Report

A New Molecular Logic for BMP-Mediated Dorsoventral Patterning in the Leech *Helobdella*

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Summary

Bone morphogenetic protein (BMP) signaling is broadly implicated in dorsoventral (DV) patterning of bilaterally symmetric animals [1–3], and its role in axial patterning apparently predates the birth of Bilateria [4–7]. In fly and vertebrate embryos, BMPs and their antagonists (primarily Sog/chordin) diffuse and interact to generate signaling gradients that pattern fields of cells [8–10]. Work in other species reveals diversity in essential facets of this ancient patterning process, however. Here, we report that BMP signaling patterns the DV axis of segmental ectoderm in the leech *Helobdella*, a clitellate annelid (superphylum Lophotrochozoa) featuring stereotyped developmental cell lineages, but the detailed mechanisms of DV patterning in *Helobdella* differ markedly from fly and vertebrates. In *Helobdella*, BMP2/4s are expressed broadly, rather than in dorsal territory, whereas a dorsally expressed BMP5–8 specifies dorsal fate by short-range signaling. A BMP antagonist, gremlin, is upregulated by BMP5–8 in dorsolateral, rather than ventral territory, and yet the BMP-antagonizing activity of gremlin is required for normal ventral cell fates. Gremlin promotes ventral fates without disrupting dorsal fates by selectively inhibiting BMP2/4s, not BMP5–8. Thus, DV patterning in the development of the leech revealed unexpected evolutionary plasticity of the conserved BMP patterning system, presumably reflecting its adaptation to different modes of embryogenesis.

Results and Discussion

Dorsoventral Patterning in Segmental Ectoderm of the Leech: The O-P Equivalence Group

Cell lineage plays a predominant role in leech embryogenesis. Segmental ectoderm and mesoderm arise from a posterior growth zone consisting of five bilateral pairs of lineage-restricted segmentation stem cells (M, N, O/P, O/P, and Q teloblasts). Each teloblast divides repeatedly and asymmetrically, giving rise to a column (bandlet) of segmental founder cells (primary blast cells). Near the surface in prospective dorsal posterior territory, the ipsilateral bandlets converge into parallel arrays, forming left and right germinal bands (Figures 1A and 1B). As the teloblasts continue adding blast cells posteriorly, the germinal bands move ventrally/vegetally, zippering together in anteroposterior progression along the prospective ventral midline to form the germinal plate. Segmental ectoderm arises from the four ectodermal lineages (N, O, P, and Q), each of which contributes segmentally iterated patterns of specific neural and epidermal cells [11]. Consistent with their definitive fates [12], the four ectodermal bandlets (designated by lowercase letters q, p, o, and n, respectively) form a dorsoventral (DV) array within each germinal band (Figure 1C).

Despite the overall determinacy of *Helobdella* development, the primary o and p blast cells in the lateral ectoderm constitute a developmental equivalence group. They are initially equipotent and assume distinct O (ventrolateral) or P (dorsolateral) fates based on their positions in the germinal band [13–18]. Previous work has revealed that contact with the dorsalmost q bandlet specifies the adjacent O/P-derived blast cells to assume the dorsolateral P fate [13, 16]. By analogy to DV patterning in other protostome embryos, we hypothesized that BMP may provide the Q-derived, DV patterning signal in the leech O-P equivalence group.

Short-Range BMP5–8 Signaling Is Necessary and Sufficient to Specify the P Fate

To test this hypothesis, we first surveyed BMPs and their antagonists in *Helobdella* (see Figure S1 and Table S1 available online); four BMPs (*Hau-admp*, *Hau-bmp2/4a*, *Hau-bmp2/4b*, *Hau-bmp5–8*) and one antagonist (*Hau-gremlin*) are expressed during stage 7/8, when patterning of the O-P equivalence group occurs (Figure S1). Whole-mount in situ hybridization (WMISH) revealed broad expression of *Hau-admp*, *Hau-bmp2/4a*, and *Hau-bmp2/4b* throughout the germinal bands, with no evident differences across the germinal band (Figure 1D). In contrast, *Hau-bmp5–8* was expressed specifically in the dorsalmost (q) bandlet (Figure 1E). These results raised the possibility that BMP5–8 is the principle ligand in DV patterning of leech segmental ectoderm; this would be a stark contrast to most other species, where BMP2/4s are the principle ligands. Furthermore, in vertebrates and in planarian flatworms, antidorsalizing morphogenetic protein (ADMP) and BMP2/4 are expressed at poles of the DV axis and form an autoregulatory loop to maintain the polarity of gene expression [19–21]; in *Helobdella* germinal bands, the expression of these genes was completely overlapping, implying that this regulatory loop does not operate in leech.

Also unexpectedly, *Hau-gremlin* was expressed at markedly higher levels in the p bandlet, immediately adjacent to the dorsalmost q bandlet, rather than in ventral territory (Figure 1F). Gremlin has not been shown to play a role in primary body axis patterning for other species. In vertebrates, its expression is first detected in neural crest cells [22], and it plays critical roles in later development [23–27].

