Scaling the *Drosophila* Wing: TOR-Dependent Target Gene Access by the Hippo Pathway Transducer Yorkie

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Abstract

Organ growth is controlled by patterning signals that operate locally (e.g., Wingless/Ints [Wnts], Bone Morphogenetic Proteins [BMPs], and Hedgehogs [Hhs]) and scaled by nutrient-dependent signals that act systemically (e.g., Insulin-like peptides [ILPs] transduced by the Target of Rapamycin [TOR] pathway). How cells integrate these distinct inputs to generate organs of the appropriate size and shape is largely unknown. The transcriptional coactivator Yorkie (Yki, a YES-Associated Protein, or YAP) acts downstream of patterning morphogens and other tissue-intrinsic signals to promote organ growth. Yki activity is regulated primarily by the Warts/Hippo (Wts/Hpo) tumour suppressor pathway, which impedes nuclear access of Yki by a cytoplasmic tethering mechanism. Here, we show that the TOR pathway regulates Yki by a separate and novel mechanism in the *Drosophila* wing. Instead of controlling Yki nuclear access, TOR signaling governs Yki action after it reaches the nucleus by allowing it to gain access to its target genes. When TOR activity is inhibited, Yki accumulates in the nucleus but is sequestered from its normal growth-promoting target genes—a phenomenon we term “nuclear seclusion.” Hence, we posit that in addition to its well-known role in stimulating cellular metabolism in response to nutrients, TOR also promotes wing growth by liberating Yki from nuclear seclusion, a parallel pathway that we propose contributes to the scaling of wing size with nutrient availability.

Author Summary

What mechanisms control the sizes of animal organs? It is known that organ growth is the product of two systems: an intrinsic system that coordinates cell proliferation with the specification of cell fate (patterning), and an extrinsic system that synchronizes growth with nutrient levels. Developing organs integrate these two inputs to ensure that properly proportioned structures develop which are of the right scale to match overall body size. However, the mechanisms used to integrate these distinct growth control systems have remained largely mysterious. In this study, we have addressed how intrinsic and extrinsic systems combine to drive growth of the *Drosophila* wing. Focusing on the Target of
Rapamycin (TOR) pathway—a major, nutrient-dependent regulator of organ growth—and Yorkie—the transcriptional activator downstream of the Hippo pathway and a key, organ-intrinsic growth regulator—we have identified a circuit in which TOR activity limits Yorkie’s capacity to promote wing growth, in part through a novel mode of transcription factor regulation that we term “nuclear seclusion.” We find that inhibiting TOR leads to the retention of Yorkie in the nucleus but diminishes its transcriptional activity by diverting it away from target genes. We posit that subjugating Yorkie in this way contributes to how fluctuations in TOR activity scale wing size according to nutrient levels.

Introduction

A universal property of animal development is the capacity to scale body size and pattern in response to environmental conditions as well as during evolution [1]. For example, when starved during the larval growth phase, Drosophila adults emerge at around one quarter of their normal size but are correctly proportioned. Likewise, Drosophila species differ over ~5-fold in body size but are highly similar in shape. The overt likeness of primate skeletons (differing 18-fold in length), as well as those of frogs (30-fold) and fish (1,600-fold) provide more dramatic demonstrations of scaling across vast taxonomic groups, but despite its generality, the genetic control of organ scaling is poorly understood. Animals possess distinct systems for controlling growth locally and systemically: organ-intrinsic signaling mechanisms couple growth to patterning and morphogenesis, defining organ shape and dimension [2–4]; conversely, humoral signals, produced on feeding, act globally to control body size [5]. Yet, the existence of scaling implies the two systems cannot be independent. Cells in developing organs must integrate local and global information and proliferate accordingly, generating organs—and entire animals—that are functioning, proportional wholes [6–9].

The Drosophila wing is a classical paradigm of organ growth [2,3]. Here, as in other animals, nutrients influence growth via Target of Rapamycin (TOR) signaling [5,10]. During larval life, this pathway is activated in wing cells by haemolymph signals produced in response to feeding, including Insulin-like peptides (ILPs) that act via the Insulin Receptor (InR)/PI3-Kinase/Akt pathway, as well as sugars and amino acids. These inputs converge to regulate TOR—an intracellular kinase with diverse roles in metabolism [5,10]. Starvation reduces TOR activity and scales wing size (and entire body size) downwards (Fig 1A and 1B), an effect mimicked by genetically inhibiting TOR (Fig 1C). Yet, wing growth is simultaneously governed by intrinsic signaling systems (e.g., Wnt, BMP, and Hh morphogens) that control wing size, shape, and pattern [2,3,6,8]. Many of these organ-intrinsic systems exert their effects at least in part via regulation of the Warts (Wts)/Hippo (Hpo) pathway [11–13]—a network of proteins that inhibit a growth-promoting transcriptional coactivator, Yorkie (Yki; orthologous to vertebrate YES-Associated Protein [YAP]) [14]. Hpo and Wts are kinases that act in sequence, Hpo phosphorylating Wts and Wts phosphorylating Yki to sequester Yki cytoplasmically. Inhibition of either kinase promotes growth by allowing Yki to evade cytosolic sequestration and gain access to the nucleus [15–17]. Nuclear Yki binds transcription factors including Scalloped (Sd, a TEAD binding domain; TEAD, Transcriptional Enhancer Activator Domain; wts, Warts; YAP, YES-Associated Protein; Yki, Yorkie).
signaling [15,31–35], the logic by which cells in growing organs integrate these inputs to achieve organ scaling remains unclear.

Here, we report a novel molecular mechanism by which Yki can integrate the control of Drosophila wing growth by the Wts/Hpo and TOR pathways. As part of this mechanism, we present evidence for what is, to our knowledge, a previously unknown mode of transcriptional regulation, which we term “nuclear seclusion”, whereby a transcription factor (Yki) is retained in the nucleus but sequestered from target loci and hence rendered unable to induce their expression.

In contrast to the Wts/Hpo pathway, which controls Yki activity by governing its access to the nucleus, our findings indicate that the TOR pathway activates Yki after it gains entry to the nucleus, by allowing it to escape nuclear seclusion and gain access to its growth-promoting target genes. We posit that this novel mechanism allows Yki to integrate local patterning inputs mediated by the Wts/Hpo pathway with systemic, humoral inputs mediated by TOR, comprising part of the system that scales wing growth in response to nutrition.

Fig 1. Effects of TOR inhibition on wing growth and Yki-driven cell proliferation. A–C: Adult Drosophila wings. (A) A wing from a well-fed wild type fly. (B) Scaled-down wing produced by raising larvae on nutrient-poor food. (C) Scaled-down wing caused by blocking TOR signaling specifically in wing cells with nubbin.GAL4 UAS.Tor\textsuperscript{TED}. (D–G) Wing discs from late third instar larvae-bearing clones of mutant tissue outlined with dashed lines, and marked negatively (“black”) by absence of the GFP marker (green): (D) wild type (control) (E) Tor\textsuperscript{AP}, (F) ex\textsuperscript{nT}, (G) ex\textsuperscript{nT} Tor\textsuperscript{AP}. Mutant clones were induced at the end of the first instar, 48±2 hr after egg laying and are associated with sibling “twin-spot” clones marked by two copies of the GFP marker (bright green) that serve as an internal control for the growth of w.t. tissue. Numbers denote mean clone size ratio compared to wt, and asterisks denote significances from t tests (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, n.s. = not significant). In (G), the bottom italicised value is a comparison with the Tor\textsuperscript{AP} genotype. Number of clones measured (\(n\)) = 32 (wt), 38 (ex), 51 (Tor), 36 (ex Tor). (H–K) Clones of the same genotypes as in (D–G) that coexpress p35 with GFP-NLS (generated using the MARCM technique [36]). Clones are positively labelled by GFP-NLS, and nuclei are counterstained with Hoechst (blue). (\(n\)) = 92 (wt+p35), 90 (ex+p35), 97 (Tor+p35), 79 (Ex Tor+p35). Numbers signify mean clone size ratio compared to wt+p35, and bottom italicised value in (K) with Tor+p35.

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Results

TOR Activity Is Required for Yki-Dependent Wing Growth

To determine the relationship between TOR and Yki in the developing Drosophila wing, we first asked whether the capacity of Yki to promote growth depends on TOR activity. To do so, we generated clones of cells mutant for Wts/Hpo pathway components that normally hold Yki activity in check and tested if the resulting increases in tissue mass and cell number depend on TOR activity. All such experiments that we performed indicate an obligate role of TOR activity for Yki-dependent wing growth. Specifically, clones mutant for the FERM (4.1 Ezrin Radixin Moesin) domain protein Expanded (Ex), which is required for normal Hpo activity, grow far larger than control clones (Fig 1F; S1 Data [37]). However, when such clones were also mutant for TOR (ex−Tor− clones), they were tiny (Fig 1G; S1 Data) and not significantly larger than Tor− clones (Fig 1D and 1E; S1 Data). The impaired growth of both Tor− and ex−Tor− clones is not due to apoptosis, since it is not detectably rescued by expressing the apoptosis inhibitor p35 in the mutant cells (Fig 1H–1K; S1 Data). Equivalent experiments, activating Yki by removing Wts and inhibiting TOR by removing the TOR-activating GTPase Rheb, gave similar results (S1A–S1H Fig). Likewise, overexpressing Yki throughout the wing primordium leads to excessive growth; however, co-overexpression of ΔP60, an inhibitor of InR signaling upstream of TOR [38], blocks this growth, yielding wings similar in size to those in which only ΔP60, alone, was overexpressed (S1I–S1O Fig and discussion in S1 Fig legend; S1 Data). Equivalent results were obtained in the developing head primordium: mutation of wts or ex leads to dramatic overgrowth of head tissue, which is suppressed when the head is also mutant for Tor or the InR pathway kinase Akt (S1P–S1X Fig). Thus, reducing the level of InR and TOR signaling, which is normally set by nutrient status, restricts Yki’s capacity to promote growth—suggesting a relationship that could contribute to scaling wing size with nutrient levels.

