Homeodomain interacting protein kinase promotes tumorigenesis and metastatic cell behavior

Jessica A. Blaquiere*, Kenneth Kin Lam Wong*, Stephen D. Kinsey, Jin Wu and Esther M. Verheyen^

Department of Molecular Biology and Biochemistry, Centre for Cell Biology, Development and Disease, Simon Fraser University, Burnaby, V5A 1S6, Canada

* These authors contributed equally to the study

^Corresponding author: everheye@sfu.ca T: 1-778-782-4665, F: 1-778-782-5583

Summary statement

The protein kinase Hipk can promote proliferation and invasive behaviors, as well as synergize with known cancer pathways, in a novel Drosophila model for tumorigenesis.
Abstract: Aberrations in signaling pathways that regulate tissue growth often lead to tumorigenesis. Homeodomain interacting protein kinase (Hipk) family members are reported to have distinct and contradictory effects on cell proliferation and tissue growth. From these studies it is clear that much remains to be learned about the roles of Hipk family protein kinases in proliferation and cell behaviour. Previous work has shown that Drosophila Hipk is a potent growth regulator, thus we predicted that it could have a role in tumorigenesis. In our study of Hipk-induced phenotypes, we observed the formation of tumor-like structures in multiple cell types in larvae and adults. Furthermore, elevated Hipk in epithelial cells induces cell spreading, invasion and epithelial-to-mesenchymal transition (EMT) in the imaginal disc. Further evidence comes from cell culture studies in which we expressed Drosophila Hipk in human breast cancer cells and show that it enhances proliferation and migration. Past studies have shown that Hipk can promote the action of conserved pathways implicated in cancer and EMT, such as Wnt/Wingless, Hippo, Notch and JNK. We show that Hipk-phenotypes are not likely due to activation of a single target, but rather through a cumulative effect on numerous target pathways. Most Drosophila tumor models involve mutations in multiple genes, such as the well-known RasV12 model, in which EMT and invasiveness occur after the additional loss of the tumor suppressor gene scribble. Our study reveals that elevated levels of Hipk on their own can promote both hyperproliferation and invasive cell behaviour, suggesting that Hipks could be potent oncogenes and drivers of EMT.

Keywords: Hipk / Metastasis / Tumor / Cancer
Introduction:

A number of evolutionarily conserved signaling pathways are used reiteratively during development to control the growth of healthy organs and tissues. Genetic aberrations in pathway components can lead to dysregulated growth signals, often resulting in uncontrolled proliferation and tumorigenesis. With time and further genetic changes, tumor cells may progress into a metastatic state by undergoing epithelial-to-mesenchymal transition (EMT), enabling cells to leave the primary tumor site and travel to other locations in the body (Reviewed in Thiery et al., 2009). Many of the cellular markers and processes involved in vertebrate tumorigenesis are conserved in Drosophila, which have been used for decades to study developmental signaling pathways and have been key in revealing molecular functions of human disease and cancer-related genes (Brumby and Richardson, 2005; Gonzalez, 2013; Potter et al., 2000; Rudrapatna et al., 2012). Tissue and organ growth are often studied using the larval imaginal discs, which are epithelial sacs composed primarily of a pseudo-stratified columnar monolayer (Aldaz and Escudero, 2010). Discs undergo extensive proliferation with subsequent patterning and differentiation to form adult structures, which requires the same key signaling pathways needed for human development and growth (Brumby and Richardson, 2005; Gonzalez, 2013; Herranz et al., 2016; Miles et al., 2011; Rudrapatna et al., 2012; Sonoshita and Cagan, 2017). Low genetic redundancy paired with powerful genetic manipulation tools makes Drosophila an excellent system for the study of tumorigenesis and metastasis.

Numerous signaling pathways have been implicated in the development of tissue overgrowth, and/or metastatic behavior in the fly. The majority of these studies have described tumor models that require the combination of multiple genetic aberrations in order to manifest hyperproliferation coupled with invasive behaviors. The earliest metastasis model involved activated Ras combined with loss of the tumor suppressor scribble (Pagliarini and Xu, 2003). Notch pathway activation coupled with alterations in histone epigenetic marks also lead to a Drosophila tumor model (Ferres-Marco et al., 2006a). Subsequent studies have identified further factors involved in both Ras and Notch driven tumorigenesis (Doggett et al., 2015). Other tumor studies involve EGFR signaling (Herranz et al., 2012) and the Sin3A histone deacetylase (HDAC) (Das et al., 2013). The Hippo pathway is a potent tumor suppressor pathway that is required to prevent hematopoietic disorders (Milton et al., 2014). Activated JAK/STAT signaling causes leukemia-like hematopoiesis defects in Drosophila (Harrison et al., 1995; Luo et al., 1997).
Homeodomain interacting protein kinases (Hipk) are evolutionarily conserved and vertebrates possess Hipk1-4, while Drosophila and C. elegans have only one Hipk each. Hipk family members are expressed in dynamic temporal and spatial patterns, highlighting their important roles during development (Reviewed in (Blaquiere and Verheyen, 2016). Hipk protein levels are highly regulated by post-translational modification and proteasomal degradation (Saul and Schmitz, 2013). Hipk family members are reported to have distinct and contradictory effects on cell proliferation and tissue growth. Overexpressing Drosophila Hipk causes tissue overgrowths in the wing, eye and legs in a dose-dependent manner (Chen and Verheyen, 2012; Lee et al., 2009a; Poon et al., 2012). In C. elegans, Hpk-1 promotes proliferation of the germline cells, and loss of hpk-1 reduces the number of proliferating cells and size of the mitotic region (Berber et al., 2013). Hipk2−/− mice have growth deficiencies and 40% die prematurely (Chalazonitis et al., 2011; Sjölund et al., 2014; Trapasso et al., 2009). In normal human skin Hipk2 protein expression is enriched in basal proliferating cells, while it is undetectable in non-proliferating cells (lacovelli et al., 2009), and expression is reactivated when cells are stimulated to proliferate, suggesting a close link between Hipk protein function and cell proliferation. Mouse embryo fibroblasts (MEFs) from Hipk2−/− knockout mice show reduced proliferation (Trapasso, 2009), while another study claimed such cells proliferated more than wild type (Wei et al., 2007). From these studies it is clear that much remains to be learned about the roles of Hipk family protein kinases in proliferation and cell behaviour.

Hipks regulate numerous signaling pathways required for the development of healthy tissues (Figure S1; reviewed in (Blaquiere and Verheyen, 2016)). Both Drosophila and vertebrate Hipks can modulate Wnt signaling in multiple ways (Hikasa and Sokol, 2011; Hikasa et al., 2010; Kuwahara et al., 2014; Lee et al., 2009b; Louie et al., 2009; Shimizu et al., 2014; Swarup and Verheyen, 2011; Wu et al., 2012). Hipk proteins modulate the Hippo pathway in Drosophila, which is an essential conserved signaling pathway regulating tissue and organ growth (Chen and Verheyen, 2012; Poon et al., 2012). Yki activity requires Hipk, as hipk loss of function can suppress the effects of constitutively active Yki (YkiS168A). Hipks have also been shown to regulate Jun N terminal Kinase (JNK) signaling in numerous contexts (Hofmann et al., 2003, 2005; Huang et al., 2011a; Lan et al., 2007, 2012; Rochat-Steiner et al., 2000; Song and Lee, 2003). (Chen and Verheyen, 2012). Hipk is required for the full effect of JAK/STAT signaling, since loss of hipk through somatic clonal analysis causes loss of Stat92E-GFP reporter and furthermore loss of hipk can suppress lethality and tumour frequency in the constitutively active hopTum-L allele (Blaquiere et al., 2016).
Hipk2 is the best-characterized vertebrate Hipk family member. Studies in cell culture and cancer samples reveal conflicting results (Blaquiere and Verheyen, 2016). For example, Hipk2 acts as a tumor suppressor in the context of p53-mediated cell death following lethal DNA damage (Hofmann et al., 2013), and reduced expression of Hipks is seen in several cancer types (Lavra et al., 2011; Pierantoni et al., 2002; Ricci et al., 2013; Tan et al., 2014). In contrast, Hipk2 is elevated in certain cancers including cervical cancers, apilocytic astrocytomas, colorectal cancer cells and in other diseases, such as thyroid follicular hyperplasia (Al-Beiti and Lu, 2008; Cheng et al., 2012; D’Orazi et al., 2006; Deshmukh et al., 2008; Jacob et al., 2009; Lavra et al., 2011; Saul and Schmitz, 2013; Yu et al., 2009). Human Hipk1 is also found at elevated levels in certain cancer cell lines and tissue samples (Kondo et al., 2003; Rey et al., 2013).

While it is known that Drosophila Hipk is a strong inducer of tissue growth, its role in tumorigenesis is less understood. We were intrigued to test whether Hipk contributes to this process in Drosophila, due to the genetic simplicity of this model system. Using various techniques, we provide evidence that elevation of \texttt{hipk} can lead to neoplasia characterized by cell invasiveness. Hipk expression drives numerous cellular changes that are hallmarks of EMT. Hipk-expressing cells can migrate through tissues, disrupt the basement membrane to exit tissues and express mesenchymal markers. Our findings are significant in that Hipk alone can promote proliferation and invasive behavior that has been previously described to arise due to perturbation of multiple pathways. We propose that Drosophila Hipk has potent oncogenic properties, and that Hipk can exert such an effect through promotion of its multiple target pathways.

