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Different Requirements for Proteolytic Processing of Bone Morphogenetic Protein 5/6/7/8 Ligands in *Drosophila melanogaster**[S]

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Background: Bone morphogenetic proteins require proteolytic processing to generate the mature ligand.
Results: The BMPs Gbb and Scw have three cleavage sites, and BMP7 has one, which are differentially required to produce the functional ligand.
Conclusion: Gbb, Scw, and BMP7 have distinct processing requirements despite being closely related.
Significance: Rapid evolution of cleavage sites is a general mechanism for fine-tuning BMP ligand activity to function.

Bone morphogenetic proteins (BMPs) are synthesized as proproteins that undergo proteolytic processing by furin/subtilisin proprotein convertases to release the active ligand. Here we study processing of BMP5/6/7/8 proteins, including the *Drosophila* orthologs Glass Bottom Boat (Gbb) and Screw (Scw) and human BMP7. Gbb and Scw have three functional furin/subtilisin proprotein convertase cleavage sites; two between the prodomain and ligand domain, which we call the Main and Shadow sites, and one within the prodomain, which we call the Pro site. In Gbb each site can be cleaved independently, although efficient cleavage at the Shadow site requires cleavage at the Main site, and remarkably, none of the sites is essential for Gbb function. Rather, Gbb must be processed at either the Pro or Main site to produce a functional ligand. Like Gbb, the Pro and Main sites in Scw can be cleaved independently, but cleavage at the Shadow site is dependent on cleavage at the Main site. However, both Pro and Main sites are essential for Scw function. Thus, Gbb and Scw have different processing requirements. The BMP7 ligand rescues *gbb* mutants in *Drosophila*, but full-length BMP7 cannot, showing that functional differences in the prodomain limit the BMP7 activity in flies. Furthermore, unlike Gbb, cleavage-resistant BMP7, although non-functional in rescue assays, activates the downstream signaling cascade and thus retains some functionality. Our data show that cleavage requirements evolve rapidly, supporting the notion that changes in post-translational processing are used to create functional diversity between BMPs within and between species.

Members of the transforming growth factor-β (TGFβ) superfamily undergo proteolytic processing to generate the active ligand (1). These proteins are synthesized as proproteins consisting of a large, N-terminal prodomain followed by a highly conserved ligand domain. Nascent polypeptides are translocated into the endoplasmic reticulum, where they form homodimers or heterodimers with other family members and then traffic to the Golgi where they are proteolytically cleaved by subtilisin-like proprotein convertases (SPCs[3] Refs. 2 and 3). Upon cleavage, the prodomain may be shed and the ligand secreted in its active form (4), or the prodomain and ligand domain may remain associated in a latent complex that is secreted from the cell and subsequently activated extracellularly (5–10).

The bone morphogenetic proteins (BMPs) are a family of TGFβ-like proteins that play key roles in development and disease (11). BMP processing has been studied in detail for members of the BMP2/4/Dpp subgroup, where it has been shown that maturation requires sequential cleavage at multiple sites that lie between the prodomain and ligand domain. In *Xenopus*, BMP4 is first processed at an optimal furin site (S1) adjacent to the ligand domain and then at a second site (S2) just upstream (12). It has been proposed that cleavage at the S1 site generates an unstable prodomain-ligand complex that acts as a short range signal, whereas processing at both sites allows for dissociation of the prodomain and liberates a stable ligand that can signal at long range (4, 9). Processing of the *Drosophila* BMP4 ortholog, Decapentaplegic (Dpp), is similar, although the order of cleavage steps is reversed with processing at the upstream S2 site preceding processing at a downstream site (13, 14). Functional studies suggest that cleavage at the S2 site is essential for long range gradient formation (13), as proposed for BMP4. Consistent with this, it has been shown that cleavage at the S1 site only occurs in tissues that require short range signaling, whereas cleavage at the S1 and S2 sites occurs in tissues that require long range signaling (14).

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[S] This article contains supplemental Figs. S1–S3 and Tables S1–S3.
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[3] The abbreviations used are: SPC, subtilisin-like proprotein convertases; BMP, bone morphogenetic proteins; Dpp, decapentaplegic; Bs, Blistered.

These studies on BMP4 and Dpp support the long-standing notion that proteolytic processing and dissociation of the ligand from the prodomain are essential steps in BMP maturation. However, processing in the BMP5/6/7/8 subgroup has not been studied in detail. The fly genome has two BMP5/6/7/8 orthologs, Scw and Gbb, both of which heterodimerize with Dpp (15, 16). Scw-Dpp heterodimers are required for the specification of the embryonic dorsal-ventral axis, whereas Gbb homodimers or Gbb-Dpp heterodimers are required for cell proliferation and patterning in imaginal discs and maintenance of the germ line stem cell fate in the ovary (17, 18).

Here we investigate the processing requirements for BMP5/6/7/8 ligands in *Drosophila*. Our data show that Gbb, Scw, and human BMP7 each have different processing requirements. Both Gbb and Scw have two cleavage sites at the junction between the prodomain and ligand domain and a novel furin cleavage site within the prodomain. Although cleavage can occur at all of these sites, the two proproteins require different cleavages to produce a functional ligand. Human BMP7, which is functional in *Drosophila*, has only one furin cleavage site that lies between prodomain and ligand domain, and cleavage at this site is essential for function. However, unlike Gbb and Scw, the cleavage-resistant BMP7 protein retains some signaling capacity. Based on these results, we propose that modulation of proprotein processing is part of the evolutionary toolkit that TGFβ-like proteins use to fine-tune ligand function in different developmental contexts.

EXPERIMENTAL PROCEDURES

Drosophila Strains—The following fly strains were used in this study: *gbb*^{D20}, *Df(2R)S246*, *gbb*^{4}, *Dp(2;2)DTD48 gbb*^{4}, and *UAS=Gbb9.1* (19–21), *Df(2L)OD16* and *scw*^{S12} (22), and *Tub=Gal80*^{ts} (from Bruce Edgar).

Rescue and Expression Constructs—Gbb and Scw cleavage mutant rescue constructs were generated in pCaSpeR4 (23) and pattB (24), respectively, using a 3.0-kb Gbb genomic fragment that rescues null *gbb* mutations or a 5.1-kb Scw genomic fragment that rescues *scw* mutations (25). FLAG tags were inserted into the Scw and Gbb coding sequences at amino acid positions 298 and 351, respectively. The Gbb-BMP7 chimera was constructed by swapping the ligand domain in the Gbb rescue construct with that of BMP7 and the full-length BMP7 construct by swapping the prodomain in the Gbb-BMP7 chimera with that of BMP7. For overexpression studies, a fragment from each FLAG-tagged Gbb construct was shuttled into the pUAS-attB vector and integrated into the attP landing site at 86Fb. For tissue culture assays, the Scw and Gbb coding sequences were cloned into pMT-V5-HisA. An N-terminal HA tag was inserted into the Scw coding sequence at residue 19.

Rescue Assays and Immunohistochemistry—For rescue assays, multiple transgene insertions on the third chromosome or single insertions at the attP landing site in 86Fb were tested for their ability to rescue the genotypes *gbb*^{D20}*/Df(2R)S246* or *scw*^{S12}*/Df(2L)OD16*, as described in Tables 1–3. For rescue of *gbb*^{4} homozygotes, males of the genotype y^{1} w^{1118}; *bw gbb*^{4}*/+; Tn***/+* were crossed to y^{1} w^{1118}*;Dp(2;2)DTD48 bw gbb*^{4} females, and homozygous *gbb*^{4} progeny were recognized by the pink eye color produced by the combination of the w^{+} transgene and the homozygous *bw* mutant. For the Pro-Main double and Pro-Main-Shadow triple, males of the genotype (*y) w;bw gbb*^{4}*/If;Tn***/+* were crossed to y^{1} w^{1118}*;Dp(2;2)DTD48 bw gbb*^{4} females, and the *If*+ progeny were scored.

