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The Hippo pathway transcription factor Yorkie: novel regulators and invasive potential in *Drosophila*.

By

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Doctor of Philosophy

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The Hippo pathway transcription factor Yorkie: novel regulators and invasive potential in *Drosophila*.

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B.S., University of California, San Diego, 2006

Advisor: Kenneth H. Moberg, Ph.D.

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Abstract

The Hippo pathway transcription factor Yorkie: novel regulators and invasive potential in *Drosophila*.

By Daniel Barron

The manner in which organisms regulate the growth of their organs is a fundamental question in biology that has broad implications for our understanding of development and cancer. *Drosophila melanogaster* is a powerful model system with which to study the signal transduction pathways governing growth. The Hippo signaling pathway has been demonstrated to play a central role in the regulation of tissue and organ size during development. The pathway is strongly conserved in humans, rendering *Drosophila* a suitable and efficient model system to better understand the molecular nature of this pathway. The LC8 family of small ~8kD proteins are highly conserved and interact with multiple protein partners in eukaryotic cells. LC8-binding modulates target protein activity, often through induced dimerization via LC8:LC8 homodimers. I find that the LC8 family member Cut up (Ctp) is primarily required to promote epithelial growth, which correlates with effects on the pro-growth factor dMyc and two genes, *diap1* and *bantam*, that are classic targets of the Hippo pathway coactivator Yorkie. Genetic tests confirm that Ctp supports Yorkie-driven tissue overgrowth and indicate that Ctp acts through Yorkie to control *bantam* (*ban*) and *diap1* transcription. Quite unexpectedly, Ctp loss elevates *ban* expression but reduces *diap1* expression. Collectively these findings reveal that that Ctp is a required regulator of Yorkie-target genes in vivo and suggest that Ctp may interact with a Hippo pathway protein(s) to exert inverse transcriptional effects on Yorkie-target genes. In addition to their role in promoting hyperplastic growth, Yorkie orthologs in humans have been associated with cancer invasion and metastasis. I present preliminary data indicating that Yorkie-overexpressing clones in the *Drosophila* wing disc extend actin-rich protrusions into neighboring tissue that are consistent with invadopodia. I also show that Yorkie overexpressing tissue have elevated levels of ECM degrading matrix metalloproteases. Together, these data suggest a new *in vivo* model with which to study invadopodia and indicate that Yorkie may be capable of driving a pro-invasive gene expression program.

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Chapter 1: I**ntroduction**

This chapter is includes sections adapted from the following published paper:

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Growth control as a biological question

The mechanisms that control the ultimate size and pattern of organisms remain a fundamental question in biology. How do we grow larger than mice and smaller than elephants? How is it that our arms grow to the same length? How do developing organs know when to *stop* growing? These types of questions have been asked by biologists for a very long time (reviewed in [1]), and while size differences may appear simple at first, the complexities involved are clear once after some fundamental observations. All animals begin life as a single-celled embryo, yet develop into an astonishingly diverse range of sizes and shapes as adults. Each species grows to a stereotypical size with proportionally sized organs. The rate at which most species grow is predicable despite the substantial variation in individual sizes. Most animals first develop their parts in miniature, and then enlarge with cellular growth and division. The sizes of cells that form the various organs of developed animals are also typically tightly controlled and consistent. Thus, organisms require mechanisms to control size not only at the gross level of entire body and organ systems, but also the finer level of individual cells.

The past several decades have provides a number of studies on growth control that have elucidated important components of this greater puzzle. Developmental growth of organisms (and organs) is controlled by both intrinsic (genetic) and extrinsic (nutrients, systemic signals) factors. Most organs rely on autonomous signals to reach their characteristic size, but the relative importance of intrinsic and extrinsic signals can vary widely for different organs. For example, multiple fetal thymus glands transplanted into a developing mouse will each independently grow to their expected adult size, suggesting that their growth is mainly dependent on intrinsic factors [2]. However, if the same

experiment is performed with fetal spleens then the total mass of transplanted spleens will reach the mass of a single adult-sized spleen, indicating that extrinsic factors are primarily responsible for its size regulation [3]. It is now appreciated that the growth and patterning of developing organs and tissues are controlled by a discrete number of signaling molecules that correspond to different signal transduction pathways, and these pathways act in a coordinated manner to determine the particular size, shape, and pattern of a structure.

Differences in the size of animals can reflect differences in cell size and cell number. Underlying these differences are three central processes that contribute to growth: biosynthetic mass accumulation, cell division (progression through the cell cycle), and cell death. These processes are controlled by molecular mechanisms that output distinct terminal signals such that *cell growth, cell division,* and *apoptosis* are capable of being regulated independently from one another. Nonetheless, the upstream growth control signaling pathways that influence these endpoint processes are capable of regulating more than one—in some cases, all three. Furthermore, these same pathways that are required for normal developmental growth are often deregulated in cancer, leading to aberrant growth.

In this introduction to my dissertation I aim to familiarize the reader with *Drosophila* as a model system for studying growth control. First I will overview the features and advantages of *Drosophila* that empower a robust model to study organ size. Next, I will discuss the processes of cell growth, division, and death that contribute to the greater regulation of organ size, highlighting the role of specific genes and signal transduction pathways involved. Finally, I will introduce the Salvador-Warts-Hippo pathway (AKA Hippo pathway), a tumor suppressor network that was initially described

in flies and functions across metazoans to regulate organ size. As you will see in later chapters, Hippo signaling and its effect on gene transcription is at the center of my thesis work. Furthermore, this pathway has emerged as an important player in multiple types of tumorigenesis and, as such, I discuss the clinical relevance of Hippo signaling.

Drosophila melanogaster as a model organism to study the regulation of organ size

Features of *Drosophila*

The use of *Drosophila melanogaster* as a model organism has a rich history beginning in the early 20th century that has contributed insights into a massive variety of foundational biological processes, including Nobel prize-winning discoveries such as sex-linked inheritance [4, 5]. Early *Drosophila* researchers valued the fruit fly for its convenient cost and fast breeding. In the time since, the fly has continued to expand its relevance due to an ever-increasing array of genetic tools, resources, and features, including the following:

- Short life-cycle, the 10 day life cycle (from embryo to adult) allows for relatively quick genetic crosses.
- Robust fecundity, allowing for high-throughput genetic analysis and screening.
- Low cost, relatively cheap to maintain.
- Visually apparent phenotypes, *Drosophila* tends to have easily scored phenotypes that can be seen under a stereo microscope, allowing for subtle variations to be characterized.
- Conservation of human genes, roughly half of fly proteins show similarity to mammalian proteins, but estimates suggest that 77% of human disease-causing

genes have orthologs in *Drosophila*. An earlier survey of cancer-related genes found 68% had a fly equivalent [6, 7].

- Sequenced, annotated genome, resources such as www.flybase.org include the fully sequenced *Drosophila* genome with mapped genetic loci including phenotypic analysis, comparative genomics to identify potential orthologs, consolidation of databases that map genetic and biochemical interactions, and lists of studies associated with a particular gene [8].
- Genetic tool-kit, an ever-increasing number of techniques allows for relatively easy manipulation of genetic expression with temporal and spatial specificity. Some of these techniques will be discussed in more detail below.

Imaginal discs

Developing *Drosophila* animals contain discrete sheets of epithelial cells, called imaginal discs, which form the developmental precursors of adult structures. There are 10 sets of imaginal discs (eye-antennae, wing, leg, humeral, haltere, labial, clypeolabral, and genital) that begin as patches of cells in the embryo and then develop into sheets of columnar epithelia in the larva (Figure 1.1). Newly hatched first instar larva have wing and eye discs that contain 20-70 cells. The discs grow expansively during development, especially during the third instar where cells double about every 10 hours, such that just before pupariation they contain from 10,000 to 50,000 cells each (reviewed in [9]). Discs have an outer layer of squamous epithelium call the peripodial membrane that make little contribution to the adult structures.

Imaginal discs have proven to be a useful platform for studying many fundamental aspects of biology, including growth and size regulation. The popularity of studies using

imaginal discs can be explained by their easy accessibility (they can be dissected and mounted for microscopy), combined with the ability to temporally and spatially manipulate the expression of genes within certain populations of disc cells (see below). For studies on growth control, the rapid and predictable growth of imaginal discs during larval development can amplify phenotypes associated with growth control genes making them more amenable to study. Furthermore, the developmental lethality of genes can be avoided using these methods, since most of the developing fly is spared the genetic manipulation of this subpopulation of non-essential cells.

Genetic manipulation toolkit

The ability to manipulate genetic expression with an array of ingenious tools and techniques is one of the most appealing features of *Drosophila* as a model system. This tool-kit is constantly expanding, including the recent addition of CRISPR/Cas9 genome editing systems [10]. However, for this dissertation I will focus on introducing two systems, originating in the yeast *Saccharomyces cerevisiae*, the GAL4-UAS system and the FLP-FRT system (reviewed in [11]).

GAL4-UAS

The GAL4-UAS system employs two different transgenic constructs in the same fly to generate spatiotemporal expression of a gene of interest. Both of these components—the GAL4 activator and the upstream activating sequence (UAS) target--are derived from yeast. To start, a transposable *P* element carrying a GAL4 transcriptional activator is randomly mobilized throughout the fly genome, including downstream of endogenous promoter elements. Using this method, a library of endogenous tissue-specific enhancers

that control the expression of GAL4 is created. Over 1000 characterized GAL4 activators, commonly referred to as 'drivers', are publicly available offering a plethora of spatial, temporal, and intensity options for GAL4 expression. The second component of the system consists of a 'target' transgene that is cloned downstream of a UAS enhancer element. When a fly's genome combines these two constructs, the GAL4 will bind to the UAS and promote the expression of the target gene in the expression pattern of the GAL4 driver (Figure 1.2).

The strengths of using the GAL4-UAS system are enormous due to its versatility and convenience. UAS transgenes can be designed with many useful tactics in mind including overexpressing proteins (by placing protein coding sequences downstream of the UAS) or knocking down proteins by expressing a targeted RNAi cassette. Transgenes can be designed to include mutant proteins, useful tags such as fluorescent markers, or human orthologs can be expressed to test for functional conservation. In addition to the vast number of publicly available GAL4 drivers, libraries containing UAS transgenes are also offered by fly community stock centers. This includes efforts by groups at Harvard and Vienna to create a comprehensive collection of UAS transgenes with short-hairpin RNAs (inverted-repeats) targeting the entire fly genome [12, 13]. Similarly, randomly inserted *P* elements containing UAS sequences (EP elements) have been mapped, covering a large percentage of the fly genome, making overexpression of many fly genes possible. Together, these techniques and resources allow researchers to overexpress or remove nearly every *Drosophila* gene, often by crossing just two fly stocks.

FLP-FRT

Another powerful technique for tissue and time specific genetic manipulation in *Drosophila* is the 'FLP-FRT' system and its variations. The core elements of this system are the Flippase (FLP) recombinase enzyme and two Flippase Recognition Target (FRT) sites encoded into DNA, either *cis* (same strand) or *trans* (same location on two different homologous chromosomes) to one another. When two FRT sites are placed in a *trans* configuration, the FLP enzyme with induce mitotic recombination of the homologous chromosomes, exchanging all the genetic material distal to the FRT sites [14]. When the FLP protein is expressed with a tissue specific promoter, this technique allows for the generation of patches of homozygous mutant cells (AKA clones) within an otherwise heterozygous organ—bypassing any embryonic lethality of a homozygous mutant fly (Figure 1.3). This system has the advantage of using genomic nulls for a gene (as opposed to less reliable transgenic knockdowns) and the opportunity to examine mutant cells in patches directly neighboring normal cells (clonal analysis).

In a variation on the FLP-FRT technique, called 'Actin-FLP-out', the FRT sites are placed *cis* to one another with a DNA insulator element in between. This FRT-insulator cassette is coded between the Actin promoter element and a downstream GAL4 coding sequence, preventing Actin-GAL4 expression. When FLP is introduced, it will prompt stochastic recombination between the FRT sites, removing the insulator element and exposing the Actin driven GAL4 transgene. When combined with a UAS transgene of choice, this method can generate clones that overexpress a protein or RNAi 'knockdown' construct. Thus, clonal analysis can be done in models requiring gene overexpression or with genes lacking genomic null alleles.

Importantly, the FLP coding sequence can be placed downstream of a variety of tissue specific promoters, and many of these constructs are publically available. For example *eyeless*-FLP permits the generation of clones in early eye imaginal discs. Furthermore, *heatshock*-FLP stocks put FLP under the control of a *HSP70* promoter such that recombinase expression can be induced at specific stages of development for specific lengths of time (allowing for more or less chance of recombination events, and thus more or fewer clones). Additionally, FRT sites inserted proximal to the centromere of each arm of three major fly chromosomes make it possible to mitotically recombine the majority of genes. Together, these tools enable the design of a wide-assortment of powerful experiments in which control tissue can be directly compared to neighboring mutant clones.

Genetic Analysis in *Drosophila*

The success of *Drosophila* as a model organism is largely due to the power of genetic interaction studies, often designed as forward genetic screens that take advantage of the fly's high-throughput and easily visualized phenotypes. Genetic interaction studies begin with a robust, reproducible phenotype produced by loss-of-function or gain-of-function for a gene of interest. In that background, a collection of candidate modifier alleles are introduced to test if the original phenotype is altered—either suppressed or enhanced. Modification of the phenotype suggests that the two genes functionally interact; when combined with other data--such as secondary genetic screens, functional reporter data, or evidence of a physical interaction—'enhancer/suppressor' screens can be an effective tool for understanding gene function. Candidate modifiers can come from a number of sources—historically genotoxic agents (such as ethyl methanesulfonate or X-rays) have

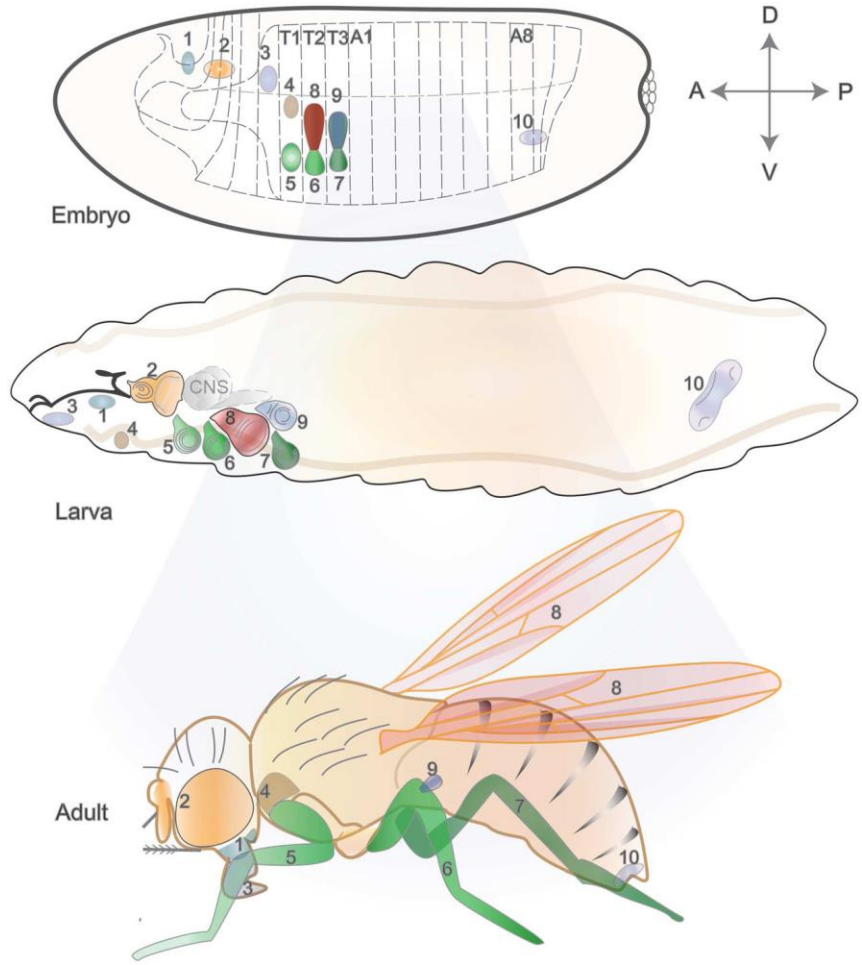

Figure 1.1. Imaginal discs are the developmental precursors to their corresponding adult tissues. Imaginal discs start as patches of cells in the embryo (top), form sheets of epithelia in the larva (middle), and eventually form adult structures (bottom). The numbers correspond to the different discs: (1) clypeolabral, (2) eye-antennal, (3) labial; (4) humeral (or prothoracic) (5) first leg, (6) second leg, (7) third leg, (8) wing, (9) haltere, (10) genital. The arrow axis represent dorsal, ventral, anterior, and posterior. T1-T3 and A1-A8 label the thoracic and abdominal segments, respectively. Adapted from [9].

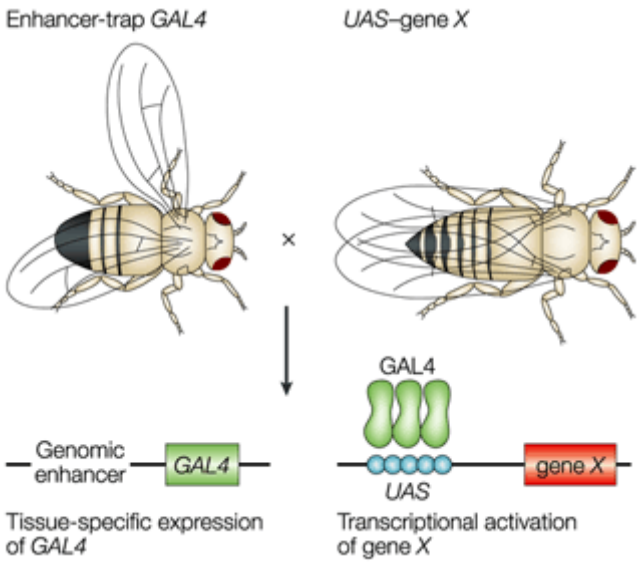

Figure 1.2. The GAL4-UAS system enables tissue specific expression of transgenes. A schematic representation of the GAL4-UAS system, adapted from yeast. Two flies are crossed, the first (left) carries a GAL4 activator downstream of a tissue-specific promoter element. The second (right), carries a UAS-sequence upstream of a transgene of interest. When combined, the GAL4 will bind to the UAS and promote the expression of the transgene in the tissue-specific pattern. Adapted from [15].

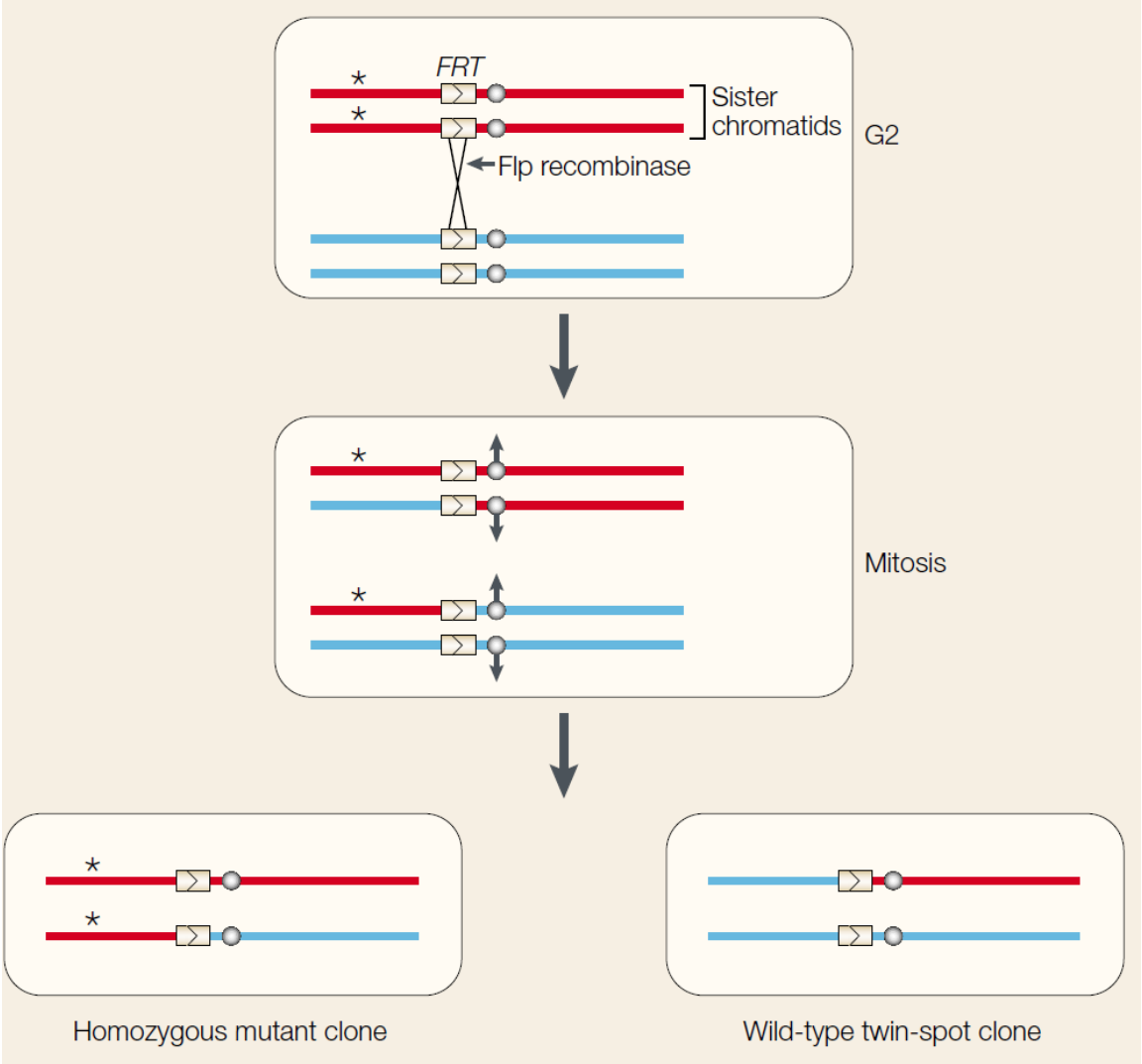

Figure 1.3. The FLP-FRT system enables mitotic recombination to generate clones of mutant cells. A schematic representation of the FLP-FRT system, adapted from yeast. A fly heterozygous for a mutant allele (*) distal to an FRT site (boxes) is exposed to a source of FLP recombinase, inducing recombination during mitosis. One of the resulting daughter cells will be homozygous mutant, while the other daughter cell will be wild-type—both will give rise to adjacent clones of their respective genotypes. Adapted from [15].

been used to generate *de novo* mutant alleles that can then be screened for modifying activity. However, newer techniques such as GAL4-UAS and FLP-FRT allow an even wider range of potential modifiers.

Growth control in *Drosophila*

The plethora of advantageous features of *Drosophila*, including its explosive developmental growth rate, have established it as one of the most popular model organisms to study growth control—in both developmental and pathologic contexts. Studies as early as 1919 have examined tumorous growth in flies [16]. Since then, a variety of methods, including forward genetic screens, have identified six or seven major signal transduction pathways responsible for the regulation of imaginal disc growth (reviewed in [15, 17, 18]). These signal transduction pathways are the insulin/PI3 kinase/Akt pathway[19], the Rheb/Tor pathway[20, 21], the receptor tyrosine kinase (RTK)/Ras pathway[22], the Myc pathway[23], the JAK/STAT pathway[24], and the Hippo pathway[25, 26]. Conserved among a diverse range of metazoans, these evolutionarily ancient mechanisms are at the center of organismal control of cell size, division, and death.

It is important to note that during the development of imaginal discs, organ growth takes place with a coordination of cell growth and division, termed '*proliferation'*. This organized cell proliferation is established by a progressively elaborate pattern of positional signals that are deployed through morphogen gradients throughout the disc, propagate through signal transduction pathways, and terminate on transcription factors responsible for growth. For example, Dpp, a BMP family member, is a diffusible morphogen that is expressed in a stripe of cells near the anterior-posterior compartment boundary in imaginal

wing discs. Dpp is both sufficient and necessary for developmental wing growth [27-29]. Despite the fact that these processes are usually coordinated as proliferation, the mechanisms that drive cell growth and division are distinct and both can operate independent of one another. In this dissertation I will briefly describe the processes that control organ size—growth, division, and death—noting how the major growth control pathways inputs into these processes. Next, I will provide a more in depth introduction to the Hippo pathway and its role in growth control and tumorigenesis.

Cell growth

Building the entire mass of an organism requires cellular biosynthesis to outpace degradation, resulting in mass accumulation and, in turn, cell growth. The instructions for biosynthesis are encoded in each cells genome, and thus replicating the genome is a key way for an organism to grow. Nonetheless, individual cells can modulate the rate of biosynthesis independent of the cell cycle allowing them to grow slower or faster (reviewed in [30]). In fact, a specific cell cycle program called endoreplication allows *Drosophila* larval body tissues (non-imaginal disc tissues specific to the larva), such as the salivary glands, to undergo cell growth without dividing [31]. The main mechanism cells employ to regulate their mass accumulation is the rate of mRNA translation, which is dependent on the number and activity of ribosomes in the cell. A key signaling molecule in the synthesis of ribosomes is the transcription factor Myc (dMyc in *Drosophila*), which promotes the transcription of ribosomal proteins and rRNA [32, 33]. Several growth regulating pathways help to control the levels of dMyc in the cell, including the RTK/Ras and the Hippo pathways [22, 34]. Correspondingly, Myc is a major proto-oncogene heavily implicated in many types of human cancer [35]. Ribosomal activity is controlled in part by

the translation factor S6 kinase and the translational repressor 4EBP. The Rheb/Tor pathway inputs into both these factors, leading to their phosphorylation and the promotion of ribosomal activity [36]. To regulate cell growth at the organismal level, the brain senses nutrient uptake which leads to the release of insulin-like peptide (dILP) family of hormones [37]. The dILPs act through the PI3K/Akt and the Rheb/Tor pathways to regulate ribosome number and activity.

Cell division

The cell cycle is the fundamental process whereby the genome is replicated, distributed into two copies, and then finally split via cell fission—resulting in two genetically identical daughter cells. This genetic material carries the blueprints for biosynthetic growth, and thus each copy increases an organism's capacity for growth. Early in *Drosophila* embryogenesis, the fertilized egg consists of giant cell that copies its genome 13 times in a syncytium, eventually dividing into all the cells of the embryo—entirely without any mass accumulation. This process illustrates that cell growth and division can be regulated independently from one another [38]. The cell cycle is controlled by oscillations in the levels and activity of cyclin proteins, which bind and activate cyclin-dependent kinases (CDKs). The rate limiting factor in the transition from the first growth phase of the cell cycle (G1) to the DNA sythnesis phase (S) is cyclin E and its partner CDK2 [39]. This G1-S transition has emerged as a key point of regulation for cell division. Cyclin E-CDK2 promote the activity of the E2F family of transcription factors (dE2F1 and dE2F2), primarily through the phosphylation and inactivation of the dE2F1-repressor Rbf1 [40]. Free dE2F1 then drives the expression of S-phase promoting genes. Several growth regulating signal transduction pathways control the levels of Cyclin E, including the

RTK/Ras and Hippo pathways. Another cell cycle transition, from the second growth phase (G2) to mitosis (M), is mediated by the rate-limiting phosphatase cdc25/string, which in turn activates Cyclin B/Cdk1 complexes [38]. Similarly to Cyclin E, signal transduction pathways, including the JAK/STAT pathway, are partially responsible for regulating the Cyclin B G2-M checkpoint [41].

Cell death

Programmed cell death, or apoptosis, culls the number of cells within an organ, and thus, can decrease organ size. For example, during normal eye imaginal disc development apoptosis removes extra cells in order to achieve the proper organization of ommatidia [42]. Programmed cell death is less important in the normally developing wing disc, with dying cells accounting for only ~1.4% of total disc cells between larval molts [43]. The mechanistic operator of programmed cell death are the caspases, a family of proteases produced as dormant pro-enzymes. When cleaved and activated, caspases begin a positive-feedback loop cascade that generates a pool of enzymatically active caspases responsible for lysing a broad number of substrates and leading to apoptotic death (reviewed in [44]). Unsurprisingly, this process is tightly regulated to prevent the unwarranted activation of caspases. In *Drosophila*, the lynchpin protein that inhibits canonical apoptosis is DIAP1 (Drosophila Inhibitor of Apoptosis 1), which binds and inhibits the caspases DRONC and DRICE. DIAP1 levels are in turn controlled by a number of upstream pathways, both transcriptionally and post-translationally (reviewed in [45]). For example, the Hippo pathway effector Yki promotes the transcription of DIAP1. At the post-translational level, DIAP1 degradation and inhibition is controlled by a complex of three proteins, Reaper,

Grim, and Hid. When Reaper, Grim, and Hid are activated, DIAP1 is disrupted, leading to DRONC and DRICE activation and death.

The Hippo pathway: growth control and human cancer

The Hippo signal transduction pathway is an essential regulator of organ size during developmental growth. Mutations in this pathway, first discovered in *Drosophila melanogaster*, have consistently demonstrated that dysfunctional Hippo pathway signaling leads to dramatic tissue overgrowth. What follows is a review of the basic components of the Hippo pathway, the evidence that deregulation of this pathway leads to well-conserved tumor-like phenotypes in model systems, and the role of Hippo signaling in cancer and human disease. A complete understanding of how the Hippo pathway contributes to disease in humans has the potential to lead to diagnostic improvements and novel therapeutics, highlighting the experimental relevancy of model systems to human disease research [46]. Overall, despite a large array of upstream mechanisms that feed into the Hippo pathway, the evidence suggests that all mechanisms of deregulation result in the common activation of the transcription factors Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). This shared downstream output of the deregulated Hippo pathway provides attractive therapeutic targets that may have the potential to treat patients that exhibit a variety of molecular alterations that feed into the Hippo pathway.