Yki Does Not Up-Regulate InR/TOR Pathway Activity to Promote Growth

Why is Yki-driven growth limited by the level of TOR activity? TOR might be required upstream to facilitate Yki activity, or in parallel to trigger other, independent growth-related processes. Alternatively, TOR might be required downstream, with Yki promoting growth at least in part by elevating InR/TOR pathway activity. To test this latter possibility, we assayed whether conditions that abnormally increase Yki activity (loss of either Ex or Wts) have a corresponding effect on the levels of phospho-Akt (pAkt-S505), which are normally elevated by enhanced InR signaling (e.g., by removal of the InR pathway inhibitor PTEN; Fig 2A). We find that pAkt-S505 levels are normal in ex− or wts− clones (Fig 2B and 2C), as well as in protein extracts from the overgrown wing discs of homozygous ex− or wts− larvae (Fig 2D). Likewise, the levels of phosphorylated S6-Kinase (pS6K-T398), a readout of TOR activity, were not affected in ex− or wts− discs (Fig 2E). We therefore infer that Wts/Hpo regulated Yki activity does not stimulate either InR or TOR pathway activity in the wing, consistent with results from Drosophila cell culture [15]. This finding argues against a downstream role of InR/TOR signaling in mediating Yki-driven growth.

Further evidence that TOR activation is not dependent on Yki comes from the observation that ectopically activating the InR/TOR pathway via upstream components can suffice to increase the growth of yki− wing clones (rescued with p35; Fig 2F, 2G and 2J; S1 Data). Specifically, expressing Rheb (to activate TOR) resulted in a 22% increase in clone size (Fig 2I and 2J; S1 Data) and expressing Dp110 (to activate InR) resulting in a 26% increase (Fig 2H and 2J; S1 Data). In both cases, clonal expansion resulted solely from increases in cell size (Fig 2L; S1...
Fig 2. Yki activity does not promote growth by up-regulating InR/TOR signaling. (A–C) Wing discs labelled for phospho-Akt S505, an indicator of InR pathway activity, bearing mutant clones marked black by absence of GFP (green); w.t. twin spots are marked by bright green. (A) pten1 (positive control), (B) exe1, and (C) wtsX1 (experiments, having elevated Yki activity owing to reduced or absent phosphorylation by Wts). Phospho-Akt S505 is not increased in (B) and (C), in contrast to (A). (D) Western blot of protein extracts derived from late third instar wing discs of the genotypes shown, labelled for phospho-Akt S505 (Total Akt and β-actin were used as loading controls; pten1/pten1ΔNls was used as a positive control; the reduction in S505 staining in the Tsc1Q87X/Tsc1PA23 lane is due to feedback of TOR activation onto Akt phosphorylation [39]). In contrast to reduced Pten, loss of either Ex or Wts does not cause an increase in pAkt S505. (E) Blot of same genotypes as in (D), labelled for phospho-S6 Kinase T398, an indicator of TOR pathway activity (β-Tubulin was used as a loading control, and runs as two species). As observed for phospho-Akt S505, Phospho-S6 Kinase T398 levels are elevated by loss of Pten and Tsc activity, but not by loss of either Ex or Wts activity. (F–I): Wing discs from late third instar larvae-bearing MARCM clones expressing UAS.p35 (labelled positively with GFP-NLS, green; nuclei are counterstained with Hoechst, blue). The genotypes of clones are (F) UAS.p35, (G) ykiΔ+p35, (H) ykiΔ+p35+Dp110, and (I) ykiΔ+p35+UAS.Rheb. J–L: Quantification of clones sizes, cell numbers, and cell sizes from genotypes in F–I. Error bars are Standard Error of the Mean and asterisks denote significances from t tests (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, n.s. = not significant), n = 42
Data), without effects on cell number (Fig 2K; S1 Data). Hence, TOR can be activated and promote detectable cell growth, albeit not cell proliferation, in the absence of Yki. Notably, control clones expressing Dp110 or Rheb with p35 that are wild type for Yki strongly overgrew (S2A–S2D Fig; S1 Data), and this was in part due to both their increased cell numbers (S2E Fig; S1 Data). We infer that although InR/TOR pathway activity appears largely independent of Yki activity, ectopic InR/TOR activation nevertheless requires Yki function as a prerequisite in order to drive cell proliferation.

TOR Inhibition Enhances Yki Nuclear Accumulation despite Inhibiting Yki-Dependent Growth

If Yki depends on, but does not activate, the InR/TOR pathway to promote growth, an alternative explanation is that the InR/TOR pathway acts in parallel to or upstream of Yki. Yki activity depends in large part on phosphorylation by Wts, which causes Yki to be sequestered in the cytosol, reducing its access to the nucleus [15–17]. Yki normally appears predominantly cytoplasmic in the developing wing (Fig 3A), consistent with its essential, but tonic, activity in sustaining wing growth. However, upon Wts inactivation, Yki accumulates in the nucleus and inappropriately up-regulates transcription of its growth-promoting target genes, causing pronounced tissue hyperplasia [15–17] (Fig 3B). Hence, inhibiting TOR activity might countermand the overgrowth phenotype caused by loss of Wts activity by compromising nuclear access of unphosphorylated Yki. In preparing to test this possibility, we were surprised to discover that reducing TOR activity has the opposite effect on Yki localization: instead of restricting nuclear access, it causes a dramatic increase in nuclear Yki, even as it blocks Yki-dependent growth.

In our initial experiments, we strongly inhibited endogenous TOR activity in the developing wing, using dpp.Gal4 to drive a stripe of expression of UAS transgenes encoding three proteins that have been widely employed to study the consequences of reducing TOR activity: a dominant negative form of TOR (TORTED) and the TOR corepressors TSC1 and TSC2 [32,40–46]. Co-overexpression of all three proteins causes strong nuclear Yki accumulation in the stripe (Fig 3C and 3D). Expressing either TSC1+TSC2, or TORTED, alone, in the stripe or in other patterns within the wing, also caused enhanced Yki nuclear localization (S3A–S3C Fig), albeit less strongly, suggesting that the level of nuclear accumulation depends on the strength of TOR inhibition, and furthermore is not region-specific. Note that we prefer the use of transgenes to inhibit TOR signaling in large sectors of tissue, rather than examining tiny Tor−−clones, where interpretation is compromised by protein perdurance. We also observed enhanced Yki nuclear accumulation when InR signaling was inhibited, upstream of TOR (S3D–S3F Fig). Conversely, removal of two downstream metabolic targets of TOR, dS6K, and 4EBP/Thor [10] did not affect Yki nuclear accumulation (S3G–S3J Fig), indicating that the control of Yki localization by TOR involves a mechanism that is not mediated by these metabolic regulators. Thus, reduced InR/TOR activity results in enhanced nuclear accumulation of Yki—a result that is counterintuitive, because nuclear Yki is usually associated with increased, rather than reduced growth [15–17].

TOR Inhibition Reduces Yki Target Gene Transcription

Typically, nuclear Yki up-regulates growth-promoting target genes, one of the best characterized of these being diap1, the gene encoding the Drosophila Inhibitor of Apoptosis protein [18,19]. However, the dramatic increase in nuclear Yki accumulation caused by TOR inhibition
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(Fig 3C and 3D) is associated with a strong reduction in DIAP protein levels (Fig 4A and 4B), consistent with reduced diap1 gene transcription. Diap1 transcription is normally driven by a Yki-Sd complex that binds ~4 kb downstream of the transcription start site of the predominant diap1-RA transcript [19]. A minimal reporter construct, 2B2C-lacZ, containing ~300 bp of this enhancer region, is up-regulated by Yki overactivation [19]. Conversely, RNAi knockdown of Yki results in reduced 2B2C-lacZ expression (S4A and S4A’ Fig). Like Yki RNAi knockdown, TOR inhibition caused a marked decrease in 2B2C-lacZ expression (Fig 4C and 4D), indicating a failure of the Yki/Sd-dependent 2B2C enhancer to maintain normal target gene expression.

TOR inhibition similarly compromises the transcription of other Yki target genes. four-jointed (fj) is the second target gene that we have confirmed is reduced by Yki knockdown (S4B Fig). Like Yki knockdown, TOR inhibition strongly reduced fj-lacZ expression (Fig 4E and 4F). A third reporter, ex1-lacZ, was unresponsive to TOR inhibition at 25°C, but raising the temperature to 29°C for several hours prior to dissection (to increase GAL4-dependent TOR TED, TSC1, and TSC2 overexpression) sufficed to repress this reporter (Fig 4G and 4H). Further, in animals homozygous for the ex1-lacZ reporter allele, ex1-lacZ expression was clearly reduced by TOR inhibition at 25°C (S4C and S4D Fig). We note that such ex1-lacZ/ex1-lacZ discs have reduced ex gene function and hence abnormally elevated levels of nuclear Yki; nevertheless, a sharp increase in nuclear Yki can still be detected in the TOR-inhibited stripe, coincident with reduced transcription of the ex1-lacZ gene (S4E–S4G Fig). Finally, the effect of TOR inhibition appears specific to Yki target genes, since other proteins expressed in the developing wing, including Vestigial (S4I Fig), Armadillo (S4J Fig), Distal-less (S4J’ Fig), a Distal-less-lacZ reporter (S4K Fig), and indeed Yki itself (Fig 3C and 3D), were not reduced by strong TOR inhibition.

Fig 3. TOR inhibition increases Yki nuclear accumulation. (A,C,D) Wing discs expressing GFP-NLS in a stripe of cells under dpp.Gal4 control that either do (C,D, experimental), or do not (A, negative control), coexpress Tor TED, TSC1, and TSC2. (B) Wing disc with wtsX1 clones (positive control), marked black by the absence of GFP. Discs are imaged at the level of nuclei and labelled for Yki (red), GFP (green), and DNA (Hoechst, blue). Coexpression of Tor TED, TSC1, and TSC2 (C,D), like the loss of Wts (B), causes enhanced nuclear accumulation of Yki, apparent at this plane of focus as increased signal at low magnification (B,C) and by coincidence of the Yki and GFP-NLS signals at high magnification (D, e.g., arrowhead).

