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Supplemental Information

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Regulation of aPKC and Hippo Signaling

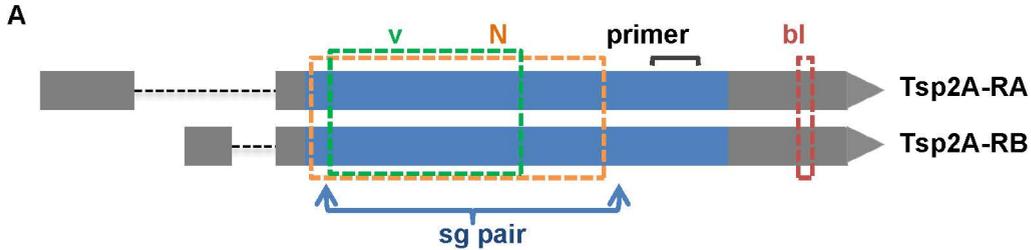
Chiwei Xu, Hong-Wen Tang, Ruei-Jiun Hung, Yanhui Hu, Xiaochun Ni, Benjamin E. Housden, and Norbert Perrimon

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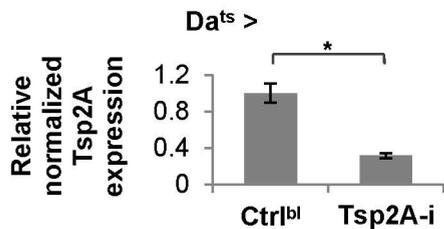
The septate junction protein Tsp2A restricts intestinal stem cell activity via endocytic regulation of aPKC and Hippo signaling

Chiwei Xu, Hong-Wen Tang, Ruei-Jiun Hung, Yanhui Hu, Xiao-chun Ni, Benjamin E. Housden, Norbert Perrimon.

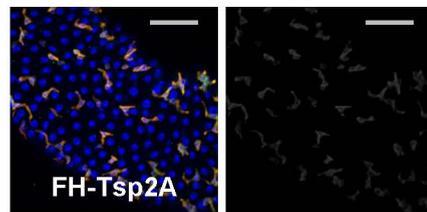
Supplemental Figures



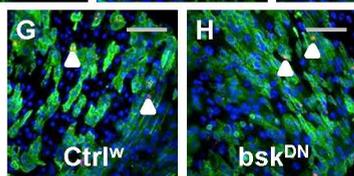
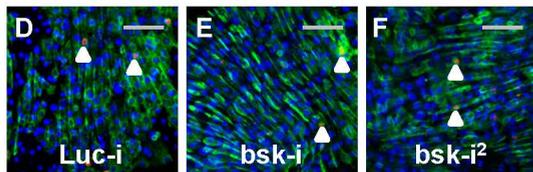
B Midgut RT-qPCR



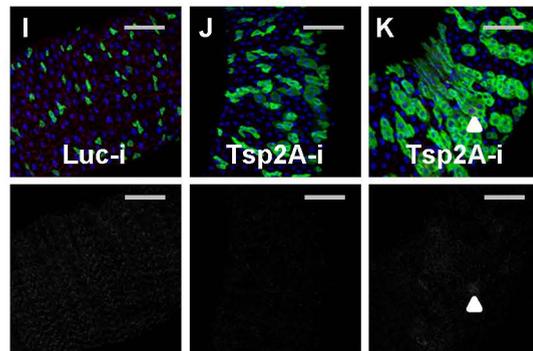
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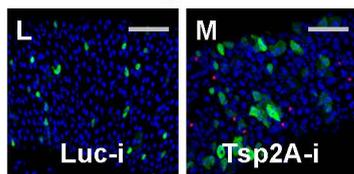
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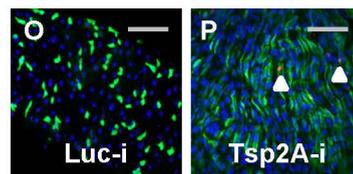
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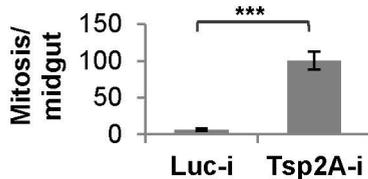
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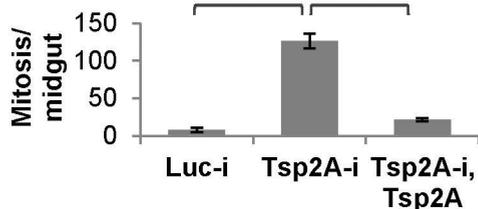
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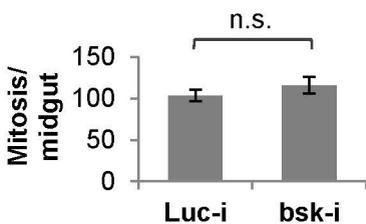
N SGT >



