Dissecting BMP signaling input into the gene regulatory networks driving specification of the blood stem cell lineage

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Hematopoietic stem cells (HSCs) that sustain lifelong blood production are created during embryogenesis. They emerge from a specialized endothelial population, termed hemogenic endothelium (HE), located in the ventral wall of the dorsal aorta (DA). In *Xenopus*, we have been studying the gene regulatory networks (GRNs) required for the formation of HSCs, and critically found that the hemogenic potential is defined at an earlier time point when precursors to the DA express hematopoietic as well as endothelial genes, in the definitive hemangioblasts (DHs). The GRN for DH programming has been constructed and, here, we show that bone morphogenetic protein (BMP) signaling is essential for the initiation of this GRN. BMP2, -4, and -7 are the principal ligands expressed in the lineage forming the HE. To investigate the requirement and timing of all BMP signaling in HSC ontogeny, we have used a transgenic line, which inducibly expresses an inhibitor of BMP signaling, Noggin, as well as a chemical inhibitor of BMP receptors, DMH1, and described the inputs from BMP signaling into the DH GRN and the HE, as well as into primitive hematopoiesis. BMP signaling is required in at least three points in DH programming: first to initiate the DH GRN through *gata2* expression, then for *kdr* expression to enable the DH to respond to vascular endothelial growth factor A (VEGFA) ligand from the somites, and finally for *gata2* expression in the DA, but is dispensable for HE specification after hemangioblasts have been formed.

BMP | GRN | HSC | *Xenopus* | hematopoiesis

Hematopoietic stem cells (HSCs) reside at the top of the hematopoietic hierarchy and serve as the reservoir for all differentiated blood cells in adult life. This regenerative capacity has been harnessed to treat a range of conditions from hematological malignancies to immunodeficiency syndromes via stem cell transplantation (1). A critical factor determining transplantation success is the number of recoverable $CD34^+$ hematopoietic stem and progenitor cells (HSPCs), which is challenging in many patients (2). Hence, de novo generation of HSCs has long been a goal to provide an ample supply of HSCs for transplantation. Transcription factor mediated reprogramming of somatic cells into induced HSCs and generation of HSCs via stepwise differentiation of pluripotent stem cells using developmental cues have both been used as methods with increasing but incomplete success (3, 4). A major caveat in both approaches is that the signaling cues and associated transcription networks required for HSC development are not yet fully understood.

HSCs are specified early during embryonic development through an intermediate stage called hemogenic endothelium (HE) from the dorsal aorta (DA) and other major arteries (5). Studies across model organisms ranging from fish and frogs to mice have shown that the hallmarks of HSC specification are conserved across species (6). In *Xenopus*, the cells fated to generate definitive blood are already segregated from primitive blood lineages at the 32 cell stage (7), a feature that allowed us to specifically characterize the HSC-generating endothelium of the DA and its precursors, definitive hemangioblasts (DHs) (8), at time points that are less feasible in other model organisms. Before the formation of the DA, these hemangioblasts are specified as bilateral populations in the lateral plate mesoderm, and express a cascade of transcription factors that are critical for hematopoietic and endothelial differentiation (see full list in ref. 9). In this 2013 study, we showed that vascular endothelial growth factor A (VEGFA)-dependent and -independent pathways synergize to specify this network of transcription factors culminating in a $fli1^+gata2^+etv2^+kdr^+tal1^+lmo2^+$ hemangioblast population. An analogous genetic network has recently been shown to operate in murine hematopoiesis (10). In *Xenopus*, fli1 resides at the top of the hierarchy and is required for *gata2* and *etv2* expression (11). The expression of scl/*tal1* requires activation of the Kdr receptor by VEGFA in addition to the presence of the transcription factors already mentioned. It was not known whether fli1 is sufficient to activate *gata2* and *etv2* or how this transcription factor network is activated other than by the VEGFA input (9). Soon after the hemangioblasts are established, a subset of these cells migrate to the midline to form the DA (7, 12). Specification of the HE toward the adult hematopoietic lineage happens in the ventral wall of the DA and can be assessed by the expression of the hematopoietic markers, *runx1*, *gfi1a*, and *spib/pu.1* (13, 14).

Bone morphogenetic protein (BMP) signaling has long been acknowledged to be pivotal in mesoderm induction and hematopoietic commitment (15). Specifically, BMP4 ligand is expressed in the mesenchyme around the DA across species (16–18). Addition of BMP4 ligand increases HSC output from cultured mouse aorta–gonad–mesonephros (AGM) tissue, and inhibition of BMP signaling by an antagonist ligand gremlin1 suggests that BMP is required for AGM hematopoiesis (19). In zebrafish, BMP signaling is required for the generation of *runx1* positive HE and temporal loss of function analysis strongly suggests that the source of BMP is the BMP4 ligand underneath the DA (18). On the other hand, loss of function experiments in mouse embryos and embryoid body (EB) cultures did not reveal a role for BMP signaling after the specification of kdr^+ mesoderm, which contains all of the precursors of blood and endothelium (20–23). Previous attempts to characterize the role of BMP signaling in primitive and definitive hematopoiesis

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in *Xenopus* lacked the temporal reagents to analyze BMP signaling specifically in the definitive blood lineage at high resolution (8). Therefore, we decided to revisit the role of BMP signaling in the definitive blood lineage to dissect the input of BMP into the updated gene regulatory network (GRN), as well as to resolve the apparent discrepancy between zebrafish and mouse with respect to BMP requirements. Here, we show that BMP signaling is required for the generation of the DH, but dispensable for the subsequent specification of HE. We identified three sequential inputs from BMP signaling into the establishment of the DH GRN, an early requirement for the expression of *gata2*, a transcription factor which together with Etv2 is at the top of the hierarchy controlling the establishment of definitive hematopoiesis, then for maintaining *kdr* expression, which is essential for *tal1* expression and finally for priming the expression of *gata2* after formation of the DA in the midline.

