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Jacob D. Kagey, University of Detroit Mercy
Jordan A. Brown, Emory University
Kenneth H Moberg, Emory University

Journal Title: Mechanisms of Development
Volume: Volume 129, Number 0
Publisher: Elsevier | 2012-09-01, Pages 339-349
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1016/j.mod.2012.05.007
Permanent URL: https://pid.emory.edu/ark:/25593/s9fzm

Final published version: http://dx.doi.org/10.1016/j.mod.2012.05.007

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Accessed November 12, 2022 8:05 AM EST
Regulation of Yorkie activity in *Drosophila* imaginal discs by the Hedgehog receptor gene *patched*

Jacob D. Kageya,b,*, Jordan A. Brownb, and Kenneth H. Mobergb

aDepartment of Biology, University of Detroit Mercy, Detroit, MI

bDepartment of Cell Biology, Emory University School of Medicine, Atlanta, GA

Abstract

The Hedgehog (Hh) pathway was first defined by its role in segment polarity in the *Drosophila melanogaster* embryonic epidermis and has since been linked to many aspects of vertebrate development and disease. In humans, mutation of the *Patched1* (*PTCH1*) gene, which encodes an inhibitor of Hh signaling, lead to tumors of the skin and pediatric brain. Despite the high level of conservation between the vertebrate and invertebrate Hh pathways, studies in *Drosophila* have yet to find direct evidence that *ptc* limits organ size. Here we report identification of *Drosophila ptc* in a screen for mutations that require a synergistic apoptotic block in order to drive overgrowth. Developing imaginal discs containing clones of *ptc* mutant cells immortalized by the concurrent loss of the *Apaf-1-related killer* (*Ark*) gene are overgrown due, in large part, to the overgrowth of wild type portions of these discs. This phenotype correlates with overexpression of the morphogen Dpp in *ptc,Ark* double-mutant cells, leading to elevated phosphorylation of the Dpp pathway effector Mad (p-Mad) in cells surrounding *ptc,Ark* mutant clones. p-Mad functions with the Hippo pathway oncoprotein Yorkie (*Yki*) to induce expression of the pro-growth/anti-apoptotic microRNA *bantam*. Accordingly, Yki activity is elevated among wild type cells surrounding *ptc,Ark* clones and alleles of *bantam* and *yki* dominantly suppress the enlarged-disc phenotype produced by loss of *ptc*. These data suggest that *ptc* can regulate Yki in a non-cell autonomous manner and reveal an intercellular link between the Hh and Hippo pathways that may contribute to growth-regulatory properties of the Hh pathway in development and disease.

Keywords

Patched; Hedgehog; Apoptosis; Yorkie; Growth; *Drosophila*

Introduction

Genetic screens in the fruit fly *Drosophila melanogaster* have identified a number of genes that are required to restrict the growth of developing tissues (reviewed in (Hariharan and Bilder, 2006; Pan, 2007)). A number of these genes control the process of tissue growth by regulating largely cell-intrinsic mechanisms that modulate rates of cell division, death, or growth. However, other genes exhibit more complex phenotypes indicative of roles in intercellular signaling and morphogen-based pathways that pattern the growth and differentiation of groups of cells in developing organs. Vertebrate orthologs of both classes of *Drosophila* anti-growth genes are mutated inhuman disease and collaborate with anti-apoptotic mutations to drive cancer e.g. (Hanahan and Weinberg, 2011; Vidal and Cagan, 2006). Similar synergy occurs in *Drosophila* (Asano et al., 1996; Herz et al., 2006;
Nicholson et al., 2009; Staehling-Hampton et al., 1999) and has been used to identify ‘conditional’ pro-growth mutations that require a collaborating block in cell death to drive tissue overgrowth (Gilbert et al., 2011).

The Hedgehog (Hh) pathway was first defined by its role in segment polarity in the *Drosophila* embryonic epidermis (Nusslein-Volhard and Wieschaus, 1980) and has since been linked to many aspects of vertebrate development and disease (Bale, 2002), including cancer (Jiang and Hui, 2008). Core Hh pathway components include the secreted morphogen Hh, a receptor complex composed of the transmembrane proteins Patched (Ptc) and Smoothened (Smo), the intracellular signaling components Protein Kinase A (PKA) and Costal-2 (Cos2), and the nuclear transcription factor Cubitus interruptus (Ci; Gli in vertebrates) (Jiang and Hui, 2008). Canonical signaling involves binding of Hh to Ptc, which relieves Ptc inhibition of the G-protein-coupled receptor Smo and allows Smo to signal via PKA/Cos2 to stimulate cleavage of Ci, which converts it from a transcriptional repressor to a transcriptional activator. In humans, loss-of-function mutations in *Patched1 (PTCH1)* occur in 90% of sporadic basal cell carcinoma (BCC) cases and in a significant subset of pediatric medulloblastomas (Corcoran and Scott, 2001). As loss of PTCH1/Ptc activates Gli/Ci, this mutational data suggests that ectopic Ci/Gli activity promotes carcinogenesis in vivo and this model is supported by the original identification of the human Gli gene as a locus amplified in glioblastomas (Kinzler et al., 1987). This model is further supported by elevated rates of tumorigenesis in mice that either lack the murine *PTCH1* homolog or overexpress Smo protein (Hahn et al., 1999).

