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Zygotic expression of Exostosin1 (Ext1) is required for BMP signaling and establishment of dorsal-ventral pattern in *Xenopus*

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ABSTRACT Exostosin 1 (EXT1) is a glycosyltransferase that contributes to the biosynthesis of heparan sulfate proteoglycans (HSPG). Loss of ext1 function leads to the human genetic disorder hereditary multiple exostoses (HME) and inhibits development in mouse, zebrafish and *Drosophila*. In *Xenopus*, loss of maternal EXT1 leads to impaired wnt11 signaling, resulting in a loss of dorsal embryonic development (Tao *et al.,* 2005), but the functions of zygotic *ext1* have not been elucidated. In this study, morpholino oligonucleotides were used to generate a zygotic partial loss of function for *ext1*, in order to evaluate the requirements for *ext1* function in gastrulation and paracrine signaling. Transcriptional profiling was carried out by microarray. Validation and subsequent analyses of gene expression were performed using Q-RT-PCR and *in situ* hybridization. Western blots were used to assess paracrine signaling pathway activity. Introduction of *ext1* MO led to gastrulation defects, which were partially rescued by co-injection of *ext1* mRNA. Microarray-based comparisons of gene expression in control vs. Ext1 MO embryos identified several developmentally significant genes that are dependent upon Ext1 function, including *brachyury* (*Xbra*). In addition, decreased Ext1 was shown to reduce the level of Wnt8 and BMP4 signaling and disrupt ventral-specific gene expression. Ext1 function is required for maintenance of normal levels of *BMP* and *wnt,* as well as their target genes. In addition, expression of *xbra* and the establishment of ventral mesoderm depend upon normal levels of Ext1. These findings suggest that *ext1*-dependent synthesis of HSPG is critical for wnt and BMP signaling, mesodermal identity, and ventral pattern.

KEY WORDS: *exostosin, microarray, BMP, Xenopus, gastrula*

Introduction

Exostosin1 and exostosin2 constitute a glycosyltransferase complex that is responsible for the polymerization of O-linked Heparan Sulfate (HS) chains (Ori *et al.,* 2008). Since Heparan Sulfate Proteoglycans (HSPGs) have been implicated in several cell signaling pathways, including wnt, FGF, BMP, TGFβ, and hedgehog, a reduction or loss of ext1 function is expected to have significant impact on a wide range of developmental processes. Mutations in human EXT1 are responsible for hereditary multiple exostosis (HME) (Ahn *et al.,* 1995), now known as multiple osteochondromas, a genetic disorder characterized by multiple cartilaginous tumors.

Ext1 is critical for paracrine signaling via several pathways, as HSPGs are known to facilitate ligand-receptor interactions or act as low-affinity co-receptors (Ori *et al.,* 2008). Mutations in the *Drosophila* orthologue of EXT1, *tout-velu*, disrupt *Wingless* signaling (Takei *et al.,* 2004). Ext1 is indirectly implicated in BMP signaling, in that the HSPG Glypican 3 modulates BMP signaling in skeletal development and renal branching morphogenesis in mouse embryos (Paine-Saunders *et al.,* 2000). HSPGs have also been extensively implicated in FGF signaling (reviewed in Matsuo and Kimura-Yoshida, 2013). Ext1 has specifically been identified

Abbreviations used in this paper: BMP, Bone Morphogenetic Protein; FGF, Fibroblast Growth Factor; GO, Gene Ontology; HME, Hereditary Multiple Exostoses; HSPG, Heparan Sulfate Proteoglycans; MAP Kinase, Mitogen-Activated Protein Kinase; MO, Morpholino Oligonucleotide; NCBI, National Center for Biotechnology Information; PCP, Planar Cell Polarity; PCR, Polymerase Chain Reaction; Q-RT-PCR, Quantitative Reverse Transcriptase Polymerase Chain Reaction; TCF, T-Cell Factor; TGF-β, Transforming Growth Factor - beta.

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as an indirect modulator of hedgehog signaling in both flies and vertebrates. Interestingly, mice heterozygous for *ext2* knockout show defects in cartilage and bone development that are independent of Indian Hedgehog (Ihh) signaling (Stickens *et al.,* 2005).

Maternally expressed Ext1 is required for wnt11 signaling in early *Xenopus* embryos (Tao *et al.,* 2005). Depletion of the maternal *ext1* transcript results in reduced expression of the Wnt11 target genes *goosecoid*, *siamois*, *chordin* and *Xnr3*, leading to disruption of the dorsal-ventral axis (Tao *et al.,* 2005). Mouse embryos display a similar early requirement for *ext1*; whole-embryo knockout of either *ext1* (Lin *et al.,* 2000) or *ext2* (Stickens *et al.,* 2005) is lethal, as homozygous knockout embryos fail to complete gastrulation.

This early maternal requirement for *ext1* makes it difficult to assess the roles of *ext1* during subsequent developmental processes. To evaluate functions of zygotically expressed *ext1* during gastrulation, we generated partial loss-of-function using a morpholino oligonucleotide (MO) directed against *ext1* and evaluated the effects of *ext1* knockdown on gene expression using microarray-based expression profiling. Our results indicate that *ext1* is required for the completion of gastrulation and paracrine signaling via the wnt and BMP pathways. Interestingly, neither FGF nor nodal signaling is significantly affected by a reduction in *ext1* function.

Results

Knockdown of Ext1 causes gastrulation defects

In order to investigate the function of zygotic *ext1* in early gastrulation, we developed a morpholino oligonucleotide directed against *ext1* (EXT1 MO), as well as a 5-base mispair MO (EXT1 MIS). To evaluate the efficacy of the morpholino oligonucleotides,

