

RESEARCH ARTICLE

Late Alk4/5/7 signaling is required for anterior skeletal patterning in sea urchin embryos

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ABSTRACT

Skeletal patterning in the sea urchin embryo requires a conversation between the skeletogenic primary mesenchyme cells (PMCs) and the overlying pattern-dictating ectoderm; however, our understanding of the molecular basis for this process remains incomplete. Here, we show that TGF- β -receptor signaling is required during gastrulation to pattern the anterior skeleton. To block TGF- β signaling, we used SB431542 (SB43), a specific inhibitor of the TGF- β type I receptor Alk4/5/7. Treatment with SB43 during gastrulation blocks anterior PMC positioning and the formation of the anterior skeleton, but does not perturb general ectoderm specification or development. This is the first example of a signaling event required for patterning of a specific part of the skeleton. Alk4/5/7 inhibition does not prevent the formation of a mouth, although SB43-treated plutei display reduced feeding ability, presumably due to the loss of the structural support for the mouth conferred by the anterior skeleton. Both Univin and Nodal are potential ligands for Alk4/5/7; however, Nodal is unilaterally expressed on only the right side, whereas Univin is bilaterally expressed in the ectoderm adjacent to the anterior skeleton during the relevant time period. Our results demonstrate that Univin is both necessary and sufficient for secondary skeletal development in a control background, consistent with the hypothesis that Univin is a relevant Alk4/5/7 ligand for anterior skeletal patterning. Taken together, our data demonstrate that Alk4/5/7 signaling during gastrulation is required to direct PMCs to the oral hood, and suggest that Univin is a relevant ligand for this signaling event.

KEY WORDS: Sea urchin embryo, TGF- β , Skeletal patterning, Univin

INTRODUCTION

The sea urchin larval skeleton is an excellent model for embryonic pattern formation, as it requires instructive interactions between two distinct cell types. The skeleton is secreted by mesenchymal cells, which are instructed by the adjacent ectoderm to conform to a characteristic pattern. In this study, we show that Alk4/5/7 signaling is required during gastrulation specifically for anterior skeletal patterning in the sea urchin embryo.

The sea urchin larval skeleton is composed of calcium carbonate and is secreted via biomineralization by the primary mesenchyme cells (PMCs). The PMCs are autonomously specified at the posterior pole of the 32-cell stage embryo in a process that is well understood (Logan et al., 1999; Oliveri et al., 2002; Sharma and Etensohn, 2010). PMCs undergo an epithelial-mesenchymal transition and individually ingress into the blastocoel at the

mesenchyme blastula stage (Lyons et al., 2011). The PMCs then migrate into a stereotypic pattern, in which the PMCs are arranged in a vegetal (posterior) ring around the archenteron, with two ventrolateral clusters (VLCs); slightly later, cords of PMCs extend from the VLCs toward the animal (anterior) pole. Skeletal secretion is initiated within the VLCs as triradiate spicules, and the ring-and-cords arrangement of PMCs defines the pattern of the primary skeleton (Etensohn and Malinda, 1993; Lyons et al., 2011). Subsequently, PMCs migrate out of this primary organization and secrete the secondary skeleton, which includes both posterior and anterior elements (Etensohn and Malinda, 1993).

Interestingly, PMCs receive skeletal patterning cues from the overlying ectoderm, an idea first proposed by von Uebisch (1937) and later supported by Wolpert and Gustafson (1961). Armstrong and McClay showed that nickel specifically impacts the ectoderm and not the PMCs to cause skeletal patterning defects (Armstrong and McClay, 1994). Additional PMC manipulation experiments provide extensive evidence that patterning cues residing in the ectoderm regulate PMC positioning (Etensohn and McClay, 1986; Etensohn, 1990; Etensohn and Malinda, 1993; Benink et al., 1997; Guss and Etensohn, 1997; Hardin and Armstrong, 1997; Tan et al., 1998).

Recent studies have identified specific gene products that regulate skeletal patterning, including the ectodermal transcription factors Otp and Pax2/5/8 (Di Bernardo et al., 1999; Cavalieri et al., 2003, 2011), fibroblast growth factor (Rottinger et al., 2008; Adomako-Ankomah and Etensohn, 2013), the tripartite motif-containing protein Strim1 (Cavalieri et al., 2011) and Wnt5a (McIntyre et al., 2013). In addition, ectodermally expressed vascular endothelial growth factor regulates PMC positioning and skeletogenesis *in vivo* (Duloquin et al., 2007; Fujita et al., 2010; Adomako-Ankomah and Etensohn, 2013), as well as skeletal morphogenesis *in vitro* (Knapp et al., 2012). Each of these factors or signals is expressed in the ectoderm directly adjacent to the PMCs that actively produce skeleton, and each is required for biomineralization, such that loss-of-function embryos exhibit little or no skeletogenesis (Cavalieri et al., 2003; Duloquin et al., 2007; Rottinger et al., 2008; Adomako-Ankomah and Etensohn, 2013; McIntyre et al., 2013). However, ectodermal cues that pattern specific parts of the skeleton have not been previously identified. Here, we show that TGF- β signaling is specifically required for patterning the secondary anterior skeleton.

The sea urchin embryo utilizes the highly conserved TGF- β signaling pathway in germ layer specification and axial patterning. Univin is a maternally expressed TGF- β ligand (Stenzel et al., 1994) that is required for dorsal-ventral (DV) axis patterning in *Paracentrotus lividus* embryos (Range et al., 2007). ActivinB is another TGF- β ligand that is essential for endomesoderm specification (Sethi et al., 2009). Nodal is the best-studied TGF- β ligand in the sea urchin, and signals to establish the ventral ectoderm (Duboc et al., 2004; Flowers et al., 2004). Nodal signaling also initiates ventral BMP2/4 expression; ventrally produced BMP2/4 protein then translocates to the opposite side of the embryo, where it signals to

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specify the dorsal ectoderm (Angerer et al., 2000; Duboc et al., 2004; Bradham et al., 2009; Lapraz et al., 2009; Saudemont et al., 2010). Following DV specification and gastrulation, Nodal expression shifts from ventral to the right side, and Nodal signaling is required to specify the left-right (LR) axis (Duboc et al., 2005; Bessodes et al., 2012). Although these ligands have very different roles throughout development, Univin, ActivinB and Nodal utilize the same TGF- β type I receptor, Alk4/5/7 (Lapraz et al., 2006).

Here, we demonstrate that Alk4/5/7 activity is specifically required during gastrulation for anterior PMC positioning and skeletal patterning. Our results strongly suggest that LvUnivin is a relevant signal for anterior skeletal patterning.

RESULTS

Alk4/5/7 signaling is necessary during gastrulation for the formation of the anterior skeletal elements

This study was initiated by the unexpected observation that inhibition of TGF- β signaling during gastrulation blocked the formation of the secondary anterior skeletal elements. We used the well-established Alk4/5/7 inhibitor SB431542 (SB43) (Inman et al., 2002) to block TGF- β signaling at different time points during embryonic development (Fig. 1). When treated with SB43 prior to or at mesenchyme blastula (MB) stage, the resulting larvae were radialized, reflecting a defect in DV axis patterning (Fig. 1Bb; supplementary material Fig. S1). This is the expected outcome, since SB43 blocks Nodal signaling, and Nodal is required for DV specification prior to MB stage in *L. variegatus* embryos (Bradham and McClay, 2006). Surprisingly, when embryos were treated with SB43 between MB and prism stage, the resulting plutei exhibited a specific block to the formation of the anterior skeletal elements, but were otherwise apparently normal (Fig. 1Bc–Be). The anterior skeleton supports the oral hood, which encompasses the larval mouth. Alk4/5/7-inhibited plutei developed oral hoods in the absence of anterior rods, indicating that the anterior skeleton is not required for oral hood morphogenesis. Finally, treatment with SB43 after prism stage resulted in normal plutei with no evident defects (Fig. 1Bf). These results indicate that Alk4/5/7 activity is required during gastrulation for the formation of the anterior skeletal elements.

Alk4/5/7 inhibition after MB stage perturbs Nodal-dependent LR, but not DV, axis specification

Because Nodal signaling is required early for DV and later for LR axis specification in sea urchin embryos (Duboc et al., 2004, 2005; Flowers et al., 2004; Bradham and McClay, 2006), we tested both DV and LR markers in embryos treated with SB43 at different time points. When treated with SB43 before or at MB stage, the Nodal-dependent ventral marker *chordin* was suppressed, whereas the expression of the dorsal marker *tbx2/3* was expanded to the entire ectoderm (Fig. 2C,D), reflecting a loss of ventral specification, as expected. SB43 treatment at later time points did not perturb DV specification (Fig. 2E,F), consistent with the results in Fig. 1. These findings agree with previous reports indicating that Nodal-dependent DV specification in *L. variegatus* is complete at MB stage (Hardin et al., 1992; Hardin and Armstrong, 1997; Bradham and McClay, 2006). SB43 treatment also perturbed LR specification at all of the tested time points, as SB43 treatment blocked the expression of the Nodal-dependent right-side marker *pix2*, and resulted in bilateral expression of the left-side marker *soxE* (Fig. 2I–L). Again, this is in agreement with previous studies (Duboc et al., 2005; Luo and Su, 2012). These data establish that SB43 treatment after MB stage does not perturb DV specification, but does perturb LR specification.