Results:

\textit{Elevated Hipk leads to overgrowths and masses}

To study the implications of elevated \texttt{hipk}, we used the GAL4-UAS system to overexpress Hipk in a variety of cell types. Using a combination of growth at different temperatures (which affects the potency of the GAL4 transcription factor) and copy number of UAS-transgenes, we have generated a range of Hipk overexpression phenotypes. Use of the \texttt{dpp-Gal4} driver to express 2 copies of Hipk at 25°C caused dramatic overgrowth of eye, wing, leg imaginal discs, characterized by tissue folds and protrusions (Fig. 1A-F'). We made use of GFP labeling to mark the \texttt{hipk}-overexpressing cells, allowing us to visualize their
behavior (Fig. 1D-F') in comparison with wild type cells (dpp>GFP; Fig. 1A-C'). Co-expression of hipk and GFP at 29°C (dpp>HA-hipk^{3M}+GFP) led to overgrown wing discs (Fig. 1J). Staining for cleaved Caspase 3 (Casp3) revealed that cell death was autonomously induced within the hipk-expressing discs (Fig. 1H-H'', Fig. S2A). When we used P35 expression to block caspase-dependent cell death, cells within the Dpp domain expanded substantially and occupied almost the entire dpp>HA-hipk^{3M}+P35+GFP discs (Fig. 1K), while dpp>P35 alone had no effect in this context (Fig. 1L). This implies that Hipk can induce both cell death and abundant proliferation to induce the gain of function phenotypes.

**Hipk induces melanotic masses in the hemocytes**

In addition to overgrown discs, darkly pigmented stationary masses were present in both dpp>HA-hipk^{3M}+2xGFP and dpp>HA-hipk^{3M}+P35+GFP larvae grown at 29°C (Fig. 2B,D), whereas control larvae dpp>GFP (Fig. 2A) and dpp>P35 (Fig. 2C) displayed none. The persistence of the masses upon P35 co-expression suggests that they were not due to cell death. Moreover, dpp>HA-hipk^{3M}+P35+GFP animals remained in the 3rd larval stage for an extended period of time (beyond 10 days) and eventually died as larvae. Arrested development in tumor-ridden animals has been reported by others and is thought to be due to alterations in ecdysone regulation (Garelli et al., 2012; Parisi et al., 2014). We hypothesized that these tumors may arise due to expression of the dpp-Gal4 driver in larval blood cells (Ayyaz et al., 2015; Clark et al., 2011).

Melanotic tumors arise due to over-amplification and melanization of hemocytes, fly hematopoietic cells (Hanratty and Ryerse, 1981). Therefore, we next tested directly whether hipk could cause tumors when overexpressed in the circulating hemocytes and lymph gland using hemolectin-GAL4 (hml-GAL4) (Sinenko and Mathey-Prevot, 2004). 91.7% of hml>HA-hipk^{3M} flies exhibited at least one clearly visible melanotic tumor, with the average being 3-4 tumors (Fig. 2F, I), compared to 0% of hml>GFP flies (Fig. 2E, I). To test if Hipk increased the number of circulating hemocytes, we isolated the total hemolymph from third instar larvae. The mean number of hemocytes in each hml>HA-hipk^{3M}+GFP sampling area (see methods) was 348, compared to 67 per hml>GFP+GFP sampling area (Fig 2G-H, J). These data suggest that the abdominal tumors induced by Hipk are derived from hyperproliferating hemocytes.
**Hipk induces cell invasiveness**

Valuable methods have been developed which allow one to assay for invasive behavior using the GAL4-UAS system (Ferres-Marco et al., 2006b; Herranz et al., 2014; Pallavi et al., 2012). In the wing disc, *dpp* is expressed in the anterior-most cells of the anterior-posterior (A/P) boundary. Thus, in *dpp>GFP* discs, a sharp border of GFP-expressing and non-GFP expressing cells is produced (Fig. 1B). In *dpp>HA-hipk^{SM}+GFP* discs, multiple isolated GFP-positive cells were found outside of the *dpp* domain, suggesting cells migrated away from their original location in the disc (Fig. 1E).

To provide further evidence of cell spreading, *dpp>HA-hipk^{SM}+GFP* wing discs were co-stained for the anterior marker Cubitus interruptus (Ci) and for the posterior marker Engrailed (En) (Fig. 3A-C). Under normal conditions, the A/P boundary is well defined, in which the Dpp domain (marked by GFP) is restricted within the anterior compartment (Fig. 3A,B). However, in discs with elevated *hipk*, GFP-positive cells originating from the anterior compartment were found in the posterior domain as isolated clusters of cells (Fig. 3C'). On rare occasions, GFP-positive clusters simultaneously expressed Ci and En, suggesting these cells have either lost their ability to interpret A/P positional cues from the tissue, or are in a period of fate transition (Fig. S3A). We also found individual GFP positive cells move from the central Dpp domain towards both anterior and posterior parts of the discs (Fig. 1E).

Another phenotype associated with metastatic behavior in Drosophila is the migration of retinal tissue into the body of the fly (Ferres-Marco et al., 2006b; Pallavi et al., 2012). When *hipk* was expressed in the eye disc using *eyeless-GAL4 (ey-GAL4)*, a large cluster of pigmented retinal cells was observed in the thorax of the adult fly (Fig. 3D). Because the endogenous eyes were fully intact, it suggests this was not likely a disc eversion defect, but rather a metastatic event where retinal tissue migrated away from the eye disc and lodged into the thorax. This phenotype also occurred in *dpp>HA-hipk^{SM}+GFP* flies, in which ectopic pigmented eye cells can occasionally be observed in the abdomen (Fig. 3E), indicating that Hipk-expressing eye disc cells can migrate within the body and proliferate.

The data presented thus far suggests that elevating *hipk* promotes proliferation, cell migration and possibly metastatic behavior. To test for this, we utilized live imaging and witnessed cell extrusion in real time. In *dpp>HA-hipk^{IM}+HA-hipk^{SM}* eye discs, cells proliferate at a high rate, and multiple cells could be seen extruding from the disc into the culture medium (Fig. S3D-F). Furthermore, the extruded cells continued to proliferate after leaving the disc. Within 60 minutes of imaging this particular disc, 12 cells left the disc into the
culture media (Fig. S3F,G) and some continued amplifying to reach a final cell count of 21 over the following 60 minutes (Fig. S3H-J). We did not observe any such occurrence in control discs (Fig. S3B-C). Together, these data suggest that cells with elevated Hipk can gain the potential to travel away from their original location in the epithelium.

**Hipk alters the integrity of the basement membrane and induces EMT**

During metastasis, cells extrude from the main epithelium through the use of various mechanisms, including degradation of the basement membrane by matrix metalloproteinases such as MMP1 (Beaucher et al., 2007; Page-McCaw et al., 2003; Srivastava et al., 2007). Expression of Hipk using either dpp-Gal4 or flip out misexpression clones leads to elevated MMP1 expression in a cell autonomous manner (Fig. 4A,B, Fig. S2B). Hipk was previously shown to induce Mmp1 expression, but only when the smt3 gene encoding Small ubiquitin-related modifier (SUMO) was simultaneously knocked down (Huang et al., 2011b). Huang et al. (2011b) suggested that in the absence of smt3 Hipk translocates to the cytoplasm and induces JNK and its target MMP1. In our experimental context, when HA-hipk3M and GFP were expressed by dpp-GAL4 at 29°C Hipk is largely nuclear (Fig. S4A), suggesting that MMP1 induction in our assay is likely due to another mechanism.

We examined the basement membrane by staining wing imaginal discs for Nidogen (Ndg), an extracellular matrix component (Fig. 4A-F) (Wolfstetter et al., 2009). In dpp>HA-hipk3M+GFP discs, disruptions in the Ndg pattern were observed in sections of wing discs (Fig. 4B) and when the basal surface was examined en face (Fig. 4F). Specifically, the location of HA-hipk3M+GFP positive cells and expression of MMP1 coincided with disruptions in Ndg (Fig. 4B). GFP-positive Hipk expressing cells appeared to be extruding through holes in the basement membrane (Fig. 4F). Cross sections of discs also revealed that hipk-expressing cells appeared to be intercalated into the basement membrane (Fig. S4K) which is never seen in wild type discs (Fig. S4I). Consistent with disruptions in Ndg, elevated hipk produced inconsistencies in Collagen IV (Viking, Vkg) in the wing disc (Fig. S4B,C). These data suggest that Hipk promotes MMP1-mediated degradation of the basement membrane.

Co-expression of P35 with Hipk caused greater abnormalities in disc morphology, with multiple folds and cell layers, due to the blocking of cell death (Fig. 4C). In this context, GFP+ cells were observed breaking through the disc surface at both the apical and basal surfaces (Fig. 4C'), suggestive of active migration processes rather than cell death being the mechanism driving cell migration and spreading. Cell autonomous alterations in MMP1 and Ndg (Fig. 4C) were seen in disc sections, and elevated MMP1 was seen within protrusions of
**Eve > HA-hipk^{3M} + P35 + GFP** eye discs (Fig. S4E'). P35 expression alone is also capable of inducing MMP1 but no defects in Ndg integrity were observed (Fig. 4D')(Rudrapatna et al., 2013).

We also observed abnormal cell behavior after staining eye discs to detect Dlg and Elav to reveal tissue architecture. In a wild type disc, the posterior margin of the disc displays a tight boundary of photoreceptor cells as detected by Dlg (Fig. 4G) and Elav (Fig. S4F). In ey\textgreater HA-hipk^{1M}+HA-hipk^{3M} discs, the cell morphology is altered and jagged cell extensions can be seen protruding towards the posterior margin using the Dlg antibody stain (Fig. 4H). Overall altered integrity of the posterior margin is also seen when staining for Elav (Fig. S4G).

EMT occurs naturally in development (Kiger et al., 2007), but can also be inappropriately induced during tumorigenesis. Characteristics of EMT are increased expression of MMP1, mesenchymal markers like Twist (Twi) and Snail, and downregulation of E-cadherin (E-cad). In dpp\textgreater HA-hipk^{3M} + GFP discs grown at 29^\circ C, levels of E-cad are reduced in a cell autonomous fashion (Fig. 4J). Twist is normally expressed within mesenchymal cells found within the notum region of the wing disc (Herranz et al., 2014) (Fig. 4K). When hipk was overexpressed, Twi expression was mildly induced along the dpp domain, and multiple cells within the wing pouch displayed ectopic expression of Twi (Fig. 4L). Others have shown that Hipk promotes epithelial remodeling of the pupal wing through an EMT-like mechanism (Kiger et al., 2007; Link et al., 2007).

