

Modeling tumor invasion and metastasis in *Drosophila*

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Conservation of major signaling pathways between humans and flies has made *Drosophila* a useful model organism for cancer research. Our understanding of the mechanisms regulating cell growth, differentiation and development has been considerably advanced by studies in *Drosophila*. Several recent high profile studies have examined the processes constraining the metastatic growth of tumor cells in fruit fly models. Cell invasion can be studied in the context of an in vivo setting in flies, enabling the genetic requirements of the microenvironment of tumor cells undergoing metastasis to be analyzed. This Perspective discusses the strengths and limitations of *Drosophila* models of cancer invasion and the unique tools that have enabled these studies. It also highlights several recent reports that together make a strong case for *Drosophila* as a system with the potential for both testing novel concepts in tumor progression and cell invasion, and for uncovering players in metastasis.

Introduction

Cancer is a leading cause of death worldwide and, according to the World Health Organization, was responsible for the death of 7.9 million people in 2007 (www.who.int). This disease is characterized by the uncontrolled malignant growth of cells; however, the vast majority of human fatalities arise from secondary metastatic tumors. These secondary tumors generally spread from the original site via the blood or lymphatic system and are highly invasive and aggressive. The metastatic process involves several discrete biological steps: loss of cellular adhesion, increased motility and invasiveness, entry and survival of tumor cells in the circulation, their exit into new tissue, and their eventual colonization of a distant site (Chambers et al., 2002; Gupta and Massague, 2006). Understanding the mechanisms that promote tumor invasion and the role of the microenvironment is important for developing therapeutic strategies to treat metastatic cancers (Yang and Weinberg, 2008; Nguyen et al., 2009; Hanahan and Weinberg, 2011).

Over the last decade, the fruit fly *Drosophila melanogaster* has become an important model system for cancer studies. Reduced redundancy in the *Drosophila* genome compared with that of humans, coupled with the ability to conduct large-scale genetic screens in this organism, has enabled its use to determine the molecular characterization of important signaling cascades, developmental processes and growth control. For example, our

understanding of the Hippo, Notch, Dpp and JAK-STAT signaling pathways, all of which are involved in tumor formation, has been enhanced by research in *Drosophila* (for reviews, see Brumby and Richardson, 2005; Vidal and Cagan, 2006; Januschke and Gonzalez, 2008). *Drosophila* genetics has revealed many genes that, when mutated or dysregulated, result in or contribute to tumorigenesis. Hyperplastic tumor suppressors, including components of the Hippo pathway, promote increased proliferation or survival, but do not disrupt tissue structure or differentiation. By contrast, *Drosophila* neoplastic tumor suppressors, such as the apical-basal cell polarity regulators (e.g. Lgl), lead to loss of tissue architecture, defects in differentiation and failure to exit the cell cycle. Elegant genetic and cell biology techniques have enabled the effects of tumor suppressors and oncogenes to be examined in the context of the whole animal. The capacity to generate patches (clones) of mutant tissue for specified genes during fly development has facilitated investigations into the role of the microenvironment in tumor development. Similarly, studies using clonal analysis in *Drosophila* have begun to elucidate cell competition mechanisms, which could potentially confer malignant cells with a growth advantage over their neighbors.

In conjunction with studies using other model organisms, flies have contributed greatly to our understanding of the mechanisms involved in cancer initiation and progression, revealing previously unknown molecular components and concepts. In turn, these have served to guide researchers who use mammalian systems to study cancer. This Perspective focuses on recent developments using fly tumor models that have been generated by tumor suppressor mutations or oncogene overexpression to induce neoplastic tumors of neuronal and epithelial origin as a means to probe the mechanisms involved in cellular invasion and metastasis.

Insights from metastatic neuronal tumors in the developing larval brain

Single gene mutations in a unique subset of genes {*lethal (3) malignant brain tumor [l(3)mbt]*, *brain tumor (brat)*, *discs large*

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(*dlg*), *scribble* (*scrib*), *lethal giant larvae* (*lgl*), *miranda* (*mira*), *prospero* (*pros*), *partner of inscuteable* (*pins*) (for reviews see Januschke and Gonzalez, 2008; Frolidi et al., 2008) cause malignant neoplastic tumors in the *Drosophila* larval brain. These genes have crucial roles in regulating proliferation and development [*l(3)mbt*] (Gateff et al., 1993) and apical-basal cell polarity (*dlg* and *scrib*) (Woods et al., 1989; Bilder et al., 2000).

The human homologs of *Dlg*, *Scrib* and *Lgl* are important regulators of cell polarity, and mutations or splice variants have been linked to poor prognosis for colorectal (Schimanski et al., 2005) and hepatocarcinoma (Lu et al., 2009) patients. Importantly, all three complex components are targets for E6 papillomavirus-mediated degradation (Nakagawa and Huijbregtse, 2000; Humbert et al., 2003; Handa et al., 2007). *L(3)mbt* has also been linked to various myeloid haemopoietic disorders (Bocconi et al., 2003) and is important for DNA replication and genomic stability (Gurvich et al., 2010).

Of these fly neoplastic tumor suppressors, *lgl* and *brat* are the best characterized. *Lgl* is localized to the cellular cortex, and functions in the same genetic pathway as *dlg* and *scrib* to maintain cellular polarity. Neuroblasts from *lgl* mutant larvae undergo aberrant symmetrical cell divisions, rather than the normal asymmetric divisions that are required for neuroblast and ganglion mother cell production (Mechler et al., 1985). *Brat* regulates ribosomal RNA (rRNA) synthesis and is a translational repressor. *Brat* is also asymmetrically localized to the ganglion mother cell after neuroblast division and is necessary for neuronal differentiation and proliferation control (Arama et al., 2000). Malignant tumors resulting from mutations in these fly neoplastic tumor suppressors cause late larval lethality but can be propagated and will metastasize when allografted into a recipient adult host (Gateff, 1978; Januschke and Gonzalez, 2008).