The molecular features of the Hippo signaling pathway

The basic molecular architecture of the Hippo signaling pathway is conserved between *Drosophila* and humans. Notably, there is a greater level of molecular complexity and

redundancy observed in the human Hippo pathway, often with multiple mammalian homologs for a single *Drosophila* protein. In humans, MST1/2 (Hippo in *Drosophila*) serve as upstream kinases that function to phosphorylate Lats1/2 (Warts in *Drosophila*) (Figure 1.4) (reviewed by Hilman and Gat [47, 48]). Lats1/2 and MST1/2 are brought into close spatial proximity by the scaffold protein SAV1 (Salvador in *Drosophila*). MOB1A and MOB1B (Mats in *Drosophila*) function to enhance the kinase activity of Lats1/2 (reviewed by Hariharan [49, 50]). Once activated, Lats1/2 continues the kinase cascade by phosphorylating the transcriptional co-activator YAP and its vertebrate-specific paralog TAZ (Yorkie (Yki) in *Drosophila*). Phosphorylated YAP (serine 127) is sequestered in the cytoplasm via a 14-3-3 protein family member, preventing it from entering the nucleus [51]. When the MST1/2 kinase cascade is inactivated, the YAP protein remains unphosphorylated, translocates into the nucleus, and activates transcription of target genes [52], and reviewed by Pobbati and Hong [53]. YAP does not have DNA binding capabilities and requires a binding partner to activate gene transcription. The most well studied transcriptional partners of YAP are the TEAD transcription factors (TEAD 1-4) (Scalloped in *Drosophila*). The TEAD proteins are unable to activate gene transcription on their own; however, when YAP is present in the nucleus they function together to promote transcription of a number of pro-proliferative and anti-apoptotic genes. Though the relationship between YAP/TEAD is the best understood, YAP has also been demonstrated to associate with additional DNA binding proteins during transcriptional activation, including SMAD family transcription factors[54]. In addition to the robust conservation of core molecular components of the pathway, the transcriptional program of an activated YAP/Yki, which promotes cellular proliferation and survival, is also conserved [55-57].

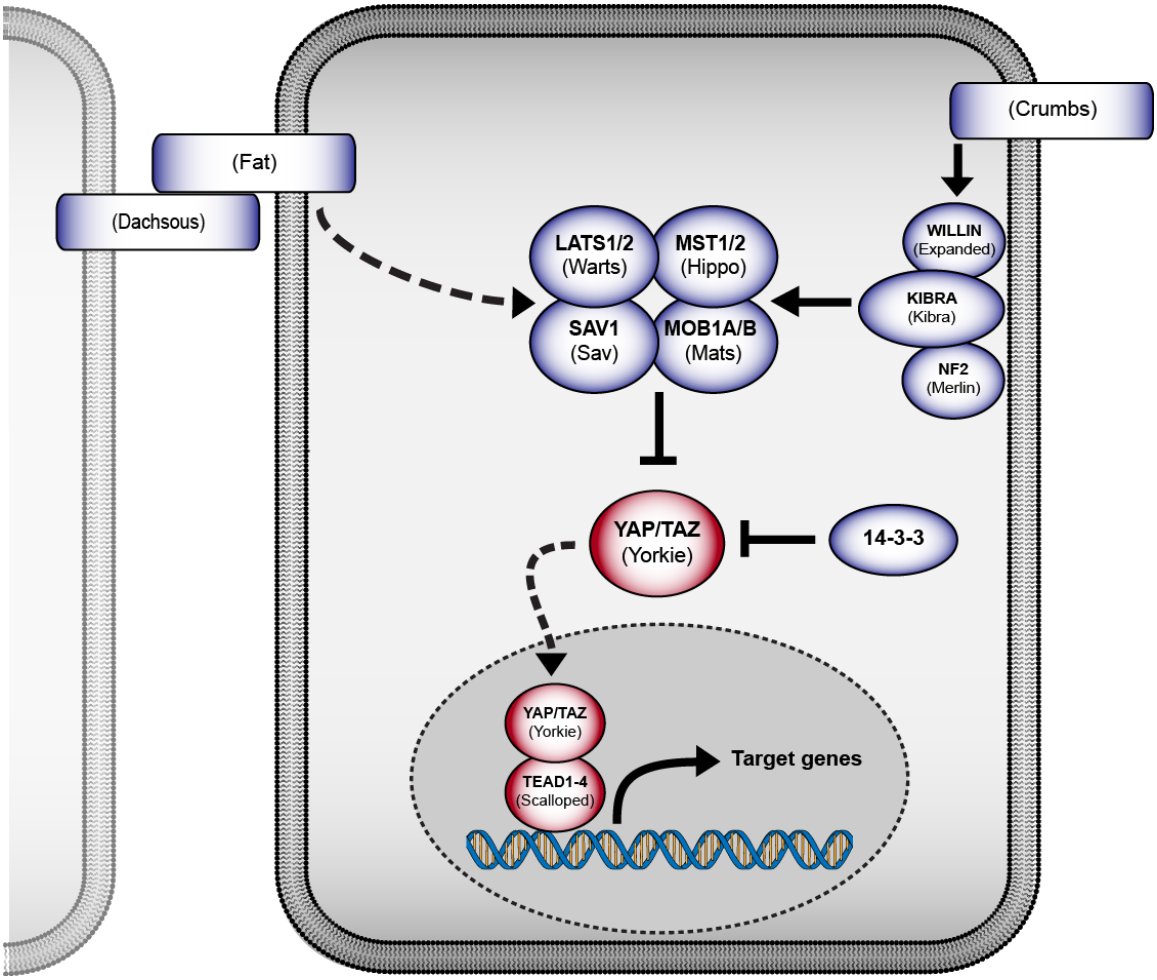

Figure 1.4. Schematic of the core Hippo signaling pathway in *Drosophila* and humans. Human molecules are in bold, *Drosophila* molecules in parentheses. Regulators, which act to restrict YAP/TAZ/Yki activation, are blue, and downstream transcriptional effectors are red.

Though flies and humans achieve this transcriptional program through the activation of different genes, the cellular outcome of the transcriptional profile remains conserved.

Though Hippo signaling converges on a singular output of YAP/Yorkie translocation to the nucleus, a variety of upstream signals converge on the core MST1/2 kinase cassette to input into the Hippo pathway. For instance, three proteins—KIBRA (Kibra in *Drosophila)*, WILLIN (Expanded in *Drosophila*), and NF2 (Merlin in *Drosophila*) —have been shown to aid in localization of the core Hippo proteins to junctional complexes, which is critical for their activation within the cell (reviewed by Grusche et al. [58])[59-62]. In *Drosophila* the most well understood upstream regulators of Hippo signaling are those related to cell polarity and cell–cell contact. Fat and Dachsous are atypical cadherins that signal as negative upstream regulators of the Hippo pathway (reviewed by Grusche et al. [58]). The expression of Fat and Dachsous integrates information encoded by morphogen gradients (Hedgehog, Wingless) to provide a molecular mechanism regulating organ size through Hippo activity [63]. Crumbs, a protein involved in maintaining apical-basal polarity, was identified as an upstream regulator of Hippo signaling which acts through the localization of Expanded [64-66]. Although homologs of Crumbs, Fat, and Dachsous have identified human homologs, their specific roles in vertebrate Hippo signaling are not as well understood. For instance, humans have four *FAT* genes (*FAT1-4*), but their role in Hippo signaling is still unclear, reviewed by Sadeqzadehet et al. [67]. In zebrafish, Fat1 has been shown to bind scribble to influence Hippo signaling [68]. However, FAT4 is arguably the closest structural and functional mammalian ortholog of *Drosophila* Fat [67, 69]. Nonetheless, recent findings have shown that a conditional knockout of FAT4 in mouse livers failed to result in liver overgrowth or

tumorigenesis, obscuring the role of FAT4 in Hippo-mediated mammalian overgrowth [70]. Recent proteomic studies in both *Drosophila* and humans have found hundreds of potential novel Hippo pathway interactors and regulators; with future mechanistic studies, these interactors could prove to be important regulators or downstream effectors of the pathway [71-74].

Cellular inputs that alter Hippo Signaling

The core of the Hippo pathway, with several kinases negatively regulating YAP, is augmented by a number of different pathways that provide input into this core cassette, including cellular polarity, cell-to-cell contact, and G-protein coupled receptor (GPCR) signaling. Different GPCR signaling pathways have been shown to both activate and inhibit YAP depending on the specific G-protein activated [75]. In addition to the alterations on the core Hippo pathway, a number of other molecular pathways commonly altered in human carcinogenesis have the ability to cross talk with the Hippo pathway (reviewed by Irvine [76]). Wnt signaling is activated through cytoplasmic β-catenin. A transcriptional target of β-catenin is CD44, which has the ability to activate of NF2, providing a mechanism in which Wnt signaling can activate the Hippo pathway [77]. Wnt pathway mutations have been shown to increase the nuclear localization of YAP, and YAP has been shown to interact physically with β-catenin[77, 78]. TGF-β signaling activates Smad1, which has the ability recruit YAP to the nucleus [54]. Moreover, Smad1/Mad is a direct binding partner for YAP/Yki in both *Drosophila* and humans [54, 79]. mTOR demonstrates crosstalk with the Hippo pathway, with the ability to both activate and be activated by Hippo signaling [80]. Mutations in the gene *patched,* the transmembrane receptor for the Hedgehog signaling pathway, have been demonstrated to activate Hippo signaling in both

Drosophila melanogaster models and human tumors [81, 82]. The Ras pathway's RASSF proteins (dRASSF in *Drosophila*), a family of proteins that have been shown to associate with Ras, serve as negative regulators of the Hippo pathway [58, 83]. Hippo signaling is also regulated in a post-translational manner through the ubiquitination of Lats1 by the E3 ligase ITCH. Alterations in the regulation of this post-translational modification can deregulate the pathway [84]. These studies point to a model, which features a centralized role for the Hippo pathway in integrating growth signals from a number of pathways that regulate tissue/organ size (Figure 1.5). Importantly, the widespread deregulation of many of these pathways in human cancer leads to the secondary inactivation of Hippo signaling, ultimately contributing to tumorigenesis (reviewed by Yu and Guan [85] and reviewed by Irvine [76]).

Deregulation of Hippo pathway alters several cancer related cellular processes

The genetic screens that led to the discovery of Hippo pathway factors in *Drosophila* relied on single recessive mutations to drive overgrowth phenotypes (reviewed by Hariharan and Bilder [86]). Some of the most dramatic mutant overgrowth phenotypes identified in these screens were mutations in negative regulators of the Hippo pathway (Hpo, Sav, Wts, Crbs, Ex), whose loss simultaneously altered several cellular processes associated with tumorigenesis including increased cellular proliferation and prevention of apoptosis [49]. Likewise, in humans, deregulation of the Hippo pathway leads to a number of cellular processes associated with cancer progression including increased cellular proliferation, inhibition of apoptosis, and the deregulation of cellular differentiation.

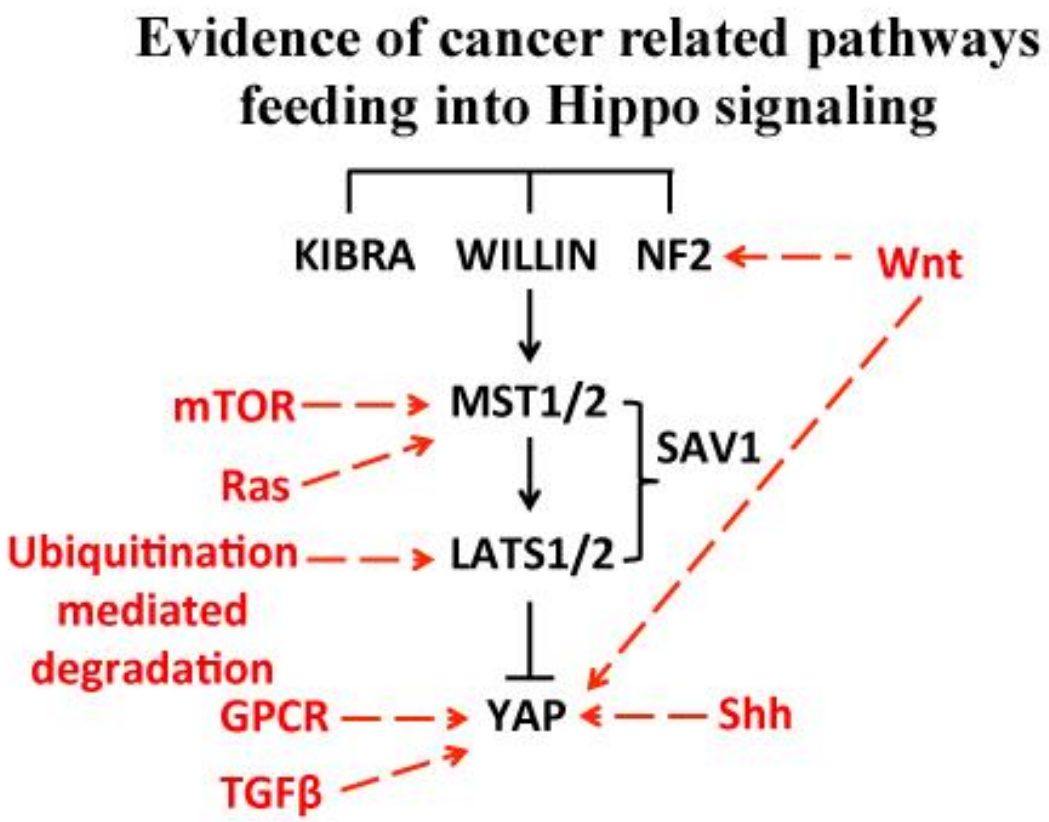

Figure 1.5. Schematic of cancer related pathways and processes that input into the Hippo pathway. Core mammalian Hippo pathway components are in black, and additional cancer related molecular pathways are in red.

Increased cellular proliferation

Mutations in Hippo pathway components in *Drosophila* consistently result in an increase in cell proliferation driven by excess Yorkie in the nucleus (reviewed by Pan [87]). Mouse models that have tissue specific activation of YAP demonstrate an increase in overall organ size, which is associated with an increase in cellular proliferation [88]. This YAP driven increase in proliferation occurs in a number of different developing murine tissues, suggesting a generalized role of YAP to promote cellular proliferation in mammalian cells [89, 90]. In addition to YAP over-expression, genetic loss of *MST1/*2 has also been demonstrated to result in over-proliferation [91]. Human cell culture models further support a mechanism in which YAP or TEAD over-expression leads to an increase in the proliferation capability of a cell [57, 92].

Inhibition of apoptosis

An increase in the levels of DIAP1 protein, a key inhibitor of cell death in *Drosophila*, has been commonly observed in mutants that inactivate Hippo signaling [56, 93, 94]. In mouse models, MST1/2 have been demonstrated to be pro-apoptotic and their loss confers a resistance to apoptosis concurrent with YAP activation [89, 95]. In human cell culture, activated YAP has been associated with an increase in survival proteins such as Survivin and IAP1 (reviewed by Dong et al. [96, 97]). Interestingly, YAP has also been identified as a binding partner of the pro-apoptotic gene *p73*, suggesting that in certain cellular contexts, YAP may be pro-apoptotic [97, 98].

Deregulation of cellular differentiation

Hippo signaling also plays a role in cellular differentiation. MST1/2 are essential components of differentiation, and inactivation of Hippo signaling leads to the induction pluripotent stem cells [99]. In mouse cell culture models, YAP expression is associated with the maintenance of embryonic stem cells, and the reduction of nuclear YAP corresponds with differentiation [85, 100]. Although the role of cancer-stem cells is controversial and not fully understood (reviewed by Brennan and Matsui [101]), deregulation of the Hippo pathway has the potential to induce stem cell–like properties such as increased proliferative capacity [102]. Therefore, the maintenance of an un-differentiated state in cells in which the Hippo pathway has been deregulated may further contribute to human cancer development.

Hippo pathway signaling suppresses cancerous phenotypes in cell culture

Given the initial discovery in *Drosophila* that Hippo signaling suppresses tissue growth, it is not surprising that Hippo pathway components are implicated in the tumorigenic transformation of mammalian cells in culture. Indeed, *in vitro* work shows that both gain and loss of Hippo signaling components in a wide range of both non-cancer and cancer cell lines can enhance or suppress cancerous phenotypes [92, 103-115]. Consistent for a central role of YAP/TAZ in the transforming properties of the Hippo pathway, the over-expression of YAP or TAZ in cell culture leads to transforming phenotypes including anchorage-independent growth, epithelial to mesenchymal transition, growth-factor independent proliferation, inhibition of apoptosis, resistance to chemotherapeutics, faster cell migration, tumor-initiation properties, invasion, and tumor formation in xenograft models [92, 103-109]. Concordantly, removal of YAP or TAZ suppresses cancerous phenotypes in cancer

cell lines [103-105, 108-113]. For example, siRNA knockdown of YAP reduces cellular proliferation, induces apoptosis, and inhibits anchorage-independent growth in pancreatic cell lines [114]. Likewise, a decrease in YAP reduces the proliferative capability of breast cancer cells in culture [116]. Collaboratively knocking down both YAP and TAZ significantly reduces the ability of colon cancer cells to proliferate, metastasize, and invade [117].

Antagonistic to YAP and TAZ, an increase in the activity of the Hippo core kinase cassette restricts cancerous phenotypes in cell culture. For example, overexpression of Mst1 in non-small-cell lung cancer (NSCLC) cells inhibits cellular proliferation and survival through YAP phosphorylation [118]. A clear cell renal cell carcinoma (ccRCC) cell line with homozygous loss of *SAV1* has reduced colony-forming capacity when SAV1 is expressed exogenously; additionally, non-tumorigenic renal cells become more proliferative upon SAV1 knockdown [119].

Hippo pathway components contribute to tumorigenesis in mouse models

Research extending from cell culture into mouse models has highlighted the ability of Hippo signaling components to drive mammalian cancer development. Overexpression of YAP in the mouse liver results in hepatomegaly, followed by tumorigenesis [89]. Furthermore, YAP expression contributes to tumor progression in mouse liver and lungs [112, 120]. Loss of YAP suppresses oncogene-induced tumor growth in mouse mammary glands [121]. Lats1 null mice develop soft-tissue sarcomas and ovarian stromal cell tumors [122]. Mouse livers missing Mst1 and Mst2, or SAV1, have elevated YAP activity leading to hepatomegaly and hepatocellular carcinoma (HCC) [65, 123-125]. Additionally, mice that are null for Mst1 and Mst2 in the intestinal epithelium develop adenomas in the distal

colon and possess an expanded undifferentiated stem cell compartment throughout their intestines [126]. Expression of the Mst1 gene in a NSCLC cell line suppresses tumor growth in a mouse xenograft model [118]. Together, these data support a conserved tumor suppressor role for Hippo signaling in mouse models of human tumorigenesis.

Molecular alterations of Hippo pathway components in human cancer

In addition to work done in cell culture and model systems, a number of studies have shown that YAP/TAZ are activated in a wide range of human cancers, bolstering the evidence that deregulated Hippo signaling contributes to carcinogenesis.. Using immunohistochemistry, YAP has been found to be either strongly expressed or highly localized to the nucleus (where it is active in gene transcription) in human cancers compared with normal tissue. Increased expression and/or nuclear accumulation of YAP has been reported in a wide array of human cancers including HCC, prostate cancer, colorectal carcinoma (CRC), NSCLC, ovarian cancer, ccRCC, pancreatic carcinoma, esophageal squamous cell carcinoma, urothelial carcinoma of the bladder, and skin basal cell carcinoma (Table 1) [77, 104-106, 110, 114, 115, 119, 126-130]. Notably, expression or nuclear localization of YAP is associated with poorer tumor differentiation and higher-grade tumors [119, 129]. Concordantly, TAZ is overexpressed in high-grade breast cancers, CRCs, and tongue squamous cell carcinomas [109, 117, 131]. Negative regulators of YAP/TAZ signaling including Mst1/2, Lats1/2, NF2, Mob1, and SAV1 exhibit loss of expression in human tumors (reviewed by Zhao et al. [88] and Harvey et al. [132]). For example, a majority of human HCCs have inactivated Mst1, and approximately 30% have reduced YAP1 phosphorylation at the inhibitory S127 site [123].

TABLE 1: Evidence of Hippo pathway dysregulation in human cancer

Cancer Type	Alterations in expression levels	Mutation	Heritable cancer syndrome	Copy Number Changes	Epigenetic Silencing	Chromosomal Fusion Protein	Prognostic indidcator in cancer patients
Acute lympocytic leukemia					KIBRA (104), FAT1 (104) LATS1/LATS2 (100),		
Breast cancers	TAZ (66)	LATS1* (96)		TAZ (66)	FAT4 (105)		TAZ (65)
Clear cell renal cell carcinoma	YAP (76)			SAV1 (76)			
Colorectal carcinoma	YAP (34,74,83,85), TAZ					YAP (74), TAZ (68,74), YAP+TAZ (74)	
Epithelioid					LATS1 (102)	TAZ-CAMTA1 (97, 98)	
Esophageal squamous cell	YAP (63)			YAP (77)			YAP (63)
Hepatocellular carcinoma	YAP (84,86),			YAP (77)			YAP (86)
Nervous system tumors (Schwannoma, meningioma, astrocytoma, etc)		NF2 (89)	NF2 (90)	FAT1 (99)	LATS1/LATS2 (101)		
Non-small cell lung cancer	YAP (61,85)	LATS1* (96)		YAP (77)			YAP (61), YAP+TAZ (69)
Oral cancers		FAT1 (95)					
Ovarian cancers	YAP (62,85)	LATS1* (96)		YAP (77)			YAP (60,62,106)
Pancreatic carcinoma	YAP (71)						
Prostate cancer	YAP (84)						
Skin basal cell carcinoma	YAP (67)						
Soft tissue sarcoma					MST1/MST2 (103)		
Tongue squamous cell	TAZ (88)						TAZ (88)
Urothelial carcinoma of	YAP (87)						YAP (87)

Table 1. Evidence of Hippo pathway deregulation in human cancer. Activating events are in green and inactivating events are in red. The asterisks indicate that this mutation is found in only a small subset of cancers (1-2%).

Given the evidence for YAP/TAZ activation, there is a surprising lack of documented genetic mutations in core Hippo components in cancer [132]. As sequencing studies continue, a greater number of mutations in Hippo pathway components may be revealed in different subsets of tumors. Nonetheless, the current dearth of *bona fide* cancerous mutations in the Hippo pathway is striking. A number of factors could help to explain this surprise. Firstly, the mammalian Hippo pathway has redundant upstream negative regulators of YAP/TAZ (*i.e.* Mst1/2 and Lats 1/2) such that multiple mutations would be required to inactivate the pathway in a tumor. Secondly, other mechanisms of Hippo pathway alteration have been discovered in various human cancers, including copy-number changes, translocations, and epigenetic silencing, which could explain the observed YAP/TAZ activation without associated mutations in Hippo pathway genes. Thirdly, the activation of YAP/TAZ could be the result of other growth regulating pathways (*i.e.* Ras, TGF-β, and Wnt) exerting suppressive pressure on Hippo signaling, such that deregulation of those pathways leads indirectly to the activation of YAP/TAZ [85, 132-138]

Nevertheless, one prominent example of genetic mutation affecting the Hippo pathway is Neurofibromatosis 2, a dominantly inherited familial syndrome caused by mutations in the *NF2* gene. This syndrome has an incidence of approximately 1:25,000 and a prevalence of approximately 1:80,000. Notably, patients with Neurofibromatosis 2 are predisposed to developing tumors of the nervous system, including schwannoma, meningioma, ependymomas, and astrocytomas [133]. Nearly all patients acquire bilateral vestibular schwannomas by age 30 [134]. In addition to germline *NF2* mutations, somatic mutations have been found in sporadic human tumors, primarily those that originate in the

nervous system [132]. Mutations of *NF2* disrupt Merlin, an upstream regulator of the Hippo pathway, which may explain the tumor predisposition seen in patients. Concordantly, NF2 acts as a tumor suppressor through Hippo signaling in mice, where conditional knockout of NF2 in mice liver resulted in HCC [135]. Furthermore, the tumor cell proliferation in human schwannomas has recently been linked to a gene expression network controlled by YAP [136].

Additional examples of somatic mutations in Hippo pathway components are rare. In the Catalogue of Somatic Mutation in Cancer (COSMIC) database, approximately 1% to 2% of the more than 5,000 unique human cancer samples contain nonsynonymous mutations in *Lats1* or *Lats2* [137, 139]. Recently, these *Lats1/2* cancer mutations were shown to disrupt a number of *Lats1/2* functions, including kinase activity, suppression of YAP activity, and tissue growth properties. These data, along with the identified mutations, serve to bolster the evidence for *Lats1/2* as tumor suppressor genes in a small subset of cancers [139]. Homozygous deletions of the *FAT1* gene, a putative upstream negative regulator of Hippo signaling, were discovered in 23% of oral cancer cell lines and 80% of primary oral cancers [138]. However, given the disproportionately large size of the FAT genes, it is still unclear if FAT gene mutations are 'passenger' or 'driver' mutations in cancer.

In addition to genetic mutations, the Hippo pathway is disrupted via other mechanisms in cancer, including copy-number changes, loss of heterozygosity, and epigenetic silencing (Table 1). In mice with inactivation of tumor suppressor genes BRCA1 and p53, an amplicon containing only the *YAP* gene was isolated from mammary tumors [92]. However, overexpression of YAP alone does not promote oncogenic growth in mouse

mammary glands, suggesting that YAP amplification requires these cooperating lesions to transform cells [121]. A similar amplicon (9qA1) containing the *YAP* gene was found at high frequency in murine tumors derived from Myc-expressing cells. These amplicons are syntenic to a larger human locus (11q22) that is amplified in 5% to 10% of tumor types, including lung, ovarian, esophageal, and liver carcinomas [120]. The *TAZ* locus is amplified in 27 of 313 breast tumors, and *TAZ* mRNA expression was specifically increased in the tumors with the amplification [109]. The *TAZ* locus has also been implicated in a disease-defining chromosomal translocation in a rare vascular sarcoma termed epithelioid hemangioendothelioma. Endothelial cells highly expressing the product of this translocation, which fuses TAZ to the calmodulin-binding transcription activator 1 (CAMTA1), are associated with this cancer, although the oncogenic mechanism of this fusion protein has yet to be elucidated [140, 141]. Studies have also shown copy number loss of *SAV1* in high-grade ccRCC [119], and loss of heterozygosity at the *FAT1* locus has been reported in primary glial tumors [142].

Epigenetic silencing affects the expression of several members of the Hippo pathway in a variety of human cancers. For example, more than half of human breast tumors have hypermethylation at either the *Lats1* or *Lats2* CpG island, resulting in lower expression of *Lats1/2* mRNA and an association with more aggressive tumors [143]. Similar results were detected in human astrocytomas and CRC [144, 145]. Analogously, *Mst1* and *Mst2* are down-regulated in human soft tissue sarcoma due to CpG island hypermethylation [146]. Upstream Hippo inputs have also been found to have hypermethylated promoters with reduced gene expression, including *FAT4* in breast cancer and *KIBRA* and *FAT1* in B-cell acute lymphocytic leukemia (B-ALL) [147, 148].

YAP/TAZ activation is a prognostic indicator in cancer patients

Expression or nuclear localization of both YAP and TAZ has been associated with poor prognostic indicators and shorter survival times for patients with a wide range of human cancers. Specifically, YAP activation is associated with reduced patient survival in CRC, NSCLC, HCC, ovarian cancer, esophageal squamous cell carcinoma, and urothelial carcinoma of the bladder [103-106, 117, 129, 130]. Recently, a *YAP* gene expression signature was validated as an independent predictor of prognosis in human ovarian cancer patients, and there was a significant association between YAP expression and tumor sensitivity to chemotherapeutic taxanes [149]. Similarly, TAZ expression has been shown to correlate with reduced survival in CRC, tongue squamous cell carcinoma, and recently breast cancer [108, 111, 117, 131]. CRC patients with overexpression of both YAP and TAZ have worse outcomes than those who have either one alone [117], and a YAP/TAZ gene expression signature was significantly associated with worse overall survival and more frequent metastasis in lung adenocarcinoma patients [112]. Future clinical application of YAP/TAZ testing may improve cancer prognosis and treatment selection,

Hippo pathway and non-cancer disease processes

In addition to its role in carcinogenesis, the Hippo pathway has been implicated in the pathophysiological processes of Sjogren's syndrome, a chronic autoimmune disorder resulting in the destruction of the salivary and lacrimal glands. In mice, Hippo signaling is required for normal salivary gland development, and salivary glands from a mouse model of Sjogren's syndrome phenocopy glands with Lats2 inhibition. Importantly, salivary

glands from human Sjogren's patients exhibit nuclear TAZ staining and upregulation of TAZ transcriptional targets [150].

The Hippo pathway is also implicated in tissue regeneration, including a requirement for YAP in the recovery of damaged mouse intestine [151]. Recently, YAP and TAZ have been shown to be upregulated in mouse wounds, and knock down of YAP and TAZ delays wound closure [152]. Further insight into the regenerative properties of downstream Hippo signals could yield important therapies for tissue healing.

