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A Large Bioactive BMP Ligand with Distinct Signaling Properties Is Produced by Alternative Proconvertase Processing

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Abstract

Dimers of conventional transforming growth factor- β (TGF- β) and bone morphogenetic protein (BMP) ligands are composed of two 100- to 140-amino acid peptides that are produced through the proteolytic processing of a proprotein precursor by proconvertases, such as furin. We report the identification of an evolutionarily conserved furin processing site in the amino terminus (NS) of the Glass bottom boat (Gbb; the *Drosophila* ortholog of vertebrate BMP5, 6, and 7) proprotein that generates a 328-amino acid, active BMP ligand distinct from the conventional 130-amino acid ligand. Gbb38, the large ligand form of Gbb, exhibited greater signaling activity and a longer range than the shorter form Gbb15. The abundance of Gbb15 and Gbb38 varied among different tissues, raising the possibility that differential processing could account for tissue-specific behaviors of BMPs. In human populations, mutations that abolished the NS cleavage site in BMP4, BMP15, or anti-Müllerian hormone were associated with cleft lip with or without cleft palate (BMP4), premature ovarian failure (BMP15), and persistent Müllerian duct syndrome (anti-Müllerian hormone), suggesting the importance of NS processing during development. The identification of this large BMP ligand form and the functional differences between large and small ligands exemplifies the potential for differential proprotein processing to substantially affect BMP and TGF- β signaling output in different tissue and cellular contexts.

INTRODUCTION

The family of transforming growth factor- β (TGF- β) and bone morphogenetic protein (BMP) signaling molecules affects various developmental processes, and a multitude of

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Author contributions: T.A. and K.A.W. designed the experiments and analyzed the data; T.A. performed most of the experiments; G.M. generated the Gbb antibody; and K.A.W. performed the phylogenetic analysis, made the figures, and wrote the paper.

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SUPPLEMENTARY MATERIALS

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Fig. S1. Signaling activity of the HA-tagged Gbb constructs.

Fig. S2. Gbb38 and Gbb15 bind to the Sax type I receptor.

Fig. S3. Signaling activity of the *hBMP4* and *hBMP4* cleavage mutants.

Fig. S4. Failure to cleave Gbb at the NS results in wing defects.

Fig. S5. The *gbb* double cleavage mutant exhibits dominant-negative behavior when expressed in *dpp*-expressing cells of the wing imaginal disc.

Fig. S6. Wild-type *gbb* (Gbb38 and Gbb15) and *gbb^{ms1}* (Gbb38) exhibit nonautonomous signaling.

Table S1. Macrochaetae and leg defects associated with misexpression of *gbb* and *gbb* cleavage mutants in *dpp*-expressing cells.

Table S2. Primers used in this study.

developmental abnormalities and disease states are associated with the misregulation of their signaling (1–3). Individual TGF- β superfamily members elicit different responses depending on tissue context. For example, patterning of the position of the longitudinal veins and the domains of intervein material in the developing *Drosophila* wing depends on a long-range signaling activity gradient established by two BMPs, Decapentaplegic (Dpp) and Glass bottom boat (Gbb) (4, 5). However, in the germline stem cell niche, these same BMPs instead act as short-range molecules to maintain stem cell identity (6). A mechanistic explanation for these differences in ligand function is still forthcoming, as is an understanding of how their activities are regulated.

The production of bioactive ligands responsible for the initiation of TGF- β and BMP signaling involves a series of poorly understood maturation events including proconvertase (furin) processing of an inactive dimeric proprotein at optimal (Arg-X-Lys/Arg-Arg) or minimal (Arg-X-X-Arg) sites to yield the C-terminally derived 100– to 140–amino acid growth factor–like peptides of ~15 to 20 kD (7). The high degree of sequence conservation observed between this C-terminal domain of more than 20 different gene products served to establish the now well-known TGF- β /BMP superfamily (8). Many of these family members have more than one furin cleavage consensus sequence just N-terminal to the first conserved cysteine of the ligand domain, and differential cleavage can influence the amount of BMP4 produced in mice and *Xenopus* and that of Dpp, the *Drosophila* ortholog of BMPs 2 and 4 (9–13). Such differences in ligand production, as well as in ligand turnover, can affect the functional range of active BMPs in developing tissues (10, 14–16). In addition, two different cleavage forms of Dpp differ in their ability to interact with the *Drosophila* heparin sulfate proteoglycan Dally, which may contribute to the difference in their in vivo stability (17).

We previously showed that the BMP signaling gradient in the *Drosophila* wing imaginal disc requires Gbb, the *Drosophila* ortholog of BMP5/6/7 (5, 18, 19). Dpp is present in a discrete stripe of cells along the anterior/posterior (A/P) boundary of the developing wing (20) where the source of morphogen responsible for the BMP activity gradient is thought to arise (21–23). We found that the activity of Gbb is critical for the breadth of the gradient (5), prompting us to investigate the basis of its long-range behavior to gain insight into how a morphogen gradient is established. As part of these investigations, we found that Gbb exists in two ligand forms. Gbb15 is the conventional ligand derived from the C-terminal domain of the proprotein and contains the conserved seven cysteine residues responsible for the cysteine knot fold characteristic of members of the TGF- β and BMP family (24, 25). Gbb38 is a previously unrecognized active ligand that is larger (with a predicted molecular mass of 38 kD) and also generated by proconvertase processing but at a furin consensus sequence that falls within the N-terminal portion of the prodomain (which we call the NS site). Gbb38 is secreted, binds to receptors, and stimulates signaling events distinct from that produced by Gbb15 alone. A survey of TGF- β family members revealed that the NS cleavage consensus sequence is conserved among many family members from cnidarians to humans. The association of NS site mutations in hBMP4, hBMP15, and AMH with developmental abnormalities [cleft lip with or without palate (CLP), premature ovarian failure (POF), and persistent Müllerian duct syndrome (PMDS), respectively (26–28)] suggests the functional importance of NS processing. Comparisons of the in vivo function of the large and small Gbb ligands uncovered distinct differences in their abilities to induce signaling and to do so over different cell distances. These findings underscore the impact that multiple ligand forms can have on signaling output and provide a new mechanistic explanation for how a single BMP family member can elicit different responses. Together, our data prompt a reevaluation of the identity of ligands that are responsible for activating TGF- β and BMP pathways in different tissue contexts, especially given the value of these molecules as therapeutics to treat and prevent various human diseases and developmental abnormalities.

RESULTS

Proconvertase processing at an unconventional site is required for signaling

To assess the importance of proprotein processing at specific furin cleavage sites in producing an active Gbb ligand, we compared the signaling activity elicited by wild-type *gbb* and *gbb* cleavage mutant constructs. BMP signaling results in the phosphorylation of Mad (the *Drosophila* R-Smad ortholog) and activation of BMP signaling transcriptionally represses *brinker* through the direct binding of a phosphorylated Mad-Med-Shn complex to *brinker* silencer elements (brkSE) in the *brinker* cis-regulatory region (29). Thus, a *brkSE-lacZ* reporter transfected into *Drosophila* S2 cells quantitatively reports the direct transcriptional response to BMP signaling induced by cotransfected ligand or activated receptor constructs (29–31) (Fig. 1, A and B). Wild-type *gbb* reduced β -galactosidase activity, thus indicating robust BMP signaling, which repressed the *brkSE-lacZ* reporter (Fig. 1, B and C) (31). *gbb* cleavage mutant constructs containing mutations at either one or both of the two furin cleavage consensus sites located between the predicted pro- and ligand domains (S2 [Arg²⁷⁷-Pro-Asp-Arg] and S1 [Arg³²²-Ser-Lys-Arg]) were assayed for signaling activity (Fig. 1, A and C). Unexpectedly, mutations abolishing the S2 site (*gbb^{mS2}*), previously shown to be essential for BMP4 and Dpp activity (11, 13, 32), had no effect on Gbb signaling activity (Fig. 1C). In addition, mutations at the S1 site (*gbb^{mS1}*) had only a slight but consistent change in signaling compared to that elicited by wild-type *gbb* (Fig. 1C). Consistent with its ability to induce BMP signaling, the phosphorylation of Mad was increased in cells expressing *gbb^{mS1}* (Fig. 1D). We considered the possibility that processing at either the S2 or the S1 furin cleavage site was sufficient for full activity and that processing at the nonmutated site compensated for the lack of processing at the mutated site. However, we found that a construct lacking both S2 and S1 furin cleavage consensus sequences (*gbb^{mS2mS1}*) did not show reduced Gbb signaling. This result suggested that either the Gbb proprotein has the ability to signal or an active Gbb ligand could be produced by some means other than the already characterized mode of proconvertase processing at sites adjacent to the conserved C-terminal domain.

