteins belonging to the EMILIN/multimerin family. Emilin1, the founding member of this family, was originally identified as an ECM component associated with microfibrils in blood vessels (Bressan et al., 1993). Emilin3 contains the characteristic cysteine-rich EMI domain at the N-terminal end, followed by a region forming three coiled-coil structures at the C-terminal end (Schiavinato et al., 2012). At variance with the other EMILIN/multimerin proteins, Emilin3 is not expressed in the cardiovascular system and it lacks the C-terminal gC1q domain, which is involved in cell attachment and integrin binding (Spessotto et al., 2003; Danussi et al., 2011). Emilin3 expression during mouse development is particularly abundant in the perichondrium of developing bones (Leimeister et al., 2002; Doi et al., 2004; Schiavinato et al., 2012). We have previously found that eight EMILIN/multimerin genes are present in the zebrafish genome in four pairs of duplicated paralogs (Milanetto et al., 2008). Thus, Emilin3 is present in zebrafish with two genetic paralogs that we termed emilin3a and emilin3b. Similarly to the murine Emilin3 ortholog, the two zebrafish paralogs are not expressed in the cardiovascular system, whereas they are abundantly expressed in the notochord and in the chordoneural hinge until 24 hpf, and in cartilage primordia of the developing craniofacial skeleton at 48 hpf (Milanetto et al., 2008).

In the present study, we focused on the role of the two zebrafish Emilin3 genes during notochord development. During the early phases of embryonic development, until the segmentation stage, the notochord is distinguishable from adjacent tissues by the expression of specific genes, such as shh, ehh and col2a1 (Yan et al., 1995; Currie and Ingham, 1996). As the notochord develops, expression of these early-transcribed genes is turned off and notochord cells differentiate in two different cell populations, an outer sheath layer and an inner vacuolated cell layer. The choice between these two fates is determined by Notch signals (Yamamoto et al., 2010). The outer cell layer is responsible for the synthesis of the notochord sheath, a thick peri-notochordal basement membrane that is composed of several ECM proteins, whereas the inner cells contain large intracellular vacuoles. Recently, the nature of these vacuoles was elegantly identified as lysosome-related organelles (Ellis et al., 2013). Both the notochord sheath and the vacuolated cells are essential for conferring the proper stiffness and rigidity to the notochord (Stemple, 2005).

Several studies in zebrafish showed that the formation of a notochord sheath is closely linked to the differentiation of notochord cells, and their reciprocal interactions are fundamental for the proper development and function of the notochord itself (Parsons et al., 2009; Pagnon-Minot et al., 2008; Mangos et al., 2010; Yamamoto et al., 2010). Here, we provide evidence that Emilin3 is a novel component of the notochord sheath that plays an essential role for the correct organization and the proper function of this structure. Moreover, we found that Emilin3 has a physiological role for limiting notochord-derived Hh signals, a function entailing the interaction of Emilin3 with the secreted protein Scube2.

RESULTS
Emilin3 is a novel essential component of the notochord sheath
To study Emilin3 protein distribution in the zebrafish embryo, we carried out whole-mount immunofluorescence with a polyclonal Emilin3 antibody. At 20 hpf, a specific signal was present throughout the notochord with a distinct posteroanterior gradient, whereas no labeling was detected in embryos at eight somites (data not shown; see also supplementary material Fig. S5D). At 24 hpf, Emilin3 became restricted to the notochord sheath, as confirmed by colocalization with collagen II. In agreement with mRNA expression data (Milanetto et al., 2008), labeling of the notochord sheath was lost at 48 hpf, indicating that Emilin3 undergoes a rapid turnover in the ECM and may fulfill a temporally restricted function during notochord development (Fig. 1A).