**Hipk can induce proliferation and cell migration in vertebrate cells**

To determine if the properties of Drosophila Hipk are conserved in a different context, we examined the effects of transient transfection of pCMV-HA-dHipk into a number of human cell lines. We first assayed proliferation induced by Hipk in HEK293T, MCF7 and MDA-MB-231 human cell lines. Following transient transfection, we used the MMT assay to measure cell proliferation and found that Hipk significantly stimulated cell proliferation in all three cell lines (Fig. 4M). Using a cell migration assay, we found that transfection of Drosophila Hipk caused MDA-MB-231 cells to exhibit a 2-fold increase in migration relative to control cells (Fig. 4N). One critical aspect of EMT is the downregulation of E-cadherin expression. In MDA-MB-231 cells transfected with Hipk, E-cad levels were reduced 3.5-fold compared to levels found in control transfected cells (Fig. 4O). Further evidence for decreased E-cad is seen following Western blotting of E-cad from HEK293T cells transfected with dHipk (Fig.
These observations support our observations from Drosophila tissues that Hipk is a potent factor that can promote proliferation and EMT in different contexts.

**Hipk-induced phenotypes cannot be attributed to a single targeted pathway**

To genetically investigate the mechanism underlying Hipk’s ability to induce cell spreading, proliferation, and migration, we assessed the effects of disruptions of individual pathways on the dpp>HA-hipk\(^{3M}\) phenotype in wing discs by assaying the extent of cell proliferation and migration from the dpp expression domain (Fig. 5A). We chose pathways that were previously shown to be promoted by Hipk in various contexts, as well as conserved tumor pathways. We evaluated the effects on proliferation and migration of the transgenes individually using dpp-Gal4 (Fig. S5). We also validated that each transgene was effective by assaying targets or downstream events specific to each pathway (Fig. S6). To test whether the Hipk-phenotype could be reverted, we first used RNAi against hipk and found that it completely rescued the abnormalities seen in dpp>HA-hipk\(^{3M}\) discs (Fig. 5B). The proliferative and invasive effects caused by each transgene and their influence on the Hipk-induced phenotype were quantified by measuring the GFP positive cell area relative to total disc area (Fig. S7A), and by assigning a ‘relative degree of invasiveness’ score to each disc (Fig. S7B).

The Wg pathway was inhibited through either knockdown of pangolin/TCF (Fig. 5C) or expression of the negative regulator Axin (Fig. 5D). We noticed that hipk-expressing discs with loss of pan (TCF) still displayed the invasive phenotype and even some overgrowth in the notum region. Similarly, expression of Axin failed to suppress the dpp>HA-hipk\(^{3M}\) phenotype. Wing discs co-expressing dominant negative EGF receptor (EGFR\(^{DN}\); Fig. 5E) or dominant negative basket (bsk\(^{DN}\), Drosophila JNK; Fig. 5F) with hipk were phenotypically indistinguishable from discs expressing hipk alone (dpp>HA-hipk\(^{3M}\)). Knockdown of yki could reduce the overgrowth effect to some degree, consistent with the effect of Hipk on Hippo signaling, but the discs still showed ectopic cell migration (Fig. 5G, G'). Expression of dominant negative Delta (Di\(^{DN}\); Fig. 5H) did not appreciably modify the Hipk overexpression phenotype. Interestingly, following the inhibition of the Hedgehog pathway through expression of the repressor form of Ci (Ci\(^{Rep}\); Fig. 5I), the cell spreading phenotype seemed suppressed and the discs only displayed a broad Dpp domain. We also noticed relatively weak GFP expression in the discs, which is most likely due to the repression of dpp-Gal4 expression, since Hh controls dpp transcription (Basler and Struhl, 1994). Reduction of JAK/STAT signaling through knockdown of hopscotch (hop; Drosophila JAK; Fig. 5J) showed
mild reduction of proliferation while knockdown of one of the unpaired ligands, Upd3, appeared to slightly increase proliferation (Fig. 5K). Together, our genetic data show that interfering with individual signaling pathways using expression of RNAi or dominant negative forms of the corresponding key effectors could not effectively suppress Hipk-induced cell proliferation and spreading.

To test whether the effects are due to multiple pathways, we simultaneously interfered with the activity of Yki and Bsk by expressing yki RNAi with bsk^{DN} in a dpp>HA-hipk^{3M} background (Fig. 5M). While expression of Hipk induces overproliferation of the dpp-expressing cells, which can be seen by staining for Hipk in dpp>HA-hipk^{3M}, following inhibition of Yki and Bsk, the number of Hipk-expressing cells in the dpp domain is drastically reduced, and no cell spreading is observed. Expression of dpp>yki^{RNAi} with bsk^{DN} alone had a mild effect on the number of GFP positive cells in the dpp stripe (Fig. 5N).

**Increasing the activity of individual signaling pathways does not phenocopy Hipk-induced phenotypes**

We next examined whether hyperactivation of pathways that are promoted by Hipk, or that are involved in growth and proliferation, can induce similar phenotypes as those caused by overexpression of Hipk (Fig. 6A). This might reveal if there are certain pathways that play a more dominant role in propagating the Hipk signal. We used UAS-controlled expression of wild type or constitutively active pathway members (Table 1). Wing discs expressing degradation resistant Arm^{S10} (β-catenin) to promote Wg signaling (Fig. 6B) displayed ectopic wing pouch-like structure in the notum, yet the Dpp stripe appeared relatively normal. Overexpression of Stat92E to elevate JAK/STAT signaling (Fig. 6C) led to oversized discs. Wing discs expressing oncogenic Ras to promote Ras/Erk signaling showed robust overgrowths (Fig. 6D). Stimulation of the JNK pathway using eiger expression primarily caused invasive phenotypes but had little effect on proliferation (Fig. 6E). Inactivation of Hippo signaling by expression of constitutively active Yki (Yki^{S168A}) led to widening of the Dpp domain, and smooth, curved edges along the domain (Fig. 6F). Activated Notch signaling (N^{act}) (Fig. 6G) and ectopic Ci (Fig. 6H), which promotes Hh, both induced very dramatic and unique cellular effects. dpp>Notch^{act} led to phenotypes in the wing disc similar to those previously seen with expression of dpp>Dl (Ferre-Marcos et al., 2006a). Together, this assay reveals that activation of different pathways leads to distinct effects on proliferation. These results suggest that Hipk-induced phenotypes likely arise as a cumulative effect of stimulating activity of multiple pathways, since no single pathway can
phenocopy the behaviour of cells in discs with elevated Hipk in the dpp domain. The proliferative and invasive effects caused by each transgene were quantified by measuring the GFP positive cell area relative to total disc area (Fig. S7C), and by assigning a ‘relative degree of invasiveness’ score to each disc (Fig. S7D).

**Htpk overexpression synergistically enhances other tumor models**

Finally, we assessed whether Htpk expression could synergize with other sensitized tumor models in Drosophila wing discs. We co-expressed Htpk with the same gain of function mutants used in the previous section (Table 1) and assayed proliferation and cell migration. The phenotype of dpp>Arm\textsuperscript{10} alone was enhanced upon co-expressing HA-hipk\textsuperscript{3M}, notably the effect was much more pronounced in the notum region of the disc (Fig. 7B). Co-expression of stat92E and hipk resulted in invasive phenotypes (Fig. 7C) whereas discs expressing stat92E alone did not (Fig. 6C). In stark contrast to the phenotype in eiger-expressing discs (Fig. 6E), Htpk cooperated with Eiger to cause a significant increase in migrating cells (Fig. 7E). Despite being smaller than dpp>yki discs, dpp>hipk+yki discs acquired noticeable cell spreading properties (Fig. 7F). Ras\textsuperscript{act} (Fig. 7D) and Notch\textsuperscript{act} (Fig. 7G) both showed a strong synergistic effect with ectopic Htpk, compared to phenotypes seen with either one alone, shown in Fig. 6. The strongest synergy was seen with Ci (Fig. 7H). Of note, the effect with Ci alone was also the most dramatic under these assay conditions. The effects of all transgenes on Hipk-induced phenotypes were quantified by measuring the GFP positive cell area relative to total disc area (Fig. S7E) and by quantifying relative degree of invasiveness (Fig. S7F). Thus Hipk expression can synergize with several well-described Drosophila tumor and metastasis models, supporting its oncogenic properties.

**Discussion:**

Accumulating evidence has strongly indicated that mammalian HIPKs are implicated in various diseases and cancers (reviewed in Blaquiere and Verheyen, 2016). However, whether HIPKs act as oncogenes or tumor suppressor genes remain ambiguous, possibly in part due to the genetic heterogeneity of different cancer types. In addition, comprehensive analyses of the four HIPK isoforms are lacking. Given the diverse expression patterns, distinct subcellular localization, and potential functional redundancy of HIPK proteins, considerable efforts are needed to identify the roles of individual isoforms in each cell context, not to mention under unstressed or stressed conditions (for example, UV induction
or hypoxia) (Schmitz et al., 2014). In light of these complications, we decided to use *Drosophila*, a simple genetic model organism containing only one, but a well-conserved Hipk, in most of our studies to unravel the roles of Hipk proteins in tumorigenesis.

Our work reveals that elevated expression of a single gene, *hipk*, in Drosophila tissues is sufficient to produce features of transformed tumors. We provide evidence that Hipk induces hyperplasia in imaginal discs and hemocytes, leading to massive tissue growth and melanotic tumor-like masses respectively (Figs. 1, 2). Importantly, cells with elevated Hipk display protruding cell shapes and gain the potential to spread away from their primary site of origin (Figs. 3, 4). We further provide evidence that Hipk induces basal invasion through mechanisms such as MMP1-mediated degradation of the basement membrane, and induction of EMT (Fig. 4). We also demonstrate that expression of *Drosophila* Hipk in the human aggressive breast cancer line MDA-MB-231 can potentiate proliferation, migratory behaviors and by extension, EMT (Fig. 4).

