The Ecdysone Receptor Coactivator Taiman Links Yorkie to Transcriptional Control of Germline Stem Cell Factors in Somatic Tissue

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In Brief
Zhang and Robinson et al. define an interaction between the Hippo pathway component Yorkie and the ecdysone receptor transcriptional coactivator Taiman. This interaction links Yorkie to mRNAs of germline stem cell factors Piwi and Nanos, identifying them as required elements of a Hippo-repressed growth program in somatic disc cells.

Highlights
- The Drosophila EcR coactivator Tai binds the Hippo pathway coactivator Yki
- Tai modulates expression of classic Yki target genes in developing imaginal discs
- Tai’s role in Yki-driven hyperplasia is independent of classic Yki target genes
- Tai links hyperactive Yki to expression of germline mRNAs in disc cells
The Ecdysone Receptor Coactivator Taiman Links Yorkie to Transcriptional Control of Germline Stem Cell Factors in Somatic Tissue

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SUMMARY
The Hippo pathway is a conserved signaling cascade that modulates tissue growth. Although its core elements are well defined, factors modulating Hippo transcriptional outputs remain elusive. Here we show that components of the steroid-responsive ecdysone (Ec) pathway modulate Hippo transcriptional effects in imaginal disc cells. The Ec receptor coactivator Taiman (Tai) interacts with the Hippo transcriptional coactivator Yorkie (Yki) and promotes expression of canonical Yki-responsive genes. Tai enhances Yki-driven growth, while Tai loss, or a form of Tai unable to bind Yki, suppresses Yki-driven tissue growth. This growth suppression is not correlated with impaired induction of canonical Hippo-responsive genes but with suppression of a distinct pro-growth program of Yki-induced/Tai-dependent genes, including the germline stem cell factors nanos and piwi. These data reveal Hippo/Ec pathway crosstalk in the form a Yki-Tai complex that collaboratively induces germline genes as part of a transcriptional program that is normally repressed in developing somatic epithelia.

INTRODUCTION
Most metazoan organisms grow during embryogenesis and a subsequent juvenile stage, which in vertebrates coincides with hormone-driven sexual maturation. The extent of growth is determined in part by extracellular signals in the form of locally acting morphogens and systemic growth factors. In the fruit fly Drosophila melanogaster, these local and systemic factors act in concert with nutrient-sensing pathways to regulate growth of pockets of diploid epithelial cells termed imaginal discs that grow during embryonic and larval stages and are reshaped during pupation into adult organs (Fristrom, 1970; Nijhout et al., 2014). The ecdysteroid 20-hydroxyecdysone (20E), the hydroxylated metabolite of ecdysone (Ec), triggers pupal metamorphosis and accompanying histolysis of most larval tissues (Riddiford, 1993) by binding a heterodimer of Ec receptor (EcR) and Ultraspiracle (Usp), which are homologs of vertebrate nuclear hormone receptors (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1992). The EcR-Usp heterodimer activates or represses gene transcription depending on bound cofactors (Hu et al., 2003; Tsai et al., 1999). 20E binding stimulates release of EcR-associated repressors such as Smrt (Tsai et al., 1999) and recruitment of coactivators that support expression of Ec-response genes (Bayer et al., 1996; DiBello et al., 1991).

In addition to stimulating larval instars and pupal metamorphosis, EcR regulates growth of larval imaginal tissue via autonomous and non-cell autonomous mechanisms. EcR activity in the larval fat body (FB) systemically suppresses growth by lowering production of insulin-like peptides (dILPs) by neuroendocrine cells (Boulan et al., 2013; Delanoue et al., 2010). Genetic reduction of EcR in the FB thus elevates insulin-like growth factor (IGF) activity in the periphery, leading to increased growth (Delanoue et al., 2010). In contrast, imaginal disc cells lacking EcR or the Ecdysoneless protein (Ecd), which is cell autonomously required for production of low levels of Ec, proliferate at reduced rates relative to normal cells (Brennan et al., 1998; Delanoue et al., 2010; Gaziova et al., 2004; Henrich et al., 1987). Ecdysteroids have been linked to expression of genes involved in cell division and growth (Ghbeish and McKeown, 2002; Ghbeish et al., 2001; Mitchell et al., 2008; Nijhout and Grunert, 2002). However, cell-autonomous mediators of EcR proliferative effects and the degree to which the EcR pathway engages in crosstalk with other proliferative pathways are not well defined. A better understanding of this aspect of EcR biology could illuminate mechanisms that modulate developmental growth and are affected in diseases of altered nuclear receptor signaling.