To investigate the role of Emilin3 during notochord development, we injected one- to two-cell-stage zebrafish embryos...
with antisense morpholino oligonucleotides targeting the splice sites between the first exon and the first intron of *emilin3a* and *emilin3b* genes. Co-injection of the two morpholinos resulted in the knockdown of the corresponding transcripts and proteins (supplementary material Fig. S1A,B), leading to a distinct distortion of the notochord with partial loss of the V-shaped conformation of somites and shortening of the main axis (Fig. 1B). Embryos injected with translation-blocking morpholinos for *emilin3a* and *emilin3b* showed a similar phenotype, and the same result was found in mixed experiments where a splice-blocking morpholino for one paralog was co-injected with a translation-blocking morpholino for the other paralog (supplementary material Fig. S1C; supplementary material Table S1). Injection of morpholinos targeting only one paralog did not cause a similar phenotype, even when injected at double concentration (Fig. 1B; supplementary material Table S1). As a further control, we performed p53 knockdown and found that co-injection of p53 morpholino was ineffective in rescuing the phenotypic defects induced by Emilin3 depletion (supplementary material Fig. S1D). Two different mechanisms could be inferred for the notochord phenotype of Emilin3 double morphants: (1) an increased pressure generated inside the notochord; or (2) a disruption of the sheath organization.

Quantification of total and vacuolated notochord cells in control and Emilin3 double morphant embryos did not reveal any significant difference, suggesting that Emilin3 deficiency is not grossly perturbing the morphology and commitment of notochord cells (Fig. 1C,D; supplementary material Fig. S2). Notch signaling is known to regulate the fate choice between vacuolated and non-vacuolated notochord cells (Yamamoto et al., 2010). Using the Tg_Hbb:EGFP Notch reporter zebrafish line (Parsons et al., 2009), we did not detect any major difference of Notch signaling activity in notochord cells of control and Emilin3 double morphant embryos (supplementary material Fig. S3).
We next investigated the structure of the notochord sheath by electron microscopy. At 30 hpf, in Emilin3 double morphants, but not in Emilin3 single morphants, the medial layer of the notochord basement membrane was not properly organized and many fibers appeared interrupted, whereas the outer and inner layers appeared normal (Fig. 1E). The medial and inner layers of the notochord sheath are thought to consist of collagen and laminin, respectively (Stemple, 2005). In situ hybridization and immunofluorescence showed that collagen II was more abundant in Emilin3-depleted embryos at both 24 and 48 hpf (Fig. 1F; supplementary material Fig. S4A,B), whereas laminin labeling was normal (supplementary material Fig. S4C). To exclude the possibility that the phenotype of Emilin3 morphants could be caused by an upregulation of collagen II, we performed concurrent knockdown of emilin3a, emilin3b and col2a1. Knockdown of col2a1 caused ventral body curvature as expected (Mangos et al., 2010), whereas simultaneous knockdown of the three genes resulted in the overlap of the two phenotypes (Fig. 1G). Altogether, these data reveal that Emilin3 is essential for the normal structure of the notochord sheath.

**Emilin3 knockdown results in the upregulation of Hh signaling**

Next, we investigated whether knockdown of Emilin3 could affect notochord patterning activity, a process that is largely mediated by the secretion of Hh ligands (Stemple, 2005). Expression of ptc1 (ptch2 – Zebrafish Information Network), which is transcriptionally regulated by Hh signaling (Concordet et al., 1996), was strongly increased in the trunk of 24 hpf Emilin3 morphants and this effect was completely blocked by treatment with 100 μM cyclopamine, an inhibitor of Hh signaling, but was unaffected by p53 (Tp53 – Zebrafish Information Network) knockdown (Fig. 2A; supplementary material Fig. S5A,B). To assess whether ptc1 upregulation was specifically due to Emilin3 ablation, we analyzed ptc1 expression at eight somites, a stage where Emilin3 proteins are not yet present in the sheath. Consistently, at this stage, ptc1 was not differently expressed in Emilin3 morphants (supplementary material Fig. S5C,D).

