

# Mutation of an upstream cleavage site in the BMP4 prodomain leads to tissue-specific loss of activity

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ProBMP4 is initially cleaved at a site adjacent to the mature ligand (the S1 site) allowing for subsequent cleavage at an upstream (S2) site. Mature BMP4 synthesized from a precursor in which the S2 site cannot be cleaved remains in a complex with the prodomain that is targeted for lysosomal degradation, and is thus less active when overexpressed in *Xenopus*. Here we report that mice carrying a point mutation that prevents S2 processing show severe loss of BMP4 activity in some tissues, such as testes and germ cells, whereas other tissues that are sensitive to *Bmp4* dosage, such as the limb, dorsal vertebrae and kidney, develop normally. In a haploinsufficient background, inability to cleave the S2 site leads to embryonic and postnatal lethality due to defects in multiple organ systems including the allantois, placental vasculature, ventral body wall, eye and heart. These data demonstrate that cleavage of the S2 site is essential for normal development and, more importantly, suggest that this site might be selectively cleaved in a tissue-specific fashion. In addition, these studies provide the first genetic evidence that BMP4 is required for dorsal vertebral fusion and closure of the ventral body wall.

**KEY WORDS:** Bone morphogenetic protein, Proprotein convertase, Proteolytic activation, Embryonic patterning, Cleavage mutant mouse

## INTRODUCTION

Bone morphogenetic proteins (BMPs) are a large subclass of the TGF $\beta$  superfamily that are involved in many aspects of development (Chang et al., 2001; Hogan, 1996; Kishigami and Mishina, 2005). *Bmp4* is widely expressed throughout embryogenesis and its earliest known function is during gastrulation. Most mice homozygous for a null allele of *Bmp4* (*Bmp4*<sup>-/-</sup> mice) die at embryonic day (E) 6.5, with little or no mesoderm (Winnier et al., 1995). In the rare mutants that survive past this time, primordial germ cells (PGCs) are not specified (Lawson et al., 1999) and lens induction does not occur (Furuta and Hogan, 1998). Chimeric analysis has shown that BMP4 is required for chorioallantoic fusion, vascularization of the allantois, migration and survival of PGCs, and establishment of left-right asymmetry (Fujiwara et al., 2001; Fujiwara, 2002). Tissue-specific inactivation of *Bmp4* has revealed additional roles for BMP4 in development of the heart, limbs and craniofacial structures (Jiao et al., 2003; Liu et al., 2005; Liu et al., 2004; Selever et al., 2004).

Strict regulation of BMP4 dosage is essential for normal development, as evidenced by patterning defects observed in mice with reduced or elevated levels of BMP4 activity. *Bmp4* null heterozygotes (*Bmp4*<sup>+/-</sup> mice) on a C57BL/6J background display several phenotypes of variable penetrance, including reduced numbers of PGCs, polydactyly, failure to maintain spermatogenesis and defects in the kidneys, eyes and craniofacial structures (Dunn et al., 1997; Katagiri et al., 1998; Lawson et al., 1999; Miyazaki et al., 2000). By contrast, mice that lack the BMP antagonists chordin and/or noggin are stillborn, show loss of ventral cell fates in the

spinal cord and defects in the development of the forebrain, somites and skeleton (Bachiller et al., 2000; Brunet et al., 1998; McMahon et al., 1998).

BMP4 dosage is regulated at multiple levels, including at the level of proteolytic activation (Miyazono et al., 2005; Nakayama et al., 2000; Yanagita, 2005). BMP4 is synthesized as an inactive precursor that is cleaved by FURIN and/or other members of the proprotein convertase (PC) family (Cui et al., 1998) at two evolutionarily conserved sites within the inactive prodomain. An initial cleavage occurs at an optimal FURIN consensus motif adjacent to the mature ligand domain (-RSKR-, denoted the S1 site) and this allows for subsequent cleavage at an upstream minimal FURIN motif (-RISR-, the S2 site) within the prodomain (Cui et al., 2001). In *Xenopus* embryos, BMP4 synthesized from exogenous precursor in which the S2 site is non-cleavable is less active, signals over a shorter range and accumulates at lower levels than does BMP4 cleaved from native precursor (Cui et al., 2001). Biochemical analysis of BMP4 cleavage in *Xenopus* oocytes revealed that mature BMP4 remains noncovalently attached to the prodomain following cleavage at the S1 site (Degnin et al., 2004). If cleavage at the S2 site does not occur, this complex is targeted to the lysosome for degradation, either within the biosynthetic pathway, or within the endocytic pathway following receptor activation and internalization. As a result, mature BMP4 in complex with the prodomain signals only at short range, to nearby cells. Cleavage at the S2 site occurs when the mature/prodomain complex traffics to a more acidic environment, which un masks and facilitates cleavage of the S2 site. This leads to dissociation of the prodomain fragments from mature BMP4, and the free ligand is stable and able to signal over long range. Cleavage at the S2 site might therefore determine how much BMP4 is available for signaling.

The ability of cleavages within the prodomain to regulate the signaling range of mature BMP4 is of particular interest because BMP4 and its *Drosophila* ortholog, decapentaplegic (DPP) can function as either short- or long-range signaling molecules depending on the tissue in which they are expressed. *Xenopus* BMP4, for example, acts over multiple cells within the embryonic

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mesoderm (Dosch et al., 1997) but acts only within the immediate environment of its synthesis in ectodermal explants (Jones et al., 1996). Similarly, DPP forms a long-range concentration gradient that specifies cell fate in a dose-dependent manner in the wing disc but signals only to adjacent cells between germ layers of the gut (Neumann and Cohen, 1997).

Tissue-specific mechanisms that regulate the range of action of BMP4 have not been identified, but it is tempting to speculate that tissue-specific use of the upstream cleavage site could contribute to this process. Accordingly, failure to cleave the S2 site in some tissues would generate a short-range ligand that accumulates at low levels, whereas cleavage at both sites would generate an identical ligand that accumulates at higher levels and possesses long range signaling properties. Given that proper regulation of BMP4 activity is critical for normal embryonic patterning, mutations that disrupt ordered cleavage of proBMP4 are expected to lead to developmental defects.

To study the physiological relevance of cleavage at the S2 site, and to begin to test the hypothesis that tissue-specific cleavage of this site regulates BMP4 activity *in vivo*, we generated mice carrying a point mutation that prevents cleavage at the S2 site. If the S2 site is normally cleaved *in vivo*, this mutation should generate a hypomorphic *Bmp4* allele. Furthermore, if the S2 site is cleaved in a tissue-specific fashion as proposed, mutants would be predicted to show phenotypic defects and lower levels of mature BMP4 protein in only a subset of tissues that are known to be sensitive to BMP dosage. As described below, biochemical and phenotypic analysis of these cleavage mutant mice provide strong support for this hypothesis.

## MATERIALS AND METHODS

### Construction of targeting vector and mouse strains

The targeting vector used to generate *Bmp4<sup>S2G/S2G</sup>* mice was constructed from previously described genomic *Bmp4* clones (Winnier et al., 1995) and introduces a single amino acid change that disrupts the S2 cleavage site, a silent point mutation that changes a *PvuI* site to a *BglII* site (to aid in screening) and a neomycin selectable marker flanked by loxP sites (Fig. 1C). Linearized vector was electroporated into R1 ES cells and homologous recombinants were selected with G418 and gancyclovir. Correctly targeted ES cell clones were identified by Southern blotting (Fig. 1D) and mutations were verified by sequencing DNA fragments PCR-amplified from genomic DNA. Heterozygous ES cells were injected into C57BL/6J blastocysts, and the resulting chimeras were mated with C57BL/6J females to obtain *Bmp4<sup>S2Gneo</sup>* heterozygotes. *Bmp4<sup>S2Gneo</sup>* females from each line were mated to male Cre deleter mice (Schwenk et al., 1995) to remove the neomycin gene. Genotypes were determined by PCR amplification of tail DNA (Fig. 1E) using the 5' primer: 5'-TCTTGAATGTCGAGATGGGGCTGTG-3' and the 3' primer: 5'-TAAGATGAGCCCGGAAGTCCCAAAC-3' under the following conditions: 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, 35 cycles.

