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Wu-Min Deng *Editor*

The Drosophila Model in Cancer

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1 *Drosophila* Model in Cancer: An Introduction

Deeptiman Chatterjee and Wu-Min Deng

Abstract

Cancer is a cumulative manifestation of several complicated disease states that affect multiple organs. Over the last few decades, the fruit fly *Drosophila melanogaster*, has become a successful model for studying human cancers. The genetic simplicity and vast arsenal of genetic tools available in *Drosophila* provides a unique opportunity to address questions regarding cancer initiation and progression that would be extremely challenging in other model systems. In this chapter we provide a historical overview of *Drosophila* as a model organism for cancer research, summarize the multitude of genetic tools available, offer a brief comparison between different model organisms and cell culture platforms used in cancer studies and briefly discuss some of the latest models and concepts in recent *Drosophila* cancer research.

Keywords

Cancer · Tumorigenesis · *Drosophila* · Animal models · Genetic tools · Cell competition · Apoptosis induced proliferation · Cachexia · Tumor hotspots · Drug discovery

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1.1 Introduction

Cancer is one of the leading causes of mortality globally, second only to cardiovascular disease in developed countries [1]. In fact, cancer has been projected to surpass cardiovascular disease in the coming years to become the leading killer in the United States [2]. Cancer is a complex set of disease states that manifests in different forms, with varying severity and diverse reactions to therapeutic approaches, making it difficult to treat. Thanks to extensive research using a wide range of approaches and model systems, we have gained great insight into the pathological and molecular mechanisms of the disease's origin and progression in the past few decades [3, 4]. These efforts are important in finding novel strategies to prevent and treat this devastating disease.

Notable among the model organisms is *Drosophila melanogaster* (hereafter referred to simply as *Drosophila*), which has gained much traction as a cancer model due to the powerful genetic tools it employs, allowing dissection of the molecular and cellular mechanisms underlying cancer initiation, progression and invasion [5–7]. As a model organism, *Drosophila* has been employed in genetic research for about a century [8–11]. It is instrumental in our understanding of the genetic basis of development, innate immunity, circadian rhythm and many other biological processes. *Drosophila* has also been crucial in

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identifying and dissecting signal transduction pathways, many of which are implicated in human diseases, including cancer [6, 9, 12, 13].

Drosophila shares 60–70% conserved sequence homology to the human genome [14, 15]. Although only 48% of *Drosophila* genes have been reported to have human homologs, more than 75% of disease-causing genes in humans have homologs in *Drosophila* [16, 17]. Compared to the mammalian genome, *Drosophila* has less genetic redundancy, enabling a more complete understanding of the role of a particular protein in the cellular processes of interest. In addition, *Drosophila* has a rapid life cycle with a generation time of 10 days at 25 °C, allowing for rapid production of strains and genotypic combinations. They can be raised in limited space, require relatively inexpensive upkeep and can generate a large number of progeny - a single female fly lays as many as 500 eggs in its lifetime. *Drosophila* has only four pairs of chromosomes, and the introduction of balancer chromosomes, which prevent genetic recombination, allows long-term maintenance of stocks with complex genotypes without requiring recurrent selection. Many of the mutant and transgenic fly lines are maintained in stock centers to assist a vibrant scientific community. A dedicated global online database named FlyBase (http://flybase.org/) hosts a variety of information on *Drosophila* genes and also offers links to associated information from the stock centers, validated gene-specific antibody resources, reference articles on PubMed and related ties to other global databases such as the NCBI DNA database and the UniProtKB protein database, allowing the scientific community open access to frequently updated information. A long history of developing genetic tools, experimental protocols and an interactive and supportive research community have made *Drosophila* one of the most popular model organisms in biological research.

1.1.1 Genetic Tools Available in *Drosophila*

A major advantage of this model organism is the arsenal of available genetic tools, which have helped uncover novel mechanisms such as cell competition and compensatory proliferation [6, 18–21], and the establishment of various cancer models, that recapitulate aspects of the disease to allow study of the underlying mechanisms in greater detail [5–7, 13, 22–24]. In recent years, cancer-related studies in the *Drosophila* model system have helped build our current understanding of the complicated nature of this disease, from its origin to subsequent application of that knowledge in the therapeutic targeting of the disease in humans, as summarized in several excellent reviews [5, 7, 13, 23, 25, 26].

Drosophila has been at the forefront of developing tools for mutagenesis and applying them to understand complex biological processes. In late 1960s, the mutagenic properties of ethylmethane sulphonate (EMS) treatment was demonstrated in *Drosophila* [27]. Along with the early success of X-ray induced mutagenesis in late 1920s [28], these techniques led to the initial push in mutant screening, resulting in the early functional annotation of several genes [29]. During the 1980s, publication of thousands of new mutant alleles using P-element derived transposable elements led to the identification of many genes involved in developmental regulation [10, 30]. More recently, a broad variety of transgenic insertions using different transposable elements has been achieved by the Berkeley Gene Disruption Project, which has been made globally available at the stock center repositories [31–33].

Many of the modern genetic tools used to manipulate gene expression in Drosophila are based around the Gal4/UAS system—dervied from the budding yeast *S. cerevisiae* [34, 35]. This tool was developed to be used in *Drosophila*, where the promoter region of a gene drives the

expression of the transcriptional activator Gal4 in cells where the driver gene is endogenously expressed. Upon Gal4 expression, it binds to the Upstream Activating Sequences (UAS) to drive the expression of any downstream transgenic element [36]. The Gal4/UAS system permits a transgene to be expressed in the same pattern as a gene of interest by placing the Gal4 transcription factor under control of the gene's DNA regulatory elements. Developed from this technique is the TARGET (Temporal And Regional Gene Expression Targeting) system, where temperature sensitive Gal4-inactivating protein Gal80 ($Gal80^{ts}$) represses Gal4 transcriptional activity at permissible temperatures, which allows precise temporal control of transgene expression [37]. The Gal4/UAS binary expression system has since incorporated RNA interference (RNAi) and CRISPR-Cas9 based gene manipulation techniques, further enriching the tool box for genetic analysis in the fly model [38, 39]. For genes whose regulatory regions are not explicitly known, a system has been recently developed that exploits the ribosomal skipping mechanism of the viral T2A peptide to co-express Gal4 with the endogenous gene of interest [40]. This T2A-Gal4 method only requires explicit knowledge of the open reading frame for the endogenous gene of interest and not its regulatory elements [40]. Recent publication of T2A-Gal4 libraries have further boosted cell-type specific transgene expression [41]. Other binary expression systems utilized parallel to the Gal4/UAS system are the LexA-lexAop and QF-QUAS systems [42].

Another powerful tool developed in *Drosophila* and well suited for studies of cancer initiation is mosaic analysis. In a mosaic analysis, homozygous mutant (−/−) cells can be generated in a heterozygous background (+/−). This not only circumvents the potential lethality associated with many mutations, but is also an apt model for studies of cancer initiation since cancer generally arises from a mosaic situation, where a small number of cells within a homotypic tissue system acquire oncogenic mutations [43]. Aided by a favorable microenvironment, cancer cells outgrow their neighbors, competing for nutrients and space, to form tumors [44–46]. While cancer-related genes in *Drosophila* were being discovered early, the study of tumorigenesis truly began with the repurposing of the mosaic analysis tool. First reported in 1993, the development of stable transgenic insertions in the fly genome along with a site-specific recombination system using FLP recombinase (FLPase) and its target FLPase Recombination Target (FRT) to catalyze mitotic recombination between homologous chromosomes, contributed to an enormous boost in cancer research in *Drosophila* [47–49]. The application of mosaic analysis has enabled us to determine cellular autonomy of gene function and intra-clonal signal transduction, and has been adapted to mammalian systems [50–53]. *Trans*-chromosomal recombination methods have been used to analyze the autonomous actions of recessive mutations that are otherwise lethal in the larval or embryonic stages. Derived from this, the Mosaic Analysis using a Repressible Cell Marker (MARCM) technique, which employs the FLP/FRT system and the Gal4/UAS system, along with Gal80-mediated repression of transgenic expression in other cells, can be used to study genetic epistasis in *Drosophila* cancer models by driving gene expression or knockdown in mutant clones [54].

Another approach to generating mosaic tissues is based on the expression of transgenes only in limited groups of cells (clones) in otherwise wild-type flies. For example, *cis*-chromosomal recombination techniques such as the FLP-out system combine the FLP/FRT and Gal4/UAS systems, and have been applied in genome-wide mosaic analysis and screens [49, 55]. Typically, the proximity of *cis*-DNA sequences can be controlled by the excision of flanking FRT sites, using FLPase expressed under a *heat-shock* promoter. This technique removes an engineered STOP codon present

between flanking FRT sites, thus allowing the expression of Gal4 downstream of FRT in the *promoter* > STOP>*Gal4* cassette (where > represents FRT sites) by the promoter that is present upstream of the FRT-STOP-FRT sequences. The FLP-out system has had a significant impact on milestone studies, such as the discovery of cell-cell cooperation, competition and non-autonomous signaling involved in cancer [18, 56–59]. Modifications of the FLP-out system such as the CoinFLP technique, have enabled the generation of a reliable ratio of mutant to non-mutant cells and the G-TRACE technique has allowed experiments involving traceable cell lineage [60, 61].

The use of tools such as Cre/loxP and CRISPR-Cas9, that were identified in other systems and developed in mammalian systems, have been adapted for use in *Drosophila* [62–65]. Another technique adapted from xenografting protocols developed in mammalian studies [66, 67], is the study of tumor migration and tumor-host interaction via tumor injection into healthy fly hosts through allografting [68]. The future might address the recent push for using *Drosophila* as a parallel platform for drug discovery [7, 14, 69, 70] and for applying single-cell transcriptomic analyses [71–73] that has recently become a popular tool to validate and identify complex concepts in human cancers such as cellular cooperation [74] and identifying tumor heterogeneity [75, 76]. The vast information database that has been built up over time in *Drosophila* makes the model organism an excellent candidate for such studies.

1.1.2 The Use of Drosophila to Identify Cancer Related Genes and Pathways

Given the vast array of genetic tools, *Drosophila* excels as a platform for genetic screens aimed at identifying genes and pathways involved in a variety of biological processes. Over time, a significant number of genes have been identified in fruit flies that have later been discovered to be homologs of human oncogenes and tumor suppressors [16, 17]. Early successes in the application of *Drosophila* as a model in cancer research led to the identification of cancer-related genes and signaling pathways. For example, in a genetic screen carried out by Gateff and Schneiderman in 1967, a recessive mutant that manifested as a malignant tumor phenotype was reported [77]. Flies with a homozygous mutation in the gene called *lethal giant larvae* (*lgl*) exhibited neoplastic overproliferation of certain internal tissues and did not survive beyond the larval stages. This observation predates that where oncogenesis by *Retinoblastoma* (*Rb*) mutants act recessively [78] and of Harris' somatic cell hybrid experiments that coined the term tumor suppressor genes [79]. Soon after the discovery of *lgl*, which was thus the first incidence of a tumor suppressor gene ever identified, another mutant, named *discs large* (*dlg*), was isolated from a similar genetic screen that shared phenotypic similarity with that of *lgl* loss-of-function (LOF) imaginal discs [80]. Decades later, after the gene *scribble (scrib)* was identified and its function was established to maintain apicobasal epithelial polarity in the same genetic pathway as the genes *lgl* and *dlg*, they were classified as neoplastic tumor suppressor genes (nTSG) and have been used to develop many single-gene models of tumorigenesis [12, 81–83].

Many signaling pathways such as Notch, Hippo, Dpp, Hedgehog and Wnt pathways were first identified in *Drosophila*, and have since been found to be conserved in humans where they play key roles in cancer development [5, 6, 12]. Studies in *Drosophila* tumor models have shown oncogenic cooperation between signaling pathways such as Notch and Ras in *scrib* mutants that result in strong neoplastic overgrowth in the eye imaginal disc [84]. Activated Notch and oncogenic Ras drive the *scrib* mutant tumors to fuse and invade posterior brain lobes and ventral nerve cord [85]. The Salvador/Warts/Hippo (SWH) pathway, or simply the Hippo pathway, was first identified in *Drosophila* through genetic mosaic screens because of its involvement in tissue and organ growth regulation [86–93]. LOF of Hippo

pathway genes gives rise to massive tissue overgrowths with a decrease in cell death and has been found to be dysregulated in human cancers [82–86, 89–91].

1.1.3 Drosophila as a "Whole Animal" Model System to Study Human Cancer

At the foundation of clinically relevant cancer research, patient biopsies and immortalized cell lines derived from surgically resected tumor tissues have contributed greatly to building our initial understanding of the disease [94, 95]. Human cancer-derived cell lines, such as the HeLa cells, serve as fundamental models used in laboratories to study cancer biology and the therapeutic efficacies of chemotherapeutic agents [94, 96]. While these tools are critical to cancer research, this sampling of single cancer cell lines represents only a snapshot of a continually evolving tumor at an advanced stage. Thus, in order to investigate the genetic and epigenetic course of cancer initiation and progression, *in vivo* "whole animal" model organisms such as Genetically Engineered Mouse Models (GEMMs) and *Drosophila* model systems were employed. GEMMs and *Drosophila* have been used simultaneously to help further our knowledge of human cancers, each having their own unique advantages over the other.

Several tumor models using genetically simplistic combinations of oncogenic overexpression and tumor suppressor knockdowns have been developed in *Drosophila* [23, 25, 26, 97]. The benefit of using *Drosophila* to study human cancers, as compared to cell culture models, is in it being a complex "whole animal" system with distant organs and tissue systems functioning synergistically in a homeostatic condition, allowing phenotypic "readouts" of cancer progression. *Drosophila*, as a model organism, has also been proven to hold certain benefits over the mouse model. Using its vast array of genetic tools, transgenic constructs and the relative ease of use, *Drosophila* has been applied to model tumor-promoting genetic cooperations in tumor cell migration and metastasis that have only later been recapitulated in mouse models [98–101].

To model tissue invasion and metastasis *in vivo,* tumor transformation has been induced in *Drosophila* expressing the oncogenic isoform $dRas1^{G12V}$ (or simply, Ras^{V12}) in the imaginal disc epithelia [102]. This oncogenic isoform has also been used in many pioneering genetic screens that have aimed to identify second-loci mutations that may cooperate with Ras^{V12} to give rise to oncogenic overgrowth. Since over 30% of all human cancers have oncogenic mutations in one of the three Ras orthologs in humans [103], the ability to design quick and unbiased genetic screens to identify tumor-promoting genetic combinations make *Drosophila* an attractive model to study cancer. In addition, *Drosophila* imaginal epithelial cell clones with oncogenic Src64B (a c-Src homolog) have also been associated with metastatic potential [104]. Several models of human cancer using oncogenic activation of Ras and Src have been reproduced in *Drosophila*, which provide an excellent platform to study Ras/Src-driven tumor progression at the whole animal level [23].

Drosophila has also been used to model complex human cancers such as the malignant brain tumor known as Glioblastoma multiforme (GBM), which is associated with increasingly poor patient outcomes due to low drug absorption, low drug efficacy, and rapid drug resistance [24]. These tumors display a constitutive tumor-driving activation of the epidermal growth factor receptor (EGFR) and PI3K pathways [105]. Activation of these pathways in the embryonic glial cells leads to their overproliferation and results in an overgrown larval brain, with upregulation of an oncogenic genetic network that is independent of the target genes for the EGFR and PI3K pathways [106]. Thus, studies in *Drosophila* have helped identify new targets for the development of better therapeutic strategies.

Recently, 3D cell culture models such as cancer spheroids have emerged as powerful tissue systems to study cancer biology and drug efficacy, as they have been shown to recapitulate key determinants such as the tumor microenvironment, tissue morphology, angiogenesis, adaptive

responses to drugs, tissue invasion and metastasis [107]. However, whole animal models such as *Drosophila* may still have certain advantages over spheroid systems, as can be demonstrated through the *Drosophila* model of cancer cachexia [108]. Cachexia is a multifactorial muscle wasting syndrome that results from a distant tumor-host interaction, culminating in a debilitating condition that affects late stage cancer related mortality [105, 109]. It is known to be caused broadly by systemic inflammation and metabolic dysfunction, and has been associated with cancer and other diseases such as sepsis [105, 109]. Studying cachexia in spheroids is not feasible as the wasting phenotype manifests in tissues distant from the cancer. Studies in *Drosophila* have revealed the Insulin signaling antagonist ImpL2 as a key mediator of the wasting phenotype and is secreted by malignant tumors; loss of ImpL2 ameliorates the wasting phenotype, providing novel targets for cancer therapeutics [108]. Thus, by using a combinatorial approach of *Drosophila* for the identification of such a factor, spheroids can be used for further validation of similar molecular signatures in mammalian systems.

1.1.4 Emerging Concepts from Drosophila Studies in Cancer

Studies in *Drosophila* have helped us identify novel mechanisms in the underlying fundamental processes that determine tissue homeostasis and how their disruption leads to tumorigenesis. The concept of cell competition, first discovered in *Drosophila* wing imaginal discs describes a biological surveillance mechanism that measures cellular fitness across neighboring cells in a tissue system, which ensures that healthy cells remain in homeostasis [19, 110–113]. Based on the levels of relative fitness - the unit of which is both context dependent and mechanistically exclusive - neighboring cells of higher fitness competitively induce apoptosis in cells of lower fitness [114]. Depending on the context, this mechanism of surveillance can be exploited by cancer cells to outcompete neighboring wild-type cells to initiate neoplastic growth, as was first shown in *Drosophila* [115–117]. Many genes and factors involved in determining cell competition and fitness levels have been identified in this model system [57]. Among them, the proto-oncogene *dmyc* and the Hippo pathway have been shown to play a role in cell competition. Activating mutations in *dmyc* and those in the Hippo pathway have been shown to cause competitive overproliferation in the mutant cells at the expense of neighboring wild-type cells in a process called supercompetition [118–120]. As Myc family genes are implicated in human cancers [121], along with the implication of Hippo pathway dysregulation in human lung, colorectal, ovarian and liver cancers [89–91, 120], supercompetition has been hypothesized to be a cancer-initiation mechanism [122].

Another important cellular phenomenon first identified in *Drosophila* is the compensatory proliferation induced by the death of a neighboring cell [123, 124]. While a generic therapeutic approach to tackling cancer has been to kill cancer cells by inducing programmed cell death, increased apoptosis has been implicated in aggravating cancer progression by proliferation signaling to neighboring cells and by promoting an inflammatory response [4, 20, 123]. Using a model of apoptosis-induced proliferation in the eye imaginal disc of *Drosophila*, accumulation of Reactive Oxygen Species (ROS) was found to signal macrophages to promote the JNK pathway activation and trigger cell proliferation [20]. Compensatory proliferation has also been implicated in the acquisition of drug resistance by cancer cells, where increased proliferation may result in increased accumulation of resistance-rendering mutations [125]. Identifying and understanding the unintended consequences of apoptosis, such as compensatory proliferation, will be important in developing more effective strategies for cancer therapy, and the "whole animal" model system of *Drosophila* fits perfectly to study such complex interactions.

Recent studies in *Drosophila* have also helped to develop the concept of "tumor hotspots" within tissues [59, 127]. In 1889, Dr. Stephen Paget proposed the "seed and soil" hypothesis that meta-

static tumor cells (seeds) grow only in a preferred organ microenvironment (soil) [126]. Recent research in *Drosophila* has revealed that primary tumor growth also depends on the tissue-intrinsic microenvironment [59, 130]. "Tumor hotspots", which contain certain tissue-intrinsic properties, such as favorable cytoarchitecture and endogenous growth-promoting signaling, are more susceptible to oncogenic signals or mutations [59, 127]. One such "tumor hotspot" is the hinge region of the wing imaginal disc where JAK-STAT signaling acts as the oncogenic driver to neoplastic overgrowth [59]. Occurrence of tumor hotspots are also seen in mammals, at the transition zones between two different epithelial cell types [128, 129]. A novel transition zone model has been recently demonstrated in *Drosophila* at the posterior boundary of the larval salivary gland imaginal ring, where JAK-STAT and JNK signaling provide a growth promoting tissue microenvironment for oncogenic Notch-driven tumorigenesis [130].

1.1.5 Translational Aspects of Cancer Research in Drosophila

Over the years, the *Drosophila* model system has directly or indirectly contributed towards drug development against cancer. In fact, *Drosophila* is the first model organism to show synthetic lethality [131, 132] which provided the theoretical foundation for identifying PARP inhibitors to kill BRCA1 and BRCA2 related tumor cells [133]. In more recent years, the *Drosophila* model system has also been used directly for drug screens.

The process of target-based drug discovery may be divided into two approaches: High Throughput Screening (HTS) and *in silico* virtual screening. While the discovery of lead compounds that elicit a pharmacological effect can be achieved through HTS to induce a desired physiological response in cultured cell lines, the development of a drug and its safe therapeutic application has traditionally required using animal models for drug testing. A persistent bottleneck in this process is the failure to recapitulate the desired effect of a test compound isolated via HTS in animal models or the failure to recapitulate in humans the efficacy of a drug that has been tested on animal models [14, 70, 134]. However, many drugs have now entered clinical trials without animal model data, by using the organ-on-a-chip [135] or organoid models [95, 107], which is but an expensive alternative to animal models, compared to the suggested use of *Drosophila* as a parallel drug testing platform [69, 70].

Due to the many tools available for genetic manipulation in *Drosophila*, fruit flies have been used to model multigenic drivers of human colon cancer that describe human cancers more comprehensively than other models. In one such study using patient data from The Cancer Genome Atlas, as many as 32 multigenic models of human colorectal cancer were generated for further investigation of drug resistance in certain genetic backgrounds [103]. Cancer models for colorectal and lung cancer have been used to support combinatorial drug cocktails for different purposes, such as to circumvent drug resistance or to synergize efficacy [103, 136]. At times, *Drosophila* has been chosen over the vertebrate models due to the lack of genetic redundancy which has allowed major pharmaceutical companies such as Novartis and AstraZeneca to test for drug specificity [134, 136–138].

Use of invertebrate models for target-based drug screening may not be able to circumvent the "lead to drug bottleneck" entirely, but they still offer value as parallel alternatives to animal models already in use for drug screening and development. Drug development studies in *Drosophila* may help reveal targets and pathways that might otherwise be missed by conventional methods, and may also help in determining drug dosage regimes in some cases [103, 136].

1.2 Concluding Remarks

In 2000, Douglas Hanahan and Robert Weinberg published the 'Hallmarks of Cancer', delineating six hallmark characteristics that could most accurately define the disease [3]. They suggested that

for a normal cell to become malignant, it has to acquire hallmark characteristics such as self-sufficiency in growth signals to enable autonomous proliferation, insensitivity to anti-growth signals, evasion of programmed cell death, acquisition of unlimited replicative potential by telomere maintenance, sustained angiogenesis for nutrients and oxygen, and tissue invasion via metastasis. In 2011, the list was updated with new hallmarks such as deregulation and misappropriation of metabolic pathways to competitively feed cancer cells and the evasion of the immune system, along with the addition of genomic instability in cancer cells and inflammation in the tumor microenvironment as enabling factors that promote cancer progression [4]. These hallmarks and enabling characteristics suggest the complicated nature of this disease, and thus warrant a multifaceted investigation using multiple modelling platforms. Most of the hallmarks of human cancer can be genetically recapitulated in *Drosophila* [139].

The fact that certain aspects of human biology are not evident in *Drosophila* such as the lack of similarities between the telomere and telomeric maintenance strategies, the inability to recapitulate the adaptive immune system, the process of angiogenesis and mammary gland development has been partially challenged from a genetic perspective. Nevertheless, it has been shown that hypoxic response in tumors induce similar HIF1α/Sima-dependent activation of signaling pathways that trigger both angiogenesis in mammals and tracheogenesis in *Drosophila,* to the same end result of obtaining increased access to oxygen [140, 141]. The genetic network that builds up the innate immune response to cancer in humans also involves similar signaling responses in the form of JNK and TOLL/NFκB pathways in *Drosophila* [13, 20]. As a consequence of obvious differences in physiology, oversimplification of signaling networks and key differences in a drug's ADME (absorption, digestion, metabolism and excretion) properties, *Drosophila* neither qualifies as a standalone model system for testing the efficacy of drugs that are ultimately meant for human trials, nor as a replacement for mammalian testing platforms. However, it has been suggested as an inexpensive screening platform parallel to other systems, and as a "whole animal" cancer screening model with phenotypic readouts to test polypharmacological approaches [69, 134, 137].

Drosophila has a long history of unraveling complex diseases using powerful genetic tools developed for use in the system. It has been an inexpensive hypothesis-building tool to identify novel mechanisms of tumor initiation and progression, as well as an unparalleled genetic screening platform that has identified numerous cancer-related genes and pathways. Given the encouraging history and a collaborative research community, the ever-expanding field of *Drosophila* cancer research will continue to find answers to complicated questions in cancer biology to identify better strategies and novel targets to counter this disease.

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2 Using *Drosophila* Models and Tools to Understand the Mechanisms of Novel Human Cancer Driver Gene Function

Santiago Nahuel Villegas, Dolors Ferres-Marco, and María Domínguez

Abstract

The formation, overgrowth and metastasis of tumors comprise a complex series of cellular and molecular events resulting from the combined effects of a variety of aberrant signaling pathways, mutations, and epigenetic alterations. Modeling this complexity *in vivo* requires multiple genes to be manipulated simultaneously, which is technically challenging. Here, we analyze how *Drosophila* research can further contribute to identifying pathways and elucidating mechanisms underlying novel cancer driver (risk) genes associated with tumor growth and metastasis in humans.

Keywords

Cancer driver genes · Drosophila · Cancer genetic toolkit

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2.1 Introduction

Cancer is an assembly of diseases driven by the dysfunction of genes often associated with cell signaling cascades. A specific mutation may confer a selective growth advantage, while additional mutations may create subclones of cells with the ability to invade, migrate, and colonize distant organs (metastasize). Therefore, identifying mutations and epigenetic alterations that increase the risk or susceptibility of developing cancer is of the utmost relevance for understanding how the disease begins and progresses, and for developing efficient therapeutic approaches. Equally important is the development of experimental models in which the complexity of gene interactions can be analyzed. Several studies have used human tumor sequencing analysis across numerous cancer types to identify common mutations. However, the high heterogeneity of mutations in tumor masses, which can include both non-oncogenic *passenger* mutations together with *driver* (cancer-promoting gene) mutations in the same tumor, often hampers the identification of cancer risk genes and makes sequencing results difficult to interpret. More recently, sophisticated bioinformatics tools have helped to single out driver cancer risk genes, but the proposed molecular mechanisms by which these genes act are yet to be confirmed in experimental models.

The fruit fly *Drosophila melanogaster* has been used to study cancer for more than one hun-

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dred years [1]. It is an exceptional model animal system for discovering and clarifying the functional outcomes of defined gene manipulations, making it an important tool for distinguishing between driver and passenger mutations. Even in cases in which the structure of the genes is not identical to cancer genes in humans, gene function can be equivalent. This provides a starting point for extrapolating discoveries made in flies to human cancers. In fact, our knowledge of the function of many cancer driver genes (CDGs) omnipresent in the oncologic clinical literature (such as TP53, APC, NOTCH1, RTK/RAS, PI3K/AKT, HIPPO, WNT, and HEDGEHOG [2]) has in part or large part been inferred from studying their homologs in *Drosophila*. Examples of cancer-causing genes first discovered in *Drosophila* that have been subsequently translated to human cancer systems, and the molecular mechanisms by which these genes function, have been exhaustively reviewed elsewhere [3–7].

In this chapter, we focus on recent and understudied conserved CDGs identified in human tumors and discuss, using examples, how studies in *Drosophila* may contribute to understanding their oncogenic molecular mechanisms. We also describe genetic tools available in *Drosophila* that can be used to study and validate the role of candidate CDGs *in vivo*.

2.2 Human CDGs and the Use of *Drosophila* to Unravel Oncogenic Mechanisms

Recent studies analyzing data compiled by the Cancer Genome Atlas have used filters to categorize and shed light on human CDGs identity. According to these studies, the number of CDGs is approximately 130–300: Bailey et al. described 299 driver mutations across 33 types of cancers [8]; Volgelstein et al. revealed 138 genes that can promote or drive tumorigenesis [9]; and Kandoth et al. identified 127 genes described as cancer drivers (from 12 cancer types) [10]. By integrating the available data, a consistent signature of mutated genes which are present across several cancer types can be obtained (Fig. 2.1). In total,

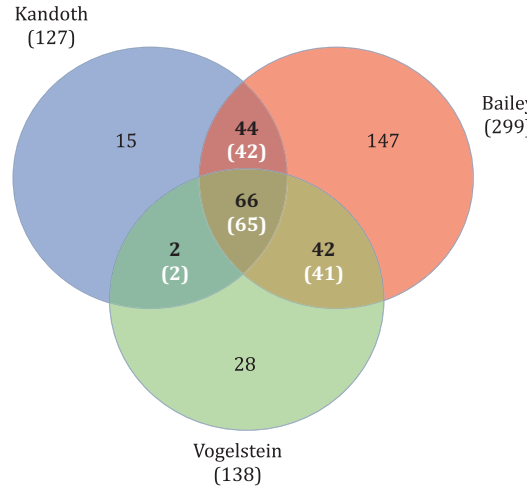

Fig. 2.1 Diagram showing CDGs coincidence between different studies and accros different cancer types. White numbers shows conserved CDGs in *Drosophila*

66 driver genes are common to the three studies and 154 are present in at least two of them, which show a high level of consistency between the different studies (Fig. 2.1 and Table 2.2). In most of the frequent solid tumors, an average of 33–66 genes exhibit somatic mutations; however, only two to eight are considered oncogenic drivers [9]. Although a common set of driver mutations can co-exist in different cancer types, how these mutations are combined varies significantly between individual patients. This complexity makes it hard to understand the epistatic interactions underlying oncogene cooperation and highlights the need to use genetically amenable animal models to systematically evaluate the interactions between candidate driver genes *in vivo*.

Remarkably, from the 154 CDGs present in at least two studies, 150 have *Drosophila* homologs (Tables 2.1 and 2.2). Importantly, the *Drosophila* system offers the unique opportunity to modulate the expression of several genes in a specific subset of cells simultaneously [11]. Therefore, using flies as a model system, it becomes possible to elucidate the molecular mechanisms underlying single and combined driver mutations and oncogenic epistatic relationships.

Mutations in most of the driver genes listed in Table 2.1 cause a selective growth advantage

Table 2.1 List of top cancer risk genes and *Drosophila* homologs

Driver[a]	Homologue	Pathway	Reported role in fly tumorigenesis
ACVR1B	*babo*	TGFβ signaling	
AKT1	*Akt1*	PI3K signaling	Y
APC	*Apc*	Wnt/β-catenin signaling	Y
ARID1A	*osa*	Chromatin SWI/SNF complex	
ASXL1	*Asx*	Chromatin other	
ATM	*tefu*	Genome integrity	
ATRX	*XNP*	Chromatin SWI/SNF complex	
BAP1	*calypso*	Transcriptional regulation	
BRAF	*Raf*	MAPK signaling	Y
BRCA2	*Brca2*	Genome integrity	Y
CDH1	*CadN2*	Wnt/β-catenin signaling	Y
CTNNB1	*arm*	Wnt/β-catenin signaling	Y
DNMT3A	*ADD1*	Epigenetics DNA modifiers	
EGFR	*Egfr*	RTK signaling	Y
EP300	*nej*	Chromatin histone modifiers	
FBXW7	*Ago*	Protein homeostasis/ubiquitination	
FGFR2–3	*Btl*	RTK signaling	Y
FLT3	*Pvr*	RTK signaling	
GATA3	*grn*	Transcriptional regulation	
IDH1–2	*Idh*	Metabolism	
KDM5C/6A	*lid*	Chromatin histone modifiers	Y
KIT	*Pvr*	RTK signaling	Y
H-, N-, KRAS	*Ras85D*	MAPK signaling	Y
MAP3K1	*Ask1*	MAPK signaling	
NCOR1	*Smr*	Chromatin histone modifiers	
NF1	*Nf1*	MAPK signaling	Y
NFE2L2	*cnc*	Transcriptional regulation	
NOTCH1	*N*	NOTCH signaling	Y
NPM1	*Nlp*	Chromatin other	
PBRM1	*polybromo*	Chromatin SWI/SNF complex	
PDGFRA	*Pvr*	RTK signaling	Y
PIK3CA	*Pi3K92E*	PI3K signaling	Y
PIK3R1	*Pi3K21B*	PI3K signaling	
PPP2R1A	*Pp2A-29B*	PI3K signaling	
PTEN	*Pten*	PI3K signaling	Y
PTPN11	*csw*	MAPK signaling	
RB1	*Rbf*	Cell cycle	Y
RUNX1	*Lz*	Transcriptional regulation	
SETD2	*Set2*	Histone modification	
SF3B1	*Sf3b1*	Splicing	
SMAD2	*Smox*	TGFβ signaling	
SMAD4	*Med*	TGFβ signaling	
SOX9	*Sox100B*	Transcriptional regulation	
SPOP	*Rdx*	Hedgehog signaling	
STAG2	*SA*	Genome integrity	
STK11	*Lkb1*	TOR signaling	
TP53	*p53*	Genome integrity	Y
U2AF1	*U2af38*	Splicing	
VHL	*Vhl*	Protein homeostasis/ubiquitination	

(continued)

Table 2.1 (continued)

Driver[a]	Homologue	Pathway	Reported role in fly tumorigenesis
WT1	*Klu*	Transcriptional regulation	
MLL3/KMT2C	*Trr*	Chromatin histone modifiers	Y
AR	*ERR*	RTK signalling	Y
CEBPA	*Irbp18*	Transcriptional regulation	
EZH2	*E(z)*	Genome integrity	Y
PHF6	*Phf7*	Transcriptional regulation	
SETBP1	*ash1*	Histone modification	
TET2	*Tet*	Genome integrity	

[a]Symbols of conserved human cancer driver genes present in all the studies analysed

Table 2.2 Human cancer driver genes and pathways and fly homologs

Studies[a]	**Hs name**	***Dm* homolog**	***Dm* symbol**	**Pathway**
1, 2, 3	*ACVR1B*	babo	baboon	TGF-β signaling
1, 2, 3	*AKT1*	Akt1	Akt1	PI3K signaling
1, 2, 3	*APC*	Apc	Apc	Wnt/B-catenin signaling
1, 2, 3	*ARID1A*	osa	osa	Chromatin SWI/SNF complex
1, 2, 3	*ASXL1*	Asx	Additional sex combs	Chromatin binding, deubiquitinase
1, 2, 3	*ATM*	tefu	telomere fusion	Genome integrity
1, 2, 3	*ATRX*	XNP	XNP	Heterochromatin
1, 2, 3	*BAP1*	calypso	calypso	Transcriptional regulation
1, 2, 3	*BRAF*	Raf	Raf oncogene	MAPK signaling
1, 2, 3	*BRCA1*	CG10916	CG10916	Genome integrity
1, 2, 3	*BRCA2*	Brca2	BRCA2, DNA repair associated	Genome integrity
1, 2, 3	*CDH1*	CadN2	Cadherin-N2	Wnt/β-catenin signaling
1, 2, 3	*CDKN2A*	Not known	Not known	Cell cycle
1, 2, 3	*CDKN2C*	CG14073	CG14073	Cell cycle
1, 2, 3	*CCND1*	CycD	Cyclin D	Cell cycle
1, 2, 3	*CTNNB1*	arm	armadillo	Wnt/β-catenin signaling
1, 2, 3	*DNMT3A*	ADD1	ADD domain-containing protein 1	Heterochromatin
1, 2, 3	*EGFR*	Egfr	Epidermal growth factor receptor	RTK signaling
1, 2, 3	*EP300*	nej	nejire	Histone modification
1, 2, 3	*FBXW7*	Ago	Archipelago	Protein homeostasis/ubiquitination
1, 2, 3	*FGFR2*	Btl	Breathless	RTK signaling
1, 2, 3	*FGFR3*	Btl	Breathless	RTK signaling
1, 2, 3	*FLT3*	Pvr	PDGF- and VEGF-receptor related	RTK signaling
1, 2, 3	*GATA3*	grn	grain	Transcriptional regulation
1, 2, 3	*IDH1*	Idh	Isocitrate dehydrogenase	Metabolism
1, 2, 3	*IDH2*	Idh	Isocitrate dehydrogenase	Metabolism
1, 2, 3	*KDM5C*	lid	little imaginal discs	Histone modification
1, 2, 3	*KDM6A*	lid	little imaginal discs	Histone modification
1, 2, 3	*KIT*	Pvr	PDGF- and VEGF-receptor related	RTK signaling
1, 2, 3	*KRAS*	Ras85D	Ras oncogene at 85D	MAPK signaling

(continued)

Table 2.2 (continued)

Studies[a]	Hs name	*Dm* homolog	*Dm* symbol	Pathway
1, 2, 3	*MAP2K4*	Mkk4	MAP kinase kinase 4	MAPK signaling
1, 2, 3	*MAP3K1*	Ask1	Apoptotic signal-regulating kinase 1	MAPK signaling
1, 2, 3	*NCOR1*	Smr	Smrter	Histone modification
1, 2, 3	*NF1*	Nf1	Neurofibromin 1	MAPK signaling
1, 2, 3	*NFE2L2*	cnc	cap-n-collar	Transcriptional regulation
1, 2, 3	*NOTCH1*	N	Notch	NOTCH signaling
1, 2, 3	*NPM1*	Nlp	Nucleoplasmin	Chromatin other
1, 2, 3	*NRAS*	Ras85D	Ras oncogene at 85D	MAPK signaling
1, 2, 3	*PBRM1*	polybromo	polybromo	Chromatin SWI/SNF complex
1, 2, 3	*PDGFRA*	Pvr	PDGF- and VEGF-receptor related	RTK signaling
1, 2, 3	*PIK3CA*	Pi3K92E	Pi3K92E	PI3K signaling
1, 2, 3	*PIK3R1*	Pi3K21B	Pi3K21B	PI3K signaling
1, 2, 3	*PPP2R1A*	Pp2A-29B	Protein phosphatase 2A at 29B	PI3K signaling
1, 2, 3	*PTEN*	Pten	Phosphatase and tensin homolog	PI3K signaling
1, 2, 3	*PTPN11*	csw	corkscrew	MAPK signaling
1, 2, 3	*RB1*	Rbf2	Retinoblastoma-family protein 2	Cell cycle
1, 2, 3	*RUNX1*	Run	Runt	Transcriptional regulation
1, 2, 3	*SETD2*	Set2	SET domain containing 2	Histone modification
1, 2, 3	*SF3B1*	Sf3b1	Splicing factor 3b subunit 1	Splicing
1, 2, 3	*SMAD2*	Smox	Smad on X	TGF-β signaling
1, 2, 3	*SMAD4*	Med	Medea	TGF-β signaling
1, 2, 3	*SOX9*	Sox100B	Sox100B	Transcriptional regulation
1, 2, 3	*SPOP*	rdx	Roadkill	Hedgehog signaling pathway/protein homeostasis/ubiquitination
1, 2, 3	*STAG2*	SA	Stromalin	Genome integrity
1, 2, 3	*STK11*	Lkb1	Lkb1 kinase	TOR signaling
1, 2, 3	*TP53*	p53	p53	Genome integrity
1, 2, 3	*U2AF1*	U2af38	U2 small nuclear riboprotein auxiliary factor 38	Splicing
1, 2, 3	*VHL*	Vhl	von Hippel-Lindau	Protein homeostasis/ubiquitination
1, 2, 3	*WT1*	CG3065	CG3065	Transcriptional regulation
1, 2, 3	*MLL3/ KMT2C*	Trr	Trithorax-related	Histone modification
1, 2, 3	*AR*	ERR	Estrogen-related receptor	Nuclear receptor transcriptional regulation
1, 2, 3	*CEBPA*	Irbp18	Inverted repeat binding protein 18 kDa	Transcriptional regulation
1, 2, 3	*EZH2*	E(z)	Enhancer of zeste	Polycomb repressor complex
1, 2, 3	*PHF6*	Phf7	PHD finger protein 7	Transcriptional regulation
1, 2, 3	*SETBP1*	ash1	absent, small, or homeotic discs 1	Histone modification
1, 2, 3	*TET2*	Tet	Ten-eleven translocation (TET) family protein	Genome integrity
1, 2	*MLL2/ KMT2D*	Trr/trx	Trithorax-related	Chromatin regulation

(continued)

Table 2.2 (continued)

Studies[a]	Hs name	*Dm* homolog	*Dm* symbol	Pathway
1, 2	*MLL3/ KMT2C*	Trr	Trithorax-related	Chromatin regulation
1, 3	RUNX3	RunxA	Runt related A	Transcriptional regulation
1, 3	*ACVR2A*	Put	Punt	TGF-β signaling
1, 3	*AJUBA*	Jub	Ajuba LIM protein	Chromatin other
1, 3	*ARHGAP35*	RhoGAPp190	Rho GTPase activating protein p190	Other signaling
1, 3	*ARID5B*	Htk	Hat-trick	Chromatin remodeling complex
1, 3	*ATR*	Mei-41	Meiotic 41	Genome integrity
1, 3	*CBFB*	Bgb	Big brother	Transcriptional regulation
1, 3	*CDK12*	Cdk12	Cyclin-dependent kinase 12	Cell cycle
1, 3	*CDKN1A*	Not known	Not known	Cell cycle
1, 3	*CDKN1B*	Not known	Not known	Cell cycle
1, 3	*CHEK2*	Lok	Loki	Genome integrity
1, 3	*CTCF*	CTCF	CTCF	Chromatin insulation
1, 3	*ELF3*	Eip74EF	Ecdysone-induced protein 74EF	Transcriptional regulation
1, 3	*ERBB4*	Egfr	Epidermal growth factor receptor	RTK signaling
1, 3	*ERCC2*	Xpd	Xeroderma pigmentosum D	Genome integrity
1, 3	*FOXA1*	Fkh	Fork head	Transcriptional regulation
1, 3	*FOXA2*	Fkh	Fork head	Transcriptional regulation
1, 3	*H3F3C*	His3.3A	Histone H3.3A	Chromatin other
1, 3	*HIST1H1C*	His1:CG33825	His1:CG33825	Chromatin other
1, 3	*KEAP1*	Keap1	Keap1	Protein homeostasis/ubiquitination
1, 3	*MAP2K4*	Mkk4	MAP kinase kinase 4	MAPK signaling
1, 3	*MECOM*	Ham	Hamlet	Transcriptional regulation
1, 3	*MTOR*	Tor	Target of rapamycin	PI3K signaling
1, 3	*NSD1*	NSD	Nuclear receptor binding SET domain protein	Chromatin histone modifiers
1, 3	*POLQ*	mus308	Mutagen-sensitive 308	Genome integrity
1, 3	*RPL22*	RpL22	Ribosomal protein L22	Other
1, 3	*RPL5*	RpL5	Ribosomal protein L5	Other
1, 3	*SIN3A*	Sin3A	Sin3A	Histone modification
1, 3	*SMC1A*	SMC1	Structural maintenance of chromosomes 1	Genome integrity
1, 3	*SOX17*	Sox15	Sox box protein 15	Transcriptional regulation
1, 3	*TAF1*	Taf1	TBP-associated factor 1	Transcriptional regulation
1, 3	*TBX3*	Bi	Bifid	Transcriptional regulation
1, 3	*TGFBR2*	Put	Punt	TGF-β signaling
1, 3	*USP9X*	Faf	Fat facets	Protein homeostasis/ubiquitination
1, 3	*PCBP1*	Mub	Mushroom-body expressed	RNA abundance
1, 3	*CCND1*	CycD	Cyclin D	Cell cycle
1, 3	*AXIN2*	Axn	Axin	Wnt/β-catenin signaling
1, 3	*CDKN2C*	CG14073	CG14073	Cell cycle
1, 3	*EGR3*	Sr	Stripe	Transcriptional regulation
1, 3	*HGF*	CG7432	CG7432	RTK/MET signaling
1, 3	*PIK3CG*	Pi3K92E	Pi3K92E	PI3K signaling
1, 3	*RAD21*	Vtd	Verthandi	Genome integrity
1, 3	*SMC3*	SMC3	Structural maintenance of chromosomes 3	Genome integrity

(continued)

Table 2.2 (continued)

Studies[a]	Hs name	*Dm* homolog	*Dm* symbol	Pathway
1, 3	*TBL1XR1*	Ebi	Ebi	Transcriptional regulation
1, 3	*TLR4*	Tehao	Tehao	NFκB signaling
2, 3	ARID2	Bap170	Brahma associated protein 170kD	Chromatin SWI/SNF complex
2, 3	AXIN1	Axn	Axin	Wnt/β-catenin signaling
2, 3	B2M	No orthologs found	No orthologs found	Immune signaling
2, 3	BCOR	CG14073	CG14073	Chromatin other
2, 3	CARD11	CG12379	CG12379	NFκB signaling
2, 3	CASP8	Dredd	Death related ced-3/ Nedd2-like caspase	Apoptosis
2, 3	CIC	Cic	Capicua	Transcriptional regulation
2, 3	CREBBP	nej	nejire	Histone modification
2, 3	CYLD	CYLD	Cylindromatosis	Protein homeostasis/ubiquitination
2, 3	ERBB2	Egfr	Epidermal growth factor receptor	RTK signaling
2, 3	FUBP1	Psi	P-element somatic inhibitor	Transcriptional regulation
2, 3	GNA11	Gαq	G protein α q subunit	GPCR signaling
2, 3	GNAQ	Gαq	G protein α q subunit	GPCR signaling
2, 3	GNAS	Gαs	G protein α s subunit	GPCR signaling
2, 3	H3F3A	His3.3B	Histone H3.3B	Chromatin other
2, 3	HRAS	Ras85D	Ras oncogene at 85D	MAPK signaling
2, 3	JAK1	hop	hopscotch	RTK/JAK/STAT signaling
2, 3	MAP2K1	Dsor1	Downstream of raf1	MAPK signaling
2, 3	MED12	Med12	Kohtalo	Transcriptional regulation
2, 3	MEN1	Mnn1	Menin 1	Histone modification
2, 3	MET	Alk	Anaplastic lymphoma kinase	RTK signaling
2, 3	MSH6	Msh6	Msh6	Genome integrity
2, 3	MYC	Myc	Myc	Transcriptional regulation
2, 3	MYCN	Myc	Myc	Transcriptional regulation
2, 3	MYD88	Myd88	Myd88	NFκB signaling
2, 3	NF2	Mer	Merlin	HIPPO signaling
2, 3	PTCH1	ptc	patched	Hh signaling
2, 3	RET	Ret	Ret oncogene	RTK signaling
2, 3	RNF43	Iru	Iruka	Immune signaling
2, 3	SMARCA4	brm	brahma	Chromatin SWI/SNF complex
2, 3	SMARCB1	Snr1	Snf5-related 1	Chromatin SWI/SNF complex
2, 3	SRSF2	SC35	SR family splicing factor SC35	Splicing
2, 3	TNFAIP3	trbd	trabid	NFκB signaling
2, 3	TSC1	Tsc1	Tsc1	PI3K signaling
2, 3	ABL1	Abl	Abl tyrosine kinase	RTK signaling
2, 3	ALK	Alk	Anaplastic lymphoma kinase	RTK signaling
2, 3	BCL2	Buffy	Buffy	Metabolism
2, 3	JAK2	hop	hopscotch	RTK/JAK/STAT signaling
2, 3	JAK3	hop	hopscotch	RTK/JAK/STAT signaling
2, 3	MSH2	spel1	spellchecker1	Genome integrity
2, 3	NOTCH2	N	Notch	NOTCH signaling
2, 3	PAX5	sv	shaven	Transcriptional regulation

[a]References of the studies in which the listed CDGs have been identified

by affecting downstream signaling cascades either directly or indirectly. Importantly, about 75% of cancer risk can be attributed to dysfunction in one of the following three domains: receptor tyrosine kinase (RTK) signaling; genome maintenance (chromatin modifiers and genome integrity); or transcriptional regulators (Fig. 2.2). Unsurprisingly, the TP53 gene, which is involved in maintaining genome integrity, accounts for the largest cancer risk, being found in 27 types of cancer. This is followed by PIK3CA, KRAS, PTEN, which are involved in RTK signaling, and ARID1A, which is involved in chromatin modification; each of these genes is associated with at least 15 cancer types. Other important nodes in the cancer network include the WNT, TGF-β and NF-κB (immune response-related) signaling pathways, as well as members of the Notch and Hedgehog pathways (Fig. 2.2 and Tables 2.1 and 2.2).

Many of the components of these pathways and their molecular mechanisms have been characterized in flies. A paradigmatic example of how *Drosophila* has contributed to the field of cancer research is the discovery and detailed characterization of Notch signaling. The Notch gene was identified a century ago, with mutation or loss of the gene resulting in characteristic notched wings [12]. Subsequently, Notch was defined as an oncogene based on a mutation found in human *NOTCH* homologs, which play a causative role in T-cell acute lymphoblastic leukemia [13]. Since then, the physiological and oncogenic actions of NOTCH1 (a major CDG; see Table 2.1) and its

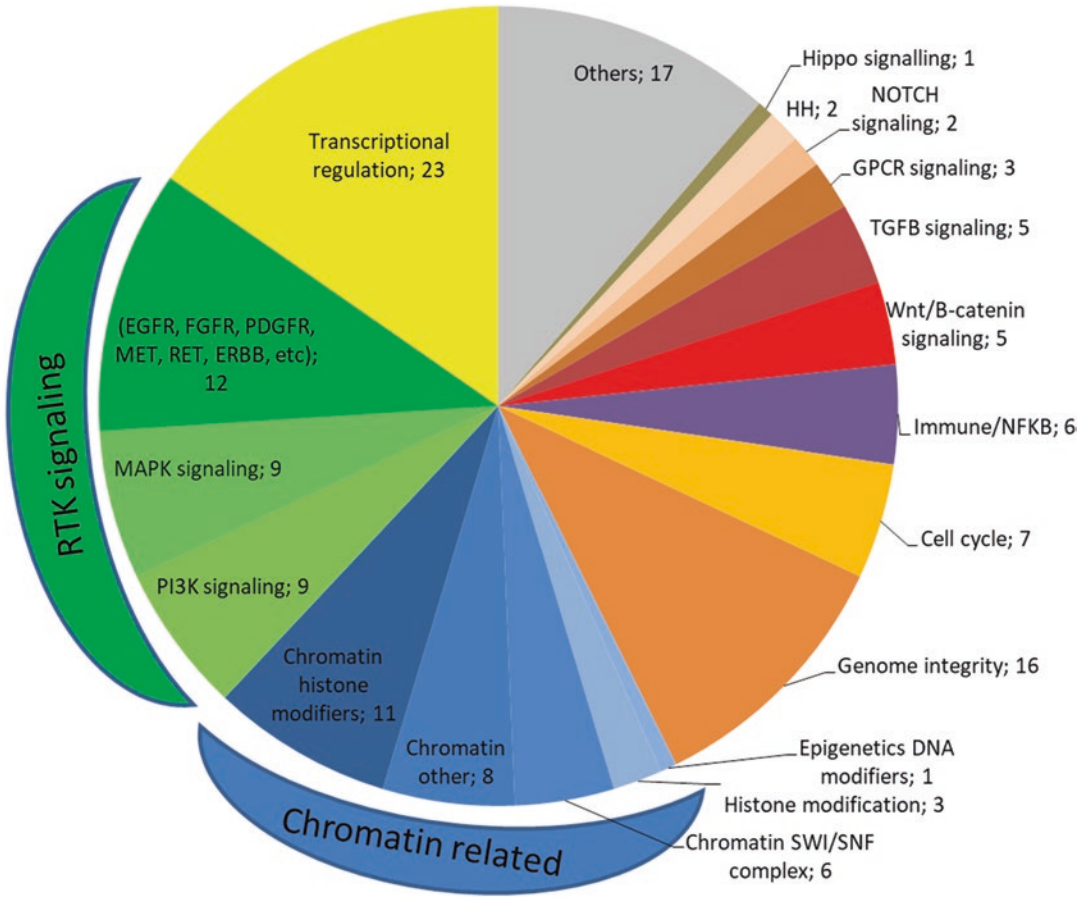

Fig. 2.2 Diagram showing the main core pathways involved in CDGs function

related pathway components have been implicated in causing many types of cancer, and flies remain a key model for identifying cooperating partners of Notch in tumorigenesis [14–16].

2.3 RTK Signaling

RTKs control a wide variety of developmental events in cell physiology and their aberrant activation is associated with several tumorigenic processes [17]. RAS mutations are among the most difficult to treat [18], and, given the therapeutic potential of RAS inhibitors, this signaling pathway is the focus of intense study. Seminal works on developmental processes in *Drosophila* have greatly contributed to elucidating and understanding the function of RTKs in normal physiology [19, 20]. Furthermore, work in *Drosophila* has identified many RTK signaling components which are conserved across species [21, 22] and has deepened our understanding of the role of RTKs during malignant cellular transformation and metastasis [23, 24].

2.3.1 RTK/RAS Signaling

The majority of the RTK intracellular signaling cascade is transduced via the RAS/MAPK and/or the PI3K/AKT pathways. The aberrant expression of many conserved RTK CDGs like EGFR, FGFRs, PDGFRA, JAKs, RET, ABL1 and ALK (Table 2.1) has been successfully modeled and characterized in *Drosophila*. There are also a number of other RTK CDGs, including RASA1 and RIT1, which have received none or little study to date.

RASA1, which encodes the Ras p21 protein activator 1, regulates cell differentiation and proliferation during angiogenesis [25, 26], with mutations of this gene associated with vascular malformation syndromes [27]. *Vacuolar peduncle* (*vap*) is the RASA1 fly homolog, mutations of which cause age-related cell death in neurons, accompanied by signs of autophagy and excessive signaling through the EGFR–Ras pathway [28]. *vap* interacts with *sprint* (*spri*) to regulate cell survival via Ras-dependent Rab5 (RAB5B) endocytic activity [29]. Whether these vap/RASA1 mechanisms are relevant to understanding tumor physiology remains to be addressed. As endocytosis and autophagy are emergent features associated with RAS-induced tumorigenesis [30, 31], these findings suggest that vap/RASA1 could be a key integrator of these processes via the RAS pathway.

RIT1 encodes the GTP-binding protein Rit1, a member of the RAS family of oncogenes. Fly studies have helped to define a fundamental and conserved link between the Rit1 homolog Ric, and p38 and Akt kinase cascades, uncovering a critical role for Ric in regulating cell survival during adaptation to oxidative stress [32]. Evidence suggests that Ric regulates cell survival in a p38-dependent manner, involving ROS-dependent Akt activation. Follow-up studies in human cells have shown that Rit1-mediated Akt activation requires mTORC2 activity [33]. The evolutionary conservation of the RIT1/Ric stress signaling pathway indicates that it is important to cell survival. As other Ras family GTPases cannot compensate for RIT1 or Ric loss, this makes RIT1 a relevant new candidate for therapeutic interventions. Furthermore, p38 modulates the expression of key inflammatory mediators which may function as cancer promoters [34], and p38 downregulation blocks tumor growth in Akt-induced, inflammation-driven tumors in flies [35]. Therefore, studying the functions of Ric may help to elucidate the role of stress signals in different tumorigenic contexts, as this protein may control whether ROS-dependent p38 activity results in cell death or recovery.

2.3.2 RTK/RAS/MAPK Signaling

Aberrant MAPK expression is linked to tumorigenic processes, with many members of this pathway, including H-, N-, and KRAS (*Ras85D*), BRAF, NF1, MAP2K1, and MAP2K4, showing the highest levels of mutation across different cancers (Table 2.1). The molecular mechanisms underlying several of these genes have been characterized by experiments performed in flies.

For instance, some of the first evidence that NF1 regulates the Ras pathway came from studies of the *Drosophila* circadian clock [36]. Recent data indicates that NF1 may cooperate with RASA1 in the induction of non-small cell lung cancer [37]. Modeling this cooperation (Nf1/vap) in flies could provide relevant information about how this oncogenic partnership can be inhibited. Early studies using *Ras85D* demonstrated that constitutively activated Ras confers only a slight proliferative advantage; however, mutations to Ras can cooperate with other mutations to powerfully drive malignant transformation [24, 38]. These findings led to an intense search for RAS oncogenic partners in *Drosophila*, resulting in the discovery of several cancer risk genes whose malfunction provokes Ras-altered cells to progress towards metastasis [39–41]. Other MAPK-related genes are also mutated at high frequencies in human cancers, but their functions are not as well understood; these include LZTR1, AR, RPS6KA3, PLCG1, RRAS2 and PTPN11.

The leucine zipper-like transcriptional regulator 1 protein LZTR1 is an adaptor for the cullin 3 (CUL3) ubiquitin ligase complex [42], which has been implicated in human diseases [43], but its mechanism of action remains underexplored. Inactivation of LZTR1 induces resistance to tyrosine kinase inhibitors (TKI) in chronic myeloid leukemia (CML) [44]. Knock-down of the *Drosophila* LZTR1 homolog Lztr1 during development results in wing vein defects, a phenotype closely resembling that derived from an increase of RAS-MAPK signaling. LZTR1 loss-of-function (LOF) mutants also show augmented RAS-MAPK pathway activation in CML cells, establishing a causal role for these cells in resistance to TKI therapy [44]. The LZTR1 LOF phenotype likely depend on interaction of LZTR1 with CUL3, another conserved CDG (*Cul3*) (Table 2.1). In *Drosophila* germ cells, *Cul3* forms a complex with *GCL* proteins (GMCL1) to target Torso/RTK for degradation [45]. As Torso is the homolog of human PDGFRB, this data point to another possible source of oncogenic signaling. The role of the LZTR1/CUL3 complex is still underexplored in human cancers; a fuller understanding of its mechanisms of action may come from analysis of existing fly data and from further *in vivo* manipulation of the components of the *Lztr1/Cul3* complex.

The androgen receptor (AR) is a nuclear receptor associated with prostate cancer that is amenable to inhibitory drug therapy. In *Drosophila,* the nuclear receptor dERR shares homology with both human AR and ERRs (oestrogen receptors). dERR regulates the expression of genes involved in glucose metabolism and switches transcription towards glycolytic metabolism, supporting cell growth [46]; this resembles the metabolic switch observed in the Warburg effect [47]. Glycolytic metabolic regulation in tumorigenic processes is also affected by mammalian ERRs [48], suggesting that signaling by AR/ERRs might control tumor growth, at least in part, by promoting a Warburg-like phenotype.

RPS6KA3, also known as RSK2 (*S6KII*), is emerging as a key signaling molecule involved in controlling human cell proliferation and transformation. RPS6KA3 activity relies on phosphorylation at different sites on the protein by activated ERK. Although there was controversy around the roles played by different phosphorylation sites [49, 50], this has been resolved more recently in flies. Research into circadian rhythms in fly clock neurons has shown that ERK binds to and phosphorylates *S6KII* at specific sites [51, 52], while studies of *Drosophila* eye differentiation have revealed a novel regulatory mechanism whereby *S6KII* negatively regulates Ras/ERK activity by acting as a cytoplasmic anchor [53]. Furthermore, *S6KII* has been shown to interact and cooperate with *CkIIα* (CK2beta) [51]. *CkIIα* regulates Hippo signaling by promoting *Wts* activity, leading to phosphorylation and inhibition of *Yki* activity. This uncovers a dual role for *CkIIα*, in that it acts in cell survival, but also as a growth inhibitor [54]. Moreover, RPS6KA3 integrates several other pathways relating to cell survival, chromatin remodeling, and inflammatory and immune reactions [55]. Therefore, further studies of the role of RPS6KA3/SK6II in cell growth control may help to shed light on as-yet-unexplored cancer interactions.

PLGC1 encodes phospholipase C-γ (PLC-γ), an enzyme which generates two intracellular

messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), following RTK phosphorylation. In *Drosophila*, the PLC-γ gene is encoded by *small wing* (*sl*) [56], which negatively regulates the MAPK cascade [56] via controlled retention of Spitz (an Egfr ligand) in the endoplasmic reticulum [57]. During cell growth, *sl* is activated by the insulin pathway, but also responds to Egfr; however, during cell differentiation, *sl* activated by the insulin receptor negatively regulates the Egfr/MAPK pathway [58]. Therefore, *sl* coordinates the switch between cell growth and cell differentiation following activation by the insulin receptor. This switch depends on components downstream of *sl*, such as the IP_3R and Pkc53E (PKC) [57]. Importantly, decreasing the expression of Egfr, Spitz and Pkc53E blocks tumor growth prompted by PI3K/Pten aberrant signaling in flies [35]. However, how these processes occur in mammals is yet to be explored.

PTPN11 encodes SHP-2, a tyrosine phosphatase which is highly conserved between species [59]. SHP-2 regulates signaling for several RTKs, such as EGFR and FGFR, through the activation of the RAS/MAPK cascade, leading to cell proliferation, differentiation and migration [60, 61]. Inhibition of PTPN11 blocks signaling from the RTK pathway and causes sensitivity to BRAF inhibitors in colon cancer [62], making PTPN11 a potential drug target. The protein Corkscrew (csw) is the *Drosophila* SHP-2 homolog. A cross-species cancer cell study in *Drosophila* and humans revealed that *csw* unexpectedly binds directly to the Pi3k21B (p60) regulatory subunit of PI3K (p50/p85 human homolog). However, it does not associate with Pi3k92E, the human homolog of the p110 catalytic subunit [63]. A similar association to that of *csw* and PI3K was also reported in BCR-ABL-positive H929 multiple myeloma cancer cells, showing that SHP-2 binds directly to free p85 (not the p85/p110 PI3K heterodimer) and impairs PI3K signaling, while enhancing ERK/MAPK signaling [63]. This new interaction may have implications for drug treatment and resistance in cancer.

While the roles of Ras85D have been extensively described in flies, the closely related protein Ras64B, the fly ortholog of the CDG RRAS2, has not yet been studied and therefore offers an additional focus for future investigations. Studies of the MAPK/RAS components discussed here, whose roles are as yet underappreciated, may help us to better understand tumor initiation, progression and acquired drug resistance in human cancers.

2.3.3 PI3K/Akt Signaling

The main components of the PI3K pathway have long been characterized as oncogenes and tumor suppressors [64]. Among the main conserved components of the PI3K signaling pathway mutated at high frequencies in human cancers are AKT1, MTOR, PIK3CA-CG, PIK3R1-R2, PTEN and TSC1–2 (Table 2.1). These genes play fundamental roles in regulating growth in response to nutrient availability and coordinate key metabolic processes which regulate cell proliferation and survival [65]. Research in *Drosophila* has ushered the way to understanding how these components control physiological, systemic and cellular growth [66, 67], as well as investigating how their dysregulation promotes tumorigenesis [68].

A lesser known component of the PI3K pathway implicated in cancer is the driver gene PPP2R1A (*Pp2A-29B*). *It* encodes one of the subunits of the protein phosphatase 2A (*PP2A*) complex, and is a tumor suppressor and a regulator of PI3K signaling via AKT inhibition [69]. PP2A is a heterotrimeric phosphatase formed by three multiprotein cores that bind to each other: a structural core (A); an invariant catalytic core (C); and a family of B regulatory subunits. PPP2R1A forms part of the subunit A. The role of the B subunit is to direct the core formed by the A and C subunits (AC core) to different substrates [70]. In *Drosophila,* subunit A is composed solely of the protein *Pp2A-29B* [71]. *Drosophila* dividing neuroblasts depleted of *Pp2A-29B* display mitotic abnormalities [71, 72], exhibiting aberrant elongation of microtubules with a high proportion of abnormal spindles [71]. Further work in *Drosophila* has shown that PP2A

regulates the balance of neural stem cell self-renewal and differentiation, which, if lost, results in improper asymmetric cell division and the development of a brain tumor [73]. However, the precise role that PP2A plays seems to depend on which B subunit interacts with the AC core. The *Drosophila* B subunit includes either *well rounded (wrd)* or *widerborst (wdb)* [71]. *Pp2A-29B* regulates stress-induced autophagy in two alternative ways depending on specific interactions with either the wdb or wrd regulatory subunits. Whereas the A/wdb/C complex acts upstream of dTOR, the A/wrd/C complex functions as a target of dTOR and may regulate the elongation of autophagosomes and their subsequent fusion with lysosomes [74]. Similarly, regulation of InR/PI3K/Akt1/Tor signaling involved in fat metabolism depends on whether Cyclin G (*CycG*) binds to either A/wdb/C or A/wrd/C [75]. Thus, the specific activity of PP2A "fine tunes" the InR/PI3K/Akt1/Tor signaling cascade in *Drosophila* [75]. This work helps to shed some light on the largely unexplored links between PPA2- and AKT-related metabolic changes during tumorigenesis.

2.4 Other Signaling Hubs: WNT, TGF-β, HH, and GPCRs

Wnt/APC/β-catenin signaling provides a further example of a highly conserved pathway whose dysregulation is associated with tumorigenesis [76]. *Drosophila* tissues have provided a powerful physiological context in which both known and novel Wnt pathway components can be investigated [77–79]. These investigations have helped to define the molecular mechanisms by which Wnt is involved in tissue growth and patterning [80, 81]. A number of conserved components of this pathway are considered CDGs, including APC and CTNNB1. Studies on their fly counterparts, *Apc* and *armadillo* (encoding β-catenin), have identified a role for APC/β--catenin signaling in controlling mitosis [82], guiding subcellular localization [83], and regulating intestinal stem cell proliferation in gut tumors [84–86].

Another signaling component discovered in *Drosophila,* which has been studied in detail during development and was later recognized as being important in a major cancer pathway, is Hedgehog (HH) [87]. HH signaling members *SPOP* and PTCH1 are relevant CDGs whose roles in tumorigenesis are only starting to be documented. Their *Drosophila* homologs, *roadkill* (*rdx*) and *patched* (*ptc*), respectively, are well characterized in flies and their further study in specific tumor contexts can continue to contribute to our understanding of the roles of *hh* proteins in cancer.

An emerging class of CDGs are those belonging to the TGF-β signaling pathway. Conserved genes such as ACVR1B (*baboon*), ACVR2A (*punt*) and SMAD2/4 (*smox/Med*), among others, are present in several cancers (Table 2.1). Recent data shows that *Drosophila* TGF-β/activins derived from the intestine can modulate fat metabolism remotely [88]. Tumor-organ communication is well described in *Drosophila* [89, 90], which provides a unique model to address long-distance regulation by secreted factors. The question of whether activins remotely control lipid and carbohydrate homeostasis to drive tumor growth in certain contexts, however, remains to be addressed. Research efforts also need to be taken towards understanding GPCR signaling, with CDGs such as GNA11, GNAQ (Gαq) and GNAS (Gαs) gaining attention. Modeling of G-protein function and interactions in flies offers some promise for understanding the role of GPCRs in tumorigenesis. Similarly, the connection between immune response pathways and driver genes is well-known [91]. Cancer drivers related to the NF-κB pathway, such as TLR4 and MYD88 (*Tehao* and *Myd88*), are responsible for activating the innate immune system across different species [92] and are very well described in *Drosophila* [93], but they have not yet been investigated in cancer studies.

2.5 Chromatin-Related Factors

The chromatin SWI/SNF (switching [SWI]/sucrose non-fermenting [SNF]) complex has attracted attention, given the association between

mutations in the genes encoding its subunits and cancer [94]. The SWI/SNF complex is a negative regulator of growth [95] composed of several subunits which was originally discovered and characterized in yeast and *Drosophila* [96, 97], and later in mammals [94, 98]. Several SWI/SNF complex subunits, such as ARID1A (*osa*), ATRX (*XNP*), PBRM1 (*polybromo*), ARID2 (*Bap170*), SMARCA4 (*brm*) and SMARCB1 (*Snr1*), are conserved CDGs (Table 2.1). In particular, ARID1A/*osa* is one of the most commonly mutated genes across different cancer types.

The main hurdle to understanding the role of mutations in specific subunits of the SWI/SNF complex is our lack of knowledge regarding how the different subunits organize and assemble. A cross-species study comparing yeast, fly and mammalian complexes found a high level of conservation for the specific modular organization and functional architecture of the complex [99]. These findings reinforce the idea that *Drosophila* studies are helpful for investigating how mutations in SWI/SNF components are involved in tumorigenesis and for elucidating the mechanisms of SWI/SNF-mediated chromatin remodeling in oncogenic states. For example, *osa* was shown to prevent tumorigenesis by inducing a transcriptional program limiting self-renewal and preventing dedifferentiation in neuroblasts, which ensures correct progression along stem cell lineages [100]. Moreover, this study provided a mechanistic explanation for the tumor-suppressing activity of SWI/SNF and showed that *osa*, as part of the SWI/SNF complex, is important for determining which subsets of NF-κB inflammatory target genes are chosen [101]. This indicates a link between the function of the SWI/SNF complex and the immune response, which is yet to be explored in mammalian systems.

Finally, numerous other conserved CDGs have great potential to be modulated in flies (Table 2.1), including those involved in the following processes: metabolism (IDH1 and 2 –*idh*-); splicing (SF3B1 –*Sf3b1*-); RNA abundance (PCBP1 –*mub*-); apoptosis (CASP8 –*Dredd*-); protein homeostasis (FWXB7 –*ago*-, KEAP1 –*Keap1*-, USP9X –*faf*-); genome integrity (MSH2 –*spel1*-, ERCC2 –*Xpd*-); and chromatin formation (H3F3A -*His3.3B*-, H3F3C -*His3.3A*-).

2.6 A Genetic Toolkit in *Drosophila* for Modeling CDG Mechanisms

Drosophila has long been at the heart of biomedical genetic research, thanks to the continuous development of ingenious and sophisticated genetic tools. The feasibility of conducting fast and low-cost large-scale unbiased genetic screens, and the high level of gene homology in CDGs (Table 2.1) [102, 103] has enabled novel genetic cooperations, interactions, and regulatory mechanisms to be tested and context-specific roles of homologs genes to be identified. *Drosophila* can also be used as a model system to express mutant versions of human cancer genes. The aggressiveness and metastatic capacity of different combinations of driver genes can be modeled for each type of cancer using innovative technologies such as RNA interference (RNAi) and CRISPR/CAS9 (see below). The use of these tools, together with clonogenic techniques available in flies, allow direct studies of complex gene-gene interactions in which the microenvironment can be also controlled. As a consequence, it becomes possible to design more efficient drug treatments to specifically target particular gene-gene interactions [104].

The *Drosophila* life cycle comprises a series of discrete stages, namely the embryonic (24 h), larval (5 days), pupal (~6 days), and adult (60–100 days) stages, which approximately correlate with the embryonic, juvenile, and adult stages in vertebrates. Similar to humans, aging flies can develop spontaneous tumors [105]. *Drosophila* larvae go through three different instars, of which the wandering third instar larval stage (LIII) is routinely used to study tumorigenesis. Tissues from the wing and the eye-antenna imaginal discs (EADs), which are groups of undifferentiated epithelial cells, are most commonly used to study tumorigenesis in the larval (juvenile) stage. The imaginal disc cells divide rapidly and exponentially during the larval stages, increasing the size

of the disc by approximately 1000-fold. The epithelial imaginal disc cells show properties similar to the mammalian epithelial cells and are amenable to cellular transformation.

Using these imaginal tissues and the tools reviewed below, the roles of CDGs can be modeled and studied, including their roles in unrestrained proliferation, invasion, genome instability and metabolic reprogramming, among others [106].

2.6.1 Traditional Methods: From Flies to Humans

Classical techniques have helped to identify cancer genes in *Drosophila*, in many cases leading to the discovery of the tumorigenic role of their human counterparts [3–7]. Historically, carcinogen ethyl methanesulfonate (EMS) and X-rays were commonly used to generate random mutations in *Drosophila* genes. Irradiating flies with X-rays is very effective at inducing genome rearrangements, especially deletions and inversions, which usually result in LOF mutations. In contrast, EMS typically causes missense or nonsense point mutations [107]. Classical EMS and X-ray genetic screens have led to the discovery of the core components of major conserved signaling pathways, such as Notch, Hippo, Wnt and Hedgehog [108–111]. Another approach to creating mutations that was introduced as a genetic tool in the 1980s involves modifying P-elements, which are transposable elements [112, 113]. Since then, P-elements have been widely used as mutagens and as tools for generating transgenic animals. In the past 30 years, several initiatives have been launched to generate a P insertion for every gene in the *Drosophila* genome [114–116] (see Box 2.1). In the Exelixis project, the transposons inserted also contain Flipase Recognition Target (FRT) sites (see below). In the presence of FLP recombinase, trans-recombination between FRT elements results in a genomic deletion between the P insertion sites, allowing "customized" aberrations to be generated in a very efficient manner [117]. Since different transposons have different specificities for their target sites, their use increases the efficiency and versatility of genomic analysis. The incorporation of a *Minos*-based transposon, MiMIC [118], has brought new advantages in terms of genomic manipulation, because of its very low site speci-

Box 2.1: Drosophila resources for tumor modeling *in vivo*

- ***CRISPR/CAS9***
  - https://fgr.hms.harvard.edu/vivo-crispr-0
    - Collection of guided RNA for KO and OE
  - https://www.crisprflydesign.org/library/
    - Reagents, protocols and results from fly CRISPR-Cas9 experiments
  - https://shigen.nig.ac.jp/fly/nigfly/
    - Protocols for vector construction and reagents
- ***P -ELEMENTS***
  - GDP (genome disruption project)
    - http://flypush.imgen.bcm.tmc.edu/pscreen/about.html
      - Provides a constant updated collection of different types of transposon insertions.
  - DrosDel project
    - http://www.drosdel.org.uk/#
      - An isogenic deficiency kit cytologically mapped
  - Exelixis
    - https://drosophila.med.harvard.edu/
      - Collection of deficiencies
  - RNAi lines
    - https://bdsc.indiana.edu/
    - https://stockcenter.vdrc.at/control/main
    - https://shigen.nig.ac.jp/fly/nigfly/rnaiListAction.do?browseOrSearch=browse
  - GAL-4/GAL-80, Split-GAL-4, LexA/LexAop, UAS, Q system, MARCM, and more
    - https://bdsc.indiana.edu/
    - https://stockcenter.vdrc.at/control/library_vt

ficity. MiMIC contains an *hsp70* promoter upstream of the GAL-4 gene (see below) and may therefore function as an enhancer detector/trap if inserted in the appropriate location. A full collection of supplies for creating deficiencies and MiMIC insertions, as well as other reagents, can be obtained from the Bloomington Drosophila Stock Center (Box 2.1) and can be found at the FlyBase website (http://flybase.org/).

P-elements have become a leading genetic tool in the field when combined with GAL-4/UAS technology [119]. This useful, highly flexible, and yet simple tool allows ectopic gene function to be systematically studied with precise temporal control and cell-type specificity. GAL-4/UAS utilizes two components: (1) yeast GAL-4, a transcriptional activator placed downstream of a promoter/enhancer region, which is expressed in a defined subset of cells; and (2) one or more copies of an upstream activating sequence (UAS), to which GAL-4 specifically binds. When flies carrying GAL-4 are crossed with flies carrying a specific UAS transgene, their progeny express the transgene selectively in the tissue driven by GAL-4. Fly lines expressing GAL-4 which direct transgene expression to very specific cell populations are available from public stock centers, such as the Vienna Tiles project [120] and the Janelia GAL-4 collection [121]. The powerful, innovative ways in which this system has been used have galvanized the development of numerous novel methods for expressing CDGs in a precise way.

The gene search (GS) system developed by Aigaki and collaborators [122] modifies the P-element by inserting two copies of UAS near the terminal inverted repeats at each end of the vector, which are oriented to direct transcription outward. The GS system has a greater sensitivity than the GAL-4/UAS system because it systematically drives gene misexpression to either side of the GS insertion. Application of the GS method has led to the discovery of several CDGs which are capable of cooperating with aberrant Notch signaling during tumorigenesis. These include epigenetic silencers and the Akt1 gene [123], which has also been shown to cooperate in human leukemia [124]. Another important tool for misexpression is the EP element [125], which enables the conditional overexpression of almost any gene of interest. One example of this approach was the use of EP lines to identify 12 novel tumor suppressor genes that alter signaling through the RAS pathway [126].

