Cell Reports

Supplemental Information

Peak BMP Responses in the *Drosophila* Embryo Are Dependent on the Activation of Integrin Signaling

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Supplemental Figures

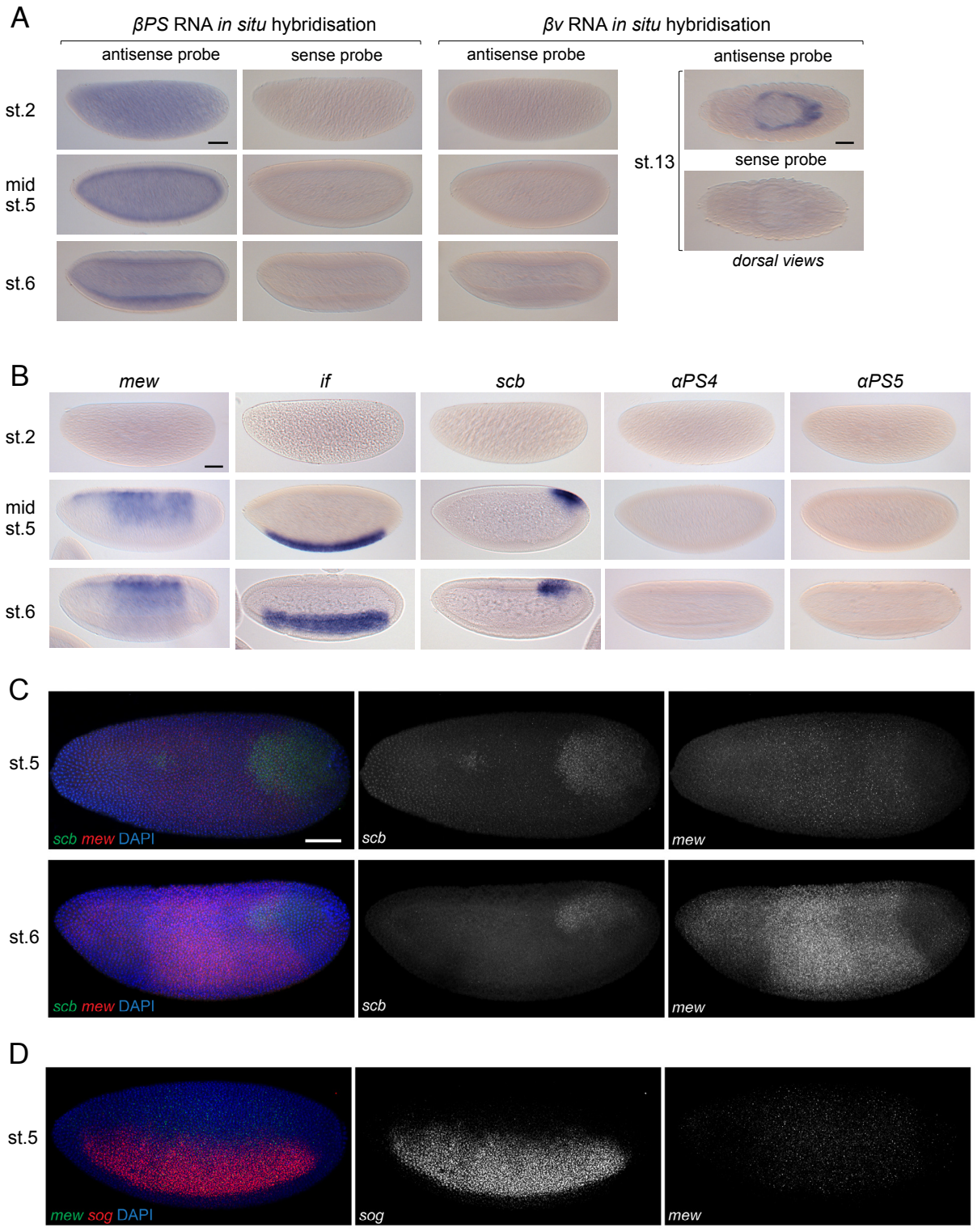

Figure S1, related to Figure 1: Expression of integrin genes during early *Drosophila* embryogenesis. RNA *in situ* hybridisations of early embryos (pre-cellularisation, stage 2; mid-cellularisation, stage 5; early gastrulation, stage 6) to visualise expression of α-integrin and β-integrin genes present in *Drosophila*. Note that transcripts detected in stage 2 embryos are maternally inherited. All images are lateral views, anterior to the left, unless otherwise stated. Scale bars = 50 μm.

