Bone morphogenetic protein 15 (BMP15) belongs to an unusual subgroup of the transforming growth factor β (TGFβ) superfamily of signaling ligands as it lacks a key cysteine residue in the mature region required for proper intermolecular dimerization. Naturally occurring BMP15 mutation leads to early ovarian failure in humans, and BMP15 has been shown to activate the Smad1/5/8 pathway in that context. Despite its important role in germ cell specification, the embryological function of BMP15 remains unknown. Surprisingly, we find that during early Xenopus embryogenesis BMP15 acts solely as an inhibitor of the Smad1/5/8 pathway and the Wnt pathway. BMP15 gain-of-function leads to embryos with secondary ectopic heads and to direct neural induction in intact explants. BMP15 inhibits BMP4-mediated epidermal induction in dissociated explants. BMP15 strongly inhibits BRE response induced by BMP4 and blocks phosphorylation and activation of Smad1/5/8/MH2-domain. Mechanistically, BMP15 protein specifically interacts with BMP4 protein, suggesting inhibition upstream of receptor binding. Loss-of-function experiments using morpholinos or a naturally occurring human BMP15 dominant-negative mutant (BMP15-Y235C) leads to embryos lacking head. BMP15-Y235C also eliminates the inhibitory activity of BMP15 on BRE (BMP-responsive element). Finally, we show that BMP15 inhibits the canonical branch of the Wnt pathway, upstream of β-catenin. We, thus, demonstrate that BMP15 is necessary and sufficient for the specification of dorso-anterior structures and highlight novel mechanisms of BMP15 function that strongly suggest a reinterpretation of its function in ovaries specially for ovarian failure.

In addition to rigorously regulating key embryological events in animals from worms to humans, the evolutionarily conserved TGFβ signaling pathway also plays a major role in homeostasis. Thus, perturbation of the pathway is causal to a variety of diseases that affect most, if not all, cells, tissues, and organs throughout life. Several landmarks can be used to classify TGFβ ligands into subgroups. First, all ligands have N-terminal signal sequences targeting a large precursor, the pre-pro form, to the secretory pathway. The precursors are cleaved at specific cleavage sites to generate a smaller “mature” ligand, which then alone or in combination with other secreted factors elicits its function in cell signaling. Second, the number of “conserved” cysteines in the mature region allows a division of TGFβ ligands into four different structural subgroups (1). Although most TGFβ ligands act as activators of the branches of the pathway, members of one subgroup, Xnr3, Lefty A, Lefty B (lefty 2 and 1, respectively in mammals), BMP3, and GDF3, have been shown to act as inhibitors. Third, TGFβ ligands act like morphogens, eliciting different outcomes based on their concentration and exposure time (2). The balance between activating and inhibitory input (provided by both TGFβ ligands and other secreted inhibitors such as noggin, chordin, follistatin, cerberus, and coco (3)), operating in different times and regions of the embryos, provides the fine-tuning of morphogen thresholds. Fourth, TGFβ ligands induce dimerization and activation of type I and type II receptors, which in turn phosphorylate the C-terminus (MH2 domain) of receptor-associated Smads (R-Smads (4)). Smad2 and -3 transduce signals on behalf of activin/nodal, whereas Smad1, -5, and -8 propagate signals on behalf of BMP/GDFs (4).

In our continuous quest to systematically address the early embryological function of TGFβ ligands, three observations draw our focus to BMP15 (also called GDF9B; Laitinen et al. (8)). First, BMP15 is structurally in the same subgroup as LeftyA, LeftyB, and GDF3; that is, missing the fourth cysteine in the mature domain, suggesting that it might act as inhibitor of the pathway (5–7). Second, it has been shown that this ligand is expressed maternally in oocytes of different mammals, including humans, and transiently during very early murine, ovine, and bovine embryogenesis, suggesting an early, perhaps evolutionarily conserved embryonic function (8–12). Third, no embryonic function has been assigned to BMP15 as of yet. BMP15−/− mice do not display an embryonic phenotype, suggesting that BMP15 early function is redundant with other ligands (13). Adult female BMP15−/− mice, however, are subfertile and display decreased ovulation and fertilization rates (13, 14). Consistently, in mouse granulosa cells BMP15 has been shown to bind the type I receptor ALK6 and activate the Smad1/5/8 pathway (15), although its phosphorylation state can alter this activity. Finally, naturally occurring BMP15 mutations in humans have highlighted much more severe phenotypes than in the mouse, with premature ovarian failure in women (OMIM #300510), leading to very early menopause.
Role of BMP15 in Xenopus Embryogenesis

(16–19). The difference in the severity of the adult phenotype between mouse and human is very likely because of major differences in ovulation and menstrual cycles between the two species. Thus, the embryonic function of BMP15 as well as its early biochemical mechanism of action remains unknown.