We identified two additional furin cleavage consensus sequences within the Gbb prodomain ([Arg¹²⁰-Gly-His-Arg] and [Arg¹²³-Ser-Arg-Arg]) at a position not previously recognized as a viable processing site (Fig. 1A). We found that signaling activity was reduced by 50% in forms of *gbb* with mutations in these N-terminal sites (NS) (*gbb^{mNS}*: R123G and R126G), suggesting the importance of processing at the NS in the production of an active Gbb ligand (Fig. 1C). When both the NS and the conventional S1 site were mutated (*gbb^{mNSmS1}*), Gbb signaling activity was attenuated (Fig. 1C), indicating that although the *gbb^{mS1}* mutant could induce signaling to nearly the same extent as the wild-type form, in the absence of processing at the NS site as well (namely, the *gbb^{mNSmS1}* double mutant) no signaling activity was retained. Thus, processing at both the S1 and the NS sites appears critical in generating wild-type amounts of Gbb signaling.

Cleavage at the N-terminal site produces a large BMP ligand

We produced a Gbb monoclonal antibody against a peptide in the C-terminal ligand domain. The specificity of the antibody for Gbb was indicated by the absence of cross-reactivity to proteins in extracts from *gbb* null larvae (*gbb¹/gbb²* and *gbb¹/gbb³*) (Fig. 2A). Three prominent protein products at 54, 42, and 15 kD were identified by the Gbb antibody in both whole larval extracts and *Drosophila* S2 cells (Fig. 2A), and the same three bands were detected with a hemagglutinin (HA) antibody in S2 cell extracts expressing an HA-tagged construct (*gbb-1xHA*) (Fig. 2B). We had previously discovered that insertion of green fluorescent protein (GFP) into Gbb renders the resulting fusion protein inactive, so we sought to verify that the HA-tagged *gbb* construct retained signaling activity to enable us to

conclude that protein products produced by this construct were functional signaling molecules (fig. S1). We found that Gbb with one HA epitope tag (Gbb-1xHA) retained considerable signaling activity, whereas the insertion of three HA tags (Gbb-3xHA) destroyed signaling activity.

As expected, the S1 mutant (*gbb^{mS1}*) resulted in the loss of the 15-kD band, henceforth referred to as Gbb15 (Fig. 2). This also occurred in wing discs from transgenic lines expressing *gbb^{mS1}* (lane 3, Fig. 2C). Extracts from wing discs expressing the NS mutant *gbb^{mNS}* lacked the higher-molecular mass 42-kD band, indicating that the 42-kD product likely results from proconvertase processing at the NS site (lane 2, Fig. 2C). The predicted size of a Gbb product produced by cleavage at NS is 38 kD; thus, we refer to the NS cleavage product as Gbb38 despite its migration at a slightly higher molecular mass, which may be due to post-translational modifications. In wing discs expressing *gbb^{mNS}*, Gbb15 is still present (as detected by a long exposure) albeit at a considerably lower amount (Fig. 2C), which may suggest that NS cleavage is important for Gbb15 production or stability. However, we observed the opposite phenomenon in S2 cells (see below; Fig. 2D).

The high signaling activity exhibited by *gbb^{mS1}* (Fig. 1C) prompted us to investigate whether Gbb38 was secreted as would be expected of a functional BMP ligand. Gbb38 was efficiently secreted from S2 cells expressing *gbb^{mS1}* (lane 4, Fig. 2D) and associated with the Sax type I receptor (fig. S2). As expected, no Gbb15 was apparent in the *gbb^{mS1}* media, although a small quantity of a 14-kD protein was detected in the media. The 14-kD protein appeared to be inactive because cells expressing the double mutant *gbb^{mNSmS1}*, which contains the 14-kD band (lane 5, Fig. 2D), showed no signaling activity (Fig. 1C). Furthermore, the HA antibody did not detect a 14-kD product from the HA-tagged *gbb* construct, indicating that the 14-kD band was derived from a nonfunctional portion of the ligand domain because it must lack the first conserved cysteine residue. Gbb15 was secreted into the media by *gbb*-, *gbb^{mNS}*-, and *gbb^{mS2}*-expressing cells (lanes 1 to 3, Fig. 2D) and, as expected, bound to the Sax receptor (fig. S2) (33). The detection of Gbb38 in S2 cell extracts expressing wild-type *gbb* varied somewhat; however, it was reproducibly observed in tissue or whole-animal extracts (Fig. 2, A and C), suggesting that some factor(s) affecting its stability could be lacking in S2 cells. For example, we detected Gbb38 in several tissues dissected from third instar larvae, with more present in wing discs and fat body (Fig. 2G). The relative amounts of Gbb38 to Gbb15 varied between tissues, possibly reflecting site-specific processing in different tissues or differences in ligand stability. Together, our results indicate that Gbb38 is produced by processing at the NS site to yield a bona fide BMP ligand that elicits robust signaling.

Several other points of interest were also revealed by our biochemical analysis. First, the amount of ligand secreted into the media did not directly correlate with the amount of signaling induced by a given construct. For example, most of the large quantity of Gbb15 produced by *gbb^{mNS}* was secreted into the media (lane 2, Fig. 2, D and F); however, *gbb^{mNS}* exhibited significantly reduced signaling activity (Fig. 1C). Second, although *gbb^{mS2}* cells produced more total Gbb15 than wild-type *gbb* (lane 3, Fig. 2, D and F), the amount of secreted Gbb15 was comparable to that secreted from wild-type *gbb*-expressing cells (lane 1, Fig. 2, D and F). No aberrant Gbb products were detected in *gbb^{mS2}* cells consistent with their wild-type signaling activity (Fig. 1C). Thus, the Gbb15 secreted by *gbb^{mS2}*-expressing cells appears to be more efficient at inducing signaling than that produced by *gbb^{mNS}*-expressing cells. Finally, when both NS and S1 sites were mutated (*gbb^{mNSmS1}*), the Gbb proprotein was secreted from cells (lane 5, Fig. 2D). Unexpectedly, we observed that proGbb could bind the Sax receptor (fig. S2) despite its inability to elicit signaling (Fig. 1C). Unlike the recent proposal that the structural “straitjacket” motif of the TGF- β 1 prodomain blocks ligand-receptor binding (34), our data indicate that the presence of the prodomain in

proGbb does not unequivocally disrupt receptor binding. Nevertheless, in wild-type cells and tissues, little to no proGbb is secreted, and therefore, we would not expect the unprocessed wild-type Gbb proprotein to occupy receptors.