2.6.2 Next-Generation Tools: From Humans to Flies and Back to Humans

Following their discovery, the manipulation of RNAi pathways [127], which are conserved in most eukaryotic species, has emerged as a powerful method for gene-specific knock-down screens. The RNAi method silences target genes by cleaving and degrading target mRNA transcripts using double-stranded (ds)RNAs homologous to the target RNA. RNAi is widely employed to perform "reverse" genetics as well as 'forward' genetic screening, in which the expression levels of specific genes are dampened. Transgenic libraries of RNAi constructs stably integrated into individual *Drosophila* stocks are publicly available (see Box 2.1) [128–130]. These RNAi lines can be expressed in any tissue under the control of GAL-4 drivers in order to restrict gene silencing and can be combined with GAL-4 repressors, GAL-80, for a controlled time and space [131]. More recently, CRISPR/Cas9, which acts as a bacterial defense system against invading viruses, was discovered and has become an invaluable tool for efficiently introducing a wide variety of genetic alterations applicable to both loss- and gain-of-function studies [132–134]. Genomic engineering using CRISPR is based on generating dsDNA breaks using the Cas9 endonuclease, which targets specific genomic sequences using crRNA (CRISPR RNA). As a result, the cell is forced to repair the breaks, allowing the original sequence to be modified during this repair process. This method eliminates one shortcoming of earlier approaches, which relied on selecting a desired mutation from a pool of randomly generated mutations. The CRISPR/Cas9 method makes it possible to mimic precise mutations in CDGs in a tissue-specific

manner by expressing Cas9 using the GAL-4/UAS system (for step-by-step instructions on CRISPR/Cas9 editing in *Drosophila*, see Box 1) and [135]. This therefore allows cooperating CDGs mutations to be studied more accurately.

2.6.3 Modeling Oncogenic Interactions Using Clonal Analysis

Clonal analysis techniques, which were pioneered in the 70s by Garcia-Bellido [136], are crucial for understanding cell–cell interactions. Generation of clones through mitotic recombination requires the exchange of chromosomal segments between homologous chromosomes. This is possible because mitotic chromosomes can pair in *Drosophila*, unlike in mammals, in which pairing is restricted to meiosis. Clonal analysis results in the generation of two individual populations of cells that originate from the same mitotic recombination event within the same tissue. This technique in *Drosophila* remains to this day a powerful research tool for geneticists, which has been pivotal in uncovering genes and signal transduction pathways involved in tumorigenesis, such as the Wnt, TGF-β and Hippo pathways. Furthermore, it allows cell-autonomous and non-cell-autonomous effects to be discriminated. This is a matter of utmost importance when it comes to clarifying cellular interactions between mutant cells and the surrounding wild-type tissue during tumour initiation or progression.

Clonal analysis techniques have been improved by the introduction of P-elements [137–139], and FLP/FRT which led to the development of a highly efficient mitotic recombination system. These are placed in a genomic region-of-interest found on the arms of every chromosome, allowing the controlled generation of mitotic clones for more than 95% of *Drosophila* genome *in vivo*. By inducing mosaic clones in a tissue, we can understand many aspects of tumorigenesis, such as how tumor cells escape, invade and colonize different tissues. One example of this approach is the introduction of mutations into clones of eye imaginal disc cells. As the eye tissue is attached to the larval brain, the metastatic potential of cancer cells can be easily measured by analyzing adjacent brain structures. This allows mosaics to be created bearing a given driver mutation surrounded by either wild-type cells or by cells bearing a different driver mutation. This approach therefore makes it feasible to address complex oncogenic interactions, such as non-cell-autonomous cancer features.

Improvements have been made to the mosaic technique by the introduction of the MARCM system [140, 141]. This method generates fluorescently labeled cells with distinct genotypes within the same tissue, allowing the effects of multiple mutations to be modeled together. MARCM allows single cells or groups of cells related by lineage to be positively labeled, in order to generate homozygous mutations and simultaneously express a driver gene of interest. MARCM employs the GAL-4/UAS system combined with the GAL-4 repressor GAL-80 (tub-GAL-4) and the FLP/FRT system. Repression of GAL-4 by the GAL-80 protein results in unmarked cells that are heterozygous for both GAL-80 and a mutation. After FLP/FRT-dependent mitotic recombination, homozygous mutant cells lack GAL-80 and, therefore, possess an active GAL-4 that can activate reporter genes, such as UAS-GFP. This method allows cooperative effects between oncogenes and tumor suppressors to be screened, closely mimicking CDGs cooperation observed in most cancers.

By combining MARCM with Flybow [142], stochastic labels in different "colors" can be applied to different clones of the same tissue, allowing their lineage to be determined and their development and interactions to be traced. As GAL-4 drivers sometimes label more than one cell subtype, the GAL-4/UAS binary system can be used in combination with the QF/QS [143] and LexA/LexAop [144, 145] systems, which results in more precise spatio-temporal control of gene expression and the creation of distant genetic mosaics. In addition, tumor transplantation in adult flies [146, 147] offers a further way to measure the metastatic potential of a given driver gene in a living organism. Lastly, clarifying the ways in which novel CDGs act may also lead to the design of new therapeutic approaches. *Drosophila* has proved to be an excellent system

for drug discovery e.g. [3, 35, 148, 149]. It therefore holds great potential as a model system for basic cancer research, as well as for testing anti-cancer therapies.

2.7 Final Remarks

Every cancer is unique, being triggered by specific oncogenic interactions and the context. Understanding the precise interactions among genetic mutations found in tumors from specific patients is of central importance in cancer treatment optimization. To effectively ascertain that a given mutation or combination of mutations is relevant for a particular cancer, it is necessary to manipulate gene expression *in vivo* in different combinations and cellular contexts. By creating genetically modified flies expressing defined combinations of genes and mutations in a temporally and spatially controlled manner, it is possible to systematically and cost-effectively test these candidate CDG mutations. This approach holds tremendous clinical promise in terms of devising novel anti-cancer strategies. The use of *Drosophila* has already led to crucial contributions in the field of cancer research and in the future, it will continue to serve as a model system for investigating particular aspects of tumorigenesis and metastasis.

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3 *Drosophila* Models of Cell Polarity and Cell Competition in Tumourigenesis

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Abstract

Cell competition is an important surveillance mechanism that measures relative fitness between cells in a tissue during development, homeostasis, and disease. Specifically, cells that are "less fit" (losers) are actively eliminated by relatively "more fit" (winners) neighbours, despite the less fit cells otherwise being able to survive in a genetically uniform tissue. Originally described in the epithelial tissues of *Drosophila* larval imaginal discs, cell competition has since been shown to occur in other epithelial and non-epithelial *Drosophila* tissues, as well as in mammalian model systems. Many genes and signalling pathways have been identified as playing conserved roles in the mechanisms of cell competition. Among them are genes required for the establishment and maintenance of apico-basal cell polarity: the Crumbs/Stardust/Patj (Crb/Sdt/Patj), Bazooka/Par-6/atypical Protein Kinase C (Baz/Par-6/aPKC), and Scribbled/Discs large 1/Lethal (2) giant larvae (Scrib/Dlg1/L(2)gl) modules. In this chapter, we describe the concepts and mechanisms of cell competition, with emphasis on the relationship between cell polarity proteins and cell competition, particularly the Scrib/Dlg1/L(2)gl module, since this is the best described module in this emerging field.

Keywords

Cell competition · Cell polarity · Drosophila · Scrib · D1g1 · L(2)gl · Hippo · Myc · PTP10D Sas · Toll · Flower · Jak/Stat · JNK · TNF · Caspase · EGFR · Ras

3.1 Cell Competition

Cell competition can be described as a biological surveillance mechanism, conserved from *Drosophila* to mammals, that allows cells to sense each other's relative fitness levels and actively eliminate the ones that are "less fit" [6, 23, 74]. These less fit cells are commonly referred to as "loser cells", while the "more fit" cells that remain in the tissue are called "winner cells" (Fig. 3.1a). A key aspect of cell competition interactions is that they are context dependent – this means that loser cells, if present in a genetically homogeneous tissue, proliferate and survive (Fig. 3.1b). This indicates these cells do not

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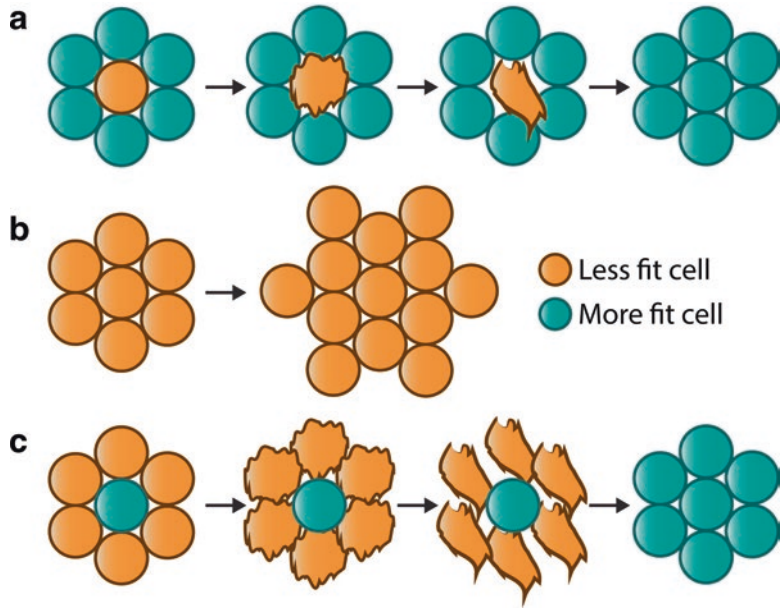

Fig. 3.1 Cell competition. (**a**) Less fit cells (losers) are recognized by more fit cells (winners) and eliminated. Compensatory proliferation by winner cells ensures tissue integrity is maintained. (**b**) Less fit cells present in a tissue populated only by cells of that genotype have equal relative fitness, and so cell proliferation, instead of elimination via cell competition, takes place. (**c**) Mutant cells that have a higher relative fitness than their *wild-type* neighbours will eliminate them and take their place in a process termed super-competition

possess an intrinsic propensity to die – only within a mosaic tissue are they recognised and actively eliminated by more fit cells. Broadly, there are two distinct types of cell competition: (1) elimination of cells due to acquired characteristics that render them less fit, and (2) elimination by cells due to acquired characteristics that render them more fit (a.k.a. "super-competition"). In the first scenario, cell competition is thought be important for preventing disease by allowing correct tissue and organ development, maintaining tissue homeostasis, or delaying aging, since it promotes the survival of the best quality cells while eliminating those that could be harmful for the individual [82, 83]. In the second case, "super-fit" mutant cells are capable of eliminating perfectly healthy *wild-type* cells (Fig. 3.1c). This form of cell competition is thought to be related to the progression of diseases such as cancer.

Cell competition was first described four decades ago in *Drosophila* wing imaginal discs [88]. It was observed that cells heterozygous for a *Minute* (*M*) mutation (a class of mutations that affect the genes encoding various ribosomal proteins [64, 78]) were less fit than their *wild-type* neighbours. In tissue consisting of only $M^{+/-}$ cells, they would persist and form adult structures, but when the $M^{+/-}$ cells were part of a mosaic tissue with *wild-type* neighbours, the resulting adult wings were exclusively *wild-type* in cellular constituency, suggesting the context-dependent elimination of $M^{+/-}$ clones [88, 121]. Initially, it was proposed that the $M^{+/-}$ cells were being outcompeted due to their slower rates of growth and division, a known consequence of cell-autonomous disruptions to ribosomal proteins [122]. However, two decades after this first description of $M^{+/-}$ cell competition, it was shown that $M^{+/-}$ clone elimination was also apoptosis dependent, as blocking apoptosis via inhibition of the c-Jun N-terminal Kinase (JNK) signalling pathway, as well as by expression of the Caspase-inhibitor p35, was sufficient for their survival [90]. Therefore, although differences in proliferation rates may contribute or

render cells sensitive to cell competition-dependent elimination, other mechanisms also contribute. Interestingly, further studies have shown that a high proliferation rate on its own – via overexpression of cell cycle regulators like Cyclin D and Cyclin-dependent kinase 4 (Cdk4) or increasing insulin signalling by expression of the catalytic subunit of the phosphatidylinositol 3-kinase *Pi3K92E* [25], or by mutation of *Phosphatase and tensin homolog* (*Pten*) [48] – is insufficient to induce cell competition. This finding confirms that cell proliferation rates are not sufficient to trigger cell competition. Furthermore, *l(2)gl* mutant clones are outcompeted in the *Drosophila* wing epithelium, despite having no significant differential in their proliferation rate compared to the *wild-type* [80].

As mentioned, in other cases, mutations can confer a winner status on cells, giving them a so-called "super-competitive" status, and the ability to eliminate their *wild-type* neighbours. Activating mutations in the proto-oncogene *Myc* lead to a classic example of this super-competitor phenotype [25, 89]. Myc is a conserved transcription factor that regulates genes involved in ribosome biogenesis and cell growth [26, 57] and, when ectopically activated in a mosaic tissue, induces super-competition and drives the elimination of *wild-type* cells via JNK-dependent apoptosis [89]. This finding led to the hypothesis that cancer cells with activating mutations in *Myc* (or similar oncogenic mutations) might utilize these genes to increase their relative fitness level and invasive capabilities [1, 30]. Similar competitive behaviours have been observed in mammals. Cells overexpressing *MYC proto-oncogene, bHLH transcription factor* (*MYC*, orthologue of *Drosophila Myc*) in a mosaic fashion outcompete their *wild-type* neighbours during the early stages of mouse development [22], during the onset of embryonic stem cell differentiation [117], or even in adult mouse cardiomyocytes [140]. Activating *Myc*/*MYC* is clearly a highly conserved method of acquiring a winner cell phenotype, as highlighted by many other studies [22, 29, 31, 75, 123]. Mechanistically, in *Drosophila*, *Myc*-initiated cell competition requires the tumour suppressor p53 in the winner cells for loser cell elimination, as Myc increases the glycolytic flux of the winner cells, a step necessary to drive their higher proliferation, and p53 is required to sustain these metabolic changes [27]. When p53 is absent, winner cells show impaired oxidative respiration, increased DNA damage, and apoptosis, and mutation of p53 in the winner cells also abolishes their ability to initiate loser cell elimination [27]. However, the apoptotic death of $M^{+/-}$ cells during competition is independent of p53, suggesting in this context that p53 may not perform such a role [60].

Since these earliest descriptions of cell competition, many more conserved genes, signalling pathways, and biological processes have been shown to induce cell competition upon altered expression or mutation: morphogens like Wingless [125, 142] or Decapentaplegic/Brinker [90, 117] (through the suggested "competition for survival factors" mechanism), the Flower code [84, 109], Salvador-Warts-Hippo pathway members [21, 40, 80, 133], innate immunity pathway members [3, 62, 85], mechanical stress [67, 76, 82, 120, 141, 143], metabolic differences (reviewed in [23]), Janus kinase-Signal Transduction and Activator of Transcription (Jak-STAT) signalling [65, 115, 150], the Src pathway [36, 51, 59, 139], endocytic pathway components (e.g. Avalanche/Syntaxin, Vps25, Erupted/TSG101, and Rab5) [7, 81, 87, 132, 135], and tumour-suppressive mechanisms and apico-basal cell polarity regulator genes [2, 13, 21, 34, 41, 55, 77, 96, 102, 127], with this final example being the focus of this chapter. However, while cell competition is broadly accepted as a form of cell elimination as a result of cell-cell interactions, there is such a diversity of mechanisms and molecules involved (notwithstanding potential interactions between them) and, as such, a consensus on the precise classification of different types of cell competition has not been reached.

Cell competition has been described using a variety of model systems. While *Drosophila* imaginal discs and cultured epithelial cells are the best-studied systems, cell competition has also been reported to occur during the development of cardiomyocytes in the mouse heart [140], and in embryonic stem cells at the onset of differentiation

[117]. Furthermore, it has been suggested that the originally described cell competition mechanism – induced via changes in ribosome biogenesis – is conserved in mammals. Cells with mutations in ribosomal protein-encoding genes during the development of mouse chimeric blastocysts are eliminated by cell competition, but survive normally in a heterozygous mutant mouse [101]. Cell competition has also been observed in non-developmental stages: for example, transplanted foetal liver cells can replace larger numbers of adult liver hepatocytes because of their high proliferation rate and capacity to induce apoptosis in the surrounding cells [97]. Similarly, in the mouse adult bone marrow, exposure to radiation induces changes in relative levels of the p53 tumour suppressor protein, and induces hematopoietic stem cells to engage in cell competition with one another based on the level of radiation-induced cellular stress (e.g. DNA damage) they have accrued [10]. There are many more examples of cell competition occurring in mammalian systems, both *in vivo* and *in vitro* [51, 58, 59, 63, 77, 143], but they are beyond the scope of this review. In this chapter, we review the literature regarding cell competition specifically involving cell polarity regulators in *Drosophila*.

3.2 Cell Polarity Regulator Proteins and Cell Competition

Cell shape is fundamentally important for the morphology, movement and function of all types of cells, and contributes significantly to the establishment and maintenance of tissue architecture. The shape of a cell depends on its polarity, which can be defined as the asymmetric distribution of its cellular components. Disturbing the balance of these components at the cellular level can lead to the mislocalization of proteins involved in signalling pathway regulation, altered cell behaviours like proliferation, differentiation, or survival, and disruptions to cell movement and migration, all of which can have far-reaching consequences on tissue architecture and homeostasis [53]. The disruption of epithelial cell polarity is considered a hallmark of epithelial cancers [49, 92, 131], and the loss of cell polarity and tissue architecture has been strongly correlated with metastatic disease [8, 92].

Apico-basal cell polarity is unique to the establishment and maintenance of polarity in epithelial tissues, contributing to the localization of cell junctions and the formation of the zonula adherens (bands of aligned adherens junctions encircling cells in an epithelium) that is critical for tissue integrity. Apico-basal cell polarity is defined by the specialised apical and basal surfaces formed in a cell – the apical surface faces the outside of the body or tissue, and the basal surface adheres to the basement membrane. Three different protein complexes/modules are required for epithelial cell polarity: the Scrib/Dlg1/L(2)gl module, the Baz/Par-6/aPKC complex, and the Crb/Sdt/Patj complex [53, 129]. These three modules act in a mutually antagonistic manner to define apical and basal-lateral membrane domains, and enable the formation and positioning of cell junctions and the correct apico-basal cytoskeletal structure (Fig. 3.2).

Of the aforementioned cell polarity genes, *scrib*, *dlg1*, and *l(2)gl* are the only ones defined as neoplastic tumour suppressors, as massive overgrowth of the highly proliferative tissues is observed in larvae homozygous mutant for any one of them. This is particularly apparent in the imaginal discs, where neoplasia leads to the tissues losing their organised structures and becoming grossly overgrown [9, 144]. As mentioned, however, the presence of cells homozygous mutant for any of these polarity regulators in a mosaic tissue initiates the process of cell competition, and the mutant cells are eliminated by their *wild-type* neighbours [13, 21, 32, 40, 46, 47, 55]. Recent research has uncovered additional roles for other polarity regulators in cell competition in *Drosophila,* and we shall discuss each relevant case in turn in this chapter.

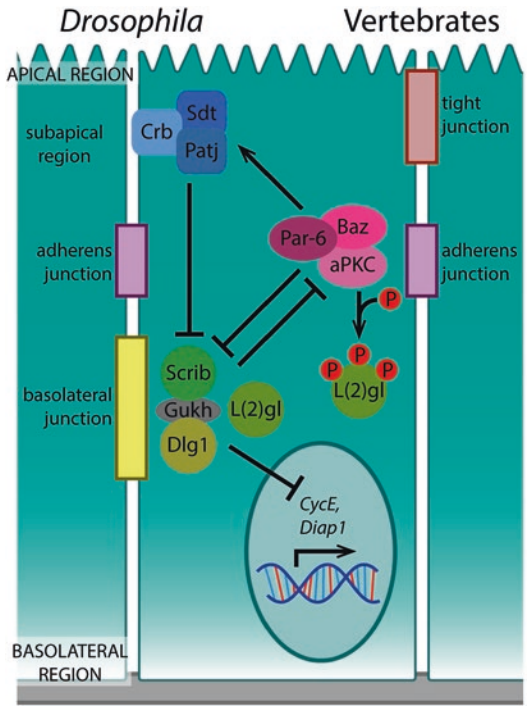

Fig. 3.2 Apico-basal cell polarity. Epithelial cells are polarized along their apico-basal axis by the localization of three protein modules. The Baz/Par-6/aPKC complex is localized to the sub-apical domain, as is the Crb/Sdt/Patj complex, while the Scrib/Dlg1/L(2)gl module is localized to the basolateral domain. Scrib and Dlg1 interact via Gukh, and do not directly complex with L(2)gl. Each complex/module regulates the activity and position of the others. For example, aPKC-mediated phosphorylation of L(2)gl excludes it from the apical cortex, and ensures that the Scrib/Dlg1/L(2)gl module remains located at basolateral/septate junctions (which serve an equivalent function to tight junctions in vertebrates). Scrib/Dlg1/L(2)gl also control tissue growth by inhibiting the expression of the cell cycle gene, *CycE*, and the cell death inhibitor, *Diap1*. Figure adapted from Humbert et al. [52]

3.2.1 Scribbled and Discs Large 1

Almost 20 years ago, Brumby and Richardson [13] published a study that would provide a foundation for the study of polarity regulators in cell competition, although it was not recognised as such at the time. Exploring cell polarity regulators in *Drosophila* epithelial tissues, their aim was to characterize the behaviour of *scrib* mutant clones in a *wild-type* background in order to mimic the early stages of mammalian tumour development. They found that although *scrib* mutant cells ectopically expressed the G_1-S-phase cell cycle regulator, *Cyclin E* (*CycE*) and showed ectopic cell proliferation, overgrowth of the mutant tissue did not occur [13]. Inducing elimination of the surrounding *wild-type* cells allowed *scrib* mutant clones to overgrow, suggesting that the "apoptosis signal" originated from these *wild-type* neighbours, and demonstrating that cell polarity regulator gene mutations could give cells a "loser" fate in a mosaic tissue [13]. This group, and others, further demonstrated that the reason for this elimination phenotype was activation of the JNK signalling pathway, specifically in the loser cells, which promoted Caspase-dependent cell death and Caspase-independent elimination of the *scrib* mutant clones [13, 21, 55, 66, 134]. Interestingly, blocking JNK signalling was capable of rescuing *scrib* mutant clone death and elimination, but blocking cell death via expression of *p35* or *Death-associated inhibitor of apoptosis 1* (*Diap1*) was only able to partially rescue the small size of *scrib* mutant clones [13, 21], suggesting a potential disconnection between cell elimination and cell death. This work has profoundly influenced the cell competition field, and continues to be applied and extended today. Indeed, it has recently been demonstrated that this mechanism also occurs in non-epithelial tissues: *scrib* mutant neuroblast clones are also eliminated via the JNK pathway by surrounding *wild-type* clones [113].

Furthermore, cell polarity-impairment can also trigger cell competition in mammalian cell culture: 50% of *SCRIB* knockdown Madin-Darby Canine Kidney (MDCK) cells die through apoptosis after 60 h of tetracycline induction when plated with *wild-type* MDCK cells [96]. Here, apoptosis of the *SCRIB* knockdown cells is dependent on the activation of JNK-family member, p38 [96], rather than JNK signalling that occurs in the *Drosophila* system [13, 55, 134]. Moreover, in *SCRIB* knockdown cells, p53 is upregulated in response to tissue crowding, and

this increase in p53 levels is necessary for loser cell elimination [143]. Thus, the role of p53 in cell competition initiated via loss of cell polarity appears to be very different to that in *MYC*-initiated cell competition [27].

3.2.1.1 Signalling Pathways Regulating Cell Competition

Tumor Necrosis Factor – JNK Signalling

The stress-inducible JNK signalling pathway is a highly conserved mitogen-activated protein kinase signalling pathway with a diversity of roles. JNK signalling involves a kinase core that is conserved regardless of context, but upstream initiators of the pathway can be highly varied. One such initiator is Eiger (Egr), the *Drosophila* orthologue of the ligand for the mammalian Tumor Necrosis Factor (TNF)-TNF Receptor signalling pathway (Fig. 3.3) [54, 91]. Egr is present in imaginal disc epithelial cells and was shown to be necessary for elimination of *scrib* and *dlg1* mutant cells from mosaic tissue [56]. Egr acts in a paracrine manner to induce TNF pathway signalling in the mutant cells [138], however expression of *egr* specifically in *scrib* mutant cells is sufficient to induce their elimination [56], suggesting it can also function in an autocrine manner. However, the source of Egr in the context of *scrib*/*dlg1* mutant clone elimination is controversial – it has been demonstrated that Egr secreted by haemocytes (macrophage-like cells) is necessary for the removal of *l(2)gl* mutant cells [24, 138], and *wild-type* haemolymph is capable of rescuing JNK activation in *scrib* mutant cells [24], suggesting that haemocyte-derived Egr is the most important source. Indeed, haemocytes are recruited to loser cells during Myc- initiated cell competition [18, 69] via the conserved mechanism of secretion and cleavage of Tyrosyl-tRNA synthetase (TyrRS), although this process requires autonomous activation of JNK signalling, suggesting both intrinsic and extrinsic JNK-activation mechanisms are at play [18]. In that vein, Egr has also been shown to be dispensable for JNK activation in some contexts. Intrinsic activation of JNK signalling in a ligand-independent manner occurs in *scrib*/*dlg1* mutant clones through elevated Rho1 activity and activation of Wallenda (Wnd), a JNKKK [72, 93]. Additional mechanisms regulating JNK activity include the apoptosis program, which is capable of initiating JNK signalling via p53 or Death regulator Nedd2-like caspase (Dronc) activity – this produces a positive feedback loop, as JNK, as mentioned, is a well-established initiator of apoptosis [119]. An important remaining question is how does TNF-JNK signalling promote death of the loser cells specifically, when it is possible that extracellularly-sourced Egr is available in equal proportions to adjacent cells? It has recently been shown that post-translational modification of Grindelwald (Grnd, a *Drosophila* TNF Receptor [4]) via glycosylation is capable of promoting Egr-Grnd binding [28]. Such a modification

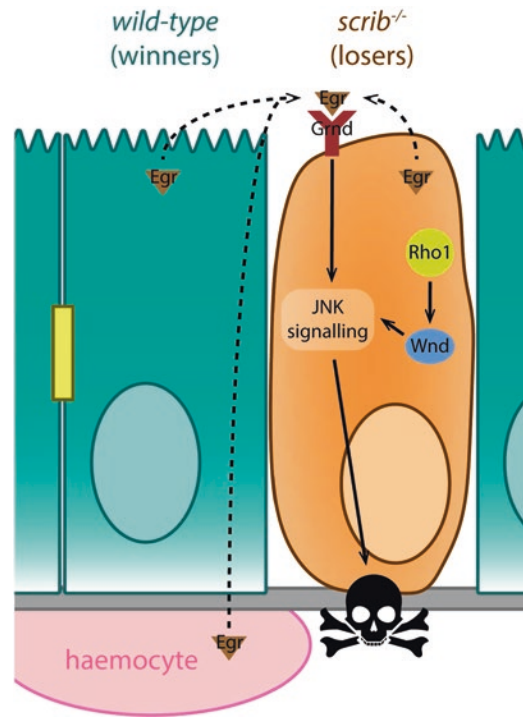

Fig. 3.3 JNK pathway activation. JNK signalling activation in polarity regulator mutant cells is necessary for their elimination. Egr is a ligand for the TNF Receptor, Grnd, and is thought to activate the JNK pathway during cell competition. Egr may act in an autocrine manner, or it may be secreted by neighbouring *wild-type* cells or haemocytes. The JNK pathway can also be activated in an Egr-independent manner by signalling via Rho1 and Wnd (a JNKKK)

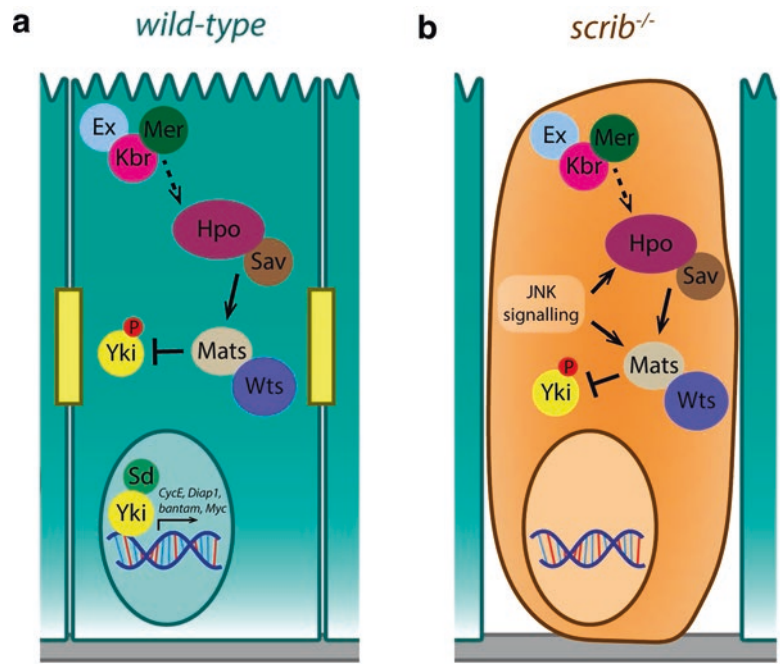

Fig. 3.4 SWH signalling and Yorkie. (**a**) SWH signalling is composed of two core protein kinases, Hpo and Wts, and their respective adaptor proteins, Sav and Mob as tumour suppressor (Mats). The activity of these proteins is positively regulated by the activity of Ex, Kbr, and Merlin (Mer). When active, SWH signalling negatively regulates tissue growth by phosphorylating, cytoplasmically sequestering, and thereby inactivating the co-transcription factor Yki. In *wild-type* cells, a balance occurs between Yki inhibition and its activity. Nuclear-localized Yki binds to Scalloped (Sd), a TEAD-family transcription factor, and initiates transcription of tissue growth/cell proliferation promoting genes, such as *CycE*, *Diap1*, *Myc*, and *bantam*. (**b**) Clones mutant for *scrib* show some upregulation of Yki activity and *CycE* expression, but also upregulate JNK signalling. Among other roles, JNK signalling promotes SWH signalling pathway activity, negatively regulating Yki, and thereby promotes *scrib* mutant cell elimination during cell competition

might enable polarity mutant cell-specific activation of TNF-JNK signalling, and promote their elimination.

Salvador – Warts – Hippo Signalling and Yorkie

Interestingly, the inhibition of JNK signalling in *scrib* mutant cells leads to larger mutant clones than when apoptosis is blocked by inhibiting Caspases in the presence of active JNK [13, 21]. This suggests that JNK could also restrain proliferation, and is not merely facilitating cell death. JNK activation in *scrib* mutant cells enhances Salvador-Warts-Hippo (SWH) activity, thereby blocking activity of the growth-promoting co-transcriptional factor, Yorkie (Yki) (Fig. 3.4a) [21, 32]. Hippo (Hpo) and Warts (Wts) are serine protein kinases, and Salvador (Sav) is an adaptor protein, and, after activation by various upstream pathways, they phosphorylate and cytoplasmically-sequester Yki (Fig. 3.4a) [86]. Yki, together with the transcription factor, Scalloped (Sd), is required for cell proliferation by upregulation of *CycE*, and for survival by induction of *Diap1* and *bantam* miR-dependent inhibition of *head involution defective* (*hid*) mRNA translation (Fig. 3.4a) [98]. *Myc* is also a Yki target [95, 149], and therefore *Myc* expression is expected to be repressed in *scrib* mutant cells through JNK-mediated enhancement of SWH activity, thereby encouraging their competitive elimination (Fig. 3.4b). Consistent with this, the competitive elimination of *scrib* mutant clones can be rescued by expression of *Myc*, likely through its conferral of a super-competitor phenotype [21]. Thus, due to its effect on key

regulators of cell growth, proliferation and survival, the activity of Yki is critical for determining whether mutant cells will undergo cell competition. Yki suppression is thought to be necessary for *scrib* mutant cell elimination and, indeed, an increase in Yki activity is able to rescue less fit cells from being eliminated by cell competition: in cells mutant for *scrib*, ectopic *yki* expression leads to the mutant cells becoming hyperproliferative and overgrowing [21], similar to what was observed for $M^{+/-}$ cells [133], while in otherwise *wild-type* cells *yki* expression transforms them into super-competitors [95].

Jak-STAT Signalling

The Jak-STAT signalling pathway, a conserved regulator of cell proliferation and tissue growth, has recently been shown to have a role in cell competition, with activated Jak-STAT signalling providing cells with a competitive advantage and transforming them into super-competitors though an unknown mechanism [115]. Conversely, cells deficient for Jak-STAT signalling are also outcompeted and eliminated by their *wild-type* neighbours [115]. However, Jak-STAT signalling also has a key role in the elimination of *scrib* mutant cells (Fig. 3.5). Activation of JNK signalling and its downstream transcription factors, Jra (Jun-related antigen) and Kayak (*Drosophila* Fos orthologue) in *scrib* mutant cells, together with Yki/Sd, drives the expression of genes encoding the IL-6-like proteins, Unpaired 1/2/3 (Upd1/2/3) [14, 145], which are secreted ligands for the Jak-STAT signalling pathway. These ligands activate Jak-STAT signalling in the neighbouring *wild-type* cells, which promotes their compensatory proliferation in the face of *scrib* mutant cell elimination [118]. Indeed, in the *wild-type* cells, Jak-STAT activity, together with Yki, is necessary for the elimination of the *scrib* mutant cells – if *Stat92E* (encoding the sole *Drosophila* STAT transcription factor) is mutated in *wild-type* cells, *scrib* mutant cells hyperproliferate and survive due to endogenous Yki activation [118]. It is believed that Jak-STAT and Yki activity in the *wild-type* cells somehow suppresses Yki activity in the *scrib* mutant cells via a "competition signal" [118], although the specifics of this relationship are as yet unclear.

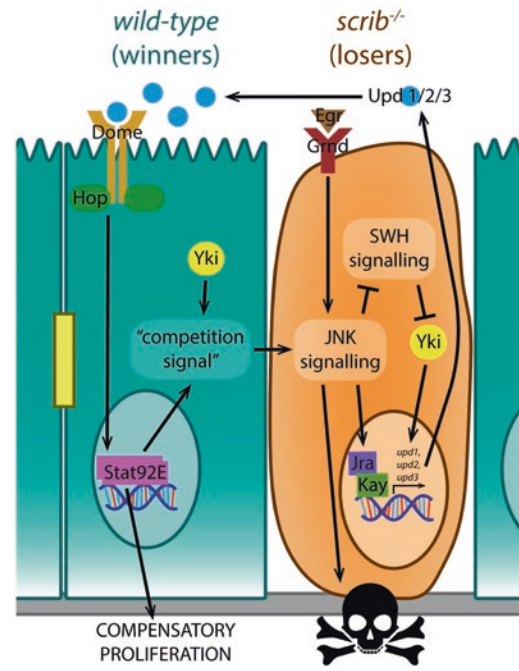

Fig. 3.5 Jak-STAT signalling. JNK signalling in *scrib* mutant cells promote gene transcription via the transcription factors Jun-related antigen (Jra) and Kayak (Kay), together with Yki and Sd. Their targets include the Upd family of ligands, which are then secreted and bind to the Jak-STAT pathway receptor, Domeless (Dome), in their *wild-type* neighbours. This activates the Jak, Hopscotch (Hop), and the STAT, Stat92E. Jak-STAT signalling drives the compensatory proliferation of the *wild-type* winner cells, but also promotes JNK-mediated activation of the SWH pathway and Yki suppression in, and apoptosis of, the *scrib* mutant losers via an unknown "competition signal"

Toll – NF-κB Signalling

In *Drosophila*, two major signalling pathways involved in innate immunity against bacteria and fungi infection are the Toll and Immune Deficiency (IMD) signalling pathways [45, 136]. Canonically, both pathways activate distinct NF-κB family transcription factors – Dorsal (Dl) and Dorsal-related immunity factor (Dif) are effectors of Toll signalling, and Relish (Rel) is an effector of IMD signalling – although there is some evidence that the regulatory relationship is not clear-cut [136]. Toll signalling has recently

been identified to be involved in cell competition to eliminate *scrib* mutant cells, via the unique mechanism of *Serpin 88Ea* (*Spn88Ea*, a.k.a. *Serpin5*) (Fig. 3.6a), which was identified in a screen for "elimination-defective mutants". Spn88Ea is a serine protease inhibitor that prevents Toll activation by the ligand, Spatzle (Spz), and is expressed in the *wild-type* neighbour cells, though it acts in a non-cell-autonomous manner [62]. Loss-of-function of Spn88Ea leads to Toll signalling activation in the *scrib* mutant cells and promotes their overgrowth via Yki activation which, unusually, occurs downstream of JNK pathway activation [62]. In other *scrib* mutant cell competition situations, JNK activation in the mutant cells blocks endogenous Yki activity, and enables the cells to undergo apoptosis, while Yki activation in the *wild-type* neighbour cells promotes their compensatory proliferation [21, 118]. However, the researchers suggest this alternative mechanism is due to the simultaneous accumulation of F-actin and JNK activation, a phenomenon known to induce Yki activity [62]. Additionally, JNK pathway activity has also been shown to activate Yki in a number of other biological contexts in *Drosophila* [126].

Interestingly, the immune system has also been described to play a role in both *Myc*- and *M*-mediated cell competition. In seminal work, differential roles for Toll signalling, IMD signalling, and NF-κB family factors were identified depending on the cell competition context, in each instance acting within the loser cells to promote their elimination [85]. In *Myc* overexpression-induced cell competition (Fig. 3.6b), the researchers identified the involvement of IMD pathway genes in *Peptidoglycan recognition protein LC* (*PGRP-LC*), *Fas-associated death domain* (*Fadd*), *Death related ced-3/Nedd2-like caspase* (*Dredd*), *caspar* (*casp*), and *Rel*, as well as the involvement of Toll pathway genes in *spz*, multiple Toll-related receptors (TRRs: *18 wheeler* (*18w*, a.k.a. *Toll-2*), *MstProx* (a.k.a. *Toll-3*), *Tollo* (a.k.a. *Toll-8*), and *Toll-9*), *Ectoderm-expressed 4* (*Ect4*) and *tube* (*tub*), but not other important factors from both pathways [85]. The researchers interpret this as being indicative of a potential pathway that begins with the TRRs, co-opts components from both canonical pathways, and ends in activity of the NF-κB Rel, and acts within the *wild-type* loser cells to facilitate their death in *Myc*-dependent cell competition via the pro-apoptotic gene *hid* [85]. Interestingly, recent work has shown that Toll-NF-κB signalling during *Myc*-initiated cell competition in the wing imaginal disc is responsible for ensuring cell competition occurs only within the necessary tissue, by restricting Spz synthesis and processing to the local environment [3]. By contrast, in cell competition induced by heterozygosity for the *M*-class gene *Ribosomal protein L14* (*RpL14*) (Fig. 3.6c), researchers identified different genes as being necessary for $M^{+/-}$ cell elimination – specifically those encoding the Toll pathway components *spz*, two TRRs (*MstProx* and *Toll-9*), *cactus* (*cact*), *dorsal* (*dl*), and *Dif*, but they also demonstrated a role for *Rel*, and determined that rather than *hid* expression driving apoptosis, the pro-apoptotic gene *reaper* (*rpr*) was responsible [85]. Detailed mechanisms of Toll-NF-κB signalling in the different modes of cell competition remain unclear, although recent work has also suggested it is dependent on tissue microorganism infection levels [42]. While there is clearly a role for the *Drosophila* innate immune system as a mechanism of cell competition, it remains to be resolved as to why the Toll pathway is pro-cell death during *Myc*- and *M*-driven modes of cell competition but pro-survival during *scrib* mutant cell competition.

3.2.1.2 Systems to Sense Cell Fitness Between Neighbouring Cells

Protein Tyrosine Phosphatase 10D – Stranded at Second Signalling

A recent genetic screen identified *stranded at second* (*sas*) as a gene necessary in *wild-type* cells to maintain their winner phenotype during cell polarity disruption-initiated cell competition [146]. Sas is a cell surface-bound ligand protein, generally localized to the apical surface of epithelial cells. Also identified was the receptor for Sas, Protein tyrosine phosphatase 10D (Ptp10D), which is an apical receptor tyrosine phosphatase. It was found that in *wild-type* cells, Sas relocal-

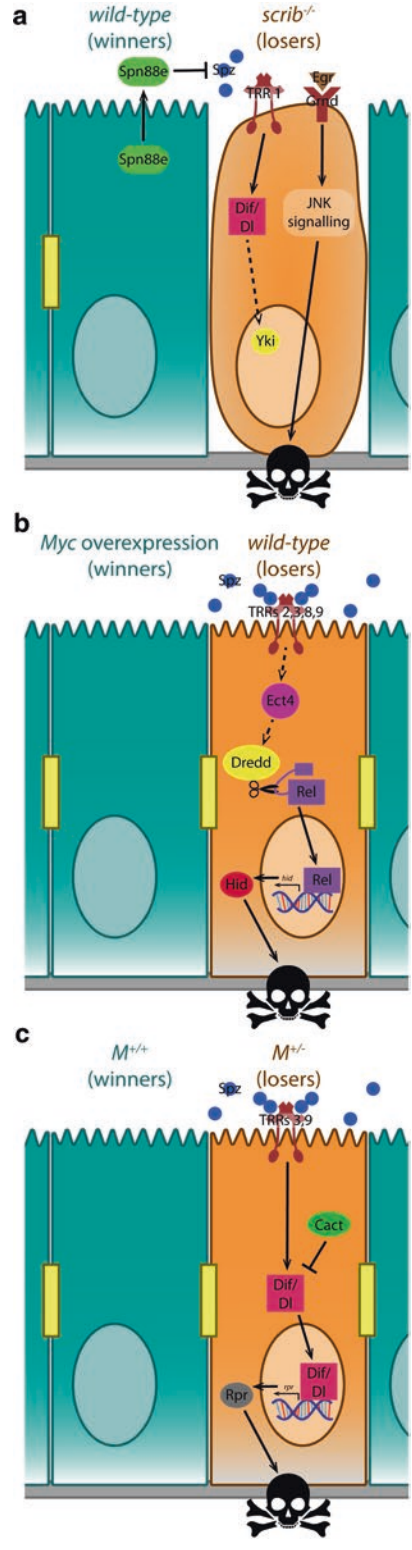

Fig. 3.6 Toll-NF-κB signalling. (**a**) Toll-NF-κB signalling in *scrib* mutant clones leads to Yki activation, but its activation is blocked by Spn88e secretion from the adjacent *wild-type* cells, which inhibit that activity of the Toll ligand, Spz. (**b**) In *Myc*-initiated cell competition, *wild-type* loser cells activate Toll-NF-κB signalling to promote their apoptosis via Rel-mediated *hid* transcription. (**c**) In *M*-initiated cell competition, the $M^{+/-}$ loser cells activate Toll-NF-κB signalling to promote their apoptosis via Dif/Dl-mediated *rpr* transcription

izes to the lateral surface of the cell, where it is adjacent to *scrib/dlg1* mutant cells that concomitantly also relocalize Ptp10D laterally. Thus, the direct transactivation of Ptp10D by Sas is enabled, which then acts to inhibit Epidermal Growth Factor (EGF) signalling via Epidermal growth factor receptor-Ras oncogene at 85D (Egfr-Ras85D) in the *scrib/dlg1* mutant clones, which in turn allows for their elimination via JNK signalling (Fig. 3.7a). However, when either *sas* or *Ptp10D* are depleted, and the transactivation process inhibited, EGF signalling is not inhibited. EGF and JNK signalling then synergistically act to inhibit SWH signalling through an F-actin dependent mechanism, which leads to the derepression of Yki and results in overgrowth of the *scrib/dlg1* mutant clones (Fig. 3.7b) [146].

The Flower Code

While the cell competition mechanisms discussed so far have largely been restricted to well-understood, conserved signalling pathways and molecules, one particularly novel exception is the "Flower code". During *Myc*-initiated cell competition, *flower* (*fwe*) was identified as being upregulated in the *wild-type* loser cells, where it acts to promote their elimination (Fig. 3.8a) [109]. Specifically, *fwe* encodes three transmembrane isoforms, which are differentially expressed, though an unknown mechanism, depending on winner/loser status: Fwe-Ubi is ubiquitously expressed in the larval eye-antennal/wing imaginal disc epithelia, while Fwe-Lose-A and Fwe-Lose-B were found to be specifically upregulated in loser cells at the expense of Fwe-Ubi [109], although there are some differences in these expression patterns in the developing *Drosophila* ommatidia, with only Fwe-Lose-B marking loser cells [84]. Ectopic expression of Fwe-Lose-A or Fwe-Lose-B is sufficient to mark cells as losers and prompt their elimination, providing they are adjacent to *wild-type* neighbour cells [109].

Another cell competition-involved gene identified by the same laboratory is Secreted protein, acidic, cysteine-rich (SPARC), a conserved multifunctional secreted protein that is believed to act in the extracellular matrix [107].

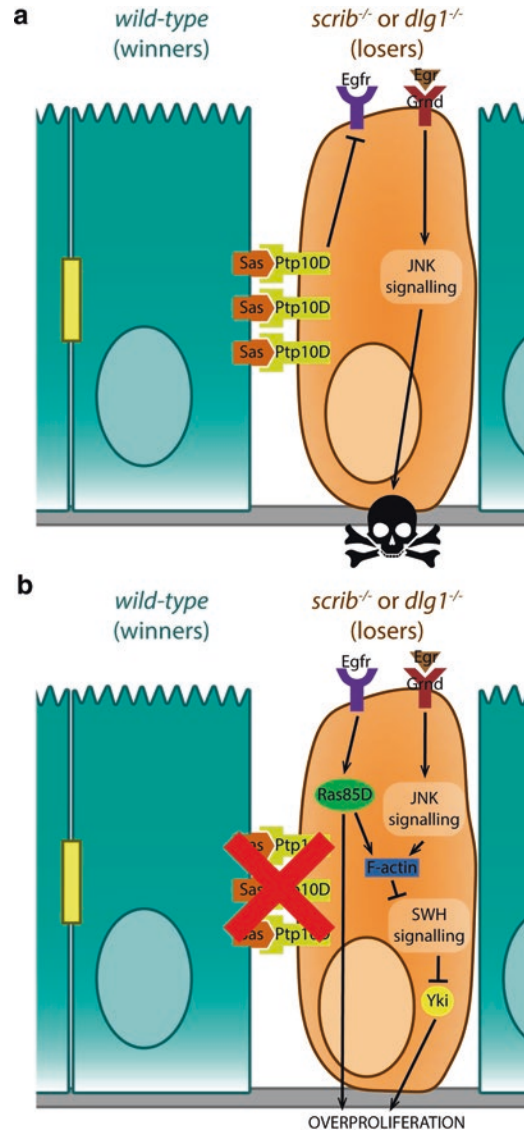

Fig. 3.7 Ptp10D-Sas signalling. (**a**) In *scrib/dlg1* mutant cells, Ptp10D is relocalized to the lateral membrane, and concomitantly Sas is relocalized laterally in the adjacent *wild-type* cells. The ligand-receptor interaction of Sas-Ptp10D then inhibits EGFR signalling, and allows JNK signalling to promote the death and elimination of the mutant cells. (**b**) If Sas-Ptp10D signalling is disrupted, EGFR-Ras85D and JNK signalling cooperate to suppress SWH pathway activity through an F-actin-dependent mechanism, leading to the upregulation of Yki activity, and thereby promoting the overproliferation and survival of *scrib/dlg1* mutant cells

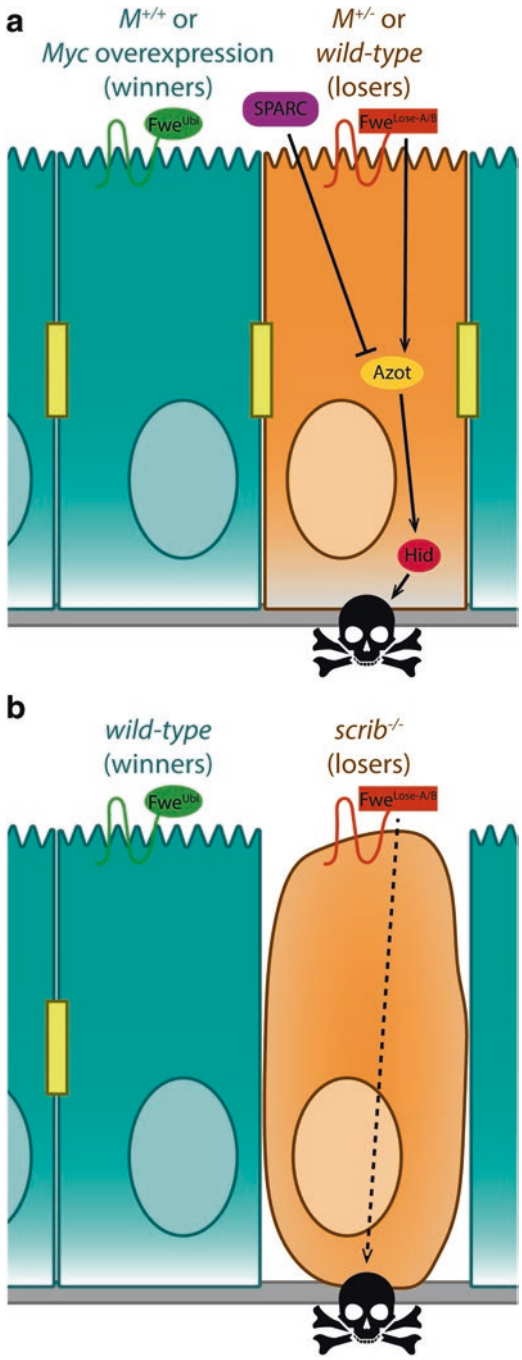

Fig. 3.8 The Flower code. (**a**) Differential expression of Fwe isoforms regulates cell fate in *M*- or *Myc*-initiated cell competition. Winner cells express the Fwe-Ubi isoform, while loser cells express the Fwe-Lose-A or Fwe-Lose-B isoforms. Cell death then proceeds via Azot-mediated Hid activity, with the extracellular protein SPARC also negatively regulating the Flower code-cell elimination process. (**b**) In *scrib* mutant cells, Fwe-Lose-A/B isoforms are expressed, but it is not known if or how they regulate the polarity regulator-initiated cell competition process

SPARC opposes cell competition-induced apoptosis in loser cells by blocking Caspase activation, but does so independently from the Flower code process (Fig. 3.8a) [107]. It has also been shown that Ahuizotl (Azot), a calcium-binding EF-hand-containing cytoplasmic protein with potential enzyme-binding activity, is upregulated specifically in the loser cells when winner and loser cells confront one another, and its mutation abolishes loser cell elimination in *M*-, *Myc*-, Wg signalling-, and Jak-STAT signalling-initiated cell competition. Azot, likely acts downstream of both Fwe and SPARC, as it is activated upon Fwe-Lose-B expression, and it is suppressed upon SPARC-mediated apoptosis inhibition [83].

However, what role does the Flower code and its related genes play in cell polarity regulator-dependent cell competition? Fwe-Lose-A and Fwe-Lose-B were also found to be upregulated in loser cells after competition initiation via the clonal induction of $M^{+/-}$ cells, *thickveins* (*tkv*, encodes a receptor of the TGF-β signalling pathway) mutant cells, and *scrib* mutant cells (Fig. 3.8b) [109], suggesting it may play a general role in all cell competition varieties. SPARC is upregulated in *l(2)gl* mutant clones in wing imaginal discs but is otherwise unexplored [107], while Azot does not appear to have a role in cell polarity-initiated cell competition [83].

3.2.1.3 Systems to Eliminate the Loser Cells During Competition

Slit – Roundabout 2 – Enabled Signalling

Signalling via Slit (Sli), a ligand, Roundabout 2 (Robo2), its transmembrane receptor, and the downstream actin polymerase Enabled (Ena, a.k.a. VASP), canonically described as a conserved neural axon guidance system important in cell repulsion and migration [12], has recently been described as being crucial in the apical or basal extrusion of *scrib/dlg1* mutant cells (Fig. 3.9) [111, 137]. Upon basal extrusion, the *scrib/dlg1* mutant cells undergo apoptosis, presumably due to their recognition by haemocytes, which reside on the basal lamina, but upon apical extrusion, they survive and overgrow [137]. Slit, Robo2, and Ena act downstream of JNK, and are in fact transcriptional targets of JNK signalling (an example of an alternative role for

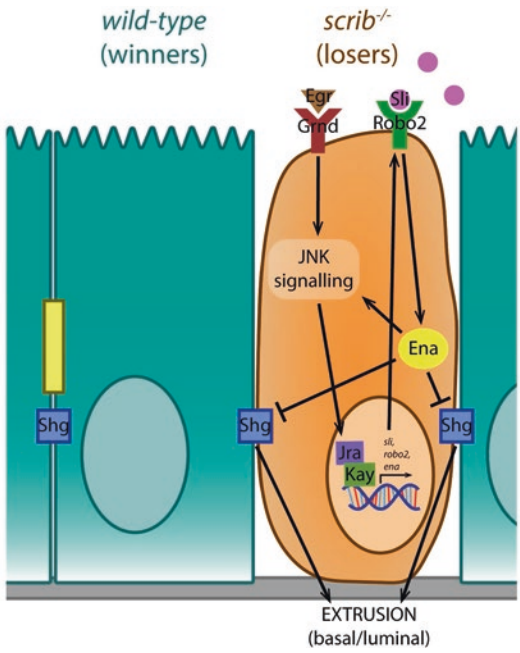

Fig. 3.9 Sli-Robo2-Ena signalling. The extrusion of *scrib* mutant clones depends on Sli-Robo2-Ena signalling pathway activity. JNK signalling transcriptionally upregulates *sli*, *robo2*, and *ena* expression. Sli then activates Robo2, which promotes Ena-mediated inhibition of Shg/E-cadherin and elevates JNK activity, thereby promoting the basal/luminal extrusion of the mutant cells

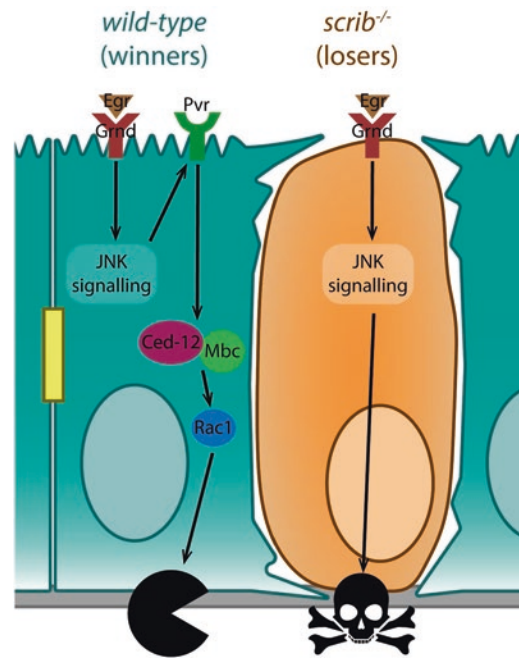

Fig. 3.10 Engulfment. The Pvr-Ced-12-Mbc-Rac1 signalling cascade is necessary for engulfment of *scrib* mutant cells by their *wild-type* neighbours. Initiated by JNK signalling, activation of the receptor, Pvr, promotes Ced-12, Mbc, and Rac1 activity to drive the engulfment process

JNK signalling during cell competition that is independent from cell death). Mechanistically, Slit-Robo2-Ena promote cell extrusion by downregulating expression of *shotgun* (*shg*, a.k.a. *E-cadherin*), a key adherens junction molecule, which is then expected to reduce cell-cell adhesion capabilities [137].

PDGF- and VEGF-Receptor Related – Ced-12 – Myoblast City – Rac1 Signalling

JNK signalling can also be activated by Egr in the *wild-type* cells surrounding polarity-disrupted cells [99]. JNK activation in these cells promotes expression of PDGF- and VEGF-receptor related (Pvr), the *Drosophila* PDGF/VEGF receptor, and activates Rac1 through Ced-12 (a.k.a. ELMO) and Myoblast city (Mbc) [99]. This mechanism promotes the apoptotic engulfment of *scrib* mutant clones by their *wild-type* neighbours (Fig. 3.10), although it is thought that this is a lesser contributing factor to cell elimination than the Slit-Robo2 system, and also than the involvement of haemocytes in JNK-mediated cell death of *scrib* mutant cells [24, 69, 70, 138]. Despite the clear necessity of Egr for JNK-mediated apoptosis of the polarity-disrupted cells, it remains unclear whether or how *scrib* mutant cells signal to activate the TNF-JNK signalling pathway in *wild-type* neighbours [99].

3.2.2 Lethal (2) Giant Larvae

Unlike the other apico-basal polarity regulators, Scrib, Dlg1 and L(2)gl do not form a traditional protein complex. Although they all act in a common process [9, 100, 106], only Scrib and Dlg1 are believed to physically associate via the mediator protein GUK-holder (Gukh) [16, 79], while *Drosophila* L(2)gl is not known to directly physically interact with its module partners. In this vein, there are differences in how disruptions to the Scrib/Dlg1/L(2)gl module members affect the process of cell competition, even though their

mutations are essentially indistinguishable in their effects on entire epithelial tissues. While mutations in *scrib* or *dlg1* are thought to be essentially interchangeable in the mechanisms of how they initiate cell competition, mutations in *l(2)gl* lead to a somewhat different cell competition process.

While *scrib* or *dlg1* mutant clones generated in developing eye epithelia possess polarity disruptions, *l(2)gl* clones do not until the latter stages of development – for example, polarity in the inter-ommatidial/pigment cells is still retained in the pupal stage [46]. It is possible that this is due to L(2)gl maternal contributions persisting beyond that of Scrib and Dlg1 (there are relatively large maternal contributions of each of these proteins [8]). *l(2)gl* mutants ectopically express CycE and overproliferate, indicating that the disruptions to cell polarity and proliferation are independently regulated in *l(2)gl* mutant clones, as this ectopic proliferation occurs without loss of apico-basal polarity (Fig. 3.11) [46]. They also upregulate another cell cycle regulator, *Cyclin A* (a downstream target of Notch signalling that is elevated in *l(2gl* mutant tissue), which similarly promotes cell proliferation [103]. However, in situations when L(2)gl is further depleted (as in *l(2)gl* mutant clones in a $M^{+/-}$ background), cell polarity disruptions appear and a more severe ectopic cell proliferation phenotype manifests [46]. Therefore, it seems that specific levels of L(2)gl are necessary to negatively regulate cell proliferation and maintain apico-basal cell polarity.

L(2)gl depletion results in the upregulation of pathway targets commonly seen upon SWH signalling inhibition: CycE, Diap1, Four-jointed (Fj), and Expanded (Ex), as well as increased levels of active Yki (Fig. 3.11) [47]. As such, it has been hypothesised that elevated Yki levels might

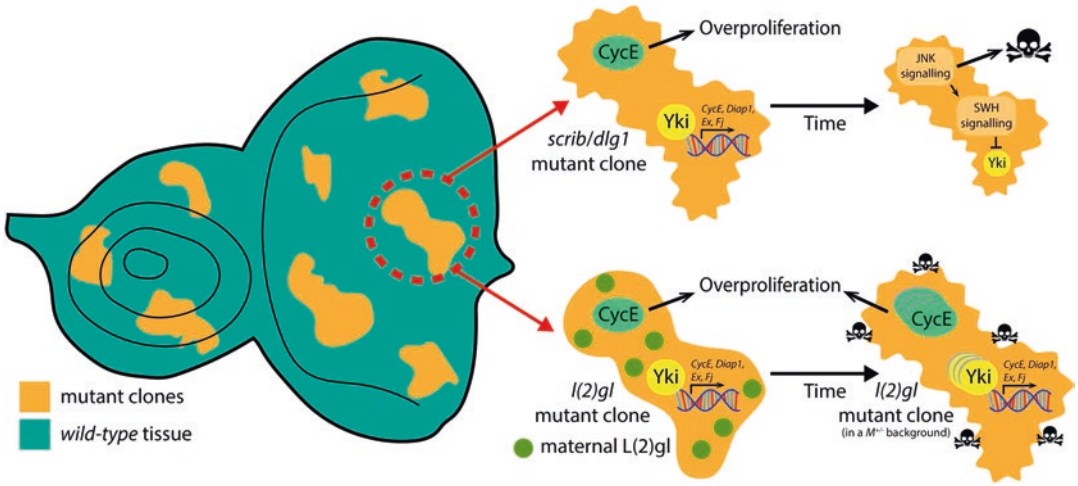

Fig. 3.11 Cell proliferation is independent of cell polarity disruption in *l(2)gl* mutant clones. When generated in third instar larval epithelial tissues (like the represented eye-antennal disc), clones homozygous mutant for *scrib/dlg1* have disrupted apico-basal polarity (represented by the rough edges), and upregulate Yki activity and several of its targets (i.e. *CycE*, *Diap1*, *Ex*, and *Fj*). However, expression of Yki targets is eventually blocked by JNK signalling, which activates the SWH pathway and consequently represses Yki activity, limiting proliferation and promoting cell death (represented with a skull). *l(2)gl* mutant clones do not initially show a cell polarity disruption phenotype, yet show elevated *CycE* expression, leading to overproliferation of the mutant clones. Thus, *l(2)gl*, but not *scrib/dlg1*, regulates cell proliferation independently from cell polarity. Persistence of maternal L(2)gl is thought to be responsible for this phenomenon, as in a $M^{+/-}$ background, when *l(2)gl* mutant clones are forced to proliferate for additional time, cell polarity is eventually disrupted, and more extensive overproliferation occurs. Loss of *l(2)gl* regulates cell proliferation by inhibiting SWH pathway signalling and derepressing Yki. Cell death occurs at the *l(2)gl* mutant clone borders at a lower level than with the *scrib/dlg1* clones (represented by the small skulls) and, as such, *l(2)gl* clones do not reduce in size, as overproliferation and apoptosis are in equilibrium

protect *l(2)gl* mutant cells from cell competition and, consistent with this, halving Yki levels in *l(2)gl* mutant clones rescues the *l(2)gl* mutant adult phenotype [47], although whether it reduces *l(2)gl* mutant clone size has not been examined. In *scrib* or *dlg1*-depleted clones (by RNAi-mediated knockdown) where cell polarity is largely not disrupted, Yki activity is not upregulated, indicating that Yki upregulation in Scrib/Dlg-depleted tissue is a consequence of cell polarity disruption [47]. In *scrib* mutants, where cell polarity is lost, Yki is upregulated, CycE is ectopically expressed and some ectopic cell proliferation occurs [13], however this in eventually counteracted by JNK signalling [21, 32].

Furthermore, it is important to highlight the relationship between aPKC and L(2)gl: as aPKC is capable of directly antagonizing L(2)gl, misregulation of aPKC could also lead to cell competition. This hypothesis is supported by the observation that the impairment of SWH signalling in L(2)gl mutant clones is dependent on aPKC, and mild aPKC activation in a whole tissue suppresses SWH signalling [47]. aPKC might regulate the SWH pathway in *Drosophila*, as occurs in mammalian cells, by directly interacting with Hpo and preventing Wts association and activation [5]. However, overexpression of a constitutively active version of aPKC in otherwise *wild-type* clones, results in cell polarity defects and a reduction in clone size relative to controls due to JNK-dependent cell death [66], although whether this is due to cell competition is not known. Finally, similar to *scrib*, an *l(2)gl* orthologue is likely to be involved in cell competition in a mammalian model system [127].

3.2.2.1 Elimination of *l(2)gl* Mutant Clones Is Tissue Dependent

As with *scrib* or *dlg1*, whole *l(2)gl* mutant tissue displays a loss of apico-basal polarity, and overgrows to produce neoplastic tumours. As mentioned, *scrib* and *dlg1* mutant clones are eliminated from the tissue when surrounded by *wild-type* cells via the activation of JNK signalling. However, in the case of *l(2)gl*, whether and how the mutant cells are eliminated is tissue dependent. Specifically, JNK-dependent *l(2)gl* mutant cell elimination has been observed in *Drosophila* wing imaginal discs, where clones are almost completely eliminated and, as such, do not contribute to the formation of the adult wing structures (Fig. 3.12a) [40]. However, similar clone elimination is not observed in the eye-antennal imaginal discs, where *l(2)gl* mutant clones undergo apoptosis at their borders (apoptosis of *wild-type* cells at the border was also observed but at lower levels), but the clone sizes do not decrease (Fig. 3.12b), and the mutant tissue contributes to adult structures [46]. It was concluded that cell competition-driven apoptosis of the *l(2)gl* mutant tissue is occurring to some degree, but ectopic cell proliferation is compensating for any losses [46].

Furthermore, in the wing imaginal disc, it has been observed that the elimination of *l(2)gl* mutant clones was much more efficient in the distal region of the disc (pouch) relative to the proximal regions (hinge and notum) (Fig. 3.12a) [40]. Also, similar to a *scrib* mutant mosaic scenario, the *wild-type* cells surrounding the *l(2)gl* mutant clones showed compensatory proliferation, offsetting the loss of mutant tissue [40]. However, unlike *scrib*/*dlg1* mutant clone elimination, it appears that *l(2)gl* mutant clone elimination does not rely on endocytosis-mediated TNF-JNK signalling, but does still require endocytosis-mediated JNK signalling (Egr was demonstrated to be dispensable for the process) [40].

3.2.2.2 Elimination of *l(2)gl* Mutant Clones in the Wing Epithelium Is Myc Dependent

Myc is an important factor in many facets of cell competition, as we have discussed, and *l(2)gl*-initiated competition is no exception: the sensitivity of *l(2)gl* mutant clones in the wing epithelium to elimination by cell competition depends on Myc levels. *l(2)gl* mutant clones express Myc at significantly lower levels compared to their neighbouring cells in the wing, and this difference in Myc expression triggers *l(2)gl* mutant elimination [40]. *Myc* downregulation in *l(2)gl* mutant clones is likely to occur at the post-transcriptional level, as it was observed that when *Myc* was overexpressed via a heterolo-

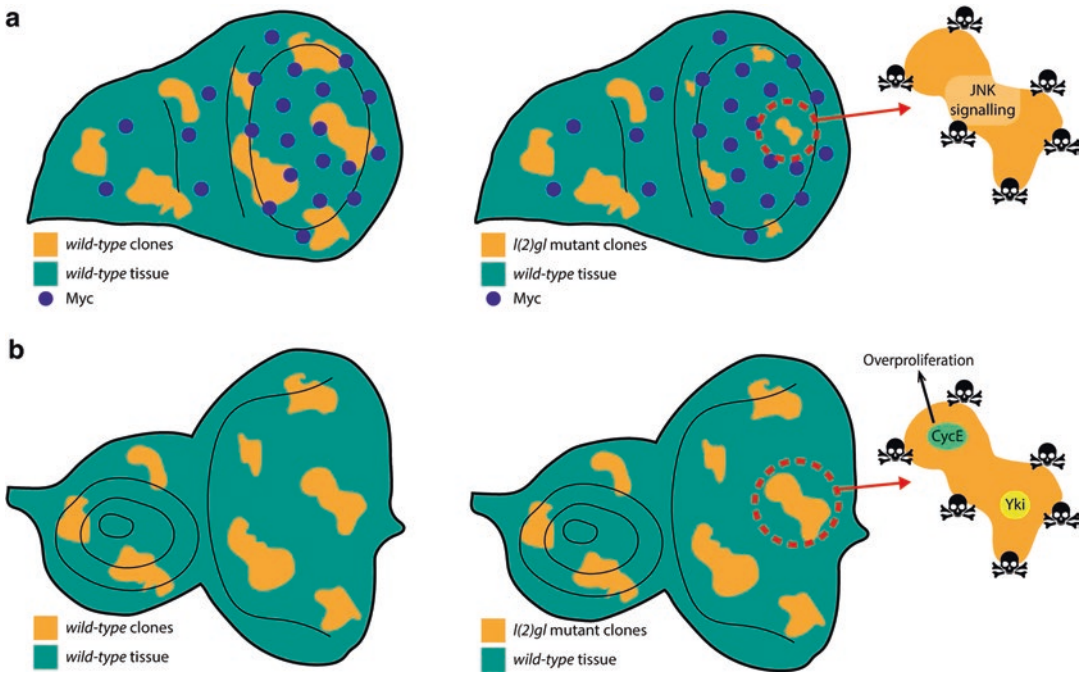

Fig. 3.12 Clone elimination is tissue- and Myc-dependent. (**a**) In third instar larval wing imaginal discs, *l(2)gl* mutant clones are eliminated through cell competition – JNK pathway activity induces apoptosis at the clone border (represented with skulls). Interestingly, a higher level of cell competition occurs in the distal region of the wing disc, which correlates with higher Myc expression in this region. (**b**) In third instar larval eye-antennal imaginal discs, *l(2)gl* mutant clones are not eliminated from the organ. While some JNK-mediated apoptosis is occurring at the clonal border in both *l(2)gl* mutant and *wild-type* cells, it is thought that any cell death is compensated for via overproliferation due to increased Yki activity and CycE expression in *l(2)gl* mutant cells

gous promoter in *l(2)gl* mutant clones, Myc levels were still low [40]. Differences in *l(2)gl* mutant cell removal efficiency in the distal and proximal regions of the wing imaginal disc also correlate with the endogenous pattern of Myc expression: while highly expressed in the distal region, Myc expression is very low in the proximal region (Fig. 3.12a) [40]. Interestingly, at the developmental stages examined, the *l(2)gl* mutant clones were eliminated despite not showing cell polarity loss, but showing reduced Myc expression, which is in contrast to observations from the eye epithelium where SWH signalling is impaired and clones are not eliminated (Myc levels have not been investigated) (Fig. 3.12b) [46]. Similarly, in another epithelial tissue, the ovarian follicular epithelium, *l(2)gl* mutant clones also do not lose cell polarity nor are they eliminated, likely due to their higher levels of Myc expression relative to the surrounding *wild-type* ($l(2)gl^{+/-}$) cells [40]. Here, *Myc* is upregulated at the transcriptional level, likely via impaired SWH signalling, which negatively regulates *Myc* transcription [95, 149], as well as *Diap1*, which was also upregulated [40]. In this setting, *wild-type* ($l(2)gl^{+/-}$) cells immediately surrounding the *l(2)gl* mutant clones show active-Caspase 3 staining and, at later stages, the *l(2)gl* mutant cells lose polarity and became invasive, a phenotype that was shown to be Myc-dependent. Interestingly, in the wing epithelium, if *l(2)gl* mutant clones were generated in a $M^{+/-}$ background, then they did exhibit cell polarity loss, upregulation of *Diap1* and *Myc*, and overgrowth of the mutant clones [40]. Thus, depending on the tissue and context, the expression of Myc in *l(2)gl* mutant clone dictates cell elimination or super-competitive behaviour. However, why Myc is upregulated in some con-

texts but not in others is unclear, and whether it involves the relative levels of JNK and SWH signalling is yet to be investigated.

3.2.2.3 Mahjong

Tamori et al. [127] identified Mahjong (Mahj) as an evolutionary conserved L(2)gl-binding protein, and another player in *l(2)gl* mutant clone cell competition. Mahj is poorly understood in *Drosophila*, but has a mammalian orthologue in VprBP (a.k.a. DCAF1), which was originally identified as physical partner of the human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) [148]. It was demonstrated that *l(2)gl* or *mahj* mutant clones underwent apoptosis when surrounded by *wild-type* cells in the wing disc epithelium, and, furthermore, that the apoptosis of *l(2)gl* mutant clones was reduced when *mahj* was overexpressed in *l(2)gl* mutant clones [127]. Loss of *mahj* did not induce observable defects to apico-basal cell polarity, suggesting that Mahj does not function with L(2)gl in cell polarity regulation. Instead, it was suggested that L(2)gl positively regulates Mahj's effector function, or possibly the signalling pathways that influence competitiveness in cells [127]. While it is still unclear precisely how L(2)gl interacts with Mahj during cell competition, and what other molecules/signalling pathways are involved in the process, it was observed that apoptosis of *l(2)gl* and *majh* mutant clones proceeds via JNK signalling, as in *scrib* mutant clones. *Mahj* knockdown cells are also subject to cell competition in mammalian cells - co-culturing of mammalian MDCK cells expressing inducible shRNAi against the ortholog of *mahj*, *VprBP* with *wild-type* MDCK cells, results in 45% of *VprBP* knockdown cells dying and being extruded from the apical surface of the monolayer after 24–52 h of induction [127]. Interestingly, VprBP has also been shown to be sequestered by Lgl2 from binding to the Cul4 ubiquitin ligase, which is required for the G_1-S-phase cell cycle transition, thereby inhibiting cell proliferation [147], but how this relates to the involvement of Mahj in *l(2)gl* mutant cell competition in *Drosophila* is unknown.

3.2.3 Crumbs

3.2.3.1 Crumbs Alteration Can Induce Competitor or Super-Competitor Behaviours

Genetic screens that identify mutations that confer increased growth potential (or, in other words, a winner phenotype) have identified many genes in *Drosophila*, including the titular SWH pathway components *salvador* and *hippo* [50, 128], and, more recently, the polarity regulator *crumbs* (*crb*) [48]. As described above, Crb is a key component of the Crb/Sdt/Patj complex, and encodes a transmembrane protein with a long extracellular domain (ECD) and short intracellular domain (ICD). The ECD of Crb contains many EGF repeats [130], which are essential for mediating protein-protein interactions. Crb is, in fact, capable of complexing with other Crb molecules on adjacent cells, which is thought to allow for Crb stabilisation at the cell border, the inhibition of apoptosis, and the undertaking of non-cell autonomous functions like, for example, cell competition. Furthermore, the ECD has also been reported to play a role in preventing the endocytic removal of Crb via recruitment of the regulators of the SWH pathway, Ex and Kibra (Kbr) [39], which bind to the FERM-binding motif (FBM) in the Crb ICD C-terminal juxta-membrane domain. Furthermore, a Crb juxta-membrane domain, the PBD (PDZ Binding Motif), recruits the aPKC complex – Baz/Par-6/aPKC – that acts to phosphorylate Crb [124], and stabilise it at the apical membrane [39]. It has been proposed that all these apically localized proteins are a positive feedback regulatory system, promoting the presence and functionality of each other at the *Drosophila* follicle cell epithelium, and that this positive feedback loop (together with antagonistic interactions with the Scrib-Dlg1-L(2)gl module) is crucial for the maintenance of correct apico-basal cell polarity [39].