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Inhibiting TOR Impedes Target Gene Access by Yki

The unexpected (and counterintuitive) effects of TOR inhibition on Yki nuclear accumulation and target gene expression are reminiscent of another scenario in which enhanced nuclear access of Yki is associated with the loss of target gene expression. In this case, Yki can be forced to accumulate in the nucleus by overexpression of Sd, the site-specific DNA binding factor that...
normally mediates target gene activation by Yki in the wing blade (S6A Fig) [18,19]. Under this condition, the abnormally high levels of Sd sequester the limited levels of Yki available, exerting a classic dominant negative "squelching" effect [47] that titrates Yki away from Sd bound to target gene enhancers. As in the case of TOR inhibition, the level of nuclear Yki increases whilst target gene expression decreases (S6B and S6C Fig). This resemblance suggests that TOR inhibition may exert its effects on Yki target gene expression via a similar mechanism: by directing Yki to the nucleus whilst reducing its access to the relevant target gene enhancers.

To test this possibility directly, we focused on diap1, the locus where the general mechanism of transcriptional control by Yki is best characterized, with a defined enhancer element, 2B2C, that is controlled exclusively by Yki in complex with Sd (henceforth Yki-Sd) [14,18,19,48]. We prepared chromatin from control and TOR-inhibited wing discs (the latter coexpressingTOR^{TED}+TSC1+TSC2 under the control of a Flp-out Act5C>CD2>Gal4 driver) and immune-precipitated using a Yki antibody [15]. To produce enough tissue despite TOR inhibition, discs were allowed to grow to a large size before a strong heat shock was used to excise the stop cassette within the Act5C>CD2>Gal4 driver and initiate Gal4 expression in nearly all cells (Fig 5A and 5B; loss of magenta CD2 marks the Act5C>Gal4 expressing tissue), with dissection of 200 discs per genotype 8–10 hr later (see Methods).

In control discs, as expected, Yki was enriched ~2–3-fold within the 2B2C diap1 element, using primers centered around the CATTCA motif to which Yki-Sd binds (relative to primers defining unrelated DNA sequences, as well as an IgG control treatment; Fig 5C). Knockdown of Yki (using the same Act5C>CD2>Gal4 protocol to drive transient expression of a UAS,ykiRNAi transgene) abolished this peak of enrichment, confirming its dependence on Yki levels and also validating the specificity of the antibody (S8A and S8B Fig). Strikingly, in experimental, TOR-inhibited discs, Yki enrichment at 2B2C was also diminished, by almost half (p < 0.05, n = 4 fully independent experimental replicates; Fig 5C; S1 Data). This reduction occurred despite the clear increase in nuclear Yki resulting from TOR inhibition (Fig 5A and 5B).

These data provide evidence that inhibiting endogenous TOR activity reduces the amount of Yki bound to a canonical Yki-Sd target gene enhancer, even as it causes an increase in Yki nuclear accumulation. Hence, we propose that TOR inhibition renders Yki subject to a phenomenon that we term "nuclear seclusion", whereby Yki is mobilized and/or sequestered in the nucleus but hindered from accessing its target enhancer sequences. We posit that this mechanism accounts at least in part for the reduction in DIAP1 expression observed when TOR is inhibited (Fig 4B) and suggest that it likewise explains the loss of expression of other TOR-dependent Yki-Sd target loci for which defined enhancers have not yet been characterized (e.g., fj, ex; Fig 4F and 4H).

It is important to note that in most of our experiments, we have strongly reduced TOR signaling by coexpressingTOR^{TED}+TSC1&2, to approximate, or at least approach, TOR loss-of-function. These experiments reveal a basic requirement in wing cells for at least some level of TOR signaling in order to override nuclear seclusion of Yki and permit it access to drive transcription of target genes. However, we additionally propose that nuclear seclusion also operates within the normal physiological range of TOR activity as set by nutrient intake. First, weaker impairment of TOR signaling, by expression of TOR^{TED} alone, produces a wing size comparable to a moderately staved animal (Fig 1C). And yet it also leads to the two hallmarks of nuclear seclusion: increased Yki accumulation (S5D Fig), with concomitant reduction in expression of target genes (S5E Fig). Second, moderate reductions in TOR activity with RNAi transgenes directed against TOR and Rheb also reduce wing blade size within the range of normal trait scaling [5] (S5A–S5C Fig). Again, these manipulations cause detectable—albeit correspondingly much less pronounced—nuclear accumulation of Yki (S5F and S5H Fig) and reduced target gene expression (S5G and S5I Fig). Hence, while our assay of strong TOR inactivation
TOR inhibition results in Yki nuclear seclusion and depends on the conserved Sd binding and WW domains but not the Wts phosphorylation sites of Yki. (A, B) Confocal sections of Act5C>CD2>GAL4 wing discs that either do (B, experimental) or do not (A, control) coexpress TorTED, TSC1, and TSC2 in most cells 8–10 hr after heat shock to excise the >CD2> stop cassette. Yki (red), CD2 (magenta), SdGFP (from a GFP knockin allele of sd; green) and DNA (blue) are shown at the level of nuclei. Control discs (expressing only GFP-NLS) have no effect on Yki localization (A) in contrast to experimental discs coexpressing TorTED, TSC1, and TSC2, which show enhanced nuclear accumulation of Yki as indicated by increased signal at low magnification (B; arrowheads show clusters of Act5C>CD2>GAL4 cells in which the >CD2> stop cassette was not excised, which coincide with reduced Yki nuclear staining). (C, D) ChIP of Yki (C) or SdGFP (D; using the sdGFP allele and anti-GFP antibody) with mock IP (IgG) at the 2B2C diap1 enhancer and a control locus (PDH: pyruvate dehydrogenase) in control and experimental discs, as in (A, B). Error bars are Standard Error of the Mean, and asterisks denote significances from t tests (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, n. s. = not significant). n = 4 independent experimental replicates. Yki and SdGFP are strongly enriched at 2B2C in control (w.t.) discs compared to PDH controls. Enrichment of both proteins is reduced in experimental (>TorTED/TSC1&TSC2) discs; (E) Conserved functional domains of Yki. (F–H) Wing discs uniformly expressing w.t. (F), P88L (G), or WW domain mutant (W292A P295A W361A P364A) (H) forms of GFP tagged Yki that also express TorTED, TSC1, and TSC2 in a stripe under dpp.Gal4 control (as in Figs 3 and 4; the dashed white lines indicate the A/P compartment boundary, which abuts the right edge of the dpp.Gal4 expressing stripe). The discs are imaged at the level of nuclei and show nuclear accumulation of the w.t. (F) but neither the P88L (G) or WW (H) mutant, forms of Yki, as indicated by signal intensity. (I, J) Confocal sections of wing discs taken at the level of nuclei to assess the nuclear accumulation of wild-type Yki-GFP (I), as well as a mutant form of Yki-GFP (J), that carries both the P88L substitution (which blocks nuclear accumulation in response to TOR inhibition, G) as well as the S111A, S168A, and S250A (S3-A) substitutions (which obviate phosphorylation of Yki by Wts and cause otherwise wild-type Yki to accumulate in the nucleus). Both proteins are expressed in clones under the direct control of the Tuba1 promoter following FLP-out cassette excision of a Tuba1>DsRed>yki-GFP transgene; the clones are marked black by the absence of DsRed expression (magenta). Wild-type Yki-GFP (I) appears predominantly cytosolic, whereas YkiP88LS3-A-GFP (J) appears much more nuclear, as indicated by the difference in staining patterns imaged at the nuclear plane—largely absent in nuclei for (I) and relatively uniform for (J):
(TOR^{TED}+TSC1&2) gives the clearest observable nuclear accumulation, and is the basis for most of our mechanistic insights regarding Yki nuclear seclusion, we posit that this phenomenon operates beyond a simple baseline requirement for TOR signaling and is modulated by fluctuations of TOR signaling within the normal range. Furthermore, we note that because Yki is predominantly cytoplasmic in wing cells (Fig 3A), the small fraction of active, nuclear, Yki might be effectively secluded by quite modest reductions in TOR signaling (without leading to the profound nuclear accumulation witnessed when TOR is strongly impeded).

TOR Inhibition Impedes Target Gene Access by Sd, in Addition to Yki

What mechanism mediates Yki nuclear seclusion in response to TOR inhibition? Given the similar effect of Sd overexpression on Yki localization and target gene transcription, we considered the possibility that the mechanism mediating Yki nuclear seclusion could be a pronounced increase in Sd protein level. However, Sd is already at high level throughout the developing wing, and TOR inhibition does not cause a further increase, as monitored by the expression of a fully functional GFP protein trap allele of the endogenous sd gene (sd\textsuperscript{GFP}; [49]) (S4J Fig; S8G and S8H Fig). Hence, we sought to assess two other possibilities: (i) that Yki, alone, is subject to nuclear seclusion, e.g., by being titrated away from enhancer-bound Sd, or (ii) that Sd, in addition to Yki, and perhaps in complex with it, is targeted for seclusion.

Using sd\textsuperscript{GFP} wing discs and a GFP antibody, we assessed Sd-GFP enrichment at the 2B2C diap1 enhancer by ChIP. Like Yki, Sd-GFP is strongly enriched at 2B2C in wild-type discs (Fig 5D). However, inhibiting TOR activity caused a reduction in Sd-GFP enrichment at 2B2C to a similar degree as it does Yki enrichment (Fig 5C and 5D). Hence, it appears that TOR inhibition results in nuclear seclusion of Sd as well as Yki, rather than just Yki. One explanation for this observation is that Sd might need to be bound by Yki to bind 2B2C — indeed, such a cooperative interaction appears to be required for enhancer binding by Sd in combination with Vg, another Sd transcriptional coactivator [50]. However, we observed that RNAi knockdown of Yki, which as expected results in reduced Yki binding at 2B2C, has no effect on Sd enrichment (S8B Fig). That Sd binding to 2B2C apparently does not depend on Yki argues against cooperative target site binding as a possibility. We therefore posit two possibilities: that Sd in complex with Yki is subject to nuclear seclusion, or, alternatively, that TOR inhibition secludes Sd in addition to, but independently of, Yki. If this second scenario were the case however, we may expect Sd in complex with transcriptional activators other than Yki to be influenced by seclusion. Conversely, expression of vg, an autoregulated target of Vg-Sd but not Yki-Sd complexes in wing cells, is not affected when TOR activity is inhibited (S4H and S4I Fig). Hence, we favor the view that nuclear seclusion acts on Sd in complex with Yki, although this additional facet of the mechanism remains to be confirmed.

