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

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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
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., TGFβ/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 an 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 oncprotein was shown to activate Yki in a JNK-dependent manner (Enomoto and Igaiki, 2013). Over-expression of Src caused Rac-Diaphanous and Ras-MAPK activation (Enomoto and Igaiki, 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 Igaiki, 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 dCskQ156Stop (from R. Cagan), yw; UASRasV12; FRT82B dCskQ156Stop (from R. Cagan), yw hsFlp; nub-Gal4 UASHpoRNAi/Cyo-GFP (from G. Halder), yw; nub-Gal4 UASHpoRNAi/CyoGros (from G. Halder), UASWts13F/Cyo (from D.J. Pan), UASWts13F (from G. Halder), UASFIRRNAi (VDRC # V9396), UASScribRNAi, UASFIRRNAi, UASHpoRNAi, UASWhsFlp, ex697- en-GAL4 UASGFP, ex597-en-GAL4 (from G.Haler), and nub-Gal4. To test the loss-of-function phenotype of dCsk, 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 UASFIRRNAi/Cyo, (2) UASdCsk-IR; ex597-lacZ/Cyo, (3) UASdCsk-IR; ff-lacZ/Cyo, (4) UASdCskIR; Diap1-4.3 GFP/TM6B, Tb, (5) MS-1096; ex-lacZ/Cyo, (6) MS-1096; ff-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 (1:2500) 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. 51, 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 by marking 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 Axiosvision 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 Wg (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). 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 and b) 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.
expression in wing discs from UASdCsk-IR; nub-GAL4/+ larvae (Fig. 5f). This effect is similar to downregulation of DIAPI 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 DIAPI 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 UASZyxRNAi) results in an overall reduction in the wing pouch (Fig. 4i and j). Co-expression of UASdCsk-IR with UASZyxRNAi phenocopies the UASZyxRNAi phenotype of reduction in wing pouch (Fig. 4k and l), suggesting that dCsk acts upstream of Zyx, and likely requires Zyx to regulate growth. Overexpression of D (UASD) leads to upregulation of DIAPI (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 DIAPI 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 DIAPI (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 DIAPI levels (Fig. 5e) and an
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 ykiB5—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 (ykiB5/+) has no obvious effects on growth (Fig. 5g and h). Heterozygosity of yki creates a sensitized background. We observed that heterozygosity of ykiB5 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
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

![Image with panels showing wing imaginal discs from wild-type (a and b), UASdCsk-IR; nub-GAL4/+, nub-GAL4/UASHpo+/+(i and j), nub-GAL4+/UASHpo+/+(m and n), and nub-GAL4/UASHpo+/+(q and u) larvae stained for DIAP1 and Wg (respectively). Phenotypes of UASdCsk-IR; nub-GAL4+/+; nub-GAL4/UASHpo+/+(r and s) show 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+/+.

Fig. 4. dCsk interacts with the Hippo pathway. Panels show wing imaginal discs from wild-type (a and b), UASdCsk-IR; nub-GAL4/+, nub-GAL4+; UASHpo+/+(i and j), nub-GAL4+/UASHpo+/+(m and n), and nub-GAL4/UASHpo+/+(q and u) larvae stained for DIAP1 and Wg (respectively). Phenotypes of UASdCsk-IR; nub-GAL4+/+; nub-GAL4+/UASHpo+/+(g and h), nub-GAL4/UASHpo+/+(k and l), nub-GAL4+/UASHpo+/+(o and p) show reduction of the wing pouch size, whereas UASdCsk-IR; nub-GAL4+/UASHpo+/+(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+/+. 
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 (j) due to heterozygosity of ykiB5. (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 ($n=5$, $p<0.05$).

**Discussion**

dCsk is 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
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, 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 p120Ctn (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 signaling 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 Paxillin), 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 then to be determined. In summary, our data uncover 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.

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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.