Next, we generated the Tg12x_Gli zebrafish line, which express the mCherry reporter under the transcriptional regulation of Gli1 (see Materials and methods). We co-injected emilin3a and emilin3b morpholinos in Tg12x_Gli embryos and investigated expression of the transgene at 24 hpf. Notably, Emilin3 morphants displayed a remarkable increase of transgene expression (Fig. 2A), as also confirmed by qPCR experiments (Fig. 2B). We investigated the expression of other genes that respond to notochord-derived Hh signals in different embryonic structures. Expression of nkx2.2a, tbx20 and myoD1 was consistently increased in Emilin3-depleted embryos (Fig. 2C). Similarly, olig2, vegf and eng2a were upregulated (supplementary material Fig. S6A). Overexpression of Hh ligands by notochord cells can lead to hyperactivation of the Hh pathway (Yamamoto et al., 2010). At 20 somites, shh and ehh, the only two Hh ligands produced in the notochord, were not Fig. 2. Hh signaling is upregulated in Emilin3 morphant embryos. (A) Wild-type (WT) and Tg12x_Gli embryos were treated with 100 μM cyclopamine (cyc) and probed at 24 hpf by in situ hybridization for ptc1 or mCherry. (B) qRT-PCR for ptc1 and mCherry transcripts in Emilin3 morphant and control embryos (*P<0.05; n=30). (C) In situ hybridization for nkx2.2a, tbx20 and myoD1 in 24 hpf injected embryos. All images are lateral views, except for bottom panels (dorsal views). (D) In situ hybridization for shh, ehh and ntl in 24 hpf injected embryos. (E) Immunofluorescence for engrailed in 24 hpf injected embryos. (F) Quantification of engrailed-positive cells at 24 hpf (*P<0.05; n=10). Data are mean+s.e.m. Ctrl MO, control morpholino; e3a MO, emilin3a morpholino; e3b MO, emilin3b morpholino; e3a+e3b MO, emilin3a + emilin3b morpholinos; MPs, muscle pioneers; MFFs, medial fast fibers.
differently expressed in Emilin3 morphants when compared with control embryos (data not shown), whereas at 24 hpf they were upregulated in the distal part of the notochord (Fig. 2D). Therefore, the sole upregulation of shh and ehh did not explain the increased Hh activity in Emilin3 morphants. Moreover, expression of ntl and of the notochord-unrelated gene fgf8 was not affected, thus excluding a developmental delay (Fig. 2D; supplementary material Fig. S6B).

We quantified engrailed-positive cells and found that both muscle pioneers (MPs) and medial fast fibers (MFFs) were significantly increased in Emilin3 morphants (Fig. 2E,F). It is known that engrailed expression in the zebrafish myotome is negatively regulated by BMP signaling (Du et al., 1997; Dolez et al., 2011), and high levels of Hh activity can prevent the accumulation of activated Smads in the medial part of the myotome (Maurya et al., 2011). Consistently, we found that expansion of the engrailed-positive domain in Emilin3 morphants was paralleled by a reduction of the pSmad1/5/8 domain in the medial part of the myotome. Treatment with low doses of cyclopamine was sufficient to rescue pSmad distribution in the somites of Emilin3 morphants, indicating that Hh pathway is primarily affected by Emilin3 knockdown (supplementary material Fig. S7A). To exclude the possibility that the observed effect on Hh signaling could be mediated by the pro-TGFβ inhibitory activity of Emilin3 (Schiavinato et al., 2012), we treated morphant embryos with two different selective inhibitors of TGFβ type I receptors, SB431542 and LY364947. Neither of these inhibitors was effective in decreasing ptc1 expression, and pharmacological inhibition of TGFβ activity also exacerbated notochord distortion in Emilin3 morphants (supplementary material Fig. S7B).