Late Alk4/5/7 activity is necessary for normal anterior PMC positioning

The loss of the anterior skeleton in embryos treated with SB43 during gastrulation, which we will refer to as ‘late’ SB43 treatment, could be the result of either a defect in the positioning of the skeletogenic PMCs or a defect in biomineralization without a positioning defect. To distinguish between these possibilities, we performed immunostaining to label PMCs in pluteus-stage embryos and tested whether the PMCs occupy the oral hood and fail to locally produce a skeleton, or whether the PMCs fail to migrate into the oral hood in SB43-treated embryos (Fig. 3). Late SB43 treatment blocked PMC migration into the anterior territory, and PMCs were not observed past the anterior branch point (Fig. 3Ab,Bb, arrowheads). Thus, late SB43 treatment interferes

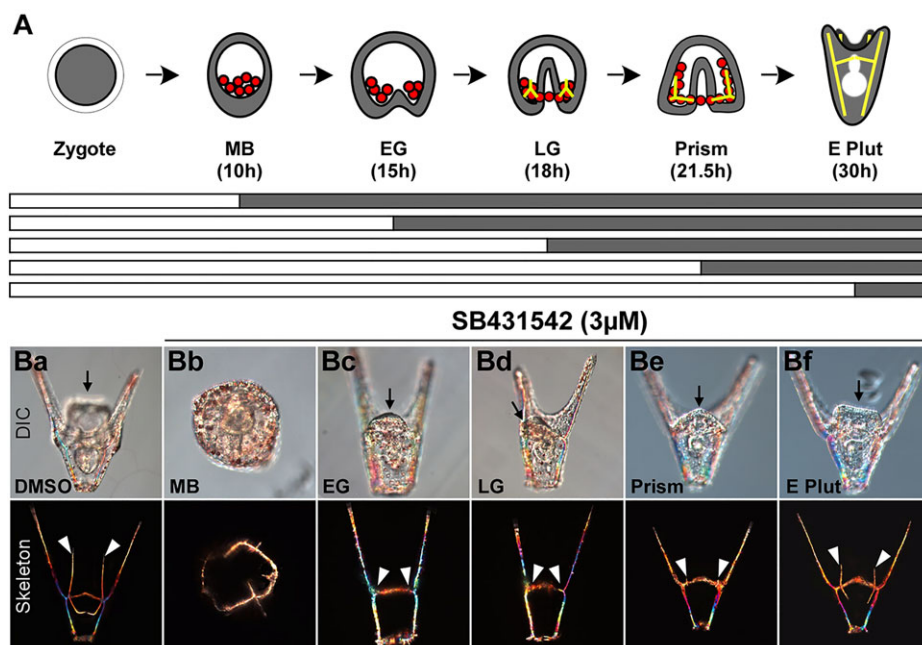


Fig. 1. Alk4/5/7 inhibition during gastrulation results in loss of the anterior skeletal elements. (A) Schematic illustrating the experimental design. The upper panel depicts stages of sea urchin development, including fertilized zygote, mesenchyme blastula (MB), early gastrula (EG), late gastrula (LG), prism and early pluteus (E Plut) stages. The skeletogenic PMCs are shown in red and the skeleton is shown in yellow. The lower panel indicates the intervals of exposure to SB431542 (SB43). (B) Sea urchin embryos were treated with DMSO (Ba) or SB43 (Bb–Bf) at the indicated stages per schematic in A, then evaluated at pluteus stage at 48 hours post fertilization (hpf) via morphology (upper panel, DIC) and skeletal development (lower panel, plane-polarized light illumination); arrowheads indicate anterior skeletal elements (lower panels) and arrows indicate oral hoods (upper panels).

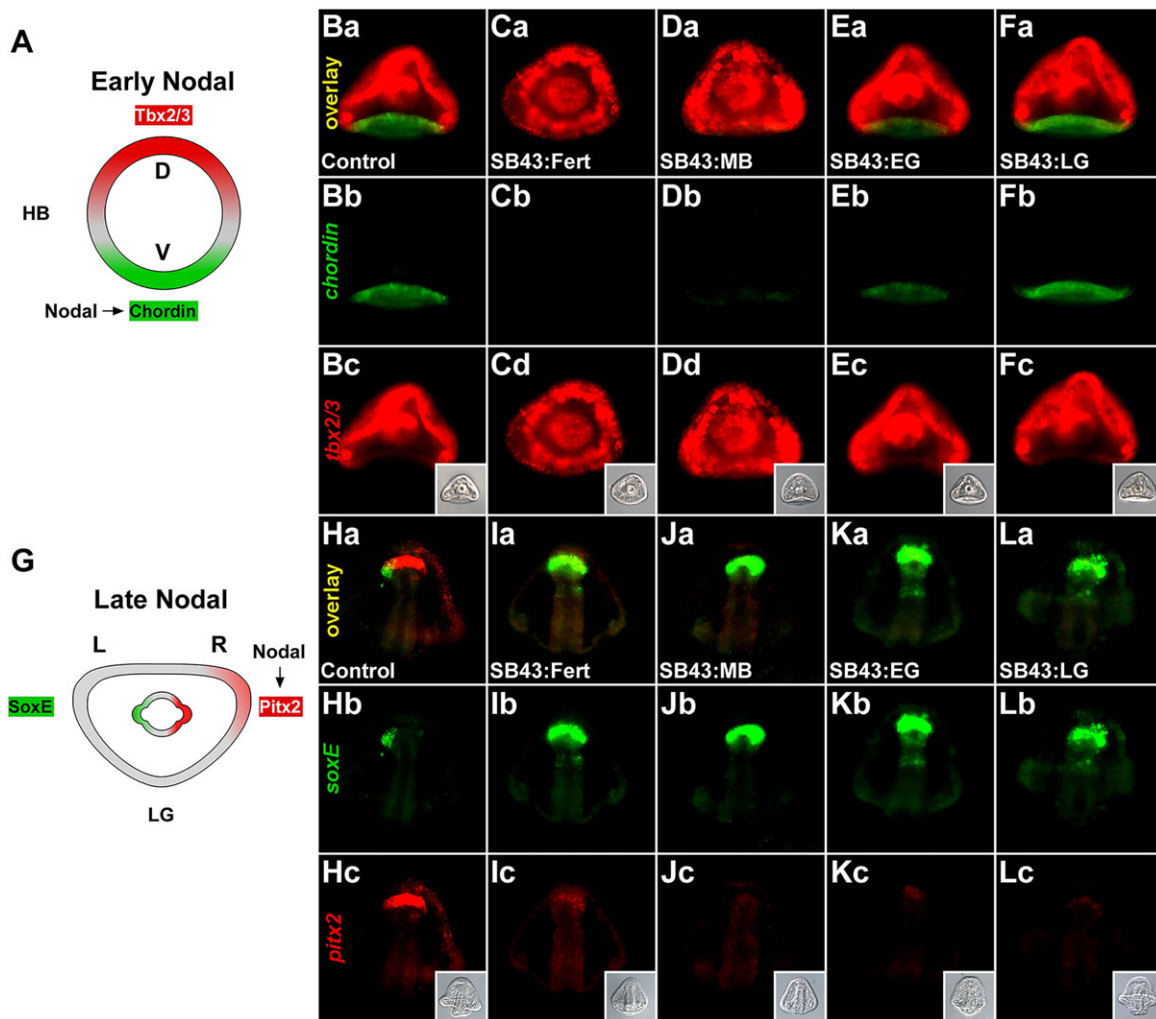


Fig. 2. Alk4/5/7 inhibition after MB stage perturbs Nodal-dependent LR, but not DV, axis specification. (A,G) Schematics represent the early (A) and late (G) roles for Nodal signaling to specify the DV and LR axes, respectively. (B-F,H-L) Spatial expression was determined by fluorescent *in situ* hybridization (FISH) for ventral *chordin* and dorsal *tbx2/3* at LG stage (B-F), or left-sided *soxE* and right-sided *pitx2* at early pluteus stage (28 hpf; H-L) in controls (B,H) or in embryos treated with SB43 (5 μ M) at the indicated time points (C-F,J-L). Insets show corresponding DIC images.

with PMC positioning within the oral hood. In addition, all PMCs appeared to be associated with skeletal elements in both controls and SB43-treated embryos, suggesting that SB43 does not impact biomineralization. We counted PMCs in SB43-treated embryos to rule out the possibility that the loss of anterior skeletal elements reflects a dramatic decrease in the number of PMCs, and found that late inhibition of Alk4/5/7 activity did not affect PMC numbers per embryo (supplementary material Fig. S2). These results demonstrate that late Alk4/5/7 activity is necessary for positioning the PMCs in the anterior region of the embryo, but not for biomineralization.