To assess for BMP pathway activation by Gbb and BMP7 cleavage mutants in the pupal wing, the Gal4/*Gal80*^{ts} system was used (26). y^{1} w^{1118}*;UAS=X/TM3, Ser GFP* males were crossed to w^{1118}*;en-Gal4/CyO,GFP;Tub-Gal80*^{ts}*/TM6b,Tb* females, and progeny were raised at 18 °C until pupariation. GFP^{-} prepupae were selected and shifted to 29 °C, aged for 32 h, and then dissected, fixed, and stained as described previously (27) using anti-DSRF (Active Motif, 1:1000) and Alexa-Fluor-488 anti-mouse (Molecular Probes, 1:1000). To detect actin, wings were incubated at room temperature for 30 min with a 1:400 dilution of Alexa-Fluor-647 Phalloidin (Molecular Probes).

Transfection—To express Gbb, Scw, and BMP7 proteins, *Drosophila* S2R^{+} cells were transfected with 2 μg of DNA using Effectene (Qiagen). Protein expression was induced 24 h after transfection with 500 μM $CuSO_4$ and cultures were harvested 72 h later. Cells were pelleted by centrifugation at 2000 $\times$ *g* for 4 min at 4 °C, and the supernatant medium was affinity-purified using anti-FLAG-M2-agarose (Sigma).

RESULTS

Furin Cleavage Sites in Drosophila BMP5/6/7/8 Orthologs Gbb and Scw—Furin recognizes the optimal core consensus R*X*(R/K)R (28), with cleavage after the final arginine, whereas generalized SPCs recognize the less strict consensus RX_nR, where *n* can be 0, 2, 4, or 6 (29). For both furin and SPCs, the probability of cleavage at a site depends on this core motif and on the residues that flank it (30). Therefore, to identify candidate cleavage sites, we scanned the amino acid sequences of Gbb and Scw with the ProP 1.0 algorithm that assigns a probability of cleavage by furin or a generalized SPC to each Arg or Lys residue in the protein (30).

Gbb and Scw both have a high probability furin cleavage site 29 amino acids N-terminal to the first cysteine of the ligand domain (Fig. 1*A*), which we refer to as the Main site (Arg-325 and Arg-271 in Gbb and Scw, respectively). Gbb has a second low probability furin site just C-terminal to the Main site, and the equivalent position in Scw has a pair of general SPC sites. We refer to these sites in Gbb and Scw as Shadow sites (Lys-335 in Gbb and Arg-276/Arg-277 in Scw). Gbb and Scw have additional furin cleavage sites within the prodomain that do not correspond to sites identified in other BMPs. In Gbb, there is a single high probability furin cleavage site at Arg-126, and there is an orthologous site in the Scw protein at Arg-91. We refer to this site as the Pro site. In Scw, there is an additional furin cleavage site in the prodomain at Arg-54, which we call the Pro2 site.

Cleavage of Gbb in Tissue Culture—To address whether the three predicted furin sites in Gbb are cleaved *in vivo*, we characterized Gbb processing in tissue culture cells (Fig. 2). Transfection of wild type Gbb produces a 19-kDa form that corresponds to the fully processed ligand domain (Fig. 2*B*, *lane 2*). This form is generated by cleavage at both the Main and Shadow sites as mutation of either does not eliminate the

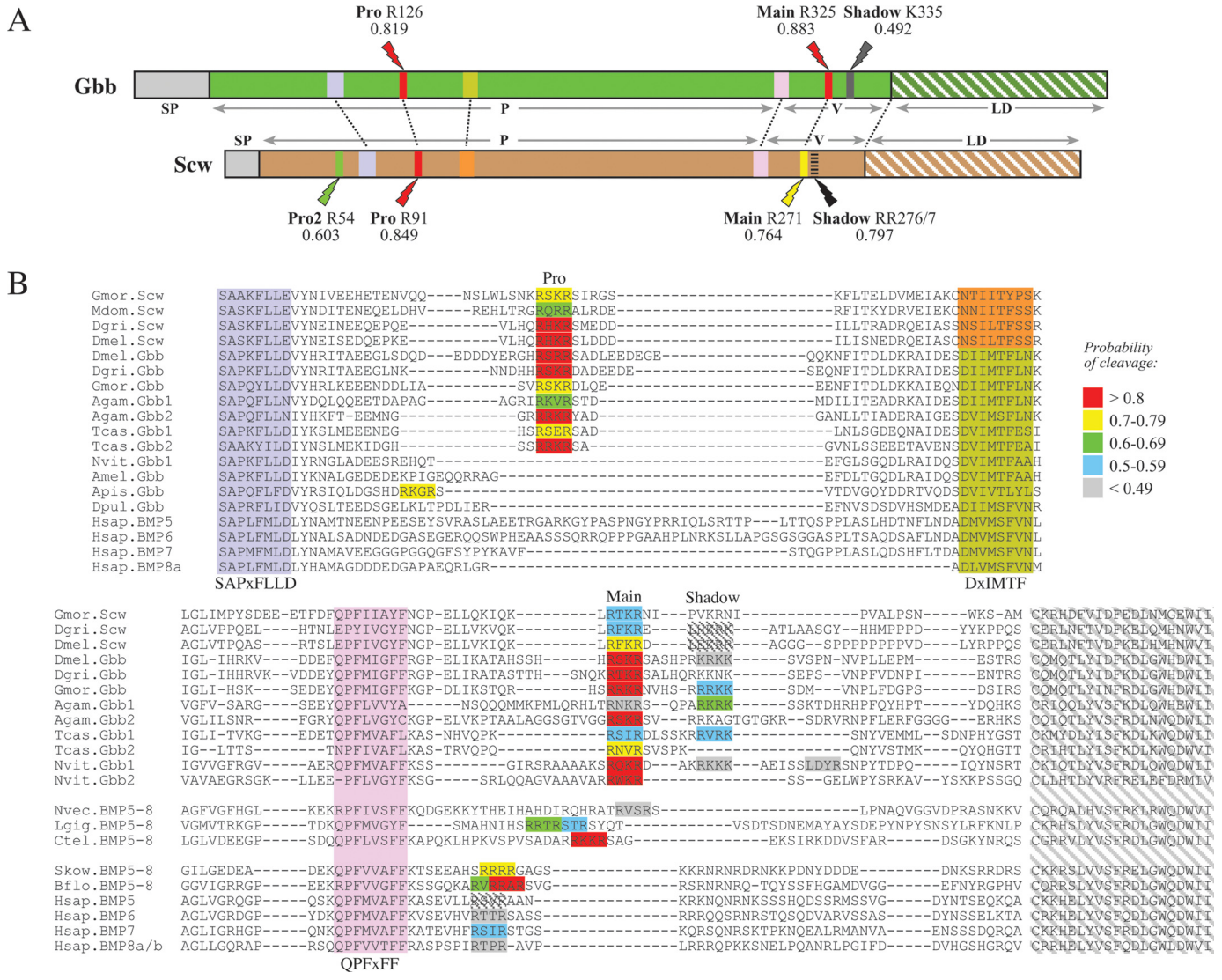

FIGURE 1. **Proprotein convertase cleavage sites in Gbb and Scw and their phylogenetic conservation.** *A*, schematic diagrams of Gbb (*green*) and Scw (*tan*) proteins show the signal peptide (*SP*, *gray box*), prodomain (*P*), variable region (*V*), and ligand domain (*LD*, *hatched box*). Potential furin cleavage sites are indicated with *lightning bolts* color-coded according to the probability of cleavage as indicated by the heat map in *B*. The SPC cleavage sites in the Scw variable region are indicated with a *black lightning bolt*. Conserved sequence motifs are shown as colored blocks (*blue*, *ochre*, *pink*) that appear in the alignments in *B*. *B*, alignments of the Pro site (*upper*) and Main and Shadow sites (*lower*) show representative sequences from the comprehensive alignments in supplemental Figs. S1 and S2.

19-kDa band, but it is lost when both sites are mutated (Fig. 2*B*, *lanes 4–6*). However, as there is only weak accumulation of the mature form when the Main site is mutated, efficient cleavage at the Shadow site requires prior cleavage at the Main site (Fig. 2*B*, *lanes 4* and *7*). The Pro site is also cleaved, as the Main site and Main-Shadow double mutant give rise to a 45-kDa band that is lost in the Pro-Main and Pro-Main-Shadow mutants (Fig. 2*B*, *lanes 7* and *8*, *cf. lanes 4* and *6*). In the latter mutants, the 45-kDa form was replaced with a 55-kDa form that corresponds to the full-length proprotein. Taken together, these data indicate that all three furin sites in Gbb are cleaved *in vivo*.