To see whether Hau-BMP5–8 is necessary for patterning the O-P equivalence group, we knocked down Hau-BMP5–8 expression by injecting the Q teloblast or a progenitor (proteloblast OPQ or NOPQ) with *Hau-bmp5–8* antisense morpholino oligomer (ASMO). In normal embryos, the o blast cell divides with marked asymmetry, giving rise to a larger anterior daughter and a smaller posterior daughter, whereas the p blast cell divides nearly equally, giving rise to two similarly sized daughters (Figure 2A; Table S2; Movie S1). In *Hau-bmp5–8* morphant embryos, however, primary blast cells in both the nominal o and p bandlets underwent the O-like asymmetric divisions (Figure 2B; Table S2; Movie S2). Furthermore, the
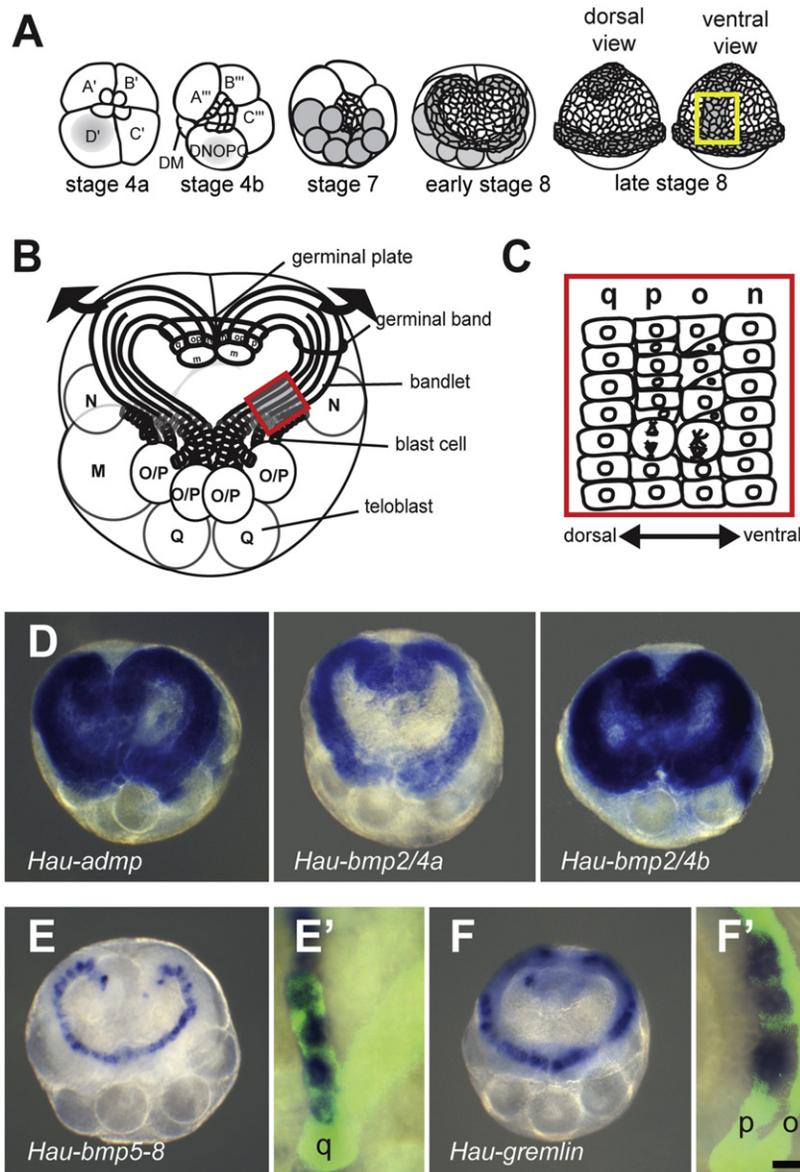
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Figure 1. BMP and Antagonist Expression during Dorsoventral Patterning of Segmental Ectoderm

In this and all subsequent figures, images depict dorsal views unless noted.

(A) Second axis specification in the *Helobdella* embryo originates with segregation of teloplasm (gray) into the D quadrant blastomeres (stages 1–6) and thence into five bilateral pairs of teloblasts (stage 7). Teloblasts give rise to the left and right germinal bands, covered by a micromere-derived epithelium (early stage 8). Germinal bands coalesce to form germinal plate (late stage 8); yellow box indicates the portion of the germinal plate illustrated in Figures 2I–2K and Figures 3A–3D.

(B) Early stage 8 embryo with epithelium removed, showing the dorsoventral (DV) array of ectodermal bandlets in each germinal band. Arrows indicate germinal band movements in germinal plate formation; red box indicates the portion of the germinal band illustrated in (C), (E′), and (F′), Figures 2A–2D, and Figures 3E–3G.

