SUPPLEMENTAL DATA

FIGURES

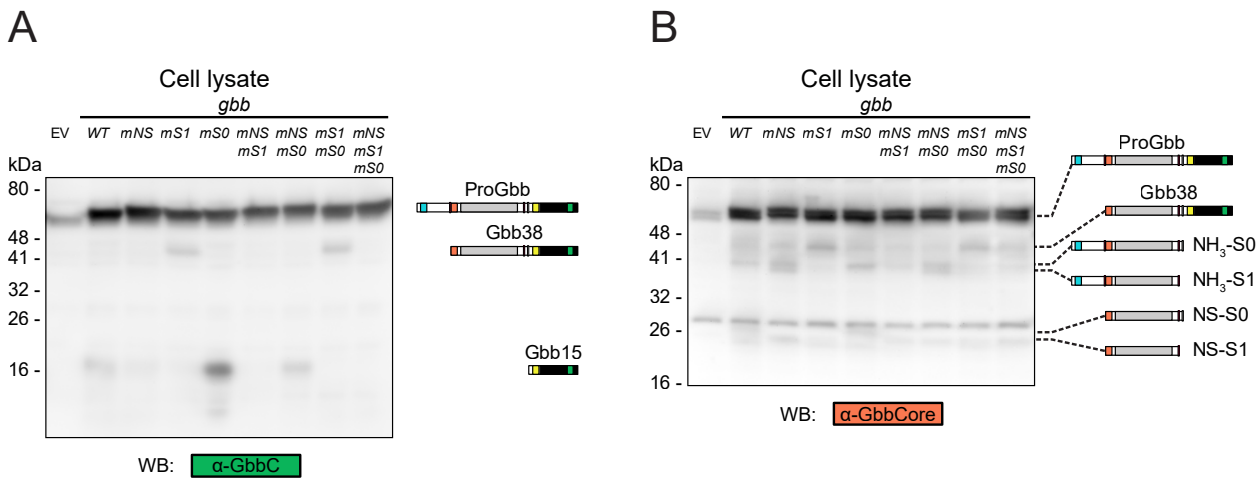

Figure S1: Gbb cleavage products are detected at low levels in cell lysates. A, Reducing Western blots of cell lysates from S2 cells expressing *gbb*, using α-Gbb to detect C-terminal cleavage products. Endogenously-expressed proGbb is detected in cells transfected with EV. In all cells transfected with *gbb* constructs, proGbb is the most abundant form. In cells expressing WT *gbb*, lower levels of Gbb15, and barely detectable Gbb38 levels are also detected. Gbb38 abundance in cell lysates is increased when S1 cleavage is blocked (*mS1* or *mS1mS0*). Gbb15 abundance is also increased when S0 cleavage is blocked (*mS0*). However, Gbb15 abundance does not increase when NS cleavage is blocked (*mNS*), opposite to the effect observed in media (Fig. 1C, D). B, Reducing Western blots of cell lysates, using α-CoreGbb to detect prodomain cleavage products. Again, endogenously expressed proGbb can be detected, and proGbb is the most abundant form in transfected cells. In cells expressing WT *gbb*, bands matching the expected size of Gbb38, NH_3-S1 and NS-S1 cleavage products are observed at low abundance. However, these bands are also visible at low levels when all cleavage sites are blocked (*mNSmS1mS0*), indicating that they may be nonspecific or that endogenous Gbb protein levels are increased by *gbb* transfection. Blocking NS cleavage reduces abundance of Gbb38 and/or NS-S1, and blocking S1 cleavage reduces abundance of NH_3-S1 and NS-S1. Therefore, these bands are at least partially produced from exogenously expressed *gbb*. We propose that these prodomain cleavage products are cleavage intermediates, and that *gbb* overexpression causes an accumulation of uncleaved exogenous and endogenous protein.

