Alternative cleavage of the bone morphogenetic protein (BMP), Gbb, produces ligands with distinct developmental functions and receptor preferences

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The family of TGF-β and bone morphogenetic protein (BMP) signaling proteins has numerous developmental and physiological roles. They are made as proprotein dimers and then cleaved by proprotein convertases to release the C-terminal domain as an active ligand dimer. Multiple proteolytic processing sites in Glass bottom boat (Gbb), the Drosophila BMP7 ortholog, can produce distinct ligand forms. Cleavage at the S1 or atypical S0 site in Gbb produces Gbb15, the conventional small BMP ligand, whereas NS site cleavage produces a larger Gbb38 ligand. We hypothesized that the Gbb prodomain is involved not only in regulating the production of specific ligands but also their signaling output. We found that blocking NS cleavage increased association of the full-length prodomain with Gbb15, resulting in a concomitant decrease in signaling activity. Moreover, NS cleavage was required in vivo for Gbb-Decapentaplegic (Dpp) heterodimer-mediated wing vein patterning but not for Gbb15–Dpp heterodimer activity in cell culture. Gbb NS cleavage was also required for viability through its regulation of pupal ec dysis in a type II receptor W i shful thinking (Wit)-dependent manner. In fact, Gbb38-mediated signaling exhibits a preference for Wit over the other type II receptor Punt. Finally, we discovered that Gbb38 is produced when processing at the S1/S0 site is blocked by O-linked glycosylation in third instar larvae. Our findings demonstrate that BMP prodomain cleavage ensures that the mature ligand is not inhibited by the prodomain. Furthermore, alternative processing of BMP proproteins produces ligands that signal through different receptors and exhibit specific developmental functions.

Bone morphogenetic proteins (BMPs), members of the TGF-β family of signaling proteins, have numerous developmental and physiological roles (1–3). Like other TGF-β family members, BMPs are synthesized as large 400–500-amino acid proproteins that form dimers linked by a C-terminal disulfide bond (4). The 110–140-amino acid ligand domain is proteolytically cleaved from the C terminus by a proprotein convertase (PC) such as furin. After secretion, the C-terminally derived ligand dimer binds and activates a complex of type I and type II transmembrane serine/threonine kinase receptors. In the active complex, the type II receptor phosphorylates the type I receptor that in turn phosphorylates downstream receptor-mediated Smad (R-Smad) signal transducers that act as transcription factors. Although the vast majority of research in the field has focused on the activity of the ligand, there is a growing appreciation of the regulatory functions of BMP and TGF-β family prodomains (4–6). In general, it has been proposed that prodomains are important for proper folding, dimerization, and secretion of the mature ligand. A comparison of prodomains between different members of the TGF-β/BMP family shows a lower sequence conservation, in contrast to the high sequence conservation observed between their ligand domains. The low degree of prodomain sequence conservation might mean that TGF-β/BMP prodomains function as mere chaperones, as has been shown for prodomains of other proteins (7). However, this divergence in TGF-β/BMP prodomain sequences across the family could instead contribute to the functional diversification of signaling exemplified by TGF-β/BMP ligands (4).

The best studied example of a TGF-β family prodomain that plays an important role in ligand activity is TGFβ1. The TGFβ1 prodomain remains non-covalently associated with the ligand dimer after secretion, producing a latent complex that is inactive until the prodomain is removed from the ligand. Receptor activation is physically blocked by the prodomain that wraps around the mature TGFβ1 ligand domain, blocking receptor-binding surfaces (8). Like TGFβ1, the non-covalent GDF8 prodomain–ligand complex is latent and prevents ligand–receptor binding (9). BMP ligands also form non-covalent complexes with their prodomains, but the consequences of these interactions are varied and less well-understood. In most cases, BMP prodomain–ligand complexes are not latent (10), and ligand activity may instead be regulated by the prodomain in

peptide; aa, amino acid; VNC, ventral nerve cord; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2- (hydroxyethyl)propane-1,3-diol; CL, confidence limit; NICD, Notch intracellular domain; CI, confidence interval; IP, immunoprecipitate; FDR, false discovery rate.
more subtle ways. In the case of BMP4, cleavage at a second PC site, S2, reduces prodomain–ligand association and is required for long-range signaling in *Xenopus* embryos (11, 12). During mouse development, cleavage at the BMP4 S2 site has been shown to be required for the development of specific tissues (13, 14).

In contrast, the BMP7 prodomain competes with the ligand for binding type II receptors in vitro, but this competition does not reduce signaling activity in the context of cell culture assays (15). Similarly, the BMP9 prodomain has been proposed to interact with type II receptors and to confer receptor specificity by blocking ligand binding to ACVR2A but not its binding to ACVR2B or BMPR2 type II receptors (16). In a separate study using real-time surface plasmon resonance, the BMP9 prodomain was instead found to be rapidly displaced from the mature ligand by all receptor types, and in this case, the prodomain did not cause latency (17). The variable findings regarding the role of BMP prodomains in receptor competition could reflect differences between the experimental approaches or the context in which prodomain–ligand or prodomain–receptor interactions were examined. Therefore, studying the consequences of prodomain–ligand interactions in vivo is of particular interest.

Our laboratory has previously reported that BMP signaling activity can be affected by processing of the Gbb proprotein at different PC cleavage sites (18). In addition to the conventional S1 site that separates the conserved ligand domain from the more divergent prodomain, the *Drosophila* BMP7 ortholog Glass bottom boat (Gbb) contains an additional PC cleavage site, NS, within the N-terminal half of the prodomain (18, 19). Cleavage at the NS site produces a large ligand, Gbb38, whereas cleavage at the C-terminal S1 site produces the conserved smaller ligand, Gbb15. In many tissues, Gbb38 is more abundant than Gbb15 (18). In the developing wing epithelium, the NS site is required for wild-type signaling activity and range, whereas the S1 site is largely dispensable. Furthermore, the NS cleavage site has been shown to be necessary and sufficient for the rescue of *gbb* null lethality (19). In cell culture, both the NS and S1 cleavage sites have been shown to be critical to achieve full BMP signaling activity (18). Together, these findings indicate that Gbb38 is an active ligand with functions distinct from Gbb15. However, it is not exactly clear how cleavage influences the maturation of either ligand or whether the prodomain impacts ligand activity and/or influences receptor preference. Furthermore, the suggestion that context-specific requirements for Gbb could be explained by the activities of different ligands produced by alternative processing requires further investigation.

The *gbb* gene is known to be required for multiple developmental and physiological processes in *Drosophila*. The roles of *gbb* in pattern formation have been shown to be mediated by receptor complexes composed of the type II receptor Punt and the type I receptors Thickveins (Tkv) and/or Saxophone (Sax), which phosphorylate the downstream R-Smad signal transducer Mothers against decapentaplegic (Mad) (20, 21). During wing development, both *gbb* and the BMP2/4 ortholog *decapentaplegic* (dpp) are required for wing patterning and differentiation (22, 21), with both homodimers and Gbb–Dpp heterodimers likely contributing to wing patterning (22–24).

and *dpp* loss-of-function mutations exhibit different effects on BMP signaling and produce distinct wing phenotypes, indicating that Gbb and Dpp are not functioning as an obligate heterodimer during larval wing patterning. However, in the case of the development of the posterior cross vein during pupal wing development, Gbb–Dpp heterodimers comprise the most likely active ligand (24–26). In the nervous system, Gbb signaling specifically requires the type II receptor Wit (27, 28). *wit* and *gbb* function is also required for the expression of peptide hormones that regulate pupal ecdisis behaviors (29). The varied roles for Gbb (cell-fate specification, synapse-growth promotion, regulation of neurotransmission, and hormone expression) could be produced by a mechanism that regulates specific outcomes of Gbb signaling, potentially by allowing activation of different sets of receptors. Given the differences in Gbb38 and Gbb15 accumulation between tissues (18), we considered the possibility that the Gbb prodomain is involved not only in regulating the production of specific ligand forms but also their signaling output.

We examined in detail the processing of proGbb, prodomain–ligand interactions, and the in vivo requirements for NS cleavage. We found that S2 cells secrete Gbb15 in complex with the NS-cleaved prodomain. Blocking NS cleavage reduced Gbb15 signaling activity and increased the association of Gbb15 and the uncleaved prodomain. Signaling activity by Gbb38 was dependent on Sax or Wit. *In vivo*, we found NS cleavage is required for wing vein patterning and *wit*-dependent pupal ecdisis. In 3rd instar larvae, O-linked glycosylation blocks S1 cleavage, and Gbb38 is produced as a ligand that preferentially activates Wit. Overall, our results demonstrate that regulated alternative cleavage of the Gbb prodomain produces ligands that preferentially activate specific receptors.

