Review Article

Drosophila as a Potential Model for Ocular Tumors

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Abstract

Drosophila has made many contributions to our understanding of cancer genes and mechanisms that have subsequently been validated in mammals. Despite anatomical differences between fly and human eyes, flies offer a tractable genetic model in which to dissect the functional importance of genetic lesions found to be affected in human ocular tumors. Here, we discuss different approaches for using *Drosophila* as a model for ocular cancer and how studies on ocular cancer genes in flies have begun to reveal potential strategies for therapeutic intervention. We also discuss recent developments in the use of *Drosophila* for drug discovery, which is coming to the fore as *Drosophila* models are becoming tailored to study tumor types found in the clinic.

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Why Use *Drosophila* as a Cancer Model?

By combining a century of genetic research resulting in a vast array of powerful genetic tools with modern cutting-edge technologies, *Drosophila* offers unparalleled opportunities to elucidate complex interactions between genes, cells and tissues [1, 2]. In recent years, *Drosophila* has been increasingly utilized as a model organism for cancer research after work in cancer cell lines had failed to reliably predict clinical relevance [3] and expensive mammalian models had struggled to provide a sufficiently sophisticated panel of genetic tools to fully capture the intricacy of tumorigenesis and metastasis. Although flies do not normally develop cancer because of their limited life span, many biological processes related to tumorigenesis

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and cancer progression are well conserved in flies, and the disruption or ectopic activation of cancer-related genes can lead to the development of several classic hallmarks of cancer, including genome instability, metabolic reprogramming, evasion of apoptosis, sustained proliferation, resisting cell death and invasive cell migration [4, 5]. Furthermore, studies in flies have yielded important conceptual insights into epithelial cancer development and progression, including cell competition, apoptosis-induced compensatory proliferation and oncogenic cooperation [6].

One of the key advantages of *Drosophila* is its relatively streamlined genome, which facilitates the conduct of large-scale genetic screens with comparative ease. The low level of redundancy means that hits are likely to provide insight into the gene function, while the high level of homology to ~75% of human disease-causing genes [7] means that *Drosophila* screens are often directly applicable to higher organisms, including humans. Indeed, such screens have identified many important and conserved cellular regulators that were later shown to be involved in tumor formation in humans, such as Notch (N), Hedgehog (Hh) and Salvador/Warts/Hippo [8]. Since nearly all of the genes implicated in the growth control from studies of human cancer are conserved in *Drosophila*, candidate genes identified in human cancers can be extensively studied in flies, where genes can be manipulated with unparalleled precision and the in vivo setting additionally enables studies to assess the role of the microenvironment in tumor development and progression. Furthermore, *Drosophila* has shown potential as a screening model for novel therapeutic compounds, putting it at the translational interface between basic science and clinical medicine [8].

How good a model is the *Drosophila* eye for ocular tumors? As with all cancer models, there is always the question of how closely the model replicates the specific cancer type. Obvious anatomical differences between fly eyes and human eyes, with the structure of the photosensitive organ (compound insect eye versus the camera-style human eye) being the most notable, naturally limit how directly applicable findings in flies are to specific cell types in human eyes. Importantly, however, numerous genes involved in eye specification (e.g. *ey*, *toy*, *so* and *eya*) or in orchestrating the proper temporal and spatial organization of the eye components (e.g. *hh* and *ato*) are highly conserved in both flies and humans, and mutations result in retinal defects in both systems alike [9, 10]. *Drosophila* therefore offers a valuable genetic model in which to test interactions between cancer-related genes found to drive the development or progression of ocular tumors in humans.

Using the *Drosophila* Toolkit for Gene Discovery

Targeted overexpression or knockdown of any number of candidate genes is the bread-and-butter methodology utilized by fly geneticists (fig. 1a, b). The targeting techniques are becoming increasingly sophisticated, allowing genes to be misexpressed or knocked down in individual cells, clones of cells or specific tissues at different developmental time points and in different genetic backgrounds [11]. Manipulated cells can be readily labeled with a fluorescent protein, facilitating the analysis of the resultant phenotypes and cellular behaviors such as invasion and metastasis. Clonal analysis (fig. 1b) enables the study of the effects of multiple genetic alterations on populations of cells alongside phenotypically normal cells, recapitulating the events that take place during the growth and spread of cancerous cells in otherwise healthy tissues and organs in humans. It can also inform us about the role of tumor microenvironments. Sophisticated functional and complementation analyses can be carried out by combining different knockdown or expression constructs, which may be wild type or carry site-directed mutations of interest, with loss-of-function mutants within the same clonal patch.