Function of Gbb Cleavage Mutants in Vivo—Previously, we generated a genomic rescue construct that fully rescues *gbb* mutants (25) (Table 1). We engineered our cleavage site mutations into this construct and introduced the mutated versions into flies to test for rescue of gene function. The wild type Gbb construct fully rescues *gbb* null mutants independent of insertion site, as do the constructs bearing the Pro, Main, or Shadow single mutations (Table 1, supplemental Table S1). Thus, none of these sites is essential for Gbb function as long as the others are present. The rescue observed with the Pro and Shadow site mutants is consistent with our tissue culture data as both of these mutants give rise to fully processed ligand (Fig. 2*B*). The Main site mutation, however, produces a low level of mature ligand, raising the possibility that the partially processed 45-kDa species is also functional. Indeed, the Main/Shadow double mutant also fully rescues, demonstrating that Gbb processed at the Pro site is capable of signaling normally *in vivo*. By contrast, the Pro/Main double mutant and the Pro/Main/Shadow triple mutant do not rescue *gbb* mutants (Table 1). Thus, Gbb must be processed at either the Pro or Main site to produce an active ligand.

Comparing the rescue data with our tissue culture data, our results suggest that the presence of either the 19-kDa fully pro-

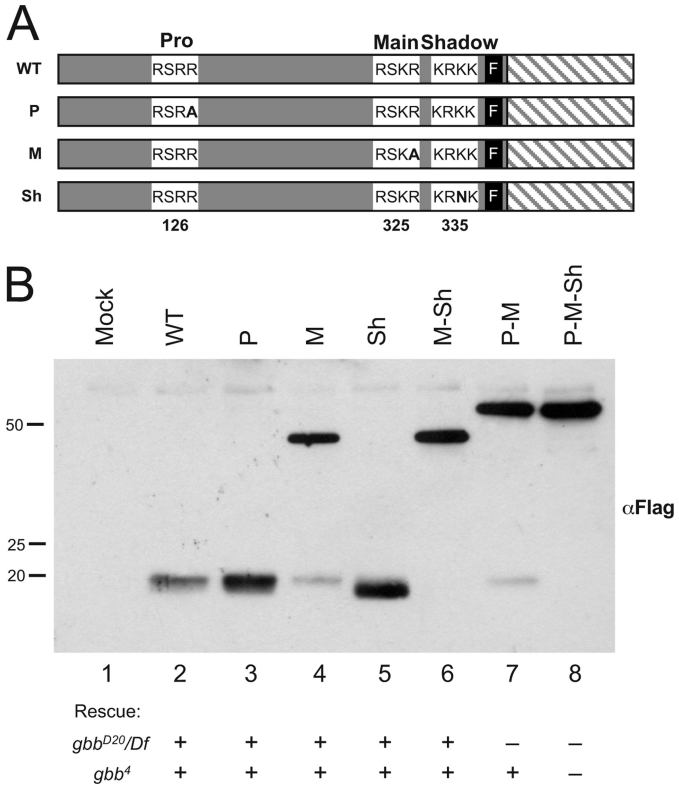

FIGURE 2. **Processing of Gbb in tissue culture cells.** *A*, diagrams of the Gbb protein show the cleavage sequences for the Pro, Main, and Shadow sites in *black*. The amino acid changes used to inactivate the site are *highlighted in bold*. The position of the last residue in the core sequence is indicated *beneath the site*. The position of the FLAG tag is indicated (*F*). *B*, a Western blot with an anti-FLAG antibody shows the FLAG-tagged Gbb proteins affinity-purified from the media of transfected S2R$^+$ cells. A summary of the rescue data shown in Table 1 and Fig. 4 is shown *beneath the blot*. *P*, Pro site mutation; *M*, Main site mutation; *Sh*, Shadow site mutation.

TABLE 1

Rescue of *gbb* mutants with transgenes bearing mutations in the Furin cleavage sites

Progeny were from a cross of $y^1\ w^{1118}$;*Df(2R)S249/Bl;Tn/+* x $y^1\ w^{1118}$;*pr cn* gbb^{D20}/*SM6a.* The percent rescue was calculated by dividing the number of gbb^{D20}/*Df;Tn+* progeny by the number of gbb^{D20}/*Bl;Tn+* progeny and multiplying the quotient by 100. For the complete data set, see supplemental Table S1.

Transgene	Rescue
	%
Gbb-FLAG-3	116
Gbb-FLAG-21	96
Gbb-R126A-4 (Pro)	110
Gbb-R126A-10 (Pro)	98
Gbb-R325A-8 (Main)	101
Gbb-R325A-26 (Main)	105
Gbb-K334N-13 (Shadow)	90
Gbb-K334N-1 (Shadow)	112
Gbb-R325A K334N-18 (M Sh)	135
Gbb-R126A R325A-9 (Pro M)	0
Gbb-R126A R325A K334N-16 (Pro M Sh)	**0**

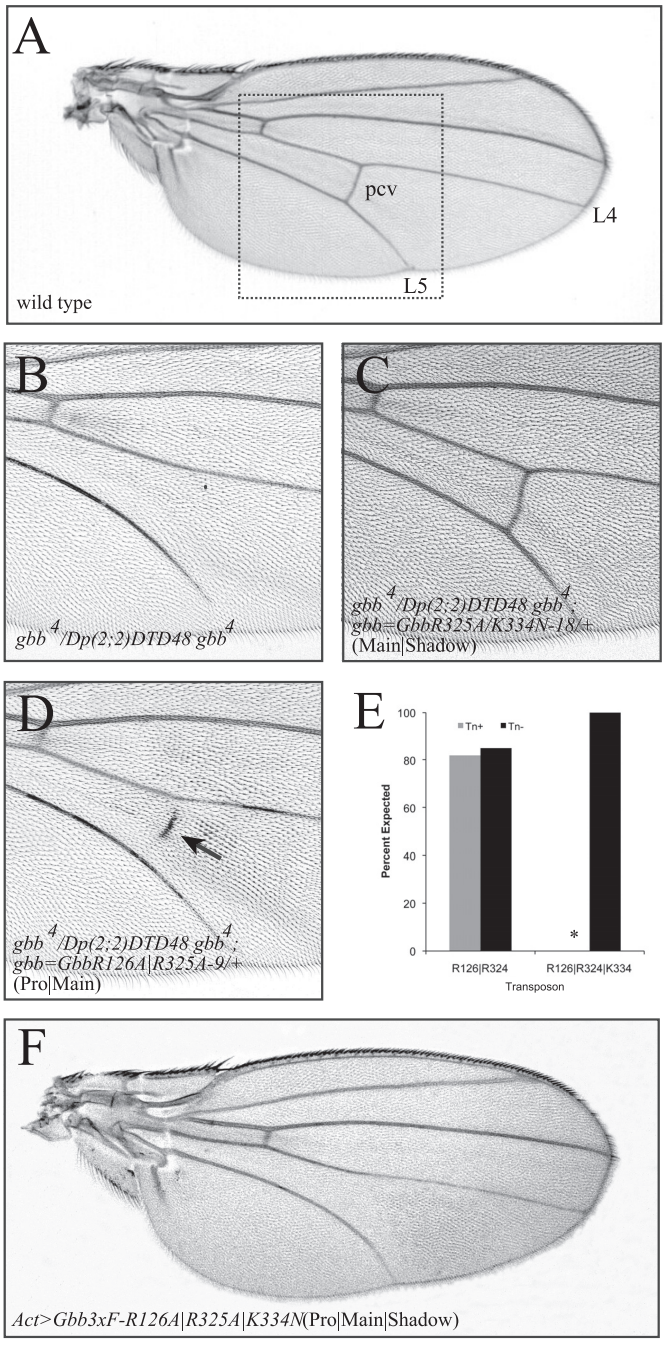

FIGURE 3. **Rescue of the cross-vein defect of *gbb*^{4} homozygotes by Gbb cleavage mutants.** *A*, shown is a wild type wing indicating the posterior cross-vein. The region of the wing shown in *panels B–D* is *boxed*. *B*, *gbb*^{4} homozygotes are semi-viable, and adults show loss of the posterior cross-vein. *C*, rescue of the *gbb*^{4} cross-vein defect by the main/shadow double mutant. *D*, partial rescue by the pro/main double mutant results in a crossvein fragment in the L4-L5 intervein (*arrow*). *E*, survival of *gbb*^{4} homozygotes in the presence (*gray*) or absence (*black*) of one copy of the pro/main double mutant or the pro/main/shadow triple mutant is shown. *F*, mis-expression of the triple mutant with the *Act5C-Gal4* driver is shown which phenocopies the *gbb*^{4} cross-veinless phenotype.