Metastasis of *lgl* and *brat* tumors

The Shearn group has utilized allograft experiments to investigate the metastatic behavior of *lgl* and *brat* mutant cells (Fig. 1A). Neoplastic brain tumors labeled with β -galactosidase (encoded by *lacZ*) from mutant larvae were dissected and allografted into the abdomens of wild-type adult flies. These tumors grew rapidly and resulted in host lethality within 12 days. Ovaries from allograft recipients were dissected and assayed for cells expressing *lacZ*. Because the ovary is contained in a non-porous epithelial sheet and muscle layers surround the ovarioles, only cells that are capable of metastasizing can invade into this organ (Fig. 1A). *lacZ*-positive cells were detected in recipient ovarioles from *lgl* and *brat* mutant tumors but not from control brains. These *lacZ*-positive cells in the ovary were shown to retain their neuronal and glial cell markers. This work provided the first evidence that *Drosophila* cells can metastasize through epithelial tissue and colonize new sites distant from the initial tumor (Beaucher et al., 2007a).

To understand the processes regulating this metastasis, a candidate approach was used to identify genes that are required for invasion by *lgl* and *brat* cells. Matrix metalloproteases (MMPs) have long been linked to metastasis in human cancer and were therefore excellent candidates. The *Drosophila* genome encodes two MMPs (MMP1 and MMP2). MMP1 expression is strongly upregulated in *lgl* mutant cells and is necessary for their metastatic

behavior (Beaucher et al., 2007b). By contrast, MMP1 expression is not required in *brat* mutant cells but is required in the host tissue, suggesting that MMP1 co-operates non-cell-autonomously with *brat* mutant cells to enable their metastasis.

Molecular profiling of *l(3)mbt* neuronal tumors

Recent studies from the Gonzalez group have taken a fresh approach to identifying genes required for *l(3)mbt* tumor growth (Janic et al., 2010). *l(3)mbt* is a substoichiometric member of the *Drosophila* dREAM-Myb complex, which is required for gene silencing, and functions in establishing and maintaining differentiation states (Lewis et al., 2004). Similar to *lgl* and *brat* tumors, those arising from *l(3)mbt* mutations result in larval lethality and can be allografted (Fig. 1B).

By conducting extensive expression arrays on larval neuronal and allograft cultured tumors, gene expression profiles were identified for tumors that result from single gene mutations [in *l(3)mbt*, *brat*, *lgl*, *mira*, *pros* and *pins*] (Janic et al., 2010). By comparing profiles, Janic and colleagues characterized an *l(3)mbt*-specific tumor signature. The concatenate sequencing of Piwi-interacting RNAs (piRNAs) and microRNAs from these tumors identified small RNA changes that are characteristic of the *l(3)mbt* tumors. Together with immunohistochemical analyses, these approaches provide a basis for understanding the gene expression changes that are specific to *l(3)mbt* tumors. The *l(3)mbt* tumor signature surprisingly contains a disproportionately high number of germline-specific genes and microRNAs. This result suggests that a germline fate is associated with *l(3)mbt* tumor growth, and is in agreement with studies from *Caenorhabditis elegans* that linked soma-to-germline transition with increased fitness and longevity (Curran et al., 2009; Wang et al., 2005).

Genetic studies then tested the capacity of mutations in upregulated germline-specific genes to modify *l(3)mbt* tumor formation and allograft metastasis (Janic et al., 2010). Interestingly, only mutations in a subset of germline-specific genes (*piwi*, *vasa*, *aub* and *nos*) could suppress *l(3)mbt* tumor formation. By contrast, other upregulated germline-specific transcripts (*zpg*, *Pxt* and *AGO*) were unable to prevent tumor formation or metastatic growth. This result suggests that only a limited number of germline-specific genes are capable of restricting tumor growth. These findings provide a detailed understanding of the gene expression and small RNA changes in *l(3)mbt* neoplastic tumors, and highlight the possibility that a soma-to-germline transition accompanies metastasis. However, this approach has not yet identified genes that are specifically required for invasion, because all metastatic suppressors also prevented *l(3)mbt* tumor growth. Further analysis of the *l(3)mbt* tumor signature will hopefully identify other metastasis-promoting factors.

Using mutations to generate neoplastic tumors has significant advantages. First, only a single mutational event is required to initiate tumor growth in an isogenic background. Second, the *l(3)mbt* alleles are particularly advantageous because they are temperature sensitive, permitting gene expression arrays to be conducted from a single stock at both permissive and restrictive temperatures. Finally, these tumors develop rapidly and metastasize when allografted. These experiments permit expansion of the tumor mass for biochemical analysis and metastasis modeling.

