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Investigation of the Genetic Interactions between Hippo Signaling Pathway and Drosophila C-terminal Src kinase (dCsk)

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Honors Thesis

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Advisor: Madhuri Kango-Singh, Ph.D.

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UNIVERSITY *of*

DAYTON

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Abstract

The focus of my research revolved around the intersection between the Hippo tumor suppressor pathway and the *Src* oncogenic pathway. Both pathways control tissue and organ size during development by regulating cell proliferation, cell death, cell migration, and cell adhesion. Aberrant functions in either pathway are often detected in human cancers and correlate with poor prognosis. The *Drosophila C-terminal Src kinase* (*dCsk*) is a genetic modifier of *warts* (*wts*), a tumor-suppressor gene in the Hippo pathway, and interacts with the *Src* oncogene. Reduction in *dCsk* expression and the consequent activation of *Src* are reported in hepatocellular and colorectal tumors. Previous studies show that *dCsk* regulates cell proliferation and tissue size during development. Given the similarity in the loss-of-function phenotypes of *dCsk* and *wts*, we investigated the interactions of *dCsk* with the Hippo pathway components. We tested if loss of *dCsk* resulted in changes in activity levels of Hippo pathway target Yki, and if *dCsk* and Hippo pathway genes genetically interact. We found multiple lines of evidence suggesting that loss of *dCsk* using RNAi mediated elimination of *dCsk* in large patches of cells causes overgrowth due to increased proliferation, due to increased Yki activity. The effects of loss of *dCsk* are cell autonomous, and our results of epistasis experiments of *dCsk* and Hippo pathway components place *dCsk* between Dachs and Zyx that function downstream of Fat in the Hippo network. Hence we concluded that *dCsk* regulates growth via the Hippo signaling pathway.

Acknowledgements

I am heartily thankful to my advisor and mentor, Dr. Madhuri Kango-Singh, whose encouragement and guidance in the last four years enabled me to develop an understanding of scientific research.

I dedicate my honors thesis to my parents, Frances M. and John C. Kwon, and my sister Valeria J. Kwon who have provided me with unconditional love and support.

Lastly, I offer my regards and blessings to all my teachers and friends who helped me in any respect during my undergraduate journey.

Sincerely,

Hailey J. Kwon

General Introduction

The growth of an organism must be tightly controlled by cellular signals during its development. The phenomenon of unregulated growth, known as cancer, is one of the major leading causes of death worldwide. Cancer arises from tumor cells that continually divide due to their inability to recognize growth regulatory signals, including signals controlling apoptosis (Kerr *et al*., 1972; Jacobson *et al*., 1997; Wyllie *et al*., 1980) and contact inhibition. Cells that repetitively undergo cell cycle are likely to become cancerous, due to multiple errors that have accumulated from the numerous DNA replications. Most cancer cells contain at least one mutated gene, *viz.,* an oncogene, which can permanently activate the cell cycle and cause malignancy. In addition, inactivation or deletion of both copies of a tumor suppressor gene can also give rise to cancerous cells. These malignant tumors evade apoptosis, or programmed cell death, and continue to proliferate by increasing in their number. These malignant tumors may spread throughout the body via metastasis, inducing the surrounding cells to also become tumorous. Studies to identify genes and genetic mechanisms that are involved in tumorigenesis and metastasis have led to the discovery of several tumor-suppressor networks (*e.g.*, the MAPK, p53, Hippo, TSC/TOR pathways) whose genes are found mutated in cancers. These studies reveal that only some cells in the tumor acquire the ability to metastasize, suggesting that additional mutations are responsible for metastatic defects. The Hippo pathway is one of the signaling pathways involved in tumorigenesis and largely contributes to organ size regulation (Harvey and Tapon, 2007) in both vertebrates and invertebrates (Figure 1).
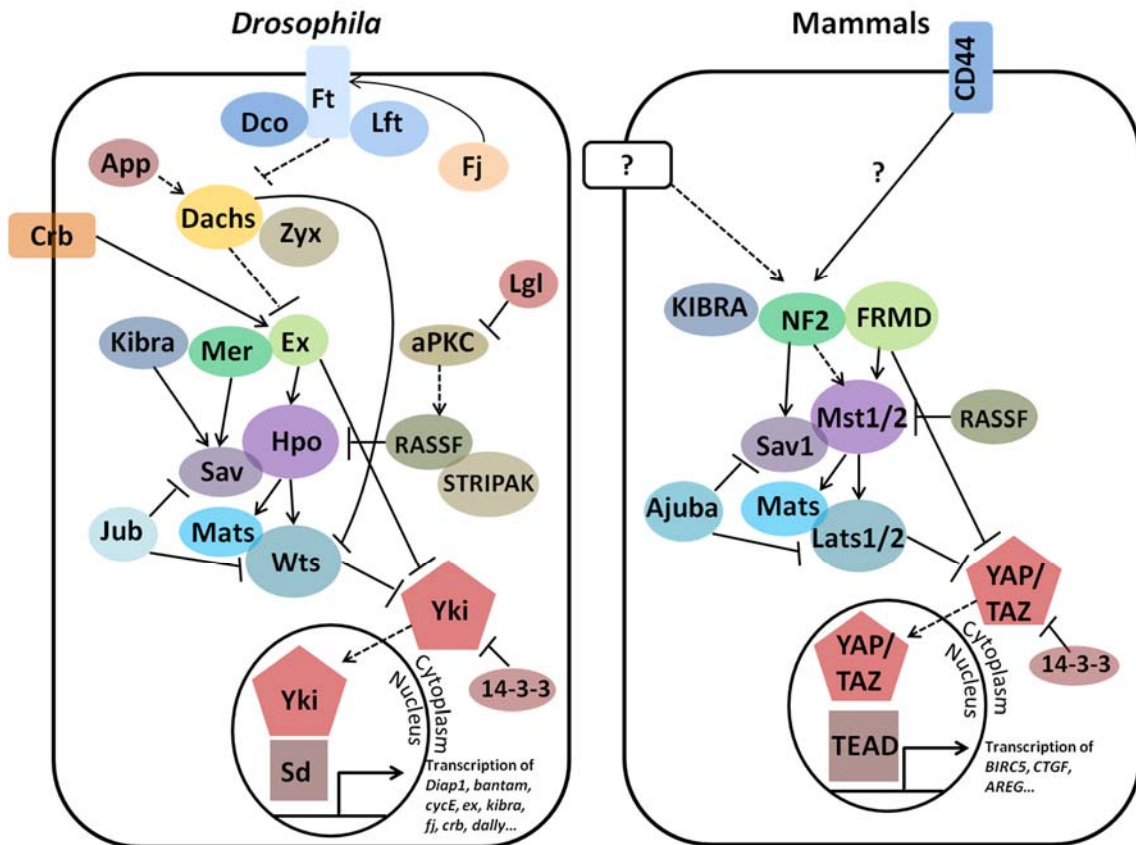
Figure 1. The Hippo Signaling Network in *Drosophila* and Mammals
Corresponding proteins in *Drosophila* and mammals are indicated by matching colors and shapes. Solid lines indicate direct biochemical interactions whereas dashed lines show interactions that may be indirect. Arrowed or blunted ends each indicate activation or inhibition. Selected target genes are shown.

Literature Review: The Hippo Tumor-Suppressor Pathway

The Hippo signaling pathway, also known as the Salvador/Warts/Hippo (SWH) pathway, is a network of tumor suppressor genes and oncogenes. It regulates organ size by inhibiting cell proliferation and promoting apoptosis in all metazoan animals, such as mammals and *Drosophila*, in addition to participating in other various cellular processes (Figure 2). The pathway attains its name from the protein kinase Hippo (Hpo), one of its key signaling components; mutations in the Hpo gene lead to a hippopotamus-like overgrowth phenotype in the tissues of the imaginal discs and adult organs (Figure 3) (Reviewed by Edgar, 2006; Pan, 2007; Saucedo and Edgar, 2007; Kango-Singh and Singh, 2009). Aberrant Hippo pathway function in humans, for example, due to mutations or amplification of genes, epigenetic silencing, and oncogenic transformation, is often detected in human cancers and correlates with poor prognosis. Although loss of Hippo signaling clearly makes cells resistant to apoptosis and promotes cell survival, the molecular mechanism by which Hippo signaling regulates apoptosis remains largely unknown (Kango-Singh et al., 2009).

Hippo pathway comprises of a core kinase cascade involving the Ste-20 family kinase Hippo (Hpo, the serine/threonine Ste20-like kinase) (Harvey *et al*., 2003; Jia *et al*., 2003; Pantalacci *et al*., 2003; Udan *et al*., 2003; Wu *et al*., 2003), and the DMPK family kinase Warts Warts (Wts, the nuclear Dbf-2-related (NDR) family kinase; also known as Lats) (Justice *et al*., 1995; Xu *et al*., 1995). Given normal Hippo signaling, Hpo forms a complex with an adaptor protein called Salvador (Sav, the WW domain scaffolding protein) (Kango-Singh *et al*., 2002; Tapon *et al*., 2002) to phosphorylate Wts. Wts, in turn, forms a complex with another adaptor protein called Mob as Tumor Suppressor

(Mats) (Lai *et al*., 2005) to phosphorylate the transcriptional co-activator Yorkie (Yki) (Huang *et al*., 2005), the major downstream target of the Hippo pathway. Upon phosphorylation by Wts, Yki creates a binding site for 14-3-3 proteins; this binding restricts Yki in the cytoplasm and leads to its degradation.

When active due to a lack of inhibition by proper Hippo signaling, Yki translocates to the nucleus where it forms a complex with the transcription factor Scalloped (Sd) [or Mothers against Dpp (MAD), Teashirt (Tsh) or Homothorax (Hth)] (Oh and Irvine, 2011) to induce the expression of target genes that promote (1) cell proliferation and cell survival like the *bantam miRNA*, *myc*, (2) cell cycle progression *e.g.*, E2F1, *cyclins A, B, E,* and (3) inhibitors of apoptosis like *drosophila inhibitor of apoptosis (diap1)*, causing tissue overgrowth (Table 1). Thus Hippo signaling regulates the expression of several genes within its pathway via a negative feedback loop.

Yki is influenced by several upstream regulators; multiple points of signal integration have been found in the Hippo Pathway, suggesting that the core kinase cassette responds to diverse stimuli. Examples of the upstream regulators include Expanded (Ex), Merlin (Mer) (Hamaratoglu *et al*., 2006), Kibra, Fat (Ft, the protocadherin) (Bennett and Harvey, 2006; Cho *et al*., 2006; Silva *et al*., 2006; Willecke *et al*., 2006), Tao1 (Boggiano *et al*., 2011; Poon *et al*., 2011), Crumbs (Crb), Ajuba (Jub), and Scribble (Scrib) (Verghese *et al*., 2012). Ex, Mer, and Kibra function together to activate the Hpo kinase cascade by directly binding to the Hpo-Sav complex (Yu *et al*., 2010). Additionally, Ex is known to directly repress Yki. The Ex-Mer-Kibra branch is modulated by the Fat/Dachsous branch, which does so by regulating Ex levels. The Fat/Dachsous branch consists of the atypical cadherins, Ft and Dachsous (Ds), and the

downstream effectors Discs overgrown (Dco, a serine-threonine kinase; aka casein kinase 1ε), Dachs (D, an atypical myosin), Approximated (App, a palmitoyltransferase), Lowfat (Lft), and Zyxin (Zyx) (Grusche *et al*., 2010; Rauskolb *et al*., 2011). Tao1, a sterile 20-like kinase, is found to phosphorylate and activate Hpo.

Furthermore, the Hippo pathway activity is thought to be also modulated by cell polarity, cell adhesion, and cell junction proteins, such as Crb, Jub, Scrib. The cell junction proteins exist in the epithelial cells of *Drosophila melanogaster* at the sub-apical region (SAR), adherens junction (AJ) or septate junction (SJ). Notably, the Hippo pathway activity is also affected by the modulation of the apical level of filamentous actin (F-actin). High accumulation of F-actin inhibits the Hippo signaling, thereby activating Yki. The activation of Yki, by phosphorylation-dependent or –independent mechanisms, results in autoregulation of some upstream genes, such as *ex, mer, kibra, crb*, and *fj* via a positive feedback loop.

Such broad spectrum of target genes confers tremendous versatility to Hippo signaling and allows context-dependent response of Hippo signaling activity. Emerging evidence demonstrates that the Hippo pathway crosstalks with other signaling pathways to regulate different target genes.
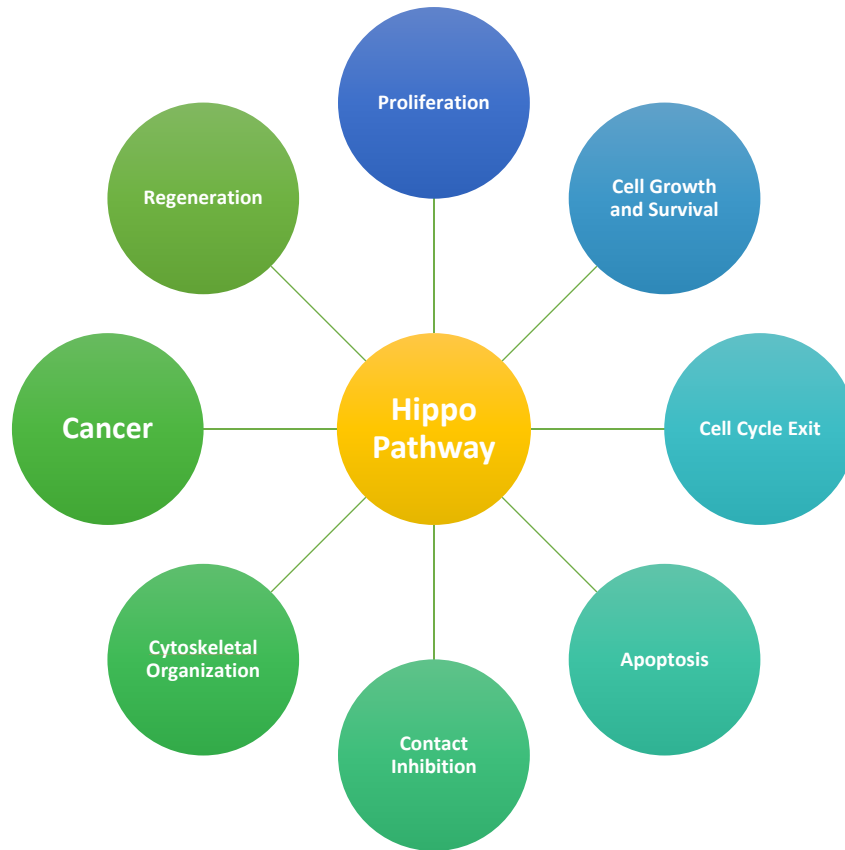
Figure 2. Roles of the Hippo Pathway
The Hippo signaling pathway regulates various cellular activities, including cell growth, proliferation , cell death, cell cycle, morphogenesis, and cell-to-cell interactions.
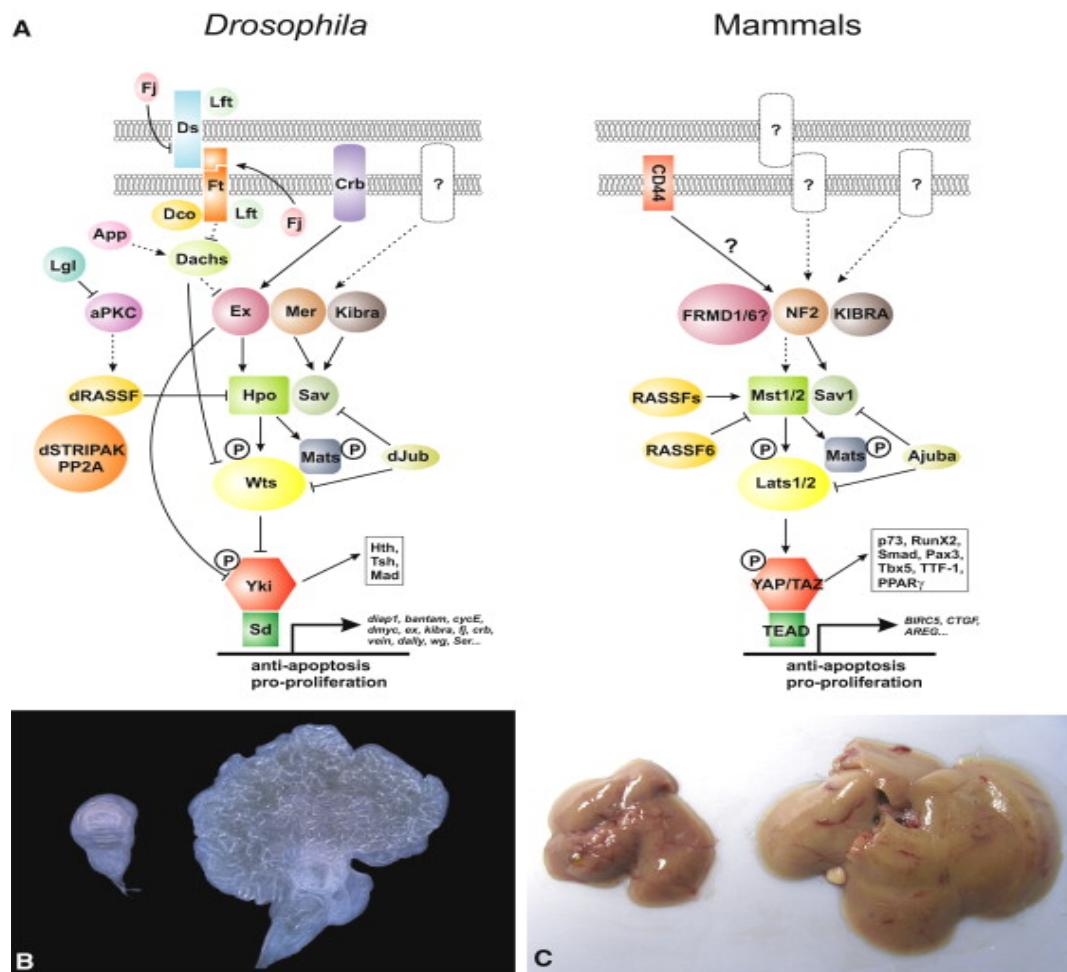
Figure 3. (A) Models of the Hippo signaling pathway in *Drosophila* and mammals. Corresponding proteins in *Drosophila* and mammals are indicated by matching colors and shapes. Arrows represent positive regulation, whereas blunted ends indicate negative inhibition. (B) A normal (left) and a *yki*-overexpressing (right) *Drosophila* wing imaginal disc. (C) A normal (left) and a YAP-overexpressing (right) mouse liver. The dramatic increase in organ size induced by Yki/YAP overexpression illustrates the potent growth-regulatory activity of Hippo signaling in *Drosophila* and mammals.