cessed form or the 45-kDa partially processed form is sufficient to rescue the lethality associated with *gbb* mutants. If true, the inability of the Pro/Main double mutant to rescue *gbb* lethality (Table 1) could be explained by the low level of mature ligand produced by this mutant (Fig. 2*B*, *lane 7*). To test this, we determined whether the Pro/Main double mutant could rescue defects associated with a hypomorphic allele, *gbb*^{4}. *gbb*^{4} homozygotes are semi-viable, and the adult escapers have venation defects that include loss of the posterior cross-vein (21). It has previously been shown that a duplication of *dpp*, *Dp(2;2)DTD48*, rescues the lethality, but not the cross-vein defect, of *gbb*^{4} homozygotes (Ref. 20; Fig. 3*B*) and that this venation defect is particularly sensitive to even low levels of functional Gbb. Consistent with our rescue data with the null allele, the Pro, Main,

Shadow, and Main/Shadow cleavage mutant constructs completely rescue the *gbb*^{4} cross-vein defect (Fig. 3*C*, data not shown), and the Pro-Main double mutant, which cannot rescue *gbb* nulls, shows partial rescue of the *gbb*^{4} cross-vein defect (Fig. 3*D*). Thus, consistent with the tissue culture data, which shows that the Pro/Main double mutant produces a small amount of mature ligand, this mutation does produce functional Gbb.

Interestingly, although we would expect the Pro/Main/Shadow triple mutant to fail to rescue the *gbb*^{4} cross-vein defect, we find that *gbb*^{4} homozygotes are in fact lethal in the presence of a single copy of this transgene (Fig. 3*E*). This result suggests that the triple mutant acts as a dominant negative that knocks down endogenous Gbb function in the sensitized mutant background. To confirm this, we determined whether overexpression of the cleavage mutant could recapitulate *gbb* mutant phenotypes in the wing. We generated a UAS=GbbPro/Main/Shadow construct and used *Act5C-Gal4* to drive expression throughout the fly. Although overexpression of wild type Gbb under control of *Act5C-Gal4* is lethal, overexpression of cleavage-resistant Gbb is semi-viable, with the survivors showing either partial or complete loss of the posterior cross-vein (Fig. 3*F*). This phenocopy of the *gbb*^{4} mutant phenotype demonstrates that cleavage-resistant Gbb antagonizes signaling by the endogenous Gbb ligand.

Cleavage of Scw in Tissue Culture—To determine whether the processing sites in Scw are cleaved *in vivo*, we transfected dual-tagged Scw (Fig. 4*A*), purified secreted Scw with FLAG affinity purification, and blotted with FLAG and HA antibodies. Wild type Scw is secreted as a single C-terminal peptide of ~19 kDa (Fig. 4*B*, *lane 1*). When the Main site is mutated, this form is lost, and a larger 42-kDa C-terminal fragment is produced, indicating that the Main site is required to produce the mature ligand (Fig. 4*B*, *lane 4*). Mutation of the Shadow site results in a small increase in the size of the C-terminal fragment (Fig. 4*B*, *lane 8*), and using peptide *N*-glycosidase treatment, we excluded the possibility that this shift was due to abnormal glycosylation (supplemental Fig. S3). Instead, these results are consistent with a model whereby mature Scw is cleaved at both the Main and Shadow sites, but processing at the Shadow site requires prior cleavage at the Main site.

Mutation of the Pro site does not affect cleavage at the Main/Shadow sites, but the level of secreted Scw is reduced (Fig. 4*B*, *lane 3*, *cf. lane 1*). In addition, the Pro site mutation leads to secretion of the N-terminal prodomain fragment, which co-purifies with the ligand domain, revealing that the prodomain remains associated with the ligand domain when cleavage at the Pro site has not occurred (Fig. 4*B*, *lane 3*). Thus, Pro site cleavage is important for both dissociation of the prodomain from the ligand domain and to promote efficient secretion or extracellular accumulation. Mutation of the Pro and Main sites results in secretion of a 50-kDa double-tagged form, corresponding to the full-length Scw proprotein, indicating that the protein is not processed at any other site independent of these (Fig. 4*B*, *lane 6*, compare with *lane 4*). Analyses of Pro2 mutants suggest that this site is not cleaved (Fig. 4*B*, *lanes 2*, *5*, and *7*). However, the Pro2 sequence may be important for prodomain function, as its mutation has both positive and negative effects on the extracellular accumulation of Pro-cleaved (Fig. 4*B*, compare *lanes 4* and *5*) and full-length (Fig. 4*B*, compare *lanes 6* and *7*) Scw proteins.

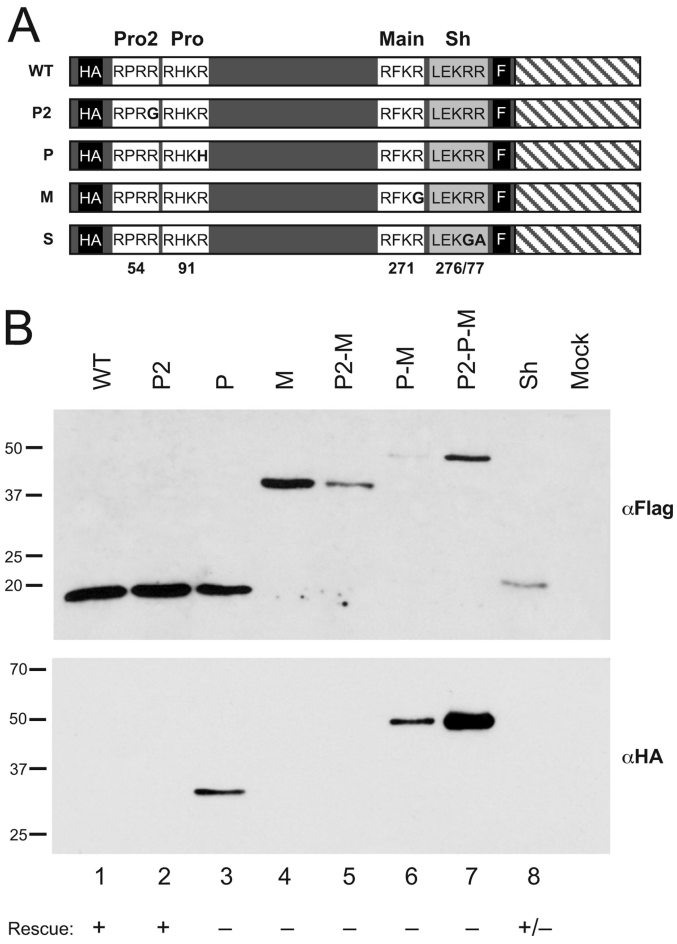

FIGURE 4. **Processing of Scw in tissue culture cells.** *A*, diagrams of the Scw protein show the cleavage sites, mutations, and FLAG tag as described for Gbb in the legend to Fig. 2. The HA tag is indicated (*HA*). *B*, Western blot of Scw proteins FLAG affinity-purified from the media of S2R$^+$ cells and detected with anti-FLAG (*upper*) and anti-HA (*lower*) antibodies. A summary of the rescue data shown in Table 2 is shown *beneath the blot*. *P2*, Pro2 site mutation; *P*, Pro site; *M*, main site mutation; *Sh*, double mutant of the dual SPC site after the Main site.