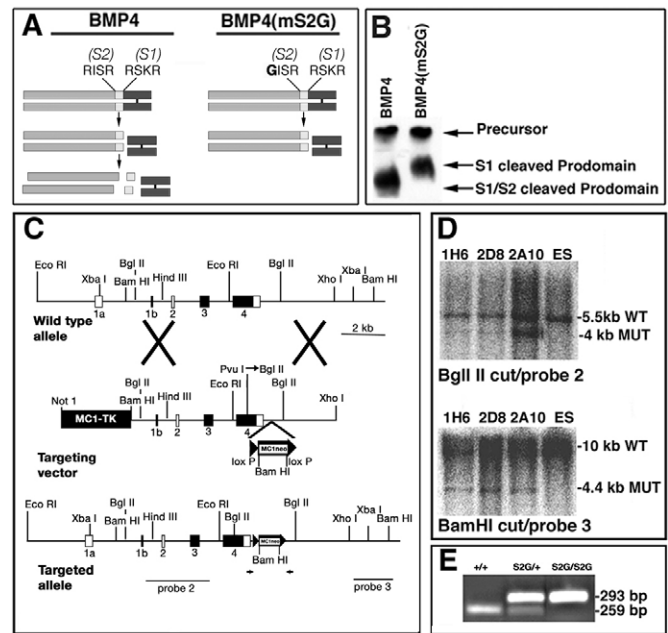
*Bmp4<sup>lacZ/+</sup>* mice were obtained from Dr B. Hogan (Duke University) and genotyped as described previously (Lawson et al., 1999). *Bmp4<sup>lacZ/+</sup>* mice were backcrossed for a minimum of six generations to C57BL/6J prior to mating with *Bmp4<sup>S2G</sup>* mice.

### cDNA constructs and transient transfections

cDNAs encoding HA- and myc-epitope-tagged forms of BMP4 or BMP4(S2G) (Degnin et al., 2004) were transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen). Seventy-two hours post-transfection, cells were lysed and supernatants collected and TCA precipitated for western blot analysis.

### Western blotting

Transfected cells and mouse tissues were homogenized in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1× Roche protease inhibitor cocktail) on ice for 20 minutes, followed by



**Fig. 1. Generation of *Bmp4<sup>S2G</sup>* mice.** (A) Schematic illustration of cleavage patterns of wild-type and S2G mutant forms of proBMP4. (B) Wild-type or mutant forms of the BMP4 precursor were expressed in HEK 293 cells and cleavage products analyzed by probing western blots of cell lysates with anti-HA antibody which recognizes an epitope tag present in the prodomain. Bands corresponding to uncleaved precursor, intact prodomain following cleavage at the S1 site, and intact prodomain following cleavage at the S2 site are indicated. Introduction of a point mutation [BMP4(mS2G)] that prevents cleavage at the S2 site still allows for cleavage at the S1 site whereas both sites are cleaved in native proBMP4. Mature BMP4 cleaved from either precursor migrates with an identical molecular mass (data not shown). (C) Genomic organization of the wild-type *Bmp4* allele, the targeting vector and the *Bmp4<sup>S2Gneo</sup>* allele. The positions of the probes used for Southern analysis, and primers for PCR genotyping (arrows) are indicated. (D) Southern blot analysis of genomic DNA from targeted (H6, 2D8 and 2A10) or non-targeted (ES) ES cells. Genomic DNA was digested as indicated and probed with the internal (probe 2) or external (probe 3) genomic fragments shown in panel C. (E) PCR genotyping of progeny generated by interbreeding *Bmp4<sup>S2G/+</sup>* mice. The PCR product generated by the mutant allele is 293 bp and that generated by the wild-type allele is 259 bp.

centrifugation to remove cellular debris. Proteins were separated on a 12% polyacrylamide gel and transferred to a PVDF membrane. Blots of transfected cells were probed with anti-HA monoclonal 12ca5 antibody (1:1000) and blots of mouse tissue lysates were probed with anti-BMP4 antibody (1:1000; R&D Systems cat. No. MAB757). Both were probed with HRP-conjugated secondary antibody (Zymed; 1:5000) and visualized by chemiluminescence.

### Detection and quantification of PGCs

PGCs were visualized and counted according to Lawson et al. (Lawson et al., 1999).

### Immunostaining, in situ hybridization, and $\beta$ -galactosidase staining

For phosphoSmad staining, E6 deciduas from *Bmp4<sup>+/+</sup>* and *Bmp4<sup>S2G/S2G</sup>* inter se crosses were dissected into phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS on ice for 30 minutes, incubated overnight in 30% sucrose in PBS at 4°C and then embedded in OCT (TissueTek). 8  $\mu$ m cryosections were incubated overnight at 4°C with an anti-phosphoSmad1/5/8 antibody (1:1000; a gift from Dr E. Laufer, Columbia

University) in PBS with 2% horse serum and 0.1% Triton X-100. Staining was visualized using anti-rabbit Alexa Fluor 488-conjugated secondary antibody (1:500; Molecular Probes). Embryos were processed for in situ hybridization with digoxigenin-labeled *Tbx5* and *Gata4* riboprobes as described previously (Wilkinson and Nieto, 1993).  $\beta$ -galactosidase staining of *Bmp4<sup>lacZ/+</sup>* and *Bmp4<sup>lacZ/S2G</sup>* placentas was performed as described previously (Lawson et al., 1999) using Red gal (Research Organics, Cleveland, OH) as a substrate. Whole-mount PECAM staining (1:500; BD Pharmingen) was performed as described (Schlaeger et al., 1995).

### Histology and TUNEL assays

Isolated embryos and organs were fixed in 4% paraformaldehyde in PBS, dehydrated and embedded in paraffin wax. Sections (10  $\mu$ m) were stained with Hematoxylin and Eosin. 6  $\mu$ m testis sections were analyzed for apoptosis using the Dead End fluorometric TUNEL system (Promega). Labeled sections were mounted with Vectashield anti-fade mounting medium containing DAPI (Vector Labs).

### Skeletal preparations

Skeletons were fixed and stained with Alcian Blue and Alizarin Red as described previously (Hogan et al., 1994). Isolated embryonic limbs were stained with Alcian Blue and cleared as described previously (Jegalian and De Robertis, 1992).

## Results

### Cleavage at the upstream site of proBMP4 is not required for viability in mice

To study the physiological relevance of cleavage at the S2 site, we generated a cleavage mutant 'knock-in' mouse (*Bmp4<sup>S2G/S2G</sup>*) by conventional gene targeting. These mice carry a single point mutation in the *Bmp4* allele that changes the amino acid sequence of the S2 cleavage site from a minimal FURIN motif (-RISR-) to a non-cleavable motif (-GISR-) (Fig. 1A). This mutation does not interfere with proper folding of the precursor, or with cleavage at the S1 site in *Xenopus* embryos (Degnin et al., 2004) or in mammalian cells (Fig. 1B). Thus, the same mature BMP4 ligand is generated from both precursors but subsequent cleavage at the S2 site of proBMP4(mS2G) does not occur.

Two independent mouse lines carrying the S2G mutation and a floxed *neomycin* cassette (*Bmp4<sup>S2Gneo</sup>*) were generated (see Materials and methods and Fig. 1C-E) and subsequently mated to Cre-expressing mice to remove the *neomycin* gene. Prior to phenotypic analysis, *Bmp4<sup>S2G</sup>* individuals from each line were backcrossed to C57BL/6J for a minimum of seven generations to remove potential modifier genes present in the 129/Sv strain that can partially rescue *Bmp4* deficiency (Dunn et al., 1997). No phenotypic differences were noted between the two backcrossed lines.