Late Alk4/5/7 is not necessary for normal ectodermal differentiation

The ciliary band and the larval nervous system are ectodermally derived tissues located at the DV boundary that are each sensitive to DV perturbations (Yaguchi et al., 2006, 2010; Bradham et al., 2009; Lapraz et al., 2009); thus, we examined the patterning of the ciliary band and the nervous system to assess ectodermal differentiation in late SB43-treated plutei. Both structures were intact and normally patterned in late Alk4/5/7-inhibited plutei (Fig. 4). Together with

DV gene expression data (Fig. 2A-F), these results indicate that late SB43 treatment does not perturb the DV axis or general ectodermal patterning and development.

Late Alk4/5/7 inhibition impairs feeding at the pluteus stage, but not the formation of the mouth

The particular embryo shown in Fig. 4Bb illustrates the effect of the absence of anterior skeletal rods on the oral hood, which, without this internal support structure, sags outward. By providing structure to the oral hood, the anterior skeleton also supports the larval mouth; thus, the loss of the anterior skeleton could impair mouth formation or function in Alk4/5/7-inhibited larvae. To test this, we performed a feeding assay, in which plutei were incubated with fluorescent beads, then scored for bead ingestion. Although SB43-treated plutei were capable of feeding, they were significantly impaired in feeding ability compared with controls (Fig. 5). Thus, late Alk4/5/7 activity is necessary for efficient feeding, but not for the formation of the mouth. These results indicate that feeding is impaired in SB43-treated larvae, presumably because the oral hood and the mouth are not supported by the anterior skeleton.

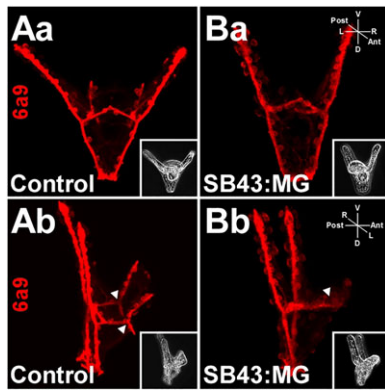


Fig. 3. Late Alk4/5/7 activity is required for anterior PMC positioning. PMC-specific immunostaining is shown for control plutei (A) and for plutei treated with SB43 (3 μ M) at mid-gastrula (MG) stage (B). Larvae are shown in *en face* (Aa, Ba) or lateral views (Ab, Bb); insets show the corresponding phase contrast images. Developmental axes are indicated. Arrowheads indicate the tips of the primary DV connecting rods, which fail to branch and elongate to form the secondary anterior skeletal elements in SB43-treated embryos.

Late Alk4/5/7 inhibition does not perturb expression of VEGF or VEGFR

Previous studies have shown that the gene encoding vascular endothelial growth factor *veg3* (VEGF), is expressed in the ectoderm adjacent to sites of skeletogenesis; the PMCs specifically express the VEGF receptor gene *vegfr-10-ig* (VEGFR), and thus can respond to VEGF signals, which are required for normal PMC positioning and for skeletogenesis (Duloquin et al., 2007; Adomako-Ankomah and Etensohn, 2013). To test the possibility that the SB43-mediated defects in anterior skeletal patterning arise from a perturbation to the expression of VEGF and/or VEGFR, we examined *veg3* and *vegfr* gene expression in vehicle- and SB43-treated embryos. This signal-receptor pair is expressed in adjacent ectoderm and PMCs, respectively, during gastrulation (Fig. 6A), and as skeletal development proceeds, expression of the receptor, then the signal, becomes dynamic, with expression of the signal appearing in an anterior region in prism-stage embryos (Fig. 6B,C) (Duloquin et al., 2007; Adomako-Ankomah and Etensohn, 2013). In embryos treated with SB43 at early gastrula (EG), *lv-veg3* and *lv-vegfr* were each expressed in their characteristic spatial patterns (Fig. 6D-F). These results indicate that late Alk4/5/7 activity is

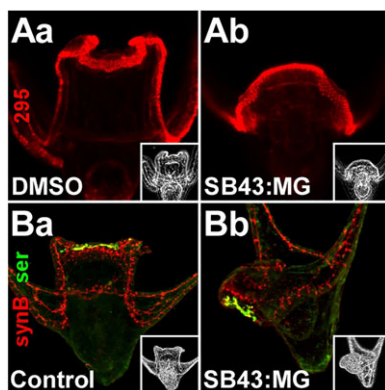


Fig. 4. Late Alk4/5/7 inhibition does not perturb ciliary band formation or neurogenesis. (A) Ciliary band-specific immunostaining is shown in a DMSO-treated control pluteus (Aa) and in a pluteus treated with SB43 (3 μ M) at MG stage (Ab). (B) Neural-specific immunostaining for synaptotagmin B (synB, red) and serotonin (ser, green) is shown in a control pluteus (Ba) and in a pluteus treated with SB43 (3 μ M) at MG stage (Bb). Insets show corresponding phase contrast images.

not required for the normal dynamic expression of *veg3* or *vegfr*. qPCR measurements demonstrate that there was no significant change in expression levels of either gene when compared with vehicle-treated controls (supplementary material Fig. S3), corroborating this result. Thus, aberrant VEGF or VEGFR expression does not explain the effects of SB43 on the anterior skeleton. These data also indicate that, in the absence of Alk4/5/7 signaling, endogenous VEGF expression is insufficient for skeletal patterning, at least in the anterior region.

Alk4/5/7 is expressed in all three germ layers

To determine whether the PMCs are competent to directly receive TGF- β signaling, we assessed Alk4/5/7 expression in control late gastrula (LG) stage embryos (Fig. 7). Alk4/5/7 transcripts were detected in all tissues, including the anteriorly positioned subset of PMCs that are appropriately localized to produce the anterior skeleton (Fig. 7, arrowheads). Thus, it is possible that PMCs directly receive TGF- β signals during anterior skeletal patterning.

Unlike Nodal, Univin is bilaterally expressed at the pluteus stage

The Alk4/5/7 receptor can be activated by multiple TGF- β ligands, including Nodal and Univin. To determine whether LvUnivin is relevant during anterior skeletal patterning, we cloned the LvUnivin cDNA. Protein domain, sequence and phylogenetic analyses confirm that LvUnivin encodes a member of the TGF- β superfamily that is most similar to other sea urchin Univins and the vertebrate GDF1/Vg1 subfamily (supplementary material Fig. S4). We examined the spatial localization of *nodal* and *univin* transcripts in control *L. variegatus* embryos. As expected, *univin* expression is spatially dynamic across development: *univin* transcripts were maternally expressed and ubiquitous through early blastula (EB) stage, were restricted from the anterior and posterior embryonic poles prior to gastrulation, and were then further restricted from the ventral and dorsal ectoderm during gastrulation (supplementary material Fig. S5), consistent with previous findings in other sea urchin species (Saudemont et al., 2010; Li et al., 2014). Interestingly, at LG and pluteus stages, *univin* transcripts were bilaterally expressed in the ectoderm adjacent to the anterior skeleton (Fig. 8A). Conversely, *nodal* transcripts were unilaterally expressed in control plutei (Fig. 8B), consistent with the established role of Nodal in specifying the LR axis at later developmental time points (Duboc et al., 2005). As late Alk4/5/7 inhibition gives rise to a bilateral defect, these results indicate that Univin, but not Nodal, is expressed in the correct location to contribute to anterior skeletal patterning. However, it is possible that earlier, ventrally expressed Nodal is indirectly required to pattern the anterior skeleton. To distinguish whether Alk4/5/7 signaling is required early and presumably indirectly, or whether Alk4/5/7 signaling is actively required during skeletal patterning, we treated embryos with SB43 at mid gastrula (MG) stage, then washed out the drug at prism stage, when skeletal patterning is underway and Nodal is strictly unilaterally expressed. SB43 washout embryos developed normal, bilateral anterior skeletons (supplementary material Fig. S6C), suggesting that the relevant Alk4/5/7 signal for anterior skeletal patterning is active during and beyond prism stage and utilizes the Alk4/5/7 receptor in a bilateral manner. This result argues against, but does not exclude, a role for Nodal in anterior skeletal patterning.

