Coordinated organ growth during development is required to generate fit individuals with fixed proportions. We recently identified *Drosophila* Dilp8 as a key hormone in coupling organ growth with animal maturation. In addition, *dilp8* mutant flies exhibit elevated fluctuating asymmetry (FA) demonstrating a function for Dilp8 in ensuring developmental stability. The signals regulating Dilp8 activity during normal development are not yet known. Here, we show that the transcriptional co-activators of the Hippo (Hpo) pathway, Yorkie (Yki, YAP/TAZ) and its DNA-binding partner Scalloped (Sd), directly regulate *dilp8* expression through a Hpo-responsive element (HRE) in the *dilp8* promoter. We further demonstrate that mutation of the HRE by genome-editing results in animals with increased FA, thereby mimicking full *dilp8* loss of function. Therefore, our results indicate that growth coordination of organs is connected to their growth status through a feedback loop involving Hpo and Dilp8 signalling pathways.
The classic reciprocal grafting experiments of Twitty and Schwind\(^1\) on salamanders have established > 80 years ago that organs follow autonomous growth programs. The principle of autonomous organ growth was later confirmed in various animals including insects. When young larval imaginal discs are transplanted in heterologous environments, they reach final sizes that are comparable to those achieved in situ\(^2\). To achieve individuals of correct size and proportions, mechanisms allowing organ growth to be coordinated and coupled with the developmental programme are essential. Evidence that such coordination mechanisms exist comes from studies showing that artificially slowing down growth of a subset of discs delays metamorphosis\(^3\) and reduces the growth rate of unperturbed discs, allowing perturbed discs to complete their growth programs before entry into metamorphosis\(^3\). We and others recently identified the hormone Dilp8 as a central organizer of organ growth coordination\(^3,5\). Dilp8 is secreted from abnormally growing organs and acts remotely on the central brain to delay entry into metamorphosis\(^6,7\). Dilp8 signals through the Laron-rich repeat-containing G-protein-coupled receptor 3 (Lgr3) in a pair of bilateral brain (GCL) neurons activating a neuroendocrine circuit that ultimately suppresses synthesis of the molting hormone ecdsyne\(^8\). Interestingly, mutations in the dilp8 and lgr3 loci produce animals that exhibit FA. FA, measured as the variance between left and right bilateral traits within individuals, is an assessment of stochastic developmental variations\(^3\). Therefore, the dilp8/lgr3 axis carries additional function in adjusting organ size and ensuring developmental stability\(^7\). Reducing lgr3 levels in the GCL neurons recapitulates the lgr3 mutant phenotype consistent with organ growth being adjusted through a central relay\(^8\). The ability of dilp8 to fine-tune organ growth suggests that its expression should be controlled by signals central to organ size assessment mechanisms.

In this study, to gain insight into how dilp8 expression might be coupled with organ growth, we searched for regulators of dilp8 expression among 120 candidates recovered in a genetic screen for molecules coupling disc growth with developmental transitions\(^6\). The condition used for this screen corresponds to a disc-specific knockdown of the syntaxin Avalanche (Avl; \(rn > avl\) RNAi), which generates neoplastic growth and a Dilp8-dependent delay in larva-to-pupa transition. We inferred that reducing the function of molecules regulating dilp8 expression should rescue the delay of \(rn > avl\) RNAi animals. Indeed, altering JNK signalling efficiently rescues the delay by suppressing the upregulation of dilp8 transcription observed in \(rn > avl\) RNAi animals\(^6\). JNK signalling induces dilp8 transcription in response to various stresses, including wound healing and tumour formation. This likely represents an important checkpoint mechanism allowing the organism to recover before entering metamorphosis, but may not be important for coordinating organ growth in normally developing animals\(^6\).