Q Myo1A^{ts} >



R Myo1A^{ts}; UAS-Tsp2A-i^N >



S Different Gal4 lines >

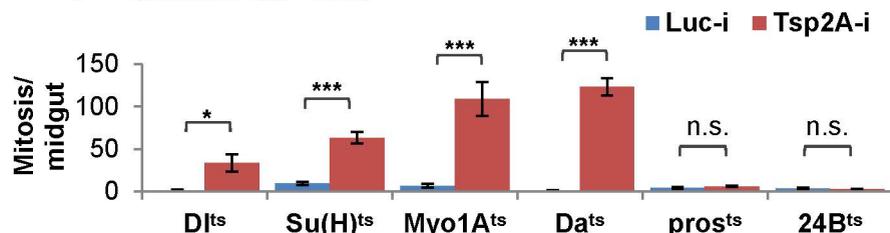
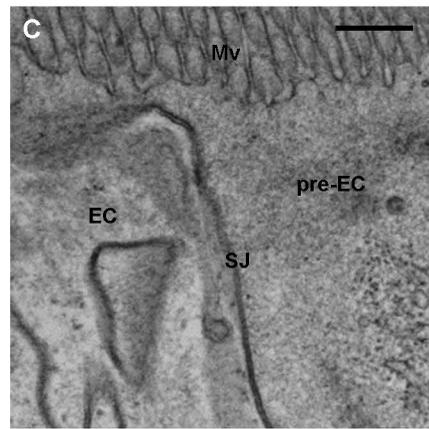
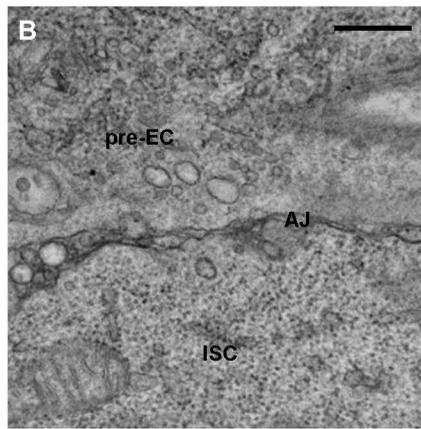
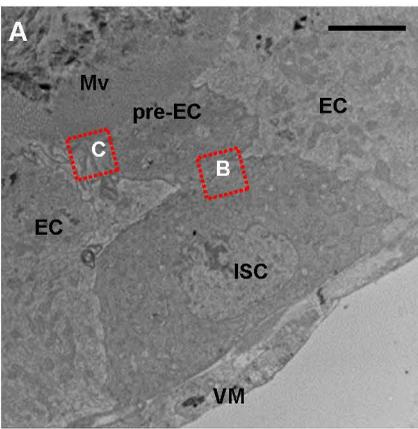


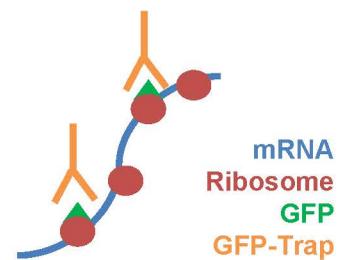
Figure S1. Further characterization of *Tsp2A* knockdown phenotype. Related to Figure 1.

(A) A diagram depicting *Tsp2A* mRNAs and reagents used in our study. The introns are shown in dashes. The exons are shown in rectangular boxes (the triangular box at the end of second exon indicates the direction of transcription/translation), with the protein-coding region (same for the two isoforms *Tsp2A-RA* and *Tsp2A-RB*) highlighted in blue. The target regions of three different RNAi lines are shown in green, yellow, and red dashed boxes for VDRC (v), NIG (N), and Bloomington (bl) stocks, respectively. The amplified region for RT-qPCR primers is marked in black bracket. Finally, the target regions for sgRNAs designed for *Tsp2A* knockout are indicated with blue arrowheads. (B) RT-qPCR measurement of *Tsp2A* expression in midguts ubiquitously expressing *Tsp2A* RNAi driven by *tubGal80^{ts}*; *daGal4* (*Da^{ts}*) for 7d. *Ctrl^{bl}* flies (genotype: *y v; attp2*, with the superscript label “bl” standing for “Bloomington”) were used as the control for *Tsp2A* RNAi. Data are represented as mean \pm SEM. (C) Anti-Flag staining of midguts expressing *Tsp2A* cDNA with Flag-HA tag (*FH-Tsp2A*) in ISCs/EBs for 5d. Scale bar: 50 μ m. The separate channel indicating Flag stainings in ISCs/EBs is shown in grayscale to the right of the merged image. (D-H) pH3 staining of midguts expressing *Tsp2A* RNAi together with *Luc* RNAi, *bsk* RNAi (two different lines), or *bsk^{DN}* in ISCs/EBs for 5d. Scale bar: 50 μ m. White arrowheads highlight examples of pH3+ cells. (I-K) Midguts expressing *Luc* RNAi or *Tsp2A* RNAi in ISCs/EBs for 3d are stained for the apoptosis marker, cleaved-caspase 3. Scale bar: 50 μ m. The red channel is presented in grayscale below each of the merged images. Some background signals could be observed in the visceral muscles (as in I). The lineages of ISCs/EBs expressing *Tsp2A* RNAi are not stained positive for cleaved-caspase 3, despite showing obvious signs of ISC/EB expansion (as in J). When the lineages of ISCs/EBs expressing *Tsp2A* RNAi expand to the extent that they almost replace the whole epithelium, some rare signs of apoptosis could be detected (as in K). The white arrowhead indicates a case of apoptosis. (L-N) pH3 staining and mitosis quantification of midguts expressing *Luc* RNAi or *Tsp2A* RNAi in differentiating progenitor cells (EBs) for 9d. Scale bar: 50 μ m. *SGT*-driven GFP expression labels EBs. N=8 midguts were analyzed for each group. Data are represented as mean \pm SEM. (O-P) pH3 staining of midguts expressing *Luc* RNAi or *Tsp2A* RNAi in ECs for 5d. Scale bar: 50 μ m. The expression of enhancer trap *esgGFP* labels ISCs/EBs. White arrowheads highlight examples of pH3+ cells. (Q) Mitosis quantification of midguts expressing *Luc* RNAi, *Tsp2A* RNAi, or *Tsp2A* RNAi together with *FH-Tsp2A* in ECs for 5d. N>5 midguts are analyzed for each group. Data are represented as mean \pm SEM. (R) Mitosis quantification of midguts expressing *Tsp2A* RNAi together with *Luc* RNAi or *bsk* RNAi in ECs for 5d. N>9 midguts are analyzed for each group. Data are represented as mean \pm SEM. (S) Mitosis quantification of midguts expressing *Luc* RNAi or *Tsp2A* RNAi for 7d in ISCs (under the control of *D^{ts}*), EBs (*Su(H)^{ts}*), ECs (*Myo1A^{ts}*), ubiquitously (*Da^s*), EEs (*pros^{ts}*), or visceral muscles (*24B^{ts}*), for comparison. N>5 midguts are analyzed for each group. Data are represented as mean \pm SEM.