Despite the high level of conservation between the vertebrate and invertebrate Hh pathways (Huangfu and Anderson, 2006), genetic analyses of the developmental effects of ptc loss in *Drosophila* have yet to show evidence of a direct role in suppressing tissue growth. ptc mutant cells in the *Drosophila* larval imaginal discs, a commonly used model of invertebrate growth control (for review see (Harhiran and Bilder, 2006)), undergo apoptotic cell death (Thomas and Ingham, 2003) and show deregulated expression of Ci target genes involved in developmental patterning, including the morphogens *decapentaplegic (dpp)* and *wingless (wg)*, the segment polarity gene *engrailed (en)*, and the ptc gene itself as part of a negative feedback mechanism (Bale, 2002). Additionally Ptc has been suggested to autonomously promote, rather than restrict, cell proliferation in the developing head capsule (Shyamala and Bhat, 2002). Cells in the *Drosophila* eye imaginal disc located just anterior to the morphogenetic furrow (MF) respond to ptc loss by elevating expression of G1 cyclins (Cyclin E and Cyclin D) and Ci protein interacts with the promoters of these genes in cultured *Drosophila* S2 cells (Duman-Scheel et al., 2002). However, the link between Ptc and G1 cyclin gene expression is spatially and temporally restricted within the developing eye and has not been shown to affect overall rates of tissue growth.

Here we report the identification of the ptc gene in a screen for *Drosophila* mutations that require a collaborating block in cell death to increase imaginal disc size. As reported previously (Thomas and Ingham, 2003) ptc mutant disc cells survive poorly into adulthood, however those lacking both ptc and the pro-apoptotic gene *Apaf-1-related killer (Ark)* (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999) survive and drive substantial overgrowth of both mutant tissue and surrounding wild type tissue. ptc,Ark mutant cells express elevated levels of the Ci target gene *dpp* and produce a gradient of Dpp signaling extending out from ptc mutant cells into surrounding wild type tissue. This Dpp gradient is coincident with activation of Yorkie (Yki), a transcriptional cofactor that acts as the primary nuclear effector of the Salvador/Warts/Hippo (SWH) tumor suppressor pathway (reviewed in (Pan, 2010)). Yki has been shown to promote growth in response to Dpp gradients in the larval wing (Rogulja et al., 2008) and interacts with the Dpp responsive transcription factor Mad to promote expression of the *bantam* microRNA (Oh and Irvine, 2011). Accordingly,
alleles of *bantam* or *yorkie* suppress disc growth associated with *ptc* loss, confirming a model in which *ptc* is required to restrict Yki activity. Increased levels of the Yki homolog Yap1 in *PTCH1*−/− medulloblastoma cells (Fernandez et al., 2009) may provide a human correlate to this *Ptcl/Yki* link in *Drosophila*. In sum these data provide insight into an intercellular link between Ptc and Yki activity in fly imaginal discs that may be significant to the growth-regulatory properties of the Hh pathway in disease. Given the emerging role of the SWH pathway in human cancers, the *ptc/Ark* genotype could serve as a useful model of tumor/stromal interactions in cancers in which Hh signaling is altered such as BCC and medulloblastoma.

**Methods**

**Genetics**

The *Ark* 82 (Akdemir et al., 2006) loss-of-function allele was used as the basis for the EMS screen. Briefly, *w;FRT42D,Ark* 82 males were fed EMS and mated to *eyeless>Flp,FRT42D* virgins. Mosaic eyes of F2 adult flies were examined for evidence of deregulated growth, which was identified by an over-representation of pigmented mutant tissue, an increase in overall organ size, or both. Over 5,000 F2 flies were screened, and stable stocks were generated for 137 mutants. One of these mapped to the *patched* locus and was designated *ptc* B.2.13. Additional genotypes used: *thread-lacZ* (*Th-Z*) (Hay et al., 1995), *expanded-lacZ* (*Ex-Z*) (Boedigheimer et al., 1993), *bantam-GFP* (*ban-GFP*) (Brennecke et al., 2003), *bantam* Δ1 (Hippner et al., 2002), *dad-lacZ* (*Dad-Z*) (Tsuneizumi et al., 1997), *UAS-ptc-IR* (TRiP), *UAS-dpp* (Bloomington), and *yki* B5 (Huang et al., 2005).