**Results**

**dilp8 expression requires the co-activators Yorkie and Scalloped.**

In addition to JNK signalling, we identified the Hpo pathway as an important regulator of dilp8 expression. The Hpo pathway is an important regulator of organ growth and is thought to play a central role in organ size assessment\(^13,14\). The core kinase module of the Hpo pathway includes the Hpo (Mst1/2 in humans) and Warts/Lats kinases, which suppress activation of the transcriptional co-activator Yorkie (Yki; YAP/TAZ in humans). When the Hpo pathway is inactive, Yki and its DNA-binding partner Scalloped (Sd) activate target genes and promote organ growth\(^15\). We observed that reducing levels of the transcriptional co-activators Yki or Sd efficiently rescues the developmental delay in \(rn > avl\) RNAi animals and normalizes dilp8 transcript levels (Fig. 1a,b). Given the substantial evidence that crosstalk takes place between the Hpo and JNK signalling pathways\(^11,22\), we tested whether Yki can regulate dilp8 expression independently of JNK signalling. Indeed, we found that dilp8 expression is still significantly upregulated by Yki overexpression in flies that are mutant for the JNK kinase Hemipterous (Hep; Fig. 1c–i). We next tested whether overexpression of yki is sufficient to activate dilp8 transcription. Using an enhanced green fluorescent protein trap inserted in the first intron of the dilp8 gene as a reporter for native dilp8 expression, we could observe increased levels of enhanced green fluorescent protein in yki-overexpressing clones (Fig. 2a,b). Consistent with this, Dilp8 protein levels were also elevated in these clones (Fig. 2c,d). In agreement with Yki-regulating gene expression through association with its co-activator Sd, we found that reducing Sd levels in Yki-overexpressing clones abolished the Yki-dependent upregulation of Dilp8 (Fig. 2e–j). We next examined dilp8 levels in clones carrying mutations in genes encoding upstream components of the Hpo pathway including expanded (ex), hpo and warts (wts). As expected, reducing the activity of upstream Hpo pathway members, which induces Yki activation, also increases Dilp8 protein levels (Fig. 2k–p).

A Hippo-responsive element controls dilp8 expression by Yki/Sd.

Genome-wide CHIPseq analyses using anti-Yki antibodies recently identified a number of potential Yki target genes\(^7,23\). Interestingly, these CHIPseq data identified a 600-bp promoter fragment localized 1.5 kb upstream of the coding region in the dilp8 locus. Close examination of this fragment reveals three potential Sd-binding sites (hereafter referred to as Hpo-responsive element (HRE))\(^23\), suggesting that dilp8 expression might be directly activated by a Yki/Sd heterodimer (see region map in Fig. 3a). To directly test this, we performed RNA pull-down assays by mixing lysates from cells expressing tagged Sd (Sd-Flag) and a 600-bp DNA fragment of the dilp8 promoter region centred around the HRE (dilp8 promoter fragment: dilp8-PF). A region in the diap promoter that is known to bind Sd (diap-PF) was used as positive control. We found that Sd-Flag binds the dilp8-PF and diap-PF with similar efficiency (Fig. 3b). Moreover, the binding of Sd-Flag to the dilp8-PF is abolished on targeted mutation of the three putative Sd-binding sites (dilp8-PF/Avl; Fig. 3b, Supplementary Fig. 1 and see the Methods section). To test the functional relevance of this binding assay, we analysed the potential of each of these fragments to promote transcription of the luciferase reporter gene in the presence of Sd and Yki. Consistent with the DNA pull-down result, both diap-PF and dilp8-PF, but not dilp8-PF/Avl, were able to activate transcription in the presence of Yki and Sd (Fig. 3c). To study the transcriptional regulation of dilp8 by Yki in vivo, we next generated transgenic fly lines carrying constructs harbouring either the full dilp8 promoter (dilp8-full-prom), dilp8-PF, dilp8-PF/Avl or intron 1 of the dilp8 gene used as a negative control, all fused to the lacZ coding sequence (Fig. 3a). We found that both dilp8-full-prom and dilp8-PF were able to promote lacZ expression in Yki-overexpressing clones (Fig. 3d–o). Moreover, the ability of Yki to induce lacZ expression depends on the integrity of the HRE, since mutation of the three Sd-binding sites prevented all lacZ expression (Fig. 3p–s). Altogether these results are consistent with Yki-activating dilp8 gene expression through Sd bound to the HRE in the dilp8 promoter.