The transcriptional coactivator protein Taiman (Tai) binds EcR and supports EcR-mediated effects in the ovary and border cells (Bai et al., 2000; Jang et al., 2009; König et al., 2011). Tai is a homolog of the vertebrate family of p160 nuclear receptor coactivators (NCOA1,2,3, also known as steroid receptor coactivator...
Tai Interacts with Yki
Tai pro-growth activity could be based on its ability to interact with proteins that act within established proliferative pathways. Proteomic analyses in cultured cells identified the Hippo pathway component and pro-growth transcriptional coactivator Yki as a candidate Tai-interacting protein (A.V. and K.H.M., unpublished data; Kwon et al., 2013). A search for motifs within Tai that could mediate Yki-binding revealed two PPxY (proline-proline-tyrosine) motifs located within the C-terminal TAD (P1432PAY and P1476PMY) (Figure 2A). Closely paired PPxY motifs in other Hippo pathway components bind WW domains present in Yki (Badouel et al., 2009; Gilbert et al., 2011; Oh et al., 2009; Salah and Aqeilan, 2011). Co-immunoprecipitation of tagged forms of Tai and Yki confirms that each protein readily associates with the other in cultured Drosophila S2 cells (Figure 2B). Yki is not co-precipitated with proteins that act within established proliferative pathways. Two sets of Yki WW domains (Yki1281A, Yki1350A, or Yki1492WW) block interaction with Tai (Figure 2B). Yki is co-precipitated with versions of Tai carrying Tyr-to-Ala mutations in key tyrosine residues within one or both of the Yki WW domains (Yki1281A, Yki1350A, or Yki1492WW) as indicated by the recovery of multiple Tai peptides in mass spectrometry analysis, with the highest possible confidence (Figure S2A). Consistent with these S2 cell data, endogenous Tai co-purifies with Yki-GFP expressed in 0- to 16-hr embryos (daGal4, UAS-Yki-GFP), as indicated by the recovery of multiple Tai peptides in mass spectrometry analysis, with the highest possible confidence (Figure S2A). In aggregate, these data indicate that paired PPxY motifs in the Tai TAD can facilitate interaction with the WW domains in Yki.

The presence of Yki and EcR-interaction motifs in the Tai protein suggests that it might be capable of forming a physical complex with both proteins. To assess whether an EcR-Tai-Yki complex can be assembled in cells, epitope-tagged EcR was expressed together with tagged forms of Tai and Yki in S2 cells. Precipitation via the V5-tag on Yki can recover EcR, but only in the presence of co-expressed Tai (Figure 2D, lane 1 versus

RESULTS
Tai Supports Tissue Growth
In view of the role of Tai as an EcR coactivator, alleles of the taiman (tai) gene were examined for tissue growth. Null tai alleles are lethal (Bai et al., 2000; König et al., 2011), but two hypomorphic alleles (tai15101 and tai15809) in trans to an uncovering genomic deficiency (ED678) yield adults that are small and show an approximate 20% reduction in adult wing size relative to controls (Figures 1A and 1B). Tai protein is detected in third instar larval (L3) wing discs and manipulating Tai levels in these cells with a UAS-tai transgene (Bai et al., 2000) or a tai RNAi line (TRIP HM05182; hereafter referred to as taiIR) (Figures S1A and S1B) produces growth effects restricted to the site of expression (Figures 1C–1F). Expression of the UAS-tai transgene with the engrailed-Gal4 (en-Gal4) posterior (P) compartment driver (en-tai) expands the P domain of L3 wing discs, particularly in the pouch (Figure 1D). Reciprocal depletion of Tai (en-taiIR) shrinks the P domain without significantly altering the size of the Cubitus interruptus (Ci)-positive anterior (A) domain (Figure 1E). Quantitation of relative A and P domain sizes among en-tai and en-taiIR L3 discs confirms the autonomous effect of Tai gain or loss (Figure 1F). Clonal analysis using the Actin>CD2>Gal4 “Flip-out” technique (Pignoni and Zimpursky, 1997) confirms that Tai-expressing clones grow larger than age-matched control clones (Figure 1G). The en-tai and en-taiIR genotypes are each lethal in the pupal phase (data not shown), precluding analysis of adult phenotypes. However, adult animals expressing the taiIR transgene from the pouch driver MS1096-Gal4 have significantly smaller wings than WT counterparts (Figures 1H and 1I). Morphologically, tai-expressing wing tissue is excessively folded (Figures S1C and S1D) with evidence of increased S-phase entry (Figure 1J). en-tai P cells also express elevated levels of the Broad Z3 protein (Figure S1E), which is induced in cultured discs by 20E (Bayer et al., 1996). In sum, these data are consistent with Tai inducing Ec-responsive genes and proliferative genes in L3 wing cells.
lane 4), indicating that Tai is required to detect an Yki-EcR complex. Thus, Tai is capable of bridging an interaction between these transcriptional regulators in cells.

Figure 1. tai Supports Organism Growth

(A) Paired control Df(2L)ED678/+ and tai mutant Df(2L)ED678/tai15101 adult females.

(B) Quantitation of adult female wing size among the indicated genotypes (SEMs are shown; n = 12 for Df(2L)ED678/+ and Df(2L)ED678/tai15101; **p = 2.9e-2, *p = 5.3e-6).

(C–E) L3 wing discs of the indicated genotypes expressing GFP (green) and immunostained with anti-Ci (blue) to mark the anterior (A, blue) and posterior (P, green) domains. Unless otherwise noted, the UAS-taiIR TRiP transgene HM05182 is used in Tai-knockdown experiments.

(F) Relative A (dark gray) versus P (light gray) areas among L3 wing discs of the genotypes shown in (C)–(E) (SEMs are shown; n = 12 for en>+, n = 15 for en-tai, n = 10 for en-taiIR; p = 3.8e-2 and p = 4.4e-6, respectively, for en-tai and en-tai-IR relative to en>+).

(G) Average 2D size of 48-hr Actin-Gal4 “Flp out” clones expressing GFP (light gray) or GFP and tai (dark gray) (SEMs are shown; n = 79 for UAS-GFP, n = 112 for UAS-GFP, tai; *p = 2.5e-2).

(H and I) Overlay and quantitation of wing size among control adult female and Tai-depleted females (taiIR) using the MS1096 driver (SEMs are shown in I; n = 11 for MS1096>+, and n = 13 for MS1096-taiIR; p = 2.2e-12).