Table 1. Function of the Hippo Pathway Target Genes

Function	Example Target Gene(s)
Cell cycle exit	*expanded*
Cell growth and survival	*homothorax* (*hth*), *diminutive* (*myc*), *bantam microRNA* (*miRNA*)
Proliferation	*cyclins A, B, E, E2F transcription factor* (*E2F1*), *hth, bantam miRNA*
Apoptosis	*merlin* (*mer*), *diap1*
Morphogenesis	*dally*, *dally-like*
Planar Cell Polarity	*crumbs, fat*
Contact Inhibition	*Mer*

Literature Review: The *Src* Oncogenic pathway and *csk*

Analogous to the role of the Hippo pathway, the *Src* family tyrosine kinases (SFKs) are involved in the regulation of normal development. Their misregulation is implicated in several types of cancer, particularly in liver, breast, and colon (Masaki *et al*., 1999; Bougeret *et al*., 2001; Cam *et al*., 2001; Frame, 2002), and is associated with metastatic behavior (Yeatman, 2004). The SFKs were originally identified in the transforming gene of the Rous-Sarcoma virus, *v-src*. *Drosophila melanogaster* has two SFK members, Src42 and Src64 (Simon *et al*., 1985; Potter *et al*., 2000). This family of proteins is regulated by signaling pathways (e.g., G protein-coupled receptors) that are involved in the regulation of cell cycle entry, cytoskeletal rearrangement and cell-adhesion.

The SFK activity is inhibited (*i.e., Src* proteins are maintained in inactive state) by phosphorylation of their Carboxyl-terminal (C-terminal) region by *C-terminal Src kinase* (*csk*), a gene first identified by its ability to negatively regulate the SFK activity (Cole, 2003). This function of *csk* is conserved in mammals and flies; flies have one *csk* homolog, *Drosophila C-terminal Src kinase* (*dCsk*) (Read, 2004; Stewart, 2003), which functions similarly to the mammalian *csk* (Hoffmann *et al*., 1983; Simon *et al*., 1985; Takahashi *et al*., 1996).

dCsk is a conserved Src family kinase (SFK) that acts as a tumor-suppressor gene by interacting with several signaling proteins via phosphorylation-mediated interactions (*e.g., paxillin, c-Jun, lats*) (Reviewed by Okada, 2012). Reduced levels of *dCsk* activate *Src* kinases (Thomas and Brugge, 1997; Schwartzberg, 1998; Bjorge *et al*., 2000), including Jun N-terminal kinase (JNK), Stat, and Btk29A (Pedraza, 2004, Read, 2004);

this *Src* activation results in organ size increase, lethality of the organism, and overproliferation due to extra cell cycles (Read, 2004; Stewart, 2003). Thus, *csk* acts as a tumor suppressor through the *Src* pathway regulation (Read, 2004), while also negatively regulating the JNK pathway.

csk is known to have Src-independent functions as well. Independently of the SFK activity, *csk* links G-protein signaling to the actin cytoskeleton (Lowry *et al*., 2002). In addition, *csk* phosphorylates a number of other downstream molecules (Autero *et al*., 1994; Hildebrand *et al*., 1995; Cloutier and Veillette, 1996; Tremblay *et al*., 1996). Within the past decade, *dCsk* has been reported to regulate cell proliferation by genetically modifying *wts* tumor suppressor gene, a core component of the Hippo pathway, by direct phosphorylation (Stewart 2003). Presumably, mammalian *wts* molecules may also be substrates of Csk, considering that the C-terminal dCsk phosphorylation site is conserved in other *wts* homologs.

Loss of *dCsk* using a RNAi approach (*UAS-dCsk^{RNAi}*) showed that effects of loss of *dCsk* are mediated through Src activation, and loss of *dCsk* in large patches leads to increased proliferation and decreased apoptosis (Read et al 2004). Whereas broad loss of *csk* results in overproliferation (Figure 4 G), inhibition of apoptosis, and decreased cell adhesion, local inactivation of *dCsk* in discrete patches surrounded by normal cells does not cause overgrowth (Figure 4 A-E), as *dCsk* mutant cells begin to delaminate and disperse from the growing imaginal disc tissue, leading to their elimination by macrophages (Vidal *et al*., 2006). Although loss-of-function phenotype of *dCsk* is strikingly similar to that of *hpo, wts, sav,* and *mats* (or gain of Yki function), clonal patches of *dCsk* cells fail to survive to adulthood unlike cells containing the others four

genes. Instead, these cells spread among the wild-type cells while simultaneously undergoing apoptosis, which may reflect the function of *Src* in promoting motility and invasion (Langton, 2007).

More recently, *Src* has been reported to control tumor microenvironment by JNK-dependent regulation of the Hippo pathway (Enomoto and Igaki, 2013). Clone cells of *Src* overexpression activate the Rac-Diaphanous and Ras-mitogen-activated protein kinase (MAPK) pathways, which induces accumulation of F-actin, one of the upstream regulators of the Hippo pathway. Highly accumulated F-actin inhibits the Hippo signaling, thus activating *yki* (Fernandez *et al*., 2011; Richardson, 2011; Sansores-Garcia *et al*., 2011) in both *Src* mutants and wild-type cells. Simultaneously, *Src* activates the JNK pathway, which signals the propagation of *yki* activity to surrounding wild-type cells; the surrounding tissue is overgrown as a result. On the other hand, activated STAT acts independently of the Hippo pathway (Rodrigues, 2012). *Src* is also known to interact with STAT. Thus we concluded that it is important to continue investigating the new, independent roles of Src and csk.
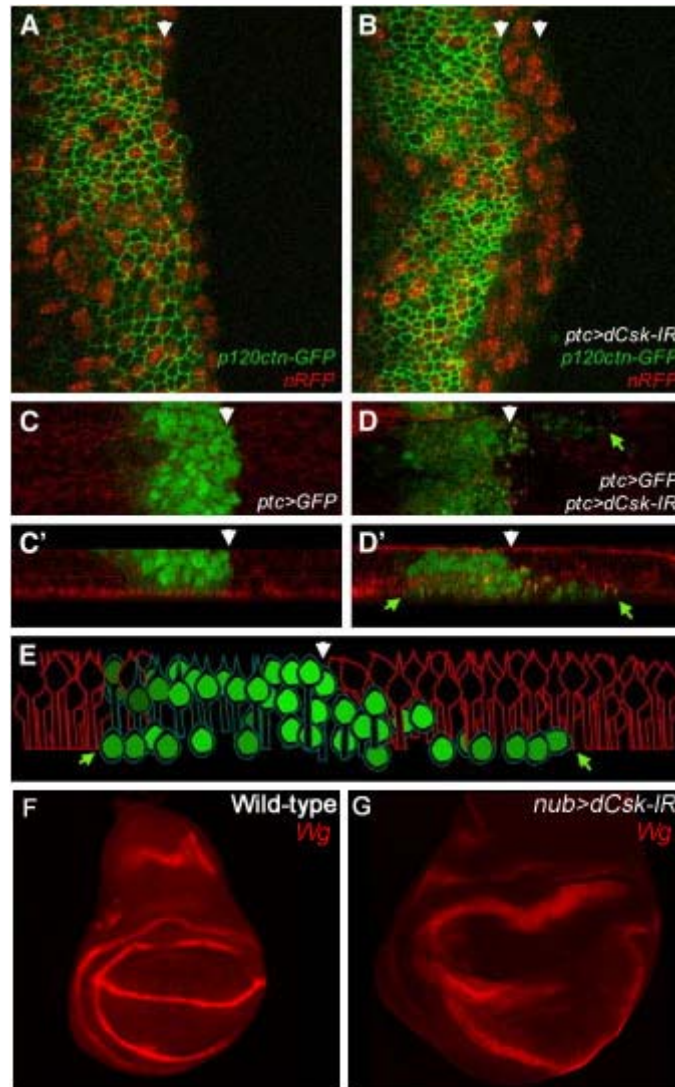
Figure 4. The outcomes of the local and broad loss of *dCsk*
(Panels A-E from Vidal *et al*., 2006; F-G from Kwon *et al*., 2014)
Arrows mark the anterior/posterior boundary in panels A-E. (A-E) Loss of *dCsk* in
discrete patches resulted in epithelial exclusion, invasive migration through the basal
extracellular matrix (green arrows in D'), and eventual apoptotic death; these events
occurred exclusively at the boundary between *dCsk* and wild-type cells. (F-G) Wing discs
were stained with Wingless. (F) Wild-type wing imaginal disc is shown. (G) Broad loss
of *dCsk* in the developing wing (*nub>dCsk*) resulted in overproliferation and disruption
of anterior/posterior polarity.

Preliminary Data

We first tested the effects of downregulation of *dCsk* on wing imaginal disc growth (*nub-GAL4; UAS dCsk^{RNAi}*) and found overgrowth in the wing pouch (Fig. 5a). Next, we tested the effects of activation (*nubGAL4;UAS Hpo$^{\Delta INH}$*) (Fig. 5b) (Udan et al., 2003; Verghese et al., 2012a, 2012b), and inactivation (*nub-GAL4 UAS Hpo^{RNAi}*) (Fig pathway (Fig. 6). Coexpression of *UAS d-csk^{RNAi}* with *UAS Hpo$^{\Delta INH}$* caused enhanced cell death (Fig. 5c) and *wg* induction (Fig. 6c), whereas coexpression of *UAS d-csk^{RNAi}*; *UAS Hpo^{RNAi}* together showed overgrowth (Fig. 5e), and expansion in *wg* expression (Fig. 5e) suggesting no additive effects. In summary, our preliminary data showed that *d-csk* interacts with *hpo*, and affects the target genes of Hippo pathway. Therefore, we proposed to test these interactions in detail. (Fig. 5d) on *d-csk* phenotype (Fig.5c, e), and regulation of Wingless, a target of Hippo.
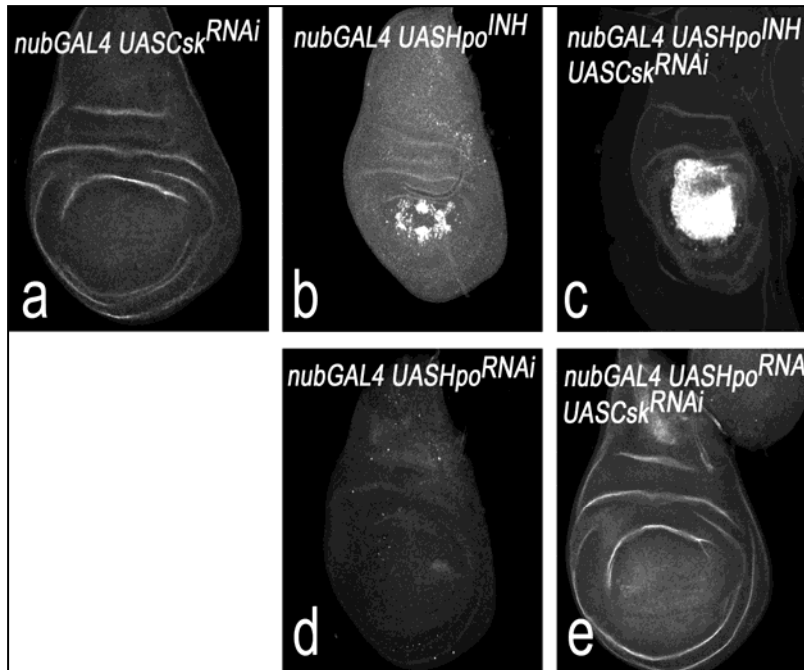
Figure 5. Caspase 3 staining of wing imaginal discs from third instar mutant larvae: Cell death induced by over-expression of Hpo (b) is unaffected by co-expression of *dCsk^RNAi* (c) suggesting that *d-csk* requires Hpo for regulating cell proliferation and growth.
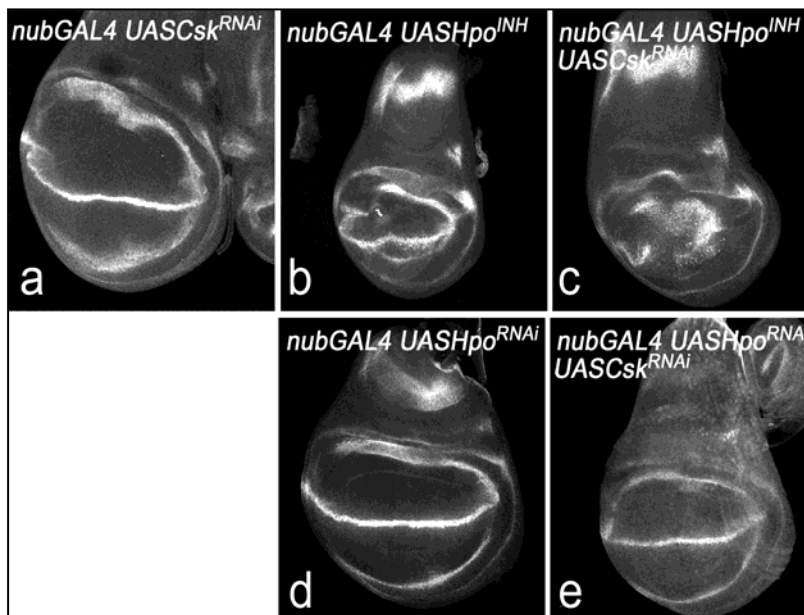

Figure 6. Wingless staining of wing imaginal discs from third instar mutant larvae: Regulation of Wg expression during *dCsk* loss of function reveals the over-proliferation of cells in the wing pouch. Over-expression of *Hpo* leads to cell death and loss of *dCsk* is unable to reverse this effect.

Why Study These Subjects

Emerging evidence supports that tumor development is regulated by cell-to-cell communication through multiple signals, namely Hippo and Src. Certain genes in each Hippo and Src pathway (*i.e.*, *wts* and *src*) have been shown to be regulated by a common kinase, Csk, suggesting that there may be a molecular link among the three (Figure 7). Although Csk activity is known to regulate metastatic behavior by decreasing cell adhesion, reduced *csk* expression alone is not
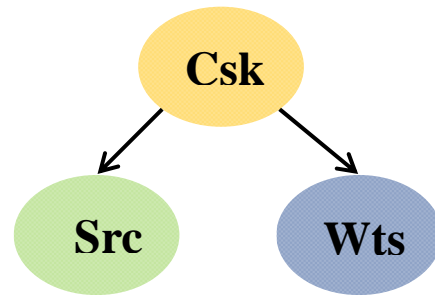
Figure 7. Csk at the Intersection between Src and Hippo Signaling Pathways
Certain genes in each Hippo and Src pathway (*i.e.*, *wts* and *src*) have been shown to be regulated by a common kinase, Csk, suggesting that there might be a molecular link among the three.

sufficient to direct stable tumor growth. The fact that discrete patches of *dCsk* fail to maintain survival of migrating cells called for further investigation of the Src-independent role of Csk in metastasis, specifically in relation to how important the Hippo signaling input is to Csk-mediated growth regulation.

Given that the growth regulatory functions of Wts occur through its interactions with Yki and the Hippo signaling pathway, we proposed that dCsk also regulates growth via the Hippo signaling pathway.

Hypotheses

Given the similarities of the loss of function phenotypes of *csk* and *wts*, we have studied the genetic interactions between *dCsk* and the Hippo signaling pathway. **We hypothesized that *dCsk* is another input into Hippo pathway.** To test this hypothesis, we took genetic approaches using mutations that modulate the levels of Hippo signaling to test two specific aims: (1) whether *dCsk* regulates the expression of transcriptional targets of Hippo signaling, *e.g.*, *ex-lacZ, fj-lacZ, dronc$^{1.7kb}$-lacZ,* and *diap1-4.3GFP,* and (2) genetic epistatic interactions between *dCsk* and components of the Hippo pathway.

> **Specific Aims:**

Aim 1: Determine whether *dCsk* regulates the expression of the transcriptional target genes of Hippo signaling, including *ex-lacZ, fj-lacZ, dronc$^{1.7kb}$-lacZ,* and *diap1-4.3GFP*. We tested if *d-csk* regulates the expression of the transcriptional targets [*ex-lacZ, fj-lacZ, dronc$^{1.7kb}$-lacZ,* and *diap1-4.3GFP*] of Hippo pathway. Our rationale was that all genes that act through Hippo pathway share a common set of transcriptional targets to regulate tissue size. At the conclusion of these experiments, we found evidence that *dCsk* requires Hippo signaling to regulate growth.

Aim 2: Determine whether *dCsk* interacts with other components of Hippo pathway. Our goal was to find the hierarchy of gene action; toward this aim, we carried out a series of genetic epistasis experiments with *dCsk* and other genes within Hippo pathway [*yki, wts, sav, D, zyx,* and *ex*]. We used combinations of the gain of function and loss of function phenotypes in order to place the genes in an epistatic pathway. These experiments were meant to provide the framework for *dCsk*-dependent input within Hippo signaling pathway.

Materials and Methods

Fly genetics: Many routine fly rearing and fly genetic techniques were used, such as the *GAL4-UAS* system to misexpress genes. Fly phenotypes were assessed for the aspects of pattern formation, growth regulation, and cell proliferation in the adult wings and wing imaginal discs.

Immunohistochemistry: Wing imaginal discs from third-instar larvae were dissected in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 20 minutes, and washed twice in PBST (PBS + 0.2% TritonX-100) for 10 minutes each at room temperature. Tissues were then blocked with 2% normal donkey serum for two hours and stained with primary antibody at 4°C overnight. Next, tissues were washed twice and incubated in secondary antibody for two hours at room temperature in foil-wrapped eppendorf tubes. Finally, tissues were mounted in Vectashield after three rounds of washing. Images were taken using confocal microscopy. The following primary antibodies were used: mouse-anti DIAP1 (1:250); mouse-anti βgal (1:100), mouse-anti Wingless (1:100), and rabbit-anti Caspase (1:250). Secondary antibodies (Jackson Immunoresearch) used were anti-mouse Cy3 (1:1000) and anti-rabbit Cy3 (1:1000). The samples were imaged using Olympus Fluoview 1000 Laser Scanning confocal microscope, and processed using Adobe Photoshop CS6.