**Yki/Sd ensures developmental stability through dilp8 expression.**

We ultimately wanted to know whether the Yki-dependent regulation of Dilp8 function is central to the mechanism allowing...
Yki or Sd efficiently suppresses the upregulation of percentage of larvae that have pupariated at the indicated hours. AED is shown (with the control dilp8-PF exhibit increased levels of FA, although flies carrying a replacement background (Fig. 4e–h). We observed that of (was efficiently suppressed in flies carrying mutations in the HRE section). As expected, Yki-dependent induction of dilp8 locus (see Supplementary Figs 2 and 3 and see the ‘Methods’ dilp8-PF with either rh3 RNAi or a replacement (Fig. 4a–d). By contrast, JNK-dependent regulation of dilp8 expression was not compromised in this genetic background (Fig. 4e–h). We observed that dilp8-PF:A123 flies exhibit increased levels of FA, although flies carrying a replacement with the control dilp8-PF do not (Fig. 4i, Supplementary Fig. 4). Finally, wing size distribution is neither modified in dilp8-PF:A123 flies, nor in dilp8KO/KO flies compared with control animals (Supplementary Fig. 5). This suggests that the FA phenotype is not due to a disruption of a general size-control mechanism operating independently in each disc. Overall, our data demonstrate that Yki-dependent regulation of dilp8 expression plays a critical role in limiting developmental variability. It also contrasts with our observation that hep75 mutant flies do not exhibit elevated FA (Supplementary Fig. 6). These results are consistent with a central role of Yki in coordinating organ growth and limiting developmental variability through its effect on dilp8 expression.

Discussion

On the basis of a flurry of experiments, different working models have been proposed to explain organ-autonomous size control operating in complex organisms. One unifying view is that the Hpo pathway plays an instrumental role in assessing and regulating organ growth. The ability of the Hpo pathway to integrate both short- and long-range signals makes it an ideal candidate for sensing organ size and regulate growth accordingly. Importantly, we demonstrate here, direct molecular and functional links between Hpo/Yki signalling and dilp8 expression during normal development. Furthermore, suppressing the sole input of Yki on dilp8 expression suffices to increase developmental instability and recapitulates the effect of a dilp8 loss-of-function mutation. The coupling between Hpo pathway activity and dilp8 expression allows the growth status of organs to be transduced into a Dilp8 signal. Dilp8 acts through its receptor Lgr3, residing in two pairs of bilateral neurons to control the levels of the steroid hormone ecdysone. This in turn affects...
the timing of maturation, allowing for a checkpoint mechanism on the developmental transition. When ectopically expressed, Dilp8 also affects organ growth, suggesting that it may also participate in a continuous feedback ensuring proper organ growth during development. Whether ecdysone acts downstream of Dilp8/Lgr3 to mediate such continuous feedback on organ growth remains to be determined. Several reports indicate that ecdysone is required for imaginal tissue growth both in flies and in Lepidoptera, and previous data suggest that organ growth coordination relies on systemic effects mediated by ecdysone. Interestingly, Taiman, a co-activator of ecdysone receptor mediates Yki-dependent dilp8 expression, therefore suggesting that the ecdysone signal itself feeds back on dilp8 expression.

On the basis of previous experimental data, two non-exclusive models can be proposed for organ growth coordination during development. In a developmental checkpoint model, each tissue autonomously follows its growth trajectory and deviations that may occur due to developmental noise are resolved at the end of the growth period by adjusting the time of the next developmental transition. Alternatively, continuous adaptation of growth rates and/or organ size could take place during development, allowing organs to adjust while growing. Although experimental evidence exists for both mechanisms in response to large growth disruptions, it is not clear whether they apply for small perturbations usually encountered by larval organs during development. Further analysis will be necessary to decipher how the Yki/Dilp8 coupling contributes to final organ size adjustment and limits developmental noise.

Methods

Fly strains and food. The following RNAi lines were from the GD or KK collections of the Vienna Drosophila RNAi Center (VDRC): yki RNAi (KK104523), sd RNAi (KK108877), avl RNAi (KK100853). The UAS-yki (ref. 15), FRT82B, LATS (ref. 28); FRT42D, hpo (ref. 29); FRT82B, ex (ref. 30) lines were provided by Nic Tapon (The Francis Crick Institute, London, UK). The elav-Gal80 was kindly provided by Alex Gould (The Francis Crick Institute). The yw, hep-2/FM7, GFP, SalPE/Cyo (ref. 31) line was kindly provided by Florenci Serras (University of Barcelona, Barcelona, Spain). The UAS-hep-2; rn-Gal4, dilp8-GFP and other lines were provided by the Bloomington Drosophila Stock Center.

Animals were reared at 25 °C (or 26.5 °C for Fau measurement) on fly food containing, per litre: 17 g inactivated yeast powder, 83 g corn flour, 10 g agar, 60 g white sugar and 4.6 g Nipagin.