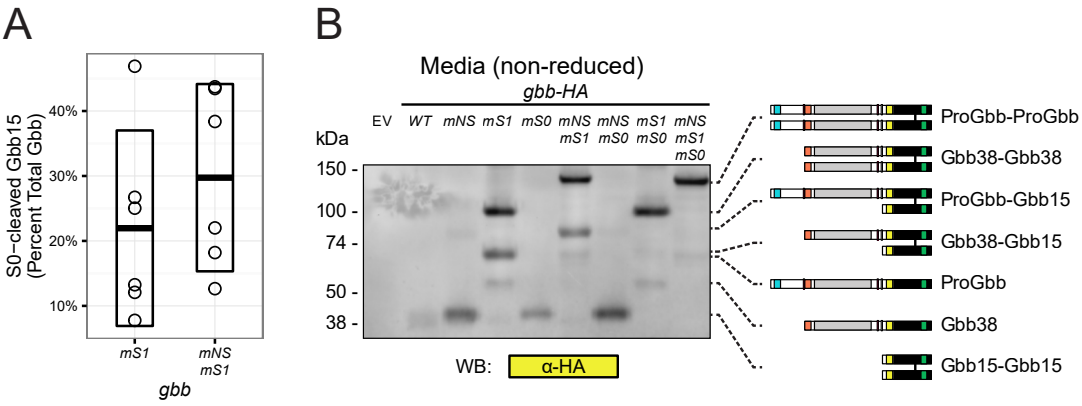

Figure S2: Gbb S0 cleavage is inefficient in S2 cells. A, Quantification of western blots shown in Figure 1C, showing the proportion of S0-cleaved Gbb in conditioned media from S2 cells expressing gbb^{mS1} or gbb^{mNSmS1}. In both genotypes, S0-cleaved Gbb is a small fraction of the total secreted Gbb. Bars indicate mean and 95% CI. B, non-reducing western blot of conditioned media from S2 cells expressing *gbb-HA*, showing Gbb dimers. In cells expressing WT *gbb-HA*, Gbb15 homodimers can be detected. Blocking S1 cleavage results in the secretion of a mix of Gbb15 and Gbb38 homodimers, as well as Gbb15-Gbb38 hemidimers. Some Gbb38 and proGbb monomers can also be detected.

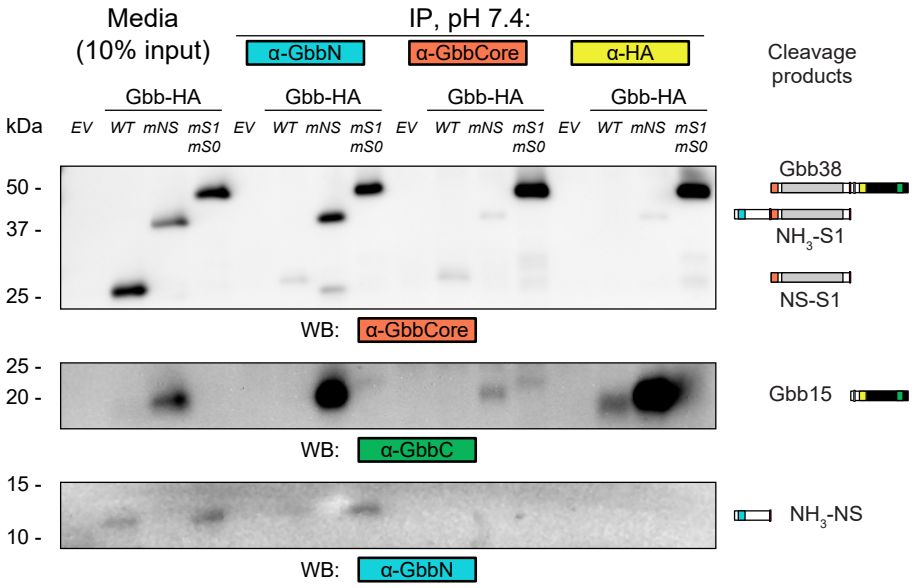

Figure S3: Association between Gbb cleavage products is reduced at pH 7.4. Reducing Western blots of Gbb immunoprecipitated at pH 7.4 from conditioned media. Compared to IP at pH 6.5 (Fig. 3), at pH 7.4 α-CoreGbb does not efficiently precipitate the NS-S1 or NH_3-S1 cleavage products. All other cleavage products are efficiently precipitated by directly-binding antibodies. Wild-type Gbb NS-S1 and NH_3-NS fragments co-precipitate, while neither co-precipitate with Gbb15. Co-precipitation of Gbb15 and the NH_3-NS fragment is also less efficient at pH 7.4.