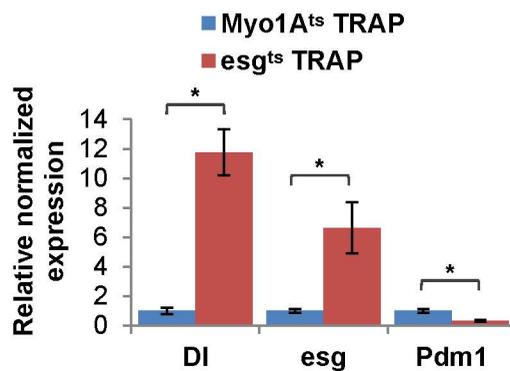
Midgut EM (genotype: *Ctrl^w*)



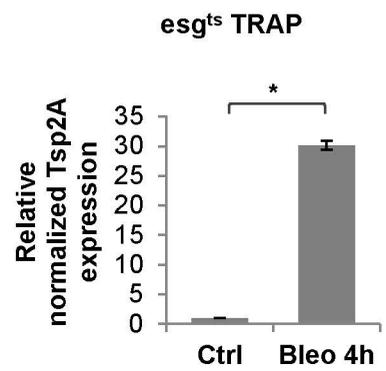
D Translating Ribosome Affinity Purification (TRAP)



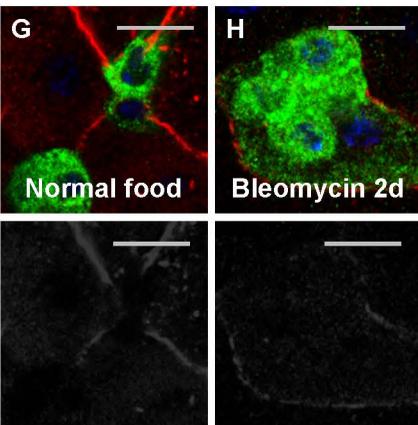
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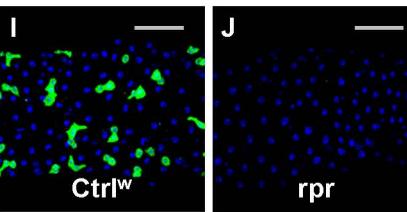
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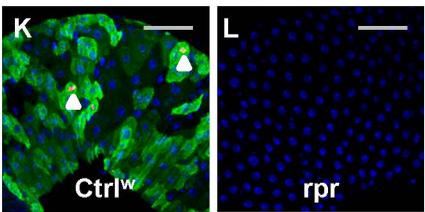
EGT **Tsp2A** **esg** **DAPI**



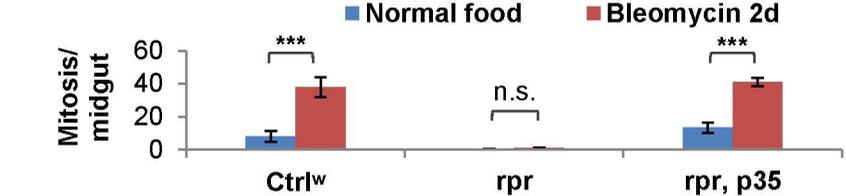
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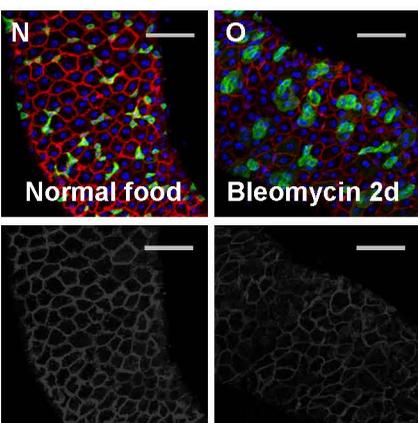
+ Bleomycin 2d **pH3** **esg** **DAPI**



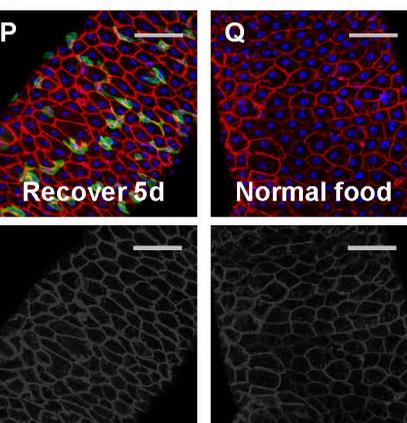
M EGT >



EGT > Ctrl^w **Tsp2A** **esg** **DAPI**



EGT > rpr **Tsp2A** **esg** **DAPI**



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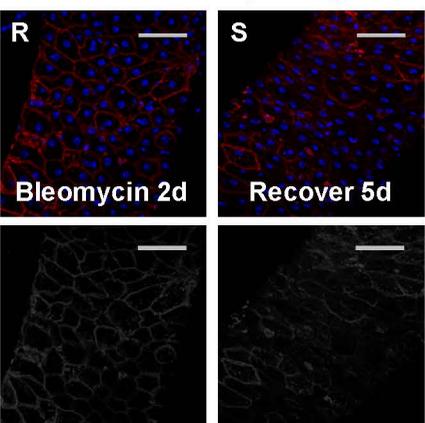


Figure S2. Further characterization of SJ formation and *Tsp2A* expression in ISCs/EBs. Related to Figure 2.