(A) RNA *in situ* hybridisation for the two β-integrin genes present in *Drosophila*, *βPS* and *βv* (Brown, 2000). *βPS* is expressed maternally and in blastula/gastrula stages, with a ubiquitous expression pattern at all stages. *βv* expression is not detected in early embryos. Stage 13 embryos, where *βv* expression is detected in the midgut, are included as a positive control for the *βv* antisense probe. **(B)** RNA *in situ* hybridisation for α-integrin genes *multiple edenomatous wings (mew), inflated (if), scab (scb), αPS4 and αPS5.* No α-integrin transcripts can be detected in stage 2 embryos, suggesting little or no maternal deposition. *mew*, *if* and *scb* transcripts are first detected in cellularising embryos (stage 5), with markedly distinct expression patterns. *if* transcripts are restricted to the presumptive mesoderm (ventral stripe), while *mew* and *scb* are expressed in the dorsal ectoderm, with *mew* transcripts localising to a broad dorsal domain and *scb* transcripts detected in a patch of posterior dorsal cells only. **(C)** RNA *in situ* hybridisation of stage 5 (top row, dorsal view) and stage 6 (bottom row, dorsolateral view) embryos hybridised with *scb* (green) and *mew* (red) probes. Merged and single channel images are shown, as labelled. **(D)** RNA *in situ* hybridisation of stage 5 embryos (lateral views) showing *mew* and *sog* expression patterns, as a merged image (*mew* green, *sog* red) or as individual channels, as labelled. Overlap between *mew* and *sog* expression raises the possibility that mew/αPS1 enhances Sog function by promoting Sog secretion or extracellular movement into the dorsal regions where it binds BMP ligands.