Based on the significantly reduced activity of an analogous cleavage mutant when ectopically expressed in *Xenopus* (Cui et al., 2001), *Bmp4<sup>S2G</sup>* is predicted to be a severe hypomorphic allele. Thus, if the S2 site is normally cleaved in all tissues in wild-type mice in vivo, *Bmp4<sup>S2G/S2G</sup>* mice are expected to have defects similar to or more severe than those observed in *Bmp4* null heterozygotes. Contrary to this prediction, whereas *Bmp4<sup>-/-</sup>* mice die early in embryonic development (Winnier et al., 1995), and *Bmp4<sup>+/-</sup>* mice display a significant degree of mortality prior to weaning (Dunn et al., 1997), *Bmp4<sup>S2G/S2G</sup>* and *Bmp4<sup>S2G/+</sup>* mice appear grossly normal and survive to adulthood in the expected Mendelian ratios (Table 1).

**Table 1. Genotype analysis of *Bmp4<sup>S2G/+</sup>* intercrosses**

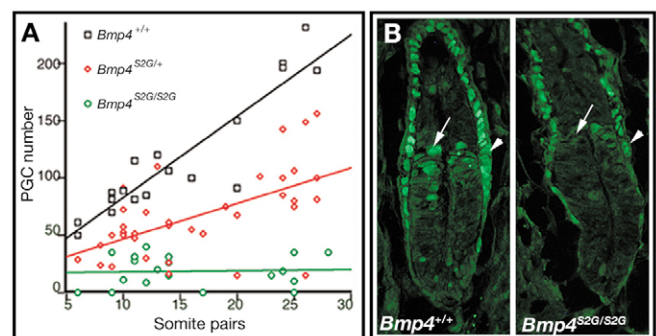
Age	<i>Bmp4<sup>+/+</sup></i>	<i>Bmp4<sup>S2G/+</sup></i>	<i>Bmp4<sup>S2G/S2G</sup></i>	Total
P21	42 (24%)	95 (54%)	39 (22%)	176

Data are presented as number (percent).

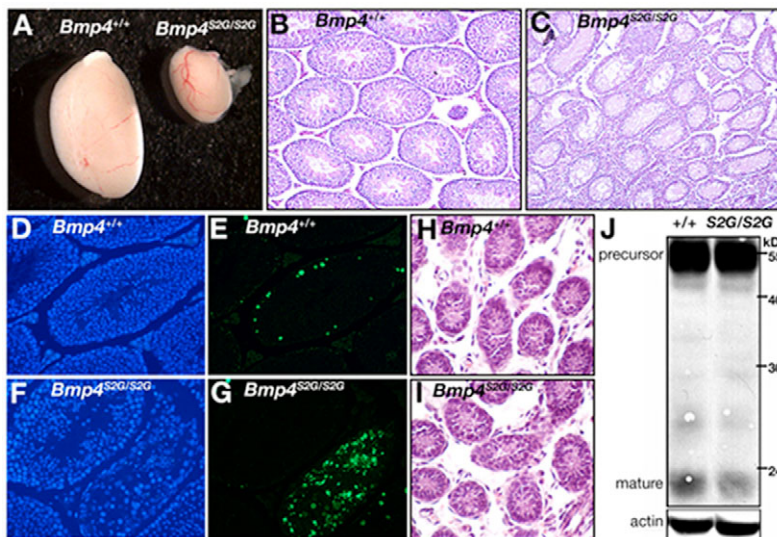
### Cleavage at the S2 site is required to generate normal numbers of PGCs

To determine whether cleavage at the S2 site is required for BMP4 function in some or all organ systems, we examined *Bmp4<sup>S2G/S2G</sup>* mice for defects in tissues that are known to require both copies of *Bmp4* for normal development. First, we analyzed PGC number, which is a sensitive indicator of BMP4 dosage since in *Bmp4* null heterozygotes they are reduced by 50%, and null homozygotes completely lack PGCs (Lawson et al., 1999). Previous studies have shown that BMP4 synthesized in extraembryonic ectoderm (ExE) prior to gastrulation (E6.5) is essential for specifying PGC fate in the epiblast (Lawson et al., 1999; Ohinata et al., 2005), whereas BMP4 expressed in the extraembryonic mesoderm (ExM) during headfold stages is required for localization and survival of PGCs (Fujiwara et al., 2001). *Bmp4<sup>S2G/S2G</sup>* and *Bmp4<sup>S2G/+</sup>* embryos had dramatically reduced numbers of PGCs relative to *Bmp4<sup>+/+</sup>* littermates (Fig. 2A). This reduction was already apparent at the presomite stage, prior to PGC migration (data not shown), indicating that the founding population of PGCs was smaller in mutants. The initial decrease in PGC number in *Bmp4<sup>S2G/S2G</sup>* and *Bmp4<sup>S2G/+</sup>* embryos was almost as severe as that reported in *Bmp4* null homozygotes and heterozygotes, respectively, suggesting that failure to cleave the S2 site almost completely ablates BMP signaling from the ExE. The steeper slope of the regression line on the plot of PGC number versus developmental age for wild-type mice relative to mutant littermates (Fig. 2A) suggests that the rate of expansion and/or survival of PGCs may also be reduced in mutants. We did not, however, detect defects in localization of the remaining germ cells (data not shown). These data demonstrate that cleavage of proBMP4 at both the S1 and the S2 sites is required in order to provide sufficient BMP4 to generate the proper number of PGCs, but is dispensable for their appropriate localization.

To determine the extent to which BMP4 signal transduction was reduced in the absence of S2 cleavage, an antibody directed against the active, phosphorylated forms of the BMP pathway-specific SMADs (SMAD1, 5 and 8) was used to detect BMP activity in E6-6.25 embryos. It has recently been shown that BMP4 produced in the ExE signals to the epiblast both directly, and indirectly via the visceral endoderm (VE), to specify germ cells (de Sousa Lopes et al., 2004). Consistent with our data demonstrating a dramatic reduction in PGC number, SMAD phosphorylation was significantly reduced both in the proximal region of the epiblast (Fig. 2B, arrows)



**Fig. 2. Reduced PGCs and phosphoSmad staining in *Bmp4<sup>S2G/S2G</sup>* embryos. (A) Graph of PGC number versus somite pairs for wild-type, *Bmp4<sup>S2G/+</sup>* and *Bmp4<sup>S2G/S2G</sup>* embryos. (B) Immunostaining of E6 *Bmp4<sup>+/+</sup>* and *Bmp4<sup>S2G/S2G</sup>* embryo sections with an antibody that recognizes the phosphorylated forms of SMAD1, 5 and 8. Staining in the proximal epiblast (arrows) and the visceral endoderm (arrowheads) is decreased in the *Bmp4<sup>S2G/S2G</sup>* embryo.**



**Fig. 3. Testicular degeneration in *Bmp4*<sup>S2G/S2G</sup> adults.** (A) Isolated testes from age-matched wild-type and *Bmp4*<sup>S2G/S2G</sup> adult males. (B, C) Hematoxylin and Eosin-stained sections from age-matched adult testis. (D-G) DAPI staining (D, F) and TUNEL staining (E, G) of sections from age-matched adult testes. (H, I) Hematoxylin and Eosin-stained P6 testis sections from wild-type and mutant littermates. (J) Western blots of equivalent amounts of protein from wild-type and mutant P6 testes probed with an antibody directed against the mature domain of BMP4. The blot was re-probed for actin as a loading control.

and in the VE (arrowheads) of *Bmp4*<sup>S2G/S2G</sup> embryos. These data demonstrate that failure to cleave proBMP4 at the S2 site reduces the ability of mature BMP4 to signal from the ExE to adjacent tissues during early gastrulation.