Emilin3 overexpression leads to downregulation of Hh target genes
To investigate whether upregulation of the Hh pathway was directly caused by Emilin3 deficiency or was a broader effect caused by disruption of the notochord sheath, we analyzed ptc1 expression in emilin3a and emilin3b single morphants. Interestingly, albeit to a lesser extent than in Emilin3 double morphants, ptc1 expression was increased in the trunk of single morphants (Fig. 3A). To further assess the role of Emilin3 in notochord-derived Hh signaling, we generated a pCS-twhh-mEmilin3 construct carrying murine full-length Emilin3 cDNA under the control of a promoter that confers notochord-specific expression (Du et al., 1997). Embryos injected with this construct developed normally but at 24 hpf they displayed mild notochord defects and a shorter axis, when compared with embryos injected with a control pCS-twhh-β-gal construct (Fig. 3B). Notably, embryos injected with pCS-twhh-mEmilin3 showed impaired myoD1 expression, together with reduced number of engrailed-positive cells. Emilin3 overexpression in the notochord did not influence ehh transcription, indicating that expression of Hh ligands by notochord cells was not grossly affected (Fig. 3B,C). Moreover, injection of the pCS-twhh-mEmilin3 construct also rescued the effects of morpholino-mediated ablation of Emilin3 both on notochord development and Hh signaling (supplementary material Fig. S8).

Emilin3 functionally interacts with Scube2
As Emilin3 paralogs are expressed by notochord cells with a pattern closely similar to that of Hh ligands, we speculated that Emilin3 could influence the rate of synthesis and/or release of Hh proteins. Interestingly, when Emilin3 and Shh were transiently co-expressed in HEK293T cells, the amount of Shh released in media was
reduced (Fig. 4A). This effect was not dependent on the EMI domain and it was lost when Emilin3 was co-expressed with ShhN, a form of Shh lacking cholesterol modification (Fig. 4B,C). Co-immunoprecipitation experiments failed to reveal any interaction between Emilin3 and Shh (Fig. 4D). Therefore, we hypothesized that Emilin3 could interact with a factor secreted by HEK293T and involved in Shh release. To test this hypothesis, we used conditioned media derived from HEK293T transiently transfected with empty vector or with Emilin3 construct to stimulate the release of Shh from stably transfected Shh-293 cells. Interestingly, the conditioned medium derived from mock-transfected cells strongly increased Shh release compared with fresh medium, whereas medium from Emilin3-transfected cells induced a much lower rate of Shh release (supplementary material Fig. S9).

Recent studies demonstrated a role for the secreted protein Scube2 in Shh release (Creanga et al., 2012; Tukachinsky et al., 2012). We therefore investigated whether Emilin3 could interact with Scube2. When HEK293T cells were co-transfected with Emilin3 and FLAG-tagged Scube2, Emilin3 was co-precipitated with Scube2 from media (Fig. 4E). Similarly, FLAG-tagged Scube2 was efficiently immunoprecipitated by HA-tagged Emilin3 (supplementary material Fig. S10A). This interaction was confirmed by in vitro-binding experiments and did not require the EMI domain or the coiled-coil region of Emilin3 (Fig. 4F,G). A similar in vitro-binding experiment showed that the EGF repeats of Scube2 are sufficient for Emilin3 binding (supplementary material Fig. S10B). To investigate whether the interaction of Emilin3 with Scube2 has any functional significance, we treated transfected HEK293T cells with conditioned media derived from Scube2-transfected cells. Notably, treatment with Scube2-conditioned media, as well as Scube2 co-transfection, were able to rescue the effect of Emilin3, whereas treatment with soluble heparin was ineffective (Fig. 4H; supplementary material Fig. S10C-E). We also prepared conditioned media derived from HEK293T transiently transfected with Scube2 alone or in combination with Emilin3, and found that the latter was less effective in releasing Shh from 293-Shh cells (Fig. 4I).