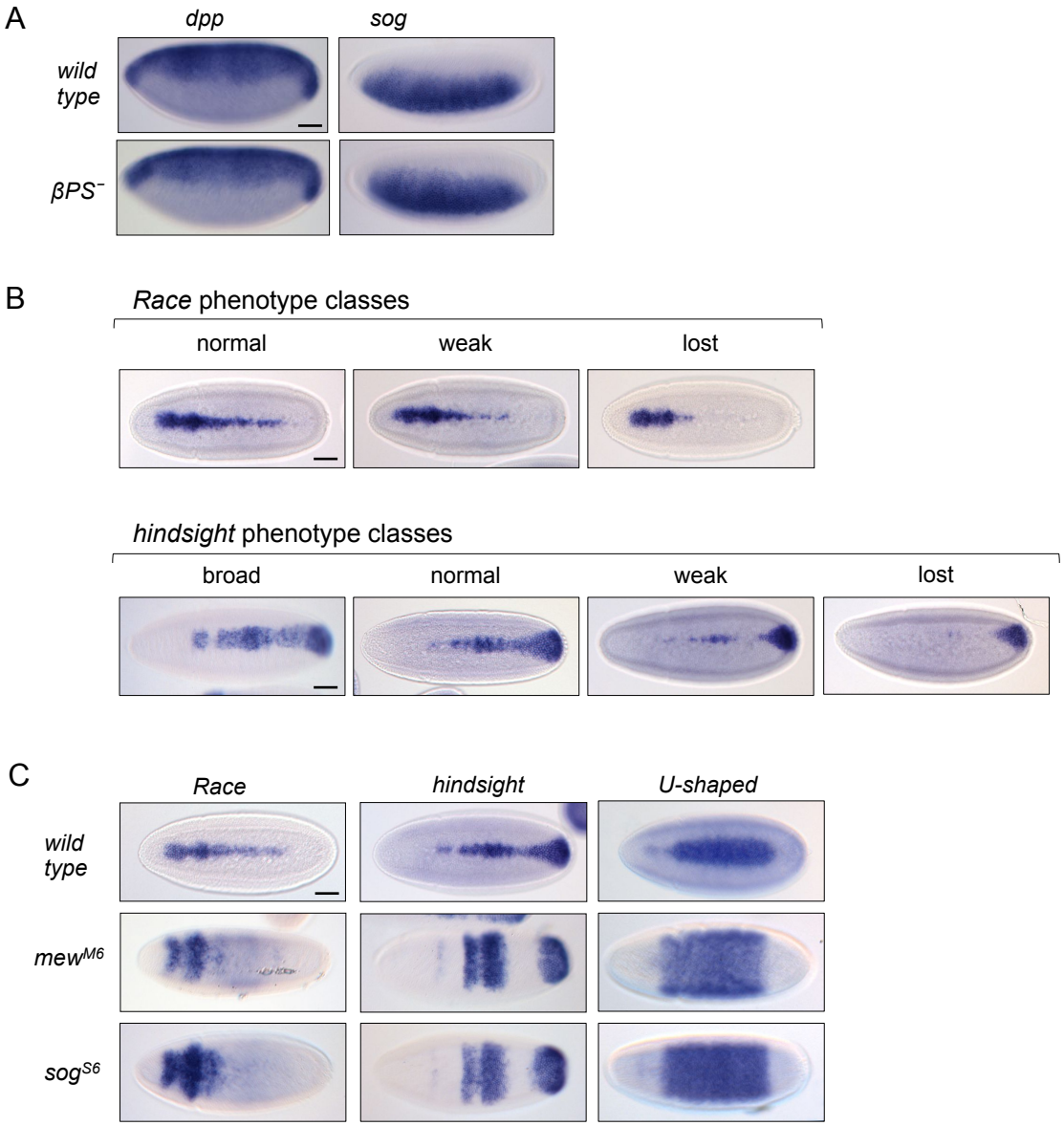

Figure S2, related to Figure 1: Expression patterns of *dpp*, *sog, Race, hnt* and *ush* in embryos lacking βPS. **(A)** RNA *in situ* hybridisations to visualise expression of *dpp* and *sog* in wild-type and *βPS*$^{-}$ embryos. All images show lateral views of stage 5 embryos, anterior to the left. The expression patterns of *sog* and *dpp* are unaffected in *βPS*$^{-}$ embryos. In a sensitised genetic background, integrins in the ovarian follicle epithelium of the mother were previously shown to affect patterning in the embryo by changing the nuclear Dorsal gradient (Negreiros et al., 2010), which sets up the expression patterns of *dpp* and *sog*. The normal expression domains of *dpp* and *sog* expression suggest that the reduction in BMP signaling observed in integrin mutant embryos (see Figure 1) is not due to an earlier defect in the formation of the Dorsal nuclear gradient. This is also consistent with our finding that zygotic expression of βPS is sufficient to rescue the BMP defect in *βPS*$^{-}$ embryos (data not shown). **(B-C)** RNA *in situ* hybridisations for the BMP target genes *Race*, *hindsight (hnt)* and *u-shaped (ush)* in

mew^{M6} and *scb*^{5J38} mutant embryos. **(B)** Expression patterns for *Race* and *hnt* exemplifying the phenotypic classes used for the scoring of phenotypes in *mew* and *scb* mutant embryos, as quantified in Figure 1C. Phenotypes in the rescue experiment of *vkg* mutant embryos in Figure 3 were scored according to the same criteria. **(C)** *sog*$^{-}$-like phenotype observed in *mew*^{M6} integrin mutants. In a small proportion of embryos (~1%), *Race* and *hnt* expression was lost in the central amnioserosa region but expanded laterally in anterior/posterior domains. In ~5% of embryos, expression of *ush* was expanded laterally. These phenotypes are indicative of disrupted BMP gradient formation, with broadened low levels of signaling, resembling the phenotype of *sog*$^{-}$ embryos. Scale bars = 50 μm.