Potential therapeutics targeting the Hippo pathway

The Hippo pathway's contribution to disease pathogenesis has sparked interest in the development of potential therapeutics that could target key effectors of the signaling cascade. No matter the mechanism of Hippo pathway inactivation, cancer cells frequently exhibit hyperactive YAP, suggesting that YAP is a central contributor to tumorigenesis. YAP's ability to also function as a tumor suppressor in certain cellular contexts, where it can act in collaboration with p73 to promote apoptosis, complicates YAP-targeted cancer therapies (reviewed by Wang et al. [97, 98, 115]). Nonetheless, YAP serves as an attractive clinical target to treat tumors with Hippo pathway deregulation. Not surprisingly, early efforts to develop YAP-targeting therapeutics have begun. For instance, in a cell-based screen, the drug dobutamine, a β-adrenergic receptor antagonist, has been shown to recruit YAP to the cytosol and inhibit YAP-dependent gene transcription through a mechanism unrelated to core Hippo signaling [153]. In addition, a small molecule drug named verteporfin, used in the laser-activated ablation of blood vessels in macular degeneration, has been identified as an inhibitor of TEAD-YAP association and YAP-induced liver overgrowth [154]. Recently, verteporfin was shown to suppress growth in breast cancer

cell lines; the cell lines with the most YAP expression were also the most sensitive to verteporfin [121]. A newly characterized tumor suppressor gene named *VGLL4* has been shown to inhibit the activity of the YAP-TEAD complex by competing with YAP for binding to TEADs via tandem Tondu domains [155, 156]. Notably, a peptide that mimics the YAP-TEAD inhibitor activity of VGLL4 was shown to suppress gastric tumor growth *in vitro* and *in vivo* [156]. Together, these early advancements in targeting YAP have generated excitement over prospective new therapeutics to treat cancer and other disease.

Conclusions: YAP/TAZ in Human Cancer

The Hippo pathway is a conserved signaling cascade that serves as a developmental regulator of organ size and, when deregulated, fuels carcinogenesis. Model systems, including *Drosophila*, mice, and cell culture, have provided insight into the molecular relationship of components within the Hippo pathway and their control of cell division, apoptosis, and ultimately, organ size. The Hippo pathway also integrates signals from a number of established growth pathways, including mTOR, Wnt, and Ras. Pertinent to human disease, the deregulation of Hippo pathway components promotes cancer development through multiple mechanisms including inactivating mutations in upstream regulators, epigenetic alterations, loss of heterozygosity, alterations in copy number, and deregulation of associated molecular pathways. Regardless of their mechanism, these alterations yield the same molecular end result—the expression of active, nuclear-localized YAP/TAZ. Targeting the constitutive activation of YAP/TAZ for therapeutic treatment may address a diverse spectrum of different molecular alterations funneling into a common transcriptional activator. This shared molecular outcome presents both

YAP and TAZ as attractive diagnostic and therapeutic markers for an array of different human cancers.

Remaining Questions and Emerging Hypotheses

Despite abundant research work on that has taken place in the field of Hippo signaling, large questions still remain to be answered. For instance, it is likely that core components of the pathway have yet to be discovered. For example, despite the prominent role of kinases in regulating Hippo signaling, a presumed phosphatase has yet to be identified [157]. Furthermore, a picture of the crosstalk between multiple cell signaling pathways and the Hippo pathway remains incomplete.

One major gap in our knowledge of Hippo signaling involves the promotion of transcriptional targets by Yki in the nucleus. Genome wide studies show Yorkie/YAP1 binding and promoting thousands of genes, but the relative contribution of Yki to the transcription of these genes and their role in growth control has not been fully elucidated [158, 159]. As discussed above, Yki does not have a DNA binding domain and cannot promote gene transcription without associating with a DNA binding transcription factor partner. Scalloped was initially discovered as one such partner and has since emerged as a focus of further study due to its robust suppression of Yorkie-driven overgrowth and promotion of known Yorkie-target gene *thread* [160, 161]. Nonetheless, the fact that *Drosophila* tissues that require Yki for normal growth do not require Sd revealed the possibility of other DNA binding partners [162, 163]. We now know that other transcription factors including Homothorax and Mad are responsible for partnering with Yki to drive the express of target genes including *bantam* [55, 164]. However, a full understanding of the logic of this transcriptional regulatory network remains a challenge.

The complete complement of Yki partner proteins and their role in the transcription of target genes is lacking [157]. How is the selection of different transcription factor partners modulated within the same cell? What other proteins exist within Yorkie transcriptional complexes? What consequences do different partners have for transcriptional output in different cellular contexts or cell types?

The project that eventually formed the basis for this dissertation began as an examination of a putative interaction between a conserved dimerization hub protein named Ctp/LC8/DYNLL1 and Archipelago, an F-box containing substrate recognition component of an E3 ubiquitin ligase responsible for degrading pro-growth proteins. When no such functional relationship could be established, I turned to a more careful analysis of Ctp phenotypes in the wing and detected genetic interactions between Ctp and Yorkie. In the process, I formed the hypothesis that Ctp acts through Yki to influence changes in target gene transcription and, thus, effect the growth of Ctp-deficient tissues. Surprisingly, I found that the loss of Ctp affected two Yki transcriptional targets, *thread* and *bantam*, in opposite directions. This result raises the implication that Ctp can shift Yki onto different target promoters, potentially by effecting the complexion of Yki transcriptional complexes. In this way, I present a case for one possible mechanism through which Yki selects its target promoters, shedding light on a prominent question within the Hippo field.

While performing epistasis experiments related to my work on Ctp and Yki, I went on to collect data that suggests a role for the Hippo pathway in driving invasion of neighboring tissue in a clonal model of tumorigenesis. I was surprised to find that clones of Yki-overexpressing cells in the *Drosophila* wing extend actin-rich projections into nearby wild-type tissue. This work never culminated into a fully realized story or

publication, but I include it here as record of exciting findings that could form the foundation of promising project.

Chapter 2: Inverse regulation of two classic Hippo pathway target genes in *Drosophila* by the dimerization hub protein Ctp

This chapter is adapted from the following published paper:

Barron DA, Moberg K. "Inverse regulation of two classic Hippo pathway target genes in Drosophila by the dimerization hub protein Ctp". *Sci Rep*. 2016 Mar. 14; 6:22726. Pubmed PMID: 26972460.

INTRODUCTION

The LC8 family of cytoplasmic dynein light-chains, which includes vertebrate LC8 (aka DYNLL1/DYNLL2) and *Drosophila* Cut-up (Ctp), are small highly conserved proteins that are ubiquitously expressed and essential for viability[165-168]. The LC8 protein is 8 kilodaltons (kD) in size and was first identified as an accessory subunit in the dynein motor complex, within which an LC8 homodimer binds to and stabilizes a pair of dynein intermediate chains (DIC)[165, 169, 170]. However, the LC8 protein has since emerged as a general interaction hub with multiple dynein/motor-independent roles and binding partners[167, 171, 172]. In fact the majority of LC8 protein in mammalian cells is not associated with either dynein or microtubules[165], and LC8 orthologs are encoded in the genomes of flowering plants that otherwise lack genes encoding heavy-chain dynein motors[173].

Accumulating evidence has reinforced the idea that the primary role of LC8 in mammalian cells is to facilitate dimerization of its binding partners via LC8 self-association, a mechanism that has been termed 'molecular velcro'[171]. LC8 can be found in association with over 40 proteins that function in diverse cellular processes, including intracellular transport, nuclear translocation, cell cycle progression, apoptosis, autophagy, and gene expression[172, 174]. LC8 exists as a homodimer at physiological conditions and binds its partners in pairs. Crystal structures show that LC8's binding partners contain partially conserved recognition motifs ~10 amino acids long in a disordered region of the protein that bind to two symmetrical grooves in the LC8 dimer (Fig 2.1 A, B). The 10 amino acid motif contains a signature Thr-Gln-Thr (TQT) sequence at the C-terminus,

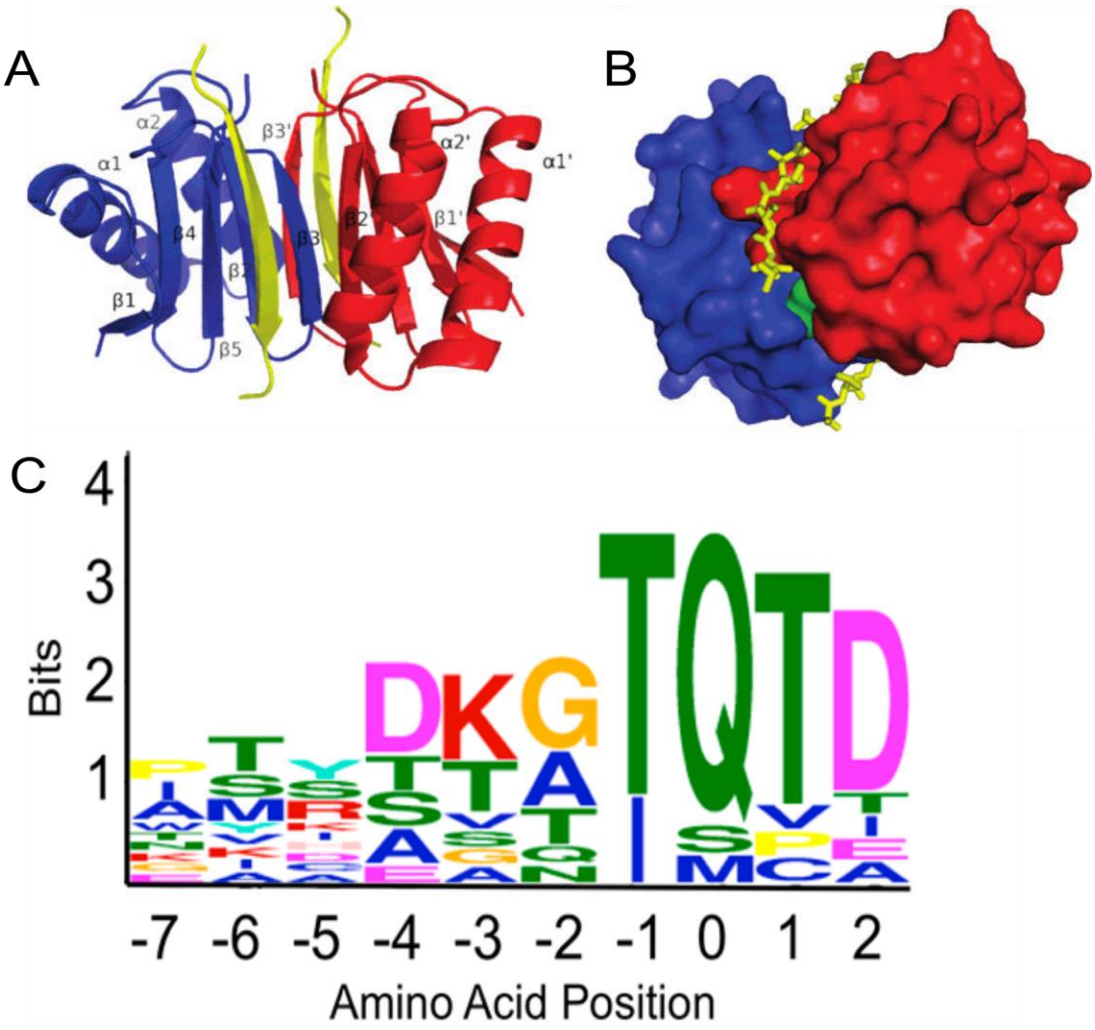

Figure 2.1. LC8 dimer binds partners in two symmetrical grooves. (**A**) LC8 dimer (blue and red) bound to Swallow peptide in ribbon diagram (yellow). Secondary elements of LC8 are labeled. (**B**) Surface representation of LC8 bound to Swallow peptide (yellow stick model). (**C**) Sequence logo of LC8 binding motifs with the height of each letter

while other positions are variable but contain some notable conservation (Fig 2.1C) [171, 175-184]. representing their relative frequency at that position, derived from 11 crystal structures of LC8/peptide complexes. Adapted from [171] and [175].

LC8 is found in both the nucleus and cytoplasm and can interact with partners in either compartment. For example the mammalian kinase Pak1 binds and phosphorylates LC8 in the cytoplasm, which in turn enhances the ability of LC8 to interact with the BH3-only protein Bim and inhibit its pro-apoptotic activity[185, 186]. Accordingly, overexpression of LC8 or the phosphorylation of LC8 by Pak1 enhances survival and proliferation of breast cancer cells in culture[186]. A recent study showed that the transcription factor ASCIZ cooperates with MYC (cMyc) to activate the expression of LC8, which is essential for the development and expansion of MYC-driven lymphomas in a mouse model [187]. LC8 also binds estrogen receptor-α (ERα) and facilitates ERα nuclear translocation, which in turn recruits LC8 to the chromatin of ERα-target genes[188-190]. In the cytoplasm, LC8 is also found in association with the kidney and brain expressed protein (KIBRA), which is an upstream regulator of the Hippo tumor suppressor pathway[191]. KIBRA binding potentiates the effect of LC8 on nuclear translocation of ERα, suggesting crosstalk may occur between LC8-regulated pathways[190]. The KIBRA-LC8 complex also interacts with Sorting Nexin-4 (Snx4) to promote dynein-driven traffic of cargo between the early and recycling endosomal compartments[192]. Thus, LC8 has been linked to a variety of proteins in both the cytoplasm and nucleus that play important roles in signaling, membrane dynamics, and gene expression.

Drosophila Ctp differs from vertebrate LC8/DYNLL by only four conservative amino acid substitutions across its 89 amino acid length. Similar to mammalian LC8,

phenotypes produced by Ctp loss in flies imply roles in multiple developmental mechanisms. *Drosophila* completely lacking Ctp die during embryogenesis due to excessive and widespread apoptosis[166, 193]. Partial loss of Ctp function causes thinned wing bristles and morphogenetic defects in wing development, as well as ovarian disorganization and female sterility[166]. Within salivary gland cells, Ctp promotes autophagy during pupation[194], while in neuronal stem cells it localizes to centrosomes and influences mitotic spindle orientation and the symmetry of cell division[195]. Testes mutant for *ctp* have motor-dependent defects in spermatagonial divisions as well as motor-independent defects in cyst cell differentiation[196]. A recent study linked *ctp* mRNA expression to the zinc-finger transcription factor dASCIZ and showed that knockdown of either Ctp or dASCIZ reduces wing size[197]. In sum, this diversity of effects produced by Ctp loss in different *Drosophila* cell types suggest that Ctp plays important yet context specific roles in vivo. However our knowledge of molecular pathways that require Ctp, and in turn underlie these developmental phenotypes associated with Ctp loss, remain poorly characterized.

Here we use a genomic null allele of *ctp* and a validated *ctp* RNAi transgene to assess the role of the Ctp/LC8/DYNLL protein family in pathways that act within the developing *Drosophila* wing epithelium. We find that clones of *ctp* null cells are quite small relative to controls and that RNAi depletion of Ctp shrinks the size of the corresponding segment of the adult wing without clear defects in mitotic progression or tissue patterning. The effect of Ctp depletion on adult wing size is primarily associated with a reduction in cell size, rather than cell division or cell number, implying a role for Ctp in supporting mechanisms that enable developmental growth. In assessing the effect

of Ctp loss on multiple pathways that control wing growth, we detect robust effects on one--the Hippo pathway. The Hippo pathway is a conserved growth suppressor pathway that acts via its core kinase Warts to inhibit nuclear translocation of the coactivator Yorkie (Yki), which otherwise enters the nucleus, complexes with the DNA-binding factor Scalloped (Sd), and activates transcription of growth and survival genes[93, 160, 161, 198]. In parallel to the effect of Ctp loss on clone and wing size, Ctp loss alters expression of the classic Yki target genes *bantam* and *thread(th)/diap-1* in wing pouch cells. Parallel genetic tests confirm a requirement for *ctp* in Yki-driven tissue growth in the wing or eye. Quite unexpectedly however, Ctp loss has opposing effects on *bantam* and *diap1* transcription in wing pouch cells: *bantam* transcription is strongly elevated while *diap1* expression is strongly decreased in cells lacking Ctp. In each case, these effects map to small segments of DNA in the *ban* and *diap1* promoters that recruit Yki transcriptional complexes[160, 161, 199]. Epistasis experiments confirm that Yki is required to activate the *bantam* promoter in Ctp-depleted cells, and that transgenic expression of Yki can overcome the block to *diap1* transcription. In sum these data argue that Ctp supports physiologic Hippo signaling in wing disc epithelial cells, and that Ctp likely interacts with an as yet unidentified Hippo pathway protein(s) to exert inverse transcriptional effects on Yorkie-target genes. These types of inverse effects have not previously been described within the Hippo pathway, and imply that distinct subsets of genes within the Yorkie transcriptome can be simultaneously activated and repressed in developing tissues via a mechanism that involves Ctp.

RESULTS

Ctp is required for imaginal-disc derived adult tissues to grow to normal size.

To test the role of *ctp* in the developing wing disc, a *UAS* transgene encoding a *ctp* RNA interference (RNAi) cassette (Vienna line #43116) was expressed in the posterior compartment of the larval wing disc at 25^{o}C using the *engrailed-Gal4* driver (*enGal4*). Depletion of Ctp protein in posterior cells of *en>ctp-IR* discs was confirmed by immunostaining with an antibody raised against the *Chlamydomonas reinhardtii* homolog of Ctp that also cross-reacts with metazoan LC8/Ctp proteins[165] (Figure 2.2). Ctp-depleted larval wing discs develop into adult wings with a fully penetrant phenotype of a shrunken posterior blade (Figure 2.3A-C). This effect occurs without obvious scarring of the adult wing or major disruption in its pattern of venation, with the exception of occasional truncated cross veins (e.g. wing in Figure 2.3B). Ctp-depletion with a second larval driver, *mirr^{DE}-Gal4*, which is active in the dorsal half of developing larval discs[200], shrinks the dorsal half of the adult eye (more darkly pigmented region above the dotted lines in Figure 2.3D-E), contracts the dorsal surface of the adult head, and leads to small, thin thoracic bristles (Figure 2.4). The Ctp-RNAi small-bristle phenotype matches a thin-bristle phenotype observed in adult flies carrying the hypomorphic *ctp* mutation, *ctp^{ins1}*, and further confirms the validity of the RNAi cassette[166]. As in the wing, Ctp depletion did not obviously scar the dorsal domain of the adult eye or strongly disrupt its surface organization, suggesting that Ctp depletion impairs growth of these organs but less so the mechanisms that drive their morphological patterning.

To better assess the effects of Ctp loss on cell proliferation and survival, *ctp* mutant clones (GFP-) and their wildtype twinspots (GFP+) were generated by combining a

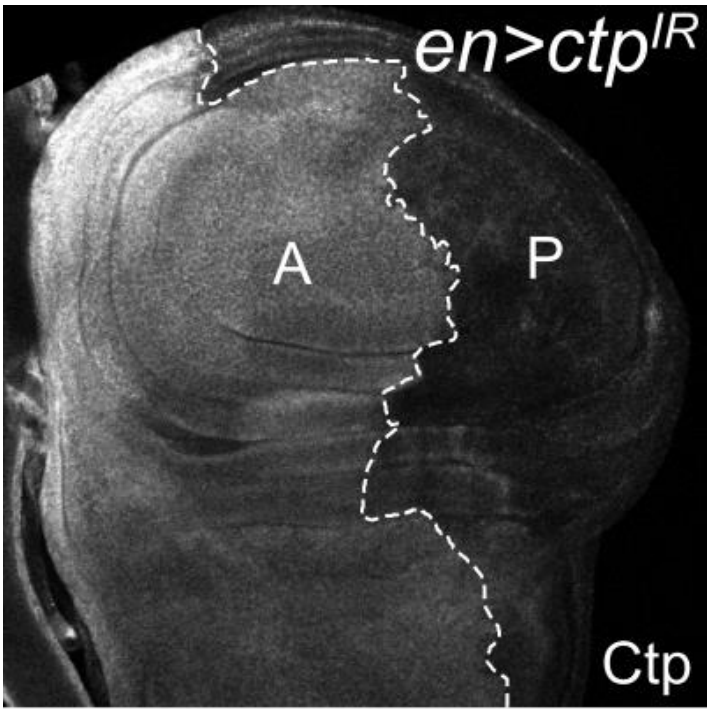

Figure 2.2. Ctp protein depletion by the *ctp^{IR}* transgene. Confocal image of an L3 *en>ctp^{IR}* wing disc immunostained with antiserum to *Chlamydomonas reinhardtii* LC3. Dotted line separates control anterior (A) cells with physiologic levels of Ctp from posterior (P) cells with reduced Ctp levels due to expression of the *ctp^{IR}* transgene.

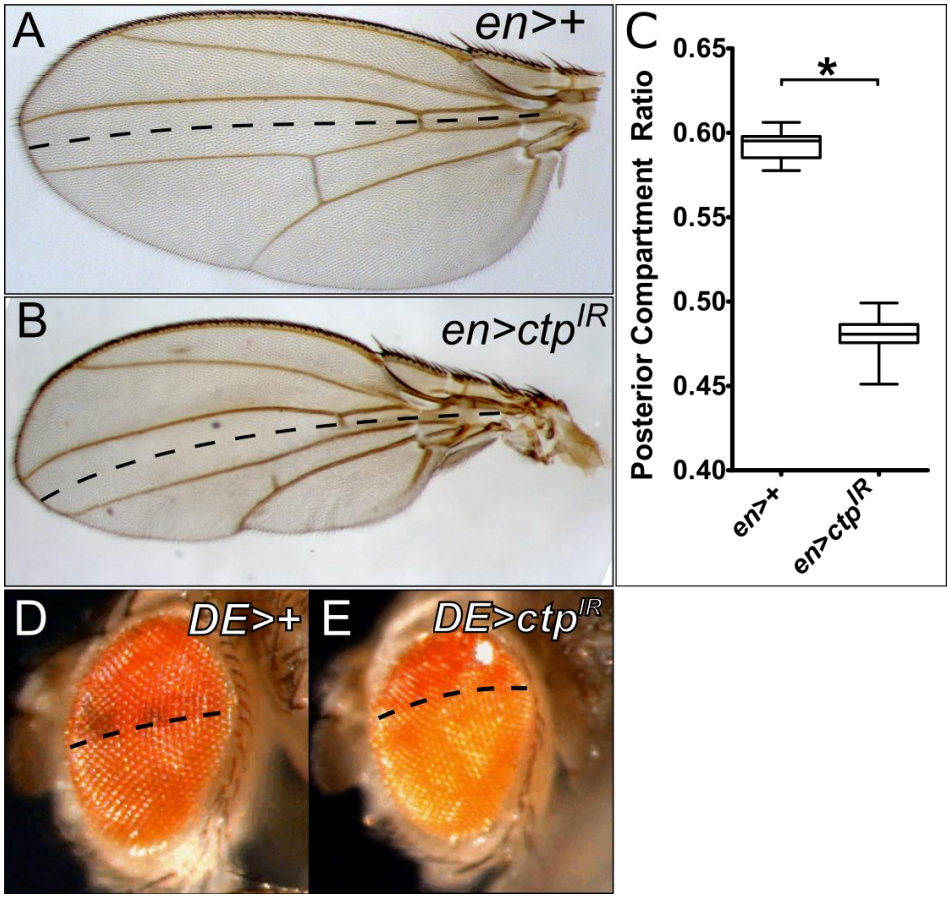

Figure 2.3. ***ctp*** **loss reduces compartment size**. Paired images of control (**A, D**) and Ctp-depleted (**B, E**) adult wings and eyes generated using the *en-Gal4* (**A, B**) or $mirr^{DE}$-*Gal4* (*AKA dorsal eye (DE)-Gal4*) (**D, E**) drivers in combination with the ctp^{IR} transgene. *en* is expressed in posterior wing cells (below dotted line in (**A, B**)). *DE* is expressed in dorsal eye cells (above dotted line in (**D, E**)). (**C**) Box-plot quantitation of the wing posterior compartment ratio (PCR = P area/P + A areas) of the genotypes in (**A**) ($n = 22$) and (**B**) ($n = 31$). Standard error of the mean (SEM) is indicated. *$p < 0.0001$.

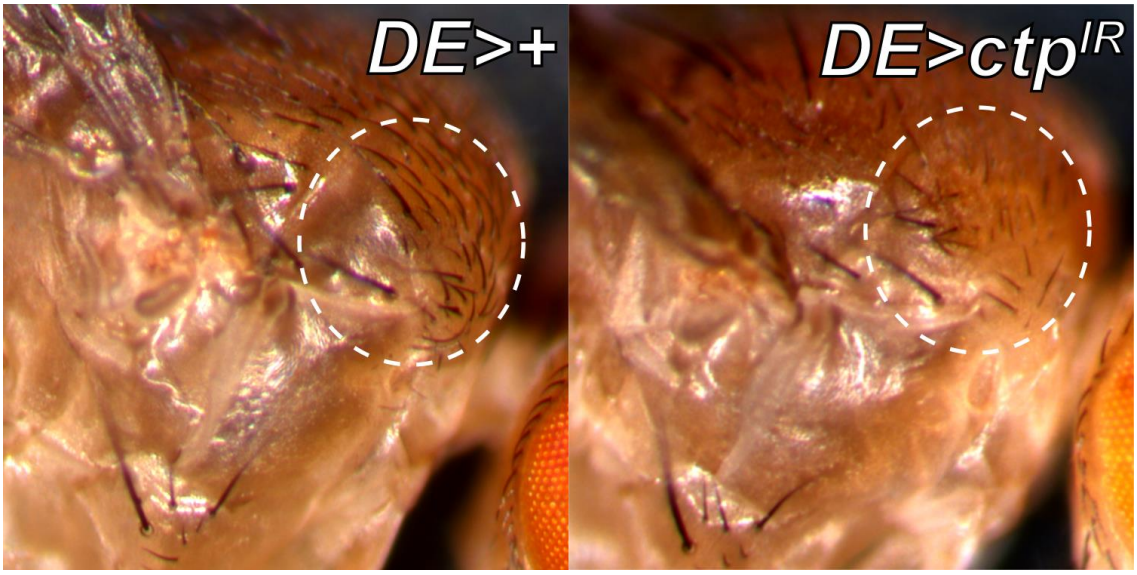

Figure 2.4. Ctp depletion shrinks the size of thoracic bristles. Light micrograph of humeral bristles (circled) in the callus region of the thorax in control (*DE>+*) or Ctp-depleted (*DE>ctp^{IR}*) adult female flies. Note the reduced size of bristles formed from Ctp-depleted cells.

heatshock(hs)-induced Flp mitotic recombination system with the *ctp*^{ex3} null allele[166]. Within dissected larval wing discs, GFP-deficient *ctp*^{ex3} clones could be found in the pouch, hinge and notum at 48hrs post-clone induction (Figure 2.5A). In the hinge and notum, these *ctp* null clones can reach a fairly large sizes (see arrows in Figures 2.5A) but are nonetheless ~2-fold smaller than their control twinspots (Figure 2.5A, B, D). This trend toward small clones is more pronounced in the pouch where *ctp*-null clones are occasionally missing and consistently quite small relative to their age-matched twinspots (e.g. see Figure 2.5A). A quantitative measurement across multiple pouch clone pairs with extant twinspots shows an approximate 5-fold reduction in *ctp* mutant clone area (Figure 2.5C-D). This apparently unequal growth deficit elicited by *ctp* loss in hinge/notum cells vs. pouch cells could nonetheless stem from perturbation of an otherwise ubiquitous cellular process, such as the mitotic spindle defects observed in *ctp* mutant neuronal stem cells[195]. Alternatively, it could indicate a role for Ctp in a molecular pathway or process that is especially important in pouch cells, similar to the cell-type specific and motor-independent requirement for *ctp* in germline and somatic cyst cells[196].

Ctp loss elevates apoptosis and division of wing disc cells and reduces their size.

To better assess the effects *ctp* loss on division and survival of larval wing disc cells, larval wing cells depleted of Ctp were probed with antibodies to cleaved caspase-3 (cC3), the incorporated nucleotide base analog bromodeoxyuridine (BrdU), and the mitotic marker phospho-histone H3 (pH3) (Figure 2.6). cC3 is moderately elevated in the posterior compartment of *en>ctp-IR* wing discs (Figure 2.6A-A'), and an antibody to the cleaved caspase Dcp-1 detects elevated apoptosis in *ctp*^{ex3} mutant wing pouch cells (Figure 2.7). This finding in disc cells parallels the widespread apoptosis that occurs in *ctp* mutant

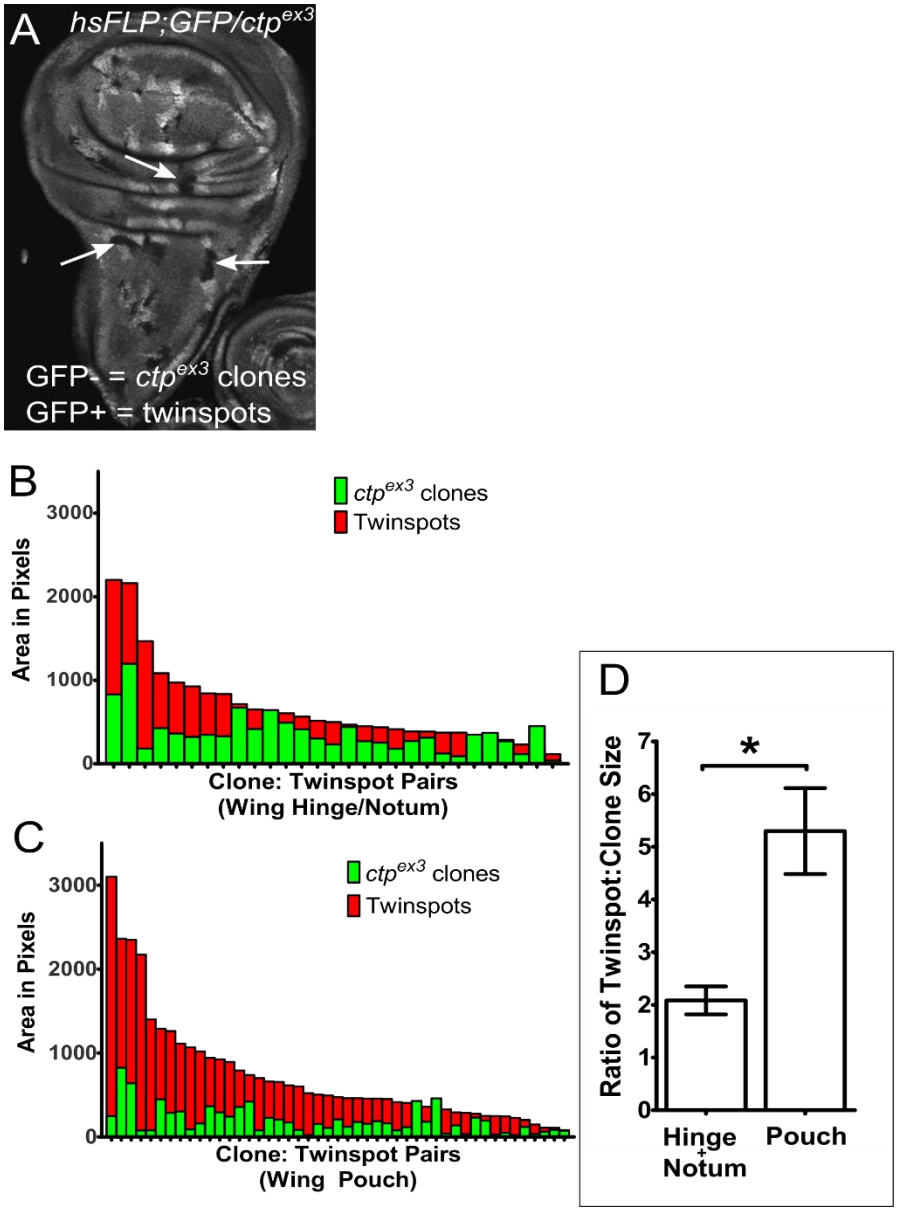

Figure 2.5. *ctp* loss reduces clonal growth. (**A**) Confocal image of a *ctp* mosaic L3 wing disc (*ctp*^{ex3},*FRT19A/ubi-GFP,FRT19A,hsFlp*) containing GFP-negative *ctp*^{ex3} clones (arrows) and their paired GFP-positive twinspots. (**B, C**) Histogram plots of the two-dimensional area of multiple *ctp*^{ex3} clone:twinspot pairs in the hinge/notum (**B**) ($n = 29$) or pouch (**C**) ($n = 47$) of L3 wing discs. Each pair of bars on the X-axis represents a distinct clone:twinspot pair. The clones are significantly smaller than

their paired twinspots, *$p = 0.0002$ for (**B**), *$p < 0.0001$ for (**C**). (**D**) Bar graph plot of average ratios of ctp^{ex3} clone:twinspot sizes in the hinge/notum versus the pouch. SEMs are indicated. *$p = 0.0034$.