### Cleavage of the S2 site is required for maintenance of spermatogenesis

To assess whether cleavage of the S2 site is required for normal BMP4 activity in additional tissues that are sensitive to *Bmp4* dosage, we examined adult *Bmp4*<sup>S2G/S2G</sup> males for testicular defects. Heterozygous deficiency of *Bmp4* results in testicular defects in 50% of males aged over 12-weeks old, as evidenced by a small decrease in testes weight and degeneration of a variable fraction (2.6-86%) of the seminiferous tubules (Hu et al., 2004). At 8-12 weeks of age, 50% of *Bmp4*<sup>S2G/S2G</sup> males examined ( $n=7/14$ ) had testes that were drastically smaller than those of age-matched controls (Fig. 3A). Histological analysis revealed extensive degeneration of a large fraction (30-100%) of seminiferous tubules in all undersized testes (Fig. 3B-C). This degeneration was due to widespread apoptosis, as revealed by TUNEL staining (Fig. 3D-G). Notably, testes from 6-day-old (P6) wild-type and *Bmp4*<sup>S2G/S2G</sup> mice were morphologically and histologically indistinguishable (Fig. 3H-I), suggesting that the appearance of 'empty' tubules at later stages (Fig. 3C) is not the result of failed colonization of the embryonic testes by PGCs, but is a consequence of defective maintenance of spermatogenesis in adults.

We have previously shown that S2 processing prevents accelerated degradation of the cleaved ligand (Degnin et al., 2004), and thus we would predict that if the S2 site is normally cleaved in the testes, then levels of mature BMP4 protein would be lower in testes of *Bmp4*<sup>S2G/S2G</sup> males. After 2 weeks of age, *Bmp4* is expressed throughout the seminiferous tubules (Hu et al., 2004), and in adults, BMP4 expression is restricted to immature germ cells (Baleato et al., 2005). Since the degeneration of BMP4-expressing testicular cells in adult *Bmp4*<sup>S2G/S2G</sup> males would confound the interpretation of our results, we analyzed protein levels in testes isolated at P6, when expression of *Bmp4* is restricted to Sertoli cells (Pellegrini et al., 2003) and testicular degeneration is not yet detectable. Less mature BMP4 was detected in individual testes from *Bmp4*<sup>S2G/S2G</sup> males compared to that of wild-type littermates (Fig. 3J), consistent with

the possibility that the S2 site of proBMP4 is normally cleaved in the testes and that failure to do so targets the mature ligand for degradation.

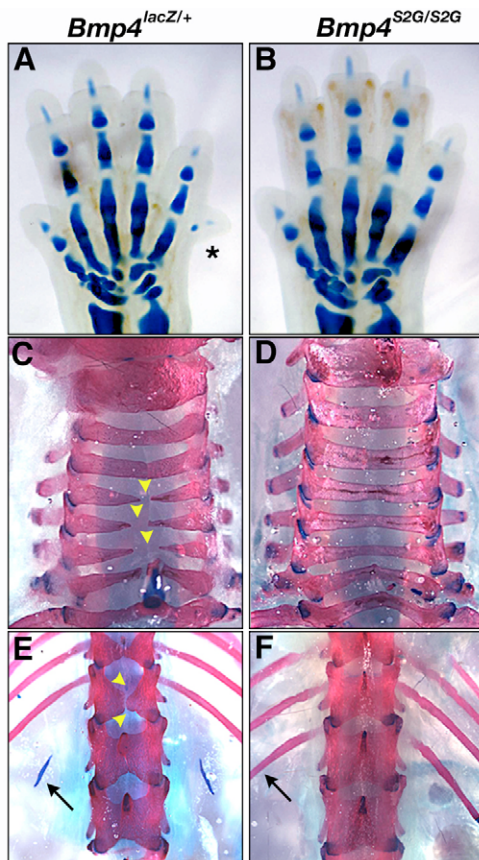
### Cleavage of BMP4 at the S1 site alone generates sufficient BMP4 activity for normal patterning of the skeleton, eyes and kidneys

The loss of PGCs and testicular degeneration observed in *Bmp4*<sup>S2G/S2G</sup> mice is more severe than that in *Bmp4*<sup>+/-</sup> mice, demonstrating that BMP4 dosage is reduced by greater than 50% in these tissues. In other tissues, however, this appears not to be the case since *Bmp4*<sup>S2G/S2G</sup> mice are fully viable (Table 1), unlike *Bmp4* null heterozygotes. To examine this issue more closely, we compared development of several other tissues (skeleton, eyes and kidneys) that are sensitive to BMP4 dosage in *Bmp4*<sup>lacZ/+</sup> mice [in which exon 3 is replaced with *lacZ* to generate a null allele (Lawson et al., 1999)] with that of *Bmp4*<sup>S2G/S2G</sup> mice.

Analysis of the appendicular skeleton of *Bmp4*<sup>lacZ/+</sup> mice revealed extra postaxial cartilaginous elements in the forelimbs of E15.5-P0 embryos (Fig. 4A,  $n=12/16$ ) and adults ( $n=20/42$ ) with a combined frequency of nearly 60%. A similar phenotype is seen in mice in which *Bmp4* is inactivated in the limb bud mesenchyme (Selever et al., 2004). We also observed right hindlimb preaxial polydactyly (data not shown,  $n=1/12$ ), as previously reported (Dunn et al., 1997). By contrast, none of the E15.5 to newborn *Bmp4*<sup>S2G/S2G</sup> mice analyzed ( $n>130$ ) displayed hindlimb or forelimb polydactyly (Fig. 4B).

Examination of the axial skeleton of *Bmp4*<sup>lacZ/+</sup> adults showed a highly penetrant ( $n=7/10$ ) defect in dorsal vertebral fusion and/or formation of the spinous processes of one or more cervical (Fig. 4C) and/or thoracic vertebrae (Fig. 4E). In addition, the 13th ribs were either missing or very small in 50% ( $n=5/10$ ) of *Bmp4*<sup>lacZ/+</sup> mice (Fig. 4E). Defects in fusion of the dorsal vertebrae were never observed (Fig. 4D, F;  $n=0/11$ ), and small or missing ribs were observed in only 1% ( $n=1/76$ ) of *Bmp4*<sup>S2G/S2G</sup> animals (Fig. 4F).

Gross visual examination of *Bmp4*<sup>lacZ/+</sup> mice revealed small or missing eyes in 13% ( $n=13/97$ ) of animals, whereas eye defects were never observed in *Bmp4*<sup>S2G/S2G</sup> mice ( $n>80$ ). Similarly, we detected polycystic or enlarged kidneys in 8% of *Bmp4*<sup>lacZ/+</sup> mice ( $n=1/12$ ),



**Fig. 4. Skeletal defects observed in *Bmp4*<sup>lacZ/+</sup> mice are not present in *Bmp4*<sup>S2G/S2G</sup> mice. (A,B)** Alcian Blue-stained E15.5 limbs. A postaxial cartilaginous element is indicated with an asterisk. **(C-F)** Dorsal views of skeletal preparations of cervical (C,D) and thoracic (E,F) vertebrae from adults. Yellow arrowheads mark vertebrae that have not fused and/or formed a dorsal spinous process. Black arrows indicate the 13th rib.

but in none of over 100 *Bmp4*<sup>S2G/S2G</sup> mice. Thus, cleavage of proBMP4 at the S1 site alone generates sufficient BMP activity for normal eye, kidney and skeletal morphogenesis.