Cleavage at Pro and Main Sites Is Essential for Scw Function in Vivo—To determine the effect that the cleavage mutations have on Scw function, we engineered the cleavage site mutations into a genomic rescue construct and assayed for rescue of the lethality associated with *scw* mutants. To neutralize position effects, we used the ϕ-C31 integration system to insert the transgenes into a single genomic site. At this site, wild type Scw rescues *scw* mutants with 85% of the expected progeny surviving to adulthood (Table 2, supplemental Table S2). The construct bearing the Pro2 mutant rescues to a similar level (72% of expected), consistent with our finding that this site is not cleaved. In contrast to our results with Gbb, we find that mutations in either the Pro site or the Main site fail to rescue *scw* mutants, whereas mutation of the Shadow site shows reduced Scw function (Table 2). Thus, processing at both the Pro and Main sites in Scw is essential to produce the active ligand. Our data show that the rescue with these transgenes directly correlates with the amount of mature Scw ligand pro-

TABLE 2

Rescue of *scw* mutants with transgenes bearing mutations in Furin and SPC cleavage sites

Progeny were derived from a cross of $y^1\ w^{1118}$; scw^{S12}, *cn bw sp/Bl;Tn/+* x $y^1\ w^{1118}$; *Df(2L)OD16/SM6a*. The percent rescue was calculated by dividing the number of *scw/Df;Tn+* progeny by the number of *Df/Bl;Tn+* progeny and multiplying the quotient by 100. Each transgene was inserted into the 86Fb attP landing site using the phiC31 transformation system, and a single line for each construct was tested. For the complete data set, see supplemental Table S2.

Transgene	Rescue
	%
Scw+/−R+B (wt)	85
Scw-R54G (Pro2)	76
Scw-R91H (Pro)	0
Scw-R271G (Main)	0
Scw-R276G R277A (Shadow SPC)	44

TABLE 3

Rescue of *gbb* mutants with human BMP7 and cleavage site mutant transgenes

Construct names are abbreviated as follows: Gbb-BMP7 ligand domain chimera, *gbb*=Gbb-BMP7LD; full-length BMP7, *gbb*=BMP7; optimal cleavage site mutant, *gbb*=BMP7-RSKR; Main site mutation, *gbb*=BMP7-R292G. Progeny were from a cross of $y^1\ w^{1118}$; *Df(2L)S246/Bl;Tn/+* x $y^1\ w^{1118}$;gbb^{D20},*pr cn/SM6a*. The statistic percent expected was calculated by dividing the number of *gbb/Df;Tn+* progeny by the number of *gbb/Bl;Tn+* progeny and multiplying the quotient by 100. For the complete data set, see supplemental Table S3.

Transgene	Rescue
	%
gbb=Gbb-BMP7LD-5.1	116
gbb=Gbb-BMP7LD-19.1	107
gbb=BMP7–15.1 (full-length)	0
gbb=BMP7–26.1 (full-length)	0
gbb=BMP7–13.3 (full-length)	2
gbb=BMP7–16.1 (full-length)	9
gbb=BMP7–6.1 (full-length)	30
gbb=BMP7–33.2 (full-length)	56
gbb=BMP7-RSKR-23 (Optimal)	0
gbb=BMP7-RSKR-F28 (Optimal)	12
gbb=BMP7-R292G-3.1 (Main)	0
gbb=BMP7-R292G-5.3 (Main)	0
gbb=BMP7-R292G-25.3 (Main)	0

duced, suggesting that only fully processed Scw is functional *in vivo*.

Conservation of SPC Cleavage Sites in BMP5/6/7/8 Proteins—Our mutational analysis has revealed three processing sites involved in the maturation of Gbb and Scw; the Pro site within the prodomain and the Main and Shadow sites between the prodomain and ligand domain. To determine whether this arrangement is more widely conserved within the BMP5/6/7/8 class, we performed a phylogenetic analysis of this group of proteins focusing on the domains that contain these sites.

The Main and Shadow sites in Scw and Gbb are flanked on the N-terminal side by the conserved prodomain motif QPF*X*FF, which is present in invertebrate and vertebrate BMP5/6/7/8 proteins, and on the C-terminal side by the first cysteine of the ligand domain (Fig. 1*B*). The "variable region" between these two landmarks differs in size and amino acid composition in different species. In arthropods, the first potential SPC cleavage site after the QPF*X*FF motif typically has the highest probability of cleavage, which prompted us to call this site the Main site; however, as the flanking sequence and core R*XX*R consensus are different in each species, the probability of cleavage at this site varies. The Shadow site, just C-terminal to the Main site, is only found in the insect lineage. In Gbb proteins, the Shadow site is variably conserved, with some species, such as *Glossina morsitans*, *Anopheles gambiae*, and *Tribolium castaneum* having a site with a significant probability of cleavage, whereas others, including *Drosophila melanogaster* and *Drosophila grimshawi*, have a low or negligible probability site. Similarly, the general SPC Shadow site in Scw is present in *Drosophila* species but not in the Scw proteins of other flies. Outside the insect lineage, the Main/Shadow site configuration is not conserved, with most BMP-5/6/7/8 orthologs having a single predicted cleavage site in the region preceding the ligand domain. Interestingly, vertebrate BMP5/6/7/8 proteins lack a high probability cleavage site in this region, although an R*XX*R motif is always present, suggesting that efficient cleavage of these proteins may not be required for function.

The Pro-site in Gbb and Scw lies in a region of variable sequence between two conserved blocks, the SAP*X*FLLD domain, which is identifiable in all BMP5/6/7/8 proteins, and the D*X*IM*X*F domain, which is less well conserved but falls just before a widely conserved intron-exon break that follows the final amino acid shown in Fig. 1*B*. The furin cleavage site in this interval is only conserved in higher insects, with Dipteran Gbb and Scw proteins and *Tribolium* Gbb1 and Gbb2 having a high probability cleavage site at this position. Although some of the vertebrate proteins have predicted general SPC sites in this interval, none have an R*XX*R motif that is orthologous to the arthropod site.

Interpreting these data in light of the phylogeny, it appears that the ancestral metazoan had a single cleavage site in the variable region between the prodomain and ligand domain, of which *Nematostella* is an example, and this single site persists in the Lophotrochozoa and Deuterostome lineages. In the arthropods, the ancestral pattern diversifies, with the Shadow site appearing at the base of the insect lineage and the Pro site appearing in the higher insects.

Processing and Function of Human BMP7 in Drosophila—After our results on Gbb and Scw, it was of interest to understand how the vertebrate BMP5/6/7/8 proteins are processed given that they only have a single low probability "Main" site (Fig. 1*B*). We have previously shown that the ligand domains of BMP5, BMP6, and BMP7 can function in *Drosophila* and rescue *gbb* mutant phenotypes (25). In these experiments we found that chimeras produced by fusing the Gbb cis-regulatory sequences and prodomain to the BMP5 or BMP6 ligand domain rescue all essential *gbb* functions but also result in sterility that prevents the rescue constructs from being stably maintained. As Gbb-BMP7 chimeras fully rescue *gbb* mutants (Table 3) without resulting in dominant phenotypes, we studied how human BMP7 is processed and functions in the *Drosophila* system.

Consistent with studies in mammalian tissue culture cells (31, 32), in $S2R^+$ cells BMP7 is cleaved at the single Main site but is secreted as two isoforms (Fig. 5*B*). The two forms arise from differential glycosylation, as peptide *N*-glycosidase treatment reduces the doublet to a single 19-kDa band (supplemental Fig. S3). Mutation of the Main site results in the loss of both mature forms, and instead the 50-kDa BMP7 proprotein is secreted into the medium (Fig. 5*B*).