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Although such analyses can be carried out in a multitude of tissues, the *Drosophila* eye in particular has attracted much attention because genetic manipulations in this nonessential tissue tend to give rise to viable animals displaying visible and easy-to-score phenotypes. For example, the *Drosophila* eye has been used to identify pathways required for neoplastic transformation and metastasis in situ. One widely used model utilizes constitutively active Ras (Ras^{V12}) targeted to the developing eye in larvae. Ras^{V12} expression results in benign hyperplastic tumors that can be readily used to screen for genetic modifiers required for neoplasia and metastasis (fig. 1c). Studies in this system revealed the critical role of the cell polarity genes *scrib*, *lgl* and *dlg* in inhibiting tumor progression and metastasis [12, 13]. In particular, cells lacking *scrib* and concurrently expressing Ras^{V12} acquire invasive properties, and affected animals display distant secondary foci of tumorous growths. Genetic
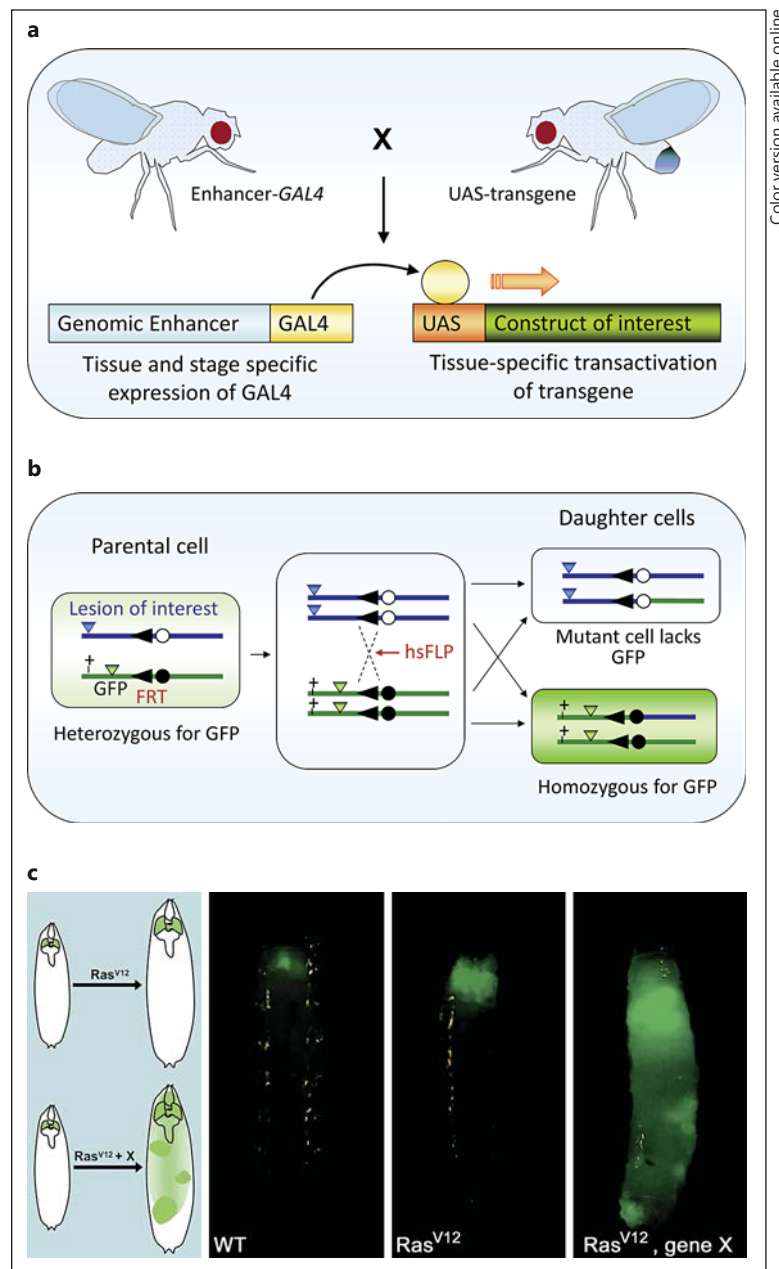
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analysis has further revealed the role of JNK activation, interclonal cooperation, extracellular matrix remodeling and the immune system for invasion and metastasis in this model (reviewed in Miles et al. [14]). Fundamental insights, such as these, into our understanding of epithelial cancer biology are likely to help guide studies of cancer in more advanced model organisms, including mammals. Indeed, mislocalization or downregulation of Dlg1, Scrib and Lgl1 have been correlated with early dysplastic stages of ocular adenocarcinoma in a mouse model [15].