### Compound heterozygotes for *Bmp4* null and S2G alleles show embryonic and/or postnatal lethality

The observation that *Bmp4*<sup>S2G</sup> functions as a severe hypomorphic allele in some tissues that are sensitive to BMP4 dosage, but as a silent mutation in others, raises the possibility that the S2 site is selectively cleaved in a tissue-specific fashion. If so, *Bmp4*<sup>S2G/S2G</sup> mutants will have wild-type levels of BMP4 activity in any tissue in which the S2 site is not normally cleaved. An alternative possibility is that *Bmp4*<sup>S2G</sup> is hypomorphic in all tissues but that S2 cleavage is less critical in some of these, such that BMP4 activity remains above the threshold required for normal patterning. To begin to distinguish between these possibilities, we reduced *Bmp4* gene dosage in all tissues of cleavage mutants by intercrossing *Bmp4*<sup>S2G/S2G</sup> and *Bmp4*<sup>lacZ/+</sup> mice to generate compound heterozygotes for null and S2G alleles. *Bmp4*<sup>lacZ/S2G</sup> mice were recovered at only 25% of the expected frequency at weaning, whereas *Bmp4*<sup>lacZ/+</sup> mice were recovered at 82% of the expected frequency when crossed with wild-type mice (Table 2). Thus, in a haploinsufficient background, the *Bmp4*<sup>S2G</sup> allele does not support viability at the same level as the wild-type allele. When analyzed between E12 and P0, *Bmp4*<sup>lacZ/S2G</sup>

**Table 2. Genotype analysis of live offspring from crosses with *Bmp4*<sup>lacZ/+</sup> mice**

<b>(A) Progeny from <i>Bmp4</i><sup>S2G/S2G</sup> × <i>Bmp4</i><sup>lacZ/+</sup></b>			
Age	<i>Bmp4</i> <sup>S2G/+</sup>	<i>Bmp4</i> <sup>lacZ/S2G</sup>	Total
P21	42 (87%)	6 (13%)	48
E12-P0	22 (62%)	13 (37%)*	35

\*Seven additional dead embryos or newborns were recovered.

<b>(B) Progeny from <i>Bmp4</i><sup>+/+</sup> × <i>Bmp4</i><sup>lacZ/+</sup></b>			
Age	<i>Bmp4</i> <sup>+/+</sup>	<i>Bmp4</i> <sup>lacZ/+</sup>	Total
P21	80 (59%)	55 (41%)	135

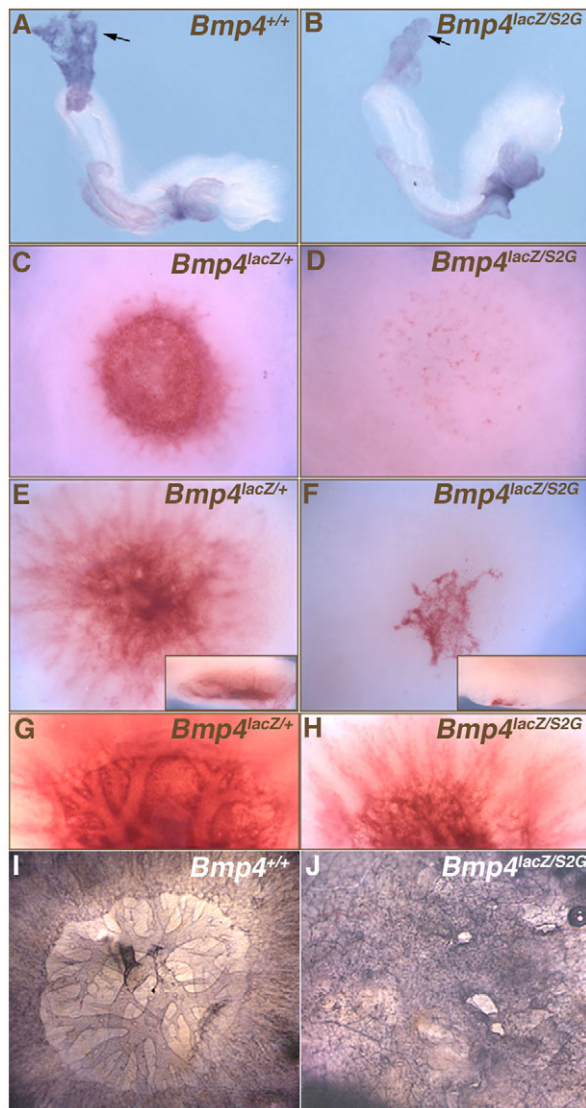
Data are presented as number (percent).

mice were recovered at a higher frequency than at weaning, and a fraction were recovered dead (Table 2), demonstrating that some of the lethality occurs late in embryogenesis or postnatally.

### Cleavage of the S2 site is required for vascular morphogenesis in the chorioallantoic placenta

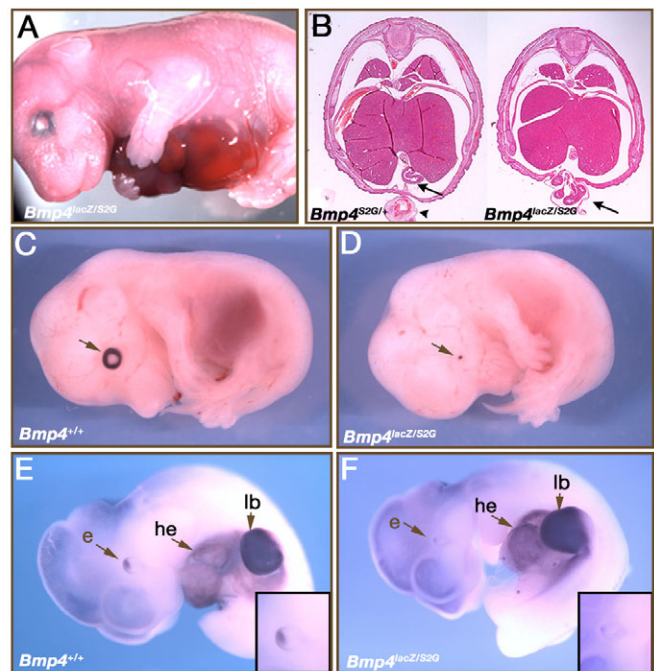
To determine when and why *Bmp4*<sup>lacZ/S2G</sup> mutants were dying, we established timed matings between *Bmp4*<sup>lacZ/+</sup> and *Bmp4*<sup>S2G/S2G</sup> or *Bmp4*<sup>S2G/+</sup> mice and analyzed embryos at various developmental ages. For all analyses, wild-type and mutant embryos were age matched based on external morphological criteria such as somite number and limb development. At E8.5-E9, *Bmp4*<sup>lacZ/S2G</sup> embryos were recovered at the expected frequency and appeared grossly normal except that in several the allantois had not yet fused with the chorion ( $n=4/8$ , data not shown). At this stage of development, the allantois of wild-type embryos had enlarged and undergone expansion by distal cavitation (Downs, 1998) (Fig. 5A), whereas the allantois of selected *Bmp4*<sup>lacZ/S2G</sup> embryos was smaller and contained a densely packed mesenchymal core, indicating that cavitation had not occurred (Fig. 5B and data not shown). In addition, expression of the BMP4 target gene, *Gata4* (Rojas et al., 2005), was decreased in the allantois of a subset of *Bmp4*<sup>lacZ/S2G</sup> embryos relative to wild-type littermates (Fig. 5A,B).