**Immunohistochemistry**

Unless otherwise noted, immunostaining and confocal imaging were performed using standard techniques. Briefly, eye and wing discs were dissected from wandering third instar larvae, fixed on ice in 4% paraformaldehyde, permeabilized in 0.3% PBST, and incubated in 0.1% PBST+10% Normal Goat Serum (NGS) and appropriate antibody dilution. For analysis of *ban-GFP* expression wing discs were dissected, fixed overnight in 0.75% paraformaldehyde and 0.01% Tween, then permeabilized in 0.3% PBST. Antibodies from Developmental Studies Hybridoma Bank (DSHB): anti-Ptc (mouse, 1:40), anti-Wg (mouse, 1:800), anti-Elav (rat, 1:800), and anti-Ci (rat, 1:100). Other antibodies: anti-β galactosidase (mouse, 1:1000, Promega), anti-phospho-Smad1/5 (rabbit, 1:100, Cell Signaling), anti-DIAP (mouse, 1:50) (Yoo et al., 2002), anti-Caspase3 (rabbit, 1:100, Cell Signaling), anti-Dpp (rabbit, 1:100) (Gibson et al., 2002), anti-GFP (chicken, 1:100, Aves Labs) and Alexa-594 Phalloidin (1:50, Molecular Probes).

BrdU incorporation was adapted from Pellock, *et al.* (Pellock et al., 2007). Briefly, wing discs were dissected in Schneider’s serum-free media and labeled in 100μM BrdU with gentle agitation for 30 minutes. Discs were then fixed overnight in 0.75% paraformaldehyde and 0.01% Tween, treated with DNase (Promega), permeabilized in 0.3% PBST for 10 minutes, and immunostained in 0.1% PBST+10% NGS and 1:50 of anti-BrdU (mouse, DSHB).

**Results**

**Patched is a conditional suppressor of growth**

The *eye>Flp,FRT* system was used to perform an EMS mutagenesis screen of *Drosophila* chromosome 2R for mutations that altered eye development. The parental *FRT42D* chromosome also carried a mutant allele of the *Apat-related killer* (*Ark*) gene, *Ark* 82 (Akdemir et al., 2006). *Ark* is a homolog of the vertebrate pro-apoptotic gene *Apa-I* and is
required for most programmed cell death in *Drosophila* (Mills et al., 2006). The *Ark* \(^{82}\) allele is null produced by imprecise excision of the *P[lacW]*\/*Ark\(^{CDM}\)* element; it retains the *w(m\(^{+}\))* element and thus can be tracked in adult eyes by the presence of red pigment. *FRT42D,Ark* \(^{82}\) mosaic eyes also show little evidence of effects on growth or patterning (Figure 1A), consistent with evidence that a block in apoptosis is insufficient to overtly alter eye or head size e.g. (Du et al., 1996)(Gilbert et al., 2011).

The *FRT42D,Ark* \(^{82}\) EMS screen identified 137 mutants with mild to severe growth defects (data not shown). A number of these alleles appeared to affect genes required for autonomous survival and non-cell autonomous growth suppression. One of these mutants, which showed strong overgrowth of wild type tissue relative to control red:white ratio in *FRT42D,Ark* \(^{82}\) control eyes (Figure 1A vs. 1B; note that mutant tissue is pigmented due to the *P[m-w\(^{+}\)]Ark* \(^{82}\) allele), was mapped by deficiency complementation to genomic interval 44D. This allele failed to complement existing alleles of the *ptc* gene, which is located in this region, and was designated *ptc* \(^{B.2.13}\). In the absence of the *Ark* \(^{82}\) mutation, *FRT42D,ptc* \(^{B.2.13}\) mosaic eyes are irregular, composed of entirely pigmented, wild type tissue (Figure 1C; wild type tissue is pigmented due to presence of the *P[m-w\(^{+}\)]* marked *FRT42D,ubi>GFP* tester chromosome), and only slightly enlarged. *FRT42D,ptc* \(^{B.2.13}\) clones accumulate cleaved caspase posterior to the morphogenetic furrow (MF) (Figure 1F-G) suggesting that *ptc* mutant cells located in this region undergo apoptosis, while *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) mosaic discs showed no evidence of cleaved caspase accumulation (Figure 1E). Previous studies have documented a similar increase in apoptosis among of *ptc* mutant cells in the eye imaginal disc (Thomas and Ingham, 2003).