In this study we dissect for the first time the embryonic function and the biochemical mechanism underlying BMP15 activity in early Xenopus embryos. First, we show that BMP15 is present in the amphibian genome and, as its mammalian ortholog, lacks the fourth cysteine in the mature region. However, unlike its mammalian ortholog, embryonic expression of amphibian BMP15 extends beyond early cleavage stages and is maintained throughout embryogenesis. Second, in contrast to traditional BMPs, gain of BMP15 function leads to a tadpole with two heads, whereas a loss-of-function leads to elimination of dorso-anterior structures, demonstrating that BMP15 function is both necessary and sufficient for the specification of head and trunk structures. Third, BMP15 function is evolutionarily conserved, as human BMP15 can substitute entirely for Xenopus. Fourth, surprisingly, BMP15 is inhibitory to both the Smad1/5/8 and the canonical Wnt pathway. Fifth, in agreement with its inhibitory effect on Smad1/5/8 and canonical Wnt pathway, we show that BMP15 neutralizes animal caps explants, inhibits epidermal induction of BMP4 at the protein level, and inhibits expression from BMP-responsive element. Finally, we show that BMP15 protein and BMP4 interact, suggesting that this inhibitory effect occurs in the extracellular space. These novel findings establish for the first time an unexpected embryological function for BMP15.

MATERIALS AND METHODS

Xenopus Embryo Manipulation—Xenopus embryos were obtained and manipulated as previously described (20). For synthetic mRNAs synthesis, specific plasmids were linearized with the Ascl restriction enzyme, and sense strand capped mRNA was obtained using the mMessage mMachine system (Ambion) using the SP6 RNA Polymerase. Embryos were injected in either animal pole or the ventral marginal zone with different amounts of human or Xenopus BMP15 mRNA. For the animal cap assay, embryos were injected at the two-cell stage in each blastomere with a total amount of 100 pg of BMP15 mRNA. Intact caps were cut at late blastula (stage 8–9) stage in each blastomere with a total amount of 100 pg of the animal cap assay, embryos were injected at the two-cell different amounts of human or injected in either animal pole or the ventral marginal zone with mRNA was obtained using the mMessage mMachine system—transcripts were performed on animal explants or whole body (Invitrogen) for the detection (supplemental Fig. 1).

Luciferase Assays—Luciferase transcription assays were performed using the Luciferase Assay kit (Promega Corp., Madison, WI) as previously described (23). For the BMP and activin response elements (Bre-Lux and Are-Lux) activity, injections were made into both blastomeres of 2-cell-stage embryos and embryos recovered for the analysis at stage 10.5, whereas for the TOP-FLASH (Wnt-responsive element) experiments, the injections were made into one animal-vegetal blastomere of 4-cell-stage embryos, and the luciferase activity was measured on lysates of embryos harvested at blastula (stage 9). For each experimental condition, 3 pools of four embryos were collected and lysed in 50 μl of passive lysis buffer 1×. The lysates were spun down, and 10 μl of the supernatant was measured. The measurement was performed in the LUMAT LB9507 using the Promega Dual Luciferase reporter assay system. All luciferase assays were performed in triplicate; one, representative of a triplicate experiment, is shown under “Results” for each condition.

Western Blot Analysis—70 μg of whole embryo protein lysates were boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE using 4–12% gradient Tris-glycine gels (Invitrogen). The proteins were transferred onto nitrocellulose membrane and then blocked in 5% milk in Tris-buffered saline-Tween at room temperature for 1 h. Anti-Smad1 (Upstate, Mil-

provided by Dr. Persani, University of Milan; Di Pasquale et al. (16) as a template and forward (5’-CATGATCGATAAGATG-GTCCCTCTCA-3’) and reverse (5’-CGTCTAGATCATCTG-

CAGGTACAAGA-3’) primers.

The PCR fragment was isolated and ligated into the ClaI/XbaI sites of the pcDNA++ vector. DNA sequencing of the PCR clone confirmed that the gene encoded the wild type version of hBMP15. The Xenopus version of BMP15 (xBMP15) was obtained by subcloning a Xenopus EST clone (no. 4677830 from IMAGE) into the pcDNA vector to generate the pcDNA++ vector. The entire sequence of the Xenopus BMP15 gene was obtained by automated DNA sequencing.

Whole-mount in situ Hybridization—Whole-mount in situ hybridizations were carried out as previously described (22). The specific Xenopus BMP15 antisense probe was made from the original IMAGE clone after linearization with EcoRI and in vitro transcribed using the T7 RNA polymerase.