To assay how BMP7 functions in the fly, we generated chimeric rescue constructs fusing wild type BMP7 and the Main site mutant to *gbb* cis-regulatory sequences. Unlike the Gbb-

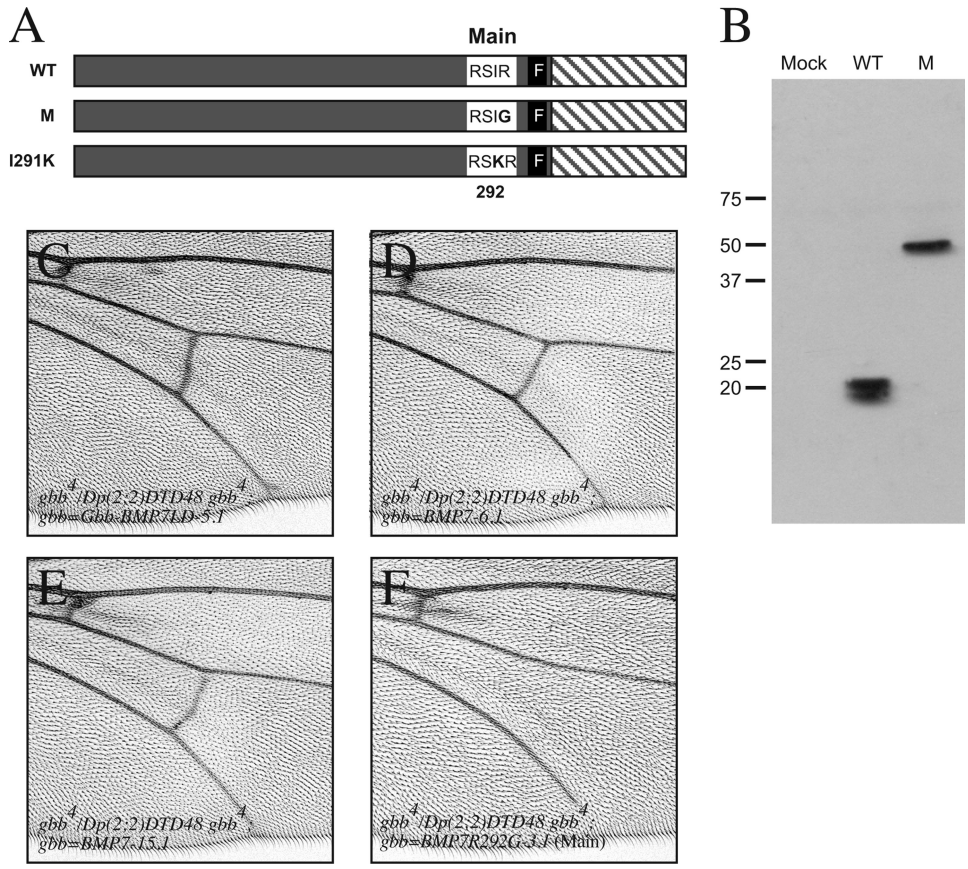

FIGURE 5. **Processing and function of BMP7 in *Drosophila*.** *A*, diagrams of the BMP7 protein, as described for Gbb in the Fig. 2 legend, are shown. *B*, anti-FLAG Western blot of BMP7 proteins secreted into the media of transfected S2R$^+$ cells are shown. *M*, main site mutation. (*C*) The *gbb*=*BMP7* ligand domain chimera (*gbb*=Gbb-BMP7LD) fully rescues the *gbb*^{4} cross-vein defect (*D*) as do the full-length BMP7 lines that partially rescue the lethality of *gbb* nulls. (*E*) Full-length BMP7 lines that do not rescue lethality of *gbb* nulls partially rescue the *gbb*^{4} cross-vein defect (*F*), whereas the Main site mutant (BMP7R-292G) does not rescue.

BMP7 ligand domain chimera, which fully rescues *gbb* mutants, full-length BMP7 only partially rescues *gbb* lethality, with the degree of rescue showing a strong dependence on insertion site (Table 3, supplemental Table S3). To determine whether the BMP7 lines are functional despite their inability to fully rescue the *gbb* null, we tested for rescue of the *gbb*^{4} cross-vein defect. We find that the Gbb-BMP7 ligand domain chimera as well as the BMP7 lines that partially rescued *gbb* lethality show complete rescue of the posterior cross-vein (Fig. 5, *C* and *D*), whereas the BMP7 insertions that could not rescue *gbb* lethality show partial rescue (Fig. 5*E*). Thus, all of the BMP7 constructs are functional, but the ability for the full-length construct to rescue is compromised, which can be attributed to differences in the Gbb and BMP7 prodomains. To rule out the possibility that this is due to the lack of a high probability Main site in BMP7, we changed the furin cleavage site in our rescue construct from RSIR to the optimal RSKR. Like the wild type constructs, the RSKR constructs show, at best, partial rescue of *gbb* mutants; thus the difference in functionality must be attributed to a different feature of the BMP7 prodomain. Not surprisingly, the BMP7 Main site mutant fails to rescue both *gbb* lethality (Table 3, supplemental Table S3) and the cross-vein defect associated with *gbb*^{4} (Fig. 5*F*), suggesting that unprocessed BMP7 is non-functional when expressed at physiological levels.

Cleavage-resistant Forms of Gbb and BMP7 Have Different Effects on BMP Signaling—We have shown above that overexpression of the cleavage-resistant form of Gbb has a dominant negative effect and recapitulates loss-of-function phenotypes of *gbb*. Given that full-length BMP7 only partially rescues *gbb* mutants, we determined how BMP7 and cleavage-resistant BMP7 behave in an overexpression assay, which offers a more sensitive test of signaling activity. To circumvent the lethality associated with overexpression of BMP7, we used the Gal4/*Gal80*^{ts} system with *en-Gal4* to drive expression in the posterior compartment of the wing specifically during pupal development (see "Experimental Procedures"). With this system, we find that overexpression of wild type Gbb results in wings showing the canonical BMP overexpression phenotype, with defects more prominent in the posterior half of the wing (Fig. 6*A*). Overexpression of cleavage-resistant Gbb recapitulates the dominant negative behavior observed with *Act-Gal4* (Fig. 3*F*), resulting in a normal wing with variable cross-vein defects (Fig. 6*B*). Overexpression of BMP7 also produces the BMP overexpression phenotype (Fig. 6*C*), and remarkably, overexpression of cleavage-resistant BMP7 produces the same phenotype (Fig. 6*D*). Thus, in *Drosophila*, the cleavage-resistant form of BMP7 retains some signaling activity despite being covalently attached to the prodomain.