Nuclear Seclusion Is Mediated by Yki’s N-Terminus and WW Domains, But Not by Its Wts Phosphorylation Site

Yki is a modular protein with three regions that are conserved with vertebrate YAP and essential for normal function (Fig 5E): (i) a Wts phosphorylation site which, when phosphorylated, mediates cytoplasmic retention of Yki by 14-3-3 anchor proteins (ii) two WW domains that
bind both Wts and Ex; and (iii) an N-terminal region, the Sd/TEAD binding domain (TBD), that mediates binding to Sd. Yki normally shuttles between the cytosol and nucleus in response to the state of phosphorylation at the Wts site [15–17], with phosphorylated and unphosphorylated forms accumulating, respectively, in the cytosol and nucleus. This shuttling is revealed by the build-up of nuclear Yki in wing cells following treatment with the nuclear export inhibitor Leptomycin B (S6D and S6E Fig [51]). However, TOR inhibition is unlikely to cause abnormal Yki nuclear accumulation by reducing Wts-dependent phosphorylation of Yki, as this would mimic the consequence of Wts/Hpo inactivation [15–17], namely target gene induction rather than the observed repression. We therefore considered an alternative possibility that a different mechanism of Yki regulation, perhaps mediated by one or both of the other conserved Yki motifs, might be at play.

To shed further light on the mechanism of nuclear seclusion, we generated Tub\(\alpha\)1.yki\(^{GFP}\) transgenes that express GFP-tagged forms of Yki at near-physiological levels (S8C Fig; S1 Data) and analyzed the consequences of mutating each of the three conserved functional domains on the subcellular accumulation of Yki. TOR inhibition caused nuclear accumulation of wild-type Yki\(^{GFP}\) as expected (Fig 5F). However, introducing a mutation, P88L, in the N-terminal TBD that diminishes binding to Sd [19] prevented nuclear accumulation in TOR-inhibited cells (Fig 5G). This block to nuclear accumulation appears to be specific to the loss of TOR pathway activity, as Yki\(^{GFP\text{-}P88L}\) protein accumulates in the nuclei of wts\(^{-}\)wing cells that have normal TOR pathway activity (Fig 5K), and the same is true for Yki\(^{GFP\text{-}P88L}\) protein that is additionally mutated for the Wts phosphorylation sites in otherwise wild type wing cells (Fig 5 and 5J). Likewise, mutating Yki’s two WW domains prevents nuclear accumulation following TOR inhibition (Fig 5H), even though the WW domains are known not to interfere with nuclear accumulation following Wts/Hpo pathway inactivation [52]. Thus, it appears that the Wts-phosphorylation site governs whether Yki has access to the nucleus: only Yki in which this domain is not phosphorylated by Wts can enter. In contrast, interactions mediated by the TBD and WW domains appear to determine whether any unphosphorylated Yki that gains access to the nucleus will be secluded there when TOR activity is inhibited. Accordingly, the abnormal nuclear accumulation of Yki resulting from TOR inhibition should represent a build-up of unphosphorylated Yki, even when Wts is present, as any unphosphorylated Yki that enters the nucleus will get trapped there. Consistent with this prediction, we observed a general reduction in phosphorylated Yki-S168 levels following TOR inhibition (S8D Fig). (Note that although we interpret this as a consequence of nuclear seclusion, we cannot rule out an additional, independent effect of TOR inhibition in dephosphorylating Yki. However, we note that if such an activity occurs, it has seemingly negligible function consequences for Yki activity; dephosphorylated Yki should increase target gene expression, but our data show that blocking TOR strongly reduces Yki target gene expression).

Based on these data, we infer that low levels of InR/TOR signaling activity result in the induction or function of a presently unidentified Nuclear Secluding Factor (NSF) that acts in wing cells to sequester Yki in the nucleus and to divert Yki-Sd from its target genes. The putative NSF might interact with Yki directly, via Yki’s N-terminus and/or WW domains and cause Yki nuclear seclusion by both increasing the binding of Yki to Sd and preventing the resulting NSF-Yki-Sd complex from accessing Yki-Sd target genes. Accordingly, NSF action would result in enhanced Yki nuclear accumulation and reduced Yki-Sd target gene expression. This mode of Yki regulation acts independently of the Hpo-Wts mediated cytosolic tethering of Yki to limit target gene expression. Indeed, even in Hpo pathway mutant tissue (e.g., loss of Ex, or Wts), where Yki is constitutively dephosphorylated, TOR inhibition still leads to increased Yki nuclear accumulation (S4E–S4G Fig) and decreased Yki target gene transcription (S4C–S4D Fig; S8E and S8F Fig).
TOR Regulates Yki Nuclear Seclusion and Cellular Metabolism in Parallel to Control Wing Growth

The requirement for InR/TOR signaling activity to allow Yki to escape nuclear seclusion is revealed by genetic manipulations that compromise InR/TOR pathway activity and cause Yki to accumulate nonproductively in the nucleus. Importantly however, we do not think that TOR signaling promotes Yki-driven wing growth solely by this mechanism. Instead, we posit that this mechanism operates in parallel with other outputs of InR/TOR signaling that regulate a range of basic metabolic processes that are necessary, independently, for cell growth as well as rate-limiting for cell proliferation [5,10,53]. A clear demonstration of this parallel requirement for InR/TOR signaling is the observation that Yki-driven overgrowth of the wing (caused by removal of wts) could be suppressed by removal of S6-Kinase (dS6K), a TOR target that catalyses cap-dependent mRNA translation (S7A–S7D Fig; S1 Data), but which is not itself involved in nuclear seclusion (S3G, S3H and S3J Fig).

Hence, while TOR/InR activity is needed to override Yki nuclear seclusion, it is not sufficient to promote proliferative growth in the absence of concomitant activation of canonical metabolic targets like dS6K. Coupled to our reciprocal finding above, that TOR activity is unable to promote cell proliferation without Yki (Fig 2F–2L), we conclude that neither output of TOR is sufficient. Rather, both outputs are required, first to unleash Yki from nuclear seclusion, and second to provide the metabolic wherewithal for Yki target genes to promote growth. By concomitantly compromising both outputs, nutritional deprivation coordinates Yki target gene expression with decreased metabolic capacity to reduce the growth rate of the wing.

Superphysiological TOR Activity Triggers a Regulatory Feedback That Circumvents Wing Overgrowth

Our results describe how inhibiting TOR signaling reduces Yki function as part of the mechanism that might scale wing growth downwards in response to nutritional deprivation. To further elucidate how TOR and Yki might function together to control wing size, we asked whether superphysiological TOR activity might alter Yki localization and/or activity to scale wing size upwards. However, we were unable to detect any such changes in Yki using either of two well-established approaches to overactivate the InR/TOR signaling in vivo. Specifically, overactivating TOR by removing the negative regulator TSC1 in wing cells did not appear to alter the nuclear–cytoplasmic distribution of Yki as assayed either by immunofluorescence in clones of Tsc1−/−mutant cells (Fig 6A) or by quantitating the nuclear–cytoplasmic ratio of Yki in fractionated whole wing discs from entirely Tsc1−/−mutant larvae (Fig 6B). Similarly, we tested whether clonal removal of TSC1, or alternatively, clonal overexpression of the positive regulator Rheb, affects the expression of any of several Yki target genes, and again, observed no differences (Fig 6C and 6D; S9A and S9B Fig). Hence, superphysiological gains in TOR activity do not appear to influence either Yki localization or activity, suggesting that peak endogenous TOR signaling is sufficient to fully override Yki nuclear seclusion as well as any other InR/TOR-dependent constraints on Yki coactivator function.