Recombinant BMP15 Protein Purification—The human BMP15 recombinant protein was obtained from the culture media of the HEK293T cell line stably transduced with a double Myc- and His-tagged version of the human BMP15 called hBMP15MycHis. The transfected cell line (HEK293T-BMP15) was previously described (16). Briefly, 3×10⁶ cells were plated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 100-mm diameter dishes in triplicate; when the cells reached 80% confluence, the medium was replaced with DMEM supplemented with 1% FBS for 24 h, then the supernatant was collected and concentrated using the Centrifugal Filter Device from Ambion (cut off, 10 kDa). The concentrated medium was then purified using the ProBond Purification System (Invitrogen), and the obtained protein was dialyzed in 4 mM HCl, 0.5% bovine serum albumin. Quantification was performed using Bradford Reagent (Bio-Rad). The obtained protein was checked in SDS-PAGE Western blot using the anti-Myc antibody (Invitrogen) for the detection (supplemental Fig. 18).

To analyze the expression of BMP15 in Xenopus embryos, we performed whole-mount in situ hybridization. The specificity of the probe was confirmed by control hybridizations. The specificity of the probe was confirmed by control hybridizations

105 cells were plated in 100-mm diameter dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 100-mm diameter dishes in triplicate; when the cells reached 80% confluence, the medium was replaced with DMEM supplemented with 1% FBS for 24 h, then the supernatant was collected and concentrated using the Centrifugal Filter Device from Ambion (cut off, 10 kDa). The concentrated medium was then purified using the ProBond Purification System (Invitrogen), and the obtained protein was dialyzed in 4 mM HCl, 0.5% bovine serum albumin. Quantification was performed using Bradford Reagent (Bio-Rad). The obtained protein was checked in SDS-PAGE Western blot using the anti-Myc antibody (Invitrogen) for the detection (supplemental Fig. 18).
lipore Corp., 1:1000), anti-phospho-Smad1 (Cell Signaling Technologies, 1:1000), anti-Smad2 (BD Transduction Laboratories, 1:2500), and anti-phospho-Smad2 (Cell Signaling Technologies, 1:1000) primary antibodies were used. Incubations were performed for 1 h at room temperature or overnight at 4 °C. For the anti-phospho Smad2 antibody, the membranes were blocked in 5% polyvinyl pyrrolidone in Tris-buffered saline-Tween overnight at room temperature, and the primary antibody was incubated for 4 h at room temperature. Anti-mouse or -rabbit IgG horseradish peroxidase-conjugated secondary antibody was used (Amersham Biosciences, 1:5000), and detection was performed using enhanced chemiluminescence reagent (Amersham Biosciences).

**Coimmunoprecipitation**—hBMP15MycHis (1 ng) was co-injected into embryos at the two-cell stage with BMP4-hemagglutinin (1 ng) or activin-hemagglutinin. Protein extracts were obtained at stage 10–11, immunoprecipitated with anti-c-Myc-conjugated beads (Sigma), and probed with anti-hemagglutinin monoclonal antibody (1:1000, Covance) as previously described (24).

**RNA Extractions and Real Time PCR**—Total RNA was extracted from HUES6 cells using Trizol reagent (Invitrogen). 1 μg of total RNA was then treated with DNase I amplification grade, and single strand cDNA was obtained using the first strand cDNA synthesis kit (both from Invitrogen). 20 ng of cDNA samples were used for the amplification reaction, performed in 20 μl containing the Light Cycler Syber Green mix (2 μM concentrations of each primer). The run was performed on the Light Cycler 480 instrument (Roche Applied Science) and the data analysis with both the Light Cycler and the Rest (Relative Expression Software Tool, Pfapp, 2001) software using either the untreated cells or the BMP4 treated cells as calibrator and the TATA box-binding protein (TBP) as the reference gene. The list of all primers used in this study is provided in supplemental Table 1.

**RESULTS**

**BMP15 Expression in Xenopus**—Although most TGFβ ligands are evolutionarily conserved among different metazoans, species-specific differences have been reported. For example, teleost and amphibians have many nodal-related TGFβ ligands, whereas mouse and human have a single nodal gene, or ligands such as GDF3 and inhibin-α-chain are mammalian inventions, which are absent in fish and frogs. We, therefore, first asked if the BMP15 ligand is evolutionarily conserved. Both the Xenopus tropicalis genome project as well as an EST clone (clone ID 4677830 from IMAGE) comprising the entire Xenopus laevis BMP15 coding region indicate that BMP15 is present in the genome of amphibians (Fig. 1). Its mammalian homolog, Xenopus BMP15 (xBMP15), is located in the X chromosome (clone ID 4677830 from IMAGE) comprising the entire Xenopus laevis BMP15 coding region indicate that BMP15 is present in the genome of amphibians (Fig. 1). Its mammalian homolog, Xenopus BMP15 (xBMP15), is located in the X chromosome.