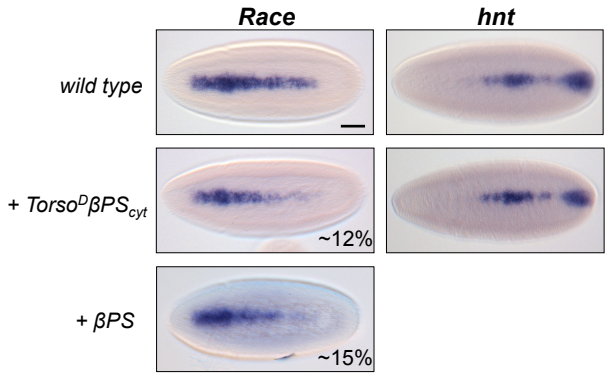

Figure S3, related to Figure 2: Overexpression of Torso^{D}βPS$_{cyt}$ in a wild-type background does not broaden the expression domains of *Race* or *hnt*. RNA *in situ* hybridisations for the BMP target genes *Race* and *hindsight* (*hnt*) in wild-type embryos and embryos overexpressing the Torso^{D}βPS$_{cyt}$ integrin signaling construct or wild-type βPS using the maternal *matαGal4-VP16* driver (see Supplemental Methods for details). *Race* or *hnt* expression is not broadened by ectopic expression of Torso^{D}βPS$_{cyt}$, suggesting that integrin signaling is not sufficient to induce peak threshold BMP target genes outside their normal expression domain. Note that in a small proportion of Torso^{D}βPS$_{cyt}$ overexpressing embryos (~12%) *Race* expression was weakened, an effect also observed in a small proportion of embryos overexpressing wild-type βPS (~15%), as shown. These effects may be due to dominant negative effects of free βPS or Torso^{D}βPS$_{cyt}$ when overexpressed in a wild-type background, as has been reported before (Tanentzapf et al., 2006). All images show dorsal views of stage 6 embryos, anterior to the left. Scale bar = 50 μm.