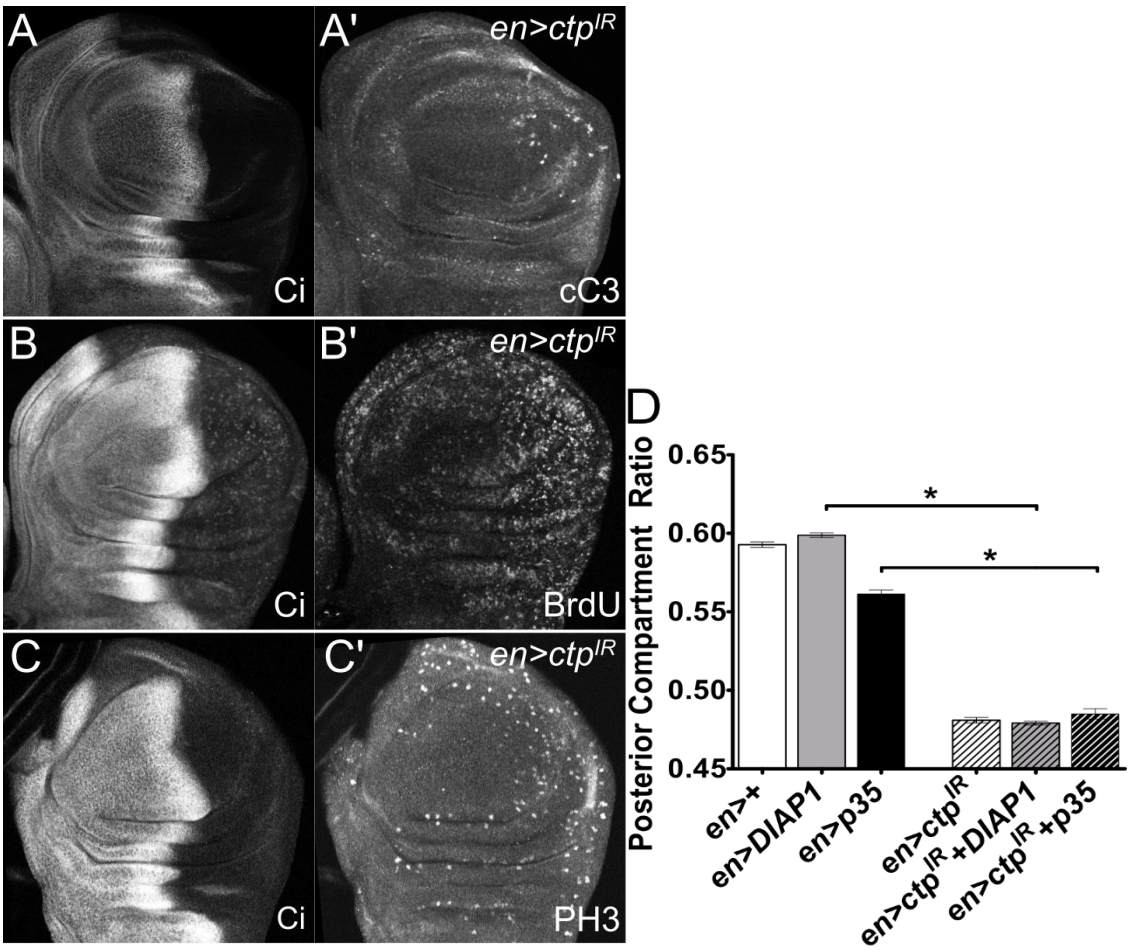

Figure 2.6. Effects of Ctp loss on the division and survival of wing cells. Confocal images of $en > ctp^{IR}$ L3 wing discs stained for (**A′**) cleaved Caspase-3 (cC3), (**B′**) the base analog BrdU, or (**C′**) phospho-histone H3 (PH3). Anti-Cubitis interruptus (Ci) staining marks the anterior domain (**A–C**). (**D**) Quantitative analysis of PCR in the indicated genotypes. $en > diap1$ ($n = 13$) is significantly larger than $en > diap1 + ctp^{IR}$ ($n = 12$) (*$p < 0.0001$), and $en > p35$ ($n = 10$) is significantly larger than $en > p35 + ctp^{IR}$ ($n = 17$) (*$p < 0.0001$).

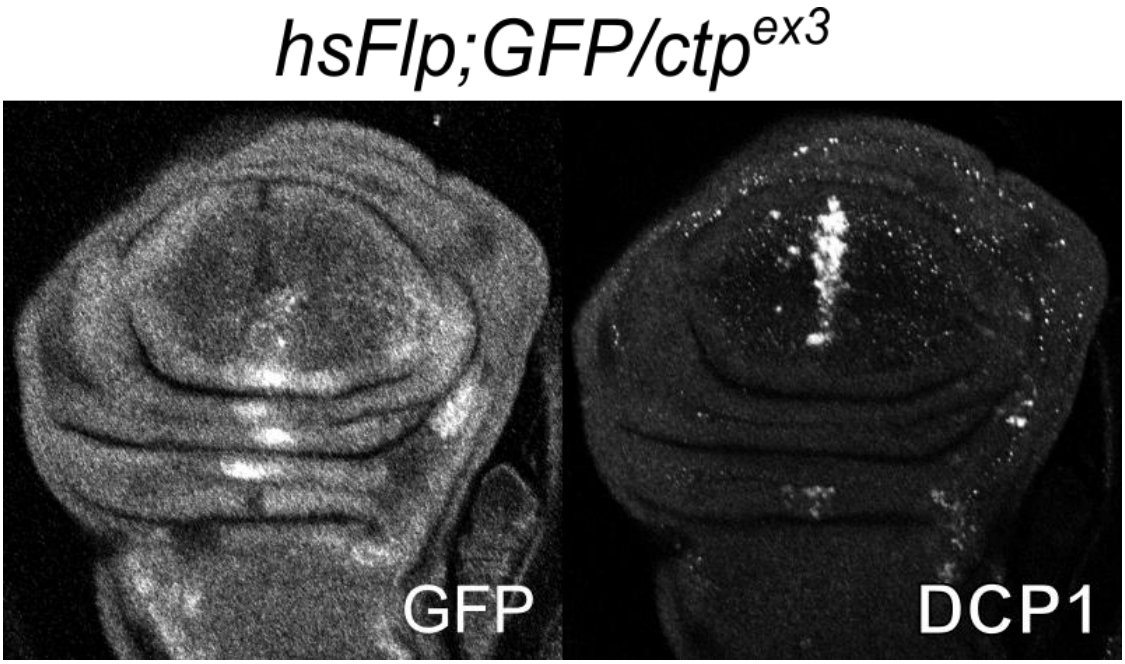

Figure 2.7. Elevated cleaved DCP1 caspase in *ctp* null wing cells. Confocal image of an L3 wing disc carrying heat-shock induced ctp^{ex3} clones (marked by the absence of GFP) immunostained with an anti-cleaved DCP1 antibody.

embryos[166] and raises the possibility that death of *ctp* mutant cells may deprive the developing wing of a sufficient pool of cells to achieve normal size. To test this hypothesis, two transgenes encoding either the *Drosophila* protein Diap1, which blocks caspase cleavage, or the baculoviral protein p35, which inhibits active cleaved caspases[201], were co-expressed with *ctp-IR* from the *en* driver (Figure 2.6D). Neither of these anti-apoptotic transgenes were able to significantly rescue small *en>ctp-IR* wings, indicating that cell death is unlikely to be the sole cause of wing blade undergrowth induced by chronic Ctp-depletion.

The early mitotic marker phospho-histone H3 (pH3) is increased among Ctp-depleted larval wing pouch cells (Figure 2.6C-C'). Taken in isolation, this higher abundance of pH3-positive *ctp-IR* larval wing cells could be indicative of an accelerated rate of mitotic entry or a slower rate of M-phase transit. Although disrupting LC8 function in cultured mammalian cells does not impede mitotic progression[202], a past study in *Drosophila* attributed an excess-pH3 phenotype in Ctp-depleted cells to dynein-motor defects that block cells in mitosis and thus enrich for M-phase markers[197]. This mitotic-block model logically predicts that Ctp-depleted cells accumulate in M-phase and thus depopulate other phases of the cell cycle. However, BrdU-incorporation analysis of *ctp-IR* wing pouch cells does not show a depletion of S-phase cells and in fact seems to show an increase in the frequency of S-phase entry (Figure 2.6B-B'). A very similar increase in cell division occurs in *Drosophila* germ cells lacking *ctp*[196]. Intriguingly *ctp* loss reduces cell division among neural stem cells[203], suggesting that Ctp plays distinct proliferative roles different cell types. In combination, the pH3 and BrdU data presented here argue that Ctp-depleted larval wing disc cells are able to actively transit between the mitotic and DNA

synthesis phases of the cell cycle, suggesting that a cell cycle block is not the prime cause of small *ctp-IR* adult wings.

Genetic manipulations that reduce cell size can also shrink *Drosophila* adult organs. Thus, the lack of experimental evidence pointing to excess apoptosis or a proliferative deficit among Ctp-depleted cells prompted analysis of the effect of Ctp loss on cell size. Each cell in the adult wing generates a single hair, enabling a quantitative determination of cell density derived from hair counts within a fixed region of the adult wing. Applying this approach to an area of the posterior wing blade between the L4 and L5 veins reveals that *en>ctp-IR* wings have significantly higher hair density relative to control *en>+* wings, indicating that Ctp-depleted cells are smaller (Figure 2.8A). The measured effect of *en>ctp-IR* depletion on cell size (~14% smaller; Figure 2.8A) is of similar magnitude to its effect on the relative size of the posterior wing blade (~17% smaller; Figure 2.3C), indicating that effects on cell size, rather than cell number, are likely be a central cause of small *en>ctp-IR* wings.

The smaller cell size of Ctp-depleted wing cells combined with the shorter, thinner thoracic bristles seen with $mirr^{DE}$*-Gal4* driven Ctp (Figure 2.4) is reminiscent of phenotypes induced by mutations in genes that support metabolic process of growth, such as the *diminutive* gene or the *Minute* ribosomal RNA genes[204] (and reviewed in [205]). The *diminutive* (*dm*) locus encodes the *Drosophila* Myc protein (dMyc), a well-established pro-growth transcription factor that promotes cell and tissue growth[23]. Notably, ctp^{ex3} null clones generated in the eye disc show reduced levels of dMyc protein as detected by immunofluorescence (Figure 2.9A-A'). Within the wing disc, dMyc protein is normally detected throughout the dorsal and ventral halves of the pouch, with a region of cells along

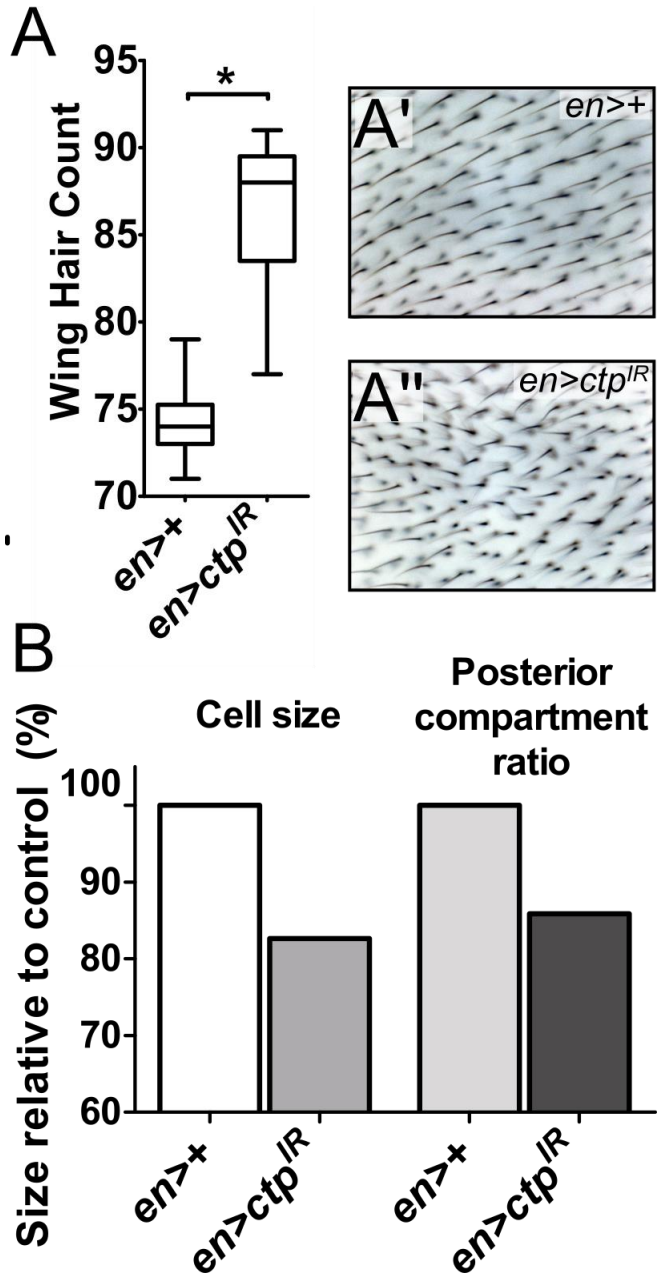

Figure 2.8. Effects of Ctp loss on size of wing cells. (**A–A″**) Box-plot quantitation and images of wing hair cell density in a fixed area in the posterior wing between the L4 and L5 wing veins in control ($en > +$) ($n = 10$) or Ctp-depleted ($en > ctp^{IR}$) ($n = 9$) adult wings. SEMs are indicated. $*p < 0.0001$. (**B**) Comparative effects of Ctp-depletion ($en > +$ vs. $en > ctp^{IR}$) on cell size (data in 2E) and PCR (data in 1C). $en > +$ values are standardized to 100%.

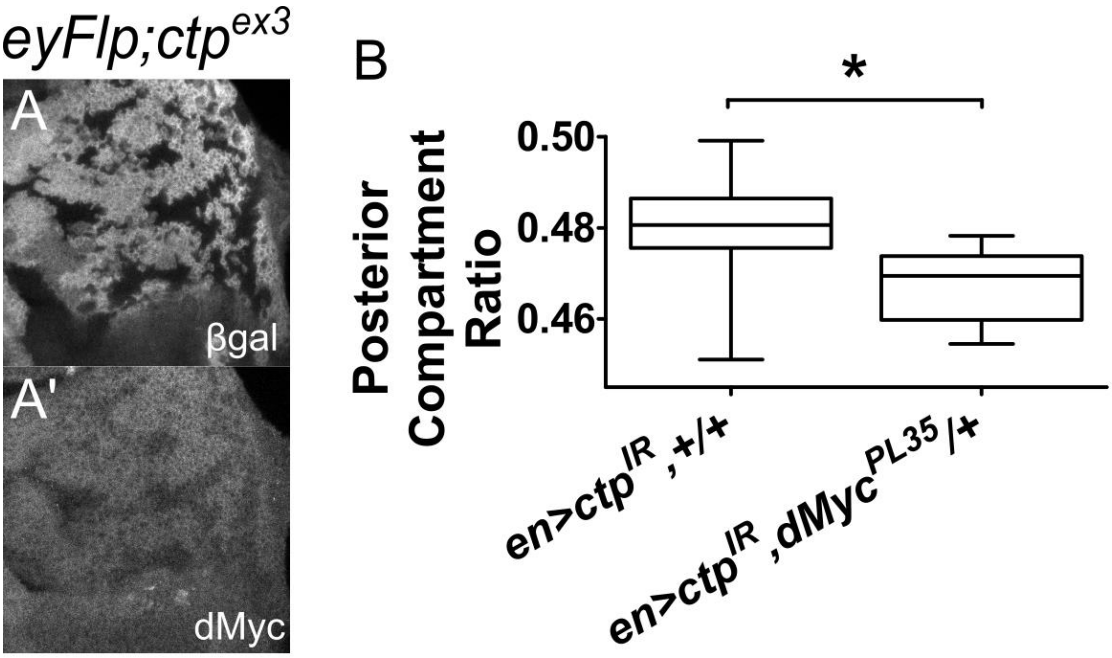

Figure 2.9. Ctp clones contain reduced levels of dMyc protein and a dMyc null allele dominantly enhances the *ctp* knockdown small wing phenotype . (**A**) Confocal image of an L3 eye disc (posterior to the right) mosaic for ctp^{ex3} clones marked by the absence of β-galactosidase (βgal) (**A**) and stained for dMyc (**A′**). Note the drop in anti-dMyc fluorescence in ctp^{ex3} clones in (**A′**). (**B**) Quantitation of PCR in the indicated adult genotypes shows that heterozygosity for the $dMyc^{PL35}$ allele ($n = 6$) has a dominant enhancing effect on the $en > ctp^{IR}$ phenotype ($n = 31$). SEMs are indicated. *$p = 0.0037$.

the dorsoventral boundary that express less dMyc (Herranz et al, 2010, EMBO). In *en>ctp-IR* discs, dMyc staining is intact in the corresponding anterior regions but decreased in these areas that express the *ctp-IR* transgene (Figure 2.10). Consistent with this link between Ctp and dMyc protein levels, heterozygosity for the null allele $dMyc^{PL35}$ further shrinks *en>ctp-IR* wings (Figure 2.9B). Notably, simultaneous overexpression of dMyc is unable to restore the small posterior compartment of *en>ctp-IR* wings, suggesting that loss of dMyc alone cannot account for the small size of Ctp depleted wings (Figure 2.11). To test the role of dMyc on the size of Ctp-depleted cells, we examined wing hair cell counts in *en>+* and *en>ctp-IR* discs, with and without co-expressed dMyc. Consistent with its known effect on cell size, *en>dMyc* wings have significantly lower cell density, and thus larger cells than controls (Figure 2.12). Interestingly, the small size of *en>ctp-IR* cells is largely rescued by exogenous expression of dMyc, supporting the hypothesis that Ctp depleted cells are small due in part to reduced levels of dMyc. (Figure 2.12). The different contribution of dMyc overexpression to wing size (posterior compartment ratio) and cell size in *en>ctp-IR* wing discs is evident when plotted relative to controls (Figure 2.13). Expression of dMyc only changes *en>ctp-IR* PCR from 81.1% to 82.2% of control, but restores *en>ctp-IR* cell size from 85.8% to 98.4% of control. Because dMyc is both pro-growth and pro-apoptosis[206], we hypothesized that the inability of dMyc expression to rescue wing size—despite its robust restoration of cell size--is due to an increase in the number of dying cells. We stained *en>+* and *en>ctp-IR* discs, with and without co-expressed dMyc, for cC3 in order to evaluate the contribution of dMyc to the levels of apoptosis. Consistent with its pro-apoptotic role, *en>dMyc* discs contained moderately elevated cC3 (Figure 2.14). When combined, *en>ctp-IR + dMyc* discs had the

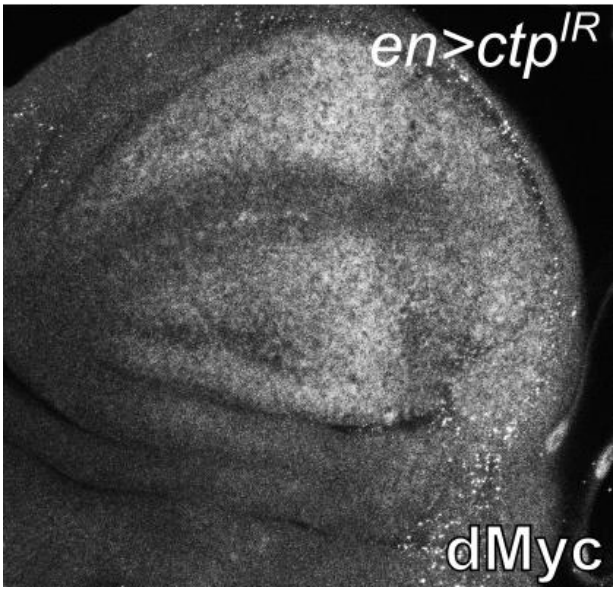

Figure 2.10. Ctp-depletion lowers level of dMyc in wing cells. Confocal image of an *en>ctp*^{IR} wing disc stained with an anti-dMyc monoclonal antibody. Note the P-domain (right) is reduced in size and that dMyc protein levels are reduced relative to the control anterior domain.

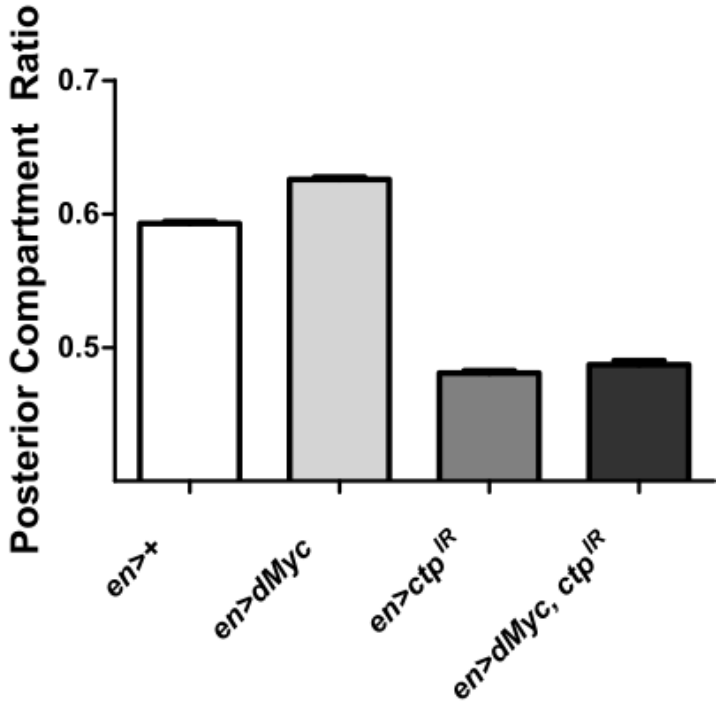

Figure 2.11. Ctp-knockdown small wing phenotype is not rescued by exogenous dMyc expression. Quantitative analysis of PCR in the indicated genotypes. *en > dMyc* ($n = 25$) is significantly larger than *en > +* ($n = 22$) (*$p < 0.0001$), and *en > dMyc + ctp^{IR}* ($n = 18$) is significantly smaller than *en > +* ($n = 22$) (*$p < 0.0001$). *en > dMyc + ctp^{IR}* is not significantly different than *en > ctp^{IR}* ($n = 31$).

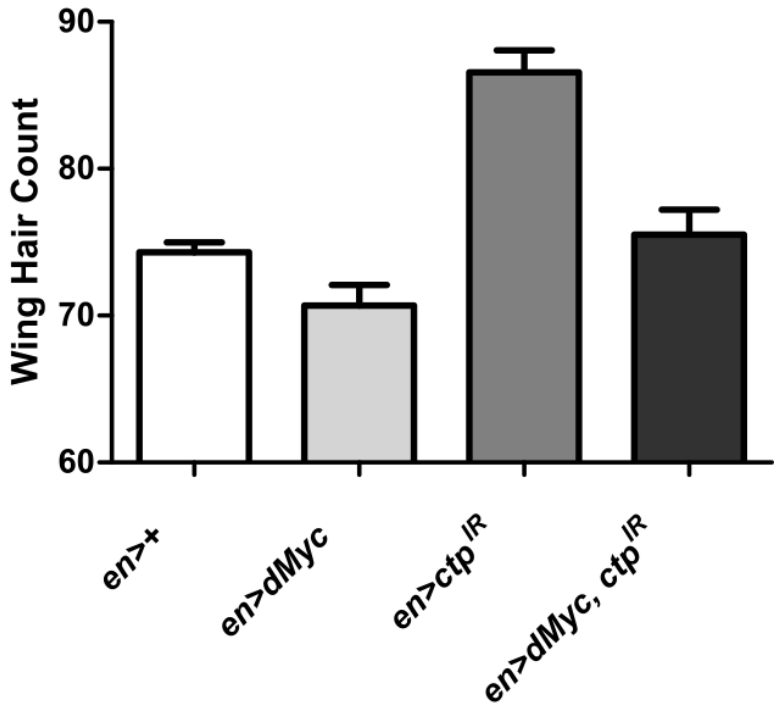

Figure 2.12. Ctp-knockdown small cell size is restored by exogenous dMyc expression. Box-plot quantitation and images of wing hair cell density in a fixed area in the posterior wing between the L4 and L5 wing veins in genotypes indicated. SEMs are indicated. $en > dMyc$ ($n = 10$) density is significantly less than $en > +$ ($n = 10$) ($*p = 0.0321$). $en > dMyc + ctp^{IR}$ ($n = 10$) density is significantly higher than $en > dMyc$ ($n = 10$) ($*p = 0.0433$) and significantly lower than $en > ctp^{IR}$ ($n = 9$) ($*p = 0.0002$).

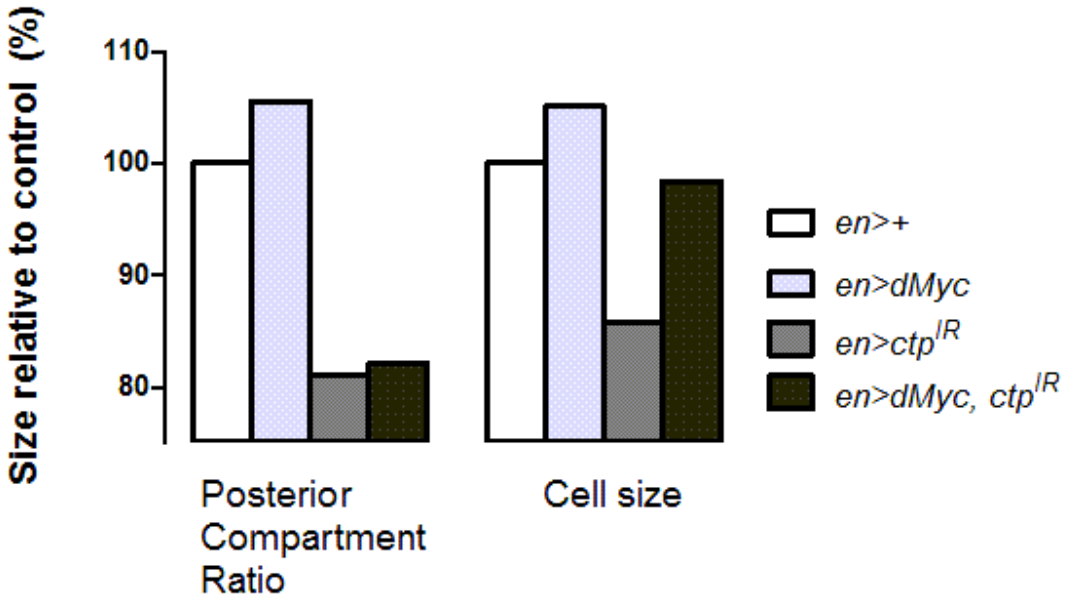

Figure 2.13. Ctp-knockdown small cell size, but not wing size, is restored by exogenous dMyc expression. Comparative effects of Ctp-depletion and dMyc overexpression on cell size and PCR. *en* > + values are standardized to 100%.