**Results**

**Gbb prodomain cleavage products are secreted**

To understand how NS cleavage in the Gbb prodomain may impact signaling, we first wanted to identify all cleavage products, their ability to be secreted, and how they were affected by mutations in specific PC sites. We raised antibodies against specific prodomain epitopes, and with the existing C-terminal mutations in specific PC sites. We were able to identify all possible products cleaved from the proGbb precursor protein (Fig. 1A). α-GbbN recognizes amino acids (aa) 46–61, near the N terminus of the prodomain, and was used to identify N-terminal cleavage products. α-GbbCore, named after the Core/Arm domain of BMP2 and TGFβ1 (8, 30), recognizes aa 127–144 and any cleavage products resulting from the removal of the N or C terminus. When wild-type *gbb* was expressed by *Drosophila* Schneider 2 (S2) cells, we detected secreted Gbb prodomain cleavage products in the media (Fig. 1B). α-GbbN identified a 10-kDa band that matched the expected size of the NH₂-NS fragment on Western blottings of the conditioned media. α-GbbCore detected a 28-kDa fragment, which matched the expected size of the NS-S1 cleavage product, and a previously unidentified prodomain fragment (31). Finally, α-GbbC detected the expected Gbb15 ligand secreted into the media (Fig. 1C). In cell lysates, we found high levels of proGbb, and we
detected Gbb38 and other prodomain cleavage products at lower abundance (supplemental Fig. S1A). Taken together, we found that in S2 cells the Gbb propeptide is cleaved at both the NS and S1 sites and that all resulting cleavage products are secreted.

We next generated gbb constructs that harbor all combinations of mutations in the following PC sites: NS, S1, and the atypical S0 (referred to as the "Shadow" site in Ref. 19). When NS cleavage is blocked (mNS), the NH3-NS product is absent; a 37-kDa band corresponding to the intact prodomain (NH3-S1) is detected; and an /H110215-fold increase in Gbb15 is observed (95% confidence limits (CL) 1.7–13.3-fold; Fig. 1D). When S1 cleavage is blocked (mS1), the most abundant product is Gbb38, and the NH3-NS fragment is also detected in the media (Fig. 1, B–D). The appearance of NS-S0 and the low abundance of Gbb15, when compared with total Gbb ligands (22, 95, and 9–35% CL, see supplemental Fig. S2A), indicate that when S1 is mutated, some cleavage most likely occurs at the atypical S0 site (Fig. 1, B and C). Blocking S0 cleavage (mS0) or S0 in combination with NS (mNSmS0) had no detectable effect on the presence or abundance of prodomain cleavage products or Gbb15 (Fig. 1, B–D). However, blocking both S1 and S0 (mS1mS0) eliminated the NS-S0 fragment present in mS1 and resulted in the secretion of Gbb38 at much higher abundance than Gbb15 produced by a wild-type construct (4.8-fold, 95% CL, 1.7–13.5-fold; Fig. 1, B–D). When cleavage at both NS and S1 is blocked (mNSmS1), the entire uncleaved proGbb was detected in the media, along with NH3-S0 and very low levels of S0-cleaved Gbb15 (Fig. 1, B–D). Finally, mutating all three PC sites (mNSmS1mS0) resulted in a loss of all cleavage products and the secretion of proGbb (Fig. 1, B and C).

To identify the putative active ligands that exist as dimers, we examined secreted Gbb products using a non-reducing Western blotting. A Gbb15 dimer, detectable as a 40-kDa band, was present in the conditioned media of S2 cells expressing wild-type gbb (supplemental Fig. S2B). As we observed using reducing Western blottings (Fig. 1), blocking NS cleavage (mNS) increased Gbb15 abundance, but blocking S0 cleavage (mS0) had little effect. Gbb38 dimers and proGbb dimers were secreted by cells expressing gbbmS1mS0 and gbbmNSmS1mS0,

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**Figure 1.** Gbb prodomain cleavage affects ligand abundance and activity. A, Gbb protein schematic, showing domains, signal peptide (SP), cleavage sites, mutations, and location of antibody epitopes. Furin cleavage motifs at NS and S1 are underlined, with the downward arrow indicating the cleaved bond. The atypical S0 cleavage site is underlined with a dashed line. B and C, reducing Western blots (WB) of Gbb cleavage products secreted by S2 cells expressing gbb cleavage mutants, using antibodies that recognize specific epitopes in the Gbb prodomain (B) or the C-terminal ligand domain (C). D, quantification of secreted ligand abundance for representative Western blot shown in C. For mutant constructs that produce multiple forms of secreted Gbb ligand, the summed total of both forms is shown. E, steady-state signaling activity produced by gbb cleavage site mutant constructs when co-expressed with the BrkSE-lacZ reporter in S2 cells. Expression of BrkSE-lacZ reporter is repressed by BMP signaling and is shown here as opposite log-transformed β-Gal activity, with the activity of empty vector (EV) transfected cells set to 0. D and E, bars indicate the mean and 95% CL. * indicates p < 0.05 compared with EV; #, p < 0.05 compared with WT gbb, using GLHT for multiple comparison testing.
NS and S1 cleavage are both required for gbb signaling activity in S2 cells

We measured the signaling activity of gbb cleavage mutants in S2 cells, using the BrkSE-lacZ reporter. In this assay, constitutive expression of lacZ is transcriptionally silenced in response to the BMP signaling-dependent brinker silencer element (34). In cells transfected with wild-type gbb, signaling activity calculated as log₂(BrkSE-lacZ) was increased compared with endogenous levels as seen in the control EV-transfected cells (Fig. 1E). When cells were transfected with a construct with all cleavage sites blocked (mNSmSmS1), signaling activity was lower than in EV-transfected cells. This likely reflects the inhibition of endogenous signaling by the formation of inactive proGbb-heterodimers with endogenously expressed BMPs (35, 36), and it is consistent with our finding that expression of gbbmNSmS1 in wing discs led to a cell autonomous loss of endogenous pMad (18). The level of BMP signaling as measured by the BrkSE-lacZ assay is significantly reduced when NS or S1 cleavage is blocked (mNS or mS1) (Fig. 1E), indicating a requirement for cleavage at each site. Blocking S0 cleavage (mS0) had no effect on signaling activity, and no activity was detectable when only S0 cleavage was permitted (mNSmS1). Therefore, cleavage at the S0 site alone was neither necessary nor sufficient for signaling activity in S2 cells.

Interestingly, although cleavage at only the NS site has been shown to fully rescue the lethality associated with gbb null alleles in vivo (19), we did not detect signaling activity in S2 cells transfected with gbbmNSmS0 using the BrkSE-lacZ assay. Even though Gbb38 is produced when proGbb is cleaved at only the NS site, NS cleavage alone is not sufficient to produce signaling activity in S2 cells (Fig. 1E). Surprisingly, when only S1 cleavage was permitted (mNSmS0), we observe signaling activity that is significantly higher than that of the activity of gbbmNS (Fig. 1E). This shows that the inhibitory effects of blocking NS cleavage can be relieved by the mS0 mutation, possibly indicating that either S0 cleavage or the Lys-334 residue mutated in mS0 is required for inhibitory interactions between Gbb15 and the prodomain. Overall, in S2 cells we find that both NS and S1 cleavage are necessary for signaling activity. We considered the possibility that NS cleavage is necessary to ensure full Gbb15 activity.

The regulation of BMP/Gbb by alternative cleavage

**Gbb15 activity is inhibited by the prodomain when NS cleavage is blocked**

The BrkSE-lacZ assay readout reflects multiple aspects of ligand production, secretion, receptor interaction, and downstream feedback, all in the context of BMP ligands endogenously expressed in S2 cells. To eliminate contributions of other signaling mechanisms that could modulate Gbb activity, we directly measured the activity of the secreted forms of Gbb15 and Gbb38 and compared their signaling kinetics. Conditioned media were produced by S2 cells stably transfected with gbb, gbbmNS, or gbbmNSmS0 expression constructs or empty vector. Because the concentration of secreted ligands (Gbb15 or Gbb38) that result from cells expressing cleavage mutant constructs (mNS or mS1mS0) is much higher than Gbb15 produced by wild-type gbb (Fig. 1, C and D), we adjusted the cleavage mutant conditioned media so that ligand concentrations were equivalent, using conditioned media from EV-expressing cells.

We measured signaling as the phosphorylation of Mad in S2 cells transiently transfected with FLAG-tagged Mad (Mad–FLAG). After a 2-h treatment of Mad–FLAG-expressing cells, wild-type conditioned media, which contains Gbb15 and the NH₃-NS and NS-S1 prodomain fragments, induced high levels of phosphorylated Mad (pMad), relative to total Mad–FLAG (Fig. 2, A and C). The activity of WT gbb-conditioned media was comparable with 10–100 ng/ml rhBMP7 in this assay (supplemental Fig. S3). Conditioned media from gbbmNS, containing Gbb15 and the uncleaved prodomain NH₃-S1, produced pMad at 55% of WT levels (p < 0.001, 95% CI 38–79%). Therefore, we conclude that the activity of Gbb15 is reduced in the presence of the uncleaved prodomain (NH₃-S1) suggesting that a failure to cleave the prodomain at the NS site can antagonize Gbb15-induced signaling. We also found that conditioned media from gbbmS1mS0, which contains Gbb38 and the NH₃-NS fragment but no Gbb15, produced pMad at 17% of WT levels (p < 0.001, 95% CI 12–25%) (Fig. 2, A and C), indicating that in this experimental system Gbb38 can induce signaling in S2 cells, albeit at lower levels than Gbb15. A similar pattern of reduced signaling activity of gbbmNS and gbbmS1mS0 is observed in S2 cells expressing endogenous Mad (supplemental Fig. S4), indicating that Mad–FLAG overexpression is not influencing gbb cleavage mutant signaling. We also examined whether the different Gbb ligands might show signaling activity transduced by Mad-independent pathways, but we found that neither WT nor cleavage mutant gbb had any effect on phosphorylation of p38 or dSmad2 (supplemental Fig. S5). The difference in Gbb38 activity between the pMad assay and the BrkSE-lacZ assay could indicate that the overexpressed gbbmS1mS0 is inhibiting the activity of endogenously expressed BMPs (35, 36), or that some feedback mechanism reduces the ability of S2 cells that are expressing gbbmS1mS0 to receive a Gbb38 signal.