The NS processing site is evolutionarily conserved and appears to be required for human development

The presence of an NS proconvertase consensus sequence is not unique to *Drosophila* Gbb. We found that a proconvertase consensus sequence is present within the N terminus of the prodomains of many invertebrate and vertebrate TGF- β and BMP family members (Fig. 3A) from *Nematostella* to humans. (We have arbitrarily defined an NS site as one that would produce a protein larger than 200 amino acids including the highly conserved C-terminal domain.) In many cases, the NS site is conserved among orthologs (Fig. 3A, gray shading). The prevalence of NS sites suggests that alternative processing of TGF- β and BMP proproteins could be functionally conserved. Indeed, we found that point mutations in three human TGF- β superfamily members, AMH, hBMP15, and hBMP4, from patients with persistent Müllerian duct syndrome, premature ovarian failure, and CLP, respectively (26–28), specifically abolish an NS furin consensus sequence (Fig. 3B). The developmental abnormalities associated with these NS mutations suggest that the wild-type function of a particular TGF- β superfamily ligand for a specific developmental function, such as palate formation, could be compromised in the absence of NS cleavage. To test for a loss of signaling activity associated with an NS mutation in hBMP4, we assayed for the BMP signaling reporter *BRE-luc* in transiently transfected human embryonic kidney (HEK) 293 cells. Although we did not detect a significant reduction in signaling due to mutations in the hBMP4 NS site, either with hBMP4-R162Q (which is associated with CLP) or with hBMP4-R162G, R165G, we found that signaling activity was significantly reduced with the S1 site mutation (hBMP4-R289G, R292G) (fig. S3). Unexpectedly, we found that a mutant S2 site (hBMP4-R253G, R256G) did not show a loss in signaling. Given that processing at the S2 site of mouse BMP4 is critical for tissue-specific functions in vivo, in that both heterozygous and homozygous *BMP4^{mS2}* mutants show reduced numbers of primordial germ cells (11), it is conceivable that we cannot detect an effect on signaling activity due to mutations at the NS or S2 sites in HEK293 cells as a result of high autocrine BMP signaling (35).

Cleavage mutants exhibit distinct signaling activities in vivo

The inability of the *gbb^{mNSmS1}* double mutant to provide functional signaling was borne out by the failure of a genomic *gbb* rescue construct with mutations in both the NS and the S1 sites (*gbbR-gbb^{mNSmS1}*) to rescue the lethality associated with a *gbb* null, *gbb^l* (36). Whereas a wild-type *gbb* genomic construct (*gbbR-gbb*) restored viability with 99% of *gbbR-gbb gbb^l/gbbR-gbb gbb^l* flies surviving ($n = 447$), 0% of the *gbbR-gbb^{mNSmS1} gbb^l/gbbR-gbb^{mNSmS1} gbb^l* class survived ($n = 363$). Other cleavage mutants yielded survivors, with 60% of *gbbR-gbb^{mNS} gbb^l*, 7.4% of *gbbR-gbb^{mS1} gbb^l*, and 0% of *gbbR-gbb gbb^l* homozygotes showing mild wing defects associated with abnormal BMP signaling (fig. S4). The higher percentage of *gbbR-gbb^{mNS} gbb^l* flies exhibiting defective wings is consistent with the lower signaling activity of *gbb^{mNS}* compared to that of *gbb^{mS1}* (Fig. 1C).

We next assessed the expression of *gbb* cleavage mutants in different tissue domains. Transgenic lines were generated such that each *gbb* cleavage mutant construct (like each *gbb* genomic rescue construct) was inserted into the same genomic position with the ϕ C31 system (37) to eliminate variation in expression between lines due to positional effects. We first tested the impact of misexpressing each cleavage mutant with different Gal4 drivers on adult viability (*hhGal4*, *ciGal4*, *apGal4*, *dppGal4*; Table 1). Inappropriate BMP signaling during development is often associated with lethality, and viability resulting from *gbb*

cleavage mutant misexpression (Table 1) inversely correlated with the different signaling activities attributed to each mutant as delineated in the S2 cell-based signaling assay (Fig. 1C). Those mutants exhibiting the highest signaling showed the lowest viability, such that wild-type *gbb* and *gbb^{mS2}* expressed under the control of *apGal4* failed to produce adult flies, whereas misexpression of the signaling-inactive *gbb^{mNSmS1}* mutant did not adversely affect viability and produced 100% of the expected number of adult progeny (Table 1). Similar trends from most to least viable (*gbb>gbb^{mS1}>gbb^{mNS}>gbb^{mNSmS1}*) were seen with the other Gal4 drivers. Misexpression of *gbb* cleavage mutants in the *dpp* expression domain was the exception (*dppGal4>UAS-gbb-CM*); for example, the expression of the double cleavage mutant (*UAS-gbb^{mNSmS1}*) was completely lethal (Table 1). Like other TGF- β and BMP cleavage mutants (38–44), we found that the double mutant *gbb^{mNSmS1}* indeed affected BMP signaling in a dominant-negative fashion. Phosphorylation of Mad was reduced compared to that of the control (*dppGal4>UAS-GFP*) when *gbb^{mNSmS1}* was specifically expressed in *dpp*-expressing cells (*dppGal4>UAS-gbb^{mNSmS1}*) (fig. S5, A to C). Pharynxes (*dppGal4>UAS-gbb^{mNSmS1}*) dissected out of their pupal cases showed macrochaetae and leg defects consistent with a reduction in BMP signaling (36, 45, 46) (table S1). We also found a cell-autonomous reduction in phosphorylated Mad when random clones overexpressing *gbb^{mNSmS1}* fell within the medial region of the wing disc (fig. S5, D and E). Defects associated with expression of uncleavable Gbb (*gbb^{mNSmS1}*; proGbb) could reflect either its ability to form a nonproductive dimer with endogenous wild-type Dpp and Gbb, which would be expected to interfere with ligand processing or secretion, or its inability to elicit signaling if it is secreted and bound to receptors (see Figs. 1C and 2D).

On the basis of the viability studies, we examined in greater detail the phenotypic consequences of expression of Gbb and the noncleavable mutant in the wings of *apGal4>gbb-CM* adult survivors and in the larval wing imaginal discs in which misexpression was directed to the dorsal compartment (*apGal4*; Fig. 4). Both *apGal4>UAS-gbb^{mS1}* and *apGal4>UAS-gbb^{mNS}* were semilethal, with *gbb^{mS1}* being more lethal, consistent with its higher signaling activity in S2 cells (Fig. 1C and Table 1). Adult wings from wild-type flies showed a stereotyped pattern with five longitudinal veins and two cross veins (Fig. 4A). Wings from adult survivors expressing the cleavage mutants showed blistered and ectopic venation phenotypes commensurate with the magnitude of ectopic signaling elicited by each cleavage mutant (Fig. 1C), with *apGal4>UAS-gbb^{mS1}* (Fig. 4B) showing the most severe wing defects of the viable progeny, followed by *apGal4>UAS-gbb^{mNS}* and *apGal4>UAS-gbb^{mNSmS1}* (Fig. 4, C and D). No adult survivors were found from *apGal4>UAS-gbb* or *apGal4>UAS-gbb^{mS2}* (Table 1).

In the wing imaginal disc, ectopic BMP signaling associated with *gbb* cleavage mutant misexpression was visible as an increase in the phosphorylation of Mad, especially in the lateral wing pouch, hinge, and notum regions of wing discs compared to the wild-type endogenous pattern of phosphorylated Mad (Fig. 4, E to P). The intensity of phosphorylated Mad, which reflects the BMP morphogen activity gradient in the wing pouch (47), was measured from the dorsal (the site of *apGal4* expression) and the ventral compartment of multiple discs for each genotype (Fig. 4, H to J and N to P). Wing imaginal discs dissected from *apGal4>UAS-gbb* and *apGal4>UAS-gbb^{mS2}* larvae showed extensive phosphorylation of Mad throughout the entire disc (Fig. 4, F, G, I, and J), indicating that ligands produced by *gbb* and *gbb^{mS2}* in dorsal cells can act nonautonomously to activate BMP signaling in cells far from where they are expressed. In addition, *apGal4>UAS-gbb* and *apGal4>UAS-gbb^{mS2}* wing discs were overgrown, a phenotype consistent with increased BMP signaling (Fig. 4, F and G). The intensity of the phosphorylated Mad signal in the notum and hinge of *apGal4>UAS-gbb* and *apGal4>UAS-gbb^{mS2}* discs was higher than that observed in the wing pouch, indicating that different regions of the disc respond differently to increased ligand concentration. Although ectopic phosphorylated Mad was evident in the lateral wing pouch