The strength of *Drosophila* as an organism of choice for sophisticated genetic screens is further demonstrated by the identification and characterization of a remarkable gain-of-function mutant, *eyeful*, which was identified as a modifier of the eye overgrowth phenotype resulting from the overexpression of the Notch ligand encoded by *Delta* [16]. As well as displaying further eye enlargement, flies overexpressing *Delta* and concurrently carrying the *eyeful* mutation in the developing eye were found to have ectopic eye tissue throughout the body that originated from the dissemination of transformed cells from the developing eye. Intriguingly, further analysis revealed that the *eyeful* mutation resulted in the hypermethylation and inhibition of the tumor suppressor gene Retinoblastoma, demonstrating the significance of epigenetic modifications in neoplastic transformation and metastasis [16].

Investigating Ocular Cancer Genes in *Drosophila*

Drosophila is increasingly being used to generate specific alterations in conserved tumor suppressors or oncogenes in an attempt to replicate pathway-specific modes of tumorigenesis or metastasis found in different tumor types. These models can then be interrogated, e.g. using forward genetic screens, to devise ways to specifically affect the growth, survival or dissemination of resulting tumor cells. This approach may be of particular relevance to relatively rare cancer types that require tissue-specific or less commonly utilized oncogenic drivers that may be overlooked in forward genetic screens. In the following section, we discuss the insights that studies on the retinoblastoma tumor suppressor gene in flies have

Fig. 1. Utilizing the genetic toolkit in *Drosophila* for cancer studies. The GAL4/UAS expression system [65] (**a**) and the FLP/FRT system [66] (**b**) are two of the most commonly used tools to manipulate gene function in *Drosophila*. **a** The GAL4/UAS bipartite expression system allows flexible overexpression or knockdown of a gene of interest in different tissues or stages of development. Flies carrying one of many available GAL4 lines are crossed to flies carrying the gene of interest under the control of the UAS element. Binding of GAL4 to the UAS element in the progeny drives the expression in the pattern of the GAL4 line. Publicly available genome-wide RNA interference and overexpression UAS libraries [67, 68] have broadened the utility of this approach. Temperature-sensitive alleles of GAL80, a repressor of GAL4-mediated gene expression, can also be employed in this system for further temporal control of expression (not shown). **b** The Flp/FRT system can be used to generate clones of mutant cells in otherwise normal tissues. Expression of the *Flp* gene, for example under the control of a heat shock *(hs)* promoter, results in recombination between two *FRT* (Flp recombination target) sites on sister chromatids. One of the resulting daughter cells is consequently homozygous mutant for the gene of interest and is negatively labeled by the absence of GFP, whereas GFP expression labels both the parental cell and the other daughter cell, which are heterozygous and homozygous wild type for the gene of interest, respectively. This system can be coupled with GAL80 to positively label mutant cells lacking the repressor (not shown). **c** Combining the approaches outlined in **a** and **b**, it is possible to generate clones of cells ectopically expressing an activated oncogene whilst simultaneously harboring additional mutations that affect the properties of the resulting tumors (see Pagliarini and Xu [12] for details). For instance, cells overexpressing Ras^{V12} in the developing eye lead to localized non-invasive tumors. The presence of additional metastasis-inducing factors (e.g. gene X) drives the formation of secondary tumors visualized by the presence of GFP in other parts of the larva.

made towards potential treatments of the corresponding disease and explore the potential for developing models of uveal melanoma, the most common malignant tumor of the eye in adults, based on what is known about the underlying genetic drivers.