With regard to cell competition, *crb* mutant cells eliminate their heterozygous (but functionally *wild-type*) neighbours via Myc-independent apoptosis, as confirmed by active-Caspase 3 staining at the borders of the clones

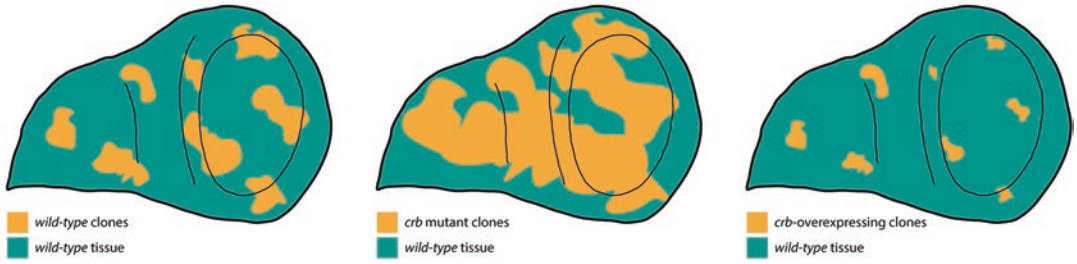

Fig. 3.13 Crumbs levels affect cell competition. In third instar larval wing imaginal discs, *crb* mutant clones (winners) eliminate their *wild-type* neighbour cells (losers). Also in third instar larval wing imaginal discs, *crb*-overexpressing clones (losers) are eliminated by their *wild-type* neighbours (winners)

(Fig. 3.13) [48]. Furthermore, it was observed that clones overexpressing *crb* adopted a loser cell fate when adjacent to *wild-type* clones (Fig. 3.13) [48]. As it had previously been reported that overexpression of *crb* in the posterior compartment of the wing imaginal disc resulted in neoplastic tissue overgrowth [71], this observation suggests that the elimination of *crb*-overexpressing cells is context dependent, and requires neighbouring *wild-type* cells. Interestingly, this elimination of *crb*-overexpressing cells was higher in the distal region of the wing imaginal disc, and when anterior to the morphogenetic furrow in the eye-antennal imaginal disc [48]. Although differences in Myc levels were not observed between *wild-type* and *crb*-overexpressing clones [48], more global Myc expression effects might be contributing to these regional elimination differences. Many *crb*-overexpressing clones were apically extruded from the disc [48], suggesting cell death and extrusion promoting pathways both contribute to the elimination of *crb*-overexpressing loser cells, as observed in other types of cell polarity disruption-initiated cell competition [13, 55, 137]. Interestingly, it was also found that *crb*-overexpressing loser cells affected the survival of surrounding *wild-type* cells (some *wild-type* cell death at the clone borders was observed), and non-autonomously affected their morphology, as evidenced by F-actin staining [48].

3.2.3.2 The Mechanism of Crb in Cell Competition

The precise involvement of Crb in cell competition remains unclear, as is whether it even effects cell competition in a manner similar to other molecules discussed in this chapter. Researchers suggested a model whereby differences in Crb levels contribute to the survival of cell populations [48]. They postulate that the intracellular association of Crb with other proteins, as well as any intercellular interactions of Crb with other Crb molecules on adjacent cells, may enable Crb to function as a surveillance mechanism between cells, where both pro- and anti-apoptotic roles for binding partners might lead to asymmetric effects in adjacent cells [48].

Almost a decade ago, Crb was identified as a regulator of the SWH signalling pathway [20, 47, 68, 114]. The ICD of Crb interacts with Ex via a C-terminal FERM-binding motif (FBM), and this interaction has been shown to regulate Ex apical localization and stability, and promote its activity as a positive regulator of SWH signalling [20, 68, 114]. However, Crb can also promote the phosphorylation-dependent degradation of Ex by ubiquitin-mediated protein degradation via the Skp/Cullin/F-box$^{Slimb/\beta TrCP}$ E3 ubiquitin ligase [110], and a second E3 ubiquitin ligase, POSH, was recently also found to have a similar role [73]. Regardless, as Ex is an established positive regulator (and transcriptional target) of SWH signalling, and since the SWH pathway is involved

in cell competition, Hafezi and colleagues analyzed SWH activity in the context of *crb*-initiated cell competition. They found that the Yki target, *Diap1*, was elevated in *crb* mutant cells in mosaic eye discs, suggesting SWH activity was reduced, and that SWH signalling was involved in *crb*-initiated cell competition [48], similar to what has been observed in wing discs [21].

With these data, Hafezi and colleagues proposed a model for *crb*-initiated cell competition. They suggest that, in a *wild-type* clonal context, Crb on adjacent *wild-type* cells physically interact, which is necessary for proper internal Crb functionality (Fig. 3.14a). There, the Crb ICD physically associates with Ex, and positively regulates SWH signalling by localizing it to the apical cortex, where it is active [48]. In the context of *crb*-overexpression, clones with higher *crb* levels were found to be eliminated (Fig. 3.14b). However, apoptosis of some *wild-type* cells at the clonal borders was also observed, suggesting an imbalance in Crb levels might be responsible. If Crb is overexpressed in one group of cells, adjacent cells might draw more Crb to where they face the Crb-overexpressing cell, leading to an imbalance in Crb levels across an epithelial tissue (Fig. 3.14b). But how does this lead to cell death? Hafezi and colleagues suggest that, aside from its role as a regulator of Ex and SWH signalling, Crb may have other pro- or anti-apoptotic proteins associated with it – an imbalance in these across the lateral plane of the cell, as caused by Crb overexpression in an adjacent cell, might result in pro-apoptotic signals overpowering anti-apoptotic proteins, leading to apoptosis [48]. Interestingly, clones overexpressing only the ICD of Crb overgrow, however, likely due to an inability to properly localize Ex, promote SWH signalling, and inhibit Yki, indicating the importance of intercellular Crb ECD interactions to the proper functioning of Crb [48]. Finally, clones mutant for *crb* acquire a winner phenotype, and outcompete their *wild-type* neighbours (Fig. 3.14c). This is likely due to a lack of proper Ex localization, which in turn would inhibit SWH signalling and promote Yki activity. Indeed, the Yki target *Diap1* is upregulated in *crb* mutant clones, promoting their survival [39, 48]. Additionally, Yki activity can upregulate *bantam* (*ban*), a miRNA encoding gene that downregulates *hid* transcription, inhibiting apoptosis [11, 94]. The recently discovered Egr receptor, Grnd, may also play a role – Grnd colocalizes with Crb, which appears to promote the ability of Grnd to activate JNK signalling via mutual interaction with an adaptor protein called Veli (orthologue of mammalian LIN7) [4]. As JNK is capable of acting as a pro-apoptotic signalling pathway, perturbing the Crb-Grnd interaction via *crb* mutation might promote *crb* mutant survival. As for the neighbouring *wild-type* cells, these are likely to have an imbalance in Crb localization, as discussed, potentially promoting their apoptosis, which together with the suppression of Yki activity via SWH signalling, reduces their relative fitness.

3.2.4 Cell Competition During Cooperative Tumourigenesis

Cooperative tumourigenesis is the phenomenon by which interaction between different genetic lesions can lead to the cancer initiation and progression, and acts as a powerful model in *Drosophila* to explore mammalian tumour development [112]. A classic example of this process is an activating mutation in *Ras oncogene at 85D* (*Ras85D*, common allele used is $Ras85D^{V12}$) coupled with a loss-of-function mutation in one of the apico-basal cell polarity regulator genes. Clones generated with these lesions in epithelial tissue, outcompete the *wild-type* tissue and hyperproliferate and overgrow into metastatic tumours, mimicking the onset of cancer [13, 102].

In the case of $l(2)gl^{-}/Ras85D^{V12}$ wing epithelial clones, they acquire a proliferation advantage through inhibition of SWH signalling, as evidenced by high expression of Diap1, a well-

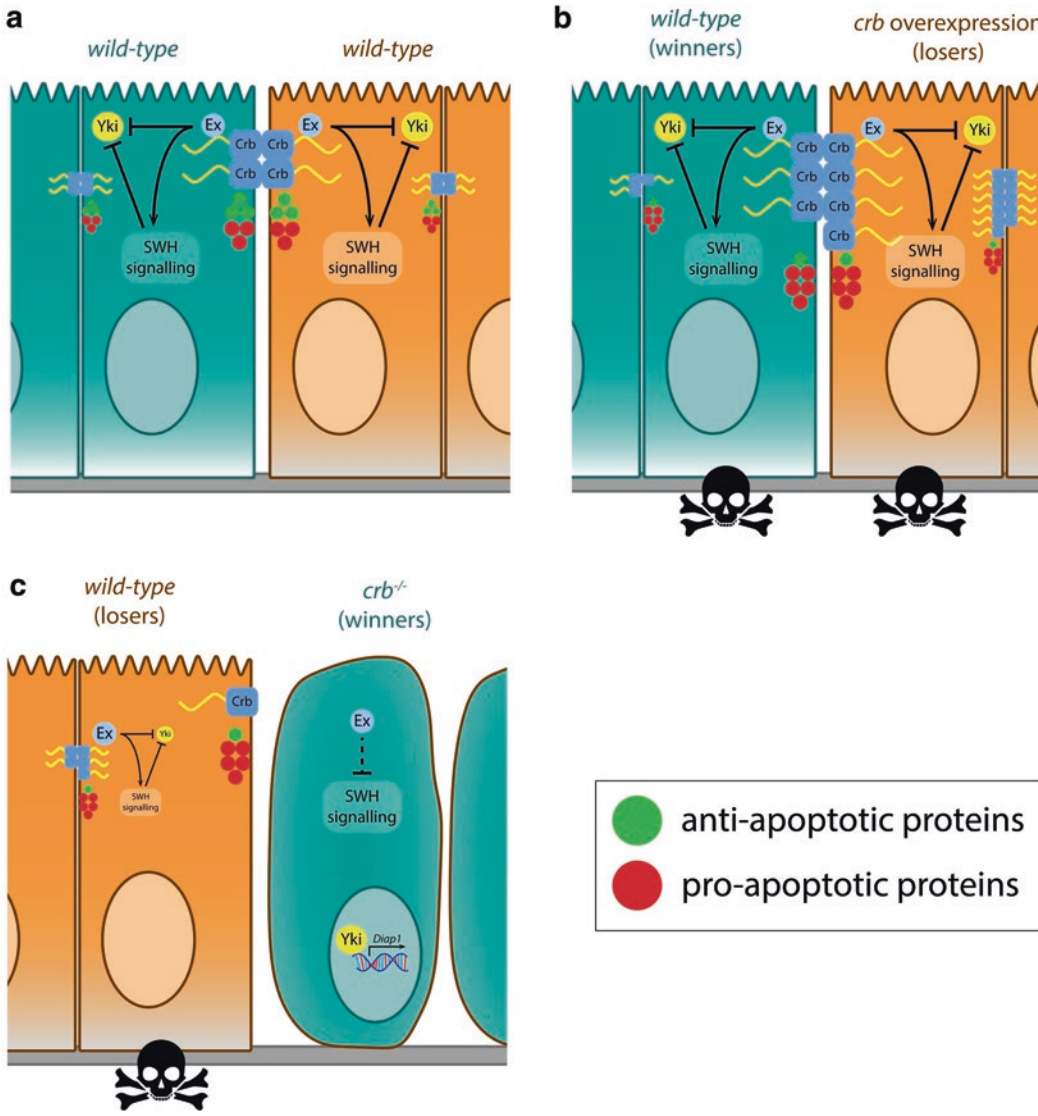

Fig. 3.14 The mechanism by which Crb regulates cell competition. (**a**) In *wild-type* clones, Crb interacts physically with Crb molecules on neighbouring cells via their ECD (blue). The ICD of Crb (yellow) acts to stabilise Ex in the apical region, activating it, and thus promoting SWH signalling. Active Ex can also isolate Yki in the apical region, and it is thought that the presence of functional Crb might balance the localization of various pro-apoptotic (red circles) or anti-apoptotic (green circles) proteins at the lateral membranes. (**b**) Clones overexpressing *crb* are eliminated from tissue by their *wild-type* neighbours. In *crb* overexpressing clones, high levels of Crb will lead to imbalanced distributions of other Crb proteins in adjacent cells. This is hypothesised to result in an imbalance in the distribution of the various pro- or anti-apoptotic proteins that associate with Crb, and lead to the cell death that is observed in both the *wild-type* and *crb* overexpressing cells at the clonal border. How or whether SWH signalling and Yki are affected is unclear, but Ex functionality is thought to increase alongside increased intercellular Crb-Crb binding, therefore it is likely upregulated in both cells. It is also unclear specifically what drives the elimination of the *crb*-overexpressing clones or the adjacent *wild-type* cells. (**c**) Clones homozygous mutant for *crb* outcompete and eliminate their functionally *wild-type* neighbours. Once again, the imbalance in Crb distributions leads to a flow-on imbalance in the adjacent *wild-type* cells, which is thought to lead to altered pro- and anti-apoptotic protein distributions and promote cell death. However, the lack of Crb in the mutant clones presumably prevents Ex from activating SWH signalling and isolating Yki – this leads to the upregulation of Yki targets, such as *Diap1*, and the survival of the *crb* mutant clones at the expense of their *wild-type* neighbours. Ex levels are also reduced in the *wild-type* cell at the *wild-type*/*crb* mutant cell interface, but this is not thought to be sufficient to alter SWH signalling in the *wild-type* cell

established Yki target. These clones possess persistent nuclear-localization of Yki, whereas normally Yki is cytoplasmic (and therefore inactive) [80]. This is similar to *scrib−/Ras85D^{V12}* clones, which also have nuclear-localized Yki [80] and upregulate Yki target genes [21, 32]. Remarkably, although more noticeable in small clones, the larger *l(2)gl^{-}/Ras85D^{V12}* clones possess apoptosis at their borders, which also occurs in *scrib^{-}/Ras85D^{V12}* clones, and results in more than half of *l(2)gl^{-}/Ras85D^{V12}* clones being eventually eliminated from the disc [80]. *l(2)gl^{-}/yki*-overexpressing cells grow faster (similar to *l(2)gl^{-}/Ras85D^{V12}* clones) [80], although, again, cells at the borders of the clones undergo apoptosis [80]. It is clear that cell competition in this context is not only due to the different tissue growth rates of the different cell populations, but also to the involvement of tissue growth rate-independent pathways, since even though *l(2)gl^{-}/yki*-overexpressing or *l(2)gl^{-}/Ras85D^{V12}* cells have higher proliferation rates, cell death is observed at the clone borders. In this study, it was shown that the fusion of individual clones enabled the generation of an internal environment within the mutant patch that is resistant to cell competition mechanisms, as it is no longer adjacent to neighbours of a different genotype, thereby enabling the mutant tissue to develop into a neoplastic invasive tumour [37].

It has recently been shown that *Drosophila* Troponin-I (TnI, a.k.a. WupA), an F-actin-binding protein involved in muscle contraction that has been recently shown to have additional roles in epithelial cell polarity and cell competition, regulates the competitive properties of *l(2)gl^{-}/Ras^{V12}* tumours [19]. In *l(2)gl^{-}/Ras^{V12}* clones in the wing disc epithelium, *TnI* overexpression promotes survival of the mutant clones, but *TnI* knockdown dramatically decreases the survival of these clones. *TnI*-defective cells undergo JNK-, Flower-, and SPARC-dependent cell competition [19], and it is likely that these cell competition mechanisms are also involved in the elimination of *l(2)gl^{-}/Ras^{V12}* clones upon *TnI* knockdown, whilst overexpression of *TnI* might antagonise these cell competition mechanisms. Since TnI facilitates the localization of, and forms complexes with, Baz/aPKC and Dlg1, and *TnI* mutant epithelial cells lose polarity and are basally extruded [17], TnI could be considered to be a new cell polarity regulator that might tether core cell polarity modules to the actin cytoskeleton. Whether other cell competition mechanisms involved in the recognition and elimination of polarity-impaired cells in an epithelium (such as Sas-Ptp10D, Spn88Ea-Toll and Slit-Robo2-Ena signalling) are also involved in the elimination of *TnI* or *l(2)gl^{-}/Ras^{V12} TnI* mutant cells remains to be determined.

3.3 Conclusions and Future Directions

During development of an organism, and while maintaining homeostasis within the adult, cells can acquire mutations. In many cases, these mutations affect the function of the cell, but are insufficient to trigger cell autonomous death, even if these mutated cells might be dangerous for tissue integrity. Cell competition is a homeostatic mechanism by which healthy cells can eliminate suboptimal cells from a tissue, avoiding possible tumourigenic growth. However, the inverse is possible – cells can acquire mutations that can make them "super-fit" and potentially oncogenic, and, as such, capable if eliminating perfectly healthy *wild-type* cells. As we have discussed, loss-of-function disruptions to the cell polarity proteins of the Scrib/Dlg1/L(2)gl module are established initiating factors of the cell competition process, where the mutant cells adopt a loser cell fate. While *scrib* remains the best understood cell competition initiator of the apico-basal polarity regulators, and various idiosyncrasies of *l(2)gl* are emerging, the involvement of cell competition mechanisms in these mutants is not as well understood compared with *Myc*- and *M*-driven cell competition. However, recent discoveries have revealed a myriad of systems that are involved in cell competition upon cell polarity regulator gene impairment: various signalling pathways, including SWH, Jak-STAT and Toll-NF-κB, cell removal systems such as Slit-Robo2-Ena and Pvr-Ced-12-Mbc-Rac1, as

well as the loser cell-recognition mechanisms, the Flower Code and Ptp10D-Sas signalling. Ptp10D-Sas signalling involves interactions between Sas on the *wild-type* cell and Ptp10D on the cell polarity regulator impaired cell, which has parallels with the involvement of the Crb cell polarity protein in cell competition, although the interaction is homophylic (between Crb molecules on adjacent cells) [48]. Although the mechanism is not well understood, it appears that relative differences in Crb levels might trigger competition by regulating SWH signalling and the localization of unknown pro- or anti-apoptotic factors [48], the identification of which is essential to dissecting the role of *crb* in cell competition.

Cell polarity regulator deficient cells die in a *wild-type* background through activation of the JNK signalling pathway. In recent years, many efforts have been made to answer the question: how is JNK being activated in these cells? Although JNK signalling can be activated cell-intrinsically through altered cytoskeletal protein signalling [72, 93], TNF signalling appears to also play a role [56, 138], although whether the source of the pathway activating ligand, Egr, is only from haemocytes or also from surrounding epithelial cells is unclear. Furthermore, details of the precise interactions between JNK signalling and the Jak-STAT and SWH signalling pathways during cell competition of polarity-impaired cells remain incomplete [21, 118]. In *scrib* mutant mosaic tissue, Jak-STAT signalling in the *wild-type* cells is involved in the elimination of the *scrib* mutant cells and in compensatory proliferation of the *wild-type* cells. However, Jak-STAT signalling is activated by Upd family ligands capable of both autocrine and paracrine activity – how then is Jak-STAT activity specifically upregulated in the *wild-type* cells, and not in the polarity mutant cells? Possibly, cell-specific expression of protein tyrosine phosphatases or suppressors of cytokine signalling might be involved, and dictate whether Jak-STAT signalling is activated, thus regulating winner and loser fates.

The potential for the cell competition mechanism to be usurped by cancerous cells to promote their proliferation and elimination of normal cells in a tissue is quite clear (for example, the proto-oncogene Myc is capable of inducing the necessary super-competitive phenotype if upregulated). The phenomenon of cooperative tumourigenesis is testament to the serious consequences of this possibility, particularly when one considers the established role that mutations in polarity regulator genes already play in that process [112]. Indeed, these mechanisms are highly conserved, from *Drosophila* to mammals. For example, cooperative tumourigenesis via *SCRIB* mutation and *H-RAS* activation (human orthologues of *Drosophila scrib* and *Ras85D*) has been observed in human epithelial cells [33], as well as mouse models of prostate cancer [105], lung cancer [35], skin cancer [104], and breast cancer [38, 44]. It seems reasonable to suggest that, at least in part, cancers must grow and proliferate at the expense of any surrounding *wild-type* tissue. This would imply that, even though cell competition is not so well understood in mammalian systems as it is in flies, the broad mechanisms might be similar and, indeed, extremely important in understanding the processes of tumourigenesis, particularly in the context of tumour-suppressive cell polarity regulator genes, which have such an extensive history of involvement in a diversity of cancers [15, 43, 61, 108, 116].

At present, the distinct molecular pathways implicated in cell competition phenomena in *Drosophila* and in mammals are numerous and complex in their interactions. Furthermore, research is just beginning to determine the relative contribution of the different mechanisms of loser cell recognition and elimination in the different types of cell competition. While it is not as well-understood as *Myc*- and *M*-initiated cell competition, recent research has uncovered a number of mechanisms, both novel and conserved, that specifically regulate cell polarity impairment-initiated cell competition. Given the importance of cell polarity regulators to human disease, and their long history of significant contributions to a wide-variety of research fields, elucidation of cell competition mechanisms that occur in polarity-impaired cells will have far-reaching implications.

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Two Sides of the Same Coin – Compensatory Proliferation in Regeneration and Cancer

4

Neha Diwanji and Andreas Bergmann

Abstract

Apoptosis has long been regarded as a tumor suppressor mechanism and evasion from apoptosis is considered to be one hallmark of cancer. However, this principle is not always consistent with clinical data which often illustrate a correlation between apoptosis and poor prognosis. Work in the last 15 years has provided an explanation for this apparent paradox. Apoptotic cells communicate with their environment and can produce signals which promote compensatory proliferation of surviving cells. This behavior of apoptotic cells is important for tissue regeneration in several model organisms, ranging from hydra to mammals. However, it may also play an important feature for tumorigenesis and tumor relapse. Several distinct forms of apoptosis-induced compensatory proliferation (AiP) have been identified, many of which involve reactive oxygen species (ROS) and immune cells. One type of AiP, "undead" AiP, in which apoptotic cells are kept in an immortalized state and continuously divide, may have particular relevance for tumorigenesis. Furthermore, given that chemo- and radiotherapy often aim to kill tumor cells, an improved understanding of the effects of apoptotic cells on the tumor and the tumor environment is of critical importance for the well-being of the patient. In this review, we summarize the current knowledge of AiP and focus our attention on recent findings obtained in *Drosophila* and other model organisms, and relate them to tumorigenesis.

Keywords

Apoptosis-induced proliferation · Caspases · Reactive oxygen species · Macrophages · *Drosophila*

4.1 Introduction – Caspase-Driven Compensatory Proliferation: Coupling Apoptosis, Regeneration and Cancer

Cancer is a multifactorial disease with an estimated 9.6 million deaths in 2018, the second leading cause of mortality in the world (WHO https://www.who.int). Consequently, it is important to understand the different aspects of tumorigenesis for developing potential therapeutic strategies. Multiple efforts have been made to define the key traits of carcinogenesis, summarized as the "hallmarks of cancer" by Hanahan and Weinberg [1]. Among these,

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increased cell proliferation and resistance to cell death are regarded as major characteristics of transformed cells [1, 2]. Indeed, the common mode of action for most chemotherapy and radiotherapy strategies is to induce cell death in the tumor cells [3–5].

Over the past decades, it has become evident that cell death, in normal as well as malignant cells, is a tightly regulated and programmed process. Many different mechanisms of programmed cell death (PCD) have been reported [6–8]. Among these, apoptosis is the best studied and evolutionarily most conserved form of PCD, important during development and for maintaining homeostasis [9, 10]. Ultrastructural studies helped define the characteristic features of apoptotic cell death under physiological conditions [11]. Morphological hallmarks of apoptosis include cytoplasmic shrinkage, DNA condensation and nuclear fragmentation, retention of membrane integrity, and membrane blebbing to form apoptotic bodies that are rapidly engulfed and eliminated by phagocytosis without an inflammatory response [9, 11]. Thus, apoptosis is considered a "silent" form of cell death, in contrast to necrosis during which cells swell and rupture in response to overwhelming damage, causing an acute inflammatory response.

Mechanistically, apoptosis requires the activation of caspases, a class of cysteine proteases that are present in the cells as inactive zymogens [12, 13]. The role of caspases in apoptosis was first discovered in *Caenorhabditis elegans* by the pioneering work of Horvitz and colleagues [14, 15]. Since then, many caspases in different model organisms have been discovered – *Caenorhabditis elegans* has 4 caspases, *Drosophila melanogaster* has 7 caspases, mice and humans contain 11 and 13 caspases, respectively [16, 17]. Apoptotic caspases are subdivided into two categories based on their location in the signaling pathways: upstream initiator or apical caspases, which include caspase−2, −8, −9, −10 in mammals and Dronc in *Drosophila*, and downstream effector or executioner caspases including caspase-3, −6, −7 in mammals as well as DrICE and Dcp-1 in *Drosophila* (Fig. 4.1) [13, 18]. Initiator caspases are defined by their long N-terminal prodomains containing motifs such as the caspase recruitment domain (CARD) or the death effector domain (DED), which mediate dimerization and activation of these enzymes by enabling their recruitment into large protein complexes, like the apoptosome or the DISC (death-inducing signaling complex). In contrast, effector caspases have short prodomains without known protein/protein interaction motifs and are activated through cleavage by initiator caspases generating large and small subunits, two of each forming the active caspase tetramer [17, 19, 20].

Activation of caspases is the result of a signaling cascade that is triggered upon an apoptotic stimulus, either in the form of developmental, homeostatic or stress cues. Initiation of the apoptotic signaling cascade occurs through either the intrinsic pathway or the extrinsic pathway. In mammals, the intrinsic pathway is regulated by the Bcl-2 family of proteins and involves mitochondrial outer membrane permeabilization (MOMP) followed by release of cytochrome c from the mitochondria (Fig. 4.1A). The released cytochrome c associates with the scaffolding protein Apaf-1 (apoptotic protease-activating factor 1) to form the apoptosome, and thus activates caspase-9 (Fig. 4.1A) [17]. In *Drosophila*, the pro-apoptotic factors Reaper, Hid and Grim initiate the intrinsic apoptotic signaling cascade by binding to the E3-ligase *Drosophila* Inhibitor of Apoptosis Protein1 (D-IAP1), thereby promoting auto-ubiquitination and proteasomal degradation of D-IAP1 (Fig. 4.1B) [21–27]. This releases the D-IAP1 inhibition of the initiator caspase Dronc, and free Dronc can now be recruited by the Apaf-1-related Dark into the apoptosome for activation [19, 27].

In contrast to the intrinsic apoptotic pathway, the extrinsic pathway is initiated at the plasma membrane upon binding of extracellular ligands (e.g. FasL and TNF) to their respective transmembrane "death" receptors (Fas for FasL, TNFR for TNF) (Fig. 4.1A). This leads to trimerization of the receptors promoting clustering of intracellular adaptor proteins (e.g., FADD, or Fas-associated death domain-containing protein), which bind the DED motifs in the prodomains of the initiator caspases-8 or −10, forming the DISC which ultimately activates caspase-8

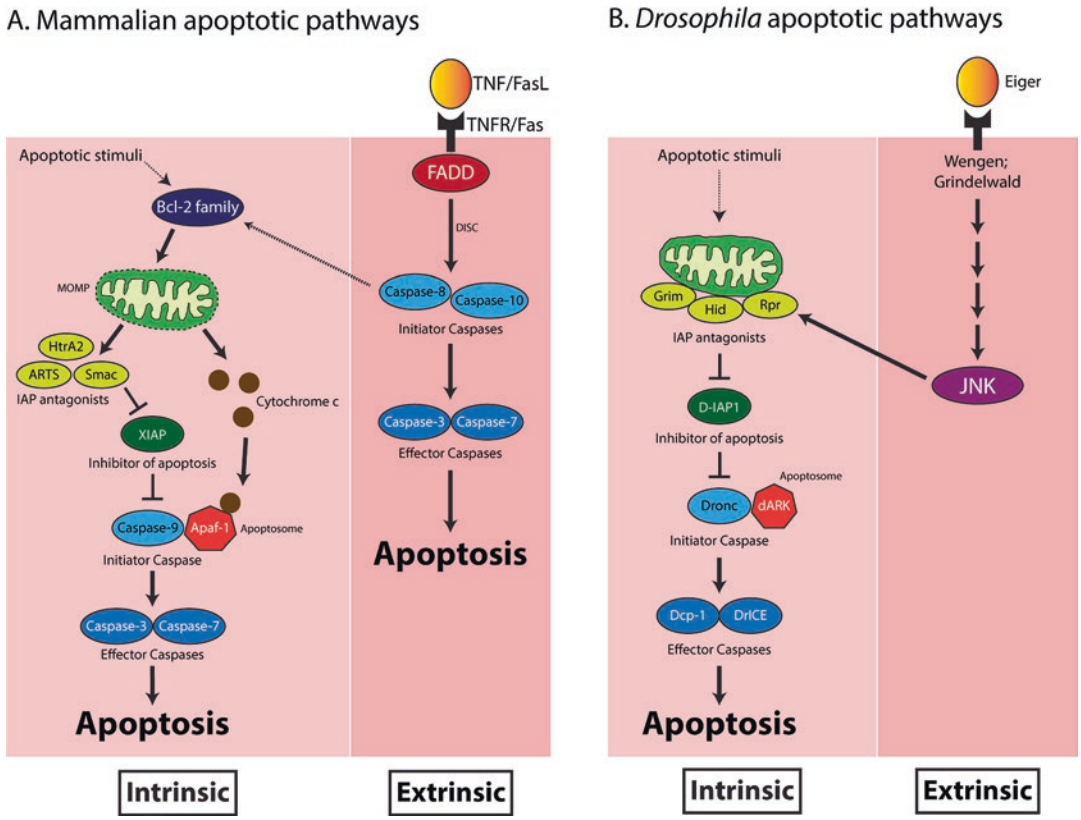

Fig. 4.1 The apoptotic pathways in mammals and *Drosophila*. (**A**) The intrinsic and extrinsic pathways in mammals. Due to the involvement of mitochondria, the intrinsic pathway is also referred to as mitochondrial pathway. Crosstalk between the intrinsic and extrinsic pathways is mediated via cleavage and activation of the pro-apoptotic Bcl-2 family member Bid by Caspase-8. *MOMP* Mitochondrial outer membrane permeabilization. (**B**) The intrinsic and extrinsic pathways in *Drosophila*. In the intrinsic pathway, mitochondria serve as a platform for insertion of the IAP antagonists Reaper (Rpr), Hid and Grim. Crosstalk between the intrinsic and extrinsic pathway is mediated through JNK-induced expression of *hid* and *reaper*

or −10 [13, 28]. In *Drosophila*, the extrinsic pathway is thought to be initiated by binding of the TNF homolog, termed Eiger, to its receptors Wengen or Grindelwald. However, in contrast to the extrinsic pathway in mammals, the Eiger/Wengen or Eiger/Grindelwald complex does not activate the caspase-8 ortholog Dredd in *Drosophila*, but rather results in activation of the stress kinase JNK (c-Jun N-terminal kinase) (Fig. 4.1B) [29–33]. Eiger-induced cell death is in part dependent on the intrinsic pathway as JNK transcriptionally induces expression of the intrinsic factors Hid and Reaper [31, 34], thereby activating Dronc (Fig. 4.1B). Once initiator caspases are active via the intrinsic or extrinsic pathways, they cleave and activate effector caspases. Finally, active effector caspases cleave a broad range of regulatory and structural proteins and important enzymes leading to the execution of the cell. Given the important role that caspases play in the death of cells, their activation as well as activity are tightly regulated. Several post-translational modifications, such as ubiquitylation and phosphorylation, and interactions with regulatory proteins, such as IAPs or FLIP family of proteins, regulate caspase activation and activity [13, 35, 36].

In recent years, accumulating evidence suggests that in addition to apoptosis, caspases function in a broad range of non-apoptotic processes including immune regulation, cell differentiation, cell migration and invasion, maintenance of tis-

sue integrity, regulation of stem cell properties, neurite pruning, non-apoptotic forms of cell death, and intercellular signaling processes [6, 37–43]. Apoptotic cells are known to secrete "find-me" and "eat-me" signals which direct their recognition and clearance by phagocytes [44, 45]. However depending on the cellular context, apoptotic cells also secrete signals that affect their environment, including pro-apoptotic signals that promote additional cell death, or mitogenic signals that induce proliferation to compensate for the cell loss [46–48] [49]. Observations that active caspases promote compensatory proliferation originally came from studies in *Drosophila*, followed by similar observations in many different model organisms (reviewed in [50]).

Compensatory proliferation is critical for tissue repair, wound healing and regeneration, and as such is important for maintaining tissue homeostasis post massive cell loss due to stress or injury. Given the strong connection between wound repair and cancer, with cancers being compared to "wounds that do not heal" [51], compensatory proliferation seems to play a role in tumor initiation and persistence as well (Fig. 4.2). In addition, the signaling pathways utilized by apoptotic cells during compensatory proliferation can be hijacked by tumorigenic cells to promote their growth and for metastasis. Studies in *Drosophila*, in conjunction with other model organisms, have contributed greatly to our understanding of the mechanisms involved in compensatory proliferation and its role in cancer. In this review, we highlight recent studies focusing primarily on *Drosophila* models of compensatory proliferation, as a means to explore the interplay between regenerative and tumorigenic contributions of compensatory proliferation.

4.2 Compensatory Proliferation: Studies in *Drosophila melanogaster*

Regeneration is a process that helps restore tissue integrity following intense trauma. This ability to repair tissue damage and maintain homeostasis is a fundamental property of various multicellular organisms [52, 53]. Cellular proliferation and tissue growth is the primary focus in the field of tissue regeneration, and one of the mechanisms by which a regenerative response is initiated is by compensatory proliferation, a process by which lost tissue is replaced via increased proliferation of uninjured neighboring cells. The earliest observation of compensatory proliferation came from studies in *Drosophila*. Haynie and Bryant demonstrated that killing 40%–60% of cells from *Drosophila* larval imaginal discs (the precursor epithelial tissue which gives rise to the adult structures) by lethal X-ray irradiation still yielded normal adult organs due to subsequent increase in proliferation among the surviving cells [54].

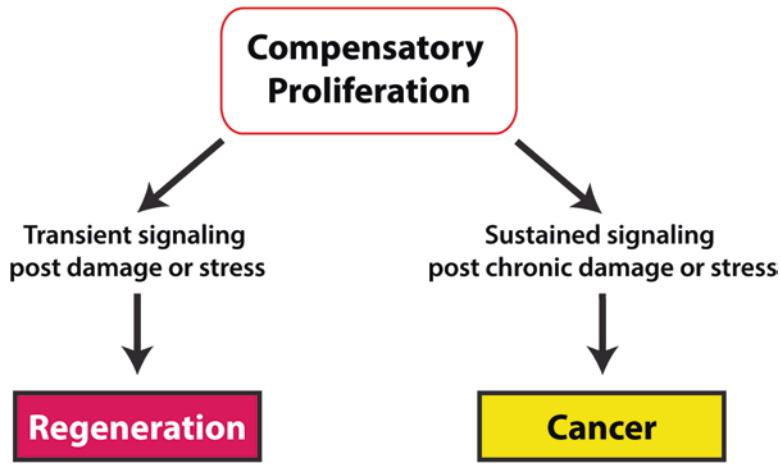

Fig. 4.2 Compensatory proliferation in regeneration and cancer

Similar observations of compensatory proliferation were made in mammalian systems, where the liver can fully regenerate in response to injury or partial hepatectomy by increased proliferation of healthy hepatocytes [55]. Studies in several model systems have now shown that apoptotic cells can secrete mitogens, thereby promoting compensatory proliferation, a phenomenon termed as "apoptosis-induced proliferation" (AiP) [49, 56].

In *Drosophila*, there are at least three distinct models of AiP: "undead", "genuine" and "post-mitotic" AiP (Fig. 4.3). In these AiP models, apoptosis is usually induced in larval eye or wing imaginal discs, either by irradiation or by expression of pro-apoptotic factors (*hid*, *reaper* or *eiger*), and the signaling events in apoptotic cells are studied. These studies in *Drosophila* provided mechanistic insights into the process and demonstrated that active caspases are important for promoting AiP (reviewed by [50, 57]). They established the role of the initiator caspase Dronc for inducing mitogenic signaling independently of its role in apoptosis, at least for "undead" AiP and possibly also "genuine" AiP [58–61].

A technical challenge in these studies was the transient nature of apoptotic processes and the rapid clearance of dead cells, making it difficult to capture the non-apoptotic signaling events. The key to circumvent this limitation was to block effector caspases by expression of the specific inhibitor protein P35 [62, 63], thus preventing execution of apoptosis. Under these circumstances, apoptotic signaling induced by *hid* or *reaper* expression, activates Dronc (which is not inhibited by P35), while cell death is blocked, thus allowing to uncouple the apoptotic and non-apoptotic functions of Dronc. Due to P35 expression, the affected cells are in an immortalized state referred to as "undead" (Fig. 4.3A), in which active Dronc persistently signals for AiP, which ultimately causes overgrowth of the tissue [64, 65]. The requirement of Dronc for AiP was confirmed by loss-of-function analysis which suppressed the overgrowth of "undead" tissue [58, 60, 61, 65].

"Genuine" (also referred to as regenerative) and "post-mitotic" AiP are P35-independent models during which apoptotic cells are allowed to complete the apoptotic process (Fig. 4.3B, C). To avoid organismal lethality due to excessive apoptosis, apoptosis is either induced for a brief period of time in a spatially-restricted manner ("genuine") or in a non-essential tissue such as the developing retina of the fly eye which is also post-mitotic at this stage [65–70]. After this apoptotic treatment, the regenerative response of the affected tissue is examined. In the following, we will summarize and compare the findings of these different models of AiP.

The "undead" model has been employed in several genetic screens for identification of genes important for AiP in *Drosophila* [65, 66, 71, 72]. Mechanistically, Dronc promotes the activation of JNK, and secretion of mitogens such as Wingless (Wg; a WNT-homolog), Decapentaplegic (Dpp; a BMP/TGFβ homolog) and Spitz (Spi; a EGF homolog) to stimulate overgrowth [59, 65, 72, 73] (Fig. 4.3A). Along with JNK, p53 was also shown to be important for AiP [61, 74]. Both JNK and p53 are known to control the expression of the pro-apoptotic genes *hid* and *reaper*. This triggers a feedback loop in "undead" cells amplifying the mitogenic signals (Fig. 4.3A) [66, 75].

An important question in the field of AiP was how an initiator caspase like Dronc can activate the stress kinase JNK. Initially, it was debated whether involvement of JNK in AiP was because of its apoptotic role as an inducer of the apoptotic process, or whether it was a downstream target of Dronc. Nevertheless, identification of the feedback amplification loop reconciled both these models [66, 75]. It was then speculated that a novel cleavage target of Dronc exists that may eventually activate JNK. It remains to be seen if this is true. However, a recent study showed that the linear pathway assumed for activation of JNK during AiP might be more complicated than it was previously thought. This study demonstrated that reactive oxygen species (ROS) act as an intermediate step between Dronc and activation of JNK. Active Dronc triggers the generation of extracellular ROS (eROS) in "undead" cells via the NADPH oxidase dDuox at the plasma membrane [66] (Fig. 4.3A). These eROS are required

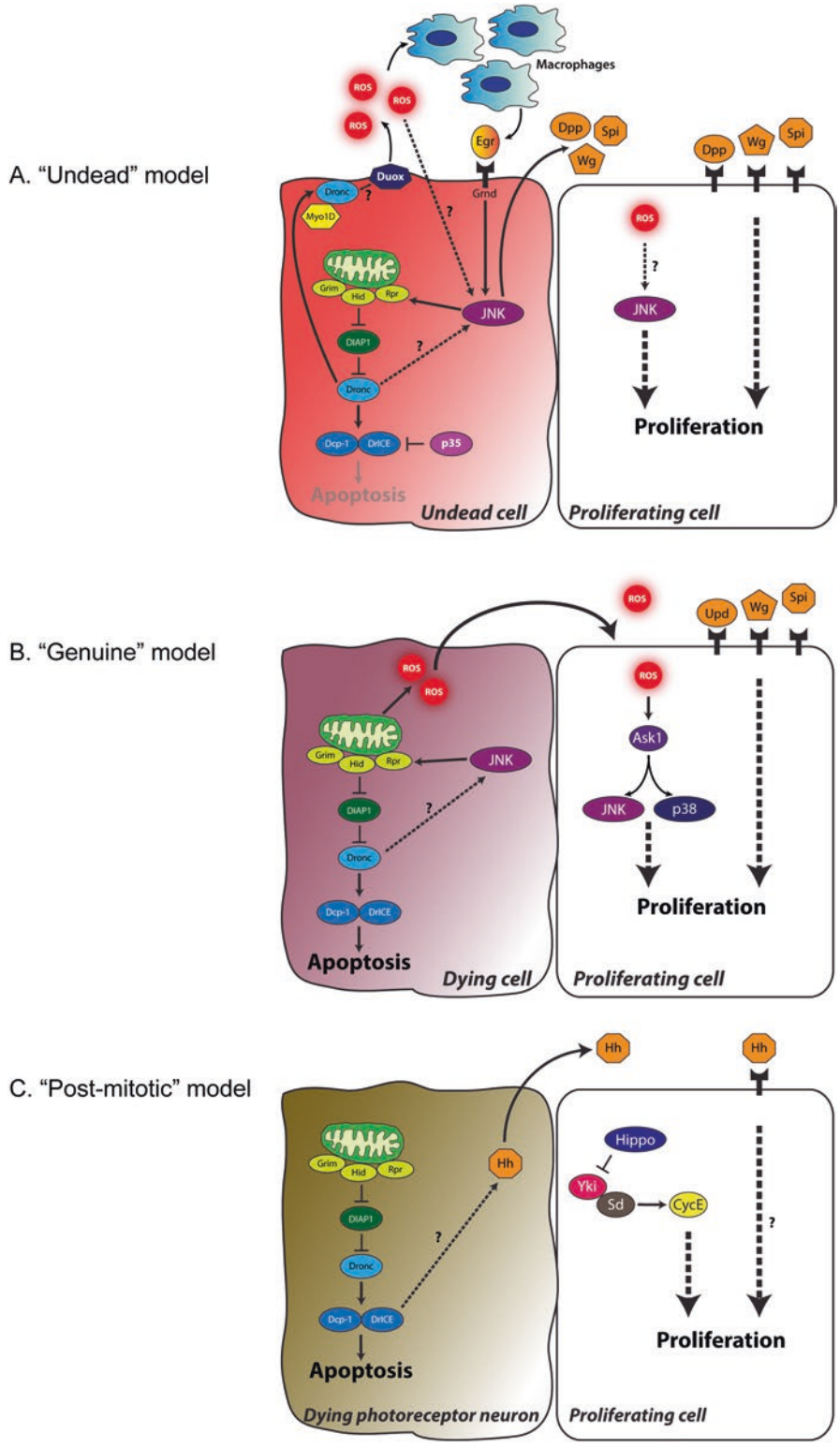

Fig. 4.3 Models of apoptosis-induced proliferation (AiP) in *Drosophila*. (**A**) The "undead" AiP model. Apoptotic cells are maintained in an immortalized state, referred to as "undead" due to expression of the effector caspase inhibitor P35. Under these conditions, the unconventional myosin Myo1D transports Dronc to the basal side of the

for AiP, as their loss impaired JNK activation and production of mitogens. One of the mechanisms by which eROS activate JNK is by recruitment of *Drosophila* macrophage-like cells, called hemocytes, to the "undead" tissue (Fig. 4.3A). Hemocytes in turn secrete Eiger which signals via its receptor Grindelwald to activate JNK back in "undead" cells [66, 76, 77]. If this is the only mechanism by which activation of JNK occurs in "undead" cells, or if any other mechanisms exist, is an area for future investigation.

A follow-up question to this work is how Dronc activates the NADPH oxidase Duox at the plasma membrane. While the final mechanistic details to answer this question are not available yet, recent work has provided more insight into this question. Dronc is usually a cytosolic protein. However, in "undead" cells, Dronc showed a prominent localization at the plasma membrane, specifically at the basal side of the plasma membrane of the disc proper of imaginal discs (Fig. 4.3A) [71]. Translocation of Dronc to the plasma membrane was mediated by Myo1D, a class I unconventional myosin. Loss of Myo1D resulted in loss of the membrane localization of Dronc and suppressed the overgrowth of "undead" tissue [71], suggesting that the membrane localization of Dronc is an integral part of the "undead" AiP pathway. The specific basal localization of Dronc is of particular interest because Duox is also enriched at the basal side, and hemocytes are recruited to the basal side of the disc proper (Fig. 4.3A) [71]. The model emerges that Dronc – directly or indirectly – activates Duox at the plasma membrane for ROS generation. It should be noted that Dronc has enzymatic activity at the plasma membrane [71], but whether it directly cleaves Duox awaits further investigation.

There is precedence for membrane localization of Dronc. Another study looked at the dynamics of Dronc localization in the *Drosophila* salivary gland during development. In late larval stage, Dronc is localized to the cortex of salivary gland cells [78]. Here, membrane localization of Dronc is not required for apoptosis or AiP, but for dismantling of the cortical F-actin cytoskeleton in a non-apoptotic role. In contrast, during early pupal stages, Dronc loses its membrane localization and becomes cytosolic where it mediates apoptosis and salivary gland cell death [78].

A common theme of these two studies is that the plasma membrane serves as a platform for non-apoptotic activities of caspases, at least of the initiator caspase Dronc. The sequestration of active caspases to specific sub-cellular locations where they can interact with targets involved in proliferation and other non-apoptotic processes, offers an answer to another critical question in caspase research – how cells escape the potential lethal activity of active caspases when they fulfil non-apoptotic functions. The aforementioned studies suggest that the basal side of the plasma membrane may provide a non-apoptotic compartment that permits Dronc to mediate non-apoptotic processes such as compensatory proliferation or cytoskeleton remodeling [71, 78, 79].

The findings obtained in the "undead" AiP model were further extended with the P35-independent "genuine" model which also showed

Fig. 4.3 (continued) plasma membrane where it directly or indirectly activates the NADPH oxidase Duox for ROS generation. *Drosophila* macrophages are attracted to "undead" cells and release the TNF ligand Eiger which activates the JNK pathway in "undead" cells. JNK activity induces expression of *hid* and *reaper*, setting up a feedback amplification loop, and of the mitogens *wg*, *dpp* and *spi* which promote proliferation. The amplification loop signals continuously, promoting tissue overgrowth. Question marks denote uncertainty. For more details, see text. (**B**) The "genuine" (or regenerative) AiP model. Temporally and spatially restricted apoptosis promotes generation of intracellular ROS, some of which propagates to neighboring surviving cells to activate JNK and p38 signaling. The role and origin of Wg, Upd and Spi is uncertain in this model. For more details, see text. (**C**) The "post-mitotic" AiP model. Induction of apoptosis in the developing retina (a largely post-mitotic tissue) triggers AiP. In this case, dying photoreceptor neurons release the mitogen Hedgehog (Hh) which promotes proliferation of surviving, undifferentiated, yet post-mitotic cells. JNK signaling is not involved, however, Hippo signaling has been implicated in this model

the requirement of JNK for proper regeneration [65–69]. Along with JNK signaling, p38 and JAK/STAT signaling pathways are also required for "genuine" AiP [80] (Fig. 4.3B). Production of ROS in response to transient pro-apoptotic signals was also observed in "genuine" models [66, 80], although in this context, these ROS appear to be intracellular, and are most likely mitochondrial in origin. Nevertheless, despite this intracellular origin, some ROS appear to propagate into neighboring surviving cells where they induce activation of JNK and p38 signaling through Akt and the redox-sensitive Ask1 factor which altogether results in expression of Unpaired (Upd), an Interleukin-6 paralog [80, 81].

However, some discrepancies do exist between the "undead" and "genuine" models, especially regarding the source and requirement of Wg signaling [67, 69, 73, 82]. These discrepancies can be explained by functional redundancy between Wg and Wnt6 which are under control of the same damage-response element [83]. However, hemocytes are not recruited to imaginal discs in "genuine" models, and neither is there a requirement for Eiger signaling nor Myo1D. These differences illustrate the context-dependent nature of AiP signals with very different consequences: overgrowth in "undead" AiP *versus* regeneration in "genuine" AiP.

In post-mitotic tissue, a completely different mechanism of AiP exists. Here, the non-apoptotic activity of effector caspases is important for inducing compensatory proliferation. Upon apoptosis induction in the differentiating *Drosophila* retina (which is largely a post-mitotic tissue), the dying photoreceptor neurons produce and secrete the mitogen Hedgehog (Hh) in a DrICE- and Dcp1-dependent manner, promoting proliferation of surrounding cells that have not yet initiated differentiation (Fig. 4.3C) [70]. JNK signaling is not involved in "post-mitotic" AiP. Hippo signaling has been implicated in this type of AiP [84]. The cells that undergo AiP in this context are usually post-mitotic; however, they are still competent to re-enter the cell cycle and divide. Interestingly, while dying photoreceptor neurons produce the Hh signal for AiP, the newly formed cells can differentiate in all accessory cell types, but not photoreceptor neurons [85]. Expression of P35 in this context blocks secretion of Hh, and thereby AiP [70], indicating that effector caspases are required for this type of AiP. Therefore, there are notable differences in the mechanisms of AiP depending on distinct cell types and developmental stages.

4.3 Compensatory Proliferation in Regeneration of Different Model Organisms

Compensatory proliferation for regeneration also occurs in a variety of different organisms, including *Hydra*, planaria, newt, *Xenopus*, zebrafish and mice. In the fresh water polyp *Hydra*, mid-gastric transverse bisection results in both head and tail regeneration [86]. Interestingly, only head, but not tail, regeneration requires proliferation. Correspondingly, apoptosis is only triggered at the head-regenerating tip via the MAPK/CREB pathway [87], which is not observed for tail regeneration. Activation of effector caspases induces secretion of the mitogen Wnt3 from dying cells, thus initiating β-catenin-driven proliferation of surrounding cells followed by regeneration of the head (Fig. 4.4A). Excitingly, ectopic activation of apoptosis at the tail-regenerating tip regenerated a head, producing a bi-headed hydra, illustrating that activation of caspases can change the regeneration program in this organism [88]. ROS are also produced immediately at the wound site, and are required for the injury-induced MAPK activation and apoptosis [89].

Fresh water planarian *Schmidtea mediterranea* also demonstrates a remarkable regenerative potential [90]. Apoptosis mediated by caspase-like effectors, DjCLg3, occurs after amputation and is required for regeneration, but whether the apoptotic cells drive AiP is currently unknown [91]. A recent study demonstrated that ROS are produced at the wound site following amputation of the head and tail compartments of planaria, and inhibition of the ROS burst impaired the regeneration capacity (Fig. 4.4B) [92]. This is reminiscent of the requirement of ROS for AiP in

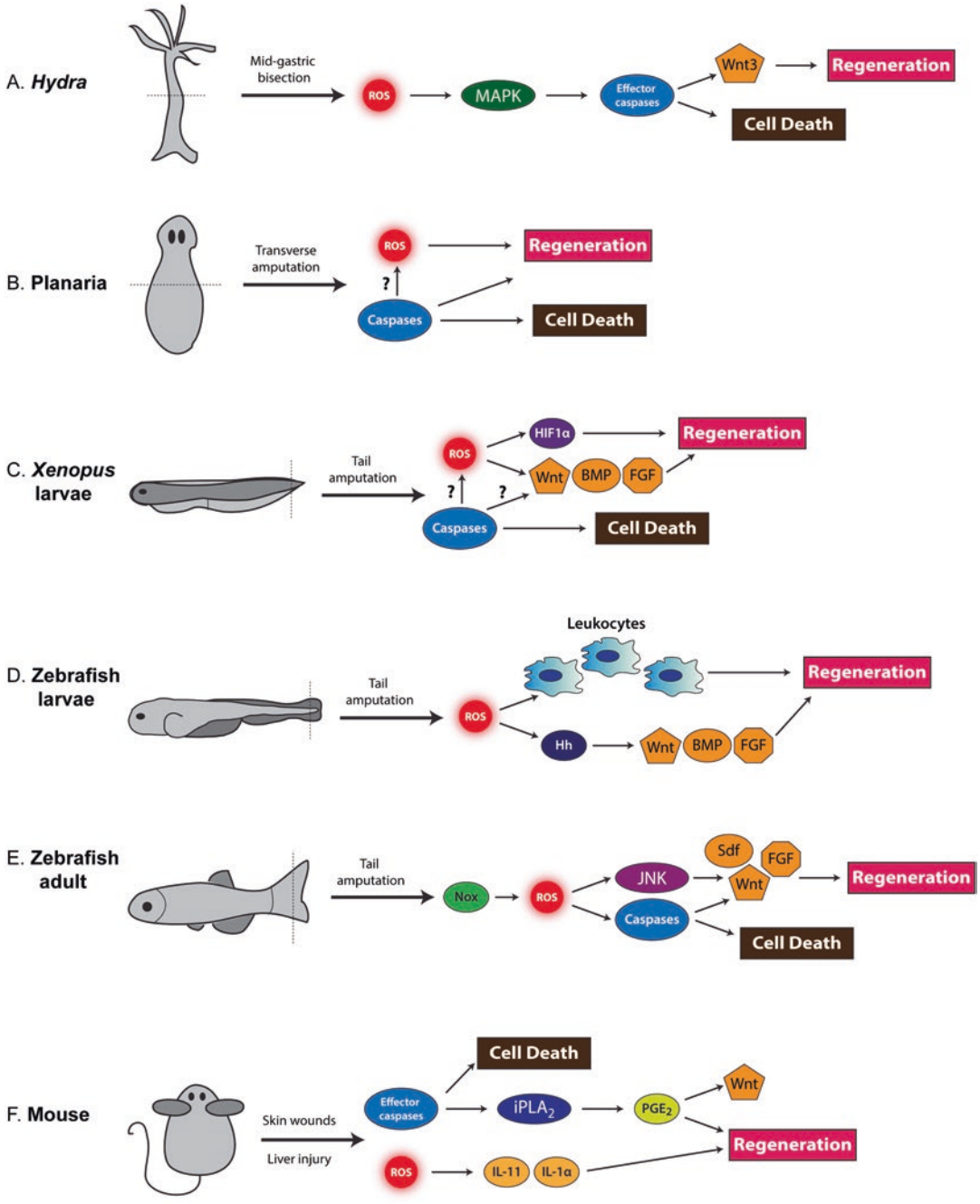

Fig. 4.4 The role of apoptosis-induced proliferation (AiP) for regeneration in different animal models. (See text for details)

the "genuine" model in *Drosophila* [66, 80]. It will be interesting to examine if caspases promote production of ROS in this regenerative context as well.

In the vertebrate *Xenopus laevis*, tadpole tail amputation induces cell death, and apoptotic cells can be detected 12 h post amputation. Caspase activity at the site of injury is essential

for regeneration of the lost tail, as effector caspase inhibitors prevent cell proliferation and regeneration [93]. Tail amputation also induces ROS production and causes an elevated oxygen (O_2) influx immediately after the injury, which is thought to sustain ROS levels over the span of regeneration (Fig. 4.4C). O_2 influx together with ROS stabilize HIF-1α levels to induce regeneration [94, 95]. Decreasing ROS levels by blocking NADPH oxidases also results in impaired regeneration, possibly due to the requirement of ROS to activate Wnt/β-catenin, FGF and BMP signaling pathways [96, 97]. It is not yet known if there is any crosstalk between the HIF-1α pathway and other signaling pathways for regeneration. It will also be interesting to understand if apoptotic caspases have any role in the signaling events following tail amputation.

Similar observations are made during fin regeneration in zebrafish, *Danio rerio*. Tail fin wounding of zebrafish larvae results in generation of a tissue-scale gradient of ROS due to activity of Duox at the site of injury. ROS, in particular H_2O_2, is important for the recruitment of blood cells to the wounds for the purpose of healing (Fig. 4.4D) [98]. This observation is similar to the "undead" fly model, where ROS attracts blood cells for the purpose of inducing overgrowth [66]. In both these contexts, it will be intriguing to identify whether the blood cells sense H_2O_2 as a chemotactic factor, or if H_2O_2 enters the cytoplasm and induces redox signaling events in these cells to direct migration. A recent study further explored the requirement of ROS for larval tail regeneration. Wounding-induced ROS rapidly repositioned notochord cells to the site of damage. These cells secreted the mitogen Hh and activated Hh signaling which is a key regulator of tail regeneration, acting upstream of the Wnt/β-catenin, FGF and Retinoic Acid signaling pathways [99]. In adult zebrafish, caudal fin amputation also causes sustained ROS production via enzymatic activity of Nox, another member of the NADPH oxidase family (Fig. 4.4E). ROS stimulated apoptosis and JNK activation in parallel, and both of these processes were required for AiP and regeneration of the fin. Expression of signaling factors involved in regenerative growth, like FGF20, SDF1, and Wnt proteins, were differentially regulated by the apoptotic pathway and JNK, suggesting these signals might be secreted from dying cells [100].

Mammals have greatly reduced regenerative potential, but do maintain the ability to regenerate a few select tissues, such as the liver and skin. In mice, liver regeneration following partial hepatectomy, along with skin wound healing, depends on the activity of effector caspases, caspase-3 and -7. Mechanistically, effector caspases cleave and activate calcium-independent phospholipase A_2 ($iPLA_2$), which leads to increased secretion of arachidonic acid and lysophosphocholine. These in turn induce the secretion of prostaglandin E_2 (PGE_2), which, in addition to its function in inflammation, promotes stem and progenitor cell proliferation, and tissue repair (Fig. 4.4F) [101]. PGE_2 has also been shown to activate Wnt signaling [102, 103]. In addition, other signaling pathways also play a role in liver regeneration, for example, dying hepatocytes secrete Hh, which induces proliferation of progenitor cells and myofibroblasts [104]. These studies highlight the role of caspases in inducing proliferation and repair. Regeneration of liver also depends on ROS (Fig. 4.4F). Following acute liver injury in mice, dying hepatocytes produced IL-11, a pro-inflammatory cytokine, in a ROS-dependent manner. IL-11 activated the JAK/STAT signaling pathway in healthy hepatocytes, thus inducing compensatory proliferation [105]. Interestingly, ROS also induces hepatocyte necrosis, which leads to release of IL-1α, and in turn induction of compensatory proliferation [106].

Taken together, the regenerative processes in the different organisms have a common theme – induction of apoptosis following amputation or wounding, a damage response such as production of ROS, and finally secretion of different mitogens to induce proliferation. In most cases, active caspases are involved in all or some of these processes. Interestingly, while many examples of effector caspase-driven proliferation exist, the only initiator caspase-dependent regenerative response so far has been described in *Drosophila*, where Dronc mediates AiP independently of its

activation of effector caspases. Given the similarities in the compensatory proliferation observed in all the different systems described above, it would be remiss not to consider the contributions of initiator caspases going forward.

4.4 The "Dark Side" of Compensatory Proliferation: Role in Promoting Cancer

Apoptosis has long been considered a process that prevents cancer. Historically, this view has been supported by the discovery of tumor suppressive roles of p53, where loss-of-function mutations in p53 inhibit apoptosis and are associated with poor cancer prognosis [107–109]. Although resistance to apoptosis is an important feature of cancers, cancer cells are not fully apoptosis-resistant [110]. Indeed, most of the therapy regimens are aimed at killing cancer cells by inducing apoptosis. Counter-intuitively, however, in some cancer types, high levels of apoptosis in tumors also correlate with poor prognosis [111–117]. This paradox can be explained by the now emerging idea that dying cells have a profound effect on their surrounding environment, which includes paracrine signaling from dying cells to stimulate proliferation, invasion and metastasis, thereby promoting cancer progression.

As outlined above, the association of apoptosis and apoptotic signaling with wound-healing and regeneration has been well established. Given the striking similarities between tissue regeneration and cancer, the involvement of AiP for tumorigenesis is of direct relevance (Fig. 4.2). Additional support for this idea comes from the finding that caspase-driven production of PGE_2, important for promoting liver regeneration, can stimulate tumor growth and repopulation following radiation therapy in mice and human cancer cells [118]. Similar observations were made in bladder cancer, where PGE_2 derived from apoptotic cells stimulated proliferation of cancer stem cells to promote resistance to chemotherapy. Importantly, in this context, inhibiting PGE_2 abrogated the AiP responses and sensitized the tumor to therapy, highlighting the contributions of AiP for tumor resistance [119]. In addition, the physiological pathways involved in regeneration may be deregulated in tumors or hijacked by cancer cells for their growth and metastasis. The *Drosophila* "undead" AiP model is an excellent example where the "undead" cells exploit the compensatory proliferation mechanism such that persistent caspase-derived mitogenic signals stimulate overgrowth of the tissue, reminiscent of how tumor cells may hijack the regenerative pathways. Moreover, the apoptosis resistance that many tumor cells have acquired make them resemble "undead" cells. Thus, "undead" cells serve as a great model to understand the multiple contributions of AiP for tumor growth and persistence. In addition to the ability to rapidly proliferate and overgrow, the "undead" model shares many similarities with tumors that extend to the signaling pathways important for promoting overgrowth, and its interaction with the microenvironment.

One key signaling event in AiP is the activation of JNK (Fig. 4.3A, B). Blocking the activity of JNK in "undead" cells suppresses the secretion of mitogens, thereby suppressing the overgrowth [65, 72]. Also, as JNK functions in the feedback amplification loop (Fig. 4.3A), blocking JNK activity also abrogates ROS production and recruitment of macrophages [66]. This makes JNK the "master-regulator" of signaling in AiP. JNK is also well studied for its tumor promoting roles in a variety of different cancer models [120–122]. The association between JNK, AiP and cancer is best established in mouse models of hepatocellular carcinoma (HCC). In humans, HCC is usually associated with chronic liver inflammation caused due to injury and cell death. Using a mouse model of carcinogen diethylnitrosamine (DEN)-induced HCC, the impact of apoptosis on HCC development was investigated [123]. They found that JNK1 activated PUMA (p53 upregulated mediator of apoptosis) to mediate apoptosis and subsequently proliferation. In mice deficient in PUMA, or treated with a JNK inhibitor, the HCC tumor burden was reduced, indicating the importance of the JNK1-

PUMA signaling axis in DEN-induced HCC [123]. Using the same HCC model, studies show that IκB kinase β (IKKβ) deficiency in hepatocytes resulted in an increase in the development of HCC caused by DEN-treatment [124]. This was due to enhanced accumulation of ROS, which are responsible for increased JNK1 activation leading to hepatocyte cell death [125]. This cell death triggered compensatory proliferation of surviving hepatocytes, and ultimately HCC. Increased JNK1 activity in the dying hepatocytes was responsible for releasing IL-1α, which stimulates surrounding Kupffer cells to secrete IL-6 for compensatory proliferation [106]. Similar observations of ROS accumulation, JNK1 activation and compensatory proliferation were observed upon hepatocyte-loss of another IκB kinase (IKKγ/NEMO) [126]. Interestingly, administration of antioxidants prevented increase of JNK activation and compensatory proliferation, thereby preventing HCC in these models [124, 126]. These examples illustrate the same principle as observed for AiP in *Drosophila*, and even though apoptotic cell death is important for compensatory proliferation here, the explicit role of caspases in this process is unknown, and an area for future research.

Analogous to the HCC mice models, *Drosophila* tumor models also show involvement of similar signaling pathways for their growth and invasion. In the $scrib^{-/-}$ Ras^{V12} tumor model, clonal mosaics with the oncogenic mutations are generated that display all the neoplastic features observed in human tumors, including overgrowth, failure to differentiate, invasion of tissues and finally death [127, 128]. A recent study demonstrated that in this tumor context, both initiator (Dronc) and effector caspases (DrICE and Dcp1) are necessary for promoting tumor growth (Fig. 4.5) [129]. This pro-tumorigenic property of caspases is dependent on generation of ROS and activation of JNK (Fig. 4.5). Akin to the "undead" model, caspase-induced ROS are necessary to recruit macrophages to the $scrib^{-/-}$ Ras^{V12} tumor tissue, which in turn signal back to the tumor to activate JNK, thus setting up an amplification loop that promotes neoplastic growth (Fig. 4.5B) [129]. This study exemplifies the similarities between tumor models and the "undead" model in *Drosophila* and highlights the importance of the caspase-ROS-JNK signaling axis for AiP and tumor growth. Why caspases do not induce apoptosis in this tumor context will be subject of future investigation.

ROS, particularly H_2O_2, act as early damage signals to initiate regenerative responses, important for compensatory proliferation as described above (reviewed in [77]). In addition to this, increased ROS production has been implicated in various cancers and is thought to be involved in the development and progression of cancer by activating pro-tumorigenic redox signaling, enhancing cell proliferation, and inducing DNA damage and genomic instability. The requirement of ROS in the *Drosophila* "undead" AiP and tumor models for mediating overgrowth emphasizes its importance [66, 129]. Extrapolating to mammalian systems, a study reported that oncogenes like activated Ras promote ROS production in a Rac1- and Nox4-dependent manner to drive the initial hyperproliferative response in human cells as well as in zebrafish [130]. Another study explored the role of Rac1-mediated ROS production and NF-κB activation in colorectal cancer to facilitate WNT-driven intestinal stem cell proliferation [131]. ROS can promote proliferative responses by regulating the mitogen activated-protein kinase (MAPK)/extracellular-regulated kinase 1/2 (ERK1/2), phosphoinositide-3-kinase (PI3K)/Akt and protein kinase D (PKD) signaling pathways [132]. Additionally, the involvement of AiP in the etiology of human cancers has been reported in multiple different studies [133–140]. Taken together, all these examples underline the importance of AiP in inducing proliferation and cancer initiation, and even though the signaling pathways may be different for each context, the conservation of key factors is remarkable.

Another key feature of cancers is the ability to invade other tissues and metastasize [2]. The metastatic process initiates after cells in a tissue migrate out of their environment, a process that mainly requires damage to the basement membrane (BM). Multiple *Drosophila* tumor models have been described, including the $scrib^{-/-}$ Ras^{V12}

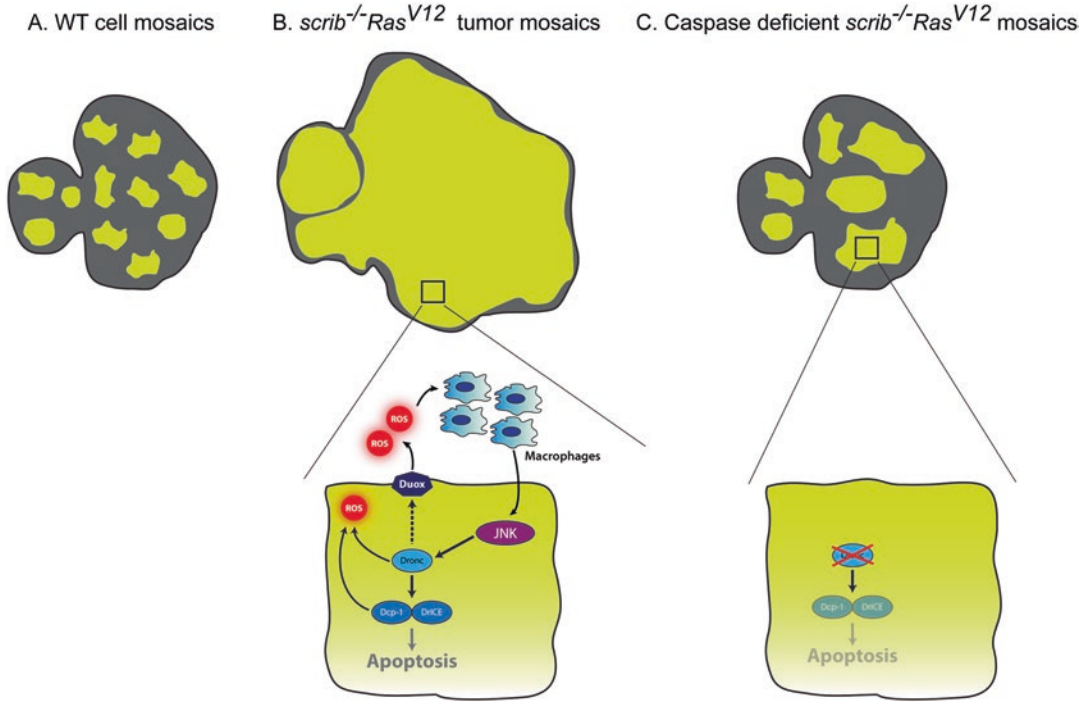

Fig. 4.5 The role of caspases for *scrib*$^{-/-}$ *Ras*^{V12} tumor growth in *Drosophila*. Shown are mosaic eye-antennal imaginal discs from late *Drosophila* larvae. Control (**A**), *scrib*$^{-/-}$ *Ras*^{V12} (**B**) and *scrib*$^{-/-}$ *Ras*^{V12} clones deficient for caspases (**C**) are indicated in green. Note the strong overgrowth of both *scrib*$^{-/-}$ *Ras*^{V12} clones and the entire *scrib*$^{-/-}$ *Ras*^{V12} mosaic disc in (**B**). Although caspases are activated in *scrib*$^{-/-}$ *Ras*^{V12} tumor tissue, they do not induce a significant amount of apoptosis. Instead, they mediate the generation of intra- and extracellular ROS which setup an amplification loop involving *Drosophila* macrophages (hemocytes), JNK activation and sustained caspase activity (**B**, bottom). Genetic loss or inhibition of caspases suppresses *scrib*$^{-/-}$ *Ras*^{V12} tumor growth (**C**)

model, which have helped to identify the mechanisms involved during cell invasion and metastasis [141]. "Undead" cells do not show the ability to invade distant tissues like other neoplastic cells; however, they do share the ability to migrate, a prerequisite for invasion. A study reported that in "undead" cells, residual effector caspase activity drives migration of cells by activating JNK [142]. The migrating cells also express the matrix metalloproteinase MMP1 [142], known for its function to degrade the BM during cell invasion [143]. The ability of "undead" cells to migrate, but not invade distal tissues is puzzling, and may indicate some inhibitory factors in play that block invasion, or the need for activation of other pathways that would promote invasion. Future work could help uncover the events that would make "undead" cells amenable for tissue invasion. However, the pro-migratory activity of caspases is conserved in many cancers. Similar to the *Drosophila scrib*$^{-/-}$ *Ras*^{V12} tumor model where activity of caspases is required for invasion and metastasis, in human cancers, caspase 3 activation promotes cell migration and invasion in glioblastoma, melanoma, and ovarian cancer [144–147].

The idea that tumors consist of a homogenous population of cancerous cells is quite restrictive, and in the past few years, the concept of "tumor microenvironment (TME)" has gained popularity. The TME is comprised of proliferating cancerous cells as well as cancer-associated fibroblasts, tumor stroma, extracellular matrix, adipose tissue, endocrine cells and blood vascular network, and infiltrating immune cells [2, 148, 149]. The infiltrating immune cells were initially thought to have anti-tumorigenic properties and function to antagonize tumor growth; how-

ever, recent evidence points towards a more tumor-promoting role for these cells in the TME. The immune cells establish a chronic inflammatory environment in the tumors, thus helping portray tumors as wounds that never heal [51, 150]. These immune cells secrete a variety of signaling molecules, which include inflammatory cytokines, growth factors, angiogenic factors and BM degrading factors that aid in tumor proliferation, progression and metastasis [151, 152]. Among the inflammatory cells present in the TME, tumor-associated macrophages (TAM) are of particular importance. Tumor-derived factors reprogram the polarization of TAMs towards the "alternatively-activated" M2 phenotype. TAMs promote cell growth, angiogenesis and matrix remodeling while inhibiting anti-tumor immune responses, thus supporting tumorigenesis [153]. Like the mammalian macrophages, *Drosophila* macrophages also have tumor-promoting functions. *Drosophila* tumor models and the "undead" model are characterized by the presence of TAMs that secrete cytokines, like Eiger [66, 129, 154]. Whether these TAMs also undergo "alternate activation" to promote tumorigenesis is an area of active interest. Intriguingly, in response to ROS, the macrophages on the "undead" cells and tumors show changes in morphology and spread [66, 129], making it quite tempting to assume ROS or some other tumor-derived factors change macrophage properties, probably making them "alternatively activated".

The "undead" cell model in *Drosophila* has been instrumental in advancing the field of compensatory proliferation, and as more evidence comes into the forefront, the contributions of the "undead" model for understanding tumorigenesis become more apparent. The "undead" model shares many of the hallmarks of cancer, namely the increased proliferation, evasion of cell death, cell migration, and tumor-promoting inflammation by TAMs. These parallels emphasize the importance of caspase-driven AiP in cancer, at the same time corroborating the efficacy of *Drosophila* as a model system to study cancer initiation and growth. It would be interesting to determine if the "undead" model shares more of the hallmark properties of tumors. Based on all these observations, the view that the "undead" model is just a hyperplastic overgrowth model needs to be revised.

4.5 Conclusions

The tumor-suppressing function of apoptosis makes a lot of sense from a logical point of view. While apoptosis certainly has this activity for some types of cancer, one would expect that tumor cells would more often inactivate the apoptotic machinery by genetic inactivation. However, this is not observed and clinical data suggest that often apoptosis correlates with poor prognosis for the patient. Although the molecular mechanisms of apoptosis are very well understood, its influence on the cellular environment is not. Apoptotic cells – before they die – communicate with and influence their environment which may be beneficial for the organism during wound healing and regeneration. In case of cancer, however, AiP may trigger tumorigenesis or relapse after therapy. Continued work probing AiP in genetically tractable model organisms will provide clues for which players and pathways may be at work in human diseases, while clinical investigations will guide the search for non-apoptotic caspase involvement in new contexts, potentially informing novel therapies and improving patient outcomes.

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The Initial Stage of Tumorigenesis in *Drosophila* Epithelial Tissues

5

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Abstract

Cancer development originates in a single mutant cell transformed from a normal cell, including further evolution of pro-tumor cells through additional mutations into malignant cancer tissues. Data from recent studies, however, suggest that most pro-tumor cells do not develop into tumors but remain dormant within or are prophylactically eliminated from tissues unless bestowed with additional driver mutations. *Drosophila melanogaster* has provided very efficient model systems, such as imaginal discs and ovarian follicular epithelia, to study the initial stage of tumorigenesis. This review will focus on the behaviors of emerging pro-tumor cells surrounded by normal cells and situations where they initiate tumor development.

Keywords

Tumorigenesis · Tumor hotspot · Cell competition · Epithelial tissues

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5.1 Introduction

Tumor progression is driven by a sequence of continually occurring genetic mutations and epigenetic alterations of DNA that affect the genes involved in cellular proliferation, apoptosis, invasion, and other traits associated with the malignant cancer phenotype [1]. Cancer biology has progressed tremendously over the past several decades with the development of molecular genetics and cell biology. Although this accumulation of knowledge in cancer research revealed various genetic backgrounds associated with processes of cancer development, it is still unclear how transformed mutant cells (pro-tumor cells) within a normal epithelial tissue behave, and what precise events occur at the crucial beginning of tumorigenesis. Tumorigenesis entails a progressive disruption of tissue organization and unleashed proliferation. This suggests that pro-tumor cells deteriorate tissue integrity or evade the robustly organized tissue environment at the initiation of tumor development. This chapter will focus on the earliest stage of tumorigenesis, such as behaviors of pro-tumor cells in epithelial layers, cell-cell interactions between pro-tumor cells and neighboring normal cells, and tumor initiation within tissue-intrinsic oncogenic microenvironments. The observations presented herein focus primarily on studies established in *Drosophila* epithelial tissues, particularly the imaginal discs in larvae and ovarian follicular

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epithelia in adult female flies, both of which have been especially popular model systems to study cellular growth control, epithelial cell polarity, and intercellular interaction between pro-tumor cells and normal cells.

5.2 Oncogenic Transformation

An epithelial tumor generally originates from a single transformed mutant cell among the highly structured and tightly regulated layer of cells which compose the tissue [1]. Despite this, an enormous number of cells in the healthy human body perpetually experience various stressors and mutagens from exogenous and even endogenous sources which may ultimately contribute to the development of genetic mutations, although primarily deleterious and leading to apoptosis. If the genetic mutation causes activation of an oncogene or inactivation of a tumor-suppressor gene, however, the mutant cell will become a pro-tumor cell with the potential to be cancerous. Nascent pro-tumor cells emerged within an epithelial layer "evolve" into malignant cells with neoplastic phenotypes through subsequent transformations over time. This process of tumor progression normally requires a multistep sequence of randomly occurring mutations to prevent apoptosis, facilitate proliferation, and promote dissemination throughout the tissue [1]. In other words, normally an initially emergent pro-tumor cell with a single mutation cannot immediately develop into a tumor, unless it subsequently gains additional driver mutations. In fact, recent studies of healthy human tissues demonstrated that somatic mutations, including nucleotide substitutions and chromosomal anomalies, increase with age [2–5]. This suggests that cells carrying cancer-causing mutations accumulate over time in various types of tissue. Considering the amount of cells in a human body, the number of transformed cells should accumulate at a significant pace every day. Nevertheless, many of these pro-tumor mutant cells are dormant and do not grow into tumors as would be expected based on the mutational load [6, 7].