Results

BMP Signaling Is Required for Primitive and Definitive Hematopoiesis. BMP signaling is required for primitive blood formation in *Xenopus* (8, 24). Previously, we attempted to determine the function of BMP signaling in primitive and definitive blood lineages using a truncated, dominant negative BMP type I receptor construct (tBR) (25) by injecting it as an mRNA, which led to extensive mesodermal patterning defects complicating analysis of the role of BMP signaling specifically in definitive hematopoiesis (8). In retrospect, this approach was crude because it lacked temporal control, and the complexity and redundancy in the BMP signaling cascade was not known at the time. BMP signaling can be activated by up to a dozen ligands binding to four type I receptors that form heterocomplexes with three type II receptors (26). Because the specificity of the signaling is determined by the localized expression of the ligands, we extensively analyzed the expression of all BMP ligands and their corresponding receptors to identify those that might be involved in HSC formation. We found that BMP ligands *bmp2*, *bmp4*, and *bmp7* are the principal BMP signaling drivers expressed in the DA as well as in the DH precursors of the HE in the dorsal lateral plate (DLP) mesoderm (Fig. 1*A*). These ligands are also expressed during gastrulation and then ventrally in primitive blood and cardiac progenitors. In addition, bmp5 is expressed in the DA. Of the four type I receptors, *alk2* and *alk3* are ubiquitously expressed at all stages analyzed, and alk1 is expressed in the DA. *Alk6* is not expressed in the DLP or DA at the times analyzed. Finally, type II receptors, *acvr2a* and *acvr2b*, are expressed broadly from gastrulation and all type II receptors including *bmpr2* are expressed in the DA (Fig. S1). Hence, BMP receptors are in place at all time points starting from gastrulation to potentially have a role in the formation of the HE. Because multiple BMP type I and type II receptors are broadly expressed, a viable approach to analyzing BMP signaling input into definitive hematopoiesis would be to knock down BMP ligands *bmp2*, *-4*, and *-7*. However, a previous study demonstrated that at least during gastrulation, the function of these ligands are redundant and to block BMP signaling one needs to knock down all three at the same time. However, triple knockdown of *bmp2*, *-4*, and *-7* in *Xenopus* results in disruption of ventral mesendoderm formation preventing further analysis (27). Moreover, because the ligands are expressed widely in the ventral and lateral plate mesoderm especially at earlier stages, and because they are soluble molecules, a tissue-specific knockdown approach is not feasible. To bypass the requirement for BMP signaling in mesendoderm induction and patterning, yet be able to block it completely and in a temporally controlled fashion, we used a heat shock-inducible Noggin transgenic line (28), as well as a specific chemical inhibitor of BMP signaling, DMH1 (29). Noggin is a well-characterized extracellular antagonist of BMP ligands that inhibits BMP signaling by sequestering ligands away from the receptors. Noggin inhibits signaling downstream of BMP2, -4, -5, and -7, all of the ligands expressed before the formation of the HE (Fig. 1 *B* and *C*), while leaving signaling downstream of BMP6, -9, and -10, the ligands expressed specifically in the heart and kidney tubules, intact (Fig. 1 *C* and *D*) (30, 31). Hence, noggin is an ideal tool to ablate all of the BMP signaling that might be required during HE formation. DMH1 is a second-generation chemical inhibitor of BMP signaling that inhibits signaling downstream of Alk2 and Alk3 (29), the main BMP type I receptors expressed before and during the formation of HE, as well as Alk1 (32), a DA-specific BMP type I receptor (Fig. 1*A*), while leaving signaling downstream of Alk4 (activin signaling), Alk5 (TGFB signaling), and Alk6 [BMP signaling in neural tissue, somites, and notochord (Fig. 1*A*)] intact (29, 32). Because Alk6 is not expressed in lateral mesoderm, in the DLP or in the DA, DMH1 is expected to block all signaling activated by BMP2, -4, -5, and -7 in these tissues. Therefore, DMH1 blocks BMP signaling in a similar way to Noggin in ventral mesoderm and lateral mesoderm derivatives but by blocking BMP type I receptor activity rather than sequestering BMP ligands. In summary, these two reagents are used to block all BMP signaling during HSC ontogeny.

Because the HS:Noggin transgenic line has not been validated for the stages we aimed to analyze, to understand the dynamics of noggin induction upon heat shock, we monitored Noggin protein levels. Noggin was detectable within 30 min of heat-shock treatment, the protein level remained steady for up to 24 h (Fig. S2*A*), and the exogenous noggin mRNA was present throughout the embryo sections 2 h after treatment in stage 33 embryos (Fig. S2*B*). Furthermore, BMP-induced phosphorylation of Smad1, -5, and -8 was blocked by Noggin induction as well as DMH1 treatment (Fig. S2*C*). Noggin and DMH1 treatments before gastrulation led to strong dorsalization, which was avoided by starting the treatment postgastrulation at stage 14. In these embryos, lateral plate mesoderm patterning is relatively normal with some posterior patterning defects evident. Hence, we adopted a treatment regime where we heat shock or chemically treat the embryos from stage 14, with iteration of the treatment every 24 h when embryos are cultured longer than a day. As expected, inhibition of BMP signaling by Noggin and DMH1 completely blocks expression of red blood cell markers, *tal1* and *hba3* (hemoglobin alpha 3 subunit), further validating the reagents (Fig. 2*A*).

BMP signaling is required for primitive myelopoiesis in zebrafish (33), but a previous study in *Xenopus* did not detect an effect on myeloid development (34). Primitive myeloid cells are specified in two waves in *Xenopus*, the first one from the embryonic hemangioblast as early as the end of gastrulation at stage 13 [*runx1* (35), *cebpa*, *spib*, and *mpo* expression profiles submitted directly to Xenbase (36) by A.C.-U.], and the second one from posterior ventral tissues around stage 30 in close association with the primitive blood islands (37). Embryonic hemangioblasts are first exposed to BMP ligands at the end of gastrulation by stage 13 (38). To probe the BMP requirement of the first myeloid wave, embryos were treated from stage 12. Indeed, DMH1 treatment resulted in a strong reduction of patrolling myeloid cells, mpo and spib, and inhibited the specification of the second wave, ventral spib staining (Fig. 2*B*). When BMP signaling was blocked from stage 14 onward, there wasn't a reduction in the numbers of patrolling myeloid cells but the *spib*-marked second wave was still absent (Fig. S2*D*). In addition, we characterized embryonic hemangioblast differentiation at stage 16 by WISH and quantitative (q)PCR, and found that myeloid markers were already strongly reduced at stage 16 (Fig. S2 *E* and *F*). Hence, BMP signaling is required for primitive myelopoiesis as well as erythropoiesis in *Xenopus*.