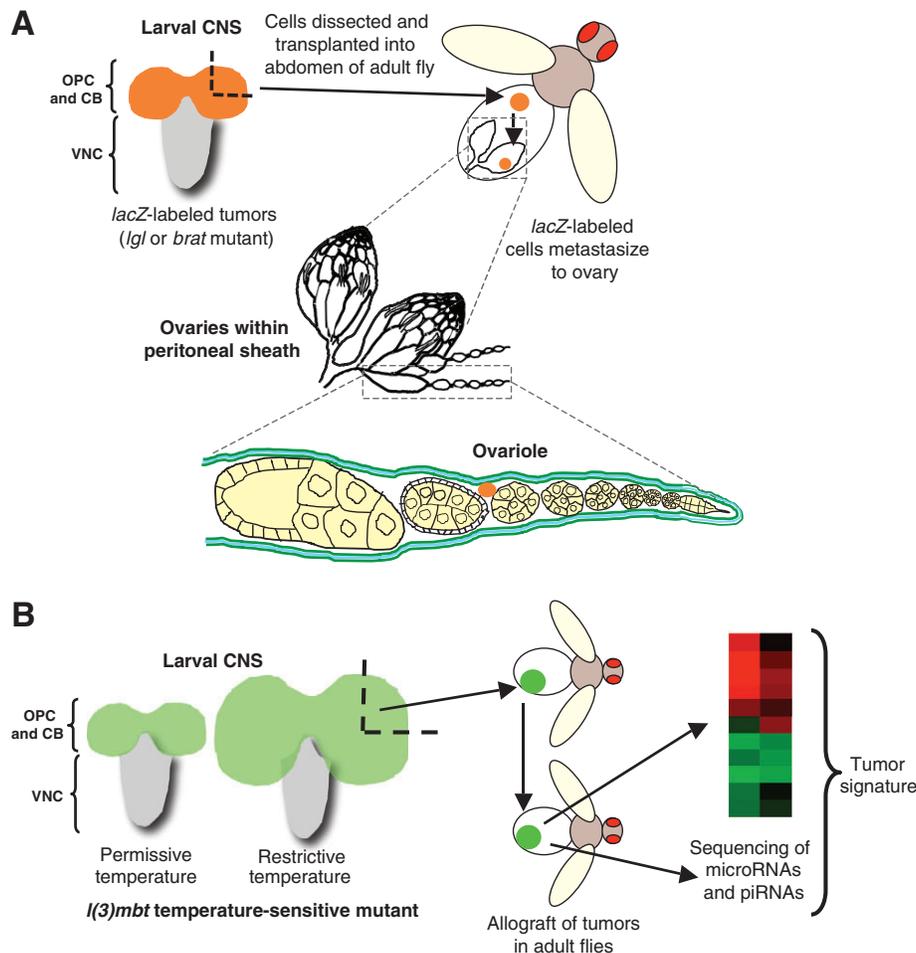


Fig. 1. *Drosophila* models of tumor metastasis caused by loss-of-function mutations in *lgl* or *brat* and *I(3)mbt*. The *Drosophila* larval brain is composed of two hemispheres and the ventral nerve cord (VNC). Mutant tumorous tissue in these models is restricted to the outer proliferative center (OPC) and central brain (CB) regions. (A) Neoplastic brain tumors caused by mutations in either *lgl* or *brat* can be engineered to express a reporter gene (*lacZ*; orange). Larval brains from these *lgl* or *brat* mutant animals are quartered and transplanted into the abdomens of adult female flies. The transplanted tissue continues to proliferate in the abdomen and, after several days, the ovaries of the host can be dissected and examined by immunofluorescence to detect labeled tumor cells that have metastasized. *Drosophila* ovaries are encased within a peritoneal sheath. Each ovary consists of 15-20 ovarioles, which are surrounded by an epithelial sheath of cells in addition to a muscle layer (blue) between two layers of extracellular matrix (green). Any *lacZ*-expressing cells found within the ovarioles must have traversed these layers and are therefore considered to have undergone metastasis. (B) Modeling metastasis using tumors caused by loss-of-function mutations in *I(3)mbt*. Temperature-sensitive mutations in *I(3)mbt* result in tissue overgrowth in the developing larval CNS when grown at restrictive temperatures (29°C). Tumor tissue (engineered to express GFP) can be dissected and transplanted into the abdomens of wild-type adult flies (allografts). These can be maintained and multiple rounds of allografts can be performed by transplantation into other flies, thereby generating sufficient material for biochemical analysis (e.g. microarray analysis, sequencing and western blotting). Comparison of the expression profiles of *I(3)mbt* and *brat* mutant tumors enabled an *I(3)mbt*-specific tumor expression signature to be obtained (Janic et al., 2010).

Insights from neuronal metastatic tumors induced by oncogene overexpression Modeling *Ras*^{V12} tumors in the larval eye neural epithelium

Overexpression of oncogenic Ras protein (*Ras*^{V12}) causes benign tumor growth in *Drosophila*. This model has been used to identify pathways that are required for tumor development and metastasis. The mosaic analysis with a repressible cell marker (MARCM) technique can be used to generate clones expressing activated *Ras*^{V12} by FLP-FRT-mediated mitotic recombination; clones are concomitantly labeled with a visible marker (*UAS-GFP*) (Theodosiou and Xu, 1998; Lee and Luo, 2001; Elliott and Brand,

2008). Using an eye-specific flippase (*ey-FLP*) to convert an inactive *GAL4* driver (*Act>y+>GAL4*) to an active conformation (*Act>GAL4*) enables the ectopic expression of *UAS* transgenes to be stimulated in the larval neural tissue, in an otherwise wild-type animal (Pagliarini and Xu, 2003). Expression of activated *Ras* (*UAS-Ras*^{V12}) causes benign tumor growth in the larval midbrain, which can be visualized through imaging living larvae or pupae directly. These benign tumors can then be used to identify genetic modifiers that are required for either initial tumor development and/or metastasis (Fig. 2A).

This system identified the cell polarity genes *scrib*, *lgl* and *dlg* as being crucial for constraining metastatic growth (Pagliarini and Xu,