![FIGURE 1. BMP15 expression in Xenopus embryos. A, alignment of the amino acid sequences for the Xenopus (xBMP15) human (hBMP15), and mouse (mBMP15) mature peptide. The mouse and Xenopus proteins show a homology of the 68 and 44%, respectively, with the human protein. B, schematic of a conventional TGFβ ligand, BMP4 (top), highlighting the prodomain (green), the mature domain (red), and the position of the seven signature cysteines. The fifth cysteines in the mature region (highlighted in red) are missing in BMP15 (bottom). C, temporal expression of BMP15 during Xenopus embryonic development, detected by reverse transcription-PCR. BMP15 expression is detected in the animal pole region of 4-cell stage embryo (D and E) and maintained in the prospective ectoderm at blastula stage (F and G). Expression then becomes ubiquitous within the ectoderm (H and I). At the neural tube closure, BMP15 is maintained in the nervous system (J and L), and at stage 26 the expression becomes exclusive to the anterior, including sensory organs and hindbrain (I). At tail-bud stage (28), peak expression is maintained in the brain and eyes (K and M).](https://example.com/figure1.png)
Role of BMP15 in Xenopus Embryogenesis

stages were examined (Fig. 1, C–G). Whole mount in situ hybridization revealed that the maternal expression of BMP15 is restricted to the animal pole region at the four-cell stage (Fig. 1, D and E); the animal expression is maintained at pre-gastrula stage (Fig. 1, F and G) and becomes ubiquitous within the ectoderm at gastrula (Fig. 1, H and I). At the neural tube closure, the expression is maintained in the central nervous system (Fig. 1, J and L) and becomes exclusively anterior at later stages, with strong expression in the telencephalon, eye, otic vesicle, cement gland, and trigeminal nerve (Fig. 1, K and M).

Gain of Function of BMP15 Induces Secondary Heads in Xenopus Embryos—To begin our functional studies on the embryological function of BMP15, we started a series of gain-of-function experiments by microinjection of synthetic mRNA encoding the full-length human and Xenopus BMP15 protein into ventral vegetal blastomeres of eight-cell-stage Xenopus embryos. Injected embryos along with uninjected controls were cultured until tail-bud stage and examined for their phenotypes. Surprisingly, we found that expression of BMP15 leads to a dramatic induction of secondary ectopic heads (Fig. 2). 84% of BMP15-injected embryos (n = 250) displayed secondary ectopic heads regardless of whether human or Xenopus BMP15 was presented. This phenotype was unexpected, because when traditional BMPs (such as BMP2, -4, -7 or GDF6) are expressed under the same conditions, the embryos display the opposite phenotype; that is, ventralization and clear loss of head structures. The two-headed tadpoles obtained by BMP15 expression instead represent a phenocopy of effects obtained with BMP/Wnt inhibitors such as Noggin, Coco, Cerberus, and Dkk (25–28). This observation, therefore, suggests that BMP15 operates differently than traditional BMPs and that at least in the context of early embryonic development might have an inhibitory effect.

BMP15 Directly Induces Neural Tissue in Intact Ectodermal Explants—Animal cap explants of a blastula stage Xenopus ectoderm give rise to epidermis when cultured intact in isolation. This is because of ongoing BMP/GDF activation of the Smad1/5/8 pathway (3). Inhibitors of the BMP4 (Smad1/5/8) pathway block epidermal differentiation and switch the cells directly to a neuronal fate (3, 29). To provide an independent readout of BMP15 activity, we tested its biological activity in intact animal cap explants (Fig. 3A). Synthetic RNA encoding hBMP15 or xBMP15 was microinjected in the animal pole of frog embryos. The animal cap explants were then dissected at blastula stage, cultured intact, and tested for the expression of cell type-specific markers at two developmental time points. Fig. 3B shows that expression of BMP15 in explants converts epidermal cells to neuronal cells. Both early and late neural-specific markers such as Sox2, neural cell adhesion molecule, Nrp-1, and Otx as well as the cement gland marker Xenopus anterior gradient are induced (Fig. 3C). This induction occurs with loss of epidermal fate, as the expression of the immediate early BMP-response gene Msx1, a robust epidermal inducer, is reduced (30). The induction of neural fate was a direct conversion within the ectodermal explants and not because of secondary or concomitant induction of mesoderm or endoderm, as neither mesodermal markers (such as Xbra, globin, muscle actin, and HoxB9) nor endodermal markers (such as Sox17a and intestinal fattyacid-binding protein, IFABP) markers were induced (Fig. 3, B and C). The same results were obtained with both human as well as Xenopus constructs, suggesting a species conser-
Role of BMP15 in Xenopus Embryogenesis