LvUnivin is necessary and sufficient for secondary skeletal development in a control background

To determine whether LvUnivin is required for skeletal patterning, we performed loss-of-function (LOF) analysis using a translation-blocking antisense morpholino (MO). Zygotic microinjection of

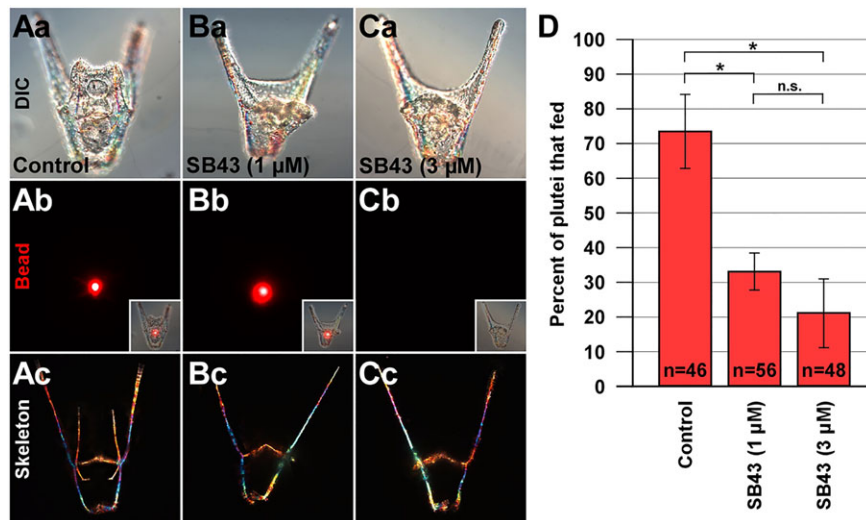


Fig. 5. Late Alk4/5/7 inhibition impairs feeding but not mouth formation. (A-C) Control plutei (A) and plutei treated with SB43 at MG stage (B,C) were assayed for feeding ability. Morphology (Aa,Ba,Ca; DIC) and skeletal development (Ac,Bc,Cc; plane-polarized light illumination) are shown for representative examples, as well as fluorescence from ingested beads (Ab,Bb,Cb). Insets show overlays of subpanels a and c. (D) Quantitation of feeding experiments, showing the average percentage of plutei that fed from three independent experiments. Error bars represent s.e.m.; *Student's *t*-test, $P < 0.05$.

LvUnivin MO reproducibly resulted in plutei that lack all secondary skeletal elements, which include the anterior skeleton (Fig. 9A,B, arrowheads) and the longer, posterior rods. Thus, Univin is required for secondary skeletogenesis. Gain-of-function (GOF) analysis via microinjection of *in vitro* transcribed LvUnivin mRNA resulted in a complementary phenotype, characterized by an excessive number of secondary skeletal elements, including ectopic anterior skeletal elements (Fig. 9C, arrowheads). Thus, Univin overexpression is sufficient to induce ectopic secondary skeletogenesis. Co-injection of LvUnivin MO and mRNA reproducibly resulted in a control phenotype (Fig. 9D,E), demonstrating that LvUnivin MO efficiently blocks LvUnivin mRNA translation.

Although the LvUnivin MO target sequence is unique in the genome, and thus is unlikely to have off-target effects, a potential concern regarding the rescue experiments is that, due to AT-rich constraints within the endogenous Univin sequence, the LvUnivin MO completely overlaps with the 5' end of the LvUnivin mRNA. As exogenous LvUnivin mRNA can directly titrate LvUnivin MO, we generated an LvUnivin mRNA construct with five silent mutations in the LvUnivin MO binding site (supplementary material Fig. S7A), which suffices to prevent LvUnivin MO from recognizing the exogenous mRNA (Eisen and Smith, 2008). Zygotic microinjection of the modified LvUnivin mRNA provoked ectopic secondary skeletal elements (supplementary material Fig. S7B,C), indicating that this reagent induces effects similar to the wild-type LvUnivin mRNA (Fig. 9C). As the endogenous Univin mRNA is spatially restricted and dynamic during development (supplementary material Fig. S5) (Stenzel et al., 1994; Lapraz et al., 2006; Range et al., 2007), it is unlikely that globally delivered mismatch mRNA could provoke a perfect rescue in a LOF context, and increased numbers of control-like phenotypes would not be expected. However, if the mismatch RNA, present globally, compensates for the effect of the MO locally, then we would expect the MO effects to diminish while the mRNA effects increase, as mRNA concentration increases. Alternatively, if the MO has off-target effects that the mismatch mRNA cannot counter, then the MO phenotype would not be reduced with increasing mRNA dose. Upon co-injection of LvUnivin MO and mutated LvUnivin mRNA, the effects of the MO diminish, while the effects of the mRNA increase, as mRNA dose increases (supplementary material Fig. S7D), suggesting that the LvUnivin MO is specific for LvUnivin mRNA. This result, together with the

strikingly complementary LOF and GOF phenotypes, their agreement with the SB43 effects and the unique nature of the Univin MO sequence in the Lv genome, strongly suggest that the LvUnivin MO is specific.

Thus, LvUnivin is necessary for secondary skeletal development and sufficient to induce ectopic secondary skeleton. These results strongly suggest that Univin is a relevant signaling ligand for the Alk4/5/7 receptor during anterior skeletal patterning. Together, these findings are consistent with a model in which bilaterally expressed LvUnivin signals from the margins of the oral hood to direct PMC migration to these locations, thereby patterning the anterior skeleton.

Univin is not required for normal DV axis specification or development in *L. variegatus* embryos

In the Mediterranean sea urchin species *P. lividus*, Univin LOF resulted in a loss of *nodal* expression and DV specification, with subsequent radialization of the ectoderm (Range et al., 2007). Surprisingly, Univin LOF in *L. variegatus* did not produce a radialized phenotype (Fig. 9B, compare with Fig. 1Bb), suggesting that Univin is not necessary for *nodal* expression in this species. To further test the relationship between LvUnivin and LvNodal, we examined expression of both *nodal* and its target gene *chordin* in control and in LvUnivin MO-injected embryos, using *in situ* hybridization at hatched blastula (HB) stage (Fig. 10). We detected a range of results, exemplars of which are shown in supplementary material Fig. S8. Expression of both *nodal* and *chordin* was overlapping and restricted to the presumptive ventral ectoderm in 77.8% of control embryos (Fig. 10A,B). In 22.2% of cases, we detected Nodal, but not Chordin, expression in control embryos (Fig. 10B); this probably occurs because LvChordin expression initiates at HB stage (Bradham et al., 2009), and some embryos in the HB samples might be slightly delayed. By contrast, normal *nodal* expression was detected in only 62.5% of LvUnivin LOF embryos, whereas 32.5% showed low and unrestricted *nodal* expression; 5% lacked Nodal expression entirely (Fig. 10A). Interestingly, we did not detect *chordin* expression in 77.5% of LvUnivin LOF morphants (Fig. 10B). These results show that LvUnivin is not necessary for *nodal* expression, but is necessary for robust expression of *chordin*.

To better quantitate these findings, we performed a more sensitive, qPCR-based analysis to measure the expression of *nodal* and its target genes *chordin* and *lefty* in control and LvUnivin MO-injected

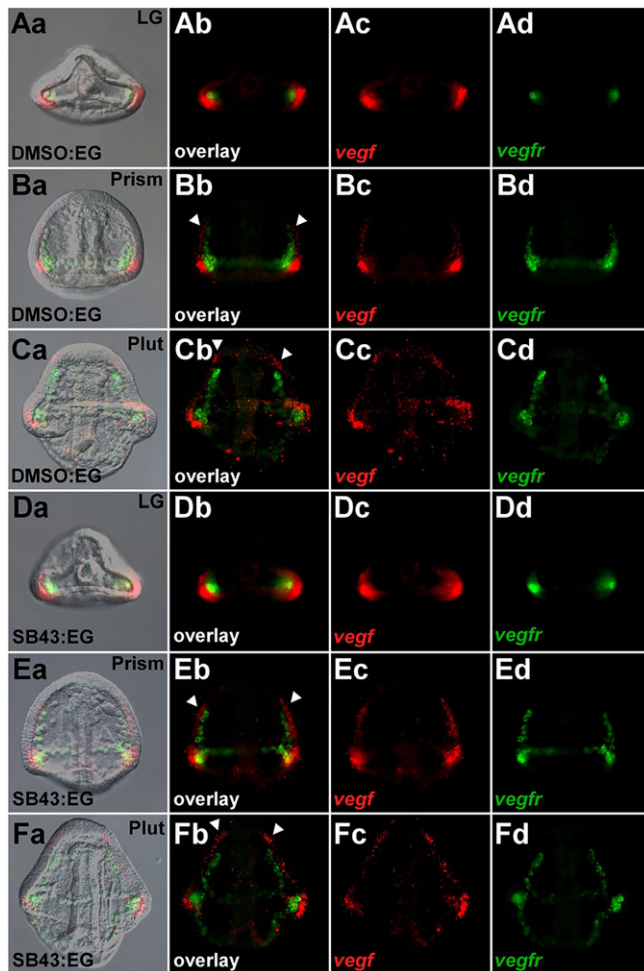


Fig. 6. Late Alk4/5/7 inhibition does not perturb VEGF or VEGFR expression. (A-F) FISH for *vegf* (red, in subpanels c) and *vegfr* (green, in subpanels d), the overlaid fluorescent signals (in subpanels b), and the expression of both genes overlaid on the corresponding DIC images (in subpanels a), is shown in embryos treated with DMSO (A-C) or with SB43 (3 μ M; D-F) at EG stage, then fixed and analyzed at LG (A,D), prism (B,E) or pluteus stage (28 hpf; C,F). Arrowheads indicate anterior *vegf* expression.

embryos at a series of early developmental time points. The results show that LvUnivin LOF depressed but did not abolish the expression of *nodal* and *chordin*, whereas the expression of *lefty* was not perturbed (Fig. 10C). These results confirm that LvUnivin is not required for expression of *nodal* or for Nodal target gene expression; however, LvUnivin function promotes peak expression of both *nodal* and *chordin*. The lack of effect on *lefty* indicates that *lefty* is more sensitive to Nodal signaling than is either *chordin* or *nodal* itself.