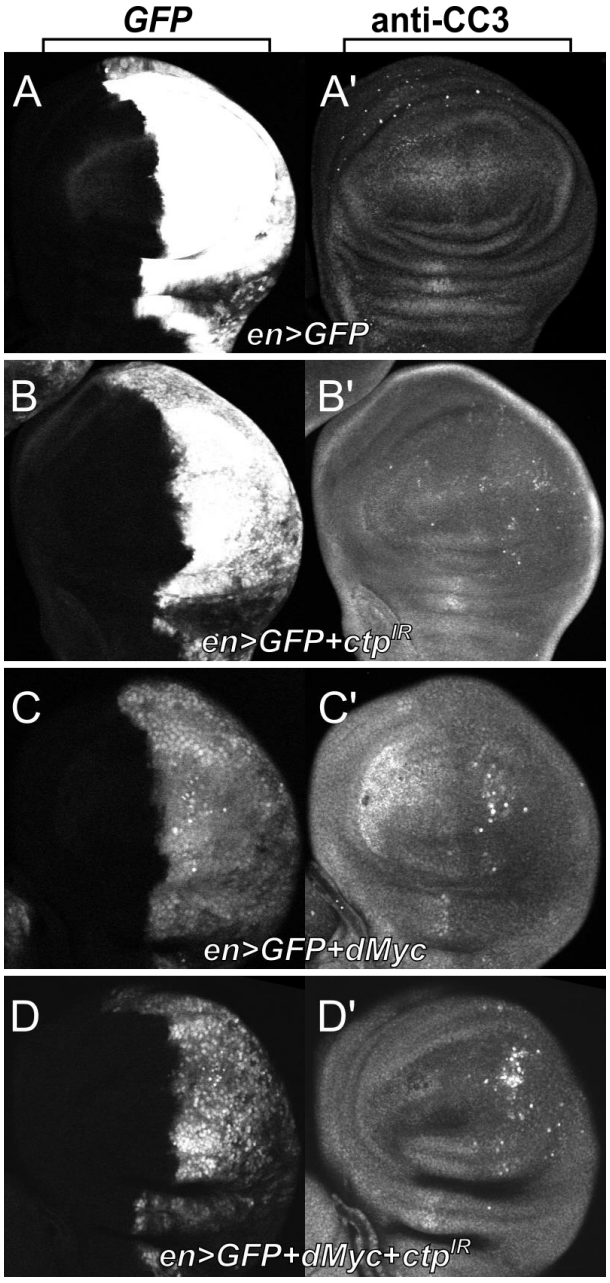

Figure 2.14. dMyc expression enhances the amount of apoptosis in Ctp depleted wings. Confocal images of L3 wing discs of indicated genotypes for cleaved Caspase-3 (cC3) (**A'-D'**). Co-expressed *UAS-GFP* transgene marks the posterior compartment (**A-D**).

highest intensity staining for cC3, supporting our hypothesis that exogenous dMyc expression is unable to rescue Ctp-depleted small wings due to an elevation of apoptosis. In sum these data suggest that reduced cell size contributes significantly to the undergrowth of *en>ctp-IR* adult wings and that reduced levels of dMyc are central to this phenotype. Furthermore, these data potentially link *ctp* to one or more of the transcriptional, translational and post-translational mechanisms that control dMyc levels in disc cells[207-209].

Ctp is dispensable for multiple signaling pathways but genetically interacts with Yorkie.

Developing imaginal discs are exposed to signals from an array of conserved developmental signaling pathways, some of which are proposed to affect dMyc levels or activity (e.g. Notch and Wg)[210]. Hence, a panel of reagents that detect activity changes in a variety of major cell pathways known to be active in the larval wing were assayed in *en>ctp-IR* discs (Figures 2.15 and 2.16). These included *Su(H)-lacZ* and *E(spl)-mß-CD2* (Notch pathway)[211,212], *PCNA-GFP* and *E2f1-lacZ* (E2F/Rb pathway)[213,214], *EcRE-lacZ* (EcR pathway)[215], *Stat-10xGFP* (Jak-Stat pathway)[216], and antibodies to the intracellular domain of Notch (N-icd), phospho-Mad (pMad), Wingless (Wg), the Wg/Notch regulated transcription factor Cut[217], the EGFR pathway component diphospho-Erk (dpErk), and the G1/S regulator CyclinE [218]. None of these markers were evidently altered in the posterior domain of *en>ctp-IR* discs (Figure 2.15C-J, K, M) or ctp^{ex3} clones in the eye disc (Figure 2.15L, N).

Notably, we did find that markers of the JNK MAP kinase signaling pathway were elevated in the posterior domain of *en>ctp-IR* discs. Both an antibody to phosphorylated

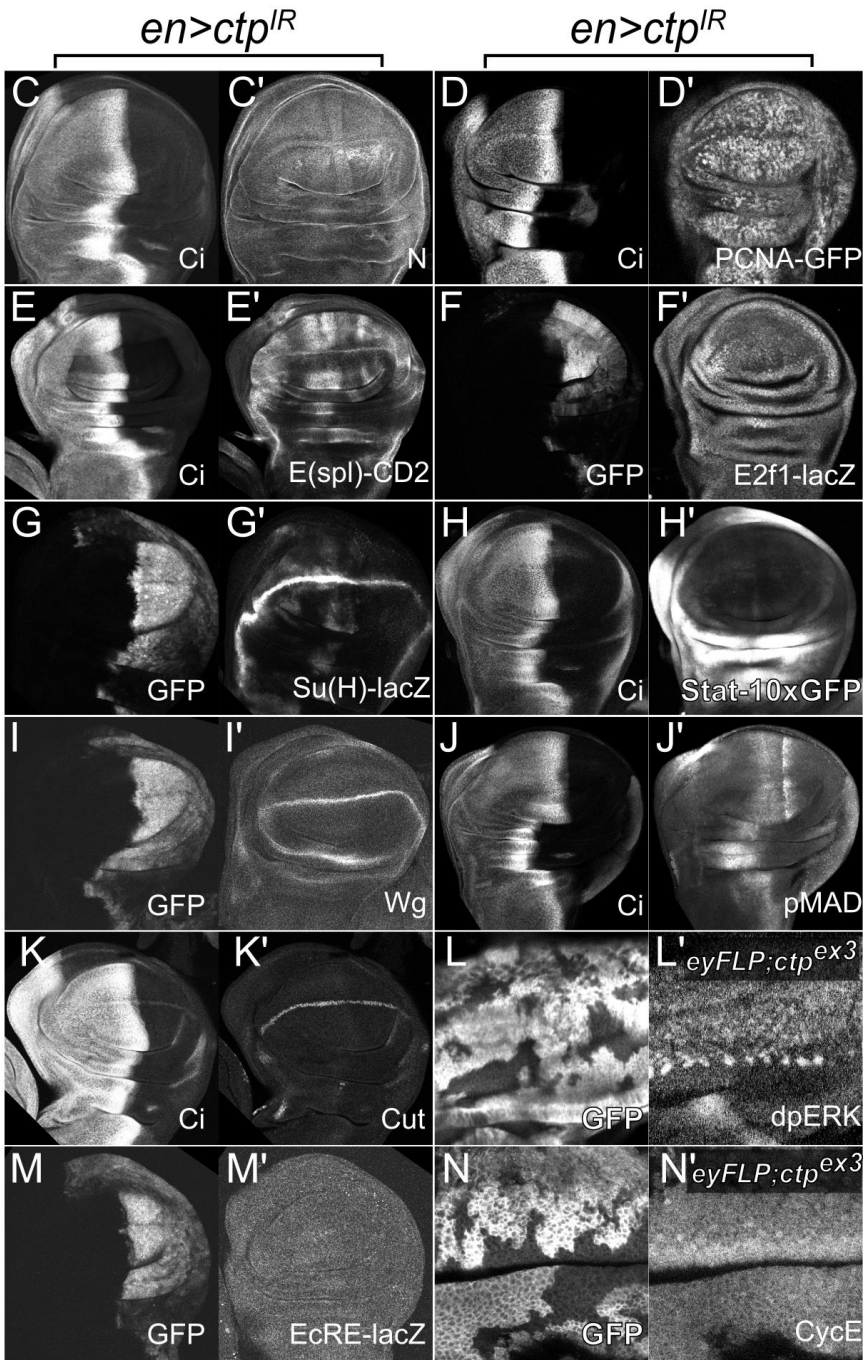

Figure 2.15. Effect of Ctp loss on a panel of disc cell proliferation and/or growth pathways. (**C–K, M**) Confocal images of L3 wing discs with Ctp depleted in the posterior domain (right-side in all images, with Ci marking the anterior domain (**C–E,H,J,K**) or transgenic *UAS-GFP* marking the posterior domain (**F,G,I,M**)) and analyzed for each of

the indicated factors: (**C′**) anti-Notch (N) to detect the C-terminal fragment of the Notch receptor; (**D′**) the E2F-activity reporter *PCNA-GFP*; (**E′**) the Notch-activity reporter *E(spl)mβ-CD2*; (**F′**) the *E2f1-lacZ* enhancer trap; (**G′**) the Notch-activity reporter *Su(H)-lacZ*; (**H′**) the Jak-Stat activity reporter *Stat10x-GFP*; (**I′**) anti-Wingless (Wg) to detect the ligand of the Wg/Wnt pathway; (**J′**) anti-phospho-Mothers against decapentaplegic (Mad) to detect signaling through the Dpp pathway; (**K′**) anti-Cut, a target of both Wg and Notch; (**M′**) the ecdysone receptor (EcR) reporter activity *EcRE-lacZ*. (**L,N**) L3 eye discs mosaic for ctp^{ex3} clones marked by the absence of GFP (**L,N**): (**L′**) anti-diphospho-Erk to detect signaling downstream of the *Drosophila* epidermal growth factor receptor (DER); (**N′**) anti-CyclinE (CycE), which is rate-limiting for progression into S-phase.

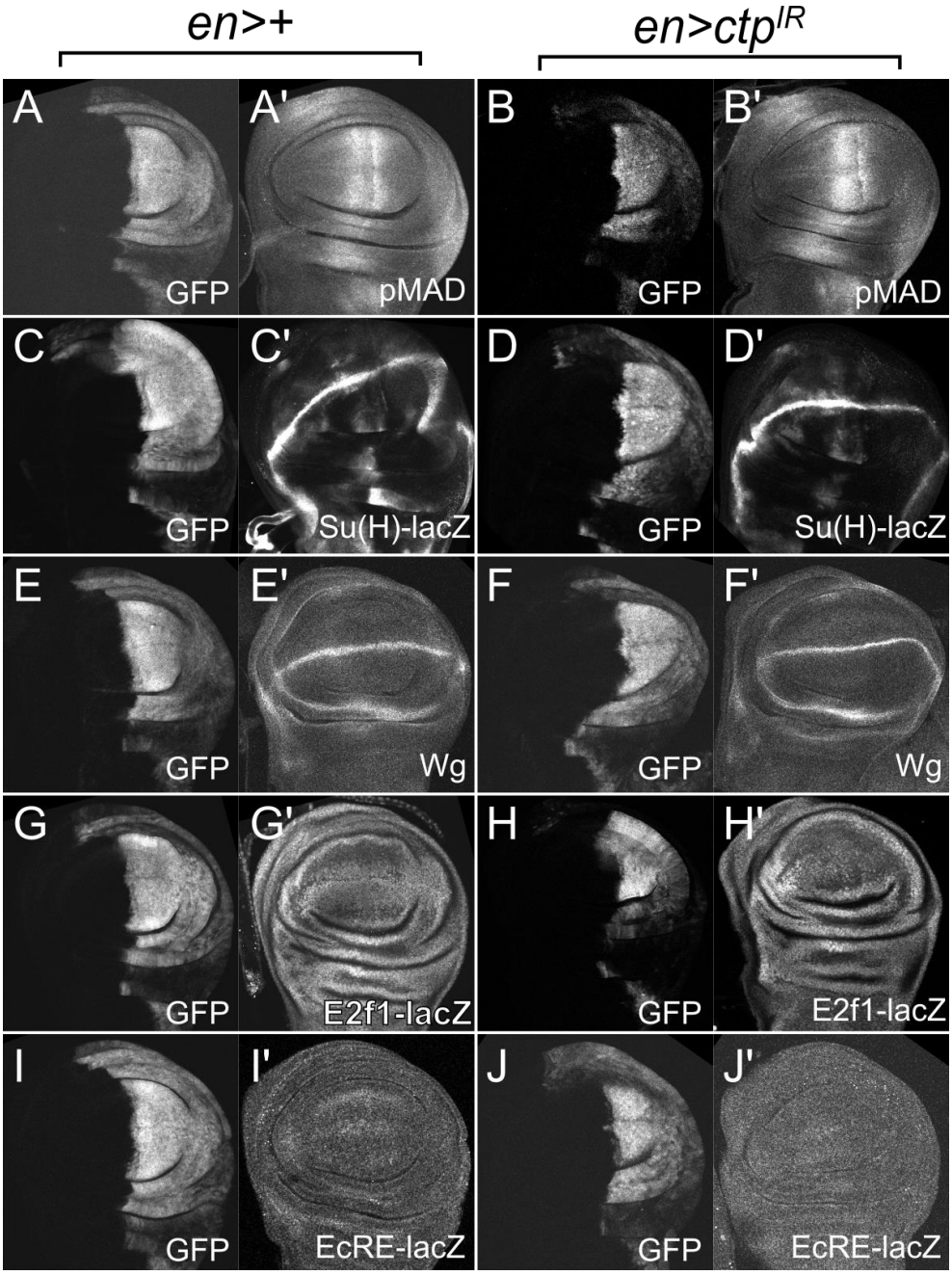

Figure 2.16. Effect of Ctp loss on a panel of proliferation and/or growth pathways. Paired confocal images of control (**A,C,E,G,I**) and Ctp-depleted (**B,D,F,H,J**) L3 wing discs analyzed for each of the indicated factors: (**A-B**) anti-phospho-MAD (pMAD), (**C-D**) *Su(H)-lacZ*, (**E-F**) anti-Wg, (**G-H**) *E2F1-lacZ*, and (**I-J**) *EcRE-lacZ*. Transgenic *UAS-GFP* marks the posterior domain.

JNK (pJNK), which indicates JNK-pathway activation, and *puckered-lacZ* (*puc-lacZ*), a downstream transcriptional target of JNK-pathway activation, were elevated in the posterior compartment of *en>ctp-IR* wing discs (Figure 2.17 and 2.18). The JNK pathway is activated through multiple upstream receptor tyrosine kinases, which act through several intermediary kinases to phosphorylate Basket (Bsk or D-JNK) that, in turn, can phosphorylate the transcription factors D-Jun and Fos or cytosolic targets. JNK pathway activation is typically noted as an indicator of cellular stress and is associated with the promotion of apoptosis through the transcriptional activation of the pro-apoptotic factor Reaper (Rpr), but in certain contexts JNK can also drive growth through poorly understood alternate mechanisms [219-222]. Despite the elevation of JNK signaling in *en>ctp-IR* wings, when we blocked JNK signaling genetically via the transgenic overexpression of either dominant negative Bsk (dnBsk), Puckered (a phosphatase which deactivates the JNK pathway), or AvrA (a *Salmonella typhrimurium* protein that potently blocks JNK activation), we did not observe any rescue of the *en>ctp-IR* small wing phenotype (data not shown) [223, 224]. These data suggest that while the JNK pathway is activated in Ctp depleted wings, it is not the main force driving wing blade undergrowth.

In contrast to the lack of genetic interaction between Ctp and JNK in the wing, we found that a loss-of-function allele of the pro-growth gene *yorkie* (*yki*) dominantly enhances the *en>ctp-IR* small-wing phenotype (Figure 2.19A). The Yki protein is the main target of the Hippo pathway and acts as nuclear co-activator for Scalloped (Sd)-dependent induction of Hippo target genes, which include *dMyc*[34]. An enlarged-wing phenotype produced by expression of a *UAS-yki-V5* transgene from the *en>Gal4* driver is significantly suppressed by co-depletion of Ctp

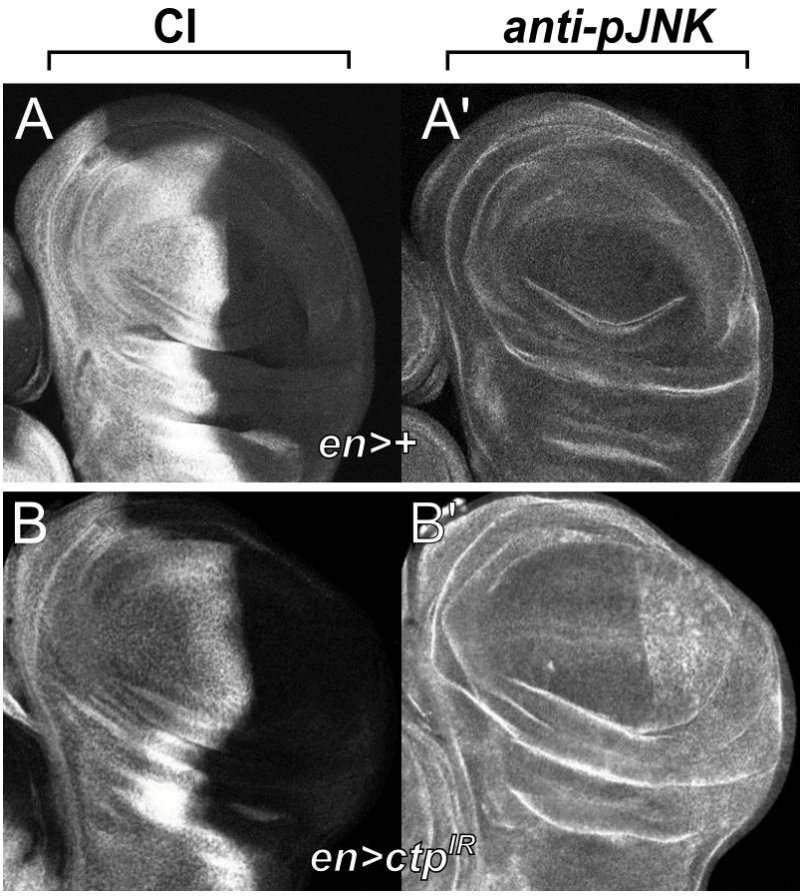

Figure 2.17. Effect of Ctp loss on phosphorylated Bsk. (**A-B**) Confocal images of L3 wing discs in control (*en* > +) (**A, A'**) or in Ctp depleted (*en* >*ctp*^{IR} ; posterior domain, right-side) (**B, B'**) with Ci marking the anterior domain and stained for anti-pJNK antibody indicating phosphorylated Bsk.

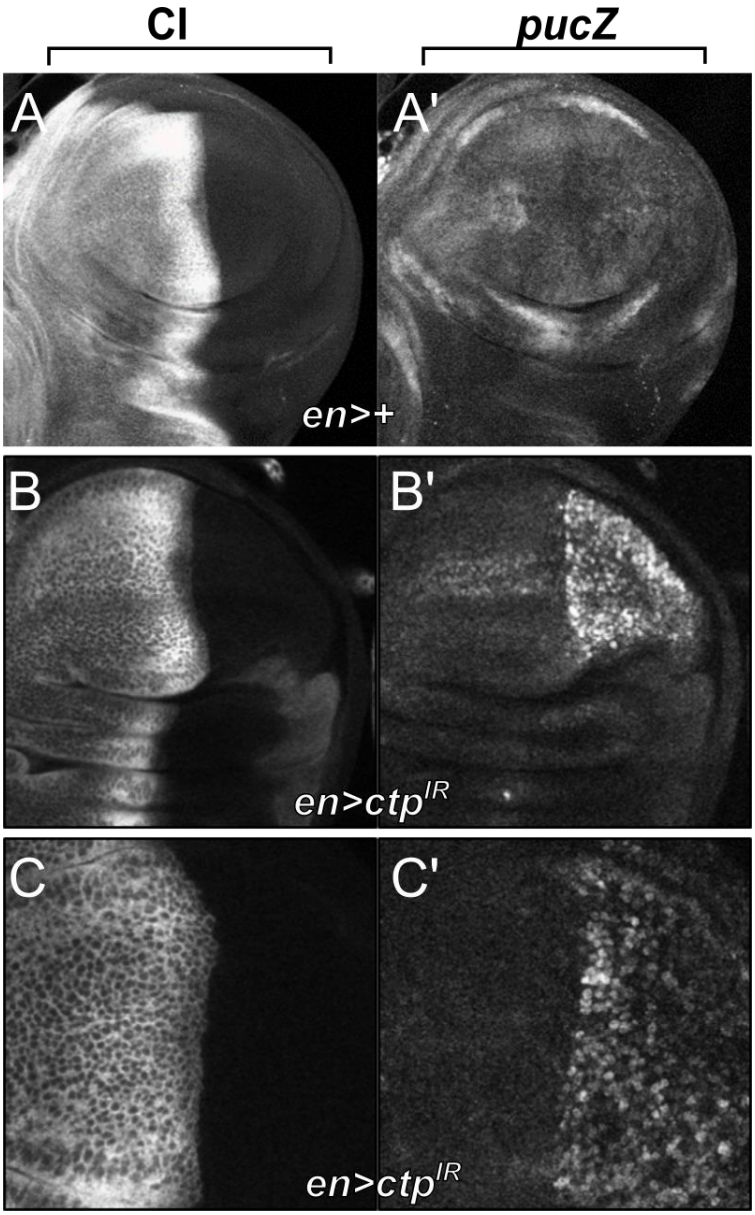

Figure 2.18. Effect of Ctp loss on *puckered* transcriptional reporter. (**A-B**) Confocal images of L3 wing discs in control (*en* > +) (**A, A'**) or in Ctp depleted (*en* >*ctp*^{IR} ; posterior domain, right-side) (**B, B', C, C'**) with Ci marking the anterior domain and stained with anti-ßgal antibody indicating activity of the *puc-lacZ* reporter.

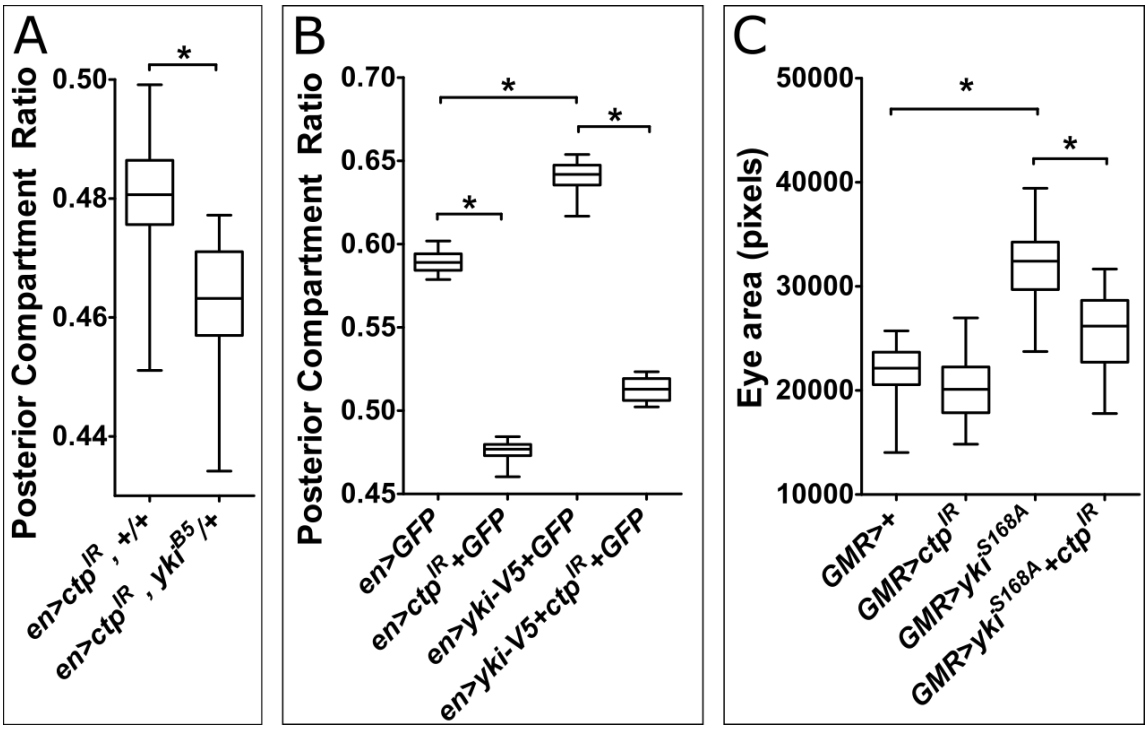

Figure 2.19 Genetic interactions between *ctp* and *yki* in control of wing and eye size. Box-plot representation of the effect of (A) the yki^{B5} allele (n=13), *$p<0.0001$ or (B) a *UAS-yki-V5* transgene (n=22, with GFP n=12, with ctp^{IR}+*GFP*), on PCR in control (*en>GFP*, n=12) or Ctp-depleted (*en>*ctp^{IR}+*GFP*, n=18) adult wings. All *p *values* <0.0001. (C) Box-plot showing the effect of *GMR-Gal4* driven Ctp-depletion at 29°C among eye cells on final adult eye area (2-dimensional *en face* circumference) and the ability of the ctp^{IR} transgene to suppress this metric in the background of the *GMR-*yki^{S168A} hyperactive allele. SEMs are indicated; (GMR, n=24; GMR> ctp^{IR}, n=22; GMR> yki^{S168A}, n=41; GMR> yki^{S168A} + ctp^{IR}, n=24), both *p *values*<0.0001.

(Figure 2.19B). Likewise *ctp* knockdown can suppress eye overgrowth induced by *GMR-Gal4* driven expression of Yki^{S168A}, a hyperactive phospho-mutant form of Yki (Figure 2.19C). Together, this evidence points towards a functional interaction between *ctp* and *yki* in disc cells destined to form the wing blade and eye.

Ctp loss reduces *thread/diap1* transcription

The proposed dynein-independent role of mammalian LC8/Ctp as a dimerization hub for cytoplasmic and nuclear complexes (reviewed in [167, 171]) suggests that Ctp could be involved in modulating activity of the Hippo pathway in vivo. This hypothesis was tested by assessing expression of the *thread(th)/diap1* gene, a key anti-apoptosis factor and canonical Yki transcriptional target[93, 225, 226] in Ctp-depleted wing pouch cells. The steady-state level of Diap1 protein is reduced but not eliminated in the posterior compartment of *en>ctp-IR* larval discs (Figure 2.20A-B), which could explain the mild increase in cC3 signal observed in *ctp* knockdown discs (see Figure 2.6A-A') as well as the sensitivity of *ctp* knockdown discs to the levels of the pro-apoptotic dMyc expression (Figure 2.14) The *th-lacZ* reporter, which is a Yki-responsive 'enhancer trap' of the bacterial *ß-galactosidase* gene inserted into the endogenous *th/diap1* locus[227], also shows reduced expression in Ctp-depleted and ctp^{ex3} mutant cells in the wing pouch (Figure 2.20C-D, K-L and Figure 2.21E-F). A series of successively smaller promoter fragments of the *th/diap1* promoter driving *lacZ* have been used to define a minimal *Hippo response element* (*HRE*) that responds to Yki hyperactivation in larval disc cells[160]. Expression of two of these reporters, *2b2-lacZ* and *2b2c-lacZ*, is strongly reduced in response to *ctp* knockdown (Figure 2.20E-H). Baseline expression of the minimal *HRE-lacZ* is fairly low, but its expression is also

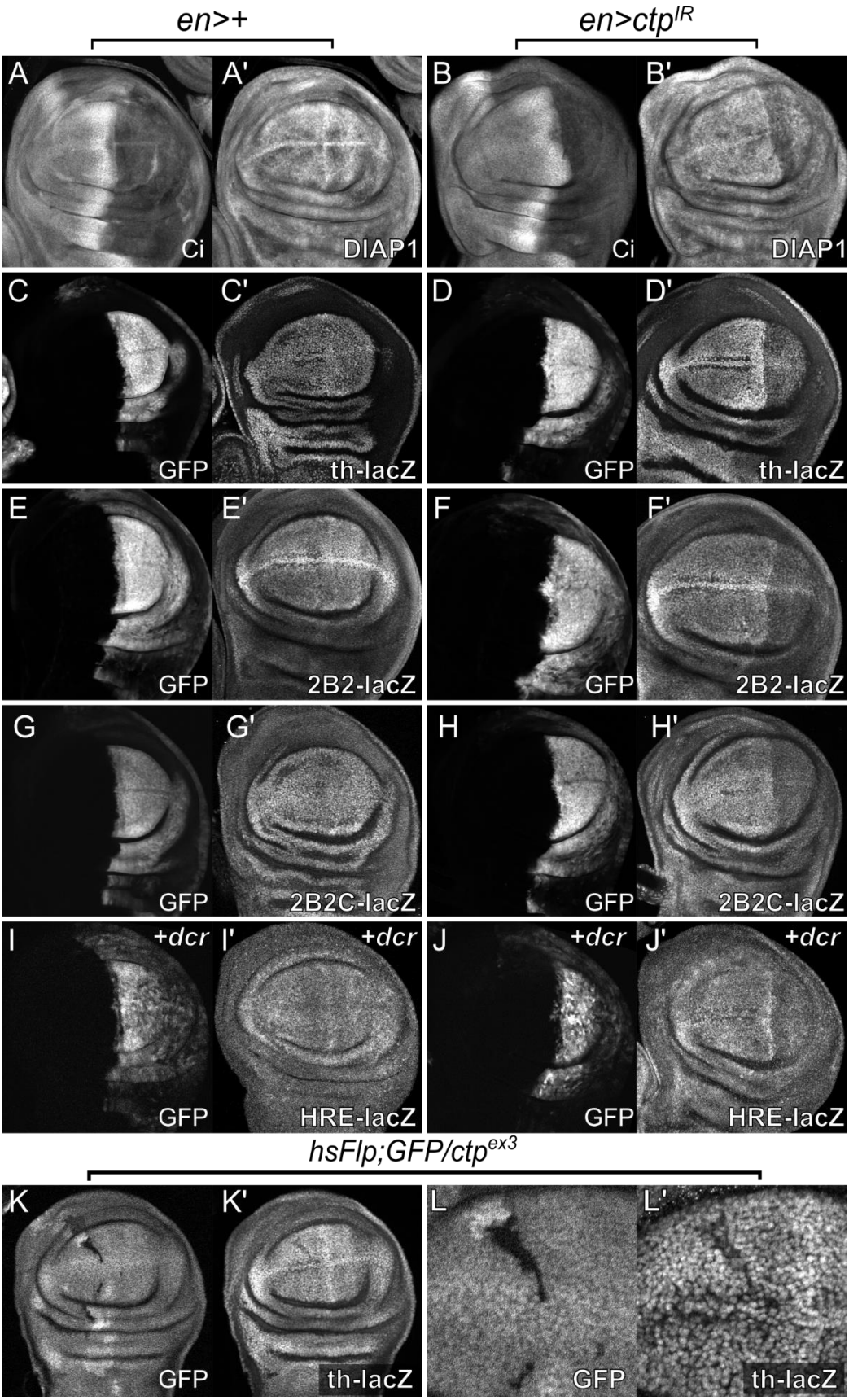
en>+
en>ctp^IR
A
Ci
A'
DIAP1
B
Ci
B'
DIAP1
C
GFP
C'
th-lacZ
D
GFP
D'
th-lacZ
E
GFP
E'
2B2-lacZ
F
GFP
F'
2B2-lacZ
G
GFP
G'
2B2C-lacZ
H
GFP
H'
2B2C-lacZ
I
+dcr
GFP
I'
+dcr
HRE-lacZ
J
+dcr
GFP
J'
+dcr
HRE-lacZ
hsFlp;GFP/ctp^ex3
K
GFP
K'
th-lacZ
L
GFP
L'
th-lacZ

Figure 2.20. Ctp supports expression of the *diap-1/thread* locus. Confocal images of control (*en > +* in (**A, C, E, G, I**)) or Ctp-depleted (*en > ctp^{IR}* in (**B, D, F, H, J**)) L3 wing discs stained for Ci (anterior domain) (**A, B** and Diap1 proteins (**A', B'**), or with anti-ßgal to detect expression of endogenous *th-lacZ* (**C',D'**) as well as sequentially smaller Yki-responsive promoter elements *2B2-lacZ* (**E', F'**), *2B2C-lacZ* (**G', H'**), and *Hippo response element (HRE)-lacZ* (**I', J'**). Co-expressed *UAS-GFP* transgene marks the posterior compartment (**C–J**), and the addition of *dicer2 (+dcr)* was used to enhance *ctp* knockdown in (**I, J**). Note decreased expression of all three Diap1 markers in cells depleted of Ctp in the posterior (right-side) compartment. (**K, L**) Heat-shock induced GFP-negative *ctp^{ex3}* null clones in an L3 wing disc in the background of the *th-lacZ* reporter. Area in L is a magnified view of the anterior clone in K. Note the drop in *th-lacZ* expression in *ctp^{ex3}* cells.