Adult Fly Wing Mounting and Imaging

Adult flies were collected in 70% Ethanol and dehydrated in an ascending alcohol series. The wings of completely dehydrated flies were clipped in 100% ethanol and mounted in Canada Balsam (3 Canada Balsam: 1 Methyl Salicylate). Wings were photographed using Olympus BX51 Microscope mounted with an Olympus XM10 camera and CellSens

Dimensions Software. Adult flies showing no-wing phenotype were maintained at 4°C overnight before they were mounted. Adult fly images were taken with the Zeiss apotome microscope and Axivision software.

Fly Stocks: The following strains were obtained from the Bloomington Stock Center unless otherwise specified: *UAS-dCsk^RNAi; Sb/ S-T (from R. Cagan)*, y w; *FRT-82B dCsk^Q156Stop (from* R. Cagan), yw; *UAS-Ras^V12; FRT82B dCsk^Q156stop (from R. Cagan)*, *yw hsFlp; nub-Gal4 UAS-Hpo^RNAi/ CyO-GFP* (from G. Halder), *yw; nub-Gal4 UAS-Hpo^ΔINH/ CyO^Roi (from G. Halder)*, *Ubx-Flp; FRT-42D yki^B5/ CyO (from G. Halder)*, *UAS-Scrib^RNAi*, *UAS-D-V5, UAS-Zyx^RNAi*, *UAS-Sav-7A* (from G. Halder), *UAS-Wts-13F* (from G. Halder), *UAS-Ex-18* (from G. Halder), *UAS-Ft^RNAi* (VDRC # V9396), *UAS-Yki-V5,* and *nub-Gal4*. To test the loss-of-function phenotype of d*Csk*, we generated the line *UAS-dCsk^RNAi; nub-Gal4* by using appropriate genetic crosses. This line was outcrossed to other UAS-bearing transgenes to study genetic interactions between dCsk and Hippo pathway genes. Other crosses were performed to create the following lines: *UAS-dCsk^RNAi; nub-Gal4 UAS-D-V5/ CyO Roi, UAS-dCsk^RNAi; dronc^1.7kb-lacZ, UAS-dCsk^RNAi; ex^697-lacZ, UAS-dCsk^RNAi; fj-lacZ, UAS-dCsk^RNAi; Diap1-4.3 GFP, MS-1096; dronc^1.7kb-lacZ, MS-1096; ex-lacZ, MS-1096; fj-lacZ*, and *MS-1096; Diap1-4.3 GFP*. Flies were grown at 25°C unless noted otherwise.

Results: Loss of *dCsk* in large patches results in overgrowth

To study the effects of loss of *dCsk* we overexpressed *UASdCsk^{RNAi}* in the wing pouch using the *nubbin-GAL4* (*nub-GAL4*) driver (Fig. 8a), and found that loss of function of *dCsk* resulted in large overgrown wing pouch (Fig. 8g-i) in the imaginal discs and in adult wings (Fig. 8f). Earlier studies have also shown that loss of *dCsk* in homozygous discs induces apoptosis (Langton et al., 2007), therefore, we tested the effects of loss of *dCsk* (*nubGAL4 UASdCsk^{RNAi}*) on cell death using antibodies against activated Caspase 3 (Casp3*) and the *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1). In addition, we used the expression of Wingless to mark the boundary of the wing pouch (Neumann and Cohen, 1998). Compared to wing imaginal discs that show wild-type expression of Wg (Fig. 8b), activated Caspase 3 (Fig. 8c) and DIAP1 (Fig. 8d), loss of *dCsk* resulted in mild induction of Caspase 3 (Fig. 8h), and a remarkable downregulation of DIAP1 (Fig. 8i) expression. On the other hand, small patches of *dCsk* mutant cells generated using MARCM approach (Lee and Luo, 1999) resulted in small clones that did not overgrow, as the mutant cells were competed out by the surrounding wildtype cells (Vidal et al., 2006; Fig. 4 A-E). Thus loss of *dCsk* results in context dependent effects on growth, together with mild effects on apoptosis despite downregulation of DIAP1.
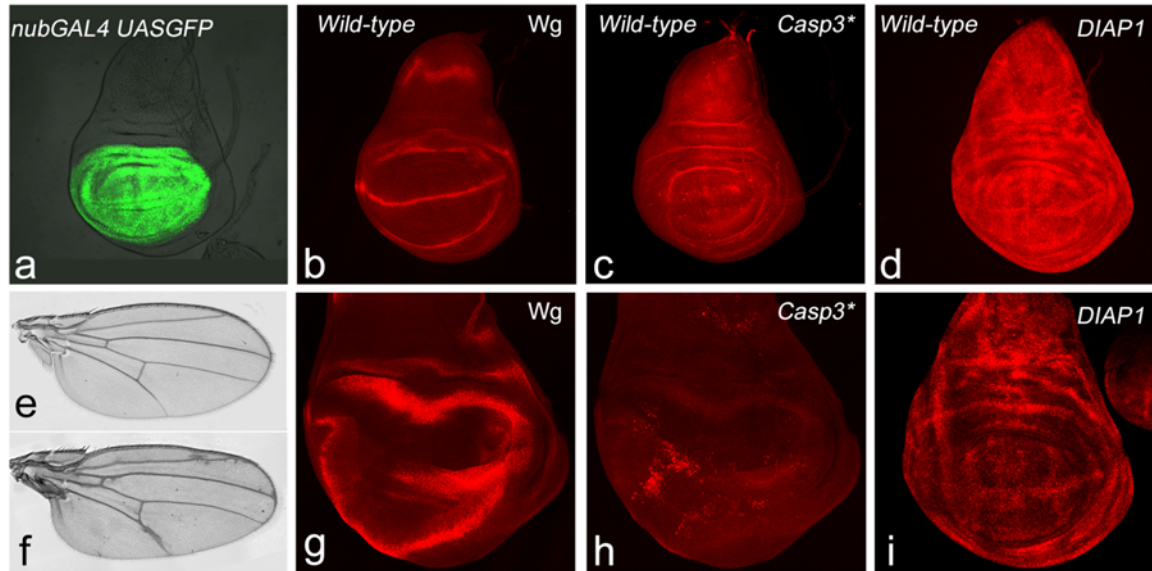
Figure 8. Comparison of wild-type and *nub-GAL4 UAS-dCsk^RNAi* (Kwon *et al.,* 2014) Panels show comparison of wing imaginal discs from third instar larvae of wild-type (a-d) and *nub-GAL4 UAS-dCsk^RNAi* (g-i) and corresponding adult wing phenotypes (e, g). The *nub-Gal* domain is shown using GFP expression (a). Loss of *dCsk* in *the nub-Gal4* expression domain causes overgrowth of the wing pouch (f). Regulation of Wingless expression during *dCsk* loss-of-function reveals over-proliferation of cells in the wing pouch (g). The overgrowth of *dCsk* mutant cells occurs despite induction of Casp3* (h) and down-regulation of DIAP1 (i).

Results: Loss of *dCsk* affects Yki activity

The increased proliferation and decreased apoptosis caused by loss of *dCsk* is similar to the effects of loss of function of Hippo pathway genes, so we tested the effect of loss of *dCsk* on transcriptional targets of Hippo signaling (Fig. 9). We tested several transcriptional targets of Hippo pathway (*ex-lacZ, fj-lacZ, dronc-lacZ* and *diap4.3GFP*). Compared to the wild-type expression of *ex-lacZ* (Fig. 9A), and *fj-lacZ* (Fig. 9C), in cells where *dCsk* is downregulated (*nubGAL4 UASdCsk^{RNAi}*) the levels of expression of *ex-lacZ* (Fig. 9B) and *fj-lacZ* (Fig. 9D) is upregulated suggesting that loss of *dCsk* leads to Yki activation. We also tested the expression of *diap1-4.3GFP,* the reporter transgene that contains the Hippo response element in *diap1* (Wu et al., 2008; Zhang et al., 2008)*,* to check if loss of *dCsk* affects *diap1* expression via the Hippo pathway. Compared to the expression of the *diap1-4.3GFP* in wild type wing discs (Fig.9E), we observed a downregulation of diap14.3-GFP expression in wing discs from *nubGAL4 UASdCsk^{RNAi}* larvae (Fig. 9F). This effect is similar to downregulation of DIAP1 protein in dCsk mutant cells. The Drosophila homolog of Caspase 9, *dronc,* is another cell death pathway gene that is transcriptionally regulated by the Hippo pathway.

Taken together, this data suggests that loss of *dCsk* results in increased Yki activity leading to upregulation of *ex* and *fj* transcription, but not *diap1* transcription. This increased Yki activity may in part explain the overgrowth phenotype of the *nubGAL4 UASdCsk^{RNAi}* wing discs.

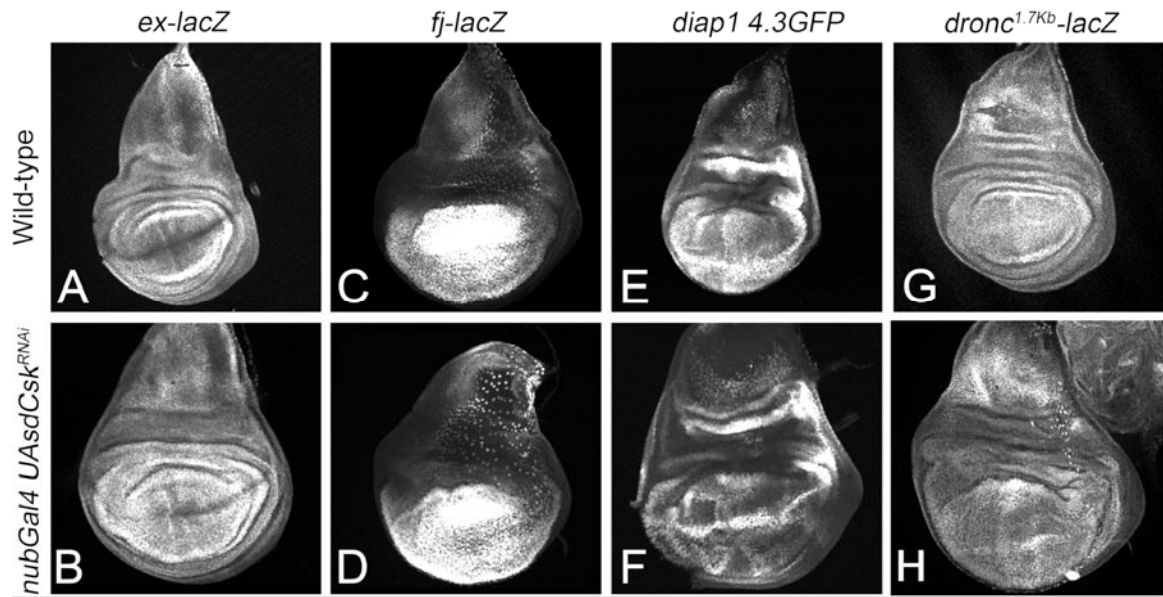**Figure 9. Hippo target genes are affected by loss of *dCsk*.**
(A, C, E, G) Panels show *ex-lacZ* (A), *fj-lacZ* (C), and *diap1-lacZ* (E) and *dronc-lacZ* (G) expression in wild-type wing imaginal discs. (B, D, F, H) Panels suggest that loss of *dCsk* affects Yki activity. *dCsk^{RNAi}* causes induction of *ex-lacZ* (B), *fj-lacZ* (D), and *dronc^{1.7kb}-lacZ* (F). Interestingly, *diap1-lacZ* (F) remains down-regulated.

Results: *dCsk* genetically interacts with the Hippo pathway

Since the phenotypes of *dCsk* suggested that *dCsk* caused increased Yki activity, we tested if *dCsk* genetically interacted with Hippo pathway genes. First we tested genetic epistasis interactions between *dCsk* and Yki. For all epistasis experiments, we used two criteria to analyze the interaction. First, we tested for the effects of epistatic interactions on DIAP1 expression, and second, we compared the size of the wing pouch. The *nub-GAL4* driver is expressed in the wing pouch, and the size of the wing pouch can be measured using the expression of Wg as a guide for the extent of the *nubbin* domain. This is because the edge of *nubbin* expression overlaps with the outer wing hinge-specific expression of Wg (Neumann and Cohen, 1998). Over-expression of Yki leads to up-regulation of DIAP1 levels (Fig. 10c) and an overgrowth of the wing pouch (Fig. 10d) in the *nub* domain. In comparison, loss of *dCsk* leads to downregulation of DIAP1 (Fig.8i) and an overgrowth of the wing pouch (Fig. 8g-i). Co-expression of *dCsk*[RNAi] and Yki resulted in up-regulation of DIAP1 levels (Fig. 10e) and an overgrowth of the wing pouch (Fig. 10f). Taken together, these data suggest that Yki may act downstream of *dCsk*.

To further clarify this epistatic relationship, we tested if the overgrowth caused by loss of *dCsk*[RNAi] is affected by heterozygosity for *yki*[B5]- the null allele for *yki*. It is well established that Hippo signaling is sensitive to dose of Yki, and reduction in Yki levels is known to affect loss of function phenotypes of other upstream genes in the Hippo pathway (Doggett et al., 2012; Verghese et al., 2012). In wild type, reduction in *yki* levels (*yki*[B5]/+) has no obvious effects on growth (Fig. 10g,h). Heterozygosity of *yki* creates a sensitized background. We observed that heterozygosity of *yki*[B5] resulted in reduction in

the overgrowth observed in the wing pouch of *nubGAL4 UASdCsk^RNAi* wing discs (Fig.

10i,j compared to Fig. 8g,i), and DIAP1 levels were restored (Fig. 10i). This suggests

that *dCsk* acts upstream of Yki, and may require Yki for regulating cell proliferation/

tissue growth.

Previous studies identified *dCsk* as a genetic modifier of loss- or gain-of-function

phenotypes of Wts (Stewart et al., 2003). Wts acts downstream of dCsk to mediate its

growth regulatory functions *in vivo*, and dCsk phosphorylates Wts *in-vitro* (Stewart et al.,

2003). We confirmed this epistatic interaction between dCsk and Wts using our

experimental system in the wing pouch (Fig. 11 a-d). Over-expression of *UAS-Wts* results

in hyperactivation of the Hippo pathway and results in smaller organs due to increased

apoptosis (Tapon et al., 2002; Verghese et al., 2012b). Over-expression of *UAS-Wts*

under *nubGAL4* results in reduction of wing pouch size (Fig. 11a, b). Co-expression of

UAS-Wts with *UAS-dCsk^RNAi* (Fig. 11c, d) resulted in a complete suppression of *dCsk*

phenotype of overgrowth (Fig. 8g,i), suggesting that *dCsk* acts upstream of Wts.

Next, we tested the genetic interaction between Hpo and *dCsk* using a similar

approach. Overexpression of *UAS-Hpo* also results in induction of apoptosis and

reduction in organ size (Udan et al., 2003; Verghese et al., 2012a; Wu et al., 2003). Over-

expression of *UAS-Hpo* under *nubGAL4* results in smaller wing pouch (Fig. 11e, f), and

coexpression of *UAS-dCsk^RNAi* and *UAS-Hpo* resulted in generation of small wings (Fig.

11 g,h) akin to the phenotype of Hpo overexpression suggesting that *dCsk* acts upstream

of Hpo. Taken together, these data suggests that dCsk interacts with the core components

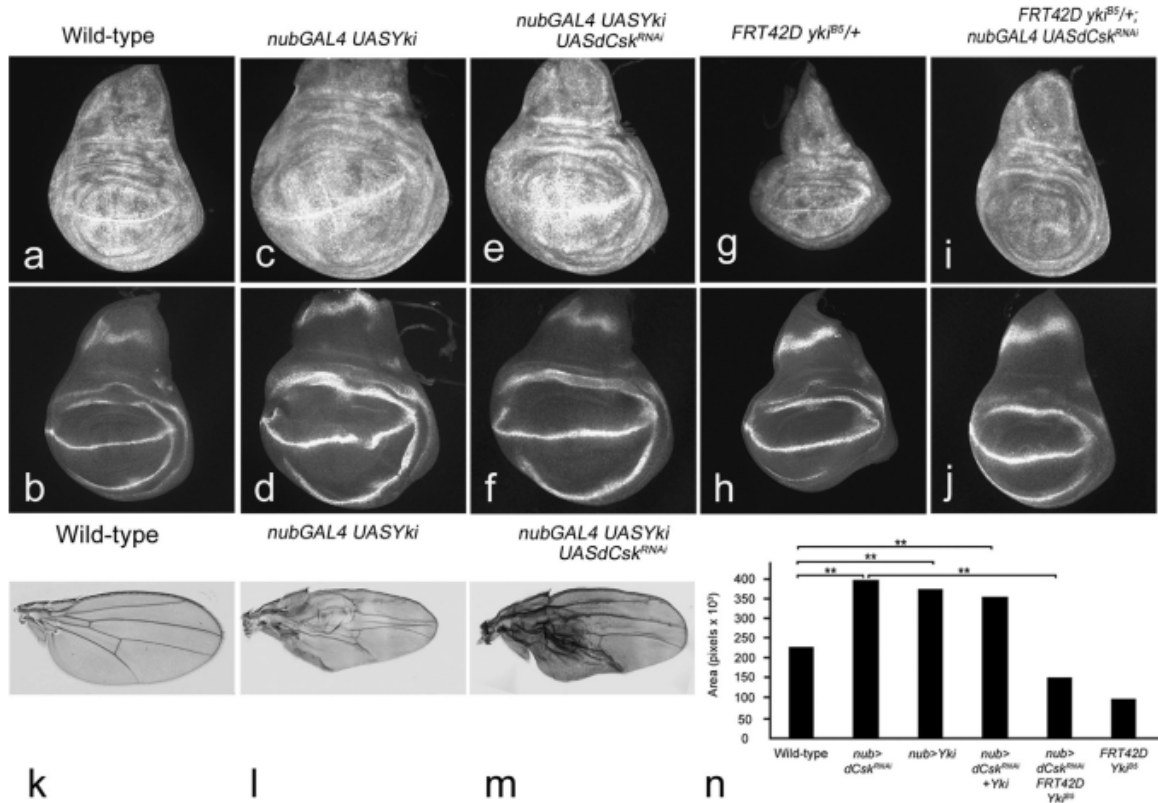of the Hippo pathway to regulate cell proliferation, and tissue sizes.