Fig. 7. LvAlk4/5/7 is expressed in all three germ layers, including the PMCs. Spatial expression of Alk4/5/7 is shown at LG stage, as determined by FISH. Arrowheads indicate ventrolateral PMC cords.

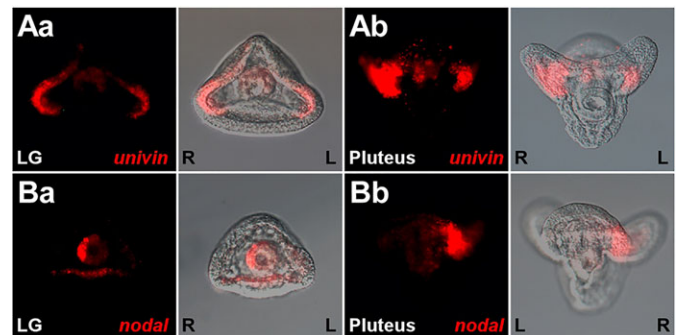


Fig. 8. LvUnivin, but not LvNodal, is expressed bilaterally within the oral hood. Spatial expression as determined by FISH for *univin* (A) and *nodal* (B) in control LG stage embryos (Aa,Ba) and early plutei (28 hpf; Ab,Bb). Overlays of fluorescent and DIC images are shown in the panels on the right. The LR axis is indicated.

The ciliary band is initially a broad ectodermal fate, the restriction of which requires ventral Nodal and dorsal BMP2/4 signaling (Duboc et al., 2004; Bradham et al., 2009; Yaguchi et al., 2010); thus, expansion of the ciliary band is a hallmark of DV perturbation. We therefore examined the ciliary band in LvUnivin morphants to determine whether the depressed *nodal* and *chordin* levels observed in these embryos have a phenotypic consequence. The results show that in both LvUnivin LOF and GOF plutei, the ciliary band was normally restricted despite the overall morphological perturbation (Fig. 10D), indicating that Nodal and its targets are expressed at sufficiently high levels to specify the DV axis and appropriately restrict the ciliary band. Together, these results demonstrate that LvUnivin is not required for normal DV axis specification and development in *L. variegatus* embryos.

DISCUSSION

In this study, we demonstrate that TGF- β signaling is specifically required during gastrulation for anterior skeletal patterning in the sea urchin embryo. This is the first example of a signaling event that is required to pattern a specific part of the sea urchin larval skeleton. We demonstrate that TGF- β signaling is required for anterior PMC positioning, but not for biomineralization, indicating that the skeletal patterning process itself, and not skeletogenesis, is disrupted by late Alk4/5/7 inhibition. We also show that ectodermal specification and development occur normally in late Alk4/5/7-inhibited embryos, indicating that the patterning defects are not a result of gross ectodermal defects. The loss of the anterior skeleton significantly impairs larval feeding ability, presumably due to the loss of structural support for the oral hood. We find that the Alk4/5/7 receptor is expressed by the PMCs, as well as by other tissues; thus, the PMCs appear to be capable of directly receiving TGF- β signaling inputs. We also show that LvUnivin, a TGF- β ligand, is expressed at the appropriate time and place to direct anterior skeletal patterning, and that Univin is necessary for secondary skeletogenesis and is sufficient to induce ectopic secondary skeletal elements. This strongly suggests that Univin is a relevant signaling ligand for anterior skeletal patterning.

In the Mediterranean sea urchin *P. lividus*, Univin is required for the onset of *nodal* expression, and thus, is required for normal DV axis specification (Range et al., 2007). As we did not observe DV defects in LvUnivin morphants, we tested the relationship between LvUnivin and LvNodal. We find that LvUnivin enhances, but is not necessary for, LvNodal expression and signaling, and subsequent DV axis specification (Fig. 10; supplementary material Fig. S8).

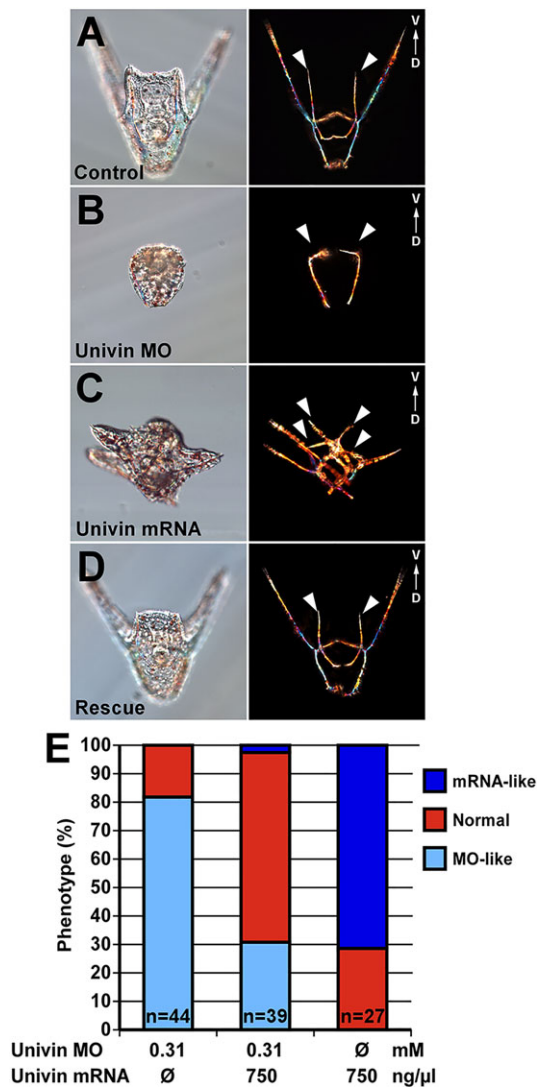


Fig. 9. LvUnivin is necessary and sufficient for secondary skeletal patterning in a control background. (A-D) Sea urchin zygotes were microinjected with LvUnivin MO (0.31 mM; B), LvUnivin mRNA (750 ng/μl; C) or combined LvUnivin MO and LvUnivin mRNA (D), and then imaged at the pluteus stage. Morphology (left panels) and skeletal development (right panels) are shown in plutei (48 hpf); the DV axis is indicated. Arrowheads indicate anterior skeletal elements. (E) Quantitation of the LvUnivin MO, LvUnivin mRNA and rescue phenotypes.

Thus, as in *P. lividus* embryos, LvUnivin is also upstream of LvNodal, but the requirement for Univin is not as stringent in *L. variegatus* embryos. Consistent with this logic, Univin LOF in *P. lividus* does not entirely abolish *nodal* expression, as determined by qPCR (Range et al., 2007), indicating that PlUnivin promotes *pl-nodal* expression, but to a different degree between these two species. There are various potential mechanisms by which Univin could enhance *nodal* expression and function. First, Univin could signal via Smad2/3 to directly promote *nodal* expression, which is responsive to Smads both in sea urchins and in vertebrates (Penheiter et al., 2002; Tian and Meng, 2006; Nam et al., 2007; Range et al., 2007; Harvey and Smith, 2009; Fleming et al., 2013). Second, Univin and Nodal could signal as heterodimers. GDF1, the mouse ortholog of Univin, directly interacts with Nodal and increases its signaling activity, thereby facilitating long-range signaling (Tanaka et al., 2007), providing a precedent for this

