Dynamic switch of negative feedback regulation in Drosophila Akt-TOR signaling.

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Supporting Information

Figure S1
Western blot of total extracts prepared from *Drosophila* Kc_{167} cells at base line (lanes 1-6) or insulin stimulation (lanes 7-12) treated with dsRNAs as indicated and blotted with anti Pan-dAkt, anti P-dAkt, and anti-Tubulin as loading control. Top and bottom panels of anti alpha-Tubulin western blots are loading controls for the anti P-dAkt and anti Pan-dAkt western blots, respectively. Note that lane 10 from the right, (insulin-stimulated, dPDK1 RNAi treated cells) is underloaded.
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(1.35 MB TIF)

Figure S2
Genome wide screen for regulators of dAkt (Ser505) phosphorylation. (A) Cartoon of the cytoblot technique used to screen 58×384 well plates containing dsRNAs covering the entire *Drosophila* genome. Each screen was performed in duplicates. Experimental values for dAkt phosphorylation are normalized to the individual cell numbers per well determined by a DNA dye staining. See experimental procedures for details. (B, C) Ranked *Z*-Scores (corresponding to relative P-dAkt levels) of genome wide RNAi screens at baseline (B) and Insulin stimulation (C) with the known components of InR and Tor signaling marked in red.
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Figure S3
Analysis of *in vivo* dAkt protein expression in various genetic gain- and loss-of-function backgrounds of the dAkt–TOR signaling pathway. Single tangential optical sections of third instar wing imaginal discs stained with DAPI (A-C, blue), anti Pan-dAkt (A-H, A'-H' red) and anti-GFP (A-H, green). Mitotic clones shown in (A,A', B,B' and G,G') are marked by the expression of GFP (green). Clones shown in (C,C' and D,D) are marked by the absence of GFP (green). All other images depict *apterous-Gal4* derived co-expression of various constructs with CD8::GFP. (A,A') Specificity control of anti Pan-dAkt. Clone of homozygously akt^{EX4} mutant cells (akt^{EX4} is a derivative of akt^{P04226}, generated by imprecise excision). Note the cell autonomous loss of the Pan-dAkt antigen. (B,B') akt^{q} clone. (C,C') $tsc2^{192}$ clone. (D,D') $s6K^{l-1}$, $tsc2^{192}$ clone. (E,E') Expression of an activated catalytic subunit of PI3 Kinase ($PI3K92E^{CAAX}$). Note the lower expression of dAkt in the $PI3K^{CAAX}$ expressing compartment, accompanied by high P-dAkt levels (Figure 1). (F,F') Ectopic expression of $Raptor^{RNAi}$. (G,G') Clone of $tsc1^{W243X}$ simultaneously expressing $Raptor^{RNAi}$. (H,H') Ectopic expression of $S6K^{STDE}$. Genotypes: (A,A'): *hs-FLP, UAS-GFP*^{nuc}*, tub-Gal4; FRT82B,* akt^{EX4}*/FTR82B, tub-Gal80, M*. (B,B'): *hs-FLP, UAS-GFP*^{nuc}*, tub-Gal4; FRT82B,* akt^{q}*/FTR82B, tub-Gal80, M*. (C,C'): *hs-Flp;* $tsc2^{192}$*, FRT80B/ubi-GFP, FRT80B*. (D,D'): *hs-Flp;* $s6K^{l-1}$*,* $tsc2^{192}$*, FRT80B/ubi-GFP, FRT80B*. (E,E'): *yw/UAS-PI3K92E*^{CAAX}*; ap-Gal4*/+. (F,F'): *yw; ap-Gal4*/+, *UAS-raptor*^{RNAi}. (G,G'): *hs-Flp, UAS-CD8::GFP; tub-Gal4/+;UAS-raptor*^{RNAi}*,FRT82B,* $tsc1^{W243X}$*/FRT82B, tub-Gal80*. (H,H'): *yw; ap-Gal4*/+,*UAS-* $S6K^{STDE}$.
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Figure S4
Negative feedback regulation of the dAkt-TOR pathway is independent of dFOXO. (A-C') Single tangential optical sections of third instar wing imaginal discs stained with DAPI (A-C, blue), anti P-dAkt (A-C, A'-C', red) and anti-GFP (A-C, green). (A, A'): Magnified view on the dorso-ventral boundary at the wing primordium. GFP expression (green) marks the dorsal expression domain of *apterous-Gal4* driver and the activated *UAS-dFOXO*^{TM} expression construct [80]. (B, B'): homozygous $foxo^{25}$ loss of function MARCM clone. Homozygous cells for $foxo^{25}$ are marked by CD8::GFP coexpression (green). (C,C'): $foxo^{25}$, akt^{q} homozygous loss of function MARCM clone. Homozygous cells for $foxo^{25}$, akt^{q} are marked by CD8::GFP (green). D/V compartment boundary as well as borders of the clones are traced by a white line in (A'-C'). Genotypes: (A, A') *yw; ap-*