Figure 10. dCsk requires Yki to regulate growth. (Kwon et al., 2014)
(a, b) Wild-type wing imaginal discs stained for expression of DIAP1 (a) and Wg (b) are shown. (c, d) The effect of overexpresion of Yki on DIAP1 (c), and Wg (d) in wing imaginal discs is shown for comparison. Note the overgrowth of the wing pouch due to increased Yki levels. (e, f) Co-expression of *UASdCsk-IR* (*UASdCsk-IR; nub-GAL4/UASYki*) leads to induction of DIAP (e) and overgrowth of wing pouch (f). (g, h) Wing imaginal discs from *FRT42D yki^{B5}/CyO* larvae stained with antibodies against Diap1 (g) and Wg (h). (i, j) *UASdCsk-IR; nub-GAL4/FRT42D yki^{B5}* wing discs showing reduced expression of DIAP (i) and reduced growth of the wing pouch (j) due to heterozygosity of *yki^{B5}*. (k-m) Adult wings from wild-type (k), *nub-GAL4/UASYki* (l) and *UASdCsk-IR; nub-GAL4/UASYki* (m) flies showing increased growth defects caused by co-expression of *Yki* and *dCsk-IR*. (n) The chart shows quantification of wing pouch growth for wing imaginal discs of the indicated genotypes. Asterisks indicate that the genotypes where there is significant difference in wing pouch growth (n=5, p<0.05).
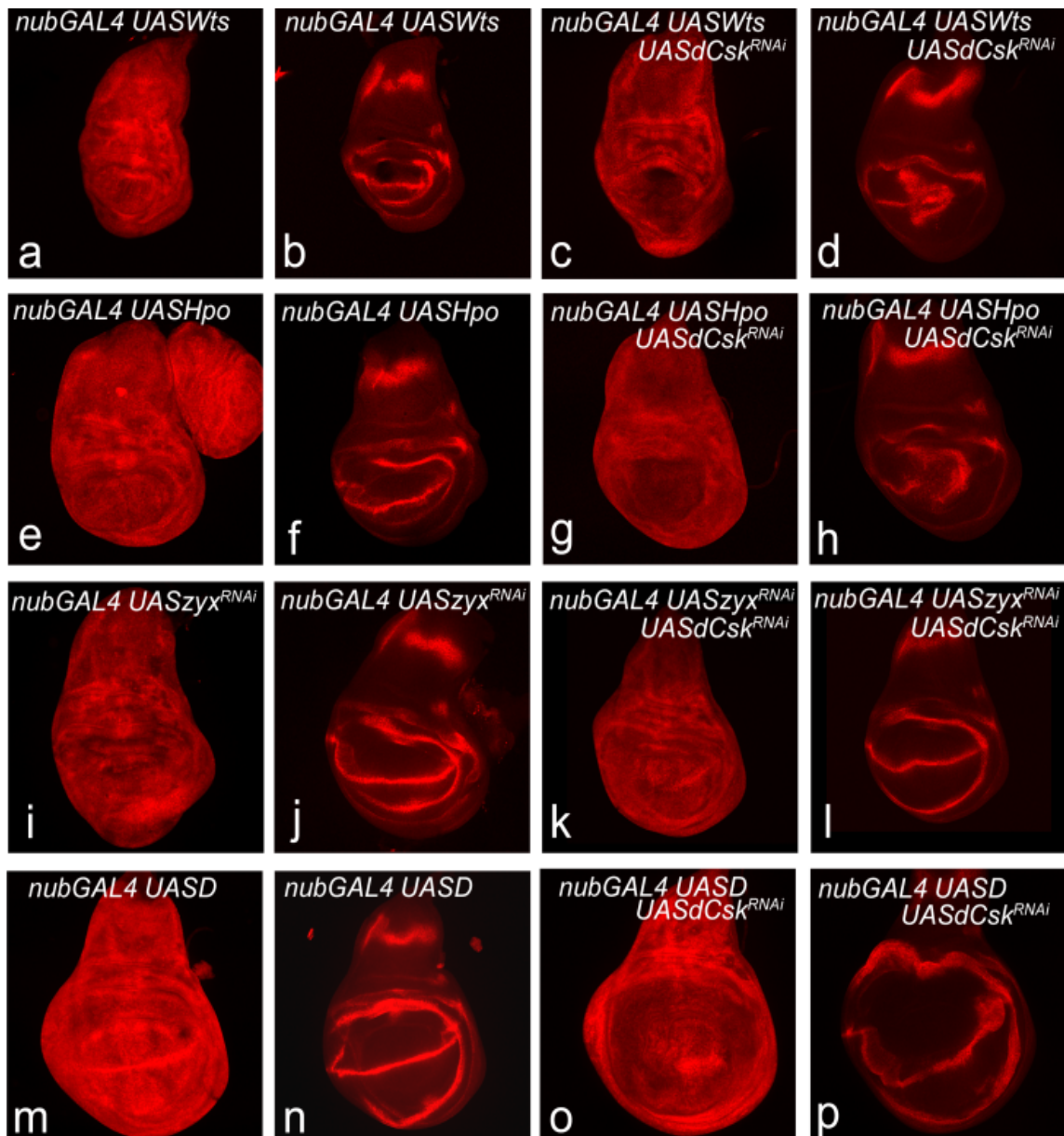
Figure 11. *dCsk* **interacts with the Hippo pathway.**
Panels show wing imaginal discs from *nub-GAL4 UAS- wts* (a, b), *nub-GAL4 UAS-hpo* (e, f), *nub-GAL4 UAS-zyx^RNAi* (I, j), and *nub-GAL4 UAS-D* (m, n) larvae stained for DIAP1 and Wg (respectively). Phenotypes of *nub-GAL4 UAS-wts UAS-dCsk^RNAi* (c, d), *nub-GAL4 UAS-hpo UAS-dCsk^RNAi* (g, h), *nub-GAL4 UAS-zyx^RNAi UAS-dCsk^RNAi* (k, l) show reduction of the wing pouch size, whereas *nub-GAL4 UAS-D UAS-dCsk^RNAi* (o, p) shows overgrowth. Interestingly, all co-expression phenotypes (c, g, k, o) with *dCsk^RNAi* show down-regulation of DIAP1, which is characteristic of *nub-GAL4 UAS-dCsk^RNAi*.

Results: *dCsk* acts upstream of *Zyx* and downstream of *Dachs*

Next, we extended our investigation of genetic interactions between *dCsk* with two other upstream components of the Hippo pathway: Zyxin (Zyx), and Dachs (D). Zyx and D act downstream of the atypical cadherin Fat and are known to negatively regulate levels of Wts protein (Rauskolb et al., 2011). Downregulation of Zyx (using UAS-Zyx^{RNAi}) results in an overall reduction in the wing pouch (Fig. 11i, j). Co-expression of *UAS-dCsk^{RNAi}* with *UAS-Zyx^{RNAi}* phenocopies the *UAS-Zyx^{RNAi}* phenotype of reduction in wing pouch (Fig. 11k, l), suggesting that *dCsk* acts upstream of *Zyx,* likely requires *Zyx* to regulate growth. Over-expression of D (*UAS D*) leads to upregulation of DIAP1 (Fig. 11m) and overgrowth of the wing pouch (Fig. 11n). Co-expression of *UAS-D* and *UAS-dCsk^{RNAi}* phenocopies the effects of *UAS-dCsk^{RNAi}* over-expression, suggesting that *dCsk* acts downstream of D. Taken together, our epistasis interactions place *dCsk* downstream of D and upstream of Wts.

To further confirm the *dCsk*-D epistasis, and to test if dCsk regulates growth through the Hippo pathway, we tested if the expression of *fj-lacZ*, the transcriptional target of Ft and Yki signaling, is affected in discs co-expressing *UAS-dCsk^{RNAi}* and *UAS-D*. *fj* is expressed in a gradient in the wing pouch, with the highest levels of *fj* expression coinciding with the presumptive (DV) wing margin, and a gradient of decreasing *fj* expression that extends in both the dorsal and ventral wing pouch (Fig. 12a) (Cho and Irvine, 2004; Ishikawa et al., 2008; Simon et al., 2010). Loss of dCsk leads to robust induction of *fj-lacZ* expression and overgrowth (Fig. 9D), whereas over-expression of D causes moderate upregulation of *fj-lacZ* and mild hyperplasia (Fig. 12b). Co-expression

of *UAS-dCsk^{RNAi}* with UAS D caused robust overgrowth of the wing pouch and robust

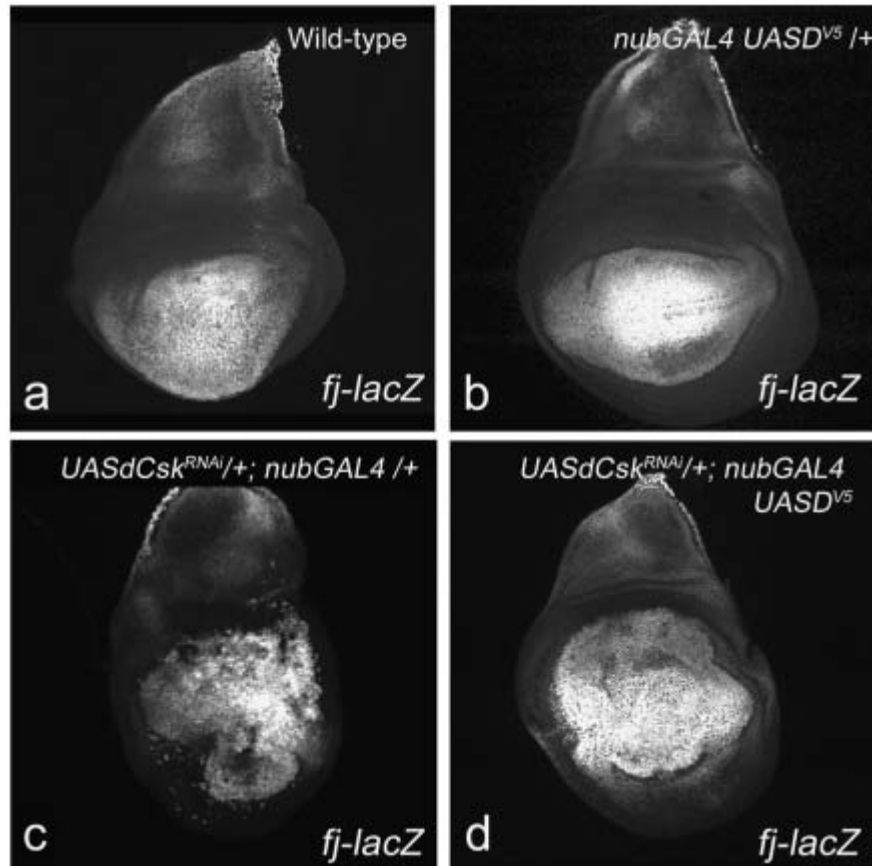induction of *fj-lacZ* expression (Fig. 12d) due to increased Yki activity.

Figure 12. dCsk acts downstream of Dachs in the Hippo pathway.
A comparison of *fj-lacZ* expression is shown for wing imaginal discs from (a) wild-type, (b) *nub-GAL4 UASD^v5/+,* (c) *UASdCsk-IR; nub-GAL4/+,* and (d) *UASdCsk-IR; nub-GAL4 UASD^v5/+*larvae.

Conclusions

The *Drosophila C-terminal src kinase* (*dCsk*) is a tumor suppressor gene, whose loss-of-function is reported to cause multiple defects in growth regulation (Read et al., 2004, Stewart et al., 2003, Vidal et al., 2006). Our characterization of the effects of loss of *dCsk* and genetic interaction analysis showed that *dCsk* mutant cells induce the transcriptional activity of the Hippo pathway effector Yki; increased Yki activity leads to uncontrolled proliferation and formation of larger organs. Our genetic epistasis places dCsk between Dachs and Warts, which corroborates with the findings from earlier studies in flies where dCsk was shown to act upstream of Wts (Stewart et al., 2003). Further, the analysis of Yki activity in cells deficient for *dCsk* revealed that loss of *dCsk* induced transcription of *ex-lacZ* and *fj-lacZ*, two well-established reporters of Yki activity.

We concluded that dCsk acts downstream of the atypical myosin Dachs, and upstream of the Ajuba LIM protein Zyx and the Wts kinase within the Hippo pathway through which it affects Yki activity. D is known to bind Zyx, and D also stimulates binding of Zyx to Wts. Zyx and D act downstream of Fat in the Hippo pathway and regulate the stability of Wts. Thus, in the future it would be interesting to investigate if dCsk is a part of the D/Zyx/Wts complex, or if it regulates Wts via phosphorylation dependent mechanisms, or if D or Zyx are involved in the mechanisms that localize dCsk to the membrane, where dCsk acts on its substrates. In summary, our data uncovers dCsk as a new input in the Hippo signaling pathway, and reveals the intersection of the Hippo and Src signaling pathways, which are of wide interest because of the roles they play in the regulation of normal development and the effects of their misregulation occurred in cancers.

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Src and Hippo Signaling: The Intersection of Two Tumor Suppressor Pathways

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Abstract

Growth of a single-celled zygote to a fully developed organism requires three processes: cell division, cell differentiation and quiescence following morphogenesis. Developmental genetic pathways control all of these processes. The molecular underpinnings of these pathways (*e.g.*, the MAPK, p53, Hippo, TSC/TOR pathways) are beginning to emerge. It is clear that not only the genes comprising these pathways play essential roles during development, but loss of these genes is responsible for several diseases including cancer. Two such pathways, *viz.*, Hippo and Src which comprise a network of tumor suppressor genes and oncogenes, are the focus of this review. These pathways control tissue and organ size during development by regulating cell proliferation, cell death, cell migration, and cell adhesion. Overall, the chief components and known interactions of the two pathways are discussed.

Introduction

The transformation of a single-celled zygote into a complex multi-cellular organism requires three processes: cell division, cell differentiation, and morphogenesis. A fertilized egg, called the zygote, undergoes multiplicative or embryonic growth via mitosis, dividing into a multi-cellular organism (Raff, 1992; Conlon and Raff, 1999).

After multiplication, these cells specialize in different structure and function due to differential gene expression, and organize themselves into three-dimensional organs. Yet, growth of an organism does not stop after embryogenesis. The growth of certain body parts, such as the muscle, is due to auxesis, in which the size of cells increases while the number of cells remains the same. In a mature organism, the differentiated cells have lost the capacity of undergoing division, but undifferentiated cells present at some locations keep dividing mitotically to replace worn-out cells; this production of reserve cells from an increase of intercellular material is called the accretionary growth. Evidently, ongoing growth is restricted to the formation of cells that are needed to be replenished. As opposed to a tightly controlled growth during development, the phenomenon of unregulated growth is named cancer, one of the major leading causes of death worldwide.

Cancer arises from tumor cells that continually divide due to their inability to recognize growth regulatory signals, including signals controlling apoptosis (Kerr *et al.*, 1972; Jacobson *et al.*, 1997; Wyllie *et al.*, 1980) and contact inhibition. Cells that repetitively undergo cell cycle are likely to become cancerous, due to multiple errors that have accumulated from the numerous DNA replications. Indeed, most cancer cells contain at
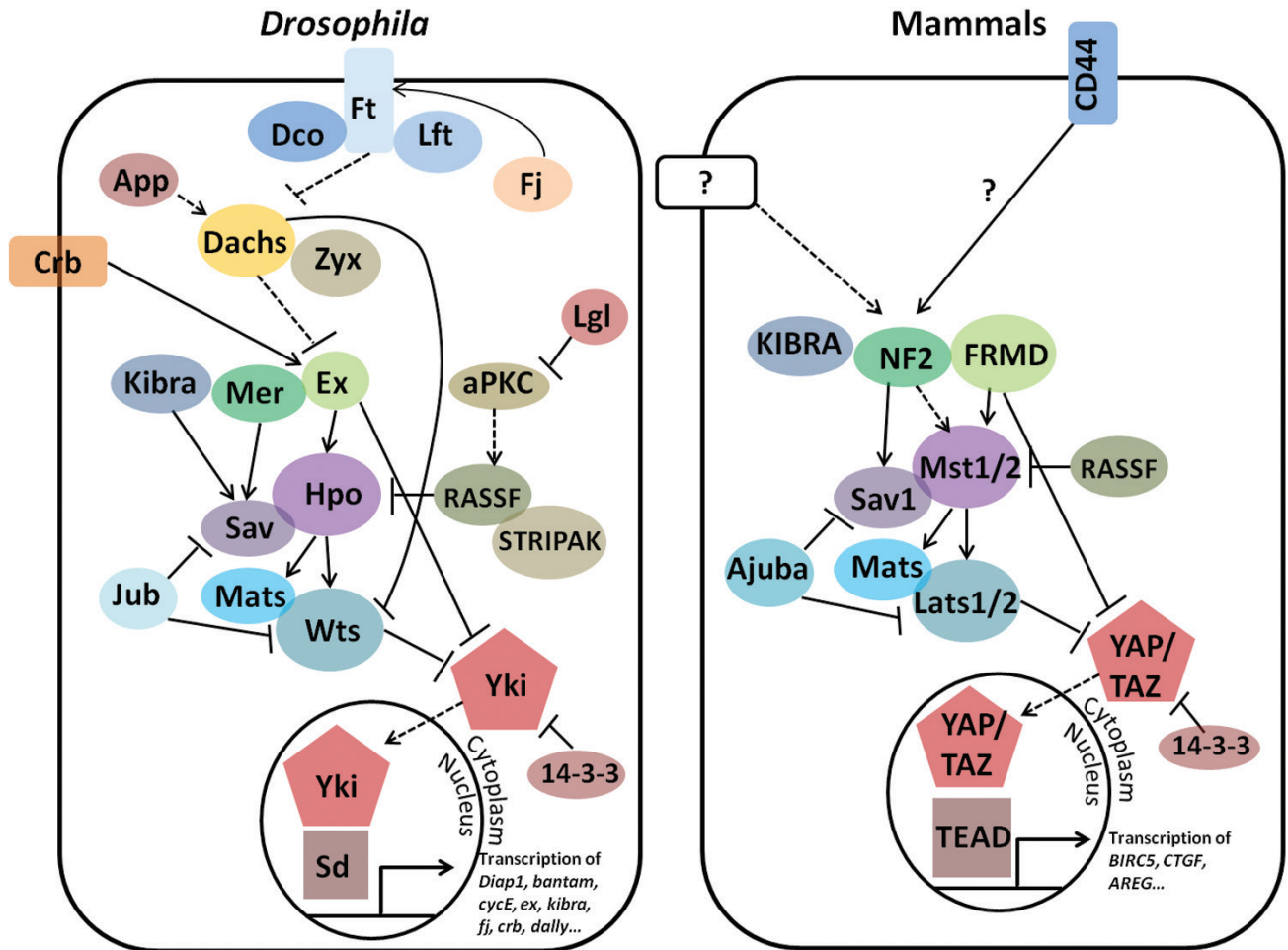
Figure 1. The Hippo Signaling Network in *Drosophila* and Mammals: Corresponding proteins in *Drosophila* and mammals are indicated by matching colors and shapes. Solid lines indicate direct biochemical interactions, whereas dashed lines show indirect interactions. Arrowed or blunted end indicates activation or inhibition. Selected target genes are shown.

least one mutated gene, or an oncogene, which can permanently activate the cell cycle and cause malignancy. In addition, inactivation or deletion of both copies of a tumor suppressor gene can also give rise to cancerous cells. These malignant tumors evade apoptosis, or programmed cell death, and continue to proliferate by increasing in their number. The more serious problem occurs when the malignant tumors spread throughout the body via metastasis, inducing the surrounding cells to also become tumorous. Studies to identify genes and genetic mechanisms that are involved in tumorigenesis and metastasis have led to the discovery of several tumor-suppressor networks (*e.g.*, the MAPK, p53, Hippo, TSC/TOR pathways) whose genes are often mutated in cancers. These studies reveal that only some cells in the tumor acquire the ability to metastasize, suggesting that additional mutations are responsible for metastatic defects. Amongst the pathways involved in tumorigenesis, the recently identified

Hippo pathway has garnered the most attention. Originally identified by studies in *Drosophila*, the Hippo signaling pathway largely contributes to organ size regulation (Harvey and Tapon, 2007) in both invertebrates and vertebrates (*Figure 1*). Loss-of-function of the genes within this pathway leads to potent tumorigenesis in flies and humans.