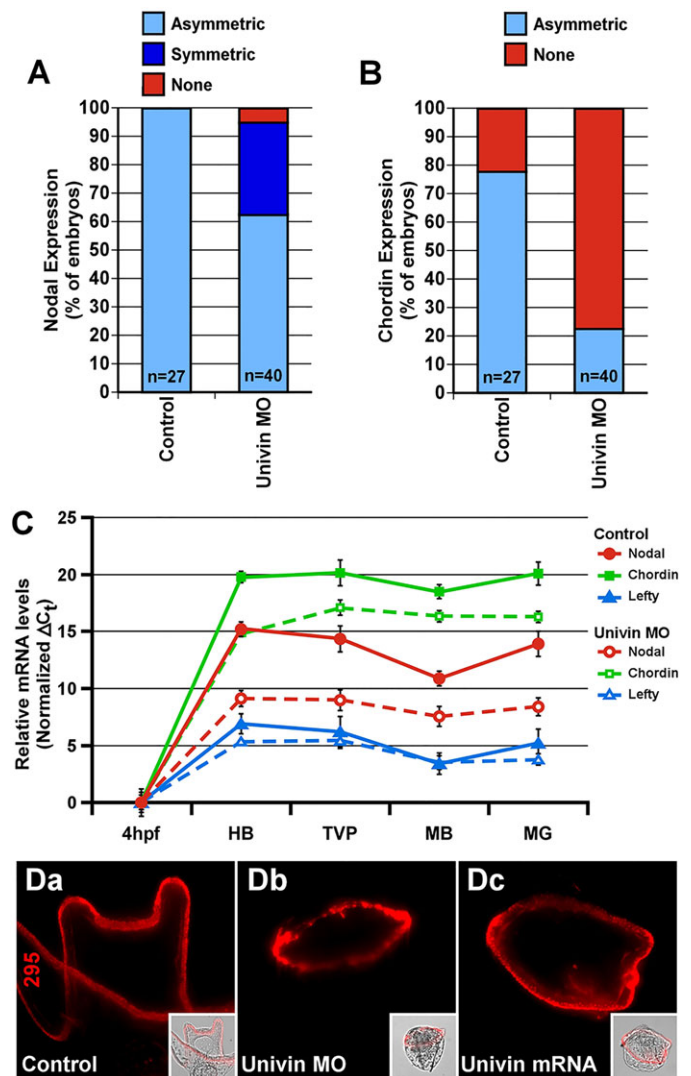


Fig. 10. LvUnivin is not required for normal DV axis specification or development in *L. variegatus* embryos. (A,B) Quantitation of spatial expression profiles for *nodal* (A) and *chordin* (B) in control and LvUnivin MO-injected (0.33 mM) embryos at hatched blastula stage. Corresponding exemplar embryos are shown in supplementary material Fig. S8. (C) Relative LvNodal, LvChordin and LvLefty mRNA expression levels were determined by qPCR in glycerol-injected control (solid lines and filled symbols) or LvUnivin MO-injected (0.36 mM, dashed lines and unfilled symbols) embryos at the indicated stages. Normalized ΔC_T values are displayed as averages \pm s.e.m. (D) Ciliary band-specific immunostaining is shown for control (Da), LvUnivin MO- (0.4 mM; Db) and LvUnivin mRNA-injected embryos (1250 ng/μl; Dc) at the pluteus stage (48 hpf). Insets show corresponding DIC images.

idea. Co-expression of Univin and Nodal is required for long-range signaling from the ectoderm to the endoderm during gastrulation in sea urchin embryos, probably also reflecting heterodimer formation (Bessodes et al., 2012).

Although our results are consistent with Univin alone signaling for anterior skeletal patterning, it remains possible that Nodal is also a relevant signal for this process, as Nodal is present in the anterior region, albeit unilaterally (Fig. 8). The Univin and Nodal expression domains are overlapping on the right side of the oral hood, and thus, these two TGF- β ligands could potentially form heterodimers in this region. In keeping with previous studies in sea urchin and mouse embryos (Tanaka et al., 2007; Bessodes et al., 2012), heterodimers

of Univin and Nodal could facilitate the transport of Nodal to pattern the opposite, left side of the anterior skeleton. Although Nodal is capable of diffusing long distances (Williams et al., 2004; Oki et al., 2007; Muller et al., 2013), Nodal/Univin heterodimers exhibit an increased signaling range compared with Nodal alone (Tanaka et al., 2007; Bessodes et al., 2012); thus, the formation of heterodimers could compensate for the spatial restriction of Nodal. Directly testing this possibility will require the ability to specifically inhibit Nodal only at later developmental stages, as zygotic inhibition of Nodal blocks DV specification and thereby interferes with normal skeletal patterning much earlier than the events in question herein.

We observed a specific block to the anterior skeleton with the Alk4/5/7 inhibitor SB431542 delivered during gastrulation (Fig. 1), whereas zygotic inhibition of LvUnivin via morpholino-mediated inhibition had a more general effect, blocking the entire secondary skeleton, including the anterior elements, posterior elements and the branched dorsal apex, while leaving the primary skeleton intact (Fig. 9). Thus, early LvUnivin inhibition blocked all secondary skeletal elements, whereas late LvUnivin inhibition (via late SB43 treatment) blocked only the anterior secondary skeleton. Conversely, ectopic secondary skeletal elements, both anterior and posterior, are produced by zygotic LvUnivin GOF (Fig. 9). Secondary skeletal elements require the PMCs to migrate out of the initial ring-and-cords arrangement that corresponds to the primary skeleton. These results indicate that LvUnivin is necessary for secondary skeletal development in general, suggesting that LvUnivin directs secondary PMC migration. Considered in conjunction with the SB43 results, these findings suggest that patterning of the posterior and anterior secondary elements is temporally separable, with the posterior elements being patterned prior to the anterior elements. Temporally controlled LOF experiments will be required to resolve the specific roles of Nodal and Univin, both early and late, in secondary skeletal patterning.

VEGF is a spatially restricted ectodermal cue that is required both for normal PMC positioning and for skeletogenesis. Zygotic inhibition of VEGF blocks skeleton formation entirely and disrupts the formation of the ventrolateral PMC clusters (Duloquin et al., 2007; Adomako-Ankomah and Etensohn, 2013). The VEGFR-inhibiting drug axitinib blocks the formation of some secondary skeletal elements when added during gastrulation (Adomako-Ankomah and Etensohn, 2013), suggesting that VEGF has a dynamic functional role in skeleton formation, as it is required for primary PMC positioning as well as primary and secondary skeletogenesis. We demonstrate that Alk4/5/7 activity is not required for normal expression of VEGF or VEGFR (Fig. 6); therefore, Alk4/5/7 activity is not upstream from VEGF expression. Furthermore, the loss of anterior skeletal elements in Alk4/5/7-inhibited embryos cannot be explained by a loss of VEGF or VEGFR. Surprisingly, these results demonstrate that, in the absence of Alk4/5/7 activity, endogenous VEGF is insufficient for anterior skeletal patterning, and does not suffice to attract PMCs into the anterior region (Fig. 3). This is an important conclusion, which argues against a model in which VEGF signaling is the only or the principle mediator of skeletal patterning in sea urchin embryos, as has been previously suggested (Duloquin et al., 2007; Lyons et al., 2011; Adomako-Ankomah and Etensohn, 2013; McIntyre et al., 2014). Late VEGFR inhibition (via axitinib) blocks secondary skeletogenesis, which has been interpreted as reflecting a block to biomineralization (Adomako-Ankomah and Etensohn, 2013). By contrast, late Alk4/5/7 inhibition blocks only the anterior skeleton (Fig. 1) and anterior PMC positioning (Fig. 3). We could speculate

that these two signaling pathways cooperate to produce the anterior skeleton, with Alk4/5/7 providing PMC guidance cues, whereas VEGFR provides biomineralization cues. In any case, these findings raise the possibility that other signaling pathways contribute to the patterning of specific skeletal structures in various regions of the developing sea urchin embryo and remain to be discovered.

MATERIALS AND METHODS

Animals, perturbations, feeding experiments and imaging

Adult *L. variegatus* sea urchins were obtained from Reeftopia (Miami, FL, USA) or from the Duke University Marine Laboratory (Beaufort, NC, USA). Gametes were harvested, and zygotic microinjections were carried out as described (Bradham and McClay, 2006). SB431542 (Sigma) was reconstituted in DMSO, and drug treatments were carried out as described (Bradham and McClay, 2006). Dose-response experiments were performed to determine optimal working doses for each perturbation reagent. The optimal SB43 concentration was determined to be 2–5 μ M, which is consistent with doses used in previous studies in sea urchins (Duboc et al., 2005, 2010; Range et al., 2007; Luo and Su, 2012). The LvUnivin MO (Gene Tools) sequence is 5'-AGATCAAGACTTTCCAGACATCCAT-3'. For feeding assays, pluteus stage larvae were incubated with fluorescein-conjugated beads (10 μ m diameter; Polysciences) for 30 min, then imaged, as previously described (Walton et al., 2009). Differential interference contrast (DIC) imaging, plane-polarized light imaging and epifluorescence imaging were carried out using a Zeiss Axioplan microscope at 200 \times magnification. Larval skeletons were photographed in multiple focal planes, which were manually collapsed using Canvas (ACD Systems) to optimally represent the full, in-focus larval skeleton.