Cell culture. Drosophila S2R+ cells (DGRC) were grown in Complete Schneider’s medium (Schneider medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker), 50 U ml−1 penicillin, and 50 μg ml−1 streptomycin) at 25 °C. Transfections were done using Effectene reagent (Qiagen).

Immunostainings of larval tissues. Tissues dissected from larvae in 1× phosphate-buffered saline (PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 8)) at the indicated hours after egg deposition (AED) were fixed in 4% formaldehyde (Sigma) in PBS for 20 min at room temperature, washed in PBS containing 0.1% Triton-X-100 (PB/T), blocked for 2 h in PB/T containing 10% fetal bovine serum (PBS-TF) and incubated overnight with primary antibodies at 4 °C. The next day, tissues were washed, blocked in PBS-TF and incubated with secondary antibodies at 1/500 dilution (Cy3-conjugated donkey
Figure 3 | Yki directly regulates *dilp8* expression through a HRE in the *dilp8* promoter. (a) Schematic of the *dilp8* promoter region and the HRE harbouring the three putative Sd-binding sites (indicated as green squares). The *dilp8* promoter fragments used to study Yki-dependent regulation of *dilp8* expression *in vivo* are shown. In *dilp8*-PF,Δ123, mutations of the three putative Sd-binding sites are indicated as red squares. (b) DNA pull-down experiments show that binding of Sd to the *dilp8*-PF is mediated by the three Sd-binding sites. The indicated DNA fragments were incubated with lysates from S2 cells transfected with Sd-Flag. Band intensities represents the average of three independent experiments: for *diap*-PF: 3.8 ± 0.7 (positive control), for *act*: 1 ± 0.2 (negative control), for *dilp8*-PF: 5.1 ± 1.0, and for *dilp8*-PF,Δ123: 1.9 ± 0.4. (c) Luciferase assay showing that Yki/Sd activate gene expression through the HRE in the *dilp8*-PF. S2 cells were transfected with Yki and Sd. The ability of Yki/Sd to induce gene expression from the indicated promoter fragments was measured (triplicate samples, error bars represent s.e.m.). (d–s) Yki induces *dilp8* transcription through the HRE *in vivo*. Wing imaginal discs carrying GFP-labelled Yki-expressing clones were dissected from transgenic flies carrying the indicated *dilp8* promoter fragments fused to the *lacZ*-encoding sequence. The full *dilp8* promoter and *dilp8*-PF, but not *dilp8*-intron1 and *dilp8*-PF,Δ123, induces *lacZ* expression as detected by β-gal staining (d,h,l in red) in the GFP-labelled yki-overexpressing clones (e,i,m,n in green). In each condition, yki overexpression leads to elevated levels of endogenous Dilp8 protein (f,j,n,r in white).
anti-rat or Alexa Fluor 647 goat anti-rat, Cy3-conjugated donkey anti-chicken from Jackson Immunoresearch) for 2 h at room temperature. After washing, tissues were counterstained with DAPI (Vector Labs). Fluorescence images were acquired using a Leica SP5 DS (×20 and ×40 objectives) and processed using Adobe Photoshop CS5 or Image J. Wing areas were measured using Image J

**Antibodies.** The following primary antibodies were used, rat anti-Dilp8: 1/500 (ref. 6) and chicken anti-beta-galactosidase: 1/1,000 (GeneTek).