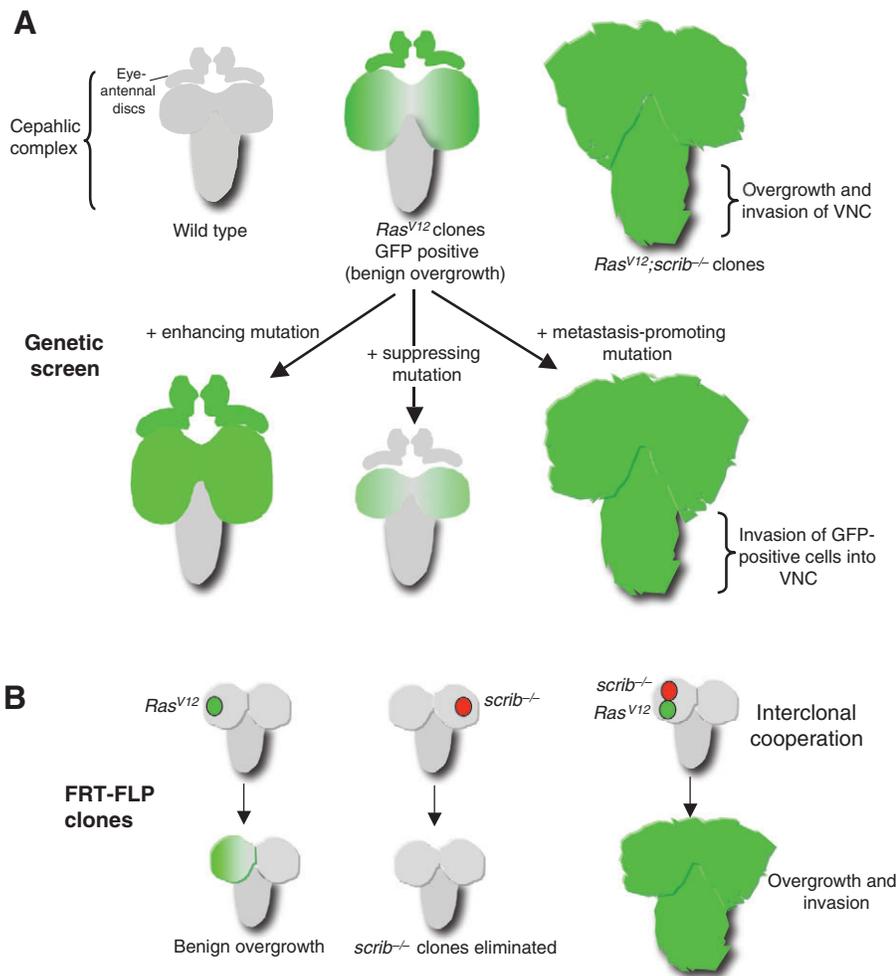


Fig. 2. Modeling tumor invasion and metastasis in the *Drosophila* larval brain.

(A) Ras^{V12} overexpression clones generated by the MARCM system in eye-antennal discs. These clones (marked with GFP, and shown in green) produce a benign overgrowth throughout the cephalic complex. When Ras^{V12} clones are also mutant for *scrib*, they become tumorigenic and metastasize, as shown by invasion of GFP-labeled cells into the ventral nerve cord (VNC). Genetic screens have identified modifiers of the Ras^{V12} phenotype [suppressor, *JNK*; enhancer, *large giant lethal (lgl)*; metastatic enhancer, *deep orange (dor)*] (Chi et al., 2010; Wu et al., 2010). (B) Interclonal cooperation of Ras^{V12} and $scrib^{-/-}$ clones. As described in A, Ras^{V12} overexpression produces benign overgrowth. $scrib^{-/-}$ clones grow at a reduced rate and are excluded via cell competition. Adjacent Ras^{V12} and $scrib^{-/-}$ clones cooperate to overproliferate and undergo metastasis. The large tumor and metastatic growth consists almost exclusively of Ras^{V12} cells, suggesting that $scrib^{-/-}$ cells actively cooperate with Ras^{V12} cells during the early stages of tumor growth and metastasis (Wu et al., 2010).

2003). All three of them genetically interact and cause overgrowth of Ras^{V12} tissue when mutated. These studies also identified *bazooka*, *stardust* and *cdc42* as factors that do not induce overgrowth when mutated singly but can strongly stimulate oncogenic Ras-mediated tumor growth and metastasis (Pagliarini and Xu, 2003). Each of these genes can also regulate cell polarity and E-cadherin expression. Crucially, although downregulation of E-cadherin is necessary for $Ras^{V12};scrib^{-/-}$ -induced metastasis, it is not sufficient, implicating other contributing factors in metastatic growth.

The relationship between oncogenic Ras and JNK signaling

Several *Drosophila* laboratories have highlighted the importance of the oncogenic cooperation between Ras and JNK signaling. Constitutive activation of the Ras signaling pathway prevents fly cells from undergoing JNK-mediated apoptosis in response to cellular stresses (Brumby and Richardson, 2003; Brumby and Richardson, 2005). The Xu group has linked JNK upregulation to cell polarity and changes in E-cadherin expression in $Ras^{V12};scrib^{-/-}$ clones (Igaki et al., 2006). They also demonstrated that overexpression of negative regulators of the JNK pathway [using a transgene to express a dominant-negative form of *Drosophila* JNK

(encoded by the *bsk* gene)] prevents tumor formation and metastasis (Igaki et al., 2006). The Bohmann laboratory has shown that the invasion potential of $Ras^{V12};scrib^{-/-}$ clones is dependent on the Fos-mediated transcriptional activation of *mmp1* downstream of JNK (Uhlirva and Bohmann, 2006). Expression of the MMP inhibitor, TIMP, or *mmp1* RNA interference (RNAi) knockdown, was able to suppress cell invasiveness (Uhlirva and Bohmann, 2006).

Recent studies of the *sds22* gene in *Drosophila* have strengthened the idea that epithelial integrity and JNK signaling cooperate to drive the metastatic growth of $Ras^{V12};scrib^{-/-}$ clones (Jiang et al., 2011). Sds22 is a conserved regulatory subunit of protein phosphatase 1 (PP1), and acts as a regulator of epithelial polarity and as a neoplastic tumor suppressor in *Drosophila* (Grusche et al., 2009). Loss of *sds22* in Ras^{V12} clones results in reduced epithelial integrity, and the clones become invasive. Overexpression of Sds22 in $Ras^{V12};scrib^{-/-}$ cells largely suppresses their tumorigenic growth, and mechanistic studies suggest that Sds22-PP1 inhibits non-muscle myosin II and JNK activity (Jiang et al., 2011). The authors of this study also showed that human *SDS22* is deleted or downregulated in multiple types of carcinoma.

A recent study by the Richardson group identified key regulators of the actin cytoskeleton and cell morphology, including Rho1-

family GTPases and RhoGEFs, as Ras-cooperating proteins (Brumby et al., 2011). The hyperplastic eye phenotype produced by driving *UAS-Ras^{V12}* with *ey-GAL4* was screened for modifiers using a collection of P-element enhancer insertion lines bearing *UAS* promoter sequences. These lines result in overexpression of the gene adjacent to the insertion element in *Ras^{V12}* cells. JNK pathway activation is crucial for the cooperation of these actin cytoskeletal regulators with *Ras^{V12}*. The relevance to human cancer of the collaboration between oncogenic Ras and JNK was demonstrated in this study by correlating JNK signaling with the upregulation of Ras in breast cancers (Brumby et al., 2011).