5.3 Competitive Interaction Between Pro-Tumor Cells and Their Neighbors

During the primordial stage of cancer initiation, a transformed pro-tumor cell emerges within the epithelial layer typically surrounded by normal cells, leading to complex interactions between pro-tumor cells and healthy neighbors [8]. One of the most important interactions is cell competition, a competitive cellular interaction which occurs when neighboring cells differ in intrinsic cellular properties contributing to selective elimination of either cell type [9, 10]. Many studies, especially in *Drosophila* epithelial tissues such as larval imaginal disc epithelia, have shown that the emergence of pro-tumor mutant cells frequently gives rise to a competitive relationship with surrounding normal cells [11–13].

Studies in *Drosophila* imaginal discs have shown that various types of mutant cells that are defective in growth rate, anabolic activity, or epithelial cell polarity trigger cell competition with surrounding normal cells and are eliminated from host tissues [12, 14]. Interestingly, it has been shown that genetically mosaic clones mutant for a group of tumor-suppressor genes identified in *Drosophila* – *lethal giant larvae* (*lgl*), *discs large* (*dlg*), and *scribble* (*scrib*)) – are outcompeted by normal neighbors and are therefore eliminated from tissues [15–18]. These tumor suppressor genes play key roles in the formation of apical-basal cell polarity and regulation of cell proliferation in developing epithelial tissues like imaginal discs [19, 20]. When imaginal-disc epithelial cells in *Drosophila* larvae have a homozygous mutation for any of these three genes, the normally monolayered epithelium loses its organized structure, fails to differentiate, and overproliferates thus becoming a multilayered amorphous masses that fuses with adjacent tissues [20]. Loss or alteration in expression of the homologs of these genes in mammals including human is also associated with development of malignant tumors [21, 22]. The neoplastic phenotypes exhibited by mutant tissues led to the classification of these three genes as conserved neoplastic tumor-suppressor genes (nTSGs) [20,

21, 23]. When sporadic nTSG mutant clones are generated in the developing imaginal disc epithelia using the FLP/FRT-mediated mitotic recombination technique, however, mutant cells adjacent to wild-type cells are eliminated through JNK (c-Jun N-terminal kinase)-dependent apoptosis and basal extrusion [15, 17, 24], or by engulfment and phagocytosis by neighbors [25]. Recently, the ligand SAS (Stranded at second) and the receptor-type tyrosine phosphatase PTP10D have been reported as the cell-surface ligand-receptor system responsible for the JNK-mediated apoptosis of nTSG mutant cells [26]. At the interface between the nTSG mutant cells and the surrounding normal cells, the SAS ligand in the wild-type cells and the PTP10D receptor in the mutant cells relocalize from apical to lateral cell membrane, leading to the trans-activation of this signaling pathway to trigger JNK activation-mediated apoptosis in nTSG mutant cells [26].

Another mechanism to induce JNK activation-mediated apoptosis in nTSG mutant cells involves circulating hemocytes, *Drosophila* blood cells which play a key role in the provision of cellular innate immune responses and in development where they secrete and remodel extracellular matrix components [27]. JNK-dependent cell death in nTSG mutant cells requires the expression of Eiger, the *Drosophila* tumor necrosis factor (TNF) [16]. Circulating hemocytes are recruited and adhere to the nTSG-deficient tumor cells as an innate immune response upon detection of basement membrane disruption [28]. These recruited hemocytes secrete Eiger to upregulate JNK signaling activity non-cell autonomously in tumor cells [29]. Thus, pro-tumor cells like nTSG mutant bearing an apico-basal polarity defect are outcompeted by the surrounding normal cells or urged to apoptose by circulating hemocytes to be eliminated from the epithelial tissues (Fig. 5.1).

Contrary to these cases, when oncogenic transformed cells mutant for the Hippo signaling pathway, Wingless (Wg) signaling pathway, or cells overexpressing proto-oncogene dMyc (*Drosophila* homolog of c-myc) or hyper-activating JAK/STAT signaling are generated as genetically mosaic clones in imaginal discs, these oncogenic mutant clones outcompete neighboring wild-type cells and overcolonize the epithelial tissues as "supercompetitors" [30–35]. Therefore, if the transformed cell emerged in an epithelium is a pro-tumor mutant cell defective in apico-basal polarity, intercellular competitive interaction with surrounding cells functions as an intrinsic tumor-suppression system to maintain homeostasis of epithelia. On the other hand, if the transformed cell is a hyperproliferative mutant cell such as a Myc-overexpressing cell or a Wg pathway-hyper-activating cell, these cells do not induce epithelial polarity defects or disorganization but outcompete neighboring wild-type cells and form a cancerization field in which possibilities of subsequent driver-mutation hits will be higher [11, 12]. In fact, ectopic expression of oncogenic signaling genes such as activated Ras, Notch, or Yorkie (Yki: *Drosophila* homolog of Yes-associated protein YAP) in nTSG mutant cells with epithelial polarity defects cooperatively induces tumorigenesis [15, 18, 24, 36] (Fig. 5.2).

5.4 Misoriented Cell Division as an Initiator of Tumorigenesis

Epithelial tissues are composed of apico-basally polarized cells. Especially in epithelial tissues constituted by a single layer of epithelial cells such as *Drosophila* imaginal discs and ovarian follicular epithelia, the direction of mitotic cell division is critical for the maintenance of tissue organization as an epithelial sheet [37]. Because of this physical constraint of a sheet-like structure, the direction of cell division is controlled to be parallel to the plane of the epithelial sheet [38]. If the cells in the epithelial monolayer divide perpendicularly, the cells may pile up and cause a disorganization of the sheet-like structure [38, 39]. Because loss of epithelial tissue architecture and uncontrolled cellular proliferation are early signs of dysplasia, mitotic misorientation-induced multilayered stratification should be one important aspect of tumor initiation [39].

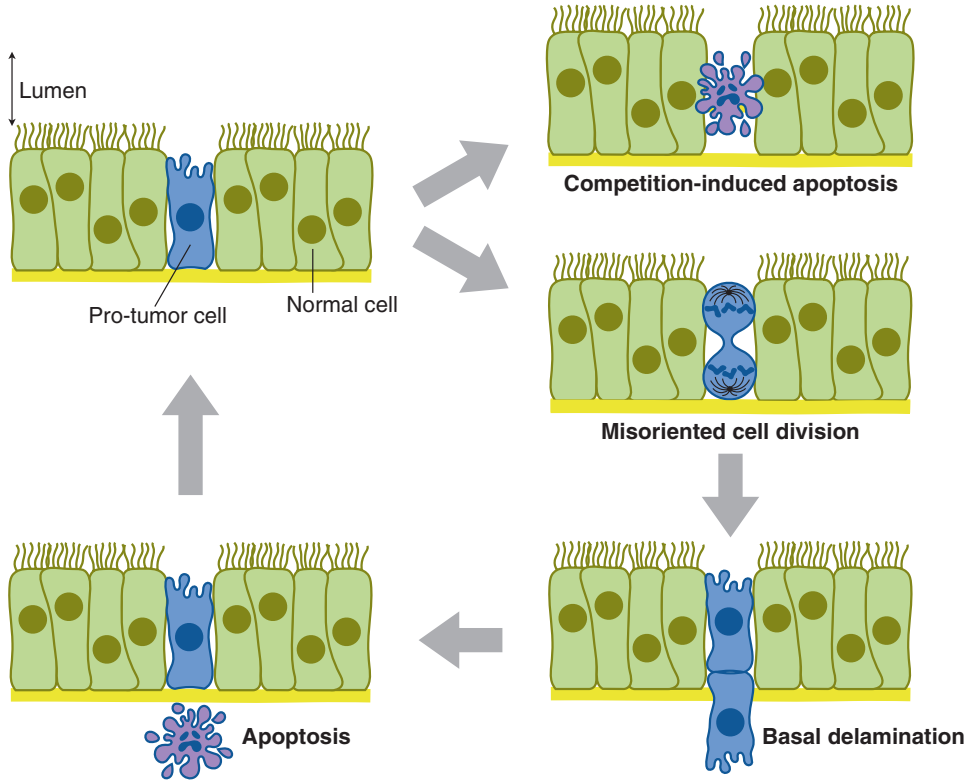

Fig. 5.1 Elimination of pro-tumor cells in an epithelial monolayer. The emergence of pro-tumor mutant cells (blue) induces a competitive relationship with surrounding normal cells (green) (upper panel). In some cases, misoriented cell division caused by spindle misorientation results in basal delamination and apoptosis of pro-tumor cells

Loss of function of genes involved in the establishment and maintenance of apico-basal polarity of epithelial cells such as *atypical Protein Kinase C* (*aPKC*), *crumbs*, *PAR3/bazooka* (*baz*) induce multilayering and uncontrolled proliferation in ovarian follicular epithelia [40]. Also, cells mutant for *integrin*, a heterodimeric transmembrane receptor involved in the adhesion of cells to the extra-cellular matrix (ECM) and signal transduction from the ECM to the cells, develop multilayers in ovarian follicular epithelia [41]. Although the *integrin* mutant cells do not show apico-basal polarity defects in the epithelial cells, they induce an aberrant orientation of the mitotic spindles; the spindle orientation is randomized and frequently perpendicular to the epithelial plane [41, 42]. Follicle cells (FCs) mutant for *α-Spectrin*, a subunit of the Spectrin cytoskeletal protein complex that lines the inner side of plasma membrane, show multilayer phenotypes similar to those of *integrin* mutants [43]. In the multilayered masses of FCs induced by *α-Spectrin* mutations, defects in epithelial polarity and differentiation are observed only in outer ectopic layers but not in the innermost layer. Furthermore, the mutilayered phenotype induced by a disruption of the Hippo signaling tumor suppressor pathway is caused by misalignment of mitotic spindles [44]. In this case, again, deterioration of apico-basal cell polarity is observed only in the ectopic layers of mutilayer masses [44]. These observations suggest that piling up of epithelial cells as an initial sign of tumorigenesis is caused by misorientation of mitotic spindles rather than apico-basal cell polarity defects (Fig. 5.3).

Recently, it has been shown in *Drosophila* that the protein products of nTSGs, *lgl*, *scrib* and *dlg*,

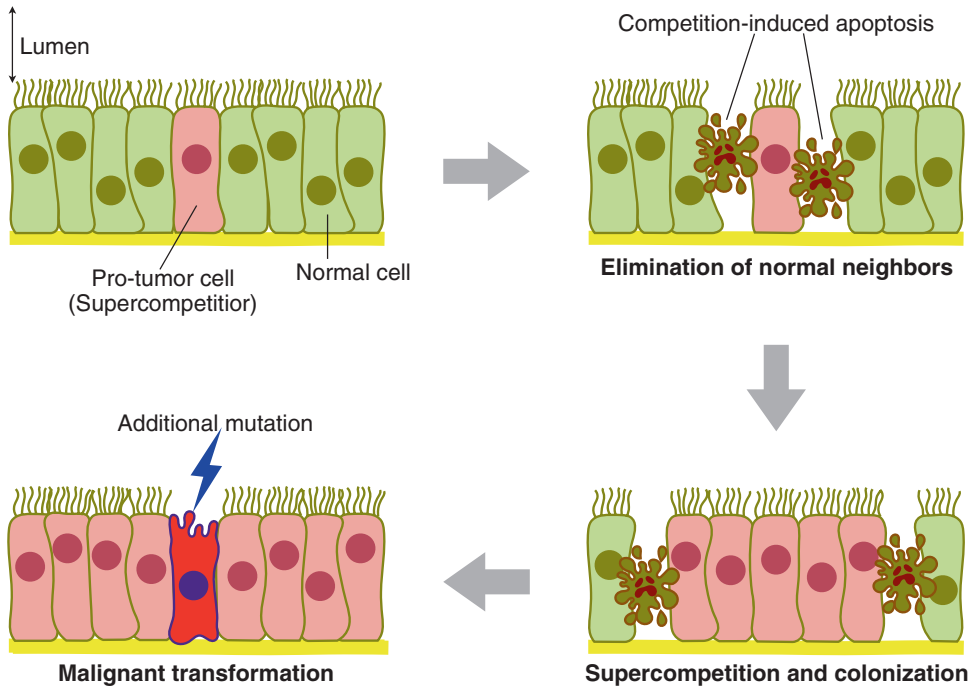

Fig. 5.2 Supercompetition and field cancerization. Hyperproliferative mutant cells (pink) such as a Myc-overexpressing cell outcompete their wild-type neighbors (green) as a supercompetitor. This process does not induce epithelial disorganization but results in a colonization of oncogenic mutant cells and formation of a cancerization field in which possibilities of subsequent driver-mutation hits will be higher

all of which act as a scaffold for the septate junction, play a key role in determining the planar orientation of the mitotic spindle that interacts with mitotic apparatuses in proliferating epithelial cells [45–48]. This function of nTSG proteins coordinates the geometry of chromosome segregation with the architecture of polarized cell-cell junctions, thereby ensuring epithelial integrity [45, 46]. In *Drosophila* epithelial tissues such as imaginal discs and ovarian follicular epithelia, mitotic spindles are aligned along the plane of the septate junction, which localizes below the adherens junction [37]. In the *Drosophila* wing imaginal epithelia, mutations of either Scrib or Dlg induces fluctuation in the direction of mitotic spindles and abnormal planar orientation, which in turn cause misoriented cell division orthogonal to the plane of the epithelium [46]. Lgl is also involved in the control of mitotic spindle orientation: during mitosis, the mitotic kinases Aurora A and B phosphorylate Lgl to promote its relocalization from the basolateral membrane to the cytoplasm [47, 48]. In fact, a mutated Lgl that does not have two phosphorylation sites for Aurora cannot be detached from the basolateral membrane. This Aurora-insensitive Lgl rescues the apico-basal cell polarity in *lgl* mutant wing discs but cannot rescue the mitotic spindle orientation defect, suggesting that the cytosolic relocalization of Lgl by Aurora-mediated phosphorylation is required for normal mitotic spindle orientation [47]. Based on these data, it has been suggested that remodeling the basolateral complex by removal of Lgl from the plasma membrane at mitosis allows Pins (Partner of Inscuteable) to bind Dlg to orient the mitotic spindle in the plane of the epithelium [37, 47].

In the case of perpendicular cell division caused by the spindle misorientation in the imaginal disc, the basally located daughter cell delaminates from the basal side of the epithelial layer and undergo apoptosis (Fig. 5.1). This spindle misorientation-induced elimination of nTSG mutant cells is triggered by live-cell delamina-

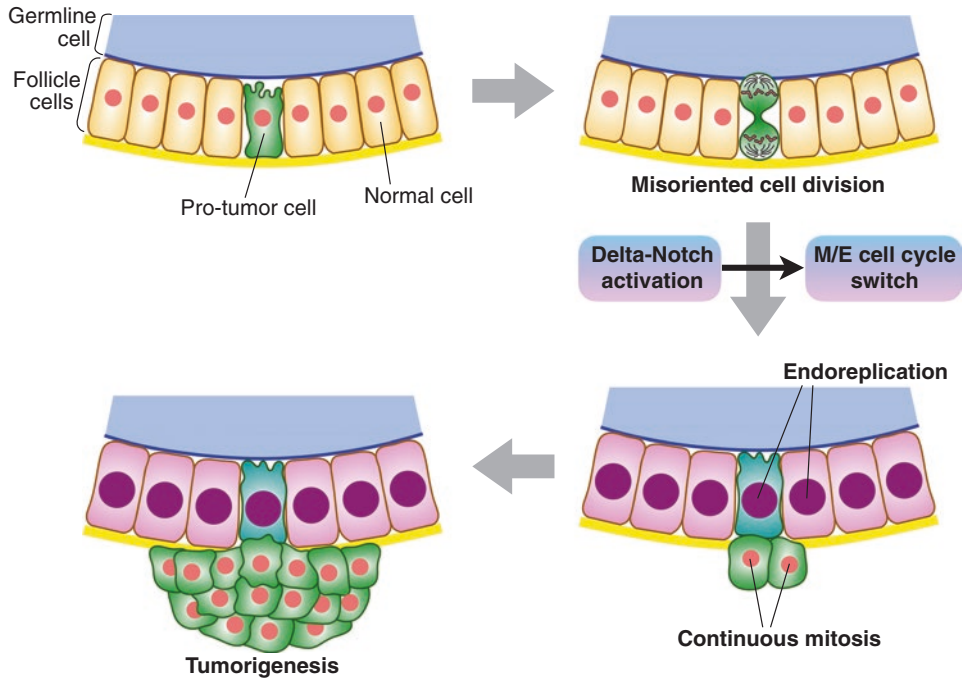

Fig. 5.3 A possible model for tumorigenesis in *Drosophila* ovarian follicular epithelia. Mutant cells (green) which induce spindle misorientation frequently causes misoriented cell division orthogonal to the plane of the epithelium. When the mutant cells divide perpendicularly, the basally-located daughter cell cannot access the ligand Delta expressed in the germline cells (blue), because its apically-located sibling will physically interrupt the juxtaposed signaling between them. This situation does not allow the basally-located daughter cell to switch cell cycle, resulting in its continuous proliferation

tion, since apoptosis of nTSG mutant cells was observed at the basal side of the epithelial layer after delamination [46]. When apoptosis of basally-delaminated *scrib*-knockdown cells are inhibited by the expression of caspase inhibitor p35, these cells form tumor masses on the basal surface of the epithelial layer [46, 49]. Therefore, in the imaginal disc epithelia, the pro-tumor nTSG mutant cells are basically eliminated from the epithelial layer through basal delamination and apoptosis, even if nTSG-defects induce misorientation of mitotic division [7] (Fig. 5.1). On the other hand, in the ovarian follicular epithelia, not only the nTSG mutant cells but also mutations for genes involved in the formation or maintenance of cell polarity and structures trigger signs of tumor initiation, such as epithelial disorganization and uncontrolled proliferation. The reason why such mutant cells have a greater chance of triggering tumorigenesis in the ovarian follicular epithelia could be attributed to the intrinsic cell-cycle regulation mechanism specific for this epithelial tissue (see below).

5.5 The Cell Cycle Regulation of the Ovarian Follicle Cells

Drosophila ovaries are composed of 16–20 ovarioles which is a string of progressively developing egg chambers. Each egg chambers contain 16 inter-connected germline cells including one oocyte and 15 nurse cells covered by a monolayer of somatic follicular epithelial cells [50]. The ovarian FCs of *Drosophila* egg chambers provide an excellent model for studies of developmental regulation of cell cycle programs, DNA replication, and epithelial cell polarity and differentiation [51]. The *Drosophila* egg chamber development is composed of 14 oogenesis stages (Fig. 5.4). Based on the cell cycle programs of the somatic FCs, oogenesis stages can be divided

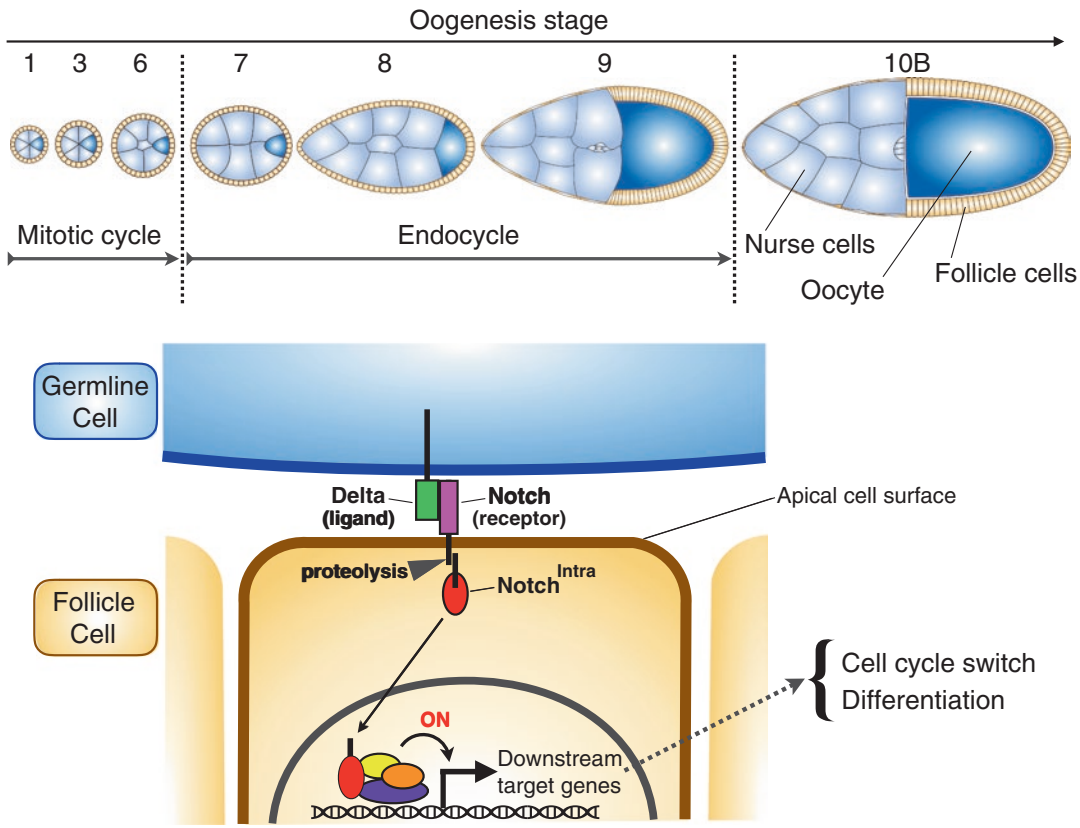

Fig. 5.4 Schematic diagrams of the *Drosophila* oogenesis (upper panel) and the Delta-Notch signaling in egg chambers. The *Drosophila* egg chamber development is composed of 14 oogenesis stages. Each egg chambers contain 16 inter-connected germline cells (blue) including one oocyte and 15 nurse cells covered by a monolayer of somatic follicular epithelial cells (orange). In stage 7, follicle cells switch their cell cycle program from mitotic to endoreplication cycle (endocycle). This cell cycle switch in the follicle cells is dependent on the Delta-Notch-mediated intercellular signaling with inner germline cells (lower panel). A transmembrane receptor Notch is cleaved after Delta binds to its extracellular domain, and its intracellular domain translocates into the nucleus and induces transcription of downstream target genes

into three different categories: the mitotic stage (stage 1–6), the endoreplication stage (stage 7–10a), and the gene-amplification stages (stage 10b-13). During the mitotic stage, the FCs with a cuboidal shape undergo 8–9 rounds of complete divisions and grow to approximately 650 FCs [51]. In stage 7, FCs switch their cell cycle program from mitotic to endoreplication cycle (endocycle), a variant cell cycle composed of DNA synthesis and gap phases without mitosis. During the endoreplication stage, FCs undergo three rounds of endocycles, which increase their genomic DNA contents from 2C to 16C [52, 53]. At stage 10B, they leave the endoreplication stage, and the main-body FCs differentiated into columnar-shape epithelial cells undergo synchronized amplification of genomic loci encoding eggshell proteins [53, 54]. Therefore, the follicular epithelium after oogenesis stage 7 is composed of nonproliferating postmitotic cells (Fig. 5.4).

Cell cycle regulation of FCs during oogenesis depends on intercellular signaling with inner germline cells [55, 56] (Fig. 5.4). At stage 5 of oogenesis, the expression of Delta, a transmembrane ligand of Notch signaling, is upregulated in the germline cells. Delta protein exposed on the surface of germline cells binds to the Notch receptor localize at the apical membrane of somatic FCs [56]. Notch is a transmembrane pro-

tein that is cleaved after Delta binds to its extracellular domain; the Notch intracellular domain migrates into the nucleus and induces transcription of downstream target genes [57] (Fig. 5.4). Therefore, the mitotic cycle-to-endocycle switch requires a physical interaction between the inner germline cells and outer FCs.

The mitotic/endoreplication cell cycle switch by Delta-Notch-mediated juxtaposed signaling between germline and FCs explains why mutant cells which induce spindle misorientation, such as nTSGs, *integrin*, or *α-Spectrin,* become tumorigenic in the ovarian FCs. As mentioned above, those mutations randomize the spindle orientation during mitosis, which frequently causes misoriented cell division orthogonal to the plane of the epithelium. When the mutant cells divide perpendicularly, the receptor Notch localized at the apical surface of the apically-located daughter cell normally binds to the ligand Delta localized on the surface of the inner germline cells. The other basally-located daughter cell, however, cannot get the ligand Delta from the germline cells, because the apically-located daughter cell will physically interrupt the juxtaposed signaling between them (Fig. 5.4). Here, the basally-located daughter cell cannot stop proliferating because the blockade of Notch signaling prevents the mitotic/endocycle switch [43]. Therefore, when those mutant cells induce the multilayered phenotype in the follicular epithelia, the mutant cells at the innermost layer of the multilayered masses normally contact germline cells and switch to endoreplication, thus maintaining a columnar structure [42]. On the other hand, those mutant cells located at the basal side of the innermost layer become tumorigenic. For this reason, the tumorigenic overgrowth of the mutant clones which induce spindle misorientation is induced in the mitotic stages of oogenesis. Consistent with this, nTSG mosaic mutant clones in the follicular epithelia generated after mitotic/endocycle switch do not induce tumorigenesis [58].

In addition, nTSG mutant cells are not outcompeted by surrounding wild-type cells in the endoreplication stages of oogenesis [58]. It is unclear why cell competition does not eliminate nTSG mutant cells in the postmitotic endocycle stages. Also, other mutations that cause supercompetition with wild-type cells in imaginal discs such as overexpression of dMyc or Yki do not induce cell competition with wild-type cells in the endocycle stages [58]. Cell competition can be considered as a replacement of unfit cells by fitter cells through apoptosis and proliferation [59]. Therefore, in a tissue composed of postmitotic cells such as the ovarian follicular epithelia in the endoreplication stages, supercompetition might not be able to occur. In the postmitotic follicular epithelia, however, mutant cells heterozygous for *Minute*, a group of dominant mutations defective in ribosomal proteins, or homozygous for *mahjong*, an evolutionarily conserved cell-competition regulator, have been shown to be outcompeted by their wild-type neighbors and undergo apoptosis. In the case of such mutation-induced cell competition in the postmitotic epithelia, remaining wild-type neighbors undergo hypertrophic cell growth to compensate for the cell loss in a process termed CCH (compensatory cellular hypertrophy) [58]. Although it is unclear why postmitotic supercompetitiors cannot take advantage of this compensatory mechanism to outcompete other cells, it might occur in other uninvestigated postmitotic tissues.

5.6 Reintegration of Misplaced Cells as an Error-Correction System

In the *Drosophila* ovarian follicular epithelia, an error-correction mechanism promotes the reinsertion of misplaced cells. Inscuteable is a protein which recruit Pins and Mud (both of which are the required for the interaction between the cell cortex and astral microtubules) to the apical cortex of neuroblasts to orient mitotic spindles along the apico-basal axis in neuroblast [60–63]. Although ectopic expression of Inscuteable in the ovarian FCs induces reorientation of mitotic spindle perpendicular to the epithelial plane, it does not disrupt epithelial architecture. Interestingly, rather than dying, misplaced daughter cells reintegrate back into the epithelial monolayer [64]. This type of error correction for misplaced cells can be

observed even in normal wild-type follicular epithelia, suggesting this is a normal feature to maintain tissue integrity in proliferating epithelial cells. Reintegration of apically extruded cells after spindle misorientation was observed when spindle misorientation was induced by Inscuteable overexpression in the ovarian FCs. While similar mitotic misorientation is induced by mutant cells defective in apico-basal polarity such as nTSG mutant clones, the end result is radically different as these cells multilayer and overgrow as tumors in ovarian follicular epithelia [65, 66]. It is still unclear why this error-correcting reintegration system does not work after mitotic misorientation of nTSG mutant cells.

5.7 Tumorigenesis Induced by Endocytic TSGs Defects

In *Drosophila*, mutations for another group of genes including *avalanche* (*avl*), *Rab5*, *erupted/tumor-susceptibility gene-101* (*tsg101*), and *vps25* have been found to show similar tumorigenic phenotypes to those of nTSG mutant cells in imaginal discs and ovarian follicular epithelia [67–70]. They are known as "endocytic tumor-suppressor genes (endocytic TSGs)," because these genes encode components of the endocytic machinery and are involved in vesicular trafficking of transmembrane proteins [71]. Each of them is required for different steps in trafficking of proteins from the plasma membrane to the lysosome, and mutation of each gene blocks endocytic degradation of certain transmembrane proteins and induces epithelial polarity defect and neoplastic overgrowth in both imaginal discs and ovarian follicular epithelia [72]. Mosaic mutant clones of these genes undergo apoptosis when they are surrounded by wild-type neighbors in imaginal epithelium [68–70, 73]. The apoptosis of these mutant clones can be prevented either by alleviation of competitive pressure by means of the *Minute* technique or by expression of the viral caspase inhibitor protein p35. In both cases, blockade of apoptosis allows the mutant clone to grow to form a tumor mass [69]. These indicate that the tumor growth induced by mosaic mutant clones of these genes results from both cell-autonomous and non-cell-autonomous mechanisms. Endocytic trafficking defects in these mutant cells result in the cellular accumulation of specific membrane proteins including the signaling receptor Notch and the epithelial-polarity determinant Crumbs (Crb) [67, 68]. Crb is an upstream regulator of Hippo signaling pathway [74]. Therefore, overexpression of Crb itself induces hyper-proliferation in imaginal disc epithelia. Increased Notch activity as a result of the endocytic trafficking defect leads to ectopic production of Unpaired, a secreted cytokine-like ligand of the JAK/STAT signaling pathway, which induces ectopic activation of JAK/STAT signaling and overproliferation of the neighboring wild-type cells [70]. Overactivation of Notch signaling itself induces hyper-proliferation in wing imaginal discs but does not result in an epithelial disorganization or dysplastic tumor growth [75], suggesting that tumorigenesis induced by a defect of endocytic TSGs results from the aberrant intracellular accumulation of two different types of membrane proteins, a receptor of growth signaling pathway (Notch) and an apico-basal polarity determinant (Crb). In the ovarian follicular epithelia, defects of Notch signaling induce uncontrolled proliferation because of the mitotic-endocycle switch, but preserve the monolayer and do not show tumor growth [55, 76, 77]. This endocytic TSG defect-triggered tumor induction mechanism demonstrates one of the general features of tumor initiation: a combination of different types of mutations causing both uncontrolled proliferation and deteriorated cellular structure is necessary for tumorigenesis.

5.8 Tumor Hotspots, a Tissue-Intrinsic Oncogenic Niche

Imaginal discs in *Drosophila* larvae homozygous mutant for nTSG or endocytic TSG do not maintain a cohesive epithelial structures and become tumor masses, likely because the primordial disc cells could not appropriately differentiate into apico-basally polarized epithelial cells [20, 71].

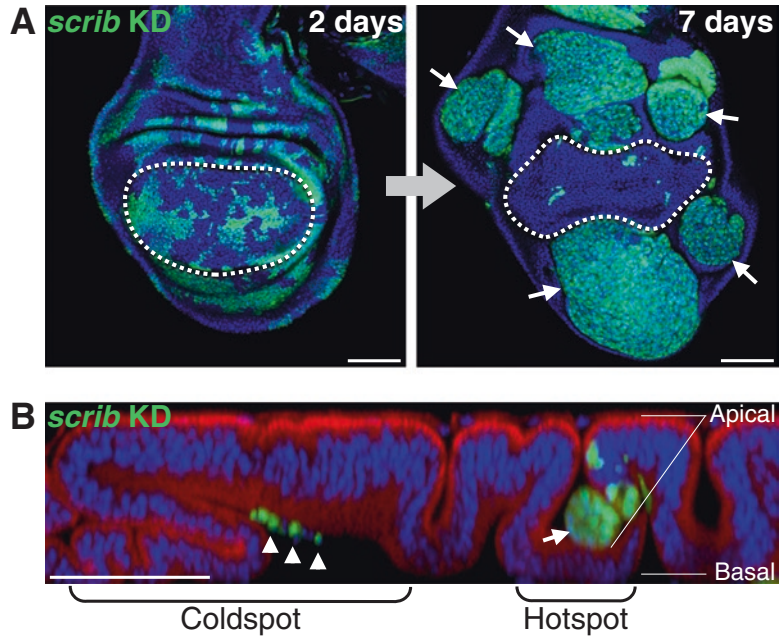

Fig. 5.5 Site-specific tumorigenesis in *Drosophila* wing imaginal discs. (**a**) Genetically mosaic wing discs with cells expressing *scrib-RNAi* (marked with GFP expression, green) at the indicated time point after RNAi induction. White dotted lines mark the boundaries between wing pouch and hinge regions. (**b**) Vertical section of a mosaic wing disc with clones expressing *scrib-RNAi* (marked with GFP expression, green) along its anterior-posterior boundary 5 days after clone induction, stained for aPKC (red). Nuclei were labeled with DAPI (blue). White arrowheads indicate apoptotic clones. White arrows indicate dysplastic tumor growths. Scale bars represent 50 μm

However, when we consider tumorigenesis in a differentiated epithelial monolayer, the situation should be different from those TSG homozygous mutant larvae. Here, when a pro-tumor cell emerges in an epithelial monolayer it triggers interactions with neighboring normal cells or induces abnormal cell division, which eventually results in the elimination of the pro-tumor cell (Fig. 5.1). In fact, pro-tumor cells such as nTSG mutant cells generally delaminate or apoptose and are eventually eliminated from the tissues [12]. A recent study in wing imaginal discs, however, showed that polarity-deficient pro-tumor cells such as nTSG mutant cells occasionally slip through the surveillance system and get a chance to survive and grow into tumors [78].

When the nTSG-deficient cells induce tumorigenesis in wing imaginal discs, it is always located at the peripheral "hinge" region and never observed in the central "wing pouch" region of the epithelial tissue [78, 79] (Fig. 5.5a). This indicates that tumorigenic potential of nTSG-knockdown cells depends on their intrinsic local environment in the epithelial tissue. In other words, the peripheral hinge region of wing imaginal discs is susceptible to tumorigenic stimuli, and conversely the wing pouch region has a strong tumor suppression system. Therefore, the wing pouch region is a "tumor coldspot", while the peripheral hinge region is a "tumor hotspot." A key difference between the behaviors of nTSG-deficient cells located in coldspots and hotspots is the direction of delamination. At the coldspot areas, nTSG-deficient cells delaminate from the basal side of the epithelial layer and undergo apoptosis. By contrast, at the hotspot areas, pro-tumor cells delaminate from the apical side of the epithelial layer and show tumor growth [78] (Fig. 5.5b). These facts suggest that apical delamination gives nTSG-deficient cells a chance to survive and proliferate in the lumen. Crucially, the determining difference in the direction of

delamination is cellular morphology, as cells in the flat wing pouch coldspot are elongated along their apical-basal axis, whereas cells in the folded hinge hotspot regions are shorter. In addition, there are some more intrinsic differences observed at the basal side of the epithelial cells: [1] in the valley-folded hotspot, cellular membranes display a complicated set of bends at the basal side, whereas in the coldspot they appear straight along the apical-basal axis; [2] hotspot cells show filopodia-like protrusions at the basal surface that elongate laterally and intertwined intricately with the protrusions of neighboring cells; and [3] the basement membrane composed of approximately ten thin laminae is organized loosely in the coldspot, but aligned tightly in the hotspot. These basal-specific structures of the hotspot have been shown to prevent delamination of pro-tumor cells from its basal surface [78].

It is still unclear why basally delaminated pro-tumor cells die at the coldspot area. One plausible explanation of the apoptosis of the basally delaminated cells is anoikis, a specialized form of apoptosis triggered by inappropriate cell-extracellular matrix interaction [80, 81]. Another possible reason is activation of the TNF-JNK signaling pathway triggered by hemocytes. JNK activation in the tumor cells is induced by Eiger, the *Drosophila* tumor necrosis factor (TNF)-α, which is produced by circulating hemocytes recruited to the site where tumor cells disrupt the basement membrane [29]. Circulating hemocytes are recruited to the site where tumor cells disrupt the basement membrane [28] and associate directly with tumor cells at the basal side of the epithelial layer to induce apoptosis. Contrary to this, apically delaminated pro-tumor cells at the tumor hotspots could be free from hemocytes, which are selectively associated with the basal epithelial side.

Cancer cells generally have strong resistance to apoptosis coupled with the ability to grow, which enables them to prevent anoikis and proliferate in the absence of appropriate adhesion to extracellular matrix [82]. This capability of cancer cells is closely related to tumorigenicity and metastaticity, and reflects the tendency of tumor cells to survive and grow in inappropriate locations *in vivo* [83]. In the case of nTSG-deficient cells in wing imaginal discs, however, these pro-tumor cells do not have additional oncogenic mutations to prevent apoptosis and promote proliferation. Although apical delamination allows pro-tumor cells to evade the suppressive epithelial environment and unleash their tumorigenicity, there should be another factor that will allows the pro-tumor cells to survive and proliferate at the luminal region in the absence of an oncogenic mutations. Indeed, the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is endogenously active specifically in the tumor-hotspot hinge region of developing wing imaginal discs [78] (Fig. 5.6). Its secreted cytokine-like ligand Unpaired (Upd) [84], a *Drosophila* homolog of mammalian Interleukin-6 (IL-6), is endogenously expressed in the hinge regions, which induces activation of JAK/STAT signaling pathway in these area including tumor hotspots [85]. The dorsal hinge region where its endogenous activity is the highest in wing imaginal discs has three epithelial folds: proximal, medial, and distal. Endogenous activity of the JAK/STAT pathway is high in the medial fold, weak in the proximal fold, and barely detectable in the distal fold (Fig. 5.5b). Indeed, tumor growth induced by nTSG-deficient cells was mostly observed in the medial fold. Furthermore, depletion of STAT blocked the dysplastic tumor growth of nTSG- deficient cells, indicating that STAT activation is necessary for the tumorigenesis. Conversely, hyper-activation of STAT in nTSG-deficient cells in tumor hotspots, including the distal fold of the dorsal hinge, dramatically enhanced tumor size [78]. These observations indicate that nTSG-deficient cells exploit local endogenous activity of the JAK/STAT pathway to survive and proliferate. Upd is secreted from the apical surface of epithelial cells to transduce the signal to the neighboring cells, where it binds the receptor Domeless, which is also localized on the apical membrane [86]. Therefore, apical delamination in the valley-folded tumor hotspot where the proinflammatory JAK/STAT ligand abundantly accumulates provides the pro-tumor cells with a crucial survival advantage [7] (Fig. 5.7).

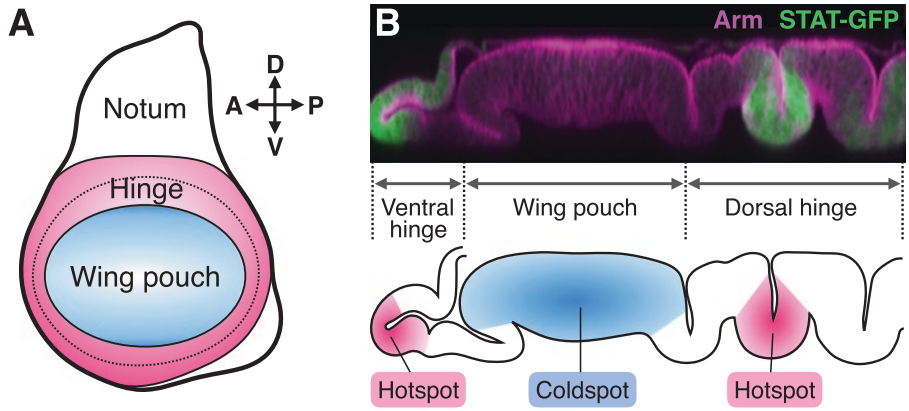

Fig. 5.6 (**a**) Schematic diagram of *Drosophila* wing imaginal discs showing the wing pouch (blue) and hinge (pink) regions. (**b**) Upper panel: Vertical section along the anterior-posterior boundary of a wing disc with 10xSTAT-GFP (green) stained for adherens junction component Armadillo (magenta). Lower panel: black line drawing traces the apical and basal sides of the epithelial layer. Tumor hotspot and coldspot regions are shown in pink and blue respectively

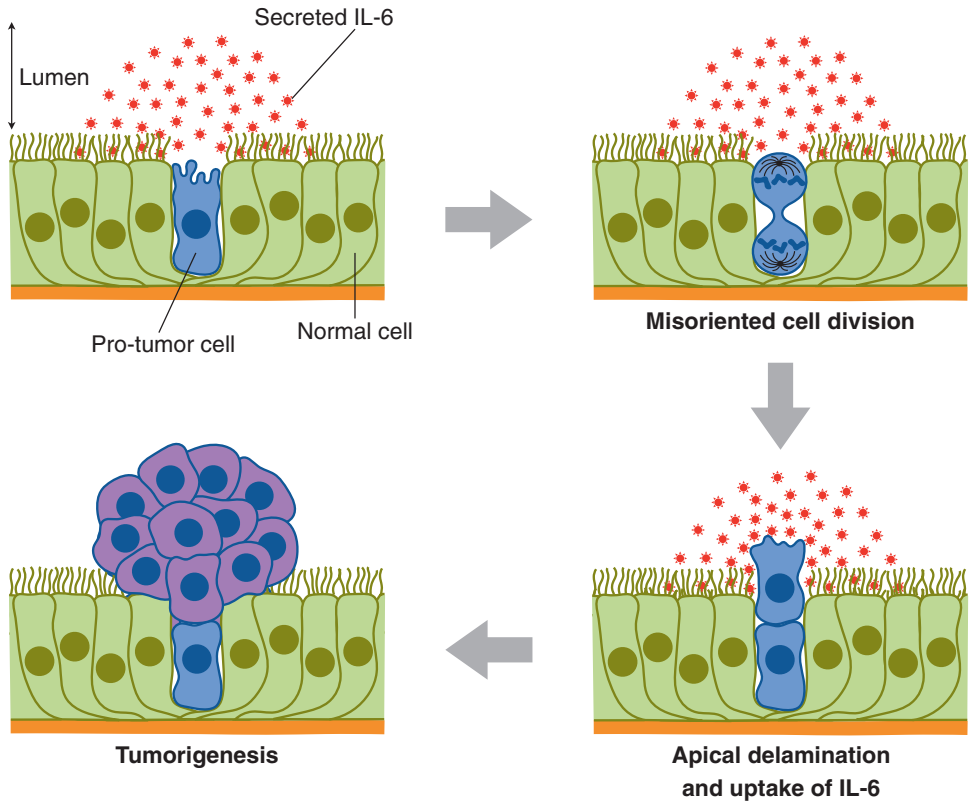

Fig. 5.7 Tumor initiation in tumor hotspots. When an nTSG mutant pro-tumor cell (blue) appears in tumor hotspots, misoriented cell division results in apical delamination of one of the daughter cells because of the hotspot-specific robust basal structures. An apically delaminated nTSG mutant cell survives and undergoes tumorigenic overgrowth by exploiting endogenous IL-6 (Upd in *Drosophila*) secreted in the lumen of tumor hotspots

These mechanisms of tumorigenesis in tumor hotspots demonstrates that two independent processes, apical delamination and endogenous JAK/STAT activation, are concurrently required for the initiation of nTSG-deficient-induced tumorigenesis. Both of these two processes result from the local environment of the epithelia, highlighting the important concept that tissue-intrinsic microenvironments have decisive roles for the behaviors and even life-or-death fate of pro-tumor cells.

In the tumor hotspots, therefore, luminal translocation is one of the key processes for the nTSG-deficient cells to initiate tumorigenesis. Luminal tumor growth following apical delamination also occurs when the ligand Slit, its transmembrane Roundabout receptor Robo2, and the downstream cytoskeltal effector Enabled/VASP (Ena) are ectopically activated in *scrib* mutant cells in eye imaginal discs [49]. Slit-Robo2-Ena signaling is best known for conserved roles in axon guidance controlling cell repulsion and migration [87, 88]. In the *scrib* mutant cells, JNK upregulation induces Slit-Robo2-Ena signaling activation, which promotes delamination of *scrib* mutant cells from the epithelial layer through disruption of E-cadherin. Thus, Slit-Robo2-Ena signaling functions as a tumor suppressor to eliminate pro-tumor *scrib* mutant cells. At the same time, hyper-activation of this signaling axis in *scrib* mutant cells enhances both apical and basal delamination. While basally delaminated mutant cells are eliminated by anoikis or hemocytes, apically delaminated mutant cells band together and cause tumor clamps at the lumen [89]. Conversely, loss of the Slit-Robo2-Ena signaling leads *scrib* mutant cells to stay in the epithelial layer and potentiates tumor formation within the epithelium [49].

5.9 Tumor Hotspots in Other Tissues

Is there a tumor hotspot in other epithelial tissues in *Drosophila*? In fact, the folded epithelial layer of hinge regions of wing imaginal discs is the only case in which intrinsic local microenvironment causes site-specific tumorigenesis. Among *Drosophila* imaginal discs, however, nTSG-deficient cells induce tumor growth more frequently in the leg discs which include many more folds in the epithelial layer than other imaginal discs. In the early second instar larvae, JAK/STAT signaling is endogenously active throughout the leg disc and become restricted to the dorsal domain during third instar [85]. Therefore, it is highly likely that the leg imaginal discs have tumor hotspots and the mechanisms of tumorigenesis might be also similar to the one described in wing discs.

Interestingly, in the ovarian follicular epithelia, a number of previous studies pointed out that tumor growth induced by mosaic mutant clones are primarily observed around the terminal regions of egg chambers. For example, mosaic clones mutant for polarity genes induce a multilayer phenotype and tumor growth at a higher rate at both anterior and posterior poles [41, 43, 65]. Also, mosaic clones mutant for Hippo signaling pathway genes disrupt posterior FCs differentiation and induce overproliferating multilayers at the peripheries of posterior polar cells [90]. In the posterior follicle clones of Hippo pathway mutants, Notch signaling is disrupted because of endocytosis defects, which disrupts the mitosis/endocycle switch [90, 91]. Although there is no study focused on causative mechanisms of such site-specific tumorigenesis in the ovarian follicular epithelia, one possible cause would be endogenous JAK/STAT signaling activity. JAK/STAT signaling is endogenously active in the FCs located at the both anterior and posterior poles, where polar cells secrete the ligand protein, Upd [85, 92]. The secreted Upd forms a concentration gradient on the apical surface of the FCs and acts as a morphogen to specify multiple FC fates in its signaling activation-dependent manner [93]. It may also be possible that a local tendency towards delamination or mitotic misorientation exists in the follicular epithelia at the poles due to geometric constraints.

5.10 Conclusions

During the past few decades, outstanding technological advances in genetics and molecular biology have made remarkable progresses on cancer research. Although a number of causative genetic background for tumor progression have been discovered, the initial stage of tumorigenesis in which transformed pro-tumor cells take to break epithelial integrity and induce tumor growth remain elusive. However, recent studies especially using the genetically mosaic analysis tools in *Drosophila* have greatly contributed to better understanding the genetic and cellular mechanisms of the tumor initiation *in vivo*. While the process of tumorigenesis induced by a single mutation in *Drosophila* epithelia, such as nTSG mutant clones, is superficially simple, the revealed deep mechanisms have shown us new conceptual developments. As described in this review, these studies in *Drosophila* have shown that epithelial tissues have intrinsic tumor suppression mechanisms such as cell competition-dependent elimination and spindle misorientation-induced delamination to prevent pro-tumor cells from tumorigenesis. Also, our novel tumor-hotspot theory helps explain how tissue-intrinsic local microenvironments play critical roles in the fate of pro-tumor cells [7]. On the other hand, phenotypes of pro-tumor mutant cells sometimes depends on the type of epithelial tissues. For example, nTSG-deficient cells do not induce tumor development in the midguts, suggesting that different mechanisms between imaginal discs and gut epithelia exist. In fact, a recent report showed that the composition of apicobasal cell polarity of midgut cells is different from the general pattern in other epithelial cells in *Drosophila*; the septate junction is basal to the adherens junction in most epithelia, whereas the order is reversed in gut cells [94]. Therefore, the detailed mechanisms of tumor suppression and initiation are dependent at least in part on tissue type. Nevertheless, numerous examples from *Drosophila* demonstrate a general concept for tumor initiation: a combination of epithelial disintegration and enhancement of survival and proliferation is required for tumorigenesis. To study the basic mechanisms of tumor initiation at the molecular and cellular levels *in vivo Drosophila* will play an increasingly significant role as an experimental model system. Further studies to identify the phenotypes of various types of pro-tumor mutant cells in different types of tissues in *Drosophila* will lead to a better understanding of tumor initiation mechanisms.

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6 P53 and Apoptosis in the Drosophila Model

Lei Zhou

Abstract

Human P53 (*HsP53*) is the most frequently mutated gene associated with cancers. Despite heightened research interest over the last four decades, a clear picture of how wild type HsP53 functions as the guardian against malignant transformation remains elusive. Studying the ortholog of P53 in the genetic model organism *Drosophila melanogaster* (*DmP53*) has revealed many interesting insights. This chapter focuses on recent findings that have shed light on how DmP53 -mediated apoptosis plays an important role in maintaining genome integrity, and how the immediate output of activated DmP53 is determined by the epigenetic landscape of individual cells.

Keywords

Apoptosis · Cancer · Corp · Epigenetics · DNA damage · Transposable element · P53 · MDM2

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6.1 Background and Overview

HsP53 is generally regarded as the most important tumor suppressor gene [1]. It is mutated in about 50–60% of all cancers. The high prevalence of *HsP53* mutation in certain types of cancers, such as lung cancers, suggests that it is the common initiating event for these cancers. The importance of P53 as the gate keeper of genome integrity is also supported by mechanistic studies conducted in mammalian animal models.

The *HsP53* gene encodes a transcription factor that binds to specific DNA sequences. A multitude of genes have been indicated as potential targets of P53 [3, 4]. These genes have a wide array of functions including cell cycle arrest, apoptosis, metabolism, and proliferation [5]. It is clear that some of these functions are mutually exclusive, such as apoptosis and cell cycle arrest. The output of activated HsP53 differs based on cellular contexts and nature of the stimuli. However, the multitude of possible targets of HsP53 complicate the task of understanding how it functions as a gate keeper for genome integrity to prevent abnormal growth and metastasis. Understanding P53 function in simpler organisms could help to reveal the mechanisms essential for its tumor-suppressive activity. It should be noted out that studies in *Drosophila* have revealed some unexpected functions of *DmP53*, such as its role in mediating apoptosis -induced proliferation, which has been reviewed previously [6]. In

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this chapter, we will focus on the role of DmP53 in mediating apoptosis in response to oncogenic stresses.

6.2 Drosophila Genome Contains One P53 Family Gene

High vertebrate and mammalian genomes have three P53 family genes. In addition to P53, there are P63 and P73. While the DNA binding and the transcription activation domains are highly conserved among the three paralogs, P63 and P73 have an additional SAM (sterile alpha motif) at their C-terminal. Unlike P53, P63 and P73 are rarely mutated in cancers, indicating that their function does not deter tumorigenesis. While P53 function is dispensable for animal development, mice mutated for P63 or P73 had clear developmental defects.

DmP53 was identified based on sequence similarity to *HsP53* at the protein level [7–9]. Similar to *HsP53*, *DmP53* has several splice forms that are predicted to encode proteins with different N-terminal sequences [10–12]. The splice form that was originally characterized, *DmP53_RA*, encodes a protein of 385 amino acids and has a single transcription activation domain (TAD) at the N-terminus. The longest form, *DmP53_RB*, is predicted to encode a protein that contains two TADs at the N-terminus. *DmP53_RC* has a longer 5'UTR but has the same ORF as *DmP53_RA*. The shorter isoform, *DmP53_RE*, is predicted to encode a protein that lacks a complete TAD.

DmP53 is extensively transcribed in most tissues through all stages of development. The predominant splice form appears to be DmP53_RA. Correspondingly, the 385 aa protein encoded by this form (and DmP53_RC) is readily detectable in most tissues. The presence of the two splice forms *RB* and *RE* are supported by corresponding cDNA clones and RNA-Seq analysis. However, their corresponding proteins, predicted to be 495 aa and 334aa, respectively, cannot be detected by western blot, possibly due to the very low expression levels. When overexpressed with transgenic constructs, *DmP53_RE* appears to inhibit rather than promote DNA damage induced apoptosis. Ectopic expression of *DmP53_RB* was actually more potent than *DmP53_RA* in inducing apoptosis. However, by inserting a transcription stop sequence to disrupt the transcription of specific isoforms, Calvi's group showed that *DmP53_RB* is dispensable and *DmP53_RA* alone is required for mediating irradiation-induced apoptosis [10]. Since the proteins predicted to be encoded by DmP53_RB and RE cannot be experimentally verified without an artificial expression construct, we will focus our attention on the 385aa protein encoded by *DmP53_RA/RC* for the following discussion.

The conservation of the DNA binding domain of DmP53 (~24% identity and 44% similarity with HsP53) was markedly better than the N-terminal transcription activation domain and the C-terminal oligomerization domain (~13% identity with HsP53). However, despite the low level of sequence conservation at the C-terminal, antibody raised against the C-terminal of HsP53 binds to DmP53 on western blot [10, 12], highlighting strong conservation of key structural characteristics.

It has been shown that DmP53 could bind to a consensus HsP53 binding site, but with considerably lower affinity [9]. A recent ChIP-Seq analysis revealed that the consensus motif shared by most DmP53 binding sites is largely similar to that shared by HsP53 binding sites from normal cells (Fig. 6.1, HsP53 binding site in normal cells was compiled by Botcheva et al. [13]). The consensus half site for DmP53 is RRR**C**-R-Y-**G**(C/A/T)3, compared to RRR**C**-A/T-T/A-**G**YYY for HsP53 (R-Purine, Y-Pyrimidine). A surprising finding on the interaction between DmP53 and chromosomes is that it may interact with target genes in *cis*, or in *trans*, i.e. with target genes located on different chromosome [14].

Several lines of evidence support the notion that *DmP53* is the functional ortholog of *HsP53*. First, none of *DmP53* isoforms encodes a protein with a discernible SAM that is shared by all P63/P73 orthologs. Secondly, *DmP53* is not required for development, but required for mediating stress-induced cell death and for maintaining the genome integrity of somatic cells [15, 16].

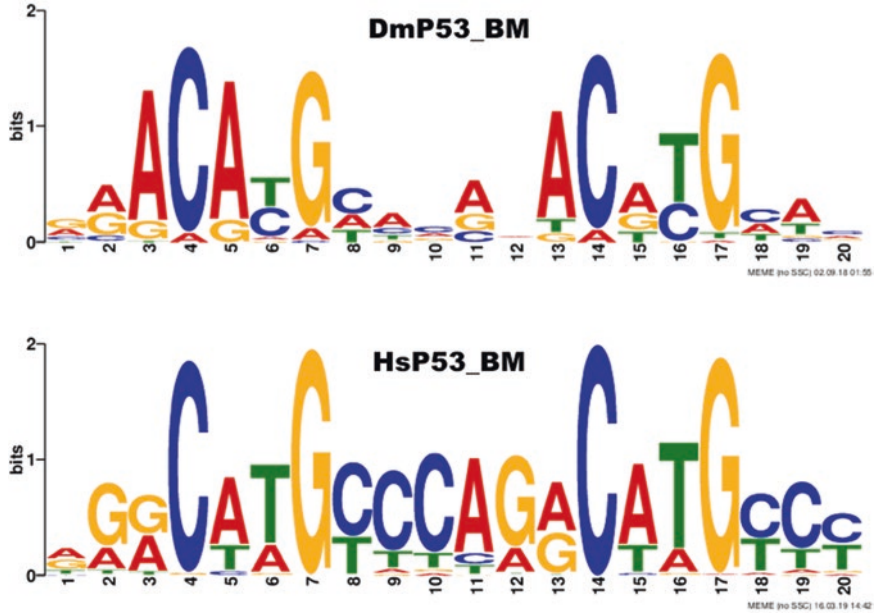

Fig. 6.1 Consensus motifs shared by most P53 binding sites identified by ChIP-Seq. DmP53_BM was based on ChIP-Seq data generated by the Zhou group (to be published), and the HsP53_BM based ChIP-Seq data compile for normal human cells

Similar to HsP53, DmP53 is activated by Chk2 following ionizing irradiation and is required for the induction of DNA repair and pro-apoptotic genes [17]. However, there also seems to be significant differences. Unlike HsP53, activated DmP53 is not responsible for the cell cycle arrest following DNA damage. In contrast to *DmP53*, the sole P53/P63 family member in *C. elegans*, *Cep-1*, encodes a protein with a SAM domain [18]. It is required for germ line development but uncoupled from the apoptosis pathway [19].

6.3 DmP53 Mediates DNA Aberration -Induced Apoptosis to Maintain Genome Stability

DNA damage induced by ionizing irradiation leads to rapid induction of apoptosis in a variety of somatic tissues in *Drosophila*. The function of DmP53 is required for this process. Several pro-apoptotic genes, including *reaper*, *sickle*, *hid*, and *eiger,* are induced within 15–30 min following x-ray treatment [17, 20]. This rapid induction of apoptosis following DNA damage is fully dependent on the function of DmP53 [15, 17]. None of these pro-apoptotic genes can be induced in homozygous *DmP53* mutant embryos following irradiation. In contrast to its essential role in mediating irradiation induced cell death, there is little change of developmental cell death in the *DmP53* mutant. When flies are irradiated at the larval stage, homozygous DmP53 adults have significantly higher load of mutated cells than wild type adults, suggesting that the pro-apoptotic function of DmP53 plays an essential role in maintaining genome integrity [15].

In human cancers, there is a strong and intriguing pan-cancer correlation between HsP53 mutation and copy number alteration (CNA) [21]. The cause of this correlation has been subject of speculation. In *Drosophila*, aneuploidy can be induced using inducible flippase and chromosomes containing FRT (flippase recognition target). By forcing the formation of dicentric chromosomes during mitosis, daughter cells will gain or lose chromosome fragments [22]. A series of work from the Golic group revealed that, in wild type flies, cells with copy number alteration

will undergo Chk2 and DmP53 -dependent apoptosis [16, 23]. Many cells with CNA will survive in DmP53 homozygous mutant flies. In certain contexts, the effect of *DmP53* loss can be semi-dominant, i.e. significantly more cells with CNA survive in *DmP53* heterozygous flies than in wild type flies.

Another interesting finding regarding CNA and aneuploidy is through the analysis of endocycling cells. Endocycling, i.e. replication of DNA without mitosis, causes genotoxic stress. During oogenesis, the follicle cells enter into endocycling after stage 7. Works from the Calvi group indicated that DmP53 is activated in response to CNA caused by endocycling [24]. However, most of the endocycling cells survive due to two mechanisms. The first is through suppression of DmP53 protein level via increased proteasome degradation [25], which is reminiscent of cancer cells with amplified MDM2. The second mechanism is through epigenetic blocking, which will be discussed in the following section.

6.4 Epigenetic Control of DmP53-Mediated Apoptosis

It has long been noticed that even for cancers with wild type *HsP53*, the sensitivity to DNA damage induced by irradiation or chemotherapy can vary dramatically. During *Drosophila* embryogenesis, cells at a particular development window, stage 9–11, are extremely sensitive to ionizing irradiation induced cell death. Three pro-apoptotic genes, *reaper*, *sickle*, and *hid* are induced rapidly within 15–30 min following irradiation in this developmental window [20]. The three genes are localized in a well conserved synteny and their stress responsiveness appears to be co-regulated. An intergenic region between *reaper* and *sickle*, termed IRER (Irradiation Responsive Enhancer Region), is required for mediating the induction of all of the three genes following irradiation at this developmental stage [20] (Fig. 6.2). However, none of these genes can be induced in embryos past development stage 12 by the same or even a higher dosage of irradiation. It turned out that this is due to epigenetic blocking of IRER. Chromatins in the IRER become enriched for both H3K27Me3 and H3K9Me3 during stage 12, and is thereafter bound by both polycomb group proteins as well as HP1 (heterochromatin protein 1) [20]. Consequently, this epigenetic shift blocks the binding of DmP53 to this region and renders the three pro-apoptotic genes irresponsive to DNA damage.

This epigenetic control of DmP53 output is unique in that it only blocks the accessibility of the intergenic regulatory region but does not silence the promoter or transcribed regions of the pro-apoptotic genes. Indeed, DNA accessibility analysis revealed that in embryos post stage 12, all the way to the end of embryogenesis (stage 17), the promoters of the *reaper*, *sickle*, and *hid* remain in open conformation. This enhancer-specific blocking is important because the transcription of these three genes are still needed to mediate cell lineage dependent apoptosis, such as the elimination of obsolete neuroblast cells, at the end of embryogenesis [26]. A detailed analysis of the DNA region between IRER and *reaper* revealed that the spread of heterochromatin formation is prevented from reaching to the promoter of *reaper* by a chromatin barrier element [27].

What is the biological significance of such an epigenetic control of DmP53 -induced apoptosis? Most cells at embryonic stage 9–11 are still dividing although they are at the last stage of the fast proliferation and are about to enter post-mitotic differentiation. Their proliferating status likely allows little time beween subsequent genome replication events for extensive DNA repair. More importantly, because cells at this stage are relatively undifferentiated, it is relatively easy for dead cells to be replaced by their proliferating sister cells. Indeed, it has been demonstrated extensively in *Drosophila* that apoptotic cells send out signals to stimulate the proliferation of the neighboring cells [28–30]. Thus the epigenetic opening of the IRER, shifting the cellular response towards rapid induction of apoptosis, would help to eliminate damaged cells which could then be replaced by neighboring

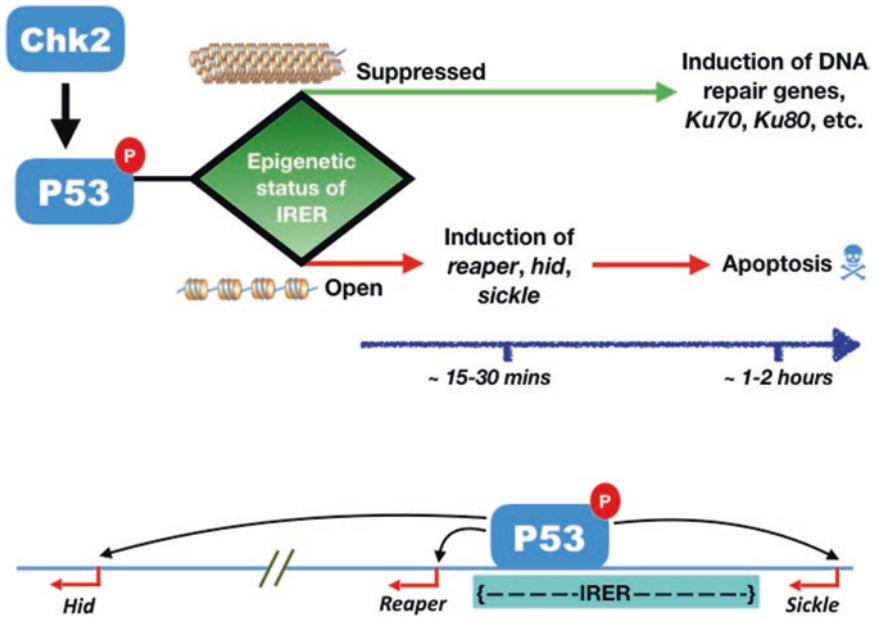

Fig. 6.2 Epigenetic control of IRER determines the outcome of activated DmP53. In cells with open IRER, the pro-apoptotic genes were activated rapidly following activation of DmP53. In cells with epigenetically blocked IRER, none of the pro-apoptotic genes was induced. A relatively slower induction and accumulation mRNAs for DNA repair genes would incur

cells. For cells that enter post-mitotic differentiation, one may speculate that cells now have more time for DNA repair. More importantly, due to the transient nature of many developmental instruction signals it may not be even feasible to replace a fully differentiated cell. Epigenetic blocking of IRER to prevent DmP53 induced pro-apoptotic gene expression is not limited to embryonic development. For instance, it is responsible for suppressing the sensitivity to CNA/aneuploidy -induced activation of DmP53 in the endocycling cells [25].

Although epigenetic regulation of IRER is related to differentiation status during embryogenesis, it is by no mean a function of cellular differentiation status in all circumstances. Using a fluorescent marker that reflects the openness of IRER (IRER{ubi-DsRed}), we found that in post embryonic development, IRER remains closed in some stem cells but open in certain differentiated cells. Hassel et al. [31] also showed that the epigenetic suppression of IRER in the endocycling cells is not tired into the differentiation process.

At the tissue level, epigenetic regulation of IRER generates variegated sensitivity to stress induced cell death among otherwise identical or similar cell populations. This was clearly reflected by using the IRER{ubi-DsRed} reporter of epigenetic status, which showed that the openness of IRER varies significantly among cells that are otherwise considered as similar or even homogenous [32]. Similar to mammalian systems, overexpression of *DmMyc* causes overproliferation. However, there is little overgrowth phenotype due to the compensatory induction of apoptosis in response to overproliferation [33, 34]. This compensatory cell death depends on the induction of *reaper* and *hid* [33]. Using the IRER{ubi-DsRed} reporter, we found that cells with relatively open IRER were selectively eliminated in response to *DmMyc* -induced overproliferation, but cells with suppressed IRER remained [32]. The functional consequence of such a variegated epigenetic landscape of IRER is that for a particular stress, be it irradiation or over proliferation, a portion of cells will die in proportion to the severity of the stress. However, under most

circumstances some cell will survive and will have the chance to repopulate the tissue. Our finding is in agreement with the theory put forward by Feinberg et al., that the stochastic nature of epigenetic regulation underlies the "nongenetic heterogeneity" and cell plasticity that is essential for development and for interaction with the environment [35]. An extension of the theory is that the disruption of this landscape, caused by repeated insults or stochastic error, could be the initiating event for diseases such as cancer [36, 37]. Epigenetic regulation of IRER, by directly controlling the sensitivity to P53-mediated apoptosis, may serve as a unique model to understand how genetic variations and environmental factors converge on regulating the epigenetic status of a locus that is important for tumor suppression. Many questions remain to be addressed, for instance, within a cell population display variegated epigenetic status of IRER, is the particular epigenetic status in a given cell purely random or linked to the fitness of the cell?

6.5 Role of DmP53 in Anti-Viral Response

There has long been speculation that apoptosis originally evolved as a defensive mechanism against intracellular pathogen infection in primitive multi-cellular organisms [38]. If so, what about P53's ability to induce apoptosis? In both mosquitoes and *Drosophila*, virus infection can induce rapid apoptotic cell death within 2 h of infection. In *Aedes aegypti*, exposure to dengue virus (RNA) or baculovirus (DNA) induced the expression of *Michelob_x* (*Mx*), which is the ortholog of *reaper* [39, 40]. In *Drosophila*, both *reaper* and *hid* were induced in response to baculovirus or flock house virus (RNA) infection. This induction of pro-apoptotic genes is responsible for halting the viral infection at the primary infection site before the first cycle of viral replication. This rapid induction of apoptosis was absent in the DmP53 mutant strain, which is much more susceptible to FHV infection [41].

Since orthologs of P53 have been identified in unicellular organisms [42], it's possible that the relationship between P53 and control of viruses existed before the emergence of multi-cellular organisms and apoptosis. Works from John Abrams group indicated that an important function of DmP53 was to constrain retrotransposon activity, in both germ line and somatic cells [43, 44]. Several LTR and non-LTR retrotransposons were much more active in the germ line of *DmP53* mutant flies than in wild type animals. This may have contributed to the lower fertility rate observed for the mutant. The mechanism of this suppression remains to be fully understood. It has long been noticed that many binding sites of HsP53 localize to repetitive sequences in the genome, some of which are retrotransposons [5, 44, 45]. Whether there is a direct interaction between DmP53 and retrotransposons remains to be revealed.

6.6 Regulation of DmP53 Activity

Works by Brodsky et al. have shown that the activation of DmP53 following DNA damage is very similar to that in mammalian systems [17], i.e. the activation of DmP53 following ionizing irradiation is dependent on phosphorylation by Chk2. The same pathway is required for mediating apoptosis following induced chromosome aneuploidy [16].

Another important regulatory mechanism controlling the activity P53 is through MDM2 mediated degradation. In mammals, *MDM2* is a transcriptional target of P53. MDM2 protein contains a N-terminal P53 binding domain and a C-terminal RING domain, which is responsible for ubiquitination and subsequent proteasomal degradation of HsP53. The identification of both MDM2 and P53 orthologs in the placozoans suggested that this relationship evolved at the emergence of eumetazoans [46, 47]. However, the absence of a clear ortholog of MDM2 in *Drosophila* has been puzzling.

A genetic screen conducted in Kent Golic's lab identified *corp* (*companion of reaper*) as the suppressor of aneuploidy -induced cell death in the eye [48]. Interestingly, *corp* is a direct

transcriptional target of DmP53 ([20] and unpublished data). The Corp protein is much smaller than MDM2 and it does not have the RING domain that is the signature to all MDM2/MDM4 family members. However, it does share a motif with MDM2 that overlaps with the P53-interacting domain. Subsequent biochemical analysis indicated that Corp interacts with DmP53 and this interaction requires the CM (Corp-MDM2) motif that is shared between these two proteins. More interestingly, Corp is able to interact with HsP53 with affinity comparable to that between MDM2 and HsP53. Overexpression of *corp* in *Drosophila* decreased the protein level of DmP53 and inhibited aneuploidy -induced apoptosis [48]. So, rather than an anomaly of P53 regulation, the relationship between Corp and DmP53 seems to reveal the essence of the MDM2/P53 relationship.

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Autophagy and Tumorigenesis in *Drosophila*

7

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Abstract

The resurgence of *Drosophila* as a recognized model for carcinogenesis has contributed greatly to our conceptual advance and mechanistic understanding of tumor growth *in vivo*. With its powerful genetics, *Drosophila* has emerged as a prime model organism to study cell biology and physiological functions of autophagy. This has enabled exploration of the contributions of autophagy in several tumor models. Here we review the literature of autophagy related to tumorigenesis in *Drosophila*. Functional analysis of core autophagy components does not provide proof for a classical tumor suppression role for autophagy alone. Autophagy both serve to suppress or support tumor growth. These effects are context-specific, depending on cell type and oncogenic or tumor suppressive lesion. Future delineation of how autophagy impinges on tumorigenesis will demand to untangle in detail, the regulation and flux of autophagy in the respective tumor models. The downstream tumor-regulative roles of autophagy through organelle homeostasis, metabolism, selective autophagy or alternative mechanisms remain largely unexplored.

Keywords

Tumor · Autophagy · ras · scrib · Scribble · yki · Yorkie · N · Notch · Raf · Autophagy · vps34 · LKB1 · TOR · ROS · Mitochondria · PI3K · Atg · Uvrag · P62 · NRF2 · Keap1 · Upd · PERK · Myc · Stem cell

7.1 Introduction

Macroautophagy (referred to as autophagy herein) is a cellular process that sequesters intracellular cargoes in a double-membrane vesicle called autophagosome, and subsequent delivery to lysosomes for hydrolytic bulk degradation (Fig. 7.1). The resultant degraded products in the form of Nucleotides, amino acids, fatty acids and sugars are recycled as cellular building blocks or utilized for energy production. Basal levels of autophagy operate in most tissues to dynamically remove dangerous, superfluous and damaged organelles, such as damaged mitochondria and protein aggre-

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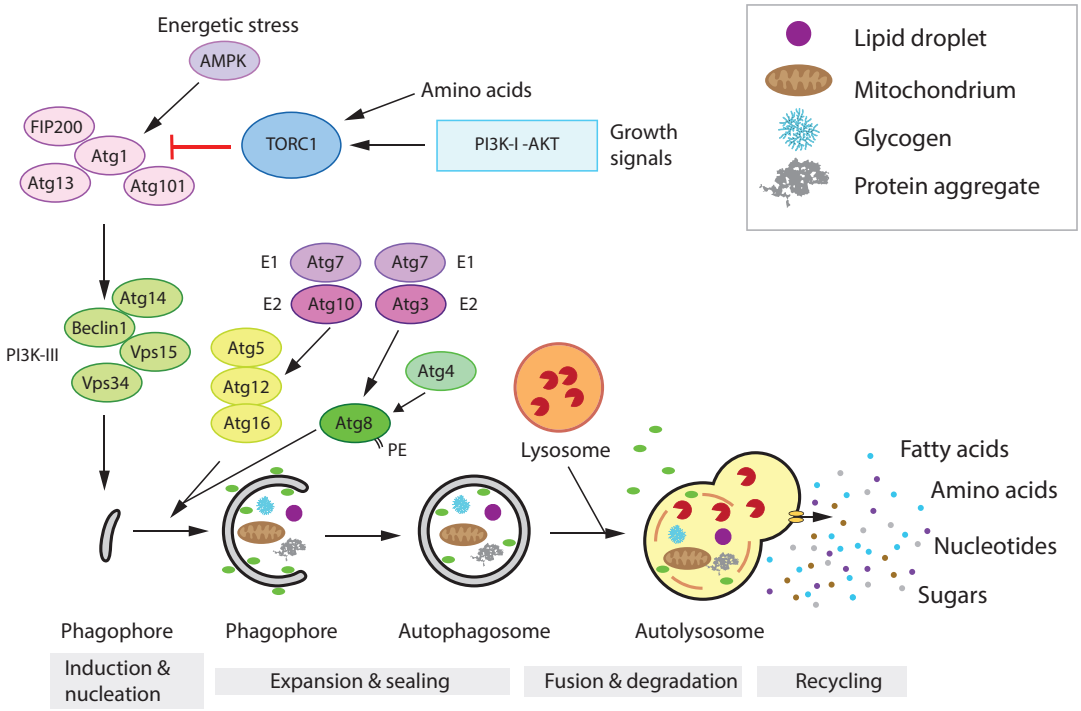

Fig. 7.1 Depiction of the autophagy process. Autophagic cargoes includes organelles, proteins and protein aggregates, lipid droplets, glycogen and other cytoplasmic constituents. Autophagy is controlled by nutrients and growth factor-TORC1 signaling and activated by stress, including energy stress through AMPK. Upon fusion with lysosomes, autophagic cargo are degraded by hydrolytic enzymes. Digested products are recycled and transported to the cytoplasm by permeases for metabolic or biosynthetic pathways

gates. Autophagy increases in response to stress, like nutrient deprivation, ER-stress, reactive oxygen species (ROS), hypoxia, or absence of growth factors. It serves as a cellular and organismal protection against insults. Indeed, mice or flies deficient for autophagy succumb during prolonged starvation, likely due to energy deprivation. The molecular machinery required for autophagy was originally defined using yeast genetics. The so-called *atg* (autophagy-related) genes are conserved to metazoa and act in distinct steps of the autophagy process [1, 2]. The induction of autophagosome biogenesis is regulated by a Ser/Thr kinase, Atg1 in complex with Atg13, Atg101 and Atg17/FIP200. Overexpression of Atg1 can initiate autophagy in flies [3]. The Atg1 complex is responsive to growth factor signaling through Phosphatidylinositol 4,5 bisphosphate 3-kinase (PI3K-class I)- AKT pathway and a direct phosphorylation target by TOR kinase and AMP kinase (AMPK) under nutrient and energy stress. In turn, the Atg1 complex stimulates phosphatidylinosityl 3-Kinase class III (PI3K-III) through phosphorylation of Atg6/Beclin1. The PI3K-III core complex consist of the catalytic subunit Vps34, the pseudokinase, Vps15 and the tumor suppressor Atg6/Beclin1. It comes in two flavors defined by Atg14 and Uvrag that regulates autophagy or endocytic trafficking respectively. Atg14-containing PI3K-III generates phosphatidylinositol-3-phosphate (PI3P) and promotes phagophore nucleation and membrane elongation through the PI3P-binding effectors; WIPI2 and DFCP, and the transmembrane Atg9 proteins that shuttle membrane vesicle to the growing phagophore. Membrane elongation and completion depends on two ubiquitin-like conjugation systems comprised of the E1 enzyme, Atg7, and E2 enzymes Atg10 and Atg3. Atg7 and Atg10 conjugates Atg5 to the ubiquitin like molecule Atg12 and associate with the membrane-bound protein Atg16. This trimeric complex stimulates Atg7 and Atg3-mediated conjugation of

Atg8 family proteins to the lipid phosphatidylethanolamine (PE) inserted in the phagophore membrane. Sealing of the autophagosome depends on vacuolar protein sorting 4 (Vps4) and endosomal sorting complex required for transport (ESCRT)-III [4]. Upon closure Atg8 on the autophagosomal surface are delipidated and recycled by Atg4, whereas luminal Atg8 is transported to the lysosomes and acts as a marker for the autophagic process. Fusion of autophagosomes and lysosomes relies on SNARE complexes, Rab7, ESCRT and HOPS complexes [5] .