**Plasmids.** *dilp8*, *dia* and *actin* promoter sequences were PCR-amplified from genomic DNA (DNA preparations made using DynaBEAD Blood and Tissue kit (Quagen)) and cloned in the pENTR/D-Topo vector (Invitrogen) using the following primers: for pENTR-dilp8-PF sense, 5′-GTA TGATACCAAGGTCTG TG-3′ and antisense, 5′-AGG TTG TAG TGG ATT TAT TAT TAT GAA GAG A-3′, for pENTR-diap-PF sense, 5′-AGG TTG TAG TGG ATT TAT TAT TAT GAA GAG A-3′ and antisense, 5′-ATC GAGCAATGATTAAACGCGT-3′, for pENTR-act-PF sense, 5′-GGACCTC GTGCATCTGCTGGTTG-3′ and antisense, 5′-CCCACT TAC GAG GCT TAT GC-3′. To generate the pENTR-dilp8-PF Δ123 plasmid, mutations were introduced in pENTR-dilp8-PF using the QuickChange site-directed mutagenesis kit (Stratagene) and the following primers: *dilp8* MutSl S1: 5′-TCT GCA GGT ATC TTG CAT GCA TCT CGT GCT GCT C-3′, *dilp8* MutSl A1: 5′-GAC GAG CAG GAC AAT CAT GAT GAA AGT AAG GAA GAA GAA GAA TAC GGT CGA A-3′, *dilp8* MutSd S3: 5′-CCA CTT CTT TGT GCT TGC TGT ATG AAC GGA TGG AAT GTT A-3′, *dilp8* mut S3: 5′-CCA CTT CTT TGT GCT TGC TGT ATG AAC GGA TGG AAT GTT A-3′, *dilp8* mut S4: 5′-ACA CCG ACT TGG CGT CTT CTG ATC GCG TGT GCC CGT T-3′, *dilp8* Mut At A4: 5′-AAG CAG CAC CGC ACA GAA TCC GAA CCG AAC TAC CGT GCC T-3′. To generate the beta-galactosidase reporter plasmids, sequences corresponding to the full *dilp8* promoter, intron I, *dilp8*-PF and *dilp8*-PF Δ123 were amplified from plasmids or genomic DNA, and cloned into the pCASPER-atbN-h43-lacZ plasmid (kind gift from Ulrike Lohr and Herbert Jaekle, Max Planck Institut für Biophysikalische Chemie, Göttingen, Germany). The sense and antisense primers were used for intron I: sense 5′-GGTG AAC GGG-3′ and antisense 5′-GGTG ATG TCG AGT TCG AGT TCG TA-3′, for *dilp8*-PF and *dilp8*-PF Δ123: sense 5′-GTA TGA TAG GAG GAT CAG GAA AGA CAA GAG CAA AAG CAA GTA G-3′ and antisense 5′-AGG TTA GAG ATT TAT TAT TAT GAA GAG A-3′, for the full *dilp8* promoter region: sense 5′-TTT AGT GGA GCT GGT CTA CAA GAA-3′ and antisense 5′-AGG TTA GAG ATT TAT TAT TAT GAA GAG A-3′. To generate the genomic DNA based reporter plasmids, *pCASPER*-atbN-h43-lacZ constructs were introduced into the germ line by injections in the presence of the PhiC31 integrase and inserted in the landing site 51C1 on the 2nd chromosome (Bloomington Drosophila Stock Center, BL24482, BestGene Inc.).

**dilp8 editing by accelerated homologous recombination.** To generate a *dilp8* null mutant, an approach combining the CRISPR technique and homologous recombination was used as described in ref. 24. Double-strand DNA breaks were introduced by the CRISPR technique using single-stranded guide (sg)RNAs and a Cas9-encoding plasmid. For optimal targeting of the *dilp8* locus, sgRNA target sequences were selected as 20 nt sequences preceding an NGG PAM sequence in the genome (GN20GG). To generate *pCD4*(*dilp8*KO), *g*RNAs targeting exon I and exon II in the *dilp8* locus were cloned into the tandem *g*RNA expression vector, *p*CFD4 (kind gift from Simon Bullock (Addgene plasmid # 49411)), using the *dilp8*-5′ KO- *pCD4*-FORWARD: 5′-ATT TAT ATA GAG ATA TCC GGG TGA ACT TCG cca taa tga gtt cca gtt TTA GAA GCT AAT AGA GCA AAG G-3′ and *dilp8*-3′ KO- *pCD4*-REVERSE: 5′-ATT TAT ATA ACT TCG TAT TAT TAT TCG TAT TAA AAT tcg aac gac cag ctc gtc gac ATG GTA AAT AGA GCT G-3′ (*dilp8*-specific sequences are in lower case) primers as described in http://www.crisprflydesign.org/wp-content/uploads/2014/06/Cloning-with-pCFD4.pdf. For homologous recombination, two homology arms were amplified from genomic DNA using the following primers: for homology arm I: sense 5′-GTA TGATACCAAGGTCTG TG-3′ and antisense 5′-GGT GAG CAG GAG TGC TGG GAAaga ggag GAA GAA GAA TAC GGT CGA A-3′ and antisense 5′-GGTG GCC GCG TTA AAT ATT CGT GCG CCC GAA GA-3′, for homology arm II, sense 5′-ACT ATG AAC TCA TTT TAG TGG CGT CTT AGG ATC A-3′ and antisense 5′-AGA TCT CAA AAT ATT ACC GAA TCA TTA AAG CTA ATC AC-3′ (in bold are the added restrictions sites used for cloning into pTV2). The resulting PCR products were digested and cloned into the pTV2 vector (kind gift from Cyril Alexandre and Jean-Paul Vincent, the Francis Crick Institute) to generate the pTV2(*dilp8*KO). To facilitate homologous recombination, embryos were injected with pTV2(*dilp8*KO) in the presence of the *p*CD4(*dilp8*KO) and a CAS9-containing plasmid. To confirm gene targeting the following primers were used: *dilp8KO S: 5′-GGG CCG GAT CTA CCT GCT TGG TGG-3′ and an antisense primer encompassing the three scalloped-binding sites (*dilp8*KO Rev: 5′-GGG CCG GAT CTA CCT GCT TGG TGG-3′). After confirming targeting, the majority of the targeting vector was removed by crossing to a strain expressing a constitutively active Cre (Bloomington stock BL1092; procedure outlined in ref. 24. The resulting strain is referred to as *dilp8*KO in the manuscript.