(A) Electron micrographs show two progenitor cells between two ECs in the wild type midgut. Scale bar: 4 μm . (B-C) The areas encircled with red dashed boxes in A were imaged at higher magnification to show cell junctions. Scale bar: 400 nm. The ISCs/EBs could be recognized by small cell size and dense cytoplasm (Ohlstein and Spradling, 2006); the ECs could be recognized by large cell size and microvilli (Mv) at the apical surface facing the lumen. One of the progenitor cells remains as an ISC, maintaining additional ISC/EB features such as basal localization in close proximity to the visceral muscles (VM), and AJ connection with neighboring cells (as in B). The other progenitor cell is differentiating into the stage of pre-EC, as it forms microvilli at its apical surface and has SJ connection with its neighboring ECs (as in C). (D) A cartoon depicting the working principle of TRAP. Following the expression of the GFP-tagged ribosome subunit (*GFP-RpL10A*) under the control of *esg^{ts}* or *Myo1A^{ts}*, polysomes and associated mRNAs from ISCs/EBs or ECs can be enriched from midgut lysates with GFP-Trap beads. (E) RT-qPCR measurement demonstrating the efficient enrichment of ISC markers (*Dl*, *esg*) and depletion of EC marker *Pdm1* with the ISC/EB-specific, *esg^{ts}* TRAP. The normalized expression for each gene is presented as the ratio to the average value measured for the EC-specific, *Myo1A^{ts}* TRAP. Data are represented as mean \pm SEM. (F) RT-qPCR measurement of ISC/EB-specific *Tsp2A* mRNA enriched by TRAP, from flies with or without 4 hrs of bleomycin feeding. Data are represented as mean \pm SEM. (G-H) Single Z-stack *Tsp2A* staining images of midguts from flies fed with normal food or bleomycin food for 2d before dissection. Scale bar: 10 μm . *EGT*-driven GFP expression labels ISCs/EBs. The red channels of *Tsp2A* staining are presented in grayscale below the merged images. (I-L) pH3 staining of midguts with or without *rpr* expression in ISCs/EBs for 6d, with or without bleomycin feeding for the last 2d before dissection. Scale bar: 50 μm . The white arrowheads highlight examples of pH3+ cells. (M) Mitosis quantification of midgut expressing *rpr* alone, or *rpr* together with the anti-apoptotic gene *p35* in ISCs/EBs for 6d, under normal feeding or tissue damage conditions. N>9 midguts were analyzed for each group. Data are represented as mean \pm SEM. (N-S) *Tsp2A* staining of midguts with or without *rpr* expression in ISCs/EBs, from young adult flies fed with normal food, bleomycin for 2d, or bleomycin for 2d followed by normal food for 5d (recover 5d). Scale bar: 50 μm . The red channels of *Tsp2A* stainings are presented in grayscale below the merged images. Note that after ISC/EB depletion, the ECs can respond to tissue damage by hypertrophic growth (size enlargement), but their *Tsp2A* stainings are much weakened.

Figure S3. The expression pattern and knockdown phenotype of different junction proteins. Related to Figure 4.

(A-D) Midguts from young adult flies on normal food or on bleomycin food for 2d before dissection were stained for the SJ protein Mesh or the AJ protein Arm. Scale bar: 50 μ m. *EGT*-driven GFP expression labels ISCs/EBs and their recent progenies due to perdurance. The red channel is presented in grayscale, below each of the merged images. (E-I) DI-lacZ staining of midguts expressing *Tsp2A* RNAi, *Mesh* RNAi, or *Ssk* RNAi (two different lines) in ISCs/EBs for 7d. Scale bar: 50 μ m. The empty vector stock (Ctrl^V, genotype: *y w; attP*) was used as the control for RNAi lines from VDRC. (J-N) DI-lacZ staining of midguts expressing *Luc* RNAi, *cora* RNAi (two different lines showing consistent phenotype, the experiment using line #2 is presented), *arm* RNAi, or *Lgl* RNAi (two different lines) in ISCs/EBs for 7d. Scale bar: 50 μ m. (O) Mitosis quantification of midguts expressing *Luc* RNAi, *arm* RNAi (two different lines), or *Lgl* RNAi (two different lines) in ISCs/EBs for 7d, with or without bleomycin feeding for the last 2d before dissection. N>5 midguts are analyzed for each group. Data are represented as mean \pm SEM. (P-V) pH3 staining and mitosis quantification of midguts expressing *Luc* RNAi or *Gli* RNAi (two different lines) in ISCs/EBs for 5d from flies on normal food, or for 7d from flies on bleomycin food during the last 2d. Scale bar: 50 μ m. White arrowheads highlight examples of pH3+ cells. N>6 midguts are analyzed for each group. Data are represented as mean \pm SEM.