To assess whether the link between *ptc*/*Ark* loss and tissue growth is generalized to other epithelial tissues, the *Ubx*\(\times\)Flp driver was used to create *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) clones in the larval wing disc. This produced dramatically enlarged 3\(^{rd}\) instar larval wing discs (Figure 1B' vs. *FRT42D,Ark* \(^{82}\) control in Figure 1A' ) and led to pupal lethality. Reintroduction of the wild type *Ark* allele into the *ptc* \(^{B.2.13}\) background suppressed this enlarged-disc phenotype (Figure 1C') and partially suppressed pupal lethality (data not shown), thus confirming the conditional nature of the *ptc* overgrowth phenotype. These differences in larval wing were quantified and found to be highly significant (Figure 1D). Furthermore, the creation of a larval wing disc entirely mutant for *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) (by flipping against a cell-lethal *Minute* allele) reduced wing disc size (Figure 1D), indicating that presence of wild type tissue enhances the *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) overgrowth phenotype. In sum these data suggest that *ptc* loss cooperates with the block in apoptosis provided by the *Ark* \(^{82}\) allele to drive both autonomous and non-cell autonomous over-growth in the eye and wing.

**ptc,Ark mutant tissue leads to an increase in cellular proliferation**

Due to the premature differentiation and subsequent mitotic arrest of *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) eye clones (Supplemental Figure 1), the wing disc was chosen as a system to investigate the molecular effect of *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) clones on cell division in a proliferating epithelium. The *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) mosaic wing discs show elevated incorporation of the S-phase marker BrdU and the mitotic marker phospho-Histone 3 (pH3) particularly in the anterior portion of the wing disc (Figure 2A-B), compared to discs carrying *FRT42D,Ark* \(^{82}\) clones, which show a more uniform pattern of BrdU incorporation and pH3 staining (Figure 2C-D). These increased proliferation markers are not restricted to *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) clones but also occur in surrounding wild type tissue (see arrows, Figure 2A-B). The effect of *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) clones on patterns of cell division coupled with the requirement for wild type tissue in maximal overgrowth of *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) mosaic wing discs (Figure 1D) suggests that there may be a non-cell autonomous component to the *ptc* \(^{B.2.13}\),*Ark* \(^{82}\) disc-size phenotype. To test this hypothesis quantitatively, the percentage of mutant (non-GFP positive) and wild type (GFP positive) tissue was measured in wandering third instar wing discs carrying either *Ark* \(^{82}\)
mutant clones, ptc mutant clones or Ark, ptc double mutant clones (all generated with the same UbxFLP transgene). On average, control Ark mosaic wing discs are composed of ∼38% mutant tissue (this percentage is likely the result of ‘unflipped’ heterozygous tissue), and ptcB.2.13 mosaic wing discs are comprised of only ∼20% mutant tissue, presumably from the apoptosis occurring within these cells. Although ptcB.2.13, Ark mosaic discs are approximately twice the size of Ark mosaic discs (see Figure 1D), they are nonetheless composed of only ∼32% mutant tissue (Figure 2E). Thus while ptcB.2.13, Ark mosaic discs are dramatically enlarged, the mutant portion of these discs is decreased relative to controls, indicating that the ptcB.2.13, Ark disc enlargement phenotype has a significant non-cell autonomous component.

**ptcB.2.13, Ark clones have increased levels of canonical Hh signaling**

The cleaved form of the Ci transcription factor is typically expressed in the anterior domain of the wing disc, with highest levels immediately adjacent to the anterior:posterior (A:P) boundary (Motzny and Holmgren, 1995). This pattern of expression is unperturbed in Ark mosaic wing discs (Figure 3D). Additionally, two transcriptional targets of Ci, the Dpp and Ptc proteins are also unaltered in the Ark mosaic discs (Figure 3E and 3F). By contrast, ptcB.2.13, Ark mutant clones show a substantial increase in active Ci levels (Figure 3A) and expression of Dpp and Ptc (Figure 3B and 3C). Similar effects on Hh signaling are observed in ptcB.2.13 clones in the anterior compartment, suggesting that the ptcB.2.13 mutation is sufficient for this phenotype (Supplemental Figure 2A). The ptcB.2.13 allele is thus similar to the ptc allele, which functionally inactivates Ptc but remains responsive to induction by Ci (Methot and Basler, 2001). The induction of active Ci and elevation in levels of Ptc and Dpp are restricted to ptcB.2.13, Ark clones located in the anterior domain of the wing disc (Figure 3A-C). Tracing the location of the A:P boundary reveals that the overgrowth of ptcB.2.13, Ark wing discs is due to enlargement of the size of the anterior domain, and this corresponds to the location of the elevated BrdU incorporation and pH3 staining (Figure 2A-B). Evidence of domain-specific enlargement is also evident in the phalloidin stains of ptcB.2.13, Ark wing discs, based on morphology of overgrown wing discs and A:P staining in Figure 3 (Figure 1B−).