**To generate a *dilp8* mutant deleting a 600 bp fragment of the promoter encompassing the three scalloped-binding sites (*dilp8*KOΔ778), *g*RNAs directed against sequences upstream and downstream of the scalloped-binding sites were
cloned into pCFD4 as described above using the following primers: dilp8-HR S: 5′-TAT ATA GGA AAG AAT ATC GCG TGA ATC GAG acc ttt cag tgt tgt GTT TTA AGA GCT AGA AAT AGC AAG-3′ and dilp8 HR REV: 5′-ATT TTA ACT TG TGT TTT CTC TAT CAA AAT ggc gct tta att tca AGC TTA AAT TGA AAA TAG TGC-3′. The primers used to generate the homology arms for the reintegration vector were: HRR S: 5′-GGG GGC GAT CCA GGT CAT CAC CAC ATC-3′, HRR AS: 5′-GGT ACC AGA AAC GAT GCT GAA GGT TCT AACTT CTC-3′, HRR S: 5′-AGA TCT CAG ACA TTA TCG TTA TCG TCT G-3′ (in bold are the added restrictions sites used for cloning into pTV2). To facilitate homologous recombination, embryos were injected with pTV2 in the presence of pCFD4 (dilp8-PF and PF) and a CAS9encoding plasmid. The resulting strain, referred to as dilp8-pTV2 PF, was used as a host for reintegration of the missing 600 bp promoter fragment harbouring point mutations in the three scalloped-binding sites (dilp8-PFΔ123) or not (dilp8-PF), control the atip site. For this purpose, dilp8-PF and dilp8-PFΔ123 were PCR-amplified from plasmids and cloned into the reintegration vector RIV cherry (kind gift from Cyrille Alexandre and Jean-Paul Vincent, the Francis Crick Institute) giving rise to RIVcherry (dilp8-PFΔ123, mini-white) and RIVcherry (dilp8-PF, mini-white). For the PCR amplification of dilp8-PF and dilp8-PFΔ123 the following primers were used: RIV S: 5′-ACT GTT TTT AGA GCT AGA AAT AGC AAG-3′, RIV AS: 5′-CGT GAC GAT ACC GTG CTC-3′ (RIVcherry). Sequencing analysis of the dilp8-PFΔ123 mutant were performed using the following primers: Dilp8 HRE S2: 5′-GGA GAT GCC ATT CGA CGG ATC TGG-3′ and Dilp8 HRE AS2: 5′-GGA-AGA CCC AAA TGG TTG TTA AAC ACC-3′.

Luciferase assays. dilp8, diap and actin promoter sequences were PCR-amplified and cloned into pGL3 basic vector (Promega) using the kit Dual Luciferase Reporter Assay System (Promega). For luciferase assays, S2 cells were transfected for 3 days with the following constructs: Firefly-luciferase pGL3-promoter (500 ng), pAct-Renilla-luciferase (100 ng), pAct-yki (600 ng), pAct-sd (400 ng). All experiments were performed in triplicate, and luciferase activities were normalized against Renilla luciferase activity following indications of the Dual Luciferase Reporter Assay System protocol (Promega).