(J) Confocal image of an en>tai,GFP L3 wing disc analyzed by BrdU incorporation (red) shows elevated S-phase entry in the P domain (green).

A Form of Tai that Cannot Bind Yki Antagonizes Yki-Stimulated Growth

The physical interaction between Tai and Yki points to potential cooperativity between the two proteins. Tai transgenes were expressed alone or in combination with a transgene encoding a hyperactive form of Yki using a GMR-Gal4 driver (GMR>ykiS168A) active in the larval and pupal eye (Hay et al., 1997). Expression of WT Tai (taiwt) with GMR-Gal4 moderately expands adult eye size and enhances eye overgrowth in GMR>ykiS168A adults (Figures 3A, 3B, S2B, and S2D versus Figures S2C and S2E). Tai depletion with the taiIR transgene leads to low adult survival in the GMR>ykiS168A background (data not shown), but shrinks adult head and eye size in both WT and GMR>ykiS168A backgrounds (Figures 3C, S2H, and S2I). ey-FLP-mediated production of clones homozygous for the null allele tai15101 (Bai et al., 2000) in the GMR>ykiS168A background also reduced adult head and eye size (Figure S2K). Suppression of GMR>ykiS168A by tai alleles is not as complete as that provided by depletion of Yki-interacting transcription factor Scalloped.
Endogenous Tai Supports Yki-Driven Tissue Growth

To assess whether taim contributes to growth of disc cells with inactive Hippo signaling, null alleles of taim and the Yki-inhibitor expanded (ex) were used in conjunction with tissue-specific Flp transgenes to generate disc cells lacking one or both genes (Figures 3M–3S). Consistent with the systemic effect of taim alleles on wing and body size (Figure 1), taim mosaic adult wings generated with the Ultrabithorax (Ubx)-Flp transgene are reduced in size relative to control organs, while ex mosaic wings are enlarged due to Yki hyperactivity (Hamaratoglu et al., 2006). Loss of Tai partially suppresses the ex phenotype such that ex, taim mosaic wings are smaller than ex mosaic wings (Figures 3M and 3N). ex, taim clones in L3 discs also appear smaller than age-matched ex clones (Figures 3O and 3P), and direct analysis of clone:twinspot ratios confirms that taim loss can suppress excess growth of ex mutant larval disc cells (Figures 3Q–3S). Thus, the ability of Tai and Yki to cooperatively induce tissue growth is based on a PPxY:WW module through which these proteins can interact, and amino acid substitutions in Tai that prevent Yki-binding convert Tai into antigrowth factor that acts in a dominant-negative fashion to block Yki-driven tissue growth.

SdJ, implying that Tai enhances rather than mediates Yki transcriptional activity in vivo. Consistent with this hypothesis, a sdIR transgene strongly suppresses the combined oncogenic effect of co-expressing Yki and Tai in the developing eye (Figure S3A).

The location of the PPxY sites in the Tai TAD implies that a physical interaction with Yki may be part of the mechanism by which Tai affects transcription of genes involved in imaginal disc development. GMR-Gal4 directed expression of a UAS-taiPPxA1,2 transgene (Ala substitutions at Tyr1435 and Tyr1479), which is expressed to equivalent levels as the UAS-taiWT transgene in larval wing disc cells (Figure S3B), shrinks the size of adult eyes (Figures S2B and S2F), and suppresses overgrowth of GMR>ykiS168A adult eyes (Figures 3D and S2G). Larval GMR>ykiS168A eye discs show precocious entry into the synthesis (S) phase of the cell division cycle and enlargement of the sheet of epithelial tissue posterior to the MF (see bracketed areas in Figures 3E–3H). Relative to GMR>ykiS168A alone, co-expression of ykiS168A with taim causes large folds of excess tissue and enhanced S-phase entry among cells behind the MF (Figures 3F and 3G). GMR>ykiS168A+taim discs also show greater spacing between adjacent F-actin enriched apical tufts of photoreceptor clusters behind the MF (Figures 3J and 3K). This combined effect of WT Tai and Yki on cell division and photoreceptor spacing is reversed when Yki is co-expressed with taimPPxA1,2. The pattern of S-phase entry in GMR>ykiS168A+taim discs resembles that in GMR-Gal4 control discs, and the distance between apical photoreceptor tufts in GMR>ykiS168A+taimPPxA eye discs is nearer to WT dimensions (Figures 3H and 3L). Thus, the ability of Tai and Yki to cooperatively induce tissue growth is based on a PPxY:WW module through which these proteins can interact, and amino acid substitutions in Tai that prevent Yki-binding convert Tai into antigrowth factor that acts in a dominant-negative fashion to block Yki-driven tissue growth.
Figure 3. Yki Requires Its Interactor Tai to Drive Tissue Hyperplasia

(A–D) Adult female heads expressing yki<sup>S168A</sup>, yki<sup>S168A</sup>+tai<sup>WT</sup>, yki<sup>S168A</sup>+tai<sup>IR</sup>, or yki<sup>S168A</sup>+tai<sup>PPxA1,2</sup>

(E–H) Paired top-bottom panels of third instar eye discs of the indicated genotypes stained with FITC-phalloidin (green) to visualize F-actin and anti-BrdU (red) to visualize S phases. Bracket in each lower panel denotes post-mitotic region posterior to the MF. Note the enhancing effect of co-expressed Tai on Yki<sup>S168A</sup>-driven ectopic S-phase entry behind the MF (F) versus (G) and the opposite suppressive effect of Tai<sup>PPxA1,2</sup> (F) versus (H).