BMP15 Protein Inhibits the Epidermal-inducing Ability of BMP4 Protein—Although intact animal cap explants cultured alone give rise solely to epidermis, dissociation of the same explants into individual cells leads to their conversion to neuronal fate (31, 32). The addition of purified recombinant BMP4 protein to dissociated explants inhibits neuronal fate while inducing and maintaining epidermal fate (2). We, thus, challenged the epidermal induction of BMP4 protein in the context of dissociated ectodermal cells with two different doses of recombinant hBMP15 protein and evaluated their choice of fate between neural and epidermal. Toward this aim, hBMP15 was tagged with both a Myc and His epitope (hBMP15MycHis) and tested for its activity in both epidermal versus neural induction assays in animal cap explants (supplemental Fig. 1A) as well as its ability to inhibit BMP4-mediated BRE transcription (Fig. 1B). Both assays established that the tags did not disturb BMP15 activity. Plasmids encoding hBMP15MycHis were used to stably transfect HEK293 cells. BMP15MycHis protein was subsequently purified by immunoaffinity and form the conditioned medium of transfected cells as shown by Western blot analysis (supplemental Fig. 1B). We hypothesized that if BMP15 protein had an inhibitory effect on BMP4 protein, cells should be neural, whereas if BMP15 had no inhibitory activity, the cells would adopt an epidermal fate. Fig. 4 shows that hBMP15MycHis inhibits the epidermal induction activity of hBMP4 protein in dissociated ectodermal explants, and the cells maintain their default neural fate. This is manifested by the reduction of the epidermal marker (Msx-1) and maintenance of neural (Sox-2) and anterior (XAG) marker expression (Fig. 4). Thus, at the protein level BMP15 blocks the activity of BMP4. BMP15 Inhibits BMP4 Activation of BRE and Activation of Smad1/5/8—To address the specificity of BMP15 activity on the two branches of the TGFβ pathway, we performed reporter assays measuring the transcriptional activity of genes that are immediate responders to activation of either Smad1/5/8 or Smad2/3. Constructs in which either the BMP-responsive element (BRE) or the activin-responsive element (ARE) drive the expression of the reporter luciferase (BRE-Lux and ARE-Lux) were injected in the animal pole of two-cell-stage embryos. Injected embryos along with controls were cultured until mid-gastrula stage (Fig. 5A). Whole embryonic extracts were used to assess the level of reporter expression driven by either BRE (Fig. 5B) or ARE (Fig. 5C). Consistent with the phenotype and neural induction in intact animal caps explants, we found a strong transcriptional inhibition of BMP-induced genes in a dose-dependent manner. That inhibition is specific to the Smad1/5/8 branch of signaling of the TGFβ pathway, as BMP15 did not affect the Smad2/3-dependent ARE transcription and confirms that BMP15 acts as an inhibitor of the Smad1/5/8 branch of the TGFβ pathway. The activation of both Smad1/5/8 and Smad2/3 was also measured directly by examining the phosphorylation state of the MH2 C-terminal domains in embryos injected with increasing amounts of BMP15 ligand. Fig. 5D shows that expression of hBMP15 leads to a specific inhibition of MH2 phosphorylation of Smad1/5/8 but does not affect the state of activation of the Smad2/3 branch of the TGFβ pathway. Taken together, the evidence presented above establishes that BMP15 is a bona fide Smad1/5/8 inhibitor.

BMP15 Can Interact Physically with BMP4 Protein—Because BMP15 and BMP4 are expressed with similar temporal and spatial coordinates in the frog embryo and display opposite activity on the Smad1 pathway, we tested whether the two secreted factors could interact. Toward this, two-cell-stage embryos were injected with different combinations of tagged BMP15, BMP4, and activin synthetic mRNAs in both blastomeres at the two-cell stage in the animal pole (Fig. 6A). Embryos were cultured until mid-blastula, and ectodermal explants were removed and cultured until sibling control embryos reached mid-gastrula stages. Total protein extracts were harvested from the cultured explants and submitted to coimmunoprecipitation and Western blot analysis to evaluate protein–protein interactions. Fig. 6B shows that tagged BMP15 is able to coimmunoprecipitate tagged BMP4 ligand. The interaction was specific, as BMP15 did not show any interaction with activin. These experiments are in agreement with the specificity of BMP15 on the Smad1 branch of TGFβ pathway and suggest that sequestering BMP4 ligand is a biochemical mechanism underlying the activity of BMP15.

BMP15 Activity Is Necessary for Head Differentiation—To complement our gain-of-function experiments, we undertook loss-of-function approaches by taking advantage of a naturally occurring dominant negative hBMP15 mutant, BMP15-Y235C (16). Tyrosine 235 in the pre-pro domain of BMP15 is evolutionarily conserved in all mammals (16). BMP15-Y235C leads to hypergonadotropic ovarian failure in women, leading to very early onset of menopause and infertility. Synthetic RNA encod-
Role of BMP15 in Xenopus Embryogenesis

![Image](image_url)