BMP signaling is essential for hemangioblast formation and enhances endothelial commitment in ES cell cultures (39, 40). Embryos treated with DMH1 from stage 12 lacked vitelline vessels and displayed disorganized expression of the endothelial

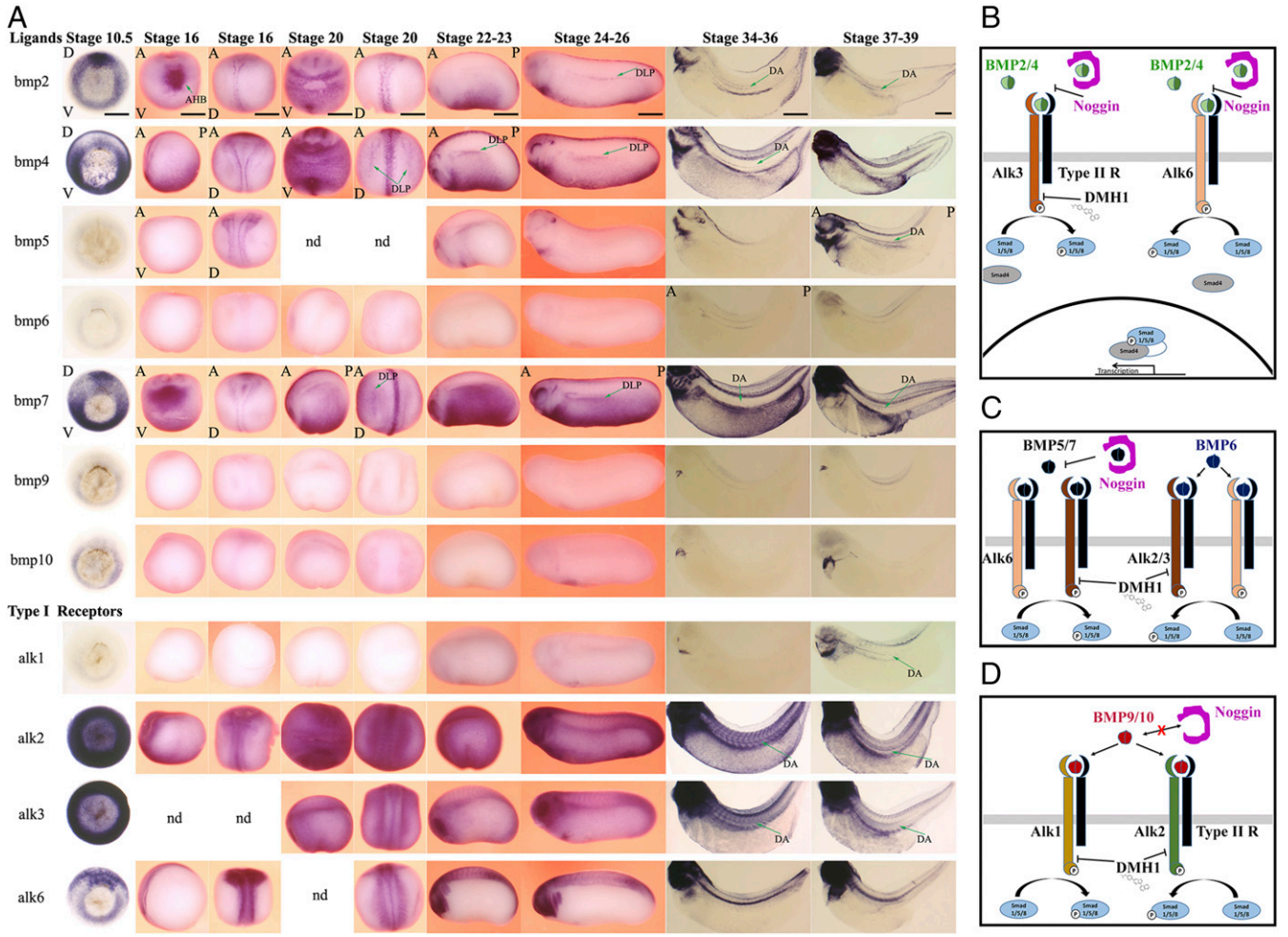

Fig. 1. Expression analysis of BMP ligands and receptors from midgastrulation stage to hemogenic endothelium formation and the description of reagents to inhibit all BMP signaling in blood ontogeny. Primarily, expression in tissues involved in blood stem cell lineage is described. (*A*) Ligands: *bmp2*, bmp4, and bmp7 are the principal BMP ligands expressed during gastrulation and onward. At stage 10.5, midgastrulation, bmp2 is expressed in the dorsal mesendoderm. At stage 16, early neurula, bmp2 expression is localized to anterior ventral mesoderm, which encompasses cardiac progenitors and anterior hemangioblasts (AHB). At stage 20–23, bmp2 is expressed along the ventral mesoderm and in lateral plate mesoderm, with signal tapering toward the DLP mesoderm. At stage 26, *bmp2* expression is localized along the DLP as well as ventral and cardiac mesoderm. At stages 34–39, bmp2 is expressed in the DA. At stage 10.5, bmp4 is expressed in mesendoderm, with expression diminishing toward dorsal mesendoderm. At stage 16–20, bmp4 is expressed extensively in ventral and lateral mesoderm. From stage 20 onward, bmp4 expression is localized to DLP, encompassing future hemangioblast. At stages 21–26, bmp4 expression is strong along the ventral and lateral plate mesoderm, specifically delineating the DLP tissue at the dorsal most expression domain. At stage 34, bmp4 is expressed in the DA. Bmp5 is not expressed in tissues related to blood lineages, except at stage 38–39, when it is expressed in the DA. Bmp6 is not expressed in HSC lineage. The only expression detectable is transiently in kidney tubules at stage 36. Bmp7 expression at stage 10.5 is localized to ventral most mesendoderm as well as a subset of dorsal mesendoderm. At stages 16–26, bmp7 is expressed along the ventral and lateral plate mesoderm, bordering the kidney field in the dorsal most extent. Bmp7 is strongly expressed in the lateral plate mesoderm for the rest of the developmental stages analyzed. From stage 34 onward, bmp7 is also expressed in the DA. Bmp8 is not expressed during *Xenopus* development. Bmp9 and bmp10 are expressed in the heart from stage 26 onward. Type I receptors: *alk1* is not expressed in early time points; later alk1 expression is primarily restricted to the heart and at stage 38–39 to DA. Alk2 and alk3 are ubiquitously expressed at all stages analyzed. From stage 34 onward, their expression is also observed in the DA. Alk6 is not expressed in blood stem cell lineage but is widely expressed early in dorsal mesoderm and later in neural tissues, neural crest, somites, and notochord. Embryos at stages 10.5 and 34–39 are cleared to enable visualization of the staining of the deeper tissues. For stage 10.5 embryos, D is dorsal and V is for ventral side. For stages 16–20, embryos are visualized from anterior-dorsal (A-D) and anterior-ventral (A-V) angles, except stage 16 bmp4 staining, which is documented from anterior–posterior (A-P) lateral view. From stage 21 onward, the pictures of embryos are taken from lateral view, with anterior–posterior labeled, where it is relevant, on the figure. Expression profile was not determined (nd) for some stages. (Scale bars: 0.5 mm.) (*B*) BMP2 and BMP4 signal through type I receptors, Alk3 and Alk6. Noggin inhibits signaling by preventing ligands from binding to receptors. DMH1 blocks signaling downstream of Alk3 but not Alk6. (*C*) BMP5, -6, and -7 signal through type I receptors Alk2, Alk3, and Alk6. Noggin blocks binding of BMP5 and -7 to type I receptors. Noggin does not inhibit signaling downstream of BMP6. DMH1 inhibits Alk2 and Alk3 but not Alk6. (*D*) BMP9 and -10 signal through type I receptors Alk1 and Alk2. Noggin does not block BMP9 or -10 from binding to receptors. DMH1 blocks Alk1 and Alk2 receptor activity.