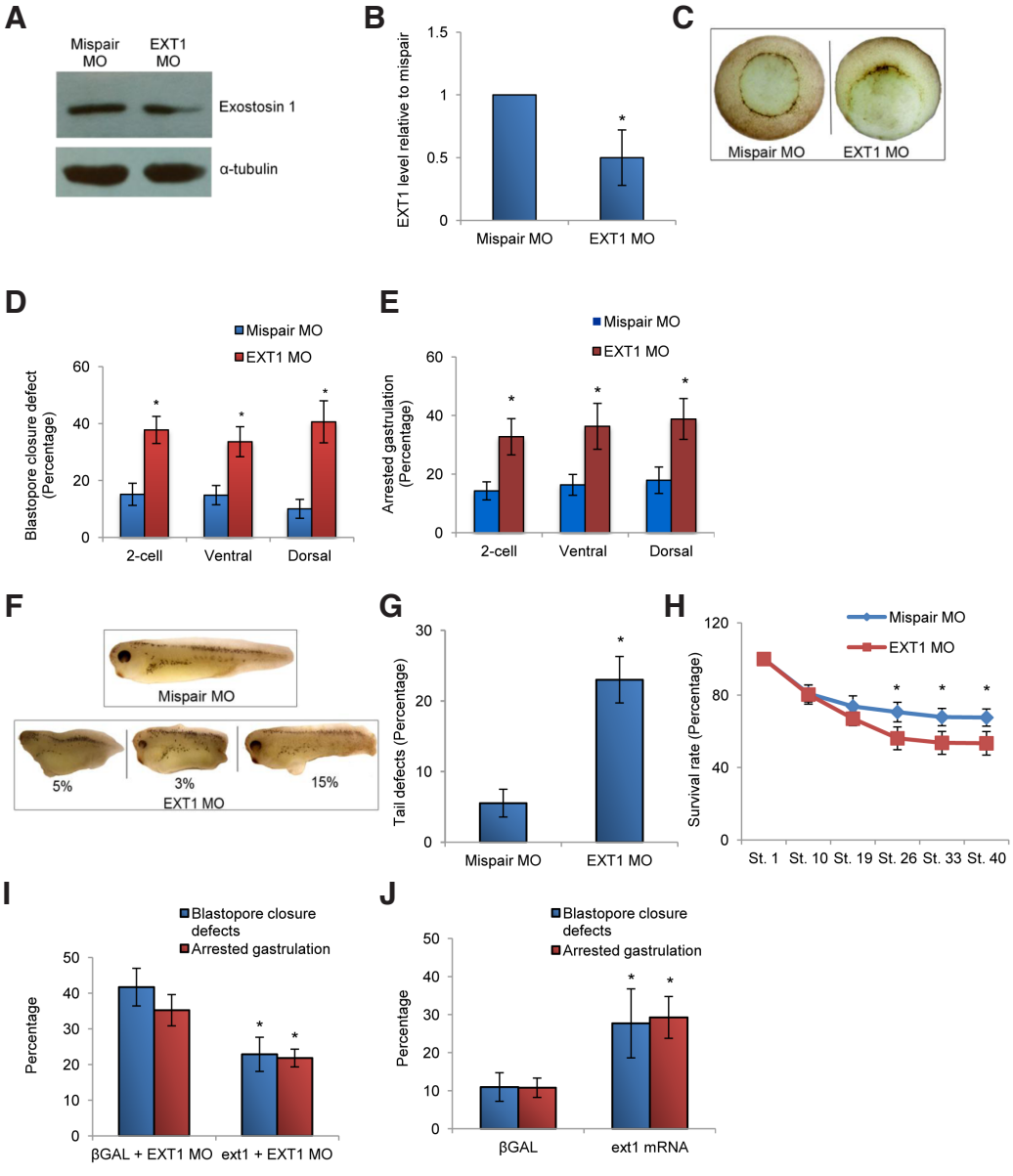

Fig. 1. Knockdown of *ext1* results in gastrulation defects. (A) *Embryos were injected with 20 ng of either the* EXT1 *MO or the mispair MO and lysed at st. 10.5 in preparation for immunoblotting to detect Ext1 and* α*-tubulin (loading control). Ext1 protein levels are decreased in* EXT1*-MO injected embryos.* **(B)** *Quantitative comparison of Ext1 protein accumulation. Relative Ext1 levels were normalized with* α*-tubulin. Bars represent mean ± S.D. N=3 independent experiments.* **(C)** *Midgastrula* EXT1 *MO-injected embryos exhibited incomplete blastopore formation and partial endoderm protrusion, whereas the mispair MO-injected embryos had a normal blastopore.* **(D,E)** *Embryos were injected with 20 ng of mispair or* EXT1 *MO at either the 2-cell stage or in either dorsal or ventral cells at the 4- cell stage; the phenotypes were compared at midgastrula (St. 10.5). A higher percentage of blastopore closure defects* **(D)** *or gastrulation arrest* **(E)** *were observed in* EXT1 *MO-injected embryos, irrespective of the stage or location of injection. N = 10 experiments, with a total of 400 embryos for each condition.* **(F)** *The phenotypes of embryos with head and tail defects in* ext1 *morphants.* **(G)** *The frequency of tail defects in* ext1 *morphants. Total number of tadpoles was 213. Bars present meant ± S.D. N=7 independent experiments.* **(H)** *The survival rates for EXT1 MO and EXT1 MIS embryos. Bars represent mean ± S.D. N=7 independent experiments (20-60 embryos/injection).* **(I)** *Blastopore closure defects and arrested gastrulation in* ext1 *morphants were partially rescued by coinjection with* ext1 *mRNA. Bars present meant ± S.D. N=5 independent experiments.* **(J)** *Embryos injected with 1ng of* β-galactosidase *or* ext1 *mRNA were collected and phenotypes were compared at gastrulation stage. The embryos overexpressing* ext1 *had higher frequencies of blastopore defects and gastrulation arrest compared to controls. Bars present meant ± S.D. N=5 independent experiments. * $p < 0.05$.*

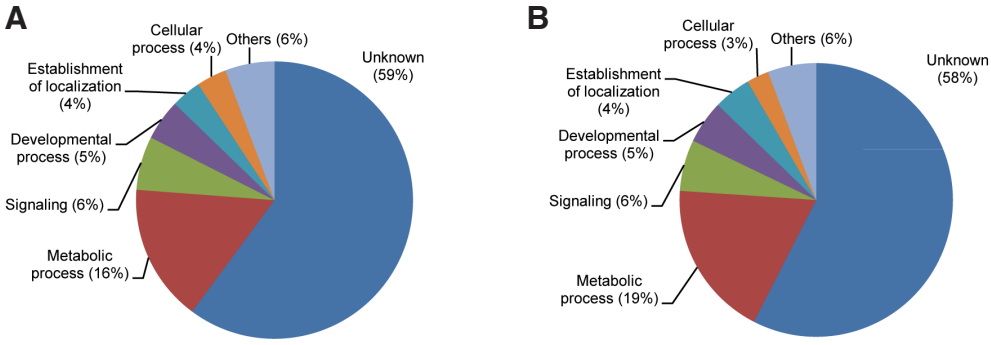

Fig. 2. Gene ontology (GO) of genes showing dysregulation in *ext1* morphants. *We characterized genes that showed a change of greater than two fold* **(A)** *or less than 0.5 fold* **(B)** *in* ext1 *morphant embryos. More than half of the genes up-regulated in response to* ext1 *knockdown had unknown functions. Among those with known functions, 16% were associated with metabolic processes, including transcriptional and translational regulation. Signaling-associated genes accounted for 6%, while 5% were involved in developmental processes. The remainder included establishment of localization (4%), cellular processes (3-4%), and others (6%). The "others" category includes multicellular organismal processes, response to stimulus, cell proliferation, cellular component organization, and reproductive processes. Similar proportions were observed for genes that were down-regulated following a reduction in Ext1 expression.*

20 ng of each MO were injected into embryos in preparation for immunoblotting to evaluate the accumulation of Ext1 protein in injected gastrula embryos. The results showed that in EXT1 MO-injected embryos, Ext1 protein levels are reduced to approximately 50% when compared to embryos injected with EXT1 MIS (Fig. 1 A,B). This concentration was also shown to produce phenotypic effects with EXT1 MO, but not EXT1 MIS; it was used in subsequent experiments, as injection of higher concentrations led to nonspecific effects.