**ptcB.2.13, Ark clones can activate Dpp signaling in adjacent wild cells**

A gradient of Dpp signaling across the A:P axis of the wing disc promotes physiologic growth of that organ, and manipulations of this gradient can enhance growth (Rogulja et al., 2008; Schwank et al., 2008; Schwank et al., 2011). Consequently, the effects of ptcB.2.13, Ark clones on Dpp expression and wing growth prompted analysis of Dpp signaling in ptcB.2.13, Ark mosaic discs. Binding of Dpp to the transmembrane receptors Punt and Thickveins results in activation of the transcription factor Mothers Against Decapentaplegic (Mad) via phosphorylation (p-Mad1/5) (Letsou et al., 1995; Sekelsky et al., 1995). p-Mad1/5 levels are elevated in the anterior portion of ptcB.2.13, Ark wing discs as compared to control discs (Figure 4A-B vs 4D). Elevated p-Mad1/5 occurs within mutant clones, but the highest levels of p-Mad1/5 are found in wild type cells immediately adjacent to mutant clones, with a declining gradient of p-Mad1/5 activation extending multiple cell diameters into wild type tissue (see arrow, Figure 4A). To better visualize this non-autonomous activation we co-stained ptcB.2.13, Ark mosaic discs with anti-Ptc to mark the mutant clones, and with anti-pMad1/5 to visualize the Dpp activity gradient (see arrow, Figure 4B). A halo of p-Mad1/5 activation can be seen ringing Ptc-positive mutant clones, and a similar effect is detected in discs mosaic for ptcB.2.13 alone (Supplemental Figure 2B). To assess why ptcB.2.13, Ark cells within mutant clones, which are presumably exposed to the high levels of Dpp, do not exhibit the highest level of p-Mad1/5, we investigated expression of the inhibitory Mad family member, Daughters against decapentaplegic (Dad), through use of the transcriptional reporter Dad-lacZ (Tsuneizumi et al., 1997). Dad is
induced by Dpp signaling in a negative feedback loop (Inoue et al., 1998) and is expressed in a domain along the anterior/posterior axis (Tsuneizumi et al., 1997). While Ar\textsubscript{k} control clones have no effect on this pattern (Figure 4E), Dad-\textit{lacZ} expression is ectopically elevated within \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} mutant clones, but not in immediately adjacent wild type tissue where pMad1/5 are highest (see arrows, Figure 4C). This autonomous increase in Dad expression may explain why the highest levels of pMad1/5 are found in areas adjacent to \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} clones where cells are closely apposed to a source of Dpp but do not express the Mad inhibitor Dad.

\textbf{\textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} mosaic wing discs show elevated Yki activity}

\textit{Drosophila} and vertebrate Mad proteins physically interact with the corresponding SWH effector proteins Yki/Yap to alter nuclear gene expression (Alarcon et al., 2009; Oh and Irvine, 2011). Furthermore, the regulation of Yki through the Fat atypical cadherin is linked to the A:P gradient of Dpp in the wing (Rogulja et al., 2008). We therefore hypothesized that exposure of wing disc cells to the ectopic gradients of Dpp around \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} clones could result in elevated Yki activity. To test the relationship between \textit{ptc} loss and Yki activity, several well-characterized reporters of Yki activity including the enhancer trap ex-\textit{lacZ} (ex\textsuperscript{697}; (Boedigheimer et al., 1993)), the apoptosis inhibitor protein DIAP-1, and the \textit{bantam} (\textit{ban}) reporter, \textit{ban-}\textit{GFP} (Brennecke et al., 2003) were analyzed in \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} mosaic wing discs. Similar to the expression pattern of pMad1/5, we find that DIAP1 levels are increased in wild type cells along the boundaries of \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} clones within the anterior portion of the wing disc (see arrow, Figures 5A). A similar effect is observed in discs co-stained for Ci protein (to mark \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} mutant cells) and DIAP1 (Figure 5B, see arrow). \textit{ex-}\textit{lacZ} is also elevated around \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} clones (see inset, Figure 5C-D). Decreased \textit{ban-}\textit{GFP}, which correlates with elevated Yki activity, is also apparent in wild types cells outside of \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} clones (see arrows, Figure 5E). These alterations in Yki targets are not seen in Ark\textsubscript{82} mosaic discs (Figure 5G-I). The pattern of Yki target activation in \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} mosaic discs correlates spatially with the p-Mad1/5 gradient (Figure 4A,B) suggesting that cells located along the steepest part of the Dpp gradient activate Yki most strongly, as has been proposed previously (Rogulja et al., 2008). Notably, levels and patterns of the protein Wingless (Wg) appear similar in \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} and Ark\textsubscript{82} control discs (Figure 5F and 5I), indicating that not all downstream readouts of Yki activity are affected by proximity to \textit{ptc} mutant cells.