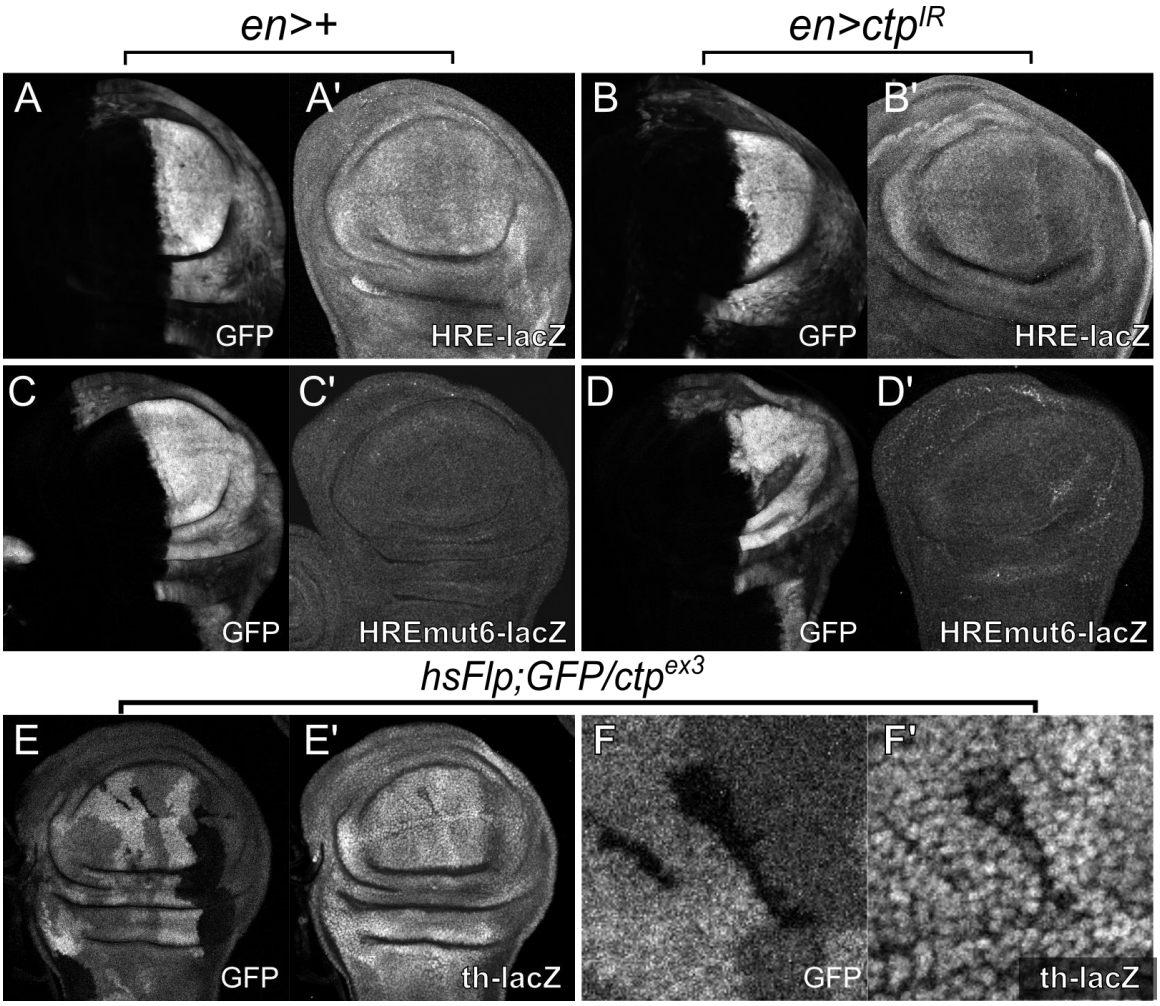

Figure 2.21. Ctp loss reduces *th* reporter transcription but does not alter Sd-binding site mutant reporter. Paired confocal images of control (*en>+* in **A, C**) and Ctp-depleted (*en>ctp^{IR}* in **B, D**) L3 wing discs stained for ßgal to visualize expression of *HRE-lacZ*. (**A-B**) and *HREmut6-lacZ* (**C-D**). (**E-F**) Confocal image of an L3 wing disc carrying heat-shock induced *ctp^{ex3}* clones (marked by the absence of GFP) immunostained with ßgal to detect expression of *th-lacZ*.

reduced in Ctp-depleted cells (Figure 2.20I-J with co-expression of *dicer2* to enhance *ctp* knockdown, and Figure 2.21A-B, without *dicer2*). Furthermore, a mutant version of the minimal *HRE* lacking the Sd-binding site necessary for Yki dependent transcription (*HREmut6-lacZ*)[160] has lowered expression and no longer responds to loss of *ctp* (Figure 2.21C-D). In sum, these data provide evidence that Ctp is required to support transcription of the Yki target gene *thread/diap1* in larval wing pouch cells.

Ctp loss elevates transcription of the *bantam* microRNA locus

Analysis of a second well-validated Yki target, the pro-growth *bantam* (*ban*) microRNA, was carried out to determine whether the requirement for Ctp is unique to *th/diap1* transcription, or can be extended to other well-validated Yki-target genes as well. Two *ban* transcriptional reporter transgenes were used for these studies: *ban3-GFP*, which contains a large proximal fragment of the *ban* promoter driving GFP, and *brC12-lacZ*, a Yki-responsive 410-bp promoter fragment that lies within the *ban3* region and contains two Yki-association sites[228],[199]. Surprisingly, and in polar contrast to *th/diap1*, expression of both *ban* reporters was strongly elevated in Ctp-depleted wing pouch cells (Figure 2.22A-D). The *brC12* reporter is also strongly induced in ctp^{ex3} null wing pouch clones (Figure 2.22E-F; two independent discs are shown with multiple ctp^{ex3} clones), confirming the link between endogenous Ctp and *ban* promoter activity. Thus, while Ctp normally supports expression of the Yki-response gene *th/diap1* in wing pouch cells, it has the inverse role of repressing transcription of the Yki-responsive locus *ban*. A third classic Yki reporter, *expanded-lacZ*, did not respond to Ctp-loss in otherwise wildtype larval wing cells or in those overexpressing Yki (Figure 2.23).

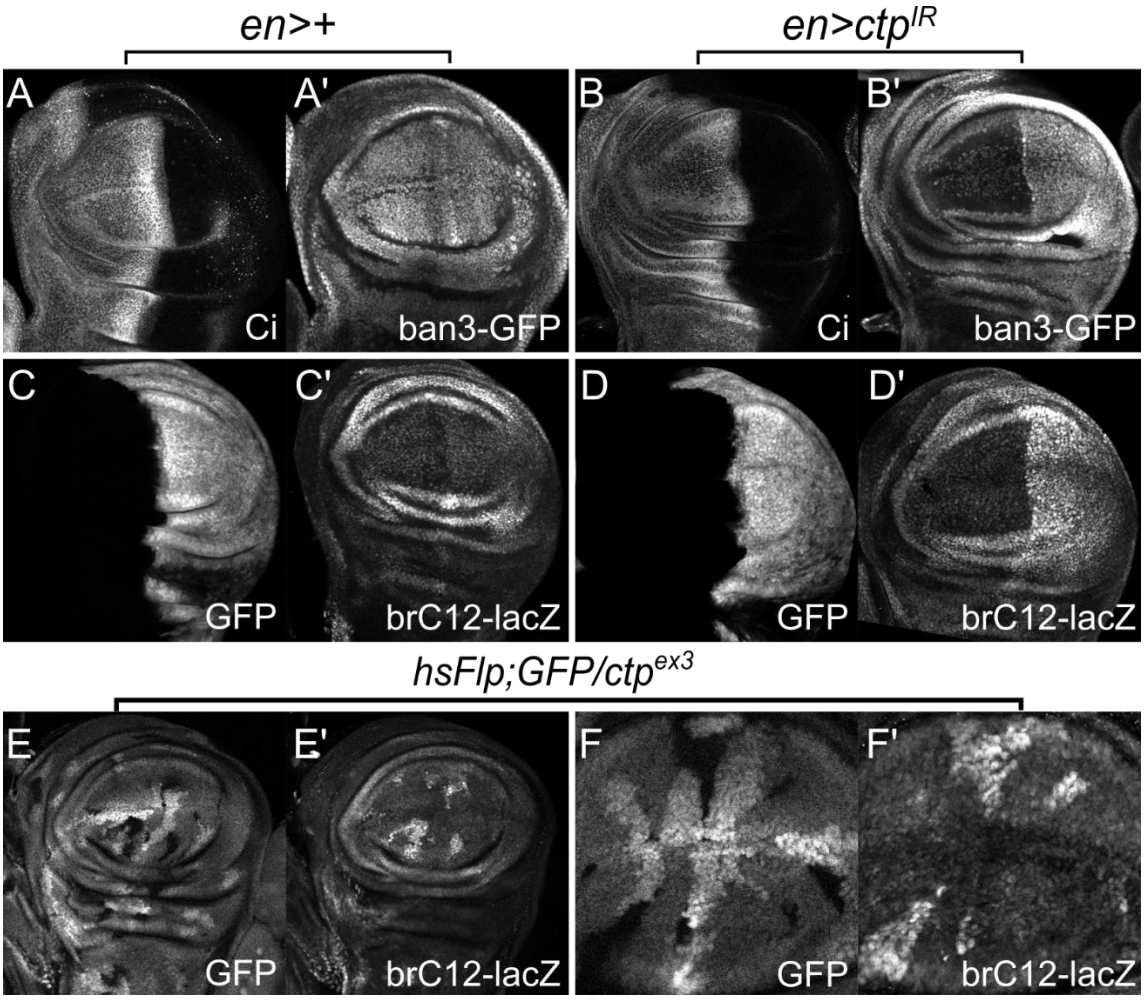

Figure 2.22. Ctp represses expression of the *bantam* locus. Confocal images of control (*en* > + in (**A,C**)) or Ctp-depleted (*en* > ctp^{IR} in (**B, D**)) L3 wing discs in the background of the*bantam* reporters *ban3-GFP* (**A', B'**) or *brC12-lacZ* (**C', D'**). Anti-Ci marks anterior cells in (**A, B**) while a co-expressed *UAS-GFP* transgene marks posterior cells in (**C,D**). Note increased expression of both *ban3* and *brC12* upon depletion of Ctp in the posterior (right-side) compartment. (**E, F**) Heat-shock induced GFP-negative ctp^{ex3} null clones in an L3 wing disc in the background of the *brC12-lacZ* reporter. Two different discs are shown. Note consistently elevated *brC12-lacZ* expression inctp^{ex3} cells, particularly within the pouch.

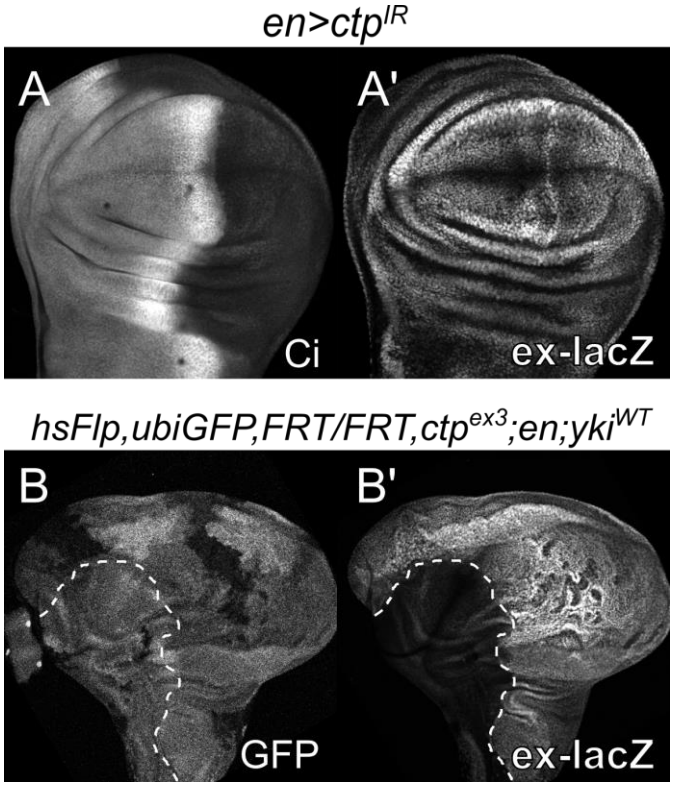

Figure 2.23. Ctp loss does not affect physiologic or Yki-induced expression of *expanded*. Confocal images of *expanded-lacZ* (*ex-lacZ*) expression in (**A-A'**) a *en>ctp*^{IR} L3 wing disc with Ctp depleted from the Ci-negative posterior domain, or (**B-B'**) a L3 wing disc with GFP-negative *ctp*^{ex3} clones embedded within the control anterior domain (left of dotted line in B) or the Yki-overexpressing posterior domain (right of dotted line in B).

Epistatic relationships between *yki* and *ctp* in control of *th/diap1* and *ban* reporters

The opposing effects of Ctp loss on the minimally Hippo-responsive *th/diap1* and *ban* promoter fragments imply an Yki-dependent mechanism links Ctp to expression of these genes. To test these relationships, the effect of Ctp loss on *brC12-lacZ* and *th-lacZ* were reassessed either in the presence of overexpressed Yki, or RNAi depletion of endogenous Yki. Elevated *brC12-lacZ* expression upon Ctp-depletion is suppressed by concurrent RNAi depletion of Yki, indicating that Yki is required for maximal induction of *ban* following Ctp loss (Figure 2.24A-D). Furthermore, expression of a *UAS-yki* transgene does not obviously elevate induction *brC12-lacZ* above the level observed in ctp^{ex3} null clones (Figure 2.25). Indeed within the center of the L3 wing pouch, *brC12-lacZ* is more highly expressed in *ctp* null cells than in those expressing a *yki* transgene (Figure 2.25B; compare centrally located ctp^{ex3} clone vs. surrounding Yki-expressing cells in the posterior domain). Thus, Ctp-depletion robustly activates *brC12-lacZ* expression in pouch cells via a mechanism that requires Yki. Because overexpression of Ctp in wing pouch cells via *UAS-ctp* has no effect on wing growth or Yki readouts (data not shown), the epistatic relationship between *ctp* and *yki* on the *diap-1* promoter is more challenging to assess. However, transgenic expression of *UAS-yki* remains capable of inducing *th-lacZ* expression in wing disc cells depleted of Ctp (Figure 2.26A-D), suggesting that *ctp* acts either upstream or parallel to *yki*. Thus, manipulating Yki levels modifies the effects of Ctp depletion on both *th/diap1* and *ban* reporters, which provides some evidence that *ctp* inversely modulates Yki activity toward each of these target genes.

Wing disc cells lacking the Yki-binding protein Myopic also show selective effects on Yki-target genes[229]. Myopic tethers Yki to endosomes for eventual degradation in

lysosomes, and its loss causes Yki to accumulate, leading to induction of *ex* and *ban* but not *thread/diap1*[229]. To test whether Ctp is required for Yki cytoplasmic trafficking in a manner similar to Myopic, endogenous Yki and a V5-epitope tagged form of Yki were visualized in Ctp-depleted pouch cells (Figure 2.27). Yki steady state levels and nuclear:cytoplasmic distribution are unaltered in Ctp-depleted cells, indicating that wholesale changes in Yki protein dynamics and trafficking in *ctp* mutant wing pouch cells are unlikely to drive downstream effects on *th/diap1* and *ban* expression

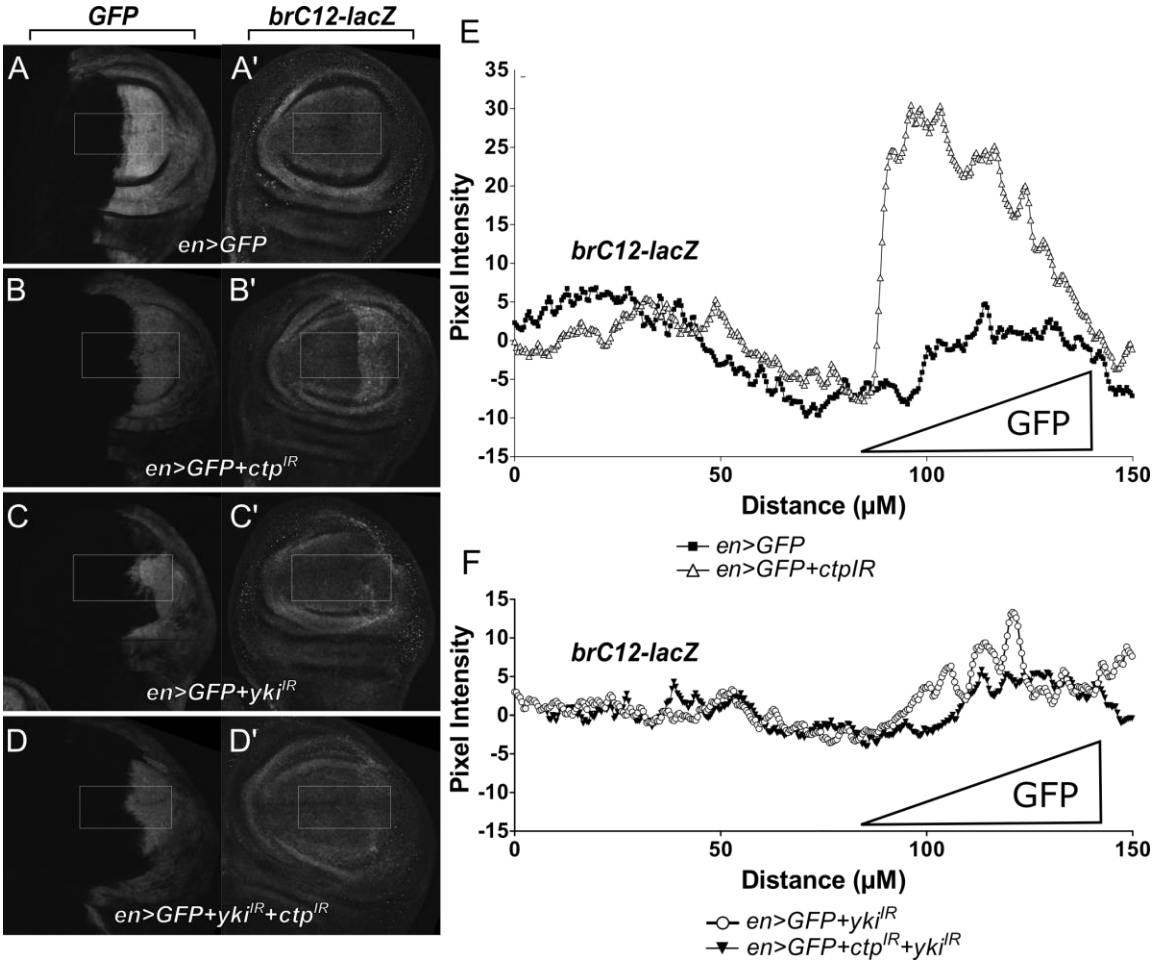

Figure 2.24. Epistatic relationship between Yki and Ctp-regulated *bantam*. (**A–D**) Anti-βgal staining of L3 wing discs to detect *brC12-lacZ* expression in the background of (**A'**) control $en > GFP$; (**B'**) Ctp-depleted, $en > GFP + ctp^{IR}$; (**C'**) Yki-depleted, $en > GFP + yki^{IR}$; or (**D'**) simultaneous depletion of Yki and Ctp, $en > GFP + ctp^{IR} + yki^{IR}$. Co-expressed *UAS-GFP* transgene marks the posterior compartment (**A–D**). (**E,F**) Quantitation of the effect of Ctp and/or Yki depletion on *brC12-lacZ* expression determined by measuring anti-βgal pixel fluorescence intensity across the anterior:posterior (A:P) boundary (left-to-right) in the white boxes in (**A–D**).

GFP-expression domain is indicated. Average fluorescence of the anterior domain was set to zero (0). (**E**) ■ *en > GFP*, Δ *en > GFP + ctp^{IR}*. (**F**) ○ *en > GFP + yki^{IR}*, ▼ *en > GFP* + yki^{IR} + *ctp^{IR}*.

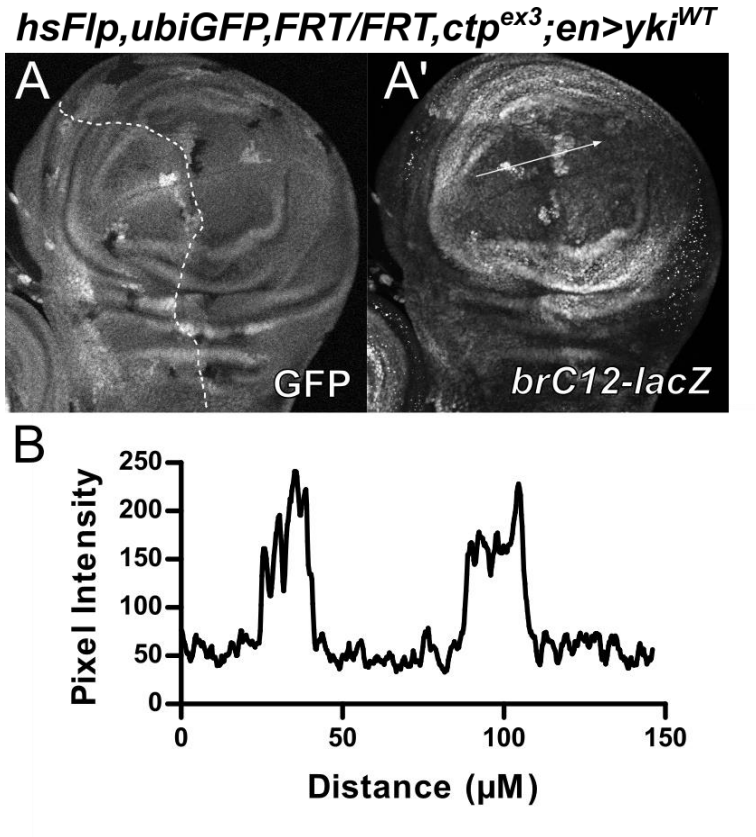

Figure 2.25. Relative effect of Yki gain and Ctp loss on *brC12* expression. (**A-A'**) Confocal image of an L3 wing disc bearing GFP-negative *ctp*^{ex3} null clones embedded within the control anterior domain (left of dotted line in A) or the Yki-overexpressing posterior domain (right of dotted line in A). The disc has been stained to visualize ßgal expression from the *brC12-lacZ* reporter. Arrow in A' corresponds to the X-axis of the anti-ßgal fluorescence (i.e. pixel) intensity graph in (**B**), which suggests that *brC12-lacZ* induction in *ctp*^{ex3} clones in both compartments is roughly equivalent.

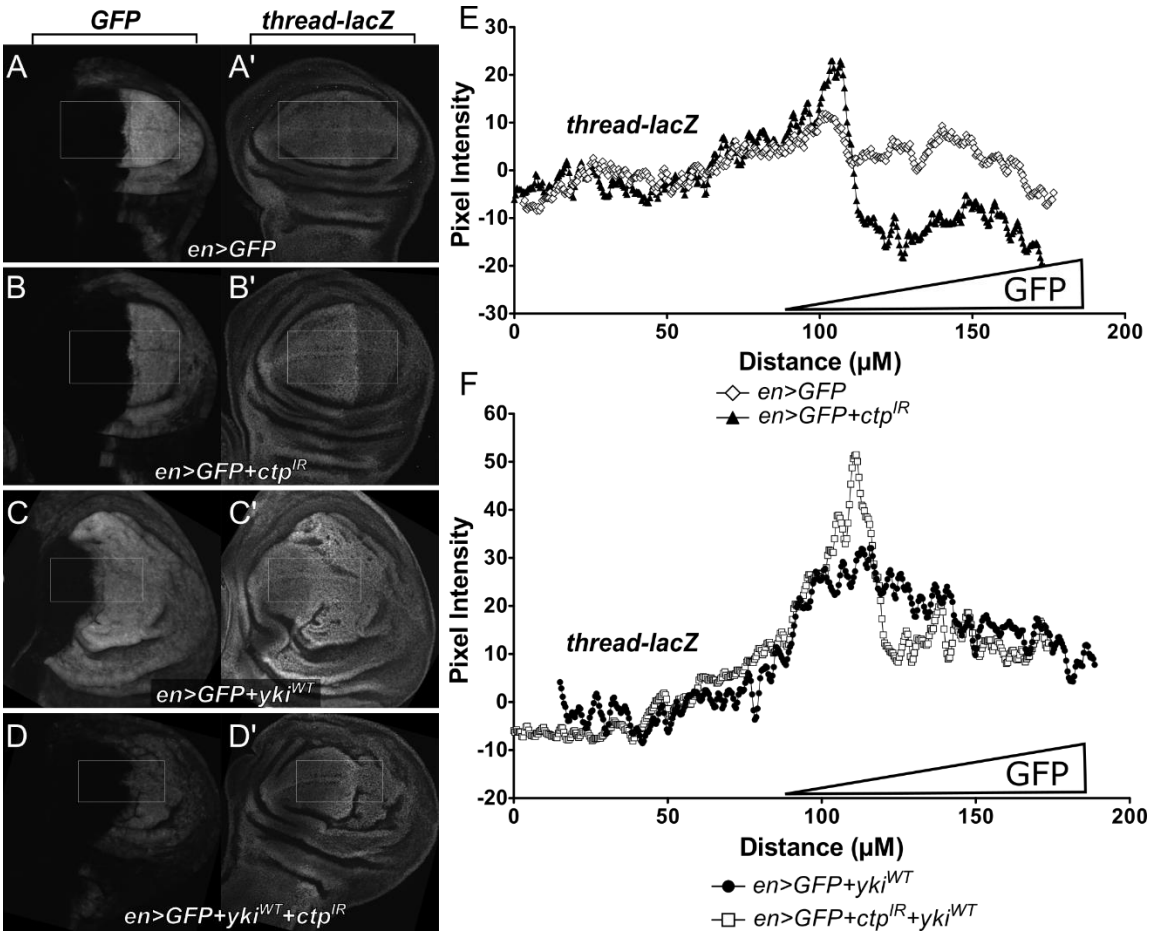

Figure 2.26. Epistatic relationship between Yki and Ctp-regulated *thread*. (**G–J**) Anti-ßgal staining of L3 wing discs to detect *th-lacZ* expression in the background of (**G'**) control $en > GFP$; (**H'**) $en > GFP + ctp^{IR}$ Ctp-depleted; (**I'**) $en > GFP + yki^{WT}$ Yki-overexpression; or (**J'**) simultaneous expression of Yki and depletion of Ctp, $en > GFP + yki^{WT} + ctp^{IR}$. Co-expressed *UAS-GFP* transgene marks the posterior compartment (**G–J**). (**K,L**) Quantitation of the effect of Ctp depletion and/or Yki overexpression on *th-lacZ* expression determined by measuring anti-ßgal pixel fluorescence intensity across the anterior:posterior (A:P) boundary (left-to-right) in the white boxes in (**G–J**) ◊ $en > GFP$, ▲ $en > GFP + ctp^{IR}$. (L) ● $en > GFP + yki^{WT}$, □ $en > GFP + yki^{WT} + ctp^{IR}$.