The Ext1 knockdown embryos showed abnormal gastrulation compared to uninjected or EXT1 MIS-injected embryos. Specifically, the blastopore of *Ext1*-deficient embryos had incomplete, irregular closure and extrusion of endoderm (Fig. 1 C,D). In addition to an incomplete or abnormal blastopore, Ext1 knockdown embryos also exhibited arrested gastrulation (Fig 1E). Targeted injections of EXT1 MO into either dorsal or ventral blastomeres at the 4 cell stage also resulted in incomplete blastopore closure, (Fig. 1D) and a significant increase in gastrulation arrest (Fig. 1E) compared with embryos injected with the mispair morpholino. These phenotypes suggest that Ext1 is required for normal gastrulation. Moreover, this requirement is not restricted to dorsal- or ventral-specific tissues.

Although there are no significant phenotypic abnormalities that emerge immediately following gastrulation, additional defects in the *ext1* morphants appear after neurulation. Embryos injected with EXT1 MIS had long straight tails, while *ext1* morphant embryos had a range of tail defects in nearly 25% of cases (Fig. 1 F-G). In approximately 1/3 of these embryos, anterior structures were reduced or absent (Fig. 1F). Knockdown of *ext1* also leads to increased mortality at later stages. The survival rate of *ext1* morphants drops significantly when the embryos start to elongate following neural tube closure (Fig. 1H).

We coinjected the EXT1 MO with mRNA encoding either *ext1* or β-*galactosidase* to determine whether overexpression of *ext1* would rescue the morphant phenotype. *Ext1* overexpression lowered the frequency of blastopore closure defects by approximately half and also reduced the incidence of gastrulation arrest as well (Fig. 1I). This result confirms the specificity of the EXT1 MO.

We evaluated the effects of *ext1* overexpression by injecting 2-cell embryos with 1 ng of mRNA encoding either β-*galactosidase* (control) or *ext1*; embryos were observed throughout gastrulation. Overexpression of *ext1* led to incomplete blastopore closure and arrested gastrulation (Fig. 1J), similar to the phenotypes observed in *ext1* knockdown embryos. The similarity between the overexpression and knockdown phenotypes suggests that the optimal level of *ext1* expression may reflect a ratio of Ext1 to other proteins, rather than an absolute or independent level. This similarity may also explain why coinjection of the EXT1 MO and *ext1* mRNA leads to a partial rescue.

Identification of genes responsive to changes in Ext1 expression

Since *ext1* has been implicated as an indirect contributor to multiple signaling pathways in vertebrate development, we sought an unbiased approach to identify genes expressed in gastrulae that would be affected by a reduction in *ext1* expression. Therefore, we carried out a microarray analysis to identify such genes. Embryos were injected with 20 ng of either EXT1 MO or EXT1 MIS at the two-cell stage, and mRNA was isolated when the injected embryos reached early gastrula (stage 10.25). The RNA samples were compared across 3 biological replicates using the Affymetrix *Xenopus laevis* genome 2.0 Gene Chip.

Ext1-regulated genes were defined as genes represented by probe sets that showed a ≥ two-fold change in expression across three independent biological replicates. We identified 405 transcripts up-regulated more than 2-fold and 609 transcripts down-regulated less than 0.5-fold out of approximately ~15,000 transcripts (See Supplementary Table 1); these transcripts were evaluated by gene ontology. The NCBI Unigene cluster assigned to the Affymetrix *Xenopus laevis* probe set was linked to the human orthologue. The human orthologues of each gene were then used as the basis for the gene ontology analysis (http://www.geneontology.org). Data collected from Gene Ontology analysis revealed that more than half of the genes affected by Ext1 knockdown were of unknown function. Among the remaining genes, the following categories were strongly represented: metabolic processes (including transcription and translation), signaling, developmental process, establishment of localization, cellular process, multicellular organismal process, response to stimulus, cell proliferation, cellular component organization, and reproductive process (Fig. 2). Most of the affected genes with known functions have primary activities unrelated to developmental processes.

Genes with known roles in embryonic development were selected for subsequent validation by q-RT-PCR. Five up-regulated genes (*isl1, pitx2, tbx5A, wnt5A, wnt7A*) and four down-regulated genes (*Xbra, pax3, wnt1, WT1*) were assayed (Fig. 3A). Significant differences in expression were found for *Xbra, isl1, pitx2, wnt1,* and

wnt7A, but not for *pax3, tbx5A, wnt5A,* and *WT1*.

Ext1 *and paracrine signaling pathways*

Given the abnormal gastrulation phenotype, we were particularly interested in the finding that the knockdown of Ext1 leads to a down-regulation of *Brachyury* (*xbra*) expression, since *xbra* activity is required for mesoderm formation, notochord development, and normal gastrulation in mouse, zebrafish and *Xenopus* (reviewed in Smith, 2004). During gastrulation, *xbra* is expressed throughout the marginal zone and is present in the notochord and tail tissue during neurula and tailbud stage (reviewed in Smith, 2004). We confirmed the downregulation of *xbra* expression in gastrula embryos via *in situ* hybridization (Fig. 3B).

Xbra activates transcription of *eFGF*, which maintains expression of *xbra*, thus establishing a positive feedback loop; several other signaling pathways, including activin/nodal, BMP, and canonical wnt, are also known to activate and/or maintain expression of *xbra* (reviewed in Smith, 2004). We assessed expression of eFGF, as well as another direct *xbra* target, *wnt11*, via Q-RT-PCR; *eFGF* expression is dramatically reduced in *ext1* morphant embryos, relative to controls (Fig. 3C). Expression of *wnt11* is also decreased, albeit to a lesser degree. This reduction in wnt11 expression may account for the gastrulation defects, since wnt11 is required for appropriate coordination of mesodermal cell movements during gastrulation.

Since several paracrine pathways contribute to the regulation of *xbra* expression, we initially asked whether these pathways are individually affected. We initially investigated FGF signaling by asking whether phosphorylation of the FGF effector Extracellular Signal Regulated (ERK) Mitogen Activated Protein (MAP) kinase was altered in *ext1* morphant embryos (Fig. 4 A,B). Quantification of immunoblots of *ext1* morphant embryos probed with antibodies to ERK MAP kinase, phospho-ERK MAP kinase, or tubulin showed that there is no discernible difference in ERK MAP kinase phosphorylation. A similar lack of effect was observed with immunoblots probed with antibodies to Smad3 and phospho-Smad3, which would have revealed any alterations in the strength of signaling via nodal or activin (Fig. 4 C,D). These findings indicate that, surprisingly, neither FGF/MAPK nor nodal signals are impaired across the embryo by a reduction in Ext1 function.