To test whether cleavage at the S2 site is required for normal differentiation or morphogenesis of allantois-derived placental blood vessels, we compared expression of *Bmp4* in the placenta of *Bmp4*<sup>lacZ/+</sup> and *Bmp4*<sup>lacZ/S2G</sup> embryos by staining for  $\beta$ -galactosidase. *Bmp4* is highly expressed throughout the mesothelium and endothelium of the allantois both before and after it fuses with the chorion (Downs et al., 2004), and it continues to be expressed in the chorioallantoic placenta in a pattern that outlines the embryonic vasculature until at least E14 (Fig. 5G and data not shown). Thus,  $\beta$ -galactosidase staining can be used to follow the development of these allantoic derivatives. In *Bmp4*<sup>lacZ/+</sup> embryos,  $\beta$ -galactosidase-positive allantoic cells spread out over the surface of the chorion (Fig. 5C,E) and penetrated into the embryonic layer of the placenta (Fig. 5E, inset) between E9.5-10.5. By E11.5, *Bmp4*-expressing cells were present at highest concentration in the mesothelium immediately adjacent to the developing blood vessels, which had branched and become organized into an extensive vascular network (Fig. 5G). By contrast, in the most severely affected *Bmp4*<sup>lacZ/S2G</sup> embryos, only a few scattered  $\beta$ -galactosidase-positive cells were observed on the surface of the placenta at E9.5-10.5 (Fig. 5D, Table 3), possibly due to aberrant chorioallantoic fusion. With few exceptions, the remaining placentas derived from E9.5-11.5



**Fig. 5. Defects in the allantois and placental vasculature of *Bmp4<sup>lacZ/S2G</sup>* embryos.** (A,B) Expression of *Gata4* in E8.75 embryos, analyzed by whole mount in situ hybridization. Arrows indicate the distal end of the allantois that has not undergone cavitation in *Bmp4<sup>lacZ/S2G</sup>* embryos. (C-H)  $\beta$ -Galactosidase expression in whole-mount placentas isolated from *Bmp4<sup>lacZ/+</sup>* or *Bmp4<sup>lacZ/S2G</sup>* embryos at E9.5 (C,D), E10.5 (E,F) or E11.5 (G,H). Inset in E and F shows a sagittal section through the placenta. (I,J) Whole-mount anti-PECAM staining of placentas from E11.5 littermates.

*Bmp4<sup>lacZ/S2G</sup>* embryos had significantly fewer stained cells and/or the embryonic placental vascular network was smaller and more poorly organized than that of age matched *Bmp4<sup>lacZ/+</sup>* embryos (Fig. 5E-H, Table 3). We confirmed these changes in the superficial placental vascular network of compound mutants by analyzing the expression of the endothelial marker PECAM in whole placentas (Fig. 5I,J). These data demonstrate that S2 cleavage is essential for the function of BMP4 in promoting differentiation and assembly of allantoic vascular endothelial cells into organized blood vessels within the placenta (Fujiwara et al., 2001). This defect in placental vascularization most probably contributes to embryonic lethality.



**Fig. 6. Defects in ventral body wall closure and eye development in *Bmp4<sup>lacZ/S2G</sup>* mutants.** (A) E17.5 *Bmp4<sup>lacZ/S2G</sup>* embryo with complete failure of ventral body wall closure. (B) Coronal sections through E16 embryos. Arrowhead indicates umbilical artery, arrow denotes midgut, which has been enclosed by the ventral body wall in the *Bmp4<sup>S2G/+</sup>* embryo but remains externalized in the *Bmp4<sup>lacZ/S2G</sup>* littermate. (C,D) E12.5 embryos showing loss of eye pigment in *Bmp4<sup>lacZ/S2G</sup>* embryos. (E,F) Expression of *Tbx5* in E10.5 embryos, analyzed by whole-mount in situ hybridization. e, eye; he, heart; lb, limb bud. Insets show staining of the eye at higher magnification.

### Cleavage of the S2 site is required for ventral body wall closure and eye development

Visual analysis of *Bmp4<sup>lacZ/S2G</sup>* mice recovered at E13.5-P0 revealed defects in ventral body wall closure and/or eye development. Nearly all *Bmp4<sup>lacZ/S2G</sup>* embryos and newborns displayed a spectrum of ventral body wall closure defects (VBD) ranging from complete failure of ventral body wall fusion leading to externalization of all of the viscera (Fig. 6A,  $n=5/18$  embryos), to umbilical hernia (Fig. 6B,  $n=9/18$  embryos), to the mildest form of VBD, in which sternal fusion fails leading to a split xiphoid process ( $n=3/3$  newborns, data not shown). The more severe forms of VBD were never observed in *Bmp4<sup>lacZ/+</sup>* embryos, although a split xiphoid process was observed in *Bmp4<sup>lacZ/+</sup>* adults at low frequency (Katagiri et al., 1998). We also noted an increase in the frequency of missing or small eyes in *Bmp4<sup>lacZ/S2G</sup>* mutants at E12-P0 ( $n=13/22$ ; Fig. 6C,D) that exceeds that observed in *Bmp4<sup>lacZ/+</sup>* mice ( $n=13/97$ ), suggesting that S2 site cleavage is also required for normal BMP function in eye development. Consistent with this possibility, expression of the dorsal retinal marker *Tbx5*, which is sensitive to *Bmp4* dosage (Murali et al., 2005), was reduced or absent in the eyes, but not in the heart or the limbs, of *Bmp4<sup>lacZ/S2G</sup>* embryos relative to wild-type littermates (Fig. 6E,F). *Bmp4<sup>lacZ/S2G</sup>* embryos showed a more severe and frequent loss of retinal expression of *Tbx5* than did *Bmp4<sup>lacZ/+</sup>* littermates (Table 4).

**Table 3. Defective placental vasculature in *Bmp4*<sup>lacZ/S2G</sup> embryos**

Genotype		+++	++	+	+/-	Total
E9.5	<i>Bmp4</i> <sup>lacZ/+</sup>	6 (75%)	2 (25%)	0 (0%)	0 (0%)	8
	<i>Bmp4</i> <sup>lacZ/S2G</sup>	0 (0%)	1 (20%)	2 (40%)	2 (40%)	5
E10.5	<i>Bmp4</i> <sup>lacZ/+</sup>	12 (100%)	0 (0%)	0 (0%)	0 (0%)	12
	<i>Bmp4</i> <sup>lacZ/S2G</sup>	0 (0%)	3 (33%)	5 (55%)	1 (11%)	9
E11.5	<i>Bmp4</i> <sup>lacZ/+</sup>	10 (83%)	2 (17%)	0 (0%)	0 (0%)	12
	<i>Bmp4</i> <sup>lacZ/S2G</sup>	1 (14%)	2 (28%)	4 (57%)	0 (0%)	7

Expression of *Bmp4* was analyzed by  $\beta$ -galactosidase staining whole placentas from embryos carrying the *Bmp4*<sup>lacZ</sup> allele. Staining intensity was scored as wild type (+++), slightly decreased (++) , severely decreased (+) or absent in all but a few cells (+/-). Data are presented as number of embryos (percent).

### Failure to cleave the S2 site enhances the frequency of ventricular septal defects in a haploinsufficient background

Tissue-specific deletion of *Bmp4* in splanchnic, branchial arch and/or myocardial mesoderm leads to defects in septation of the embryonic atrium (Jiao et al., 2003), ventricle, and/or outflow tract (Jiao et al., 2003; Liu et al., 2004) raising the possibility that heart anomalies also contribute to the late embryonic or perinatal lethality observed in *Bmp4*<sup>lacZ/S2G</sup> and *Bmp4*<sup>lacZ/+</sup> embryos. Histological analysis of mice recovered at E15-E17.5 revealed that approximately 10% of *Bmp4*<sup>lacZ/+</sup> embryos examined (1/11) had defects in closure of the membranous portion of the ventricular septum (Fig. 7E, arrow) whereas these defects were more frequent in *Bmp4*<sup>lacZ/S2G</sup> embryos (Fig. 7F; *n*=4/8). Membranous ventriculoseptal defects were never observed in *Bmp4*<sup>S2G/S2G</sup> embryos (Fig. 7D; *n*=0/11) or wild-type controls (data not shown). Defects in septation of the outflow tract (Fig. 7A-C) or the atrium (Fig. 7G-I) were not detected in any mice that were examined. These data demonstrate that cleavage at the S2 site is essential for normal BMP function in closure of the membranous ventricular septum. Furthermore, they suggest that septation of the ventricle is more sensitive to BMP dosage than is septation of the atrium or outflow tract.