NS site cleavage of the Gbb ortholog, Scw, has been shown to be required for wild-type signaling kinetics (37). Unlike Gbb, Scw homodimers have very low signaling activity in S2 cells and is instead thought to function primarily as a part of Scw–Dpp heterodimers (38). Scw–Dpp heterodimers show relatively rapid signaling kinetics and exhibit peak activity at 1 h (37).
Mutating the Scw NS cleavage site was shown to alter heterodimer such that activity reached wild-type levels only after 3–5 h, suggesting that a failure to cleave the Scw prodomain at the NS site impacted the signaling kinetics of Scw–Dpp heterodimers. Given the reduced level of signaling observed from gbbmNS conditioned media described above, we also tested the effect of alternative processing of Gbb on signaling kinetics. We examined pMad levels produced over a time course of 0.5–10 h in cells treated with conditioned media produced by different gbb constructs (Fig. 2). Activity of wild-type gbb conditioned media was detectable at 0.5 h, peaked at 2 h, and gradually decreased through hour 10. When NS cleavage was blocked, gbbmNS conditioned media had reduced activity at nearly all time points, with peak activity at 2–4 h. When S1/S0 cleavage was blocked and only Gbb38 was present in conditioned media, we again detected low levels of signaling activity, with a peak at 2–4 h. The low level of signaling at each time point was consistent with our finding that in S2 cells Gbb38 has less activity than Gbb15, although it signals with kinetics similar to Gbb15. In fact, preventing cleavage at NS or at S1/S0 did not affect the overall signaling kinetics. Given that blocking NS cleavage enabled secretion of the uncleaved prodomain with Gbb15, and that this led to a reduction in the ability of Gbb15 to signal (Figs. 1 and 2A), results from the time course indicate that NS cleavage does not affect Gbb15 signaling kinetics.

Cleavage at the NS site reduces Gbb15-prodomain association

Cleavage of the GDF8/GDF11 prodomain by Tolloid metalloproteases, at a site that aligns near the NS site of Gbb, releases the ligand from the latent complex (39, 40). We next hypothesized that cleavage of the Gbb prodomain at the NS site may enable full levels of Gbb15 signaling by preventing prodomain–Gbb15 association. To examine prodomain–ligand interactions, Gbb with a C-terminal HA tag was co-immunoprecipitated from conditioned media using antibodies that recognize epitopes in each cleavage product. α-GbbN directly precipitates the NH3-NS fragment and co-precipitates Gbb15 from conditioned media produced by cells expressing WT gbb-HA (Fig. 3). Similarly, α-GbbCore directly precipitates the NS-S1 fragment and co-precipitates Gbb15. However, when Gbb15 is precipitated with α-HA, no co-precipitation of the NH3-NS or the NS-S1 cleavage product was observed. When NS cleavage is blocked, co-precipitation of Gbb15 using either α-GbbN or α-GbbCore to pull down the uncleaved prodomain is more efficient than with the NS-cleaved prodomain, and α-HA effectively co-precipitates the NH3-S1 product. Finally, when S1/S0 cleavage is blocked the NH3-NS fragment co-precipitates with Gbb38 more efficiently than with Gbb15. Therefore, we conclude that Gbb15 forms a loosely associated complex with both NS-cleaved prodomain fragments (NH3-NS and NS-S1), and a failure to cleave at the NS site increases Gbb15-prodomain association. These co-immunoprecipitations were carried out at pH 6.5, the physiological pH of S2 cell culture media and larval hemolymph (41). Interestingly, at pH 7.4, we observed reduced co-precipitation of prodomain cleavage products with either Gbb15 or Gbb38, suggesting that these complexes are sensitive to pH (supplemental Fig. S6). We also observed pH-dependent interactions between Gbb ligands and prodomain cleavage products using heparin chromatography (supplemental Fig. S7). Together with the observation that blocking NS cleavage increases Gbb15 abundance but reduces its ability to signal (Figs. 1 and 2), we propose that NS cleavage is required to prevent latency of Gbb15.

Gbb alternative cleavage generates ligands that exhibit receptor preference

Given that S2 cells express the type II receptor ACVR2A/B ortholog Punt but not the BMPR2 ortholog Wit (42), we con-
Regulation of BMP/Gbb by alternative cleavage

Figure 3. Cleavage of the NS site reduces Gbb15–prodomain association. A, reducing Western blots (WB) of co-immunoprecipitating Gbb cleavage products. Conditioned medium from S2 cells expressing gbb-HA cleavage mutants was immunoprecipitated with α-proGbb and α-CoreGbb to directly pull down prodomain cleavage products and α-HA to pull down C-terminal cleavage products. Immunoprecipitate was analyzed by Western blotting using α-proGbb, α-CoreGbb, and α-GbbC to identify each cleavage product. B, summary of relative co-IP efficiencies between cleavage products for WT and cleavage mutant gbb-HA.

Considered the possibility that different behaviors of mNS, mS1, and mS1mS0 in S2 cells, wing discs, and in null rescue (18, 19) could reflect the usage of different receptors. To measure signaling activity in the context of different type II receptors, we co-transfected S2 cells with constructs expressing punt or wit and Mad–FLAG, and we measured the level of pMad induced by conditioned media from cells expressing gbb. In cells transfected with punt, wild-type gbb conditioned media induced a 5-fold increase in pMad, compared with untreated cells (Fig. 4A). However, neither gbbmNS (NH3-S1 + Gbb15) nor gbbmS1mS0 (NH3-NS + Gbb38) conditioned media induced a detectable increase in pMad over the empty vector conditioned media control. This failure to induce Mad phosphorylation suggests that, in the case of gbbmNS media, the ability of Gbb15 to induce Punt-mediated signaling is prevented when NS cleavage is blocked, and in the case of gbbmS1mS0 media, Gbb38 cannot activate Punt-dependent signaling. In cells transfected with wit, conditioned media from all gbb-expressing constructs elicited a significant increase in pMad with both wild-type gbb and gbbmNS conditioned media inducing an 8-fold increase, and gbbmS1mS0 conditioned media resulting in a 5-fold increase in pMad. Taken together, these results indicate that activation of Punt by Gbb requires both NS and S1/S0 cleavage and that neither Gbb38 associated with the NH3-NS fragment nor Gbb15 with the uncleaved prodomain can activate Punt. In contrast, both Gbb38 (mS1mS0) and Gbb15 with the uncleaved prodomain (mNS) can activate Wit-mediated signaling. Thus, ligands resulting from a mutation in either of the proconvertase processing sites can signal through the Wit type II receptor, and Gbb38 appears to function as a ligand that preferentially activates Wit.

Gbb has been proposed to form high-affinity complexes with the type I receptor Sax but not with Tkv (43). Consistent with this report, our laboratory previously found that Gbb38 co-immunoprecipitates with Sax but not Tkv (18). We further investigated the ability of different type I and type II receptor combinations to mediate signaling elicited by different Gbb cleavage products. gbb cleavage mutant constructs were co-expressed in S2 cells with constructs expressing type I receptors encoded by tkv or sax, or type II receptors encoded by punt or wit, and signaling activity was measured using the BrkSE-lacZ assay. WT gbb showed high activity in cells transfected with tkv, punt, and wit (Fig. 4B). The lower activity of gbb in sax-transfected cells is consistent with previous reports of inhibitory effects of sax in this assay (44). Surprisingly, blocking Gbb NS cleavage only caused a significant reduction in signaling activity in punt-transfected cells. Blocking S1/S0 cleavage also led to a loss of detectable signaling activity in punt-transfected cells, but it only caused a partial reduction in signaling activity in tkv- and wit-transfected cells. The data indicate that Gbb alternative cleavage products have distinct receptor preferences. However, these preferences are not indicative of absolute requirements. In tkv-transfected cells, for example, endogenously-expressed punt (42) is providing the type II receptor function that allows high activity from either gbbmNS (Gbb15 + NH3-NS) or gbbmS1mS0 (Gbb38 + NH3-NS). Similarly, co-transfection of punt and sax can also permit gbbmS1mS0 signaling (supplemental Fig. S8). Therefore, the ability of Punt to transduce the signal of Gbb different cleavage products appears to be sensitive to type I receptor stoichiometry, which may explain context-dependent responses. Regardless, these results show that blocking Gbb NS cleavage has the greatest effect on Punt-mediated signaling. Overall, we conclude that the latency caused by blocking NS cleavage can be overcome by Wit expression or high levels of type I receptor expression and that Gbb38 can most effectively elicit signaling through receptor complexes that contain the type II receptor Wit.
Regulation of BMP/Gbb by alternative cleavage