7.2 Autophagy and Tumorigenesis

The interest surrounding autophagy and tumorigenesis originally stemmed from two major lines of observation. Early findings suggested a tumor suppressive role of autophagy as mouse models of Beclin-1, ATG4C, and the autophagy PI3K-III associated regulator BIF1, showed a high incidence of spontaneous malignancies [6–8]. Beclin-1 was also found frequently mutated in human breast and prostate cancer. The involvement of Beclin-1 in breast cancer has later been called into question due to its genomic proximity to the breast cancer susceptibility gene BRCA-1 [9]. Another line of observations showed that tumor suppressive (PTEN) and oncogenic mutations (PIK3CA) of the PI3K class I-TORC1 pathway suppressed autophagy. Even though genes for core autophagy proteins rarely are mutated in human cancers, a tumor suppressive role of autophagy was relatively early on demonstrated in mice. Tissue-specific deletion of Atg5 or Atg7 produces benign liver hepatoma that fails to progress towards malignancy [10, 11]. Readers are advised to consult one of several excellent reviews summarizing the extensive literature that exists on the roles of autophagy in cancer progression, primarily derived from human cell and mouse studies [1, 2, 47]. Here, we review the literature related to tumorigenesis and autophagy in *Drosophila*. We employ a liberal definition of tumor growth; as cell growth and overproliferation when the models used depend on known oncogenes and tumor suppressors.

7.3 Tumor-Suppressive Effects of the Autophagy Machinery in Flies

Flip recombinase target site (FRT)-mediated mosaic analysis of recessive alleles emulates loss of heterozygosity in flies. This lends itself to an analysis of potential tumor suppressive gene functions in mitotic tissues of larval and adult stages. This approach was used to investigate potential tumor suppressive roles of the PI3K-III complex required for autophagy initiation. Stem cell-derived mutant clones of *vps34*, *vps15* or *Uvrag* leads to dysplasia-like loss of epithelial integrity and mixing of polarity markers in the follicular epithelium [12, 13]. This phenotype was not observed for $atg14^{-/-}$ mutant clones, suggesting that the endosomal function of PI3K-III, rather than the autophagy function carries tumor suppressive capacity. In line with this, $atg13^{-/-}$ mutant clones showed no epithelial integrity defects. Using human spheroid cell culture to model epithelial structure, the authors extended the observations to a human epithelium where Beclin1 and PIK3C3/Vps34 knockdown produced epithelial integrity defects, whereas Atg14 did not. Mechanistically, the epithelial integrity defects were found to be caused by failure endosomal trafficking of the Peutz-Jeghers syndrome kinase, Liver Kinase B1 (LKB1/STK11). A small screen for proteins harboring PX and FYVE domain containing PI3P-binding domains identified WD repeat and FYVE domain encoding protein 2 (WDFY2) in controlling LKB1 activity and epithelial integrity in flies and human cells. WDFY2, is a recurrent fusion gene with CDKN2D in ovarian carcinoma raising the possibility that this cancer type, in part is caused by LKB1 miss-regulation [14]. WDFY2 exists in a complex with LKB1 in flies and human cells where it controls LKB1 levels, activity and epithelial integrity through its PI3P-binding activity [13]. Although loss of PI3K-III does not cause bona fide tumor growth by itself, it cooperated with Ras^{V12} in forming tumors of the eye-antennal disc. Cooperative tumor formation depends on LKB1, in part through JNK activation that is a downstream effector of LKB1 in flies [13, 15].

UV radiation resistance-associated gene (Uvrag) is a tumor suppressor involved in endocytosis, DNA damage repair, and endocytic trafficking. Mutations in Uvrag are found in microsatellite-unstable colon cancers, but its mechanistic role as a tumor suppressor is controversial. Uvrag is a component of the "endosomal" PI3K-III complex PtdIns3KC2. It has also been reported to be required for autophagosome formation and maturation, possibly indirectly through its function in endocytosis. RNAi-mediated knockdown of *Uvrag* in adult intestinal stem cells (ISC) leads to intestinal hyperplasia, expansion of the intestinal stem cell pool and increased the thickness of the intestinal wall [16]. Clonal analysis of Uvrag$^{-/-}$ cells corroborated these results with duplication of ISCs and overall increased proliferation. Uvrag-deficient cells showed increased JNK activation and upregulation of Unpaired (Upd1) within the ISCs and Upd3 in the immediate niche. Both Jak-Stat and JNK signaling was found to contribute cell autonomously in the ISCs and descendants to hyperplasia. Despite earlier reports that Uvrag is required for autophagy in mammalian cells, the authors did not find evidence of an effect on autophagy in intestinal cells, suggesting that hyperplasia arises due to the endosomal role of Uvrag. In line with this idea, knockdown of autophagy-specific genes, *atg14, atg2, atg9, atg12, atg3*, or overexpression of a dominant negative form of *atg4* (*Atg4^{DN}*) in ISCs produced opposite effects to loss of *Uvrag* with midguts containing less ISCs [17]. The authors traced the root cause for ISC depletion to be due to age-dependent stem cell exhaustion as a result of accumulated DNA damage, checkpoint kinase 2 activation and JNK-mediated cell elimination [17]. In support of the idea that *Uvrag* defects lead to hyperplasia due to defects in endocytic trafficking, Rab7 knockdown produced hyperplasia.

Homozygous loss of *Drosophila atg6/beclin1* results in pupal lethality. As predicted, *atg6* mutant clones lack phosphatidylinositol-3-phosphate production. As a result, atg6$^{-/-}$ cells have defects in several vesicle trafficking pathways. *atg6*$^{-/-}$ cells display reduced endocytic uptake and trafficking, and lack starvation-induced autophagy [18]. Interestingly, *atg6* is additionally required for the vesicle-mediated secretion. In preparation for pupation, larvae secrete glue proteins from salivary gland cells to attach to a suitable surface before pupariation. Release of the GFP-tagged glue protein, Sgs3, was defective in both *atg6*$^{-/-}$ and *vps34*$^{-/-}$ cells. This defect in secretion may represent defects in so-called secretory autophagy, as *atg1*$^{-/-}$ cells also failed to efficiently secrete Sgs3-GFP [18]. Defects in early endocytic trafficking by loss of *rab5* of the *avalanche* (*avl*) or endocytic sorting by Endosomal Sorting Complex Required for Transport (ESCRT) components, have been shown to act as tumor suppressors in several organs, including eye-antennal imaginal discs (EAD) [19–22]. Despite the predicted role of PI3K-III/Vps34 upstream of ESCRT function, clonal loss of *atg6*$^{-/-}$ did not produce tumor overgrowth like that of *vps25* (ESCRT-II) or *vps32* (ESCRT-III) [18]. In fact, eye discs carrying double mutant *vps25*$^{-/-}$, *atg6*$^{-/-}$, or *vps32*$^{-/-}$,*atg6*$^{-/-}$ clones grew less than either ESCRT mutant discs alone. This may mean that *atg6* is required for tumor cell fitness, growth promoting signaling or secretion or growth promoting factors. Although the inhibitory effect by *atg6* loss on tumor growth is likely due to a function at the endosome, it remains a formal possibility that autophagy is required for growth or survival of ESCRT mutant cells. *atg6*$^{-/-}$ mutant animals display an appearance of so-called melanotic tumors, often found in larvae with an elevated number of hemocytes, in particular, crystal cells that release phenol oxidases and initiate the cascade leading to melanization. Melanotic tumors were not observed in *atg7*$^{-/-}$ or *atg13*$^{-/-}$ larvae and rescue experiments expressing atg6 in hemocyte compartments of atg6$^{-/-}$ mutant animals failed to prevent melanotic mass formation. This suggests that the cause of supernumerary blood cells may be an indirect effect. A root cause for this phenotype may be due to an enlarged lymph gland with failure of Nimrod C1 (NimC1) positive plasmatocytes and an increase of L1 positive Lamellocytes that are rare in healthy larvae. The cause for supernumerary blood cells and differentiation

does not to depend on NFkB activation as triple mutants for the three NFkB transcription factors, $Dif^{-/-}$, $Rel^{-/-}$, $Dorsal^{-/+}$ did not reverse supernumerary hemocytes.

Collectively, these studied supports tumor suppressive functions of PI3K-III in multiple tissues ascribed to endocytic defects, rather than autophagy.

7.4 Myc Induces Autophagy to Mediate Overgrowth

The *Drosophila* orthologue of the proto-oncogene c-MYC encodes a basic Helix Loop Helix transcription factor, best known to drive anabolic cell growth and cell competition in *Drosophila* [23]. It, therefore, came as a surprise when Drosophila Myc was shown to be required for starvation-induced autophagy in two main nutrient-responsive cell types; adipose cells of the fat body and enterocytes of the midgut (Fig. 7.2). Conversely, overexpression of Myc can induce autophagy in the fat body, enterocytes of the gut, and epithelial cells of the wing imaginal disc [24, 25]. The control of autophagy by Myc does therefore not appear restricted to nutrient-responsive tissues. The marked cell overgrowth induced by Myc in both adipose cells and wing disc cells was suppressed in both tissues by inhibiting the Atg1 initiation complex (*FIP200, atg1*), *atg9*, *atg18a* or *vps34*. Thus, autophagy is required to sustain Myc-induced overgrowth (Fig. 7.2). What induces autophagy in this context and how does it mediate overgrowth? Mechanistically, Myc overexpression was shown to contribute to cell growth in two ways. 1. Expression of dMyc increased cytoplasmic levels of the autophagy adaptor and cargo protein, Ref (2)P/P62 despite increased autophagic flux. In mammals, increased levels of P62 ectopically triggers oxidative stress responses. KEAP1 serves as a Cul3-Rbx1 ubiquitin complex adaptor for NRF2 leading to its ubiquitination and proteasomal breakdown. Upon oxidative stress, Keap1 is released from NRF2, leading to nuclear translocation. NRF2 mediates transcriptional oxidative stress responses and metabolic reprogramming. High levels of P62 sequesters KEAP1, and as a result the NRF2 transcription factor enters the nucleus and drives unwarranted oxidative stress responses [26–28]. In the liver, this eventually results in inflammatory signaling and liver tumor formation [26–28]. Similarly, *Nagy* et al. found that Ref (2)P/P62 binds Keap1 and activate cap-n-collar (CncC)/Nrf2 target genes as judged by the reporter gene GstD-GFP. Ref (2)P/p62 and CncC are both required for Myc-induced overgrowth. As Ref (2) P is also a direct transcriptional target of CncC/NRF2 in flies, it is possible that NRF2 engages Ref (2) P in a feed forward loop of NRF2-mediated overgrowth as suggested in mammals [27, 29]. A second way by which Myc overexpression mediates overgrowth is through activation of ER stress. Myc induced increased levels of phosphorylated eIF2a and activity of the Xbp1 reporter *in vivo,* indicating ER stress. Suppressing the ER stress response by PERK or Gadd34

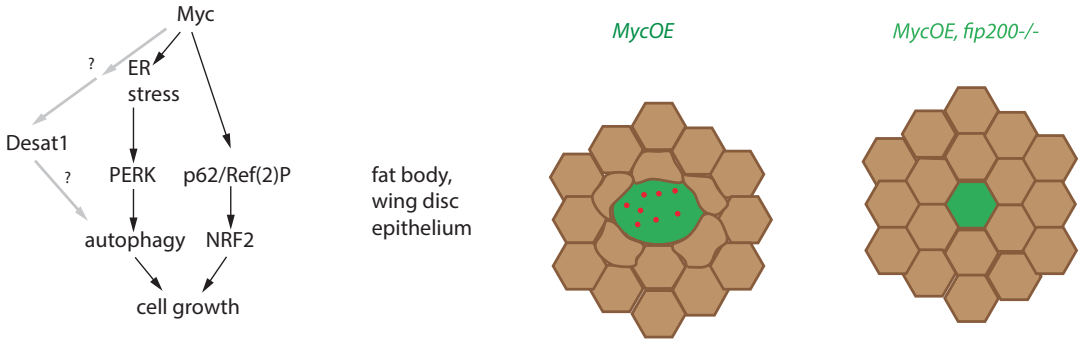

Fig. 7.2 Myc and cell growth. Myc expression induce ER stress that regulate autophagy, and ROS independent CncC/NRF2 transcriptional responses. NRF2 and Autophagy, in turn are both required for Myc-induced growth

knockdown reduced overgrowth of cells and autophagy induction in adipose cells and wing imaginal discs. Another mediator of Myc induced cell growth may be governed through altered lipid metabolism. Myc indirectly leads to increased levels of proteins involved in lipid metabolic processes, including Stearocyl-CoA Desaturase-1 (Desat1) [24]. As Desat1 is predicted to be essential for generation of monounsaturated fatty acids utilized for triglycerides and phospholipids, the authors tested its role during Myc-induced overgrowth and autophagy regulation. A reduced overgrowth of Myc-transformed epithelial cells was observed upon *desat1* knockdown. Myc-induced autophagy in the gut, wing and adipose tissue was modestly reduced upon *desat* knockdown. It remains to be established whether Desat indeed regulates lipid metabolism in this setting and whether autophagy flux is affected and how. Thus, Myc is a *bona fide* regulator of autophagy under physiological starvation-induced autophagy. Abnormal levels of Myc engage ER- and oxidative stress pathways, of which autophagy is one, that all contribute to increased cell mass and growth.

7.5 Autophagy and Stem Cell Tumors

In adult flies, stem cells of the ovary, testis, and gut serve as models for stem cell-derived tumors. Stem cells are resilient to many cancer treatments, likely due to inherently distinct properties like quiescence. In part due to these properties, cancer stem cells are believed to be responsible for tumor recurrence and progression. Distinct from differentiated cells of the gut, adult ISCs are refractory to the Reaper (Rpr)-induced apoptosis [30, 31]. Activation of cell cycling of ISCs and resulting tumor overgrowth through overexpression of Upd1, Ras^{V12}, Raf^{GOF} or loss of Notch signaling through MARCM loss of function clones or expression of N^{DN} ($N^{264\text{-}39}$, Dl) sensitized cells and ISCs to Rpr-induced cell death suggesting that ISCs, once activated for proliferation, can respond to apoptotic stimuli similar to differentiated tissue. One underlying distinction of ISCs to proliferating and differentiated cells appear to be the metabolic state. In an elegant study, ISCs were found to be exquisitely sensitive to defects in lipolysis [31]. Genetic or chemical inhibition of lipolysis selectively killed ISCs through necrosis. Curiously, Atg1 overexpression, which is known to activate autophagy, also counteracts midgut tumor growth due to loss of Notch signaling ($N^{264\text{-}39}$, Dl^{RevF10}) and Raf^{GOF} expressed under esg-Gal4 control (Fig. 7.3a). This suggests that excessive autophagy may be a way to counteract tumorigenesis arising from stem cells. In addition to activating autophagy, *atg1* overexpression in the fat body or imaginal discs leads to cell shrinkage and elimination preceded by caspase activation and TUNEL positive small nuclei, suggesting that these cells are eliminated by apoptosis [3]. This may mean that the cause of reduced clonal growth derived from transformed ISCs should be interpreted with some caution. Co-expression of the apoptosis inhibitor, P35 did not rescue the Atg1-mediated reduction of Raf^{GOF} -induced tumor growth in the adult intestine, suggesting that, either P35 is incapable of blocking apoptosis in this context or that Atg1 reduces cell fitness and growth by another mechanism. Raf^{GOF} expression increased mito-GFP signal suggesting that transformed cells increase mitochondrial mass to support growth and proliferation. In support of this idea, feeding animals with the mitochondrial uncoupler, oligomycin or genetic inhibition of mitochondrial respiration by knockdown of the Complex I subunit ND75 reduced Raf^{GOF}-mediated growth. Autophagy degrades cytosolic material including organelles like mitochondria. Atg1 expression reduced the level of the mitochondrial marker mito::GFP and increase of Cytochrome C indicating mitochondrial leakage. These results suggest that Raf^{GOF} -stimulated cell growth and proliferative capacity rely on intact mitochondrial respiration and that intense autophagy induced by Atg1 may reduce mitochondrial mass or respiratory capacity. Direct metabolic measurements of Atg1-expressing cells with or without autophagic capacity is needed to resolve whether this effect is mediated by autophagy or an alternative function of Atg1.

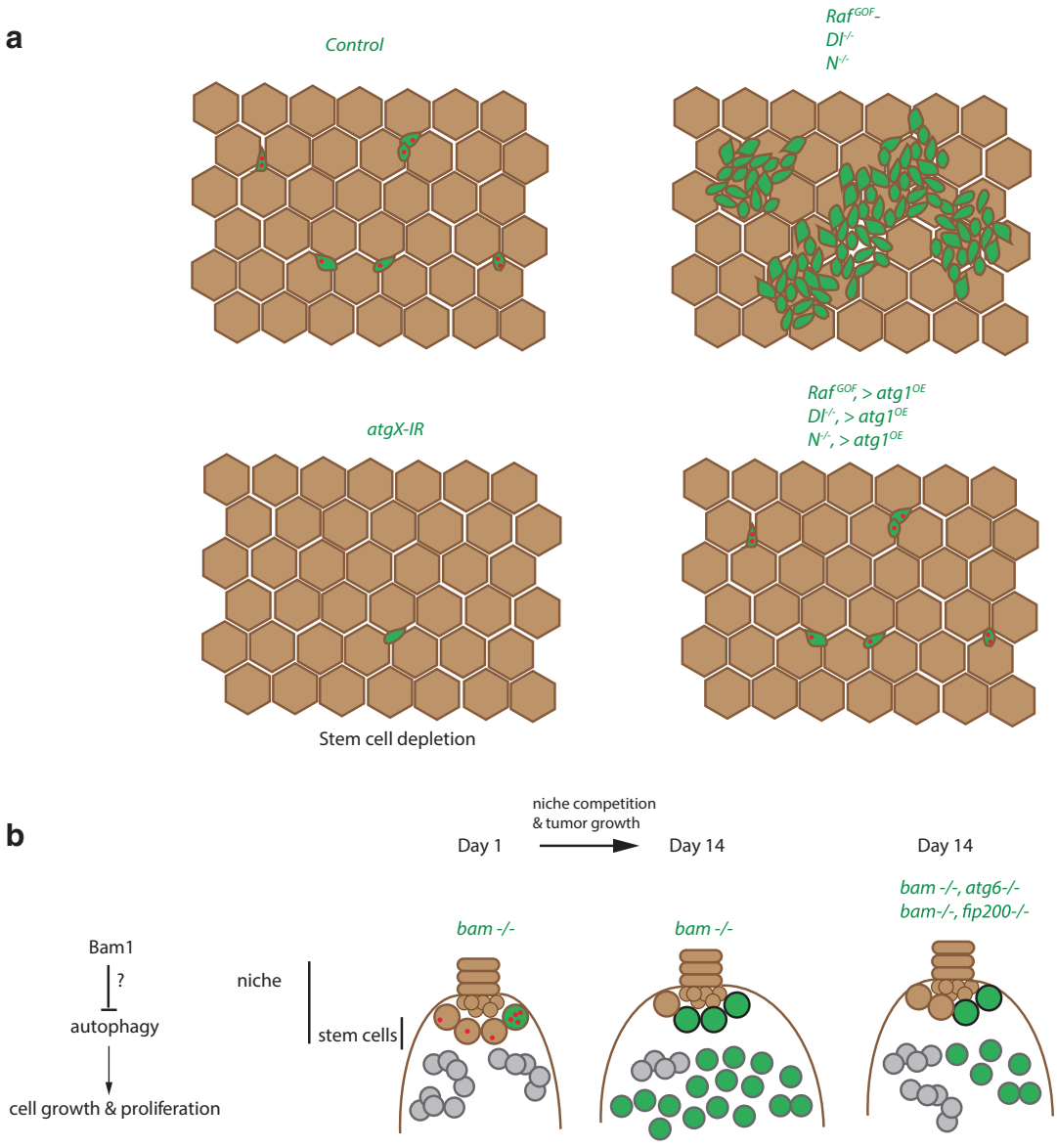

Fig. 7.3 Stem cell derived proliferation and autophagy. (**a**) Autophagy is required for adult stem cell survival. Overproliferation due to loss of Notch signaling or activation of the Ras-Raf-ERK pathway is counteracted by Atg1 overexpression

Niche occupancy is believed to favor tumor initiation in stem cell-derived tumors, likely due to access to niche factors. Zhao S, et al. used the *bag of marbles* (*bam*) ovarian stem cell cancer model to address the role of autophagy in niche occupancy and tumor growth. *Bam*$^{-/-}$ mutant stem cells outcompete normal stem cells for niche occupancy over time and show increased autophagic activity (Fig. 7.3b). In agreement with a role of autophagy in this process, removing autophagy by the Atg1 complex mutant, *fip200* or *atg6* of the PI3K-III complex, reduced the cell size and *bam1*$^{-/-}$ −induced competitiveness. Removing autophagy alone (*atg7*$^{-/-}$) had no such effect. The underlying reason was identified to be caused by reduced cell cycling and not

increased cell death upon autophagy deficiency of transformed cells. Reducing cell cycling by removing cdk2, or reducing insulin signaling in *bam*$^{-/-}$ cells, which is known to result in autophagy activation, phenocopied loss of autophagy with reduced niche occupancy. The details of reduced niche appropriation or slower cell cycling governed by PI3K-III and the ULK1 complex remain unknown.

There seems to be no simple relationship between autophagy and differentiation and homeostasis in stem cells. Whereas autophagy is dispensable in ovarian stem cells for niche occupancy and differentiation, its necessity becomes apparent upon *bam*-mediated transformation where it becomes required for efficient cell growth. It does not affect cell death, nor differentiation. In the intestinal stem cells of the gut, autophagy is needed for sustained ISC function and survival. Upon oncogenic transformation, hyperactive autophagy represses proliferation.

7.6 The Role of Autophagy in Tumor Growth Is Context-Specific and Can Act Cell Autonomously and Non-autonomously

Expression of oncogenic Ras^{V12} leads to moderate benign overgrowth when expressed ubiquitously in the eye-antennal disc (EAD) using eyeless-Gal4. In an RNAi-mediated genetic screen for tumor suppressors that can cooperate with Ras^{V12}, several genes required for autophagy were identified. Widespread co-expression of Ras^{V12} and simultaneous knockdown of *atg8a, atg7* or *atg9* led to strong neoplastic overgrowth with loss of epithelial architecture (Fig. 7.4a). Knockdown of genes acting in all steps of the autophagy pathway was found to produce similar overgrowth, including the autophagy initiation complex (*atg1, atg101,atg13,atg17*), PI3K-III nucleation complex (*vps34, atg6, vps15, atg14*), elongation complex (*atg3, atg4a, atg4b, atg8b*) and completion (*atg5, atg10, atg12*). Knockdown of genes required for autophagosome-lysosome fusion; *syx17, snap29, vamp7*, also cooperated with *Ras^{V12}*. This aligns with earlier findings where *Ras^{V12}* was found to cooperate with knockdown of *vps33/car, vps16*, and *vps18/dor*, that are required for autophagosome-lysosome fusion [32]. Overexpression of Ras^{V12} moderately induces Cherry-Atg8a structure in imaginal discs, suggesting that Ras^{V12} may induce metabolic stress leading to autophagy. Indeed, accumulation of reactive oxygen species and ROS-induced JNK stress signaling were activated in cells expressing Ras^{V12} with simultaneous inactivation of *atg8a*, but not in clones with either manipulation alone. This is reminiscent of the findings where Ras^{V12} was found to cooperate with mitochondrial dysfunction to produce overgrowth of EADs [33]. Mutant lesions in components of the oxidative phosphorylation machinery accumulate reactive oxygen species that is further aggravated under simultaneous Ras^{V12} stimulation. As a main function of autophagy is the removal of depolarized damaged mitochondria through mitophagy, it is tempting to speculate that ROS generation in Ras^{V12}, *atg8-IR* cells is due to ROS generated by non-cleared mitochondria. In both models, overgrowth is primarily due to non-autonomous upregulation proliferation. Indeed, ROS scavenging through the expression of superoxide dismutase (SOD) or inhibition of JNK downstream, block cooperation of Ras^{V12} and inhibition of autophagy flux to induce tissue overgrowth. Although the factors inducing non-autonomous overgrowth were not identified, they are likely to include Unpaired, Wingless and Dpp mitogens that execute non-autonomous compensatory proliferation during wounding to reestablish tissue integrity and size.

Neoplastic tumor suppressors include *scribble* (*scrib*), *lethal giant larvae* (*l(2)gl*) and *disc large* (*dlg*). These proteins act as junctional scaffold proteins to regulate cell polarity, in part through regulating retromer endocytic trafficking [34, 35]. Homozygous larvae unleash neoplastic growth of imaginal discs. A series of elegant studies have shown that in a clonal setting, *scrib*$^{-/-}$, *lgl*$^{-/-}$ or *dlg*$^{-/-}$ clones are eliminated through tissue- intrinsic tumor suppressive cell elimination [36, 37]. This is executed by tumor necrosis factor α (TNFα)- mediated activation of

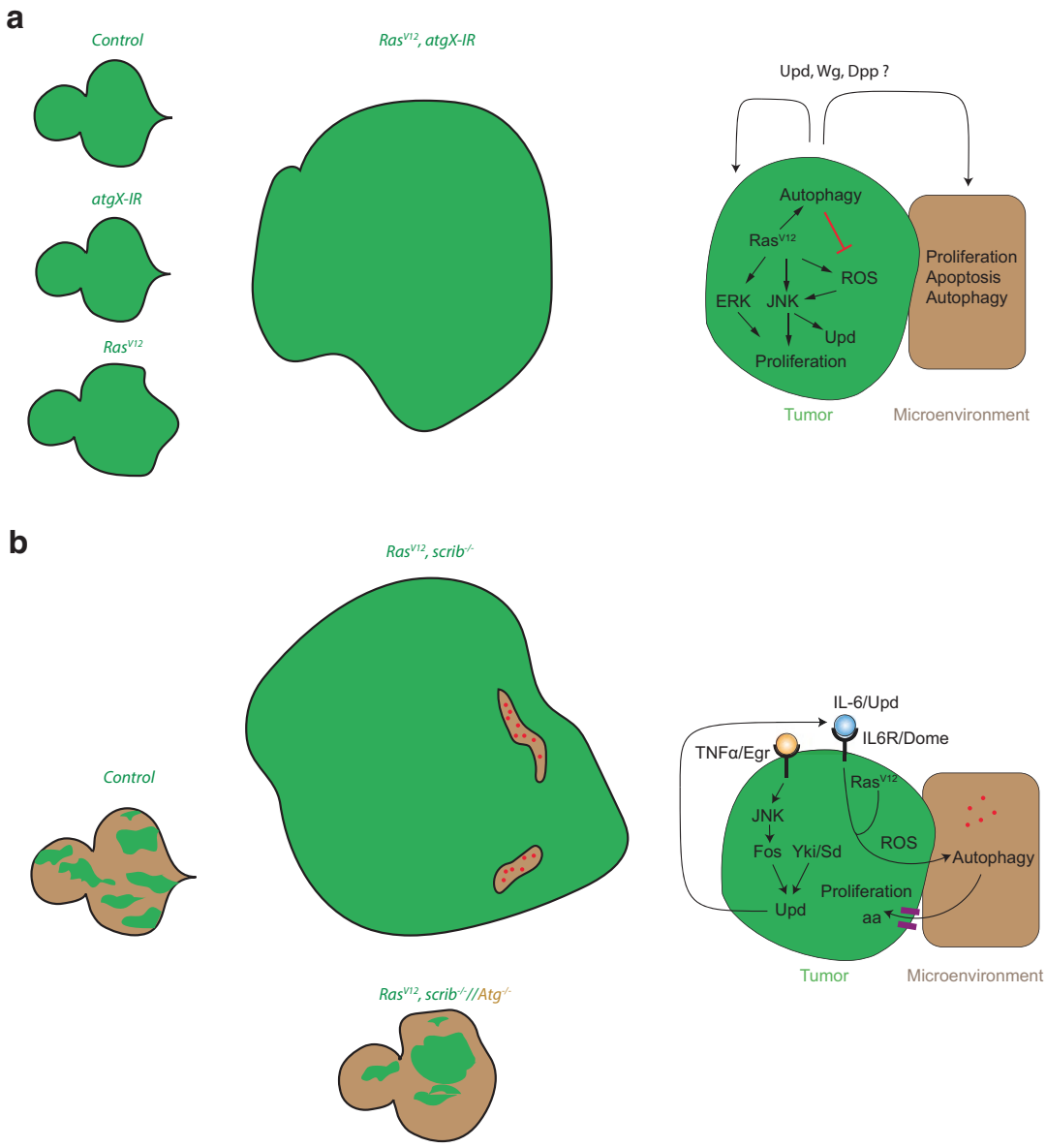

Fig. 7.4 Ras-driven tumor models and autophagy. (**a**) Cell autonomous cooperation between Ras^{V12} and loss of autophagy acts through ERK and ROS-JNK stress activated signaling resulting in overproliferation. (**b**) Malignant Ras^{V12}, *scrib*$^{-/-}$ tumors activate a non cell autonomous stress response of autophagy in the microenvironment downstream of inflammatory responses. In this model, autophagy supports tumor growth from the microenvironment, likely through nutrient provisioning

cell death through the TNFR receptors Grindelwald and Wengen and downstream TRAF2-JNK signaling [38, 39]. Inhibition of JNK or downstream apoptosis by JNK^{DN} (Bsk^{DN}) or expressing baculovirus protein, P35 that inhibits caspase-induced cell death, blocks elimination of *scrib*$^{-/-}$ clones and unleashes overgrowth. Interestingly, simultaneous inhibition of autophagy by *atg1* knockdown, leads to a marked increase in *scrib*$^{-/-}$ tumor size with a marked elimination of neighboring wild type clones in the EAD [40]. This suggests that loss of

autophagy in concert with JNK/apoptosis inhibition may endow *scrib*$^{-/-}$ cells with supercompetitor status during cell competition. As the autophagy status was not evaluated in this study, this remains to be explored.

In two defining publications for the field of tumorigenesis in flies, the labs of Tien Xu and Helena Richardson established that Ras^{V12} cooperate with loss of *scribble* (*scrib*) or *disc large* (*dlg*). These models result in malignant tumors with failure of differentiation, loss of E-Cadherin, epithelial cell polarity, expression of matrix metalloproteases and invasion into the neighboring central nervous system. Inhibition of autophagy by knockdown of *atg1*, or blocking of autophagy by simultaneous removal of *atg13*$^{-/-}$ or *atg14*$^{-/-}$ reduce tumor growth [40, 41].

Analysis of autophagy activity in animals carrying clones of *Ras*^{V12}*,scrib*$^{-/-}$ or *Ras*^{V12}*, dlg-IR* in EADs surprisingly revealed non cell-autonomous upregulation of autophagy in neighboring cells of the microenvironment rather than in the tumors themselves (Fig. 7.4b). Compartment- specific genetic analysis revealed that autophagy within the tumor microenvironment was required to support tumor growth and that this effect was stronger than within the tumor itself. Epistatic established that this non-autonomous autophagy regulation depends on *TNFR-JNK-Fos/Jun* signaling within the tumor cells. *Scrib*$^{-/-}$ cells also inactivate the hippo signaling allowing Yki to enter the nucleus and together with *scalloped* (*sd*) activate a pro-growth transcriptional program. Inactivating the transcriptional response of either AP1 (Jun/Fos) downstream of the JNK signaling pathway, or Sd of the Hippo pathway both inhibit non-autonomous stress responses arguing the involvement of transcriptional targets. In support of this expression of active Yki in clones of the EAD which leads to hyperplastic growth and elicit non-autonomous autophagy response. Co-expression of known transcriptional targets of both pathways, Upd1 or Upd3 with Ras^{V12} is sufficient for autophagy induction. Surprisingly, Upd signaling through the Dome receptor was required in the tumor cells for non-autonomous response arguing that the signal to induce autophagy is downstream of Jak-Stat. The signaling activity triggering autophagy may be Reactive Oxygen Species production activated downstream, but this remains to be explored.

Ras^{V12}*, scrib* $^{-/-}$, and *Ras*^{V12}*, dlg-IR* tumors transplanted to adult flies have been shown to induce systemic cachexia-like organ wasting. Similar responses were observed to stem cell -generated *Yki*^{act} tumors. In both cases insulin signaling was inhibited through secretion of the Insulin-binding peptide IMPL-2 leading to reduced ovary size. Whether this represents *bone fide* organ wasting or a lack of ovary development is unclear. Systemic responses to tumor growth appear to be multifactorial, as secreted Pdf1 also drive degeneration of muscle structure and function, lipid mobilization, as well as blood sugar increase instigated by *Yki*^{act} gut tumors. As autophagy is upregulated in muscle and adipose tissue of *Ras, scrib*$^{-/-}$ larvae it will be interesting to explore whether autophagy is contributing to cachexia-like responses in flies [41].

Notch signaling acts as an oncogene or tumor suppressor depending on context in both mammals and flies. In imaginal disc tumors, Notch drives tumor growth in neoplastic tumors caused by defective endocytosis and sorting [20, 42–44]. In a genetic overexpression screen for tumorigenesis, eyeful was identified [45]. This turned out to be a result of UAS-driven co-expression of the Notch ligand, Delta (Dl) and two Chromatin modifiers pipsqueak and lola. Knockdown of genes of any stage of autophagy increased growth of eyeful imaginal discs and resulting adult eyes (Fig. 7.5a). Conversely, overexpression of Atg1 reduced tumor size. Atg1-mediated growth restriction appears to be mediated by autophagy and as simultaneous autophagy inhibition by *atg8* or *atg12* knockdown restored tumor growth. Similarly, co expression of P35 increased eyeful growth and had an additive effect with autophagy inhibition. As eyeful tumors show strong upregulation of autophagy and cleaved caspase 3, this suggests that autophagy supports and apoptosis restrains eyeful-induced overgrowth.

Inactivation of the Hippo-Warts pathway leads to increased hyperplasia in imaginal discs and other tissues due to Yki-driven hyperproliferation and cell competition. Activation of autophagy by

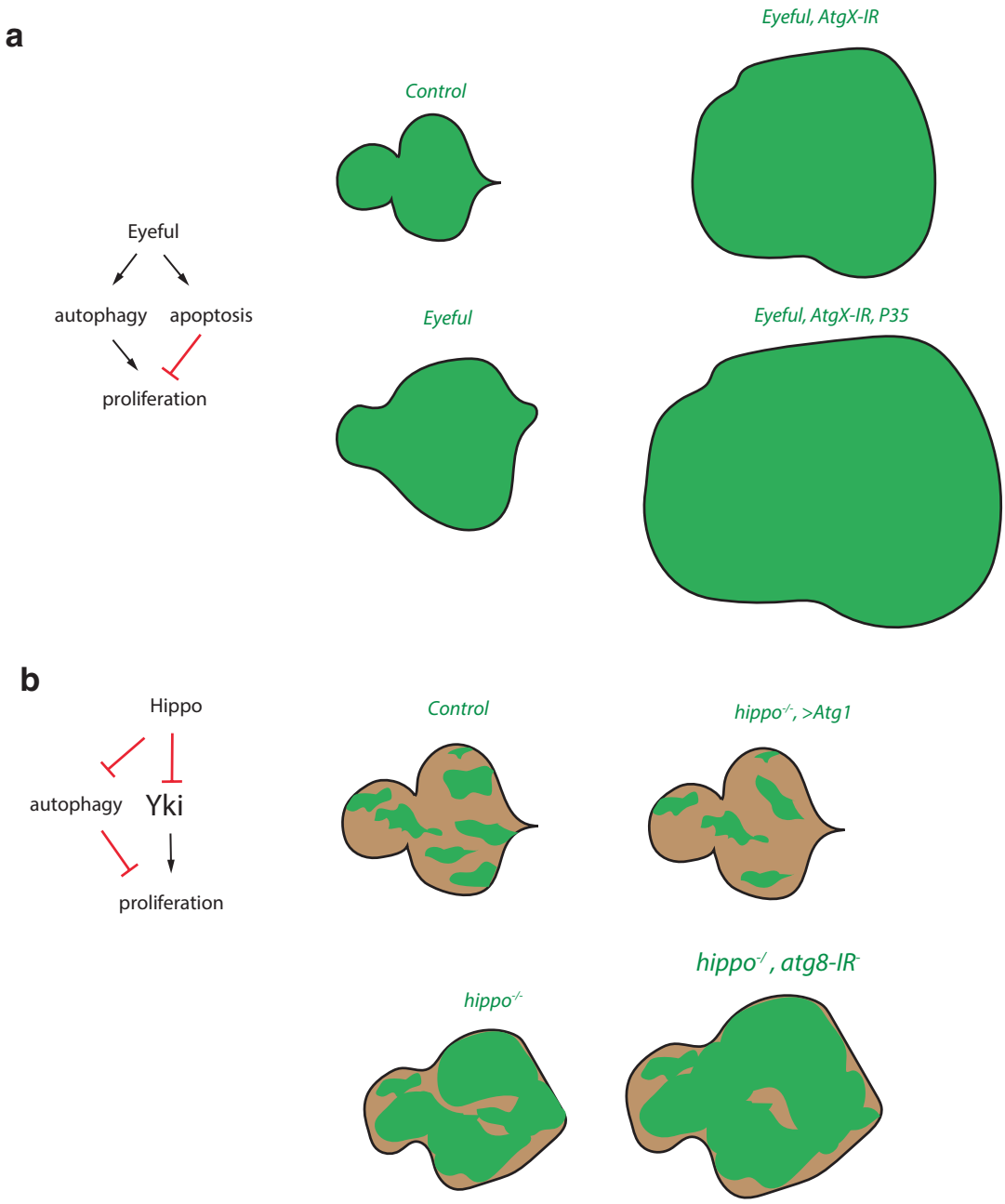

Fig. 7.5 Autophagy and tumors of the eye. (**a**) Notch-driven eyeful tumors activate autophagy and apoptosis. Inactivation of autophagy and apoptosis show that they both serve to restrain growth. (**b**) Hyperplastic overgrowth and cell competition by defects in the Hippo-Warts pathway are exacerbated upon simultaneous cell autonomous loss of autophagy and counteracted by autophagy stimulation

way of Atg1 overexpression inhibits the growth of *hippo*$^{-/-}$ clones, whereas autophagy inactivation had no effect in hyperplastic eye discs [40] (Fig. 7.5b). The activation status of autophagy was not evaluated in these experiments, but overexpression of Yki, was shown to induce non-autonomous autophagy in the eye disc [41]. In contrast to *Ras*^{V12}, *scrib*$^{-/-}$ tumors, selective

inhibition of autophagy in the microenvironment of Yki-transformed discs did not compromise Yki-driven hypertrophy and cell competition [41]. This suggests that not all tumor growth depend on microenvironmental growth support through autophagy.

The relationship of Hippo pathway inactivation and autophagy appear complex, as responses are distinct in different cell populations. Similar to imaginal discs, Yki overexpression leads to expansion of glial cell populations when expressed under repo-Gal4 control. In contrast to the situation in imaginal discs, stimulating autophagy through Atg1 overexpression led to an increase of glial expansion.

7.7 Conclusions and Perspectives

Although autophagy is considered a tumor-preventive process, genetic analysis of core autophagy components in flies does not so far provide proof for a classical tumor suppressive role for autophagy. This does not mean that autophagy does not harbor tumor suppressive activities. Loss of autophagy leads to reactive oxygen species generation that is exacerbated in transformed cells, and DNA damage likely arises due to the accumulation of defective and leaky mitochondria. Over time, this may drive mutating events that lead to tumorigenesis. With the short lifespan of the fly, this tumor evolution is challenging to address. Nevertheless, it is clear that loss of autophagy with subsequent ROS generation can cooperate with other tumor drivers, like oncogenic Ras^{V12} to drive tumor growth [46]. It is unlikely that this cooperation is due to mutating events by ROS, but rather ROS-driven transcriptional responses and secretion of mitogenic molecules with autonomous and non-autonomous effects on proliferation. Several tumor models support a role of autophagy in sustaining or preventing tumor growth. This can occur cell autonomously, or non-autonomously, highlighting the necessity for careful analysis of autophagic activity in both models and compartment-specific manipulations. It will be important to measure autophagic flux carefully and to delineate the regulation of autophagy in any given tumor model. Autophagy is dynamically regulated by several stress signaling pathways some of which are activated or inhibited by oncogenic or tumor suppressive lesions. Regulation can be complex and sometimes through parallel pathways as during Myc-driven overgrowth where two stress responses act in concert. The ROS response transcription factor CncC/NRF2 stimulates autophagy independent of ROS generation. Parallel ER-stress caused by Myc overgrowth drives autophagy through PERK signaling. It is important to stress that the processes downstream of autophagy that regulate tumor growth are so far unexplored in tumor models of the fly. This is a critical, as a large body of literature from studies employing genetically engineered mouse models (GEMMs) for cancers demontrate that the most central role of autophagy in tumorigenesis is maintaining tumor cell metabolism (reviewed in [1, 2, 47]). Autophagy can also directly regulate abundance of oncogenic proteins, such as the PML-RARA fusion protein in acute promyelocytic leukemia [48]. The stress signaling pathways regulating autophagy in the variety of fly tumor models, remain largely unidentified. Moving on, it is imperative to carefully assay autophagic flux and identify autophagic cargoes that impact tumor progression. As autophagy is the only mechanism to remove damaged mitochondria and acts to recycle macromolecules that can are utilized as nutrients or building blocks in growing cells, metabolic analysis will undoubtedly enlighten our evolving understanding of autophagy and tumorigenesis. *Drosophila* offers a multitude of sophisticated methods to analyze the roles of autophagy during carcinogenesis. Having several tumor models at hand where autophagy is central, *Drosophila* with its strong genetic toolbox is poised to provide further valuable mechanistic insights (Table 7.1).

Table 7.1 Tumor models and autophagy function

Tumor model, mutations	Autophagy manipulation	Mechanism, biological role	References
Ovarian stem cell tumor (*bam*$^{-/-}$)	*Fip200, atg6, atg7*	Niche occupancy, cell growth, cell cycle progression	[49]
Cell overgrowth (*myc*^{OE}) Wing epithelium, adipose tissue	*Fip200*, chloroquine, *atg1, vps34, atg18*	Autophagy induced through ER stress/ PERK. Cell growth, proliferation. Lipid metabolism regulation by Myc may affect autophagy induction.	[24, 25]
Adult gut tumors Raf^{GOF}, *Dl*$^{-/-}$, *N*$^{264\text{-}39}$ *N*^{DN}, *Ras*^{V12}, *Upd*, *Raf*^{GOF}, adult intestinal stem cell tumors	*atg1, atg5*	Autophagy stimulation by Atg1 can counteract ISC-derived tumor growth.	[30, 31]
Ras^{V12}, follicle cell epithelium, eye-antennal disc	*vps34, vps15, Uvrag,*	PI3K-III restricts LKB1 activity through endosomal trafficking to control epithelial integrity. PI3K-III cooperate with Ras^{V12} in tumor growth through LKB1 and JNK activity. UVRAG restricts N signaling.	[12, 13]
Uvrag, adult intestinal stem cell tumors	*Uvrag, rab7*	Loss of *Uvrag* function in intestinal stem cells leads to hyperplasia with expansion of the stem cell pool in adult midgut due defects in endosomal trafficking. Resulting JNK activation and Upd1 upregulation both contribute cell autonomously to hyperplasia.	[16]
ESCRT (*vps25, vps32*) eye disc tumors	*atg6/beclin1*	atg6 function is required for tumorous overgrowth of ESCRT mutant eye discs. *atg6*$^{-/-}$ *animals have* increase blood cell numbers and "melanotic tumors". The origin of this phenotype may be non-cell autonomous.	[18]
Ras^{V12}	*atg1, atg101, atg13, atg17., vps34, atg6, vps15, atg14, atg3, atg4a, atg4b, atg8b, atg5, atg10, atg12), syx17, snap29, vamp7*	Accumulation of reactive oxygen species is high in imaginal disc cells expressing Ras^{V12} in conjunction with autophagy inhibition. ROS-induced JNK stress signaling mediates non-autonomous proliferation and tissue overgrowth	[46]
Scrib−/−, Ras^{V12}, *scrib*$^{-/-}$ or *Ras*^{V12}, *dlg-IR*	*atg13, atg14, atg1, atg8*	Epithelial-intrinisic elimination of *scrib*$^{-/-}$ cells limit their tumorigenic capacity and depends on TNFR-mediated apoptosis and autophagy. Autophagy is induced in *Ras*^{V12}, *scrib*$^{-/-}$ and *Ras*^{V12}, *dlg-IR* microenvironment downstream of TNFR, JNK and Upd-Dome-DN and supports tumor growth.	[40, 46, 50]
Hpo$^{-/-}$, *Yki*^{act}	*atg8, atg1*	Yki-driven hypertrophy activates autophagy in the microenvironment. Stimulation of autophagy in hpo$^{-/-}$ or Yki^{act} expressing epithelia by Atg1 suppress tumor growth. Yki- driven glial cell expansion is stimulated by Atg1 overexpresssion.	[40, 41]
Eyeful (*Dl, lola, pipsqueak*)	*Atg1, atg6, atg12, atg5, atg5, atg7, atg4a, atg4b, atg8a, atg8b, atg3, atg9, atg18*	Autophagy and caspase-mediated apoptosis serve to restrict eyeful driven hyperplasia in the eye,	[40]

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8 Understanding Obesity as a Risk Factor for Uterine Tumors Using *Drosophila*

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Abstract

Multiple large-scale epidemiological studies have identified obesity as an important risk factor for a variety of human cancers, particularly cancers of the uterus, gallbladder, kidney, liver, colon, and ovary, but there is much uncertainty regarding how obesity increases the cancer risks. Given that obesity has been consistently identified as a major risk factor for uterine tumors, the most common malignancies of the female reproductive system, we use uterine tumors as a pathological context to survey the relevant literature and propose a novel hypothesis: chronic downregulation of the cyclin-dependent kinase 8 (CDK8) module, composed of CDK8 (or its paralog CDK19), Cyclin C, MED12 (or MED12L), and MED13 (or MED13L), by elevated insulin or insulin-like growth factor signaling in obese women may increase the chances to dysregulate the activities of transcription factors regulated by the CDK8 module, thereby increasing the risk of uterine tumors. Although we focus on endometrial cancer and uterine leiomyomas (or fibroids), two major forms of uterine tumors, our model may offer additional insights into how obesity increases the risk of other types of cancers and diseases. To illustrate the power of model organisms for studying human diseases, here we place more emphasis on the findings obtained from *Drosophila melanogaster*.

Keywords

Obesity · Endometrial cancer · Uterine leiomyomas · The CDK8 module · *Drosophila*

8.1 Introduction

The major challenges to modern medicine include diseases such as cancer, diabetes, obesity, cardiovascular diseases, neurodegenerative diseases, and neurological disorders. Given that the initiation and progression of these illnesses are determined by genetic factors, environmental factors (such as diets, stress, and life styles), and the complex interplay among these factors, these common medical problems are also known as complex or multifactorial diseases. Accordingly, one of the major tasks of biomedical research is to define the causal relationships of the intertwined interplays and correlations among different factors, thereby revealing the underlying the molecular and cellular mechanisms. This knowledge is essential for developing efficient

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treatments of these diseases and ensuring the best interests of the public health.

Here, we focus on recent progresses related to the complex relationships between obesity and the risk of developing cancer, particularly the progresses based on the basic researches using *Drosophila* as a model system. The reported prevalence of obesity has been rising steadily over the last decades in many countries in the world, with nearly one-third of global population being obese or overweight in recent years [76, 139]. Large-scale epidemiological studies have consistently identified obesity as the single most important risk factor for a number of cancers, but the mechanisms of how obesity increases the risk for these cancers remain poorly understood and controversial [31, 62, 79].

Using uterine tumors as an example, we propose a novel model that may provide a simple explanation of how obesity increases the risk of uterine tumors: chronic downregulation of the CDK8 activities in obese women increases the chances of dysregulating the activities of a number of transcription factors in different tissues of the uterus, thereby increasing the risk of uterine tumors. In this model (Fig. 8.1), CDK8 serves as a regulatory node linking upstream signaling pathways to different transcription factors, but its activities are chronically dysregulated by obesogenic hormones in obese women. We focus on the malignant endometrial cancer and the benign uterine leiomyomas (or uterine fibroids), which represent the most common gynecological tumors with increasing frequencies in recent decades. This pathological context may provide an example to illustrate how the humble fruit flies can continue contributing to biomedical research.

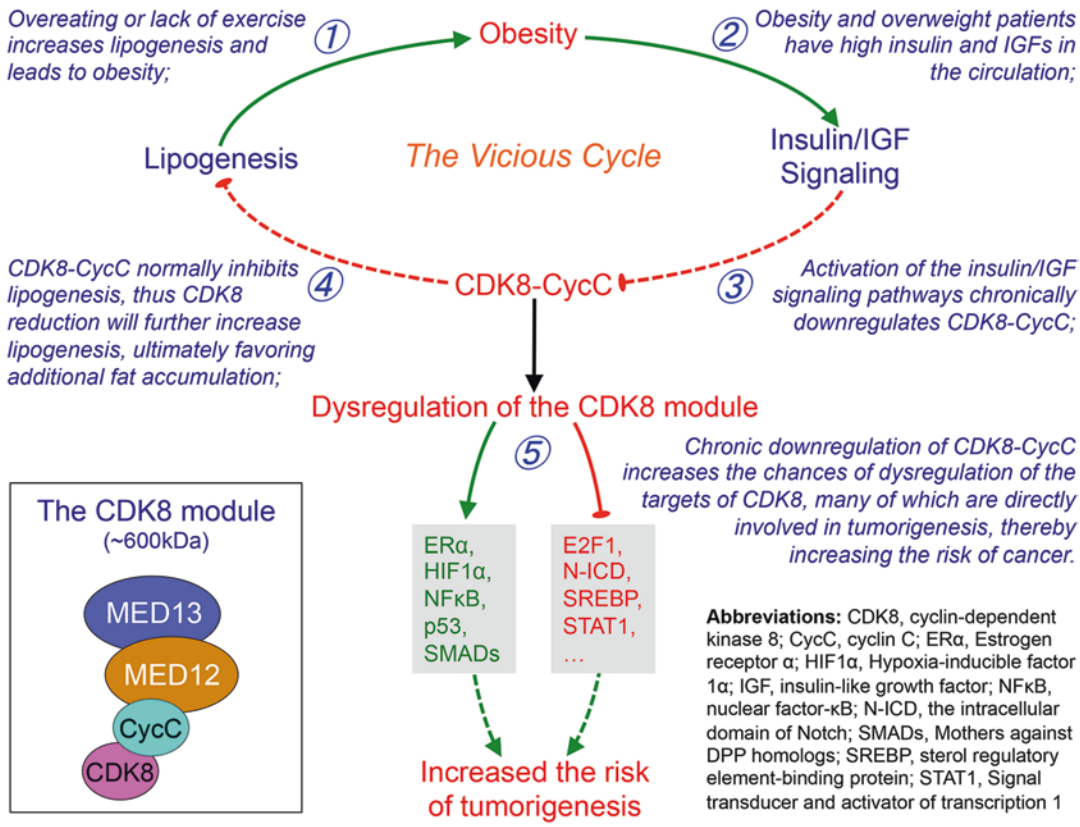

Fig. 8.1 Model: Chronic downregulation of the CDK8 module by obesity increases the risk of uterine tumors. Inset: the subunit organization of the CDK8 module, based on the cryo-EM structure of the yeast CDK8 module discovered by Tsai et al. [191]

Given that a number of excellent reviews on the applications and advantages of *Drosophila melanogaster* in cancer research have been published in recent years [66, 74, 155, 168, 176, 190, 198], here we focus on the reports that are relevant to our hypothesis on the relationship between obesity and risk of uterine tumors, instead of providing an extensive review of the literature.

8.2 Obesity and Elevated Risks for Uterine Tumors

Multiple large-scale epidemiological studies have established a clear link between obesity and the increased cancer risk [62, 79]. Among these, perhaps the most impressive study to date was the one published in Lancet in 2014, where the researchers examined the correlation between body-mass index (BMI) and risks of cancers [17]. This research team analyzed the BMI of a cohort of 5.24 million adults in the United Kingdom over 25 years, and approximately 3.2% of them (166,955 individuals) developed 22 specific cancers. They found that excess weight was significantly associated with increased risks of 17 of 22 types of site-specific cancers, such as uterine, gallbladder, kidney, liver, colon, ovary, cervix, esophageal, pancreas, thyroid, and postmenopausal breast cancers, as well as leukemia [17]. Exactly how obesity (BMI of 30 or higher) and overweight (BMI of 25–29.9) increases the risks of these specific cancers remains unknown. Interestingly, the effects vary by site, and the strongest correlation was found between high BMI and uterine cancer [17]. This result is consistent to other large-scale epidemiological studies in that they all identify strong correlation between obesity and increased risk of uterine cancer (Table 8.1) [17, 18, 26, 100, 110, 112, 113, 160, 163, 165, 178, 218].

The body of the uterus in mammals has three layers: the inner layer is the endometrium, the outer layer tissue coating the outside of the uterus is the serosa, separated by a muscular middle layer known as the myometrium. According to their tissue origins, uterine cancers are generally classified into the following four types. The most common type of uterine cancer is derived from the epithelial cells in the endometrium, and they are catogorzied as endometiral carcinoma or endomtrial cancer, accounting for approximately 90% of uterine cancers [159]. The second type is endometrial sarcoma, which accounts for about 5% of uterine cancers. They arise within mesenchymal tissues and are further classified as endometrial stromal sarcoma, leiomyosarcoma, and other nonspecific sarcomas. The third type of uterine cancers have a mixed tissue origin and include carcinosarcoma and adenosarcoma, representing about 3% of cases. Finally, about 2% of cancers found in the uterus are secondary tumors metastasized from other organs such as cervix, colon, and ovary [159]. Aside from these malignant uterine cancers, severeal types of benign tumors can also grow in the uterus, including endometrial hyperplasia and fibroid tumors such as uterine leiomyomas, adenofibromas, and adenomyomas. Of these, uterine leiomyomas (or uterine fibroids) is the most common tumor in women [21, 147, 183–185]. Therefore, the maligant endometrial cancer and the benign uterine leiomyomas represent the vast majority of cases of uterine tumors.

Known risk factors that may increase a woman's risk of developing uterine tumors include age, race, diet, nulliparity, extended exposure to estrogen, and usage of chemotherapy drug tamoxifen and radiation therapy. In addition, disease conditions such as obesity, diabetes, and other types of cancers such as breast cancer, colon cancer, ovarian cancer, polycystic ovarian syndrome, and hereditary non-polyposis colorectal cancer (or the Lynch syndrome), may also increase the risk of uterine tumors [48, 49, 148, 167, 195, 197]. Two major models have been proposed to explain how obesity increases the risk of cancers. First, excessive fat, particularly the visceral fat, may result in higher levels of hormonal factors such as estrogen, insulin, and insulin-like growth factors (IGFs), which may stimulate cell proliferation [25, 62, 79]. Second, excessive fat accumulation in adipocytes may damage adipocytes and create hypoxia conditions, which can trigger inflammation and attract immune cells, such as macrophages, into adipose tissue. These

Table 8.1 Large-scale epidemiological studies have consistently identified obesity as the top etiologic risk factor for uterine cancer

Reference (Sample source)	Total population	Number of females	Types of cancer	Relative Risk (95% confidence interval)								Notes
				Overweight		Obese I		Obese II		Obese III		
				BMI: 25.0–29.9		BMI: 30.0–34.9		BMI: 35.0–39.9		BMI ≥40.0		
Lauby-Secretan et al., 2016 (France)	>1,000		**Uterine cancer**							7.1	(6.3–8.1)	
			Esophagus cancer							4.8	(3.0–7.7)	
			Gastric cancer							1.8	(1.3–2.5)	
			Liver cancer							1.8	(1.6–2.1)	
			Kidney cancer							1.8	(1.7–1.9)	
Bhaskaran et al., 2014 (UK)	5,243,978	2,864,658	**Uterine cancer**	1.52	(1.33–1.74)	2.65	(2.29–3.06)	5.86	(5.08–6.76)∗			∗ ≥35.0
			Liver cancer	1.25	(1.08–1.44)	1.71	(1.43–2.04)	2.38	(1.87–3.03)∗			
			Gall bladder cancer	1.37	(0.97–1.96)	1.77	(1.15–2.73)	2.11	(1.16–3.81)∗			
			Kidney cancer	1.18	(1.03–1.36)	1.48	(1.24–1.77)	1.99	(1.56–2.52)∗			
			Colorectal cancer	1.16	(1.10–1.22)	1.32	(1.23–1.41)	1.36	(1.23–1.51)∗			
Setiawan et al., 2013 (US)		49,381	**Endometrial cancer**	1.57	(1.46–1.68)∗	2.56	(2.35–2.80)∗	4.75	(4.22–5.34)∗	6.88	(5.95–7.96)∗	∗OR, odds ratio
Dossus et al., 2010 (Europe)		879$	**Endometrial cancer**	1.23	(0.82–1.84)∗	2.02	(1.26–3.23)∗#					$ case control; ∗OR, odds ratio # ≥30.0
Lindemann et al., 2009 (Norway)		36,755	**Endometrioid adenocarcinomas**	2.1	(1.4–3.0)∗	2.1	(1.3–3.5)∗	5.8	(3.3–10.3)∗	11.1	(5.2–23.8)∗	
			Endometrial cancer	1.8	(1.3–2.4)∗	2.1	(1.4–3.2)∗	5.6	(3.5–9.1)∗	8.3	(4.1–16.7)∗	
			Uterine cancer	1.6	(1.2–2.2)∗	2	(1.3–2.9)∗	5.3	(3.4–8.2)∗	6.7	(3.4–13.4)∗	
Renehan et al., 2008	282,137 ∗	127,804 ∗	**Endometrial cancer**	1.59	(1.50–1.68)#							∗incident cases #Risk ratio (per 5 kg/m2 increase)
			Gall bladder cancer	1.59	(1.02–2.47)#							
			Oesophageal cancer	1.51	(1.31–1.74)#							
			Renal cancer	1.34	(1.25–1.43)#							
			Leukaemia	1.17	(1.04–1.32)#							
Reeves et al., 2007 (UK)		1,222,630	**Endometrial cancer**	1.43	(1.29–1.58)∗	2.73	(2.55–2.92)#					∗27.5–29.5, # ≥40.0
			Esophagus cancer	1.57	(1.04–2.36)∗	2.54	(1.89–3.41)#					
			Kidney cancer	1.19	(0.99–1.44)∗	1.52	(1.31–1.77)#					
			Pancreatic cancer	1.2	(1.00–1.44)∗	1.37	(1.18–1.60)#					
			Postmenopausal breast cancer	1.21	(1.13–1.29)∗	1.29	(1.22–1.36)#					

Lundqvist et al., 2007 (Sweden and Finland)	70,067	37,264	**Uterine cancer (elder)**	1.2	(0.8–1.6)∗	3.2	(2.1–4.8)∗#					∗OR, odds ratio, #≥30.0
			Breast cancer (elder)	1.2	((1.0–1.4)∗	1.3	(1.0–1.7)∗#					
			Colon cancer (elder)	1.2	(0.9–1.5)∗	1.3	(0.9–1.8)∗#					
			Uterine cancer (younger)	1.6	(1.0–2.5)∗	2.9	(1.4–5.9)∗#					
			Prostate cancer (younger)	1.1	(0.9–1.4)∗	1.3	(0.7–2.2)∗#					
			Colorectal cancer (younger)	1.0	(0.7–1.4)∗	1.1	(0.5–2.5)∗#					
Lukanova et al., 2006 (Sweden)	74,207	38,530	**Endometrial cancer**	1.45	(0.93–2.24)	2.93	(1.85–4.61)∗					∗≥30.0
			Skin cancer	0.74	(0.34–1.52)	2.55	(1.27–4.93)∗					
			Colorectal cancer	1.28	(0.78–2.18)	2.25	(1.25–3.98)∗					
			Urinary tract cancer	0.76	(0.26–2.02)	2.12	(0.77–5.43)∗					
			Kidney cancer	0.92	(0.31–2.58)	1.79	(0.55–5.27)∗					
Bjørge et al., 2006 (Norway)		1,036,909	**Uterine cancer (Type I)**	1.39	(1.32–1.47)	2.72	(2.56–2.90)∗					∗≥30.0
			Uterine cancer (Mixed tumors)	1.48	(1.14–1.92)	1.97	(1.44–2.71)∗					
			Uterine cancer (Type II)	1.26	(1.09–1.46)	1.94	(1.64–2.30)∗					
			Uterine cancer (Sarcomas)	1.22	(0.99–1.50)	1.88	(1.46–2.41)∗					
Rapp et al., 2005 (Austria)	145,931	78,484	**Uterine cancer**	1.29	(0.90–1.86)	2.13	(1.38–3.27)	3.93	(2.35–6.56)∗			∗≥35.0 hazards ratio, #≥30.0
			Gastric cancer	0.78	(0.51–1.20)	1.28	(0.76–2.15)	1.34	(0.57–3.13)∗			
			Breast cancer	0.96	(0.83–1.10)	1.07	(0.88–1.31)	1.01	(0.72–1.42)∗			
			Non-Hodgkin's lymphoma	1.64	(0.89–3.01)	2.86	(1.49–5.49)#					
			Gall bladder cancer	1.35	(0.74–2.48)	1.6	(0.76–3.36)#					
Calle et al., 2003 (US)	900,053	495,477	**Uterine cancer**	1.5	(1.26–1.78)	2.53	(2.02–3.18)	2.77	(1.83–4.18)	6.25	(3.75–10.42)	Mortality from Cancer
			Kidney cancer	1.33	(1.08–1.63)	1.66	(1.23–2.24)	1.7	(0.94–3.05)	4.75	(2.50–9.04)	
			Pancreatic cancer	1.11	(1.00–1.24)	1.28	(1.07–1.52)	1.41	(1.01–1.99)	2.76	(1.74–4.36)	
			Breast cancer	1.34	(1.23–1.46)	1.63	(1.44–1.85)	1.7	(1.33–2.17)	2.12	(1.41–3.19)	
			Colorectal cancer	1.1	(1.01–1.19)	1.33	(1.17–1.51)	1.36	(1.06–1.74)	1.46	(0.94–2.24)	

Note: Types of cancer are ranked based on the Relative Risk of the most severe obesity
Blank cells: Data not available

immune cells may also secrete growth factors that can stimulate cell proliferation [63, 172]. This scenario is further complicated by the cross-talks among insulin, estrogen, and inflammation in adipose tissue. For examples, adipocytes contain enzymes for estrogen production; insulin can stimulate further fat accumulation within adipocytes (see below); and inflammation may also affect how adipocytes respond to insulin [25, 62, 79]. However, the exact molecular mechanisms underpinning how obesity and these endocrine factors contribute to the increased risks of uterine tumors remain not fully understood.

By focusing our discussions on the links between obesity and the increased risk of endometrial cancer and uterine leiomyomas, we propose that chronic dyregulation of the CDK8 module in obese women may play a number of currently underappreciated roles in the pathologenesis of uterine tumors (Fig. 8.1). We discuss the relevant observations to evaluate the idea that chronic downregulation of the CDK8 module by the hyperactive insulin/IGF signaling and the mechanistic target of rapamycin (mTOR) pathways in obese women contributes to increased risk of uterine tumors (Fig. 8.1). In this pathological context, the CDK8 module may serve as a regulatory node linking these hormonal factors and different transcriptional activators in different tissues.

8.3 Role of the CDK8 Module and Mediator Complex in Regulating Gene Expression

The transcription cofactor Mediator complex serves as a molecular bridge between DNA-bound transcription factors and RNA polymerase II (Pol II) in eukaryotes [32, 95]. The Mediator complex is composed of up to 30 different subunits that can be divided into the head, middle, tail, and the CDK8 modules [7, 19, 120]. The head, middle, and tail modules can be biochemically purified as the small Mediator complex, which can interact with Pol II and is transcriptionally active. The CDK8 module (Fig. 8.1 inset), comprised of CDK8 (or its paralog CDK19, a.k.a., CDK8L in vertebrates), CycC, MED12 (or MED12L in vertebrates), and MED13 (or MED13L in vertebrates), can reversibly associate with the small Mediator complex, forming the large Mediator complex, which is transcriptionally inactive [47]. Thus, the CDK8 module may regulate Pol II-dependent gene expression by physically blocking the interaction between the small Mediator complex and Pol II [7, 120]. It is thought that the Mediator complex is involved in most, if not all, of RNA Pol II-dependent transcription [95]. However, it is unlikely that a simple mechanism can be generalized to explain how different classes of transcription factors work *in vivo*. As discussed below, it is evident that CDK8 has pleiotropic effects on the activities of different transcription factors, suggesting that the actual modes of regulation by CDK8 are complex and context-specific.

Besides serving as a physical block, the kinase activity of CDK8 (or CDK19), the only enzymatic subunit of the Mediator complex [19], also plays important roles in regulating Pol II-dependent gene expression. A number of CDK8 substrates in metazoans have been identified, including a few factors of the general transcription machinery can be phosphorylated by CDK8, including the carboxyl-terminal repeat domain of Pol II, Cyclin H subunit of the TFIIH (CDK7-CycH), Histone H3 Ser10, and CDK8 itself [3, 93]. More importantly, a number of transcription factors have been identified as direct targets of CDK8 kinase. These include the intracellular domain of Notch (N-ICD), the tumor suppressor p53, the transcription factor SMADs that function downstream of the bone morphogenetic proteins (BMP) signaling pathway, signal transducer and activator of transcription 1 (STAT1) that functions downstream of the JAK/STAT signaling cascade, E2F1, and sterol regulatory element-binding protein (SREBP) [5, 11, 43, 53, 73, 94, 136, 149, 217]. Interestingly, CDK8 plays a positive role in regulating the transcription activities of p53, STAT1, and SMADs, but negatively regulates the activities of N-ICD, E2F1, and SREBP. CDK8 can also modulate the activities of transcription factors such as

Hypoxia-inducible factor 1α (HIF1α), MYC, NF-κB, *Drosophila* nuclear hormone receptor Ecdysone receptor (EcR), and the GATA factor Serpent in *Drosophila* [1, 29, 54, 97, 205], but it is still unclear whether these transcription factors are direct substrates for CDK8.

It is unlikely that the effects of CDK8 on these transcription factors and cofactors can adequately explain the full spectrum of CDK8 functions *in vivo*, thus it is expected that there are additional CDK8 targets that remain unidentified. *Drosophila* may serve as a powerful experimental system to identify CDK8 targets and facilitate the subsequent analyses of the physiological relevance of these interactions. The initial observations that led to the discovery of E2F1 and SREBP as the direct targets of CDK8 were made in *Drosophila*, and later validated in mammalian systems [136, 216, 217]. The transcription factor E2F1 plays critical roles in regulating the G1 to S phase transition of the cell cycle in *Drosophila* and mammalian cells [44, 82]. Genetic interactions between CDK8 and E2F1 spurred the subsequent biochemical analyses, which revealed that CDK8 directly binds to and phosphorylates E2F1 [136]. The phosphorylation site was subsequently mapped in mammalian cells [216]. These studies suggest that CDK8 indirectly regulates G1-S phase transition of the cell cycle by negatively regulating E2F1-dependent transcription. Interestingly, ectopically overexpress wild-type, but not kinase-dead, CDK8 in *Drosophila* wing disrupted vein patterns, while depleting CDK8 or CycC resulted in ectopic venation in the intervein regions [106]. These phenotypes, representing the first *in vivo* readout for CDK8-specific activities in any organism, may aid the studies to elucidate the function and regulation of CDK8 *in vivo*.

8.4 Role of the CDK8 Module in Mediating the Effects of Insulin on Lipogenesis

Analyzing the mutant phenotypes of the *cdk8* and *cycC* null mutants allowed us to identify another direct substrate of CDK8, SREBP [217]. SREBP plays a critical role in the maintenance of lipid and cholesterol homeostasis [61, 142, 162]. SREBP is a unique transcription factor in that its precursor is localized in the Golgi apparatus. When intracellular levels of sterol are low, its precursors translocate from the Golgi apparatus to the endoplasmic reticulum, where it will be cleaved by proteases, resulting in the release of the amino terminal basic helix–loop–helix leucine zipper domain of SREBP from endoplasmic reticulum. This mature form of SREBP translocates into the nucleus, where it binds to the promoters of SREBP target genes as homodimers [61]. The nuclear or mature SREBP activates transcription by directly interacting with the KIX domain of MED15 subunit of the Mediator complex [208].

There are three SREBP family members in vertebrates, SREBP1c mainly controls the expression of genes encoding lipogenic enzymes, SREBP2 more specifically activates the transcription of genes encoding cholesterogenic enzymes, while SREBP1a can regulate the expression of both lipogenic and cholesterogenic genes [41, 61, 142, 162]. SREBP2 and SREBP1a are not present in invertebrates, and the *Drosophila* SREBP resembles the vertebrate SREBP1c in activating the expression of lipogenic enzymes [162].

The *cdk8* and *cycC* mutant *Drosophila* larvae accumulate significantly higher levels of triglycerides than the control, accompanied with elevated transcription of SREBP target genes, such as *ACC* (encoding acetyl-CoA carboxylase, the rating limiting enzyme for lipogenesis) and *FAS* (encoding the fatty acid synthase, the key enzyme for lipogenesis) [217]. This effect is dependent on SREBP, since knocking down SREBP in *Drosophila* adipocytes rescues the effects of CDK8 or CycC depletion on lipogenic gene expression. These observations can be recapitulated in cultured mammalian cells, and notably, depleting CDK8 in mouse liver resulted in fatty liver and hyperlipidemia [217]. Mechanistically, CDK8 directly phosphorylates SREBP at a Thr residue that is conserved from *Drosophila* to humans, and phosphorylated SREBP are destabilized through the ubiquitin proteasome pathway

[217]. These results have revealed the molecular mechanisms of how SREBP-activated gene expression is subsequently turned off in the nucleus.

It is well documented that insulin stimulates lipogenesis by activating SREBP [39, 41, 61, 156, 180, 182]. The components of insulin signaling and their functions are highly conserved during evolution [45, 59, 60]. Because CDK8-CycC inhibits SREBP-dependent gene expression, the physiological relevance of this regulation was further analyzed using several approaches. First, treating cultured mammalian cells with insulin destabilizes CDK8 and CycC proteins, while ectopic expression of CycC abolishes the effects of insulin in stimulating the accumulation of nuclear SREBP and the expression of SREBP target gene *FASN* [217]. Second, starvation of the early third-instar *Drosophila* larvae, which presumably inactivates insulin signaling, significantly increases the levels of CDK8 protein compared to the control of the same developmental stage. In contrast, when the starved larvae are put back in food, which is expected to activate insulin signaling, the levels of CDK8 protein are reduced in less than one hour of re-feeding [205]. Third, analysis of CDK8 and CycC levels in liver of re-fed mouse revealed that re-feeding reduced the levels of CDK8 and CycC proteins compared to the liver samples from the fasted mouse [217]. These results show that CDK8-CycC is negatively regulated by insulin signaling, allowing SREBP to stimulate the expression of lipogenic genes and lipogenesis (Fig. 8.2).

Although the exact mechanism of how insulin signaling destabilizes CDK8-CycC is still not fully understood, several recent reports suggest that the mTOR plays a key role in this process. The mTOR signaling pathway plays critical roles in sensing nutrients in the environment and responding to growth factors such as insulin [12, 170]. The conserved Ser/Thr kinase mTOR is a shared subunit of two distinct complexes, mTORC1 and mTORC2. First, treating cultured HEK293T cells with insulin can stimulate mTORC1 activity, as evidenced by the phosphorylation of S6K1, S6, and 4E-BP, accompanied with significant reduction of CDK8 and CycC [50]. However, these effects are abolished when mTOR is depleted by shRNA treatment, suggesting that mTOR is required for the destabilizing effects of insulin on CDK8 and CycC [50].

Second, the inhibitory effect of mTOR on CDK8 was also observed in both *Drosophila* and mammalian cells during starvation-induced autophagy and cell metabolism [187]. In this work, the authors identified subunits of the cleavage and polyadenylation (CPA) complex that can genetically modify rough eye phenotype caused by overexpression of *Atg1* in the *Drosophila* eye. During starvation, the CPA complex stimulates autophagosome formation by regulating the length of the 3′-untranslated region and the alternative splicing of *Atg1* (encoding an autophagy-related protein kinase) and *Atg8a* (encoding a ubiquitin-like protein) transcripts thereby increasing the levels of these ATG proteins. Mechanistically, mTORC1 regulates autophagy and cell metabolism by inhibiting the CDK8 and Darkener of apricot (DOA), the two kinases that phosphorylate cleavage and polyadenylation specificity factor 6 (CPSF6), a key component of the CPA complex [187]. During starvation, mTORC1 activity is inhibited, allowing CDK8 and DOA to phosphorylate CPSF6, which stimulates the formation of authophagosomes but inhibits lipid storage and protein synthesis. This mechanism has been validated in mammalian cells upon starvation [187]. These results are consistent with the previous reports showing that CDK8 negatively regulates lipogenesis by destabilizing nuclear SREBP and that CDK8 proteins are destabilized by re-feeding, but stabilized by starvation [205, 217].

Third, the link between mTOR and CDK8 is further strengthened by the observation that the third instar *cdk8* and *cycC* mutant larvae are hypersensitive to the levels of dietary proteins [57]. Interestingly, the *cdk8* and *cycC* mutant larvae do not display the same sensitivity to different amino acids. Instead, increasing the levels of seven specific amino acids (arginine, glutamine, isoleucine, leucine, methionine, threonine, and valine) in the diets appears particularly potent in disrupting the development of the *cdk8* and *cycC*

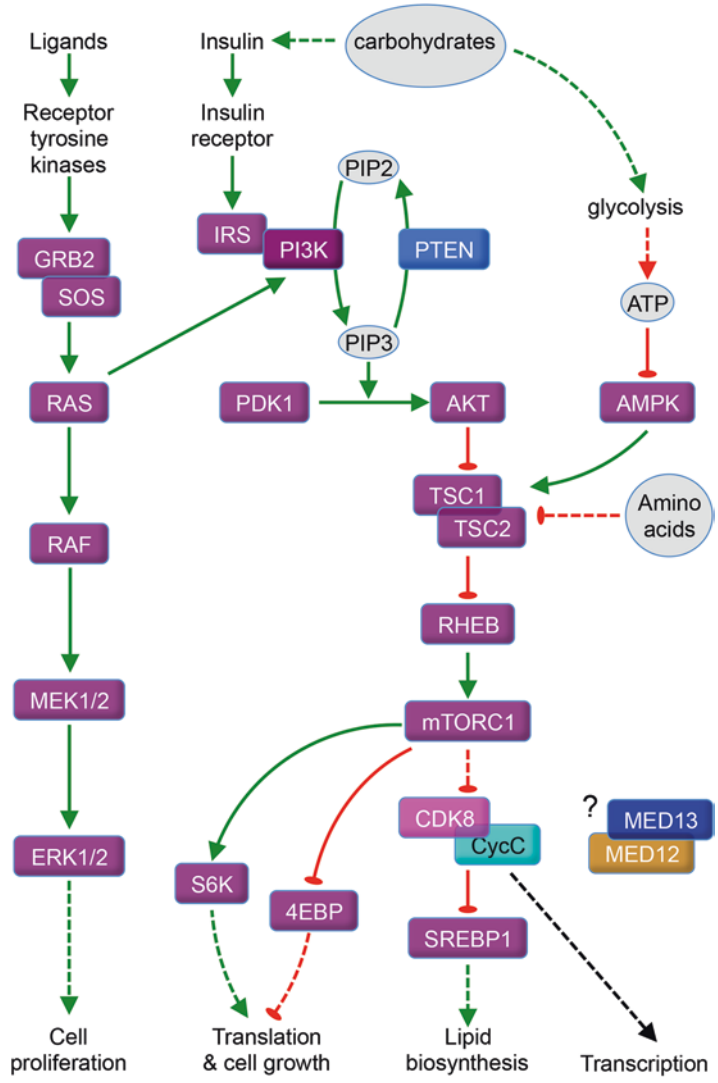

Fig. 8.2 Schematic representation of the RTK signaling pathway and CDK8-CycC

mutants, while other 13 amino acids do not display obvious effects [57]. Numerous studies have shown that branched-chain amino acids (leucine, isoleucine, and valine) and the other four amino acids (arginine, glutamine, methionine, and threonine) can stimulate mTOR activities [51, 56, 98, 114, 138, 170, 202, 212, 213, 215]. The tuberous sclerosis complex (TSC) plays a critical role in integrating signals from PI3K/AKT and the availability of amino acids with the inactivation of TORC1 (Fig. 8.2) [40]. The simplest model to explain these observations is that these amino acids may inhibit the residual maternal CDK8 or CycC in the *cdk8* and *cycC* mutant larvae through mTOR (Fig. 8.2). The *cdk8* and *cycC* mutants are also sensitive to increased dietary sugars, consistent with the idea that CDK8 acts downstream of the insulin/mTOR signaling pathway [57].