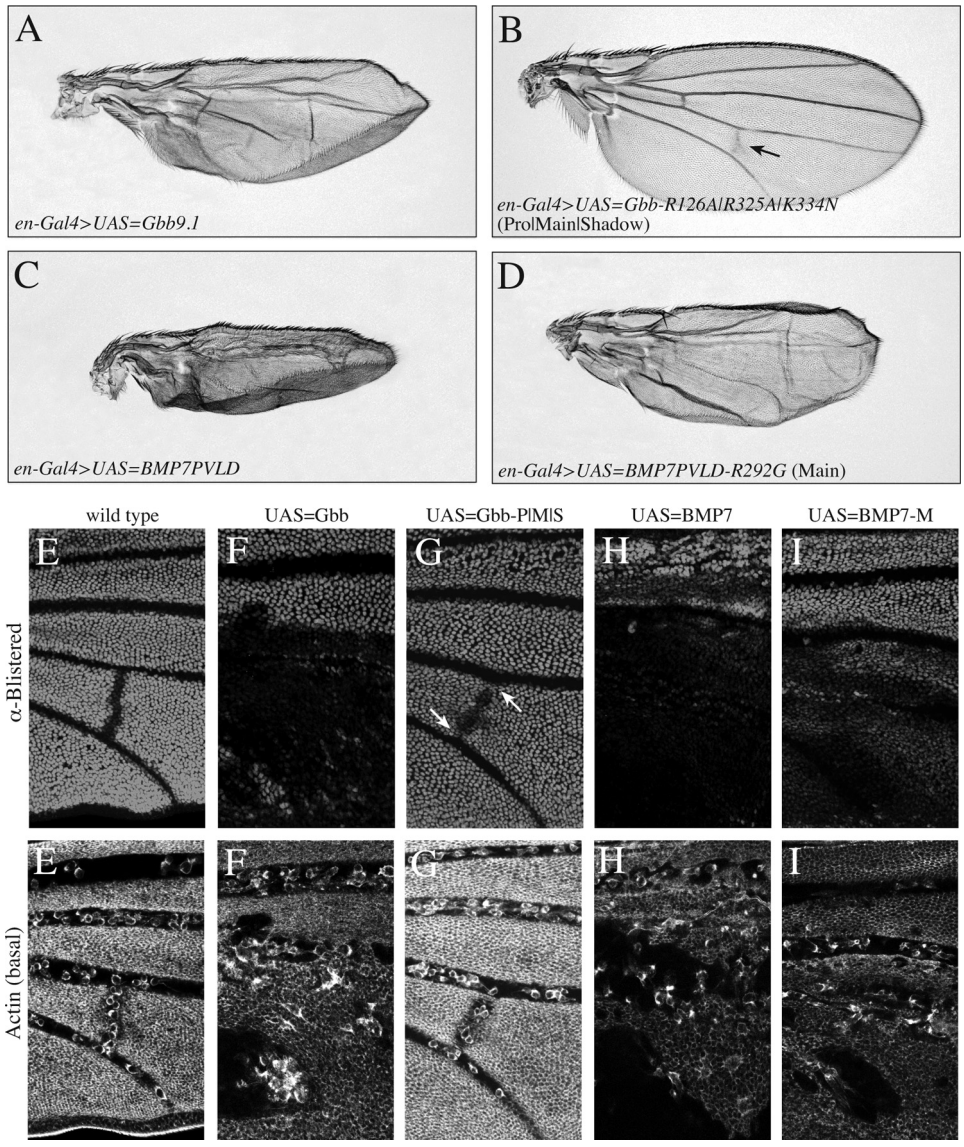

FIGURE 6. **Different effects of cleavage-resistant forms of Gbb and BMP7 on BMP signaling in the pupal wing.** *A–D*, adult wing phenotypes produced by overexpression of Gbb (*A*), cleavage-resistant Gbb (*B*), BMP7 (*C*), and cleavage-resistant BMP7 (*D*) driven by *en-Gal4* from the onset of pupal development. Over-expression of cleavage-resistant Gbb phenocopies *gbb* mutants and results in a crossvein defect (*arrow* in *B*) while over-expression of cleavage-resistant BMP7 phenocopies the gain-of-function phenotype. In the pupal wing expression of Bs (*E*) and apposition of the dorsal and ventral wing epithelia as revealed by phalloidin-stained actin (*E′*) is characteristic of intervein cells, and both are antagonized by BMP signaling. Gbb overexpression down-regulates Bs (*F*) and interferes with apposition (*F′*), whereas cleavage-resistant Gbb does not affect Bs expression (*G*) and leaves apposition intact (*G′*) but does result in defects in cross-vein specification (*arrows* in *G*). Expression of BMP7 (*H* and *H′*) and cleavage-resistant BMP7 (*I* and *I′*) results in repression of Bs and a failure to appose.

To test directly whether cleavage-resistant BMP7 activates the BMP signaling pathway, we assayed whether the gain-of-function phenotype associated with overexpression of this form induces changes in gene expression and cell behavior that are known outputs of pathway activation. In the pupal wing, BMP signaling specifies vein fates and blocks apposition of the dorsal and ventral wing surfaces (33). A direct target of BMP signaling in this context is repression of the *Drosophila* serum response factor Blistered (Bs) (27, 34). In the wild type wing, Bs is expressed in intervein cells but not in vein cells where the BMP pathway is active (Fig. 6*E*), and cells of the dorsal and ventral wing epithelia that express Bs form actin-rich focal adhesions that can be visualized by staining with phalloidin (Fig. 6*E′*) Using the *en-Gal4/Gal80*^{ts} system to confine the overexpression temporally and spatially, we determined the effect of overexpressing wild type and cleavage-resistant forms of Gbb and BMP7 on these two read-outs of BMP signaling.

Overexpression of wild type Gbb results in the down-regulation of Bs and a failure to form focal adhesions between the dorsal and ventral wing epithelia in the posterior compartment (Fig. 6, *F* and *F′*). Cleavage-resistant Gbb cannot activate the signaling pathway, and Bs expression and the formation of focal adhesions are normal (Fig. 6, *G* and *G′*), but it antagonizes signaling by endogenous Gbb, resulting in posterior cross-vein defects (Fig. 6*G*, *arrows*). Analogous to what we observe with Gbb, overexpression of wild type BMP7 results in down-regulation of Bs and a failure to form focal adhesions, consistent with activation of the BMP signaling pathway (Fig. 6, *H* and *H′*).

Significantly, the same effect is observed upon overexpression of cleavage-resistant BMP7 (Fig. 6, *I* and *I′*). Taken together, our data reveal that cleavage-resistant Gbb antagonizes signaling by endogenous Gbb, whereas cleavage-resistant BMP7 does not but rather activates the downstream signaling cascade.

DISCUSSION

Different Ways to Process BMP5/6/7/8 Proproteins—We have investigated the processing requirements for the *Drosophila* BMP5/6/7/8 orthologs Gbb and Scw and human BMP7 in the *Drosophila* system. Although our results support the conventional dogma that proteolytic processing and dissociation of the prodomain are essential steps in BMP activation, we show that different BMPs, even if closely related, can have distinct processing requirements. In particular, for the BMP5/6/7/8 proteins, we have identified a novel mechanism of prodomain shedding involving furin-mediated cleavage at a site within the prodomain, and we provide evidence that some BMPs can signal with part or all of the prodomain covalently attached.

Gbb processing occurs at three sites to give rise to an N-terminal pro-fragment, a C-terminal pro-fragment, and the ligand domain. The processing event that must occur to activate Gbb is the separation of the ligand domain from the N-terminal pro-fragment, which can occur by cleavage at either the Pro site or the Main site. Thus, Gbb can function either as a fully processed ligand or with the C-terminal fragment of the prodomain covalently attached (Fig. 7). An inhibitory role for the N-terminal part of the prodomain is not without precedent; functional studies on Myostatin/GDF8 have mapped the inhibitory domain of the protein to amino acids 42–115 (35), and the recent crystal structure of TGFβ1 has also implicated this part of the protein in blocking ligand function (36) (see below).

Scw is processed at three cleavage sites orthologous to those in Gbb (Pro, Main, and Shadow), but in this case cleavage at both the Pro site and the Main site are required to produce a functional ligand (Fig. 7). Cleavage at the Pro site is required for dissociation of the prodomain and for stable accumulation of the secreted ligand. Thus, when complexed with the prodomain, Scw is either inefficiently secreted or rapidly internalized after secretion, which is similar to what has been reported for members of the BMP2/4/Dpp group (4, 12). Cleavage at the Main site is required for dissociation of the ligand domain from the C-terminal prodomain fragment, which in Scw inhibits ligand function. The inhibitory effect of the C-terminal prodomain region in Scw is a recent evolutionary adaptation and could reflect either a newly evolved property of the prodomain or a newly evolved function of the Scw ligand domain with which this prodomain fragment interferes. Notably, a similar rapid functional divergence in the prodomain has also been reported for BMP15, where the prodomains of the mouse and human orthologs confer differential processing efficiencies to the proprotein that may underlie the distinct functional properties of the ligand in these two species (37).

Our data support the model that human BMP7 is processed at a single, low probability furin cleavage site that lies between the prodomain and ligand domain and that cleavage at this site is essential for function (Fig. 7). Curiously, although the Gbb-BMP7 ligand domain chimera fully rescues *gbb* mutants, full-length BMP7 does not, suggesting that the BMP7 prodomain is incompatible with essential features of Gbb processing and signaling. Thus, although the Gbb and BMP7 ligands are functionally interchangeable, their prodomains have diverged, presumably to fine-tune their activity to fulfill their endogenous functions.