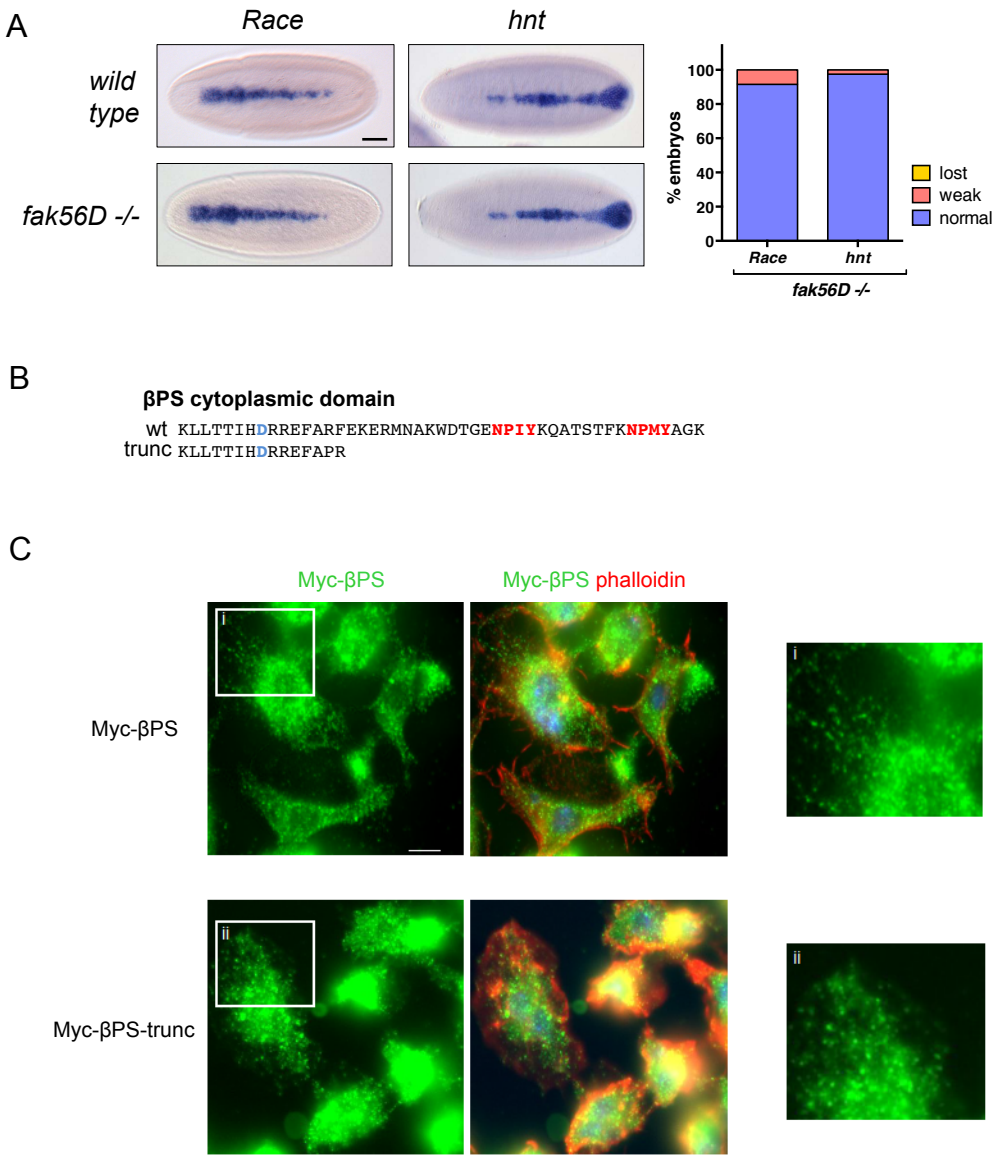

Figure S4, related to Figure 4: Dissecting the mechanism of integrin signaling-mediated enhancement of BMP responses. (A) RNA *in situ* hybridisations for *Race* and *ush* in wild-type and *Fak56D* mutant embryos (dorsal views, stage 6). The *Race* and *hnt* expression patterns are unaffected in the mutant embryos, suggesting that peak BMP expression is FAK-independent. Quantification of phenotypes show counts performed on two repeats. Scale bar = 50μm. **(B)** Amino acid sequence of the cytoplasmic domain of wild-type βPS and the truncation mutant, βPS-trunc, used in the co-immunoprecipitation experiment in Fig. 4J, with the two conserved NPXY motifs highlighted in red, and the salt bridge aspartate (D) residue implicated in the interaction with the α-integrin cytoplasmic domain highlighted in blue. The final two amino acids in βPS-trunc were introduced during the cloning procedure. **(C)** Immunoflourescence images of S2R+ cells expressing Myc-tagged wild-type βPS or the βPS-trunc mutant and plated on collagen IV, and stained with anti-Myc antibody

(green) and phalloidin (red). βPS and βPS-trunc show similar subcellular distribution. Right panels show higher magnification views of regions at the cell periphery outlined in the main figures as as i) (βPS) and ii) (βPS-trunc). Scale bar = 5 μm.

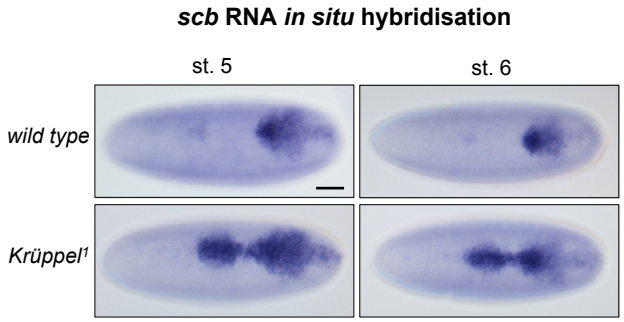

Figure S5, related to Figure 5: *scb* expression in the central domain is repressed by Krüppel.
RNA *in situ* hybridisation for *scb* in wild-type and homozygous *Krüppel*[1] mutant embryos (dorsal views, stage 5 or 6 as indicated). In *Krüppel*[1] mutant embryos, expression of *scb* extends into the central region, consistent with a function for Krüppel in repressing *scb* in this region. Scale bars = 50μm.