Although mTOR is known to stimulate lipid biosynthesis through SREBP1, the mechanisms linking mTOR and SREBP1 are still not fully understood [99, 156, 170]. The observations summarized above suggest that mTOR may stimulate SREBP activities by inhibiting CDK8. In mammals, the mTORC1 and mTORC2 complexes share three subunits, mTOR, DEPTOR, and mLST8. In addition, the mTORC1 complex contains two unique subunits RAPTOR and

PRAS40, while the mTORC2 complex has three unique subunits RICTOR, mSIN1, and PROCTOR. Deleting the mTORC1 subunit *Raptor* abolishes the effects of re-feeding on mTOR activity, as well as the levels of CDK8 and nuclear SREBP1 in mouse liver, suggesting that the mTORC1 complex is required for feeding induced effects on CDK8 and SREBP1 [50]. Although it is unknown whether mTOR inhibits CDK8 by directly phosphorylating it, the Thr31 and Thr196 residues of CDK8 have been speculated as the potential mTORC1 phosphorylation sites [187]. Both CDK8 and CycC are phosphorylated proteins [13, 93], but the kinase(s) that targets them and the biological contexts of such modifications remain unknown. It is also unknown whether MED12 and MED13 are involved in this process. Taken together, the studies summarized above show that insulin/mTOR signaling inhibits CDK8-CycC activities, which allows the mature SREBP1 to activate the expression of lipogenic genes and promote lipid biosynthesis (Fig. 8.1).

8.5 Dysregulation of the CDK8 Module in Endometrial Cancer

CDK8 was initially discovered as the kinase partner of CycC that regulates RNA Pol II-dependent transcription in *Saccharomyces cerevisiae* and *Drosophila* [102, 188]. Thereafter, a majority of the studies on CDK8-CycC have focused on their roles in regulating development and Pol II-dependent transcription in different model systems. The clinical relevance of these studies was shown by the discovery that *CDK8* gene is amplified in nearly half of the colorectal cancer patients, and gain of CDK8 drives tumorigenesis by potentiating Wnt/β-catenin signaling [52, 136]. Mutations of a number of Mediator subunits, particularly the CDK8 module, have been observed in cancer and cardiovascular diseases, as reviewed previously [30, 36, 173, 174, 207]. Here we survey the literature and focus on evidence for aberrant functions of the CDK8 module in two major types of uterine tumors, the malignant endometrial cancer and the benign uterine leiomyomas. Mutations of the *MED12* gene in uterine smooth muscle tumors have also been summarized a few years ago [34].

Epidemiological studies have identified obesity as an important risk factor for uterine cancer (Table 8.1), and our previous work has revealed that CDK8 represses lipid biosynthesis by inhibiting SREBP activity [217]. Thus, we started to explore the role of CDK8 in endometrial cancer. Specifically, we examined whether CDK8-CycC regulates endometrial cancer cell growth using cultured endometrial cancer cell lines, including KLE, which express low levels of CDK8, as well as AN3 CA and HEC-1A cells, which have high levels of endogenous CDK8 [64]. Ectopic expression of CDK8 in KLE cells inhibited cell proliferation and potently blocked tumor growth in an *in vivo* mouse model. Gain of CDK8 in KLE cells blocked cell migration and invasion in transwell, wound healing, and persistence of migratory directionality assays. Conversely, we observed the opposite effects in all of the aforementioned assays when CDK8 was depleted in AN3 CA and HEC-1A cells [64]. These results show a reverse correlation between CDK8 levels and several key features of the endometrial cancer cells, including cell proliferation, migration and invasion, as well as tumor formation *in vivo*. Although gain of CDK8 activity is oncogenic in melanoma and colorectal cancers [52, 89, 136], the observations based on cultured endometrial cancer cells and nude mouse models suggest that CDK8 plays a tumor suppressive role in endometrial cancer cells, and disprove the idea that gain of CDK8 plays a general oncogenic function in different type of cancers.

The notion that the CDK8 module has tumor suppressive functions in uterine tumors is further strengthened by the data summarized below. For example, a recent report analyzed the expression of CDK8 and MED12 in extrauterine leiomyosarcomas, a type of uterine sarcoma derived from the extrauterine smooth muscle [210]. Immunohistochemical analyses have revealed a prevalent loss of CDK8 expression in leiomyosarcomas, suggesting that the level of CDK8 could serve as a predictive parameter for leio-

myosarcomas [210]. Aside from these reports, there is a paucity of data available on status of CDK8 and other subunits of the CDK8 module in endometrial cancer or endometrial carcinoma.

Although the subunits of the CDK8 module may not be mutated in endometrial cancer patients, downregulation of the CDK8 activity can be caused by mutations encoding the upstream regulators of the CDK8 module. For example, approximately 85% of newly diagnosed cases of endometrial carcinoma are classified by histology as endometrioid carcinoma, or type I endometrial cancer [15]. Loss of *PTEN* (encoding Phosphatase and Tensin homolog) mutations has been identified as the most frequent somatic mutations in endometrioid carcinoma, occurring in 70–80% of cases [27, 125]. PTEN coverts phosphatidylinositol-3,4,5-trisphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2), thereby negatively regulating the PI3K-AKT pathway (Fig. 8.2). Besides *PTEN*, high-freuency mutations of *PIK3CA* (encoding the catalytic subunit p110α of PI3K), *PIK3R1* (encoding the regulatory subunit p85α of PI3K), and *KRAS* (encoding Kirsten rat sarcoma viral oncogene homolog) have also been identified in endometrial carcinoma [27]. Importantly, mutations in these genes cause the aberrant activation of the AKT kinase, which activates mTORC1 and mTORC2 through the TSC2 tumor suppressor and Rheb in endometrial carcinomas [15]. In addition, loss of *TSC2* and *LKB1* expression has been found in 13% and 21%, respectively, of primary endometrial carcinomas, correlating with activation of mTOR [111]. The critical roles of PI3K and PTEN in driving endometrial carcinoma have been demonstrated in mouse models [84, 153]. These findings suggest that aberrant activation of the PI3K-PTEN-AKT-mTOR pathway plays a major role in promoting endometrial carcinomas [15].

Besides these somatic mutations, the PI3K-PTEN-AKT-mTOR pathway can also be hyperactivated by elevated upstream regulators, chiefly receptor tyrosine kinases (RTKs) such as insulin receptor (InR), epidermal growth factor receptor (EGFR), and vascular endothelial growth factor receptor (VGFR) (Fig. 8.2). Obesity is often accompanied with insulin resistance and hyperinsulinemia [25, 62, 79]. Moreover, higher levels of insulin correlate with increased risk for endometrial carcinoma [48, 49]. Expression of EGFR and VGFR-C in endometrial carcinoma was associated to the stage, differentiation degree, myometrial invasion depth, and lymphatic metastasis [24]. Therefore, inappropriate activation of the RTK signaling pathway by either elevated ligands such as insulin or loss of function mutations in genes such as *PTEN* can lead to aberrant activation of mTOR, which drives endometrial carcinoma.

Because of hyperactive RTK signaling, particularly the PI3K-PTEN-AKT-mTOR pathway, in endometrial carcinomas, a number of inhibitors that target RTKs, mTOR, PI3K, AKT, or both mTOR and PI3K have been tested in preclinical trials to treat endometrial cancer [15, 46]. Given that mTOR destabilizes CDK8-CycC, our model predicts that CDK8-CycC is downregulated in the majority of endometrial carcinomas, while inhibitors that targeting the PI3K-PTEN-AKT-mTOR pathway can increase the levels of CDK8 and CycC (Fig. 8.2). To test these predictions, it will be necessary to determine whether the levels of CDK8 and CycC correlate with those somatic mutations and mTOR activities in samples from patients with endometrial carcinoma.

8.6 Dysregulation of the CDK8 Module in Uterine Leiomyomas

Related to the links between dysregulation of the CDK8 module and tumorigenesis, one of the major breakthroughs in the past decade is the identification of somatic *MED12* mutations in exon 1 and exon 2, particularly the highly conserved codon 44 in exon 2, in nearly 70% of uterine leiomyomas by the Lauri Aaltonen laboratory [119]. The prevalence of *MED12* mutations, either missense changes or in-frame indels, in uterine leiomyomas has been subsequently confirmed by many studies across countries, including Australia [80], Austria [108], Brazil [108,

131], China [199, 204, 214], Finland [71, 72, 118, 128], France [150], Germany [121], Japan [123], Iran [171, 179], Italy [42], Netherlands [38], Rassia [144], Saudi Arabia [2], South Africa [116], South Korea [81, 104], Spain [55], and the United States [16, 67, 69, 127, 145, 161, 175, 209]. As summarized in Table 8.2, *MED12* gene is mutated in approximately 62% of 3,445 uterine leiomyoma samples that were analyzed, regardless of the race or ethnicity of the patient.

In addition to *MED12* mutations, several recurrent mutations have been revealed in uterine leiomyomas, including rearrangements of high mobility group AT-hook 1 and 2 (*HMGA1* and *HMGA2*), biallelic inactivation of fumarate hydratase (*FH*), and deletions in collagen type IV α5 and type IV α6 (*COL4A5-COL4A6*) [128–130]. Mutations of these genes occur in a mutually exclusive manner, with the *MED12* mutations representing the most frequent genetic alteration in uterine leiomyomas [119, 128–130]. Moreover, somatic *MED12* mutations were also identified in nearly 15% of 218 uterine leiomyosarcomas samples analyzed (Table 8.3) [16, 38, 87, 108, 115, 118, 123, 150, 214]. Furthermore, *MED12* mutations were also found in ovarian and other adnexal leiomyomas [106, 107], leiomyomas with bizarre nuclei [108], and uterine intravenous leiomyomatosis [22]. These findings suggest that dysregulated MED12, thereby the functions of the CDK8 module (see below), may play important roles in the pathogenesis of uterine leiomyomas and other types of uterine tumors.

Besides these uterine tumors, rare *MED12* mutations have also been identified in ~6% of patients who suffer from chronic lymphocytic leukemia (CLL) [85, 203], prostate cancer [85, 86], breast fibroadenomas and phyllodes tumors [109, 137, 186], and colorectal cancer patients [81, 87, 181], but the mutation rates are much lower in these cancers compared to uterine leiomyoma.

These point mutations appear to be unique to MED12, as no mutations of genes encoding other subunits of the CDK8 module (such as *CDK8*, *CDK19*, *CCNC*, *MED12L*, *MED13*, and *MED13L*) in uterine leiomyomas have been identified [117, 204]. However, most of the aforementioned studies have only analyzed the *MED12* mutations in uterine leiomyomas after the initial breakthrough by Makinen et al. [119]. Compared to the extensive analyses of *MED12* mutations in clinical samples of uterine tumors (Table 8.2), little is known about potential mutations of other subunits of the CDK8 module in uterine tumors. Nevertheless, in uterine leiomyomas that do not harbor *MED12* mutations, gene copy number loss was detected in genomic regions uncovering genes encoding the Mediator subunits such as *CDK8*, *MED8*, *MED18*, and *MED15* [209]. In addition, rare cases of *MED12L* deletions have been identified in uterine leiomyomas [75]. These discoveries suggest that compromised functions of the Mediator complex, particularly those of the CDK8 module, may have key influence on pathogenesis of uterine leiomyomas.

8.7 *MED12* Mutations and CDK8 Activities in Uterine Leiomyomas

Mutations idenfitied in tumor samples can be either "driver" mutations that confer a fitness advantage essential for tumor formation and survival, or "passenger" mutations that are not essential for tumorigenesis [9, 154]. It is thus critical to determine the functional relevance of the *MED12* mutations identified in uterine leiomyoma samples. These *MED12* mutations have been proposed to cause "oncogenic stress" and upregulation of the expression of *RAD51B*, which encodes RAD51 paralog B, a key player in DNA repair and homologous recombination, thereby contributing to the development of uterine leiomyoma [128, 129].

The critical role of *MED12* missense mutation in exon 2 in promoting the formation of uterine leiomyomas has been elegantly demonstrated using a mouse model [133]. Conditional expression of a *Med12* missense variant (c.131G > A, which causes the p.Gly44Asp mutation as in many uterine leiomyomas) in either the wild-type or *Med12* heterozygous mouse uterus was sufficient to drive chromosomal rearrangements and genomic instability, thereby promoting uter-

Table 8.2 The prevalence of *MED12* mutations in uterine leiomyomas across countries

Author and year of publication	The nationality of the patients	Number of patients	Number of samples	# with *MED12* mutations	% with *MED12* mutations
Mäkinen et al., 2011	Finland	80	225	159	70.7
Mäkinen et al., 2011	South Africa	18	28	14	50.0
Je et al., 2012	South Korea	53	67	35	52.2
Perot et al., 2012	France	NA	9	6	66.7
McGuire et al., 2012	USA	NA	148	100	67.6
Markowski et al., 2012	Germany	50	80	47	58.8
Matsubara et al., 2013	Japan	NA	55	39	70.9
Ravegnini et al., 2013	USA	NA	19	3	15.8
de Graaff et al., 2013	Netherlands	NA	19	11	57.9
Heinonen et al., 2014	Finland	28	164	138	84.1
Bertsch et al., 2014	USA	134	178	133	74.7
Schwetye et al., 2014	USA	NA	28	15	53.6
Di Tommaso et al., 2014	Italy	NA	36	12	33.3
Zhang et al., 2014	China	NA	40	30	75.0
Halder et al., 2015	USA	135	143	92	64.3
Shahbazi, et al., 2015	Iran	NA	23	11	47.8
Wang et al., 2015	China	NA	181	93	51.4
Sadeghi et al., 2016	Iran	NA	103	32	31.1
Mehine et al., 2016	Finland	NA	94	34	36.2
Osinovskaya et al., 2016	Rassia	NA	122	63	51.6
Liegl-Atzwanger et al., 2016	Austria	15	20	9	45.0
Wu et al., 2017	China	NA	362	158	43.6
Heinonen et al., 2017	Finland	244	763	599	78.5
Mäkinen et al., 2017	Finland	NA	65	37	56.9
Lee et al., 2018	South Korea	NA	60	40	66.7
Mello et al., 2018	Brazil	56	69	34	49.3
Galindo et al., 2018	Spain	NA	20	15	75.0
Jamaluddin et al., 2018	Australia	14	65	39	60.0
Hayden et al., 2018	USA	NA	40	30	75.0
Park et al., 2018	USA	76	219	121	55.3
Total		**903**	**3,445**	**2,149**	**62.4**

Note: Case reports based on less than 5 cases or samples were not included in this Table
NA: Data not available

ine leiomyoma formation and hyperplasia [133]. However, conditional removal of the entire *Med12* locus in uterine myometrial cells did not cause leiomyoma formation or hyperplasia in adult uteri. These observations suggest that the *MED12* missense mutation in codon 44 is a dominant or gain-of-function mutation that drives genomic instability [133]. It is unclear whether the effect of the *MED12* exon 2 mutations on genomic instability is dependent on elevated *RAD51B* transcription and how wild-type and mutated MED12 proteins regulate the expression of *RAD51B* gene.

A key breakthrough in our understanding of these *MED12* mutations in exon 2 is the demonstration that these MED12 mutant proteins specifically disrupt the direct interactions between MED12 and CycC [88, 146, 192]. The cryo-EM

Table 8.3 Somatic *MED12* mutations identified in uterine leiomyosarcomas

Author and year of publication	The nationality of the patients	Number of samples	# with *MED12* mutations	% with *MED12* mutations
Perot et al., 2012	France	10	2	20.0
Kampjarvi et al., 2012	Finland	41	3	7.3
Matsubara et al., 2013	Japan	12	2	16.7
de Graaff et al., 2013	Netherlands	7	1	14.3
Zhang et al., 2014	China	38	4	10.5
Bertsch et al., 2014	USA	32	3	9.4
Liegl-Atzwanger et al., 2016	Austria	8	2	25.0
Mäkinen et al., 2016	Finland	19	4	21.1
Mäkinen et al., 2017	Finland	51	11	21.6
Total		**218**	**32**	**14.7**

structure of the CDK8 module in *Saccharomyces cerevisiae* have revealed that Med13 and Cdk8 do not directly interact with each other, they are localized at the opposite ends of the CDK8 module and interact through Med12 and CycC (Fig. 8.1, inset) [191]. This subunit organization of the CDK8 module is likely conserved from yeasts to humans, considering the conservation of these four subunits in eukaryotes [19]. CycC directly interacts with the N-terminus of MED12 [145]. Consistent with the notion that MED12 is required for stimulation of the kinase activity of the CDK8-CycC complex, disrupting the interaction between MED12 and CycC reduces CDK8 kinase activities [93, 145, 192]. Interestingly, a nonsense mutation in *MED12* (c.97G > T, p. E33X), identified in acute lymphoblastic leukemia, dirsupts nuclear localization signal of MED12, resulting in mislocalised MED12 in cytoplasm [70]. Considering that all subunits of the CDK8 module are predominantly localized in nucleus, these mislocalized MED12 mutant proteins are also expected to reduce CDK8 kinase activities in the leiomyomas tissues. Therefore, we would expect that CDK8 also play a tumor suppressive role in leiomyomas, similar to endometrial cancer cells [64].

One puzzling observation that cannot be easily reconciled with this model is that conditional deletion of the *MED12* locus in uterine myometrial cells did not lead to leiomyoma formation in adult mice [133]. One possibility is the presence of MED12L, which may compensate the complete loss of MED12, but may not be capable of doing so in MED12 mutants because the MED12 missense mutant proteins are expected to retain the ability of binding MED13 or MED13L. Consistent with this idea, depleting MED12 reduces the expression of WNT4 in cultured uterine fibroid cells, but elevated expression of WNT4 was observed in MED12 missense mutants [4, 121]. In addition, given that the stabilities of the subunits of the CDK8 module could be interdependent, complete removal of MED12 may have different impacts on other subunits compared to the MED12 missense mutant proteins.

In *Drosophila*, we have observed asymmetric interdependencies in the stabilities of the four subunits of the CDK8 module. The stability of CycC is dependent on CDK8 but not vice versa [205]; the stabilities of MED12 and MED13 are interdependent but not affected by the levels of CDK8 or CycC (X. Li and J.Y. Ji, unpublished observations). In mammalian cells, the stability of CDK8 and CycC are interdependent [217]. This relationship has not been systematically analyzed with all other subunits of the CDK8 module, but it is likely more complex than in *Drosophila* considering the presence of paralogous proteins (CDK19, MED12L, and MED13L). Thus, it is possible that MED12 missense mutant proteins gain the abilities in promoting hyperplasia and leiomyoma formation, but they cause loss or reduction of the CDK8 kinase activity at the molecular level. Analyzing the CDK8 activities, the kinetics among the subunits of the CDK8 module, and gene expression profiles of uterine

myometrial cells with complete deletion of *MED12* and comparing those aspects with the *MED12* missense mutants may reveal the exact nature of the dominant effects of these MED12 missense mutants.

8.8 The Vicious Cycle of Chronic Downregulation of CDK8 by Obesity

Based on findings summarized above, we propose the hypothesis that chronic downregulation of the CDK8 module by the hyperactive insulin/IGF signaling pathway in obese women will increase the chances to dysregulate the transcriptional activities of different transcription factors, thereby increasing the risk for and promoting the progression of uterine tumors.

The key steps of this hypothesis are illustrated in Fig. 8.1: (1) overeating, lack of exercise, or both are expected to increase lipogenesis, resulting in excessive of fat accumulation over time; (2) obese women have higher obesogenic factors including insulin and IGFs in the circulation than women with normal weight; (3) Insulin and other growth factors activate RTK signaling, particularly the PI3K-PTEN-AKT-mTOR pathway, which downregulates CDK8 and CycC (Fig. 8.2); (4) CDK8-CycC directly inhibits lipogenesis by destabilizing SREBP1, thus reduced CDK8 by obesity is expected to further increase lipogenesis and fat accumulation. Importantly, these processes form a feed-forward mechanism that stimulates fat accumulation, which exacerbates the overall disease state of obesity. The key problem is that over time, this vicious cycle is expected to cause a chronic downregulation of CDK8-CycC in obese women. (5) Given that the CDK8 module plays important roles in regulating the activities of different transactivators, downregulation of CDK8 increases the chances of dysregulating the expression of their target genes, thereby increasing the risk of endometrial cancer (Fig. 8.1). Several key transactivators pertinent to uterine tumors are further discussed below.

In this model, the CDK8 module serves as the central regulatory node linking hormonal factors such as insulin and other growth factors that can activate the PI3K-PTEN-AKT-mTOR pathway, lipid biosynthesis through SREBP1, and other tissues-specific transactivators downstream of different signaling pathways in the uterus. Inhibition of CDK8 by aberrant activation of mTOR and RTK signaling seems to be a key event in endometrial cancer, while the somatic mutations of *MED12* represent a major defect in uterine leiomyoma. However, the net effects of either CDK8 inhibition or MED12 mutations on the CDK8 module are similar, consistent with the idea that the CDK8 module functions as a tumor suppressor in uterine tumors in general. Nevertheless, it creates a puzzle as to why the evolution of these two major types of uterine tumors would preferentially impinge on two different subunits of the CDK8 module.

Perhaps the answer lies within the different cellular properties and origins of these two types of uterine tumors. Endometrial cancer cells are mainly derived from endometrial epithelial cells, which undergo monthly growth and shedding cycles before menopause. Cells of uterine leiomyoma are originated from uterine smooth muscle cells, which are normally quiescent. Dysregulated functions of the CDK8 module may have different impacts on signaling pathways and transcriptional factors that are active in a tissue-specific manner. We discuss several transcription factors that are regulated by CDK8 and their potential impacts on initiation and progression of the uterine tumors in the next section.

It is also noteworthy that uterine leiomyomas with *MED12* missense mutations express significantly higher levels of *IGF-2* gene [42]. Consistently, IGF1 and IGF2 are required to promote the growth of uterine leiomyomas with *MED12* missense mutations [177]. This autocrine effect caused by *MED12* missense mutations is also consistent with the notion that elevated levels of insulin or IGFs correlate with increased risk for uterine cancer (Fig. 8.1) [65, 141, 164, 201].

8.9 Multiple Transcription Factors Pertinent to Uterine Tumors that Could Be Affected by Chronic Downregulation of CDK8 in Obese Women

Activation of RTK signaling is primarily relayed through the PI3K-PTEN-AKT-mTOR and RAS-RAF-MEK-ERK signaling pathways [15]. Besides the prominent roles of the RTK signaling pathway, the Wnt/β-catenin pathway and the transforming growth factor β (TGFβ) signaling pathway are also dysregulated in uterine tumors [15, 46]. Unlike the RTK signaling that functions upstream of CDK8 through mTOR, the CDK8 module may regulate the activities of Wnt/β--catenin and TGFβ signaling through specific downstream transcription factors of these signaling pathways (Fig. 8.1). Here we further discuss how chronic downregulation of the CDK8 module by obesity may impact the activities of a few transcription factors and cofactors pertinent to uterine tumors, including SMADs, p53, T-cell factor/lymphoid enhancing factor (TCF/LEF) family transcription factors and their cofactor β-catenin, and estrogen receptor (ER).

SMADs SMAD proteins (homologs of the *Drosophila* protein Mad, or mothers against Dpp) comprise a family of transcription factors act downstream of the TGFβ signaling pathway [37]. As ligands, the 32 members of TGFβ superfamily can be mainly classified as the TGFβ subfamily and the BMP subfamily. Receptor-regulated SMAD proteins (R-SMADs, including SMAD1/2/3/5/8 in mammals) and common partner SMADs (co-SMADs, SMAD4 in mammals or Medea in *Drosophila*) are characterized with a conserved amino-terminal MH1 (Mad homology 1) domain that binds to DNA and a carboxyl-terminal MH2 (Mad homology 2) domain that harbors the transactivation activity, separated by a serine- and proline-rich linker region [37, 157]. Receptor phosphorylated R-SMADs form heterodimer with the co-SMAD to modulate the expression of target genes in the nucleus. The MED15/ARC105 subunit of the Mediator complex directly interacts with the MH2 domain of Smad2/3 in *Xenopus*, which is required for the SMAD2/3-SMAD4-dependent transcription [90]. In *Drosophila*, MED15 is also required for the expression of genes regulated by the TGFβ homolog Decapentaplegic (Dpp) [106, 189]. CDK8 and a few additional Ser/Thr protein kinases, such as CDK7, CDK9, glycogen synthase kinase-3β (GSK3β), and mitogen-activated protein kinases (MAPKs), have been reported to phosphorylate the flexible linker region of R-SMADs in mammalian cells [37, 206]. It has been proposed that phosphorylation in the linker region by CDK8 and CDK9 not only facilitates the recruitment of additional cofactors, but also primes the R-SMADs for further phosphorylation by GSK3β and subsequent proteasome-dependent degradation [37]. Because there are multiple Ser/Thr residues within the linker region, it has been challenging to decipher exactly which site(s) is phosphorylated by each of these kinases *in vivo*.

In *Drosophila*, Dpp and its primary downstream transcription factor Mad control diverse biological processes such as patterning and growth during organ formation [166, 193]. CDK8 and Shaggy (the *Drosophila* homolog of GSK3β) can also phosphorylate three Ser residues in the Mad linker region. Depleting CDK8 in S2 cells reduced the phosphorylation level of Ser212 within the linker region, while knocking down Shaggy decreased the phosphorylation level of Ser204 and Ser208 residues [6]. In addition, depleting Shaggy increased the peak intensity and range of the BMP signal in *Drosophila* embryos and wing imaginal discs [6]. These observations show important roles of Mad linker phosphorylation by CDK8 and Shaggy in BMP/Dpp signaling. We have observed that CDK8 and CycC genetically interact with the components of the Dpp signaling pathway in *Drosophila*. CDK8 directly interacts with the linker region of Mad protein; CDK8, CDK9, and multiple Mediator subunits, including CycC, Med12, Med13, Med15, Med23, Med24, and Med31, are required for Mad-dependent transcription in wing discs [106]. However, depleting CDK7, MAPKs, or

Shaggy did not obviously affect the expression level of a Mad target gene *spalt* in wing discs [106]. These results are consistent to a conserved role of CDK8 and CDK9 in stimulating SMAD-dependent gene expression [5], suggesting that the Mediator complex is a critical cofactor required for the expression of TGFβ and BMP target genes in the nucleus.

During tumorigenesis, TGFβ signaling can function as either a tumor suppressor in premalignant cells or a tumor promoter in malignant cells, depending on the stage and cellular contexts [122]. Dysregulated expression of TGFβ isoforms and mutations of the components of the TGFβ signaling pathway have been observed in endometrial cancer cells, and attenuated TGFβ signaling seems to be important for endometrial tumorigenesis [46]. Recently, the tumor suppressive role of TGFβ signaling in uterine cancer was unambiguously demonstrated using conditional knockout mouse models by the Matzuk laboratory [96, 134]. Conditional deletion of *Smad2* and *Smad3* in mouse uterus using progesterone receptor-cre (*Pgr-cre*, which is expressed in uterine muscle, stroma, and epithelium) led to uterine hyperplasia and increased cell proliferation [96]. Similarly, conditional ablation of *Alk5* (encoding activin receptor-like kinase 5) using *Pgr-cre* caused metastatic endometrial cancers with metastases to the lungs [134]. These results, along with the observation that concurrent deletion of TGFβ receptor 1 (*Tgfbr1*) and *Pten* using *Pgr-cre* promotes endometrial cancer formation and pulmonary metastasis [58], demonstrate tumor suppressive roles of TGFβ signaling in the mammalian uterus [105].

Given that CDK8 positively regulates SMAD-activated transcription, our model predict that chronic downregulation of CDK8 by obesity or insulin/mTOR signaling would reduce the expression of SMAD target genes, thereby compromising the tumor suppressive functions of TGFβ signaling in uterus (Fig. 8.1).

P53 In response to genotoxic stresses such as DNA damage induced by ultraviolet light and ionizing irradiation, the transcription factor p53 activates the expression of factors involved in cell-cycle arrest, apoptosis, or senescence. P53 has two transactivation domains (TADs) that can directly interact with a number of transcription cofactors that regulate different steps of transcription [158]. The p53 TAD at the N-terminus directly interacts with MED17, while its C-terminus TAD interacts with MED1; these different interactions induce distinct structural shifts within the Mediator complex, thereby activating transcription elongation [78, 132]. CDK8 positively regulates the expression of p53 target genes such as *p21* and *Hdm2* when p53 is activated by nutlin-3 [43]. Recruitment of three subunits of the CDK8 module (CDK8, CycC, and MED12) to *p21* promoter correlates with strong *p21* transcriptional activation [43]. Depleting CDK19 also reduced the effects of nutlin-3 induced *p21* expression, yet this effect of CDK19 is independent of its kinase activity [10]. These results indicate that the role of the CDK19 module is also involved in regulating *p21* transcription by p53.

In the context of endometrial cancers, mutations causing p53 inactivation are found in over 85% of serous carcinoma, 31–50% of clear cell carcinomas, and approximately 50% of high-grade endometrioid carcinomas [15, 101]. Inactivation of p53 drives the tumorigenesis of these types of endometrial cancers, which account for approximately15% of newly diagnosed cases of endometrial cancers [15]. It is unknown whether CDK19 is also downregulated by insulin/IGF and mTOR signaling. Given the positive role of CDK8 in regulating p53-dependent gene expression, we expect that chronic downregulation of CDK8 in obese women may compromise the transactivation activity of p53, thereby favoring tumorigenesis of the uterus (Fig. 8.1).

TCF/LEF Family Transcription Factors and β-Catenin Similar to TGFβ signaling, the Wnt signaling pathway also plays critical roles in regulating normal development and diseases [140]. The TCF/LEF family transcription factors and their cofactor β-catenin act downstream of the Wnt signaling pathway to control the expression

of the Wnt target genes [23, 194]. A number of mutations, including gain-of-function mutations in *CTNNB1* (encoding β-catenin) and loss of function mutations in negative regulators of Wnt signaling such as *GSK3β*, *RNF43*, *Sox7*, and *SOX17*, are often observed in endometrial cancers ([15]; [46]). These mutations result in constitutive activation of Wnt signaling [15, 46], suggesting oncogenic roles of Wnt signaling in endometrial cancer. Consistent with this notion, elevated Wnt activities are also observed in uterine leiomyomas [33]. In addition, uterine leiomyomas with *MED12* mutations display increased expression of WNT4, indicating that the mutated MED12 may cooperate with elevated Wnt activities in promoting leiomyoma [121]. However, knocking down MED12 in the immortalized human uterine fibroid cells lead to reduced expression of WNT4 and β-catenin activities [4]. As discussed earlier, one likely explanation for these observations is that the MED12 missense mutant proteins have a dominant negative effect on WNT4 expression.

The oncogenic role of CDK8 in colorectal cancer is mainly based on the following observations: *CDK8* gene is amplified in nearly half of the colorectal cancers, ectopic expression of CDK8 can transform immortal murine cells, and CDK8 positively regulates β-catenin activated gene expression and colon cancer cell proliferation [52]. However, conditional deleting *CDK8* in the murine Apc^{Min} intestinal tumor model significantly increased intestinal tumor size and growth rate, and this increased tumor burden also correlated to the shortened survival of the mice [124]. These observations suggest that CDK8 may act as a tumor suppressor in early stages of intestinal tumorigenesis, but functions as an oncogene in invasive colorectal cancers through β-catenin [36]. Thus, it is critical to consider the pathological contexts when evaluating the consequence of CDK8 dysregulation.

In *C. elegans*, the MED12 and MED13 homologs repress the expression of Wnt target genes [211]; while in *Drosophila* and mammals, these two subunits of the CDK8 module are required to activate the transcription of Wnt/β-catenin targets [28, 92]. The *Drosophila* Med12 and Med13 homologs can directly interact with Pygopus, a transcription cofactor that directly binds to Armadillo (β-catenin ortholog in *Drosophila*) [28]. In addition, MED12 can also directly interact with carboxyl-terminal domain of β-catenin [92]. Depleting MED12 or MED13 in heart and muscle increased fat accumulation in adipocytes of adult flies, and the crosstalk between muscle and fat body is mediated by Wingless (Wnt in *Drosophila*) [103]. Furthermore, CDK8 and CycC may indirectly stimulate β-catenin activities via E2F1 in flies and mammals [136]. These results suggest that the CDK8 module can modulate Wnt/β-catenin signaling through multiple mechanisms. Chronic downregulation of CDK8 or the CDK8 module may increase the chances to dysregulate Wnt/β-catenin signaling in the uterus.

Estrogen Receptor By directly regulating gene expression, steroid hormones and their nuclear receptors play critical roles in a wide variety of physiological processes during metazoan development. The Mediator complex serves as a transcription cofactor for a variety of nuclear hormone receptors [14]. In *Drosophila*, CDK8 plays a positive role in regulating ecdysone receptor (EcR), the major steroid hormone receptor that controls developmental transitions in insects and other arthropods. CDK8 directly interacts with EcR-AF2, and loss of CDK8 or CycC cause developmental defects that are reminiscent of *EcR* mutants [205]. Interestingly, the LXXLL motifs in MED1 are only present in vertebrates; instead, CDK8 has a LXXLL motif that is conserved from yeasts to human [205]. The LXXLL motif of CDK8 is required for CDK8 to regulate EcR and the timing for the larval-pupal transition (our unpublished observations). In mammals, estrogens and ERs play central roles in the development, physiology, and pathology of female organs such as the breast, uterus, and ovaries [68, 196]. ERα can directly interact with MED1 subunit of the Mediator complex, likely through interactions between the LXXLL motifs in MED1 and ligand-dependent AF2 (activation function) domain of ERα [14, 83, 200]. Inhibition of CDK8 and CDK19, or loss of CDK8, abol-

ishes the effects of estrogen in stimulating the expression of ER target genes, suggesting that CDK8 plays a positive role in regulating ERα-dependent gene expression [126]. Although exactly how MED1 and CDK8 coordinately stimulate ER-dependent transcription and the role of the LXXLL motif in this process are still unclear, these observations prompted the idea of inhibiting CDK8 and CDK19 to treat cancers in which estrogen and ER play prominent roles in promoting tumorigenesis, such as ER-positive breast cancer [35, 126].

If CDK8 is downregulated in the uterine tissues of obese women, then ER activity would be reduced considering the positive role of CDK8 on ERα. This appears to be contradictory to the notion that prolonged estrogen action and ER activity increase the risk for endometrial hyperplasia and endometrial cancer [91]. One likely explanation is that chronic downregulation of CDK8 increases the chances to dysregulate a number of transcription factors, the net effect of which may increase the risk for tumorigenesis. In this section, we have discussed the potential impacts of CDK8 downregulation on TGFβ/Smad signaling, p53 activity, Wnt/β-catenin signaling, and ER activity, but additional transactivators that may also be affected by reduced CDK8 in different tissues of the uterus, include E2F1, Notch, SREBP1c, STAT1, and other nuclear hormone receptors such as progesterone receptor (PR) and vitamin D receptor (VDR). Like estrogen and ER, progesterone and PR also play critical roles in pathogenesis of uterine leiomyomas [15, 135], but it remains unexplored whether the CDK8 module is involved in PR-regulated transcription. In addition, Vitamin D can antagonize with Wnt/β-catenin activity and inhibits the proliferation of cultured human uterine leiomyoma primary cells [33]; VDR can interact with multiple Mediator subunits, although the role of the CDK8 module on VDR-dependent gene expression is also not clear [77]. Therefore, the scenario of chronic downregulation of CDK8 by obesity does not necessarily require favorable impacts of all the transactivators on tumorigenesis; instead, the net impact is determined by the dynamics of these dysregulated interactions.

8.10 Conclusions and Future Perspectives

In this essay, we have summarized discoveries leading us to propose that chronic downregulation of the CDK8 module in obese women may increase the chances to dysregulate the activities of transcription factors regulated by CDK8, thereby increasing the risks for uterine tumors. In this model, the CDK8 module serves as a regulatory node linking genetic and pathophysiological perturbations with dysregulated gene expression in different tissues, resulting in initiation and progression of endometrial cancer and uterine leiomyoma. Such a model may provide a unified view of how obesity is so closely associated to uterine tumors.

Because of the complex factors involved in obesity and pathogenesis of the uterine tumors, no single model is capable of answering all questions related to how obesity increases the risk of uterine tumors. Nevertheless, the answers to this question require probabilistic, rather than deterministic, models. Therefore, it is hoped that our model, which considers the stochastic factors and offers several testable predictions, will advance the understanding of these complex diseases by complementing the existing models and stimulating rigorous assessment of these ideas in uterine tumors and other types of human cancers in the future.

In addition to uterine tumors, obesity is also a risk factor for other types of cancers and diseases such as diabetes, cardiovascular and neurodegenerative diseases [8, 20, 152]. We favor the idea that this model may also be applicable to other obesity-related diseases, and it is essential to understand how downregulation of CDK8 may impact the tissue-specific signaling and the specific pathological contexts.

The identification of oncogenic effects of CDK8 amplification or overexpression in melanoma and colorectal cancers had fueled the interest in developing CDK8-specific inhibitors in

treating cancers [143, 151, 169]. However, observations discussed in this essay suggest tumor suppressive functions of the CDK8 module in uterine tumors, and potentially in other types of tumors as well. To avoid unintended consequences, cautious considerations are clearly required for clinical applications of these CDK8-specific inhibitors in the future. There is an unmet need to gain more contextual information about the function and regulation of the CDK8 module *in vivo*. We expect that studies using model organisms such as *Drosophila* will continue to offer fundamental insights into the complex gene-environment interactions, which will be directly or indirectly applicable to human physiology and medicine.

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MicroRNAs in *Drosophila* Cancer Models

9

Moritz Sander and Héctor Herranz

Abstract

MiRNAs are post-transcriptional regulators of gene expression which have been implicated in virtually all biological processes. MiRNAs are frequently dysregulated in human cancers. However, the functional consequences of aberrant miRNA levels are not well understood. *Drosophila* is emerging as an important *in vivo* tumor model, especially in the identification of novel cancer genes. Here, we review *Drosophila* studies which functionally dissect the roles of miRNAs in tumorigenesis. Ultimately, these advances help to understand the implications of miRNA dysregulation in human cancers.

Keywords

Drosophila · Cancer · Animal models · miRNAs · Oncogenic cooperation · *Bantam* · *let-7* · *miR-7* · *miR-8*

Abbreviations

Ago-1	Argonaute-1
Brat	Brain tumor
CSC	Cancer stem cell
Dcr-1	Dicer-1
Dl	Delta
Dpp	Decapentaplegic
EGFR	Epidermal growth factor receptor
GSC	Germline stem cell
JAK/STAT	Janus kinase/Signal transducer and activator of transcription proteins
let-7	lethal-7
Lgl	Lethal giant larvae
Pnut	Peanut
RNAi	RNA interference
Scrib	Scribbled
Socs36E	Suppressor of cytokine signaling at 36E
YAP	Yes associated protein
Yki	Yorkie

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9.1 Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that repress gene expression by regulating the stability and translation of target messenger RNAs (mRNAs) [1]. Approximately 1% of genes in different organisms encode for miRNAs. However, in mammals, more than 60% of mRNAs are predicted to be regulated by miRNAs [2]. MiRNAs can thus target multiple mRNAs modulating gene expression programs in virtually every biological process [3].

This contrasts with the observation that only few miRNA mutants are associated with obvious developmental defects [4–8]. Instead, many miRNAs are thought to function to fine-tune gene activity providing robustness to gene regulatory networks [9–11]. This serves as a mechanism to ensure proper signaling responses in the face of environmental and genetic stresses, which are often the cause of disease. Consistent with that, and despite the small number of examples associated with strong loss-of-function phenotypes, miRNAs have been shown to play important roles in human pathologies, including cancer [12]. The complexity of their regulation and the high number of potential targets for each miRNA poses the challenge of elucidating the specific targets associated with miRNA-related phenotypes and diseases.

Although bioinformatic prediction tools have been helpful in finding potential miRNA-target interactions [13], these approaches predict many false positives [14]. Thus, to establish the important miRNA-mRNA interactions—which are relevant in different cellular contexts—putative targets need to be tested and validated *in vivo*. The use of animal models including worms (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), zebrafish (*Danio rerio)* and mice (*Mus musculus*) has been crucial for the identification of miRNA functions in development and disease [15, 16]. We focus this review on the use of *Drosophila* as an *in vivo* model to study how miRNAs influence cancer.

9.2 MiRNAs in Human Cancer

MiRNAs are frequently dysregulated in human cancers; however, the specific functions of miRNAs in tumorigenesis are often elusive [17–19]. Aberrant miRNA expression levels are caused by chromosomal abnormalities, changes in transcriptional control, epigenetic changes or defects in the miRNA biogenesis machinery [20]. Oncogenic miRNAs, "called oncomiRs", are often upregulated in cancer, and facilitate tumorigenesis and disease progression. On the contrary, "tumor suppressor" miRNAs counteract tumor growth and are frequently downregulated in cancer. In fact, miRNAs have been associated with various cancer-related processes such as DNA damage response, differentiation, angiogenesis, senescence, invasion and metastasis [18–23]. MiRNA signatures can be discriminated between different types of cancer [24, 25]. Thus, miRNAs can be used as diagnostic and prognostic tools in the clinic [26]. Moreover, miRNAs are considered as tools and targets for cancer therapy. In numerous preclinical studies, miRNA expression levels are modulated via the delivery of miRNA mimics, to replenish miRNAs with tumor suppressive functions, and antimiRs, to repress oncogenic miRNAs [27, 28].

Despite the progress in understanding the role of miRNAs in cancer, there is still a gap between the observations of widespread miRNA dysregulation in cancer and functional data proving causality of aberrant miRNA expression. Thus, *in vivo* animal models are key to dissect the underlying mechanisms of individual miRNAs in cancer.

9.3 *Drosophila* Tumor Models

Cancer is a genetic disease that involves the accumulation of mutations causing, among others, increased cell proliferation, reduced apoptosis and differentiation, and the activation of invasion and metastasis [29]. Mutations affect-

ing "driver" genes, which provide the cells with the initial potential to form tumors, have been identified. However, the identification of genes that cooperate with known cancer drivers in malignancy remains a major challenge in cancer research [30–32].

Drosophila is emerging as a useful model to identify genes that cooperate with driver mutations in malignancy [33–36]. Despite the obvious differences between flies and humans, using *Drosophila* to model cancer has distinct advantages: (1) a reduced complexity due to a lower genetic redundancy and simpler biology; (2) a short generation time that, among other benefits, allows to quickly test hypotheses and generate large scale *in vivo* screens; (3) a powerful genetic toolkit for targeted gene modulation in a tissue and stage-specific manner. Moreover, many of the pathways that control key cellular and physiological processes are highly conserved. In fact, nearly 75% of human disease genes have orthologs in the fly [37]. Remarkably, several signaling pathways relevant to cancer such as the Hippo [38, 39], Notch [40], and Hedgehog pathways [41] were first described in *Drosophila*, contributing to our understanding of the molecular mechanisms underlying tumor formation [42].

Loss of tumor suppressors such as elements of the Hippo pathway, or activation of oncogenes like Ras or Notch, leads to benign tissue overgrowth in fly imaginal tissues [39, 43, 44]. However, combining Ras or Notch activation with mutants affecting the apical-basal polarity genes *scribbled (scrib)*, *discs large* (*dlg*) or *lethal giant larvae* (*lgl)*, drives transformation into neoplastic tumors [45, 46]. These early screens showcased the utility of *Drosophila* models to study oncogenic cooperation in tumorigenesis. Interestingly, loss of apical-basal polarity is a key characteristic of malignancy in human cancers, and the Scrib/Dlg/Lgl polarity module is frequently dysregulated and is associated with tumor metastasis [29, 47].

Since these seminal works, studies in *Drosophila* have identified numerous oncogenes and tumor suppressors involved in oncogenic cooperation. Apart from key signaling elements controlling cell growth and proliferation, other factors regulating additional cancer traits have been described in fly tumors. These include apoptosis and compensatory cell proliferation, genome stability, metabolic reprogramming, actin cytoskeletal changes, inflammation, cell competition, the tumor microenvironment, and even angiogenesis [33, 34, 36].

According to miRBase release 22 (mirbase.org), the *Drosophila* genome contains 258 miRNA loci, which are processed to form 469 mature miRNAs [48]. To dissect the roles of miRNAs in tumorigenesis, methods to manipulate miRNA activity in a tissue-specific fashion without affecting the animal globally are required. To that end, resources which provide a genome-wide collection of miRNA overexpression and miRNA depletion ("miRNA sponges") transgenes are available in flies [49–51]. Different approaches have been used to determine the roles of miRNAs in tumorigenesis where these tools have been central. These strategies—described in detail below—include tumor miRNA transcriptome profiling followed by functional analyses (illustrated in Fig. 9.1a) and screens for modifiers of tumor-related phenotypes (illustrated in Fig. 9.1b, c).

9.4 MiRNA Expression Changes in *Drosophila* Tumors

MiRNAs are aberrantly expressed in human cancers and miRNA profiles are associated with tumor development and progression. However, functional analyses to examine these correlations remain limited. *Drosophila* provides a tractable system to perform this kind of analysis.

Expression of oncogenic Ras^{V12} together with loss of tumor suppressive *lgl* in the imaginal tissues of *Drosophila* leads to the formation of malignant tumors [45]. The levels of approximately 11% of all mature miRNAs (51 miRNAs)

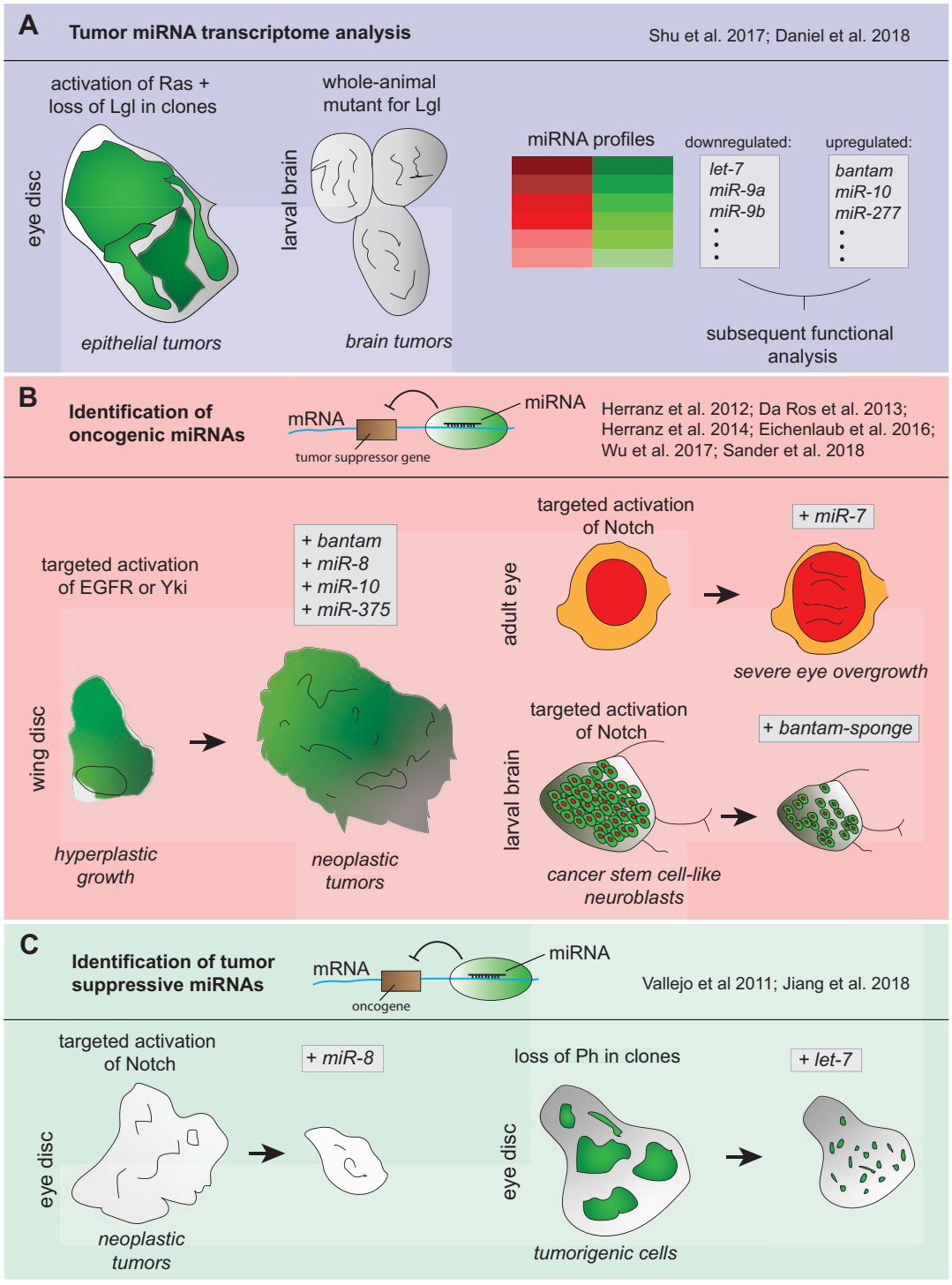

Fig. 9.1 Overview of the different systems used to identify miRNAs that play a role in *Drosophila* tumors. (**a**) Representation of the miRNA transcriptome profiling studies in loss-of-*lgl*-induced tumors. (**b** and **c**) Representation of the different studies that identify miRNAs which enhance or repress tumor phenotypes

in those tumors present robust changes [52]. Clonal depletion of *lgl* in the wing disc results in tumorous overgrowths. In contrast, tumors are not formed when *lgl* is specifically depleted in the *dpp* domain, a band of cells adjacent to the anterior-posterior boundary of the wing disc (hereafter referred as *dpp* > *lgl-RNAi*). These backgrounds served to assess the implications of miRNAs dysregulated in Ras^{V12}-*lgl* tumors. Among the 28 miRNAs upregulated, 10 induce tumorigenic overgrowth when expressed in *dpp* > *lgl-RNAi* discs. Furthermore, depletion of these miRNAs in *lgl* clones limits tumor growth. Similarly, the miRNAs downregulated in the Ras^{V12}-*lgl* tumors were tested for their potential to repress tumor formation in *lgl* clones. In that context, 11 of the 23 miRNAs downregulated, when expressed in *lgl* clones, repress tumor formation and restore normal tissue organization. Interestingly, the upregulated, tumor enhancing miRNAs *bantam* and *miR-10*, and the downregulated, tumor suppressive miRNA *let-7* were also identified in other *Drosophila* tumor models and will be discussed below. Furthermore, nearly 50% of the miRNAs identified in this study are conserved and their human homologs are involved in various cancers [52]. This analysis shows that tumor formation goes hand in hand with miRNA dysregulation and, more importantly, that many of these differentially expressed miRNAs contribute to tumorigenesis.

lgl mutant brain and imaginal discs develop neoplastic tumors [53–55]. Transcriptome analysis also revealed widespread changes in miRNA expression [56]. To improve the temporal resolution of the miRNA profiles, this analysis was performed at three different time-points of tumor development. 10 miRNAs were dysregulated in all tumor stages analyzed. Amongst these, *let-7*, *miR-210*, and *miR-9a* were downregulated—all of which have been functionally implicated in human cancers [57–59]. *miR-9a* was amongst the top downregulated miRNAs suggesting tumor suppressive functions. Consistently, overexpression of *miR-9a* limited the growth of *lgl* mutant wing discs [56]. At the stage when tumors were fully developed, *bantam* levels were highly enriched [56]. This is consistent with observations from another study where *bantam* levels are also upregulated in lgl^{-}, *scrib*−, or $brat^{-}$ brain tumors [60].

9.5 *let-7* and *bantam*: Old Dogs with New Tricks—in Cancer

let-7 and *bantam* were amongst the first miRNAs discovered and their analysis provided important insights into miRNA mechanisms [61, 62]. More recently, *let-7* and *bantam* have been implicated in tumorigenesis in cooperation with cancer drivers.

9.5.1 *let-7*

In human cancers, *let-7* is the most frequently downregulated tumor suppressor miRNA and repression of *let-7* is correlated with poor prognosis [63]. Furthermore, *let-7* has been shown to reduce proliferation and tumor growth in cancer cell lines [58, 64]. One of the tumor suppressive mechanisms used by *let-7* has been elucidated in *Drosophila* and involves the *let-7* target *chinmo* [65, 66], a transcription factor involved in tumorigenesis [67, 68]. In the *Drosophila* eye-antennal disc, clones mutant for the epigenetic silencing regulator Polyhomeotic generate neoplastic tumors [69, 70]. These tumors show malignant traits and continue to grow when transplanted into an adult wild-type fly [69]. On the contrary, tumors generated in the larval tissue are repressed after metamorphosis and eventually eliminated in the adult fly, revealing tumor suppressive signaling during larval–adult transition [65]. The steroid hormone Ecdysone, a crucial signal coordinating metamorphosis [71], induces the expression of *let-7*, and *chinmo* downregulation by *let-7* is key for tumor eviction downstream of steroid signaling during metamorphosis [65, 72].

9.5.2 *bantam* in Tumors of Epithelial Origin

bantam was the first miRNA discovered in flies as an element that, when overexpressed, induces tissue growth [61, 73]. *bantam* is a developmentally regulated miRNA and its expression is controlled by different signaling pathways such as the Hippo [74, 75], Notch [76, 77], Dpp [78], and EGFR [79] signaling pathways. *bantam* promotes tissue growth by inducing cell proliferation and repressing apoptosis, two processes frequently dysregulated in cancer [29, 80].

Activation of the oncogene EGFR in the wing epithelium activates the Ras/MAPK pathway and induces tissue hyperplasia [79]. However, this does not cause malignancy; cooperating factors are required for cellular transformation and neoplasia. The miRNAs *miR-10*, *miR-375* and *bantam* have been found to, individually, synergize with EGFR to facilitate neoplastic transformation [81, 82]. The *bantam* target *Suppressor of cytokine signaling at 36E* (*Socs36E*) plays a central role in this context. EGFR induces *Socs36E* expression. In turn, Socs36E antagonizes EGFR signaling [83, 84], which provides a negative feedback that limits the growth-promoting role of EGFR. Socs36E also dampens the JAK/STAT pathway [84] and JAK/STAT cooperates with oncogenic Ras in malignancy [85]. Thus, *bantam*-mediated repression of *Socs36E* inactivates this homeostatic feedback and drives neoplasia (Fig. 9.2a). In analogy to this, repression of the human Socs36 ortholog SOCS5, in combination with activated RAS, promotes colony formation in a cell transformation assay [81]. In agreement with these findings, subsequent studies in human cell lines showed that the transforming activity of oncogenic RAS relies on its ability to downregulate SOCS5/6 [86].

9.5.3 *bantam* in Brain Tumors

bantam is embedded in a similar regulatory loop in neuroblasts, neural stem cells in *Drosophila*. In those cells, Notch plays a conserved role coordinating self-renewal and differentiation [87, 88]. Notch promotes dMyc-dependent nucleolar and cellular growth, which is key for neuroblast self-renewal [89]. *bantam* controls neuroblast proliferation where it targets the Notch repressor *Numb* [90–92]. Notch hyperactivation induces the formation of cancer-stem-cell (CSC)-like neuroblasts that can initiate tumors [89]. *bantam* is required, downstream of Notch, for the formation of CSC-like neuroblasts and tumorigenesis [93]. In this context, via repressing *Numb, bantam* establishes a positive feedback that reinforces Notch signaling. Furthermore, *bantam*-dependent repression of *Numb* induces Myc signaling. Thus, *bantam* helps to maintain neuroblast homeostasis in two ways: a) by promoting Notch signaling, and b) by facilitating Myc-dependent nucleolar and cellular growth. Interestingly, overexpression of *bantam* is not sufficient to drive CSC-like formation and hence *bantam* acts to fine tune Notch-mediated neuroblast homeostasis [93] (Fig. 9.2b).

Although *bantam* is not obviously conserved in mammals, its functions likely are. *bantam* is proposed to functionally mimic mammalian *miR-130a* [94]. The mammalian Yki homolog YAP controls *miR-130a* expression and this regulation mediates over-proliferation and tumorigenesis. *miR-130a* targets VGLL4, which is an inhibitor of YAP [95, 96]. Thus, by repressing a negative regulator of YAP, *miR-130a* provides a positive feedback loop that is critical in YAP-mediated tumorigenesis. Intriguingly, analogous to this mechanism, the *Drosophila* VGLL4 homolog SdBP/Tgi is regulated by *bantam*. Thus, *bantam* and *miR-130a* share functional characteristics: both are involved in a conserved feedback that ensures robust Hippo pathway signaling in growth control and tumorigenesis [94] (Fig. 9.2c, d).

9.5.4 *bantam* and Invasion

Apart from promoting cell proliferation and repressing apoptosis, *bantam* has been proposed to repress cell invasion [97]. Hippo signaling

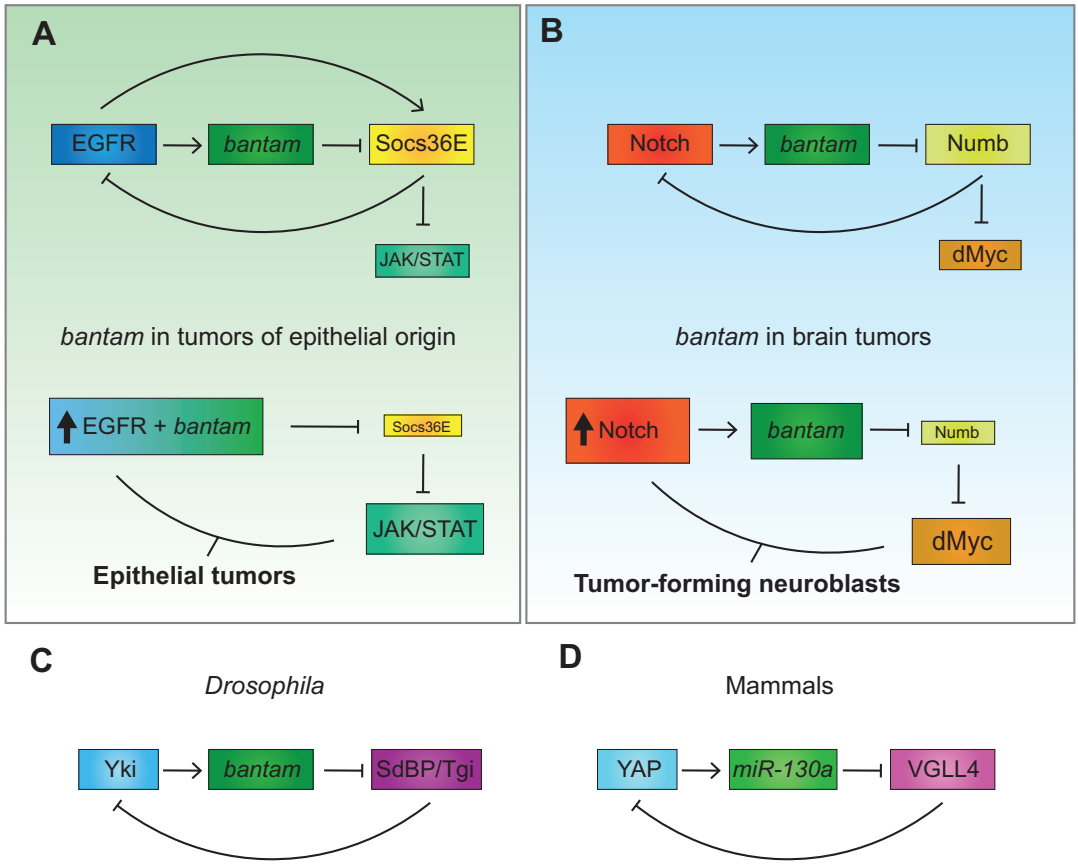

Fig. 9.2 *bantam* is involved in positive feedback loops downstream of major growth regulatory pathways to reinforce their outputs via alleviation of inhibitory elements. (**a**) *bantam* represses the EGFR and JAK/STAT-inhibitory element Socs36E. Thus, In the wing epithelium, upregulation of *bantam* removes this homeostatic element and facilitates the formation tumors in cooperation with EGFR. (**b**) In the neuroblasts, *bantam* represses the Notch and dMyc inhibitor Numb. Notch overactivation leads to tumor-forming neuroblasts due to *bantam*-mediated depletion of Numb. (**c** and **d**) Hippo pathway-mediated *bantam* functions are possibly conserved in mammalian *miR-130a*. Both *miR-130a* and *bantam* act downstream of YAP/Yki to repress the YAP/Yki inhibitory elements VGLL4 or SdBP/Tgi respectively

appears to modulate invasion and epithelial-to-mesenchymal transition in the wing epithelium through JNK. Overexpression of the Yki target *bantam* impairs cell invasion upon *yki*-depletion, while other Yki targets such as *diap1* and *dMyc* do not alter that. In that situation, reducing *bantam* also phenocopies the loss of *yki*. *Rox8* has been identified as a *bantam* target involved in JNK regulation downstream of the Hippo pathway [97].

9.6 MiRNAs Affect Tumorigenesis in a Context Dependent Manner

Notch signaling promotes growth in various tissues and organs, and Notch hyperactivation in *Drosophila* epithelial tissues leads to hyperplasia [45, 69, 98, 99]. Overexpression of the Notch ligand *Delta* (*Dl*) in the developing eye results in mild tissue overgrowth [100]. This genetic background has been used to screen for genes that

cooperate with Notch in malignancy and neoplasia [101]. By using this strategy, the conserved miRNAs *miR-7* and *miR-8* were identified as modulators of Notch-mediated growth and tumorigenesis [102, 103]. While *miR-7* was found to cooperate with Notch, *miR-8* functions as a tumor suppressor inhibiting Notch-mediated tumor formation.

9.6.1 *miR-7*

To identify *miR-7* targets contributing to the synergism between *miR-7* and Notch, RNAis depleting predicted *miR-7* targets were coexpressed with *Dl* [103]. This showed that depletion of the Hedgehog receptor *interference hedgehog* (*ihog*) reproduces the *miR-7*/*Dl* overgrowth. Direct targeting of *ihog* by *miR-7* was validated *in vivo*. Moreover, repression of core Hedgehog signaling components drives tumorigenesis in the *Dl*-overexpression background. Reciprocally, increase in Hegdehog signaling prevents *miR-7*/*Dl* tumorigenesis. This study unraveled an unknown tumor suppressive aspect of the Hedgehog pathway in Notch-driven tumors [103].

miR-7 also controls growth of the wing epithelium, as loss of *miR-7* results in small wings with defects in cell size and the cell-cycle [104]. *miR-7* targets the cyclin-dependent kinase inhibitor *dacapo* in the germline [105]. In agreement with that, reduction in the levels of *dacapo* or *Notch* rescues the wing defects associated with loss of *miR-7* [104].

In human lung and skin cancers, *miR-7* is upregulated and acts as an oncogene [106]. However, *miR-7* tumor suppressive functions have also been reported in numerous cancers [107]. Interestingly, these also involve *miR-7*-dependent regulation of the Hedgehog pathway [108]. In fact, it is frequently observed that miRNAs may act as tumor suppressors in one context and as oncogenes in another [109]. Understanding these phenomena is especially relevant in miRNA-based cancer therapy. Another miRNA showing this context dependent behavior is the member of the miR-200 family, the *Drosophila* miRNA *miR-8*.

9.6.2 The Tumor Suppressor Side of *miR-8*

Overexpression of *Dl* in combination with the epigenetic repressors *pipsqueak* and *lola* leads to the formation of malignant tumors—this characteristic phenotype has been referred to as "*eyeful*" [101]. Expression of *miR-8* in the *eyeful* background reduces tumor growth and represses metastasis formation [102]. *miR-8* overexpression in the wing and eye imaginal disc induces apoptosis and growth defects, phenotypes reminiscent of a reduction in the Notch ligand Serrate [110]. These observations led to the identification of *Serrate* as the *miR-8* target responsible for its tumor suppressive role in the eye epithelium [102]. Importantly, the human *Serrate* ortholog JAGGED1 is also targeted by the *miR-8* orthologs *miR-200c* and *miR-141,* and, similar to the *Drosophila* tumor, JAGGED1-mediated prostate cancer cell proliferation is inhibited by *miR-200c* and *miR-141* [102].

The *miR-200* family is frequently dysregulated in various types of cancer and has been functionally implicated in tumorigenesis and metastasis [111, 112]. Several studies support that *miR-8*/200 targets and functions are conserved between flies and mammals. *miR-8* in flies and *miR-200* in mammals inhibit epithelial-to-mesenchymal transition (EMT) by repression of zhf1/Zeb1 and Zeb2 [113–115]. Furthermore, the *miR-200* family inhibits cell invasion by targeting regulators of the actin cytoskeleton; similarly, *miR-8* modulates the actin cytoskeleton in the neuromuscular junction and the wing epithelium [116–121]. The pesticide component transnonachlor was shown to inhibit *miR-141* in human melanocytic cells, facilitating malignant transformation [122]. In *Drosophila*, transnonachlor also represses *miR-8*. Strikingly, transnonachlor-induced downregulation of *miR-8* is epigenetically inherited over multiple generations and leads to a loss-of-weight phenotype in the offspring [122].

Despite the fact that numerous studies demonstrate tumor suppressor functions of *miR-200* miRNAs, clinical data on *miR-200* levels are inconsistent and suggest cancer type or even sub-

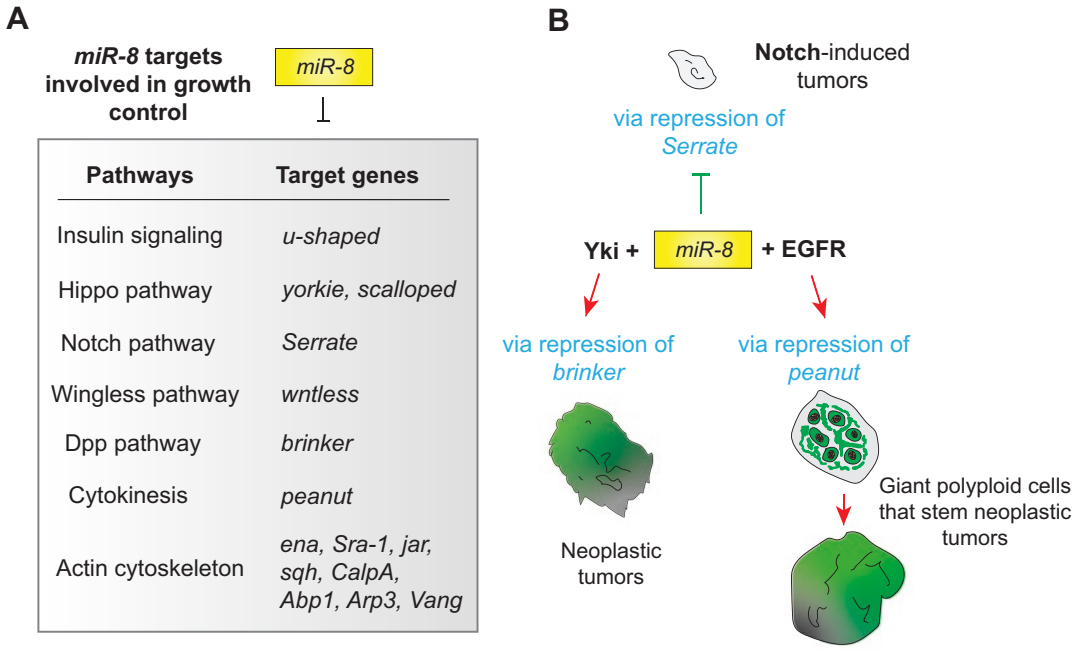

Fig. 9.3 *miR-8* generally acts as a repressor of growth, but in some contexts, it promotes tumorigenesis. (**a**) List of *miR-8* targets relevant in tissue growth. (**b**) The dual role of *miR-8*: it inhibits Notch-induced tumors; however, *miR-8* facilitates tumorigenesis together with Yki or EGFR

type dependent roles [111, 123]. For instance, high *miR-200* levels are associated with improved clinical outcome in ovarian, lung, renal, basal-like breast adenocarcinomas and certain colorectal cancers [123, 124]. However, high *miR-200* correlate with worse outcome in luminal breast, certain ovarian and pancreatic cancers [124–126]. Furthermore, functional studies suggest that *miR-200* family members can act as oncogenes by repressing the tumor suppressor *PTEN* [127, 128]. In contrast to the tumor suppressive function of *miR-8* in the context of Notch-induced growth, *miR-8* was shown to cooperate with the tumor drivers EGFR [129] and Yki [130] respectively, suggesting that the dual role of *miR-8*/200 was maintained between flies and humans.

9.6.3 *miR-8* as an Oncogenic Factor

Multiple studies show that *miR-8* limits tissue growth in imaginal tissues. *miR-8* represses numerous genes required for normal growth including elements involved in cytokinesis, Hippo signaling, Wingless pathway, Notch signaling, insulin signaling and cytoskeletal regulators [102, 120, 121, 129–131] (Fig. 9.3a). Strikingly, when *miR-8* expression is combined with oncogene activation (EGFR or Yki) the observed effect is the opposite and *miR-8* fuels oncogene-driven growth resulting in the development of tumors (Fig. 9.3b).

As *bantam*, *miR-8* cooperates with EGFR in tumorigenesis. Coexpression of *EGFR* and *miR-8* causes the formation of tumors and metastasis in *Drosophila* larvae. These tumors are heterogeneous and are composed of a mix of normal epithelial cells and giant polyploid cells. The latter show defects in epithelial polarity, which is a common trait in neoplastic tumors [132]. During tumor progression, giant polyploid cells get selected and, in late stages of tumor development, they stem the formation of metastasis. A closer analysis revealed the presence of apoptotic corpses within giant cells suggesting that these kill and engulf surrounding cells. Consistently, genetic suppression of engulfment in those discs (*EGFR* + *miR-8*) abolishes the formation of giant

cells, tumor development and metastasis. Giant tumor cells hence grow at expenses of their surrounding neighbors in a process resembling cell competition—a cell-cell interaction process first described in *Drosophila* by Morata and Ripoll in the early 70s [133].

The *miR-8* target gene *peanut* (*pnut*) plays a central role in the formation of these tumors. *Pnut* encodes a Septin that is required for normal cytokinesis [134]. *pnut* depletion is required for *miR-8* + *EGFR*-driven tumorigenesis, as *pnut* overexpression in this background rescues tumor formation. Thus, via repressing *pnut*, *miR-8* induces cytokinesis failure and thereby, in concert with EGFR, facilitates the emergence of polyploid cells that hijack cell competition mechanisms to propagate themselves and eventually form malignant metastatic tumors [129]. Cytokinesis failure has been described to be tumorigenic in mammals and it is proposed that approximately 40% of human tumors have gone through a round of gene duplication [135]. This work [129] provides a new example whereby defective cytokinesis is associated with the formation of malignant tumors.

One of the *miR-8* targets required for normal growth is the oncogene Yki. *miR-8*, in addition to dampen Yki levels, acts as an oncogenic partner of Yki [130]. Reminiscent of the *EGFR* + *miR-8* tumors, a subset of *yki* + *miR-8* cells display aberrant ploidy, possibly due to defective cleavage as a consequence of *pnut* downregulation. Consistently, Yki can also induce neoplasia in discs with cytokinesis failure, generated via RNAi-mediated depletion of *pnut* [136]. However, *yki* + *miR-8* tumors grew bigger in size than the *yki-pnut-RNAi* ones suggesting that additional Yki targets are involved in the formation of those tumors. This led to the identification of the growth repressor *brinker* as a *miR-8* target gene contributing to tumor formation downstream *miR-8* [130].

Taken together, these studies demonstrate a context-dependent impact of miRNAs in tumorigenesis, which is an important consideration for the application of miRNA therapeutics.

9.7 MiRNA Biogenesis Pathway and Tumorigenesis

The canonical pathway of miRNA biogenesis is a multistep process at the end of which the mature ~22 nucleotide long miRNA is incorporated in the RNA-induced silencing complex (RISC), directing it to the target mRNA for post-transcriptional repression. A global depletion of miRNAs by alterations in the miRNA biogenesis machinery has widespread implications in human cancer [137].

MiRNAs are transcribed into long primary transcripts (pri-miRNAs), which are further processed by an RNase III enzyme, Drosha, to form miRNA precursors (pre-miRNA) [138, 139]. In the cytoplasm, pre-miRNAs are further processed [140] by another RNase III enzyme, Dicer-1 (Dcr-1), to form a duplex, which is subsequently loaded into the Argonaute-1 protein (Ago-1) [141, 142]. The duplex is then unwound, one of the strands discarded—the ssRNA guide strand is retained—and eventually the mature silencing complex is formed [143]. The exoribonuclease Nibbler has been shown be important for 3′ end trimming of longer miRNA intermediates produced by Dcr-1 [144, 145]. Nibbler has been recently associated with tumorigenesis in flies [146]. As discussed previously, *lgl* mutant tumors show broad changes in the miRNA transcriptome [52, 56]. Interestingly, *lgl* interacts with Fragile X protein (FMRP), and with Ago-1, both of which are involved in the miRNA biogenesis machinery [56, 147, 148]. These findings insinuate that changes in miRNA expression upon loss of *lgl* could be a direct consequence of a dysregulated miRNA biogenesis pathway. Further studies will be required to validate this interesting hypothesis.

9.7.1 The Proto-Oncogene dMyc Senses miRNA Levels

Dcr-1 mutants show a general depletion of miRNAs and this background has been used to study

how cells with reduced miRNAs behave in different developmental contexts. Even though miRNAs control nearly every biological process, *Dcr-1* mutant cells in the wing primordia are viable, differentiate normally, and do not show major patterning defects [149]. The most obvious outcome of global miRNA depletion is a reduction in the levels of the proto-oncogene dMyc. As a consequence, these cells are smaller in size and show reduced proliferation rates. Mechanistically, miRNA reduction results in an accumulation of the TRIM-NHL protein Mei-P26, which triggers proteasome-dependent degradation of dMyc [149]. At the same time, dMyc induces Mei-P26 as a means to buffer its own levels, which has been shown to be a mechanism to ensure epithelial tissue homeostasis [150]. *bantam* is one of the miRNAs that controls Mei-P26 levels, and overexpression of *bantam* in cells with reduced *Dcr-1* restores dMyc levels and cell size defects [149]. Thus, dMyc appears to serve as a sensor of general miRNA levels in the cell.

Cell competition is a cell-cell interaction mechanism that senses cellular fitness and mediates the elimination of suboptimal cells in a tissue [151]. Cell competition is not only relevant in normal development and homeostasis, but in some contexts it also influences tumor formation [152]. Importantly, dMyc is a central mediator of this competitive interaction. In this scenario, cells with reduced dMyc are referred to as losers and are eliminated by cells with higher dMyc, reffered to as winners [153, 154]. Consistent with the reduction in dMyc, *Dcr-1* mutant cells acquire the loser status and are eliminated from the wing primordia [149]. In sum, this study suggests that cells with reduced miRNAs are identified as less fit, which causes a reduction in dMyc and their consequent elimination by cell competition.