genes *aplnr* and *tie2*/*tek* in the cardinal vein. When treatment started at stage 14, this effect was largely absent, and crucially as in Myers and Krieg (34), a fraction of embryos displayed expanded expression of endothelial markers in the ventral periphery, where erythrocyte precursors would have been located (Fig. S2*D*), albeit in a disorganized manner. In conclusion, in addition to validating heat-shock Noggin and DMH1 as appropriate reagents to block BMP signaling, we confirmed the predicted requirement, based on zebrafish embryos and embryonic stem cell (ESC) cultures, for BMP signaling in primitive myeloid and endothelial specification in *Xenopus*.

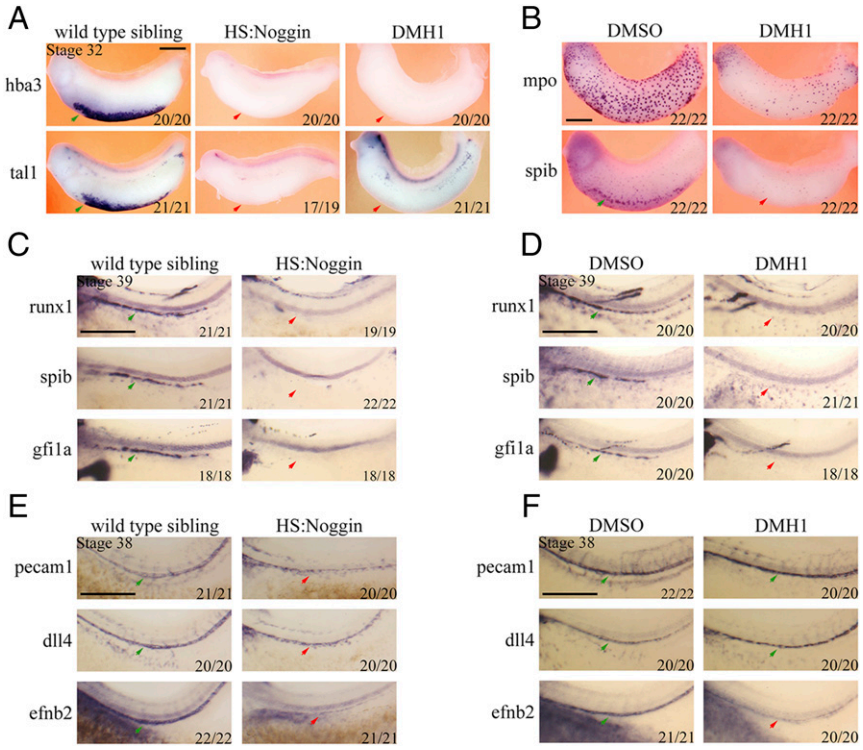

Fig. 2. BMP signaling is required for definitive and primitive hematopoiesis in *Xenopus laevis*. (*A*) Noggin protein induced by heat-shock treatment at stage 14 leads to a complete loss of *tal1* and hba3 expression in ventral blood islands (arrows) in HS:Noggin transgenic *Xenopus* stage 32 embryos. Similarly, DMH1 treatment starting from stage 14 blocks *tal1* and hba3 expression. (*B*) Inhibition of BMP signaling by DMH1 from stage 14 abrogates expression of myeloid blood markers mpo and spib at stage 33 compared with DMSO-treated control. (*C* and *D*) Inhibition of BMP signaling from stage 14 by Noggin (*C*) or DMH1 (*D*) results in the loss of expression of hemogenic endothelium markers *runx1*, spib, and *gfi1a* in the DA (red arrow for absence of in treated, green arrows for normal expression in control embryos). (*E* and *F*) The endothelium marker *pecam1* and arterial gene *dll4* is expressed in control as well as treated samples, albeit slightly lower in HS:Noggin embryos. The arterial marker efnb2 is severely reduced in either HS:Noggin-expressing or DMH1-treated embryos (red arrows in *E* and *F*). The stages at which the in situ hybridization was performed are indicated on the top left corner. Embryos were photographed from a lateral view, with anterior on the left and dorsal at the top. Numbers in bottom right corner indicate proportion of embryos displaying the phenotype. (Scale bars: 0.5 mm.)

Next, we investigated whether BMP signaling is required for definitive hematopoiesis. *Runx1*, a marker of HE and later of HSCs, is expressed along the ventral wall of the DA at stage 39. *Gfi1a* and *spib*, a *Xenopus* analog of *pu.1*, are direct downstream targets of Runx1 in the murine system (41) and are expressed in the hemogenic endothelium of the DA in *Xenopus*. Inhibition of BMP signaling from stage 14 by misexpression of Noggin or by treatment with DMH1 results in the loss of hemogenic endothelium marker expression in the DA of stage 39 embryos (Fig. 2 *C* and *D*). Indeed, *runx1*, *gfi1a*, and *spib* expression is completely eliminated in all of the treated embryos analyzed. On the other hand, the endothelium marker *pecam1* and arterial marker *dll4* are expressed along the trunk in treated embryos, albeit at slightly lower levels, indicating that the DA precursors are present (Fig. 2*E*). In addition, the arterially expressed gene efnb2 is severely reduced both in HS:Noggin misexpressed and DMH1-treated embryos (Fig. 2 *E* and *F*). Hence, we conclude that BMP signaling is required for the formation of hemogenic endothelium of the HSC lineage as well as for the proper specification of the DA.