when *gbb* and *gbb^{mS2}* were overexpressed, the absolute amount of phosphorylated Mad in the medial wing pouch was reduced compared to that measured in control discs (Fig. 4, H to J), although the characteristic two peaks in the phosphorylated Mad gradient remained (47). Wing discs from *apGal4>UAS-gbb^{mS1}* larvae similarly exhibited a high degree of over-proliferation, but curiously, the wing pouch of this genotype was not expanded (Fig. 4K). The gradient of phosphorylated Mad in the dorsal compartment of larvae expressing *apGal4>UAS-gbb^{mS1}* extended into the lateral domains similar to larvae expressing *apGal4>UAS-gbb* and *apGal4>UAS-gbb^{mS2}*. However, this ectopic phosphorylated Mad signal was not apparent in the ventral compartment (Fig. 4N). Both the amplitude and the breadth of the phosphorylated Mad gradient in the ventral compartment of *apGal4>UAS-gbb^{mS1}* discs were reduced, indicating that although Gbb38 produced by the *gbb^{mS1}* mutant can promote signaling in dorsal lateral cells, signaling is affected differently than in the ventral compartment. Similar to *apGal4>UAS-gbb* and *apGal4>UAS-gbb^{mS2}* wing imaginal discs, stronger ectopic phosphorylated Mad signals were detected in the hinge and notum compared to the wing pouch in *apGal4>UAS-gbb^{mS1}* discs (Fig. 4, K and N), again revealing a region-specific response. Consistent with its ability to induce signaling, albeit at a reduced amount, the notum and hinge regions of *apGal4>UAS-gbb^{mNS}* wing discs showed a moderate increase in phosphorylated Mad, but within the wing pouch, the gradient of phosphorylated Mad was narrower, and the intensity of phosphorylated Mad was reduced in the dorsal compartment (Fig. 4, L and O). The double cleavage mutant *apGal4>UAS-gbb^{mNSmS1}* resulted in an overall reduction in the size of the wing disc, and as expected from the cell-based signaling assay (Fig. 1C), this construct did not induce ectopic phosphorylated Mad at other sites in the disc such as the notum or hinge domains (Fig. 4, M and P). The decrease in cell-autonomous phosphorylated Mad in the dorsal wing pouch of these discs was consistent with the expectation that an uncleavable form of Gbb would behave dominantly to disrupt the function of endogenous BMPs (Dpp and Gbb) or the Sax receptor, all of which are critical for signaling in the medial wing pouch (5, 18, 19). Strikingly, despite reduced phosphorylation of Mad in the dorsal wing pouch (Fig. 4, M and P), adult *apGal4>UAS-gbb^{mNSmS1}* wings (Fig. 4D) showed minimal patterning defects, suggesting that loss of BMP signaling in the dorsal cells of the larval wing disc could be compensated for by events during the pupal stage to generate a relatively normal wing pattern.

Signaling activity of cleavage mutants is controlled in a domain-specific manner

We next considered the effects of the Gbb cleavage mutants when expressed in the posterior (*hhGal4*) and anterior (*ciGal4*) compartments (Fig. 5, A to P) on BMP signaling by visualizing the distribution of phosphorylated Mad. We found that the expression of wild-type *gbb* led to ectopic phosphorylated Mad both within and outside *gbb*-expressing cells (Fig. 5, A, B, and J). Similar to our observations in *apGal4>UAS-gbb* discs (Fig. 4, F and I), when *gbb* was expressed throughout either the anterior (Fig. 5J) or the posterior (Fig. 5B) compartments, BMP signaling was induced in all cells of the wing disc. As predicted by our previous studies (5, 18, 19), the ectopic signaling induced in *hhGal4>UAS-gbb*, when Gbb was produced by posterior compartment cells where Dpp is not present, indicates that Gbb homodimers can act over a long range. Consistent with both the signaling activity and the long-range activity of Gbb homodimers, the expression domain of BMP target gene *Spalt* (*Sal*) was expanded in both the posterior and the anterior compartments of the wing pouch in *hhGal4>UAS-gbb* wing discs compared to its endogenous pattern (Fig. 5, F and G).

We observed minor but reproducible differences in the ability of Gbb38 (*gbb^{mS1}*) and Gbb15 (*gbb^{mNS}*) to induce signaling in posterior cells when expressed in those cells (*hhGal4>UAS-CM*), as well as in the cells of the anterior compartment (Fig. 5, C to E). When *gbb^{mNS}* was expressed in posterior cells, the width of the phosphorylated Mad

gradient and the expression of Sal were slightly reduced (Fig. 5, D, E, and I). Although the narrowing in the phosphorylated Mad gradient could reflect a reduction in the size of the wing pouch given that the gradient of phosphorylated Mad scales with disc size (48, 49) and the average width of the *hhGal4>UAS-gbb^{mNS}* wing pouch was 0.77 times that of the *hhGal4>UAS-GFP* wing pouch ($n = 7$ discs, $P < 0.05$), the significant increase in the amplitude of the anterior peak of phosphorylated Mad suggested that Gbb15 is active and may alter the dynamics of signaling in this medial-most portion of the disc. The reduction in phosphorylated Mad in the lateral regions may indicate a dominant-negative effect. As we have observed previously, *dpp* and *gbb* genetically interact, and the balance of Dpp to Gbb is likely critical to the generation of the phosphorylated Mad gradient (5, 18, 19). When *gbb^{mNS}* is expressed in the posterior compartment, it is possible that its mild negative impact on signaling could result from a shift in the stoichiometry of Gbb15 to Gbb38, although further studies will be required to test this hypothesis.

When expressed in the anterior compartment, *gbb^{mS1}* induced ectopic signaling in the lateral cells of the anterior compartment as well as throughout the posterior compartment (Fig. 5, K and O). However, *gbb^{mNS}* induced increased signaling only in the lateral anterior cells (Fig. 5, L and P). As summarized in Fig. 5M, wild-type Gbb (Gbb38 and Gbb15) induced phosphorylated Mad in cells far from where it was produced, whereas Gbb38 induced ectopic phosphorylated Mad at a reduced amount and in a more selective pattern. In contrast, Gbb15 did not exhibit the ability to induce signaling outside of the anterior compartment where it was produced (*ciGal4>UAS-gbb^{mNS}*); furthermore, it induced minimal ectopic phosphorylated Mad outside of the notum region. The narrow signaling range of Gbb15 and the long-range signaling ability of both Gbb38 and wild-type Gbb are further supported by the induction of ectopic phosphorylated Mad outside random clones expressing *gbb* (wild-type Gbb) and *gbb^{mS1}* (Gbb38) compared to *gbb^{mNS}* (Gbb15)–expressing clones (fig. S6, A to F). Together, all of our results from the domain-specific expression of *gbb* cleavage mutants indicate that (i) the response of cells varies with the region within the disc; (ii) Gbb38 can influence cells distant from where it is produced, but Gbb15 in general does not; (iii) the expression of wild-type *gbb* elicits a more pronounced, long-range influence on cells; and (iv) the phosphorylated Mad gradient within the medial wing pouch is resilient to increases in ligand abundance.