(C) Primary o and p blast cells divide with differing degrees of asymmetry (see text for details).

(D) *Hau-admp*, *Hau-bmp2/4a*, and *Hau-bmp2/4b* were expressed throughout the germinal bands.

(E and E′) *Hau-bmp5–8* is specifically expressed in a single bandlet (E). Combined whole-mount in situ hybridization (WMISH) and lineage tracing revealed that the bandlet expressing Hau-bmp5–8 is the q bandlet (E′).

(F and F′) *Hau-gremlin* is specifically upregulated in one bandlet (F), revealed by combined WMISH and lineage tracing as the p bandlet (F′); fluorescent lineage tracer was injected into the OP proteloblast to label o and p bandlets (O/P teloblasts not visible). Scale bar represents 150 μm in (D), (E), and (F) and 30 μm in (E′) and (F′).

itself was not affected, suggesting that *Hau-pax6a* expression in the ventral n bandlet is insensitive to BMP signaling. This is similar to *Platynereis*, a polychaete annelid, where expression of ventral midline genes is not as sensitive to ectopic BMP as the lateral neuroectoderm genes [30].

nominal p bandlet ceased to express the P fate marker *Hau-six1/2a* (Figures 2E and 2F) [28] and expressed *Hau-pax6a*, a gene normally expressed in O and N sublineages (Figures 2I and 2J) [29]. These results indicated that knockdown of Hau-BMP5–8 results in a P-to-O fate change. No such fate change was observed when a control morpholino oligomer was injected (Table S2), and a subsequent injection of mutated, ASMO-resistant *Hau-bmp5–8* mRNA into the Q teloblast rescued the morphant phenotype (i.e, restored *Hau-six1/2a* expression and nearly equal blast cell divisions to the nominal p blast cells; Figures 2D and 2H; Table S2).

We next determined the effects of expressing Hau-BMP5–8 ectopically in the n bandlet. In embryos injected with *Hau-bmp5–8* mRNA in an N teloblast, primary blast cells in both of the ipsilateral o/p bandlets underwent P-like division patterns (Figure 2C; Table S2; Movie S3); *Hau-six1/2a* was upregulated in nominal o blast cells (Figure 2G), and *Hau-pax6a* expression disappeared from the nominal o bandlet (Figure 2K). Interestingly, expression of *Hau-pax6a* in the n bandlet