We speculated that Hipk might trigger EMT in *Drosophila* tissues and vertebrate cells through conserved molecular mechanisms. Our studies uncover previously unrecognized functions of *Drosophila* Hipk in mediating metastasis. Our conclusions are in agreement with some studies reporting that human HIPK2 promotes EMT in renal fibrosis (Jin et al., 2012, Huang et al. 2015). HIPK2 expression has been shown to be remarkably upregulated in kidneys of patients with HIV-associated nephropathy, diabetic nephropathy, and severe IgA nephropathy (Huang et al. 2015). Moreover, certain human cancers display elevated levels of HIPK2 within tumorous tissue (Al-Beiti and Lu, 2008; Deshmukh et al., 2008b; Jacob et al., 2009b). We infer that *Drosophila* Hipk mimics human HIPK2 in these fibrosis and tumor models. In contrast, another study found that in bladder cancer metastasis downregulation of HIPK2 induced EMT and cell invasion (Tan et al. 2014). What causes the switch of roles of HIPKs between EMT-promoting and EMT-suppressing needs further investigation.

To elucidate the molecular mechanism of how Hipk can confer both proliferative and migratory properties on cells, we examined genetic interactions between Hipk and tumorigenic pathways that are known, or proposed, to be regulated by Hipk. First, we noticed that interfering with individual signaling pathway activity is not sufficient to suppress both Hipk-mediated cell spreading and invasive phenotypes (Fig. 5). Second, stimulation of single pathways fails to recapitulate all the phenotypes induced by Hipk overexpression (Fig. 6). Of note, we do find that inhibition of individual pathways can suppress a subset of Hipk-induced phenotypes. For example, knockdown of Yki in a Hipk overexpression background inhibited cell proliferation, but did not have a strong effect on cell spreading. Conversely, inhibition of
Hedgehog signaling did not affect proliferation, but appeared to reduce cell spreading. We were able to observe that the combined inactivation of Yki and Bsk were able to ameliorate most Hipk phenotypes. We propose that elevation of a single protein kinase, Hipk, even without accumulation of additional mutations, is likely potent enough to perturb multiple signaling pathways and ultimately cause a cumulative effect of oversized, proliferative and protruding phenotypes. This mechanism, in effect, mimics tumor initiation due to multiple activating mutations in distinct pathways. Previously described *Drosophila* tumor models involve concomitant mutations that enhance proliferation such as activated Ras, along with loss of cell polarity genes such as *scribble*, to drive invasive behavior (Pagliarini and Xu, 2003).

Consistent with our proposed mechanism, HIPK2 is thought to mediate EMT through activating EMT-promoting pathways including TGFβ, Wnt and Notch (Huang et al. 2015). We believe that, in the future, profiling the transcriptome, the protein/protein interactions, protein/DNA interactions in Hipk-expressing cells will give us an unbiased and thorough analysis of alterations of signaling network upon Hipk overexpression.

The versatility of Hipk functions raises concerns of how we can block Hipk-induced phenotypes efficiently. Although inhibiting multiple downstream effectors of Hipk may be an option, we notice that impeding Hipk expression through *hipk-RNAi* can strongly reverse the overgrowth and cell spreading phenotypes (Fig. 5B). In line with our suggestion, a previous study proposed that exogenous overexpression of *miR-141*, which targets the 3'UTR of HIPK2, represented a potential approach to hinder HIPK2-mediated EMT (Jin et al., 2012). Given the large roles of post-translational modifications in Hipk protein turnover and localization (reviewed in (Saul and Schmitz, 2013), we consider that mutations in other genes encoding Hipk regulators may also contribute to tumorigenesis even in the absence of Hipk gene mutations or changes in transcription levels of Hipk. Thus, revealing the regulation of Hipk activity is critical to avoid Hipk-induced deleterious effects and to develop promising therapeutic interventions for Hipk-related disorders.

Lastly, we notice that Hipk is able to cooperate with other sensitized tumor models, probably in both additive and synergistic manners (Fig. 7). This implies that Hipk itself can elicit tumor-like transformations during the early phases of tumorigenesis. At the later phases when multiple genetic alterations occur, Hipk might play an important role in accelerating tumor progression and metastasis. Future research would need to validate whether the human counterparts, HIPK1-4 can play roles in cancer initiation and progression in specific cancer types, and whether the functions of HIPK isoforms are redundant or disparate.
Acknowledgements:
We are grateful to A. Hölz, M. Leptin, M. Miura, N. Perrimon, H. Richardson, G. Tanentzapf, the Developmental Studies Hybridoma Bank, The Vienna Drosophila Resource Centre, the Bloomington Drosophila Stock Center, and Jackson Immunoresearch Laboratories Inc. for providing fly strains and antibodies. We thank J. Parker, A. Kadhim and E. Hall for experimental help. This work was funded by operating grants from the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada.

Competing interests: The authors declare that they have no competing interests.

Materials and Methods:

Genetic crosses and fly stocks: Flies were raised on standard media and w^1118 was used as wild type. A commonly used assay for proliferation and cell invasion is the use of ey-Flp to induce clones of tumor suppressors or expression of oncogenes in the eye-antennal disc (Pagliarini and Xu, 2003). We could not use this assay due to the inhibition of early eye specification mediated by Hipk (Blaquiere et al., 2014). All crosses were raised at 29°C to increase the effectiveness of GAL4-driven UAS constructs unless otherwise noted. All genetic interaction studies included controls for GAL4 titration through the use of benign UAS lines such as UAS-GFP, UAS-RFP or UAS-lacZ to match the UAS construct dose in experimental crosses. Fly strains used in study: (1) ;dpp-GAL4/TM6B (Staehling-Hampton et al., 1994), (2) ;vgk-GFP; (Flytrap), (3) ;UAS-HA-hipk^1M; and (4) ;UAS-HA-hipk^3M which are both wild type Hipk transgenes inserted on different chromosomes, which were previously reported as UAS-Hipk (II) and UAS-Hipk (III), respectively (Swarup and Verheyen, 2011), (5) ;dpp-GAL4, UAS-HA-hipk^3M/TM6B [recombinant derived from stocks (1) and (4)], (6) ;UAS-eGFP; (BL#5431), (7) ;UAS-eGFP (BL#5430), (8) ;UAS-P35; (BL#5072) (Hay et al., 1994), (9) ;hml-GAL4; (BL#30139), (10) ;ey-GAL4; (BL#5535), (11) ;UAS-Axin-GFP (BL#7225), (12) UAS-EGF^DN (dominant negative) with inserts on both II and III, (13) UAS-bsk^DN; (BL# 6409), (14) ;UAS-ykRNAi (BL#34067), (15) ;UAS-D^DN; (BL#26697), (16) UAS-C^Rep, (17) ;UAS-arm^10; generated in our lab (Mirkovic et al., 2011), (18) ;UAS-Stat92E; (19) UAS-Ras^act (from H. Richardson), (20) ;UAS-Eiger, (21) ;UAS-yki^S168A::GFP; , (22) ;UAS-N^act, (23) ;UAS-Ci^SM.; Strains obtained from the Bloomington Drosophila Stock Center, Bloomington, IN, have BL# stock numbers indicated. RNAi lines were primarily obtained from the Vienna
Antibodies and microscopy: Third instar imaginal discs were dissected and stained using standard protocols, and in most cases we analyzed equal to or greater than 20 discs per genotype. The following primary antibodies were used: mouse anti-MMP1 (1:100; 3A6B4, 3B8D12, 5H7B11 DSHB; Rubin, G.M.), rat anti-Ci (1:20; 2A1 DSHB; Holmgren, R.), mouse anti-En (1:10; 4D9 DSHB; Goodman, C.), mouse anti-Dlg (1:100; 4F3 DSHB; Goodman, C.), mouse anti-HA (1:200 ABM), rabbit anti-Cas3 (1:100; 9661S Cell Signaling), rabbit anti-Ndg (1:500; gift of Anne Hölz; (Wolfstetter et al., 2009)), rabbit anti-Twi (1:3000; gift of Maria Leptin (Roth et al., 1989)), mouse anti-beta-Galactosidase (1:50, 40-1a DSHB; Sanes, J.R.), rabbit anti-CycE (1:100, d-300 Santa Cruz), mouse anti-Wg (1:40, 4D4 DSHB; Cohen, S.M.), mouse anti-Cut (1:50, 2B10 DSHB; Rubin, G.M.), mouse anti-Ptc (1:40, Apa1 DSHB; Guerrero, I.). Rabbit anti-Hipk antibodies were generated in our lab and used at 1:200 dilution. The following secondary antibodies were obtained from Jackson Immunoresearch: DyLight649 anti-rabbit, DyLight649 anti-rat, Cy3 anti-mouse, and Cy3 anti-rabbit. Nuclei were detected by staining with Dapi, and F-actin was detected by staining with Rhodamine phalloidin (R-415 Molecular Probes). Immunofluorescent images were acquired using a Nikon Air laser-scanning confocal microscope. For live imaging, dissected eye discs were placed on a depression slide containing insect media and 2 larval brains. Discs were imaged using differential interference contrast microscopy (DIC) once per minute over two hours (n=5 for each genotype). Whole larvae were mounted in Hoyers media, allowed to sit for 2 minutes, and imaged with a Canon Rebel T1i. Images were processed with Nikon Elements, Adobe Photoshop, Adobe Illustrator, ImageJ and Helicon Focus. For a subset of fluorescent images channel colours were converted to accommodate colour-blind viewers.

Hemocyte counts: Prior to hemolymph collection, third instar larvae (hml>GFP+LacZ and hml>HA-hipk3M+GFP) grown at 29°C were washed thoroughly with 1X PBS, dried, and placed in glass dissection wells containing 10μL of 1X PBS. The cuticle of single larvae was carefully punctured ventrally with forceps and hemolymph was allowed to drain into the well for 30-60 seconds. The hemolymph was mixed with a pre-wetted pipet, and 1.5μl of the hemolymph mixture was transferred to a poly-d-lysine treated 8-well chamber-slide (BD Disease Models & Mechanisms • DMM • Accepted manuscript)
Falcon CultureSlides, Product #354108). Five 1.5µl droplets were plated per larva, after which they were air-dried. The dried samples were washed with 4% formaldehyde for five minutes, washed with PBS, and stained with DAPI. For each sample (n=16), five cell counts were performed from images taken at the center of each droplet at 200X magnification, and means of the five cell counts were plotted; values were subjected to an unpaired two-tailed t-test.