(I–L) Magnified views of FITC-phalloidin staining posterior to the MF in the same genotypes as in (E)–(H) showing opposing effects of Tai and Tai<sup>PPxA1,2</sup> on spacing between the F-actin-enriched apical tufts of adjacent Yki<sup>S168A</sup> photoreceptor clusters.

(M and N) Overlay and quantitation of Ubx>Flp adult wings mosaic for a control chromosome (FRT40A; n = 6; set as 1.0) versus a tai null (tai<sup>61G1</sup>; n = 11) or an ex allele (ex<sup>e1</sup>; n = 7) versus double mutant for ex and tai (ex<sup>e1</sup>, tai<sup>61G1</sup>; n = 6). SDs are shown in (N) (*p = 3.0e-5, **p = 1.0e-5).

(O and P) L3 wing discs containing heat shock-FLP induced clones of ex<sup>e1</sup> (O) or ex<sup>e1</sup>, tai<sup>61G1</sup> (P) homozygous cells (lacking GFP) and control twinspots (strong GFP signal).

(Q–S) Quantitation of individual ex<sup>e1</sup> and ex<sup>e1</sup>, tai<sup>61G1</sup> clone:twinspot size ratios plotted in (R) and (S) relative to a hypothetical linear 1:1 ratio (black line). SEMs are shown in (Q) (n = 18 for both genotypes, *p = 2.98e-11).
Expression of a version of Crumbs containing only the transmembrane and cytoplasmic domains (Crbintra) in the developing wing induces an adult blistering effect related to its role in apico-basal polarity and a separable enlarged-wing phenotype that is suppressed by heterozygosity for yki (Robinson et al., 2010). A tai null allele and two tai hypomorphs (ta161G1, ta15101, and tai01351) also dominantly suppress the enlarged size of Crbintra (en>crbintra) wings (Figures S4F–S4I). Although tai dosage could theoretically also affect Crbintra-induced blistering, the common sensitivity of Crbintra-expressing cells to yki and tai gene dosage is consistent with Tai interacting with Yki and co-regulating a pro-growth transcriptional program in developing wing and eye tissue.

Tai Requires EcR to Activate ex and thread Transcription

In view of the physical and functional interactions between Tai and Yki, tai alleles were tested for effects on expression of canonical Yki-target genes in imaginal disc cells. Depletion of Tai with en>taiIR lowers expression of the expanded (ex-lacZ), thread/Diap1 (th-lacZ and diap4.3-GFP), and e2f1 (e2f1-lacZ) transcriptional reporters in the P compartment of L3 wing discs (Figures 4I–4K and S5A). A second tai RNAi transgene (TRiP HMS00673) with a distinct shRNA targeting sequence also reduces expression of the ex-lacZ and th-lacZ reporters, although more weakly (Figures S5D and S5E). Homozygosity for the tai61G1 null allele reduces expression of th-lacZ and e2f1-lacZ in the L3 eye disc posterior to the morphogenetic furrow (MF) (Figures 4L and S5C). Reciprocally,
ex and th reporters show elevated expression in the P compartment of en->tai wing discs (Figures 4A–4F and S5B). A transgene encoding a version of Tai lacking the N-terminal bHLH domain (Jang et al., 2009) retains the ability to activate ex-lacZ in disc cells (Figures S5F and S5G), indicating an interaction with the BTB-domain protein Abrupt is not required for Tai to induce ex. These Tai-expressing clones project slightly away from the surface of the epithelium (data not shown), and sections across their base thus appear as “rings” of Yki hyperactivity (e.g., compare the two clones highlighted by arrows in Figure S5G). Tai overexpression also induces activity of the pro-growth miRNA bantam (ban) as detected by reduced expression of a GFP-ban sensor (Figures 4G and 4H), which contains ban complementary sites in the 3’UTR of a GFP mRNA (Brennecke et al., 2003).

Chromatin immunoprecipitation (ChIP) studies by the modENCODE Project have identified EcR-association peaks within the D. melanogaster genome in close proximity to the Yki-induced genes ex, thread, ban, and e2f1 (modEncode Consortium et al., 2010). Within the ban promoter, one of these EcR-association peaks overlaps binding sites for Yki:Mad heterodimers (Oh and Irvine, 2011). ChIP analysis of an inducible, tagged form of Tai detects significant enrichment for this segment of the ban promoter (ban-C12) (nomenclature according to Oh and Irvine, 2011) in induced cells versus untreated cells (Cu versus NT; Figures S5J and S5K). Control primer sets corresponding to two randomly selected areas of the ban promoter (ban-upstream1, ban-upstream2) show little to no enrichment, as does a ban promoter region that interacts with Yki but lacks a coincident EcR-association peak (banA4) (mod-Encode Consortium et al., 2010; Oh and Irvine, 2011). Although the degree of Tai association with the ban-C12 region is relatively moderate, it is nonetheless equivalent to the degree of association of the Tai cofactor with the EcR-binding site in the Broad promoter (EcB in Figures S5J and S5K) (Bernardo et al., 2014), suggesting similar levels of Tai occupancy on each EcR-interaction site.