**FIGURE 5.** BMP15 inhibits transcriptional response from BRE. A, schematic of experimental design. B, luciferase measurements of total embryo lysates from Xenopus embryos injected with the reporter plasmids BRE-Lux (20 pg). hBMP15 strongly inhibits the activation of the BRE in Xenopus embryos. C, ARE (25 pg) with different doses of hBMP15 mRNA and BMP4 (100 pg), activin (ACV, 100 pg) transcripts. BMP15 has no effect on the ARE. D, increasing amounts of hBMP15 mRNA (10, 50, 100, 250 pg) were injected into one blastomere of two-cell-stage frog embryos, and protein was extracted at the onset of gastrulation (stage 10) from 25 whole embryos/experimental condition. Protein extracts from early cleavage embryos (stage 6) were used as the negative control, and uninjected embryos at stage 10 were used as the positive control. Expression of hBMP15 in embryos strongly decreases Smad1 MH2 domain phosphorylation, demonstrating inhibition of the Smad1/5/8 pathway. BMP15 expression, on the other hand, had no effect on the Smad 2 MH2 phosphorylation, demonstrating the specificity of inhibition within the TGFβ signaling pathway. IB, immunoblot.

Intriguingly, BMP15-Y235C protein was microinjected in the animal pole of two-cell-stage embryos followed by phenotypic and biochemical analysis of the state of Smad1/5/8 activation. Fig. 7, D, G, and I, shows that expression of dominant negative BMP15 leads to a dramatic loss of the dorso-anterior structure, leading to embryos lacking heads. This phenotype was obtained in 76% of embryos (n = 80). Co-injection of wild type BMP15 together with the BMP15-Y235C mutant rescued the phenotype, providing stringent control for this type of approach (Fig. 7H).

Additionally, transcriptional response from the BRE-Lux reporter increases when BMP15-Y235C is expressed alone (Fig. 7F). This is consistent with the elimination of the inhibitory activity of endogenous BMP15. Coexpression of BMP4 and BMP15 leads to a dramatic inhibition of BRE response induced by BMP4 alone. Finally, triple coexpression of BMP4, BMP15, and BMP15-Y235C restores BRE response to BMP4 by wild type and dominant-negative BMP15, canceling each other’s activity. An independent morpholino-based loss of function targeting the xBMP15 also led to the same phenotype (Fig. 8), although with a lower penetrance, highlighting qualitative differences in inhibiting BMP15 at the RNA level (morpholinos) versus inhibition at the protein level (dominant negative). In addition and consistent with our hypothesis, embryos injected with BMP15-morpholino showed elevated levels of Smad1 activation as revealed by an increase of Smad1-MH2 phosphorylation (Fig. 8G). Taken together the results of these experiments establish that BMP15 activity is necessary for proper head development.

**BMP15 Inhibits Wnt Signaling—** All secreted inhibitors of Wnt ligands directly induce neural fate in ectodermal explants. These inhibitors, however, can be classified into two categories; that is, those that affect only the TGFβ pathway and those that inhibit other pathways in addition to TGFβs. For example, Noggin and the Dan domain proteins (such as Gremlin and SOS) specifically inhibit the Smad1/5/8 branch of the TGFβ pathway, whereas other inhibitors such as Cerberus (26) and Coco (27) inhibit the canonical Wnt pathway in addition to Smad1/5/8. To address to which of the two groups BMP15 belongs, we asked if BMP15 had any effect on the Wnt pathway using two different approaches. In the first approach, RNAs encoding hBMP15 and Wnt8 were injected in the animal pole of two-cell-stage embryos, and animal caps were examined for the expression of the immediate response genes activated by the canonical branch of the Wnt pathway. Fig. 9A shows that hBMP15 could block the Wnt8-dependent and immediate-early induction of Siamois and Xnr3 expression. In the second approach we measured at the transcriptional level the behavior of the Wnt-responsive promoter (TOP-FLASH) in vivo in the presence of BMP15 (Fig. 9, B and C). hBMP15 encoding synthetic RNA was co-injected with Wnt-8 synthetic RNA and TOP-FLASH DNA in four-cell-stage frog embryos, and transcriptional responses were measured at late blastula stages. Fig. 9C shows that BMP15 strongly represses Wnt8-responsive element. To control for the specificity of the BMP15 inhibitory action on TOP-FLASH, we performed another transcriptional assay, co-injecting BMP15 with a stabilized form of β-catenin (T2) (33). This β-catenin mutant has an N-terminal truncation that constitutively activates the Wnt pathway (33). BMP15 had no effect on the β-catenin T2 activity when co-expressed together (Fig. 9D). This suggests that the inhibitory effect of BMP15 on the canonical branch of Wnt pathway occurs upstream of β-catenin. Thus, in both marker-induction assays as well as in our transcriptional assays, BMP15 behaved as a strong inhibitor of the canonical branch of
the Wnt pathway. This inhibition also provides a molecular explanation for the formation of secondary head phenotypes, a common property of Wnt-specific inhibitors such as Dkk (34). BMP15 seems to behave more like Cerberus and Coco rather than other specific BMP inhibitors such as Noggin and Chordin.