DISCUSSION

To date, active TGF- β and BMP family dimeric ligands are known to consist of two peptides processed from the C-terminal 100 to 110 amino acids of two larger proproteins. All ligands adopt a similar “inverted clasped hands” or “butterfly” fold because of the high conservation in this domain, especially of the hallmark cysteine residues. Here, we report the identification of a BMP ligand form containing the highly conserved C-terminal domain contiguous with a portion of the less conserved prodomain. This large ligand (Gbb38), one of two forms produced by *gbb*, arises from proconvertase processing at a previously unrecognized furin consensus sequence (NS) at residue 123 of the 455–amino acid Gbb proprotein. A second ligand, Gbb15, is produced by cleavage at the canonical S1 site located at residue 322, which is N-terminal to the conventional ligand domain. Both Gbb38 and Gbb15 are active, with Gbb38 exhibiting a higher level of signaling activity both in cell culture (Fig. 1C) and in vivo (Figs. 4 and 5 and fig. S6). The ability of Gbb38 and Gbb15 to signal is influenced by the tissue domain in which they are expressed. When expressed in either the dorsal or the anterior compartment of the wing imaginal disc, Gbb38 and Gbb15 induce ectopic signaling commensurate with their relative signaling abilities as measured in cell culture (Figs. 1C, 4, and 5). However, when expressed in the posterior compartment, their ability to elicit ectopic signaling is impaired (Fig. 5). This finding is particularly interesting in light of our previous observations that Gbb produced in the anterior

compartment behaves differently from that generated in the posterior compartment (5). On the basis of the fact that Dpp is produced solely in the anterior compartment, one could envision that the compartmental difference in the behavior of Gbb may be explained by the action of Dpp-Gbb heterodimers formed only in the anterior compartment. However, we found that Gbb38 induced signaling from clones lacking Dpp (fig. S6, C and D) and that Gbb15 induced a significant increase in the amplitude of the anterior phosphorylated Mad peak when expressed in the posterior compartment (Fig. 5, D and E). Furthermore, the expression of wild-type *gbb* (*hhGal4>UAS-gbb*) in the posterior compartment, which produces both Gbb38 and Gbb15 (Fig. 2C), induced considerable ectopic phosphorylated Mad in all cells of the disc in both posterior and anterior compartments (Fig. 5, B and E). Thus, the differential response to expression of *gbb* and *gbb* cleavage mutants in various wing disc domains cannot be explained solely by the presence of Dpp. Given that both Gbb38 and Gbb15 are produced by posterior cells (Fig. 2C), the factors responsible for this compartment-specific behavior are likely to be involved in the reception or integration of their independent signals.

In addition to compartment-specific behavior, we also found that Gbb38 promoted signaling in cells far from where it is produced (Fig. 5, K and O, and fig. S6, C and D), as did wild-type Gbb (Figs. 4, F and I, and 5, B, E, J, and N, and fig. S6, A and B). However, Gbb15 in general did not because it induced high signaling in the notum and hinge of the anterior compartment only when it was expressed in the anterior compartment (Fig. 5, L and P). Tissue-specific processing at the conventional linker domain cleavage sites can regulate the amount of BMP4 and Dpp ligand produced and thus influence signaling strength and ligand function in *Xenopus* and *Drosophila* (11, 13). In the case of the larger Gbb38 ligand, the presence of considerable additional “pro-domain” sequences not found in the conventional ligands adds a new set of parameters to be considered in understanding the mechanisms that regulate TGF- β signaling. The additional protein domains Gbb38 could allow for or block interactions with extracellular factors that regulate ligand movement, such as the heparan sulfate proteoglycans Dally and Dally-like protein (Dlp), Pentagone (Pent) and Larval Translucida (Ltl), and different combinations of receptors (50, 51). In this vein, the existence of an active large ligand form is of particular interest in light of the structure of the TGF- β 1 prodomain (34). Cleaved prod domains are thought to assist in intracellular ligand folding and secretion, but for some family members, the ligand remains noncovalently associated with its two prod domains after secretion. In most cases, this association confers latency (inactivity) as a result of structural changes, with ligand activation requiring proteolytic cleavage of the prod domain in the latent complex or a force-dependent mechanism through integrin interactions (such as is the case for TGF- β , BMP2, and GDF8) (34, 52–55). The prod domain-BMP7 complex is not latent, and the prod domains are displaced upon ligand binding to the BMP type II receptor (56). In this case, ligand activation does not require extracellular matrix molecules or proteases, an observation that may hold true for AMH as well (56, 57). Despite the apparent differences in TGF- β and BMP family members and their extracellular associations with their prod domains, structural studies indicate that prod domains in TGF- β and BMP family ligands are expected to share the same cysteine knot fold (58). In the case of TGF- β 1, the prod domain dimer envelops the C-terminally derived ligand dimer to prevent ligand activation, with a straitjacket motif created by the overlapping N-terminal 45 residues of the prod domain (Fig. 6). If processing occurs at the NS site in Gbb, the N-terminal 90 amino acids would be removed, eliminating the straitjacket domain and the “latency lasso” that encircles the residues critical for receptor binding. As our analyses have shown, the removal of this N-terminal-most domain must allow for receptor binding of the Gbb ligand without requiring any further processing and subsequent signaling. Indeed, the 73 amino acids at the extreme N terminus of the GDF8 prod domain confer its inhibitory effect over GDF8 (53). Although the structural conformation of the large ligand is not yet known, it is conceivable that the butterfly structure of the C-terminally derived ligand domain will fold

on the basis of the conserved cysteine knot structure, with the receptor interaction faces unimpeded by the linker domain (Fig. 6). Given the more robust signaling elicited by Gbb38 than Gbb15 and the therapeutic uses of BMPs, it will be interesting to resolve the structure of a large ligand form.

Although we found that the signaling ability of cleavage mutants that exclusively produced Gbb38 (*gbb^{mS1}*) or Gbb15 (*gbb^{mNS}*) depended on the domain in which they were expressed, the expression of wild-type *gbb* led to increased BMP signaling in all cells of disc with the exception of the medial wing pouch, which appears to be under tight regulation. Although the phosphorylated Mad gradient in the medial wing pouch is sensitive to the loss of *gbb* and other signaling components, it is resistant to their increases most likely in part due to a negative feedback mechanism involving Dad, an inhibitory Smad (5, 59). In addition, the phosphorylated Mad gradient scales with disc size in the medial wing pouch (48, 49), an effect that we did not observe in other regions of the disc upon overexpression of *gbb* or the *gbb* cleavage mutants. Furthermore, we found that scaling was disrupted by wild-type *gbb* expression, which produced an overgrown disc with relatively minor changes to the phosphorylated Mad gradient (Figs. 4 and 5 and fig. S6). Future studies will be important to resolve the molecular basis of these domain-specific signaling responses and the inability of either Gbb38 or Gbb15 to recapitulate the effects of wild-type *gbb*, but it is clear that the different Gbb ligands exhibit distinct effects.

The inability of either Gbb38 or Gbb15 to recapitulate wild-type *gbb* when expressed under endogenous *gbb* control (fig. S4) indicates that to attain full Gbb signaling, processing must be possible at both the S1 and the NS sites. The simplest explanation for this observation is that wild-type signaling requires the presence of both Gbb38 and Gbb15 ligands either as homodimers or as Gbb38:Gbb15 heterodimers. An alternative explanation is that to produce a canonical active Gbb ligand (presumably Gbb15), processing must occur at both sites either sequentially or simultaneously. Our data do not favor the latter alternative because Gbb38 alone exhibits robust signaling and both forms are present endogenously with differential enrichment in various tissues, suggesting that the cell type or tissue environment may influence proGbb processing (Fig. 2G). Instead, we envision that the production, maturation, and stability of the array of active ligands under normal conditions depends on the presence of both NS and S1 cleavage sites and that the reception and integration of the various combinations of ligands occurs in a domain-specific manner.