Dats >

Tsp2A DAPI

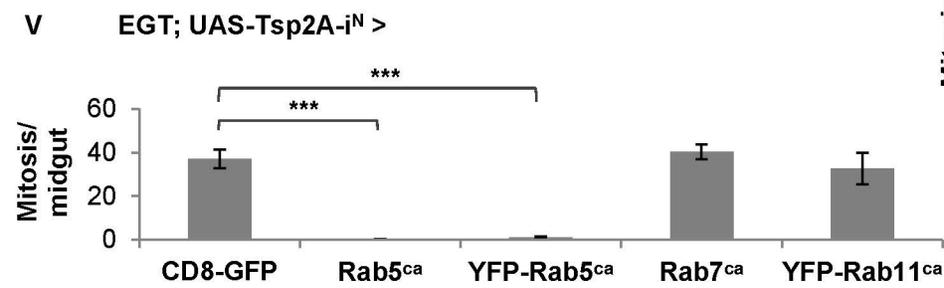
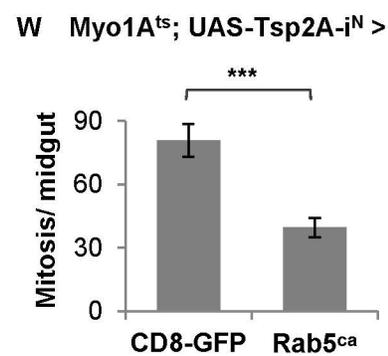
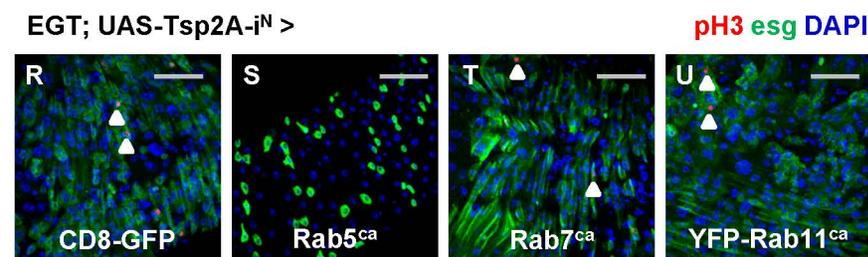
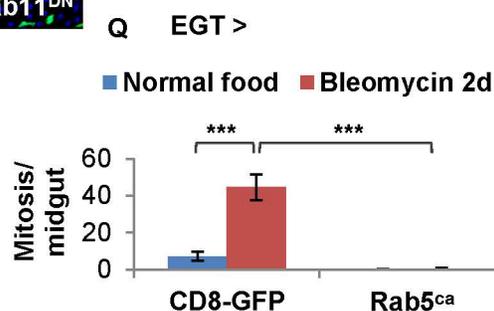
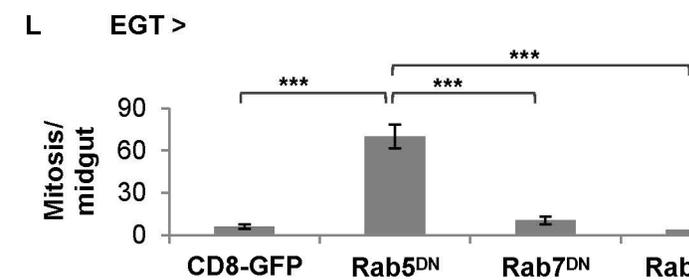
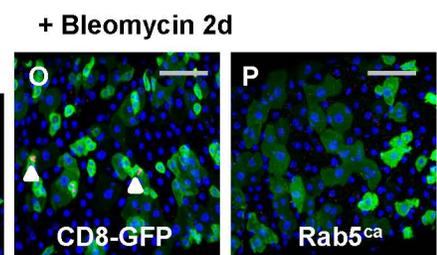
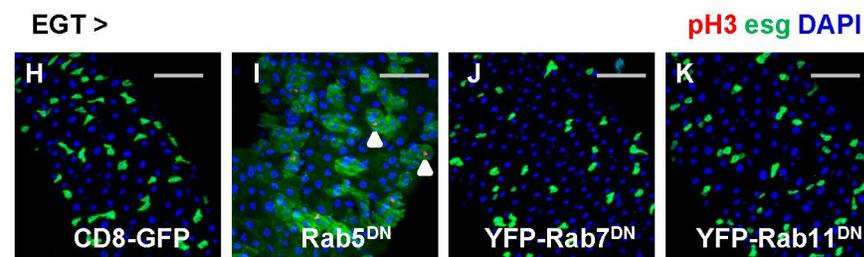
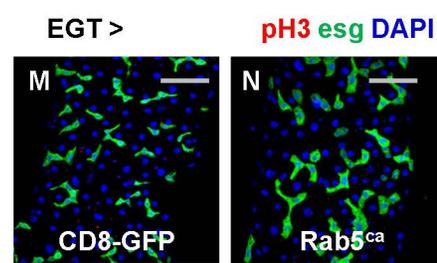
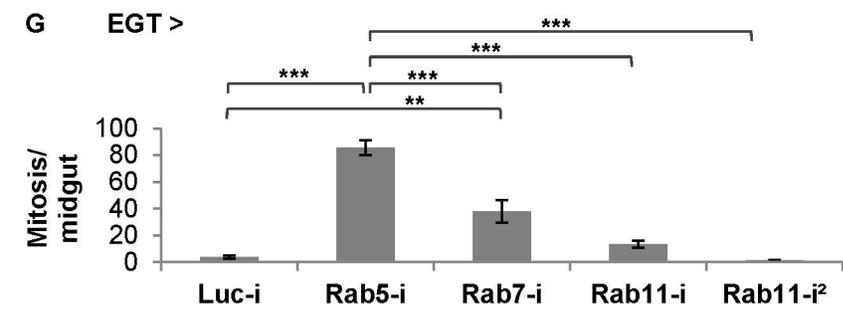
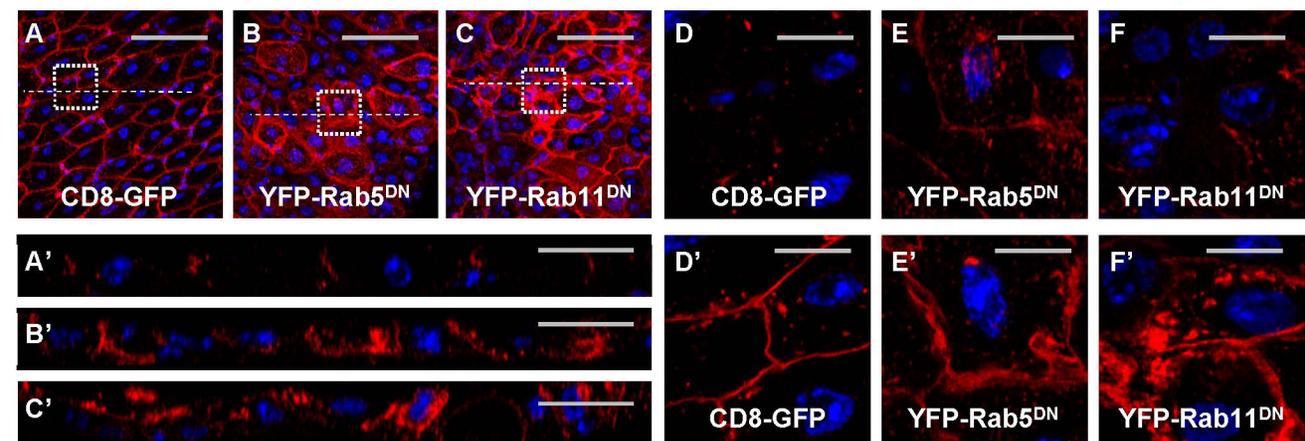


Figure S4. Functional analysis of different Rab proteins in the midgut. Related to Figure 5.