The role of JNK signaling in promoting metastatic behavior seems to be context specific. In contrast to the promotion of Ras-induced metastasis by JNK, analysis of *lgl^{-/-}* clones suggests that the activity of Diap1 (a caspase inhibitor) and JNK loss are essential for invasion of mutant cells out of the clone (Grzeschik et al., 2010a; Grzeschik et al., 2010b). Although Diap1 expression is sufficient to inhibit widespread apoptosis, cells at the boundary of *lgl^{-/-}* clones undergo cell death in a JNK-dependent manner. By blocking the JNK signaling cascade, apoptosis of these cells is specifically inhibited, stimulating de-differentiation and cellular invasion.

Lysosome dysfunction in *Ras^{V12}* tumor growth and invasion

Recent studies from the Xu laboratory have used genetic screens to identify mutations that promote *Ras^{V12}* cell metastasis (Fig. 2A). The authors tested 3119 ethyl-methanesulfonate-mutagenized lines (on the X chromosome) for their capacity to modify *Ras^{V12}* larval tumors. This screen identified 516 suppressors and 351 enhancers of tumor growth, of which 23 enhanced tumor growth as well as metastasis (Chi et al., 2010). Two of these metastasis-promoting mutations occurred within the class C vacuolar protein sorting (VPS) complex member *deep orange* (*dor*). By testing mutations in genes encoding other components of this lysosome complex, namely *carnation* and *vps16A*, they confirmed that loss of lysosome activity stimulates metastatic growth of the *Ras^{V12}* tissue (Chi et al., 2010). Characterization of other modifiers from this screen might provide a clearer understanding of the mechanisms underlying Ras-mediated tumor growth in *Drosophila*. This study also confirmed their genetic findings: feeding chemical inhibitors of lysosomal function to larvae with *Ras^{V12}* overgrowths promoted tumor development and metastasis. This work illustrates the potential of *Drosophila* to screen for therapeutic cancer drugs in vivo, a system that might one day provide a cheap and rapid means for drug discovery.

Interclonal cooperation between *Ras^{V12}* and *scrib* mutant cells

Exciting recent work using the *Ras^{V12}scrib^{-/-}* system has demonstrated a non-cell-autonomous effect between neighboring clones (called interclonal cooperation; Fig. 2B). Adjacent *Ras^{V12}* clones and *scrib^{-/-}* clones actively cooperate to form tumors and can metastasize (Wu et al., 2010). Interclonal tumors and metastatic growths are smaller than those produced from a single *Ras^{V12}scrib^{-/-}* clone but still represent a significant tumor burden to the hosts (Fig. 2B). During later stages of tumor development, these interclonal tumors are made up almost exclusively of *Ras^{V12}*-expressing cells, suggesting that the *scrib^{-/-}* mutant cells are only

required for the initial stages of tumor formation and metastasis (Wu et al., 2010). These studies also identified the JAK-STAT signaling pathway as being a crucial downstream target of JNK activity, implicating JNK and JAK-STAT as important oncogenic drivers for Ras-mediated tumor growth (Wu et al., 2010). In agreement with these findings, the authors went on to demonstrate that JNK activation after wounding is also able to promote the overgrowth of *Ras^{V12}* cells. This study potentially forges a link between tissue damage and Ras-stimulated JNK activity – a situation resembling the chronic inflammation that has been reported to contribute to tumorigenesis in humans (Mantovani, 2010). Other recent *Drosophila* studies have also highlighted the role of the immune response in tumor growth (Box 1).

Screening for regulators of invasive growth caused by activation of the Notch pathway

The Notch signaling cascade was originally identified as an important regulator of proliferation and differentiation in flies. Extensive genetic and biochemical studies have identified and characterized the components and regulators of this pathway (Artavanis-Tsakonas and Muskavitch, 2010). *Drosophila* studies have revealed that, similar to oncogenic Ras, Notch signaling cooperates with JNK to promote dysregulation of epithelial integrity (Brumby and Richardson, 2003; Leong et al., 2009). Aberrant Notch signaling is associated with several human cancers, including skin, breast, lung and ovarian cancer (for a review, see Allenspach et al., 2002). Tumors containing amplifications of genes encoding Notch signaling components (e.g. Notch and Jagged) tend to be highly aggressive and metastatic.

Overexpression of the Notch activator Delta (Dl) using the *eyeless-GAL4* driver (*ey-GAL4>UAS-Dl*) stimulates a non-metastatic overproliferation of eye tissue. Flies treated with a γ -secretase inhibitor to prevent Notch receptor proteolysis showed complete rescue of the *ey-GAL4>UAS-Dl* phenotype (Palomero et al., 2007). This model has been employed by the Dominguez group to screen for modifiers of this phenotype using a library of P-element UAS insertion lines that result in gene overexpression

Box 1. The role of the immune system in tumor growth in *Drosophila*

Several recent reports suggest that the immune system plays a crucial role in *Drosophila* tumor models, as is the case in mammalian tumors. Circulating blood cells, known in *Drosophila* as hemocytes, are part of the fly immune system and have been found to associate with *Ras^{V12}scrib^{-/-}* tumors and to reduce tumor growth in *scrib^{-/-}* animals (Pastor-Pareja et al., 2008). The *Drosophila* genome encodes a single member of the tumor necrosis factor (TNF) family, named Eiger (Egr). Egr has been shown to be required for the JNK-dependent cell death of *scrib* or *dlg* clonal tissue (Igaki et al., 2009). In the absence of *egr*, these mutant clones grow aggressively and develop into tumors (Igaki et al., 2009). By contrast, another study showed that loss of *egr* in *Ras^{V12}scrib^{-/-}* tumors prevented invasive overgrowth, which was correlated with reduced JNK activation and a failure to express MMP1 (Cordero et al., 2010). Therefore, in the presence of oncogenic Ras, Egr seems to play a role as a tumor promoter. This study also revealed that Egr is produced in the hemocytes associated with *Ras^{V12}scrib^{-/-}* tumors (Cordero et al., 2010). Together, these *Drosophila* models might provide an excellent parallel to mammalian tumors, in which TNF is produced in both tumor cells and associated immune cells, where it has been shown to have both oncogenic and tumor-suppressive roles (Balkwill, 2009).