The differential requirement for different sets of ligands could explain the apparent specificity of the NS mutation in hBMP4 (hBMP4-R162Q) associated with CLP (28). The associated failure in the fusion of the medial nasal and maxillary processes during orofacial development is a fairly specific temporal and spatial disruption of hBMP4 signaling. BMP4 is required for mouse lip and palate fusion (60) as well as many other fundamental roles in early development including axis determination and the specification of different germ layers (3, 61, 62). Given that BMP4 is essential for embryonic development, this relatively minor defect suggests that either processing at the NS site of hBMP4 occurs only in a specific set of cells, at a specific time in development, and individuals carrying the *hBMP4^{R162Q}* allele fail to produce the proper hBMP4 ligand type(s) necessary to elicit the proper magnitude of BMP signaling required for lip and palate fusions, or compensatory mechanisms, such as BMP redundancy and feedback regulation, restore BMP signaling activity to its normal magnitude in other tissues of patients with CLP. *Bmp4^{S2G/S2G}* mice also survive to adulthood with severe defects in only some tissues, such as the testes, with other tissues or organs that require BMP4 signaling, such as the limb and kidney, developing normally (11). It is conceivable that ligand produced by cleavage at NS and S2 sites does not affect the magnitude of signaling but rather the signaling between specific sets of cells. Consistent with this possibility, we found that although the S1 site is required for hBMP4

signaling, we could not detect a difference in the amount of signaling induced by mutations in S2 and NS, as well as hBMP4-R162Q in transfected cells (fig. S3), where the production or reception of a putative large hBMP4 ligand form may not occur, especially over the basal extent of autocrine signaling. Instead, it will be of greater interest to test for the effect of the CLP mutation hBMP4-R162Q on BMP signaling specifically in the orofacial primordia because this tissue may be most sensitive to a large form of the hBMP4 ligand or a prodomain-ligand complex with a shorter prodomain segment. BMP4 plays an instrumental role in the morphological differences in the beaks of Darwin's finches, as well as in jaw development of the rapidly evolving species of cichlid fishes occupying Lakes Victoria, Malawi, and Tanganyika of the East African Rift valley (63–65). Although the existence of a large BMP4 ligand has yet to be determined, it is intriguing to consider that among the East African THMV (Tropheini, Haplochromine, Lake Malawi, and Lake Victoria) group of cichlids, the prodomain of BMP4 contains a high number of amino acid substitutions not seen in the signal peptide or ligand domain (66). Either the regulatory function of the prodomain in a BMP4 latent complex could be critical for modulating ligand activity during jaw development or amino acid changes in a putative large BMP4 ligand could alter its signaling abilities in orofacial development of cichlids.

In conclusion, our studies have identified a proconvertase cleavage site that is essential for the full activity of the *Drosophila* BMP5/6/7 ortholog Gbb, as well as for that of several human BMPs. Given the evolutionary and functional conservation of alternative processing in the N-terminal region of the proprotein, it is of interest to note that despite lower sequence conservation between BMP prodomains, they are thought to share a folded core domain (58). TGF- β and BMP family members that are processed to yield both large and small ligand forms may exhibit an expanded range of signaling activities because of differences in ligand structural motifs or interaction faces. Thus, the posttranslational regulation of BMP ligands is more intricately controlled than previously anticipated. A role for proconvertase processing as a mechanism to control both quantity and quality of ligand production is now more strongly supported by the identification of the NS cleavage site critical for signaling activity, by its evolutionary conservation, and by the fact that mutations in this site appear to disrupt human development (Fig. 3). Our results raise the possibility that context-dependent behaviors reported for various TGF- β and BMP ligands could be explained by the action of such alternate ligand forms.

MATERIALS AND METHODS

Drosophila strains

All *Drosophila* strains have been described previously (31, 36) or are in FlyBase and were obtained from Bloomington Stock Center (67). Crosses were performed at 25°C on standard sugar, cornmeal, and yeast extract media. *UAS-gbb* and *UAS-gbb-CM* were inserted at chromosomal location 86Fb with the ϕ C31 system (37). The null allele *gbb^l* was recombined onto chromosomes bearing *gbbR-gbb* or *gbbR-gbb-CM* insertions at the ϕ C31 landing site at 53B2. The presence of *gbb^l* in the resulting *gbbR-gbb* gbb^l* lines was verified by sequence analysis. Random clones expressing *gbb*, *gbb^{mS1}*, and *gbb^{mNS}* were generated by the FLPout system (68, 69) in *hsFLP/+; AyGal4 UAS-GFP/+; UAS-gbb*/+* with 10 min heat shock at 37°C.

DNA constructs

The Gateway system (Invitrogen) was used to clone most DNA constructs. *gbb* and *gbb* cleavage mutants (*gbb-CMs*) were generated by first amplifying the *gbb* coding sequence (cds) with the entire 5' untranslated region (UTR) (70) by polymerase chain reaction (PCR) with primers containing the *attB1* sequence at the 5' and *attB2* at the 3' end (table S2). The

PCR product was cloned into *pDONR221* (Invitrogen) with the BP reaction. Cleavage site mutations were generated in *pDONR221-gbb* by Quik-Change (Stratagene). After confirming the DNA sequence of *pDONR221* clones, the insertions were transferred into *pAW* [Drosophila Genomics Resource Center (DGRC), <http://dgrc.cgb.indiana.edu/>] by the LR reaction to generate *pAW-gbb* and the various *pAW-gbbCMs* for *Drosophila* cell culture. *gbb* and *gbb-CM* were also amplified from *pDONR221* constructs by PCR with primers containing an Eco RI site at the 5' end and an Xba I site at the 3' end (table S2) and were cloned into *pUAST attB* to make *pUAS-attB-gbb* and *pUAS-attB-gbb-CMs* for generating transgenic lines with the ϕ C31 system (37). HA-tagged *gbb* constructs were generated by the introduction of either a single HA tag between Thr³⁵¹ and Arg³⁵² of the Gbb, Gbb^{mS1}, and Gbb^{mNSmS1} ligand domain by PCR. The amino acid sequence of the junction is EPMEST(351)-GG-1x HA-GG-R(352) SCQMQ. A 3xHA tag was obtained from *pBRacpA dpp 3xHA* (71) and introduced between Ser³⁵³ and Cys³⁵⁴ of the Gbb ligand domain by PCR, resulting in MESTRS(353)-GGG-3xHA-GGG-C(354)QMQL. A C-terminally GFP-tagged *gbb* with *attB1* and *attB2* sites was generated by PCR with *pUAST-gbb-GFP* (72) as a template DNA. All PCR products were cloned into *pAW* (DGRC) vector with the Invitrogen Gateway system as described above.

We had previously shown that a 6.8-kb Sal I genomic fragment contains all the necessary information for *gbb* expression and function during development (36). To generate a series of *gbb* rescue constructs (*gbbR*), we generated a genomic subclone lacking only the *gbb* coding sequences. First, a Not I site was engineered in place of the *gbb* coding region by PCR amplification of a 3.8-kb upstream (A) and 2.0-kb downstream (B) genomic region with the following primers: 5'-GCGACGAGCTCCTCTAGAACGTCTGGCTGGGATGGC-3' (sense primer for A with Sac I-Xba I sites), 5'-CAGGCGGCCGCTTTGGACGGATCGGTTGC-3' (antisense primer for A with Not I site), 5'-CAGGCGGCCGCTTTGGACGGATCGGTTGC-3' (sense primer for B with Not I site), and 5'-GGACCTGGGTACCATCTAGATCCAGAACAGCATC-3' (antisense primer for B with Xba I-Kpn I sites). A and B fragments were cloned into Sac I/Not I and Not I/Kpn I sites of *pBluescript II SK⁺*, resulting in *pBS gbb AB*. The AB fragment was excised with Xba I and subcloned into *pattB* (<http://flyc31.frontiers-in-genetics.org/index.php>) with the multiple cloning Not I site destroyed. Finally, the wild-type *gbb* or various *gbb* cleavage mutant sequences generated in *pDONR221-gbb* were transferred into *pattB-gbb-AB* with Not I to generate *gbbR-gbb* or *gbbR-gbb-CMs*.

A Flag-tagged form of *sax* was generated with *pAWF* (DGRC; an S2 cell expression vector with C-terminal 3 × Flag). The *sax-RA* (FlyBase, <http://flybase.org/>) transcript without the stop codon was amplified by PCR and cloned into *pAWF*. The coding sequence of human *Bmp4* (*hbmp4*) was cloned with a 5' primer containing *attB1*-Hind III-Kozak sequences and a 3' primer with Xho I-*attB2* sequences (table S2) to generate *pDONR221-hbmp4*. Mutations in the hBMP4 furin cleavage consensus sequences and the R162Q mutation were generated in *pDONR221-hbmp4*. Each insert was transferred into *pcDNA3* (Invitrogen) for mammalian cell culture with digested *hbmp4* Hind III and Xho I.