RB1 and Retinoblastoma

Retinoblastoma, the most common malignant tumor of the eye in children, is an aggressive neoplasm that develops from immature cells of the retina following biallelic loss of the RB1 gene [17, 18]. Like RB1, the *Drosophila* ortholog *rbf1* is best known for the regulation of the G1/S transition via the repression of E2F-dependent transcription [19]. Mutations in *rbf1* have only a modest effect on eye development and are not sufficient to drive tumor formation in *Drosophila*. By investigating context-dependent effects, it has been found that both *rbf1* and RB1 cooperate with the hippo tumor suppressor pathway in cell proliferation control and cell specification. Cells that are double mutant for *rbf1* and *hippo* dedifferentiate from a neuronal state and become uncommitted eye-specific cells with an increased potential to contribute to tumor growth [20]. It was subsequently shown that Yki, a transcriptional coactivator that is inactivated by Hippo signaling in flies, requires the transcription factor E2F to transcribe genes necessary to bypass exit from the cell cycle [21]. Similarly, the Hippo pathway and the RB1-mediated regulation of the G1/S transition in human cells are required for RB1-mediated repression of E2F target genes and consequent RB1-induced inhibition of cell proliferation [22].

A further constraint on tumor development is that *rbf1* mutant cells are sensitized to undergo apoptosis, which can be triggered for example by signaling from the cellular micro-environment [23] or by further genetic insults to the defective cells [24]. Using clonal genetic screens in the fly eye, loss-of-function mutations in the Tsc2 tumor suppressor [24] or a highly conserved peptidyl prolyl isomerase [25] were independently found to eliminate cells lacking *rbf1* but allow wild-type cells to survive. It was subsequently found that inactivation of human Tsc2 in cancer cells inhibited the growth of RB1 mutant cells and induced cell death under stress conditions [24], suggesting potential therapeutic strategies for treating cancers in which RB1 has been inactivated. Importantly, the confirmation of synthetic lethality in a cancer cell line clearly illustrates the power of *Drosophila* as a discovery tool.

Uveal Melanoma Cancer Genes

Whereas retinoblastoma is largely considered to be a single-gene disease, in uveal melanoma, several genetic and epigenetic lesions have been variously associated with tumor development or progression [26]. As illustrated above, multigenic models of tumorigenesis can be readily generated in the fly. Activated oncogenes can be readily expressed ectopically; however, modeling the effects of the loss of human tumor suppressor genes is limited by the requirement for the presence and expression of the relevant homologs in the fly. Importantly, almost all of the genes implicated in uveal melanoma are conserved in *Drosophila* (table 1), including ubiquitous oncogenes or tumor suppressors, such as IGFR1, B-RAF and PTEN, which play conserved roles in growth and proliferation control in all metazoans, as well as highly conserved tissue-specific factors, such as G protein α_q subunits GNAQ/GNA11 [27], which in *Drosophila* is required for the termination of phototransduction and prevents retinal degeneration [28]. The insult most closely associated with metastasis of uveal melanoma cells is somatic loss-of-function mutations in the BRCA1-associated protein 1 (*BAP1*) tumor suppressor, which have been reported in nearly 50% of primary uveal melanomas and 84% of metastasizing tumors [29, 30]. Both human *BAP1* and the *Drosophila* counterpart encoded by *calypso* act as the catalytic subunits of a Polycomb repressive deubiquitinase (PR-DUB) complex, which remove ubiquitin from histone H2A in nucleosomes to repress Hox and other genes [31]. This is particularly interesting given the well-established role of certain Polycomb Group (PcG) genes as tumor suppressors in the *Drosophila* eye, including Sex combs extra (Sce, an E3 ligase

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Table 1. Uveal melanoma cancer genes and their counterparts in *Drosophila*