Thus, we have shown that BMP5–8 signaling is both necessary and sufficient to direct O/P-derived blast cells to assume the P fate. Moreover, three aspects of our results suggest that the spatial range of BMP signaling within the germinal band is restricted to one cell diameter or less: (1) each of the o/p bandlets responds only to BMP5–8 expressed in the adjacent n or q bandlet; (2) when ASMO-induced knockdown of BMP5–8 expression in the q bandlet was rescued by subsequent mRNA injection, the rescued expression of *Hau-six1/2a* in the p lineage was restricted to cells immediately adjacent to the q blast cells inheriting the ASMO-resistant *Hau-bmp5–8* mRNA; and (3) similarly, when BMP5–8 was expressed ectopically in the n bandlet by mRNA injection, *Hau-six1/2a* expression was detected only in the o blast cells immediately adjacent to n blast cells containing *Hau-bmp5–8* mRNA (Figure S2). The spatially restricted nature of BMP5–8 signaling also suggested that it should be possible to invert the normal O and P fates within the germinal band by combining ASMO injection in the Q teloblast with *Hau-bmp5–8* mRNA injection
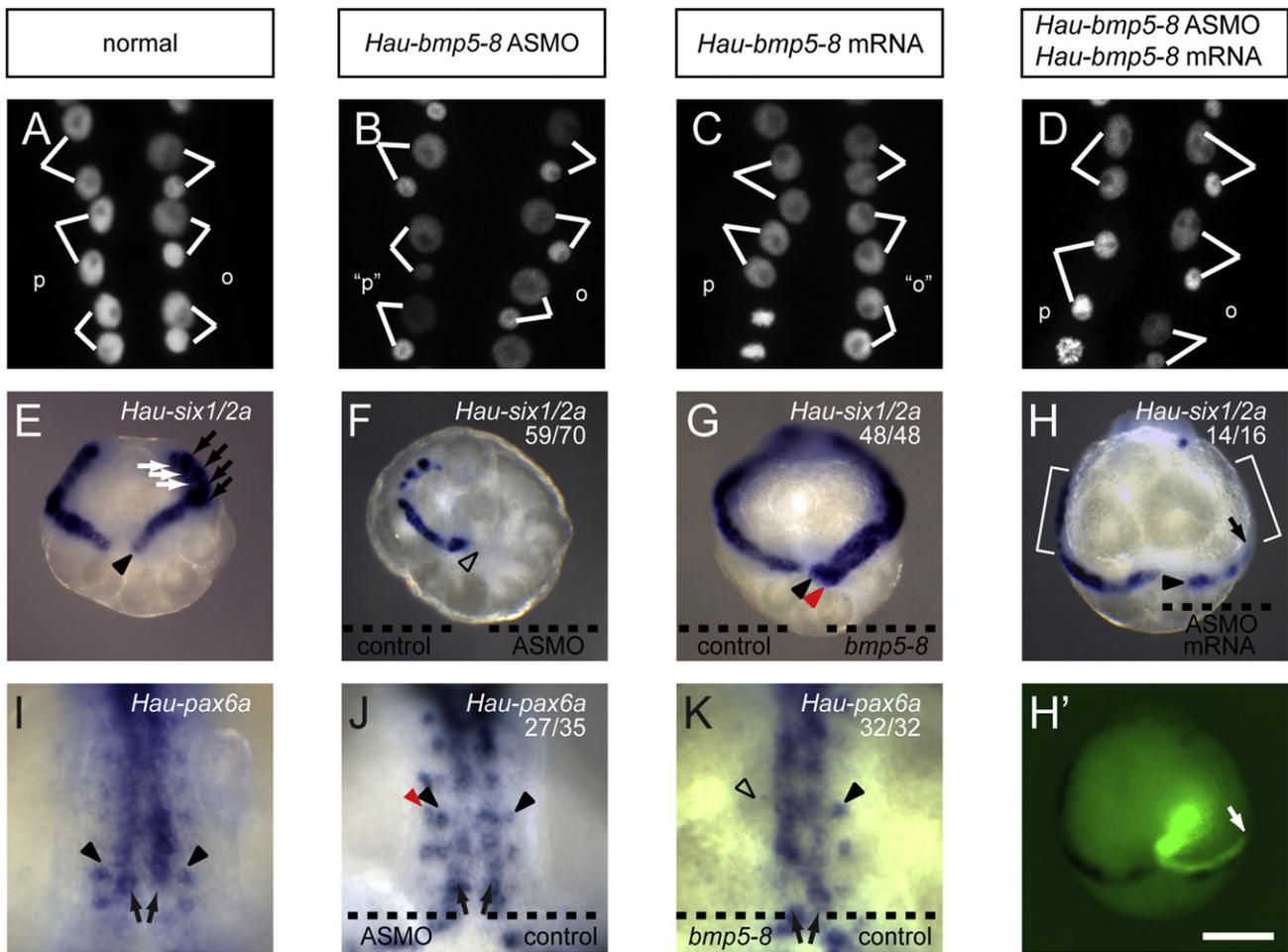
Figure 2. Hau-BMP5–8 Regulates Morphological and Molecular Markers of O and P Fates

Blast cell division patterns in right germinal band revealed by histone 2B:GFP fluorescence (A–D), *Hau-six1/2a* expression in germinal bands at early stage 8 (dorsal view) (E–H), and *Hau-pax6a* expression in germinal plate at late stage 8 (ventral view; see Figure 1A) (I–K).

(A) Lineage-specific o and p blast cell divisions in normal embryo.

(B) Nominal p blast cells ("p") made O-like divisions in embryos injected with *Hau-bmp5–8* ASMO.

(C) Nominal o blast cells ("o") made P-like divisions in embryos whose N teloblast was injected with *Hau-bmp5–8* mRNA.

(D) Normal blast cell divisions in embryo coinjected with *Hau-bmp5–8* antisense morpholino oligomer (ASMO) and an ASMO-resistant *Hau-bmp5–8* mRNA.

(E) Normal expression of *Hau-six1/2a* in the primary p blast cells (arrowhead) and also in OP (rostral segments, black arrows) and Q (white arrows) sublineages within older blast cell clones.

(F) *Hau-six1/2a* expression was not detected in the right germinal band (open arrowhead), containing *Hau-bmp5–8* ASMO, but was normal in the left germinal band, containing control MO.

(G) Misexpressing Hau-BMP5–8 in the n bandlet induced ectopic *Hau-six1/2a* expression in the adjacent nominal o bandlet (red arrowhead); control mRNA in the left n bandlet had no effect.

(H and H') *Hau-six1/2a* expression in the p bandlet was rescued (arrowhead) by introducing ASMO-resistant *Hau-bmp5–8* mRNA into Q_R 48 hr after injection of *Hau-bmp5–8* ASMO into proteloblast $NOPQ_R$ (H). Rescuing mRNA was coinjected with fluorescent lineage tracer (H'). The anterior boundary of the clone inheriting rescuing mRNA (arrows) aligned with the anteriormost *Hau-six1/2a*-expressing cells in the p bandlet. *Hau-six1/2a* expression was absent from cells anterior to this boundary (compare brackets on the left and right sides).

(I) Normal expression patterns of *Hau-pax6a* within the n (arrows) and o (arrowheads) bandlets.