Cleavage of NS or S1/S0 is sufficient for activity of Gbb–Dpp heterodimers

BMP family members can associate and form active heterodimers (45). Given that Gbb–Dpp heterodimers are thought to contribute to patterning and differentiation of the developing wing (22–24), and that Gbb38 is the most abundant ligand form in the larval wing imaginal disc (18), we examined whether alternative Gbb processing may impact Gbb–Dpp heterodimer formation and activity. HA-tagged dpp (dpp-HA) was co-expressed in S2 cells with different gbb-HA wild-type and cleavage mutant constructs. The composition of Gbb and Dpp protein products secreted into the media was examined using α-HA on non-reducing Western blottings (Fig. 5A). When we compared secreted products produced by cells expressing gbb-HA wild-type or cleavage mutant constructs with dpp-HA versus gbb-HA constructs alone, we detected three new bands corresponding to heterodimers composed of various Gbb cleavage products and the processed C-terminally derived Dpp ligand domain. A Gbb15–Dpp heterodimer is present when wild-type gbb or gbbmNS are co-expressed with dpp. Blocking NS cleavage (mNS) appears to increase the abundance of the Gbb15–Dpp heterodimer. A Gbb38–Dpp heterodimer is enriched in media from cells expressing the S1/S0 cleavage mutant (gbbmS1mS0). When all three Gbb-processing sites are mutated, a proGbb–Dpp heterodimer is evident. Cleaved Dpp can be secreted as a heterodimer with Gbb15, Gbb38, or proGbb. We note that when dpp-HA is expressed alone, Dpp-HA is not detected in media but is instead observed in cell lysates at low abundance (data not shown). Therefore, the relatively high abundance of Gbb–Dpp heterodimers does not necessarily indicate preferential heterodimer formation or production, and instead it may reflect increased secretion or decreased turnover.

Next, we measured the activity of the ligands secreted from cells expressing each gbb construct co-expressed with dpp or empty vector, using the BrkSE-lacZ signaling assay (Fig. 5B). Similar to our previous observations, blocking NS cleavage (mNS) reduces signaling activity of Gbb, and blocking S1/S0 cleavage (mS1mS0) or all three cleavage sites (mNSmS1mS0) reduces activity below endogenous levels (Fig. 1E, 5B). dpp alone produces significantly higher signaling activity than gbb alone (Fig. 5, B and C), as also seen when each is overexpressed in the developing wing (20), and when co-expressed a higher level of signaling activity is achieved. Whereas expression of gbbmNS alone fails to induce signaling, when co-expressed with dpp the signaling activity is equivalent to the levels produced by co-expression of dpp and wild-type gbb. Based on the cleavage products produced by gbbmNS (Gbb15 + NH3-S1; see Fig. 1 and supplemental Fig. S2) and the ligands detected in media from the co-expression of gbbmNS and dpp (Gbb15–Dpp; Fig. 5A), we attribute the higher level of signaling to Gbb15–Dpp heterodimers. Because the uncleaved Gbb prodomain (NH3-S1) is present in the media of cells co-expressing gbbmNS and dpp (data not shown), we also conclude that the Gbb prodomain does not compromise Gbb15–Dpp signaling. Whereas the production of Gbb38 failed to induce signaling in S2 cells and appeared to antagonize endogenous BMP signaling following the expression of gbbmS1mS0 (Fig. 5B), we found that the co-expression of gbbmS1mS0 and dpp produced a high level of signaling activity, likely due to the formation of Gbb38–Dpp heterodimers (Fig. 5C). Finally, when all Gbb cleavage sites are mutated (gbbmN3mS1mS0), the ability of dpp to induce signaling is inhibited, most likely as a result of sequestering Dpp into inactive proGbb–Dpp heterodimers. Thus, cleavage of Gbb at S1/S0 or at NS enables the formation of heterodimers with Dpp leading to significant signaling activity.

Gbb NS cleavage is required for wing vein patterning

Mutations in the Gbb NS cleavage site have different effects on signaling activity in S2 cells, depending on whether gbb is expressed alone or co-expressed with dpp. Our laboratory previously observed that mutations in the Gbb NS cleavage site led to wing vein defects, including ectopic posterior cross vein (PCV) spurs (18). To generate animals that lack cleavage at the NS site, we made use of a mutant form of a gbb genomic rescue construct (gbbRmNS) in a gbb1 null mutant background. Development of the PCV requires both gbb and dpp, likely acting as
Gbb–Dpp heterodimers (24, 25). We assessed the requirement for the NS cleavage in Gbb–Dpp heterodimer function by making use of a sensitized genetic background where overall BMP gene dosage was reduced while preserving ligand stoichiometry. A single functional copy of \( gbb \) was provided by \( gbb^R \) in a homozygous \( gbb^1 \) null in the context of reduced \( dpp \) function in animals heterozygous for \( dpp^{d12} \) or \( dpp^{hr4} \) mutations \( dpp^0/gbb^{R^0}/gbb^1/dpp^{hr4}/gbb^1 \). This had no effect on wing patterning. In contrast, when Gbb NS cleavage is blocked in combination with \( dpp^{hr4} \) or \( dpp^{d12} \), wing patterning defects, including ectopic vein formation at the tip of the 2nd longitudinal vein (L2), were significantly enhanced (Fig. 6, A and B). The PCV spur phenotype was enhanced in \( dpp^0/gbb^R^0/gbb^1/dpp^{hr4}/gbb^1 \) (Fig. 6, A and C). In summary, wing patterning defects caused by blocking NS cleavage can be enhanced by \( dpp \) loss-of-function mutations. Because Gbb NS cleavage and Gbb–Dpp heterodimers are required for PCV morphogenesis (18, 24, 25), we conclude that in vivo pro-

Figure 5. Dpp signaling activity is enhanced by forming heterodimers with Gbb cleavage mutants. A, non-reducing Western blots (WB) of media from cells co-expressing \( dpp-HA \) and \( gbb-HA \), showing secreted homodimers and heterodimers (indicated with †). B and C, steady-state BrkSE-lacZ signaling assay, measuring activity of \( gbb-HA \) cleavage mutants co-expressed with EV (B) or \( dpp-HA \) (C). All signaling experiments were performed in parallel and are presented separately to emphasize the relative effects of \( gbb-HA \) expression. Bars indicate mean and 95% CI. * indicates \( p < 0.05 \) compared with EV; # indicates \( p < 0.05 \) compared with WT \( gbb-HA \), using GLHT multiple comparison test.

A

| EV (ng) | 200 | 100 | 100 | 100 | 100 |
|--------|-----|-----|-----|-----|-----|
| \( dpp-HA \) (100 ng) | -   | -   | -   | -   | -   |
| \( gbb-HA \) (100 ng) | -   | WT  | mNS | mS1 | mS1 |

Media (non-reduced)

| EV (ng) | 100 | 0   | 0   | 0   | 0   |
|--------|-----|-----|-----|-----|-----|
| \( dpp-HA \) (100 ng) | -   | +   | +   | +   | +   |
| \( gbb-HA \) (100 ng) | -   | WT  | mNS | mS1 | mS1 |

WB: α-HA

B

C

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cessing at the Gbb NS site is necessary for the activity of heterodimers in wing vein formation.

**Gbb NS cleavage is required for pupal viability**

We examined the course of gbbRmNS gbb\(^1\) development from egg to adult to identify other developmental processes that may require NS cleavage. We observed significant pupal lethality, which was not previously described as a gbb phenotype. We scored the pupal viability of a gbb allelic series and found that inter se crosses between mild loss-of-function alleles (gbb\(^3\), gbb\(^4\), or gbb\(^5\)) or with the gbb\(^1\) null allele result in different amounts of pupal lethality (Table 1). Notably, gbbRmNS gbb\(^1\) exhibits higher pupal lethality than gbb\(^3\)/gbb\(^1\) and gbb\(^5\)/gbb\(^1\). However, gbb\(^1\)/gbb\(^4\) and gbb\(^5\)/gbb\(^4\) have more severe wing patterning defects than gbbRmNS gbb\(^1\), including loss of the PCV and distal portions of the L4 and L5 longitudinal veins (18, 21, 23). Therefore, we hypothesized that the functions of gbb during pupal development require NS cleavage. Most of the dead gbbRmNS gbb\(^1\) pupae exhibited developmental arrest at early stages of pupation, prior to head eversion and pigment development (Fig. 7A) (49). gbbRmNS gbb\(^1\) pharates, which have undergone metamorphosis, failed to fully extend their legs (Fig. 7, A and B). The failure in both head eversion and leg extension is indicative of a defect in pupal ecdysis behaviors during pupation, the process by which anatomical structures are inflated to take on their adult form (50–52). Additionally, some gbbRmNS gbb\(^1\) pharates arrested at late developmental stages, just prior to or during the process of exiting the pupal case. Thus, processing of proGbb at the NS site is required in vivo for functions of gbb during pupal ecdysis and development.

To examine how blocking Gbb NS cleavage affects cleavage products present in vivo, we immunoprecipitated Gbb from gbbR-HAgbb\(^1\) or gbbRmNS-HAgbb\(^1\) expressing HA-tagged Gbb, or WT larvae expressing untagged Gbb (Fig. 7C). We observed
HA-tagged proGgb in gbbR-HA gbb and gbbRmNS-HA gbb, and we only detected HA-tagged Gbb38 in gbbR-HA gbb. In these experiments, HA-tagged Gbb15 was not immunoprecipitated, consistent with its very low abundance in most larval tissues except the fat body (18), which was not retained in the aqueous larval lysates used in this experiment. Thus, the phenotypes caused by blocking NS cleavage are preceded by loss of Gbb38.