We also evaluated the effects of Ext1 knockdown on BMP signaling by comparing levels of activated (phospho-) Smad1 in control and *ext1* morphant embryos. MO-mediated reduction in Ext1 decreased the amount of phospho-Smad1 by approximately 50% (Fig. 4C). We also assessed the expression of the BMP4 target genes *vent1*, *vent2*, and msx1 in *ext1* morphant embryos via Q-RT-PCR; *BMP4* itself was also included, since BMP4/Smad1 signals activate *BMP4* transcription, forming an autoregulatory loop. In *ext1* morphant embryos *vent1*, *vent2*, *msx1* and *BMP4* are down-regulated (Fig. 4D), and BMP4 shows a dramatic reduction to less than 10% of levels observed in controls. These findings demonstrate that *ext1* is required for BMP4 signaling and the maintenance of BMP4 expression.

Overexpression studies suggest that BMP4 is sufficient (Northrup *et al.,* 1995) but not necessary (Kumano and Smith, 2000) for *xbra* expression. Thus, these findings do not account for the reduction of *xbra* in *ext1* morphant embryos. Wnt8, however, is an upstream regulator of *xbra* at late blastula and gastrula stages (Vonica and Gumbiner, 2002): binding sites for TCF have been found in the *xbra* promoter, and mutation of TCF3 decreases the expression of *Xbra*, suggesting that *xbra* is a direct target of canonical Wnt signaling (Vonica and Gumbiner, 2002). We assessed expression of *wnt8* and the wnt8 target genes *myoD, HoxA1, HoxB1*, and *HoxD1* in *ext1* morphant embryos; expression of these genes is substantially reduced in the morphants, and expression of *wnt8* decreases to approximately 10% of controls (Fig. 4E).

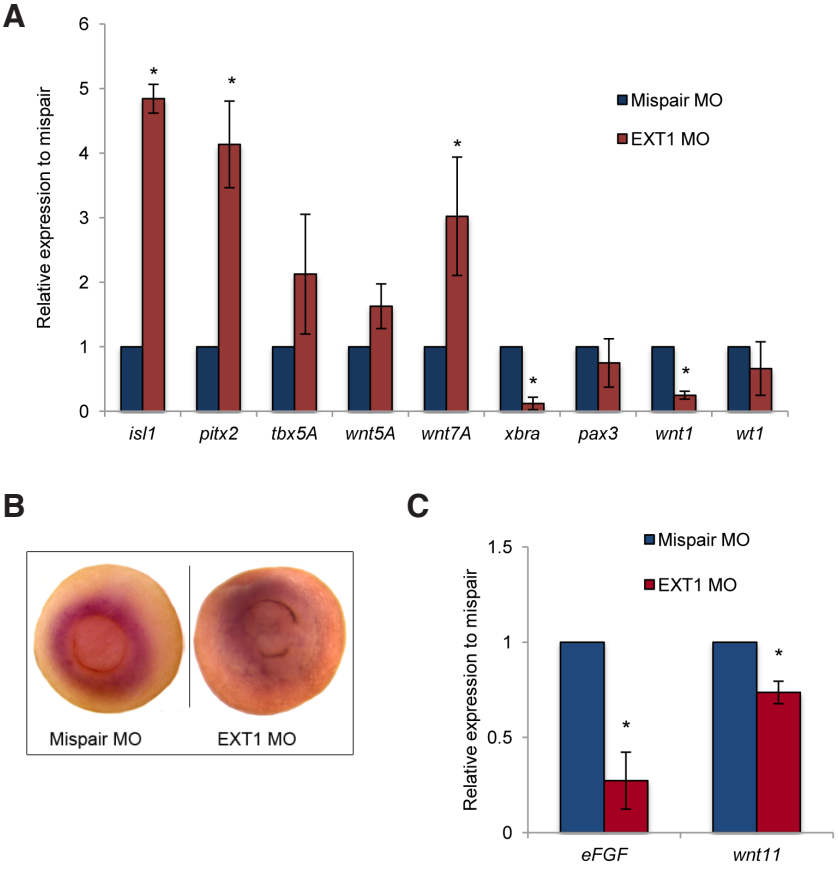

Fig. 3. Validation of *xbra* and other genes affected by *ext1* knockdown. (A) *Selected genes identified by microarray were validated using Q-RT-PCR. Embryos injected with 20 ng of EXT1 MO or EXT1 MIS were collected at stage 10.5 for RNA extraction and Q-RT-PCR.* **(B)** In situ *hybridization shows differences in* xbra *expression in control vs* ext1 *morphant embryos. Results are representative of over 2/3 of embryos from 3 independent experiments.* **(C)** *Q-RT-PCR assays for expression of the* xbra *target genes* eFGF *and* Wnt11. *Relative fold enrichment of each target gene was calculated after normalization with the housekeeping gene ornithine decarboxylase (*ODC*) using the ΔΔCt method. Bars represent mean ± S.D. N=3 independent experiments. *: p < 0.05.*

Ext1 *and dorsal-ventral pattern*

To understand how a reduction in *ext1* affects dorsal-ventral patterning during gastrulation, we examined the expression of the organizer genes *not1* and *otx2* and the ventrally expressed BMP target genes v*ent1* and *vent2*. As expected from our earlier Q-RT-PCR results, the regions expressing *vent1* and

vent2 are diminished in the *ext1* morphants, and the overall level of expression is also reduced (Fig. 5 A-D). Interestingly, however, there is no corresponding expansion of expression for the organizer genes; indeed, expression of the organizer genes *not1* and *otx2* is diminished across the dorsal region (Fig. 5 E-H). Organizer gene expression was also evaluated via Q-RT-PCR; expression levels for the organizer genes *chordin, goosecoid*, and *otx2* are decreased by over 50% in *ext1* morphants.

Discussion

Our results indicate that a reduction in the expression of Ext1 leads to defects in gastrulation. This conclusion is consistent with earlier findings indicating that HSPGs are required for both the cell migratory behavior characteristic of the head mesoderm (Smith *et al.,* 2009) and for the convergence extension movements observed in the axial mesoderm (Ohkawara *et al.,* 2003). Gain or loss of the *Xenopus* HSPGs syndecan-4 (xSyn4) or glypican4 (xgly4) result in convergent extension defects in activin-treated animal caps and a shortened anteroposterior axis in intact embryos (Ohkawara *et al.,* 2003; Munoz *et al.,* 2006). The zebrafish mutation *knypek* represents a loss of function of *glypican4*; mutant embryos show impaired convergent extension movement and abnormal cell polarity (Sepich *et al.,* 2011). Moreover, since HSPGs are essential for wnt signaling, loss of *ext1* function might disrupt the non-canonical wnt signals that regulate the planar cell polarity (PCP) pathway responsible for the coordination of cell behavior during convergent extension. It should be noted that although expression of wnt11 is decreased, the major components of the PCP pathway do not show changes in expression in ext1 morphant embryos (Suppl. Table 1). Impairment of HSPG synthesis and the resulting reduction in wnt11, however, presumably lead to attenuated signaling through the PCP pathway, which could underly the observed defects in gastrulation.