(J) *Hau-pax6a* was normally expressed in the n (arrows) and o (black arrowheads) bandlets on both sides and was ectopically expressed in the nominal p bandlet (red arrowhead) on the right side, containing *Hau-bmp5–8* ASMO. The left side received a control MO.

(K) Misexpressing Hau-BMP5–8 in the right n bandlet and Hau-myostatin in the left n bandlet; *Hau-pax6a* expression was blocked in the right o bandlet (open arrowhead) but was normal in n bandlets (arrows) and left o bandlet (black arrowhead). Scale bar represents 15 μm in (A)–(D), 120 μm in (E)–(H'), and 50 μm in (I)–(K).

in the N teloblast, and this was indeed the case (Figure S2; Table S2).

Gremlin Expression Is Upregulated by BMP Signaling

Similarities in the expression patterns of *Hau-six1/2a*, a target of BMP signaling, and *Hau-gremlin* suggest that *Hau-gremlin* is upregulated by BMP signaling. To test this hypothesis, we assayed *Hau-gremlin* expression by WMISH in the Hau-BMP5–8 misexpression and knockdown experiments. As with *Hau-six1/2a*, *Hau-gremlin* expression was downregulated in the nominal p bandlet when the Q teloblast or its progenitor had been injected with *Hau-bmp5–8* ASMO (Figure S3A) and upregulated in the nominal o bandlet when the N teloblast had been injected with *Hau-bmp5–8* mRNA (Figure S3B).
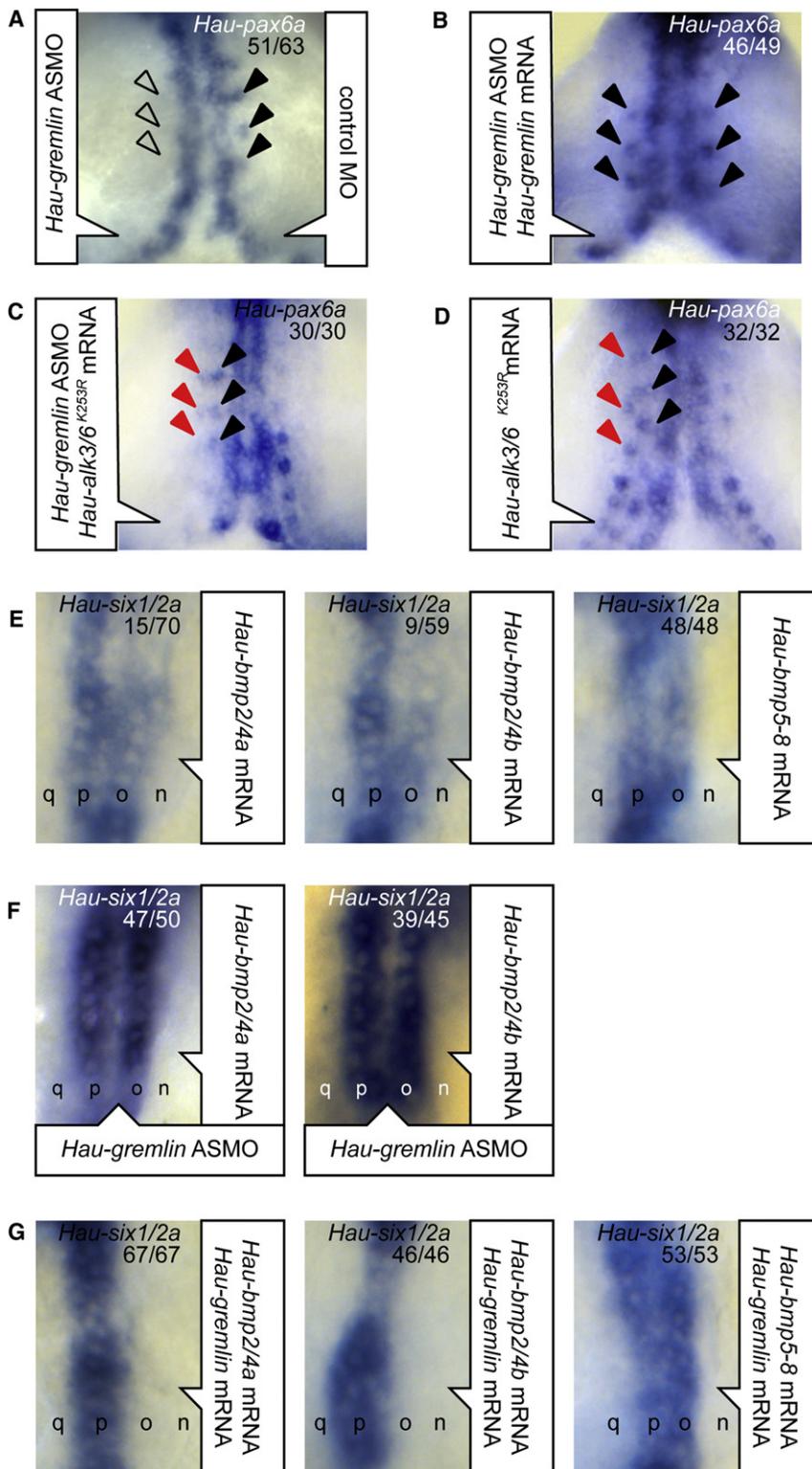
Figure 3. *Hau-gremlin* Is Required for Normal O Fate in the O-P Equivalence Group and Is a BMP2/4-Specific Antagonist

(A)–(D) are ventral views of the germinal plate; (E)–(G) are dorsal views of the right germinal band.