**Cell culture**: MDA-MB-231 (ATCC, CRM-HTB-26), MCF7 (ATCC, HTB-22), and HEK293T (ATCC, CRL-3216) cell lines were grown in DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Gibco, Cat: 11330032) with 10% FBS (fetal bovine serum).

**Cell transfection**: MDA-MB-231 cells were transiently transfected with pCMV-myc empty vector (control) and pCMV-HA-dHipk vector using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instruction. 2 µg plasmid was used for each well in a 6-well plate.

**Cell proliferation**: Cell counts in transfected MDA-MB-231 cells were determined using an MTT assay. MDA-MB-231 cells were seeded into 6-well plates (VWR, Cat# 10062-892) on day one to allow cells to attach, and transfections were performed on day two. On day three, 5000 cells were seeded into a 96-well plate, and on day five, 10µl 12mM MTT stock solution was added into 100µl medium in each well and incubated at 37°C for three hours. Culture medium was then removed and 100µl DMSO was added to each well and incubated for ten minutes at 37°C. We then read the OD at 540nm. Three replicates were performed for each condition in triplicate. Values were calculated as the mean with standard error of the mean. Significance between samples was assessed using unpaired two-tailed t-tests.

**Migration assay**: MDA-MB-231 cells were seeded at 80% confluence into 6-well plates for 24 hours and then transfected with pCMV-Myc empty vector or pCMV-HA-dHipk for six hours, after which the medium was changed to starving medium (DMEM/F-12 without FBS) for 24 hours. Then transfected cells were trypsinized (0.25% Trypsin-EDTA, Gibco) and counted using Trypan blue, and 20,000 cells were suspended in 200 µl serum free DMEM/F-12, seeded into the upper chamber of each insert (24 well insert, pore size is 8µm, Greiner Bio-one). 800µl DMED/F-12 containing 50% FBS was added to the bottom wells. After 24 hours at 37°C, the culture medium was replaced with 450µl serum free medium with plus 8µm calcein-AM, incubated for 45 mins at 37°C and then 500µl trypsin was used to release the cells which had migrated through the membrane, incubating for ten minutes. 200µl
trypsin solution with detached migrated cells was transferred into a black flat bottom 96 well plate and fluorescence was measured with an excitation wavelength of 485nm and an emission wavelength of 520nm. Three replicates were performed for each condition in triplicate. Values were calculated as the mean with standard error of the mean. Significance between samples was assessed using an unpaired two-tailed t-test.

**RNA extraction and qPCR:** total RNA was isolated from cells using RNeasy Mini kits (Qiagen Cat: 74101). First strand cDNA was synthesized from 0.5μg RNA by PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Cat: 6110A). qPCR was performed using FastStart SYBR Green Master (Roche, Cat: 04673484001) on StepOne real time PCR (ABI). HPRT was used as a housekeeping gene control. Primers used were: hHPRT forward GCTATAATTTCTTGCTGACCTGCTG; hHPRT reverse AATTACTTTATGTCCCCTGTTGACTGG; hE-Cad (CDH1) forward GGACTTTGGCGTGGGCCAGG; hE-Cad (CDH1) reverse CCTGTCCAGCTCAGCCCGA. Relative fold levels were determined by the 2^(-ΔΔCt) method. Statistical significance was confirmed with a one-sample two-tailed t-test, with a theoretical mean set to 1.

**Western Blot:** Whole cell lysates were prepared with Cell lysis buffer (#9803, Cell Signaling Technology), supplemented with 1X Protease Inhibitors (#04693132001 ROCHE) and 1mM PMSF before use. Protein lysates with 1xSDS sample buffer were subjected to 8% SDS-PAGE, followed by western blotting. The blots were detected by using the Pierce ECL Western Blotting Substrate (#32209). Images were captured with the use of FujiFilm LAS-4000 Chemi-luminescent Scanner. Rabbit anti-E-cadherin (1:1000, #3195 CST) and mouse beta-Actin (1:1000, G043 Abm) were used as primary antibodies. Anti-mouse and anti-rabbit HRP light-chain specific were used as secondary antibodies at 1:5000 (Jackson ImmunoResearch).

**Imaginal disc size measurements and invasiveness scoring:** Experimental sets from Figs. 5 – 7 were quantified for two parameters. First, the proliferative effects of each transgene were assessed by measuring the area of the GFP positive cells (driven by dpp-GAL4) and dividing it by the total disc area. Area measurements were taken in Image J from the .nd2 file for each disc. Measurements were calculated as ratios of the dpp stripe area to the total disc area (dpp/total) (Fig. S7, Tables 5 and 7). The difference in ratios was then quantified for figures 5 and 7 using one-way ANOVA (Fig S7-5. F(12,136) = 41.88, p<0.0001) (Fig S7-7. F(7,81) = 49.94, p<0.0001), with Holm Sidak’s post hoc test applied for multiple
comparisons to 5A or 7A in Fig. S7-5 or Fig. S7-7 respectively. The scores for Holm Sidak’s tests are depicted in Fig. S7 as “ns” = not significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and **** = $p<0.0001$. Second, the invasive effects caused by each transgene were assessed by assigning a ‘relative degree of invasiveness’ score to each disc. We defined the ‘relative degree of invasiveness’ scores as follows: “None” = no cells found outside the normal dpp>GFP region, “Weak” = a few cells emerging from the dpp>GFP region, not just a widened GFP region due to proliferation, “Moderate” = extensions of cells that have travelled to edges of the hinge region, “Strong” = all of the above and some solitary GFP masses found distinct from the main dpp>GFP region.
References


Deshmukh, H., Yeh, T.H., Yu, J., Sharma, M.K., Perry, A., Leonard, J.R., Watson, M.A.,
reveals frequent HIPK2 amplification and increased expression in pilocytic astrocytomas.
Oncogene 27, 4745–4751.

Doggett, K., Turkel, N., Willoughby, L.F., Ellul, J., Murray, M.J., Richardson, H.E., and
Brumby, A.M. (2015). BTB-zinc finger oncogenes are required for ras and notch-driven

Ferres-MARCO, D., Gutierrez-GARCIA, I., Vallejo, D.M., Bolivar, J., Gutierrez-AVIÑO, F.J., and
Domínguez, M. (2006a). Epigenetic silencers and Notch collaborate to promote malignant

Ferres-MARCO, D., Gutierrez-GARCIA, I., Vallejo, D.M., Bolivar, J., Gutierrez-AVIÑO, F.J., and
Domínguez, M. (2006b). Epigenetic silencers and Notch collaborate to promote malignant

Discs Secrete Insulin-Like Peptide 8 to Mediate Plasticity of Growth and Maturation. Science
(80-. ). 336, 579–582.


(JAK) causes hematopoietic neoplasia and developmental defects. EMBO J. 14, 2857–2865.


cooperation between SOCS family proteins and EGFR identified using a Drosophila epithelial

Mesenchymal Tissues in Tumorigenesis and Imaginal Disc Development. Curr. Biol. 24,
1476–1484.


of TCF3 by Wnt-dependent phosphorylation during vertebrate axis specification. Dev Cell 19,
521–532.

transforming growth factor-beta-induced c-Jun NH(2)-terminal kinase activation and

homeodomain-interacting protein kinase 2 (HIPK2) effector function through dynamic small

Hofmann, T.G., Glas, C., and Bitomsky, N. (2013). HIPK2: A tumour suppressor that controls
DNA damage-induced cell fate and cytokinesis. Bioessays 35, 55–64.