(A-C) Tsp2A staining of midguts ubiquitously expressing *CD8-GFP*, *Rab5^{DN}*, or *YFP-Rab11^{DN}* for 3d. Scale bar: 50 μ m. (A'-C') Orthogonal view showing the cross-sections indicated by dashed lines in A-C. Scale bar: 20 μ m. (D-F) High magnification, single Z stack images near the cell surface, corresponding to regions encircled with white dashed squares in A-C. Scale bar: 10 μ m. (D'-F') High magnification, single Z stack images near the cell center, corresponding to regions encircled with white dashed squares in A-C. Scale bar: 10 μ m. (G) Mitosis quantification of midguts expressing *Luc* RNAi, *Rab5* RNAi, *Rab7* RNAi, or *Rab11* RNAi (2 different lines) in ISCs/EBs for 5d. N>6 midguts are analyzed for each group. Data are represented as mean \pm SEM. (H-L) pH3 staining and mitosis quantification of midguts expressing *CD8-GFP*, *Rab5^{DN}*, *YFP-Rab7^{DN}*, or *YFP-Rab11^{DN}* in ISCs/EBs for 5d. Scale bar: 50 μ m. White arrowheads highlight examples of pH3+ cells. N>8 midguts are analyzed for each group. Data are represented as mean \pm SEM. (M-Q) pH3 staining and mitosis quantification of midguts expressing *CD8-GFP* or *Rab5^{ca}* in ISCs/EBs for 7d, with or without bleomycin feeding for the last 2d before dissection. Scale bar: 50 μ m. White arrowheads highlight examples of pH3+ cells. N=10 midguts are analyzed for each group. Data are represented as mean \pm SEM. (R-V) pH3 staining and mitosis quantification of midguts expressing *Tsp2A* RNAi together with *CD8-GFP* (control), *Rab5^{ca}*, *Rab7^{ca}*, or *Rab11^{ca}* in ISCs/EBs for 5d. Scale bar: 50 μ m. White arrowheads highlight examples of pH3+ cells. N>12 midguts are analyzed for each group. Data are represented as mean \pm SEM. (W) Mitosis quantification of midguts expressing *Tsp2A* RNAi together with *CD8-GFP* or *Rab5^{ca}* in ECs for 5d. N>7 midguts are analyzed for each group. Data are represented as mean \pm SEM.

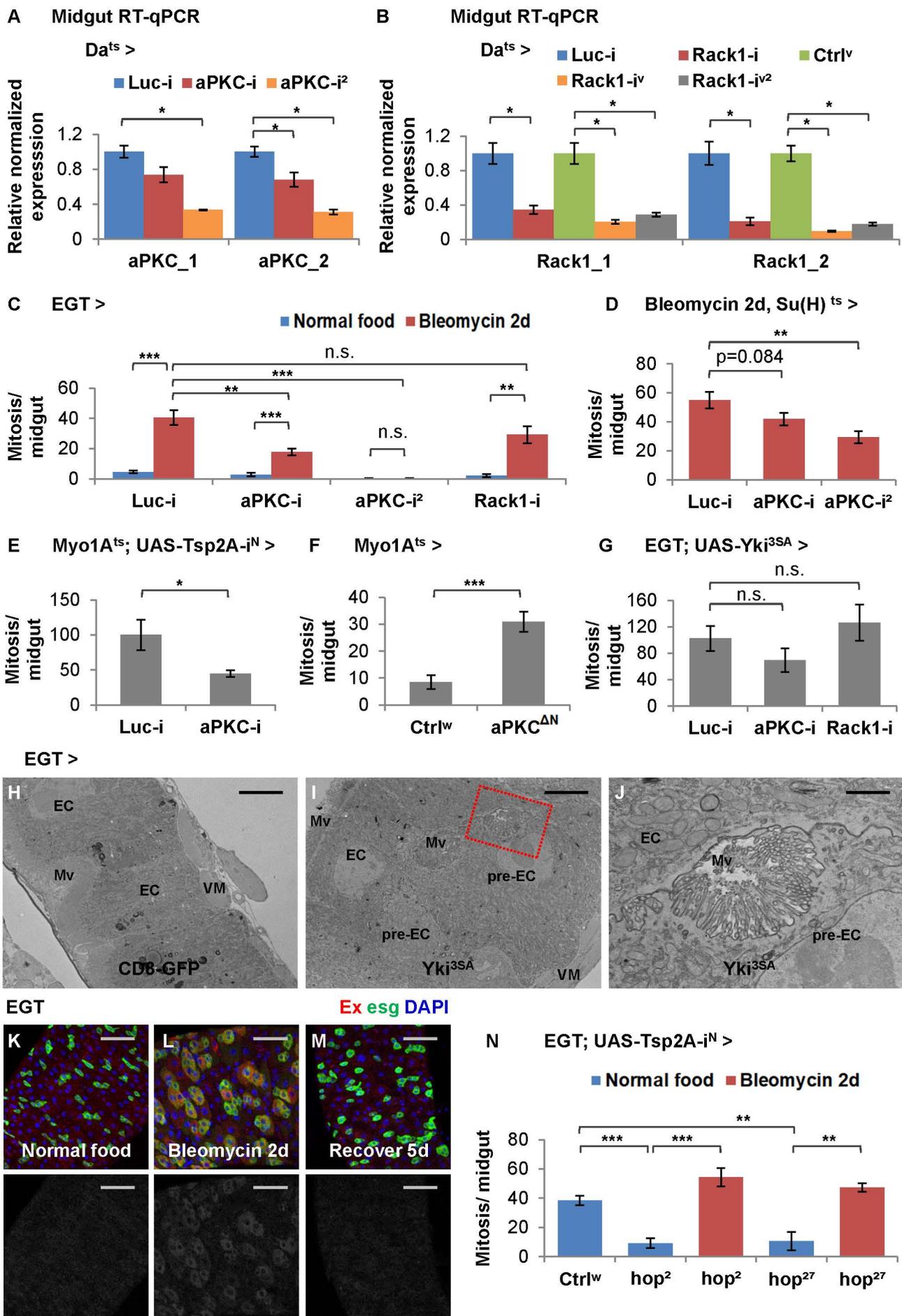


Figure S5. Further characterization of *aPKC/Rack1* knockdown and *Yki* activation in the midgut. Related to Figure 6.