BMP Signaling Is Required for Definitive Hemangioblast Programming but Dispensable After Their Formation. Having shown the requirement for BMP signaling for definitive hematopoiesis, we sought to dissect whether it is required at one specific stage or repetitively for key events. All HSCs in *Xenopus* are derived from DLP mesoderm (42, 43). The DLP first starts expressing angiogenic genes at stage 20 and is further matured into the full hemangioblast program during stages 24–27 by VEGFA signaling emanating from within the DLP as well as the somites (9). A subset of the DH starts migrating to the midline starting from stage 28, coalescing to form the DA at stage 31–32. The ventral floor of the DA becomes *runx1*$^{+}$ at stage 36. By stage 39, HE expresses *gfi1a*, spib, *tal1*, and *lmo2*, as well as *runx1*. Runx1 expression persists until HSPCs are budding from the DA at stage 43. We therefore started Noggin and DMH1 treatment at progressively later stages to identify for which specific event BMP is necessary. Misexpression of Noggin starting from time points before stage 28 completely eliminates *runx1* expression in the DA at stage 39 (Fig. 3*A*). Coincidentally, there was a strong reduction in the expression of DA marker efnb2 in treatments starting before stage 28 (Fig. 3*B*). However, another arterial marker *dll4* is expressed in the DA, regardless of the time BMP signaling is blocked, showing that not all arterial programming requires BMP (Fig. 3*C*). A subset of stages describing the embryo-wide phenotype along with *runx1* and *dll4* expression, and how HS:Noggin embryos are sorted from wild-type siblings, are described in Fig. S3. In short, we observed loss of *runx1* expression together with defects in the DA patterning when treatments were started before stage 28 (Fig. S3 *A* and *B*). Similarly, inhibition of BMP signaling by the drug, DMH1, starting from any time point before stage 28, results in loss of *runx1* expression (Fig. S3*C*). Runx1 is continuously expressed in and underneath the floor of the DA until HSPCs are budding off from the DA at stages 43–44. Similar to the observation at stage 39, abolition of BMP signaling starting from stage 30, does not affect *runx1* expression at stage 43 (Fig. S3 *D* and *E*). As in earlier time points (Fig. S2*C*), DMH1 inhibits BMP signaling in later stages effectively (Fig. S3*F*), and Noggin can be efficiently induced by heat shock in stage 32 embryos (Fig. S2*B*), which shows that the reagents work as expected at later stages. Therefore, we conclude that BMP signaling is required for specification of the definitive blood lineage during the hemangioblast stages before the formation of the DA, but is dispensable afterward.

BMP Signaling Is Required for *gata2* and *kdr* Expression in DHs. Because we observed that BMP signaling is required only during DH specification, we sought to understand its input into the GRN of the definitive hemangioblast. Previously, we described the hemangioblast transcriptional network in great detail (9). An updated version of this GRN is presented here as a guide (Fig. S4*A*). Importantly, it shows that *fli1* and *gata2* are at the top of the hierarchy, and are required early for *flk1*/*kdr* expression. Fli1 and Gata2 are later required for *etv2* expression at stage 22. Crucially, the requirement for Gata2 in *etv2* induction is partial (9). Once the expression of the core transcription factors, *fli1*, *gata2* and *etv2*, is established, VEGFA signaling through

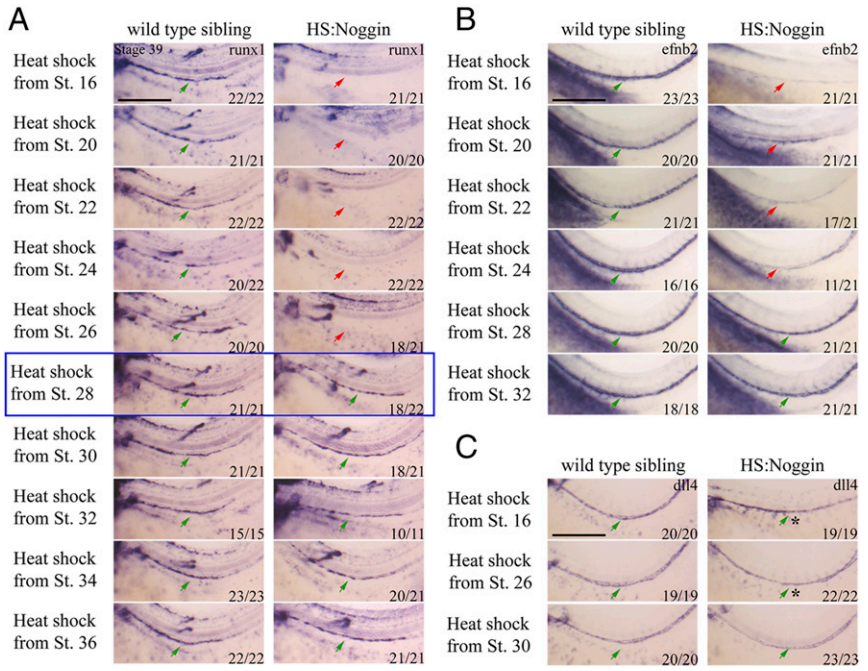

Fig. 3. BMP is required for the specification of hemogenic endothelium before stage 28. (*A*) Runx1 WISH of heat-shock time course. Runx1 expression is abolished in HS:Noggin siblings when BMP signaling is inhibited before stage 28 (blue box). Arrows mark *runx1* expression along the DA, with green arrows indicating normal expression levels and red arrows indicating absent or decreased expression. Expression of *runx1* is unaffected when the treatment is started at stage 28 or afterward. Runx1 is also expressed in lateral line nerves (*) as well as in some motor neurons (*), the staining of which in some images is overlaid with *runx1* staining in the DA due to embryo positioning and focus. (*B*) Efnb2 expression is strongly reduced in HS:Noggin siblings when BMP signaling is blocked before stage 28. (*C*) Dll4 expression is relatively normal in HS:Noggin siblings. However, the DA is not properly lumenized (*) when BMP signaling is inhibited before stage 28. Embryos were visualized from the lateral view, zoomed in to the trunk region, with anterior to the left and dorsal at the top. Proportion of embryos which the image represents is shown in bottom right corner. (Scale bars: 0.5 mm.)

Flk1/Kdr completes the circuit culminating in the induction of *tal1*/scl and *hhex* transcription factors. Tal1/Scl then controls the downstream gene expression cascade together with Lmo2. When BMP signaling is blocked by HS:Noggin (Fig. 4*A*) or DMH1 (Fig. S4*B*) from stage 14, there is no significant change in fli1 expression levels, but the expression of *gata2* is completely abolished. The level of *kdr* decreases drastically whereas expression of *etv2* is reduced. Furthermore, the expression of *tal1*/scl and *lmo2* is completely abolished in the DLP. We then quantitated the levels of expression of DLP genes by qPCR using cDNA from DLP tissue dissected at stage 25. Similar to the observations in WISH, all of the hemangioblast genes except fli1 display significantly decreased expression in HS:Noggin misexpressed DLPs (Fig. 4*B*). Because some of the DH genes are already expressed at stage 22, we probed the effect of BMP inhibition at this stage as well. As in stage 25 embryos, *gata2* and *kdr* are not expressed at stage 22 in treated embryos. Again, there is no effect on fli1 expression, but the level of *etv2* is strongly reduced at stage 22 (Fig. S4 *C* and *D*). Thus, we conclude that BMP signaling is required to initiate the DH network through induction of *gata2* expression.