Figure 1. Hipk induces overgrowths in Drosophila imaginal discs. Control eye-antennal (A), wing (B), and leg (C) imaginal discs stained for actin to reveal tissue morphology and GFP to reveal the dpp-Gal4 expression domain. Expression of two copies of wild type HA-hipk (HA-hipk$^{3M}$ and HA-hipk$^{1M}$) within the dpp domain leads to overgrown eye-antennal (D), wing (E), and leg (F) imaginal discs. (G) A control wing disc pouch stained for Casp3. (H) Casp3 is autonomously induced in dpp>HA-hipk$^{3M}$+GFP wing discs. (I) A control wing imaginal disc showing the dpp-GAL4 expression domain marked by GFP. (J) dpp>HA-hipk$^{3M}$+GFP leads to overgrown wing discs. (K) Loss of cell death in dpp>HA-hipk$^{3M}$+GFP+P35 discs worsens hipk induced overgrowths, while (L) dpp>GFP+P35 appears normal. Scale bars equal 50μm.
Figure 2. Hipk induces hemocytes-derived melanotic tumors. (A) A control dpp>GFP third instar larva. (B) Stationary melanized masses are observed in 65% of dpp>HA-
hipk\textsuperscript{3M}+GFP+GFP larvae (blue arrowheads; n=40) and (D) persist when apoptotic cell death is inhibited in dpp>HA-hipk\textsuperscript{3M}+P35+GFP larvae. (C) dpp>P35 larvae show no tumours. (E) The abdomen of a control hml>GFP fly. (F) Melanized tumors are present in hml>HA-hipk\textsuperscript{3M} flies (blue arrowheads). Spermathecae were not counted (magenta arrowhead). Smears of total hemolymph collected from (G) hml>2xGFP and (H) hml>HA-hipk\textsuperscript{3M}+GFP third instar larvae. (I) Quantification of the number of tumors scored from dissected abdomens of flies shown in (E) and (F), n=36 for both groups. (J) Quantification of mean number of hemocytes per defined sampling area counted from genotypes in (G) and (H). Each data point represents the mean of 5 cell counts from one larva, hml>GFP+LacZ (n=16 samples, n=80 cell counts) and hml>HA-hipk\textsuperscript{3M}+GFP (n=16 samples, n=80 cell counts), P<0.0001.
Figure 3: Hipk induces cell spreading. (A,B) A dpp>GFP wing disc stained for GFP, Ci and En. (C) In dpp>HA-hipk$^{3M}$+GFP discs, anterior fated cells are found within the posterior wing compartment (arrowheads). (D) An ectopic eye seen within the thorax of an ey>HA-hipk$^{1M}$+HA-hipk$^{3M}$ fly (arrowhead; frequency 1%, n=100). (D') dissection of this region (white box) reveals the size of the ectopic eye. (E) A smaller ectopic eye is seen in the abdominal region of a dpp>HA-hipk$^{3M}$,GFP fly (frequency 2%, n=100).
Figure 4: Hipk alters epithelial integrity and induces EMT. (A) A control wing disc and a cross section of central region of the disc (A') stained for GFP, MMP1 and Ndg. (B, B') dpp>HA-hipk$^{1M}$+HA-hipk$^{3M}$+LacZ+GFP stained to detect GFP, MMP1 and Ndg. (C, C') dpp>HA-hipk$^{1M}$+HA-hipk$^{3M}$+P35+GFP stained to detect GFP, MMP1 and Ndg. (E) Ndg is expressed in a uniform pattern along the basement membrane. (F) Gaps in Ndg expression are present in dpp>HA-hipk$^{3M}$+GFP discs (F'), and a zoom-in (F'') shows that the location of HA-hipk$^{3M}$+GFP expressing cells coincides with regions where Ndg is perturbed (arrowheads). (G) Wild type eye disc stained for Dlg to reveal cell membranes. (H) ey>HA-hipk$^{1M}$+HA-hipk$^{3M}$ eye disc stained for Dlg showing defects in Dlg stain and apparent cell extensions towards posterior margin of disc (chevrons). Cross-sections of (I) dpp>GFP and (J) dpp>HA-hipk$^{3M}$+GFP expressing cells in the center of the wing pouch, stained for E-cad. (K) Twi is expressed in the adepithelial myoblasts, located in the notum region of the wing disc. (L) Twi-positive mesenchymal cells are present in the wing pouch region of dpp>HA-hipk$^{3M}$+GFP discs (arrowheads), and Twi is induced in a swathe of cells along the dpp domain (asterisk). Boxed-in regions represent areas of corresponding zoom-ins. Scale bars equal 50μm. (M) Drosophila Hipk (dHipk) promotes significant proliferation of three cell lines. dHipk transfected HEK293T cells (M=2.097, SEM=0.02803) display increased proliferation compared to empty vector transfected (M=1.161, SEM=0.01619) conditions; t(4)=28.92, p<0.0001 (****). dHipk transfected breast adenocarcinoma MCF7 cells (M=1.098, SEM=0.01217) display increased proliferation compared to empty vector transfected (M=0.8671, SEM=0.0035) conditions; t(4)=18.25, p<0.0001 (****). dHipk transfected breast adenocarcinoma MDA-MB-231 cells (M=1.067, SEM=0.0037) display increased proliferation compared to empty vector transfected (M=0.6457, SEM=0.0074) conditions; t(4)=50.83, p<0.0001 (****). (N) dHipk transfected MDA-MB-231 cells (M=1.953, SEM=0.2277) demonstrated significantly increased migration compared to empty vector transfected (M=0.9999, SEM=0.0375) conditions; t(19)=4.759, p=0.0001 (**). (O) qRT-PCR was used to quantify the expression of the human E-Cadherin gene (CDH1) following transfection of MDA-MB-231 cells with dHipk (t(2))=34.86, p=0.0008 (**). (P) Western blot analysis of E-cad expression in HEK293T cells following dHipk transfection.
Figure 5: Loss of individual signaling pathway components cannot suppress the Hipk-over-expression phenotype. We assessed the ability of knockdown of the activity of individual pathways to suppress phenotypes induced by overexpressed Hipk, by F-actin staining (magenta) to reveal morphology and GFP (green, white) to indicate cells in which genotypes were manipulated. As proof of concept, hipkRNAi (B) suppressed effects seen in dpp>HA-hipk3M wing discs (A). The following pathways were targeted with the indicated transgenes: Wg, using (C) UAS-panRNAi [dTCF] and (D) UAS-Axin-GFP; EGFR, using (E) UAS-EGFRDN; JNK, using (F) UAS-bskDN; Hippo, using (G) UAS-ykiRNAi; Notch, using (H) UAS-DlDN; Hedgehog, using (I) UAS-CiRep; JAK/STAT using (J) UAS-hopRNAi and (K) UAS-upd3RNAi. (L) dpp>HA-hipk3M wing disc stained for nuclei (DAPI) and Hipk. (M) Expression of dpp>HA-hipk3M + UAS-ykiRNAi + UAS-bskDN stained to reveal Hipk and F-actin. (N) dpp>UAS-ykiRNAi+ UAS-bskDN stained to reveal GFP and F-actin. All crosses were done at 29°C degrees. Scale bars equal 50μm.
Figure 6: Over-expression of individual signaling pathway components does not phenocopy the cell spreading phenotype induced by elevated Hipk. (A) A third instar wing imaginal disc with HA-hipk^{3M}+GFP expressed along the dpp domain serves as the baseline phenotype/control disc. Individual pathway activators were expressed using dpp-Gal4, UAS-GFP, namely: (B) UAS-Arm^{S10}, (C) UAS-Stat92E, (D) UAS-Ras^{act}, (E) UAS-eiger, (F) constitutively active UAS-yki^{S168A}, (G) UAS-N^{act}, (H) UAS-Ci. Discs were stained for F-actin (magenta) to reveal tissue morphology and for GFP (green, white) to mark cells in which transgenes were ectopically expressed using dpp-Gal4. Scale bars equal 50μm. All crosses were done at 29°C degrees.
Figure 7: Hipk overexpression synergistically enhances other tumor models

Individual pathway activators were expressed by crossing to dpp-Gal4, UAS-GFP; UAS-HA-hipk^{3M}/TM6B namely: (A) Gal4 titration control crossed to UAS-RFP, (B) UAS-Arm^{S10}, (C) UAS-Stat92E, (D) UAS-Ras^{act}, (E) UAS-eiger, (F) constitutively active UAS-yki^{S168A}, (G) UAS-N^{act}, (H) UAS-Ci. Discs were stained for F-actin (magenta) to reveal tissue morphology and for GFP (green, white) to mark cells in which transgenes were ectopically expressed using dpp-Gal4. Scale bars equal 50μm. All crosses were done at 29°C degrees.
Figure S1. Hipk regulates numerous signaling pathways. A schematic diagram depicting Hipk’s known relationships with many of the conserved signaling pathways.
Figure S2: Hipk induces cell autonomous expression of Cleaved Caspase 3 and MMP1. 
Actin flp-out clones expressing HA-hipk$^{3M}$ and marked with UAS-RFP were generated in wing imaginal discs. Anti-cleaved Caspase 3 antibody (shown in blue, A, A") and anti-MMP1 staining (shown in green, B, B") reveal low level cell autonomous induction.
**Figure S3: Hipk induces cell spreading.** (A) A zoom-in of a dpp>HA-hipk^{3M}+GFP wing disc (similar to Fig. 3C) stained for Ci and En. Arrowheads mark a cluster of cells that simultaneously express GFP (A'), Ci (A''), and En (A'''). (B) A DIC image of a control eye disc. Live imaging stills of the zoomed-in control disc show that cells do not leave the disc after 60 mins (B', C). (D) At t = 0 mins a ventral overgrowth is present in the dpp>HA-hipk^{1M}+HA-hipk^{3M} eye disc. Zoomed-in images (D', E) reveal that cells leave the disc over a 60-min period (arrowheads). (F-J) At t = 65 mins, 12 extruded cells are next to the disc. Over the next 55 mins, those 12 cells amplify to reach a cell count of 21 (J). Boxed-in regions represent areas of corresponding zoom-ins. Scale bars equal 50µm.
Figure S4: Hipk alters epithelial integrity.

(A) Anti-HA antibody reveals that UAS-HA-hipk\textsuperscript{3M} expression is nuclear (inset shows zoom-in).
(B) A control disc shows the expression pattern of a basement membrane component Collagen IV (called Viking, Vkg, in Drosophila) using Vkg-GFP.
(C) In dpp\textgreater HA-hipk\textsuperscript{3M} wing discs, inconsistencies in the normal Vkg pattern are present in the dpp-Gal4 expression domain. (C') anti-Hipk stain in dpp\textgreater HA-hipk\textsuperscript{3M} reveals that effects on Vkg are cell autonomous. (D-D'') When cell death is blocked in dpp\textgreater HA-hipk\textsuperscript{3M}+P35+GFP wing discs, MMP1 is dramatically up-regulated and Ndg is disrupted (compared to normal pattern seen in Fig. 4C). (E-E''') Zoom in on white box shown in D reveals high levels of MMP1 in GFP positive cells, as well as abnormal Ndg stain indicating disruptions in basement membrane. (F) Control eye disc stained for Elav (blue) and Dlg (red). (F') zoom of posterior region of eye disc. (G) ey\textgreater HA-hipk\textsuperscript{1M}+HA-hipk\textsuperscript{3M} disc stained for Elav to reveal photoreceptors (blue) and Dlg to highlight apical domains (red). (G') zoom of posterior region of eye disc. (H) dpp\textgreater GFP expressing cells in the center of the wing pouch, stained for Dlg to highlight apical domains and Ndg to reveal basement membrane, and GFP to show dpp expression domain. (I) Cross section of tissue shown in Fig. 4H. (J) A dpp\textgreater HA-hipk\textsuperscript{3M}+GFP disc stained for Dlg, Ndg and GFP. (K) A cross section of disc shown in Fig. 4J. Chevrons in K, K'' reveal sites of intercalation of GFP positive cells with basement membrane. Scale bars equal 50\textmu m.
The phenotypes of various UAS lines used in genetic analyses when driven by dpp-GAL4 were determined. Genotypes are indicated in panels. Discs were stained for GFP to reveal cells expressing transgenes and F-actin to reveal disc morphology. The following pathways were targeted with the indicated transgenes: Wg, using (A) UAS-panRNAi [dTCF] and (B) UAS-Axin-GFP; EGFR, using (C) UAS-EGFRDN; JNK, using (D) UAS-bskDN; Hippo, using (E) UAS-ykiRNAi; Notch, using (F) UAS-DlDN; Hedgehog, using (G) UAS-CiRep; JAK/STAT using (H) UAS-hopRNAi and (I) UAS-upd3RNAi.