**Gbb NS cleavage is required for expression of the pupal ecdysis hormone CCAP**

Pupal ecdysis involves a series of bodily movements that result in shedding of the larval cuticle, eversion of the head and appendages, and extension of the everted wings and legs to reach their adult shape (50–52). This behavioral sequence is coordinated by hormones produced by neurosecretory neurons and endocrine cells (53). The neuropeptide CCAP is expressed in a specific set of neurons in the ventral nerve cord (VNC) of the central nervous system; ablation of these neurons, or mutations in ccap, produces pupal ecdysis defects (52, 54). It has been shown that wit is required for pupal ecdysis and for the expression of CCAP and other ecdysis-regulating hormones in CCAP neurons (29). Similarly, it has been shown that the presence of nuclear pMad in CCAP neurons is dependent on wit and that the number of CCAP-expressing neurons is also reduced in gbb1 1st instar larvae. Because gbbRmNS gbb pupal phenotypes resemble those of wit mutants or CCAP neuron ablation, we hypothesized that Gbb NS cleavage is required for wit-dependent CCAP expression. We assayed for the expression of CCAP in gbbRgbb1 or gbbRmNS gbb wandering 3rd instar larvae using CCAP-Gal4, UAS-GFP (CCAP > GFP), and we used nuclear pMad immunostaining to measure BMP signaling activity in CCAP neurons.

CCAP neurons are comprised of several distinct classes of neurons in late 3rd instar larvae just prior to pupariation: interneurons (CCAP-IN), efferent neurons (CCAP-EN), late efferent neurons (CCAP-ENL), and posterior lateral neurons (CCAP-PL) (Fig. 8A) (55). Because CCAP-IN neurons are not required for pupal ecdysis, and do not exhibit regulation of CCAP expression by BMP signaling, we focused our analysis on the other classes of CCAP expression neurons. Of these, only the CCAP-ENL and CCAP-PL neurons are required for pupal ecdysis. CCAP-EN and ENL neurons are marked with nuclear pMad and Dachshund (Dac), although CCAP-PL neurons are marked with only nuclear pMad. In the VNC of wandering 3rd instar gbbRgbb1 larvae, we detected CCAP-EN and CCAP-ENL neurons marked with nuclear pMad and Dac (pMad+ Dac+), and CCAP-PL neurons marked only with nuclear pMad (pMad + Dac−) (Fig. 8B). CCAP expression has not been examined in gbb mutants at stages beyond 1st instar larvae (29); therefore, we first assessed the expression of CCAP > GFP in the VNC of gbb1 null mutant 3rd instar larvae. CCAP-PL and CCAP-ENL neurons were almost completely undetectable in gbb1 3rd instar larvae (supplemental Fig. S9, A and B). Wild-type numbers of CCAP-EN neurons could be identified in gbb4 VNCs, but the expression of CCAP > GFP and nuclear pMad in the CCAP-EN neurons was greatly reduced (supplemental Fig. S9, C and D). Therefore, we conclude that Gbb signaling is required for Wit-dependent expression of CCAP in CCAP-EN neurons. This indicates that either gbb is required for CCAP expression in these neurons or the developmental delay associated with gbb1 nulls (21) prevented the mutant larva from progressing to the late 3rd instar when CCAP expression begins in the CCAP-PL and CCAP-ENL neurons (55).

To determine the consequences of blocking NS cleavage during pupal development, we examined the expression of CCAP in the VNC of gbbRmNS gbb1 late 3rd instar larvae. We detected CCAP-EN neurons, and the level of CCAP > GFP expression in the CCAP-EN neurons was significantly reduced (Fig. 8, C and E). In gbbRmNS gbb1 VNCs, we found significantly fewer CCAP-PL and CCAP-ENL neurons with detectable CCAP > GFP expression, compared with gbbRgbb1 (Fig. 8, B–D). Of the remaining detectable CCAP neurons, there was a significant reduction of CCAP > GFP expression in CCAP-PL neurons (Fig. 8E). We quantified the signal intensity of nuclear pMad in CCAP neurons in gbbRmNS gbb1 VNCs, and compared with gbbRgbb1, we observed a reduction in BMP signaling in CCAP-EN neurons (Fig. 8F). In detectable CCAP-ENL and CCAP-PL neurons with visible CCAP > GFP expression, we did not see a significant change in nuclear pMad intensity. In summary, we find that NS cleavage of Gbb is required to activate pMad in a subset of CCAP neurons and for the wit-dependent expression of CCAP. Because Gbb38 preferentially activates Wit in S2 cells (Fig. 4A), and gbbRmNS gbb1 shows defects in wit-dependent signaling (8) and a loss of Gbb38 (7C), we propose that NS cleavage is required in vivo for production of Gbb38 as a ligand that preferentially activates Wit.

**Cleavage of the Gbb S1/S0 site is blocked by O-linked glycosylation**

The endogenous abundance of Gbb38 and Gbb15 varies between tissues, which suggests that the production of Gbb38 could be a regulated process whereby S1/S0 cleavage is prohibited and only NS cleavage is permitted. O-Glycosylation has been shown to prevent PC processing (56). Therefore, we considered the possibility that the modification of residues near Gbb PC sites could influence the production of one ligand form over the other. Based on NetOGlyc 4.0 (57), proGgb is predicted to contain O-glycosylation sites near its PC cleavage sites (Fig. 9A). Of particular note is the cluster of six high-score predicted glycosylation sites within three residues of the S1/S0 sites. Gbb38 is the most abundant Gbb ligand form in most 3rd instar larval tissues (18). Therefore, we used an in vitro cleavage assay to determine whether proGgb or Gbb38 isolated from 3rd instar larvae could be cleaved at the S1/S0 site. An HA-tagged
Regulation of BMP/Gbb by alternative cleavage

A rescue transgene was inserted in a gbb<sup>1</sup> null background, generating gbb<sup>RHAgbb</sup><sub>1</sub> and ensuring that Gbb-HA would be produced at endogenous levels.

Gbb-HA was immunoprecipitated from lysates of gbb<sup>RHAgbb</sup><sub>1</sub> wandering 3rd instar larvae. Similar to our previous observations of Gbb cleavage product abundance (18), proGbb was the most abundant form, with very low levels of Gbb38 and no Gbb15 detectable by Western blotting (Fig. 9B).

Treatment with recombinant furin resulted in a decrease of proGbb and an increase of Gbb38 indicating an NS cleavage. No Gbb15 resulting from an S1/S0 cleavage was detected. To determine whether S1/S0 cleavage may be blocked by O-glycosylation, immunoprecipitated Gbb was treated with glycosidases prior to the addition of furin. In Drosophila, Core 1 glycans represent the most abundant form of O-glycans, whereas most of the remaining O-glycans consist of a HexNAc or GlcA extension of the Core 1 glycan (58). The unmodified Core 1 O-glycan can be removed with O-glycosidase, and most extensions of the Core 1 O-glycan can be removed with exoglycosidases/HexNAcase and β-glucuronidase (β-Glcase). Treatment of Gbb with each glycosidase, in the absence of furin, had no effect on the presence or abundance of Gbb cleavage products. Additionally, treatment of Gbb with O-glycosidase alone had no effect on furin cleavage. However, following treatment with all three glycosidases, addition of furin produced Gbb15. We conclude that in Drosophila 3rd larval instar, S1/S0 cleavage of Gbb is blocked by an extended O-glycan, resulting in the production of Gbb38 by cleavage of the NS site. Because S1/S0 cleavage occurs in S2 cells, it is conceivable that specific glycosyltransferases are expressed in 3rd instar larvae that are responsible for the O-glycosylation of the Gbb S1/S0 site and that these enzymes are not expressed in S2 cells. Thus, S1/S0 cleavage is allowed, and Gbb38 is not produced as a mature ligand by S2 cells.

Discussion

Regulation of Gbb signaling by alternative cleavage

Alternative processing of the Gbb proprotein can produce ligands of different sizes, Gbb38 and Gbb15 (Fig. 10). These ligands vary by the inclusion of the Core region of the prodomain, which appears to confer receptor preference. In S2 cells, Gbb15, generated by PC processing at the S1/S0 site, is secreted as a loosely associated complex with the two prodomain fragments (NH<sub>3</sub>-NS and NS-S1) produced by cleavage at the NS site. In this configuration, Gbb15 can activate signaling via either type II receptor, Punt or Wit. When NS cleavage is blocked, Gbb15 forms a tightly associated latent complex with the uncleaved prodomain (NH<sub>3</sub>-S1) with reduced Punt-mediated signaling activity. In this case, more of the resulting Gbb15 is found in the media than in wild type when NS cleavage occurs, suggesting that the Gbb15-uncleaved prodomain latent complex influences either protein turnover, secretion, and/or binding to cell-surface receptors that can also affect ligand turnover. Interestingly, when S2 cells are induced to express Wit, the Gbb15-uncleaved prodomain latent complex is not restricted in its ability to signal and exhibits full activity. In the absence of S1/S0 cleavage, NS-cleaved Gbb38 is secreted in complex with the small N-terminal fragment of the prodomain (NH<sub>3</sub>-NS) and is only able to signal through Wit. These findings indicate that the Gbb prodomain influences type II receptor usage. Signaling through Punt is restricted by either the Core domain as part of Gbb38 or by the intact prodomain when it is non-covalently associated with Gbb15, whereas Wit can mediate all ligand–prodomain cleavage fragment combinations.