Tai influences gene expression via its cognate transcription factor EcR in germline cells and oocyte border cells (Bai et al., 2000; König et al., 2011). To test the requirement for EcR in Tai-driven induction of Yki-responsive genes, an EcR RNAi transgene directed at all three EcR isoforms (EcRβ) was used to deplete EcR from wing disc cells. EcR depletion alone had minimal effect on the ex-lacZ and th-lacZ reporters in L3 wing discs, but eliminated their induction by co-expressed Tai (Figures 4M–4T). RNAi depletion of the EcR-associated transcriptional repressor Smrter (Smr) (Tsai et al., 1999) strongly induces expression of ex-lacZ, th-lacZ, and suppresses expression of the GFP-ban sensor in the larval wing disc (Figures 4U–4W), but has no obvious effect on the unrelated reporter transgene SerratelacZ (Figures S5H and S5I). The opposing effects of tai and smr alleles suggest that EcR contributes to both repression and activation of Yki-regulated genes in L3 wing cells. Consistent with this hypothesis, the EcR.A–F645A dominant-negative allele, which is deficient in EcR-mediated gene activation but not repression (Cherbas et al., 2003), moderately lowers th-lacZ expression among P-domain cells (Figure 4X). These effects of EcR and smr alleles on Yki-activity reporters are consistent with a role for Tai in modulating Hippo-regulated gene expression in L3 disc cells.

Tai Is Dispensable for Yki Induction of Classic Hippo-Regulated Genes

One explanation for the role of Tai in Yki-driven organ overgrowth is that the Yki:Tai interaction is required for Yki to efficiently stimulate transcription of its established target genes. To test this hypothesis, the effects of tai depletion were assayed on transcriptional reporters for the classic Hippo target genes ex and ban. Although Tai can affect ex expression in otherwise WT disc cells (see Figure 4J), RNAi depletion of Tai does not prevent transgenic Yki from activating the ex-lacZ transcriptional reporter in most of the L3 wing disc, despite suppressing P-domain overgrowth (Figures 5A–5C). Cells along the dorsoventral boundary of the pouch appear to be an exception to this rule, as ex-lacZ expression in en>yki,taiIR discs is mildly reduced in this region (arrows, Figure 5C). In a reciprocal test, depletion of Yki effectively blocks ex-lacZ induction by Tai throughout a majority of the L3 wing disc (Figures 5D–5G), indicating that Yki inputs on ex expression are dominant over those from Tai. To test the epistatic relationship between Yki and Tai in control of a ban transcriptional reporter, expression of the ban2.5-lacZ reporter was analyzed in clones of ex, tai, or compound mutant ex,tai cells in L3 wing discs. Similar to the ex-lacZ reporter, Yki-dependent activation of the ban2.5-lacZ reporter in ex mutant cells is not appreciably suppressed by concurrent loss of tai (Figures 5H–5J).

To further test whether suppression of Yki phenotypes by the taiR and taiPPA1,2 transgenes can be uncoupled from expression of canonical Yki targets, qPCR analysis of the ex and cyclin E (cycE) mRNAs was carried out from RNAs harvested from L3 wing discs expressing either Yki alone, Yki with Tai co-depleted, or Yki with the TaiPPA1,2 dominant-negative allele (Figures 6A and 6B). Removal of Tai did not significantly suppress Yki induction of either of these classic Hippo target mRNAs. Co-expression of TaiPPA1,2 also had no effect on ex and cycE mRNA induction despite suppressing Yki-driven P-compartment overgrowth. These data indicate that Yki induces the ex and cycE mRNAs independent of its interaction with Tai.

nanos and piwi Are Induced by Hyperactive Yki and Tai in Wing Discs

The uncoupling of a role for Tai in Yki-driven growth from its role in induction of classic Hippo target genes implies that Tai supports an as yet undefined portion of a transcriptional program induced by hyperactive Yki in disc cells. To identify Yki-induced/Tai-dependent genes, RNAs harvested from L3 wing discs expressing either Yki (en>yki), or Yki in combination with Tai-depletion (en>yki+taiR) were analyzed by high-throughput sequencing. This analysis generated mapped reads corresponding to more than 12 × 10⁶ transcripts (Figure 6C; Table S1). A subset of these wing disc mRNAs was designated as “candidate Yki-induced mRNAs” based on a greater than 2-fold increase (log2D0.8) upon co-depletion of Tai (Table S3). This group includes factors with diverse predicted functions, including a set of RNA regulatory factors expressed in the germline (e.g., nanos, piwi, blanks, CG17018, Argaonate-3, sister of Yb, qin, and krimper) (Chintapalli et al., 2007), but lacks...
ex and kibra, consistent with a lack of a requirement for Tai induction of classic Hippo-responsive genes.

Direct analysis of select candidate Yki-induced mRNAs by qPCR confirmed that depletion of Tai in Yki-overexpressing L3 wing disc cells blocks induction of the RNA regulatory factors nanos and piwi, the Ec-induced gene Eig71Ee, and the secreted insulin-like peptide dILP-8 (Figure 6D). A second, weaker taiR transgene (HMS00673) also partially suppressed Yki-induction of nanos, dILP-8, and Eig71Ee but did not alter induction of piwi, perhaps indicative of a lower threshold of Tai required for Yki to activate piwi transcription (Figure S6A). Importantly, co-expression of Tai enhances Yki-driven induction of the nanos and piwi mRNAs, while TaiPPxA1,2 blocks induction of nanos and dILP-8 and shows reduced ability to enhance piwi expression. Tai thus requires its PPxY motifs, which can bind to Yki, to support Yki-driven induction of these mRNAs. RNAi depletion also supports a selective role for EcR isoforms in the Yki-dependent segment of the Yki-induced transcriptome in L3 wing disc cells (Figure S6B). Transgenes targeting all three EcR isoforms (EcRΔ10), the EcR.A isoform (EcR.AΔ10), or the EcR.B1 isoform (EcR.B1Δ10) do not affect Yki-induction of the ex or cycE mRNAs, but can individually suppress induction of nanos and piwi. Induction of dILP-8 was effectively inhibited only by pan depletion of EcR, whereas individual depletion of EcR.A or EcR.B1 induced induction of Eig71Ee, suggesting that the remaining EcR isoform, EcR.B2, may contribute to activation of dILP-8 and repression of Eig71Ee. In sum, the lack of an effect of taiPPxA1,2 or taiR on Yki-induction of the classic Hippo targets ex and cycE (Figure S6B) versus Figure 6D) contrasts with the requirements for Tai, and apparently EcR as well, in Yki-mediated control of the nanos, piwi, dILP-8, and Eig71Ee mRNAs in L3 wing disc cells.