Associated human gene	Contribution to uveal melanoma	Frequency	*Drosophila* orthologue	Effect in *Drosophila*
Tumor suppressors				
BAP1	Mutational inactivation of BAP1 implicated in uveal melanoma metastasis [29, 30]	Inactivating somatic mutations in up to 84% of tumors	calypso	Control of cellular differentiation
PTEN	Submicroscopic deletions lead to loss of PTEN expression in uveal melanoma [39]	Mutations in the PTEN coding region identified in 11.4% of tumors; up to 76.3% of tumors with loss of heterozygosity of at least one PTEN marker	PTEN	PTEN mutants exhibit eye overgrowth, whereas PTEN overexpression inhibits cell proliferation and promotes cell death during eye development [40]
p53	Altered expression in uveal melanomas following plaque radiotherapy, with elevated p53 levels [41]	Strong p53 staining observed in 50% of eyes containing posterior uveal melanomas	p53	Ectopic expression in the eye disc causes cell death and leads to a rough eye phenotype [42]
SF3B1	Mutations in a distinct molecular subset of uveal melanomas [43]	Mutations in 18.6% of tumors	CG2807-PA	No phenotypic data
Potential diagnostic markers				
MC1R	Highly overexpressed in uveal melanoma [44]	Detected in 95% of tested melanoma tissues	No clear orthologue identified	
SSR2, SSTR3 and SSTR5	Expressed in human uveal melanomas; better prognosis for patients with high levels of SSR2 in melanoma tissue [45]	Immunohistochemical staining positive for SSR2 in all uveal melanomas, whilst SSR3 was found in 29% and SSR5 in 58% of tumors	AlCR2	AlCR2 is closest to SSR2; no data for the effect on the eye
Proto-oncogenes				
GNAQ and GNA11	Constitutively active in uveal melanoma [27, 46]	Activating mutations affecting GNA11 in up to 49% of primary uveal melanomas and in 45% of uveal melanomas for GNAQ	Gαq	Loss leads to retinal degeneration [28]
MYC	Amplification of the c-myc oncogene detected in 43% of uveal melanomas [47]	70% of uveal melanomas with extra copies of c-myc	diminutive	Myc overexpression in the eye induces both growth and apoptosis, yielding large rough eyes [48]
CCND1	Cyclin D1 protein expression was observed in 65% of uveal melanomas [49] and is associated with more aggressive disease [50]	Expression observed in 65% of uveal melanomas	Cyclin D	Clonal expression of CycD-Cdk4 promotes cellular hypertrophy in post-mitotic cells of differentiating eyes [51]
DDEF1	DDEF1 is commonly overexpressed in high-grade uveal melanomas [52]	Elevated expression in high-grade (class 2) tumors, corresponding to 50% of uveal melanoma patients more likely to die of metastatic disease	Asap1	No phenotypic data are available
NBS1	High expression of Nbs1 associated with increased metastatic death in uveal melanoma patients [53]	Elevated expression in high-grade (class 2) tumors, corresponding to 50% of uveal melanoma patients more likely to die of metastatic disease	nbs	Null mutant flies die as pharate adults with rough eyes [54]
IGF1R	High expression associated with metastases of primary uveal melanomas [55]	High IGF1R expression in 50% of patients who died following metastasis but in only 25% of patients who survived 15 years	InR	Increased activity leads to overgrowth of the eye, reduced activity leads to reduced eye growth [56]
Jag2	May promote Notch activity, growth and metastasis in uveal melanoma [57]	Elevated expression in high-grade (class 2) tumors, corresponding to 50% of uveal melanoma patients more likely to die of metastatic disease	Serrate	Loss of function has no discernible effect on eye development [58] but is required for cell survival with Lobe during early eye development [59]
B-RAF	Low prevalence of mutations but frequent downstream activation of the MAPK pathway associated with uveal melanoma [60–62]	Rare; mutations detected in 14.3% of uveal melanoma cell lines and primary specimens	phl	Rough eye phenotype due to loss of photoreceptor cells [63]; photoreceptors fail to differentiate in mutant somatic clones of the larval eye disc [64]

MC1R = Melanocortin 1 receptor; *AlCR2* = allatostatin C receptor 1; *Gαq* = G protein α q subunit; *IGF1R* = insulin-like growth factor 1 receptor; *InR* = insulin-like receptor; *phl* = pole hole.