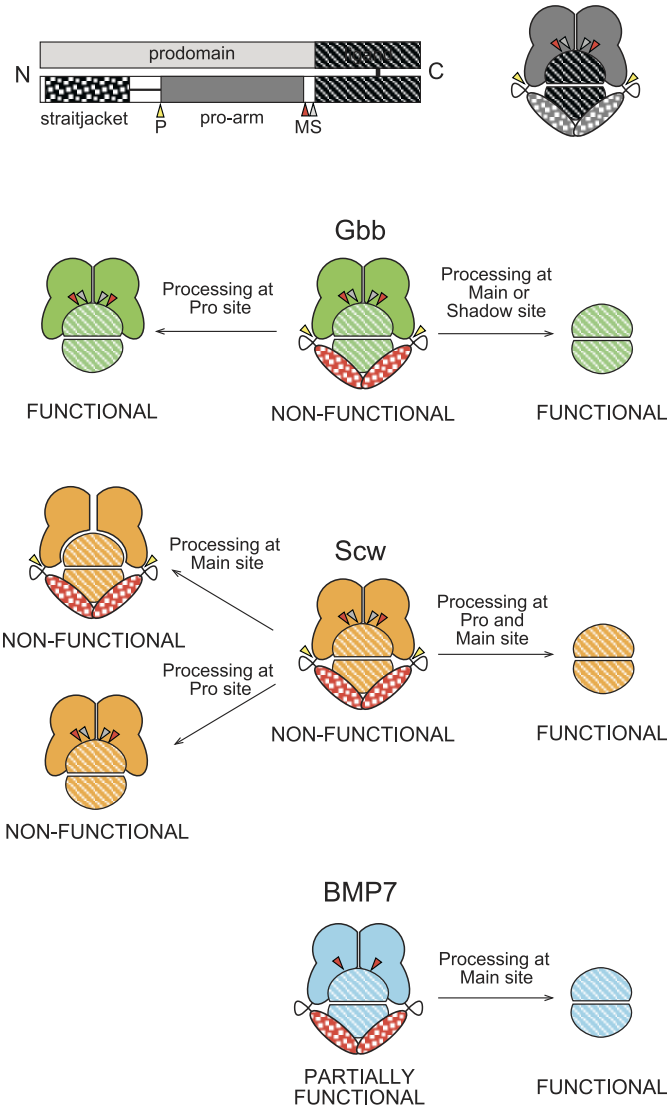

FIGURE 7. **Processing of BMP5/6/7/8 proteins.** The different BMP proteins have different processing requirements depending on the number and position of their cleavage sites and the function of particular protein domains. *Top left*, shown is a schematic of a BMP dimer with the upper monomer showing the division of the protein into the N-terminal prodomain and C-terminal ligand domain and the lower monomer showing the structural features including the N-terminal "straitjacket" (*stippled*), prodomain arm (*solid gray*), and ligand domain (*hatched*) The Pro (*P*), Main (*M*), and Shadow (*S*) processing sites are indicated with *arrowheads* in *yellow*, *red*, and *gray*, respectively. *Top right*, shown is a schematic of the folded BMP dimer based on the crystal structure of TGFβ1 (36) showing the straitjacket and prodomain arms wrapped around the ligand dimer. *Lower panels* illustrate the processing requirements for Gbb (*green*), Scw (*orange*), and BMP7 (*blue*). Generation of active Gbb can be achieved by cleavage at either the Pro site or the Main site, whereas Scw requires cleavage at both the Pro and Main sites to produce the active ligand. BMP7 has only a Main site, and although cleavage at this site is essential to produce fully active ligand, the proprotein retains some signaling activity.

Taken together our findings on Scw, Gbb, and BMP7 illustrate how evolutionary plasticity in the prodomain sequence serves to modulate the activity of the ligand, which may be

subject to stronger evolutionary constraints and thus unable to diverge sufficiently to provide functional diversity. The furin-mediated cleavage of Gbb and Scw we have characterized here is one of a number of different prodomain processing events that have been shown to modulate the function of TGFβ superfamily ligands. For many ligands, the prodomain has been shown to remain non-covalently associated with the ligand, and a range of different functions have been ascribed to it, including targeting the complex for degradation (4) or to the extracellular matrix (8, 38). Indeed, the prodomains of BMP-4, -5, -7, and -10 and GDF5 have been shown to interact with Fibrillin (8, 38, 39) and the GDF8 prodomain with Perlecan (39), and various strategies are deployed to dissociate this complex and release the active ligand. *In vitro* evidence suggests that for BMP7, the prodomain can be displaced by the Type II receptor (9). A more general strategy may be proteolytic cleavage of the prodomain. GDF11, GDF8, and BMP10 require proteolytic cleavage by the metalloproteinase Tolloid/BMP-1 to activate signaling *in vivo* or in tissue culture assays (7, 10, 40). Our data suggest that an additional mechanism for activation is furin/SPC cleavage of the prodomain.

BMP5/6/7/8 Cleavage Requirements in Light of Crystal Structure of Pro-TGFβ1—The recent crystal structure of the pro-TGFβ1 dimer reveals that the two prodomains form a ring-like shape that wraps around the ligand dimer, altering its structure and shielding it from interaction with receptors. The prodomain ring is subdivided into C-terminal "arms," an extended loop that encircles the tip of each ligand monomer, and two N-terminal, α-helical forearms that cross one another, forming a "straitjacket" around the ligand (see Fig. 7). In the TGFβ1 model, the N-terminal straitjacket locks the ligand dimer into the prodomain ring, and mechanical force induced by interaction with extracellular matrix proteins unfastens the straitjacket to release the active ligand (36).

This model for the prodomain-fold provides insight into the molecular basis for our results on the processing requirements for Gbb, Scw, and BMP7 and also raises intriguing questions. The Pro site in Gbb and Scw lies between the arm and straitjacket and thus is in a key position to influence ligand accessibility and function. In the case of Gbb, cleavage at this site effectively opens the straitjacket and reveals the ligand dimer irrespective of cleavage at the Main/Shadow sites. Scw, on the other hand, cannot function even with the straitjacket removed in this way. This distinction suggests there is a difference between the arm domains of Gbb and Scw, the former of which is neutral to ligand function and the latter of which is inhibitory. Moreover, Scw requires cleavage at the Pro site to shed the prodomain and activate the ligand, whereas Gbb functions without this cleavage. Thus, Scw follows the TGFβ1 paradigm of requiring more than just cleavage at the Main site to shed the prodomain, whereas Gbb presents a distinct situation where cleavage at the Main site is sufficient for prodomain shedding.

Our results with BMP7 also depart from the TGFβ1 paradigm where the prodomain locks the ligand into an inactive form. Although cleavage-resistant BMP7 only retains a low level of signaling activity, our data show that it can activate the signaling pathway despite having the prodomain covalently attached. Although the ability for a BMP proprotein to bind to its receptor has been previously shown for BMP2 (41) and BMP7 (8) *in vitro*, our data provide the first evidence that a BMP proprotein can signal *in vivo* without displacement of the prodomain.

The Dominant Negative Behavior of Cleavage-resistant Mutants—The expression of cleavage-resistant TGFβ superfamily proteins has been shown to generate dominant negative effects by blocking secretion of the wild type protein (42–45). In these studies it was assumed that the mutant proteins formed heterodimers with the wild type monomers, thus promoting their degradation within the cell (43). Our results with cleavage-resistant Gbb do not support this type of model for two reasons. First, cleavage-resistant Gbb is secreted at normal levels and thus is not degraded within the cell. Second, we find that cleavage-resistant Gbb knocks down the function of endogenous Gbb, but not Dpp, with which it forms heterodimers. Thus, the dominant negative effect is exclusive to the homotypic ligand. This specificity has been reported previously (43), but it is not clear how it might arise for a protein that is known to function by forming heterodimers with other ligands. This suggests that either the relationship between dimerization and processing is different than currently thought or that the mechanism underlying the dominant negative behavior is not exclusively due to heterodimerization.

The Evolution of Furin/SPC Cleavage Sites in BMP Proteins—Our phylogenetic analysis of the BMP5/6/7/8 proteins has shown that the processing sites are embedded in blocks of sequence that are poorly conserved even between closely related species. This feature is also a characteristic of the cleavage sites in the BMP2/4/Dpp proteins (13), and thus the domains that include the cleavage sites in the BMPs appear to be in constant flux, with the core and flanking sequences changing from one species to the next. Indeed, in both subgroups there is evidence that the arrangement of cleavage sites can change, presumably influencing the processing mechanism. For example, in the vertebrate BMP2/4 proteins, the S1 site is a high probability site and is cleaved first, whereas the S2 site is a low probability site and is only cleaved after processing at the S1 site. In arthropods, these cleavage probabilities are reversed (and an additional cleavage site is added), and the order of cleavage is correspondingly inverted (13). Similarly, in the Gbb proteins the Main site is typically a high probability site and the Shadow site is a low probability site, but in *Glossina*, *Anopheles*, and *Tribolium*, the cleavage probabilities are reversed, indicating that in Gbb it is the presence and not the position of the site that is important. In this light, the furin cleavage sites appear to evolve like transcription factor binding sites in a promoter where the key feature is maintaining the function of the element irrespective of the position, number, or affinity of the binding sites that comprise it (46). Given this, the plasticity in processing requirements we observe for the BMP5/6/7/8 proteins may well apply to other BMPs and be a general mechanism whereby the ligands are fine-tuned for their particular functions.

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