Nevertheless, manipulations that generate superphysiological levels of TOR activity do reveal a further facet of the TOR-Yki relationship in the wing. We noticed that despite strongly elevating TOR signaling in wing discs (Fig 2E), loss of Tsc1 activity failed to alter overall wing disc size (S9C and S9D Fig). Instead, it appears to cause excess growth that is counterbalanced by apoptosis (S9C and S9D Fig). The same was true of discs overexpressing rheb in all cells under the control of the imaginal disc-specific headcase.GAL4 driver (hdc.G4; Fig 6J, 6K and 6N; S1 Data), which produces an equivalent level of TOR activation to loss of Tsc1 (compare phospho-S6K levels in S7I Fig to those of Tsc1−/−discs in Fig 2E). This increase in apoptosis can
Fig 6. Superphysiological TOR activity causes excess, Yki-independent growth that is offset by a negative feedback that down-regulates the anti-apoptotic factors DIAP and bantam. (A) Wing disc carrying a clone of Tsc1<sup>Q87X</sup> mutant cells (marked black by absence of GFP, green, and outlined with a dotted line) expressing Yki<sup>lacZ</sup>. (B) Western blot analysis of Yki and Tubulin expression in control (C) and Tsc1<sup>−/−</sup> (N) wing discs. (C) Wing disc expressing f<sub>j</sub>-lacZ. (D) Wing disc expressing ex-lacZ. (E) Wing disc expressing DIAP1 and bantam sensor. (E') Wing disc expressing DIAP1 and bantam sensor with ptc>rheb. (F) Wing disc expressing DIAP1 and bantam sensor. (F') Wing disc expressing DIAP1 and bantam sensor with ptc>rheb. (G) Western blot analysis of DIAP1, pYki, Yki, and β-actin expression in control (wt), Tsc1<sup>−/−</sup> (Tsc1<sup>−</sup>), wt<i>CIP</i>, and Tsc1<sup>−/−</sup>i<i>CIP</i> wing discs. (H) ChIP analysis of PDH binding to chromosomes in control (wt), Tsc1<sup>−/−</sup> (Tsc1<sup>−</sup>), wt<i>CIP</i>, and Tsc1<sup>−/−</sup>i<i>CIP</i> wing discs. (I) Bar graph showing % of Input for PDH ChIP in control (wt), Tsc1<sup>−/−</sup> (Tsc1<sup>−</sup>), wt<i>CIP</i>, and Tsc1<sup>−/−</sup>i<i>CIP</i> wing discs. (J-M) Images showing disc size (pixels x 10<sup>4</sup>) for wt, >rheb, >DIAP1, >rheb, >DIAP1, >rheb, >DIAP1, and >rheb, >DIAP1, >rheb, >DIAP1 conditions.
nucleocytoplasmic distribution is similar in wild-type and Tsc1−/− discs. Effective separation of cell fractions was confirmed by Tubulin (cytoplasm) and Histone 3 (nucleus). (C, D) Wing discs carrying Tsc1Q73X mutant clones (marked and imaged as in A): expression of the Yki target genes fj-lacZ (C) and ex-lacZ (D) (red) is not affected. (E, F) Compared to wild type discs (E), expression of Rheb in a stripe under ptc:Gal4 control (F) causes a reduction in DIAP accumulation (red), as well as bantam micro-RNA activity, the latter indicated by relief of repression of a bantam-GFP sensor (green) [54]; peak activity of the ptc:Gal4 driver is indicated by expression of β-galactosidase from a UAS:Gal4 transgene, magenta). (G) Protein extracts of wild type, Tsc1Q73X/Tsc1Δ23, and homozygous wts1Δ2 (positive control) discs probed for DIAP1 protein reveal that DIAP1 is strongly reduced in Tsc1−/− discs, β-actin was used as a loading control. (H) Phospho-Yki S168 levels Tsc1−/− homozygous mutants are not elevated (and are in fact mildly reduced) compared to wild type control discs (total Yki and β-actin were used as loading controls; CIP treatment was not used to ensure the correct product was being observed). (I) Tsc1−/− discs do not show a reduction in Yki enrichment in a Yki responsive enhancer in the bantam locus compared to wild-type discs. IgG mock IP and enrichment at the PDH (pyruvate dehydrogenase) locus were included as controls. (J, K) hdc-Gal4 wing discs that either do (K, experimental) or do not (J, control) express a UAS: rheb transgene, labelled for active caspase III (green); both discs are approximately the same size (counterstained with Hoechst, blue), but the experimental disc shows pronounced Caspase activity in contrast to the control. (L–N) Coexpressing a UAS: diap transgene together with UAS:rheb (L) prevents cell death caused by expression of UAS:rheb (K) and results in tissue hyperplasia, as indicated by the increase in disc size. Hyperplasia is further increased by the addition of bantam expression under the direct control of the Tubα1 promoter (M). (N) Quantification of disc sizes. Error bars are Standard Error of the Mean and asterisks denote significances from t tests (* = p < 0.05, ** = p < 0.01, *** = p <0.001, n. s. = not significant). n = 20 (wt), 24 (Rheb), 18 (diap1), 10 (tub-ban), 20 (rheb+diap1), 15 (rheb+tub-ban), 22 (rheb+diap1+tub-ban).

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be attributed to a repressive effect of superactivation of TOR on the expression of DIAP1 protein and the bantam microRNA (Fig 6E–6G; S9F and S9F’ Fig), both targets of Yki regulation that have antiapoptotic activity [55]. However, the repression of DIAP1 and bantam does not appear to be due to enhanced Wts kinase activity, as Tsc1−/− discs showed a mild reduction rather than an increase in phospho-Yki S168 levels (Fig 6H). Moreover, the effects on DIAP1 and bantam are seemingly Yki-independent, since the repression of DIAP1 appears to be post-transcriptional (S9A and S9B Fig), and ChIP experiments indicate that Yki binding to its target enhancer in the bantam gene is not reduced in Tsc1−/− wing discs (Fig 6I) [56]. In principle, DIAP repression could be a secondary consequence of bantam repression, since DIAP1 protein is negatively regulated by the Head involution defective protein (Hid) [57], a bantam target [58]. However, we failed to observe any effect of overexpressing or removing bantam on DIAP1 protein levels (S9G and S9H Fig), from which it appears that TOR superactivation does not act via bantam to repress DIAP. Thus, TOR overactivation appears to cause cell death independently of the Wts/Hpo/Yki pathway, possibly mediated by increased expression of both DIAP1 and bantam.

To gauge if abnormally high levels of TOR activity indeed repress DIAP and bantam to offset tissue overgrowth, these gene products were resupplied to discs overexpressing rheb under hdc.GAL4 control. Overexpression of diap1, alone, under hdc.GAL4 control produced no significant effect on wing disc size (Fig 6N; S1 Data). However, co-overexpression of diap1 together with rheb fully suppressed cell death that would otherwise result from the overexpression of rheb alone (Fig 6L), and disc size increased by 77% (Fig 6N; S1 Data). To resupply bantam, GAL4-dependent transgene expression was not used, since bantam overexpression itself induces growth [58,59], rendering synergistic outcomes difficult to interpret. Instead, a transgene was constructed that expresses GFP at low levels under the direct control of the Tubα1 promoter, with the 3’UTR containing a minimal 100 bp genomic bantam fragment that includes the 21 bp bantam microRNA [58]. The resulting Tubα1.GFP-bantam transgene does not itself stimulate overgrowth (Fig 6N; S1 Data) but produces enough bantam to rescue a homozygous null bantam animal to adulthood, producing a normally sized and patterned animal (S9O and S9P Fig). This suggests that a single copy of Tubα1.GFP-bantam produces a level of bantam activity comparable to homozygosity for the endogenous bantam gene. Strikingly, when introduced as a single copy into the hdc.G4→UAS:rheb background, disc size increased 70% (Fig 6N; S9N Fig; S1 Data), similar to the effect of co-overexpressing DIAP with rheb, although the addition of the Tubα1.GFP-bantam did not fully suppress cell death (compare Caspase staining in S9L and S9M Fig). Finally, combining co-overexpression of rheb and
diap1 with Tubel1.GFP-bantam led to still more growth, with the wing disc more than doubling in size (110% larger; Fig 6H and 6N; S1 Data).

In sum, the effects of reintroducing DIAP1 and bantam reveal a novel effect of superphysiological TOR activity: the repression two key Yki target genes, albeit by Yki-independent mechanisms, to induce cell death and counterbalance the excessive tissue growth that would otherwise result from TOR hyperactivity. We suggest that this regulatory circuit normally safeguards against organ overgrowth and overriding it exposes a hidden capacity of superphysiological TOR signaling to stimulate tissue hyperplasia.

Discussion

Organ size depends on organ-intrinsic mechanisms that couple cell proliferation to the specification of pattern, as well as on external signals such as nutrients that scale overall body size [6–9]. Our study reveals one way in which these two distinct developmental phenomena can be integrated in the developing Drosophila wing, via a circuit linking nutrient-controlled TOR (and upstream InR) activity to Yki target gene access. A key finding is our discovery that, in addition to the well-known cytoplasmic anchoring mechanism employed by the Wts/Hpo tumour suppressor pathway to constrain Yki nuclear access, the InR/TOR pathway operates by a wholly different mechanism to allow nuclear Yki to activate its target genes. Inhibiting each pathway results in the accumulation of Yki in the nucleus, but with opposite effect. Nuclear Yki resulting from reduced Wts/Hpo activity drives Yki target gene expression and promotes growth. But, strikingly and counterintuitively, nuclear Yki resulting from reduced InR/TOR activity causes a loss of target gene access and impedes growth.

Based on our results, and as summarised in Fig 7A, we propose that reduced TOR signaling causes Yki to bind a secluding factor that sequesters Yki in the nucleus but diverts it from acting in complex with Sd to bind target loci, thereby reducing gene transcription. We posit that, by combining TOR-dependent relief from Yki nuclear seclusion with canonical Wts/Hpo phosphoregulation of Yki nuclear access, wing cells are able to integrate nutrient levels (via TOR) with patterning/morphogenetic inputs (via Wts/Hpo) to achieve a level of Yki-Sd activity that matches metabolic capacity and scales wing size appropriately. Elucidating this mechanism further will depend on identifying the proposed NSF and determining how its action is mediated by Yki’s N-terminus and WW domains. NSF may bind Yki that shuttles into the nucleus, titrating it away from target genes; one possibility, raised by a reviewer, is that NSF acts by modifying Yki in some way, stabilizing the protein and preventing its degradation (thereby contributing to its observed accumulation in the nucleus) with a modification that blocks it from accessing target loci.

A model for how TOR and Wts/Hpo inputs are integrated during normal Drosophila wing growth is depicted in Fig 7B. When the native TOR pathway is signaling at its peak, the level of Wts/Hpo activity dictates the amount of unphosphorylated Yki that is free to enter the nucleus. TOR activity liberates the Yki-Sd complex from a default state of nuclear seclusion, permitting nuclear Yki-Sd to access and transcriptionally activate loci that promote proliferative growth. In parallel, TOR also stimulates cellular metabolic processes such as protein translation (mediated by targets such as dS6K), which are preconditions for tissue growth. Both outputs of TOR are required for wing cells to divide and gain mass under Yki-Sd control. Given the central role of TOR signaling in nutrient sensing, [5,10], we further propose that this circuit may contribute to the capacity of the developing wing to scale downwards under conditions of nutrient deprivation. Specifically, under nutrient-limiting conditions, we suggest that the consequent reduction in TOR activity renders the Yki-Sd complex subject to nuclear seclusion, thereby decreasing transcription of Yki-Sd target genes that promote growth. Reduced TOR activity
would also diminish metabolism, matching metabolic capacity with the reduced activity of Yki-Sd target genes. Such a circuit would provide a sensitive means for developing tissues to integrate systemic and local inputs into growth. Our finding that excess activity of TOR appears to cause excess growth that is compensated by cell death via reduced DIAP1/bantam expression (Fig 7C) reveals that the circuit linking TOR and Yki is additionally stabilized by negative feedback, constraining organ growth in the event of erroneous, superphysiological TOR activity. Hence, a previously unknown facet of nutrient sensing by TOR is the incorporation of a self-limiting tumor suppression mechanism.