9.7.2 Proliferation Defects in *Dcr-1* Mutant Stem Cells

Multiple studies demonstrate essential roles of the miRNA machinery for self-renewal in germline stem cells (GSCs) [155–157]. Loss of *Dcr-1* in GSCs leads to defects in cell cycle control. In that context, the cell cycle regulator *dacapo* is increased and a reduction of *dacapo* partially rescues loss of *Dcr-1*-dependent cell cycle defects [155]. *dacapo* is regulated by *miR-7* and *miR-278,* and loss of these individually in GSCs leads to cell-cycle aberrations [105]. Loss of *Dcr-1* in GSCs of adult animals leads to defective stem cell maintenance—a phenotype mimicked by loss of *bantam* [158]. Similarly, in neuroblasts, depletion of *Dcr-1* or *bantam* leads to a decrease in neuroblast number due to cell proliferation defects [92]. Interestingly, similar to *Dcr-1* mutant GSCs [155], these cells display elevated *dacapo* expression levels. Since *bantam* also targets *dacapo* in GSCs [105], the *bantam-dacapo* axis might contribute to the proliferation defects observed in *bantam* mutants.

Similar to the observations in *Drosophila*, the mouse ortholog of Mei-P26, TRIM32, regulates stem cell self-renewal by targeting c-Myc for proteasome-mediated degradation and by binding to Ago-1 [159]. Moreover, TRIM32 is frequently upregulated in human cancers [160] and it has been shown to target tumor suppressor p53 to promote tumorigenesis [161].

9.7.3 p53

p53 is a central tumor suppressor that mediates the response to numerous types of stress by inducing cell cycle arrest, cellular senescence, and apoptosis. Besides, p53 can also control other biological processes involved in disease progression such as metabolism, stem cell maintenance, invasion and metastasis [162]. Therefore, scrutinizing the mechanisms involved in p53 regulation is crucial towards our understanding of cancer. MiRNAs are central players suppressing tumor formation downstream of p53 [163], and downstream targets of p53 are modulated by miRNAs [163, 164]. Importantly, studies in flies showed that p53 is sensitive to miRNA levels [165]. Depletion of *Dcr-1* in *Drosophila* leads to

an increase in the expression of a transgene consisting of the *dp53-3'UTR* fused to GFP (*dp53-sensor*). The analysis of the *dp53 3'UTR* led to identify *miR-305* as a direct regulator of *dp53*. [165]. *dp53* is upregulated under starvation, which mediates a metabolic adjustment that increases survival in nutrient deprivation conditions. Importantly, *miR-305* contributes to this adaptive response. Upon nutrient deprivation, *Drosha*, *Dcr-1*, and *Ago-1* are downregulated, which leads to a reduction in *miR-305* levels. This, consequently, alleviates *miR-305*-mediated repression of *dp53* and facilitates metabolic adaptation [165]. Metabolic reprogramming is central in cancer [166]. Thus, analyzing whether *miR-305* regulates *dp53* and the potential implications of this axis in tumorigenesis remain to be determined.

9.8 Conclusions and Perspectives

Since the discovery of miRNAs, these regulatory molecules have been associated with virtually every cellular process. As a consequence of this, changes in miRNA expression can contribute to the initiation and development of human diseases including cancer. The main challenge in the field is to identify the relevant miRNA targets in normal development and different pathological contexts. For this, the use of animal models is key.

Studies in *Drosophila* tumor models establish direct implications of miRNAs as regulators of different hallmarks of cancer such as cell proliferation, apoptosis, differentiation and metabolism. However, we are likely still in the first stages towards understanding the roles that miRNAs play in disease initiation and progression. Thus, insights from *Drosophila* models will continue to unravel molecular mechanisms underlying miRNA-mediated tumorigenesis. Ultimately, these advances will help understanding the implications of miRNAs in human cancer.

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Cancer Stem Cells and Stem Cell Tumors in *Drosophila*

10

Shree Ram Singh, Poonam Aggarwal, and Steven X. Hou

Abstract

Accumulative studies suggest that a fraction of cells within a tumor, known as cancer stem cells (CSCs) that initiate tumors, show resistance to most of the therapies, and causes tumor recurrence and metastasis. CSCs could be either transformed normal stem cells or reprogrammed differentiated cells. The eventual goal of CSC research is to identify pathways that selectively regulate CSCs and then target these pathways to eradicate CSCs. CSCs and normal stem cells share some common features, such as self-renewal, the production of differentiated progeny, and the expression of stem-cell markers, however, CSCs vary from normal stem cells in forming tumors. Specifically, CSCs are normally resistant to standard therapies. In addition, CSCs and non-CSCs can be mutually convertible in response to different signals or microenvironments. Even though CSCs are involved in human cancers, the biology of CSCs, is still not well understood, there are urgent needs to study CSCs in model organisms. In the last several years, discoveries in *Drosophila* have greatly contributed to our understanding of human cancer. Stem-cell tumors in *Drosophila* share various properties with human CSCs and maybe used to understand the biology of CSCs. In this chapter, we first briefly review CSCs in mammalian systems, then discuss stem-cell tumors in the *Drosophila* posterior midgut and Malpighian tubules (kidney) and their unique properties as revealed by studying oncogenic Ras protein (Ras^{V12})-transformed stem-cell tumors in the *Drosophila* kidney and dominant-negative Notch (N^{DN})-transformed stem-cell tumors in the *Drosophila* intestine. At the end, we will discuss potential approaches to eliminate CSCs and achieve tumor regression. In future, by screening adult *Drosophila* neoplastic stem-cell tumor models, we hope to identify novel and efficacious compounds for the treatment of human cancers.

Keywords

Cancer stem cell · Stem cell tumor · Transformed stem cell · Drosophila · Intestinal stem cells · Renal and nephric stem cells

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10.1 Introduction

Significant advances over the last decade have improved the quality of life for cancer patients. However, globally people still die because of this disease, which is because of the relapse of this disease. Accumulative evidence suggests that tumor metastasis, relapse and death of patients are due to a rare population of cells reside in the tumors, called cancer stem cells (CSCs) [11, 19, 41, 42, 44, 79, 80]. CSCs have unlimited self-renewal capacity and multilineage differentiation ability [65]. Because of these characteristics, these specialized cells are thought to be mainly responsible for the initiation, growth, spread, and recurrence of cancer. However, the cells from which CSCs are derived, called tumor initiating cells (TICs) or the cells-of-origin for cancer (COCs), appear to vary in different types of tumors. Some CSCs are generated from the transformation of normal stem cells, and others develop from the reprogramming of non-CSC cancer cells into a stem cell state (Fig. 10.1; [4]).

CSCs often reside as largely dormant cells in a hypoxic storage niche surrounded by dense extracellular matrix, and show resistance to traditional radiation and chemotherapies, which mostly target dividing and actively metabolizing cells [78]. Because of this resistance, conventional treatment can significantly enrich CSCs in breast and pancreatic cancers [12, 37, 49, 60, 86]. Thus, the ablation of CSCs should reduce the risk of cancer recurrence, therefore, new CSC-targeting therapies are needed. However, the properties of quiescent stem cells and CSCs, including the molecular mechanisms regulating metastasis, dormancy, and resistance to treatments, are poorly understood. The eventual goal of CSC research is to identify pathways that selectively regulate CSCs and then target these pathways to eradicate CSCs. In this review, we will first briefly summarize the distinctive properties of CSCs in mammalian systems and then review the unique properties of stem cell tumors in *Drosophila* genetic system.

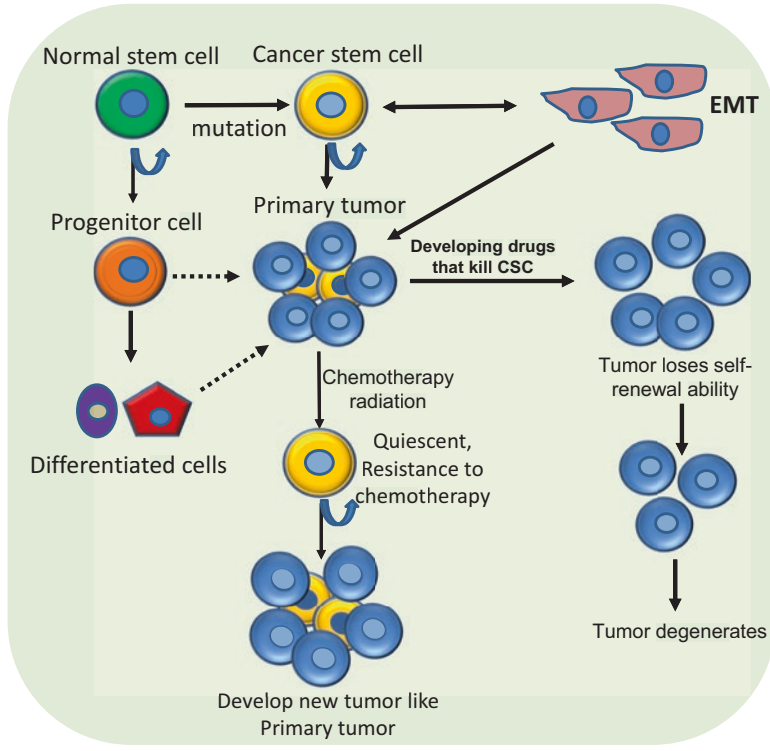

Fig. 10.1 Possible origin of cancer stem cells (CSCs) and tumor resistance and strategies to eliminate CSCs

10.2 Properties of Cancer Stem Cells

10.2.1 CSCs Are at Stem Cell States and Display Normal Stem Cells Properties

CSCs can be generated either by transforming normal stem cells or through reprogramming non-CSC cancer cells to stem cell states. CSCs are at stem cell states and also transformed (transformed stem cells) cells [51]. CSCs display characteristics of normal stem cell as they self-renew and differentiate to form the bulk of the tumor mass. CSCs also express normal stem cell markers. Like normal stem cells, CSCs reside in a specialized niche. Similar to normal stem cells, but unlike cancer cells, CSCs generate less reactive oxygen species (ROS) [20]. Normal stem cells and CSCs share major signaling pathways as well, such as Notch, WNT/β-Catenin, Hedgehog, JAK/STAT (janus kinase/signal transducers and activators of transcription), and NFκ-B (nuclear factor kappa-light-chain-enhancer of activated B cells) to maintain their stemness [8, 62, 64, 71]. In studies of hematological malignancies, it was initially found that chronic myeloid leukemia (CML) arose only when the *BCR–ABL* (Abelson murine leukemia viral oncogene homolog-breakpoint cluster region) mutation occurred in stem cells [13], this paradigm was later found also applying to other hematological cancers. In glioma, Dufour et al. [15] reported that malignant astrocytes maybe originated from neural stem cells or progenitors. In mouse intestinal tumor model, large tumor mass develops only deletion of the tumor-suppressor gene *Apc* (adenomatous polyposis coli) in Lgr5^{+} (leucine rich repeat containing G protein-coupled receptor 5^{+}) stem cells [67]. Majority of the current CSC surface markers are known to express in normal embryonic or adult stem cell system.

10.2.2 CTCs and DTCs Have CSC Characters

It was found that some tumor cells exist in cancer patients many years after the initial cancer therapy. They are either circulating tumor cells (CTCs) or disseminating tumor cells (DTCs) after setting down in distant sites. It has been demonstrated that dormant tumor cells could hide for years or even decades after surgical resection or radio/chemotherapy and then suddenly reappear and lead to metastatic cancer.. In the case of breast cancer survivors who were free of clinical disease detected circulating tumor cells (CTCs) 7–22 years after mastectomy [45].

The CTCs are short life cells and can exist for many years, suggesting that some clinically undetectable disseminating cells from the primary tumors can regenerate CTCs. Further, patient-derived CTCs were cultured and expanded for long period of time [10, 87]. Grillet et al. [23] shown that CTCs derived from colorectal cancer patients display CSCs characteristics. Recently, Gkountela et al. [21] reported that CTC clusters have stem cell signatures, which help CTC to become tumorigenic. They found that binding sites for stemness factors were hypomethylated in CTC clusters [21]. Most of DTCs are generally in state of quiescence [74]. DTCs and CTCs could be detected in asymptomatic patients with various cancers [27, 82]. DTCs maintain dormancy display chemo- and -radiotherapy resistance [40]. These studies together clearly demonstrated significant overlap of dormant cancer cells and CSCs, specifically after various cytotoxic therapies.

10.3 Therapy Resistance of CSCs in Mammals

Several mechanisms have been proposed to explain the resistance of CSCs to chemo- and radiotherapy, including a more robust DNA repair activity, localization to a low oxygen microenvironment, resis-

tance to apoptosis, a low level of ROS, a slow cell cycle, and maintenance in a quiescent state [6]. Particularly, it was recently demonstrated that CSCs are responsible to tumors' immune evasion.

In the recent years, immunotherapies have been utilized in the treatment of cancer. Accumulative studies suggest that both DTCs and CSCs can actively escape immune-mediated elimination. Malladi et al. [43] isolated stem-like latency competent cancer (LCC) cells and found that LCC cells self-impose a slow-cycling state, acquire immune evasion, maintain long-term survival and tumor-initiating ability during the latent metastasis by expressing the Wnt inhibitor DKK1 (Dickkopf WNT Signaling Pathway Inhibitor 1) that downregulate UL16-binding protein (ULBP) ligands for natural killer (NK) cells. Studies also suggest that microenvironment of quiescent DTCs may be helping to immune evasion [22]. Further, Pommier et al. [61] reported that an unresolved endoplasmic reticulum (ER) stress helps DTCs to escape immunity and establish latent metastases. Down-regulation of major histocompatibility class I (MHC I) antigen expression, which is crucial for $CD8^+$ T cell recognition was found in DTCs isolated from patients [56]. Recently, a correlation between stem cell quiescence, antigen presentation, and immune evasion was identified, which suggest that cancer cells evade immune surveillance by systemic down-regulation of the antigen presentation machinery [1, 48]. These data together suggest that quiescent CSCs are major components of immune dormant tumor cells and source of cancer relapse after therapy.

10.4 CSCs Are Metabolically Unique

The precise goal of CSC research is to eradicate CSCs. Accumulated evidence suggests that CSCs are metabolically unique. In recent years, researchers have found that CSCs from multiple tumor types rely on one kind of metabolism, called oxidative phosphorylation, more than any other method. It was recently reported that leukemia stem cells (LSCs) isolated from *de novo* acute myeloid leukemia (AML) patients uniquely rely on amino acids for oxidative phosphorylation (OXPHOS) to survive [30], given that AML blasts and HSCs but not LSCs can upregulate glycolysis to compensate for the loss of OXPHOS. Treatment with a combination of venetoclax and azacytidine, which inhibits amino acid uptake and catabolism, leads to deep and durable remission in most AML patients. We previously reported that the Arf1-mediated lipolysis pathway selectively sustains stem cells and transforms stem cells in *Drosophila,* and that knockdown of this pathway kills stem cells through necrosis [70]. Both studies suggest that CSCs are less "metabolically flexible" than other cells, they cannot adapt when their preferred fuel source is cut off, the cells die. Muscle stem cells (satellite cells) rely on mitochondrial fatty acid oxidation (FAO) but switch to glycolytic metabolism when they progress toward more committed states [65]. Some CSC-enriched disseminated tumor cells also obtain energy from fatty acids delivered through the fatty acid receptor CD36 expressed on a subset of highly aggressive CSCs [57, 85]. These data together suggest that targeting the unique metabolism of CSCs, such as by blocking amino acid or lipid metabolism, may be a promising general strategy for killing CSCs and inducing other systemic reactions.

10.5 CSCs Plasticity and Therapeutic Resistance

CSC is a plastic entity whose phenotype and function are regularly modified by the tumor microenvironment, epigenetic regulation, and different experimental systems. One property of the dormant tumor cells is that they continuously evolve and become more potent and finally generate more aggressive heterogenetic tumors once re-emerge. Cancer cells may acquire stem cell properties by regulating the signaling cues from tumor microenvironment or because of anti-tumor therapeutic intervention or intrinsically oncogene/tumor suppressor gene mutations [18]. Ionizing radiation was shown to reprogram cancer cells lacking stem cell properties to become CSCs [34]. It was found that a single melanocyte can reactivate progenitor

transcription factors to become a tumor-initiating cell for melanoma [31]. In summary, CSCs and non-CSCs are mutually convertible in response to different signals or microenvironments [4]. Therefore, CSCs can be re-created as long as non-CSCs and the tumor microenvironment remain intact, and only killing CSCs is not sufficient to elicit tumor regression.

10.6 Stem Cell Tumors in *Drosophila* Digestive System

In the *Drosophila* digestive system, three organs, the posterior midgut, the hindgut, and the Malpighian tubules (MTs), meet and join at the junction of the posterior midgut and hindgut. Stem cells in these organs exhibit different degrees of quiescence (reviewed in Zeng et al. [90]). The intestinal stem cells (ISCs), located in the posterior midgut, divide once every 24 h [46, 53]; the renal and nephric stem cells (RNSCs), located in the MTs, divide about once a week [69, 89]; and the quiescent hindgut intestinal stem cells (HISCs), found at the midgut/hindgut junction, divide only during stress-induced tissue repair [17, 76]. ISCs and RNSCs can be transformed to produce tumors: ISCs by knocking down their Notch (N) activity [53, 54], and RNSCs by forcing their expression of a constitutively activated Ras [89]. These transformed stem cells thus true CSCs.

10.6.1 Stem-Cell-Based Tumorigenesis in the Adult *Drosophila* Midgut

10.6.1.1 Intestinal Stem Cells in the Adult Drosophila Posterior Midgut

The *Drosophila* posterior midgut is similar in function to mammalian small intestine [25]. They are maintained by intestinal stem cells (ISCs) [46, 53]. ISCs reside near basement membrane (BM) and have ability to divide every day. ISCs can generate absorptive enterocytes (ECs) or secretory enteroendocrine (EE) cells. The *Drosophila* midgut is an attractive model system for studying adult stem-cell-mediated tissue homeostasis and regeneration, due to its well-defined cell lineages, the ease of performing genetic analyses, and the availability of large collections of *Drosophila* mutants.

Through asymmetric division, ISCs generate new ISCs as well as an enteroblast (EB) cells, which in turn differentiates into an EC or EE cell [46, 53]. The behavior of ISCs are regulated by Notch (N) signaling [46, 53, 54]. Recent studies demonstrated that ISCs can directly differentiate into EC or ee cells [24, 88, 93].

10.6.1.2 Spontaneous Somatic Mutations of Notch Results in Neoplasia in Aged Flies

Somatic genetic variation is well known in various cancers, however, how they are associated to stem-cell induced tumor have not been well documented. Recently, Siudeja et al. [72] connected somatic mutation to stem-cell tumor using *Drosophila* ISC model system. They found two unique mechanisms of genome instability in *Drosophila* ISCs, which led to phenotypic alterations in the aging intestine. First, they reported frequent loss of heterozygosity that develop because of mitotic homologous recombination in ISCs. Further they found that somatic deletion of DNA sequences and large structural rearrangements causes frequent gene inactivation that induced somatic inactivation of the X-linked tumor suppressor Notch in ISCs that resulted in neoplasias in 10% of the aged wild-type males [72].

10.6.1.3 Niche Appropriation Drives ISC Tumor Initiation and Progression

Mutations that limit the self-renewal ability of stem cell or differentiation in stem cell lineages are thought to be common early step in cancer development. However, how these mutations (such as *N* mutation) initiate tumorigenesis is unclear. Using *Drosophila* ISC system, Patel et al. [58] demonstrated that a single mutation in the *N* gene can trigger stem-cell tumor develop-

ment in the posterior midgut. They also noticed that some of the *N*-depleted guts had no stem-cell tumors, which suggest that restricting differentiation was not sufficient for tumor initiation. Previous studies showed that stress or enteric infection affect epithelial homeostasis and ISC tumor outgrowth [2, 28]. Consistent with this, Patel et al. [58] reported that enteric infection can enhance the tumor initiation in *N*-depleted flies and cell division. Further, they found that activation of JNK (c-Jun N-terminal kinase) signaling in ECs can also initiate tumor in the *N*-depleted flies [58]. These studies together suggest that blocking differentiation and stress-induced stem-cell division are important for tumor initiation.

Siudeja et al. [72] have shown that the *N*-depleted tumors in the *Drosophila* midgut grow over time. To understand how these tumors are further progressed after initiation, Patel et al. [58] demonstrated that the EGFR (epidermal growth factor receptor) and MAPK (mitogen-activated protein kinase) pathways are responsible for their progression [58]. Patel et al. [58] noticed that during tumor growth, some of the ECs close to and overlying the tumors get away from BM and died. They found that the growing tumor induced pro-apoptotic genes such as *reaper* and *grim* in non-tumor cells, and that apoptosis supported tumor growth but not required for EC detachment. Further, Patel et al. [58] found the expression of JNK and Yki (yorkie) signaling components near the detached ECs as well as apical to the ISC tumors but they found no expression in inside the ISC tumors. They further reported that knockdown of JNK or Yki signaling in the ECs block tumor growth, however, tumors were enlarged when signals were increased for these signaling. These results together suggest that during tumor progression, tumors first push the adjacent ECs away, then JNK and Yki signaling are activated in these detached ECs, which in turn enhances stem-cell tumor growth. In addition to above findings, Patel et al. [58] also identified the direct stimulator of stem-cell tumor growth. They found high expression of *vn (vein)*, *upd2 (unpaired2)*, and *upd3* in the N-defective tumors, specifically in the ECs and visceral muscle (VM), which suggest that tumor growth induces mitogenic signals in the niche. They further found that Upd3 is induced by the detachment of ECs from the VM and plays a crucial role in the EC-detachment-stimulated tumor growth.

The above studies together suggest that stem-cell-based tumorigenesis in the adult *Drosophila* posterior midgut are coordinated through several steps. Tumor initiation: (i) spontaneous mutation of the tumor suppressor *N* blocks stem-cell differentiation, and (ii) environmental stress-activated JNK signaling and stem-cell division. Tumor progression: (i) ISC-like cells in the small tumor clusters express *spi (spitz)* and activate EGFR signaling to promote their autonomous expansion. Tumor pushes adjacent ECs away, which activate JNK and Yki signaling and *upd3* expression, and (ii) the Upd3 then activates JAK/STAT signaling in the stem cells to promote tumor expansion (Fig. 10.2).

10.6.1.4 The Stem Cells in Malpighian Tubules

Drosophila renal tubules are the functional analog of the mammalian kidneys. They provide a best model organ system to study adult stem cell regulation and tumor formation. *Drosophila* has two pairs of renal tubules [75, 81]. Each tubule is divided into four compartments: initial, transitional, main (secretory), and proximal (reabsorptive). The proximal segment has two parts: the lower tubule and the ureter. We have identified renal and nephric stem cells (RNSCs) in the lower tubule and ureter of the renal tubuels ([69]; Fig. 10.3a). RNSCs self-renew to generate new RNSC and renablast (RB). RBs differentiate to form renalcytes (RC) at the lower tubules. RBs then migrate to the upper tubules and produce type I (principal) or II (stellate) cells (Fig. 10.3b). The RNSC self-renewal and differentiation are controlled by an autocrine JAK/STAT signaling [69] and other signaling pathways [7, 38, 84, 89]. RNSCs are marked by the expression of Escargot (Esg), a transcription factor of the Snail/SLUG family [46] and STA92E [69].

10.6.1.5 Ras-Transformed RNSCs

Mutations that activate the oncogene Ras have been identified in more than 30% of all types of human tumors [3]. In *Drosophila* MTs, Ras acti-

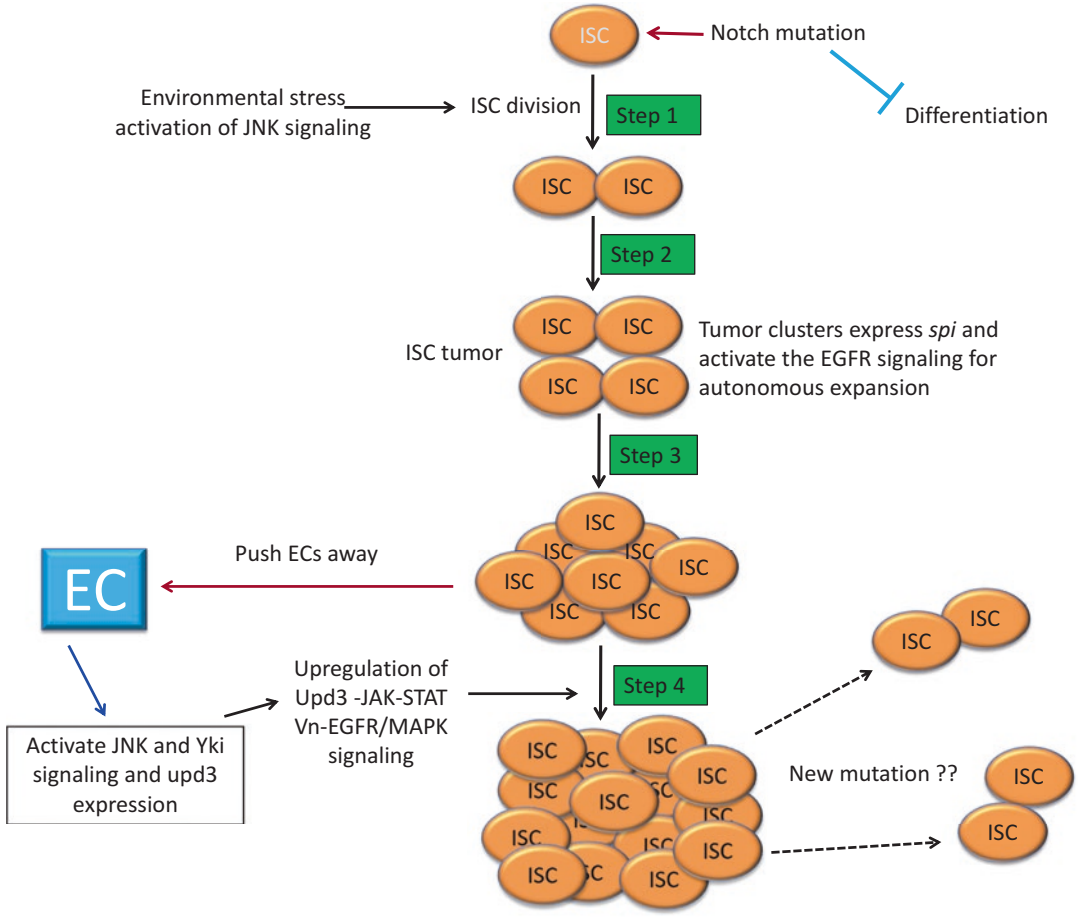

Fig. 10.2 Model of the sequence of events involved in N-dependent tumorigenesis in the *Drosophila* adult posterior midgut. (Modified after [58])

vation causes RNSCs to lose their differentiation and to overproliferate, resulting in neoplastic tumorous growth ([89]; Fig. 10.3c).

10.6.1.6 Ras-Transformed Stem Cells Exhibit Hallmarks of Cancer

In their advanced stages, human cancers exhibit several hallmarks [26], including supplying their own growth/proliferation signals, insensitivity to anti-proliferative signals, evasion of apoptosis, failure to differentiate, invasion/metastasis, activation of a telomerase to allow unlimited replicative potential, and increased angiogenesis. Among these hallmarks, the last two are not seen in *Drosophila* cancer models, because *Drosophila* regulates its DNA replication by a different system from mammalian telomere maintenance, and because *Drosophila* has an open circulation system, so tumor growth does not rely on angiogenesis [9]. However, Zeng et al. [89] reported that the Ras-transformed RNSCs display all of the first four hallmarks of human cancers.

First, Zeng et al. [89] showed low expression of cell-cycle and proliferation regulators (Cyclin E (Cyc E), phosphorylated Cdc2 (pCdc2), *Drosophila* Myc (dMyc), and phosphorylated ERK (pERK)) in normal RNSCs but their expression was dramatically increased in Ras^{V12}-transformed RNSCs [89]. Second, significantly high expression of the apoptosis inhibitor DIAP and its transcriptional reporter *diap1-lacZ* [66] was found in Ras^{V12}-transformed RNSCs compared to wild-type RNSCs [89]. Third, a few Ras^{V12}-transformed RNSCs were able to migrate

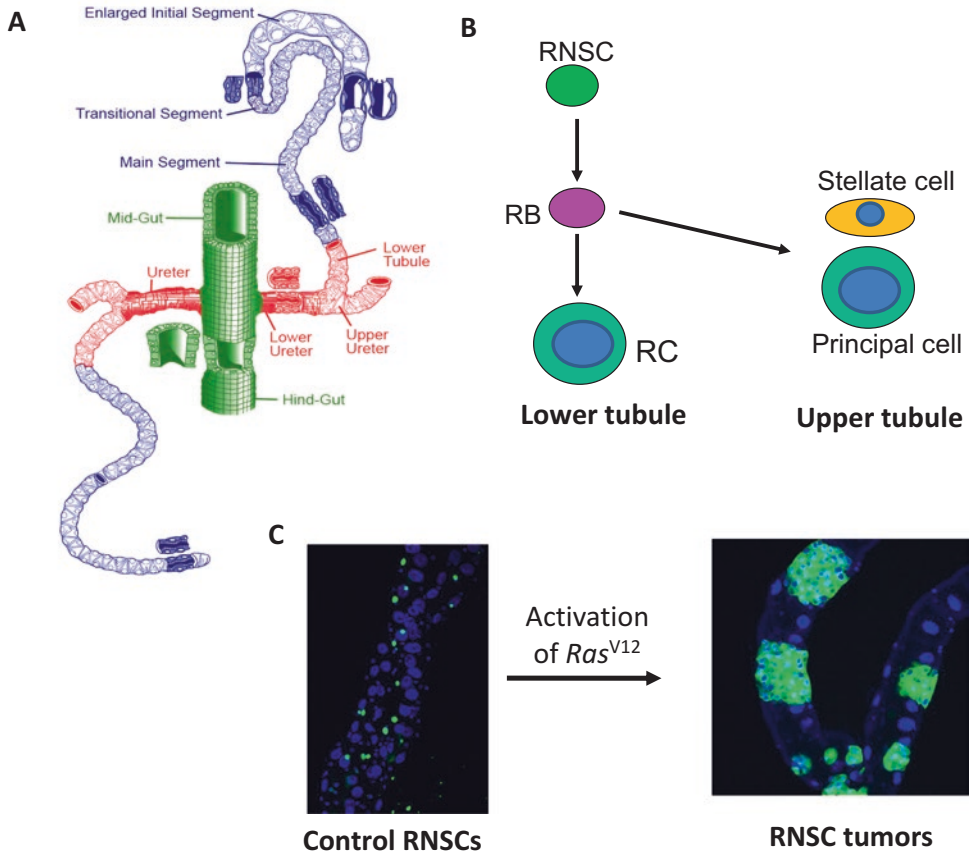

Fig. 10.3 (**a**) Drawing of the *Drosophila* MTs (adapted and modified from [81]). (**b**) Model of the RNSC lineage (Modified after [69]). (**c**) Normal RNSC (left panel) and activation of the oncogene Ras results in stem cell tumor in adult *Drosophila* kidney

to the main segments compared to wild-type RNSCs, which remained in the lower tubule and ureter of the MTs [89]. In addition, the metalloproteinase 1 (MMP1) was also highly expressed in the Ras^{V12}-transformed RNSCs compared to wild-type RNSCs. Zeng et al. [90] further showed that an MMP1 inhibitor suppressed the Ras^{V12}-transformed RNSC phenotypes, which suggest that transformed stem cells were very motile, but their mobility was restricted to main segments. Even though these transformed stem cells were highly proliferative, their transplantation studies suggest that these tumors were not metastatic, but need further long-term investigation [89]. Fourth, the cortical expression of cell-polarity markers (Bazooka (Baz) and *Drosophila* atypical protein kinase C (DaPKC)) were found in Ras-transformed RNSCs but these markers were restricted to apical crescent at metaphase in the normal RNSCs that suggest that activated Ras may disrupt the polarity and asymmetric division of RNSCs [89].

In conclusion, Ras^{V12}-transformed RNSCs are highly proliferative, have low cell death, disrupted cell polarity, poor differentiation, and highly migrating nature compared to normal RNSCs. These together suggest that the Ras^{V12}-transformed RNSCs display most of the hallmarks of human cancer and could portray true CSCs [89].

10.6.1.7 Signaling Downstream of Ras Regulates RNSC Transformation

In the last several decades, many Ras downstream effectors that control complex signaling networks have been identified [63]. To identify which signaling pathways mediate the Ras activity in RNSC transformation, Zeng et al. [89] preformed a screen and found that the expression of a dominant-negative form of Raf or Rho A or an RNAi of MEK (Dsor1) inhibited the Ras^{V12} phenotypes in MTs. Zeng et al. [89] also performed a screen on 22 commercially available protein kinase or proteinase inhibitors and reported that inhibitors of protein kinase A (PKA), Tor, or MMP1 significantly inhibited the Ras^{V12} phenotypes in MTs. Further, they found that the genetic loss of PKA or Tor also inhibited the Ras^{V12} phenotypes in MTs.

Further, by expressing constitutively active Raf, RhoA, or ERK (rl^{Sem}) or cbl RNAi (cbl^{RNAi}, unpublished result) in RNSCs we found that only the constitutively active Raf and cbl^{RNAi} were able to form stem cell-tumor in MTs.

In summary, the above results suggest that RNSC transformation has the following properties (Fig. 10.4). (i) Raf loss suppresses the Ras^{V12} phenotype, and the expression of a constitutively active form of Raf or cbl^{RNAi} transforms RNSCs into CSCs (just like Ras^{V12}), suggesting that Raf is an immediate target of Ras and that Cbl negatively regulates the Ras/Raf signaling. (ii) The down-regulation of RhoA or MEK activity or the inhibition of Tor or PKA activity suppressed the Ras^{V12} phenotype, but the expression of a constitutively active form of RhoA or ERK did not transform RNSCs into CSCs, suggesting that these signaling pathways cooperatively mediate Ras' function in RNSC transformation, and that none of them alone is enough to transform the stem cells. (iii) An autocrine JAK/STAT signaling system regulates the *Drosophila* RNSCs because both the ligand Upd and its receptor Dome express in MTs, which activates JAK/STAT signaling. This autocrine JAK/STAT signaling regulates the RNSCs' self-renewal. However, mutations in *stat92E* (signal-transducer and activator of transcription protein at 92E) did not inhibit the Ras-transformed RNSC tumor phenotype, which suggest that the growing Ras-transformed RNSCs are not dependent on JAK-STAT signaling for their growth and that these transformed stem cells are niche-independent.

10.6.1.8 New Genes that Mediate the Ras Activity in RNSC Transformation

To dissect the molecular mechanism that regulates the Ras-mediated transformation of RNSCs,

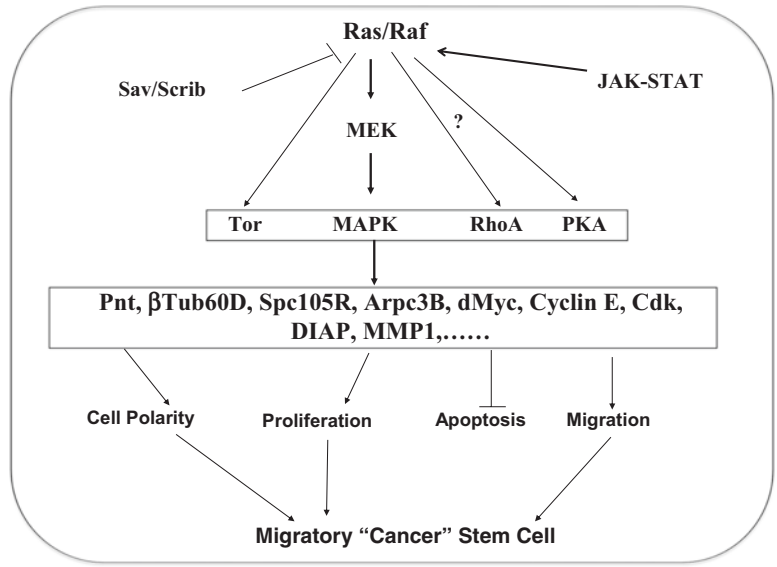

Fig. 10.4 Signaling pathways downstream of Ras that regulate RNSC transformation in MTs

Zeng et al. [89] performed transcriptome comparisons of wild-type RNSCs and Ras-transformed RNSCs using a microarray assay and identified 186 genes, which showed highly different expression. Zeng et al. [89] further examined the genetic interactions between Ras^{V12} and 147 genes using UAS-RNAi lines and identified 20 genes whose RNAi dramatically inhibits the Ras-transformed phenotypes. Some of these genes include replication protein A2 (RPA2), β-tubulin at 60D (βTub60D), actin-binding protein Arpc3B, mitotic spindle protein Spc105R, transcription factor point (pnt), and lipid phosphatase Wun2. Further investigation of these genes could be useful in the development of specific anti-tumorigenic stem-cell cancer therapies.

10.7 Therapy Resistance of Normal and Tumorigenic Stem Cells in *Drosophila*

10.7.1 Strategy to Protect Female Germline Stem Cells from IR-Induced Death in Drosophila

Normal stem cells and CSCs share various features, such as self-renewal and multi-lineage differentiation and show resistance to radio-and chemotherapies. Xing et al. [83] using *Drosophila* ovary system reported that female germline stem cells (GSCs) use a "dying daughters protect their mother" strategy to maintain GSCs under pathological conditions. After exposure to radiation, dying daughter cells release a protein, Pvf1 that is like a human angiopoietin and which binds to Tie receptors on nearby mother stem cells. This protein-receptor binding causes the GSCs to produce a microRNA bantam, that represses the generation of Hid/Diablo/Smac, needed to trigger apoptosis and protect the GSCs from IR-induced apoptosis. It is possible that a similar mechanism may function in CSCs, which can be investigated in future experiments.

10.7.2 Intestinal Stem Cells Are Resistant to Apoptosis but Sensitive to Blockage of Lipolysis in Drosophila

To examine the response of the different cells to cell-death effectors, we overexpressed *reaper* (*rpr*, an inhibitor of *Drosophila inhibitor of Apoptosis-1; DIAP-1*) in differentiated enterocytes (ECs), the intestinal stem cells (ISCs), the renal and nephric stem cells (RNSCs), and the quiescent hindgut intestinal stem cells (HISCs), using the cell-type-specific Gal4s [70]. The induction of *reaper* in differentiated ECs for 12 h causes widespread apoptosis but has little effect on stem cells (i.e., ISCs, RNSCs, HISCs), even after 7 days of induction in stem cells, suggesting that stem cells are internally resistant to apoptosis [70].

10.7.3 Activation of Proliferation Accelerates Apoptotic Cell Death of Normal but Not Transformed Stem Cells

In mammals, quiescent normal hematopoietic stem cells (HSCs) and leukemic stem cells (LSCs) show significant resistance to radiation and anti-proliferative chemotherapy but may be eliminated by a two-step protocol involving initial activation by IFNα (interferon alpha) or G-CSF (granulocyte-colony stimulating factor), followed by targeted chemotherapy [16]. In the *Drosophila* adult digestive system, JAK/STAT signaling regulates the activation and proliferation of ISCs, RNSCs, and HISCs [29, 39, 69, 76]. Drawing on Essers and Trumpp [16], we therefore conducted a two-step protocol in the *Drosophila* stem cells consisting of an initial activation by overexpressing the JAK/Stat pathway ligand *unpaired* (*upd*), followed by *rpr* induction. Induction of *upd* + *rpr* in stem cells precisely killed all of the ISCs and RNSCs in 4 days [70].

Overexpression of Ras (Ras^{V12}) in RNSCs or and the knockdown of N activity in ISCs can

transform them into actively proliferating CSC-like cells [54, 89]. We found that expressing *rpr* in Ras^{V12}-transformed RNSCs or in ISCs expressing a (N^{DN} killed only a portion of the transformed RNSCs and of few transformed ISCs. However, a significant populations of cells remained even after 1 week of rpr induction [70].

These data together suggest that when normal stem cells proliferation are induced they can be eliminated through apoptotic cell death pathway, however, actively proliferating transformed stem cells of MTs and gut are still mainly resistant to apoptotic cell death [70].

10.7.4 Knockdown of the COPI/Arf1-Mediated Lipolysis Pathway Kills Normal and Transformed Stem Cells but Not Differentiated Cells

In a genome-wide screen of the adult *Drosophila* digestion system using transgenic RNAi lines, we found that knockdown of the COPI/Arf1 (coat protein complex I/ADP-ribosylation factor 1)-mediated lipolysis pathway kills normal and transformed stem cells but not differentiated cells [70, 91].

The COPI and COPII (coat protein complex II) complexes are essential components of the trafficking machinery for vesicle transportation between the ER and the Golgi (reviewed in Lee et al. [36]). The COPII complex mediates vesicle cargo transport from the ER to the Golgi, while the COPI complex mediates cargo transport from the Golgi back to the ER. In addition, the COPI complex regulates the transport of lipolysis enzymes to the surface of lipid droplets for lipid droplet usage [5, 73]. In our screen, we did not identify any COPII complex components whose RNAi-mediated knockdown resulted in stem-cell death, suggesting that lipid droplet usage (lipolysis) rather than the general trafficking machinery between the ER and Golgi is important for stem-cell survival. We found that knockdown of almost all components of the COPI/Arf1 complex selectively kill stem cells but not differentiated cells. These components include Arf79F [*Drosophila* homologue Arf1 of guanine-nucleotide-binding (G) proteins], Garz (*Drosophila* homologue of Arf1 Guanine nucleotide exchange factor GBF1), and most components of the vesicle-mediated COPI transport complex [91].

Acyl-CoA synthetase long-chain (ACSL) [55, 92] and a very long-chain fatty acid-CoA ligase (bubblegum, bgm) [47] are enzymes that convert free fatty acids into acyl-CoA in the *Drosophila* lipolysis/β-oxidation pathway. We reported that knockdown of ACSL and Bgm also effectively killed ISCs and RNSCs [70]. Scully (scu) is the *Drosophila* ortholog of hydroxy-acyl-CoA dehydrogenase [55], an enzyme in the β-oxidation pathway. Hnf4 regulates the expression of several genes involved in lipid mobilization and β-oxidation [55]. Overexpression of Scu or Hnf4 could significantly rescue stem cell death induced by knocking down Arf1, suggesting that the whole lipolysis pathway is required for stem cell survival [70].

We further generated *δ-COP* and *γ-COP* mutant clones using the mosaic analysis with a repressible cell marker (MARCM) technique [35] and found that the COPI complex cell-autonomously regulates stem cell survival [70]. In addition, we found that a lipolysis reporter is specifically expressed in stem cells but not differentiated cells [70]. These data together suggest that the COPI/Arf1-mediated lipolysis pathway selectively sustain stem cells.

10.7.5 Knockdowns of the COPI/Arf1-Lipolysis Pathway Kill Stem Cells through Necrosis

We further investigated the intracellular pathway that directly regulates stem-cell death induced by knocking down the COPI/Arf1 complex genes. We found that the stem cell death induced by knocking down of the COPI/Arf1-lipolysis pathway is not through caspase-mediated apoptosis and autophagy-regulated cell death [70].

A third type cell death, necrosis, is defined by early plasma membrane rupture, activation of lysosomal catabolic enzymes causing reactive oxygen species (ROS) accumulation, and intracellular acidification [33, 77]. Propidium iodide (PI) detects necrotic cells with compromised membrane integrity, the oxidant-sensitive dye dihydroethidium (DHE) indicates cellular ROS levels, and LysoTracker staining detects intracellular acidification [77]. We examined markers of necrosis and found that knocking down the COPI/Arf1 complex induces stem cell death through necrosis [70].

10.7.6 Dying ISCs Are Engulfed by Neighboring ECs through the Draper-Rac-JNK-Autophagy Pathway

We noticed cases where the GFP (green fluorescent protein)-positive material of the dying ISCs was present within neighboring ECs, suggesting that these ECs had engulfed dying ISCs [70]. We further investigated whether engulfment genes, JNK pathway, autophagy, or caspases are required for ISC death. We found that the phagocytotic receptor Draper-Rac-JNK-autophagy pathway in ECs controls the engulfment of dying ISCs (Fig. 10.5; [70]).

10.7.7 Arf1 Inhibitors Selectively Suppress Cancer Stem Cells

Our finding that the COPI/Arf1 complex regulated transformed stem-cell survival in the fly led us to ask whether the COPI/Arf1 complex has a similar role in CSCs. We tested the Arf1 inhibitors on the human prostate cancer DU-145, colon cancer HT29 and breast cancer MCF7 cells [70]. We found that the inhibitors inhibited tumor sphere formation by cancer cells [70], a widely used in vitro technique for assessing CSC self-renewal capacity [14]. These results suggest that inhibiting Arf1 activity or blocking the lipolysis pathway can kill CSCs and block tumor growth in mammal.

10.8 Summary and Prospective

Normal stem cells and CSCs share various features, such as self-renewal and multi-lineage differentiation and show resistance to radiation and chemotherapies. Accumulating evidence suggests that the behavior of normal stem cells and CSCs is controlled by a tissue-specific niche microenvironment. Although the strategies so for suggested would be helpful in developing anti-CSC drugs for cancer, not all strategies should be active in each CSC in tumor tissues. Therefore, identifying novel and effective drugs will be crucial to CSCs for treating various types of cancer

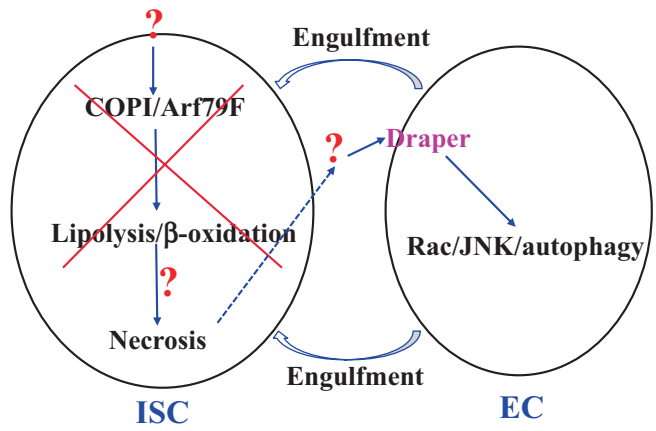

Fig. 10.5 The lipolysis pathway regulates stem cell survival

[11]. We investigated stem cell death in the adult *Drosophila* digestion system. We found that apoptosis activation selectively killed differentiated cells but not quiescent or transformed stem cells, while knockdowns of the COPI/Arf1 complex killed quiescent and transformed stem cells (but not differentiated cells) through necrosis, by attenuating the lipolysis pathway. Furthermore, Arf1 inhibitors selectively blocked CSCs in human cancer cell lines. Our results suggest that quiescent stem cells or CSCs are metabolically unique; like hibernating animals, they mainly rely on lipid reserves for their energy supply and blocking lipolysis can starve them to death.

Quiescent stem cells often reside in a secluded location surrounded by dense extracellular matrix and a dormant hypoxic storage niche [78]. Such an environment makes them less accessible to sugar and amino acid nutrition from the body's circulatory system. Like hibernating animals, the quiescent stem cells may mainly rely on lipid reserves for their energy supply and blocking lipolysis may starve them to death.

Most of the current therapeutic strategies targeting CSCs involve inhibiting the self-renewal or survival pathways in these cells, including Notch, Hedgehog, Wnt, AKT, and TGFβ (transforming growth factor beta) [32, 50]. Because these pathways play important roles in normal cells, inhibiting them could result in systemic toxicities, limiting the clinical usefulness of this approach. Normal cells mostly rely on sugar and amino acids for their energy supply, with lipolysis playing only a minor role in their survival. Thus, blocking lipolysis may selectively kill CSCs but not normal cells. In support of this idea, the depletion of COP subunit ζ1 induces the death of tumor cells but not normal cells [68], and oral administration of the Arf1 inhibitor AMF-26 (M-COPA [2-methylcoprophilinamide]) elicits strong antitumor activity without severe body weight loss in mouse xenografts of BSY-1 human breast cancer cells [52]. Therefore, targeting the COPI/Arf1 complex or the lipolysis pathway may prove to be a well-tolerated, novel approach for eliminating CSCs.

The plasticity of tumor cells is well known [4]. CSCs can be re-created if non-CSCs and the tumor microenvironment remain intact, and only killing CSCs is not enough to elicit tumor regression. The ideal therapy should not only kill CSCs but also alternate their microenvironment. As described above, we found that ablation of the Arf1-mediated lipid metabolism not only kill stem cells but also activate the neighboring differentiated ECs in *Drosophila*. Further study the detail molecular mechanism may uncover the means that not only kill CSCs but also alternate their microenvironment to elicit tumor regression. Adult *Drosophila* stem cell tumor systems will be useful models to understand the properties of CSC and together with drug screening, it will ultimately help in developing novel drugs to treat resistant cancer.

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Drosophila as a Model for Tumor-Induced Organ Wasting

11

Pedro Saavedra and Norbert Perrimon

Abstract

In humans, cancer-associated cachexia is a complex syndrome that reduces the overall quality of life and survival of cancer patients, particularly for those undergoing chemotherapy. The most easily observable sign of cachexia is organ wasting, the dramatic loss of skeletal muscle and adipose tissue mass. Estimates suggest that 80% of patients in advanced stages of cancer show signs of the syndrome and about 20% of cancer patients die directly of cachexia. Because there is no treatment or drug available to ameliorate organ wasting induced by cancer, cachexia is a relevant clinical problem. However, it is unclear how cachexia is mediated, what factors drive interactions between tumors and host tissues, and which markers of cachexia might be used to allow early detection before the observable signs of organ wasting. In this chapter, we review the current mammalian models of cachexia and the need to use new models of study. We also explain recent developments in *Drosophila* as a model for studying organ wasting induced by tumors and how fly studies can help unravel important mechanisms that drive cachexia. In particular, we discuss what lessons have been learned from tumor models recently reported to induce systemic organ wasting in *Drosophila*.

Keywords

Drosophila · Cachexia · Muscle · Fat body · Organ wasting

11.1 Cancer-Induced Cachexia

Cachexia induced by cancer is characterized by increased systemic inflammation, general metabolic dysfunction, and elevated resting energy expenditure; it can be accompanied by anorexia and loss of appetite but it is not usually reversed by increasing nutritional intake [55]. All of these symptoms lead to a progressive loss of body weight due to organ wasting, particularly of the skeletal muscle and, in many cases, of adipose tissue and fat reserves [6, 56, 113, 133, 147]. Even though cachexia is often observed in a high proportion of cancer patients and correlates with poor life expectancy and reduced quality of life, the mechanisms driving this syndrome are poorly understood and efficient treatment therapies are needed [56, 91].

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Most of our knowledge of cachexia induced by cancer has been acquired from studies using rodent models [13, 17]. The induction of cachexia has no clear correlation with either tumor mass or tumor type [49, 71, 98], yet it is widely accepted that circulating factors secreted directly by tumor cells or from normal host cells cause wasting [56, 147]. Lysates of Krebs2 carcinoma samples, when injected in mice, reduce fat levels and cause weight loss, arguing for the presence of a wasting-inducing factor [42]. In a study using extracts of mouse thymic lymphoma, a soluble lipid-mobilizing factor (LMF) derived from the cancer cells also induced lipolysis [83]. A distinct LMF, isolated from human melanoma A375 cells [143], and later found also in MAC16 mouse adenocarcinoma cells and urine samples of cancer patients, was further identified as zinc-a2-glycoprotein [150]. Interestingly, MAC13 cells, derived from the same tumor type as MAC16, are histologically similar and have a similar growth rate, but do not produce zinc-a2-glycoprotein and do not provoke weight loss, arguing that similar types of tumor can have different potential to induce cachexia [98]. Further, a secreted proteolysis-inducing factor (PIF) isolated from both MAC16 cells and human tumor samples was shown to cause muscle-specific proteolysis [148, 149]. Taken together, these examples shed light on the heterogeneity of tumor-secreted factors that can induce cachexia independently of the type of tissue that originates the tumor.

Cachexia involves systemic inflammation, and several pro-inflammatory cytokines, either derived from host tissues or from tumors, have been shown to have a relevant role in cachexia [7]. Muscle samples incubated with interleukin-1 (IL-1), a cytokine usually produced by human leukocytes in the context of sepsis, exhibit increased proteolysis and signs of wasting [15]. Tumor necrosis factor alpha (TNF-α), initially called "cachectin" [19], was also shown to induce organ wasting in mouse models [103], as was IL-6 [25, 141]. Although rodent models have been crucial to understanding cachexia, data from human patients has not always correlated with mouse models, particularly since there is no clear link between circulating levels of TNF-α in the serum of cancer patients and their respective weight loss [93]. Further, the observation that antibodies against TNF-α in cancer patients do not improve prognosis puts in question the role of TNF-α in cachexia [77]. Regarding IL-6, higher circulating levels of this cytokine in patients with lung cancer correlates with lower survival rates [99, 134, 142]. In mouse models, higher levels of IL-6 are linked to higher tumor burden and decreased survival in the presence of certain tumor types [141]. However, overexpression of IL-6 in tumor-free mice does not cause organ wasting, indicating a tumor-dependent role of IL-6 in organ wasting [14]. Moreover, circulating cytokines can directly promote tumor growth [31] or stimulate production of tumor-derived factors [24] and, consequentially, influence the development of cachexia. These synergistic interactions emphasize the difficulty in understanding the role of cytokines and other secreted factors in cancer-induced cachexia when relying only on data from rodent models [13, 108]. It also highlights that using other model organisms to study organ wasting might help to expand our understanding of cachexia.

11.2 Muscle Wasting

Loss of skeletal muscle mass is a hallmark manifestation of cancer-induced cachexia and results from an imbalance in the coordination between protein synthesis and protein degradation [52, 63, 79, 132]. In skeletal muscle, protein synthesis is mainly regulated by the insulin-like growth factor 1 (IGF1) [39, 99]. IGF1 activates the PI3K/AKT pathway, which stimulates expression of downstream target genes involved in protein synthesis and hypertrophy of muscle fibers [27, 120]. Conversely, proteolysis can be caused by different catabolic inputs such as starvation, denervation, or cachexia, which typically lead to increased activity of the ubiquitin-proteasome system and the autophagy/lysosome pathway [10, 88, 94, 125].

The proteasome system acts in the muscle by two muscle-specific E3 ubiquitin ligases, the muscle RING finger-containing protein 1, MuRF1, and the muscle atrophy F-box protein, MAFbx [38]. Both are upregulated under several catabolic states and are extensively used as mark-

ers of muscle wasting; deletion of either of these two ubiquitin ligases in mice ameliorates skeletal muscle atrophy [26, 64]. In addition, both are transcriptionally controlled by Forkhead box O (FoxO) transcription factors, which are negatively regulated by the insulin/AKT pathway, putting both MuRF1 and MAFbx under direct control of the IGF-1 pathway [96, 130, 140]. MuRF1 and MAFbx act in the muscle by ubiquitinating specific proteins and targeting them for degradation by the proteasome system: MuRF1 mediates ubiquitination of myosin heavy chain (MyHC) and other thick filaments that compose the muscle fibers [35, 37]; MAFbx targets both eIF3-f, a translation initiation factor, and MyoD, a key regulator of myoblast identity and differentiation [87, 145]. Notably, the latter finding suggests that MAFbx acts by suppressing protein synthesis rather than by increasing proteolysis of muscle fiber components [11].

The autophagy/lysosome pathway can also be elevated during muscle wasting [129]. Muscle denervation or starvation induces FoxO3-mediated expression of autophagy-related genes in mouse skeletal muscle [94, 96] and in *in vitro* models of C2C12 myoblast cells [163]. Importantly, the autophagy/lysosome pathway has also been shown to be upregulated in muscles of mice with cachexia [10, 110]. Bnip3, a member of the Bcl-2 family of apoptosis regulators, is a mediator of autophagy [67, 94, 151], and is upregulated during wasting [10], making it a relevant gene involved in muscle wasting.

11.3 Adipose Tissue Wasting

In many cases of cancer-induced cachexia there is loss of adipose tissue [50, 124, 152]. Fat accumulates in the form of triglycerides in lipid droplets (LDs) located in the cytoplasm of adipocytes. Brown adipocytes form the brown adipose tissue, whereas white and beige adipocytes constitute white adipose tissue (WAT) [36]. Brown and beige adipocytes have higher mitochondria content than white adipocytes, and brown adipocytes express higher levels of uncoupling protein 1 (UCP-1) to generate heat in response to cold stress through a process called thermogenesis [107, 155]. In rodent models of cachexia, browning of WAT by activation of UCP-1 in beige cells increases energy expenditure [112], and is induced by the parathyroid-related peptide (PTHrP), a tumor-secreted factor [82].

WAT is the storage tissue in mammals for energy reserves, and works as an endocrine organ that controls general metabolic homeostasis [60]. In cancer patients with cachexia, loss of adipose tissue seems to be due to excessive lipolysis in LDs, rather than reduction of lipid synthesis [44, 123]. Lipolysis is driven by a cascade of three lipases, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase, which sequentially process triglycerides into diacylglycerol, monoacylglycerol, and finally glycerol and fatty acid that are further released into circulation [159]. The specific removal in adipose tissue of ATGL or HSL prevents excessive lipolysis and wasting of adipose tissue in a mouse model of cachexia [45]. Furthermore, in cancer patients with cachexia, some tumors may lead to increased activity of ATGL and HSL [45], whereas other tumors only induce activity of HSL [3], arguing for a role of both lipases in induction of adipose tissue wasting by stimulating lipolysis [44, 123].

Adipose tissue loss has been shown to precede skeletal muscle wasting in patients with cachexia [59]. In addition, the removal of either ATGL or HSL from adipose tissue also protects skeletal muscle from wasting [45], suggesting a link between excessive lipolysis in adipocytes and subsequent induction of muscle wasting. An excessive rate of lipolysis increases the cellular levels of lipids in the muscle, leading to insulin resistance and glucose metabolism impairment [127, 158]. Although the mechanisms of insulin resistance induced by lipid accumulation are unclear, it has been hypothesized that intracellular accumulation of diacylglycerol in muscles activates a subgroup of protein kinase C (PKCs) that inhibit the insulin receptor, causing skeletal muscle insulin resistance [33, 77, 159]. Interestingly, insulin resistance is present in both human patients and mouse models of cachexia [73, 158], although in some clinical cases of cachexia there is a lack of correlation between tumor-secreted factors and insulin resistance [2]. Nevertheless,

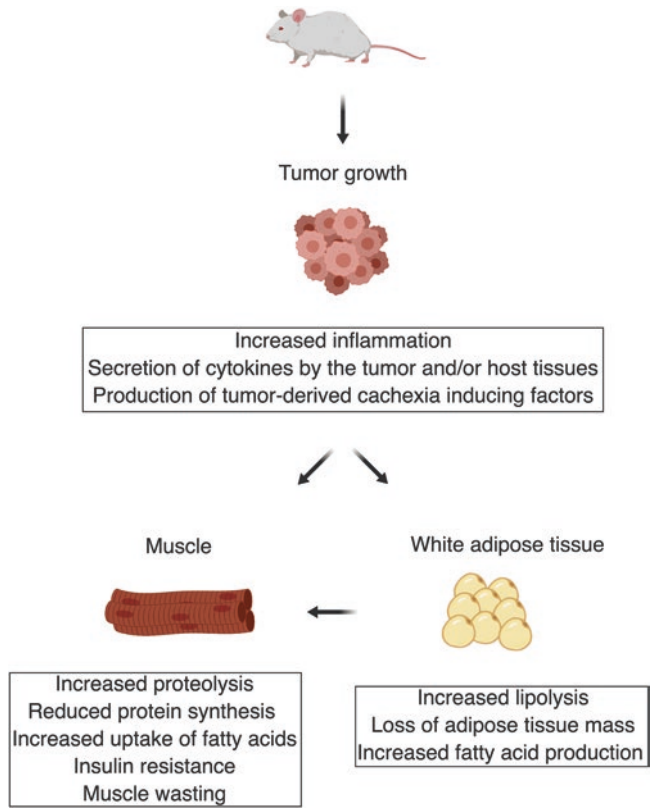

Fig. 11.1 Cancer-induced cachexia in mammals. Tumors promote a systemic inflammatory response and pro-inflammatory cytokines produced by either tumor or host tissues induce organ wasting. Tumors can also produce other cachexia-inducing factors that promote muscle and white adipose tissue wasting. In the muscle, wasting is caused by excessive proteolysis and reduction of protein synthesis. Wasting of the white adipose tissue consists of increased lipolysis, loss of fat, and production of circulating fatty acids. The excessive uptake of fatty acids by muscles leads to tissue-specific insulin resistance that can also contribute to muscle wasting

changes in glucose metabolism in skeletal muscle and adipose tissue correlate with organ wasting [73]. Therefore, excessive lipolysis causing insulin resistance and impaired glucose metabolism in the muscles may lead to a synergistic effect that, when combined with the activity of circulating factors derived from tumors or host tissues, induces muscle wasting [6] (Fig. 11.1).

In summary, cachexia is a syndrome with several layers of complexity. Tumors and host tissues can produce different types of circulating factors that cause systemic metabolic impairment and organ wasting. These factors induce catabolism, lipolysis, or proteolysis directly in muscles and adipose tissues, and/or work synergistically to promote tumor growth. Excessive lipolysis in adipose tissues can lead to insulin resistance in the muscle and decreased anabolism, which may contribute to the wasting process. Moreover, data from rodent models of cachexia does not always reflect what is observed in human patients. As such, as well as due to the extreme heterogeneity of cancer and cachexia, it has been very difficult to pinpoint if there is a general mechanism of systemic organ wasting, and therefore other models of research are necessary.

11.4 *Drosophila* as a Model to Study Cancer

The *Drosophila* genome encodes orthologs of many human genes associated with diseases, and several fly organs have analogous functions to human organs [119, 122, 153]. The repertoire of genetic tools available in *Drosophila* includes the GAL4/UAS system for tissue specific modulation of gene expression and the FLP/FRT MARCM system to induce and label mosaics of genetically distinct cells in a specific tissue [28, 46, 157]. In addition, an extensive collection of fly strains for RNAi and overexpression, covering most fly genes, allows for spatially-controlled knockdown or ectopic expression of any gene of interest. Moreover, some of the most important signaling pathways involved in cancer were first discovered in flies [111], making *Drosophila* an invaluable model for the study of human diseases, including cancer [34, 153].

Despite being short-lived animals, flies can spontaneously develop tumors [139]. Tumors can also be readily induced by ectopic expression of oncogenes or disruption of tumor-suppressor genes in target tissues [23, 62]. *Drosophila* tumors display the typical hallmarks of cancer, namely resistance to apoptosis, chronic mitogenic signaling, evasion of tumor suppressor action, genomic instability, metabolic alterations, and invasion of tissues [68]. Given this, the use of *Drosophila* as a model to study cancer has revealed new genes involved in tumorigenesis and contributed to our understanding of the mechanisms of tumor growth and metastasis [54, 97, 137, 146].

Both fly larvae and adult stages have been used to study tumor development [65, 146]. In larvae, tumor models have been established in various tissues: lung cancer in the trachea [90], gliobastoma in the brain [118, 154], rhabdomyosarcoma in muscle [61], and leukemia in hemocytes [43]. However, the imaginal discs have been the most-used tissues for study of tumorigenesis [69]. Imaginal discs are epithelial tissues composed of highly proliferative diploid cells, making them an easy tissue in which to induce gene knockout or overexpression mosaics of cells [157]. Several signaling pathways that can drive cancer in mammals have been manipulated in the imaginal discs to induce tumors, including EGFR-Ras-Raf, Hippo-Salvador-Warts, TGFbeta, Notch, JAK/STAT, and have been extensively studied in the context of tumorigenesis [69].

Most genetic manipulations used to model tumors in larvae lead to hyperplastic tumors but some become metastatic and invade other tissues [22, 62, 97]. Expression of an activated form of Ras ($Ras^{[V12]}$) or activated Notch ($Notch^{[ACT]}$) in clones in imaginal discs, for example, induces overproliferation and hyperplastic growth [30, 80]. Similarly, ablation of the cell polarity genes *scribble (scrib), discs-large-1 (dlg1)* or *lethal (2) giant larvae (lgl)* drives loss of apico-basal polarity and induces hyperplasia [23]. However, when a mutation for a polarity gene is combined with overexpression of either $Ras^{[V12]}$ or $Notch^{[ACT]}$, cells become severely malignant, invade other tissues, and induce secondary tumor growths [30, 104].

The study of tumor progression in adult flies has mainly consisted of dissecting and transplanting larval tumors into adult flies [121]. Unlike malignant tumors, benign tumors transplanted into adult flies do not display metastatic behavior, such that the transplantation method provides a way to distinguish between neoplastic and hyperplastic tumors [62]. An alternative to the transplantation method is to generate tumors directly in adult fly tissues [65]. One example of this strategy is the overexpression of an activated form of Yorkie (Yki), a transcriptional co-activator of the Hippo pathway, in adult stem cells of the intestine, which generates gut tumors [81, 135]. Other adult tissues used as sites for induction of tumor formation include the Malpighian tubules [161], germline [126], brain [16, 92], and hemocytes [5], arguing that induction of tumors in adult flies is a valid alternative to the transplantation method of larval-induced tumors.

11.5 *Drosophila* as a Model of Cachexia

Developmental biology studies in *Drosophila* have unraveled important signaling pathways that are implicated in cancer [111]. Moreover, *Drosophila* has been used as model to study tumorigenesis, but it has only recently been considered as a model of organ wasting [54, 69, 137]. Two independent studies have described how tumor progression in adult flies induces phenotypes consistent with organ wasting, in a manner similar to what is observed for rodent and human models [57, 86]. In one study, the authors induced tumors in the adult midgut by specifically expressing Yki in intestine stem cells (gut yki-tumors) [86]. In the other study, the authors generated neoplastic tumors in the eye disc by inducing clones of cells mutant for *scribble* while ectopically expressing $Ras^{[V12]}$ ($Ras^{[V12]}$/*scribble*) and transplanted the tumors into adult flies [57].

In both tumor models, flies display a loss of muscle function and severe wasting of the ovaries and fat body. Interestingly, both tumors secrete high levels of *Imp-L2*, an insulin-like binding peptide, and flies show reduction of systemic insulin signaling, while being hyperglycemic, suggesting that the peripheral tissues become insulin resistant [57, 86]. Ectopic expression of *Imp-L2* is sufficient to induce wasting, whereas suppressing expression of *Imp-L2* specifically in the tumors significantly ameliorates the wasting phenotype [57, 86]. Transplanted discs with yki-induced tumors have lower production of Imp-L2 and do not cause wasting, even though their tumor burden is larger than that of $Ras^{[V12]}$/*scribble* tumors [57]. Therefore, high levels of Imp-L2 induce organ wasting independently of the tumor burden, making Imp-L2 a novel tumor-secreted factor that can cause organ wasting. Notably, IGF-binding protein-3 (IGFBP-3) was found to be upregulated in pancreatic cancer samples of human patients, supporting the possibility of a role for insulin binding peptides in induction of organ wasting [74].

Imp-L2 is a circulating peptide that forms a ternary protein complex with the acid-labile subunit of the IGF1-binding protein, dALS, and with the *Drosophila* insulin-like peptides (Dilps) [9]. Increased levels of circulating Imp-L2 correlate with systemic insulin signaling reduction, although it is unclear how binding of Imp-L2 to circulating Dilps modulates insulin signaling [4, 58]. Under starvation, the fat body, the fly counterpart organ of WAT in mammals, produces Imp-L2 to protect flies from starvation by reducing systemic insulin signaling [72]. The fact that Imp-L2 is induced by starvation raises the question of whether yki-tumors drive organ wasting by simply disrupting the basic gut functioning of food intake, inducing general starvation [86]. However, the feeding behavior of flies with gut yki-tumors and the expression of *pepck,* which is upregulated during starvation conditions, are not severely affected, suggesting that Imp-L2 is not simply increased due to starvation caused by the impairment of gut function [86]. More importantly, both yki-tumors in the gut and imaginal disc tumors produce Imp-L2, suggesting that tumor-driven wasting by Imp-L2 might be a general mechanism to "starve" the peripheral tissues by reducing insulin signaling (Fig. 11.2).

Interestingly, the insulin pathway, and in particular the rate-limiting enzymes involved in the glycolytic pathway, are highly active in yki-tumors in the gut, despite the fact that the peripheral organs show reduced insulin signaling [86]. This evidence highlights the importance of glucose metabolism in supporting growth of yki-tumors, and is in accordance with other tumor models in which high-sugar diets promote malignant growth in imaginal discs [53, 70].

Another study reported that yki-induced tumors in the intestine of adult flies also secrete a PDGF- and VEGF-related factor 1 (Pvf1) ligand that leads to a pathological activation of ERK/MAPK signaling non-autonomously in host tissues to induce wasting of muscles and the fat body [136]. Similarly, in a mouse model of cachexia, ERK signaling was increased in skeletal muscle, leading to upregulation of MAFbx and increased proteolysis [109]. In both studies, pharmacological inhibition of the ERK pathway ameliorated the wasting phenotype caused by tumors, independently of affecting tumor growth [109, 136]. As such, these results support a role for ERK signaling in promoting catabolism in peripheral tissues like the muscle [109, 136].

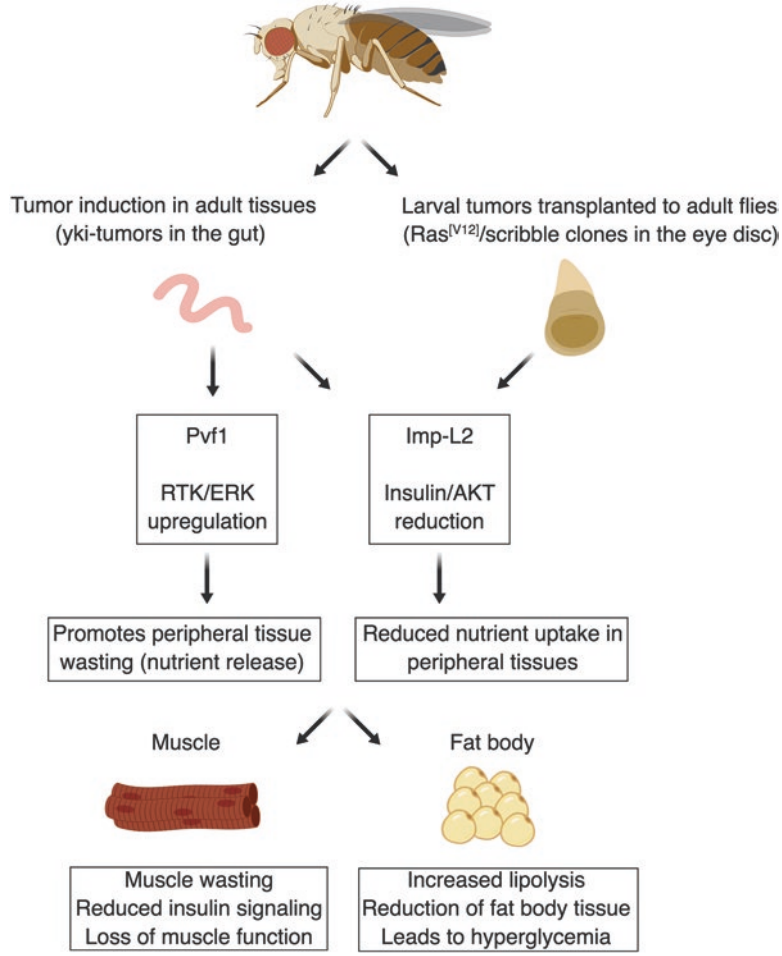

Fig. 11.2 Putative model of organ wasting induced by Pvf1 and Imp-L2 in *Drosophila*, using two different types of tumors. One tumor is generated by overexpressing Yki in the stem cells of the adult intestine. The other type of tumor is formed by transplantation to adult flies of larval imaginal discs that have clones of cells that express an activated form of Ras ($Ras^{[V12]}$) and are mutant for the polarity gene *scribble* ($Ras^{[V12]}$/*scribble*). Both tumor types produce Imp-L2, but yki-tumors in the adult gut also secrete Pvf1. $Ras^{[V12]}$/*scribble* transplanted discs induce organ wasting by overproducing Imp-L2. However, in gut yki-tumors, wasting is thought to be driven by a combination of Pvf and Imp-L2. Increased levels of circulating Imp-L2 reduce systemic insulin signaling, which leads to a reduction of nutrient uptake by muscle and adipose tissue, and further drives organ wasting. Pvf1 activates ERK signaling in the peripheral tissues and possibly reinforces the wasting of muscle and fat body already caused by Imp-L2

In summary, the studies describing organ wasting in the fly [57, 86, 136] suggest that *Drosophila* can be a useful model to study tumor-induced organ wasting (Fig. 11.2). Furthermore, the fly genetic tools, short generation time, and conservation of signaling pathways that induce tumors make *Drosophila* an important alternative to rodent models of cachexia.

11.6 *Drosophila* as a Model for Studying Muscle Wasting

Drosophila muscles are composed of actomyosin cables formed by repetitive contractile units – the sarcomeres – and share both functional and structural similarities with mammalian skeletal muscles [114, 116, 138]. Growth and atrophy of muscle are regulated by insulin signaling in a

manner similar to what is observed for vertebrate skeletal muscle [48]. Moreover, specific reduction of insulin signaling in muscles promotes FoxO-mediated expression of autophagy genes [12, 48]. However, although MAFbx and Bnip3 orthologs are present in the *Drosophila* genome (*CG11658* and *CG5059*, respectively), there is no apparent ortholog for MuRF1, arguing whether other ubiquitin ligases might be involved in muscle wasting in *Drosophila*.

In both Imp-L2-secreting tumors described above, flies show reduced climbing ability and defects in wing position [57, 86], indicating impaired muscle function. Also, AKT activity is reduced in muscles due to a decrease in insulin signaling [86]. Interestingly, whereas in mice models of cachexia, Bnip3 and MuRF1 are increased in muscle wasting [10], the expression levels of *CG11658* and *CG5059* are not significantly elevated during wasting [57], suggesting that either these genes are post-transcriptionally regulated during wasting or that other genes related to the proteasome system or the autophagy/lysosome pathway are involved. Furthermore, since FoxO transcription factors play a role in mammalian muscle wasting [129], and in *Drosophila* FoxO modulates expression of autophagy genes [12, 48], it remains to be addressed if FoxO activity is required in both fly tumor models to induce organ wasting.

11.7 *Drosophila* as a Model for Studying Adipose Tissue Wasting

The *Drosophila* fat body has an analogous function to the white adipocytes in mammals, while there is no apparent counterpart to brown adipose tissue. The fat body is the main organ where energy is stored in the form of fat and glycogen, with fat being stored in the form triglycerides in lipid droplets of the fat body [8]. *Brummer* (*bmm*), the fly ortholog of ATGL, and dHSL drive the hydrolysis of triglycerides in free fatty acids and glycerol that are further released into circulation [21, 66]. Mutations in any of these genes lead to accumulation of triglycerides in LD and obesity in flies [21, 66]. Conversely, feeding flies a high-sugar diet leads to hyperglycemia, insulin resistance, and obesity [101] – hallmarks of type 2 diabetes – suggesting that flies are a useful model to study lipid and glucose metabolism [20, 102, 144].

In the adult gut yki-tumor model, several rate-limiting enzymes of the glycolytic pathway are downregulated in muscles, and flies have lower levels of triglycerides stored in the fat body [86]. Since flies with yki-tumors are hyperglycemic, the systemic reduction of insulin signaling combined with an increase in lipolysis of triglycerides could indicate a dramatic accumulation of diacylglycerides in the muscle and induction of insulin resistance, similar to what occurs in mammalian skeletal muscle as discussed previously [33, 127]. However, it is unclear if *bmm* and dHSL mediate lipolysis in both fly models of organ wasting, and further studies are needed to understand the interactions between the fat body wasting and muscle wasting.