The abundance of Gbb38 and Gbb15 varies in different tissues of the developing animal, suggesting that either the production or turnover of the two ligand types is regulated. Here,
Regulation of BMP/Gbb by alternative cleavage

Figure 8. Gbb NS cleavage is required for expression of the ecdysis-regulating hormone CCAP. A, schematic of CCAP-expressing peptidergic neurons in the larval VNC, adapted from Veverysa and Allan (55). Prior to the 3rd larval instar wandering stage, CCAP is expressed in interneurons (IN) that lack nuclear pMad and Dac (CCAP-IN) and in efferent neurons that have nuclear pMad and Dac (CCAP-EN). At later stages, CCAP expression is activated in additional pMad+ nuclei. B and C, representative confocal image stacks of VNC from wandering 3rd instar larvae. CCAP > GFP is used as a reporter of CCAP expression, and nuclear Dac and pMad serve as markers and indicators of BMP signaling. Insets, CCAP neurons with pMad nuclei are indicated with arrowheads and dashed outline. D, number of late CCAP neurons per VNC, *p < 0.05 using Wilcoxon rank-sum test and FDR multiple comparison adjustment. E, quantification of mean cell body CCAP > GFP signal intensity for each VNC and neuron class. F, quantification of mean nuclear pMad signal intensity for each VNC and neuron class. E and F, bars indicate mean and 95% CI, *p < 0.05 using GLHT multiple comparison test. D–F, n = 8, VNCs for gbbR gbb1, n = 7, VNCs for gbbRNS gbb1.

we show in 3rd instar larvae that proteolytic processing at the S1/S0 can be influenced by O-glycosylation leading to preferential processing at the NS and an abundance of Gbb38 (Fig. 10). Furthermore, we find that NS cleavage is required in vivo for wit-dependent functions in pupal ecdysis and the regulation of CCAP hormone in specific CNS neurons that are critical for metamorphosis. We propose that at least one mechanism responsible for regulating the alternative cleavage of Gbb involves O-glycosylation of residues near the S1/S0 site, resulting in the production of Gbb38 as a ligand that preferentially activates Wit.

Gbb NS cleavage is necessary for Gbb–Dpp heterodimer activity patterns

We found that gbbRNS gbb1/gbb1 adult wings have a low occurrence of vein-patterning defects. However, in combination with dpp loss-of-function mutations (gbbRNS gbb1/gbb1/dpp+gbb1), a substantial enhancement of defects is apparent, including PCV spurs and ectopic vein material. These phenotypes do not resemble those displayed by loss-of-function mutations in gbb or dpp, which typically cause the absence of longitudinal veins and cross veins, rather than the formation of ectopic veins (21–23, 59). Instead, the wing phenotypes of gbbRNS gbb1/dpp+gbb1 resemble those caused by mutations in or overexpression of several secreted BMP extracellular regulators. During PCV morphogenesis, Gbb–Dpp heterodimers expressed in longitudinal vein primordia are bound by the extracellular regulators Crossveinless (Cv) and Short gastrulation (Sog), the Drosophila Chordin ortholog (60). When complexed with Cv and Sog, the Gbb–Dpp heterodimer is inactive. However, Crossveinless-2 (Cv-2) serves to localize the complex to the PCV primordia between longitudinal vein L4 and L5, where cleavage by the Tolloid (Tld) metalloprotease releases Gbb–Dpp for signaling. Together, these extracellular regulators comprise a system that shuttles Gbb–Dpp heterodimers from the longitudinal veins to the PCV primordia. Experimental disruption of the stoichiometry or spatial distribution of these extracellular regulators produces PCV spurs and ectopic
**Regulation of BMP/Gbb by alternative cleavage**

**A. Predicted Gbb O–glycosylation**

![Figure 9](image1.png)

**B. Gbb cleavage in vitro**

![Figure 10](image2.png)

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**Functions of Gbb in regulation of ecdysis hormones**

We demonstrate that NS-cleaved Gbb is required during metamorphosis for the proper expression of hormones that control pupal ecdysis, the process by which imaginal tissues assume their adult form. In addition to the pupal ecdysis defects we described, we have also observed failure of eclosion, wing inflation, and defects in tanning, whereby newly emerged adults remain soft for many hours after heterozygous siblings have hardened their cuticle (data not shown). We also note that the gbbR<sup>NS</sup> gbb<sup>1</sup> pupal phenotype includes failure to develop pigment, but pigment development is not affected by ablation of CCAP neurons or mutations in CCAP and related hormones (52, 54). Our laboratory has also observed larval ecdysis defects in gbb loss-of-function mutants, including significant developmental delays and failure to shed cuticle during each molt. Together, these phenotypes all suggest that gbb has a broader and previously unrecognized role in the regulation of ecdysis in both larval and pupal development. Regulation of ecdysis by gbb could be related to its role in the regulation of energy homeostasis (65), which is intimately tied to the organism’s ability to undergo or delay ecdysis (53), or to the role of gbb in circadian neural circuit development (66, 67). With regard to the regulation of pupal ecdysis hormones, wit signaling is required specifically in CCAP neurons for CCAP expression (29). 1st larval instar gbb<sup>1</sup> has fewer CCAP-expressing neurons and a resulting reduction in the expression of other ecdysis hormones, Myo-inhibiting Peptide and Bursicon β (29). Because NS cleavage of Gbb is required for the regulation of CCAP, it is conceivable that NS cleavage is required for the production of active Gbb38 when S1/S0 cleavage is blocked. Gbb38 may be produced as a ligand that preferentially activates Wit in the nervous system and acts to regulate hormonal expression throughout development.

**Regulation of receptor binding and preference by Gbb and TGF-β family prodomains**

Our data suggest that the Core domain of the Gbb prodomain influences the preferential use of type II receptors. When the Core domain fragment (NS-S1) is non-covalently associated with Gbb15, signaling activity is permitted with any receptor. In contrast, when the Core domain remains attached to the C-terminal ligand domain (in Gbb38) or to the NH<sub>3</sub>-terminal domain (in the NH<sub>3</sub>-S1 uncleaved prodomain), signaling via the type II receptor Punt is inhibited, whereas signaling via the type

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3 S. Chien, M. Psotka, and K. A. Wharton, personal communication.
II receptor Wit is permitted. These findings are consistent with previous reports that have found that the prodomain from several TGF-β family members can interact with type II receptor-binding surfaces on the ligand. The Core/Arm domain occupies the type II receptor-binding surfaces of the mature ligand in the crystal structures of ligand–prodomain complexes of TGFβ1, Activin A, and BMP9 (8, 16, 68). The type II receptors ACVR2A/B/Punt and BMPR2/Wit primarily differ by the sequence and conformation of the “A loop,” which is adjacent to receptor- and prodomain-binding sites on the mature ligand, and may change conformation upon ligand binding (4, 69). We speculate that the A loop determines the relative ability of each type II receptor to displace prodomains from ligands.

Competition for ligand binding between type II receptors and prodomains has been demonstrated in vitro for BMP7, BMP9, Activin A, Inhibin A, and AMH (15–17, 70–72). However, until now, the in vivo consequences of prodomain–receptor competition have not been established. Although type II receptor usage depends on the alternative cleavage of Gbb, the prodomains of other BMPs and TGF-β family ligands may be able regulate type II receptor preference in vivo even without alternative cleavage. For example, the differential expression of type II receptors with varying abilities to displace prodomains might explain why the BMP10 ligand–prodomain complex is latent in C2C12 myoblasts but not in multiple endothelial cell lines (10, 73).

We found that when NS cleavage is blocked, Gbb forms a latent prodomain–ligand complex that has reduced Punt-dependent signaling activity. Although we observed this latency by mutating the NS cleavage site, cleavage at this site could be regulated in vivo by an as yet unknown mechanism. Latent Gbb could be produced when NS cleavage is blocked by predicted O-glycosylation. Latency of Gbb (Gbb15 + uncleaved prodomain) represents a novel finding for the BMP5/6/7/8 subfamily, which has been shown to form active prodomain–ligand complexes (10, 15, 74). Instead, the activation of Gbb by NS cleavage is analogous to the activation of latent GDF8, GDF11, and BMP10 complexes by Tolloid family metalloproteinases, which cleaves at a site that aligns near the NS site on Gbb (10, 39, 40). From this perspective, the functional similarity of Gbb NS cleavage and GDF8/11 Tld cleavage could represent convergent evolution of a ligand activation mechanism. Additionally, there may be other mechanisms that regulate prodomain–ligand association and latency. We saw that the Gbb prodomain–ligand association was higher at pH 6.5 than at pH 7.4, even when the prodomain is cleaved at the NS site (Fig. 3 and supplemental Figs. S3 and S4). This observation is intriguing because latent TGFβ1 can be activated by physiologically relevant shifts in pH (75). Although the hemolymph of Drosophila larvae is acidic (41), there could certainly be neutral extracellular microenvironments within developing tissues. In such an environment, neutral pH could promote dissociation of the prodomain from Gbb15 or Gbb38, to allow other protein interactions and alter signaling output.