While our study delineates a circuit that integrates TOR and Yki activity during Drosophila wing growth, we note that it is only in this tissue that we find unequivocal evidence for nuclear seclusion of Yki, the means by which we posit this integration is achieved. Blocking TOR signaling in two other imaginal disc types, the eye and leg, does not appear to affect either Yki localization or target gene expression (S8I–S8K Fig). The same is also true even for other cell populations within the wing imaginal disc, notably those giving rise to the wing hinge and body wall. Hence, nuclear seclusion of Yki does not appear to be a general consequence of inhibiting TOR activity, and may occur primarily (or possibly only) in presumptive wing cells.

Intriguingly, the wing primordium is unique amongst imaginal disc tissues in expressing high levels of Sd under the control of Vg, a transcriptional coactivator that forms a complex with Sd to “select” the wing state. Although Yki-Sd complexes regulate growth control downstream of the Wts/Hpo pathway in the other Drosophila imaginal discs, Sd is expressed in these other tissues at tonic, low levels. Further, in the wing, Vg-Sd and Yki-Sd coexist, regulating distinct cohorts of target genes, and our present evidence suggests that inhibiting TOR does not alter the capacity of the Vg-Sd complex to gain access to, or activate, its distinct set of target genes (e.g., vg and sd itself). Hence, nuclear seclusion of Yki may represent an organ-specific mechanism to integrate nutrient-regulated InR/TOR and morphogen-dependent Wts/Hpo activities under conditions in which Sd plays a major, independent role in specifying cell state.
It remains possible that TOR activity regulates nuclear seclusion of Yki to control growth in other tissues, and possibly in other animals, albeit in complex with other transcriptional effectors and primarily through the control of target gene access rather than nuclear access. Alternatively, TOR may limit growth in these other systems exclusively through its regulation of cellular metabolism, without additionally modulating the level of Yki activity. Studying how global chromatin occupancy by Yki changes in response to TOR pathway activity, and varies by tissue type, will be needed to evaluate these scenarios.

To our knowledge, nuclear seclusion represents a novel paradigm of transcription factor regulation. Although experimental overexpression of nuclear Yki cofactors (e.g., Sd [18,52]) can exert similar effects on Yki localization and target gene expression, thus far it has not been demonstrated that such a mechanism functions as a biologically relevant mode of transcription factor regulation operating in vivo. Presently, the identity of the factor mediating Yki nuclear seclusion is unknown. However, in both yeast [60] and mammals [61,62], mTOR functions as a classical kinase that phosphorylates diverse substrates, leading to their retention in the cytoplasm. We hypothesize that our proposed NSF is one such substrate. A potential avenue of future exploration will be systematically testing how TOR inhibition influences the increasing number of identified nuclear modulators of Yki—some of which, like Sd, affect Yki localization and function when overexpressed (e.g., Hipk [63,64], MASK [65,66] and Tgi [48,67]). As we have demonstrated for the regulation of Yki by TOR, nuclear seclusion offers a complementary mechanism for controlling transcription factors that are already subject to phosphorylation-dependent partitioning between the cytosol and nucleus. Nuclear seclusion may therefore be used more widely to achieve signal integration, both during development as well as in mature tissues.

**Methods**

**Drosophila Genetics**

The following mutant alleles, Flp Recombination Targets (FRTs), UAS transgenes, Gal4 drivers and reporter transgenes were used (source indicated in parentheses; see Flybase [http://flybase.org/ for details]).

- **Mutations and FRTS.**  
  TorΔP FRT40A (T. Neufeld), exε1 FRT40A (G. Halder), FRT82B rhebΔA4 (Bloomington), bantamΔ1 FRT2A, FRT82B wtsX1, FRT82B akt1Δ (S. Cohen), FRT82B foxo25 (E. Hafen), FRT42D ykiB5 (D. Pan), pten1 FRT40A (C. Wilson), pten1Δ189 FRT60A (D. Pan), FRT82B Tsc1Q87X (I. Hariharan), FRT82B Tsc1PA23 (G. Halder), wtsA2 (G. Halder), ds6k1–1 (E. Hafen), Thor2 and sdGFP (Bloomington).

- **UAS transgenes.**  
  UAS-p35 (B. Hay), UAS-ΔP60 (S. Levers), UAS-Yki (D. Pan), UAS-Dp110 (S. Levers), UAS-Rheb (B. Edgar), UAS-TSC1+2 (I. Hariharan), UAS-TorTED (T. Neufeld), UAS-TorRNAi (P[TRiP.HMS0114]attP2; Bloomington), UAS-RhebRNAi (P[TRiP.HMS00923]attP2; Bloomington), UAS-YkiRNAi (VDRC), UAS-HA-Sd (J. Jiang), UAS.GFP, and UAS.GFP-NLS.

- **Gal4 drivers.**  
  nubbin.GAL4, tubulin.GAL4, headcase.GAL4 (C. Lee) act>CD2> GAL4, dpp.GAL4, and ptc.GAL4.

- **Reporter transgenes.**  
  dll-lacZ, exε1-lacZ (exε1, G. Halder), fj-lacZ, diaph2BC-lacZ (D. Pan), diaph3C-lacZ, and the bantam sensor [58] were used.

For generation of mutant heads, the EGUF/Hid technique was used [68]. For generation of negatively marked mutant clones, hsp70.flp; mut FRT/ubi.GFP FRT larvae (“mut” standing for the mutation(s) of interest) were heat shocked to induce by Flp-mediated mitotic recombination. Similarly, positively marked MARCM clones were generated by heat shocking hsp70.flp UAS.GFP-NLS Tubal.GAL4 / Y or hsp70.flp; mut FRT / Tubal.GAL80 FRT larvae. For Minute+ clones on 3R, an FRT ubi.GFP M(3)95 A chromosome was used.
Quantification of Wing and Clone Sizes and Cell Numbers

All measurements were made blind, without knowledge of the genotype. When performing multiple t-tests involving the same treatments, Bonferroni-corrected p-values were used to assess the level of significance. Clone and wing sizes were measured in Adobe Photoshop using the “lasso” tool, and cell numbers counted using the “count” tool. For whole wing cell number estimates, setae were counted within a 170 x 170 pixel box within the posterior intervein region. Total wing cell number = (wing size in pixels / (170 x 170)) x cell number in 170 x 170 pixel box.

Immunohistochemistry and Scanning Electron Microscopy

Discs were dissected, fixed in 4% PFA and stained with the following antibodies: rabbit anti-Phosho-AKT (S505) (1:200, Cell Signalling Technology), rabbit anti-Distal-Less (1:3,000), rabbit anti-Yki (1:500; D. Pan), mouse anti-DIAP1 (1:500), rabbit anti-β-galactosidase (1:6,000; Cappel), mouse anti-Ptc (1:150; DHSB), mouse anti-Armadillo (1:10; DSHB), guinea pig anti-Distal-less (1:3,000; R. Mann), mouse anti-CD2 (1:500; BD Biosciences). Alexa Fluor secondaries (Invitrogen) were used, and Hoechst was used to label nuclei. A Leica SP5 confocal microscope was used to capture all immunofluorescence images. For SEM, adult heads created by the EGFU/Hid technique were dissected into 70% ethanol, dried with an AUTOSAMDRI critical point drier, gold coated and imaged using the Hitachi S-3400N Variable Pressure SEM installed at the Microscopy Imaging Lab, University of Toronto.

Protein Blotting

For standard blotting of total wing protein, ~80 wing discs/lane were dissected from wandering third instar larvae into laemmli sample buffer supplemented with DTT, Complete Mini EDTA-Free protease inhibitor (“CPI” Roche) and Phosphatase Inhibitor Cocktail III (Calbiochem). Samples were boiled for 10 min and protein was quantified using the Pierce 660nm assay. Equal amounts of protein were loaded per lane. For fractionation of wing disc cells, ~80 wing discs were dissected per genotype, and washed twice with ice cold PBS+CPI. Discs were then washed briefly in 10μl hypotonic buffer (10 mM HEPES + 1.5 mM MgCl₂ + 10 mM KCl + CPI), centrifuged (1,500 rpm, 5 min, 4°C), and incubated in 30 μl hypotonic buffer on ice for 10 min. Discs in buffer were then dounced ~30 times in a glass homogeniser. Nuclei were separated by centrifuging (15 min, 3,750 rpm, 4°C) and removing the cytoplasmic fraction. Nuclei were washed three times in hypotonic buffer (spinning down and resuspending each time), and finally resuspended in 30 μl hypotonic buffer. 40μl laemmli sample buffer + DTT + CPI was added to both nuclear and cytoplasmic fractions, which were then boiled for 10 minutes and quantified using the Pierce 660 nm assay. Equal amounts of nuclear and cytoplasmic protein were loaded per lane. Antibodies used were rabbit anti-Phospho-Drosophila Akt S505 (1:500; Cell Signalling), anti-Akt (1:1,000; Cell Signalling), anti-Phospho-Drosophila p70 S6 Kinase T398 (1:500; Cell Signalling), rabbit anti-Yki (1:1,000, K. Irvine), rabbit anti-phospho Yki S168 (1:1,000; D. Pan), mouse anti-DIAP1 (1:1,000; B. Hay), mouse anti-β-actin (1:10,000; Abcam), rabbit anti-Histone 3 (1:10,000) and mouse anti-β-tubulin (1:500; DSHB). HRP-conjugated secondaries (Invitrogen) were used, and blots were developed with an ECL Plus detection kit (Amersham).