(Ferres-Marco et al., 2006). One of these lines, *eyeful*, strongly promoted the metastatic growth of *ey-GAL4>UAS-Dl* eye tissue, resulting in secondary eye growths throughout the body. Using various tissue-specific *GAL4* drivers, the authors demonstrated that co-expression of *Dl* and *eyeful* could stimulate massive overgrowth and metastatic invasion in multiple tissue settings. The *eyeful* construct was mapped within the divergently transcribed genes of *longitudinals lacking (lola)* and *pipsqueak (psq)*, which produce a myriad of alternatively spliced BTB (BR-C, ttk and bab) proteins, which are required for the recruitment of histone deacetylases and Polycomb complexes to promoter regions. Further biochemical studies demonstrated that silencing of Retinoblastoma (Rb) expression via promoter hypermethylation strongly contributed to the metastatic phenotype (Ferres-Marco et al., 2006).

This screen also identified the P-element insertion GS1D233C as an enhancer of the *ey-GAL4>UAS-Dl* phenotype. This insertion was mapped to the *Akt1* locus, which encodes an important serine/tyrosine kinase linked to phosphatase and tensin homolog (PTEN) and Notch signaling (Palomero et al., 2007). These results suggest that flies will provide a useful system to test new pharmacological reagents targeted against the Notch pathway.

The Hassan group used the *ey-GAL4>UAS-Dl/eyeful* phenotype to screen for mutations that could further enhance metastatic growth (Bossuyt et al., 2009). This screen identified *atonal (ato)*, a transcription factor required for retinal terminal differentiation, as a crucial tumor suppressor. Mutations affecting *ato* dramatically enhanced tumor burden and metastasis rates, and tumors displayed elevated levels of proliferation markers (such as phosphorylated histone H3). Conversely, overexpression of *atonal* upregulated both Decapo (also known as p21 cell-cycle inhibitor) and phosphorylated-JNK levels, inhibiting proliferation and inducing apoptosis. Furthermore, overexpressing dominant-negative JNK (*bsk*) partially mimics *ato* downregulation in the *eyeful* (*ey-GAL4>UAS-Dl/eyeful*) model, indicating that JNK signaling is downstream of *atonal* and that *atonal* requires active JNK signaling to inhibit overgrowth (Bossuyt et al., 2009).

Modeling glioma in Drosophila

Developing *Drosophila* models of specific human tumor types is limited in many cases owing to the lack of directly homologous organs (e.g. pancreas, liver and lung). However, recent studies have capitalized on the similarity of mammalian and *Drosophila* glial cells to model glioblastoma in flies (Read et al., 2009; Witte et al., 2009). Glioblastomas are the most common tumors of the central nervous system (CNS), and their rapid proliferation and malignancy result in poor patient prognosis.

Mutation or amplification of the gene encoding epidermal growth factor receptor (EGFR) tyrosine kinase, loss of PTEN [which antagonizes the growth promoting effects of phosphoinositide 3-kinase (PI3K) signaling] or activating mutations in PI3K (Furnari et al., 2007) are genetic lesions that are commonly associated with gliomas. Consequently, the glial-specific *Repo-GAL4* driver (incorporating the promoter *reversed polarity*) was used to simultaneously express transgenes encoding constitutively active EGFR and PI3K in larval glia. Co-activation of EGFR (or Ras) and PI3K in larval glia results in neoplasia, neurological defects and lethality (Read et al., 2009; Witte et al., 2009). The overproliferation and neoplastic transformation seems to be specific for glia, because

overexpression of EGFR and PI3K in neurons, neuroblasts or other glial cells (such as oligodendrocyte-like neuropil glia and astrocyte-like cortex glia) failed to transform them.

The neoplastic glia induced in this *Drosophila* model mimic the highly proliferative anaplastic glia from high-grade human gliomas. They ectopically express Cyclin-B (CycB), Cyclin-E (CycE) and MMP1, promoting cell cycle entry and invasive growth. These studies demonstrated that glia expressing activated EGFR or Ras and PI3K invade into inappropriate areas of the brain, such as along Bolwig nerves, which are not normally accompanied by glial cells (Read et al., 2009; Witte et al., 2009).

Genetic experiments have revealed that the malignant neoplastic transformation of larval glia induced by EGFR and PI3K occurs via a complex network of genetic factors that are commonly mutated or activated in human gliomas. These downstream effectors include Tor, Myc, G1 cyclin-Cdk complexes (such as those including Cyclin B or E) and the Rb-E2F pathway. Interestingly, pharmacological inhibitors, including compounds that are used to treat patients, rescued these phenotypes in larvae. An EGFR inhibitor (gefitinib) partially reduced the migration of EGFR- and PI3K-transformed glia, whereas a PI3K inhibitor (wortmannin) and an Akt inhibitor (tricitriline) completely prevented invasion (Witte et al., 2009). Together, these studies suggest that this *Drosophila* model is useful for deciphering the signaling cascades underlying the abnormal behavior of glioma cells, including their metastatic potential.

Modeling tumor invasion in the epithelia of the Drosophila wing disc

Roles for both receptor and non-receptor tyrosine kinases in cell transformation and progression towards malignant phenotypes are well established (Blume-Jensen and Hunter, 2001). The SRC family kinases (SFKs) are membrane-linked non-receptor tyrosine kinases that are required for regulating adhesion and cytoskeleton reorganization, cell cycle progression, and migration (Guarino, 2010; Thomas and Brugge, 1997). Elevated SRC levels have been reported in a wide variety of human cancers, including those of the colon, liver, lung, breast and pancreas (Ishizawa and Parsons, 2004; Summy and Gallick, 2003). SFK activity is inhibited by the C-terminal SRC kinase (CSK) family of tyrosine kinases [CSK and CSK homologous kinase (CHK)] (Chong et al., 2005); both CSK and CHK phosphorylate and inactivate SFKs, and mutations disrupting this activity have been implicated in a plethora of cancers. Elevated SRC levels (either by amplification of SRC or loss of CSK function) promote anchorage-independent cell growth, tumor cell invasion and metastasis (Guarino, 2010). Although SRC is a crucial regulator of epithelial-mesenchymal transition (EMT), the exact mechanism of how SRC promotes metastatic growth remains elusive.