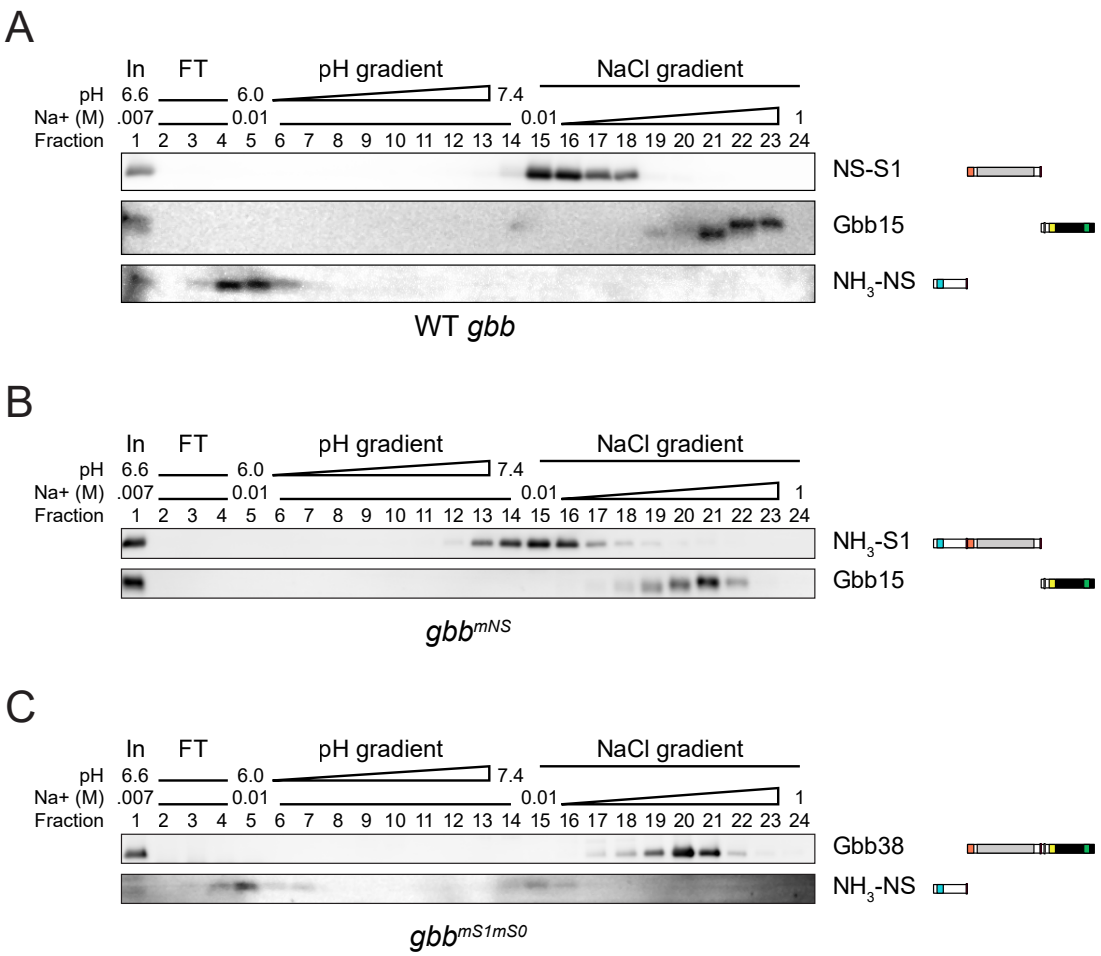

Figure S4: Heparin affinity chromatography of Gbb prodomain-ligand complexes. A, heparin affinity chromatography of media from cells expressing wild-type *gbb*, analyzed by reducing Western blot. Conditioned media was loaded on a heparin column. Fraction 1 contains 10% input (In), and fractions 2-4 are media flow-through. Fractions 5-14 were eluted using a sodium phosphate pH 6.0-7.4 gradient, and fractions 15-24 were eluted using a 0-1 M NaCl gradient. Gbb15 is eluted by > 400 mM NaCl, while the NS-S1 fragment begins to elute at pH > 7. The NH_3-NS fragment is eluted in the flow-through and initial wash columns. B, heparin chromatography of gbb^{mNS} conditioned media. Gbb15 is eluted by NaCl > 0.3 M, while the NH_3-S1 fragment is eluted by pH > 7.4. C, heparin chromatography of gbb^{mS1mS0} conditioned media. Gbb38 is eluted by NaCl > 0.2 M. Part of the NH_3-NS fragment is found in the flow-through, while another part retained until elution by pH > 7. A-C, Since prodomain cleavage products have a predicted net negative charge at pH > 6 (NH_3-NS pI = 5.07, NS-S1 pI = 5.60, NH_3-S1 pI = 5.41), and BMP ligands have well-characterized heparin binding, retention on the negatively charged heparin column is likely indirect via binding to Gbb15 or Gbb38.