### A single copy of *Bmp4*<sup>S2G</sup> generates sufficient BMP4 activity for patterning of the skeleton and kidneys

Although analysis of BMP4 compound mutants carrying null and S2G alleles revealed additional tissues in which the *Bmp4*<sup>S2G</sup> allele is hypomorphic, we did not detect any evidence for this in the skeleton or kidney. *Bmp4*<sup>lacZ/S2G</sup> mutant embryos did not show an increase in the frequency of polycystic kidneys (*n*=0/9) relative to that found in age matched *Bmp4*<sup>lacZ/+</sup> mice (*n*=1/12). Likewise, the presence of the *Bmp4*<sup>S2G</sup> allele did not enhance the severity or frequency of hindlimb preaxial polydactyly (*n*=1/12) or forelimb postaxial duplications (*n*=8/12) compared to that seen in *Bmp4*<sup>lacZ/+</sup> controls (*n*=1/12 and 13/16, respectively). Thus, even when endogenous BMP4 levels are halved, the S2G mutation is silent in the kidney and limbs, suggesting that S2 site processing does not occur, or is not required for normal BMP4 activity in these tissues.

### DISCUSSION Tissue-specific regulation of BMP4 activity by selective proteolysis

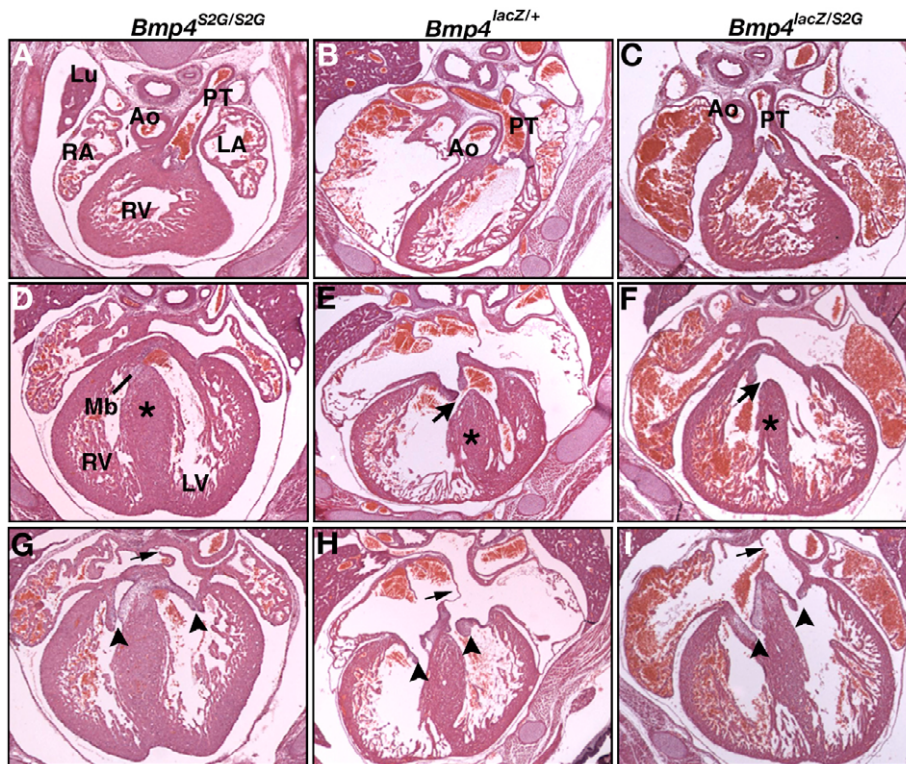
Previous work in our lab has demonstrated that proBMP4 is sequentially cleaved at two sites within the prodomain, and that failure to cleave at the upstream site targets the ligand for lysosomal degradation (Degnin et al., 2004) such that it is less active and signals at shorter range (Cui et al., 2001). These studies involved overexpression of exogenous protein, however, and did not address whether cleavage of the S2 site was physiologically relevant. In the current studies, we show that mice expressing a single allele of *Bmp4* containing a point mutation that prevents S2 processing have defects in multiple organ systems and die during embryogenesis and early postnatal life. Thus, cleavage of the S2 site of endogenous proBMP4 is essential for normal development in vivo.

An intriguing possibility is that BMP4 activity and/or signaling range is regulated in a tissue-specific fashion by cleavage of the S2 site. Consistent with this idea, inability to cleave the S2 site of proBMP4 in all tissues leads to phenotypic defects in only a subset of tissues where full *Bmp4* dosage is required. The lack of defects in some tissues could potentially be explained if these tissues have a lower threshold requirement for *Bmp4*, or express functionally redundant *Bmp* family ligands. This is inconsistent, however, with the spectrum of phenotypes observed in *Bmp4*<sup>+/-</sup> mice relative to those in *Bmp4*<sup>S2G</sup> mutants. Specifically, we show that *Bmp4*<sup>S2G</sup> functions as a silent mutation in some tissues that are sensitive to a 50% reduction in *Bmp4* dosage, such as the limb, dorsal vertebrae and kidney, yet leads to a much greater than 50% reduction in *Bmp4* dosage in other tissues, such as the testes and PGCs. Furthermore, levels of mature BMP4 are reduced in some tissues (e.g. testes), but are equivalent in other tissues (e.g. kidneys and limbs, D.C.G., S.S. and J.L.C., data not shown) of *Bmp4*<sup>S2G/S2G</sup> mice relative to wild-type littermates. The simplest interpretation of our data is that the S2 site is cleaved in a tissue-specific manner and that reduced levels of mature BMP4 protein and/or phenotypic defects are observed only in tissues where the S2 site is normally cleaved. A less likely possibility is that the S2 site is ubiquitously cleaved and generates a long range gradient of BMP4 activity in all tissues, but that S2 cleavage is irrelevant in some tissues because a short range signal is

**Table 4. Loss of *Tbx5* expression in the dorsal retina of *Bmp4*-deficient embryos**

Genotype	<i>Tbx5</i> staining in dorsal retina				Total
	+++	++	+	-	
<i>Bmp4</i> <sup>+/+</sup>	17 (100%)	0 (0%)	0 (0%)	0 (0%)	17
<i>Bmp4</i> <sup>lacZ/+</sup>	1 (6%)	8 (50%)	7 (44%)	0 (0%)	16
<i>Bmp4</i> <sup>lacZ/S2G</sup>	1 (9%)	2 (18%)	6 (55%)	2 (18%)	11

Expression of *Tbx5* was analyzed by in situ hybridization at E9.5-10.5. Staining intensity in the dorsal retina was scored as wild type (+++), slightly decreased (++) , severely decreased (+) or absent (-). Data are presented as number of embryos (percent).



**Fig. 7. Ventricular septal defects in *Bmp4*<sup>lacZ/+</sup> and *Bmp4*<sup>lacZ/S2G</sup> mutants.** Hematoxylin and Eosin-stained coronal sections of an E16.5 *Bmp4*<sup>S2G/S2G</sup> embryo (A,D,G), an E16.5 *Bmp4*<sup>lacZ/+</sup> embryo (B,E,H) and an E16.5 *Bmp4*<sup>lacZ/S2G</sup> embryo (C,F,I). (A-C) Anterior sections at the level of the pulmonary trunk (PT) and aorta (Ao) show normal outflow tract septation. (D-F) Sections through the muscular (\*) and membranous (Mb) portion of the ventricular septum. Arrows indicate an aberrant opening where the membranous portion of the septum has not formed. (G-I) Sections through the atrioventricular valves (arrowheads) and atrial septa (arrows) showing normal development of these structures. LA, left atrium; Lu, lung; LV, left ventricle; RA, right atrium; RV, right ventricle.

sufficient for patterning. At present, it is not possible to distinguish between these possibilities in mammals since the range of action of BMP4 cannot be tracked *in vivo* and we have been unable to detect endogenous cleaved prodomain on western blots using any of four antibodies generated against epitopes in this region. These studies may not be feasible if the prodomain is rapidly degraded *in vivo*, or may require the development of more sensitive reagents.