(A) *Hau-pax6a* expression in the o bandlet was blocked in the right side, injected with *Hau-gremlin* ASMO (open arrowheads), but is normal in the left side (black arrowheads), injected with a control MO.

(B) *Hau-pax6a* expression in the o bandlet is rescued (compare arrowheads on left and right) by coinjecting ASMO-resistant *Hau-gremlin* mRNA with *Hau-gremlin* ASMO.

(C) *Hau-pax6a* expression was detected in the nominal o bandlet (black arrowheads) and the nominal p bandlet (red arrowheads) on the right side and coinjected with *Hau-alk3/6^{K253R}* mRNA (encoding a dominant negative receptor) and *Hau-gremlin* ASMO.

(D) Injection of *Hau-alk3/6^{K253R}* mRNA alone similarly induces ectopic *Hau-pax6a* expression in the p bandlet (red arrowheads) in addition to normal *Hau-pax6a* expression in the nominal o bandlet (black arrowheads).

(E) Misexpressing Hau-BMP2/4a, Hau-BMP2/4b, or Hau-BMP5–8 in the n bandlet induced ectopic *Hau-six1/2a* expression in the adjacent nominal o bandlet. The differing efficacies indicate that Hau-BMP2/4a and Hau-BMP2/4b are not as efficient as Hau-BMP5–8 in inducing *Hau-six1/2a* expression.

(F) Ectopic *Hau-six1/2a* expression induced by misexpressing Hau-BMP2/4a or Hau-BMP2/4b in the n bandlet was enhanced in germinal bands containing *Hau-gremlin* ASMO.

(G) Injecting N teloblasts with equimolar mixtures of *Hau-gremlin* plus *Hau-bmp2/4a* or *Hau-bmp2/4b* failed to induce ectopic *Hau-six1/2a* expression in the adjacent o bandlet, but equimolar mixtures of *Hau-gremlin* plus *Hau-bmp5–8* induced *Hau-six1/2a* expression in the nominal o bandlet. Scale bar represents 50 μm in (A)–(D) and 30 μm in (E)–(G).

asymmetric O manner (Table S2) but failed to express the O fate marker *Hau-pax6a* (Figure 3A). *Hau-pax6a* expression in the o bandlet was rescued by coinjecting ASMO-resistant *Hau-gremlin* mRNA along with the *Hau-gremlin* ASMO (Figure 3B), suggesting that the loss of *Hau-pax6a* expression in morphant embryos was not due to nonspecific toxicity of the ASMO. Moreover, *Hau-pax6a* expression in morphant embryos was also rescued by injecting the proteloblast OP with mRNA encoding a dominant negative (DN) type I BMP receptor kinase (*Hau-alk3/ 6^{K253R}*) (Figure 3C). The same pattern of *Hau-pax6a* expression was observed in embryos expressing DN receptor alone (Figure 3D). Thus, the disruption of *Hau-pax6a* expression in the o bandlet in the Hau-gremlin knockdown was caused by hyperactive BMP signaling, consistent with Hau-gremlin functioning as a BMP antagonist.

Gremlin Expression in P Lineage Is Required for the O Fate

To determine the roles of gremlin in specifying the O and P fates, we knocked down Hau-gremlin expression with ASMO. In *Hau-gremlin* morphant embryos, the nominal o blast cells exhibited an intermediate fate: they still divided in the

To test the sufficiency of Hau-gremlin for determining the O fate, *Hau-gremlin* mRNA was injected into the OP proteloblast. In embryos overexpressing Hau-gremlin, primary blast cell division patterns in both the nominal o and p bandlets exhibited the O-like division pattern (Figure S3C; Table S2), but Hau-gremlin overexpression did not lead to a complete or permanent P-to-O transformation. In these embryos, *Hau-six1/2a* expression in the p bandlet was delayed but not abolished (Figure S3D). Thus, overexpressing Hau-gremlin only delayed expression of the P fate and was not sufficient to specify an ectopic O fate; the definitive P fate was presumably restored by a gremlin-insensitive mechanism or after ectopic gremlin had decayed. These results are consistent with previous work showing that the O lineage can be respecified to the P fate even after the blast cell has undergone the O-specific asymmetric division [14, 31]. Together, our results suggested that the cells in the O-P equivalence group can assume fates that are intermediate between the normal O and normal P fates, and that the BMP antagonist Hau-gremlin is required for specifying the normal O.