To test whether Yki activity might contribute to the overgrowth effect of \textit{ptc} loss, the \textit{ban}\textsuperscript{A1} loss-of-function allele was tested for a dominant effect on the size of \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} mosaic 3rd instar larval wing discs. Heterozygosity for \textit{ban}\textsuperscript{A1} potently suppressed of the \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} enlarged wing phenotype (Figures 5J), but had no effect on the size of Ark\textsubscript{82} mosaic wing discs. The location of \textit{yki}, Ark, and \textit{ptc} on the same chromosome arm (2R) prevented similar analysis of the requirement for \textit{yki} in the background of the \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} genotype. To circumvent this, the \textit{yki}\textsuperscript{B5} loss-of-function allele was tested for it's ability to alter the growth of wing discs carrying heat-shock induced clones of cells depleted of Ptc by an RNAi transgene (\textit{UAS-}\textit{ptc-IR}; Bloomington). While \textit{yki}\textsuperscript{B5/+} has no effect on otherwise normal discs (data not shown), it substantially suppresses the enlarged size of wing discs carrying clones of Ptc-depleted cells (Figure 5J) without any overt effect on the relative size of the \textit{ptc-IR} clones (Figure 5K’-K’’; GFP marks the \textit{UAS-}\textit{ptcIR} clones). Together these data suggest that \textit{ptc}\textsuperscript{B.2.13,Ark}\textsubscript{82} mosaic imaginal discs overgrow due to an increase in Yki activity.

To determine if simply overexpressing Dpp could elicit a similar effect on Yki in proximal wild type cells as loss of \textit{ptc} clones of wing disc cells were generated that expressed Dpp from a \textit{UAS-}\textit{dpp} transgene. These wing discs are overgrown and show evidence of elevated DIAP1 levels surrounding Dpp-expressing clones, particularly in the pouch (see arrows,
Supplemental Figure 3A-B). This effect is consistent both with the parallel effect of Ptc loss on Yki documented here, and the prior finding that cells that cells located on steep gradients of Dpp signaling activate a Yki-dependent growth program (Rogulja et al., 2008).

**Discussion**

**Ptc is a conditional suppressor of growth**

Some cancer-causing mutations are proposed to reactivate proliferative programs normally associated with early stages of metazoan development (e.g. (Lipinski and Jacks, 1999). Understanding how factors encoded by these genes can influence cell proliferation pathways during development, or interface with other factors that directly control cell division, growth or survival, is thus a key step in understanding carcinogenic mechanisms. Here we identify the Drosophila gene ptc, a homolog of the PTCH1 tumor suppressor gene, as a conditional suppressor of growth in developing epithelia via an intercellular link between the Hh and Hippo pathways. Immortalization of ptc mutant cells by removal of the Apaf-related gene Ark allows these cells to survive and drive sustained activation of the Hippo-responsive transcriptional co-activator Yki in surrounding cells. The resultant overgrowth can be suppressed by an allele of either yki or the Yki target and pro-growth microRNA bantam. The strength of this suppression suggests Yki hyperactivity is a key contributor to the excess growth elicited by ptc loss in this fly model. Yki activation in wild type cells adjacent to ptc/Ark mutant clones may be linked with elevated expression of the Dpp morphogen, and a concurrent increase in phosphorylation of the Dpp-responsive transcription factor, and Yki-binding partner, Mad. The resultant p-Mad gradient radiating out from ptc/Ark clones correlates with activation of multiple Yki reporters (DIAP1, ban, and expanded) in surrounding wild type cells. These data suggest a model in which blocking the death of ptc mutant disc cells collaborates with ptc mutant cells to create a sustained source of Dpp, which activates Mad in surrounding cells and allows p-Mad:Yki complexes to promote expression of a pro-growth transcriptional program that includes bantam (Oh and Irvine, 2011).