DNA pull-down. The method was adapted from ref. 32. Drosophila S2 cells were plated in a six-well plate at a density of 2 × 106 cells per well. Each well was transfected with 400 ng of pAC-Flg-S (kind gift from Clara Sidero and Barry Thompson, the Francis Crick Institute). In all, 48 h later, cells were washed once in PBS and lysed in 500 µl IP buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA) in the presence of protease (complete mini tablets from Roche) and phosphatase (phosphatase inhibitor cocktail 1, Sigma) inhibitors for 10 min on ice. Cell extracts were then spun down at 13,000 × g for 10 min at 4°C to remove nuclei and cell debris. Extracts were incubated for 1 h at 4°C on a rotating wheel. Pre-cleared cell extracts and probe-bound beads were combined and incubated overnight at 4°C on a rotating wheel. Beads were then washed 3 × 5 min in IP buffer and resuspended in 30 µl SDS loading buffer for western blot analysis. DNA probes were amplified from genomic DNA using the following primers: for the DIAP1 probe (508 bp): 5′-ATT TTA ACT TCC TCT GTG TTA CTC TGG C-3′ and R: 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-GGA GCT GTC GTC CTT GCT GCT G-3′ and R: 5′-[Bnt]ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′, for the Act5c probe (606 bp): 5′-Bnt[ATC GAC GAA TTA AAC AAT GAC GAG-3′.

Quantitative RT-PCR. Larvae were collected at the indicated number of hours AED. Whole larvae or dissected larval wing discs were frozen in liquid nitrogen. Total RNA was extracted from whole larvae or dissected tissues using a QIAGEN RNasey lipid tissue minikit or microkit according to the manufacturer’s protocol. RNA samples (2 μg per reaction) were treated with DNase and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen), and the generated cDNAs were used for real-time RT-PCR (StepOne Plus; Applied Biosystems) using PowerSYBRGreen PCR mastermix (Applied Biosystems), with 8 ng of cDNA template and a primer concentration of 300 nM. Samples were normalized to levels of ribosomal protein (rp19) transcript levels. Three separate biological samples were collected for each experiment and triplicate measurements were performed. Primers were designed using PrimerExpress software (Applied Biosystems) as follows: dilp8 5′-CGA CAG AAT GTC CAT CAA GT-3′, dilp8 5′-GTG CCA GTG CCA AAT C-3′.

Western blotting. Proteins were resolved by SDS-PAGE using 12% gels (NuPAGE Novex gel, Invitrogen) and transferred electrophoretically to polyvinylidine difluoride membranes (Amersham). The membranes were incubated for 1 h in blocking buffer (PBS, 5% milk) and incubated overnight at 4°C in the same buffer containing primary antibodies at 1:1,000 dilutions (mouse anti-Flag F3165 Sigma). Membranes were washed three times in PBS-T, blocked for 1 h, and probed with secondary antibodies in blocking buffer for 1 h at room temperature. After three washes in PBS-T, chemiluminescence was observed using the ECL-Plus western blotting detection system (Amersham Biosciences). Images were generated using the Fujifilm Multi Gauge software. The uncropped western blot (for Fig. 3) can be found in Supplementary Fig. 1.

Purification curves. L1 larvae were collected 24 h AED on agar plates with yeast (w3111, isogenic) and reared in a closed (three) jars containing standard food (see above). The number of larvae that had pupariated at a given time AED was scored every 6 h.

Measurement of the FA index. L1 larvae were collected 24 h AED (4-h egg collections) and reared at 30 animals per tube at 26.5°C. Adult flies of the appropriate genotypes were collected, stored in ethanol and mounted in an Euparal solution. Measurements of dissected wings were acquired using a Leica Fluorescence Stereomicroscope MZ16 F with a Leica digital camera DFC 490. Wing areas were measured using Image J. We used the FA index to assess intra-individual size variation between left and right wing areas as described in ref. 12. FA index = Z(A)/n2, where A is the normalized differences between left and right wing area with a given individual (n=22). P values are the results of a F Test provided by Microsoft Excel.

Statistics. P values are the results of a Student’s t-test provided by Microsoft Excel (P<0.05; *P<0.01).

Genotypes. In Fig. 1a: w-; elav-Gal80 ++; +nt-Gal4 ++, +w-; elav-Gal80 ++; +nt-Gal4 ++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80/r3h RNAi KK; +nt-Gal4, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++, +w-; elav-Gal80 RNAi GD/++. See uncropped image of the western blotting experiment in Supplementary Fig. 1.

Data availability. All data are available in the article or its Supplementary Files or available from the authors on request.

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