Figure S5: Phenotypes of signaling pathway disruptors used in study
Figure S6: Validation of signaling pathway disruption by reagents used in study.

To validate that all transgenes used in the study targeted their respective pathways, we examined either phenotypes or changes in target genes following GAL4-driven expression of either GFP or the indicated transgenes. Discs were stained for targets, GFP (to reveal cells expressing transgenes) and F-actin to reveal disc morphology:

(A) dpp>GFP, (B) UAS-panRNAi +GFP [dTCF] and (C) UAS-Axin-GFP +GFP stained for Wg target Dll-lacZ. (D) MS1096-GAL4 control adult wing. (E) MS1096>UAS-EGFR<sup>DN</sup> adult showing typical EGFR loss of function vein loss phenotype. (F) dpp>GFP, (G) UAS-ykiRNAi +GFP stained using antibody for Hippo target gene CycE. (H) Control wing disc stained for JNK target puc-lacZ. (H') Inset shows expression in stalk of disc. (I) dpp>UAS-bsk<sup>DN</sup>+GFP stained to reveal reduced puc-lacZ. (J) dpp>GFP and (K) dpp>UAS-Dl<sup>DN</sup>+GFP stained for the Notch target Wg. (L) dpp>GFP and (M) dpp>UAS-Dl<sup>DN</sup>+GFP stained for the Notch target Cut. (N) dpp>GFP and (O) UAS-CiRep +GFP stained to reveal Hedgehog target Ptc. (P) Control disc, (Q) dpp>UAS-hopRNAi and (R) dpp>UAS-upd3RNAi expressing the 10xStat92EGFP reporter for JAK/STAT signaling, stained for GFP.
Figure S7: Quantification of proliferative areas in discs and degree of invasiveness in Figs. 5-7

Imaginal disc size measurements and invasiveness scoring were performed as described in methods. The genotypes in the following charts are:

Panels A-B
5A  dpp>HA-hipk$^{3M}$, UAS-RFP, UAS-GFP
5B  dpp>HA-hipk$^{3M}$, UAS-hipk$^{RNAi}$, UAS-GFP
5C  dpp>HA-hipk$^{3M}$, UAS-pan$^{RNAi}$, UAS-GFP
5D  dpp>HA-hipk$^{3M}$, UAS-Axin-GFP, UAS-GFP
5E  dpp>HA-hipk$^{3M}$, UAS-EGFR$^{DN}$, UAS-GFP
5F  dpp>HA-hipk$^{3M}$, UAS-Bsk$^{DN}$, UAS-GFP
5G  dpp>HA-hipk$^{3M}$, UAS-yki$^{RNAi}$, UAS-GFP
5H  dpp>HA-hipk$^{3M}$, UAS-D$^{DN}$, UAS-GFP
5I  dpp>HA-hipk$^{3M}$, UAS-Ci$^{Rep}$, UAS-GFP
5J  dpp>HA-hipk$^{3M}$, UAS-hop$^{RNAi}$, UAS-GFP
5K  dpp>HA-hipk$^{3M}$, UAS-upd3$^{RNAi}$, UAS-GFP
5L  dpp>HA-hipk$^{3M}$
5M  dpp>HA-hipk$^{3M}$, UAS-yki$^{RNAi}$, UAS-Bsk$^{DN}$

Panels C-D
6A  dpp>HA-hipk$^{3M}$, UAS-GFP
6B  dpp>UAS-Arm$^{S10}$, UAS-GFP
6C  dpp>UAS-stat92E, UAS-GFP
6D  dpp>UAS-Ras$^{act}$, UAS-GFP
6E  dpp>UAS-eiger, UAS-GFP
6F  dpp>UAS-yki$^{S168A}$-GFP, UAS-GFP
6G  dpp>UAS-N$^{act}$, UAS-GFP
6H  dpp>UAS-Ci, UAS-GFP

Panels E-F
7A  dpp>HA-hipk$^{3M}$, UAS-RFP, UAS-GFP
7B  dpp>HA-hipk$^{3M}$, UAS-Arm$^{S10}$, UAS-GFP
7C  dpp>HA-hipk$^{3M}$, UAS-stat92E, UAS-GFP
7D  dpp>HA-hipk$^{3M}$, UAS-Ras$^{act}$, UAS-GFP
7E  dpp>HA-hipk$^{3M}$, UAS-eiger, UAS-GFP
7F  dpp>HA-hipk$^{3M}$, UAS-yki$^{S168A}$-GFP, UAS-GFP
7G  dpp>HA-hipk$^{3M}$, UAS-N$^{act}$, UAS-GFP
7H  dpp>HA-hipk$^{3M}$, UAS-Ci, UAS-GFP
Table 1

Signaling pathway reagents used in study

<table>
<thead>
<tr>
<th>Pathway name</th>
<th>Loss of function</th>
<th>Gain of function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wingless</td>
<td>UAS-panRNAi (TCF)</td>
<td>UAS-ArmST0</td>
</tr>
<tr>
<td></td>
<td>UAS-Axin</td>
<td></td>
</tr>
<tr>
<td>EGFR/Ras</td>
<td>UAS-EGFRDN</td>
<td>UAS-Rasact</td>
</tr>
<tr>
<td>JNK</td>
<td>UAS-bskDN</td>
<td>UAS-Eiger</td>
</tr>
<tr>
<td>Hippo</td>
<td>UAS-YkiST68A</td>
<td>UAS-ykiRNAi</td>
</tr>
<tr>
<td>Notch</td>
<td>UAS-DeltaDN</td>
<td>UAS-Notchact</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>UAS-Citrhop</td>
<td>UAS-CitySM</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>UAS-hopRNAi</td>
<td>UAS-Stat92E</td>
</tr>
<tr>
<td></td>
<td>UAS-upd3RNAi</td>
<td></td>
</tr>
</tbody>
</table>

See Methods for details on strains
### Supplementary Table 2: Raw data

qPCR Ct, ΔCt, ΔΔCt, and 2^(-ΔΔCt) values for MDA-MB231 cells transfected with either pCMV-myc (control) or pCMV-myc-dHipk (transfected)

<table>
<thead>
<tr>
<th>Test genes</th>
<th>HPRT control</th>
<th>HPRT transfected</th>
<th>dHipk control</th>
<th>dHipk transfected</th>
<th>ECAD control</th>
<th>ECAD transfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate #</td>
<td>Ct</td>
<td>ΔCt</td>
<td>ΔΔCt</td>
<td>2^(-ΔΔCt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20.458896</td>
<td>0.865526835</td>
<td>3.067857742</td>
<td>0.119256703</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>22.92055257</td>
<td>1.199629784</td>
<td>3.777639389</td>
<td>0.744480933</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>23.66424179</td>
<td>0.858194987</td>
<td>5.203167597</td>
<td>0.02714504</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Supplementary Table 3:

Migration RFU data normalized to the average of the control

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>Control</th>
<th>pCMV-myc-dHipk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.097433015</td>
<td>2.138842046</td>
</tr>
<tr>
<td>2</td>
<td>0.977562301</td>
<td>2.333286491</td>
</tr>
<tr>
<td>3</td>
<td>0.924957841</td>
<td>1.624554993</td>
</tr>
<tr>
<td>4</td>
<td>0.895780078</td>
<td>2.839919541</td>
</tr>
<tr>
<td>5</td>
<td>1.039014374</td>
<td>2.683526799</td>
</tr>
<tr>
<td>6</td>
<td>1.065205548</td>
<td>2.445585216</td>
</tr>
<tr>
<td>7</td>
<td>1.0151289</td>
<td>1.031069312</td>
</tr>
<tr>
<td>8</td>
<td>0.817120002</td>
<td>1.148883447</td>
</tr>
<tr>
<td>9</td>
<td>1.167736606</td>
<td>1.331502601</td>
</tr>
<tr>
<td>10</td>
<td>1.149188902</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.096471069</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.754339601</td>
<td></td>
</tr>
</tbody>
</table>
**Supplementary Table 4: Raw data**

Proliferation measured by OD @ 540nm

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>Control</th>
<th>pCMV-myc-dHipk</th>
<th>Control</th>
<th>pCMV-myc-dHipk</th>
<th>Control</th>
<th>pCMV-myc-dHipk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.1751</td>
<td>2.0414</td>
<td>0.8615</td>
<td>1.074</td>
<td>0.6322</td>
<td>1.0617</td>
</tr>
<tr>
<td>2</td>
<td>1.179</td>
<td>2.1293</td>
<td>0.8662</td>
<td>1.1075</td>
<td>0.6578</td>
<td>1.0742</td>
</tr>
<tr>
<td>3</td>
<td>1.1286</td>
<td>2.1211</td>
<td>0.8735</td>
<td>1.1129</td>
<td>0.647</td>
<td>1.0655</td>
</tr>
</tbody>
</table>