Gremlin Preferentially Antagonizes BMP2/4a and BMP2/4b but Not BMP5–8

Based on its expression and the knockdown and overexpression phenotypes, we postulated that Hau-gremlin may participate in patterning the O and P fates by antagonizing the broadly expressed BMPs but not the dorsally localized BMP5–8. To test this hypothesis, we first asked whether any of the broadly expressed BMPs could induce ectopic P fate when overexpressed in the n bandlet. Hau-ADMP overexpression failed to affect normal O and P fates in the germinal band (data not shown). In contrast, both Hau-BMP2/4a and Hau-BMP2/4b efficiently induced an O-to-P change in blast cell division patterns (Table S2). But, ectopic *Hau-six1/2a* expression was detected in only 15%–20% of embryos misexpressing BMP2/4s (Figure 3E; Table S2), and in those embryos, the level of ectopic *Hau-six1/2a* expression in the nominal o bandlet was significantly lower than that induced by Hau-BMP5–8 and lower than in the normal p bandlet (Figure 3E). Thus, Hau-BMP2/4a and Hau-BMP2/4b are weaker than Hau-BMP5–8 at inducing the P fate, and Hau-ADMP cannot induce P fate at all.

In embryos misexpressing *Hau-bmp2/4a* or *Hau-bmp2/4b* in the n bandlet, Hau-gremlin knockdown resulted in increasing levels and frequencies of ectopic *Hau-six1/2a* expression in the nominal o bandlet (Figure 3F), suggesting that endogenous Hau-gremlin normally inhibits signaling activity of BMP2/4s. To further test interaction between BMP2/4s and gremlin, we injected equimolar mixtures of mRNAs encoding *Hau-gremlin* and one of these BMPs into the N teloblast, then scored cell division patterns and *Hau-six1/2a* expression patterns in the adjacent nominal o bandlet. As expected, Hau-gremlin effectively blocked P-inducing activity of Hau-BMP2/4a and Hau-BMP2/4b (Figure 3G; Table S2). In contrast, Hau-gremlin did not block P-inducing activity of Hau-BMP5–8 (Figure 3G; Table S2), indicating that Hau-gremlin specifically antagonizes Hau-BMP2/4a and Hau-BMP2/4b but not Hau-BMP5–8.

Baseline-Level BMP Signaling Is Required for Maintaining Teloblast Stem Cells

The expression patterns of the BMP2/4s and their receptors suggested that low-level BMP signaling operates in all four ectodermal bandlets. To determine the role of baseline-level BMP signaling provided by the broadly expressed BMP2/4s,
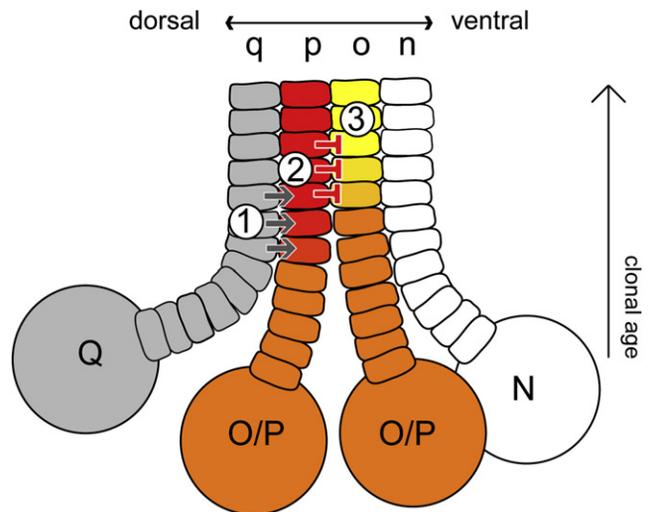

Figure 4. A Three-Step Model for BMP Signaling in the *Helobdella* O-P Equivalence Group

(1) Short-range signaling from q bandlet to p bandlet mediated by BMP5–8; (2) BMP5–8 signaling specifies the P fate (red), including upregulation of *gremlin*; (3) gremlin signaling specifies O fate (yellow) in the neighboring bandlet by inhibiting broadly occurring BMP2/4 signaling (see text for further details).

we sought to indiscriminately inhibit BMP signaling in ectodermal bandlets by expressing high levels of a DN receptor. Because expression of DN receptor by mRNA injection had failed to completely prevent nuclear phosphoSMAD accumulation (data not shown), we used a transient transgene system [32] to achieve higher sustained levels of expression and thus a stronger DN effect. When proteloblast OP was coinjected with tracer and the plasmid containing the DN receptor construct Hau-ALK3/6^{K253R}, blast cell production by teloblasts was disrupted; this was evidenced by the truncation of bandlets and irregularly shaped "teloblasts" (Figure S4A). When the wild-type Hau-ALK3/6 construct was injected at similar dosage, the bandlets remained intact and the teloblast morphology appeared normal (Figure S4B). Hence, in addition to DV patterning, BMP signaling is also required for the stem cell activity in the posterior growth zone.