Recent studies from the Cagan group have utilized the pseudostratified epithelia of the *Drosophila* larval wing imaginal disc to model invasive cell growth (Vidal et al., 2006). Global depletion of the sole *Drosophila* CSK/CHK ortholog, *Csk*, using either RNAi or *Csk* mutations, elevates active levels of Src, leading to significant overgrowth in developing larvae. By contrast, targeting *Csk* depletion to a discrete stripe along the anterior-posterior (A-P) boundary of the larval wing disc using a *patched-GAL4* driver (*ptc-GAL4*) to express a *Csk* RNAi transgene (*UAS-Csk-RNAi*) produces a metastatic phenotype (Vidal et al., 2006).

Src-transformed cells lose their apical profile and are excluded from the epithelium. These cells invasively migrate through the basal extracellular matrix, and eventually apoptose (Fig. 3). This invasive migration has been used to model tumor metastasis. Invasion occurs only at the boundaries between *Csk* mutant cells and the adjacent wild-type cells, suggesting that the microenvironment is crucial in determining the outcome of the Src-activated cells (Vidal et al., 2006).

By utilizing this model of metastasis, Vidal and co-authors examined the capacity of GFP-labeled *Csk-RNAi* cells to invasively migrate in different mutant backgrounds. These studies implicate JNK signaling in the apoptotic response of *Csk-RNAi* cells. Mutations in *puckered* (*puc*), a *Drosophila* JNK-specific phosphatase, cause an upregulation in JNK signaling and enhance both the apoptotic and the invasion phenotype of *ptc>Csk-RNAi* cells. Conversely, *puc* overexpression (*UAS-puc*) using *ptc-GAL4* prevents apoptosis within the stripe. Similar experiments revealed that the small GTPase Rho1 is a positive mediator of the JNK signal in *Csk-RNAi* boundary cells, similar to that seen in activated *Ras^{V12}* tumors in the eye epithelia (discussed above) (Brumby et al., 2011).

Cadherin-containing complexes are required for maintaining adherens junctions, which are important for cell adhesion and tissue structure. Src modulates the integrity of these adhesion sites: E-cadherin-dependent adhesion is reduced in *ptc>Csk-RNAi* tissue. These results implicated E-cadherin in the recognition and elimination of *Csk-RNAi* cells. Depletion of E-cadherin suppresses both the migratory and apoptotic phenotypes of *ptc>Csk-RNAi* boundary cells. Using candidate approaches, p120-catenin and both MMPs (MMP1 and MMP2) were found to be important for the *Csk-RNAi* invasive phenotype (Vidal et al., 2006; Vidal et al., 2010).

mmp1 transcript levels were specifically upregulated at the leading edge of *Csk-RNAi* migrating cells (Singh et al., 2010; Vidal et al., 2010), suggesting that rearrangement of the extracellular matrix is crucial for this invasive growth.

In a separate study, the capacity of Src to contribute to *Ras^{V12}*-induced tumor growth was tested (Vidal et al., 2007), and malignant overgrowth of *Ras^{V12}* tumors was found to correlate with elevated Src levels. These studies suggest a progressive role for Src, whereby low levels promote proliferation during early tumorigenesis and high levels are required for the later stages of invasive migration and metastasis (Vidal et al., 2007).

Invasive migration phenotypes similar to those of *ptc>Csk-RNAi* cells are produced by the overexpression of the oncogene *Abl* in the A-P boundary of the wing disc (Singh et al., 2010). Co-expression of *ptc>Csk-RNAi* and *UAS-Abl* results in synergistic enhancement of the invasive phenotype. Conversely, the migration phenotype induced by *UAS-Csk-RNAi* is suppressed by reducing *Abl* function using RNAi. Together, these findings suggest that *Abl* functions downstream of *Csk* and *Src* to mediate cell invasion. In addition to activating JNK (required for cell invasion and apoptosis), *Abl* overexpression also stimulates ERK signaling, further promoting cellular proliferation. Interestingly, the authors of this study defined a positive feedback loop whereby *Abl* increases the activity of *Src*, resulting in signal amplification (Singh et al., 2010).

This system provides an excellent model in which to study the function of genes in cell proliferation, survival and invasive behavior in the context of cell polarity. Although these reports only tested candidate genes for their ability to modify the invasion phenotype induced by *Csk* RNAi, the system should prove amenable to genetic screening using *UAS-RNAi* fly lines to identify genes involved in