Chromatin IP

For ChIP of Yki and Sd-GFP at the 2B2C locus, the following procedure was undertaken four times to generate four fully independent experimental replicates. Female larvae of the experimental genotype (sd-GFP/y w hs-flp; UAS-TorTED/+; act>CD2>GAL4/Tsc1&2), negative
control female larvae (sd-GFP/y w hs-flp; act>CD2>YkiRNAi) or YkiRNAi control (sd-GFP/y w hs-flp; UAS-YkiRNAi/+; act>CD2>YkiRNAi) were allowed to reach the late larval stage before a 1 hr/37°C heatshock. This caused widespread >CD2> cassette excision leading to an extremely high frequency of clones expressing TORTED and TSC1 + 2, or YkiRNAi. Eight to ten hours later, 200 experimental and control larvae were into PBS + Complete Mini EDTA-Free protease inhibitor (denoted "CPI" Roche). Following the protocol of Estella et al. [69], batches of 25–30 larvae/tube were fixed (25 min; 1.8% formaldehyde in 50 mM HEPES + 1 mM EDTA + 0.5 mM EGTA + 100 mM NaCl + CPI), quenched (6 min; 0.125 M glycine in PBS + 0.01% Triton) and passed through a buffer series to prepare nuclei for lysis (2 washes 10 min Buffer A: 10 mM HEPES + 10 mM EDTA + 0.5 mM EGTA + 0.25% Triton + CPI, 2 washes 10 min Buffer B: 10 mM HEPES + 200 mM NaCl + 1 mM EDTA + 0.5 mM EGTA + 0.01% Triton + CPI). Individual wing discs were then dissected from larvae and placed in sonication buffer (10 mM HEPES + 1 mM EDTA + 0.5 mM EGTA + CPI), and sonicated on ice. For each genotype, 10% of the fresh chromatin was removed for the input control, and the remainder was split into two samples, each diluted 1:1 with 2xRIPA buffer (280 mM NaCl + 20 mM HEPES + 2 mM EDTA + 2% glycerol + 2% Triton + 0.2% Sodium Deoxycholate). Samples were incubated overnight with either anti-Yki (1:315; D. Pan), anti-GFP (1:300; molecular probes) or anti-Rabbit IgG control (Cell Signalling Technology) antibodies. Antibody-bound chromatin was pulled down for 3–4 hr with Protein A Agarose (Roche), washed several times in 1xRIPA and once in TE. Chromatin was then eluted twice with elution buffer (50 ul 1% SDS, 0.1 M NaHCO₃), once at room temperature and once at 55°C. Eluted chromatin was placed at 65°C to reverse crosslinks. Following a 3 hr treatment with Proteinase K, ChIP DNA was extracted with phenol/chloroform, ethanol precipitated and resuspended in TE. Quantitative real time PCR was performed using HotStart-IT SYBR Green qPCR Master Mix (2x) and an Applied Biosystems 7300 RT-PCR machine. For both genotypes from each of the three experimental replicates, DNA was amplified from total input control (serially diluted to avoid saturating the reaction), IgG-, GFP- and Yki-precipitated treatments, using primers for the 2B2C region of diap1 (2B2CF3 5'–AGAAAACTCGAAAGGCAGCTC/2B2CR3 5'–CCAAAACCAAACCAAC-GAAC) and a control locus (pyruvate dehydrogenase; PDH_F 5'–CGGAAGTGAAGCTGAC-CAAG/PDH_R 5'–GTAGGTCCATCCGTGGA CAC). The ΔCt method was used to calculate the degree of enrichment in each treatment relative to the input control DNA.

For ChIP at the bantam locus, chromatin was prepared from ~150 wild type and Tsc1Q87X/Tsc1PA23 mutant wing discs, and subjected to pulldown with anti-Yki or IgG control antibodies as explained above. An ~7 kb region around the microRNA was screened for Yki enrichment, and a strong peak was determined within a region 1,861–1,621 bp 5' to the 21 bp hairpin, corresponding to positions 640419–640646 on chromosome 3L of the April 2006 assembly of the Drosophila melanogaster genome (BDGP R5/dm3). This region was targeted in qPCR using the primers: bantam3F 5'–CGGGAACAGTCATAAAAGTTGC/bantam3R 5'–CTTTGCCTGTTCTGCCATCC. Note that this region lies inside the C12 enhancer region bound by Yki identified by Oh and Irvine [56].

Transgene Construction

**tubulin-Yki-GFP alleles.** A full-length yki cDNA was amplified with KpnI and SpeI linkers, and C-terminally tagged with SpeI-EGFP-XbaI. Site directed mutagenesis was used to create YkiP88L, YkiS111A S168A S250A (Yki3S-Δ), and YkiW292A P295A W361A P364A (YkiΔWW), and combinations thereof. Yki-GFP alleles were inserted as KpnI-XbaI fragments into p(Tuba1>DsRed, y2>AttB, where >DsRed, y2> is a flip-out cassette containing DsRed and yellow2 markers. Transgenic animals were generated with phiC31 Integrase-mediated
recombination, by transforming a Drosophila stock containing an ATTP landing site at 25C7 [70]. Excision of the intervening >ds-Red, y2+/> cassette leads to ubiquitous, low level expression of Yki-GFP from the alpha-tubulin promoter.

**tubulin-bantam.** EGFP with a 3’UTR containing a 100 bp fragment encompassing the bantam hairpin [58] was inserted as a KpnI-XbaI fragment into p(Tuba1>DsRed, y2+>)AttB, and the construct was inserted into the genome at 25C7. The >DsRed, y2+/> cassette was excised to generate Tuba1>ban. This flipped out form was introduced into the null banΔ1 homozygous background to assess rescue.

**Quantification of Transgenic Yki expression**

To quantify Yki expression from the tub-Yki-GFP transgene, the cassette was removed in clones, and discs were stained for total Yki. The staining intensities of five wild type regions and five regions of clonal tissue were quantified in ImageJ and averaged. The ratio between clonal and wild type tissue was calculated as 1.69.

**Supporting Information**

**S1 Data.** Raw numerical values for quantification of wing, clone and cell sizes, chromatin immunoprecipitation, and fluorescence intensity measurements.

(XLSX)

**S1 Fig.** TOR inhibition blocks Yki-driven overgrowth of the wing and head. (A–D) Late third instar wing discs with mutant clones marked negatively by absence of GFP (green; wild type sibling clones appear as bright green). (A) wild type (control) (B) rhebAV4, (C) wtsX1, (D) rhebAV4 wtsX1. Note that in b and d, mutant clone tissue is not recovered. (E–H) Mutant clones of the same genotypes as in A–D, except given a survival advantage by making them Minute+ in a Minute heterozygous background (clones are labelled negatively, by absence of GFP, as in (A–D); Hoechst labels nuclei (blue). rheb− clones can now be recovered as single cells or small groups of cells (F; arrowheads). Double mutant rheb−wts clones are slightly larger (H), but nowhere near as large as wild type (E) or wts− clones (G). (I–L) Wings from adult males expressing the following transgenes with nubbin.GAL4. (I) no transgene (wild type) (J) UAS.ΔP60, (K) UAS,yki, (L) UAS,yki+UAS.ΔP60. Numbers are size ratios compared to wild type; in (L), bottom italicised number is the size ratio compared to the ΔP60-expressing wing in (J). (M–O) Quantification of wing sizes, cell numbers, and cell sizes from genotypes in (I–L). Error bars are Standard Error of the Mean and asterisks denote significances from t tests (" = p < 0.05, "" = p < 0.01, """" = p < 0.001, n. s. = not significant). Number of wings measured = 10 (wt), 11 (ΔP60), 11 (Yki), 11 (Yki+ΔP60). Expressing yki increased blade size by 34% (K, M) via increased cell number (N), indicating that Yki accelerates cell growth and cell division rates equally. Expressing a strong inhibitor of the InR pathway component PI3-Kinase (ΔP60; [38]) decreased blade size by 66% (J, M), due to reductions in both cell size and cell number (N, O). When yki and ΔP60 were coexpressed, blade size was still 57% smaller than wild type (L, M, the slight size increase being manifested in a modest increase in cell number (N). The accelerated rates of cell growth and cell division caused by Yki expression are thus equally hindered by ΔP60 coexpression. (P–X) Standard Error of the Mean images of heads composed predominately of mutant tissue generated using the EGUF/hid technique [68]. (P–S) Images are to scale; (P) wild type (control). (Q) TorAP causes a “pinhead” phenotype composed of fewer and smaller cells. (R) exΔ caused substantial overgrowth of the head (note that overgrowth of the eye field does not occur in ex mutants [71,72], but the rest of the head capsule is strongly affected). (S) exΔ TorAP again produces a pinhead phenotype, indistinguishable from (J). (T–
InR signalling activates TOR at least in part via the kinase Akt [73], but Akt also controls the phosphorylation and cytoplasmic sequestration of the transcription factor FOXO [74–76]. FOXO is dephosphorylated when InR/Akt activity is diminished and accumulates in the nucleus to suppress cell growth and proliferation [76,77]. Loss of akt decreases head size (T), an effect which can be partially rescued by simultaneous removal of foxo (U) [77]. akt loss was able to block the wts− overgrowth phenotype (V, W), and importantly, overgrowth was still mostly suppressed when foxo was also removed (X). These results show that blocking InR pathway activity inhibits Yki-driven cell proliferation largely in a FOXO-independent manner.

S2 Fig. Effects of TOR and Rheb overexpression on clone size, cell size, and cell number. (A–C) Wing discs from late third instar larvae bearing MARCM clones expressing UAS.p35 (labelled positively with GFP-NLS, green; nuclei are counterstained with Hoechst, blue). The genotypes of clones are (A) UAS.p35, (B) UAS.Dp110+UAS.p35, (C) UAS.Rheb+UAS.p35. D–F: Quantification of clones sizes, cell numbers, and cell sizes from genotypes in A–C. Error bars are Standard Error of the Mean, and asterisks denote significances from t tests (′ = p < 0.05, ** = p < 0.01, *** = p < 0.001, n.s. = not significant). n = 50 (p35), 34 (UAS.Dp110+UAS.p35), 26 (UAS.Rheb+UAS.p35). Expression of either UAS.Dp110 or UAS.Rheb leads to enlarged clone size, through increased cell size and cell number.