We note that the latency conferred by the uncleaved Gbb prodomain is not absolute. In the presence of endogenously expressed receptors in S2 cells, the uncleaved prodomain reduces but does not completely abolish Gbb15 activity. However, this inhibitory effect is increased when Punt is overexpressed and is alleviated when either Tkv or Sax is overexpressed with Punt. Although the Core/Arm domain of TGF-β family prodomains occupies the type II receptor-binding sites, the N-terminal portion of prodomains forms an α-helix that occupies type I receptor-binding sites (8, 68, 76). Therefore, it is possible that type I receptors cooperate with Punt to effectively displace the uncleaved prodomain from Gbb15. Alternatively, NS cleavage could convert the Gbb prodomain–ligand complex from a closed, inactive form into an open, active form (16, 77).

**Regulation of BMP/Gbb by alternative cleavage**

O-Glycosylation regulates cleavage of several known PC substrates (78). To our knowledge, Gbb represents the first known TGF-β family member where alternative processing is influenced by O-glycosylation. However, there are several indications that O-glycosylation could be a more general regulatory mechanism for TGF-β family member processing. In vitro cleavage of peptides containing the cleavage sites of BMP7 and Inhibin α can be blocked, at least partially, by O-glycosylation (56). Like Gbb, the Inhibin α proprotein contains multiple cleavage sites. Cleavage at only the N-terminal site produces Inhibin αNaC, a Gbb38-like protein that is produced when Inhibin is expressed in 293T cells (79). Alternative processing of Inhibin α could potentially be controlled by expression of specific O-GlcNAc transferases, which differ in their ability to block cleavage of an Inhibin α peptide in vitro (56). Protein processing can also be regulated by phosphorylation that blocks O-glycosylation but not PC cleavage. The Golgi kinase Fam20c permits FGF23 cleavage by furin by blocking inhibitory O-glycosylation (80). Intriguingly, the PCV spur and ectopic L1 vein phenotype we observe in gbbRmNS gbb1 also have been observed in mutants of the Drosophila Golgi kinase Fj (81, 82). Conceivably, Fj might be necessary to block O-glycosylation that is predicted at the NS site (Fig. 9A) and thus permit PC cleavage. Further studies are warranted to examine the potential impact of Fj and protein phosphorylation on Gbb processing and activity during pupal wing vein formation.

**Conclusion**

We find that the maturation of different BMP ligand forms by alternative processing can lead to differential activation of receptors and specific signaling outputs. Cleavage of proGbb at its S1/S0 site is blocked by O-glycosylation in 3rd instar larvae, resulting in the production of Gbb38 that has preference for Wit-mediated signaling. Cleavage at the NS site is required for Gbb15 homodimer signaling via the type II receptor Punt but not for Gbb–Dpp heterodimers. Because many other TGF-β family prodomains have predicted PC cleavage sites within their prodomains, and mutations in these predicted sites are associated with human developmental abnormalities (18), we suggest that alternative processing is an important mechanism for regulating TGF-β family signaling activity and receptor preference.
Regulation of BMP/Gbb by alternative cleavage

Experimental procedures

Drosophila strains and cell lines

Wild-type strains used in lines were w1118 or b pr cn bw. Rescue strains gbbRgbb and gbbR\textsuperscript{SENS} gbb, produced by inserting a gbb genomic fragment at 53B2 using the dC31 system, were described previously (referred to as gbbR-gbb gbb\textsuperscript{2} in Ref. 18). gbbR\textsubscript{AA} gbb\textsuperscript{2} was generated in the same way, with an HA tag inserted in the gbb coding sequence between residues 351 and 352. dpp\textsuperscript{112} gbb\textsuperscript{1}/CyO and dpp\textsuperscript{phor} gbb\textsuperscript{1}/CyO strains were created by recombination. CCAP-Gal4, obtained from the Bloomington Drosophila Stock Center (BDSG no. 25686), and UAS-GFP were recombined with gbb\textsuperscript{1}, gbbRgbb\textsuperscript{1}, and gbbR\textsuperscript{SENS} gbb\textsuperscript{2}. Drosophila strains were maintained on standard cornmeal/sugar/yeast media supplemented with live yeast.

Schneider 2 (S2) cells were obtained from the Drosophila Genomics Resource Center (DGRC no. 6), and were maintained in M3 media supplemented with 10% Insect Media Supplement (Sigma, I7267) and 2% FBS (Corning 35-010CV). Cells were passaged weekly and used for up to 25 passages.

DNA constructs

Constructs containing the gbb-coding sequence and UTR, with mNS (R123G/R126G), mSI (R322G/R325G), and an HA tag inserted between residues 351 and 352, were described previously (18). The m50 mutation, K334N (19), was introduced in pDONR221-gbb constructs by site-directed mutagenesis using primers 5’-CACGCAGGCAATAGGCGGTCG-3’ and 5’-CGACACCAGCATTATGGCGGTG-3’. Correctly mutated transfomants were confirmed by sequencing. Gateway cloning was used to transfer pDONR221 (Invitrogen no. 12536017) inserts into pAW (DGRC no. 1127) for constitutive expression and into pMT-DEST48 (Invitrogen no. 11282018) for metallothionein-induced expression.

Expression constructs for type I receptors, pAWF-sax and pAWF-tkv, were described previously (18). Expression constructs for type II receptors, pAWH-punt and pAWH-wit, were created by in-frame insertion of punt and wit CDNA (a gift of M. O’Connor) into the pAWH expression vector containing a C-terminal 3×HA tag (DGRC no. 1096). pAW-Dpp-HA was constructed by site-directed mutagenesis of pAW-Dpp, inserting a 1×HA tag between residues 485 and 486 of Dpp. Expression constructs pBRACP A-dSmad2-FLAG, pBRACP A-Daw, and pAC-Mad-FLAG were kindly provided by M. O’Connor (42). pCoPuro was used for puromycin selection of stably transfected cells (83).

Antibodies

Rabbit polyclonal antisera was raised against peptides corresponding to specific regions within the Gbb prodomain (Pierce Custom Antibody Services). α-GbbN was raised against peptide GKDQTIMHRLVSEDDKD, corresponding to aa 46–61 of pre-proGbb. α-GbbCore was raised against peptide SADLEE-DEGEQQKNFIDT, corresponding to aa 127–144 of pre-proGbb. Antisera from multiple animals and batches were selected for maximum specific signal. For use in immunoprecipitations and to reduce background on Western blottings, antibodies were purified by affinity to their respective peptide antigens.

Remaining primary antibodies include mouse α-Dac (Developmental Studies Hybridoma Bank (DSHB) 1-1), mouse α-FLAG (Sigma, M2), mouse α-GbbC (DSHB 3D6-24 (18)), chicken anti-GFP (Invitrogen, A10262), and rat α-HA (Roche Applied Science, 3F10). Rabbit α-pSmad2 (Cell Signaling Technologies, 3108) was used to detect phosphorylated Smad2, and rabbit α-phospho-p38 (Thermo Fisher Scientific, MA5-15182) was used to detect phosphorylated p38. Rabbit α-pMad was an antibody raised against human pSmad3, which has an identical phosphorylated C-terminal SSVS motif that is not shared with the Drosophila Smad3 ortholog (Abcam, EP823Y (84)).

Secondary antibodies used were goat α-chicken-IgY:AF488 (Invitrogen, A11039), goat α-mouse-IgG:AF568 (Invitrogen, A11004), goat α-mouse-IgG:HRP (Jackson ImmunoResearch, 115-035-146), goat α-mouse-IgGLC-HRP (Millipore, AP200P), goat α-rabbit-IgG:AF633 (Invitrogen, A21070), mouse α-rabbit-IgG:CHC:HRP (Abcam, 2A9), and goat anti-rat-IgG:HRP (Jackson ImmunoResearch, 112-135-167). Clean-Blot (Thermo Fisher Scientific, 21230) was used to specifically detect folded primary antibodies on Western blottings without interference from denatured antibodies used for immunoprecipitation.

Statistical analysis

All statistical analysis was done in R 3.3.1. Data transformations were determined using the Box-Cox procedure. All statistical inference uses multiple-comparison adjusted tests. Inference of continuous numerical data was done using the general linear hypothesis test (GLHT) using the glht() function in the package multcomp (85). To control the family-wise error rate, the family of hypothesis was explicitly defined for each analysis, and it included all orthogonal pairwise comparisons, including a control condition. When comparing multiple treatments with a single control, this approach is identical to Dunnett’s multiple comparison test. Observations with studentized residuals of >5 were rejected as outliers. For experiments that were repeated multiple times, data were pooled and analyzed using “experiment” as a co-variate. The Wilcoxon rank-sum test was used for discrete numerical data, and Fisher’s exact test was used for categorical data, controlling for multiple comparisons using the false discovery rate (FDR) (86).