required for H2A monoubiquitylation), which operates synergistically with the PR-DUB complex to transcriptionally repress a subset of PcG target genes [32, 33]. Armed with the knowledge of some of the genetic events driving uveal melanoma [26] and given the conservation of the affected genes in flies, it will be possible to begin to devise *Drosophila* models with which to help determine the key players in uveal melanoma development and progression.
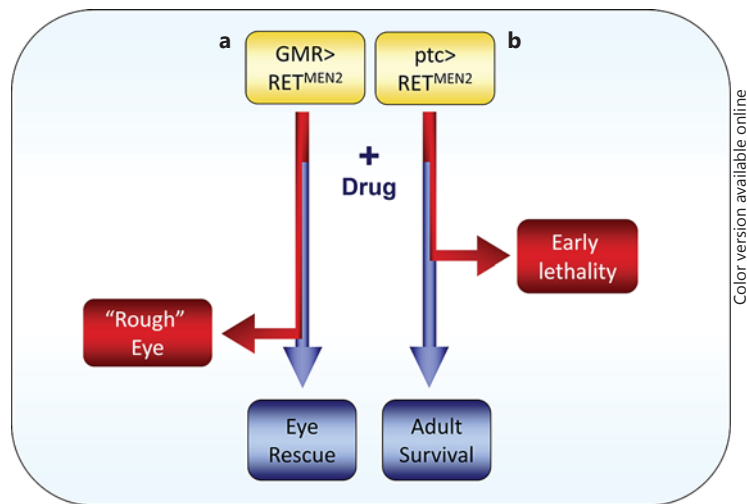
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Fig. 2. Strategies for testing the ability of chemical inhibitors to suppress the effects of RET[MEN2] overexpression in *Drosophila*. **a** Eye-specific expression: ectopic overexpression of *RET[MEN2]* under the control of the *GMR* promoter results in a fully penetrant rough eye phenotype in adults (red lines). Flies fed with vandetanib (ZD6474) strongly rescue defective eye development (blue line) [35]. **b** Expression in developing epithelia (wing, eye and leg) and other tissues: ectopic expression of UAS-RET[MEN2] under the control of *ptc-GAL4* results in lethality early in development (red line). This assay was used to screen a library of polypharmacological compounds capable of restoring viability (blue line). Further chemical refinement of initial hits identified a compound, AD80, with optimal efficacy and toxicity compared to analogs [37]. Colors refer to the online version only.

Future Perspectives

Future efforts to develop *Drosophila* strains that are specifically modeled on human data will ensure that insights can be more quickly taken back into mammals or cancer cell lines for validation and ultimate translation into clinical practice. With respect to translational potential, *Drosophila* has begun to emerge as a time and cost-efficient model for whole-animal drug testing to find suitable compounds capable of targeting cancer signaling in a multicellular context. A particularly successful example of the utility of *Drosophila* for drug development has come from studies of activated forms of the RET (rearranged during transfection) proto-oncogene, which are associated with several types of cancers, including type 2 multiple endocrine neoplasia (MEN2), an inherited condition that can result in the development of cancers such as medullary thyroid carcinoma (fig. 2). Ectopic expression of oncogenic RET *(RET[MEN2])* in the developing fly eye results in overproliferation, cell fate defects and cell death, leading to the formation of visibly 'rough' adult eyes [34]. Using this simple assay, flies were fed ZD6474 (vandetanib) to test whether this compound, which had previously been shown to inhibit RET, was capable of modulating *RET[MEN2]* function in the whole organism when administered orally [35]. Notably, ZD6474 suppressed the *RET[MEN2]*-mediated phenotypes at doses much lower than those required to cause toxicity to the animal. Following validation in flies, this compound subsequently passed clinical trials for the use in treating medullary thyroid carcinoma [36] and became the first drug to be approved by the FDA for the treatment of late-stage medullary thyroid cancer in adult patients who might be considered ineligible for surgery due to metastasis. This success has recently prompted further efforts to better understand the pathways responsible for drug efficacy and dose-limiting toxicity in the *Drosophila* RET[MEN2] model as well as to develop new compounds with improved therapeutic

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indices [37] (fig. 2b). Semi-automated methods for high-throughput screening are also being trialed to screen chemical compounds in larvae. A recent screen for suppressors of Ras^{V12} $scrib^{-/-}$ tumor overgrowth in the developing eye identified clinically active drugs with known activity against human tumor cells that are similarly effective in flies [38]. These studies illustrate how models of cancer involving multiple genetic alterations in flies can serve as a starting point for pharmacological interrogation and how *Drosophila* can provide an important function in preselecting the most useful compounds prior to testing in more expensive mammalian models.

Conclusion

There is a long tradition of using *Drosophila* to identify universal regulators of basic cellular processes such as proliferation, differentiation and death. As more genetic data from clinical samples become available, *Drosophila* models offer the potential to recapitulate the properties of ocular tumors with increasing accuracy and therefore applicability. Aside from providing valuable mechanistic insight, tailored cancer models are increasingly being seen to have promise in drug discovery efforts, where they can help to bridge the gap from bench to bedside.

Acknowledgements

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