The T-box gene brachyury (*xbra*) is required for mesoderm formation and the completion of gastrulation (reviewed in Smith, 2004). Our transcriptional profiling revealed that *xbra* is down-regulated in *ext1*-morphant embryos. Similarly, in *EXT1* knockout mouse embryos, mesoderm does not form and *brachyury* expression is not detected (Lin *et al.,* 2000).

Xbra expression is initiated *in vivo* by nodal-related signals, and maintained in large part via a positive feedback loop involving transcriptional activation of *eFGF*. Our results indicate that neither the nodal/activin pathway nor the FGF/erk MAPK pathway show reduced activity in *ext1* morphant embryos, which suggests that *Xbra* expression is decreased in spite of nodal-related or FGF signaling. Wnt signals also contribute to the activation and maintenance of *Xbra* expression (Vonica and Gumbiner, 2002), so

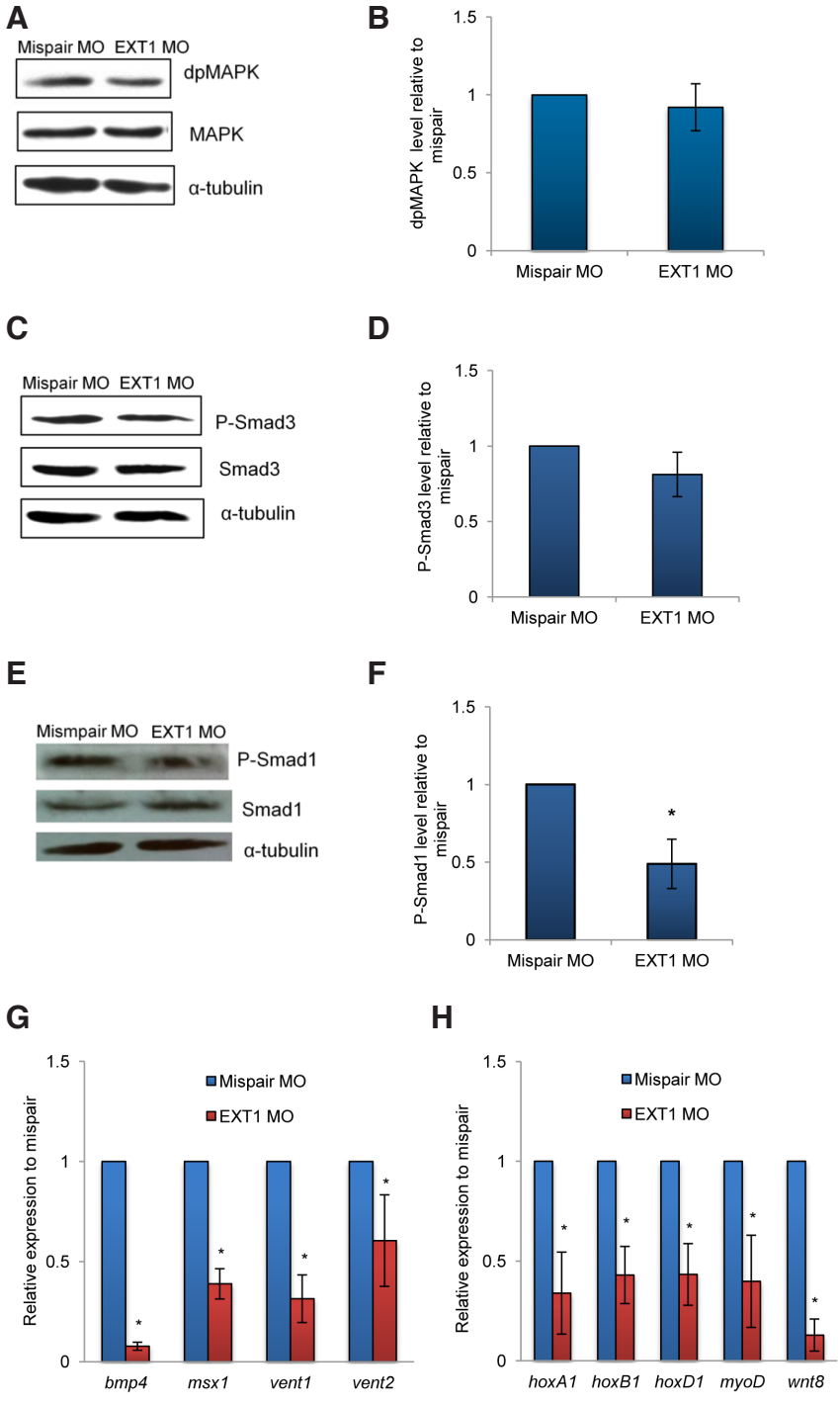

Fig. 4. The effects of Ext1 knockdown on paracrine signaling pathways. *Embryos were injected with EXT1 or mispair MO and lysed at stage 10.5.* **(A,C,E)** *Immunoblots showed levels of diphospho- Erk MAPK (dpMAPK), phospho-Smad3, and phospho-Smad1 in* EXT1 *or mispair MO-injected embryos.* **(B,D,F)** *Relative levels of phosphorylated forms of MAPK, Smad3, and Smad1 in* EXT1 *or mispair MO-injected embryos. Phospho-Smad1 is reduced in* EXT1 *morphant embryos, whereas levels of dpMAPK and phopho-Smad3 are unchanged.* **(G)** *Q-RT-PCR assays of* BMP4 *and the BMP4 target genes* msx1, vent1, *and* vent2 *in control or* ext1 *morphant embryos.* **(H)** *Q-RT-PCR assays of* wnt8 *and the wnt8 target genes* HoxA1, HoxB1, HoxD1, *and* myoD. *The relative -fold change of each target gene in* EXT1 *MO-injected embryos was calculated as described in Fig. 3. (B,D,F-H) Bars represent mean ± S.D. N=3 independent experiments. *: $p < 0.05$.*