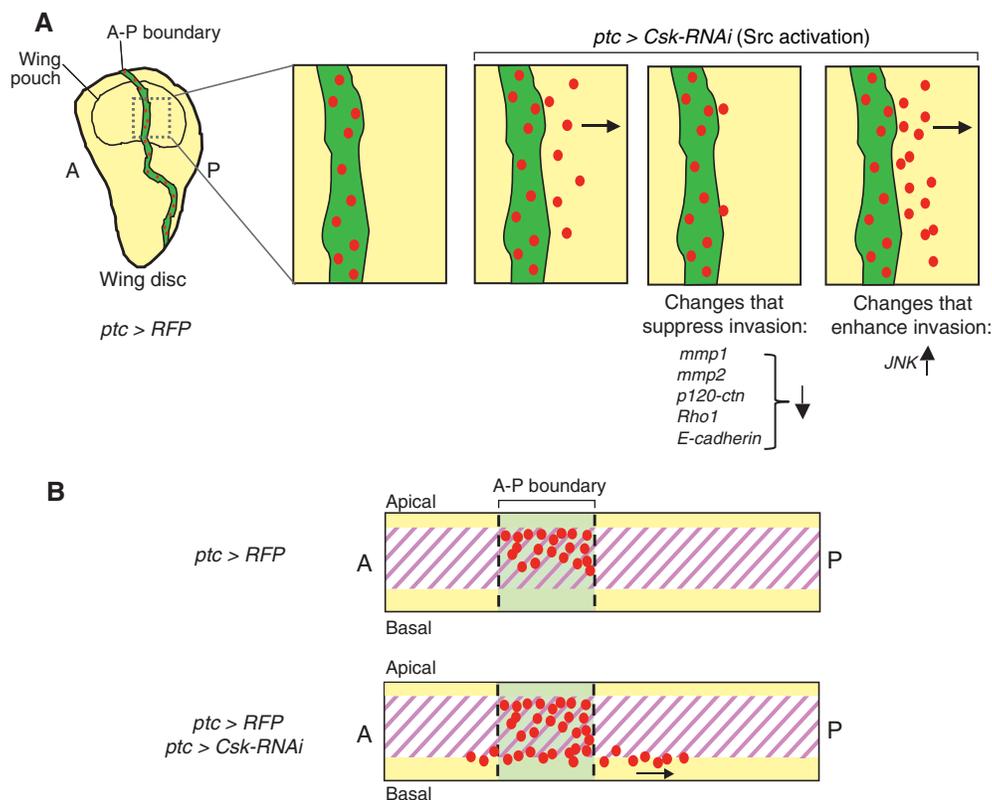


Fig. 3. Modeling tumor invasion in the *Drosophila* larval wing disc. (A) The larval wing disc consists of a sheet of epithelial cells (yellow). A *ptc-GAL4* driver is used to knock down *Csk*, a negative regulator of *Src*, specifically in a stripe along the A-P boundary, using an RNAi transgene (*UAS-Csk-RNAi*). The *Src*-transformed cells are labeled with red fluorescent protein (RFP). The *ptc* domain is shown in green. The *Csk-RNAi* cells at the boundary of the *ptc* domain have the potential to invade into the surrounding tissue. Activation of the JNK pathway or knockdown of regulators of the cytoskeleton (*mmp1*, *mmp2*, *p120-ctn*, *Rho1* and *E-cadherin*) using transgenic overexpression, different genetic backgrounds or RNAi, can either enhance or suppress the invasion of the *Csk-RNAi* cells, highlighting the importance of the tumor microenvironment (Vidal et al., 2006). (B) Horizontal cross-section of the boxed area of the wing disc shown in A. On silencing of *Csk*, labeled cells (red) are basally excluded and migrate from the boundary through the extracellular matrix (purple hatch).

metastasis. In addition, the identification of the factors that collaborate to control cell migration suggests possible approaches for dual therapeutic targeting in combating metastasis in various cancers.

Conclusions, limitations and perspectives

This review has highlighted the many advantages of *Drosophila* as a model for studying tumor progression. A streamlined genome, coupled with powerful genetic tools, provides a unique system in which to explore the mechanisms regulating the metastatic growth of tumor cells. However, as with any model organism, it has several limitations that should be considered. In mammals, malignant cells undergoing metastasis enter a local blood or lymph vessel before colonizing a distant tissue and forming secondary tumors. This is difficult to model in *Drosophila* because flies have rudimentary hematopoietic systems and a dramatically different lymphatic system compared with mammals. In addition, the metastatic potential of tumors induced in *Drosophila* is greatly reduced compared with their mammalian counterparts; tumor cells tend to invade the local surrounding tissue distally (e.g. in the cephalic complex in the case of *Ras*^{V12} overexpression).

Tumor formation is generally stimulated by a single mutation or activated oncogene expression in *Drosophila*. However, as the examples outlined here demonstrate, *Drosophila* has also proved to be a powerful experimental system for examining ‘two-hit’ models of tumor overgrowth and invasion. Although the *Drosophila* models discussed here are largely limited to tumors that develop in the larval stage, adult flies can be employed to examine the metastatic potential of these tumors using allografts, providing an ideal way to propagate tumors for extended studies.

The systems of modeling tumor development in *Drosophila* capitalize on the unique advantages of this model organism coupled with recent innovative technologies (see Box 2). Undoubtedly, the major strength of *Drosophila* is the ease of conducting large-scale genetic screens, which can now make use of the large publicly available collections of isogenic deficiencies and RNAi lines that cover the entire genome (Dietzl et al., 2007; Parks et al., 2004). Elegant targeting techniques – including the UAS/GAL4 system, FLP-FRT-mediated recombination and MARCM clonal analysis – enable gene knockdown in specific tissues or patches of cells, bypassing issues of organism lethality. Because these cancer models

involve an in vivo setting, they are also ideally suited for studies that examine the role of the microenvironment. In addition, *Drosophila* offers the potential for drug screening. Together, these approaches make *Drosophila* an excellent model organism to elucidate the basic mechanisms governing tumorigenesis and tumor progression. Validation of this research in mammalian cancer models and human cancer cell lines could lead to new insights into tumor invasion. Metastasis modeling in *Drosophila* is in its infancy but, as better tools and models of specific tumor types are developed, it offers the potential to probe the basic mechanisms regulating cancer cell invasion.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

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Box 2. Advantages of *Drosophila* for modeling tumor invasion and metastasis

- The signaling pathways controlling growth, differentiation and development that are involved in tumorigenesis and tumor progression are largely conserved between *Drosophila* and humans.
- Models of tumor formation and cell invasion have been created in *Drosophila* using a wide variety of gene targeting strategies, such as loss-of-function mutations and tissue-specific RNAi knockdown, as well as transgenic overexpression of activated oncogenes found in human cancers.
- The ability to generate clones that are mutant for specific genes juxtaposed with wild-type cells using the FLP-FRT and MARCM systems allows the genetics of the tumor microenvironment required for invasion and metastasis to be examined.
- Genome-wide screens using either de novo mutagenesis or tissue-specific knockdown by RNAi in *Drosophila* can identify genes with previously unidentified roles in cancer progression.
- *Drosophila* tumor models can be used for pharmacological screening.

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