Cell-based signaling assays

BMP signaling assays were performed in *Drosophila* S2 cells or in HEK293 cells as described previously (29, 31, 73, 74). In brief, the S2 signaling assay uses a *lacZ* reporter construct under the control of Notch activation through Su(H) binding sites and BMP signaling-mediated repression through the brkSE (silencer element). Direct binding of phosphorylated Mad-Med-Shn to the brkSE and a quantitative response to BMP signaling have been demonstrated previously (29, 31). S2 cells were transfected (Effectene, Qiagen) with 100 ng of *gbb* and *gbb-CM* constructs and incubated at 25°C for 60 to 72 hours before lysis ($\sim 3 \times 10^6$ cells) to measure β -galactosidase activity. β -Galactosidase activity was

measured with the Dual-Light system (Applied Biosystems). Differences in signaling activity between wild-type *gbb*, *gbb*-CMs, and control were tested for significance with the Tukey-Kramer HSD (honestly significant difference) procedure and Hsu's MCB (multiple comparisons with the best mean) test for multiple comparisons at $P < 0.05$.

In HEK293 cells, the Id1 promoter luciferase reporter (BRE-luciferase) and simian virus 40 (SV40)-*Renilla* luciferase were used to examine BMP signaling activity and transfection efficiency, respectively. To measure BMP signaling activities of each hBMP4-CM and hBMP4^{R162Q}, we transiently transfected 293 cells with 1.1 μ g of each hBMP4 construct and 250 ng of BRE-luciferase and SV40-*Renilla* luciferase. After 48 hours of incubation, cells were lysed and BMP signaling activities were measured with the Dual-Luciferase Reporter Assay System (Promega).

Sample preparation, coimmunoprecipitation, and Western analysis

S2 cells transfected with 2 μ g of *pAW gbb* or *pAW gbb*-CM were grown for 4 days at 25°C. Either the entire cell culture was recovered and used as "total fraction" or it was centrifuged at 2000 rpm for 5 min at room temperature to separate the pellet ("cell fraction") and supernatant fractions. The supernatant fraction was recentrifuged at 13,000 rpm for 5 min at room temperature to completely remove debris and used as "conditioned media fraction." To assess BMP signaling by measuring phosphorylated Mad, we transfected S2 cells with *pAc-Mad-Flag* (a gift of M. O'Connor) and incubated with either 1 $\times$ or 1/10 $\times$ conditioned medium (prepared as described above) from *pAc*-, *pAW-gbb*-, and *gbb^{MS1}*-transfected S2 cells for 3 hours at room temperature. After incubation, cells were collected by centrifugation at 2000 rpm for 5 min at room temperature and were lysed in SDS sample buffer. *yw*, *gbb¹/gbb²*, and *gbb¹/gbb³* third instar larvae or dissected tissues were homogenized in SDS sample buffer.

Protein samples were analyzed by Western blot with ECL-Plus (GE Healthcare Life Sciences). Monoclonal antibody 3D6-24 against Gbb peptide 424-440 was generated at the UAB Epitope Recognition and Immunoreagent Core Facility. Anti-Gbb (1:1000), anti-actin (1:10,000; Millipore), anti-Flag (M2, 1:2000, Sigma), anti-pSmad3 [EP823Y, 1:2000, Epitomics (51)], and horseradish peroxidase-conjugated secondary antibodies (1:10,000; Jackson ImmunoResearch) were used for Western blot analyses.

S2 cells were transfected with 1 μ g of *pAW gbb*-HA, *pAW gbb^{MS1}*-HA, or *pAW gbb^{MS1}SmS1*-HA and 500 ng of *pAWF-sax* and grown for 4 days at 25°C. Cultures were solubilized in PBST (phosphate-buffered saline containing 1% Triton X-100, pH 7.0) and incubated with Dynabeads proteins G (Invitrogen)-conjugated mouse anti-Flag antibody (M2, Sigma) for 20 hours at 4°C with rotation. Proteins bound to Flag antibody were washed with TBST (50 mM Tris-HCl, 150 mM NaCl, and 0.2% Triton X-100, pH 7.4) and eluted with SDS sample buffer. Then, protein samples were analyzed by Western blots with mouse anti-Flag (1:1000, M2, Sigma) and rat anti-HA antibodies (1:1000, 12CA5, Roche).

Immunohistochemistry

Immunostaining of wing discs was conducted as described previously (17) with anti-pSmad3 [EP823Y, 1:1,000, Epitomics (51)], anti-Sal [1:200 (75)] and Alexa Fluor secondary antibodies (1:500, Invitrogen). Images of wing discs were collected on either a Leica SP2 or a Zeiss 510 Meta confocal microscope as Z stacks of 1- μ m sections. For each experiment, the same confocal settings were used during image capture. Except where noted, fluorescence intensity values were obtained from a fixed segment across the dorsal wing pouch (20% offset from the dorsal-ventral boundary) of six SUMed confocal slices

with Plot Profile (Fiji). The average intensity profile was plotted from more than three discs with the SE.

Sequence and phylogenetic analysis

Potential proconvertase cleavage sites were predicted with ProP (76). Phylogeny.fr (<http://www.phylogeny.fr/version2.cgi/index.cgi>) was used to build a phylogenetic tree. BMP and TGF- β protein sequences were aligned with MUSCLE. The tree was calculated with the maximum likelihood method of PhyML with WAG substitution model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Furin cleavage site mutations differentially affect Gbb signaling activity. **(A)** Schematic of Gbb pre-proprotein with amino acid (aa) position of furin cleavage consensus sequences. NS, S2, and S1 and disrupting mutations are noted. SP, signal peptide. **(B)** Schematic representation of cell-based BMP signaling assay. *brkSE-lacZ* reporter is activated in response to active Notch signaling (N*) through Su(H) binding sites (BS). Repression of *brkSE-lacZ* is a direct response to active BMP signaling (BMP*) resulting from binding of the *Drosophila* BMP signal transducer pMad-Med-Shn complex to brkSE. **(C)** Signaling activity induced by *gbb* cleavage mutants (*gbb*-CMs). In the absence of Gbb-induced signaling (–), β-galactosidase activity is set to 100%. Repression of *brkSE-lacZ* in response to BMP signaling is measured as reduction in β-gal activity. Horizontal lines indicate significance at $P < 0.05$ with the Tukey-Kramer HSD procedure for multiple comparisons. *gbb^{mS1}* is marginally different from *gbb* (dotted horizontal line) on the basis of its significant difference using Hsu's MCB test but not at $P < 0.05$ with Tukey-Kramer. Each bar represents the average of three independent experiments; error bars indicate SE. **(D)** Induction of phosphorylated Mad (pMad) measured in S2 cells transfected with Mad-Flag

and treated with 0.1× and 1× conditioned media from S2 cells expressing *gbb*, *gbb^{ms1}*, or empty vector (pAc).

Fig. 2.

Large and small ligand forms are produced by differential processing of Gbb. **(A)** Extracts from *gbb* null (*gbb*¹/*gbb*² and *gbb*¹/*gbb*³) and wild-type (+/+) third instar larvae (left) and S2 cells (right) probed for cross-reactivity to a Gbb monoclonal antibody (α -Gbb). In addition to Gbb proprotein (proGbb) and expected ligand (Gbb15), a 42-kD protein specific to Gbb was detected (arrow). Anti-actin (α -Actin) served as a loading control. **(B)** Extracts from *gbb*-1xHA- and *gbb*^{mS1}-1xHA-transfected S2 cells probed with anti-HA (α -HA). Gbb15 product is absent from *gbb*^{mS1}-1xHA extracts. **(C)** Extracts from wing discs expressing *gbb*, *gbb*^{mNS}, and *gbb*^{mS1} under the control of *hhGal4* probed with α -Gbb and α -actin. The 42-kD product is lost when the NS cleavage site is mutated (*gbb*^{mNS}). Predicted size of a product produced by processing at NS is 38 kD. Lower part of blot was exposed for 15 times longer to reveal Gbb15 in *gbb*^{mNS} extracts and its absence in *gbb*^{mS1} extracts. **(D to F)** Extracts from S2 cells transfected with wild-type *gbb* and *gbb*-CM constructs analyzed for Gbb products. Media (D) and cell (E) fractions indicate secreted and cellular products,

respectively. Total extracts (F). The abundance of endogenous Gbb38 and Gbb15 differs between different tissues from isolated third instar larval tissues, including brain (B), salivary glands (SG), fat body (FB), and wing discs (WD), which were probed with α -Gbb and α -actin (G). Actin is the loading control. The bottom panel of each Western blot was exposed twice as long as the top panel to visualize the lower-molecular weight products.