(A) RT-qPCR measurement of *aPKC* expression (with two sets of primers) in midguts ubiquitously expressing *Luc* RNAi or *aPKC* RNAi (two different lines) for 5d. (B) RT-qPCR measurement (with two sets of primers) for knockdown efficiency of different *Rack1* RNAi lines, all of which can rescue *Tsp2A* RNAi-induced overproliferation (Table S1). (C) Mitosis quantification of midguts expressing *Luc* RNAi, *aPKC* RNAi, or *Rack1* RNAi in ISCs/EBs for 7d, with or without the last 2d on bleomycin food. N>6 midguts are analyzed for each group. Data are represented as mean \pm SEM. (D) Midgut mitosis quantification of flies expressing *Luc* RNAi or *aPKC* RNAi (two different lines) in EBs for 7d, with the last 2d on bleomycin food. N=10 midguts are analyzed for each genotype. Data are represented as mean \pm SEM. (E) Mitosis quantification of midguts expressing *Tsp2A* RNAi together with *Luc* RNAi or *aPKC* RNAi in ECs for 5d. N>6 midguts are analyzed for each group. Data are represented as mean \pm SEM. (F) Mitosis quantification of midguts with or without *aPKC^{ΔN}* expression in ECs for 5d. N=8 midguts are analyzed for each genotype. Data are represented as mean \pm SEM. (G) Mitosis quantification of midguts expressing constitutively active *Yki* (*Yki^{3SA}*) together with *Luc* RNAi, *aPKC* RNAi, or *Rack1* RNAi in ISCs/EBs for 3d. N>6 midguts are analyzed for each group. Data are represented as mean \pm SEM. (H) A representative electron micrograph showing the single-layered epithelium of midguts expressing GFP in ISCs/EBs for 4d. Scale bar: 4 μ m. (I) A representative electron micrograph showing the multi-layered epithelium of midguts expressing *Yki^{3SA}* in ISCs/EBs for 4d. Scale bar: 4 μ m. (J) A zoomed-in view of the region encircled with the red dashed box in I, showing the formation of microvilli at the apical surface of a basally localized pre-EC. Scale bar: 1 μ m. (K-M) Ex staining of midguts that are intact, damaged by 2d bleomycin feeding, or recovered on normal food for 5d after damage. Scale bar: 50 μ m. The red channel is presented in grayscale, below the merged images. (N) Mitosis quantification of midguts expressing *Tsp2A* RNAi in ISCs/EBs for 5d, with or without bleomycin feeding for the last 2d before dissection. The heterozygous genetic backgrounds containing a null allele of *hop* (*hop²* or *hop²⁷*) were used to reduce JAK/Stat signaling activity. N>5 midguts are analyzed for each group. Data are represented as mean \pm SEM.

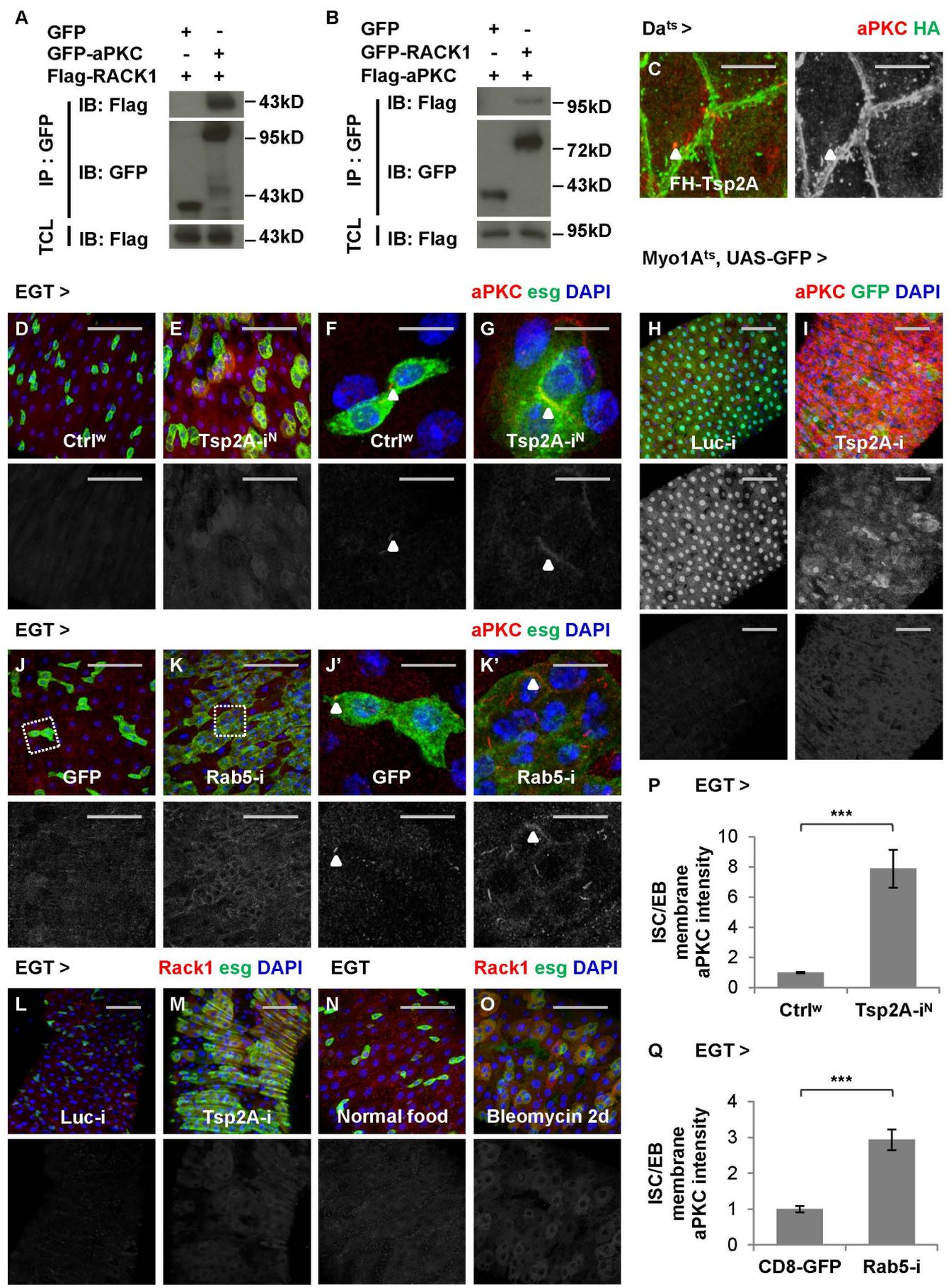


Figure S6. Further characterization of aPKC/Rack1 interaction and expression. Related to Figure 7.