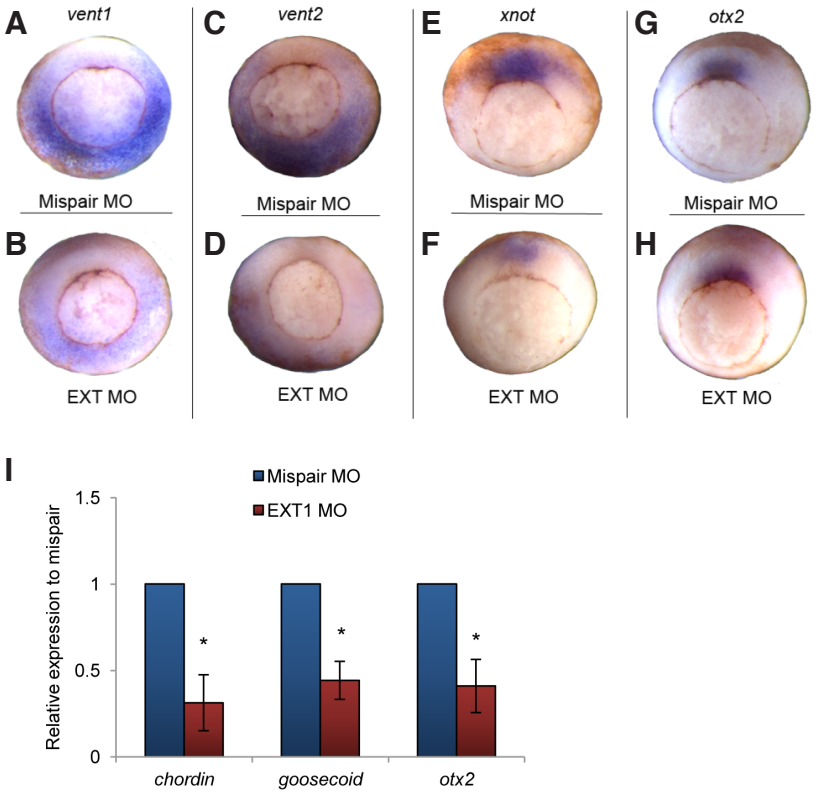

Fig. 5. Establishment of dorsal- or ventral-specific patterns of gene expression in *ext1* morphant embryos. *Embryos were injected with 20 ng of EXT1 or mispair MO.* In situ *hybridization of* vent1 **(A,B)***;* vent2 **(C,D)***;* not1 **(E,F)***; and* otx2 **(G,H)***. Expression patterns shown are representative of at least 2/3 of embryos from 3 independent experiments.* **(I)** *Q-RT-PCR assays of organizer-specific gene expression in* ext1 *MO or mispair MO embryos. Relative –fold changes were evaluated as described in Fig. 3. Bars represent mean ± S.D. N=3 independent experiments. *: p < 0.05.*

decreased wnt signaling activity may account for the reduction in *Xbra* expression in *ext1* morphants.

A related conundrum offered by these findings is that erk MAPK phosphorylation is unaffected, despite a dramatic reduction in the expression of *eFGF*. However, erk MAPK activity in gastrula embryos reflects inputs from other FGFs, as well as stimulation by Insulin-like Growth Factor (IGF) and other receptor tyrosine kinase-dependent paracrine signals. Thus, while eFGF activity may be depressed in the marginal zone, this may have only a limited effect on the erk MAPK activation averaged across the entire embryo.

Brachyury has been shown to play a critical role in the regulation of specific cell behaviors during vertebrate gastrulation (reviewed in Smith, 2004): in mouse embryos, cells lacking functional *brachyury* were unable to migrate out of the primitive streak, while in *Xenopus*, *xbra* function is required for the mediolateral intercalation behavior that underlies convergent extension (Conlon and Smith, 1999; Kwan and Kirschner, 2003). Brachyury function is not required for the migratory behavior characteristic of anterior mesoderm, however (Conlon and Smith, 1999; Kwan and Kirschner, 2003). Thus, the gastrulation defects observed in *ext1* morphant embryos may result directly from the reduction in *Xbra* expression.

The transcriptome comparisons of Yanai *et al.,* (2011) indicate that expression of *X. laevis ext1* mRNA drops by nearly 20% between st. 9 and the end of gastrulation; this decrease presumably reflects the loss of the maternal *ext1* transcript. Our results indicate that an additional loss of 50% of the total accumulated Ext1 protein is sufficient to disrupt signaling through at least two major paracrine signaling pathways, wnts and BMPs. The timing of the drop in *ext1* transcript accumulation is noteworthy because heparan sulfate accumulation increases significantly during gastrulation (Yamada *et al.,* 2009), and HSPGs are required for both signaling and cell movement during gastrulation. Thus, gastrulation may be a period of increased sensitivity to reductions in *ext1* accumulation or activity. Although depletion of maternal *ext1* does not affect expression of *vent1* (Tao *et al.,* 2005), our results indicate that a loss of zygotic expression leads to decreased *vent1* expression. This difference suggests that the critical interval, during which HSPGs are required for the BMP signals that activate *vent1*, follows the loss of maternal transcripts after the onset of gastrulation.

Our findings suggest that *ext1* function is required for the cellular signals that establish dorsal-ventral pattern within the mesoderm. Although primary pathways for both FGF and nodal-related signals are apparently unaffected, reduction in Ext1 expression disrupts both BMP and wnt signals. Biochemical studies have demonstrated that BMPs bind to HSPG (Ohkawara *et al.,* 2002), and knockdown of HSPG results in reduction of BMP signaling in *Xenopus* early development (Olivares *et al.,* 2009). The HSPG glypican3 modulates BMP signaling (Paine-Saunders *et al.,* 2000), and Wnt8 and Wnt11 bind directly to glypican4 (Ohkawara *et al.,* 2003). Tissue-specific knockout of *EXT1* leads to abnormalities in BMP signaling in mouse skeletal development (Matsumoto *et al.,* 2010).

In *ext1* morphants, both dorsal and ventral tissue identities are affected, as shown by a reduction in tissue-specific gene expression. The effects on ventral identity are more pronounced, as shown by the loss of regionalized expression of *vent2*: both BMP4 and Wnt8 regulate patterning of ventral mesoderm (Hoppler and Moon, 1998) and both pathways are impaired in *ext1* morphant embryos. In contrast, organizer-specific gene expression is still properly regionalized, reflecting proper function of the maternal wnt11 pathway, although expression levels are lower than in controls. This may result from the reduction in *Xbra* and the concommitant loss of mesoderm.

Our results add to the already extensive findings demonstrating roles for HSPGs and the enzymes responsible for their biosynthesis and modulation during early vertebrate development. Syndecans and glypicans have been strongly implicated as low-affinity receptors for several paracrine signals (Matsuo and Kimura-Yoshida, 2013), as regulators of the distribution of paracrine signals (Billoni *et al.,* 2013), and as modulators for the shape of morphogen gradients in embryonic tissues (Yan and Lin, 2009). Perlecan promotes shh localization and activity (Palma *et al.,* 2011). Moreover, the extracellular HSPG remodeling endosulfatases contribute to the regulation of wnt, BMP, and FGF signals (Freeman *et al.,* 2008). Additional studies have demonstrated that heparan sulfate itself regulates pluripotency and self-renewal in embryonic stem cells (e.g., Sasaki *et al.,* 2008). HSPGs and their modulators are essential

components for the fine-tuning of cell signaling and cell behavior.