**Supplementary Table 5: Raw data**

*dpp* Stripe Area / Disc Area Ratio Data for Figure 5

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>5A</th>
<th>5B</th>
<th>5C</th>
<th>5D</th>
<th>5E</th>
<th>5F</th>
<th>5G</th>
<th>5H</th>
<th>5I</th>
<th>5J</th>
<th>5K</th>
<th>5L</th>
<th>5M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.282</td>
<td>0.289</td>
<td>0.294</td>
<td>0.321</td>
<td>0.250</td>
<td>0.336</td>
<td>0.176</td>
<td>0.248</td>
<td>0.270</td>
<td>0.239</td>
<td>0.418</td>
<td>0.243</td>
<td>0.100</td>
</tr>
<tr>
<td>2</td>
<td>0.357</td>
<td>0.209</td>
<td>0.355</td>
<td>0.250</td>
<td>0.238</td>
<td>0.352</td>
<td>0.191</td>
<td>0.325</td>
<td>0.324</td>
<td>0.263</td>
<td>0.335</td>
<td>0.332</td>
<td>0.113</td>
</tr>
<tr>
<td>3</td>
<td>0.241</td>
<td>0.214</td>
<td>0.377</td>
<td>0.315</td>
<td>0.316</td>
<td>0.305</td>
<td>0.221</td>
<td>0.292</td>
<td>0.266</td>
<td>0.301</td>
<td>0.358</td>
<td>0.262</td>
<td>0.141</td>
</tr>
<tr>
<td>4</td>
<td>0.277</td>
<td>0.169</td>
<td>0.391</td>
<td>0.314</td>
<td>0.233</td>
<td>0.349</td>
<td>0.162</td>
<td>0.229</td>
<td>0.300</td>
<td>0.281</td>
<td>0.430</td>
<td>0.236</td>
<td>0.111</td>
</tr>
<tr>
<td>5</td>
<td>0.303</td>
<td>0.218</td>
<td>0.334</td>
<td>0.282</td>
<td>0.291</td>
<td>0.384</td>
<td>0.176</td>
<td>0.317</td>
<td>0.307</td>
<td>0.310</td>
<td>0.369</td>
<td>0.307</td>
<td>0.135</td>
</tr>
<tr>
<td>6</td>
<td>0.235</td>
<td>0.206</td>
<td>0.413</td>
<td>0.158</td>
<td>0.235</td>
<td>0.386</td>
<td>0.182</td>
<td>0.213</td>
<td>0.301</td>
<td>0.333</td>
<td>0.394</td>
<td>0.226</td>
<td>0.127</td>
</tr>
<tr>
<td>7</td>
<td>0.306</td>
<td>0.339</td>
<td>0.235</td>
<td>0.297</td>
<td>0.336</td>
<td>0.220</td>
<td>0.307</td>
<td>0.313</td>
<td>0.297</td>
<td>0.341</td>
<td>0.301</td>
<td>0.155</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.276</td>
<td>0.407</td>
<td>0.241</td>
<td>0.258</td>
<td>0.334</td>
<td>0.195</td>
<td>0.303</td>
<td>0.350</td>
<td>0.329</td>
<td>0.238</td>
<td>0.184</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.297</td>
<td>0.392</td>
<td>0.222</td>
<td>0.318</td>
<td>0.389</td>
<td>0.225</td>
<td>0.265</td>
<td>0.296</td>
<td>0.400</td>
<td>0.275</td>
<td>0.167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.330</td>
<td>0.386</td>
<td>0.303</td>
<td>0.255</td>
<td>0.278</td>
<td>0.220</td>
<td>0.298</td>
<td>0.322</td>
<td>0.427</td>
<td>0.257</td>
<td>0.087</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.294</td>
<td>0.347</td>
<td>0.162</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.236</td>
<td>0.353</td>
<td>0.223</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.274</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.218</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.190</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.147</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.209</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Supplementary Table 6: Raw data

*dpp* Stripe Area / Disc Area Ratio Data for Figure 6

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>6A</th>
<th>6B</th>
<th>6C</th>
<th>6D</th>
<th>6E</th>
<th>6F</th>
<th>6G</th>
<th>6H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.388</td>
<td>0.229</td>
<td>0.445</td>
<td>0.711</td>
<td>0.213</td>
<td>0.569</td>
<td>0.531</td>
<td>0.622</td>
</tr>
<tr>
<td>2</td>
<td>0.389</td>
<td>0.302</td>
<td>0.395</td>
<td>0.688</td>
<td>0.249</td>
<td>0.557</td>
<td>0.635</td>
<td>0.605</td>
</tr>
<tr>
<td>3</td>
<td>0.308</td>
<td>0.250</td>
<td>0.287</td>
<td>0.544</td>
<td>0.208</td>
<td>0.464</td>
<td>0.546</td>
<td>0.713</td>
</tr>
<tr>
<td>4</td>
<td>0.366</td>
<td>0.225</td>
<td>0.295</td>
<td>0.789</td>
<td>0.226</td>
<td>0.449</td>
<td>0.546</td>
<td>0.681</td>
</tr>
<tr>
<td>5</td>
<td>0.231</td>
<td>0.295</td>
<td>0.427</td>
<td>0.287</td>
<td>0.511</td>
<td>0.530</td>
<td>0.653</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.234</td>
<td>0.315</td>
<td>0.466</td>
<td>0.313</td>
<td>0.558</td>
<td>0.642</td>
<td>0.584</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.186</td>
<td>0.271</td>
<td>0.441</td>
<td>0.246</td>
<td>0.581</td>
<td>0.605</td>
<td>0.734</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.334</td>
<td>0.262</td>
<td>0.438</td>
<td>0.234</td>
<td>0.608</td>
<td>0.668</td>
<td>0.641</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.369</td>
<td>0.395</td>
<td>0.381</td>
<td>0.291</td>
<td>0.657</td>
<td>0.503</td>
<td>0.521</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.230</td>
<td>0.375</td>
<td>0.592</td>
<td></td>
<td>0.500</td>
<td>0.614</td>
<td>0.593</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.230</td>
<td>0.303</td>
<td>0.373</td>
<td></td>
<td>0.490</td>
<td>0.554</td>
<td>0.801</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.301</td>
<td>0.291</td>
<td>0.345</td>
<td></td>
<td>0.527</td>
<td>0.615</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.397</td>
<td>0.328</td>
<td>0.412</td>
<td></td>
<td></td>
<td></td>
<td>0.597</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.361</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.241</td>
<td></td>
</tr>
</tbody>
</table>

### Supplementary Table 7: Raw data

*dpp* Stripe Area / Disc Area Ratio Data for Figure 7

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>7A</th>
<th>7B</th>
<th>7C</th>
<th>7D</th>
<th>7E</th>
<th>7F</th>
<th>7G</th>
<th>7H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.282</td>
<td>0.506</td>
<td>0.552</td>
<td>0.769</td>
<td>0.324</td>
<td>0.577</td>
<td>0.722</td>
<td>0.671</td>
</tr>
<tr>
<td>2</td>
<td>0.357</td>
<td>0.543</td>
<td>0.474</td>
<td>0.854</td>
<td>0.403</td>
<td>0.652</td>
<td>0.771</td>
<td>0.838</td>
</tr>
<tr>
<td>3</td>
<td>0.241</td>
<td>0.649</td>
<td>0.670</td>
<td>0.761</td>
<td>0.379</td>
<td>0.610</td>
<td>0.850</td>
<td>0.818</td>
</tr>
<tr>
<td>4</td>
<td>0.277</td>
<td>0.556</td>
<td>0.410</td>
<td>0.790</td>
<td>0.354</td>
<td>0.647</td>
<td>0.576</td>
<td>0.544</td>
</tr>
<tr>
<td>5</td>
<td>0.303</td>
<td>0.519</td>
<td>0.456</td>
<td>0.749</td>
<td>0.346</td>
<td>0.562</td>
<td>0.614</td>
<td>0.630</td>
</tr>
<tr>
<td>6</td>
<td>0.235</td>
<td>0.583</td>
<td>0.506</td>
<td>0.806</td>
<td>0.325</td>
<td>0.531</td>
<td>0.561</td>
<td>0.748</td>
</tr>
<tr>
<td>7</td>
<td>0.306</td>
<td>0.545</td>
<td>0.419</td>
<td>0.621</td>
<td>0.365</td>
<td>0.472</td>
<td>0.370</td>
<td>0.796</td>
</tr>
<tr>
<td>8</td>
<td>0.276</td>
<td>0.546</td>
<td>0.568</td>
<td>0.689</td>
<td>0.337</td>
<td>0.506</td>
<td>0.613</td>
<td>0.681</td>
</tr>
<tr>
<td>9</td>
<td>0.297</td>
<td>0.526</td>
<td>0.432</td>
<td></td>
<td>0.265</td>
<td>0.474</td>
<td>0.726</td>
<td>0.729</td>
</tr>
<tr>
<td>10</td>
<td>0.330</td>
<td>0.335</td>
<td>0.484</td>
<td></td>
<td>0.303</td>
<td>0.666</td>
<td>0.549</td>
<td>0.774</td>
</tr>
<tr>
<td>11</td>
<td>0.294</td>
<td>0.482</td>
<td></td>
<td></td>
<td></td>
<td>0.402</td>
<td></td>
<td>0.569</td>
</tr>
<tr>
<td>12</td>
<td>0.236</td>
<td>0.494</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.763</td>
</tr>
<tr>
<td>13</td>
<td>0.274</td>
<td>0.579</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.555</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.483</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Supplementary Table 8: Raw data

**dpp Invasion Severity Data for Figure 5**

<table>
<thead>
<tr>
<th>Severity</th>
<th>5A</th>
<th>5B</th>
<th>5C</th>
<th>5D</th>
<th>5E</th>
<th>5F</th>
<th>5G</th>
<th>5H</th>
<th>5I</th>
<th>5J</th>
<th>5K</th>
<th>5L</th>
<th>5M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>12</td>
<td>0</td>
<td>8</td>
<td>15</td>
<td>10</td>
<td>10</td>
<td>14</td>
<td>9</td>
<td>0</td>
<td>11</td>
<td>12</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Supplementary Table 9: Raw data

**dpp Invasion Severity Data for Figure 6**

<table>
<thead>
<tr>
<th>Severity</th>
<th>6A</th>
<th>6B</th>
<th>6C</th>
<th>6D</th>
<th>6E</th>
<th>6F</th>
<th>6G</th>
<th>6H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>11</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

### Supplementary Table 10: Raw data

**dpp Invasion Severity Data for Figure 7**

<table>
<thead>
<tr>
<th>Severity</th>
<th>7A</th>
<th>7B</th>
<th>7C</th>
<th>7D</th>
<th>7E</th>
<th>7F</th>
<th>7G</th>
<th>7H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>12</td>
<td>15</td>
<td>10</td>
<td>8</td>
<td>11</td>
<td>4</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>