Cloning and sequence analysis

The LvUnivin (GenBank accession number KJ607975), LvAlk4/5/7 (accession number KJ906599) and LvVEGFR (*Lv-vegfr-10-ig*) (accession number KJ906600) cDNAs were cloned from LG stage Lv cDNA into pGEM-T Easy (Promega) for *in situ* probe synthesis using standard techniques. Gene-specific primers used for cloning are provided in supplementary material Table S1. The LvUnivin open reading frame (ORF) was cloned from the cDNA library into pCS2 between *Clai* and *XbaI* restriction sites, and mRNA was *in vitro*-transcribed using an SP6 mMACHINE kit (Life Technologies). To produce a version insensitive to the Univin MO, five synonymous mutations were introduced into the LvUnivin ORF using PCR. The cDNA sequences used for phylogenetic analysis were collected using the Ensembl genome browser. Sequence alignment was carried out using MultAlin (Corpet, 1988), and phylogenetic analysis was performed by Bayesian inference using MrBayes (<http://mrbayes.sourceforge.net>), and visualized using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

Fluorescent *in situ* hybridization

Probes for LvChordin, LvTbx2/3, LvNodal, LvSoxE and LvVEGF have been previously described (Bradham et al., 2009; Walton et al., 2009; McIntyre et al., 2013). The LvPitx2 probe was a kind gift from David McClay (Duke University, Durham, NC, USA). The LvUnivin, LvAlk4/5/7 and LvVEGFR probes were generated from our cloned sequences described above. All probes were transcribed using SP6 or T7 RNA polymerases (New England Biolabs) and labeled with digoxigenin (DIG, Roche) or DNP-11-UTP (PerkinElmer). *In situ* hybridization and washes were carried out as described (Sethi et al., 2009). Hybridized probes were detected using HRP-conjugated anti-DIG (Roche) or anti-DNP (PerkinElmer) Fab fragments, both at 1:750 dilutions, and were developed sequentially using TSA Plus System kits (PerkinElmer) with Cy3 or Fluorescein (1:100 dilutions).

Immunostaining and confocal microscopy

Embryo fixation and immunostaining were carried out as described (Bradham et al., 2009). Primary antibodies used were PMC-specific 6a9 (1:3), ciliary band-specific 295 (undiluted) and neural-specific 1e11 (1:10) [gifts from Charles Etensohn (Carnegie Mellon University, Pittsburgh, PA, USA), David McClay (Duke University, Durham, NC, USA) and Robert

Burke (University of Victoria, Canada)] and anti-serotonin (Sigma, S5545, 1:500). Confocal imaging was performed using an Olympus FV10i laser-scanning or an Olympus DSU spinning-disk confocal microscope. Confocal z-stacks were projected using Olympus software, and full projections are presented.

qPCR analysis

Total RNA was collected from 60 embryos per sample using TRIzol (Invitrogen), and precipitated along with glycogen carrier (Ambion). After DNase treatment (DNA-free, Ambion), samples were reverse-transcribed using the TaqMan reverse transcription kit (Life Technologies). qPCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7900ht qPCR thermocycler. Primer sequences used for qPCR analysis are provided in the supplementary material Table S1. Results were determined by subtracting sample C_T values from control C_T values to determine ΔC_T , and then normalized to LvSetmar expression.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.L.P. and C.A.B. designed, and M.L.P. and J.R. performed the experiments in this study. M.L.P. and C.A.B. wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.114322/-/DC1>

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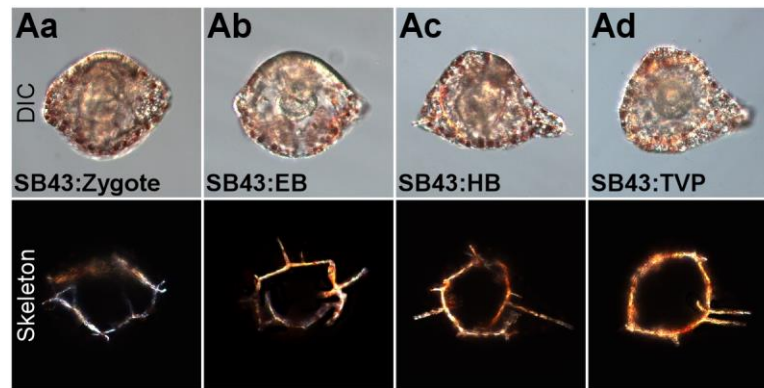


Figure S1. Early Alk4/5/7 inhibition results in plutei that display radialized skeletons. Sea urchin embryos were treated with SB43 at the indicated stages. The resulting morphology (upper panels, DIC) and skeletal development (lower panels, plane-polarized light illumination) are shown at pluteus stage (48 hpf). EB, early blastula; HB, hatched blastula; TVP, thickened vegetal plate.

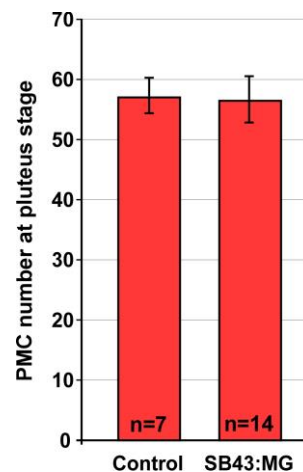


Figure S2. Late Alk4/5/7 inhibition does not affect PMC number at the pluteus stage. PMC counts from control and late SB43-treated (2-4 μ M) plutei are depicted as average \pm standard deviation.

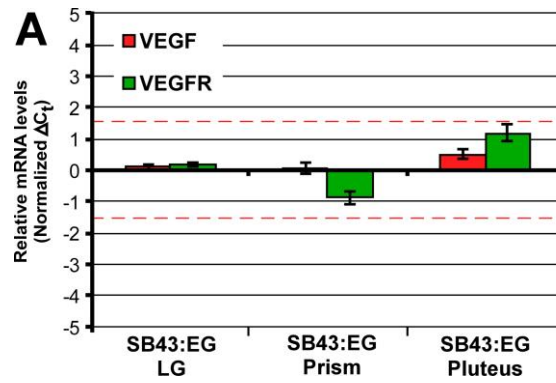


Figure S3. Late Alk4/5/7 inhibition does not affect *veg* or *vegfr* expression levels. Relative *veg* and *vegfr* mRNA levels in embryos treated with SB43 (3 μM) at the indicated time points. Normalized ΔC_T values are displayed as averages \pm SEM relative to controls. Dashed red lines indicate the threshold of significance at a ΔC_T of 1.6 (approximately 3-fold).