**DISCUSSION**

Our study demonstrates that in the context of early embryogenesis BMP15 ligands belonging to the subfamily of BMP/GDF ligands of the TGFβ superfamily act as a double inhibitor, blocking conventional BMP/GDF ligands as well as Wnts. Specifically, we find that in *Xenopus* embryos, BMP15 overexpression leads to ectopic head structures and direct induction of neural markers, which are because of BMP and Wnt inhibition. We show that BMP and Wnt reporter constructs are strongly inhibited in the presence of BMP15. Consistently, expression of BMP15 inhibits the MH2 domain phosphorylation of Smad1/5/8 but not the Smad2/3 branch of the TGFβ pathway. One of the mechanisms responsible for the observed inhibitory function of BMP15 would be its ability to directly bind to BMP ligands. Thus, BMP15 joins the ever-growing list of secreted factors with BMP inhibitory activity, expressed very early during embryonic development, highlighting the remarkable functional diversity of this conserved family. This provides the first evidence of an embryological function for BMP15, which is in contrast with its activity described in mouse granulosa cells. In these cells, BMP15 has been shown to be an activator of the Smad1/5/8 pathway, although that activity has very recently been shown to be dependent on the state of phosphorylation of the ligand (35). Surprisingly, however, we have previously shown that Smad1 is not activated by phosphorylation of its MH2 domain in the early-cleaving embryo where BMP15 is expressed (36). Although different states of phosphorylation of BMP15 ligands seem to switch its activity from agonist to antagonist of the Smad1/5/8 pathway in granulosa cells, in *Xenopus* embryos the sole activity of BMP15 is as an antagonist. These three new findings, (i) the embryological function of BMP15 and (ii) inhibition of Wnt pathway as well as (iii) the inhibitory role on the Smad 1/5/8 pathway, allow us to reinterpret some of the conclusions reached for (i) other members of the BMP15 subgroup of the TGFβ family, (ii) embryonic function of the evolutionarily conserved BMP15, and (iii) both the adult function of BMP15 in the context of ovarian development and the molecular basis of premature ovarian failure in humans because of naturally occurring dominant negative mutation of BMP15 in humans.

**The Peculiar Subgroup of BMP15 within the TGFβ Family**—BMP15 belongs to a subgroup that is structurally different from the other TGFβ ligands in that it has a crucial cysteine (4) missing from the mature region. This cysteine is in charge of establishing intermolecular disulfate bonds necessary for proper dimerization of TGFβ ligands. In classic ligands, this cysteine is involved in dimer formation of the mature domain of TGFβ ligands, and its absence suggests a lack of typical dimerization potential. Other members of this subgroup include LeftyA, LeftyB, GDF3, and GDF9. Lefties have already been shown to have nodal and Wnt inhibitory properties, and GDF3 acts as a BMP inhibitory (5, 7, 37). This study demonstrates the same for...
Role of BMP15 in Xenopus Embryogenesis

BMP15, suggesting that inhibition is the attribute of this entire subfamily. Preliminary examination of the role of GDF9 in the same context as the assays described above also shows that GDF9 is also a potent inhibitor of BMPs. Thus, inhibition rather than activation seems to be a biochemical property of BMPs.

4 E. Di Pasquale and A. H. Brivanlou, manuscript in preparation.
shared by all members of this subgroup. The fact that in granulosa cells BMP15 seems to act sometimes as an agonist and sometimes as an antagonist might be due not to a direct effect of BMP15 but, rather, to what type of ligands BMP15 is inhibiting during the differentiation of granulosa cells. When BMP15 inhibits an inducer such as BMP4, Smad1/5/8 are inhibited; however, if BMP15 inhibits another inhibitor, this will lead to activation of the Smad1/5/8 pathway. Regardless, this subgroup of five TGFβ ligands need to be added to the ever-growing list of secreted TGFβ inhibitors (3). What is emerging is that inhibitory input, at least during cell fate determination events, seems to fulfill an instructive a role, not only in the context of early embryonic germ layer specification but also during oocyte maturation.

Embryonic Function of BMP15—In Xenopus, TGFβ and Wnt ligands provide early inducing signals that induce and pattern the three embryonic germ layers. The first inducing signals during early cleavage stages form the prospective endoderm and mesoderm in the vegetal pole and equatorial regions of the embryo respectively. This occurs while the ectoderm is specified at the opposite end, in the animal pole (Fig. 8). The spread of the vegetal-derived TGFβ and Wnt signals needs to be inhibited above the equator so that the animal pole territory can differentiate into ectodermal derivatives. Therefore, the simplest explanation of early animal pole-localized secreted inhibitors is to block the spread of these signals to protect the presumptive ectoderm. In addition, ongoing BMP4 signals in the dorsal ectoderm will have to be inhibited for the establishment of neural territory, a few hours later. The same argument can be made about controlling the spread of the organizer in the dorsal animal territory that needs to be tightly regulated to protect the ectoderm from becoming axial mesoderm. Thus, Wnt immediate early responsive genes, such as Xnr3 and Siamois, also need to be shut down in the dorsal ectoderm. There are two maternally encoded TGFβ inhibitors expressed specifically in the early presumptive ectoderm: BMP15 and Coco. They are both secreted BMP/Wnt inhibitors (27). It is tempting to speculate that together they block the invasive mes-endodermal specification forces emanating from the vegetal pole (Fig. 8).