**Fig. 4.** Emilin3 functionally interacts with Scube2. (A-C) HEK293T were transiently co-transfected with the indicated constructs. Conditioned serum-free media and cell lysates were analyzed by western blot. (D) Cell lysates of HEK293T transfected with the indicated plasmids were analyzed by western blot or subjected to immunoprecipitation. (E-G) HEK293T were either co-transfected (E,G) or separately transfected (F) with the indicated plasmids. Media (E,F) and cell lysates (G) were analyzed by western blot or subjected to immunoprecipitation. (H) Transfected HEK293T were incubated for 24 hours with conditioned media derived from control or Scube2-transfected cells. (I) Stably transfected 293-Shh cells were incubated for 6 hours with fresh medium (DMEM) or with conditioned media derived from HEK293T co-transfected with the indicated plasmids. Media and cell lysates were analyzed by western blot. (J) Lateral views of 24 hpf embryos injected with the indicated morpholinos and immunostained for engrailed. Scale bar: 50 μm. (K) Quantification of engrailed-positive cells in injected embryos (*P<0.05; n=6). Data are mean+s.e.m. CM, conditioned media; E3, murine full-length Emilin3; E3Δ1, murine Emilin3 lacking the EMI domain; E3Δ2, murine Emilin3 lacking the EMI domain and the coiled-coil region; ev, empty vector; IP, immunoprecipitation; PTC1, human FLAG-patched-1; WB, western blot; Ctrl MO, control morpholino; e3a+e3b MO, Emilin3a+Emilin3b morpholinos; Scube2 MO, Scube2 morpholino; MPs, muscle pioneers; MFFs, medial fast fibers.
Finally, to investigate whether the interaction of Emilin3 with Scube2 was relevant in vivo, we injected embryos with morpholinos directed against emilin3a/emilin3b and scube2, as well as their combination. Emilin3 knockdown resulted in a robust increase of both MFFs and MPs, whereas Scube2 morphants lacked engrafted-positive cells as expected (Woods and Talbot, 2005). Interestingly, although co-injection of the three morpholinos resulted in the overlap of the two phenotypes (supplementary material Fig. S11A), the number of engrafted-positive cells was rescued almost to wild-type levels in triple morphants (Fig. 4J,K). qRT-PCR experiments showed that Scube2 expression was slightly, albeit not significantly, upregulated in Emilin3-depleted embryos (supplementary material Fig. S11B). Overall, these results support a physiological role for Emilin3 in limiting the activity of Scube2 on the release of Hh ligands by notochord cells.

**DISCUSSION**

In this work, we provided the first in vivo functional characterization of Emilin3, revealing both structural and functional roles for notochord development in zebrafish. Electron microscopy showed that Emilin3 is required for proper structural organization of the peri-notochordal basement membrane. In the absence of Emilin3, the sheath is not resistant enough to withstand the pressure generated by vacuolization of notochord cells, and this defect provides an explanation for the notochord distortion we observed in Emilin3 morphants.

Interestingly, the notochord phenotype of Emilin3 double morphant embryos is similar to that described for other ECM components. In particular, the ultrastructural alterations we found in the medial layer of the notochord sheath are reminiscent of those described for the zebrafish gulliver mutants, which carry a missense mutation in the globular C1q domain of the colba1 gene (Gansner and Gitlin, 2008). Intriguingly, unlike other EMILIN proteins, this domain is missing in Emilin3. It has been shown that the globular C1q domain is involved in the supramolecular organization of EMILINS by interacting with the EMI domain (Doliana et al., 2000), thus suggesting the possibility that Emilin3 and collagen VIII are interacting partners in the medial layer of the notochord sheath.

Our data also support previous findings, which indicated that defects in the notochord sheath can influence the gene expression pattern of notochord cells. However, at odds with what is observed in other models of notochord disruption (Parsons et al., 2002; Pagnon-Minot et al., 2008; Yamamoto et al., 2010), we found that col2a1 and Hh ligands are not co-regulated. Indeed, in Emilin3 double morphants, col2a1 expression is upregulated throughout the length of the notochord, whereas shh and ehh are overexpressed only in the expression field of emilin3a and emilin3b, at the level of the chordoneural hinge. Conversely, laminin mutants and collagen XV morphants display a similar upregulation of col2a1 and ehh throughout the notochord (Parsons et al., 2002; Pagnon-Minot et al., 2008). This implies that notochord cells can respond to extracellular signals in a region-specific way.