Materials and Methods

Embryos collection and treatments

Embryos were obtained by the methods described in Sive *et al.,* (2000). Morpholino oligonucleotides were obtained from Gene Tools. Embryos were injected at the 2- or 4-cell stage with 20 ng/embryo of either the *ext1* MO (5' - AGCGTTTTTTCGCCTGCATGTGTCC - 3') or the mispair MO (5'-AGGGTATTTTCCCCTGGATGTCTC -3' - altered bases are underlined). Capped mRNAs for microinjection were synthesized using the mMessage mMachine *in vitro* transcription kit (Applied Blosystems).

Microarray

For each sample, RNA was isolated from five embryos at st 10.5 using the RNeasy mini kit (Qiagen). RNA samples were prepared from three independent sets of *ext1* MO- and mispair MO- injected embryos. Probe synthesis and hybridization to Affymetrix *Xenopus laevis* genome 2.0 GeneChip were performed by the Baylor College of Medicine Microarray Core Facility. The mispair and *ext1* MO-injected samples were processed in parallel to reduce inter-sample variation. Raw CEL and DAT files were generated using Affymetrix Microarray Suite 5.0 (MAS 5). The values for the control and experimental array were normalized, and the -fold change of each gene between mispair and *ext1* MO was calculated. Genes for which the standard deviation exceeded the -fold change were omitted from further consideration. Those genes with a -fold change bigger than 2 or smaller than 0.5, were chosen for further analysis. Gene Ontology (GO) analysis of each gene was carried out by using the NCBI Unigene set to identify the human orthologue, which was used as the basis for GO analysis (GO Consortium; http://www.geneontology.org).

Quantitative RT-PCR (Q-RT-PCR)

Embryos were lysed in TRIzol (Invitrogen), and RNA was isolated according to manufacturer's instructions. For each sample, 2 micrograms of total RNA were treated with DNase (RQ1 RNase-free DNase, Promega); cDNA synthesis was performed using either the superscript III (Invitrogen) or First-Strand cDNA Synthesis Kit (Roche). The qPCR primer sequences are listed below:

BMP-4 (F): GCATGTAAGGATAAGTCGATC;
BMP-4 (R):GATCTCAGACTCAACGGCAC
HoxA1 (F): ACCAGCAGCACCAGTCTTAC;
HoxA1 (R): GGAGGCTGGATACATTGTTG
HoxB1 (F): GATGGAAGGTTTGTTGTTGG;
HoxB1 (R): GTGAGGGTAACTGGGGTTCT
HoxD1 (F): TTTCCAGGCAATGGATCTTA;
HoxD1 (R): GCCCCCAGGATAAACTTCTA
isl1 (F): GGGTCAGGACGGTTCTTAAT;
isl1 (R): ACGAGCTGTTCCTTCATCAG,
myoD (F): TTGAGACCCTGAAGCGATAC;
myoD (R): TAATGTTCCAGAACCGGGTA
msx1 (F): ACTGGTGTGAAGCCGTCCCT;
msx1 (R): TTCTCTCGGGACTCTCAGGC
pax3 (F): CGCTAGATGGAGAAAGCAAG;
pax3 (R): CTGGCATAGCTGTAGGAGGA
pitx2 (F): CAGTGTGGACCAATCTGACA;
pitx2 (R): TGGTTCCTCTCCCTCTTTCT
tbx5A (F): CATCCTGAATTCAATGCACA;
tbx5A (R): CAAAGCCATTGTTCTCGTCT
vent1 (F): TGGTTCAACAGGGATTCTC;
vent1 (R): CTGCTAAGGAAGGATTTGC
vent2 (F): CCTCTGTTGAATGGCTTGCT;
vent2 (R): TGAGACTTGGGCACTGTCTG
wnt1 (F): AATGGTGGGGGATAGTGAAT;
wnt1 (R): TCTAGCACCAAAGGAACAGG
wnt5A (F): TTAAAACCTGTTGGCTCCAG;
wnt5A (R): CGCACTGTCGTATTTCTCCT
wnt7A (F): GAGAGCAAGCAGAAACAAGC;
wnt7A (R): TAGACCAGATCGGTGTCCAT
WT1 (F): CCTTGACCCCTCATTCTTTT;
WT1 (R): TAAATGCGCTGAGACACTGA
xbra (F): AGACATCTTGGATGAGGG;
xbra (R): GAAGGGTACTGACTTGAG

Whole mount in situ *hybridization*

In situ hybridization was carried out according to Sive *et al.,* (2000). The antisense RNA probes were synthesized and labeled with digoxygenin using the Maxiscript kit (Applied Biosystems).

Western Blot

For each sample, 10 embryos were lysed at st. 10.5 in 100 μl Kinase Buffer (20 mM HEPES pH7.5, 40 mM $MgCl_2$, 20 mM EGTA, 1 mM DTT, 80 mM glycerol-2-phosphate, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM microcystin, and 2.5X protease inhibitor cocktail). Samples were centrifuged at 13,000 rpm for 20 minutes in 4°C and then prepared for SDS-PAGE. Following electrophoresis, the protein was transferred from gel to membrane using a Bio-Rad semi-dry apparatus. The membrane was incubated with primary antibody overnight in 4°C. The primary antibodies are Exostosin 1 (Abbiotech, 1:100); Phospho-Smad1 (Cell Signaling, 1:500); Smad1 (Abcam, 1:1000); Phospho-Smad3 (Cell Signaling, 1:1000); Smad3 (Cell Signaling, 1:1000), and α-tubulin (Abcam, 1:2000). Proteins were visualized using HRP-conjugated secondary antibodies (Sigma-Aldrich) and the ECL plus kit (Amersham/ GE Healthcare Life sciences). The intensity of each band on the membrane was quantified using the "area measurement" function of Image J (Schneider *et al*., 2012), a Java-based image processing program (http://rsb.info.nih.gov/ij/index.html).

Accession number

The microarray data have been deposited into the NCBI Gene Expression Omnibus and are available under accession number GSE51562.

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References

AHN J, LUDECKE H J, LINDOW S, HORTON W A, LEE B, WAGNER M J, HORSTHEMKE B, WELLS D E. (1995). Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (*EXT1*). *Nat Genet* 11: 137-143.

BILIONI A, SÁNCHEZ-HERNÁNDEZ D, CALLEJO A, GRADILLA AC, IBÁÑEZ C, MOLLICA E, CARMEN RODRÍGUEZ-NAVAS M, SIMON E, GUERRERO I. (2013). Balancing Hedgehog, a retention and release equilibrium given by Dally, Ihog, Boi and shifted/DmWif. *Dev Biol* 376: 198-212.