The Hippo Signaling Pathway

The Hippo signaling pathway is a complex network of tumor suppressor genes and oncogenes, whose mutations lead to large, Hippopotamus-like phenotype (reviewed by Edgar, 2006; Pan, 2007; Saucedo and Edgar, 2007; Kango-Singh and Singh, 2009). It regulates organ size by inhibiting cell proliferation and promoting apoptosis (*Figure 2*). At the core of the pathway reside two kinases, Hippo (Hpo, the serine/threonine Ste20-like kinase) (Harvey *et al.*, 2003;
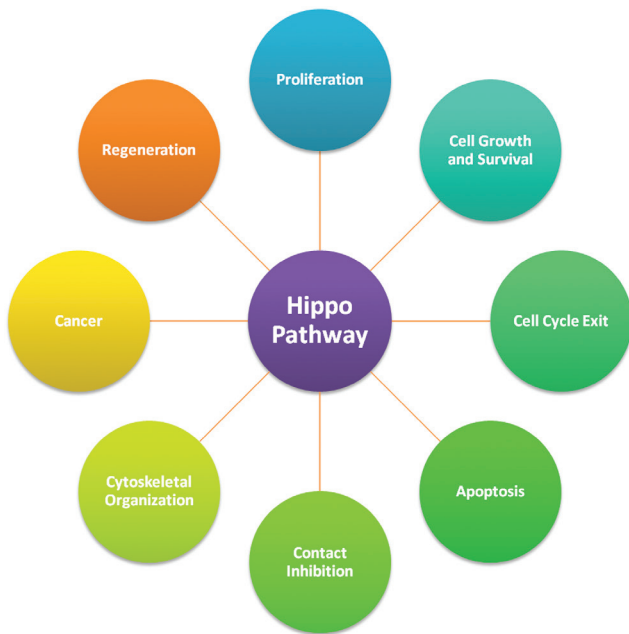
Figure 2. Roles of the Hippo Pathway: The Hippo signaling pathway regulates various cellular activities, including cell growth, proliferation, cell death, cell cycle, morphogenesis, and cell-to-cell interactions.

Jia *et al*., 2003; Pantalacci *et al*., 2003; Udan *et al*., 2003; Wu *et al*., 2003) and Warts (Wts, the nuclear Dbf-2-related (NDR) family kinase; also known as Lats) (Justice *et al*., 1995; Xu *et al*., 1995), and two adaptor proteins, Salvador (Sav, the WW domain scaffolding protein) (Kango-Singh *et al*., 2002; Tapon *et al*., 2002) and Mob as Tumor Suppressor (Mats) (Lai *et al*., 2005). Initially, Hpo forms a complex with Sav to phosphorylate Wts. Wts, in turn, forms a complex with Mats to phosphorylate Yorkie (Yki) (Huang *et al*., 2005), a transcriptional coactivator, retaining Yki in the cytoplasm. In the absence of Hippo signaling, the dephosphorylated Yki translocates into the nucleus and binds to the transcription factors (*e.g*., Scalloped, Sd), inducing the expression of target genes that (1) promote cell proliferation (*e.g*., bantam microRNA, myc), (2) induce cell cycle progression (*e.g*., E2F1, cyclins A,B, E), or (3) inhibit apoptosis (*e.g*., diap1), which results in tissue overgrowth (Huang *et al*., 2005) (*Table 1*).

Multiple points of signal integration have been found in the Hippo Pathway, suggesting that the core kinase cassette responds to diverse stimuli. Examples of the upstream regulators include Expanded (Ex), Merlin (Mer) (Hamaratoglu *et al*., 2006), Kibra, Fat (Ft, the protocadherin) (Bennett and Harvey, 2006; Cho *et al*., 2006; Silva *et al*., 2006; Willecke *et al*., 2006), Tao1 (Boggiano *et al*., 2011; Poon *et al*., 2011), Crumbs (Crb), Ajuba (Jub), and Scribble (Scrib) (Verghese *et al*., 2012). Ex, Mer, and Kibra function together

to activate the Hpo kinase cascade by directly binding to the Hpo-Sav complex (Yu *et al*., 2010). Additionally, Ex is known to directly repress Yki. The Ex-Mer-Kibra branch is modulated by the Fat/Dachsous branch, which does so by regulating Ex levels. The Fat/Dachsous branch consists of the atypical cadherins, Ft and Dachsous (Ds), and the downstream effectors Discs overgrown (Dco, a serine-threonine kinase; aka casein kinase 1ε), Dachs (D, an atypical myosin), Approximated (App, a palmitoyltransferase), Lowfat (Lft), and Zyxin (Zyx) (Grusche *et al*., 2010; Rauskolb *et al*., 2011). Tao1, a sterile 20-like kinase, is found to phosphorylate and activate Hpo. Furthermore, the Hippo pathway activity is thought to be also modulated by cell polarity, cell adhesion, and cell junction proteins, such as Crb, Jub, Scrib. The cell junction proteins exist in the epithelial cells of *Drosophila melanogaster* at the sub-apical region (SAR), adherens junction (AJ) or septate junction (SJ). Notably, the Hippo pathway activity is also affected by the modulation of the apical level of filamentous actin (F-actin). High accumulation of F-actin inhibits the Hippo signaling, thereby activating Yki.

Yki is the major downstream target of the Hippo signaling pathway. Upon phosphorylation by Wts, Yki creates a binding site for 14-3-3 proteins; this binding restricts Yki in the cytoplasm and leads to its degradation. Without proper phosphorylation, however, Yki can bind to several transcription factors, such as Scalloped (Sd), Homothorax (Hth) (Peng *et al*., 2009), Teashirt (Tsh), and Mothers against DPP (Mad) (Oh and Irvine, 2011), to promote tissue growth. In addition, the activation of Yki results in autoregulation of some upstream genes, such as *ex, mer, kibra, crb,* and *fj* via a positive feedback loop. Such broad spectrum of target genes confers tremendous versatility to Hippo signaling. Emerging evidence demonstrates that the Hippo pathway crosstalks with other signaling pathways to regulate different target genes.

Table 1. Function of the Hippo Pathway Target Genes

Function	Example Target Gene(s)
Cell cycle exit	*expanded*
Cell growth and survival	*homothorax (hth), diminutive (myc), bantam microRNA (miRNA)*
Proliferation	*cyclins A, B, E, E2F transcription factor (E2F1), hth, bantam miRNA*
Apoptosis	*merlin (mer), diap1*
Morphogenesis	*dally, dally-like*
Planar Cell Polarity	*crumbs, fat*
Contact Inhibition	*Mer*

The Src Pathway and Csk

The Src-family protein tyrosine kinases (SFKs) are implicated in various cellular processes, such as cell cycle exit, cell proliferation, survival, differentiation, adhesion, and cytoskeletal rearrangement. For instance, abnormal activation of the SFKs is involved in proliferative disorders such as cancer, particularly in liver, breast and colon (Masaki *et al.*, 1999; Bougeret *et al.*, 2001; Cam *et al.*, 2001; Frame, 2002), and is associated with metastatic behavior (Yeatman, 2004). The SFKs were originally identified in the transforming gene of the Rous-Sarcoma virus, v-src. *Drosophila melanogaster* has two SFK members, Src42 and Src64 (Simon *et al.*, 1985; Potter *et al.*, 2000). The SFK activity is inhibited by phosphorylation of their Carboxyl-terminal (C-terminal) region by C-terminal Src kinase (Csk) (Cole, 2003). Flies have one Csk homolog, *dCsk* (Read, 2004; Stewart, 2003), which functions similarly to the mammalian Csk (Hoffmann *et al.*, 1983; Simon *et al.*, 1985; Takahashi *et al.*, 1996). In fact, Csk was first identified by its ability to negatively regulate the SFK activity. Reduced levels of *dCsk* activate Src kinases (Thomas and Brugge, 1997; Schwartzberg, 1998; Bjorge *et al.*, 2000), including Jun N-terminal kinase (JNK), Stat, and Btk29A (Pedraza, 2004, Read, 2004); this Src activation results in organ size increase, lethality of the organism, and overproliferation due to extra cell cycles (Read, 2004; Stewart, 2003). Thus, Csk acts as a tumor suppressor through the Src pathway regulation (Read, 2004), while also negatively regulating the JNK pathway.

Csk is known to have Src-independent functions as well (*Figure 3*). Independently of the SFK activity, Csk links G-protein signaling to the actin cytoskeleton (Lowry *et al.*, 2002). In addition, Csk phosphorylates a number of other downstream molecules (Autero *et al.*, 1994; Hildebrand *et al.*, 1995; Cloutier and Veillette, 1996; Tremblay *et al.*, 1996). Within the past decade, *dCsk* has been reported to regulate cell proliferation by genetically modifying wts tumor suppressor gene, a core component of the Hippo pathway, by direct phosphorylation (Stewart 2003). Presumably, mammalian Wts molecules may also be substrates of Csk, considering that the C-terminal *dCsk* phosphorylation site is conserved in other Wts homologs.

Broad loss of *csk* results in overproliferation (*Figure 4 G*), inhibition of apoptosis, and decreased cell adhesion. However, local inactivation of *dCsk* in discrete patches surrounded by normal cells does not cause overgrowth (*Figure 4 A-E*), as *dCsk* mutant cells begin to delaminate and disperse from the growing imaginal disc tissue, leading to their elimination by macrophages (Vidal *et al.*, 2006). Although loss-of-function phenotype of *dCsk* is strikingly similar to that of hpo, wts, sav, and mats (or gain of Yki function), clonal patches of *dCsk* cells fail to survive to adulthood, unlike cells containing the others four genes. Instead, these cells spread among the wild-type cells while simultaneously undergoing apoptosis, which may reflect the function of Src in promoting motility and invasion (Langton, 2007).
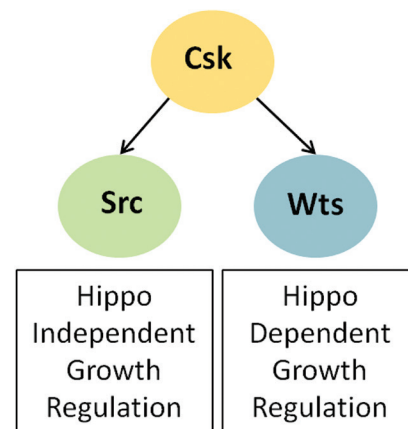

Figure 3. Csk at the Intersection between Src and Hippo Signaling Pathways: Certain genes in each Hippo and Src pathway (i.e., *wts* and *src*) have been shown to be regulated by a common kinase, Csk, suggesting that there might be a molecular link among the three.

More recently, Src has been reported to control tumor microenvironment by JNK-dependent regulation of the Hippo pathway (Enomoto and Igaki, 2013). Clone cells of Src overexpression activate the Rac-Diaphanous and Ras-mitogen-activated protein kinase (MAPK) pathways, which induces accumulation of F-actin, one of the upstream regulators of the Hippo pathway. Highly accumulated F-actin inhibits the Hippo signaling, thus activating Yki (Fernandez *et al.*, 2011; Richardson, 2011; Sansores-Garcia *et al.*, 2011) in both Src mutants and wild-type cells. Simultaneously, Src activates the JNK pathway, which signals the propagation of Yki activity to surrounding wild-type cells; the surrounding tissue is overgrown as a result. On the other hand, activated STAT acts independently of the Hippo pathway (Rodrigues, 2012). Src is known to interact with STAT. Thus, it is important to continue investigating the new, independent roles of Src and Csk.
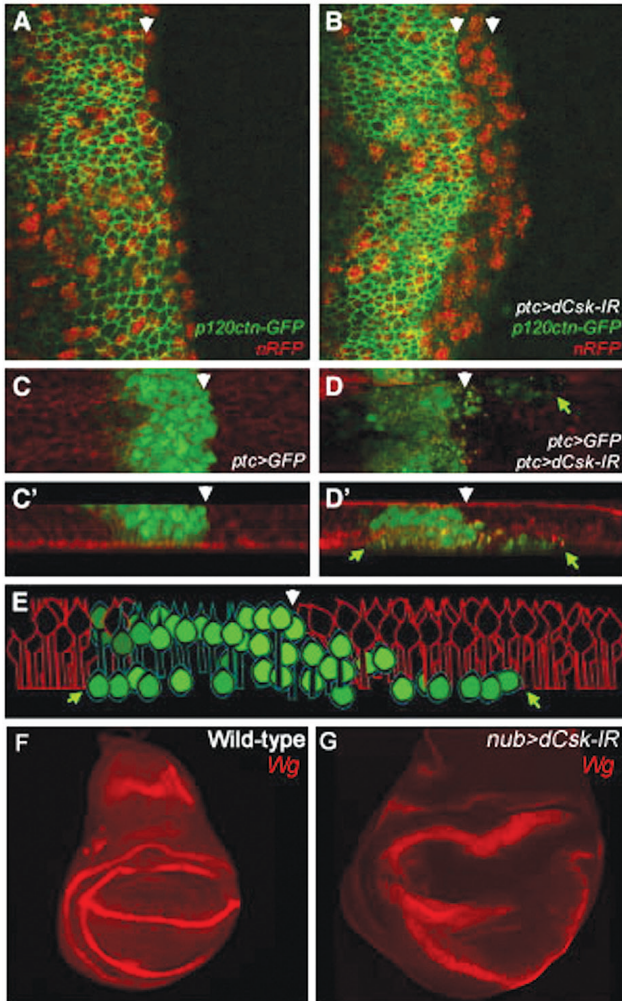
Figure 4. The Outcomes of the Local and Broad Loss of *dCsk*: (Panels A-E from Vidal *et al.*, 2006; F-G from Kwon *et al.*, *manuscript in preparation*.) Arrows mark the anterior/posterior boundary in panels A-E. (A-E) Loss of *dCsk* in discrete patches resulted in epithelial exclusion, invasive migration through the basal extracellular matrix (green arrows in D'), and eventual apoptotic death; these events occurred exclusively at the boundary between *dCsk* and wild-type cells. (F-G) Wing discs were stained with Wingless. (F) Wild-type wing imaginal disc is shown. (G) Broad loss of *dCsk* in the developing wing (nub>*dCsk*) resulted in overproliferation and disruption of anterior/posterior polarity.

Discussion

Emerging evidence supports that tumor development is regulated by cell-to-cell communication through multiple signals, namely Hippo and Src. Certain genes in each Hippo and Src pathway (*i.e.*, *wts* and *src*) have been shown to be regulated by a common kinase, Csk, suggesting that there might be a molecular link among the three (*Figure 3*). Although Csk activity is known to regulate metastatic behavior by decreasing cell adhesion, reduced *csk* expression alone is not sufficient to direct stable tumor growth. The fact that discrete patches of *dCsk* fail to

maintain survival of migrating cells calls for further investigation of the Src-independent role of Csk in metastasis, specifically in relation to how important the Hippo signaling input is to Csk-mediated growth regulation.

Csk may have specific roles as a tyrosine kinase by modifying proteins within the Hippo Pathway, a hypothesis that remains untested. These modifications may reveal the molecular circuitry that enhances the migratory behavior of certain types of tumors, which can then be targeted for therapeutic intervention. For example, suppressing Csk activity could revert defects, or disrupting the binding domains of Csk with the Hippo Pathway components could prevent the cellular changes required for metastasis. Therefore, understanding the genetic relationship between Csk with the Hippo Pathway would enhance the therapeutic approaches to relevant diseases such as cancer.

Acknowledgements

Notation
In keeping with *Drosophila* nomenclature, gene names are italicized (*e.g.*, *dCsk*), proteins coded by the corresponding genes are capitalized (*e.g.*, *dCsk*). Species names are also italicized (*e.g.*, *Drosophila*).

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Drosophila C-terminal Src kinase regulates growth via the Hippo signaling pathway

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ABSTRACT

The Hippo signaling pathway is involved in regulating tissue size by inhibiting cell proliferation and promoting apoptosis. Aberrant Hippo pathway function is often detected in human cancers and correlates with poor prognosis. The *Drosophila C-terminal Src kinase* (*d-Csk*) is a genetic modifier of *warts* (*wts*), a tumor-suppressor gene in the Hippo pathway, and interacts with the *Src* oncogene. Reduction in *d-Csk* expression and the consequent activation of *Src* are frequently seen in several cancers including hepatocellular and colorectal tumors. Previous studies show that *d-Csk* regulates cell proliferation and tissue size during development. Given the similarity in the loss-of-function phenotypes of *d-Csk* and *wts*, we have investigated the interactions of *d-Csk* with the Hippo pathway. Here we present multiple lines of evidence suggesting that *d-Csk* regulates growth via the Hippo signaling pathway. We show that loss of dCsk caused increased Yki activity, and our genetic epistasis places dCsk downstream of Dachs. Furthermore, dCsk requires Yki for its growth regulatory functions, suggesting that dCsk is another upstream member of the network of genes that interact to regulate Wts and its effector Yki in the Hippo signaling pathway.