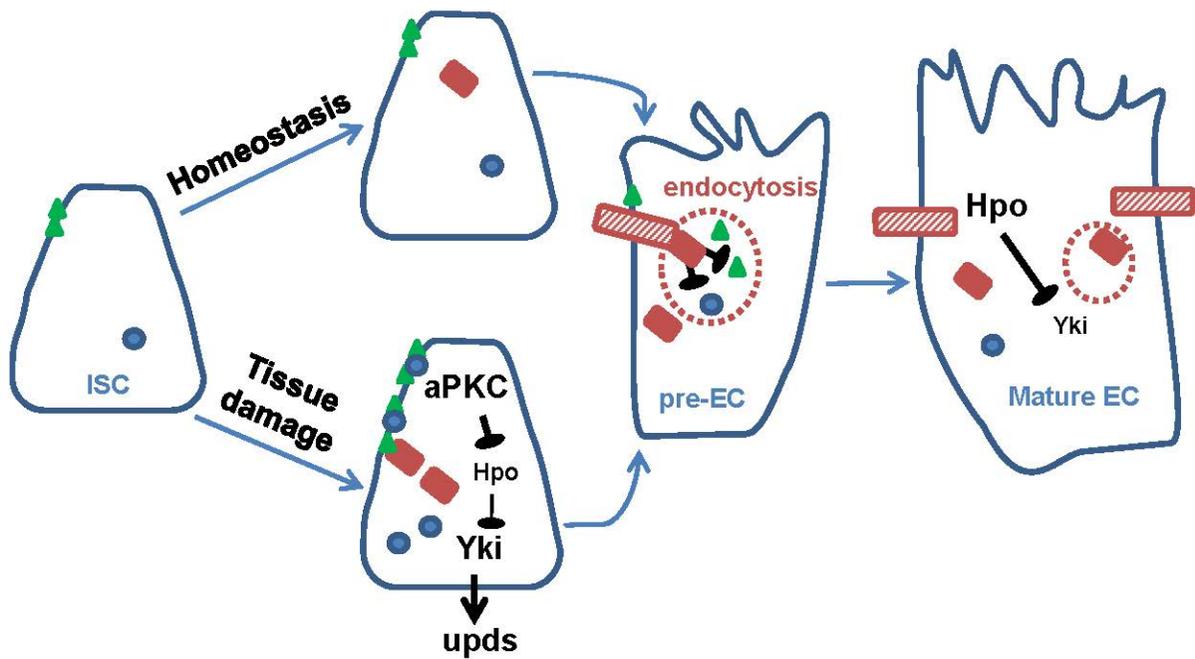
(A-B) Co-IP in S2R+ cells. GFP-aPKC co-precipitates with Flag-tagged Rack1, and GFP-Rack1 co-precipitates with Flag-tagged aPKC. (C) Midguts ubiquitously expressing Flag-HA-tagged *FH-Tsp2A* are co-stained with anti-aPKC and anti-HA antibodies. Scale bar: 10 μ m. The green channel of HA staining is presented in grayscale to the right of the merged images. The arrowhead indicates a case of internalized aPKC found in *Tsp2A* punctae. (D-G) Regular (D-E, Scale bar: 50 μ m) or high magnification (F-G, Scale bar: 10 μ m) confocal images showing aPKC staining of midguts expressing *Tsp2A* RNAi (the NIG line), or not, in ISCs/EBs for 3d. White arrowheads highlight concentrated aPKC staining on the membrane. The red channels are presented in grayscale below the merged images (in D-O, J'-K'). (H-I) aPKC staining of midguts expressing *Luc* RNAi or *Tsp2A* RNAi in ECs for 4d. Scale bar: 50 μ m. The separate channels indicating *Myo1A^{ts}>GFP* and aPKC signals are presented in grayscale, below the merged images. *Myo1A^{ts}>GFP* expression in the *Tsp2A* RNAi group is much weaker than the control, suggesting that *Myo1A* expression, as a feature of EC maturation, could be suppressed by *Tsp2A* RNAi. (J-K) aPKC staining of midguts expressing *CD8-GFP* (control) or *Rab5* RNAi in ISCs/EBs for 5d. Scale bar: 50 μ m. (J'-K') A zoomed-in view of regions encircled with white dashed squares in J-K. Scale bar: 10 μ m. White arrowheads highlight examples of concentrated aPKC stainings on the membrane. (L-M) Midguts expressing *Luc* RNAi or *Tsp2A* RNAi in ISCs/EBs for 3d are stained for Rack1. Scale bar: 50 μ m. (N-O) Rack1 staining of midguts from young adult flies on normal food or on bleomycin food for 2d before dissection. Scale bar: 50 μ m. (P) Quantification of membrane-localized aPKC stainings in ISCs/EBs with or without *Tsp2A* RNAi (the NIG line) expression for 3d. N=24 cells from 3 midguts were analyzed for each genotype. Data are represented as mean \pm SEM. (Q) Quantification of membrane-localized aPKC stainings in ISCs/EBs with *CD8-GFP* or *Rab5* RNAi expression for 5d. N=18 cells from 3 midguts were analyzed for each genotype. Data are represented as mean \pm SEM.

Tsp2A

SJ

aPKC

Rack1



Myo1AGal4, Pdm1, trypsins

Su(H)Gal4, Su(H)Gbe-GFP

DIGal4, DI-lacZ

esgGal4, esgGFP

DaGal4, tubGal4

Figure S7. SJ assembly and endocytic degradation of Tsp2A mediate the down-regulation of aPKC-Yki-JAK/Stat signaling during ISC-EC differentiation. Related to Figure 7.

The cell polarity determinant protein aPKC disrupts Hippo signaling and sustains Yki-JAK/stat activity to support proliferation. As ISCs/EBs differentiate towards ECs, Tsp2A is expressed for *de novo* SJ assembly. The active internalization of Tsp2A from the cell surface brings its interacting cargo proteins aPKC and Rack1 into endosome/lysosome for degradation. Under tissue homeostasis condition, Tsp2A-mediated degradation is sufficient to eliminate membrane-localized aPKC during the relatively infrequent events of ISC-EC differentiation. Under tissue damage or *Tsp2A* knockdown condition, aPKC accumulates on the membrane of ISCs/EBs to stimulate hyperactive Yki-JAK/Stat signaling. The gradient charts below the main chart of ISC-EC lineage match different Gal4 lines, reporter lines, or cell type markers to the stages of differentiation when they are expressed (red means high, yellow means low), based on literature and our characterization.