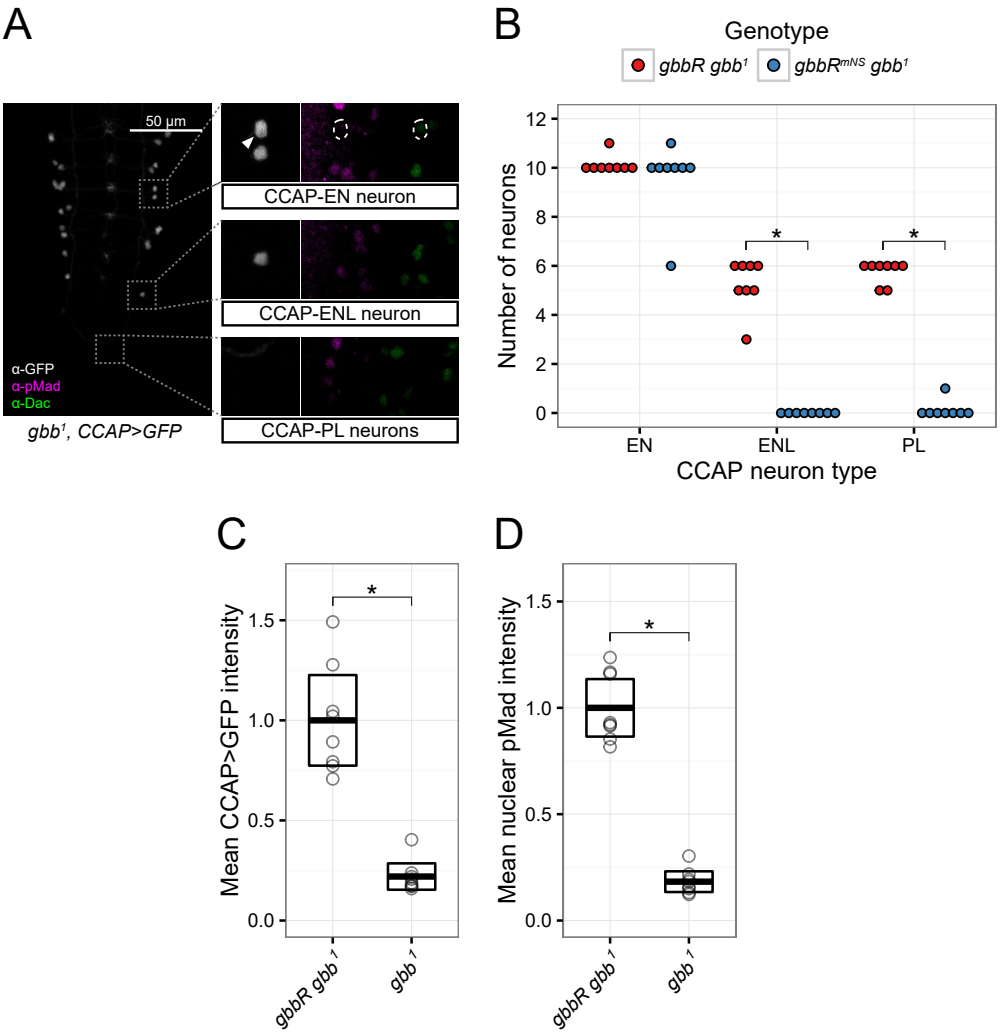

Figure S5: Reduced CCAP>GFP and nuclear pMad in *gbb*^{1} 3rd instar larvae, at 8-11 days post egg-lay. A, VNC of 3rd instar CCAP>GFP *gbb*^{1} larvae. Dac+ CCAP-ENs can be detected, with very low levels of nuclear pMad. No ENL or PL neurons are detectable in this particular VNC. B, total count of each CCAP neuron type. In *gbb*^{1} VNCs, late CCAP neurons are almost completely absent, with no detectable ENL neurons and only a single PL neuron found in one of eight *gbb*^{1} VNCs. * indicates $p < 0.001$ using Wilcoxon rank-sum test and FDR multiple comparison adjustment. C, quantification of mean CCAP>GFP cell body intensity, per VNC, in CCAP-EN neurons. CCAP>GFP is dramatically reduced in *gbb*^{1} VNCs. D, quantification of mean nuclear pMad intensity, per VNC in CCAP-EN neurons, measured within the volume of Dac+ nuclei. Nuclear pMad is absent in *gbb*^{1} CCAP-EN neurons, with no discernable nuclear pMad staining above background levels. B-D, control *gbbR gbb*^{1} data is the same shown in Fig. 8; n = 8 VNCs for *gbbR gbb*^{1} and *gbb*^{1}. C, D, bars indicate mean and 95% CI. * indicates $p < 0.001$ using GLHT multiple comparison test.