The nanos, piwi, and dILP-8 mRNAs all share a pattern of highly enriched expression in the germline (Chintapalli et al., 2007). The nanos and piwi gene products act cell autonomously to support self-renewing divisions of GSCs (Cox et al., 1998; Forbes and Lehmann, 1998; Kobayashi et al., 1996; Lin and Spradling, 1997), and in the case of piwi, ectopic expression is sufficient to increase somatic cell division (Cox et al., 2000). nanos and piwi expression were analyzed in L3 wing disc cells using an anti-Nanos antibody (Asaoka-Taguchi et al., 1999) and a lacZ enhancer trap inserted into the piwi locus (piwiIR or piwi-lacZ) (Lin and Spradling, 1997). Nanos protein is expressed at very low levels in lysates of control discs but induced in Yki-expressing discs in a Yki-dependent manner similar to its mRNA (Figure 7A). Expression of piwi-lacZ is also induced in P-domain wing disc cells that express the yki transgene (Figures 7B–7E). This induction of piwi-lacZ by transgenic Yki is blocked in cells also depleted of Tai (Figure S6C), and this correlates with a reduced degree of tissue expansion and folding in the areas of the dorsal and ventral wing hinge. Significantly, activating endogenous Yki by RNAi depletion of the Wts kinase (wtsKK101055) leads to mild disc growth, consistent with a partial loss of Wts, and also elevates piwi-lacZ expression (Figure 7F). Induction of piwi-lacZ by Yki expression or Wts loss is most robust in the dorsal and ventral regions of the wing hinge, suggesting that cells in these areas activate a GSC-like program most strongly. Individual depletion of Nanos or Piwi, with either a nanosIR line (IR-1) that efficiently reduces Nanos protein levels in en>yki discs (Figure S7A) or two piwiIR lines (IR-1 and IR-2), suppresses the

Figure 5. Tai is Not Required for Induction of ex or ban by Yki Hyperactivity

(A–F) L3 wing discs stained with anti-β-gal to detect expression of the ex-lacZ enhancer trap in the indicated genetic backgrounds. Arrows in (C) denote reduced ex-lacZ expression along the dorsal-ventral boundary.

(G) Corresponding quantitative analysis of the posterior compartment ratio (P area/total area) among L3 wing discs in (A–F) (SDs are shown; n = 10 for en> + and en>taii, n = 4 for en>ykiRI, n = 11 for en>taiiykiRI, n = 9 for en>yki, n = 12 for en>taiiRI, n = 8 for en>yki,ykiRI, nonsig = not significant).

(H–J) Anti-β-gal staining (red) to detect ban2.5-lacZ expression in taIC1 mutant (H), exm1 mutant (I), or taiRIP2,exm1 double-mutant (J) clones in the L3 wing pouch marked by the absence of GFP (green).
growth of en>yki L3 wing discs and GMR-ykiS168A transgenic adult eyes with little effect on corresponding WT organs (Figures 7G–7I; see also Figure S6C). Moreover, combining a nanosl7 hypomorphic allele and a deficiency covering the nanos locus (Df(3L)Exel6183) also partially suppresses en>yki L3 wing disc overgrowth (Figure S7B). These requirements for Nanos and Piwi for Yki-driven growth supports a model in which these GSC factors are ectopically induced by a hyperactive Yki-Tai complex as one element of a transcriptional program that is normally repressed by the Hippo pathway in developing somatic epithelia.

**DISCUSSION**

Studies of the Drosophila Hippo pathway have uncovered an array of cytoplasmic regulators and nuclear factors that modulate expression of a fairly small set of transcriptional targets. Yet the association of the Hippo nuclear effector Yki with a large number of sites in the fly genome (Oh et al., 2014) implies links to a wider array of targets and cellular processes. Here we show that the EcR-coactivator protein Tai plays a dual role as a regulator of Yki-induced genes during normal development and a key mediator of the effect of hyperactive Yki on cells with disrupted Hippo signaling (model, Figure 7J). Tai supports normal developmental growth by controlling expression of classic Yki targets ex, thread, bantam, and e2f1, but its role on these genes is largely subordinate to inputs from Yki. EcR is required by Tai to induce ex and thread and the EcR-bound repressor Smrter is also required to repress ex, thread, and ban, suggesting that Tai and other EcR components provide parallel, but subordinate, inputs to Yki on these developmentally regulated genes. Tai can interact with Yki via PPxY motifs present in the Tai TAD, and this interaction links hyperactive Yki to a previously undefined segment of the Yki-induced transcriptome that includes the GSC factors nanos and piwi. The ability of the TaiPPxA1,2 protein to mildly retard the growth of otherwise WT tissues (e.g., Figure S2B versus Figure S2F) suggests that a Tai-Yki complex plays a small role in normal developmental growth, perhaps during developmental stages other than those examined here. Critically, under conditions of Yki hyperactivity, the Yki-Tai interaction becomes central to induction of an additional set of transcriptional targets in L3 discs, including nanos and piwi, which support tissue overgrowth. These findings reveal that Tai plays a key role in determining the transcriptional output of hyperactive Yki and significantly expand knowledge of Yki-induced genes by highlighting a division within the Yki-induced transcriptome into Tai-dependent and -independent target genes.