Having established that BMP signaling is required at the level of *gata2* expression, we sought to determine the end point of this requirement for the DLP GRN. So we blocked signaling at stage 20 just when the first expression of *fli1*, *gata2*, and *kdr* commences, and analyzed expression of DH markers at stage 26. Because the HS:Noggin transgenic siblings are morphologically inseparable from wild types, we sorted transgenic siblings by counterstaining for misexpressed Noggin mRNA. In these embryos, *fli1*, *gata2*, *etv2*, and *lmo2* are all expressed as in wild-type embryos. However, there is a strong decrease in *kdr* and *tal1* expression (Fig. 4*C*). Finally, when BMP signaling is blocked from stage 23, there is no effect on the expression of hemangioblast genes at stage 26 (Fig. S5). Accordingly, we conclude that BMP signaling is required for *kdr* expression in the hemangioblast GRN after its requirement for *gata2* expression ceases.

When probing for the requirement for BMP signaling in HE, we concluded that BMP signaling is required until stage 28. Therefore, BMP must be instructive for the HE between stages 23 and 28 even though there is no apparent effect on gene expression in the DH after treatment at these stages. Between stages 28 and 31, precursors of the DA migrate from the DLP to the midline of the embryo, during which expression of *gata2*, *tal1*/scl and *lmo2* is switched off. Gata2 is reexpressed from stage 32. So, we sought to understand whether BMP signaling has a separate requirement for *gata2* expression at this stage in addition to its input during hemangioblast stages. Indeed, HS:Noggin siblings do not express *gata2* in the DA, when treated from stage 23, but express *gata2* like wild-type siblings when treated from stage 28 (Fig. 4*D*). Thus, we conclude that BMP signaling is required at two time points for *gata2* expression, first to initiate *gata2* expression in DH precursors, and then in DH for priming the expression of *gata2* in the DA, a requirement for the emergence of HE/HSCs.

Discussion

Generation of HSCs as replacement therapy for malignancies and age-related degeneration of blood output has been a holy grail of the hematopoiesis field for decades. Achieving this goal requires an understanding of the signaling cascades and associated transcriptional networks, but this understanding has proved challenging. Indeed, the seeming complexity of signaling cascades prompted many researchers to attempt to generate HSCs using mixtures of transcription factors rather than their de novo generation from pluripotent cells, which comes with its own drawback as many of the transcription factors are oncogenic proteins. Hence, we aim to understand the signaling events in the setting up of the transcription factor networks necessary for HSC generation. To this end, we have characterized the GRN architecture before HE specification extensively, and identified positive inputs from vegfa splice isoforms into this network as well as an inhibitory effect by TGFB signaling at early stages of hemangioblast formation (9, 14, 44). The current analysis furthers our understanding by dissecting the role of BMP signaling with regard to the definitive blood transcription network. BMP signaling is required at three instances in the DH, first to initiate the DH network through *gata2*, second for *kdr* expression to enable the DH to respond to VEGFA ligand from somites, and third for *gata2* expression in the DA, but is dispensable for HE gene expression after hemangioblasts have been formed (summarized in Fig. 5). Gata2 is required repetitively for the specification of the blood stem cell lineage in DHs and in the DA (9, 45, 46) and is required