Gal4/+, *UAS-FOXO-TM*/+. (B, B') *hs-Flp, UAS-CD8::GFP/+; tub-Gal4/+; FRT82B, foxo^{25}/FRT82B, tub-Gal80*. (C,C') *hs-Flp, UAS-CD8::GFP/+; tub-Gal4/+; FRT82B, foxo^{25}, akt^{q}/FRT82B, tub-Gal80*.
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Figure S5
P-dAkt levels are not elevated in *s6K^{l-1}* whole larval extracts. Western blot of total lysates prepared from whole third instar larvae of *wt* (left lane) and *s6K^{l-1}* (right lane) genetic backgrounds. Western blots probed with anti Pan-dAkt (total Akt), anti P-dAkt, anti Pan-S6K (total S6K), anti P-S6K and anti alpha-Tubulin as loading control.
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Figure S6
Activated S6K is sufficient to drive negative regulation of P-dAkt. (A-D') Single tangential optical sections of 3rd instar wing imaginal discs expressing *wild-type* and activated alleles of S6K expressed by *apterous-Gal4*. Stainings with DAPI (A-D, blue), anti P-dAkt (A-D, A'-D', red) and anti-GFP (A-D, green) are shown. GFP expression (green) marks the expression domain of the *apterous-Gal4* driver and the various *UAS-S6K* expression constructs. A'-D' show P-dAkt channel only, the boundary of *apterous-Gal4* expressing vs. non-expressing cells are marked with by a white line. Genotypes: (A,A') *yw; ap-Gal4/+, UAS-S6K^{WT}*, (B, B') *yw; ap-Gal4/+, UAS-S6K^{TE}* (substitution Thr398Glu in the linker region). (C, C') *yw; ap-Gal4/+, UAS-S6K^{STDETE}* (combined substitutions Thr398Glu in the linker region and Ser418Asp and Thr422Glu in the autoinhibitory domain). (D,D') *yw; ap-Gal4/+, UAS-S6K^{STDE}* (substitutions Ser418Asp and Thr422Glu in the autoinhibitory domain).
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Figure S7
Dominant active S6K is sufficient to inhibit P-dAkt under low TORC1 activity. (A-B') Single tangential optical sections of 3rd instar wing imaginal discs co-expressing Tsc1, Tsc2 and CD8::GFP (A, A'); and Tsc1, Tsc2, CD8::GFP and a constitutively activated allele of S6K (S6K^{TE}) (B, B'). Expression of the transgenes is driven by *apterous-Gal4*. Staining with DAPI (A-B, blue), anti P-dAkt (A-B', red) and anti-GFP (A, B, green) are shown. GFP expression (green) marks the expression domain of the *apterous-Gal4* driver and the of the various expression constructs used. A' and B' show the P-dAkt channel only, the boundary of *apterous-Gal4* expressing vs. non-expressing cells are marked with by a white line. Genotypes: (A, A') *yw*; *UAS-CD8::GFP, ap-Gal4/+; UAS-Tsc1, UAS-Tsc2/+*. (B, B') *yw*; *UAS-CD8::GFP, ap-Gal4/UAS-S6K^{TE}; UAS-Tsc1, UAS-Tsc2/+*.
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Figure S8
Raptor and S6K dependent negative feedback on P-dAkt. (A) Single confocal section of *S6K*, (B) *Raptor*, (C) *Luciferase* and (D) *Pten* RNAi treated *Drosophila* Kc$_{167}$ cells stained with DAPI (blue) anti P-dAkt (green) after 10 minutes of insulin stimulation. Images were recorded and processed using identical conditions. Note the highest level of anti P-dAkt signal in the *Raptor* dsRNA treated cells.
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(2.64 MB TIF)

Table S1
Amplicons identified in the RNAi screens that enhance or suppress P-dAkt levels. Averaged Z-Scores from the two screen replicates of the baseline (no stimulation) and insulin-stimulated screens are shown. The DRSC amplicon identifies individual dsRNAs from the genome wide dsRNA set. Primer and sequence information available at www.flyRNAi.org. With the exception of the InR pathway components, the hits indicated in this Table were identified using a single dsRNA and therefore need further validation to eliminate false positives. Fbgn: Fly base gene number.
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