The second function we report here for Emilin3 concerns the regulation of notochord-derived Hh signals. We show that Emilin3 interacts with Scube2 in the extracellular environment. Recently, Scube2 has been identified as a secreted, permissive factor, acting non-cell autonomously in the release of lipidated Shh from producing cells (Creanga et al., 2012; Tukachinsky et al., 2012). Scube2 is a multi-domain protein, with a signal peptide sequence at the N-terminal end followed by nine EGF repeats, one spacer region, three cysteine-rich motifs and one CUB domain at the C-terminal end. The CUB domain was found to interact with Shh and

**Fig. 5. Hypothetical model of Emilin3 and Scube2 interaction in the notochord sheath.** Emilin3 is located in the extracellular matrix of the notochord sheath and interacts with Scube2, a secreted factor that mediates the release of lipid-modified Hh ligand. In wild-type embryos, Emilin3 ensures the integrity and function of the notochord sheath, which is necessary for proper notochord patterning activity. Emilin3 deficiency results in the disruption of the notochord sheath and affects Scube2 localization and activity in the extracellular milieu, thus leading to increased Hh signals.

**MATERIALS AND METHODS**

**Zebrafish lines**

Wild-type (AB/TU) zebrafish were maintained as described previously (Westerfield, 1995). For the generation of the Tg(12xGli-HSVTK:nsfCherry)ia10 zebrafish line (Tg12xGli, Gil), a cassette containing twelve multimerized Gli1-binding sites was isolated from the 12GLI-RE-TKO-luc construct (Kogerman et al., 1999) and ligated with the Herpes simplex virus thymidine kinase minimal promoter in the p5E-MCS Tol2 5′ entry vector. Positive clones were sequenced in both strands and recombined with a middle entry vector carrying the mCherry open reading frame and a 3′ entry vector carrying a SV40 poly-A tail. Entry plasmids were finally recombined into the pTol2 destination vector as previously described (Kwan et al., 2007). A total of 25 pg of Tol2 recombinant plasmids and 25-50 pg of in vitro synthesized transposase mRNA (Kawakami et al., 2004) were co-injected into zebrafish embryos at the one- to two-cell stage. Microinjected embryos were raised to adulthood and outcrossed to wild-type fish. Six out of 30 screened fish were identified as founders for the reporter line. The Tg(EYFP:TP1-Mmu.Hbb:EGFP)ia12 zebrafish line (Tg_Hbb:EGFP) has been previously described (Parsons et al., 2009).
Zebrafish embryo injection

Sequences of the morpholinos used in the study are reported in supplementary material Table S2. All morpholinos were splice blocked, except where indicated. Morpholinos were dissolved in 1×Danieu’s buffer and injected at the doses reported in supplementary material Table S1. p53 morpholino (Robu et al., 2007) and Scube2 morpholino (Woods and Talbot, 2005) were injected at 6 ng/embryo and 2 ng/embryo, respectively. For DNA microinjection, linearized DNA was dissolved in deionised H2O to a final concentration of at 50 ng/µl (Du et al., 1997), except where indicated. Murine Emilin3 cDNA was cloned into the pCS-twhh-fgal vector (Du et al., 1997).

Pharmacological treatments

Cycloamine hydrate (Sigma) was dissolved in DMSO and embryos were incubated at the indicated concentrations beginning at shield stage until fixation at 24 hpf. LY364947 (Zhou et al., 2011) and SB431542 (Ho et al., 2006) (Sigma) were dissolved in DMSO and diluted into embryo medium to a final concentration of 30 µM and 100 µM, respectively, and embryos were incubated with these compounds from 8 hpf until fixation at 24 hpf. Embryos incubated with DMSO were used as control.