Interestingly in Xenopus BMP15 is expressed maternally and maintained until very late stages of development, whereas in the mouse and cow pre-implantation expression declines after morula and the eight-cell stage, respectively (10, 38). As no cell fate decision or movement occurs at these very early stages, it is tempting to speculate that the sole function of BMP15 is to keep BMP4 from signaling. Signaling by BMP4 has been linked to the earliest cell fate decision in the mouse embryo: inner cell mass versus trophectoderm. By keeping BMP4 in check, BMP15 might prevent premature cell fate assignment. In agreement with this hypothesis we have previously shown that the Smad1/5/8 pathway is not activated during very early mouse embryogenesis (36). Knock-out of BMP15 in the mouse has been reported to give rise to very mild and specific, but late, aberrations in female germ cells, suggesting that its embryonic function might be redundant with other inhibitors, such as lefties and GDF3 (13). Thus, BMP15, GDF3, and lefties are the earliest BMP inhibitors expressed during mouse embryogenesis, highlighting the critical, if not preponderant, role of inhibition during early development of both amphibians and mammals.

Reinterpretation of the Molecular Basis of Ovarian Failure—As mentioned above, dominant mutation of the BMP15 locus

FIGURE 9. BMP15 inhibits Wnt signaling. A, BMP15 reduces the expression of Wnt responsive genes. Experiments setting are the same as shown in Fig. 3A. Ectodermal explants expressing either 50 pg of Wnt8 or 100 pg of xBMP15 or both were submitted to reverse transcription (RT)-PCR to evaluate the level of expression of Xnr3 and Siamois. B, schematic of experimental setting for measurement of Wnt-responsive element TOP-FLASH. Embryos were injected at two cell stages, with 50 pg of Wnt8 or 200 pg of β-catenin T2 alone or together with different concentrations of BMP15. Luciferase assays were performed at early gastrula stage. C, BMP15 inhibits Wnt8-induced transcription from TOP-FLASH. D, BMP15 does not affect the activity of β-catenin T2. This was not because of the ratio of BMP15 to β-catenin, as a 10-fold increase in BMP15 still did not lead to inhibition (data not shown).

5 A. J. Levine and A. H. Brivanlou, submitted for publication.
Role of BMP15 in Xenopus Embryogenesis

on the X chromosome has been associated with premature ovarian failure leading to infertility in women by causing premature menopause. The molecular mechanism proposed for explaining this dramatic outcome has rested on the assumption that BMP15 is a Smad1/5/8-activating ligand. BMP15 has been previously reported to be a conventional BMP, transducing signal through the type I receptor ALK6 and activating the Smad1/5/8 cascade by phosphorylation of the MH2 domain (15). Our studies demonstrate that BMP15 acts as an inhibitor of both conventional BMP as well as the canonical branch of the Wnt signaling pathway. Although there is ample evidence that BMP15 can regulate granulosa cell proliferation and differentiation, the biochemical mechanism of this biological activity might be based on inhibition of ongoing conventional BMPs, also expressed in these cells, rather than direct activation of the Smad1/5/8 pathway. Alternatively, it can be argued that BMP15 has opposite activities in embryonic cells versus granulosa cells. For example, in a scenario in which heterodimer formation between BMP15 with other TGFβ ligands might activate different sets of receptors cannot be excluded. This will present an exception, as no TGFβ ligand activity has been so far reported to be diagnostically opposite in one cell type versus the others. Finally, it can also be argued that BMP15 activity forms a morphogen gradient with very sharp thresholds emanating from the cells surrounding the oocyte. Different thresholds will dictate different outcomes, including opposite responses of different cell types depending on the expression of cell type-specific receptors and co-factors. In support of this possibility, the inactivation of only one copy of BMP15 in sheep gives rise to a completely opposite phenotype of increased ovulation rate and super fertility (39, 40). Thus, it is important to stress that the complete opposite phenotype of increased ovulation rate and super fertility (39, 40). Thus, it is important to stress that the presence of BMP or Wnt inhibitors does not necessarily imply the cause of ovarian failure might be because of a loss of BMP4 inhibition rather than the loss of a direct activation of Smad1/5/8 pathway. This would de-repress the Smad1/5/8 pathway, affecting thresholds of morphogen, rather than complete loss of Smad1 activation.

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