**Figure 6. Identification of Yki-Induced, Tai-Dependent RNAs in Larval Wing Discs**

(A) Late L3 enGal4, UAS-GFP wing discs from control (wt) animals or those expressing the indicated transgenes stained with phalloidin-AlexaFluor-594 (gray) to visualize F-actin (images to scale).

(B) Quantitative real-time PCR (qPCR) analysis of expanded and cyclin E mRNA levels in late L3 wing discs carrying the indicated transgenes in combination with enGal4.

(C) Color-coded heat map illustrating changes in abundance of individual RNAs between control versus Yki-expressing (Yki:Ctrl), and Yki-expressing versus Yki-expressing/Tai-depleted (Yki:Yki+TaiIR) late L3 wing discs (see Tables S1, S2, and S3). The Yki:Ctrl heatmap contains 9,303 RNAs, of which 555 are induced >0.8(log2)-fold. Of these, 160 RNAs (bracketed) are suppressed by >0.8(log2) upon Tai depletion.

(D) qPCR analysis of AGO3, Eig71Ee, nanos, piwi, and dILP-8 mRNAs in wing discs of the indicated transgenes in combination with enGal4. For all qPCR data, SEMs are shown (n = 3; *p values see Statistics in Supplemental Experimental Procedures; ns = not significant).
Induction of the normally germline-specific nanos and piwi RNAs in imaginal disc cells by hyperactive Yki implies that inactivation of the Hippo pathway engages a transcriptional program that diverges from normal development. In addition to nanos and piwi, a significant fraction of the candidate Yki-induced/Tai-dependent genes (Table S3) display germline-enriched patterns of expression (Chintapalli et al., 2007). The dILP-8 mRNA falls into this category as well; it is normally expressed mainly in the adult ovary, but is ectopically induced by Yki in a Tai-dependent manner in wing discs (this study) and by mutations that produce neoplastic wing disc tumors (Colombani et al., 2012; Garelli et al., 2012), suggestive of common mechanisms linking expression of germline genes to abnormal disc growth. However, unlike the secreted factor dILP-8, Nanos and Piwi proteins act autonomously in cells that express them. Nanos complexes with other RNA binding proteins such as Pumilio to block translation of germline mRNAs encoding differentiation factors (Asaoka-Taguchi et al., 1999; Forbes and Lehmann, 1998) and supports survival of germ cells by repressing translation of the pro-apoptotic factor Hid (Sato et al., 2007). Intriguingly, the miR bantam is required to maintain female GSCs (Yang et al., 2009) and directly represses the hid mRNA (Brennecke et al., 2003), suggesting that Nanos and bantam may co-regulate the hid mRNA downstream of hyperactive Yki. Piwi interacts with piRNAs in the germ cell cytoplasm to repress target mRNAs but also has a critical nuclear role in formation of repressive chromatin on specific genomic loci (Klenov et al., 2014; Le Thomas et al., 2013). Both Nanos and Piwi play key roles in blocking differentiation and supporting self-renewing divisions in the germline (Cox et al., 1998, 2000; Forbes and Lehmann, 1998; Kobayashi et al., 1996; Lin and Spradling, 1997), suggesting that disc cells with hyperactive Yki autonomously adopt elements of a germline transcriptional program. As depletion of either factor in disc cells blunts Yki-driven growth but has no effect on control tissues, Nanos and Piwi behave as required elements of a larger transcriptional program that is engaged by hyperactive Yki in disc cells. The physiologic correlate to this GSC-like growth program...
is unclear, although a similar program is engaged in larval brain cells lacking the insulator accessory protein l(3)Mbt (Janic et al., 2010) and may be associated with Yki-mediated regeneration of damaged epithelia (Gru{sschke et al., 2011; Sun and Irvine, 2011). Piwi-related proteins are expressed in somatic stem cells of the planarian flatworm S. mediterranea and are required for these cells to drive regenerative growth (Reddien et al., 2005), further supporting a link between Yki and Piwi in regenerative tissue growth. The ectopic expression of piwi and nanos homologs in human cancer cells and their roles in supporting cancer cell proliferation (reviewed in Bonnomet et al., 2008; Ross et al., 2014; Strumane et al., 2006) imply that GSC factors can also support aberrant tissue growth in vertebrates, perhaps by promoting self-renewing divisions of cancer stem cells as proposed for the vertebrate Yki homolog Yap1 (reviewed in Mo et al., 2014).

In addition to effects on disc growth, co-overexpression of Yki and Tai appears to enhance levels of F-actin in L3 wing discs, as detected by phalloidin staining (e.g., Figure 6A), suggesting that Yki and Tai modulate expression of factors involved in actin cytoskeleton dynamics. Given that F-actin can modulate Yki activity (Fern{ández et al., 2011; Sansores-Garcia et al., 2011), this phenomenon could further augment the effect of Yki and Tai on the Hippo transcrismote. Alternatively, effects of Yki and Tai on the actin cytoskeleton could occur via a shared downstream target that stimulates F-actin polymerization during motility or cell-shape changes associated with tissue remodeling, as occurs during pupal morphogenesis.