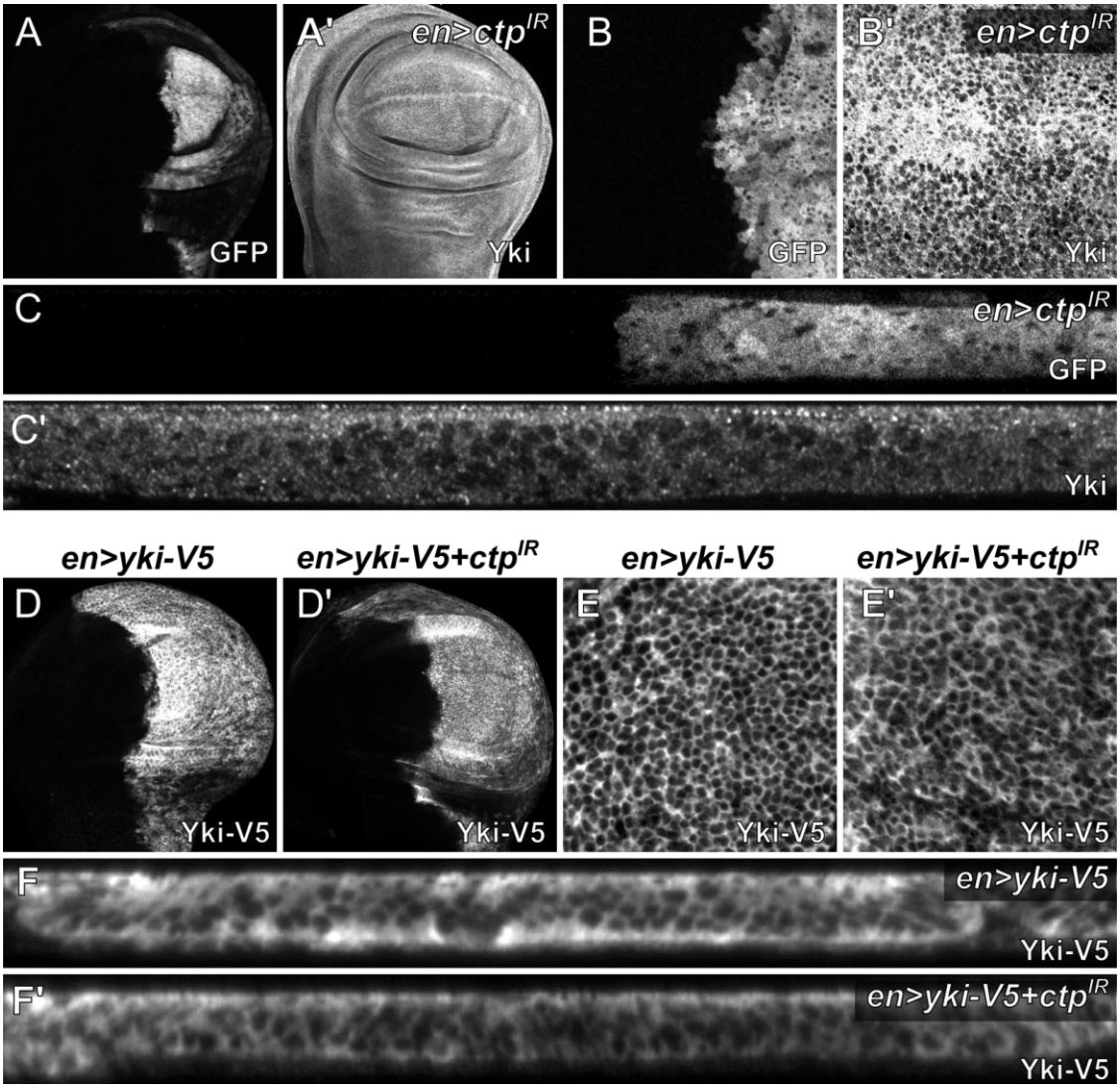

Figure 2.27. Ctp is not required to control the steady-state distribution or levels of Yki within wing disc cells. Projection images of immunolocalization of (**A-C**) endogenous Yki or (**D-F**) transgenic V5-tagged Yki in L3 wing discs lacking Ctp in posterior cells (*en>ctp*^{IR}). GFP and V5 respectively mark the posterior domains in A-C and those in D-F. Magnified and transverse sections along the dorsoventral axis of each pouch are shown in B-C and E-F.

DISCUSSION

Here we define a role for the *Drosophila* protein Ctp, a member of the LC8 protein family, in regulating expression of two Hippo target genes, *th/diap1* and *bantam*, in larval disc cells. Ctp is a member of a highly conserved family (Ctp, Dlc1, and DYNNL1/DYNNL2) of small proteins that were first identified as components of cytoplasmic dynein motors[165, 169, 170] but are now recognized to also act as interaction hubs for many proteins with roles in diverse processes such as autophagy, signal transduction, cell:cell adhesion, and transcription[167, 171, 172] . Largely because of this diverse set of potential effector pathways, the role of LC8 proteins in specific cellular and developmental contexts is not particularly well defined. We find that larval wing compartments depleted of Ctp by RNAi give rise to smaller compartments in the adult wing that are populated by smaller cells. Importantly, the Ctp-depleted larval precursors of these adult wing cells express markers of both M and S-phase, and thus do not appear to undergo cell cycle arrest as reported elsewhere. Parallel analysis with a *ctp* genomic allele confirms that Ctp supports clonal growth in the larval wing disc, particularly in the pouch, and that levels of the pro-growth transcription factor dMyc are reduced in *ctp* mutant epithelial cells. Testing candidate growth regulatory pathways active in the wing pouch uncovers a specific role for Ctp in regulating expression of genes regulated by the Hippo pathway target Yorkie (Yki), which is can positively regulate *dMyc* transcription. Activity reporters of two Yki-responsive genes, *thread/diap1* and *bantam*, each respond to *ctp* loss in pouch cells and these effects map to smaller regions of the *thread/diap1* and *bantam* promoters that contain Yki-responsive elements. Reduction of Yki activity enhances the Ctp-small wing phenotype and depletion of Ctp in turn blunts eye and wing growth driven by *yki*

transgenes. These data are consistent with Ctp normally regulating Yki activity and Yki-dependent growth in imaginal disc epithelia.

The effects of Ctp on growth and Yki-target gene transcription are complex and intriguing. While Ctp supports Yki activity on the *thread/diap1* promoter, it restricts activity of the *bantam* promoter, and has no effect on a third Yki reporter, *ex-lacZ*. Moreover transgenic expression of *ban* drives tissue overgrowth[230, 231] but upregulation of *ban* transcription in *ctp* mutant cells is paradoxically associated with tissue undergrowth. This apparent *ban* paradox may be explained by the finding that *ban* promotes tissue growth through dMyc[232, 233], so that Ctp-depleted cells with reduced dMyc levels may be resistant to *ban*-induced growth.

The opposing effects of Ctp on *thread/diap1* and *ban* transcription differ from core Hippo components, which affect these targets in a uniform way. The mechanism through which Ctp achieves its unique effects on *thread/diap1* and *bantam* is not known. Intriguingly human LC8 interacts with the Kibra protein, a conserved element of the Hippo pathway[190]. *Drosophila* Kibra forms a complex with Merlin and Expanded, and together these proteins promote phosphorylation of Warts[59]. However Warts uniformly represses expression of Yki-target genes, making it unlikely that Ctp acts via a Kibra-Warts axis to exert inverse effects on *thread/diap1* and *ban*. The Kibra-LC8 complex interacts with Sorting Nexin-4 (Snx4) to promote dynein-driven traffic of cargo between the early and recycling endosomal compartments[192]. Yki associates with endosomes[229] and endosomal traffic modulates levels of Yki protein and the transcription of its nuclear targets[71, 229]. However, Ctp loss does not obviously alter steady state levels of Yki or its distribution in wing disc cells (see Figure 2.27), and although vertebrate LC8 (aka

DYNLL1) is as a high-confidence interactor of the Yki vertebrate homolog Yap1[72, 73], an equivalent association was not detectable in cultured *Drosophila* S2 cells (data not shown).

In addition to cytoplasmic effects, Ctp/LC8 proteins also have nuclear roles in transcriptional control[167]. For example, LC8 interacts with the estrogen receptor (ER), can promotes ER activity, and is found with ER on the promoters of induced genes[189]. A recent study confirmed that ER dimerizes when bound to DNA and can activate target gene transcription either as a monomer or dimer[234]. *Drosophila* Ctp may thus interact with Yki, or alternatively with a nuclear effector in a distinct pathway that converges on Yki, to bias Yki promoter selectivity and /or to modulate formation of higher order transcriptional complexes with promoter-specific roles *in vivo*. Future studies will be required to identify Ctp interacting proteins within the Hippo pathway and to resolve the precise link between Yki and the multifunctional and highly conserved Ctp/LC8 protein.

MATERIALS AND METHODS

Fly Strains

All crosses were maintained at 25°C unless otherwise noted. Alleles used in these studies (Bloomington stock number indicated) are as follows: *thread-lacZ* (#12093), *ex697* (*ex-lacZ*, #44248), *e2f1rM729* (*e2f1-lacZ*, #34054), *UAS-yki-V5* (#28819), yki^{B5} (#36290), *mirr^{DE}-Gal4* (#29650), *EcRE-lacZ* (#4517), *Stat92E-10XGFP* (#26198) (courtesy of R.Read), *UAS-diap1* (#6657), *UAS-p35* (#5073), *UAS-dcr2;enGal4,UAS-GFP* (#25752) obtained from the Bloomington *Drosophila* Stock Center. *UAS-yki-IR* (v104523) and *UAS-ctp-IR* (v43116) were obtained from the Vienna Drosophila Resource Center (VDRC). Other alleles used were *ctp^{ex3}* (Gift of Bill Chia), *enGal4/CyO, HRE-lacZ, HREmut6-lacZ, 2B2-lacZ, 2B2C-lacZ* (all gift of D.J. Pan), *brC12-lacZ* (K. Irvine), *GMR-Gal4, UAS-ykiS168A:GFP* (K. Harvey), *E-spl(m)ß-CD2, Su(H)-lacZ, PCNA-GFP, ban-3xGFP, UAS-dMyc* (P. Gallant), *UAS-BskDN, UAS-puc, puc-lacZ, UAS-AvrA* (all gift of R. Jones), and *UAS-yki* (D.J. Pan).

Immunofluorescence Microscopy

Immunostaining and confocal microscopy performed as described previously on a Zeiss 510 inverted confocal microscope [208]. Primary antibodies include mouse anti-β-Gal 1:1000 (Promega); rabbit anti-LC8 (1:100) (W. Sale), mouse anti-Ci (1:50), mouse-α-Notch 1:10, mouse anti-Wg (1:800), mouse anti-Cut (1:100) and mouse anti-DIAP1 (1:50) (DSHB); rabbit α-yki 1:1000 (K. Irvine); mouse α-rat CD2 1:100 (Research Diagnostics, Inc.); mouse anti-V5 (1:200, Invitrogen); mouse anti-dpERK (1:10,000, Sigma); rabbit anti-phospho histone H3 (1:100), rabbit anti-phospho-Smad1/5 (1:100), rabbit anti-DCP1 (1:150), rabbit anti-Caspase3 (1:100) (Cell Signaling); mouse anti-dMyc (monoclonal

P4C4-b10); mouse anti-Cyclin E (H. Richardson). BrdU assays performed as described previously (Robinson et al., 2010) with mouse anti-BrdU (1:100; Becton Dickinson).

Wing/Eye Measurements

Eyes/wings were imaged on a Leica DFC500 CCD camera and quantified with Image J. Posterior compartment ratio (PCR) = posterior compartment size/total wing size.

Chapter 3: Yki-overexpressing clones extend actin-rich protrusions and secrete matrix metalloproteases

INTRODUCTION

The Hippo pathway is a conserved tumor suppressor pathway that acts to restrict the activity of the activating transcription factor Yorkie (YAP/Taz in humans). Increased levels or activity of Yki or its orthologs leads to impressive hyperplastic growth via the transcription of pro-growth target genes [235]. In *Drosophila,* imaginal wing disc clones mutant for Hippo components that suppress Yki-activity are overgrown, but usually have well-circumscribed borders [236-238]. In addition, Hippo pathway components localize to border cell contacts and act through a non-Yki-mediated mechanism to promote collective border cell migration during development [239]. Furthermore, evidence has accumulated which indicates a role for YAP/Taz in cancer invasion and metastasis. Pro-invasive properties of YAP/Taz have been associated with multiple types of cancer, including breast, prostate, melanoma, mesothelioma, pancreatic, and oral cancers [240-246]. In mice for example, the expression of an activating-mutant form of YAP in breast cancer, melanoma, or non-transformed mammary epithelial cell lines dramatically enhanced their ability to grow and metastasize *in vivo* [247]. Human mammary epithelial cells overexpressing YAP undergo epithelial-to-mesenchymal transition (EMT) and exhibit invasive morphology in three-dimensional cultures, with spike-like projections that penetrate the basement membrane [92]. Despite the fact that YAP/Taz appear to play a role in cancer invasion and metastasis, little is known about the transcriptional targets or mechanisms that trigger these events.

Cancer metastasis is a complex process that requires a series of sequential steps including invasion of adjacent tissues, transport through the circulatory system, and growth at the secondary organ. Metastases are of incredible clinical significance, since they

account for 90% of human cancer deaths [248]. Invadopodia are actin-rich, finger-like projections that protrude out of metastatic tumor cells to degrade the surrounding extracellular matrix (ECM)—an important first step of metastasis [249]. Invadopodia are similar to podosomes, which are analogous structures formed during normal development in non-cancerous cells, such as dendritic cells, macrophages, and osteoclasts [250]. Multiple types of human cancer cells form matrix-degrading invadopodia that are important for their invasive function [251-255]. Both invadopodia and podosomes are regulated by a myriad of adhesion, scaffolding, signaling, and actin-remodeling molecules, including integrins, FAK kinase, Src kinase, WASP, and ARP2/3 (reviewed in [250]). Early in their formation, invadopodia recruit matrix metalloproteases (MMPs), a key family of proteases responsible for degrading the ECM [250]. Despite the well-understood local signaling environment within invadopodia and podosomes, we have an incomplete picture of how upstream signal transduction pathways influence their formation and regulation.

Invadopodia are typically studied in 2D cultures of cancer cell lines, and multiple challenge face researchers attempting to study the structures *in vivo* (reviewed in [256]). Here, I present data indicating that Yki-overexpressing clones in the *Drosophila* wing disc extend actin-rich protrusions into neighboring tissue that are consistent with invadopodia. Furthermore, I show that driving Yki in the wing leads to MMP expression. These data are preliminary, but suggest that Yki-driven overgrowths are primed to degrade surrounding ECM and may form invadopodia as part of an invasive program of gene expression. Although more evidence is needed to definitively characterize these protrusions as

invadopodia, these results hint at a potential new *Drosophila* imaginal disc model to study these invasive structures *in vivo*.

RESULTS

Clones overexpressing Yki in the wing disc usually exhibit hyperplastic overgrowth with well circumscribed borders. However, occasionally I have observed what appear to be membrane protrusions from the edge of Yki-positive clones that extend into neighboring wild-type tissue (see Figure 3.1). The appearance of these protuberances is reminiscent of structures such as invadopodia or cytonemes [257, 258]. In order to characterize these extensions, I have used a heatshock inducible actin-FLP out system to stochastically generate clones throughout the early *Drosophila* larva that drive wild type Yki. Then, I dissected the wing discs and examined them for the presence of Yki-positive clones in addition to other molecules associated with invasive membrane structures. Notably, this system is not without its complications and drawbacks. Firstly, the use of heatshock induction means that the Yki-overexpressing clones are not tissue specific to the wing. Therefore, other means would be necessary to exclude the possibility of marked clones being generated in non-wing cell types. Of particular concern are tracheal cells, which as a part of normal development, partially invade into imaginal wing disc tissue [259]. Secondly, these extensions do not emerge in every disc with Yki-overexpressing clones, and seem more prominent, but not restricted to the notum region of the disc (as opposed to the pouch or hinge). This characteristic requires one to use a relatively high-throughput experimental design in order to reliably observe of the structures. Despite these drawbacks, I have captured multiple images of these Yki-driven structures in addition to staining Yki-positive wing clones with increased levels of invasion-associated proteins.

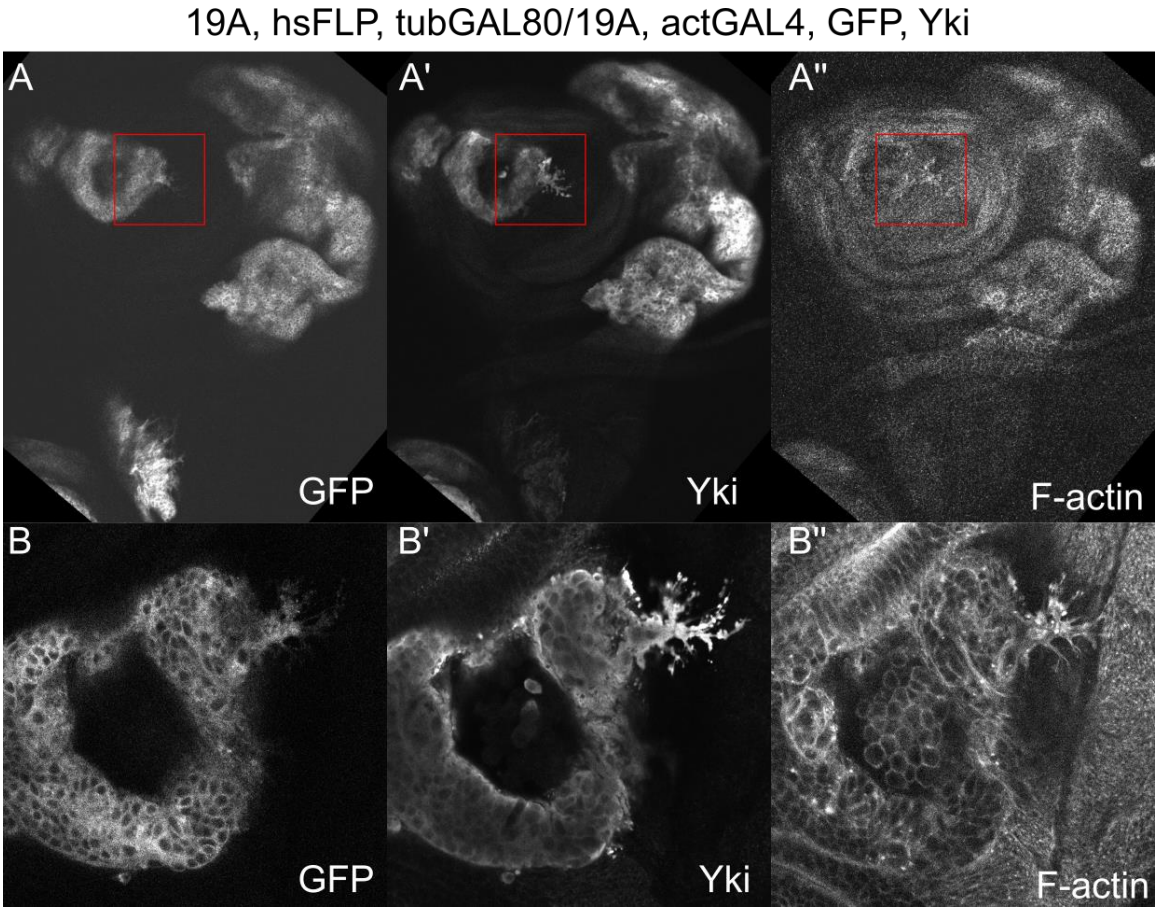

Figure 3.1. Wing clones overexpressing Yki produce actin rich protrusions. Confocal images of L3 wing discs with heatshock induced clones overexpressing Yki via an Actin>GAL4 driver, stained for (**A', B'**) Yki or (**A'', B''**) phalloidin. GFP marks the clones (**A, B**). The panels in B are magnified images the disc in A, indicated by the red box.

In a first step to characterize these structures, I used a fluorescent phalloidin to label filamentous actin (F-actin) in wing discs with Yki-overexpressing clones (Figure 3.1). As typical, most of the clones were overgrown, with bulging, but well-circumscribed borders (see the large clone on the right half of the disc in Figure 3.1A). However, a disc with clones in both the pouch and notum exhibited branched extensions staining brightly with both an anti-Yki antibody and phalloidin (Figure 3.1A'). In particular, the clone in the pouch possessed a highly branched, actin-rich projection that appears to be extending into neighboring wild-type tissue (see Figure 3.1B), consistent with descriptions of invadopodia in other experimental systems. Also of note, Yki protein intensely decorates the tips of the branches in a punctate staining pattern. Yki is known to be recruited to plasma membrane-associated complexes via the FERM domain protein Expanded [260], but has not previously been shown to be present at the tips of long membranous extensions.

A key step required for local cellular invasion in both developmental and cancerous contexts is the degradation of extracellular matrix (ECM) proteins. In order to test whether Yki-overexpressing clones were primed to perform this invasive process, I stained wing discs for the presence of matrix metalloproteases (MMPs). MMPS are a conserved family of extracellular proteases that degrade ECM components such as collagen [261]. The *Drosophila* genome contains two MMP homologs, MMP1 (secreted) and MMP2 (membrane-associated) [262]. Yki-overexpressing clones throughout the wing disc stain positively for MMP1, whether they contain putative invadopodia or not (Figure 3.2). It is important to note that tracheal cells express MMP1 as part of tracheagenesis [263]; thus, streaks of MMP1 can be seen in normally developing wing discs, which accounts for the MMP1 staining outside clones in Figure 3.2. Nonetheless, wing derived, Yki-positive cells

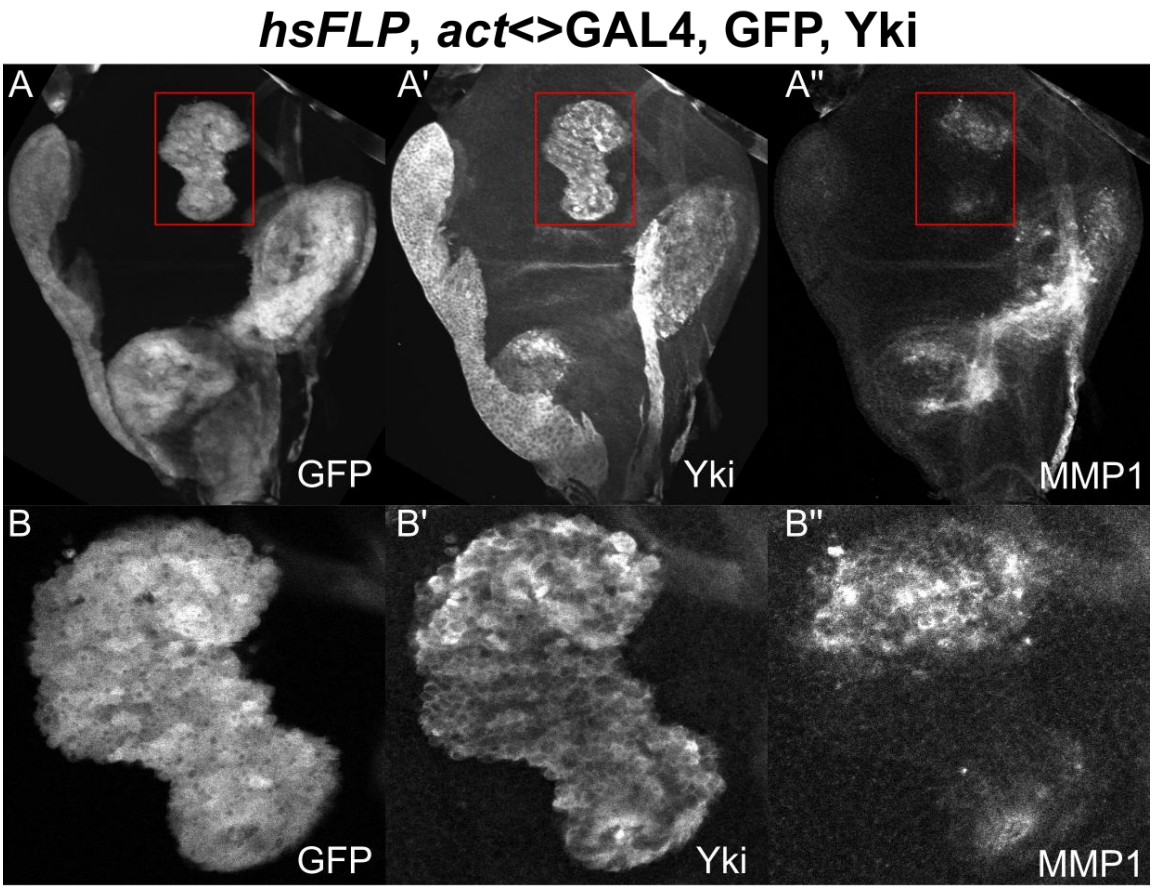

Figure 3.2. Wing clones overexpressing Yki stain positively for MMP1. Confocal images of L3 wing discs with heatshock induced clones overexpressing Yki, stained for (**A', B'**) Yki or (**A'', B''**) MMP1. GFP marks the clones (**A, B**). The panels in B are magnified images the disc in A, indicated by the red box.

appear to produce their own discrete pool of MMP1 (see red box in Figure 3.2). Yki promotes transcription of its targets by binding its partner Scalloped (Sd), which in turn binds to DNA promoter elements. In order to test whether actin-driven Yki overexpression clones require Yki-Sd transcriptional activity to express MMP1, I generated the clones in the background of an inverted repeat to simultaneously knockdown the levels of Sd (Sd-IR). Clones overexpressing both Yki and Sd-IR are not noticeably overgrown and do not stain positively for MMP1 (Figure 3.3), suggesting that MMP1 is either a direct target of Yki transcription or a target of a Yki-dependent downstream pathway. In order to test whether Yki-driven expression of MMP1 results in ECM degradation, I used *engrailed*-GAL4 (enGAL4) to drive Yki in the posterior compartment of wing discs in the background of a GFP labelled copy the *viking* gene (Vkg-GFP), the fly ortholog of collagen IV, a major component of the ECM, and target of MMP1 [261]. In these wing discs, Yki-expressing cells in the posterior compartment stained positively for MMP1 (Figure 3.4A). However, I did not detect any degradation in the Vkg-GFP signal proximal to the MMP1 signal in cross-section confocal images (Figure 3.4B), suggesting that the levels or activity of extracellular MMP1 is not sufficient to substantially degrade the imaginal disc ECM in this genotype. MMP2, the other MMP homolog in *Drosophila*, is a membrane associated protein with its catalytic domain oriented extracellularly [263]. Clones overexpressing Yki appear to have mildly elevated levels of MMP2 staining in their surrounding membranes (Figure 3.5). This effect is less pronounced than the expression of MMP1, and more experiments are needed to conclude whether MMP2 is Yki-driven—however, this result indicates that Yki may induce a multifactorial program of invasive gene expression.

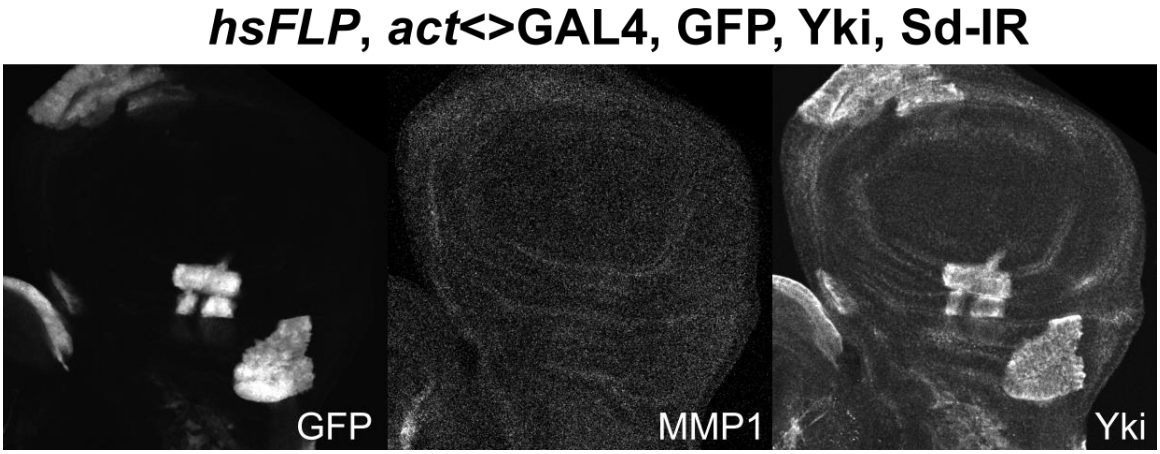

Figure 3.3. Yki-overexpressing clones require Sd to express MMP1. Confocal images of L3 wing discs with heatshock induced clones, overexpressing Yki and knocking down Sd, stained for Yki or MMP1. GFP marks the clones.