**A Drosophila model of human PTCH1 cancer**

Although the ptc gene was identified in Drosophila a number of years ago and the human homolog PTCH1 has been implicated in numerous cancers, there is little literature linking ptc and development in Drosophila. One possibility for this gap may be tied to our finding that ptc mutant cells require a collaborating block in cell death in order to significantly enlarge developing organs. At a genetic level, the collaboration of ptc-driven tissue growth with an apoptotic block is conserved in mammals: a high percentage of BCCs with mutations in PTCH1 also have mutations in the pro-apoptotic transcription factor p53 (Ling et al., 2001), and a p53 knockout allele accelerates the rate of tumorigenesis in PTCH1 mouse models (Epstein, 2008).

Data here show that wing discs composed mainly of immortalized ptc mutant cells are moderately enlarged relative to controls (Figure 1D), but are smaller than discs which contain immortalized ptc mutant cells mixed with genetically normal cells, indicating that the presence of wild type cells in the same organ enhances the ability of ptc,Ark mutant cells to drive tissue overgrowth. The requirement for genetic heterogeneity may be due to the inhibitory effects of Dad, which is elevated within ptc,Ark cells, or by a requirement for a Dpp gradient to activate Yki in cells surrounding ptc,Ark clones (e.g. (Rogulja et al., 2008). These type of cell:cell interactions are important determinants of cancer cell survival, growth and metastasis in mammals (Hanahan and Weinberg, 2011), and the ptc,Ark fly model may provide an opportunity to study this relationship in vivo defined genetic mosaics.
Our finding of a link between Ptc and Yki in *Drosophila* wing development may be
generalizable to other contexts and organisms. Vertebrate homologs of Yki and Dpp, Yap1
and the TGFβ morphogen, are altered in human tumors with deregulated Hh signaling. One
study found an increase in expression of TGF-β and several associated Smad proteins in
human BCC samples, while another documented an increase in Yap1 levels in
medulloblastoma cells and tissue samples (Fernandez et al., 2009; Gambichler et al., 2007).
Data presented here suggest that these phenomena could be linked, and that mutations that
alter Hh activity in vertebrates could alter Yap1-driven proliferation via paracrine or
autocrine mechanisms. Insight into these mechanisms could provide important opportunities
for therapeutic intervention in the treatment of human tumors lacking *PTCH1*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We apologize to those whose work we could not cite due to space constraints. We thank many members of the
*Drosophila* community for gifts of reagents and stocks including J. Abrams for the *Ark82* allele. We thank the
Bloomington Drosophila Stock Center, and Developmental Studies Hybridoma Bank for fly stocks and antibodies.
We thank members of the Moberg lab, the Emory fly community, and the UDM biology department for helpful and
insightful discussions. KHM is supported by NIH 2R01-CA123368; JDK is supported by NIH/NIGMS K12
GM000680.

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Figure 1. A mosaic screen for conditional suppressors of growth identifies a novel allele of patched, ptc<sup>B.2.13</sup>, as a conditional suppressor of growth.

Mosaic adult eyes and imaginal wing discs from L3 larvae, stained with phalloidin: (A-A') Ark<sup>82</sup> (mutant tissue is pigmented) (B-B') ptc<sup>B.2.13</sup>,Ark<sup>82</sup> (mutant tissue is pigmented) (C-C') ptc<sup>B.2.13</sup> (wild type tissue is pigmented); (D) Quantification of mosaic wing discs size from L3 larvae demonstrates the conditional nature of the ptc<sup>B.2.13</sup>,Ark<sup>82</sup> overgrowth; Mosaic larval eye discs stained with anti-Cleaved Caspase 3 (cCasp) shows ptc<sup>B.2.13</sup>,mutant clones accumulate cCasp without Ark<sup>82</sup> (E-E') ptc<sup>B.2.13</sup>,Ark<sup>82</sup> (mutant tissue is GFP negative) (F-G '') ptc<sup>B.2.13</sup> (inset is amplified in G-G '') (mutant tissue is GFP negative).
Figure 2. *ptc*\(^{B.2.13,Ark^{82}}\) wing discs have increased proliferation in both mutant cells and adjacent wild type cells

Imaginal mosaic wing discs dissected from wandering L3 larvae stained with the proliferation markers BrdU and phospho-histoneH3 (pH3) in the anterior of the wing disc; *ptc*\(^{B.2.13,Ark^{82}}\) (A-A\(^{''}\)) stained with BrdU, see arrows for examples of non-autonomous increased proliferation (B-B\(^{''}\)) stained with pH3, see arrow for example of non-autonomous increased proliferation; *Ark^{82}\) (C-C\(^{''}\)) stained with BrdU (D-D\(^{''}\)) stained with pH3; (E) quantification of the portion of larval wing discs composed of mutant tissue reveals a non-autonomous component of *ptc*\(^{B.2.13,Ark^{82}}\) overgrowth. Mutant tissue is GFP negative.
Figure 3. mutant tissue has an autonomous increase in canonical Hedgehog signaling