S3 Fig. TOR and InR pathway inhibition causes nuclear accumulation of Yki. (A–C) Wing discs expressing UAS.GFP in the dorsal compartment with ap.GAL4 labelled with anti-Yki (red), GFP (green) and nuclei (blue; Hoechst). (A) Yki is largely cytoplasmic in wild type discs, as indicated by modest staining imaged at low magnification. Expressing TorTED (B) or TSC1+2 (C) in the dorsal compartment with ap.GAL4 domain causes some Yki to become nuclear (note gain in staining intensity within the GFP-positive region). (D–F) Tuba1.Gal80ts wing discs expressing UAS.GFP with dpp.GAL4 for 9 hrs, labelled as in (A–C); weak GFP expression results from the short window of GAL4 activity. (D) Yki is primarily cytoplasmic in otherwise wild type discs. (E) Inhibiting InR signalling in GFP cells by coexpressing UAS-ΔP60 causes some Yki to accumulate in the nucleus. (F) Magnification of portion of disc in (E) to confirm nuclear accumulation. (G–J) Yki localisation (red) is similar in wild type (G), dS6k− (H), thor− (I) and thor−; dS6k− double mutant (J) wing discs. Blue in G–J labels Hoechst-stained nuclei.

S4 Fig. Effects of Yki knockdown and TOR inhibition on gene expression. (A, B) RNAi-mediated knockdown of Yki under dpp.GAL4 control lowers DIAP1 protein (A), diap1R282C.P60.lacZ (A′) and fj-lacZ expression (B). (C–G) Expression of ex-lacZ (C, D) and Yki (E–G) in exel homzygous mutant wing discs. UAS-GFP (green) is driven with dpp-GAL4 (dotted line indicates the A/P boundary) and Hoechst-labelled nuclei are in blue. (D, F, G) Expression of TorTED and TSC1&2 under dpp.GAL4 control represses ex-lacZ (D) while elevating the level of nuclear Yki above that normally seen in exel homzygous mutant wing discs (F), magnified in (G). Control discs, expressing only GFP, are shown in (C, E). (H, I) Neither YkiRNAi nor TOR inhibition repress Vestigial protein in the central blade region, (red: Vg protein). (J) TOR inhibition in the Dpp domain has no effect on Armadillo protein (J; Arm: magenta), Distal-less protein (J'; Dll: red), and a Sd-GFP protein chimera (J; Sd-GFP: green). (K) TOR inhibition has no effect on a Dll-lacZ reporter UAS-GFP was not coexpressed in the discs shown in (J, K); dotted line indicates approximate position of the anteroposterior compartment boundary.
S5 Fig. Moderate TOR inhibition leads to Yki nuclear accumulation and reduced Yki target gene expression. (A–C) Wings from adult females expressing the following transgenes with nubbin.GAL4: (A) y+ (wild type) (B) UAS.RhebRNAi, (C) UAS.TORRNAi. (D–I) Moderate TOR inhibition in the Dpp domain by expressing TORRNAi (D, E), TORRNAi (F, G) and RhebRNAi (H–I). Dpp domain marked with UAS-GFP (green and arrowheads) and Hoechst-labelled nuclei are in blue. Moderate to weak nuclear accumulation of Yki is evidenced as gain in Yki staining intensity within the GFP-positive region (D, F, H). Expression of the Yki target gene fj-lacZ is reduced (E, G, I).

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S6 Fig. Effect of Sd overexpression and Leptomycin B treatment on Yki. (A–C) Overexpression of Sd under dpp.Gal4 control causes strong nuclear accumulation of Yki (A), and modest repression of the Yki targets DIAP1 (B) and fj-lacZ (C). Yki, DIAP1, and fj-lacZ are labelled in red, UAS.GFP expression (green) indicates the region of dpp.Gal4 expression, counterstained with Hoechst. (D,E) Cytosolic/nuclear shuttling revealed by incubating wild type wing discs in Leptomycin B (LMB) for 1.5 hrs, which inhibits nuclear export: LMB treatment causes increased nuclear accumulation of Yki (red; counterstained with Hoechst).

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S7 Fig. Parallel metabolic stimulation by TOR is necessary for Yki to drive wing growth. (A–D) wing discs from late third instar larvae are shown labelled for Distalless (green), which marks the wing primordium and adjoining portions of the prospective wing hinge, counterstained with Hoechst. (A) wt, (B) wtsP2, (C) ds6k1-1, (D) ds6k1-1; wtsP2. Numbers denote disc size ratios compared to wild type; in (D), the bottom italicised value is a comparison with the ds6k genotype. Asterisks denote significances from t tests (⁎ = p < 0.05, ⁎⁎ = p < 0.01, ⁎⁎⁎ = p < 0.001, n.s. = not significant). Number of discs measured = 12 (wt), 5 (wts), 13 (ds6k), 12 (ds6k; wts). Yki-driven overgrowth of the wing (caused by removal of wts) can be suppressed by removal of S6-Kinase (ds6k; compare discs in (B) and (D)), a TOR target that catalyses cap-dependent mRNA translation, but which is not involved in the nuclear sequestration of Yki (S2H Fig).

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S8 Fig. Hpo pathway-independent nuclear seclusion of Yki-Sd following TOR inactivation. (A) Confocal section of a wing disc taken at the level of nuclei shows Yki protein (red), CD2 (magenta) and Hoechst and Sd-GFP (blue and green respectively) 9 hours following a 1 hr heat shock to excise the Flp-out cassette from an Act5C>CD2>GAL4 driver (the CD2-expressing cassette has been removed from virtually all cells). GAL4 drives expression of YkiRNAi, which strongly depletes Yki protein in clonal tissue (arrows indicate no clonal tissue where normal Yki levels remain). (B) ChIP of Yki (black bars) or Sd (blue bars; using an Sd-GFP protein trap chimera in the endogenous Sd locus, and anti-GFP antibody) with mock IP (IgG; green bars) at the 2B2C diap1 enhancer and a control locus (PDH) in wild type (Sd-GFP/ y w hs-flp; Act5C>CD2>GAL4/+; YkiRNAi discs (Sd-GFP/ y w hs-flp; UAS-ykiRNAi/+; Act5C>CD2>GAL4/+);Act5C>CD2>GAL4/+;Act5C>CD2>GAL4/+) following 1 hr heatshock and 8–10 hrs of GAL4 expression. Yki and Sd-GFP are enriched at 2B2C in wild type discs compared to PDH controls. YkiRNAi strongly reduces enrichment of Yki but not Sd-GFP. (C) Excising the cassette (magenta) in a tub>Ds-RED>yki-GFP transgene produces Yki-GFP expression (green), raising total Yki levels 1.69 fold (red), close to the normal physiological range. (D) Western blot of phospho-Yki.S668 with total Yki in wild type control discs (y w hs-flp; Act5C>CD2>GAL4/+;TOR-inhibited (y w hs-flp; UAS-TOR-TED/++; Act5C>CD2>GAL4/UAS-TSC1&2) discs following 1 hr heatshock and 8–10 hr of GAL4 expression. 50 discs per lane were dissected; β-actin is a loading control.
F) fj-lacZ expression (magenta) is still repressed in the Ptc domain (green in rightmost panels of E and F) by expression Tor\textsuperscript{TED} with ptc-Gal4\textsuperscript{> in wts\textsuperscript{X1} clones (E; marked by absence of GFP in second panel), and wts\textsuperscript{X1}/wts\textsuperscript{P2} homozygous mutant wing discs (F). (G, H) An Sd-GFP protein trap line (green) shows similar expression in wild type discs (G) and discs expressing Tor\textsuperscript{TED} and TSC1\textsuperscript{e2} in the Dpp domain (H). Ptc staining approximately marks the Dpp expression domain. (I–J) Expressing TOR\textsuperscript{TED} and TSC1\textsuperscript{+2} in the eye (I, K) and leg (J) has no clear effect on Yki localisation or Yki target gene expression. The driver dpp.GAL4 UAS-GFP was used in I and J, and Act5C>CD2>GAL4 UAS-GFP in K.

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S9 Fig. Superphysiological TOR activity does not affect Yki activity but triggers a Yki-independent tumour-suppressive mechanism. (A, B) TOR overactivation caused by overexpression of rheb with dpp.GAL4 (A) or Act5C>CD2>GAL4 (B) (regions marked positively with GFP in green and counterstained with Hoechst in blue) does not influence expression of two different diap-lacZ reporters (red). (C, D) Wild type discs (C) and discs experiencing TOR overactivation (D; Tsc1\textsuperscript{Q87X}/Tsc1\textsuperscript{A22}) are similar in size, but Tsc1\textsuperscript{−} discs show extensive cell death (cleaved caspase III in green; blue: Hoechst). (F) Tsc1\textsuperscript{−} clones (marked by absence of magenta ß-galactosidase) in the eye repress DIA1 (F; red) and bantam (F'; bantam sensor in green). (G, H) Overexpressing bantam using the EP line EP(3)3622 with ptc-GAL4 (G; domain labelled by coexpressed ß-galactosidase; magenta), strongly diminishes expression of the bantam sensor (G') without noticeable effects on DIA1 protein levels (G; red). Likewise, in H, bantam homozygous mutant clones (labelled by absence of magenta ß-galactosidase) show a very strong increase in bantam sensor levels (green) but no discernable change in DIA1 protein levels (red). This indicates that native DIA1 levels are regulated independently of bantam in the wing. (J–N) hdc.

GAL4 discs labelled for cleaved Caspase III (green) and Hoechst (blue). hdc>rheb discs (K) are the same size as wild type (J) but show highly elevated levels of cell death. When diap1 and rheb were coexpressed, cell death is fully suppressed (L), and disc size increases by 77%; introducing tub.GFP-bantam while coexpressing rheb increases disc size by 70% (M), without obviously suppressing cell death. Simultaneously combining rheb, diap1, and tub.GFP-bantam together caused the disc to more than double in size (N). (O, P) Wings from adult female flies of the genotypes: bantam/+/ (O) and bantam−/bantam−/tub.GFP-bantam (P). A single copy of tub.GFP-bantam rescues a bantam homozygous mutant animal, producing a normally sized adult.

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Author Contributions

Conceived and designed the experiments: JP GS. Performed the experiments: JP. Analyzed the data: JP. Contributed reagents/materials/analysis tools: JP GS. Wrote the paper: JP GS.

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