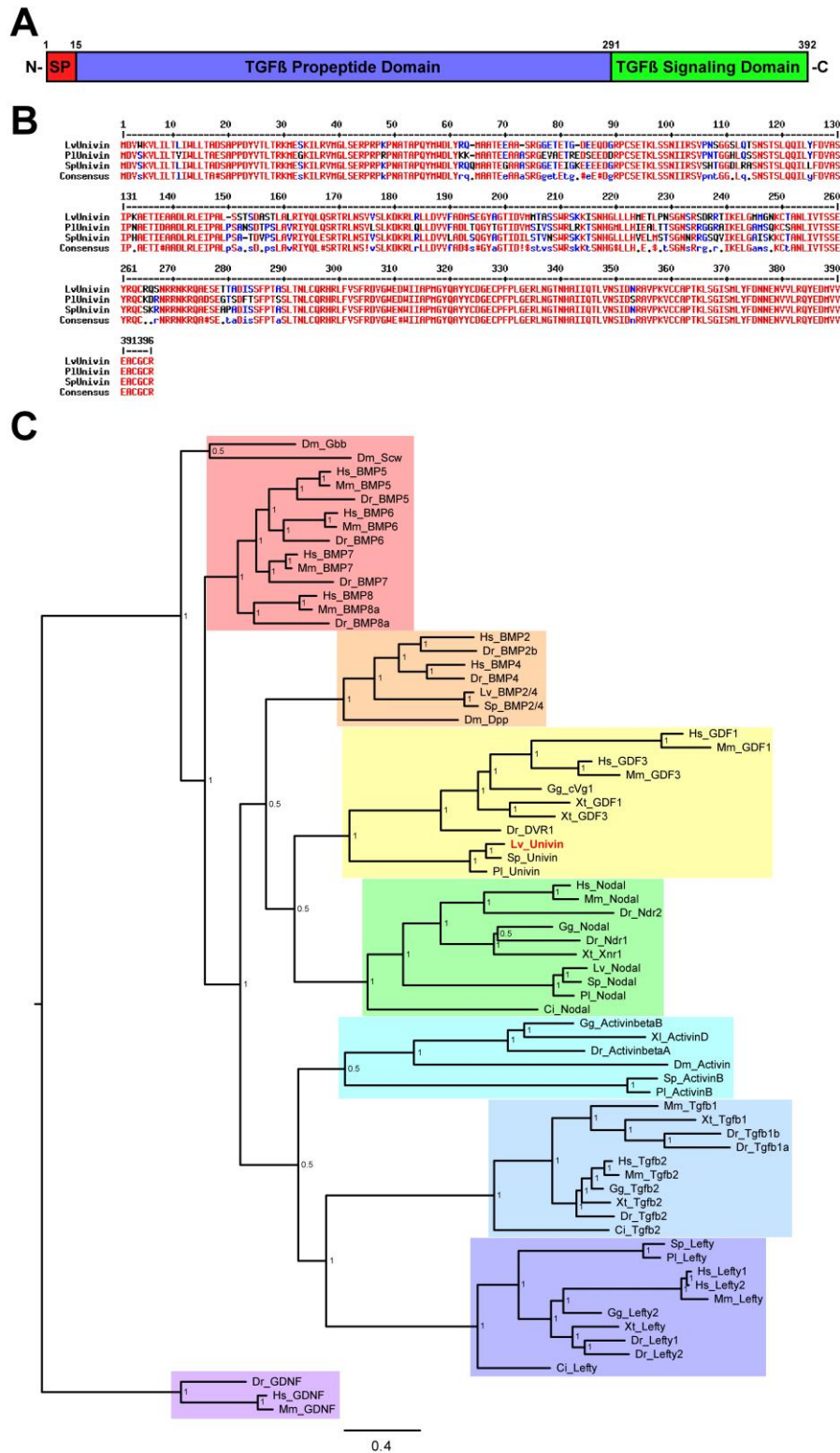


Figure S4. LvUnivin clades with other sea urchin Univins within the vertebrate Vg1/GDF1 subfamily. **A.** Domain structure of LvUnivin. The signal peptide (SP), the TGF- β prodomain, and the mature TGF- β signaling domain are displayed. Amino acid numbers above schematic indicate domain boundaries. **B.** Predicted Univin amino acid sequence alignment between three echinoderm species. **C.** Phylogenetic analysis of TGF- β superfamily members from various species is shown as a distance tree produced by Bayesian inference, with node probabilities indicated. GDNFs were used as an outgroup. Species included are echinoderm: Lv, *Lytechinus variegatus*; Sp, *Strongylocentrotus*

purpuratus; Pl, *Paracentrotus lividus*; vertebrate: Hs, *Homo sapiens*; Mm, *Mus musculus*; Gg, *Gallus gallus*; Xt, *Xenopus tropicalis*; Xl, *Xenopus laevis*; Dr, *Danio rerio*; invertebrate chordate: Ci, *Ciona intestinalis*; and arthropod: Dm, *Drosophila melanogaster*.

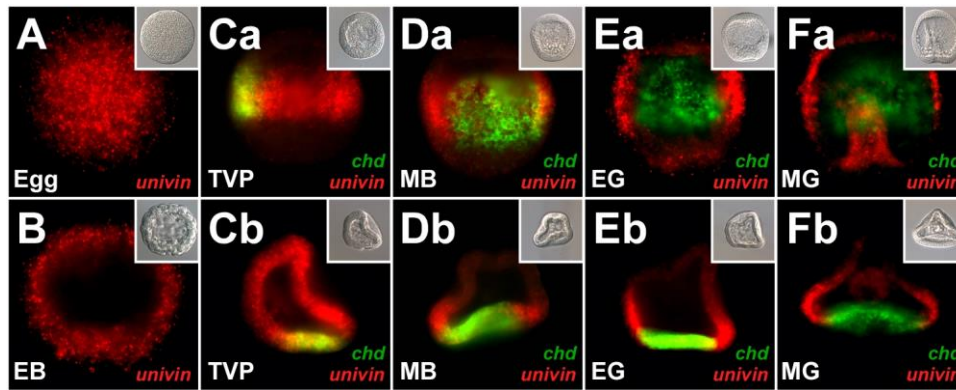


Figure S5. LvUnivin expression is spatially dynamic during gastrulation. Spatial expression of *univin* (red, A-F) and *chordin* (green, C-F) in control embryos at the indicated stages. Ca is shown in a lateral view; Da-Fa are shown in *en face* views, and Cb-Fb are shown in vegetal views. Insets show corresponding DIC images. Stage abbreviations as in Fig. S1.

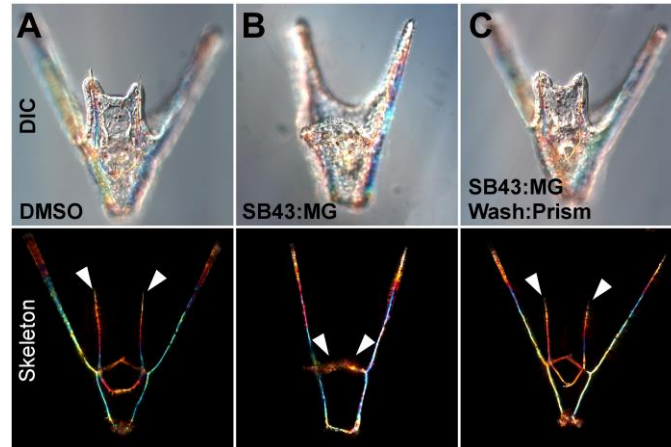


Figure S6. SB43 washout demonstrates that anterior skeletal patterning perturbation is reversible after the prism stage. Sea urchin embryos were treated with DMSO (A) or with SB43 (B, C) at MG stage; embryos were maintained in SB43 (B) or the drug was washed out at the prism stage (C). Morphology (upper panels) and skeletal development (lower panels) are shown for the resulting plutei (48 hpf); arrowheads indicate anterior skeletal elements.

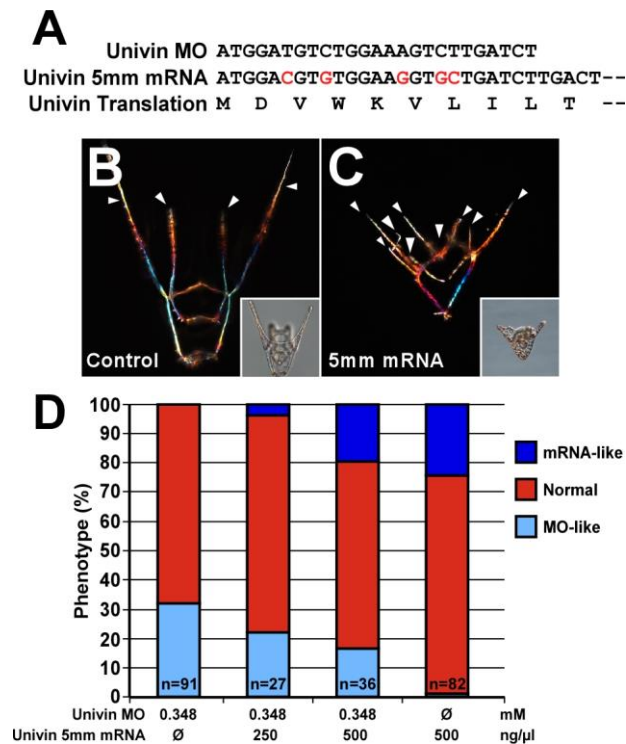


Figure S7. A non-binding, mutated LvUnivin mRNA inhibits LvUnivin morpholino. **A.** Schematics representing the sequence of the LvUnivin morpholino (MO), LvUnivin mRNA with five silent mutations (5mm, mutations in red), and the corresponding amino acid translation. **B-C.** Representative pluteus stage skeletons from control (B) and LvUnivin 5mm mRNA-injected (C) embryos. Insets show corresponding DIC images. Arrowheads indicate secondary skeletal elements. **F.** A graph depicting the percent distribution of phenotypes resulting from microinjections of LvUnivin 5mm mRNA and LvUnivin MO at the indicated concentrations.

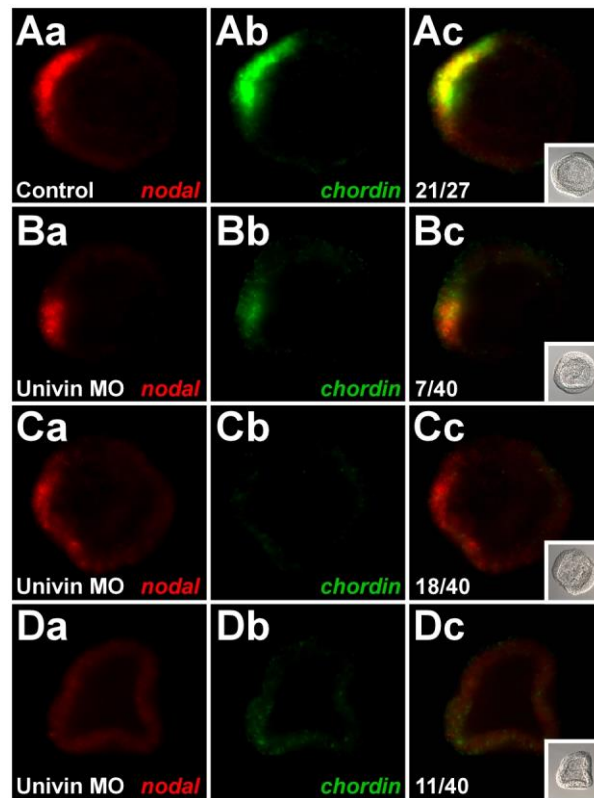


Figure S8. LvUnivin LOF has variable effects on Nodal and Chordin expression in *L. variegatus* embryos. *nodal* and *chordin* gene expression in control (A) and three exemplar LvUnivin MO-injected (0.33 mM) embryos at hatched blastula (HB) stage, chosen to depict the range of observed effects (B-D). Insets show the corresponding DIC images.