Supplemental Experimental Procedures

Fly strains, crosses and phenotype analysis

The following fly strains were used in this study: *mys^{XG43} FRT101* (Leptin et al., 1989), *mew^{M6}* (Bloomington #1483), *scb^{5J38}*, *UAS-mys UAS-srcEGFP*, *UAS-Torso^{D}βPS$_{cyt}$ UAS-mCD8GFP* (Schock and Perrimon, 2003), *UASp-Tkv^{QD}* (Casanueva and Ferguson, 2004), *vkg^{k00236}* (Bloomington #10473), *Fak56D^{CG1}* (Grabbe et al., 2004), *LanB1^{1B1} FRT40A* (Urbano et al., 2009), *matα-Gal4-VP16* (Bloomington BL7062), *dpp^{Hin37}/GlaDp(2;2)DTD48*, *Kr^{1}* (Bloomington #3493). Bloomington strains #1813, #1929 and #2121 were used to generate *mys^{XG43} or LanB1^{1B1}* germ-line clones using the FLP-DFS technique (Chou and Perrimon, 1996). *y^{67c23}w^{118}* flies were used as wild-type controls.

To generate *βPS^{-}* germ-line clones, 3rd instar larvae of the genotype *mys^{XG43} FRT101/ovo^{D1} FRT101; hsFlpase/+* were heat-shocked on two consecutive days for 2h at 37°C. The resulting adult females were crossed as follows to obtain *βPS^{-}* embryos (marked by absence of a *ftz-lacZ* balancer):

♀ *mys^{XG43} FRT101/mys^{XG43} FRT101* x ♂ *FM7c ftz-lacZ/Y* → *mys^{XG43} FRT101/Y*

LanB1^{1B1} germ-line clones were generated similarly by heat-shocking larvae of the genotype *hsFlpase/+; LanB1^{1B1} FRT40A/ovo^{D1} FRT40A*. Resulting adult females were crossed as follows to obtain *LanB1^{1B1}* embryos (marked by absence of *ftz-lacZ* balancer):

♀ *LanB1^{1B1} FRT40A/LanB1^{1B1} FRT40A* x ♂ *LanB1^{1B1} FRT40A/CyO ftz-lacZ*→ *LanB1^{1B1} FRT40/LanB1^{1B1} FRT40A*

For rescue experiments of *βPS^{-}* embryos, *mys^{XG43}* germline clones in females were generated as described above, but with inclusion of a matα4-Gal4-VP16 transgene to allow expression of paternally derived rescue constructs in the embryo. Embryos were collected from the following crosses (presence of UAS-Torso^{D}βPS$_{cyt}$ marked by absence of *ftz-lacZ*):

a) ♀ *mys^{XG43} FRT101 / FRT101 mys^{XG43}; hsFLPase/matα4-Gal4-VP16* X ♂ *yw*

b) ♀ *mys^{XG43} FRT101 / FRT101 mys^{XG43}; hsFLPase/matα4-Gal4-VP16* X ♂ *UAS-mys*

c) ♀ *mys^{XG43} FRT101 / FRT101 mys^{XG43}; hsFLPase/matα4-Gal4-VP16* X ♂ *UAS-Torso^{D}βPS$_{cyt}$/TM3, ftz-lacZ*

For *vkg* rescue experiments, embryos were collected from the following crosses (presence of UAS-Torso^{D}βPS$_{cyt}$ marked by absence of *ftz-lacZ*)::

a) ♀ *vkg^{k00236}/matα4-Gal4-VP16* X ♂ *yw*

b) ♀ *vkg^{k00236}/matα4-Gal4-VP16* X ♂ *UAS-mys*

c) ♀ *vkg*^{k00236}*/matα4-Gal4-VP16* X ♂ *UAS-Torso*^{D}*βPS*$_{cyt}$*/TM3, ftz-lacZ*

To overexpress βPS or Torso^{D}βPS$_{cyt}$ in a wild-type background, embryos were collected from the crosses (presence of UAS-Torso^{D}βPS$_{cyt}$ marked by absence of *ftz-lacZ*):

♀ *matα4-Gal4-VP16 x* ♂ *UAS-Torso*^{D}*βPS*$_{cyt}$*/TM3, ftz-lacZ*

♀ *matα4-Gal4-VP16 x* ♂ *UAS-mys/UAS-mys*

Embryos overexpressing Tkv^{QD} were obtained by collection from mothers with the genotype *matα4-Gal4VP16/+, UASp-Tkv*^{QD}*/+*, allowing Tkv^{QD} expression in the germline and developing oocyte/embryo.