CONLON F L, SMITH J C. (1999). Interference with brachyury function inhibits convergent extension, causes apoptosis, and reveals separate requirements in the FGF and activin signalling pathways. *Dev Biol* 213: 85-100.

FREEMAN S D, MOORE W M, GUIRAL E C, HOLME A D, TURNBULL J E, POWNALL M E. (2008). Extracellular regulation of developmental cell signaling by XtSulf1. *Dev Biol* 320: 436-445.

HOPPLER S, MOON R T. (1998). BMP-2/-4 and Wnt-8 cooperatively pattern the *Xenopus* mesoderm. *Mech Dev* 71: 119-129.

KUMANO G, SMITH W C. (2000). FGF signaling restricts the primary blood islands to ventral mesoderm. *Dev Biol* 228: 304-314.

KWAN K M, KIRSCHNER M W. (2003). *Xbra* functions as a switch between cell migration and convergent extension in the *Xenopus* gastrula. *Development* 130: 1961-1972.

LIN X, WEI G, SHI Z, DRYER L, ESKO J D, WELLS D E, MATZUK M M. (2000). Disruption of gastrulation and heparan sulfate biosynthesis in *EXT1*-deficient mice. *Dev Biol* 224: 299-311.

MATSUMOTO Y, MATSUMOTO K, IRIE F, FUKUSHI J, STALLCUP W B, YAMAGUCHI Y. (2010). Conditional ablation of the heparan sulfate-synthesizing enzyme *Ext1* leads to dysregulation of bone morphogenic protein signaling and severe skeletal defects. *J Biol Chem* 285: 19227-19234.

MATSUO I, KIMURA-YOSHIDA C. (2013). Extracellular modulation of Fibroblast Growth Factor signaling through heparan sulfate proteoglycans in mammalian development. *Curr Opin Genet Dev* 23: 399-407.

MUNOZ R, MORENO M, OLIVA C, ORBENES C, LARRAIN J. (2006). Syndecan-4 regulates non-canonical Wnt signalling and is essential for convergent and extension movements in *Xenopus* embryos. *Nat Cell Biol* 8: 492-500.

NORTHROP J, WOODS A, SEGER R, SUZUKI A, UENO N, KREBS E, KIMELMAN D. (1995). BMP-4 regulates the dorsal-ventral differences in FGF/MAPKK-mediated mesoderm induction in *Xenopus*. *Dev Biol* 172: 242-252.

OHKAWARA B, IEMURA S, TEN DIJKE P, UENO N. (2002). Action range of BMP is defined by its N-terminal basic amino acid core. *Curr Biol* 12: 205-209.

OHKAWARA B, YAMAMOTO T S, TADA M, UENO N. (2003). Role of glypican 4 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. *Development* 130: 2129-2138.

OLIVARES G H, CARRASCO H, AROCA F, CARVALLO L, SEGOVIA F, LARRAIN J. (2009). Syndecan-1 regulates BMP signaling and dorso-ventral patterning of the ectoderm during early *Xenopus* development. *Dev Biol* 329: 338-349.

ORI A, WILKINSON M C, FERNIG D G. (2008). The heparanome and regulation of cell function: structures, functions, and challenges. *Front. Biosci* 13: 4309 – 4338.

PAINE-SAUNDERS S, VIVIANO B L, ZUPICICH J, SKARNES W C, SAUNDERS S. (2000). Glypican-3 controls cellular responses to Bmp4 in limb patterning and skeletal development. *Dev Biol* 225: 179-187.

PALMA V, CARRASCO H, REINCHISI G, OLIVARES G, FAUNES F, LARRAÍN J. (2011). Shh activity and localization is regulated by perlecan. *Biol Res* 44: 63-67.

SASAKI N, OKISHIO K, UI-TEI K, SAIGO K, KINOSHITA-TOYODA A, TOYODA H, NISHIMURA T, SUDA Y, HAYASAKA M, HANAOKA, K, HITOSHI S, IKENAKA K, NISHIHARA S. (2008). Heparan sulfate regulates self-renewal and pluripotency of embryonic stem cells. *J Biol Chem* 283: 3594-3606.

SCHNEIDER CA, RASBAND WS, ELICEIRI KW (2012). "NIH Image to ImageJ: 25 years of image analysis". *Nature Methods* 9: 671-675.

SEPICH, D S, USMANI M, PAWLLICKI S, SOLNICA-KREZEL L. (2011). Wnt/PCP signaling controls intracellular position of MTOCs during gastrulation convergence and extension movements. *Development* 138: 543-552.

SIVE H, GRAINGER R, HARLAND R. (2000). *Early Development of Xenopus laevis: A Laboratory Manual.* Cold Spring Harbor Laboratory Press.

SMITH, E M, MITSI M, NUGENT M A, SYMES K. (2009). PDGF-A interactions with fibronectin reveal a critical role for heparan sulfate in directed cell migration during *Xenopus* gastrulation. *Proc Natl Acad Sci U S A* 106: 21683-21688.

SMITH, J C. (2004). Role of T-box genes during gastrulation. In *Gastrulation: From Cells to Embryo*. (ed. C.D. Stern) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

STICKENS D, ZAK B M, ROUGIER N, ESKO, J D, WERB Z. (2005). Mice deficient in ext2 lack heparan sulfate and develop extostoses. *Development* 132: 5055-5068.

TAKEI Y, OZAWA Y, SATO M, WATANABE A, TABATA T. (2004). Three *Drosophila* EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. *Development* 131: 73-82.

TAO Q, YOKOTA C, PUCK H, KOFRON M, BIRSOY B, YAN D, ASASHIMA M, WYLIE C C, LIN X, HEASMAN, J. (2005). Maternal Wnt11 activates the canonical Wnt signaling pathway required for axis formation in *Xenopus* embryos. *Cell* 120: 857-871.

VONICA, A, GUMBINER, B M. (2002). Zygotic Wnt activity is required for Brachyury expression in the early *Xenopus laevis* embryo. *Dev Biol* 250: 112-127.

YAMADA S, ONISHI M, FUJINAWA R, TADOKORO Y, OKABAYASHI K, ASASHIMA M, SUGAHARA K. (2009). Structural and functional changes of sulfated glycosaminoglycans in *Xenopus laevis* during embryogenesis. *Glycobiology* 19: 488-498.

YAN D, LIN X. (2009). Shaping morphogen gradients by proteoglycans. *Cold Spring Harb Perspect Biol* 1: a002493.

YANAI I, PESHKIN L, JORGENSEN P, KIRSCHNER M W. (2011). Mapping gene expression in two *Xenopus* species: evolutionary constraints and developmental flexibility. *Dev Cell* 20: 483-496.

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