Probes and plasmids

For in situ hybridization experiments, the used riboprobes were: emilin3a and emilin3b (Milanetto et al., 2008), col2a1 (Yan et al., 1995), ndl (Shulte-Merker et al., 1994), shh (Krauss et al., 1993), ehh (Currie and Ingham, 1996), ptc1 (Concordet et al., 1996), nks2.2a (Barth and Wilson, 1995), tbx20 (Ahn et al., 2000), vegf (Liang et al., 1998), olig2 (Park et al., 2002), eng2a (Ekker et al., 1992), and fgf8 (Reiers et al., 1998). The cDNA constructs for murine full-length Emilin3 and EMILIN-3Δ constructs for murine full-length Emilin3 and EMILIN-3Δ constructs (amino acid residues 54-189) and the coiled-coil region (amino acid residues 534-758), was prepared by PCR using the EMILIN-3Δ construct, which lacks amino acid residues 54-189, has been described previously (Schiavinato et al., 2012). Murine EMILIN-3Δ2 cDNA construct, which lacks both the EMI domain (amino acid residues 54-189) and the coiled-coil region (amino acid residues 534-758), was prepared by PCR using the EMILIN-3Δ1 construct as a template and the following primers: SP6 (forward) and 5’-CTAAACGCTTGAACATCGTATGGTGACAGGCCCTACCCCTTGCA: (reverse). The EMILIN-3-ΔA cDNA construct was generated by adding the human influenza hemagglutinin (HA) tag sequence at the C-terminal end of murine full-length Emilin3. Murine full-length Shh, ShhN and Scube2 cDNAs were obtained by PCR and cloned into the pcDNA3+ vector. The Scube2-FLAG construct was generated by adding the FLAG tag sequence at the C-terminal end of murine Scube2. The EGF-FLAG construct, encompassing amino acids 1-479 of murine Scube2, was generated by PCR using the Scube2 full-length construct as a template with the following primers: 5’-GTTGTCGACTGATGGGAAAAGGGACGACCACTCCTACCCCTTGCA: (forward) and 5’-TTCTCAGTACACTGTCTGTACATGCCCTTCTGCTGTACTGCTGACGAGA-GTTGATGCCC (reverse, coding for the FLAG tag sequence). The PCR product was then subcloned into the pcDNA3.1+ vector, followed by selection with G418 (Invitrogen) at a concentration of 400 µg/ml.

Western blot and immunoprecipitation

Cells and conditioned media were harvested 24 hours (western blot) or 72 hours (immunoprecipitation) after incubation in serum-free medium. Cells were lysed in ice-cold lysis buffer [25 mM Tris (pH 7.5), 150 mM NaCl, 2.5 mM EDTA, 10% glycerol, 1% Nonidet P-40] supplemented with proteases inhibitor (Roche) and media were TCA precipitated. Immunoprecipitation, SDS-PAGE and western blot were performed as previously described (Schiavinato et al., 2012). Used antibodies were: rabbit polyclonal anti-Emilin3 (Schiavinato et al., 2012), rabbit polyclonal anti-Shh (Santa Cruz), mouse monoclonal anti-FLAG (Sigma) and mouse monoclonal anti-HA (Sigma).

Statistical analysis

Data are expressed as mean±s.e.m. Statistical significance was determined by unequal variance Student’s t-test. P<0.05 was considered significant.

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Competing interests

The authors declare no competing financial interests.

Author contributions

D.C. and A.S. conceived and performed the majority of the experiments, and analyzed data. V.T. performed experiments and analyzed data. E.M. and F.A. provided zebrafish transgenic lines, contributed new reagents/analytic tools. P.B. and A.S. designed the study, analyzed data and wrote the paper. All authors discussed the results and commented on the manuscript.

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Supplementary material

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