Protein sample preparation

S2 cells were transiently transfected with pAW-gbb-HA or dpp-HA expression constructs containing the indicated cleavage site mutations, using Effectene transfection reagent (Qiagen). At 3 days post-transfection, cells were resuspended and pelleted by centrifugation. Media samples were taken from the supernatant, and 0 or 100 mM DTT and NuPAGE 4× LDS sample buffer (Novex) was added. Cell lysate samples were made by adding 1× LDS sample buffer with 100 mM DTT to cell pellets, to a volume equivalent to media samples. We observed significant and potentially confounding protein degradation when using Laemmli sample buffer or 95 °C heating, so instead samples were heated for 65 °C for 10 min using the NuPAGE sample buffer.
**Western blottings**

SDS-PAGE was run on NuPAGE bis-tris 12% acrylamide gels with MOPS buffer (Novex) or modified Tris/Tricine 10% acrylamide gels (87). Protein was transferred to PVDF membranes using a Trans-Blot semi-dry transfer apparatus (Bio-Rad) at 10 V for 1 h, with transfer buffer containing 100 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked with 5% BSA in tris-buffered saline with 0.1% Tween 20 (TBSTw) or with protein-free blocking solution (Thermo Fisher Scientific, 37570) supplemented with 0.1% Tween 20. Primary antibodies and secondary antibodies were diluted in blocking buffer. Primary incubations were done shaking overnight at 4 °C, followed by washes in TBSTw. Secondary incubations with HRP-conjugated antibodies were done for 1 h at 4 °C, followed by TBSTw washes. Blots were imaged using a chemiluminescent substrate (Pierce Supersignal West Dura, no. 34075) and a digital imaging system (Kodak IS4000S or Bio-Rad Chemidoc XRS). For reprobing, blots were stripped with Restore acidic glycine stripping buffer (Thermo Fisher Scientific). Bands were quantified with ImageJ (National Institutes of Health), using the gel tool to take lane profiles and measure the integrated peak signal.

**Immunoprecipitation**

S2 cells were transiently transfected with the indicated pAW-gbb-HA expression construct. At 3 days post-transfection, 1 ml of conditioned media was concentrated ×50 with 10,000 MWCO centrifugal ultrafiltration units (Millipore Amicon Ultra-4) and diluted with IP buffer (25 mM bis-tris, pH 6.5, or Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5% glycerol). Primary antibodies were added to the media concentrates and incubated for 1 h rotating at 4 °C. Antibodies were pulled down with magnetic protein G Dynabeads (Novex), washed three times with IP buffer and one time with water. Samples were prepared by adding 1 × NuPAGE LDS sample buffer with 100 mM DTT and heating for 10 min at 65 °C. Western blottings were done as described previously, except using secondary detection reagents with minimal cross-reactivity to the immunoprecipitating antibodies. For α-GbbN and α-GbbC, Clean-blot (Thermo Fisher Scientific) was used to detect natively folded primary antibodies. For α-GbbG, goat α-mouse-IgG-LC:HRP (Millipore AP200P) was used to detect specific signals without interference from the mouse IgG heavy chain and with minimal interference from rat IgG heavy or light chains.

**Heparin affinity chromatography**

Conditioned media from S2 cells expressing gbb-HA were loaded on a 1-ml HiTrap heparin HP column (GE Healthcare) operated with a peristaltic pump at 1.0 ml/min. The column was washed with 10 volumes of a 10 mM sodium phosphate, pH 6.0−7.4, gradient, followed by 10 volumes of a 10 mM sodium phosphate, 0−1 mM NaCl gradient. 1-ml fractions were manually collected, precipitated with TCA, and resuspended in 100 μl of sample buffer for analysis by Western blotting.

**Furin and glycosidase assay**

HA-tagged Gbb was immunoprecipitated from gbbHAgbb3 wandering 3rd instar larvae. Larvae were ground in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5% glycerol, 0.1% SDS) using a glass tissue grinder on ice. Crude lysate was centrifuged to remove cuticle, debris, and fat. Cleared lysate was incubated for 1 h with α-HA magnetic beads (Pierce no. 8836). Each IP contained 100 μg of α-HA beads and the lysate of 68 mg of larvae (~20 larvae). Beads were washed twice with lysis buffer, and twice with furin/glycosidase reaction buffer (25 mM Tris, pH 7.5, 50 mM KCl, 5 mM CaCl2, 0.5% Triton X-100). Samples were briefly denatured by heating for 5 min at 65 °C with 1 mM β-mercaptoethanol. Samples were treated with the indicated glycosidases for 1 h at 37 °C, using 20,000 units of O-glycosidase (New England Biolabs, P0733), 5 units of β-N-acetylhexosaminidase (New England Biolabs, P0721), or 0.1 unit of β-glucuronidase (Sigma, G8295). After another 5-min 65 °C incubation, samples were treated overnight at 37 °C with 4 units of furin (P8077S) and analyzed by Western blotting.

**Cell signaling assays**

The BrkSE-lacZ assay was used to measure BMP signaling activity in S2 cells (34). In summary, the lacZ reporter expression is activated by co-transfected Notch intracellular domain (NICD) and Su(H). lacZ expression is quantitatively silenced by binding of the pMad−Med−Shn complex to the Brinker silencer element (BrkSE). For signaling assays, 2 × 105 S2 cells were plated in 96-well plates. Cells were transfected with 5 ng of BrkSE-lacZ, 7 ng of NICD, 7 ng of Su(H), 1 ng of pAc-luciferase as a transfection control, and a total of 50 ng of gbb, dpp, or receptor expression constructs, using Effectene transfection reagent (Qiagen). At day 3 post-transfection, luciferase and β-galactosidase activity was measured with the Dual-Light assay (Novex). Repeated assays of identical samples yielded consistent β-galactosidase activity (R² = 0.93), but luciferase activity was far less consistent (R² = 0.57), so we only include β-galactosidase activity in our analysis. Each BrkSE-lacZ signaling experiment used 3−6 technical replicates per condition and was repeated three times in total. For analysis, data from all three replicates was pooled and fit to a model of the form −log(BrkSE-lacZ) = βtransfection + βexperiment and GLHT was used for multiple-comparison-adjusted inference of differences between transfections.

For measurement of BMP signaling activity by pMad levels, pAc-Mad−FLAG transfected cells were treated with conditioned media containing Gbb or with rHBMP7 (Thermo Fisher Scientific, PHC9544). Conditioned media were collected from S2 cells stably transfected with pMT empty vector or pMT-gbb-HA constructs, and induced with CuSO4 in serum-free media. Ligand concentration was measured by Western blotting. Cleavage mutant conditioned media were diluted to match wild-type ligand concentration, using pMT (empty vector)-conditioned media. At the indicated time points, media were aspirated from centrifuged cell pellets, and cell lysate samples were prepared for Western blotting. Signaling activity was measured as the ratio of pMad to FLAG-tagged Mad band intensities. Phosphorylation of p38 was measured by stripping and re-probing using an phospho-p38 antibody, using 200 mM NaCl-treated cells as a positive control for p38 activation (88). Measurements of phosphorylated dSmad2 were performed by...
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treating pBRAcpA-dSmad2-FLAG-transfected cells with Gbb- or Daw-conditioned media and examining phospho-dSmad2 by Western blotting.

Microscopy

For measurements of wing phenotypes, gbbR* gbb\(^{-}\)/T2;3 females were crossed with gbb\(^{+}\)/CyO, dpp\(^{112}\) gbb\(^{+}\)/CyO, or dpp\(^{-}\)/gbb1/SM6A males. Wings were dissected from male and female Cy\(^{+}\) progeny and mounted in Gary’s Magic Mounting Media between two coverslips to permit imaging from both sides. The dorsal face of each wing was imaged using a Microphot FXA compound microscope (Nikon), using a Plan 4/0.13 objective (Nikon 208135) and a D600 digital SLR camera (Nikon). Images were blinded for scoring the presence and severity of wing vein abnormalities. Representative images were selected from female wings with median phenotypes and good image quality.

For measurements of pupal phenotypes, pupae were mounted on 1% agarose plates and observed each day. At 90 – 116 h after pupariation, pupae were imaged from the ventral side with an oblique transmitted light source with a Zeiss Lumar V12 stereo microscope and AxioCam Mrc5 camera. Images were blinded for measurements of leg extension in ImageJ, taken as the ratio between the pharate head to tail and head to leg tip length.

For examination of CCAP expression the larval VNC, CCAP-Gal4 gbbR gbb\(^{+}\)/T2;3 was crossed to UAS-GFP gbbR gbb\(^{+}\)/CyO GFP, containing either the wild-type or mNS rescue construct. Tb\(^{+}\), CyO GFP-wandering 3rd instar larvae were selected for dissections. Larvae were filleted, and all organs that might obscure the VNC, including the lobes of the CNS, were removed. Fillets were marked according to genotype and fixed for 20 min in PBS plus 4% paraformaldehyde, and all genotypes were combined in a single tube. Fillets were blocked for 60 min with 10% normal goat serum in PBS plus 0.3% Triton X-100 (PBSTr). Primary incubation with α-GFP, α-Dac, and α-pMad was done rotating at 4 °C overnight, followed by washes in PBSTr. Secondary incubation with fluorophore-conjugated secondary antibodies was done rotating for 1 h at 4 °C, followed by washes with PBSTr and a 10-min stain in 1 μg/ml Hoechst. Fillets were equilibrated overnight in 80% glycerol, 0.5% n-propyl gallate mounting media at 4 °C, and mounted the next day. The VNC was imaged on a Zeiss LSM800 confocal laser-scanning microscope, with a ×40/1.3 Zeiss Plan-APOCHROMAT objective. Image stacks were obtained with 0.16-μm horizontal resolution and 1.0-μm slice thickness and blinded for analysis. For each CCAP neuron from the 3rd thoracic segment to the 9th abdominal segment, CCAP > GFP signal intensity was quantified in cell bodies, and pMad nuclear intensity was quantified for each CCAP neuron using ImageJ. Images with approximately median numbers of CCAP neurons, CCAP > GFP intensity, and nuclear pMad intensity were selected as representative images.

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