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Introduction

Growth regulation requires a balance between cell proliferation and cell death (Raff, 1996; Stanger, 2008). Amongst these pathways the Hippo pathway regulates organ size by inhibiting cell proliferation and promoting apoptosis in all metazoan animals from *Drosophila* to mammals (Schroeder and Halder, 2012; Staley and Irvine, 2012; Tumaneng et al., 2012; Yu and Guan, 2013). The Hippo pathway is a network of tumor suppressor genes and oncogenes, and mutations in Hippo pathway lead to overgrowth of the imaginal discs and adult organs (Kango-Singh and Singh, 2009). Aberrant Hippo pathway function in humans (due to amplification of genes, epigenetic silencing and oncogenic transformation) is

often detected in human cancers and correlates with poor prognosis (Halder and Camargo, 2013; Harvey et al., 2013; Johnson and Halder, 2014; Pan, 2010; Zeng and Hong, 2008).

The Hippo pathway comprises of a core kinase cascade involving the Ste-20 family kinase Hippo (Hpo) (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003) and the DMPK family kinase Warts (Wts) (Justice et al., 1995; Xu et al., 1995), which acts upon the transcriptional co-activator Yorkie (Yki) (Huang et al., 2005). Nuclear availability of Yki is regulated by phosphorylation-dependent and -independent mechanisms (Badouel et al., 2009; Oh and Irvine, 2008, 2010; Oh et al., 2009). Wts-mediated phosphorylation of Yki causes its cytoplasmic localization and degradation. When Hippo signaling is down-regulated, active Yki translocates to the nucleus where it forms a complex with the transcription factor Scalloped (Sd) [or Mothers against Dpp (MAD), Teashirt (Tsh) or Homothorax (Hth)] to induce the expression of target genes that promote (a) cell proliferation and cell survival like the *bantam miRNA*, *myc*, (b) cell cycle progression e.g., E2F1, *cyclins A*, *B*, *E*, and (c) inhibitors of apoptosis like *drosophila inhibitor of apoptosis* (*diap1*) (Edgar, 2006; Enderle and McNeill, 2013; Halder and Camargo, 2013; Halder and Johnson, 2011; Kango-Singh and Singh, 2009; Saucedo and Edgar, 2007). Hippo signaling also regulates the expression of several

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genes within its pathway via a negative feedback loop (Edgar, 2006; Kango-Singh and Singh, 2009; Saucedo and Edgar, 2007). Thus, there are several upstream regulators of Yki in the Hippo pathway, and the Hippo pathway activity shows context-dependent response to these upstream inputs (Grusche et al., 2010; Halder and Johnson, 2011). Recent studies have revealed that the Hippo pathway cross-talks with other signaling pathways (e.g., TGFb/Dpp, Wnt/Wg, EGFR/MAPK, G-Protein Coupled Receptors[GPCRs]) in several contexts(Boggiano and Fehon, 2012; Mauviel et al., 2012; Schroeder and Halder, 2012; Staley and Irvine, 2012). We tested the interaction of *C-terminal Src kinase* (*Csk*), a Src-family protein tyrosine kinase (SFK) (Okada, 2012; Read et al., 2004; Stewart et al., 2003) with the Hippo pathway, to investigate the mechanism of growth regulation by SFKs—an area that remains poorly understood.

Analogous to the role of the Hippo pathway, the SFKs are involved in the regulation of normal development, and their misregulation is implicated in several types of cancers (Ingley, 2008; Okada, 2012). This family of proteins is regulated by signaling pathways (e.g., GPCRs) that are involved in the regulation of cell cycle entry, cyto-skeletal rearrangement and cell-adhesion. Src proteins are maintained in inactive state by *C-terminal Src kinase* (*Csk*), a conserved Src family kinase (SFK) (Imamoto and Soriano, 1993; Okada, 2012). C-terminal Src kinase (Csk) maintains SFKs in an inactive state by an inhibitory phosphorylation (e.g., Tyr527 in avian c-Src) (Cole et al., 2003). *Csk* acts as a tumor-suppressor gene by interacting with several signaling proteins via phosphorylation-mediated interactions (e.g., Paxillin, c-Jun, Lats) (reviewed by Okada (2012)). Mammalian Src is well known for mitogenic signaling, and can act as a proapoptotic or anti-apoptotic signal by its context-dependent interactions with the Ras-MAPK, PI3 Kinase/Akt, and Stat3 signaling pathways (Alexander et al., 2004; Martin, 2006; Thomas and Brugge, 1997). Thus, tissue context decides the outcome of Src activation.

The *Drosophila Csk* homolog, *dCsk*, acts as a tumor suppressor gene because loss of *dCsk* in homozygous mutant animals results in hyperplasia of imaginal discs (Read et al., 2004; Stewart et al., 2003), whereas loss of *dCsk* in somatic clones results in poor growth of the mutant cells (Read et al., 2004; Vidal et al., 2006). Loss of *dCsk* using a RNAi approach (*UASdCsk-IR*) showed that large patches of *dCsk* mutant cells lead to increased proliferation and decreased apoptosis (Vidal et al., 2006), and effects of loss of *dCsk* are mediated through Src activation(Read et al., 2004). Although Src expression is elevated in several tumors (Yeatman, 2004), Src overexpression in epithelial cells in flies is known to cause both proliferation and apoptosis (Vidal et al., 2007). Recently, activation of Src oncoprotein was shown to activate Yki in a JNK-dependent manner (Enomoto and Igaki, 2013). Over-expression of Src caused Rac-Diaphanous and Ras-MAPK activation (Enomoto and Igaki, 2013), which cooperatively activate F-actin a Hippo pathway target gene (Fernandez et al., 2011; Richardson, 2011; Sansores-Garcia et al., 2011). These studies revealed that both the cell autonomous and the non-cell autonomous effects of Src activation depend on JNK mediated Yki regulation (Enomoto and Igaki, 2013). Furthermore, *dCsk* is known as a genetic modifier of Wts, and is known to phosphorylate Wts (Pedraza et al., 2004; Stewart et al., 2003).

Given that the growth regulatory functions of Wts occur through its interactions with Yki and the Hippo signaling pathway, we proposed that dCsk regulates growth via the Hippo signaling pathway. We tested if loss of *dCsk* resulted in changes in Yki activity levels, and if *dCsk* and Hippo pathway genes genetically interact. We present multiple lines of evidences suggesting that loss of *dCsk* using RNAi mediated elimination of *dCsk* in large patches of cells causes overgrowth due to increased proliferation – due to increased Yki activity. The effects of loss of *dCsk* are cell autonomous, and genetic interactions between *dCsk* and Hippo pathway components place *dCsk* between Dachs and Zyx that function downstream of Fat in the Hippo network.

Material and methods

Fly stocks

The following strains were obtained from the Bloomington Stock Center unless otherwise specified: *UASdCsk-IR*; +; *TM3Sb/SM6a-TM6B, Tb* (from R. Cagan), y w; *FRT82B dCsk^{Q156Stop}* (from R. Cagan), yw; *UASRas^{V12}; FRT82B dCsk^{Q156stop}* (from R. Cagan), *yw hsFlp*; *nub-Gal4 UASHpo^{RNAi}/CyO-GFP* (from G. Halder), *yw*; *nub-Gal4 UASHpo^{ΔINH}/CyO^{Roi}* (from G. Halder), *Ubx-Flp*; *FRT42D yki^{B5}/CyO* (from D.J. Pan), *UASWts^{13F}* (from G. Halder), *UASFt^{RNAi}* (VDRC # V9396), *UASScrib^{RNAi}*, *UASD^{V5}*, *UASZyx^{RNAi}*, *UASYki^{V5}*, *en-GAL4 UASGFP*, *ex697 en-GAL4* (from G.Halder), and *nub-Gal4*. To test the loss-of-function phenotype of d*Csk*, we generated the line *UASdCsk-IR*; *nub-Gal4/CyO* by using appropriate genetic crosses. This line was outcrossed to other UAS-bearing transgenes to study genetic interactions between *dCsk* and Hippo pathway genes. Other crosses were performed to create the following lines: (1) *UASdCsk-IR*; *nub-Gal4 UASD^{V5}/CyO*, (2) *UASdCsk-IR*; *ex^{697}-lacZ/CyO*, (3) *UASdCsk-IR*; *fj-lacZ/CyO*, (4) *UASdCsk-IR*; *Diap1-4.3 GFP/TM6B, Tb*, (5) *MS-1096*; *ex-lacZ/CyO*, (6) *MS-1096*; *fj-lacZ/CyO*, and (7) *MS-1096*; *Diap1-4.3 GFP/TM6B*. Flies were grown at 25 °C unless noted otherwise.

Immunohistochemistry

Immunohistochemistry was done following the previously published protocol (Kango-Singh et al., 2002). Briefly, wing imaginal discs from third-instar larvae were dissected in phosphate buffered saline (PBS), fixed for 20 min in 4% paraformaldehyde, and washed twice for 10 min each in PBST (PBS + 0.2% TritonX-100) at room temperature. Tissues were then blocked with 2% normal donkey serum for 2 h and stained with primary antibody at 4 °C overnight. Next, tissues were washed twice and incubated in secondary antibody for 2 h at room temperature in foil-wrapped eppendorf tubes. Finally, tissues were mounted in Vectashield after three rounds of washing. Images were taken using confocal microscopy. The following primary antibodies were used: mouse-anti DIAP1 (1:250); mouse-anti βgal (1:100), mouse-anti Wingless (1:100), and rabbit-anti Caspase (1:250). Secondary antibodies (Jackson Immunoresearch) used were anti-mouse Cy3 (1:1000) and anti-rabbit Cy3 (1:1000). The samples were imaged using an Olympus Fluoview 1000 Laser Scanning confocal microscope, and the images were processed using Adobe Photoshop CS6.

Quantification of disc size

The size of the wing pouch was measured using the expression of Wg as a guide for the extent of the *nub-GAL4* domain (Fig. S1, Fig. 4). The size of the wing pouch was measured by marking the edges Wg expression in the wing disc corresponding to region of the wing margin. The area of the wing pouch was measured (in pixels) using the histogram function in Adobe Photoshop CS6.0 for each genotype ($n=5$). A two-tailed *T*-test was performed using Excel 2013 to quantify if the observed differences between pouch sizes were significant ($p < 0.05$).

Adult fly wing mounting and imaging

Adult flies were collected in 70% ethanol and dehydrated in an ascending alcohol series. The wings of completely dehydrated flies were clipped in 100% ethanol and mounted in Canada Balsam (3 Canada Balsam: 1 Methyl Salicylate). Wings were photographed using an Olympus BX51 Microscope mounted with an Olympus XM10 camera and images acquired using the CellSens Dimensions Software. Adult flies showing no-wing phenotype were maintained at 4 °C overnight before they were mounted. Adult fly images were

taken with the Zeiss Apotome microscope, and Z-stack projections were generated using Axiovision software.

Results

Loss of dCsk in large patches results in overgrowth

To study the effects of loss of *dCsk*, we overexpressed *UASdCsk-IR* (Vidal et al., 2006) in the wing pouch using the *nubbin-GAL4* (*nub-GAL4*) driver (Fig. 1a). The UASdCsk-IR is an inverted repeat (IR) containing transgene that targets the dCsk transcript (Vidal et al., 2006). The *nub-GAL4* driver is expressed in the wing pouch (Neumann and Cohen, 1998). We used the expression of Wingless (Wg) to mark the boundary of the wing pouch, as the edge of *nub* expression overlaps with the outer wing hinge-specific expression of Wg (Neumann and Cohen, 1998). Compared to wild-type (a–d), loss of function of *dCsk* resulted in large overgrowths in the wing pouch in imaginal discs (Fig. 1g–i) and in adult wings (Fig. 1 compare e to f). Earlier studies have also shown that loss of *dCsk* in homozygous discs induces apoptosis (Langton et al., 2007); therefore, we tested the effects of loss of *dCsk* (*UASdCsk-IR*; *nub-GAL4*/+) on cell death using antibodies against activated Caspase 3 (Casp3∗) and the *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1). Compared to wing imaginal discs that show wild-type expression of Wg (Fig. 1b), activated Caspase 3 (Fig. 1c) and DIAP1 (Fig. 1d), loss of *dCsk* (*UASdCsk-IR*; *nub-GAL4*/+) caused overgrowth of the wing pouch accompanied by mild induction of activated Caspase 3 (Casp3∗) (Fig. 1h), and a remarkable downregulation of DIAP1 (Fig. 1i) expression.

On the other hand, compared to wild-type clones (Fig. 2a–c), small patches of *dCsk* mutant cells generated using an MARCM approach (Lee and Luo, 1999), resulted in small clones that did not overgrow (Fig. 2d green, gray in e). *dCsk* mutant clones resulted in strong upregulation of Casp 3∗ in the eye (Fig. 2c) and wing discs (data not shown). This phenotype of loss of *dCsk* in somatic clones differs from the effects of loss of *dCsk* in larger regions of the discs where *dCsk* mutant cells can overcome apoptosis and cause overgrowth (compare Fig. 2d to Fig. 1g). Earlier studies have shown that loss of *dCsk* causes microenvironment specific phenotypes, e.g., loss of *dCsk* over larger areas of the wing discs using *769-GAL4* or *omb-GAL4* results in enlarged wings, whereas somatic clones of *dCsk* fail to survive (Vidal et al., 2006). Consistent with these reports, further analysis of the *dCsk* mutant clones (Fig. 2) revealed that the mutant cells were competed out by the surrounding wild-type cells, and resulted in no developmental defects in the adult fly. Taken together, loss of *dCsk* results in context dependent effects on growth, together with mild effects on apoptosis despite downregulation of DIAP1.

Loss of dCsk affects Yki activity

The overgrowth phenotypes resulting from loss of *dCsk* are similar to the effects of loss of function of Hippo pathway genes like *expanded* (*ex*), *merlin* (*mer*) and *fat* (*ft*), that induce excess cell proliferation and mild effects on apoptosis in mutant cells resulting in formation of larger structures(Bennett and Harvey, 2006; Pellock et al., 2007; Silva et al., 2006; Willecke et al., 2006). Therefore, we tested the effect of loss of *dCsk* on transcriptional targets of Hippo signaling (*ex-lacZ*, *fj-lacZ*, and *diap4.3GFP*) (Fig. 3, Fig. S1). Compared to the wild-type expression of *ex-lacZ* (Fig. 3a–c), and *fj-lacZ* (Fig. 3g–i), downregulation of *dCsk* using *en-GAL4* in the posterior compartment of the wing disc results in upregulation of *ex-lacZ* (Fig. 3d–f) and *fj-lacZ* (Fig. 3j–l) suggesting that loss of *dCsk* leads to Yki activation. We confirmed this observation as *dCsk* downregulation in the wing pouch under *nub-GAL4* (*UASdCsk-IR*; *nub-GAL4*/+) upregulates the levels of expression of *ex-lacZ* (Fig. S1a andb) and *fj-lacZ* (Fig. S1c and d). We also tested the expression of *diap1-4.3GFP*, the reporter transgene that contains the Hippo response element in *diap1* (Wu et al., 2008; Zhang et al., 2008), to check if loss of *dCsk* affects *diap1* expression via the Hippo pathway. Compared to the expression of the *diap1-4.3GFP* in wild type wing discs (Fig. S1e), we observed a downregulation of diap14.3-GFP
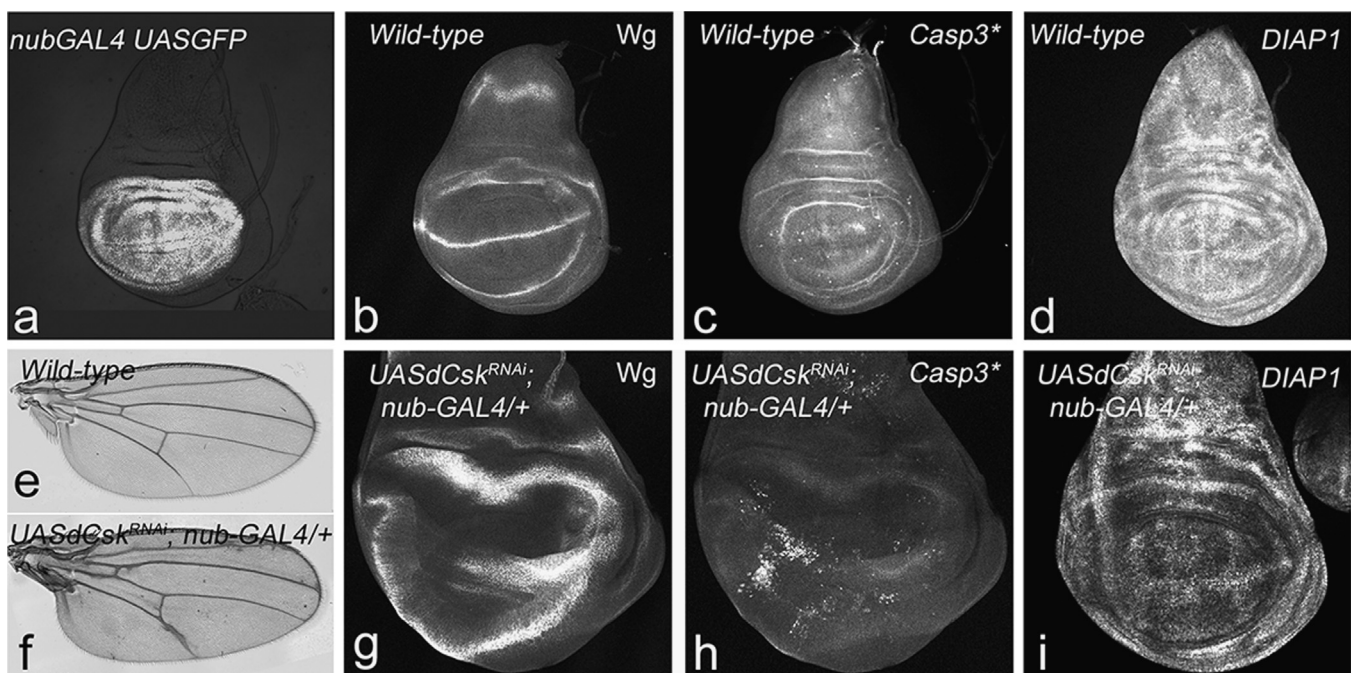