A Feed-Forward Mechanism for BMP-Mediate DV Patterning in *Helobdella*

On the basis of this work, we propose the following model for patterning the *Helobdella* O-P equivalence group (Figure 4): (1) short-range Hau-BMP5–8 signaling from the dorsalmost q bandlet elevates BMP signal activity specifically in the adjacent dorsolateral p bandlet and not in the ventrolateral o bandlet; (2) elevated levels of BMP signaling in the p bandlet activate transcription of genes that specify the P fate, such as *Hau-six1/2a*, and also upregulate *Hau-gremlin*; (3) upregulation of gremlin in the p bandlet further dampens BMP signal activity in the adjacent o bandlet to permit normal O fate development (e.g., *pax6a* expression), whereas signaling in the p bandlet remains high as a result of the gremlin-insensitive Hau-BMP5–8 signal from the q bandlet. Because *Hau-gremlin* expression is positively regulated by BMP signaling, it is possible that gremlin and BMP2/4s form homeostatic feedback to maintain low-level BMP signaling activity throughout the germinal band. The functional significance of such low-level BMP signaling in the DV patterning is not entirely clear,

because knocking out BMP signaling entirely interferes with the blast cell-forming divisions of the teloblasts.

We have shown that the BMP2/4-specific antagonist gremlin is the principle BMP antagonist in DV patterning of leech ectoderm. Unlike gremlin, chordin—the principle antagonist in DV patterning of other species—does not discriminate different classes of BMPs [33]. Henceforth, deploying chordin in the same context as gremlin in leech ectoderm would be expected to block both BMP2/4 and BMP5–8, as in sea urchin embryos, where cells coexpressing chordin and BMPs sit at the low end of the signaling gradient [34]. The use of gremlin in leech DV patterning, however, allows gremlin-insensitive BMP5–8 signaling in the very cells expressing the highest levels of gremlin. Thus, specificity of ligand-antagonist interactions is critical for using this unusual combination of ligand and antagonist in the DV patterning of leech segmental ectoderm.

Evolutionary Significance

BMP-mediated DV patterning in *Helobdella* is consistent with the overall conservation of BMP signaling in bilaterian DV patterning but shows significant differences from that in other systems: (1) BMP5–8, rather than BMP2/4, exhibits dorsally localized expression and is the principle ligand in *Helobdella* DV patterning; (2) gremlin, rather than chordin, is the principle BMP antagonist and is expressed dorsolaterally, rather than ventrally; and (3) critical signaling interactions require cell-cell contact. These differences in specific patterning mechanisms among species may reflect the requirements for patterning different types of embryos (indeterminate lineages with large fields of equipotent cells as opposed to fixed lineages with limited sets of equipotent cells).

We note that *Capitella teleta*, the only other annelid for which whole genome sequence is available, also lacks a *chordin* homolog, whereas both *gremlin* and *chordin* are present in the mollusk *Lottia gigantea* (Table S1). Thus, pending the sequencing of other annelid genomes, we speculate that the loss of chordin may be an annelid synapomorphy. Because the role of cell lineage in *Capitella* DV patterning remains to be determined, we cannot rule out the possibility that the loss of chordin preceded the evolution of lineage-driven embryogenesis, in the branch leading to leeches. In any event, given that BMP signaling in DV patterning appears to have been lost completely in *C. elegans* [35] and in ascidians [36], representing the convergent evolution of lineage-driven embryogenesis in Ecdysozoa and Deuterostomia respectively, the leech provides a unique opportunity for exploring the evolutionary plasticity of BMP signaling in patterning the DV axis.

Experimental Procedures

Molecular cloning was performed following standard procedures, and details are given in Supplemental Experimental Procedures. Procedures for WMISH are described elsewhere [37]. Cell lineage tracing was carried out by injecting identified blastomere(s) with fluorescent dextrans (Molecular Probes). Synthetic mRNA [38], plasmid constructs [32], and custom morpholino oligomers (Gene Tools) were used to perform misexpression and knockdown experiments. Capped RNA was transcribed from linearized DNA template using mMESSAGE mMACHINE kit (Ambion). Wild-type *Hau-myostatin1* and a mutant form lacking the mature TGFβ cysteine knot domain (*Hau-myostatin1ΔC*) were used as negative control for BMP misexpression experiments; both yielded similar results. *h2b:gfp* mRNA was used to label cell nuclei to reveal the size ratios of the daughters of primary blast cells [32]. Transfection-grade plasmid DNA was prepared following standard procedure. Unless otherwise indicated, each mRNA was injected at 1 μM in the micropipette. Plasmid DNA was injected at 0.2 μg/μL in the

micropipette. Morpholino oligomer was injected at 1 mM in the micropipette. Teloblasts or proteloblasts were injected with a volume corresponding to ~0.5% of the cell volume.

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