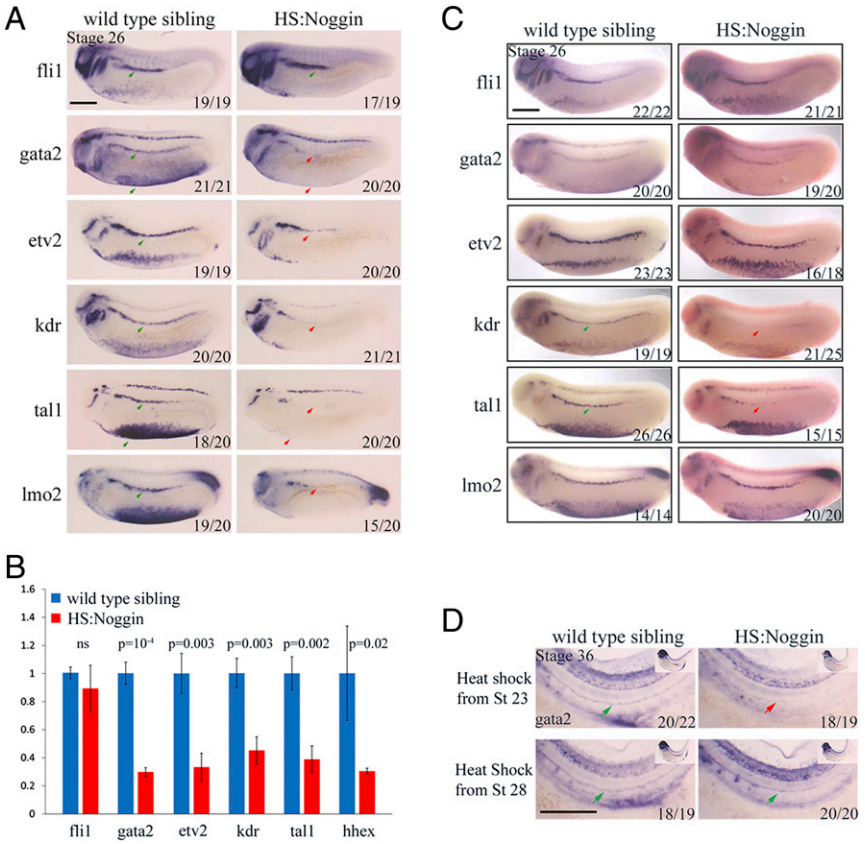

Fig. 4. BMP signaling is required for *gata2* expression in the DLP transcriptional network. (*A*) Hematopoietic programming in the DLP, at stage 25, following heat shock-induced noggin expression at stage 15. Embryos were heat-shocked for 15 min at 35 °C at stage 15 and were collected for analysis at stage 25. Fli1 expression is unaffected in the DLP (green arrows), whereas *gata2*, *kdr*, *tal1*, and *lmo2* expression is absent in the DLP as well as ventral blood island (VBI) (red arrows) of HS:Noggin transgenic siblings. Etv2 expression is decreased in the DLP and is absent in VBIs (red arrow). Numbers on individual images show the proportion of embryos for which that image represents (bottom right). (*B*) qPCR analysis of hemangioblast genes in stage 25 DLPs dissected from control and heat-shock noggin transgenic embryos. Fli1 is not significantly affected, whereas all of the other genes quantified show significant decreases of expression. (*C*) Hematopoietic programming in the DLP, at stage 26, following heat shock-induced noggin expression at stage 20. The expression of fli1, *gata2*, *etv2*, and *lmo2* is unaffected. The expression of *kdr* and *tal1* is reduced in the DLP hemangioblasts. HS:Noggin transgenic siblings were identified by the expression of Noggin transcripts (uniform Fast Red staining in HS:Noggin embryos). (*D*) Gata2 expression in the DA, at stage 36, following heat shock–induced noggin misexpression at stages 23 and 28. Gata2 expression is absent in the DA of HS:Noggin transgenic siblings when heat shock is done at stage 23 (red arrow), whereas *gata2* is expressed in the DA of HS:Noggin siblings when heat shock is done at stage 28, compared with wild-type siblings (green arrows). The stage of the embryos are indicated in the top left corner of the top left image. Numbers on individual images shows the proportion of embryos that the image represents (*Bottom Right*). Images and numbers are from one experiment and are representative of two biological replicates. (Scale bars: 0.5 mm.)

specifically for *runx1* expression after the DA forms (47). Therefore, we propose that the main input from BMP signaling into the HSC lineage is through *gata2*. Additionally, inhibition of BMP signaling and consequent disruption of the DH GRN leads to a strong reduction in *efnb2* expression in the DA. Efnb2 is a transmembrane ligand expressed in arterial but not venous endothelial cells before the onset of circulation and is critical for proper sorting of arterial and venous fated endothelium into distinct vascular beds but is not required for the specification of the DA (48, 49). A recent study revealed an unexpected role for Efnb2 in the DA for HE emergence (50). Hence, we conclude that BMP signaling is required for blood stem cell lineage upstream of EFNB2 signaling.

Previous analysis of BMP signaling requirements in zebrafish and mouse suggests that BMP signaling is required for definitive hematopoiesis in the DA, pointing to signaling emanating particularly from the mesenchyme around the aorta (16–19, 51). However, the reagents used in these papers have certain caveats. First of all, even though the truncated BMP receptor (Alk3), the main proof for a temporal requirement for BMP in the DA in Wilkinson et al., does not block activin signaling (24), because later studies showed that TGFB ligands signal through heteromeric complexes containing a BMP receptor (52), it is possible that truncated Alk3 blocked TGFB signaling as well. Second, in mouse, Gremlin1, a soluble inhibitor of BMP signaling, was used to address the requirement of BMP signaling for definitive hematopoiesis (19). In this study, the authors also noted that they didn't observe a convincing decrease in AGM hematopoiesis when Noggin was used instead of Gremlin1. Gremlin1 was later shown to be an agonist for VEGFA signaling in addition to its many other roles in other signaling pathways (53, 54). Furthermore, when the requirement for BMP signaling in ESC cultures was probed by using soluble BMP receptors, which block signaling by competing for the ligands, BMP signaling was indeed shown to be dispensable in ESC cultures once flk1/kdr^{+} hemangioblasts have been formed (21) as in *Xenopus* embryos. Moreover, mouse embryos lacking Alk3 receptor within the flk1 lineage (20), or lacking smad1 and -5 proteins within all hematopoietic precursors of the vav1 lineage (22), do not display hematopoietic defects. In addition, in chicken embryos, the DA and surrounding mesenchyme is already positive for phospho-Smad1/5 long before *runx1* expression, suggesting that BMP signaling is already active at an earlier time point (55). Finally, the role of BMP signaling in the AGM has been revisited in a recent study in which the authors show that BMP signaling is down-regulated in the HSC lineage by BMP inhibitors and, unexpectedly, supplementing these precursors with BMP was inhibitory, whereas addition of noggin enhanced the production of HSCs (56). Thus, we conclude that, as in *Xenopus*, BMP signaling is likely to be only required for hemangioblast specification but not beyond.

Recently, in zebrafish embryos, we showed that TGFB signaling is involved in HSC generation (57). Interestingly, TGFB ligands can activate BMP signaling type Smad transcription factors such as Smad1 as well as its canonical targets such as Smad2, in a BMP receptor-dependent as well as -independent manner (52, 58). Considering that our analysis didn't reveal a role for BMP signaling after the hemangioblast stage, and the fact that TGFB ligands can trigger Smad1 phosphorylation, we suggest that the role attributed to BMP signaling in AGM hematopoiesis after the hemangioblast stages might in fact be driven by TGFB ligands, or alternatively loss of BMP signaling is being compensated by TGFB signaling in *Xenopus*.

In this study, while analyzing the requirements for BMP signaling in the programming of hemogenic endothelium and definitive blood, we also revisited the role of BMP signaling in primitive hematopoiesis. In addition to its well-established role in primitive erythropoiesis, we showed that BMP signaling is required for