Fig. 1. Loss of function phenotypes of *dCsk*. Panels show comparison of wing imaginal discs from third instar larvae of wild-type (a–d) and *UASdCsk-IR*; *nub-GAL4*/+ (g–i) and corresponding adult wing phenotypes (e and f). The *nub-Gal* domain is shown using GFP expression (a), and wild-type expression of Wg (b), activated Caspase 3 (Casp3∗) (c), and DIAP1 (d) is shown. Loss of *dCsk* in the *nub-Gal4* expression domain causes overgrowth of the wing pouch (f). Regulation of Wingless expression during *dCsk* loss-of-function reveals over-proliferation of cells in the wing pouch (g). The overgrowth of *dCsk* mutant cells occurs despite induction of Casp3∗ (h) and down-regulation of DIAP1 (i). The resulting adult wing phenotype is shown in panels e and f.
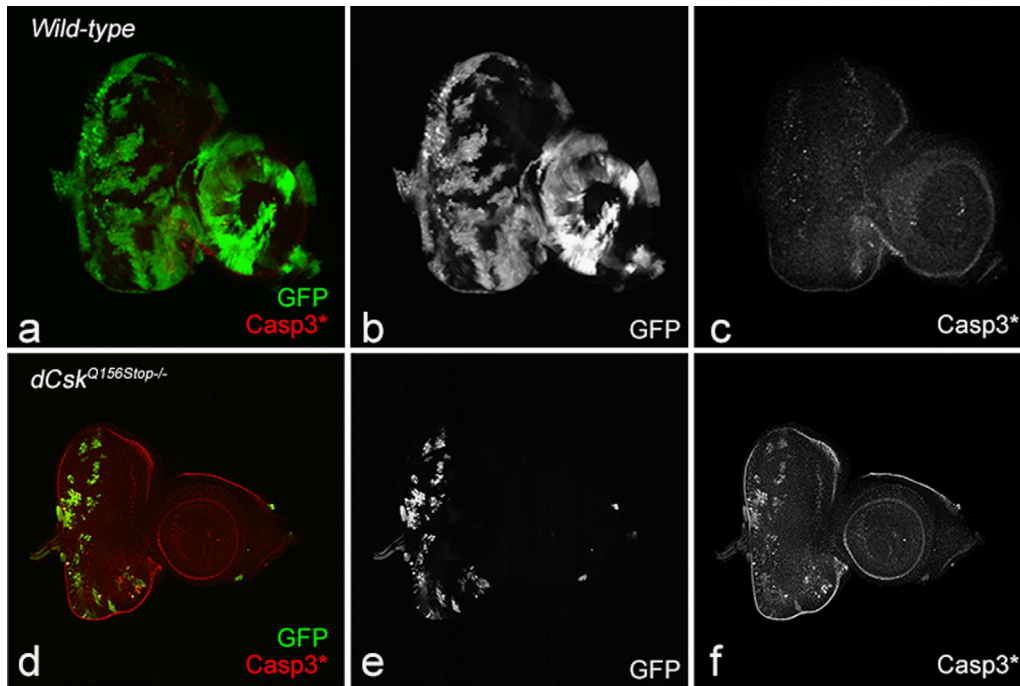
Fig. 2. *dCsk* mutant cells are out competed by neighboring wild type cells. GFP labelled somatic clones of wild type (a–c) and loss of *dCsk* (d–f) is shown. Wild-type clones (green in a) were induced in the eye imaginal discs using the MARCM approach, and stained with antibodies against activated Caspase 3 (Casp 3*) to assess the extent of cell death (red in a). MARCM clones of dCsk (d–f) fail to cause large overgrowths, and Casp3* (red in d) is induced in *dCsk* mutant cells (green in d), which may account for their elimination from the tissue due to cell competition.

expression in wing discs from *UASdCsk-IR; nub-GAL4*/+ larvae (Fig. S1f). This effect is similar to downregulation of DIAP1 protein in dCsk mutant cells. Taken together, this data suggests that loss of *dCsk* results in increased Yki activity leading to upregulation of *ex* and *fj* transcription, but not *diap1* transcription. This increased Yki activity may in part explain the overgrowth phenotype of the *UASdCsk-IR; nub-GAL4*/+ wing discs.

dCsk genetically interacts with the Hippo pathway

Since the phenotypes of *dCsk* suggested that *dCsk* caused increased Yki activity, we tested if *dCsk* genetically interacted with Hippo pathway genes using genetic epistasis approaches (Figs. 4, 5, S2). For all epistasis experiments, we used two criteria to analyze the interaction. First, we tested for the effects of epistatic interactions on DIAP1 expression, and second, we compared the size of the wing pouch. Previous studies identified *dCsk* as a genetic modifier of loss- or gain-of-function phenotypes of Wts (Stewart et al., 2003). Wts acts downstream of dCsk to mediate its growth regulatory functions in vivo, and dCsk phosphorylates Wts in-vitro (Pedraza et al., 2004; Stewart et al., 2003). We confirmed this epistatic interaction between dCsk and Wts using our experimental system in the wing pouch (Fig. 4a–d). Over-expression of *UASWts* results in hyperactivation of the Hippo pathway and results in smaller organs due to increased apoptosis (Tapon et al., 2002; Verghese et al., 2012b). Over-expression of *UASWts* under *nub-GAL4* results in reduction of wing pouch size (Fig. 4a and b). Co-expression of *UASWts* with *UASdCsk-IR* (Fig. 4c and d) resulted in a complete suppression of *dCsk* phenotype of overgrowth (Fig. 1g–i), suggesting that *dCsk* acts upstream of Wts.

Next, we tested the genetic interaction between Hpo and *dCsk*. Overexpression of *UASHpo* results in induction of apoptosis and reduction in organ size (Udan et al., 2003; Verghese et al., 2012a; Wu et al., 2003). Over-expression of *UASHpo* under *nub-GAL4* results in smaller wing pouch (Fig. 4e and f), and co-expression of *UASdCsk-IR* and *UASHpo* resulted in generation of small wings (Fig. 4g and h) akin to the phenotype of Hpo overexpression suggesting that *dCsk*

acts upstream of Hpo. Taken together, these data suggests that dCsk interacts with the core components of the Hippo pathway to regulate cell proliferation, and tissue sizes.

Next, we extended our investigation of genetic interactions between *dCsk* with two other upstream components of the Hippo pathway—Zyxin (Zyx) (Rauskolb et al., 2011), and Dachs (D) (Mao et al., 2006). Zyx and D act downstream of the atypical cadherin Fat and are known to negatively regulate levels of Wts protein (Mao et al., 2006; Matakatsu and Blair, 2008; Rauskolb et al., 2011). Downregulation of Zyx (using *UASZyx*[RNAi]) results in an overall reduction in the wing pouch (Fig. 4i and j). Co-expression of *UASdCsk-IR* with *UASZyx*[RNAi] phenocopies the *UASZyx*[RNAi] phenotype of reduction in wing pouch (Fig.4k and l), suggesting that *dCsk* acts upstream of *Zyx*, and likely requires *Zyx* to regulate growth. Over-expression of D (*UASD*) leads to upregulation of DIAP1 (Fig. 4m) and overgrowth of the wing pouch (Fig. 4n). Co-expression of *UASD* and *UASdCsk-IR* phenocopies the effects of *UASdCsk-IR* over-expression (Fig. 4o and p), suggesting that *dCsk* acts downstream of D. A quantification of the wing pouch size further confirmed the epistatic interactions between loss of *dCsk* and over-expression of Wts, Hpo, Zyx or D (Fig. S2). Taken together, our epistasis interactions place *dCsk* downstream of D and upstream of Wts in the Hippo signaling pathway.

dCsk requires Yki to regulate growth

Since the signaling inputs in the Hippo pathway converge on the Yki oncoprotein, we tested genetic epistasis interactions between *dCsk* and Yki using similar genetic epistasis approaches. Compared to wild-type (Fig. 5a and b), over-expression of Yki (*nub-GAL4*/*UASYki*) leads to up-regulation of DIAP1 levels (Fig. 5c) and an overgrowth of the wing pouch (Fig. 5d) in the *nub* domain. In comparison, loss of *dCsk* (*UASdCsk-IR; nub-GAL4*/+) leads to downregulation of DIAP1 (Fig. 1i) and an overgrowth of the wing pouch in imaginal discs (Fig. 1g–i). Co-expression of *dCsk-IR* and Yki (*UASdCsk-IR; nub-GAL4*/ *UASYki*) resulted in up-regulation of DIAP1 levels (Fig. 5e) and an
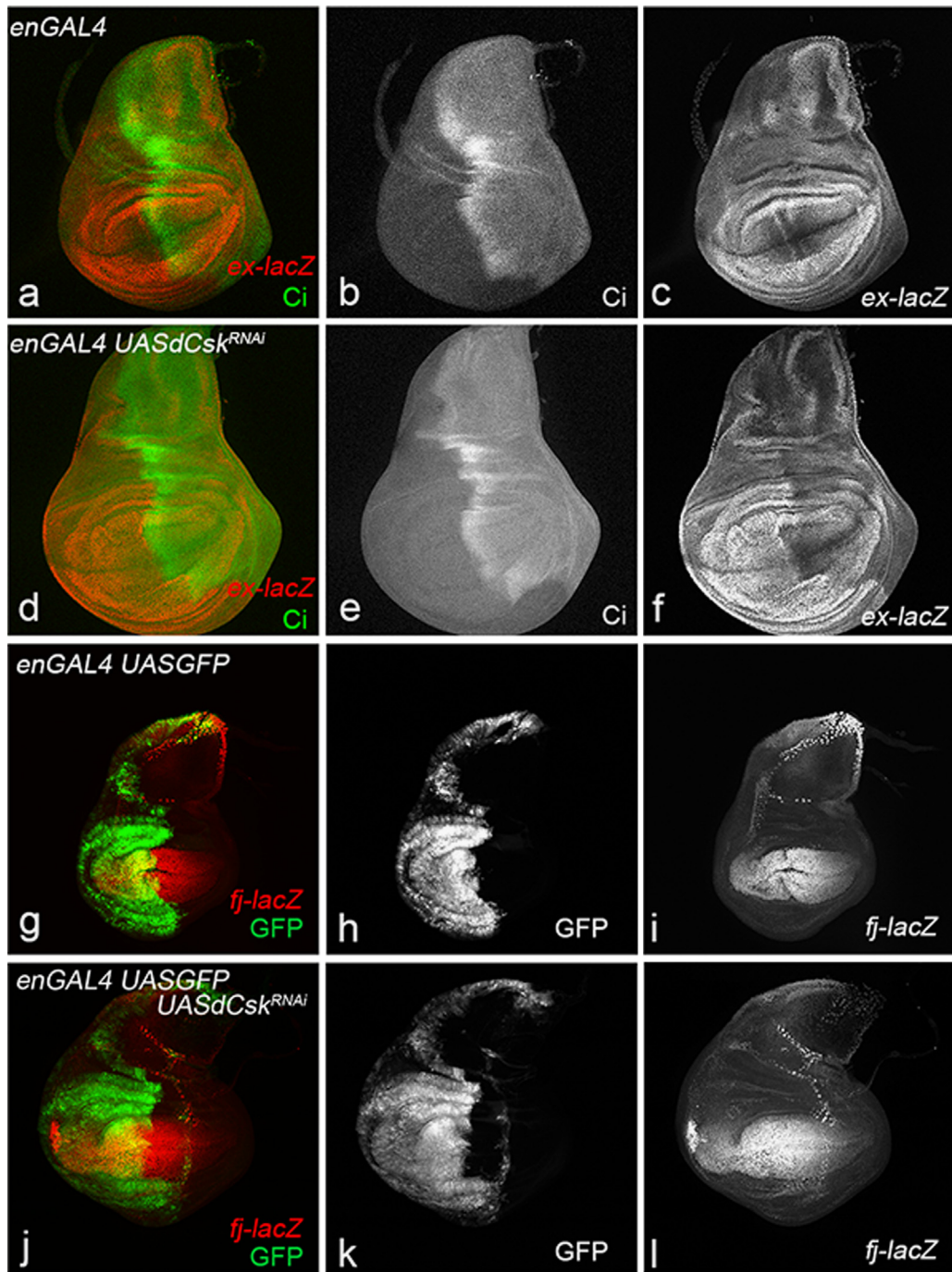
Fig. 3. Yki activity is upregulated in *dCsk* mutant cells. (a–c) Wing imaginal discs from *enGAL4* larvae stained for the anterior compartment marker Ci (green in a, gray in b) and wild-type expression of *ex*[697]-*lacZ* (red in a, gray c) is shown. (d–f) Downregulation of dCsk using enGAL4 causes upregulation of *ex*[697]-*lacZ* (red in d, gray f). Ci expression (green in d, gray in e) marks the anterior compartment where dCsk levels are wild-type. (g–i) Panels show enGAL4 domain marked using UASGFP (green in g, gray in h), and wild-type expression pattern of *fj-lacZ* (red in g, gray in i). (j–l) Loss of dCsk in the enGAL4 domain (GFP positive- green in j, gray in k) leads to upregulation of fj-lacZ (red in j, gray in l) in the posterior compartment.

overgrowth of the wing pouch (Fig. 5f). The wild type adult wing (Fig. 5k) is presented as reference. The size of the wing pouch and the resulting adult wings appear very similar when comparing *UASdCsk-IR*; *nub-GAL4/UASYki* (Fig. 5m) to either *nub-GAL4/UASYki* (Fig.5l) or *UASdCsk-IR*; *nub-GAL4/*+ (Fig. 1g–i). However, the *UASdCsk-IR*; *nub-GAL4/UASYki* double mutant wings show upregulation of DIAP1 expression similar to effects of over-expression of Yki. Taken together, these data suggest that Yki may act downstream of *dCsk*.

To further clarify this epistatic relationship, we tested if the overgrowth caused by loss of *dCsk-IR* is affected by heterozygosity for *yki*[B5]-the null allele for *yki*. It is well established that Hippo

signaling is sensitive to dose of Yki, and reduction in Yki levels is known to affect loss of function phenotypes of other upstream genes in the Hippo pathway (Baumgartner et al., 2010; Doggett et al., 2011; Gilbert et al., 2011; Poernbacher et al., 2012; Sun and Irvine, 2011; Verghese et al., 2012b; Wu et al., 2008). In wild type, reduction in *yki* levels (*yki*[B5]/+) has no obvious effects on growth (Fig. 5g and h). Heterozygosity of *yki* creates a sensitized background. We observed that heterozygosity of *yki*[B5] resulted in reduction in the overgrowth observed in the wing pouch of *UASdCsk-IR*; *nub-GAL4/*+ wing discs (Fig. 5i and j compared to Fig. 1g and i), and DIAP1 levels were restored (Fig. 5i). A quantification of the wing pouch size also
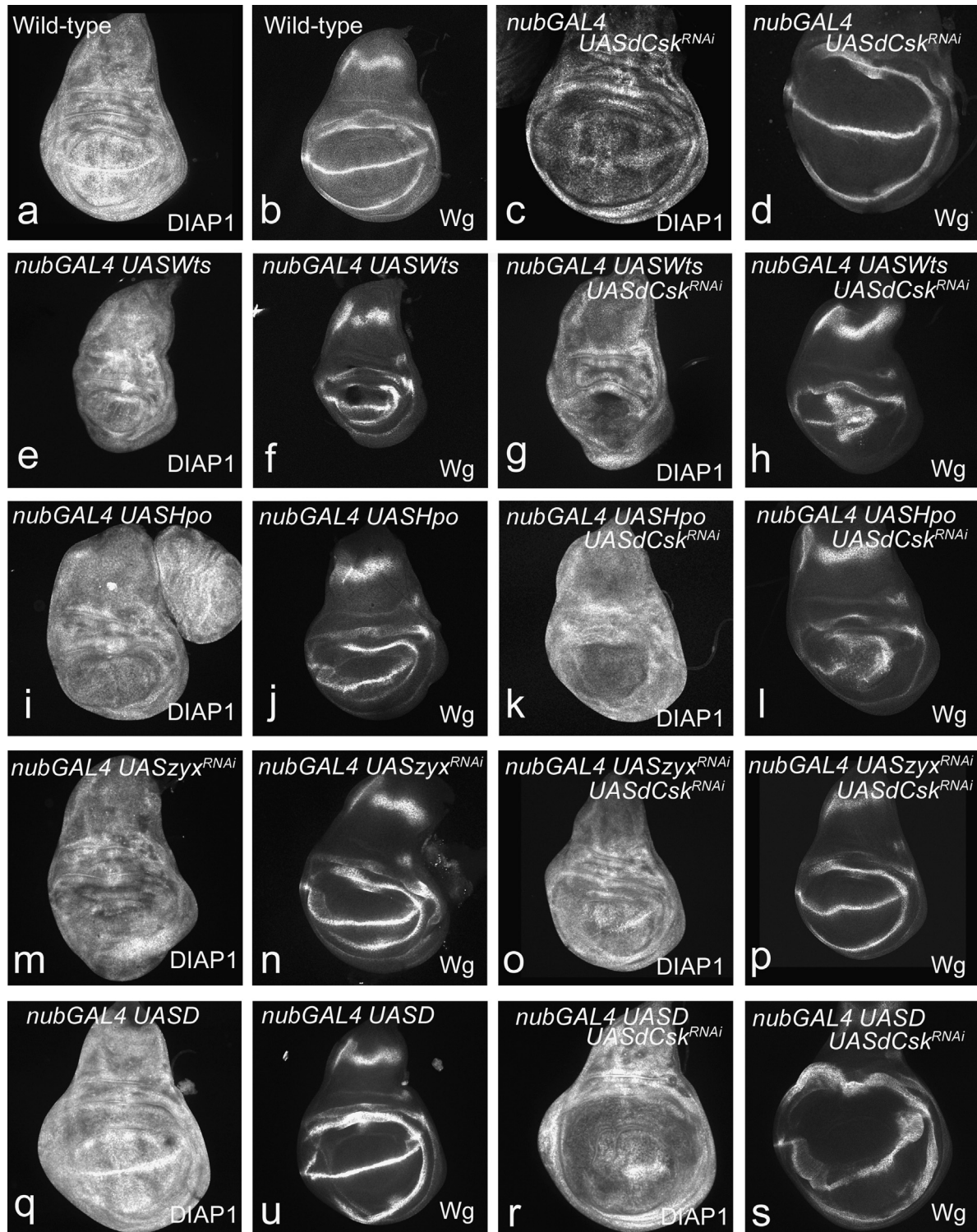
Fig. 4. *dCsk* interacts with the Hippo pathway. Panels show wing imaginal discs from wild-type (a and b), *UASdCsk-IR; nub-GAL4/+* (c and d), *nub-GAL4/+UASWts/+* (e and f), *nub-GAL4/UASHpo* (i and j), *nub-GAL4/+UASZyx^{RNAi}/+* (m and n), and *nub-GAL4UASD^{V5}/+* (q and u) larvae stained for DIAP1 and Wg (respectively). Phenotypes of *UASdCsk-IR; nub-GAL4/+; UASWts/+* (g and h), *UASdCsk-IR; nub-GAL4/UASHpo* (k and l), *UASdCsk-IR; nub-GAL4/+; UASzyx^{RNAi}/+* (o and p) show reduction of the wing pouch size, whereas *UASdCsk-IR; nub-GAL4 UASD^{V5}/+* (r and s) shows overgrowth. All co-expression phenotypes (g, k, o, and r) with *dCsk-IR* show down-regulation of DIAP1, which is characteristic of *UASdCsk-IR; nub-GAL4/+*.