For all other embryo collections, embryos were collected from heterozygous (*vkg*^{k00236}, *mew*^{M6}, *scb*^{5J38}, *dpp*^{Hin37}*/GlaDp(2;2)DTD48*) or homozygous (*Fak56D*^{CG1}) stocks, which were sometimes derived from available stocks by introducing a *ftz-lacZ*-marked balancer.

For the quantification of phenotypes, expression patterns for *Race* and *hindsight* in the central presumptive amnioserosa region were scored as either normal or disrupted, with a disrupted pattern defined as the clear loss of a continuous expression stripe along the dorsal midline. For *mew* and *scb* mutants and the rescue experiments with *vkg* mutant embryos, *Race* and *hindsight* expression was scored in additional categories as weak or broadened (see Figure S2B for examples of each category). For all rescue experiments, the % rescue was calculated as the percentage reduction in the proportion of embryos with a disrupted expression pattern (weak + lost) in the progeny of the rescue cross relative to the proportion seen in the non-transgene control (i.e. crosses to yw, crosses a) above), according to this formula: $\%\ rescue\ for\ (b) = \frac{\%\ disrupted\ in\ (a) - \%\ disrupted\ in\ (b)}{\%\ disrupted\ in\ (a)} \times 100.$

To measure the width of *ush* and *Race* expression (Figures 1B, 3C and 3D), the width of the expression stripe at ~0.5 embryo length in pixels was measured manually for each embryo using Image J. Pixel measurements were then converted into measurements of cell numbers by determining the average cell width in pixels through repeated pixel measurements of a defined number of cell rows along the dorsal midline.

pMad imaging and quantification

Embryos were imaged using a Leica TCS SP5 AOBS inverted confocal microscope using a 20x objective and 1.7x confocal zoom. Images for direct comparison were imaged on the same day with identical settings. Embryos were imaged in 18 consecutive coronal optical sections covering the total depth of the pMad stain, followed by maximal intensity projections of 3D stacks. Quantification was

performed as previously described (Umulis et al., 2010). The analysis comprised three image sets, (images obtained from three independent stainings). Each image set contained four groups: (1) maternal *βPS*$^{-}$, zygotic *βPS*$^{+}$ stage 5; (2) maternal *βPS*$^{-}$, zygotic *βPS*$^{+}$ stage 6, (3) m/z *βPS*$^{-}$ stage 5, (4) m/z *βPS*$^{-}$ stage 6. Stage 6 was marked by appearance of the cephalic furrow. Each group from each set contained at least 6 embryos. One image set was chosen as a reference and intensity values of other sets were scaled (normalised) to this set to allow combination of the data. To calculate the width of the pMad stripe above a certain intensity threshold (Figure 1F, G), the width for each individual embryo was first determined in pixels from each image thresholded at the given intensity. The typical cell diameter in pixels was determined and used to convert the pixel width measurement into a measurement in unit of cells.

DNA constructs and dsRNA preparation

The following expression plasmids have been previously described: pAC-Flag-Mad, pAC-Flag-Tkv^{QD} (Muller et al., 2003). pMT-frizzled-V5 was a gift from Jean-Paul Vincent. To generate pAC-Tkv^{QD}-V5, the coding sequence of Tkv^{QD} from pAC-Flag-Tkv^{QD} was cloned into the XbaI site of pAC5.1A in frame with the C-terminal V5-His tag. For pMT-Myc-Torso^{D}βPS$_{cyt}$, the Myc-Torso^{D}βPS$_{cyt}$ CDS was PCR-amplified from genomic DNA of *UAS-Torso^{D}βPS$_{cyt}$* flies and inserted into pMT (Invitrogen). The βPS (*Drosophila* Genomics Resourse Center) and *α*PS3 (a gift from Nick Brown) cDNAs were inserted into a pMT-Myc vector using In-Fusion HD cloning kit (Clontech) to allow expression of N-terminally tagged integrin subunits. βPS and Torso^{D}βPS$_{cyt}$ cytoplasmic domain mutations (Y831A, Y843A and the double mutant) were generated from pMT-Myc-Torso^{D}βPS$_{cyt}$ by site-directed mutagenesis. The βPS cytoplasmic tail truncation (comprising the last 34 residues) was generated by PCR around the pMT-Myc-βPS using the below primers followed by restriction digestion (Avr2) and ligation.