#### BMP redundancy may mask the requirement for S2 site processing in some tissues

Although functional redundancy cannot account for the lack of skeletal or kidney defects in *Bmp4*<sup>S2G</sup> mice, it is probable that related BMP family members do compensate for decreased levels of BMP4 in other tissues. *Bmp4* is highly expressed in mesenchymal cells of the intestine and loss of BMP function leads to ectopic crypt formation (Haramis et al., 2004; He et al., 2004). *Bmp4* mRNA and protein levels are significantly reduced in the intestine of *Bmp4*<sup>lacZ/S2G</sup> mice, yet we do not detect any reduction in phosphoSmad staining, nor do we see histological abnormalities in intestinal architecture (D.C.G., R.H. and J.L.C., unpublished). It is probable that the related family members *Bmp2* and/or *Bmp7* can compensate for reduced levels of *Bmp4* in the intestine, given that their expression domains overlap extensively in this tissue (Lyons et al., 1995). Consistent with this possibility, expression of *Bmp4* from the *Bmp7* locus can rescue kidney defects in *Bmp7* mutants suggesting that these ligands can function interchangeably in at least some tissues (Oxburgh et al., 2005). Furthermore, S1 and S2 FURIN consensus motifs are conserved in all BMP2 and BMP4 precursor proteins (Cui et al., 2001), suggesting that cleavage of proBMP2 is most probably regulated in a fashion identical to that of proBMP4. Thus, in tissues of *Bmp4*<sup>S2G/S2G</sup> mice where cleavage of the S2 site is required, and where *Bmp2* is co-expressed, the S2 site of proBMP2 is also likely to be cleaved and this may generate sufficient levels of mature protein to compensate for reduced levels of BMP4.

#### Analysis of cleavage mutant mice reveals novel roles for BMP4

In addition to confirming and/or extending previous studies showing that *Bmp4* is required for specification of PGCs, maintenance of spermatogenesis, and development of other organ systems such as the allantois, heart and eye, our studies provide the first genetic evidence that *Bmp4* function is required for dorsal fusion of the vertebrae and for ventral body wall closure.

The dorsal portion of the vertebral body, which gives rise to the spinous process, is derived from mesenchymal cells of somitic origin. These cells migrate and begin to express the *Bmp4* target genes, *Msx1* and *Msx2*, after taking a position between the surface ectoderm and the roof plate of the neural tube, both of which express *Bmp4* (reviewed by Christ et al., 2004). Classical embryological experiments in chick showed that grafts of BMP2- or BMP4-producing cells dorsal to the neural tube led to hypertrophy of the spinous process (Monsoro-Burq et al., 1996; Watanabe et al., 1998; Watanabe and Le Douarin, 1996), whereas grafts of a ventral structure, the notochord, or of recombinant sonic hedgehog (SHH), which is produced by the notochord, inhibited expression of *Bmp4* and prevented differentiation of the spinous process (Watanabe et al., 1998). These studies led to a model in which dorsal patterning of the vertebrae is accomplished by BMP4, which is opposed by ventrally derived SHH. Our data showing that formation of the spinous process is defective in *Bmp4* null heterozygotes provide strong genetic evidence in support of this model. Interestingly, *Bmp2* is co-expressed with *Bmp4* in the surface ectoderm and dorsal neural tube (Dudley and Robertson, 1997), and we observe dorsal vertebral defects at a similar frequency in *Bmp2*<sup>+/-</sup> mice (D.C.G. and J.L.C., unpublished data), underscoring the importance of achieving full BMP dosage for development of the axial skeleton. This exquisite dosage sensitivity, together with the lack of vertebral defects in *Bmp4*<sup>S2G/S2G</sup> mice, suggests that BMP signals at short range from the dorsal ectoderm or roof plate to the cells that will form the spinous process, and that S2 site processing is not essential to generate these signals.



Defects in closure of the ventral body wall are fairly common in humans and yet the underlying genetic and environmental causes are poorly understood (Brewer and Williams, 2004). Members of the TGF $\beta$  family, including BMPs, have been implicated in this process since deletion of *Tgfb2* plus *Tgfb3*, or of *Bmp1* (a metalloproteinase that boosts BMP activity by inactivating a BMP antagonist) leads to defects in ventral body wall fusion (Dunker and Kriegelstein, 2002; Suzuki et al., 1996). In *Drosophila*, *Dpp* is required for dorsal closure, a process that has been proposed to be analogous to ventral closure in mammals (Brewer and Williams, 2004). DPP is responsible for the induction of cell shape changes that draw together and 'zipper' close the two epithelial sheets during dorsal closure (Ricos et al., 1999). It plays an analogous role in regulating cytoskeletal organization during pupal thorax closure (Martin-Blanco et al., 2000) and morphogenesis of the wing (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). These observations have led to the suggestion that DPP plays a broadly conserved role in driving cell shape changes that are required for patterned morphogenesis of developing epithelia. Our demonstration that *Bmp4* is required for ventral closure in the mouse supports the assertion that this role is conserved in vertebrates as well.

### Regulation of proBMP4 cleavage

This analysis of *Bmp4*<sup>S2G</sup> mutant mice illustrates the importance of proteolytic processing in regulating BMP activity, yet the identity of the proprotein convertase (PC) that cleaves the S1 and/or S2 sites of proBMP4 remains ambiguous. In vertebrates, seven PCs have been identified and, among these, FURIN, PACE4 (PCSK6 – Mouse Genome Informatics), PC6 and PC7 are eligible to cleave proBMP4 since they are all broadly expressed and function within the constitutive secretory pathway. Earlier studies involving the use of a selective PC inhibitor in *Xenopus* embryos suggested that FURIN and/or PC6 are the best candidates for endogenous proBMP4 convertases (Cui et al., 1998). Consistent with this possibility, mouse embryos lacking *Furin* die by E11.5 and show an early defect in chorioallantoic fusion similar to that observed in *Bmp4*<sup>lacZ/S2G</sup> mutant mice (Roebroek et al., 1998). Loss of *Furin* has less severe consequences than loss of *Bmp4*, however, suggesting that other PCs function redundantly to cleave proBMP4. *Pc6* (*Pcsk5* – Mouse Genome Informatics) mutant mice die prior to E7.5 (Essalmani et al., 2006), precluding analysis of its requirement in later BMP4-dependent patterning events. By contrast, proteolytic maturation of proBMP4 is reported to be intact in mice lacking both *Furin* and *Pace4*, at least during pregastrula stages of development (Beck et al., 2002), eliminating PACE4 as an essential BMP4 convertase in the early embryo. *Pc7* (*Pcsk7* – Mouse Genome Informatics) mutants develop normally (Taylor et al., 2003), ruling out the possibility that this protease plays a dominant role in the maturation of proBMP4. Furthermore, we have previously shown that recombinant PC7 can cleave the S1, but not the S2 site of proBMP4 in vitro (Cui et al., 1998), consistent with other studies showing that PC7 has a strict requirement for a basic residue at the P2 position (van de Loo et al., 1997). It is possible, however, that PC7 functions redundantly with FURIN in cleaving the S1 site of proBMP4. The generation of mice carrying tissue-specific mutations in *Pc6*, *Furin* and/or *Pace4* will facilitate analysis of the potential role of these proteases in cleaving proBMP4.

We thank B. Hogan for providing the *Bmp4* genomic clones and *Bmp4*<sup>lacZ</sup> mice, E. Laufer for the anti-Psmad antibody, Y. Furuta and L. Sussel for in situ probes, and N. Donley for excellent technical assistance. We are indebted to G. Thomas for significant intellectual contributions throughout the development of this project, E. Meyers for assistance with heart analysis, and B. Hogan, Y.

Mishina and members of the Christian laboratory for helpful comments on the manuscript. D.C.G. and T.N. were recipients of postdoctoral fellowships from the American Heart Association, R.H.P. was supported by an NIH NRSA, and J.L.C. is supported by NIH grants RO1 HD42598 and RO1 HD37976.

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