The physical interaction between the Yki and Tai proteins, and the genetic requirements for EcR and Smrter in control of Hippo target genes, suggests that the Ec and Hippo pathways could contribute to the 20E pulse at the L3-pupal transition (Graveley et al., 2013; Mohan et al., 2011; Sedkov et al., 2003). In addition, the EcR-IR and DNP-IR in disc nuclei (Figure 7B) suggests that the EcR transcriptional complex (Carbonell et al., 2013; Mohan et al., 2011; Sedkov et al., 2003). In addition, the EcR-IR and DNP-IR in disc nuclei (Figure 7B) suggests that the EcR transcriptional complex (Carbonell et al., 2013; Mohan et al., 2011; Sedkov et al., 2003).

EXPERIMENTAL PROCEDURES

Genetics

All crosses were maintained at 25°C unless otherwise noted. For RNA analyses, 24-hr embryos and L1 larvae were shifted to 28°C, and discs were collected from late L3 wandering-stage larvae. Alleles used in these studies (Bloomington stock number indicated) are as follows: D(2L)ED678 (#8906), taiP610 (12172), tai11677 (10453), nanos1 (#28300), Df(3R)Exel183 (#7662), UAS-tai (#6379), UAS-tai-JB (#28273), UAS-tai-IR-1/2 (#28371, #32868, FRT40A,tai11677-FRT40A (#6379), exF1-FRT40A (#44249), tai11677-exF1-FRT40A, UAS-ykiP610a:V5 (#28818), UAS-EcR-A-F645A (#9542), UAS-EcR-IR (pan-EcR, #29374), UAS-EcR-A-IR (#9452), UAS-EcR-B1-IR (#9329), UAS-smrter-IR (7068), exF1-B (nuc-lacZ; #12093), piwi1 (#43637), UAS-nanos-IR (#28300), UAS-piwi-IR-1/2 (#34866, #33724), exF1 (ex-lacZ, #44428), exF1MT29 (ex-lacZ, #44428), da-Gal4 (#55851), and M51965-Gal4 (#8860) obtained from the Bloomington Drosophila Stock Center. UAS-yki-IR (104523) and UAS-warts-IR (v106174) were obtained from the Vienna Drosophila Resource Center (Vigro). Other alleles used were enGal4/CyO, ey-FLP;ubi-GFP-FRT40A, Ubx-Fpl;ubi-GFP-FRT40A, UAS-yki-GFP, Serrate-lacZ (R. Read), ban25-lacZ, (K. Irvine), UAS-sd-IR and DIAP43-3-IR (J. Jiang), ban-sensor GFP (G. Haider), GM4-Lar4, UAS-ykiP610a:GFP (K. Harvey), UAS-yki (D. Pan), and hsFLP:Act>CD2>Gal4 (J. Treisman). The UAS-taiP610a:2 transgene was generated by standard approaches; transgenic services were provided by BestGene.

Immunofluorescence Microscopy

Immunostaining and confocal microscopy performed using standard procedures. Primary antibodies include mouse anti-Gal80 (Robinson et al., 2010) with mouse anti-BrdU (1:100; Becton Dickinson). Secondary antibodies are goat anti-mouse-Cy3 and goat anti-mouse-Cy5 (1:100; Jackson Labs). Phalloidin-AlexaFluor-594 and 488 (1:100; Life Technologies) were used to detect F-actin.

Immunoprecipitation and ChiP in Cultured Cells

Drosophila S2 cell culture, transfection, and immunoprecipitation analysis were performed as described in Gilbert et al. (2011), Hip expression constructs were generated from a ta ORF plasmid (gift of D. Montelli) into the streptavidin binding peptide (SBP), CuSO4-inducible pMK33 vector (Kiyakakis et al., 2008), tai-pMK33 variants generated via site-directed mutagenesis. WT and WW mutant versions of HA-tagged Yki are described previously in Gilbert et al. (2011). For ChiP, an S2 clone stably transfected with pMK33-SBP-tai was induced with 0.5 mM CuSO4, cross-linked in 1% formaldehyde, and quenched in 125 mM glycine. Lysates prepared in ice-cold nuclei lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCl, 0.5% NP-40) plus protease inhibitors (Roche), followed by equal volume of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.0]) plus protease inhibitors (Roche), and then sonicated prior to centrifugation. Streptavidin-agarose beads (Thermo Scientific) were mixed with lysates and then sequentially washed (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl, then 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 500 mM NaCl, then 0.25 M LiCl, 1% NP 40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]; 2× with TE) and eluted (50 mM Tris-HCl [pH 7.4], 250 mM NaCl 0.5% NP-40, 0.1% sodium deoxycholate, 4 mM biotin), extracted, and suspended in TE buffer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.05.010.

AUTHOR CONTRIBUTIONS

C.Z. and B.S.R. conceived and carried out experiments, analyzed data, and contributed to writing the paper. H.Z., L.Z., and W.X. designed and carried...
out protein:protein interaction assays. P.K.B. contributed data for revision. P.J. and B.Y. carried out and analyzed RNA-seq experiments. A.V. and K.H.M. conceived experiments, analyzed data, and wrote the paper.

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