FW - GCCCTAGGTAGGGCGCGCCTAGATTCGCAATCA

REV - GCCCTAGGAGCGAACTCCCGCCGATCGTGGATC

dsRNA was generated from PCR fragments amplified with the following primers using the Megascript kit (Invitrogen):

bPS FW-TAATACGACTCACTATAGGGTGGACACAGACGATCCACAT

bPS REV-TAATACGACTCACTATAGGGCAACTGGTTCCTGTTCCGTT

aPS1 FW- TAATACGACTCACTATAGGGTCATCGCCACAGTCTTTCTG

aPS1 REV-TAATACGACTCACTATAGGGTTGGCATCTCTATATCCGGC

aPS3 FW-TAATACGACTCACTATAGGGTGGACACAGACGATCCACAT

aPS3 REV -TAATACGACTCACTATAGGGCAACTGGTTCCTGTTCCGTT

RNA was recovered using ammonium acetate and EtOH, dried, resuspended in 20μl of nuclease free water.

DNA Transfections of S2R+ cells

For pMad signaling assays, $3x10^6$ *Drosophila* S2R+ cells were transfected using effectene (Qiagen) and the following amounts of DNA: 250ng pAC-Flag-Mad, 100-500ng of pAC-Flag-Tkv^{QD} and 500ng of pAC-Flag (EV-Flag), pMT-Myc (EV-Myc), pMT-Myc-βPS, pMT-Myc-βPS-trunc or pMT-Myc-$Torso^{D}βPS_{cyt}$ (wild-type or mutant $βPS_{cyt}$ region). For co-immunoprecipitation experiments, $3x10^6$ cells were transfected with 5μg DNA per plasmid. When plating cells on a collagen IV substrate prior to transfection, tissue culture wells were coated with collagen IV by adding 50μg human placental collagen IV (Advanced Biomatrix), diluted with sterile PBS, for 1h at 37°C. Excess unbound collagen IV was removed by rinsing in PBS and cells were then plated as appropriate. For laminin competition experiments, collagen IV-plated cells were treated with a final concentration of 1 or 2μg/mL of mouse laminin (Corning). Laminin was added in serum-free medium 72h post-transfection and cells were harvested 2h after treatment.

Immunostaining of S2R+ cells

S2R+ cells transfected with pMT-Myc-βPS, pMT-Myc-βPS-trunc and plated on collagen IV-coated coverslips were fixed with 4%PFA, followed by incubation overnight with mouse anti-Myc (1:250, Millipore) and Phalloidin dye (1:500, Life Technologies), washes with PBT (PBS with 0.1% Tween 20) and incubation in anti-mouse secondary antibody (Alexa Fluor® 488 dye, 1:500). Stained cells were mounted with Prolong gold antifade reagent with DAPI (Life technologies). Images were captured with an Olympus BX51 upright microscope using a 63x oil immersion objective and Coolsnap EC camera and processed through MetaVue software (Molecular Devices).

Co-Immunoprecipitation experiments

48h after transfection cells were harvested and lysed in NP-40 buffer. Lysates were pre-cleared by incubation with protein G-coupled Agarose beads (Pierce) for 30 min at 4°C. Cleared lysates were incubated with 3ug of anti-αPS1 antibody (DK.1A4, Developmental Studies Hybridoma Bank) for 2h at

4°C followed by incubation with Protein G-coupled Agarose beads for 30 min at 4°C. Lysates were than washed four times in lysis Buffer. For Myc-tagged proteins, complexes were precipitated using anti-Myc agarose beads (Sigma) for 1.5 h at 4°C. Beads were then washed four times with chilled PBS. Protein complexes were resolved by SDS-PAGE and detected by Western blotting.

Supplemental References

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