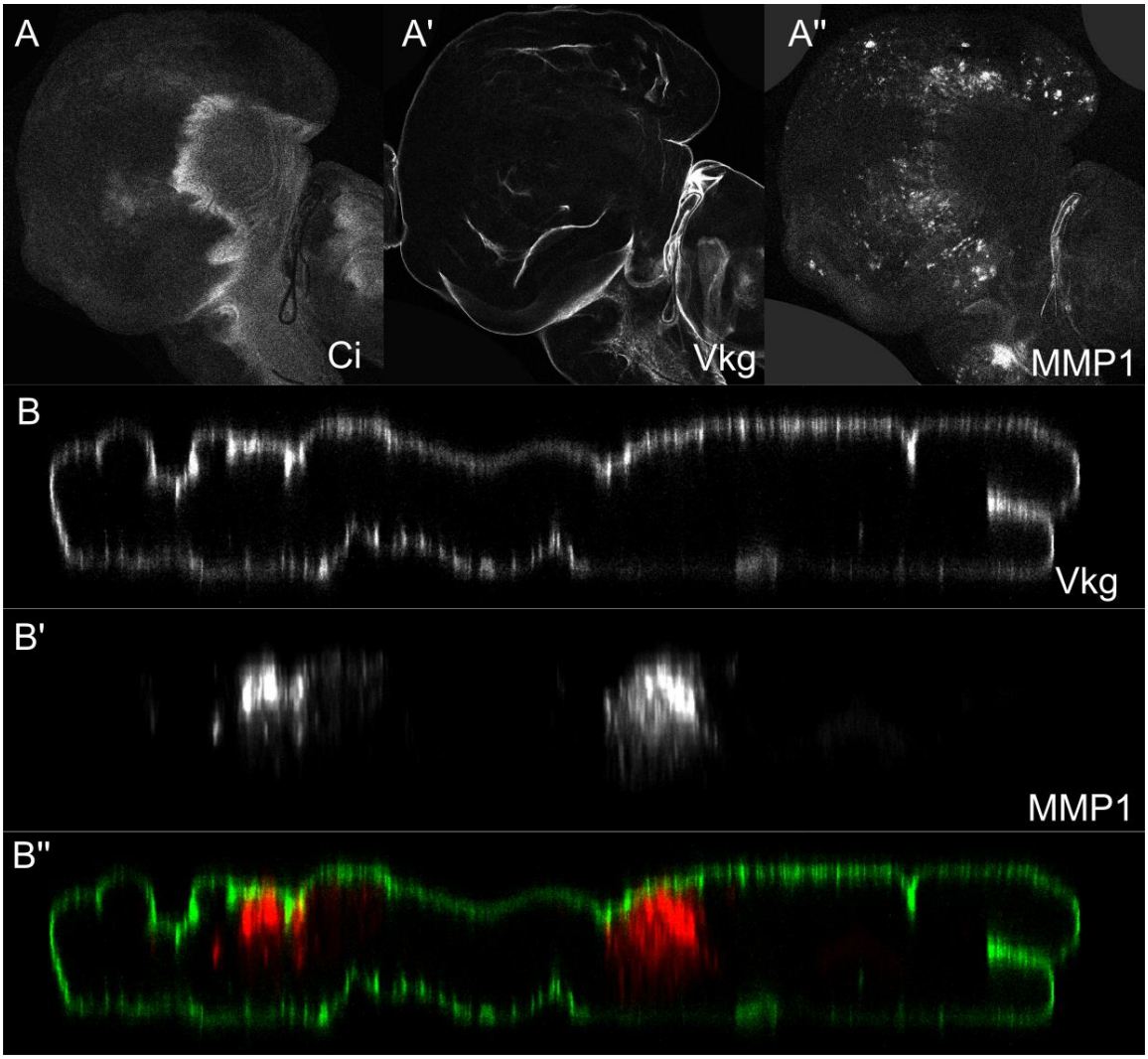

Figure 3.4. The ECM surrounding the wing is not degraded by MMP1 protein from Yki-overexpressed cells. (A) Confocal images of L3 wing disc (*en >yki*; posterior domain, left-side) with staining for **(A)** Ci marking the anterior domain. **(B)** Orthogonal line-scan (in the anterior-posterior direction) confocal images of L3 wing disc with Yki overexpressed in the posterior compartment, stained with **(B**), Viking-GFP, and **(B')** MMP1. **(B'')** is a composite image of Viking-GFP and MMP1.

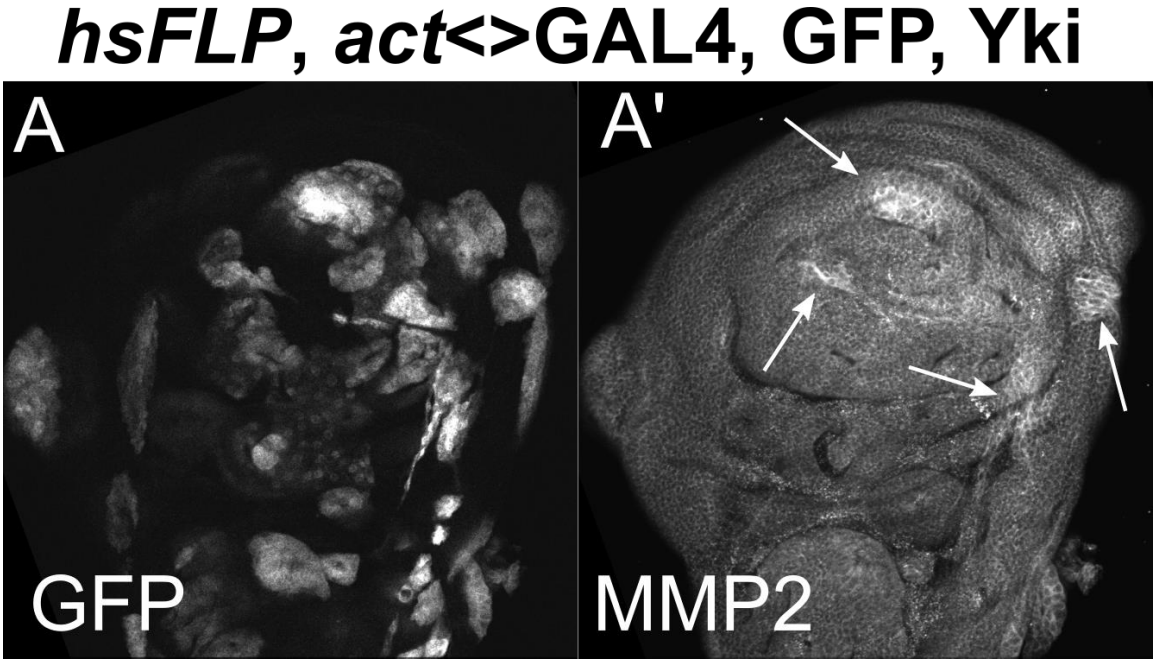

Figure 3.5. Wing clones overexpressing Yki stain positively for membrane bound MMP2. Confocal images of L3 wing discs with heatshock induced clones overexpressing Yki, stained (**A'**) MMP2. GFP marks the clones (**A**). The arrows highlight areas within clones showing increased MMP2 staining.

Invadopodia and podosome formation require a complex series of signaling events and mechanisms, including rosette formation, recruitment of MMP1, and actin-dynamics (reviewed in [264] and [250]). Integrins are a family of adhesion molecules that anchor cells to the ECM and can recruit MMPs [265]. Integrin proteins, including β1 and β2 integrin, localize to invadopodia and are required for their formation [264]. In order to test whether the observed actin-rich, Yki-driven protrusions are consistent with invadopodia/podosomes, I stained wing disc clones with an antibody to β2 integrin (Figure 3.6). A Yki-expressing clone in the wing notum shows a highly branched extension decorated with β2 integrin (see red box is Figure 3.6), consistent with the description of invadopodia. Another early player in the formation of membrane extensions is focal adhesion kinase (FAK). Phosphorylated FAK (pFAK) indirectly regulates invadopodium formation by sequestering active Src kinase at focal adhesions, localizes to invadopodia, and is required for normal invadopodia formation (reviewed in [258]). To assess the activity of FAK in Yki-overexpressing clones, I stained wing discs with an antibody to pFAK (Figure 3.7). A wing pouch clone appeared to contain mildly elevated levels of pFAK, including in string-like extensions, and with particularly strong puncta of staining in individual cells (see arrows in Figure 3.7). This result is not conclusive, but it suggests that Yki may impact the activity and function of kinases involved in the early stages of invadopia/podosome formation.

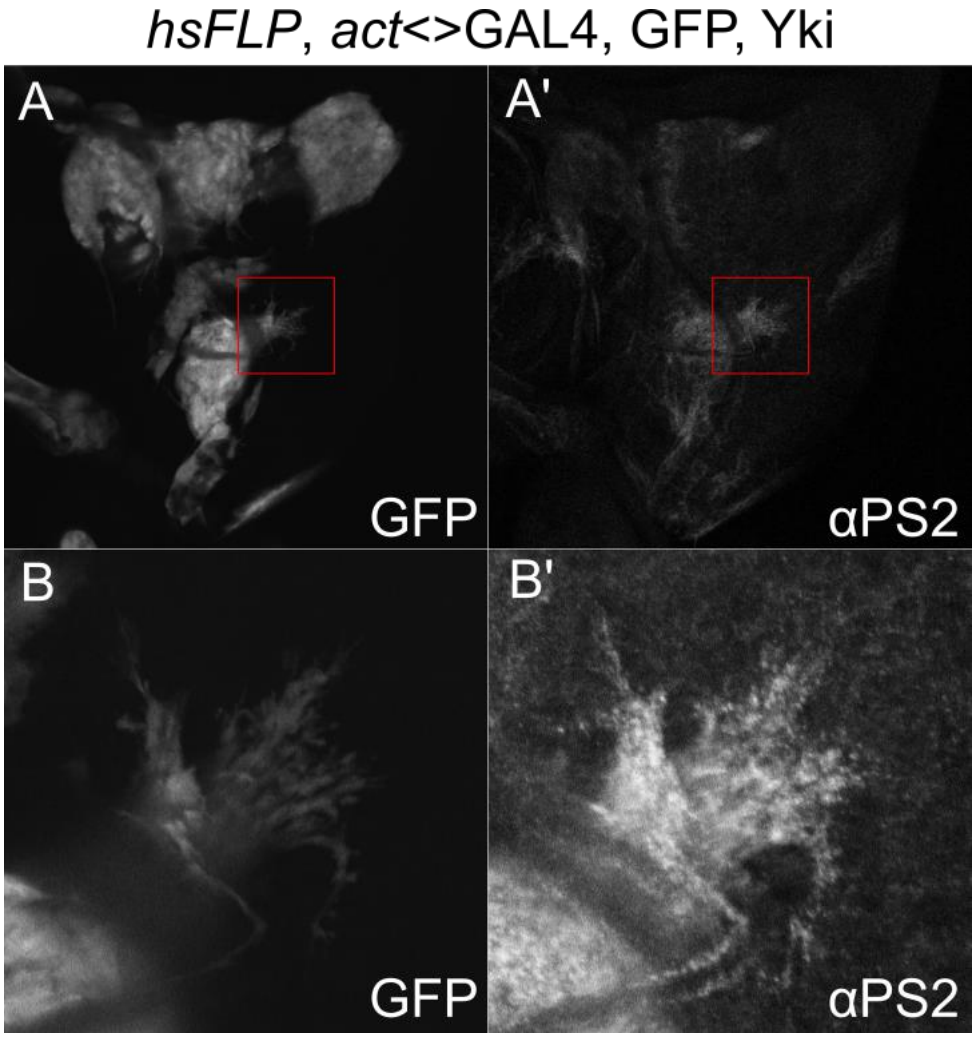

Figure 3.6. Yki-overexpressing clonal protrusions stain positively for αPS2 integrin. Confocal images of L3 wing discs with heatshock induced clones overexpressing Yki, stained for (**A', B'**) αPS2 integrin. GFP marks the clones (**A, B**). The panels in B are magnified images the disc in A, indicated by the red box.

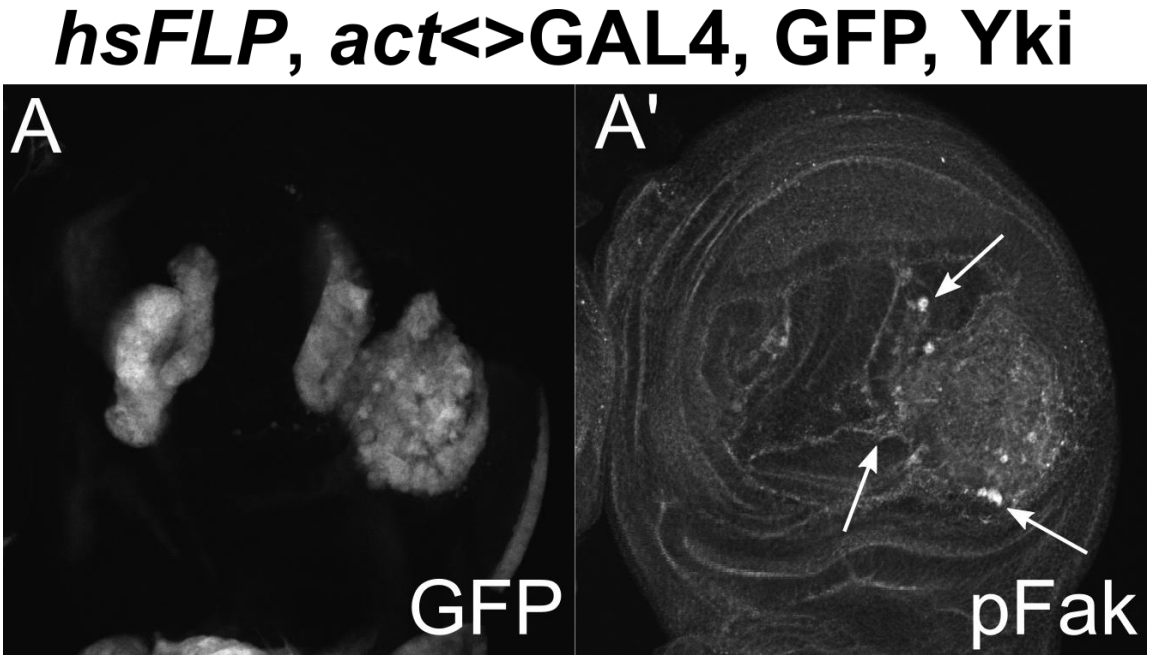

Figure 3.7. Wing clones overexpressing Yki stain positively for pFAK. Confocal images of L3 wing discs with heatshock induced clones overexpressing Yki, stained (**A'**) pFAK. GFP marks the clones (**A**). The arrows highlight areas within clones showing increased pFAK staining.

DISCUSSION

Here, I show that clonal Yki-overexpression in the *Drosophila* wing disc leads to the extension of putative invadopodia into neighboring wild-type tissue. These protrusions are enriched for F-actin, β2 integrin, and phosphorylated FAK—consistent with observations of invadopodia in other experimental systems. However, a complete characterization of these structures is lacking and future studies are need to fully define them as invadopodia. One strategy would be to probe the structures for the presence of other invadopodia-associated molecules. For instance, actin remodeling proteins such as cortactin, ARP2/3, and WASP are enriched in invadopodia and are required for invadopodia formation and function (reviewed in [266]). Another fundamental question regarding these Yki-driven protrusions is whether or not they degrade the ECM and invade neighboring tissues. Here, novel data reveals that Yki-overexpressing cells are enriched for MMPs, proteases that are key for degradation of ECM proteins. However, in a compartmental expression system, I did not observe the degradation of collagen IV (Viking in flies, and a major component of the ECM), despite proximal pools of MMP1. The reasons for this discrepancy are not clear; perhaps the MMP1 is not present at high enough concentrations, has insufficient levels of enzymatic activity, or is not secreted and exposed to the ECM. Future studies could use the clonal expression system in the background of *viking-GFP* in an attempt to capture images of Yki-positive protrusions in contact with the ECM. Do these putative invadopodia actively degrade the ECM and invade through it? The fact that Yki overexpression leads to the robust induction of MMP1 also begs more questions. I have shown that Yki-driven MMP1 expression requires Yki's transcription factor partner Sd, but it is not clear whether MMP1 is a direct transcriptional target of Yki or an indirect target of a downstream signal

transduction pathway. MMP1 expression is a commonly used marker of JNK activation [267]. Although crosstalk between the Hippo and JNK pathways has been reported previously [222], the Yki-driven activation of JNK signaling to promote MMP1 expression would be an important result.

The presence of putative invadopodia in a *Drosophila* clonal expression system presents an exciting new platform in which to study these structures *in vivo*. Although some successful work has used *C. elegans* and zebrafish to examine invadopodia *in vivo* (reviewed in [256]), most studies have been limited to cell culture with cancer cell lines. Previous studies have provided evidence of local invasion in *Drosophila* during tracheogenesis [268], but to date no models of tumorigenic invadopodia exist in flies. One confounding limitation of the heatshock induced clonal expression system I have presented is the lack of tissue-specificity. The clones I have generated for these experiments are not specific to the wing and could potentially be born from non-imaginal disc cells such as tracheal or peripodial cells. Tracheal cells express MMPs and partially invade into imaginal discs as part of normal development [259, 269]. Nonetheless, the putative invadopodia I have presented here arise from clones with morphology consistent with wing cell clones and particular examples (Figure 3.1B) are located in regions of the disc not normally associated with tracheal invasion [269]. Future studies could address these concerns by further defining the specific cell type of these protrusion-containing clones by using cell-specific markers or a wing-specific driver of clonal generation.

The Hippo pathway was first discovered in *Drosophila* and has since been shown to be highly conserved among metazoans [25, 26, 270]. While normally associated with hyperplastic growth, disregulation of the Hippo pathway can lead to invasive phenotypes

and promotes metastasis in humans [92, 236-238, 240-247]. However, *Drosophila* models of these invasive phenotypes have not been well-developed. Here, I have presented evidence that overexpression of the major Hippo pathway effector, Yki, is sufficient to prime cells for invasion by expressing MMPs and extending putative invadopodia. One curious question that arises given this data is how these invasive phenotypes have gone undetected until now. *Drosophila* researchers have long used clones of mutants of Hippo components (i.e. Hippo, Warts, Salvador, Fat, Expanded) to activate Yki and generate hyperplastic overgrowths, but invadopodia-like structures have never been described. It is possible that strongly overexpressing Yki itself engages an invasive program of gene expression not available in other types of Hippo pathway deregulation. Hippo-associated human cancers commonly contain elevated levels of YAP/Taz, but rarely have mutations in the core Hippo components (reviewed in [132] and [271]). Therefore, the overexpression of Yki could present a more accurate model of YAP/Taz-driven tumorigenesis and invasion. Future studies could take advantage of this model by screening the putative-invadopodia phenotype for genetic interactions. A myriad of candidate genes could be used to test for enhancement or suppression of this phenotype, including actin cytoskeleton regulators (i.e. cortactin, ARP2/3), integrins, MMPs, Hippo pathway components, and JNK pathway components. Together, this evidence could help to shed light on the role of Yki in invasion and metastasis, potentially furthering our understanding of YAP/Taz driven cancers.

MATERIALS AND METHODS

Fly Strains

All crosses were maintained at 25°C. Alleles used in these studies are as follows: *UAS-yki* (D.J. Pan), *UAS-Sd-IR* (Bloomington stock center), *Viking-GFP*.

Immunofluorescence Microscopy

Immunostaining and confocal microscopy performed as described previously on a Zeiss 510 inverted confocal microscope [208]. Primary antibodies include mouse anti-Ci (1:50), guinea pig α-yki; mouse anti-MMP1 (DSHB), mouse anti-MMP2 (DSHB), mouse anti- β2 integrin (DSHB), and anti-pFAK (Abcam).

Chapter 4: Concluding remarks

This dissertation has detailed a project that started as an examination of Ctp and its putative role in regulating a component of an E3 ubiquitin ligase named Archipelago. When a link between Ctp and Archipelago was not detected, I remained interested in the cause of the small organ size of Ctp depleted tissues. The subsequent experiments I performed to pursue this question lead to many interesting and surprising results. For instance, I was surprised to find that small wings resulting from the knockdown of Ctp has increased levels of proliferation markers BrdU and Ph3. This apparent contradiction remains an unresolved question, but given the background levels of apoptosis in these wings, it could indicate a process of compensatory proliferation to replace dying cells. This phenomenon has been well studied in *Drosophila* and, in certain contexts, involves Yorkie signaling [272].

The project propelled forward once I identified genetic interactions between *ctp* and *yki* as part of a candidate genetic screen for modifiers of the Ctp-knockdown small wing phenotype. This data coupled with the reduced levels of dMyc protein, a downstream target of Yorkie, in *ctp* null clones, focused the project on a potential role for Ctp in affecting the Hippo pathway. The result that Ctp depleted cells had reduced levels of the Yki target *DIAP1/thread*, both transcript and protein, solidified this notion. However, a surprising result came when I found that the levels of a *bantam* transcriptional reporter, another Yki-target, were increased in Ctp-knockdown wings and Ctp null clones. Paradoxically, the *bantam* locus encodes a pro-growth, pro-survival microRNA, but its upregulation in Ctp-knockdown wings is associated with undergrowth. This apparent paradox may be explained by the finding that *ban* promotes tissue growth through dMyc [232, 233], so that Ctp-depleted cells with reduced dMyc levels may be resistant to *ban*--induced growth.

The fact that *thread* and *bantam* transcription are effected in opposite directions by the loss of Ctp is a significant finding that begs additional study. One would predict that if Ctp acted upstream to effect Yki transcriptional output, the target transcripts would be altered in a uniform direction. For example, if Ctp altered the translocation of Yki from the cytoplasm to the nucleus, as has been reported for other Ctp-interactors such as Pak1 and ERα, the effect on both *thread* and *bantam* would be predicted to be the same [189, 273]. Likewise, if Ctp interacted with an upstream component of the Hippo pathway, the ultimate result would be typically be a change in Yki output that would affect *thread* and *bantam* equally. While it is not uncommon for upstream Hippo pathway effectors to result in changes to some, but not all, Yki transcriptional targets, this type of opposing interaction has not been reported before. The fact that Ctp's impact on *bantam* requires Yki, and that loss of Ctp cannot suppress Yki-driven *thread* transcription, places Ctp at or above Yki in an epistatic hierarchy. Together, this data suggests that Ctp is effecting Yki signaling in the nucleus.

As discussed above, many unanswered questions remain in the field in regards to the complexities of Yki's role in promoting target transcription. Yki does not have a DNA-binding domain, and must partner with other DNA-binding transcription factors including Scalloped, Mad, and Homothorax to promote gene transcription. Furthermore, while Yki contains canonical phosphorylation sites that regulate it's binding to 14-3-3 proteins and retention in the cytoplasm, additional phosphorylation sites have been identified with unknown roles for Yki function (reviewed in [157]). The full gamut of Yki transcriptional targets and the components of Yki-containing transcriptional complexes required to promote specific targets has not been identified. Recently, our lab published findings that

Yki interacts with the ecdysone coactivator Taiman to promote the expression of canonical Yki transcriptional targets. Interestingly, loss of Taiman suppresses Yki-driven overgrowth, with no resulting reduction in canonical Yki targets. Instead, a novel set of germline stem cell factor genes were found to require Taiman for transcription in Yki-induced tissues [274]. These findings are an example of the potential for Yki to exist in different nuclear complexes with different effects on the transcription of specific sets of target genes.

Together, this information has led to the construction of my preferred model of Ctp-Yki interaction, whereby Ctp is part of a transcriptional complex with Yki that shifts Yki-driven promotion onto specific transcriptional targets. This model presents a potential mechanism that could explain the opposite effect that Ctp-loss has on *thread* and *bantam*. As seen in Figure 4.1, when Ctp is available it is a member of a transcriptional complex containing Yki and Scalloped that supports the transcription of the *thread* gene. When Ctp is depleted, this complex is disrupted, leading to a decrease in Yki-mediated *thread* transcription. Instead, the pool of Yki previously responsible for sustaining *thread* is shifted into an alternative Yki transcriptional complex, perhaps with a different DNA-binding partner such as Stat or Mad, to promote the transcription of *bantam*. A role for the Ctp ortholog LC8 in transcriptional complexes has already been described in mammalian cells, where LC8 is bound to estrogen-receptor-α and is enriched in the chromatin of ERα target genes [188-190]. Interestingly, vertebrate LC8 (aka DYNLL1) is as a high-confidence interactor of the Yki vertebrate homolog Yap1 [72, 73]. Despite the fact that we were unable to detect an equivalent association in cultured *Drosophila* S2 cells, it is possible that this LC8-YAP1 interaction is conserved in flies and mediates the promoter

choice of Yki as described in this model. It is also possible that Ctp and Yki do not physically interact in *Drosophila*; instead, perhaps Ctp affects the activity of another component of Yki transcriptional complexes that is capable of shifting Yki's choice of promoter (i.e. from *thread* to *bantam*). Another potential mechanism would involve Ctp influencing the post-translational modifications of Yki in the cytoplasm. As noted previously, Yki contains phosphorylated residues of unknown functional consequence. Feasibly, Ctp could alter the phosphorylation pattern of Yki, leading to downstream changes in nuclear protein-protein interactions that affect the assembly of different Yki transcriptional complexes. Regardless, it seems likely that the mechanism of Ctp's effect on Hippo signaling results in changes to nuclear Yki's binding partners, and thus, target promoters.

Needless to say, future studies are necessary to solidify this speculative model and detail the mechanism of the Ctp-Yki interaction. In particular, biochemistry experiments would help to elucidate the role of Ctp in Yki transcription. One way to directly test my preferred model would be to do chromatin immuno-precipitation (ChIP) of Yki in Ctp-depleted cells. This model would predict that Yki would shift from *thread* promoter chromatin to the *bantam* promoter when Ctp is removed. Furthermore, it would be interesting to ChIP Ctp to show if Ctp itself mapped to chromatin containing Yki target genes in wild-type cells. Although we were unable to detect a physical association between Yki and Ctp, the orthologous interaction has been repeatedly found between LC8 and Yap1, suggesting that it could be present. A more rigorous approach to identifying a Ctp-Yki physical interaction would help to inform the proposed model. Another possibility is that Ctp physically binds to another Hippo pathway component, and detecting such an

interaction would shed light on the mechanism by which Ctp effects the transcription of Yki-target genes.

To summarize, amid many surprising results this project has uncovered a fascinating connection between the multifunctional dimerization hub protein Ctp and the conserved pro-growth transcription factor Yki. The novel result that loss of Ctp elevates *bantam* transcription while simultaneously reducing *thread* transcription has important implications for understanding how Yki selects the promoters of its many target genes. By coincidence, in the course of investigating this project I found that Yki overexpressing clones in the wing disc extend actin-rich protrusions that are consistent with invadopodia. This exciting finding suggests that Yki is capable of engaging an invasive program of gene expression and provides a potential *in vivo* model for studying Yki's role in promoting invasion and the formation of invadopodia.

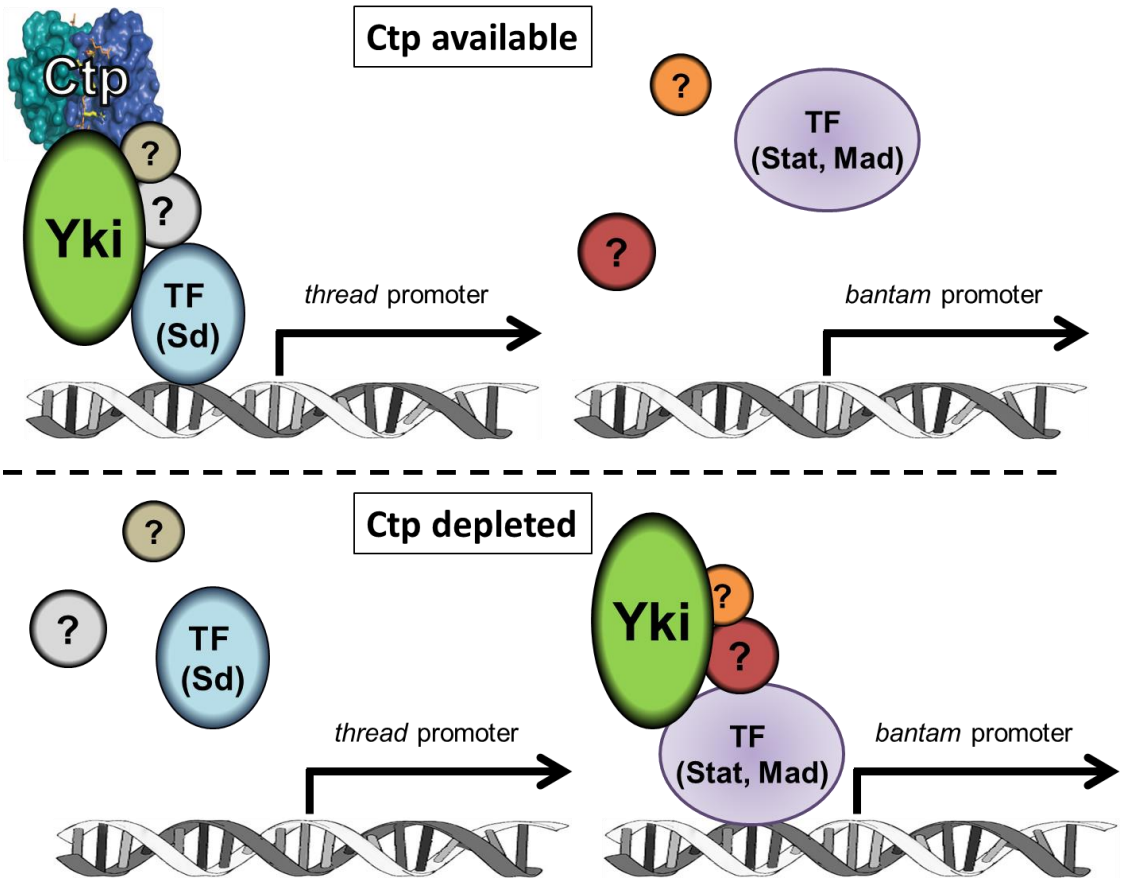

Figure 4.1. Model of Ctp as a member of a Yorkie (Yki) transcriptional complex affecting promoter choice. Schematic showing Ctp as a member of putative transcriptional complex with Yki. When Ctp is available (top), it supports the transcription of the *thread* locus in as a component of a complex with Yki and Scalloped (Sd). When Ctp is depleted (below), this complex dissociates leading to reduced *thread* transcription. Yorkie instead shifts into an alternative complex with another DNA-binding partner such as Stat or Mad to promote the transcription of *bantam*.

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