supported the genetic interaction data, where the overgrowths caused by loss of *dCsk* or over-expression of Yki are similar to the effect of the double mutant (Fig. 5n); and the overgrowth caused by loss of dCsk is strongly suppressed by reduction in Yki levels (Fig. 5n). This suggests that *dCsk* acts upstream of Yki, and may require Yki for regulating cell proliferation/tissue growth.

dCsk acts downstream of Dachs

Our genetic epistasis places dCsk downstream of D. Therefore, to further confirm the *dCsk*-D epistasis, and to test if dCsk regulates growth through the Hippo pathway, we tested if the expression of *fj-lacZ*, the transcriptional target of Ft and Yki
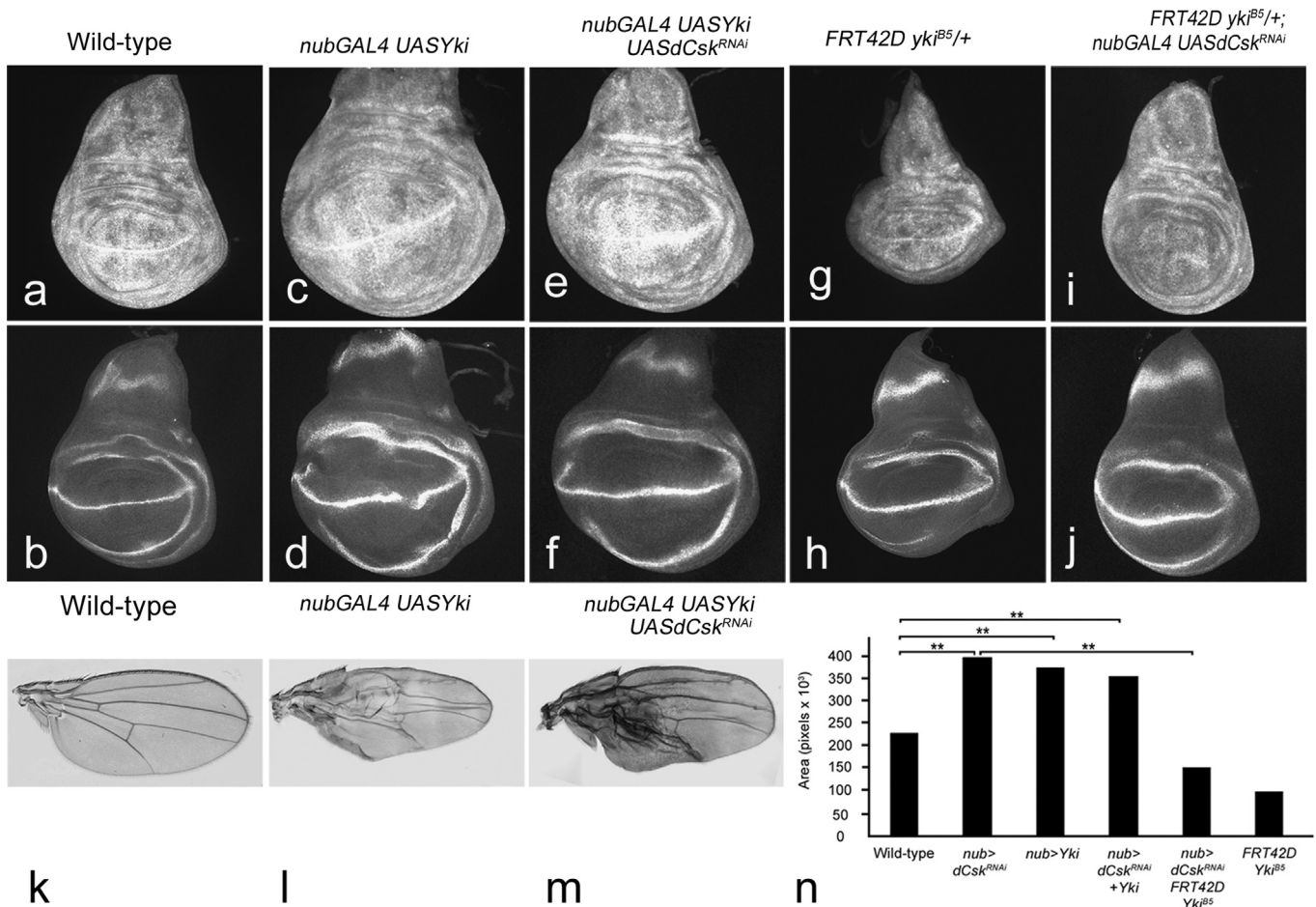
Fig. 5. *dCsk* requires Yki to regulate growth. (a and b) Wild-type wing imaginal discs stained for expression of DIAP1 (a) and Wg (b) are shown. (c and d) The effect of overexpression of Yki (*nub-GAL4/UASYki*) on DIAP1 (c), and Wg (d) in wing imaginal discs is shown for comparison. Note the overgrowth of the wing pouch due to increased Yki levels. (e and f) Co-expression of *UASdCsk-IR* (*UASdCsk-IR*; *nub-GAL4/UASYki*) leads to induction of DIAP1 (e) and overgrowth of wing pouch (f). (g and h) Wing imaginal discs from FRT42D *yki^{B5}*/CyO larvae stained with antibodies against Diap1 (g) and Wg (h). (i and j) *UASdCsk-IR*; *nub-GAL4*/FRT42D *yki^{B5}* wing discs showing reduced expression of DIAP1 (i) and reduced growth of the wing pouch (j) due to heterozygosity of *yki^{B5}*. (k–m) Adult wings from wild-type (k), *nub-GAL4/UASYki* (l) and *UASdCsk-IR*; *nub-GAL4/UASYki* (m) flies showing increased growth defects caused by co-expression of *Yki* and *dCsk-IR*. (n) The chart shows quantification of wing pouch growth for wing imaginal discs of the indicated genotypes. Asterisks indicate that the genotypes where there is significant difference in wing pouch growth ($n=5$, $p<0.05$).

signaling, is affected in discs co-expressing *UASdCsk-IR* and *UASD. fj* is expressed in a gradient in the wing pouch, with the highest levels of *fj* expression coinciding with the presumptive (DV) wing margin, and a gradient of decreasing *fj* expression that extends in both the dorsal and ventral wing pouch (Fig. 6a) (Cho and Irvine, 2004; Ishikawa et al., 2008; Simon et al., 2010). Over-expression of D causes moderate upregulation of *fj-lacZ* and mild hyperplasia (Fig. 6b), whereas loss of dCsk leads to robust induction of *fj-lacZ* expression and overgrowth (Fig. 3j–l; Fig. 6c). Co-expression of *UASdCsk-IR* with UAS D caused robust overgrowth of the wing pouch and robust induction of *fj-lacZ* expression (Fig. 6d) due to increased Yki activity. These data also support a role of *dCsk* downstream of D but upstream of Wts in the Hippo pathway.

Discussion

dCsk is as an upstream regulator of the hippo pathway

The *Drosophila C-terminal src kinase* (*dCsk*) is a tumor suppressor gene, and loss of *dCsk* is reported to cause multiple defects in growth regulation dependent on the tissue microenvironment, for example, loss of *dCsk* in homozygous mutants causes extensive hyperplasia of tissues due to increased proliferation and decreased apoptosis (Read et al., 2004; Stewart et al., 2003; Vidal et al., 2006). Loss of *dCsk* in a

narrow band of cells or loss of *dCsk* in small patches in somatic clones (Fig. 2) results in increased proliferation, decreased cell adhesion, epithelial exclusion, upregulation of invasive/ cell migration related markers, and eventually apoptosis (Vidal et al., 2006). Our characterization of the effects of loss of *dCsk* and genetic interaction analysis showed that *dCsk* mutant cells induce the transcriptional activity of the Hippo pathway effector Yki (Fig. 3). Increased Yki activity leads to uncontrolled proliferation and formation of larger organs (Huang et al., 2005). Our genetic epistasis places dCsk between Dachs and Warts, which corroborates with the findings from earlier studies in flies where dCsk was shown to act upstream of Wts (Stewart et al., 2003). Further, the analysis of Yki activity in cells deficient for *dCsk* revealed that loss of *dCsk* induced transcription of *ex-lacZ* and *fj-lacZ*, two well-established reporters of Yki activity (Fig. 3). Thus, taken together these findings suggest that dCsk is another upstream component of the Hippo pathway that exerts its effects on tissue growth by affecting Yki activity through Wts.

DIAP1 suppression and its implications on dCsk mediated growth regulation

Interestingly, loss of *dCsk* shows a strong suppression of DIAP1 —as revealed by downregulation of DIAP1 protein (Fig. 1h), and these effects on DIAP1 regulation likely occur through the Hippo
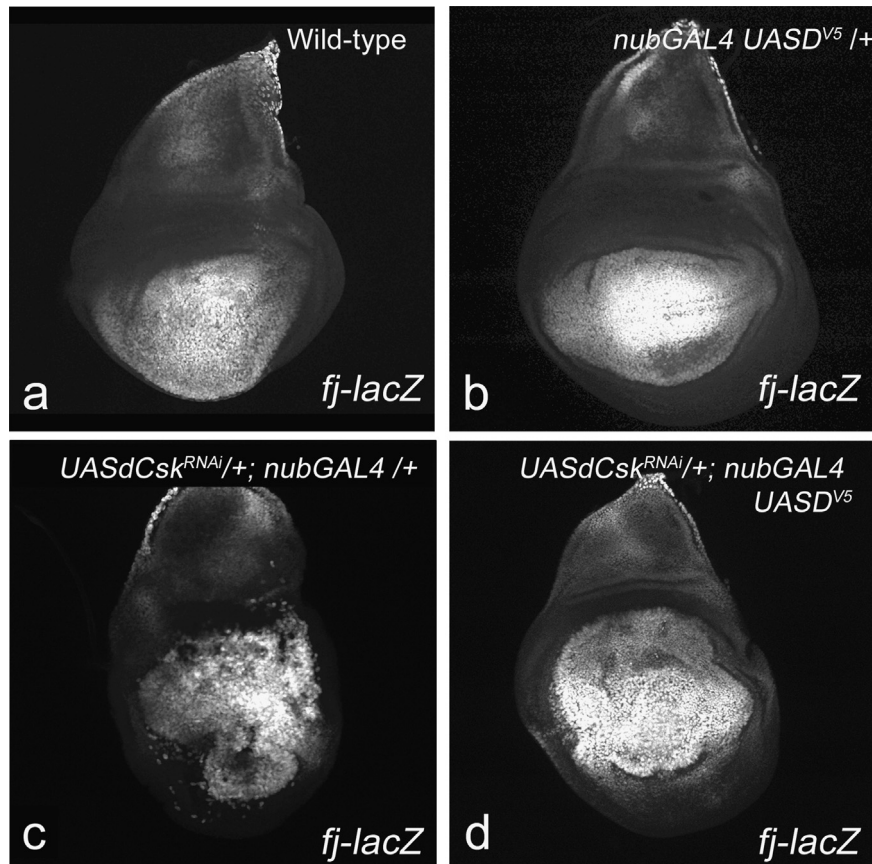
Fig. 6. dCsk acts downstream of Dachs in the Hippo pathway. A comparison of *fj-lacZ* expression is shown for wing imaginal discs from (a) wild-type, (b) *nub-GAL4 UASD^{V5}/+*, (c) *UASdCsk-IR*; *nub-GAL4/+*, and (d) *UASdCsk-IR*; *nub-GAL4 UASD^{V5}/+* larvae.

signaling pathway as the Hippo response element in *diap1—diap1 4.3GFP—*is downregulated in *dCsk* mutant cells (Fig. S1). These findings have many implications on the known effects of loss of *dCsk*. First, loss of DIAP1 in small clones of *dCsk* may present them for elimination (due to cell competition). Second, increased apoptosis observed in *dCsk* homozygous mutant discs may be due to downregulation of DIAP1 (Langton et al., 2007). Third, the overgrowth of cells despite increased apoptosis and reduced DIAP1 levels in *dCsk* mutant discs may be explained by the faster rate of proliferation which outcompetes the effects of cell elimination from the developing tissue. Alternatively, the overgrowth in the mutants may be caused by activation of Caspases that result in apoptosis-induced proliferation, a well-documented phenomenon in several growth contexts (Fan and Bergmann, 2008; Levayer and Moreno, 2013). These effects of loss of *dCsk* are very similar to two other tumor suppressor genes, *scribble* (*scrib*) and *discs overgrown* (*dco*) that show similar effects on growth regulation. Homozygous mutant animals cause dramatic overgrowths while generation of small patches of mutant clones in somatic mosaics grows poorly when compared to their wild-type twin clones, or neighboring heterozygous cells (Chen et al., 2012; Enomoto and Igaki, 2011; Guan et al., 2007; Verghese et al., 2012b). Furthermore, DIAP1 is downregulated in all three mutants (Fig. 1) (Guan et al., 2007; Verghese et al., 2012b), and genetic and molecular analyses place these genes downstream of Fat in the Hippo pathway(Feng and Irvine, 2009; Sopko et al., 2009; Verghese et al., 2012b).

dCsk requires Yki and acts upstream of Zyx to regulate growth

Although previous studies had identified some similarities in the Csk loss of function phenotype to the loss of function phenotype of

tumor suppressor genes within the Hippo pathway (e.g., Sav or Wts), a clear link between dCsk and the Hippo pathway was not established (Pedraza et al., 2004; Read et al., 2004; Stewart et al., 2003; Vidal et al., 2006). Our work links dCsk to the Hippo pathway, as our findings suggest that dCsk requires Yki for growth regulation (Fig. 5). This is because the overgrowth caused by loss of *dCsk* is strongly suppressed by heterozygosity for *yki*. Loss of *dCsk* or overexpression of Yki induces overgrowth; however, these genes interact synergistically as the UASdCsk-IR, UASYki double mutant wings are larger than those of the single mutants [dCsk-IR or Yki overexpressing] animals. Thus, our data suggests that dCsk requires Yki to regulate growth, and may also have other Yki-independent functions.

Our data place dCsk downstream of the atypical myosin Dachs, and upstream of the Ajuba LIM protein Zyx and the Wts kinase within the Hippo pathway (Fig. 4). D is known to bind Zyx, and D also stimulates binding of Zyx to Wts (Mao et al., 2006; Rauskolb et al., 2011). Zyx and D act downstream of Fat in the Hippo pathway and regulate the stability of Wts (Rauskolb et al., 2011). Thus, in the future it would be interesting to investigate if dCsk is a part of the D/Zyx/Wts complex, or if it regulates Wts via phosphorylation dependent mechanisms, or if D or Zyx are involved in the mechanisms that localize dCsk to the membrane, where dCsk acts on its substrates.

SFK and the Hippo pathway

Csk is a known regulator of the activity of Src-family kinases (SFKs) (Okada, 2012; Okada et al., 1991). Elevation of Src activity, and increased Src expression is associated with increased proliferation, invasion and metastasis in several cancers (e.g., colon cancer) (Cao et al., 2008; Cole et al., 2003). One mechanism that promotes

Src mediated tumor progression involves activation of focal adhesion kinases (FAKs), JNK and matrix metalloproteases (MMPs), which together cause decreased cell adhesion and degradation of the basement membrane, two important criteria for tumor progression and metastasis (Je et al., 2014; Okada, 2012). Loss of *dCsk* in a *Drosophila* model of tumor invasion revealed that the mutant cells at the boundary (that contact the normal cells) show reduced cell adhesion, which promotes their basal exclusion and migration. Further studies showed that JNK, Rho1, E-Cadherin and p120Cn (alpha Catenin) all promote increased cell migration and apoptosis (Vidal et al., 2006, 2007). Furthermore, increased Src activity was shown to induce JNK and the Hippo pathway, via Rac-Diaphanous, and RAS-Mitogen activated protein kinase (MAPK) pathways, leading to propagation of Yki activity to non-cancer cells, which contribute to the tumor phenotype (Enomoto and Igaki, 2013). Taken together, our studies suggest that dCsk may reveal another signal integration point between the SFK and the Hippo pathway, two potent tumor suppressor networks.

In mammalian models, Csk is known to regulate SFK via phosphorylation of a C-terminal regulatory tyrosine (equivalent to Tyr-527). Besides, SFKs, substrates of Csk include Paxillin, c-Jun and Lats (Okada, 2012; Pedraza et al., 2004; Stewart et al., 2003). These proteins are part of different signaling pathways, supporting a role of Csk in multiple signaling interactions during development. Another important aspect of Csk function that remains unclear is its localization with respect to that of its target substrates (e.g. SFK or Lats or Paxilin), which are all preferentially expressed on the cell membrane. Thus, Csk needs to be translocated to the membrane, and several scaffolding/adapter proteins (e.g., Caveolin-1, Paxillin) have been identified for their role in anchoring Csk to the membrane (Martin, 2006; Okada, 2012). Interestingly recent studies have emphasized the importance of localization of several key components of the Hippo pathway to the apical membrane for their regulation and function (Ho et al., 2010; Schroeder and Halder, 2012). Overall, if Csk is localized to the membrane via conserved mechanisms, and if Csk interacts with other signaling pathways using phosphorylation-independent mechanisms remain to be determined. In summary, our data uncovers dCsk as a new input in the Hippo signaling pathway, and reveals the intersection of the Hippo and Src signaling pathways. Both these pathways are of wide interest because of the roles they play in the regulation of normal development, and the effects of misregulation of these pathways in diseases like cancer.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.10.010.

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