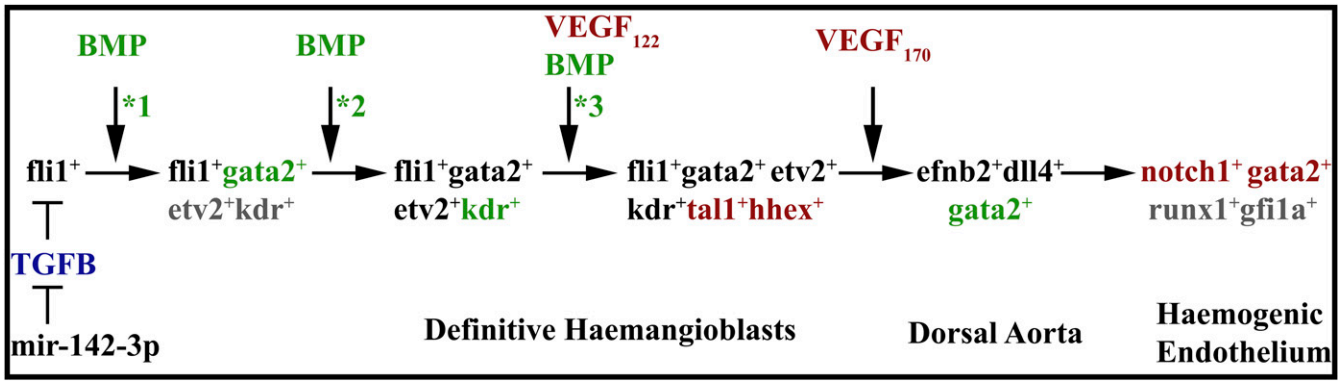

Fig. 5. Summary of signaling events and downstream transcription factors driving specification of the blood stem cell lineage. The hemangioblast GRN commences with the expression of fli1, which is genetically upstream of *gata2* and flk1/*kdr* (9). Meanwhile, TGFB signaling has an inhibitory effect on fli1 expression (44). BMP signaling, presumably through activation Smad transcription factors, together with Fli1, is first required for *gata2* expression before stage 20 (*1) and then for *kdr* expression at stage 20 (*2) (current study). The genes that are affected consequentially at those stages are colored gray. VEGFA small isoform ($VEGFA_{122}$) then activates Kdr receptor which lead to the expression of scl/*tal1* and *hhex* (14). Finally, BMP signaling is still required at stage 23–26 for the initiation of *gata2* expression later in the DA (*3) (current study). Later, VEGFA medium isoform ($VEGFA_{170}$) from somites induces Notch1, which in turn is required for the maintenance of *gata2* expression and induction of *runx1* (14). The requirements for each signaling pathway are indicated by color-matching the text with the transcription factors. The three inputs from BMP signaling are shown with asterisks (*).

differentiation of primitive myeloid cells in *Xenopus*. Both in primitive and definitive hematopoiesis, the main role of BMP signaling is to induce the hemangioblast program through *gata2*. Here and in our previous studies, we noticed that the same set of hemangioblast markers are expressed both in primitive blood and HE precursors. However, the immediate outcomes of these two populations differ, as the first one starts differentiating to myeloid and erythroid progeny immediately, whereas the latter population holds off the hematopoietic potential until after the DA forms. Whereas BMP ligands such as *bmp4* are expressed in both populations (A.C.-U., direct submission to Xenbase), *vegfa* is only expressed in adult hemangioblasts (13) [see extensive profile in Xenbase (36)]. Previously, Walmsley et al. (38) and Myers and Krieg (34) proposed that in the ventral blood island, sustained BMP signaling to hemangioblast precursors ensures commitment to the erythroid lineage and inhibition of endothelial cell fate. Conversely, VEGFA ligand is shown to be inhibitory to blood formation across species and, at least in the murine system, this act is through inhibition of gata1 expression (59-61). Thus, despite being induced in a similar way by BMP signaling, DHs are likely to be kept from differentiating into primitive blood by the inhibitory effect of VEGFA, most probably through inhibition of gata1, because this gene is one of the first hallmarks (9) of differential fate between the ventral blood island and the DLP DHs.

The expression of *fli1*, and partially *etv2*, is not dependent on BMP or VEGFA signaling in the DHs. Thus, an unresolved question in the initiation of the genetic program leading to the establishment of HSCs, how *fli1* is activated in the DLP, remains open. Wnt signaling operates upstream of BMP in primitive hematopoiesis (62). In the same study, the authors also detected strong canonical Wnt reporter activity in the DLP DH. Therefore, Wnt signaling could be involved earlier than BMP signaling in the DLP to induce *fli1* and *etv2*.

Materials and Methods

***X. laevis* Transgenic Line, Embryo Culture, and Treatments.** *Xenopus* embryos were obtained, cultured, and staged according to standard procedures Xenbase (xenbase.org/entry, RRID:SCR_003280). HS:Noggin transgenic line was kindly provided by Caroline Beck (28) as embryos, and raised to adulthood in house. Eggs collected from heat-shock inducible noggin transgenic frogs were fertilized using wild-type testes. Because the HS:Noggin transgenic frogs are heterozygous, this outcross results in ∼50% transgenic, 50% wild-type embryos. Wild-type siblings underwent the same heat-shock treatment to act as a control. The hsp70 promoter driving noggin expression was activated by transferring the embryos into prewarmed 35 °C 0.1× MBS and placing in a 35 °C incubator for 15 min. After this heat-shock treatment, the embryos were washed into 19 °C 0.1× MBS to continue developing before collection but heat-shocked again every 24 h if the collection point was longer than a day away. Transgenic embryos were separated from their wild-type siblings based on general morphology, or by costaining for misexpressed noggin mRNA post fixation using WISH. DMH1 Inhibitor (Sigma) was dissolved in DMSO, and the working concentration was optimized to a level that it leads to strong dorsalization, as is the case for heat-shocked HS:Noggin embryos, when treated before gastrulation. Embryos were treated with 100 μM DMH1, which needs to be titrated to this concentration from a 10mM stock solution dropwise to avoid precipitation. TFGB signaling inhibitors, SB-505124 (Sigma) and SB-431542 (Sigma) were dissolved in DMSO to make a 25 mM stock solution and used at 25 μM and 100 μM final concentration, respectively (63). All animal work was carried out according to UK Home Office regulations under the appropriate project license and approved by the University of Oxford Animal Welfare and Ethical Review Body.

In Situ Hybridization. Whole-mount in situ hybridization (WISH) and in situ hybridization on sections (ISHS) were performed as previously described (7, 64) except when noggin probe was fluorescein labeled for double WISH, and detection was performed using Fast Red Tablets (Roche). For details of probes used see supplementary material in refs. 9 and 14. Images and numbers shown in figures are from one experiment and are representative of three experiments.

RNA Extraction and qPCR Analysis. RNA from stage 26 excised DLPs was isolated using the RNeasy Micro Kit (74004; Qiagen) following the protocol for fibrous tissues, cDNA was synthesized using SSIV RT (ThermoFisher), and qPCR was performed using the ABI Prism 7700 with the SYBR green dye. Primers were designed to amplify both copies of the duplicated *Xenopus* genes (Table S1). The mRNA transcript levels were normalized to the ODC gene and changes were calculated using the Standard Curve method generated with cDNA made from a stage matched wild-type embryo as reference. Error bars represent SEM, and those treatments showing a significant change in mRNA levels relative to the controls are indicated, with their *P* value shown. The data shown summarize the results of three biological replicates for control and transgenic embryos.

Western Blotting. Protein extraction and Western blot analysis were done as previously described (9); phospho-Smad1/5/8 proteins were detected using an anti-PSmad158 (AB3848; Millipore) and normalized to total smad1 (6944P; Cell Signaling) and beta-actin (A3854; Sigma) protein levels. Myc-noggin was detected using myc-HRP antibody (11814150001; Roche).

Software. Network schematics were generated using Biotapestry and figures were prepared using Photoshop. qPCR results were analyzed using Microsoft Excel.

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