Fig. 3. NS cleavage sites are conserved in the TGF- β superfamily. **(A)** Phylogenetic tree of TGF- β family members from bilateria [*Drosophila melanogaster* (Dm, protostome), *Strongylocentrotus purpuratus* (Sp), and *Homo sapiens* (Hs; deuterostomes)] and radiata [*Nematostella vectensis* (Nv)]. Shaded proteins contain a putative NS cleavage site. **(B)** Specific mutations at NS sites in human BMP4, BMP15, and AMH proteins are associated with the indicated developmental abnormalities.

Fig. 4.

Differential response of wing disc cells to dorsal expression of *gbb* cleavage mutants. (A to D) Wings from (A) *apGal4>UAS-GFP*, (B) *apGal4>UAS-gbb^{mS1}*, (C) *apGal4>UAS-gbb^{mNS}*, and (D) *apGal4>UAS-gbb^{mNSmS1}* adults. Ectopic venation, abnormal wing size, and the improper apposition of the dorsal and ventral wing surfaces are phenotypes that correlate with altered BMP signaling. (E to P) Distribution of pMad (red) in third instar larval wing discs expressing *UAS-gbb* CMs with inset of same disc at 0.4× shows *UAS-GFP* (green) under the control of *apGal4* in the (E) dorsal compartment (d). v, ventral compartment; N, notum; H, hinge; WP, wing pouch. (H to J and N to P) pMad quantification [in arbitrary units (a.u.)] across the wing pouch (WP) in the dorsal (red) and ventral (black) compartments of multiple discs for each genotype as indicated by open boxes in (E). Tracing is the average intensity from multiple discs with SEs shown (vertical lines). *n* = number of discs. (F and I) *apGal4>UAS-GFP, UAS-gbb* (*n*=5); (G and J) *apGal4>UAS-GFP, UAS-gbb^{mS2}* (*n*=5); (K and N) *apGal4>UAS-GFP, UAS-gbb^{mNS}* (*n*=3); (L and O) *apGal4>UAS-GFP, UAS-gbb^{mS1}* (*n*=4); (M and P) *apGal4>UAS-GFP, UAS-gbb^{mNSmS1}* (*n*=4). Tracing of wild-type endogenous pMad gradient characterized by two peaks of higher pMad separated by a trough of lower accumulation is shown for comparison (blue, *n*=3) in (I) and (J) and (N) to (P). Asterisks (*) highlight regions of ectopic pMad in the hinge (H) and notum (N) regions of the wing disc. All images obtained at the same magnification under identical confocal microscope settings.

Fig. 5.

Long-range activity of Gbb38 is domain-dependent. BMP signaling [distribution of pMad (gray)] assessed in third instar larval wing discs expressing *UAS-gbb* and *UAS-gbb*-CMs in posterior (*hhGal4*) and anterior (*ciGal4*) compartments. Dorsal up, posterior (P), right. Insets of same disc at 0.4 $\times$ show distribution of pMad (red) and GFP (green). (A) Control wild-type disc, *hhGal4*>*UAS-GFP*. (B) *hhGal4*>*UAS-gbb*, *UAS-GFP*. (C) *hhGal4*> *UAS-gbb^{ms1}*, *UAS-GFP* (Gbb38). (D) *hhGal4*> *UAS-gbb^{msNS}*, *UAS-GFP* (Gbb15). All images collected at the same confocal settings. (E) pMad quantification in arbitrary units (a.u.) collected from dorsal wing pouch (open rectangle) from multiple discs. [*hhGal4*>*UAS-GFP* (black), *n* = 4; *hhGal4*> *UAS-gbb* (red), *n* = 6; *hhGal4*> *UAS-gbb^{ms1}* (blue), *n* = 4; *hhGal4*> *UAS-gbb^{msNS}* (purple), *n* = 4]. SE indicated. (F to I) pMad and Sal distribution in the wing pouch of *hhGal4*>*UAS-GFP* (F), *hhGal4*>*UAS-gbb* (G), *hhGal4*>*UAS-gbb^{ms1}* (H), and *hhGal4*>*UAS-gbb^{msNS}* (I) wing discs. Arrows in (G) denote expanded Sal expression. (J to M) *ciGal4*>*UAS-gbb*, *UAS-GFP* (J), *ciGal4*>*UAS-gbb^{ms1}*, *UAS-GFP* (Gbb38) (K), *ciGal4*>*UAS-gbb^{msNS}*, *UAS-GFP* (Gbb15) (L), schematic of sites of ectopic BMP signaling induced by *gbb^{ms1}* (Gbb38), *gbb^{msNS}* (Gbb15), and wild-type *gbb* (Gbb38 + Gbb15) when expressed in the anterior compartment (gray) (M). Portion of wing pouch refractory to *gbb* overexpression, red. (N to P) pMad quantification in arbitrary units (a.u.) collected from dorsal wing pouch. (N) *ciGal4*>*UAS-GFP* (black), *n* = 2; *ciGal4*>*UAS-gbb* (red), *n* = 3. (O) *ciGal4*>*UAS-GFP* (black), *n* = 2; *ciGal4*>*UAS-gbb^{ms1}* (blue), *n* = 3. (P) *ciGal4*> *UAS-gbb^{ms1}* (blue), *n* = 3; *ciGal4*>*UAS-gbb^{msNS}* (purple), *n* = 3. All images collected at the same confocal settings. (O and P) X marks anomaly in pMad tracing due to fold at edge of wing pouch [see also (K)]. *n* = number of discs.

Fig. 6. Model summarizing the signaling capacity and range of action for products produced by TGF- β and BMP family members. **(Top)** Linear schematic of TGF- β /BMP protein domains (prodomain, linker, and ligand domains), NS and linker cleavage sites (vertical lines, scissors), and cleavage products. **(Bottom)** Model schematic of structure of products based on Shi *et al.* (34) (color-coded to linear representations) with secretion, signaling activity, and range of action properties noted. No structure is known for the linker region, which is designated in the large ligand model.

Viable progeny resulting from misexpression of *gbb* and *gbb* cleavage mutants. Transgenes were integrated at chromosomal position 86Fb with the ϕ C31 system (37). *n* = total number of *GAL4/UAS* progeny (including viable adult flies and/or dead pupae).

Table 1

<i>GAL4</i> lines	Transgenic UAS lines				
	<i>gbb</i>	<i>gbb^{mS2}</i>	<i>gbb^{mSI}</i>	<i>gbb^{mNS}</i>	<i>gbb^{mNSmSI}</i>
<i>ap-GAL4</i>	0% (<i>n</i> = 57)	0% (<i>n</i> = 52)	14% (<i>n</i> = 45)	64% (<i>n</i> = 58)	100% (<i>n</i> = 31)
<i>hb-GAL4</i>	0% (<i>n</i> = 72)	0% (<i>n</i> = 86)	0% (<i>n</i> = 93)	18% (<i>n</i> = 66)	94% (<i>n</i> = 70)
<i>ci-GAL4</i>	0% (<i>n</i> = 40)	0% (<i>n</i> = 42)	0% (<i>n</i> = 52)	0% (<i>n</i> = 65)	3% (<i>n</i> = 67)
<i>dpp-GAL4</i>	39% (<i>n</i> = 107)	0% (<i>n</i> = 68)	97% (<i>n</i> = 117)	99% (<i>n</i> = 100)	0% (<i>n</i> = 172)