$ptc^{B.2.13}$. $Ark^{82}$ mosaic wing discs have a strong autonomous increase in Hedgehog signaling confined to the anterior portion of wing discs as demonstrated by $(A-A^{‴})$ anti-Cubitus Interruptus (Ci), $(B-B^{‴})$ anti-Patched (Ptc), $(C-C^{‴})$ anti-Decapentaplegic (Dpp). The phenotype is dependent on $ptc^{B.2.13}$, as $Ark^{82}$ mosaic wing discs have normal Hedgehog signaling as demonstrated by $(D-D^{″})$ Ci, $(E-E^{″})$ Ptc, $(F-F^{″})$ Dpp. Mutant tissue is GFP negative.
Figure 4. *ptc*<sup>B.2.13</sup>,*Ark*<sup>82</sup> clones activate Dpp signaling most robustly in wild type cells immediately adjacent to mutant clones

*ptc*<sup>B.2.13</sup>,*Ark*<sup>82</sup> mosaic wing discs have an increase of Dpp signaling within the anterior of the wing disc, the signaling is highest in those cells immediately adjacent to mutant clones. Increases in Dpp signaling in *ptc*<sup>B.2.13</sup>,*Ark*<sup>82</sup> mosaic discs measured by levels of phosphorylated-Mothers Against Decapentaplegic (p-Mad1/5) as marked by (A-A″) anti-p-Mad1/5 (p-Mad1/5) see arrows, (B-B″) Ptc denotes mutant clones (Figure 3B) and pMad1/5 levels create a ring round the mutant clones, see arrow; these same mutant discs have an autonomous increase in the inhibitory smad, Daughters Against Decapentaplegic (Dad) (C-C″) measured by the Dad transcriptional reporter Dad-LacZ (Dad-Z), see arrow; these phenotypes are dependent upon *ptc*<sup>B.2.13</sup>, as mosaic discs for *Ark*<sup>82</sup> have normal pattern of (D-D″) p-Mad1/5 and (E-E″) Dad-Z. Mutant tissue is GFP negative, unless otherwise noted.
Figure 5. *ptc*\(^{B.2.13} \text{Ark}^{82}\) mutant clones result in gradient dependent activation of a subset of Yorkie target genes, which necessary for the *ptc*\(^{B.2.13} \text{Ark}^{82}\) overgrowth phenotype. *ptc*\(^{B.2.13} \text{Ark}^{82}\) larval mosaic wing discs have elevated Yki activity in the anterior portion of the wing discs, with the highest levels occurring in wild type cells immediately adjacent to mutant clones. Increases of several Yki targets were elevated within *ptc*\(^{B.2.13} \text{Ark}^{82}\) mutant clones and in those wild type cells adjacent to the clones (A-A″) anti-Drosophila Inhibitor of Apoptosis Protein 1 (DIAP1), see arrow, (B-B″) Ci denotes mutant clones (Figure 3B) and DIAP1 levels are most elevated just outside the mutant clones, see arrow, (C-C″) Expanded levels as measured by the reporter Expanded-lacZ (Ex-Z), (D-D″) Enlarged image of inset from 5C of Ex-Z, (E-E″) Ci denotes mutant clones and bantam levels are highest immediately adjacent to mutant clones as see by decreasing levels of the bantam reporter, ban-GFP. Not all Yki targets are altered by *ptc*\(^{B.2.13} \text{Ark}^{82}\) as Wingless pattern and levels are unchanged (F-F″) anti-Wingless (Wg). The alteration of Yki targets is dependent on the *ptc*\(^{B.2.13}\) mutation given that we find no alteration of Yki reporters in wing discs mosaic for Ark\(^{82}\), (G-G″) DIAP1, (H-H″) Ex-Z, (I-I″) Wg. Dominant suppression of the *ptc*\(^{B.2.13} \text{Ark}^{82}\) overgrowth phenotype by a single copy of ban\(^{A1}\) (J). A single copy loss of function yki allele (yki\(^{B5}\)) dominantly suppresses the overgrowth of larval wing discs generated from driving *UAS-ptcIR* in clones (K), despite the relative clone size remaining the same in both (K′) *UAS-ptcIR* and (K″) *UAS-ptcIR*, yki\(^{B5}/+\).