The fat body functions as an endocrine tissue that regulates systemic metabolism and organismal growth [40, 89]. In addition to Imp-L2, cytokines and hormones such as Unpaired-2 [117]; the ortholog of TNF-α, Eiger [1]; Stunted, the ligand of the Methuselah receptor [47]; growth-blocking peptides (GBP) [85, 95]; and the peptide hormone CCHamide-2 [131] are secreted from the fat body in response to nutritional cues to modulate systemic insulin signaling.

A link between cytokines, inflammatory responses, and tumorigenesis has been established in larvae [41, 76, 105, 106]. JNK signaling induction in $Ras^{[V12]}$/*scribble* disc tumors upregulates the cytokine-encoding *unpaired* genes *(upd1*, *upd2,* and *upd3)*, which further activate JAK/STAT signaling and promotes tumor growth and metastasis [106, 156]. Curiously, the release of Upd cytokines from the tumor also induces a systemic inflammatory response that limits tumor growth [106]. Circulating hemocytes produce Eiger, which activates JNK pathway in tumors to induce apoptosis and suppress growth, highlighting the complex interaction between cytokines and JNK signaling in tumorigenesis [41]. Nevertheless, it remains unknown if circulating cytokines induce organ wasting in *Drosophila*.

11.8 Considerations for Studies of Organ Wasting in *Drosophila*

Perturbation of different conserved signaling pathways in tissues, either in larvae or adult stage, generates different types of tumor in *Drosophila*. Though two types of tumor have already been identified to induce organ wasting, it remains unknown if more tumor types are capable of inducing wasting. While it is easy to generate tumors in imaginal discs with mosaic induction [157], the developmental time of the larva before reaching pupariation is short and, therefore, organ wasting might only be detected with very aggressive tumors. Thus, adult flies seem more suitable for studying organ wasting. However, transplantation of larval tumors into adults is technically demanding and often lethal, making it difficult to obtain a high number of surviving individuals to study [121]. In addition, it is difficult to control the amount of tumor sample transplanted, raising a concern that there could be considerable variability among individuals.

An alternative approach is to induce tumors directly in adult flies, as in the case of the gut yki-tumors [86]. In larvae the imaginal discs, in which tumors are induced, are formed of highly proliferative diploid cells [51]. Alternatively, the yki-tumors in adults are formed by stimulating overproliferation of intestinal stem cells by using a GAL4 driver, specific to gut stem cells, to ectopically express an active form of Yki [81, 135]. This indicates that other adult stem cells might be suitable for generating tumors when using GAL4 drivers specific of particular stem cell populations, and in combination with temperature-sensitive GAL80 transgenes that allow temporal control of gene expression [160].

One problem to consider with this strategy is that tumors might compromise the function of the organ in which they are being induced and rapidly cause lethality or affect systemic metabolic homeostasis, independently of tumor-secreted factors. Tumors induced in tissues that are not essential for adult viability, such as the germline, might be more compatible with long-term viability, unlike tumors induced, for example, in the brain [16, 92] or in the Malpighian tubules [162]. Nevertheless, the demonstrated ability to induce organ-specific tumors in adult tissues opens the door to testing which tumors are prone to induce systemic organ wasting. More importantly, testing various types of tumors generated in different tissues for their abilities to induce wasting in flies might help understand changes in target tissues during organ wasting, and unravel possible conserved mechanisms that also induce cachexia in humans.

Although neither tumor burden nor tumor type correlates with induction of cachexia [49, 71, 98], tumor-secreted factors can drive lipolysis and/or proteolysis [56, 147]. In flies, Imp-L2 was the first tumor-secreted factor to be identified that induces organ wasting [57, 86], followed by identification of Pvf1 [136]. While both Imp-L2 and Pvf1 were discovered by transcriptomic analysis of tumor samples [136], one additional method to identify novel factors produced by the tumor is to apply proximity-based labeling of proteins specifically in tumors, followed by purification of the labeled proteins from the hemolymph or target tissues of wasting and identification by mass spectrometry [29, 32]. This approach would help identify novel tumor-derived circulating factors with potential to induce organ wasting in peripheral tissues and with a conserved function in humans.

Cachexia is a syndrome that induces general metabolic dysfunction and increased catabolism in muscle and adipose tissue. In addition, muscle tissue can show signs of insulin resistance, an indicator of impaired glucose metabolism [33, 126]. In the two fly models of organ wasting described above, muscle function, adipose tissue mass, and glucose metabolism are affected [57, 86], showing similarities with human patients with cachexia and with rodent models. The development of genetic tools to monitor metabolite levels in flies at cellular resolution will sig-

nificantly facilitate the characterization of metabolic pathways in real time. An ingenious FRET sensor for measuring pyruvate levels [128] was used in the fly brain and unraveled a role for energy consumption in driving long-term memory formation [115]. Additional fluorescent reporters have been developed for measuring the ratio of ATP to ADP [18] and NADH to NAD [75]; however, these need to be tested in *Drosophila*.

In summary, combining *Drosophila* genetic tools with proteomic and transcriptomic approaches in both tumors and in target tissues, as well as with a detailed analysis of the changes in metabolic pathways in muscle and fat body, would produce a more complete and broader picture of the process of organ wasting induced by tumors.

11.9 Conclusion

As there is no treatment for cancer-induced cachexia and because of discrepancies in data obtained from cancer patients with cachexia and rodent models, new models for studying cachexia are needed. In addition, the pace, scale and genetic manipulation of rodent model studies have limitations that a model organism like *Drosophila* does not have. *Drosophila* has been used as a model for studying tumor biology [65] and more recently has emerged as a model to dissect the mechanisms underlying organ wasting induced by tumors [57, 86, 136]. Combining the genetic potential of the fly with protein-labeling techniques may help uncover novel tumor-derived factors with potential to induce organ wasting. More importantly, high-throughput studies of proteomics and metabolomics in *Drosophila* provide a unique opportunity to create a rapid approach to identify the types of tumors that induce metabolic changes similar to organ wasting observed in human patients with cachexia. These studies should help uncover new cellular and molecular mechanisms that drive organ wasting induced by tumors and shed light on the process of cachexia.

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12

Drosophila melanogaster as a Model System for Human Glioblastomas

Alexander S. Chen and Renee D. Read

Abstract

Glioblastoma multiforme (GBM) is the most common primary malignant adult brain tumor. Genomic amplifications, activating mutations, and overexpression of receptor tyrosine kinases (RTKs) such as EGFR, and genes in core RTK signaling transduction pathways such as PI3K are common in GBM. However, efforts to target these pathways have been largely unsuccessful in the clinic, and the median survival of GBM patients remains poor at 14–15 months. Therefore, to improve patient outcomes, there must be a concerted effort to elucidate the underlying biology involved in GBM tumorigenesis. *Drosophila melanogaster* has been a highly effective model for furthering our understanding of GBM tumorigenesis due to a number of experimental advantages it has over traditional mouse models. For example, there exists extensive cellular and genetic homology between humans and *Drosophila*, and 75% of genes associated with human disease have functional fly orthologs. To take advantage of these traits, we developed a *Drosophila* GBM model with constitutively active variants of EGFR and PI3K that effectively recapitulated key aspects of GBM disease. Researchers have utilized this model in forward genetic screens and have expanded on its functionality to make a number of important discoveries regarding requirements for key components in GBM tumorigenesis, including genes and pathways involved in extracellular matrix signaling, glycolytic metabolism, invasion/migration, stem cell fate and differentiation, and asymmetric cell division. *Drosophila* will continue to reveal novel biological pathways and mechanisms involved in gliomagenesis, and this knowledge may contribute to the development of effective treatment strategies to improve patient outcomes.

Keywords

Glia · Glioblastoma · EGFR · Phosphatidylinositol-3-kinase · PI3K · Neoplasia

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12.1 Glioblastoma

Glioblastoma multiforme (GBM) is the most common primary malignant adult brain tumor. GBM originates primarily in the cerebral hemispheres, though tumors can also arise in the brain stem, cerebellum, and spinal cord [1]. GBMs are classified by the World Health Organization (WHO) as grade IV gliomas due to their highly aggressive, malignant, invasive, and undifferentiated nature [2]. Standard of care for GBM involves surgical resection followed by concomitant radiotherapy with adjuvant chemotherapy with the DNA damaging agent temozolomide, and median survival for GBM remains poor at 14–15 months [1]. As such, it is imperative to better understand the biology of GBM in order to develop more effective treatments.

To discover genetic mutations essential to gliomagenesis, large-scale genomic projects such as The Cancer Genome Atlas (TCGA) analyzed hundreds of GBM tumor tissue specimens [3, 4]. Common alterations in GBMs include genomic amplification, activating mutations, and overexpression of receptor tyrosine kinases (RTKs), such as *EGFR, PDGFRA,* and *FGFR,* and genes in core RTK signal transduction pathways, such as the *phosphatidyl-inositol-3-kinase (PI3K)* pathway, and inactivation of the *TP53/RB* pathway [3, 4]. Genetic alterations in RTKs occur in over 80% of GBMs [3, 4]. One of the most frequent genetic alterations is amplification and mutation of *EGFR* [3, 4]. The most common *EGFR* mutation is variant III ($EGFR^{vIII}$), a constitutively active version of EGFR created by an internal deletion of the ligand-binding domain (exons 2–7) [5, 6]. Constitutive activation of RTKs through amplification and/or mutation contributes to GBM pathogenesis by promoting proliferation, migration, and resistance to apoptosis [7–10].

Common genetic alterations in GBM occur in the PI3K pathway, including activating missense mutations in *PIK3CA*, which encodes the p110α catalytically active subunit of PI3K [11, 12], and *PIK3R1*, which encodes the p85α regulatory subunit of PI3K [3, 13]. Loss of *PTEN* phosphatidylinositol-3-phosphate (PIP3) lipid phosphatase, which is inactivated through point mutations, deletions, and epigenetic silencing, results in constitutive activation of PI3K signaling as a consequence of elevated PIP3 levels [14–16]. As a response to PI3K activation, AKT kinases are upregulated in approximately 70% of GBMs [12, 14]. Other common mutations inactivate the TP53/RB pathways, including homozygous *CDKN2A/CDKN2B* or *RB1* loss, *TP53* mutations, *MDM4* and *MDM2* amplification, and *CDK4* amplification [3, 4]. Another common genetic alteration in GBM are activating mutations in the promoter of telomerase reverse transcriptase (*TERT*), which occurs in 70% of GBM patients [4, 17]. These mutations inactivate cell cycle checkpoints, immortalize glial cells, and, together with RTK alterations, promotes GBM tumor initiation and progression (reviewed in [18]). Studies in mice indicate that co-mutation of these pathways cooperates to promote GBM tumorigenesis [19–23]. However, due to limited drug absorption into tumors, cellular and genetic heterogeneity in tumors, and emergence of drug resistance over time, efforts to therapeutically target these genetic alterations have shown limited efficacy in the clinic [24–27]. To improve outcomes for GBM patients, there must be a concerted effort to translate these data to develop new therapies that target underlying biological mechanisms that drive GBM.

12.2 *Drosophila melanogaster*: An Effective Tool to Model GBM

One of the most effective models for advancing our understanding of cancer is *Drosophila melanogaster* (reviewed in [28]). As a cancer model, *Drosophila* has numerous advantages over traditional animal models, including a relatively short lifespan, a large number of progeny, a fully sequenced and annotated genome, and powerful modalities to analyze complex tissues. Furthermore, there are tools available for targeted or insertional mutagenesis and RNA interference (RNAi) for almost all genes in the genome. The binary UAS/GAL4 system allows for Gal4-

driven cell-type-specific gene manipulation in fly tissues, including the central nervous system (CNS), and expression of multiple UAS-containing transgenes within the same cell type allows for complex genetic manipulation [29–33]. Finally, 75% of genes associated with human diseases have functional fly orthologs, and 30% of them have enough similarity such that human and fly proteins have identical functions [34].

Drosophila is also an excellent model organism for neurological diseases due to extensive cellular homologies between *Drosophila* and humans. The *Drosophila* CNS consists of two bilaterally symmetrical brain hemispheres and a central nerve cord that, like humans, is comprised of glia and neurons. *Drosophila* glia share similar developmental origins, cellular properties, and physiological functions of human glia, such as astrocytes, oligodendrocytes, and Schwann cells [35]. In *Drosophila*, there are several types of CNS glia that are derived from neural stem cells, known as neuroblasts, and that are defined by their morphology and associations with neurons, including astrocytes, cortex glia, and peripheral glia (reviewed in [35]). Astrocytes, which restructure neuronal circuits and regulate synapses, similar to mammalian astrocytes, primarily localize in the cortex and extend processes into the neuropil, a histologically distinct region of the *Drosophila* CNS where dendrites and axons project and connect [35]. Cortex glia, which have similarities to mammalian astrocytes, localize in the cortex, a histologically distinct region of the CNS where neuronal cell bodies are localized, and support development and maturation of neuroblasts, neurons, and other glial cell types in response to local and systemic signals [36–38]. Perineural glia along with the subperineural glia act as a chemical and physical barrier for the CNS and make up the blood-brain barrier (BBB) [35]. Perpherial glia, similar to Schwann cells, ensheath the peripheral sensory and motor nerves [39, 40].

In support of their relevance as a cancer model, *Drosophila* do in fact develop neoplastic tumors. *Drosophila* develop malignant neoplasms that share key features of vertebrate neoplasms: rapid autonomous growth, invasion into adjacent healthy tissue, metastasis into distant organs, lethality to host, loss of tissue structure, lethal autonomous growth after transplantation, and lack of contact inhibition [28]. Studies show that subjecting wild-type *Drosophila* to genetic manipulation, carcinogens, and X-ray irradiation can result in heritable tumor-causing mutations that cause uncontrolled cell growth that produce malignant cells [41–43]. Furthermore, *Drosophila* models adhere to a crucial characteristic of malignant tumors, unlimited growth after transplantation: pieces of larval wild-type brains transplanted into the abdominal cavity of female adult flies neither grew nor killed their hosts, whereas pieces from tumor suppressor mutant brains grew rapidly up to 300 times the size of the original transplant and prematurely killed their hosts [41].

Several genetic pathways involved in gliomagenesis are conserved between *Drosophila* and humans, and single orthologs exist for many genes involved in gliomagenesis, simplifying complex genetic analysis. For example, there are single functional orthologs for genes such as *EGFR (dEGFR), PIK3CA (dp110), PTEN (dPTEN), RAS(dRas)*, *RAF(dRaf)*, and *AKT (dAkt)*. Moreover, many essential genes involved in human gliomagenesis were first discovered in flies: for example, the gene *Notch* is named for *Drosophila* mutant phenotypes [44], and human orthologs of Notch are involved in GBM tumorigenesis [45].

12.3 *Drosophila* Models for RTK-Driven GBM

Given the homologies between *Drosophila* and mammals, Read et al. developed a *Drosophila* GBM model to investigate how signaling pathways cooperate during neural tumorigenesis [46]. Similar to published mouse models, in *Drosophila*, co-activation of EGFR and PI3K signaling in a glial-specific manner resulted in glial neoplasia (Fig. 12.1) [46]. This was achieved using glial-specific *repo-Gal4* to co-overexpress constitutively active versions of dEGFR and dp110, the catalytic subunit of PI3K, or downstream effectors, such as dRas or dRaf combined

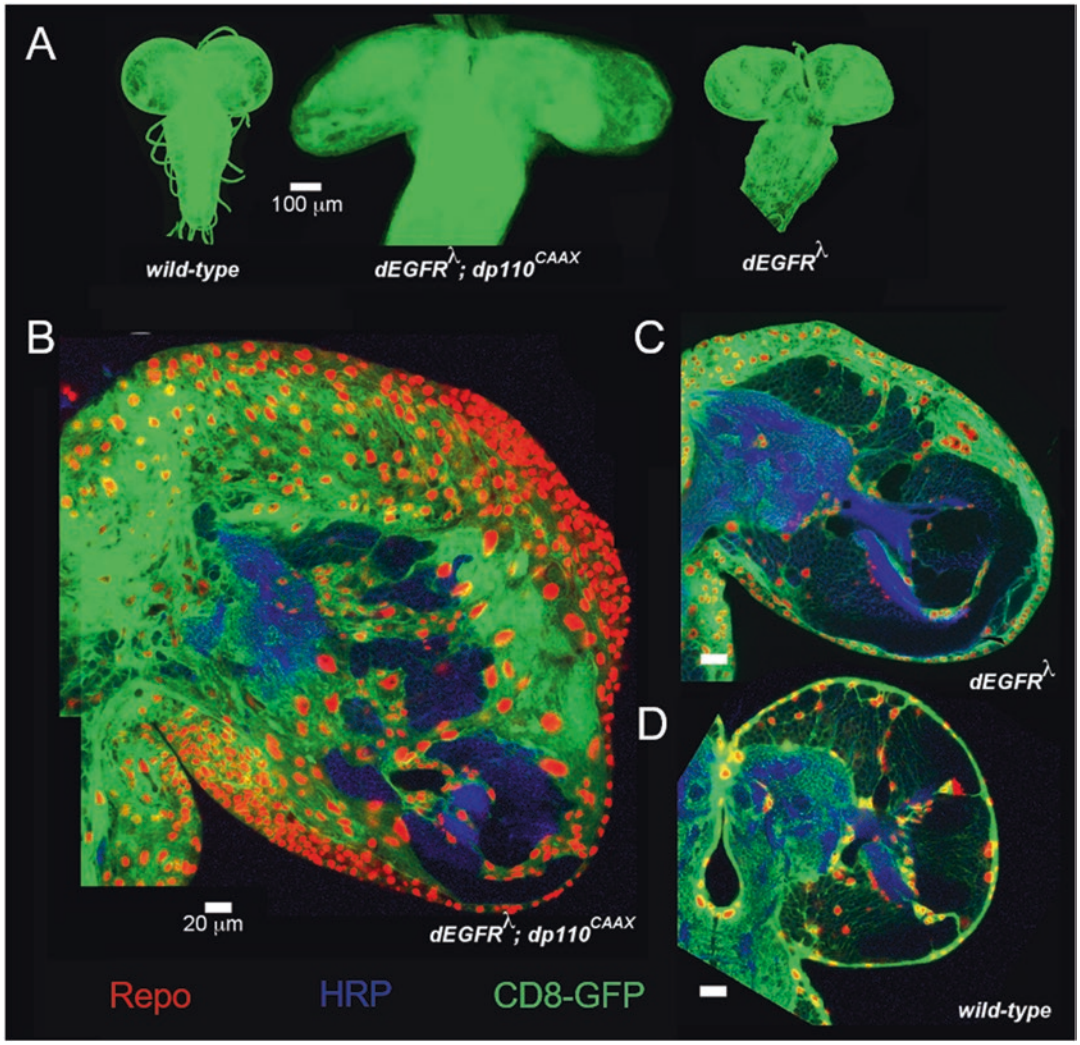

Fig. 12.1 Co-activation of EGFR and PI3K in *Drosophila* glia causes neoplasia. (**a**) Optical projections of whole brain-nerve cord complexes from late 3rd instar larvae, displayed at the same scale. Glia are labeled with CD8-GFP (green) and constitutively activated EGFR(dEGFR$^{\lambda}$) and PI3K(dp110^{CAAX}) driven by repo-Gal4. Each brain is composed of 2 symmetrical hemispheres attached to the ventral nerve cord. In *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* larvae, both brain hemispheres are enlarged and elongated relative to other genotypes. (**b**–**d**) 2 μm optical sections of larval brain hemispheres from late 3rd instar larvae approximately, displayed at the same scale. Glial cell nuclei labeled with Repo (red); anti-HRP for neuropil (blue). In *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* brains (**b**) there is a dramatic increase in glia relative to *repo>dEGFR$^{\lambda}$* (**c**) or wild-type animals (**d**). (Figure adapted from [35])

with *dPTEN* RNAi. In contrast, glial-specific activation of EGFR-Ras or PI3K signaling alone did not induce neoplasia, indicating that EGFR and PI3K work together synergistically to drive glial tumorigenesis [46]. Mutant variants of EGFR found in GBM, such as EGFR^{vIII}, also produced neoplastic phenotypes in *Drosophila* [47]. Moreover, co-activation of either dEGFR or dRas with PI3K did not induce neoplasia in neuroblasts or neurons, indicating that neural tumorigenesis in response to these pathways is glial-specific [46].

Read et al. used transplant assays and FLP-FRT clonal analysis to investigate developmental origins, malignant properties, and invasive potential of neoplastic glia. EGFR-PI3K mutant glia

transplanted into the abdomen of adult host flies yielded large lethal tumors in which neoplastic glia invaded adjacent tissues [46]. These tumors were associated with excess trachea, which are oxygen delivery tubules, suggesting that they stimulated tracheal growth in a process similar to angiogenesis [46]. FLP recombinase was used to induce clones of mutant glia in otherwise normal tissue in late development or young adult flies. While single *dPTEN*^{null} mutant clones or clones overexpressing dEGFR showed a slight increase in glial cells compared to wild-type control clones, dEGFR or dRas - dPTEN^{null} double mutant clones created highly proliferative and invasive tumor-like neoplastic growths [46].

This *Drosophila* GBM model recapitulates a key aspect of human GBM in that tumor formation relies on activation of multiple pathways that work synergistically to promote uncontrolled proliferation and migration [46]. At least four pathways downstream of EGFR and PI3K co-activation promote glial neoplasia, all of which have orthologous gliomagenic human genes [46]: dMyc (MYC) promoted cell cycle entry, Pnt (ETS-family transcription factor) promoted cell cycle progression, and the Tor-S6K pathway promoted protein translation and cellular growth [46]. Thus, *Drosophila* GBM models show evolutionary conservation of oncogene cooperativity.

Read et al. established that, in *Drosophila*, glial progenitor cells are prone to malignant transformation in response to oncogenes that drive human gliomagenesis, and that *Drosophila* models of RTK-driven glial neoplasia are effective tools to characterize gliomagenic pathways. These models have now been used by several groups to evaluate the capacity of other RTK and Ras pathway components to promote glial neoplasia, to identify novel genes and pathways that contribute to neuro-glial tumorigenesis, and to identify subtypes of glia prone to transformation (Table 12.1) [37, 47–56].

GBM is by nature a highly invasive tumor, and RTK and PI3K signaling pathways promote invasive behavior of tumor cells; *Drosophila* can be used to investigate this aspect of tumor biology [18]. To better understand how these signaling pathways govern migration and invasion in GBM, Witte et al. used imaging to track changes in proliferation and migration of EGFR-PI3K transformed glia in the visual system [49]. During larval development, repo-positive glia originate in the optic stalk and migrate into the retina but, not the Bolwig's organ, at predictable times during development [57, 58]. Neoplastic transforma-

Table 12.1 *Drosophila* GBM models are an effective tool for discovery and characterization of many genes involved in a variety of important pathways involved in GBM tumorigenesis

Drosophila genes	Human orthologs	Function in glioblastoma
dRIOK1, dRIOK2	RIOK1, RIOK2	Atypical serine/threonine kinases, responds to and promotes AKT signaling in glial tumorigenesis
Drak, Sqh, Anillin	STK17A, MRLC, ANLN	Kinase-dependent pathway that acts downstream of EGFR to regulate cytokinesis in glial tumor cells
dPiezo	PIEZO1	Mechanosensory ion channel, regulates ECM deposition and tissue stiffness through effects on Integrin
ACAT1 (CG8112)	ACAT1	Cholesterol acyltransferase, regulates cholesterol and lipid homeostasis
dPink1	PINK1	Serine/threonine kinase, regulates glycolytic metabolism
Pvr	PDGFR/VEGFR	Receptor tyrosine kinase, promotes tumor cell proliferation and invasion/migration
Fd59a, dALDH	FOXD1, ALDH1A3	Transcriptional pathway, regulates cancer stem cell maintenance and tumor cell growth
achaete	ASCL1	bHLH transcription factor, regulates neural stem cells and tumor cell differentiation
brat	TRIM3	Multifunctional protein that attenuates Notch nuclear localization
dCdk5	CDK5A	CDK family kinase, regulates GBM cell self-renewal

tion by EGFR and PI3K signaling dramatically increased the number of migratory glia in the optic stalk, with some glia invading along the Bolwig's organ nerves, and importantly, this increase was rescued using pharmacological inhibitors of EGFR and PI3K, consistent with the conclusion that, in *Drosophila* glia, these pathways are sufficient to drive neoplastic invasion and migration [49]. Moreover, Witte et al. observed that overexpressing other RTKs such as *Pvr* (*PDGFR/VEGFR*), *htl* (*FGFR1*), and *Inr* (Insulin receptor), also increased the proportion of migratory and invasive neoplastic glia [49]. This study indicates that *Drosophila* can be effectively used to model GBM migration and invasion in response to constitutive activation of RTKs and PI3K signaling. However, pharmacological agents that target a single RTK show limited efficacy in GBM patients [59]. Therefore, RTK effector pathways in glial tumorigenesis, which could be identified in *Drosophila*, may be a more relevant to therapeutic development.

Due to the ease of cell-type-specific genetic manipulation, *Drosophila* are also effective at identifying glial cells-of-origin in GBM tumorigenesis and investigating their propensity to be transformed in relation to their developmental roles. Mouse models have identified glial progenitors as cells-of-origin in gliomas, including astrocytic and oligodendrocyte progenitor cells (OPCs), because driver mutations associated with human GBM can transform these cells [60]. As discussed previously, *Drosophila* possess glia and glial progenitor cells that share many features with their mammalian counterparts. In a study of neuroblast development, Pvr ligands produced by neuroblasts were found to stimulate Pvr signaling in cortex glia, supporting their survival and morphogenesis, which, in turn, is required for their ability to support neuroblast and neuron development [37]. Importantly, constitutively active Pvr or EGFR-PI3K induced neoplastic transformation of cortex glia, and, as they grew during early stage tumorigenesis, neoplastic cortex glia colonized the developing brain at the expense of neuroblasts and neurons [37]. Like cortex glia, OPCs similarly depend on PDGFR signaling for their normal development, become transformed by constitutive PDGFR or RTK-Ras signaling, and outcompete neural stem cells in the mouse brain [60, 61]. However, the pathways that mediate cell-cell interactions to allow tumor cells to kill neuronal cells and colonize the brain during early-stage tumorigenesis are not clear. In the future, *Drosophila* GBM models may help elucidate the mechanisms involved in competition between normal and RTK-driven tumor cell populations.

12.4 *Drosophila* GBM Models as Tools to Identify Novel Signaling Pathways in GBM

Drosophila GBM models have proven critical to elucidating pathway components and cellular processes involved in glial tumorigenesis. One of the key discoveries using *Drosophila* GBM models is the identification of novel kinases that operate downstream of EGFR-PI3K signaling specifically in tumorigenic glia but not in normal glia. Read et al. utilized previously described *Drosophila* GBM models in a kinome-wide modifier screen for kinases that are ectopically activated in a tumor cell-specific manner [47]. This screen identified that non-redundant atypical serine/threonine kinases open reading frame 1 and 2 (dRIOK1 and dRIOK2) promote EGFR-PI3K-dependent glial tumorigenesis [47]. Glial-specific knockdown of *dRIOK1/2* dramatically reduced neoplastic glial proliferation in the context of EGFR-PI3K co-activation, but not in wild-type glial cells, with *dRIOK2* knockdown yielding synthetic growth reduction in the context of EGFR activation [47].

To determine if novel kinases identified from this screen represent new regulators of pathways involved in human gliomagenesis, orthologous kinases were subject to functional validation in mammalian GBM model systems. In human GBM tumor tissue and cells, oncogenic constitutive RTK-PI3K-AKT signaling drove RIOK1/2 overexpression [47], whereas, in normal control glial cells and brain tissue, RIO kinase expression was undetectable in astrocytic glia. Moreover, RIOK1/2 expression was required for

proliferation and survival of EGFR-driven GBM cells. *RIOK1/2* knockdown resulted in decreased AKT kinase activity, whereas RIOK2 overexpression transformed immortalized murine *PTEN*$^{-/-}$*; Ink4a/arf*$^{-/-}$ astrocytes and resulted in increased AKT activity. In both cell types, we observed phosphorylation of AKT on serine-473 [47], which is mediated by the TORC2 signaling complex to stimulate AKT kinase activity. The TORC2 complex, which includes the Tor kinase, is ectopically activated in RTK-PI3K mutant tumor cells and is required for tumor cell growth but not normal glial cell growth in *Drosophila* [46]. Reduced TORC2 signaling, which is required for RTK-PI3K mutant human GBM tumor cell growth and survival, likely contributes to apoptosis and growth reduction induced by RIOK1/2 knockdown [62–65]. Thus, constitutive RTK-PI3K-AKT signaling drives RIO kinase overexpression which in turn drives AKT signaling to create a tumor cell-specific feedforward loop that promotes and maintains oncogenic AKT activity to drive tumorigenesis (Fig. 12.2).

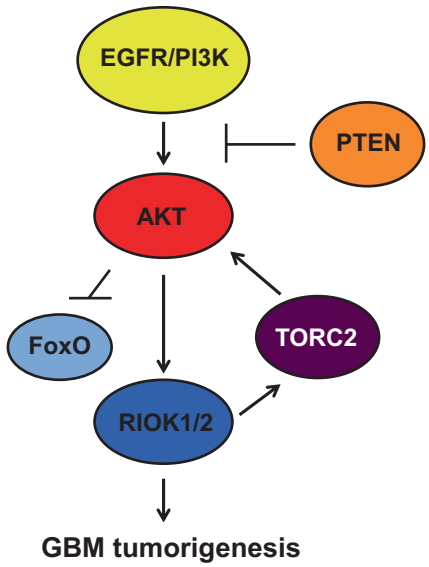

Fig. 12.2 RIO kinases are required for EGFR- and PI3K-mediated tumorigenesis. Diagram showing RIOK1 and RIOK2 function in relation to AKT downstream of EGFR and PI3K signaling in GBM. RIOK2 mediates signaling both upstream and downstream of AKT via stimulation of TORC2. (Figure adapted from [36])

This study demonstrates that *Drosophila* models can be used to identify novel RTK-PI3K effectors that mediate tumorigenesis, although many questions remain regarding the mechanisms by which RIO kinases promote tumorigenesis. Given that RIO kinases promote maturation of the 40S ribosomal subunit in yeast [66–71], we postulated that RIOK1/2 may drive tumorigenesis by altering ribosome assembly; although, our recent unpublished experiments show that RIOK2 knockdown does not significantly alter ribosome assembly in GBM cells, indicating a yet unidentified novel protein functions. We are confident that using forward genetic approaches in *Drosophila*, RIOK1/2 function will be determined in both normal and neoplastic glia.

12.5 *Drosophila* Models Identify New Regulators of Cytokinesis in GBM

Death-associated protein kinase related (Drak), which is a serine/threonine kinase and a member of the death-associated protein (DAP) family of cytoplasmic protein kinases [72], was another candidate identified in the kinome-wide modifier screen in *Drosophila* GBM models [47]. DAP family kinases primarily regulate cytoskeletal dynamics, cytokinesis, and cell adhesion [73]. In the context of EGFR-PI3K co-activation, glial-specific knockdown of *Drak* with RNAi dramatically and significantly reduced proliferation of neoplastic glia [50]. While Drak loss or overexpression had little effect on glial development, Drak co-overexpression with EGFR caused glial neoplasia: thus, Drak exerts its modifier effects only in the context of constitutive EGFR activation [50]. Furthermore, Spaghettisquash (Sqh), a known Drak substrate [72], was required for proliferation of EGFR-PI3K mutant neoplastic glia [50]. Sqh phosphorylation, which was reduced upon Drak loss in EGFR-PI3K mutant glia, promoted glial neoplasia in the context of constitutively active EGFR [50]. Anillin, a known Sqh binding partner, is an actin-binding scaffolding protein important for cytoskeletal reorganization during cytokinesis [74], and is also required for

EGFR-PI3K-driven glial neoplasia [50]. Previous studies show that phosphorylation of MRLC family proteins, including Sqh, activates non-muscle myosin type II (NMII) motor proteins to modulate cytoskeletal contractility to mediate cellular processes such as cytokinesis [75, 76]. In *Drosophila* development, Sqh recruits Anillin during mitosis to coordinate cytokinesis [74, 77]. Our studies show that, in EGFR-PI3K mutant glia, phosphorylated Sqh co-localized with Anillin in the cortex and cleavage furrow of mitotic glia in a Drak-dependent manner [50], indicating that this pathway functions in cytokinesis in neoplastic glia. In EGFR-mutant human GBM cells and tissues, we found that STK17A, the human ortholog of Drak, is required for their proliferation, and that STK17A is co-localized and is coordinately upregulated with phosphorylated MRLC and ANLN in mitotic cells, particularly during cytokinesis [50]. Moreover, in patient specimens, *STK17A* mRNA is upregulated in both GBMs and lower-grade gliomas (LGGs), and STK17A expression is correlated with EGFR amplification and poor prognosis, supporting a role for STK17A as a modifier of EGFR tumorigenic activity [50]. Together, these results reveal a novel mechanism in which aberrant activation of the STK17A/MRLC/ANLN pathway differentially regulates cytokinesis in glioma cells relative to normal glia and neural stem cells (Fig. 12.3). While additional research is required to understand how cytokinesis is regulated in GBM cells, this study validates *Drosophila* GBM models as an effective tool to elucidate new aspects of glioma biology.

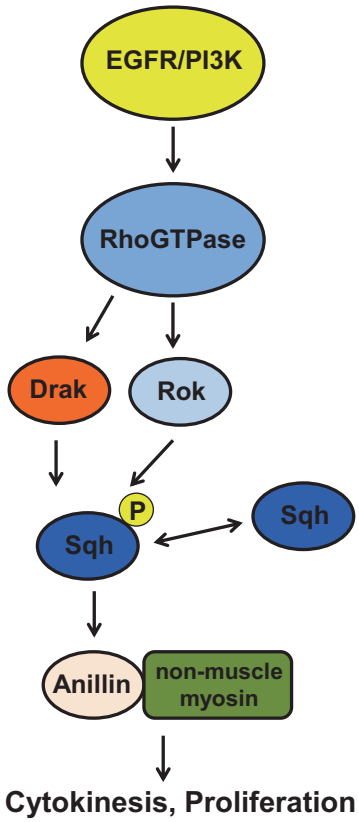

Fig. 12.3 Drak interacts with Sqh and Anillin to drive cytokinesis and proliferation in GBM. Diagram depicting the functional role of Drak/STK17A and its effector pathway in promoting gliomas. (Figure adapted from [32])

12.6 *Drosophila* Models Identify Mechanosensory Mechanisms in GBM

As genomic analyses of GBMs continues, new RTK mutations are being uncovered. For example, a subset of adult GBMs carry gene fusion mutations in which the C-terminal tyrosine kinase domains from FGFR RTKs are fused in frame to the N-terminal regions of the TACC family proteins, which drive oligomerization and kinase activation [78, 79]. Recent studies show that FGFR-TACC fusion proteins drive glioma cell proliferation and invasion, and that tumor cells positive for FGFR-TACC fusion proteins can be sensitive to FGFR kinase inhibitors *in vitro* and *in vivo* [78, 79]. FGFR-TACC proteins drive tumorigenesis in mammalian astrocytes, although they localize to the nucleus and fail to activate canonical FGFR signaling pathways, indicating that they have aberrant activity distinct from full-length FGFR proteins [78, 79]. Thus, there is great enthusiasm for understanding how these lesions contribute to tumorigenesis and for using such knowledge to therapeutically target FGFR-TACC mutant tumors.

To understand how FGFR-TACC proteins promote glial transformation, the human FGFR3-TACC3 fusion was overexpressed spe-

cifically in *Drosophila* glia [51]. Like co-activation of EGFR and PI3K, FGFR3-TACC3 induced neoplastic transformation of larval glia, and tumorous glia showed increased deposition of extracellular matrix (ECM) proteins and increased ECM stiffness [51]. Glial tumor tissues have altered ECM stiffness that contributes to altered mechanosignaling within tumor and stromal tissues [80]. These ECM changes actively promote tumor progression by increasing cellular proliferation, survival, migration, drug resistance, and angiogenesis [81–83]. However, the molecular mechanisms involved in this process are not well understood.

In *Drosophila* models of EGFR-PI3K and FGFR-TACC driven GBM, function of the dPiezo ion channel was required for GBM tumorigenesis, and required for increased ECM deposition and tissue stiffness in neoplastic glial tissues but not in normal glia [51]. Research studies have shown that ion channels play a critical role in regulating ECM stiffness and cellular rigidity [84]. The evolutionary conserved PIEZO transmembrane ion channels, which normally function in sensory neurons, are mechanosensitive and open in response to membrane tension to allow for permeation of potassium, calcium, and sodium ions [85–88]. Loss of dPiezo function was rescued by increased Integrin signaling or overexpression of ECM proteins, indicating that these pathways act downstream of dPiezo function genetically in glial neoplasia [51]. The human ortholog of dPiezo, PIEZO1, is overexpressed in human GBM cells and tissues, and its overexpression is inversely correlated with patient survival [51]. In cell-culture and xenograft models of human GBM, PIEZO1 is required for tumor cell growth and sustained Integrin signaling through pathways that regulate tissue and ECM stiffness, such as the FAK kinase pathway, in a manner dependent on channel activity [51]. Thus, key aspects of dPiezo function in *Drosophila* GBM models are recapitulated in human GBM tumor models. Given that ion channels are well-established pharmacologic drug targets [89], these results open a way forward to the possible development of new therapeutics for GBM.

In a related imaging-based study, Kim et al. identified Lysyl oxidase (Lox), which mediates ECM stiffness and regulates cell migration, as a potential mediator of Pvr-dependent neoplastic glial migration [53]. They found that Lox operates through Integrin signaling in a positive feedback loop that leads to changes conducive to cell migration in the local extracellular microenvironment [53]. These observations were repeated in mammalian GBM model systems [53]. Mechanisms that influence ECM stiffness are of particular interest, because increased ECM rigidity favors migration of glioma cells [90, 91]. Moreover, Integrins mediate ECM stiffness by providing mechanical coupling to the matrix, adhesion to surrounding cells, and signal transduction to the cytoskeleton and nucleus [92, 93]. Collectively, these studies show that *Drosophila* models and modifier screens are invaluable tools for uncovering novel biological pathways involved in GBM.

12.7 *Drosophila* Models Identify Metabolic Mechanisms in GBM Tumorigenesis

In his seminal work on cancer metabolism, Otto Warburg discovered that tumor cells generate the majority of their ATP through aerobic glycolysis regardless of extracellular signals, and that tumor cells use this altered metabolism to generate biosynthetic precursors, which in turn allow for production of nucleotides, fatty acids, membrane lipids, and proteins to increase tumor cell proliferation and survival [94–96]. This shift in metabolic processes is particularly important in adult GBM tumors [95]. GBM is a late onset disease with a median age of 62 years [1], as such, an adult *Drosophila* GBM model may better reflect adult human GBM biology. Dr. Hueng's group has expanded on the aforementioned *Drosophila* GBM model developed by Read et al., and designed a *Drosophila* model that uses a temperature sensitive GAL80^{ts} temporal expression system to allow for the induction of glioma formation in adult flies [54]. In adult flies, EGFR-PI3K neoplastic glia induced grossly enlarged brains, a

progressive decline in neurological function, and shorter lifespans [54]. Using a bioinformatics approach to analyze the REMBRANDT tumor genomics database, Chi et al. identified four key metabolic genes that are correlated with poor prognosis in adult GBM patients, *ALDOA*, *ACAT1*, *ELOVL6*, and *LOX* [54]. Of particular interest was ACAT1, a key metabolic enzyme involved in ER-cholesterol homeostasis and lipid metabolism and is highly expressed in many different types of cancers. Although the mechanisms by which ACAT1 promotes GBM tumorigenesis remain poorly understood, *ACAT1* knockdown in the adult GBM *Drosophila* model dramatically reduced glioma brain enlargement and improved lifespan [54]. These results were recapitulated in human models wherein *ACAT1* expression was upregulated in human GBM cell lines in a EGFR-PI3K-dependent manner, and *ACAT1* knockdown dramatically reduced GBM tumorigenesis [54]. This study shows that *Drosophila* models are well suited to test the biological relevance of metabolic pathways and alterations identified from tumor genomic databases.

Drosophila GBM models are also effective at elucidating evolutionarily conserved roles for metabolic genes in glioma. Agnihotri et al. conducted a screen for gliomagenic mutations and found PINK1, a mitochondrially localized serine/threonine kinase that regulates mitophagy, reactive oxygen species (ROS) production, and OXPHOS [55]. In primary murine and human astrocytes, PINK1 loss promotes aerobic glycolysis and an altered metabolic state that is conducive to GBM tumor progression [97, 98]. Subsequent experiments indicate that PINK1 expression is downregulated in human GBM tumors, and that PINK1 restoration in human GBM cell lines reduced ROS and blocked tumor cell growth [55]. In the *Drosophila* GBM model developed by Read et al., dPink1 overexpression similarly reduced EGFR-PI3K dependent glial neoplasia [55], demonstrating an evolutionarily conserved metabolic function of PINK kinases in glioma. Thus, *Drosophila* GBM models can provide an efficient system to functionally characterize poorly understood metabolic enzymes that impact tumorigenesis.

12.8 *Drosophila* Models and Glioma Stem Cells

Despite treatment with surgery, radiation, and chemotherapy, GBM tumors invariably recur [1]. These tumors display considerable cellular heterogeneity, and recent studies show that there exists a subset of cells within GBMs called glioma stem cells (GSCs) that have neural stem cell-like self-renewing properties [99–102]. GSCs derived from human tumors express many of the same transcription factors and receptors present in neural stem/progenitor cells, maintain capacity to generate tumors resembling human GBM in serial transplantation, and exhibit radiation and chemotherapy resistance (reviewed in [103]). RTK and PI3K signaling are required for normal development of neural stem/progenitor cells and for maintenance of stem cell-like properties of GSCs, and gliomagenic mutations in RTKs and PI3K pathway components confer GSC-like properties to neural stem/progenitor cells in mouse GBM models [103]. Given that neoplastic *Drosophila* glia transformed by RTK-Ras and PI3K display many properties of GSCs, namely the ability to form malignant tumors upon transplantation and ectopic expression of factors that confer neural stem cell-like properties like dMyc, *Drosophila* GBM models are attractive systems for identifying and characterizing factors that govern the biological properties of GSCs [46]. For example, the previously described RTK-PI3K pathway components RIOK1, RIOK2, and STK17A are all required for GSC proliferation, maintenance, and survival [47, 50].

In particular, RTK-Ras driven *Drosophila* GBM models have been used to evaluate functional requirements for transcription factors altered in GSCs. For example, Cheng et al. identified several transcription factors ectopically upregulated in patient-derived GSCs relative to human normal neural stem/progenitor cells [56]. One of these transcription factors, FOXD1, which is a member of the Forkhead family of transcription factors, regulates organogenesis, mediates induced pluripotent stem cell dedifferentiation, and promotes tumor cell proliferation in various cancers [104–106]. In GSCs, FOXD1 transcrip-

tionally upregulates Aldehyde dehydrogenase 1A3 (ALDH13), which is involved in glycolytic pathways [107] and may provide a link between GSC identity and metabolic reprogramming. Using RNAi, Cheng et al. showed that fly orthologs of *FOXD1* (*fd59a)* or *ALDH1A3* (*dALDH)* were required for tumor cell proliferation in neoplastic *Drosophila* larval glia transformed by co-activation of the Ras and PI3K pathways [56], thereby demonstrating that FOXD1 and ALDH1A3 form an evolutionary conserved gliomagenic pathway.

Drosophila models are also useful for testing the function of transcription factors that normally promote neural differentiation. For example, overexpression of Achaete-scute homolog 1 (ASCL1), which is orthologous to *Drosophila acheate*, is correlated with improved patient prognosis [48]. ASCL1 promotes cell cycle exit and neuronal differentiation of human neural progenitors cells [108, 109]. To understand how ASCL1 functions in GBM, Park et al. overexpressed either fly Acheate or human ASCL1 in EGFR-PI3K mutant *Drosophila* glia [48], which dramatically reduced neoplastic proliferation and induced a switch from glial-to-neuronal cell fate in neoplastic glia [48]. Subsequent experiments revealed that ASCL1 reduces GBM tumorigenicity by transcriptionally suppressing glial cell and neuronal progenitor cell fate and promoting differentiation [48]. Thus, *Drosophila* models can reveal how genes that regulate differentiation can limit gliomagenesis.

During development, stem cells undergo asymmetric cell division in which one daughter cell self-renews and retains the stem cell fate and the other daughter generates a more specialized and differentiated cell. GSCs are thought to undergo both symmetric cell division, wherein both daughter cells generate GSCs, as well as asymmetric cell division, wherein one daughter cell maintains GSC fate and the other daughter cell of limited proliferative potential gives rise to tumor bulk [110, 103]. While processes of symmetric and asymmetric cell division are not well understood in *Drosophila* glia, much of the pioneering work on mechanisms of asymmetrical cell division have been carried out in *Drosophila* neuroblasts [111, 112]. Because another chapter in this volume reviews how defects in asymmetric cell division contribute to tumor formation in *Drosophila*, we will only briefly review research on this topic that has specifically led to the discovery and characterization of *Drosophila* genes with human orthologs that are mutated in GBM.

In *Drosophila* neuroblasts, asymmetric cell division requires setting up an axis of polarity and differential segregation of cell fate determinants between daughter cells [111, 112]. Among those determinants is Brat (encoded by the *brain tumor* gene), which normally asymmetrically segregates into the differentiating daughter cell during neuroblast division, where Brat inhibits self-renewal and promotes cell cycle exit and differentiation [112]. *brat* loss-of-function clones or RNAi dysregulate differentiation of neuroblasts and their daughter cells, causing these cells to maintain self-renewal and to express neuroblast and glial markers, with *brat* mutant cells maintaining the ability to create malignant tumors in adult brains and in transplantation assays [113, 114]. The human Brat ortholog TRIM3, which is only normally expressed in the brain, is genomically deleted in 25% of GBMs and is not detectably expressed in nearly all GBMs [115]. In *Drosophila* and human GSCs, loss of Brat/TRIM3 function led to increased active cleaved Notch (NICD), and to NICD nuclear accumulation [116], whereas restored Brat/TRIM3 expression suppressed Notch signaling and attenuated NICD nuclear localization due to direct interactions between TRIM3 and nuclear import proteins [116]. Furthermore, in a *Drosophila* modifier screen designed to identify downstream factors that mediate the tumorigenic effects of *brat* mutation, Mukherjee et al. discovered that the *brat* mutant phenotype is rescued by *Cdk5a* knockdown or pharmacological kinase inhibition, and that Cdk5a overexpression promoted the neuroblast-like fate [117]. In human GBM, *CDK5A* is frequently genomically amplified and overexpressed, and in GSCs *CDK5A* RNAi or pharmacological kinase inhibition decreased tumorigenicity and reduced expression of stem cell markers, indicating that CDK5A regulates GSC self-renewal [117]. These studies reveal a

novel and evolutionarily conserved connection between Brat/TRIM3 and dCdk5a/CDK5A, and demonstrates that GSCs are particularly sensitive to targeting of this pathway, although the connections between CDK5 and Notch signaling remain to be determined. Collectively, these studies demonstrate that *Drosophila* is an effective model for studying how defects in asymmetrical cell division and neural differentiation can contribute to tumorigenesis.

12.9 Concluding Remarks and Future Directions

As outlined in this review, *Drosophila melanogaster* as a model organism has proven to be uniquely suited to experimentally investigate the signaling pathways and cellular mechanisms involved in gliomagenesis. Subsequent studies have expanded upon initial development of versatile RTK-driven tumor models to apply forward genetic screens to identify novel regulators of tumorigenesis. Our studies showed how kinases such as RIOK1/RIOK2 and Drak/STK17A are involved in complex signaling pathways that promote RTK-dependent tumorigenesis. These models have also been used to address evolutionarily conserved hallmarks of cancer, including uncontrolled proliferation, invasion and metastasis, and altered cellular metabolism (Fig. 12.4).

Drosophila models have a number of well-documented advantages that will be crucial for investigating many emerging areas in glioma biology. Due to the ease of genetic manipulation and the availability of powerful imaging modalities, *Drosophila* models are well suited to study tumor microenvironments, where a complex network of different cell types operate within distinct microenvironments that, through local and systemic cues, regulate normal and tumor stem/progenitor cell proliferation and differentiation [118]. Studies show that *IDH1/2* mutations are a powerful predictor of GBM patient outcomes [2], and by manipulating *Drosophila* Idh, the ortholog of human IDH1/2, researchers may better understand how *IDH1/2* mutations contribute to glial tumorigenicity [119]. Moreover, many human epigenetic regulators were first discovered in *Drosophila* and have functional orthologs in *Drosophila*, such that *Drosophila* models could be effective for determining how mutations in epigenetic regulators, such as *TET2*, promote GBM tumorigenesis [120–122]. *Drosophila* models may also be effective tools to test complex, multi-targeted combinations of pharmacological agents in order to discover effective combinations that can be translated to treat human GBM patients [123]. Of note, while *Drosophila* lack an adaptive immune system, they possess a functional innate immune system where *Drosophila* glial cell types perform

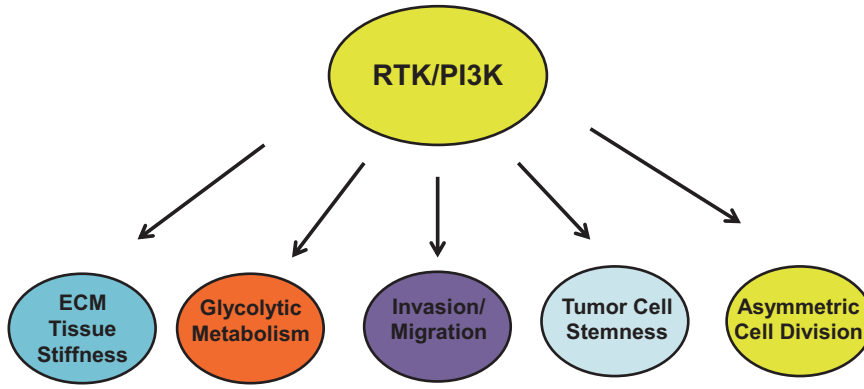

Fig. 12.4 RTK and PI3K signaling regulate a wide range of gliomagenic pathways. Diagram depicting that ectopic constitutive RTK and PI3K signaling is responsible for a range of functions including but not limited to ECM-based regulation of tissue stiffness, glycolytic metabolism, invasion/migration, tumor stem cell self-renewal, and asymmetric cell division that drive GBM tumorigenesis

microglia-like functions during development and injury [35], and as such, *Drosophila* may be an effective tool to better understand how the innate immune system responds to GBM tumors [124]. In the future, *Drosophila* will likely continue to reveal novel biological pathways and mechanisms involved in gliomagenesis, and eventually this knowledge may contribute to the development of effective treatment strategies to improve patient outcomes.

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What *Drosophila* Can Teach Us About Radiation Biology of Human Cancers

13

Tin Tin Su

Abstract

Ionizing radiation (IR) is used to treat more than half of human cancer patients. The therapeutic effect of IR is due to its ability to induce apoptosis. Success of radiation therapy relies not only on apoptosis induction but also on whether surviving cancer cells proliferate and regenerate a tumor. *Drosophila melanogaster* is a premier genetic model and, relevant to radiation biology of cancer, *Drosophila* larvae display an amazing capacity to regenerate. IR doses that kill more than half of the cells in larval tissues still allow complete regeneration to produce an adult fly of normal size and pattern. It is by understanding not only the initial effects of IR such as DNA damage and cell death but also longer-term regenerative responses that we may manipulate and improve radiation therapy of cancer. In this regard, *Drosophila* offers an unparalleled model to study both types of responses.

Keywords

Drosophila · Cancer · Ionizing radiation · Apoptosis · Regeneration

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Abbreviations

AiP	Apoptosis-induced Proliferation
F1 and F2	Filial 1, Filial 2
IR	Ionizing Radiation
JAK	Janus kinase
JNK	c-Jun N-terminal Kinase
PGE2	Prostaglandin E2
ROS	Reactive Oxygen Species
STAT	Signal Transducer and Activator of Transcription

13.1 Introduction

Ionization Radiation is radiation with sufficient energy to dislodge electrons from a target atom, to produce ions. Types of IR include γ-rays, x-rays and particle radiation, all of which are used in radiation therapy of cancer. Therapeutic effect of IR relies on its ability to kill cells. The main cell killing mechanisms by IR are apoptosis and clonogenic or reproductive death in which irradiated cells lose their ability to multiply. Paradoxically, IR exposure can also stimulate the proliferation of some surviving cells. Proliferation of surviving cells repopulates the tumor to confer resistance to radiation therapy. Understanding how IR kills cells but also stimulates proliferation and repopulation is key to improving radiation therapy. As discussed in sections below,

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Drosophila melanogaster, provides a useful model to study these seemingly opposing effects of IR.

13.2 Basic Understanding of What IR Does; X-Rays Induce Mutations

In the early 1900s, *Drosophila* geneticists had been studying naturally occurring mutations such as those affecting eye color and eye shape. Many wanted to go beyond spontaneous mutations and wanted to instead induce mutations so that more gene functions may be studied. When others failed to induce mutations using chemicals, Hermann Joseph Muller succeeded using X-rays. Radiation had been tried for mutagenesis by Muller's PhD mentor, Thomas Hunt Morgan, and others, but those efforts had been unsuccessful [3]. Muller thought that lack of success was not because radiation lacked activity but because detection methods for mutants were not optimal. He therefore chose recessive lethal mutations as the read out, as opposed to visible phenotypes such as eye color or wing shape. He designed the original stocks and subsequent genetic crosses such that induced recessive lethal mutations could be detected readily by simply examining the progeny for the absence of certain classes. For example, he used a stock carrying a ClB chromosome which is an X chromosome with three genetic elements: a dominant visible mutation called Bar (B), a recessive lethal mutation (l), and a crossover suppressor (C) [Female parent in Fig. 13.1, [3, 24]]. The properties of these genetic elements are as follows. Bar mutation changes the eye shape so that animals carrying the CIB chromosome could be identified readily simply by inspecting their eyes. A recessive lethal chromosome meant CIB animals that also carried a wild type X chromosome, such as the female parent in Fig. 13.1, were viable whereas males with just the CIB X chromosome were lost. The cross-over suppressor was known genetically to do exactly that, to suppress crossing over in meiosis such that homologous chromosomes were inherited intact from one generation to the next

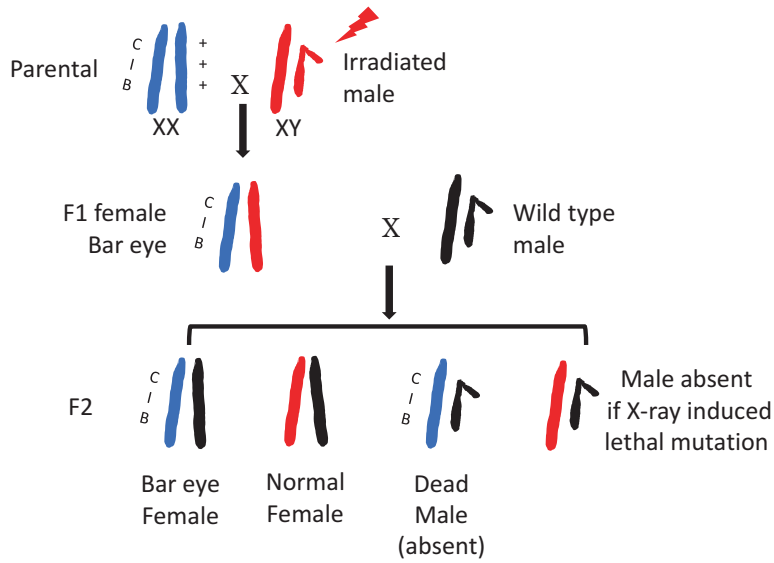

Fig. 13.1 One of the crossing schemes used by Muller to determine whether X-rays induce mutations. Bar eye females are crossed to irradiated males in the parental generation. In the F1 progeny, only the Bar eye females among all possible classes is shown. Crossing these females to wild type males produce four possible progeny classes in the F2. Males with the CIB chromosome (blue) are absent because of the recessive lethal on this chromosome. If the irradiated X chromosome (red) carries a recessive lethal mutation, non-Bar males would also be absent in F2

without recombination and exchange of alleles. We now know chromosomes with a crossover suppressor as Balancer Chromosomes. Balancer Chromosomes contain multiple inversions such that crossing over produces severely rearranged chromosome products that do not support viable gametes or off-springs. Thus, it is not that crossing over is suppressed, rather, any product of crossing over is not represented in the progeny.

Muller irradiated males and crossed them to a female carrying one CIB chromosome [Fig. 13.1 'Parental' cross, [24]]. F1 female progeny that inherited the irradiated X chromosome (red) from their father and the CIB X chromosome (blue) from their mother were recognized by their Bar eyes. When these F1 females were crossed to wild type males, the progeny in F2 included males with the irradiated X chromosome (red). If X-rays induced recessive lethal mutations, such males would be absent among the viable F2 population. Alternatively, If X-rays induced viable but visible recessive mutations, the phenotype will be manifested in these F2 males. Muller observed both of these outcomes, concluding that X-rays induced mutations, an important and fundamental insight into how IR works [39–41].

In his earlier work with X-rays, Muller used them as a tool to understand what exactly genes were and how they behaved. He discovered the phenomenon of dosage compensation; a gene on the X chromosome when present in two copies in an XX female produced the same phenotype as when it is present in one copy in XY males. Thus, he concluded, there must be mechanisms to compensate for the different gene dose in males and females for genes on the X-chromosomes [3, 42]. He discovered 'position effect'; a gene from the X chromosome that translocated to another chromosome (e.g. after X-ray induced chromosome breakage and repair) and remained intact could be functional or not depending on the new location [48]. He studied the location of X-ray-induced breakpoints cytologically and correlated their effects on the resulting phenotype, reaching the conclusion that there are regions of chromosome between genes that are not functional [48]. These are fundamental insights into what genes are, how they are organized and how they function.

It was in later work that he used genes/mutations to understand radiation. Muller's PhD student S. P. Ray-Chaudhuri found that the a given dose of IR was equally mutagenic whether the dose was administered acutely (in 30 min) or split into smaller doses delivered over a longer period of time (a month) [49]. The conclusion that even low, diagnostic doses of radiation could be harmful remains controversial now as it was when Muller first disclosed it [66], but has led to the current regulations concerning exposure monitoring of radiation workers; we now monitor total exposed dose over time.

13.3 Cytological Responses to IR

Muller was the sole recipient of the 1946 Nobel Prize in Physiology or Medicine 'for the discovery of the production of mutations by means of X-ray irradiation'. After his seminal findings, there followed many decades of deeper studies of *Drosophila* and IR, including studies that analyzed how environmental factors, dose rate, and organism age influence X-ray mutagenesis [for example, [4, 56]], how IR affects aging and fertility [for example, [58]], the effect of IR on developmental patterning [for example, [46, 64]], and X-ray-induced somatic crossing over [for example, [18]]. The results of many of these studies laid the ground for the next level of investigation in the 1970's in which *Drosophila* geneticists added cell biological tools to phenotypic observations at the organism level. Peter Bryant and colleagues carefully quantified cell death and mitoses in irradiated larval imaginal discs, and measured the size of cytologically marked clones of cells that formed as irradiated discs regenerated [17, 22]. Clonal analysis revealed cells that died by apoptosis as well as cells that were alive but suffered clonogenic death in that these cells did not proliferate during regeneration [17]. X-rays first inhibited mitosis, which we now know to be due to cell cycle checkpoints [22]. But mitosis recovered eventually and surviving cells were even more proliferative than un-irradiated cells [22].

The data led Bryant and others to suggest that extra proliferation served to compensate for cells killed by IR [17, 22], a phenomenon we now call compensatory proliferation [6, 7, 38, 51]. In short, collective work from this era defined cell biological phenomena that are conserved in mammals. As summarized in the next sections, *Drosophila* has been an extremely useful model to dissect the molecular mechanisms responsible for these phenomena.

13.4 Apoptosis-Induced Proliferation and Accelerated Proliferation

Many tissues such as the skin and gut epithelia regenerate using dedicated stem cells. But tissues and organs without dedicated stem cells also regenerate. *Drosophila* larval imaginal discs are one such example. Imaginal discs are precursors of adult organs. Each imaginal disc is composed of a single layer of columnar epithelium juxtaposed with a single layer of squamous epithelium. Exposure to IR doses that kill half of the columnar epithelial cells [17, 22] or surgical removal of up to 25% of the disc [23, 52, 69] is still compatible with complete regeneration to produce a viable adult fly of normal size and patterning. Regeneration of damaged discs occur by proliferation of the surviving cells as opposed to the use of dedicated stem cells (Fig. 13.2a). This model of regeneration resembles, for example, how the mammalian liver regenerates after surgery, by proliferation of remaining hepatocytes [14, 36, 37].

Wing discs in 3rd instar larvae exposed to 25 or 40 Gy (2500 or 4000 R) of γ-rays show reduced mitotic index as early as 1 h after irradiation [22, 68]. We have found a similarly rapid block of M and S phases using 20–40 Gy (2000–4000 R) of X-rays, with these responses requiring conserved check point proteins encoded by *mei-41* (*Drosophila* ATR) and *grapes* (*Drosophila* Chk1) [21, 31]. Mitotic index recovers to pre-irradiation levels at 6–8 h after irradiation [22, 68], and at 48 h after irradiation, mitotic index in the wing disc exceeds the levels found in unirradiated controls [22]. Higher than normal frequency of mitoses was observed also in the larval eye discs at 24 h after exposure to 20 Gy of X-rays [21]. In other words, at longer time during recovery, irradiated cells proliferate faster than unirradiated

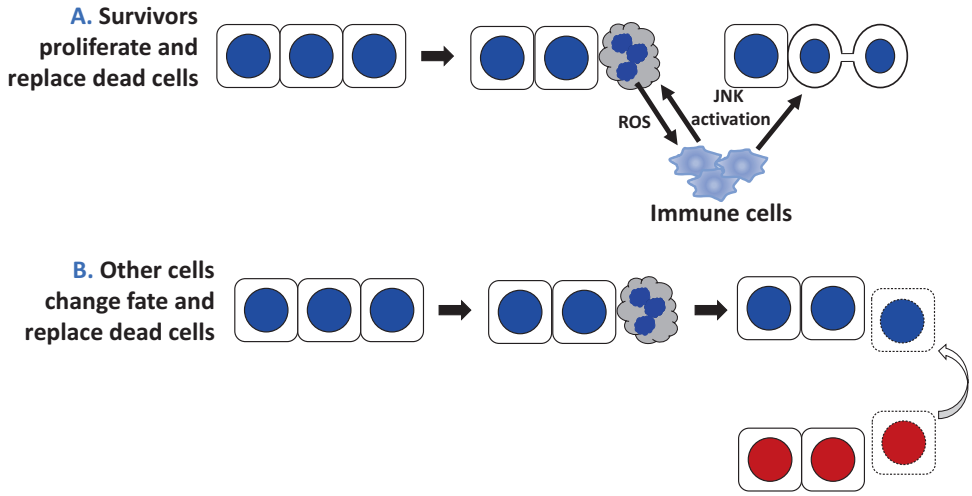

Fig. 13.2 Two sources of regenerative cells in systems that lack dedicated stem cells. (**a**) In response to cell death (grey cells), survivors proliferate to regenerate the tissue. Dying cells produce Reactive Oxygen Species (ROS) to recruit immune cells. Immune cells stimulate JNK signaling in the dying cells (for a positive feedback loop) and JNK signaling in surviving cells (to stimulate proliferation). (**b**) Unrelated cells (red) change fate to replace dying cells

cells. Irradiated wing discs contain 30% fewer cells than unirradiated discs even at 48 h after irradiation [22]. Therefore, extra proliferation observed may be stimulated by the need to replace cells lost to IR-induced apoptosis, which can be detected for as long as 48 h after irradiation in these experiments. The phenomenon in which surviving cells in irradiated tissues proliferate faster than unirradiated cells is conserved in mammalian tumors and is called 'accelerated proliferation' [page 384 of [15]]. Accelerated proliferation provides one explanation for the greater success of fractionated radiation therapy in multiple small doses given at regular intervals than delivery as a single large dose; each fractionated dose could kill proliferative cells stimulated by the preceding dose.

What molecular mechanisms stimulate surviving cells to proliferate when their compatriots have been killed by IR? The signals that instruct survivors to proliferate, we now know, come from the dying cells themselves in a process called Apoptosis induced Proliferation or AiP, a phenomenon seen also in human cancer models [reviewed in [6, 7, 9, 38, 51]]. In *Drosophila* where AiP is best understood, the required components in the dying cells include death regulators p53, JNK and apical caspase Dronc (see Fig. 13.3 for apoptosis signaling in *Drosophila*). AiP in some contexts also requires mitogens Wg and Dpp (for AiP from dying epithelial cells) or Hh (for AiP from dying photoreceptors in the eye disc). These mitogens are thought to be produced in the dying cells, with their production being dependent on p53, Dronc and JNK.

Most experiments in *Drosophila* that addressed AiP employed apoptosis induction with genetic means rather than IR. In these experiments, expression of pro-apoptotic genes such as *hid* and *reaper* are targeted to a subset of cells in imaginal discs. Regulation of their expression temporally with the Gal80-Gal4 system allows a burst of apoptosis followed by a period of regeneration. In a variation of this protocol, co-expression of caspase inhibitor p35 generates 'undead cells' (see Fig. 13.3). In these cells, apoptosis program has been initiated and apical caspase Dronc is active because it is refractory to inhibition by p35. But effector caspase activity is inhibited so that the cell does not die but persists in a sustained apoptotic state. Both cells that complete genuine apoptosis and undead cells elicit AiP. When AiP occurs in response to cells that complete apoptosis, the product of induced proliferation serves to replace the dead cells and is considered to be 'compensatory pro-

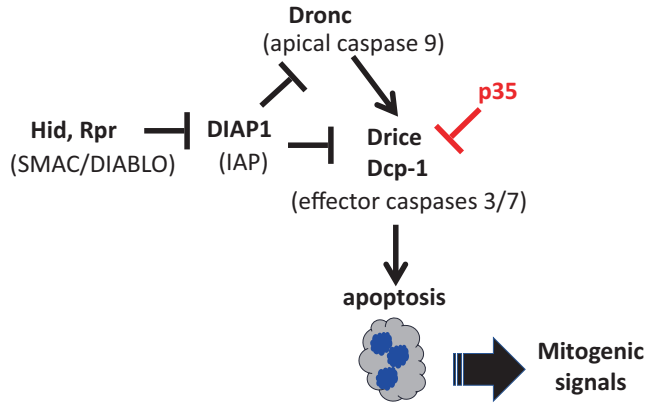

Fig. 13.3 Basic components of apoptotic signaling in *Drosophila*. Mammalian homologs are shown in brackets. Apoptosis requires caspase activity, which is normally kept in check by Inhibitor of Apoptosis Proteins (IAPs). Upon apoptosis induction, for example by X-rays, pro-apoptotic proteins Hid and Rpr neutralize IAPs to result in caspase activation. Apoptotic cells produce mitogenic signals to maintain tissue homeostasis. Viral caspase inhibitor p35 inhibits effector caspases but not apical caspases. A cell exposed to both death stimuli and p35 activates apical caspases and initiates the apoptotic program, but cannot complete it. Such an 'undead' cell remains alive and shows sustained mitogenic signaling

liferation' that restores normal structures. Genetic screens for mutations that fail to restore normal structures have identified many components of AiP as well as regulators that ensure precise growth control and tissue repatterning during regeneration [for example, [2, 27, 53–55]]. When AiP occurs in response to undead cells, the product of induced proliferation creates supernumerary cells. Because undead cells produce sustained mitogenic signaling, AiP from undead cells results in tissue overgrowth and hyperplasia. Genetic screens for mutations that suppress such overgrowth have identified new components of AiP [for example, [7, 8, 10]].

Caspase-driven mitogenic signaling by dying cells is conserved in mammals in a phenomenon called Phoenix-Rising which has proved to be highly relevant to radiation therapy [19, 33]. Here, mitogenic signaling by lethally irradiated cancer cells or fibroblasts stimulate other cells to proliferate, both in culture and in mice. This effect requires effector caspase 3, which cleaves calcium-independent Phospholipase A2, ultimately leading to the production of Prostaglandin E2 (PGE2), a signaling molecule known to stimulate stem cell proliferation, tissue regeneration and would healing [33]. Caspase 3−/− mutant mice show attenuated skin wound-healing and liver regeneration [33], and fail to repopulate the tumors after radiation treatment [19]. This is as expected if caspase-mediated mitogenic signaling is important for regeneration after IR damage. In human head and neck or breast cancer patients, activated caspase 3 staining in the tumor correlates with recurrence and reduced survival [19], suggesting that findings from *Drosophila* and mice are likely relevant to human cancers. PGE2 is not the only mitogen from dying cells. Another study identified WNT16B as the mitogen released by dying fibroblasts that promote survival and proliferation of prostate cancer cells [59]. Yet another study identified Shh signaling as a component of mitogenic signaling from irradiated cancer cells to unirradiated cancer cells [34]. PGE2 or similar molecules have not been implicated in AiP in *Drosophila* but Wg (*Drosophila* Wnt1) and Hh (founding member of the conserved family that includes Shh) are both known mediators of AiP and compensatory proliferation as described in a preceding section. Thus, *Drosophila* models can predict not only conserved phenomena but also conserved molecular mechanisms.

13.5 Cross Talk Between Radiation Responses and the Immune System

Tissue damage in multicellular organisms stimulates the immune system. A study of AiP that results from undead cells in the *Drosophila* larval eye imaginal discs found that innate immune system is activated upon tissue damage and plays a role in AiP [10]. The study was designed to investigate how caspase activity leads to JNK activation. The data identified an extra-cellular signaling loop that involves Reactive Oxygen Species (ROS). Specifically, apical caspase Dronc is required cell-autonomously to activate a membrane-associated NADH oxidase Duox. Duox activity results in the production of extracellular ROS. Indeed, mis-expression of enzymes that reduce cytoplasmic ROS had little effect on AiP while mis-expression of enzymes that reduce extracellular ROS reduced JNK activation and AiP [10]. In agreement with these results, an independent study in regenerating larval wing discs found that up-regulation of a co-factor for Duox was required to sustain ROS production and regenerative signaling [27].

Duox was required for the recruitment of hemocytes to undead cells and for the induction of a JNK activity reporter [10], suggesting that extracellular ROS was required to recruit circulating hemocytes and activate JNK. An allele of transcription factor Srp that specifically inhibits hemocyte differentiation also reduced JNK activation and AiP. Ectopic JNK activation, however, did not recruit hemocytes, suggesting that hemocyte recruitment is upstream of JNK activation. These data led to the model in which hemocytes activate JNK in the dying cells for mitogen production, trigging a positive feedback loop, and hemocytes activate JNK in surviving neighbors, to stimulate

proliferation (Fig. 13.2a). *Drosophila* TNF-α homolog Eiger and its receptor Grnd were identified as possible mediators of hemocyte-to-epithelial cell signaling [10]. Thus immune cell presence and activity at the site of damage promotes regenerative proliferation. In *Drosophila* neoplastic tumors, where oncogenic RAS activity maintains tumor cells in an undead state, caspase activity like-wise produces both intracellular and extracellular ROS, hemocyte recruitment, and further proliferation of tumor cells [43].

The above-described studies employed cells dying or undead because of genetic ablation. In the context of cell killing by IR in *Drosophila*, there is very little known about immune cell involvement. In a study using UV radiation instead of IR, damage to the retina results in the production of Pvf1 (a *Drosophila* PDGF/VEGF-like ligand) production, which in turn activates its receptor Pvr in hemocytes and induces a macrophage-like morphology [25]. Components of this paracrine signaling is required to prevent tissue loss after UV exposure, suggesting that stimulation of the immune cells by signals from the dying cells somehow contribute to regeneration. We have shown that exposure of larval discs to ionizing radiation (IR) also results in transcriptional up-regulation of Pvf1 and Pvf2 [60]. Pvf1, we found, is likewise needed to limit IR-induced apoptosis [1]. It remains to be seen of Pvf1 from IR-damaged cells also stimulates immune cells.

IR is known to induce intracellular Reactive Oxygen Species [50]. Whether IR also induces extracellular ROS and whether such induction has similar consequences as AiP in genetic ablation models remain to be investigated. But IR activates both apical and effector caspases, as well as JNK. IR also induces AiP [28, 44]. Thus all indications are that IR exposure also engages in immune-cell-mediated paracrine signaling described in preceding paragraphs for experiments using genetic ablation, but this possibility has not been tested experimentally. But if such an interaction exists, then it would parallel the cross talk between IR responses and the immune system seen in mammalian tumors [for example, [67]].

13.6 Cell Fate Changes Induced by IR

In studying the effect of X-rays on larval wing discs, we identified a second mode of regeneration in addition to AiP [61–63]. We found that cells of the future wing hinge region are protected from IR-induced apoptosis by the actions of Wg (*Drosophila* Wnt1) and JAK/STAT activity acting cell-autonomously within these cells [61]. Lineage tracing shows that as the disc regenerates during a 3 day period after IR, some hinge cells lose the hinge fate, translocate to the future pouch area that suffers more cell death, and express pouch markers [61, 63]. This represents a mode of regeneration in which one cell type changes into another to help replace the lost tissue (Fig. 13.2b). IR-induced cell plasticity here acts to restore the organ but parallels IR-induced cell plasticity that produces tumor-initiating cells after radiation therapy as explained below.

'Tumor initiating cells' or 'Cancer Stem-like Cells' (CSCs) are defined operationally as cells within a tumor with particularly high ability to regenerate the tumor. Their existence is controversial even with the operational definition, and their numbers in some cancer types appear to depend on experimental conditions. For example, in melanoma, one in a million cancer cells are able to initiate new tumors if implanted into NOD/SCID mice but this number increases to one in three if more immune-compromised NSD (NOD/SCID interleukin 2-receptor gamma chain null) mice were used [47]. What is generally agreed upon is that within a given tumor, cells vary widely in their ability to produce new tumors [35, 71]. In Head and Neck Cancer models where radiation is a major therapy choice, most tumorigenic cells within patient-derived samples show high CD44 expression and the presence of ALDH [26]. Such CSCs represent 0.1% to 4.1% of tumor cell population depending on the patient and can produce tumors nearly 70% of the time when implanted at 1000 cells/mouse. In contrast cells that are ALDH- and show low CD44 expression produced tumors <5% of the time even when 100,000 cells were used per implant. Cancer Stem-like Cells with superior tumor initiating

ability have been identified in multiple types of solid tumors, although associated molecular markers differ for different cancer type, for example CD133 and NPM1 in glioblastoma [70]. Eradication of tumor initiating CSCs is considered necessary for successful therapy and for prevention of metastases to a distant site.

In a hierarchical view of cancer, CSCs produce non-stem cancer cells. In addition, it is now recognized that, non-stem cancer cells are also capable of converting to CSCs. The plasticity that allows non-stem cancer cells and CSCs to interconvert presents a major challenge to any therapy that targets CSCs. Even more concerning, cancer treatments themselves promote the conversion of non-stem cancer cells into CSCs [5, 45]. In particular, IR converts non-stem cancer cells from a variety of cancer types into cells with CSC markers that can initiate new tumors in culture and *in vivo* [30, 32, 65]. An estimated 50% of cancer patients receive IR, alone or as part of their treatment (www.cancer.org). Therefore, it is essential that we understand what aspects of IR exposure induce fate conversion or what factors, cell-internal or external, regulate IR-induced regenerative behavior.

Using the *Drosophila* hinge-to-pouch system to monitor cell fate changes after irradiation, we have been systemically identifying genes needed for cell fate plasticity and cell movement after IR exposure. We have identified signaling molecules [e.g. Wg and STAT, [61]], epigenetic regulators [e.g. Nurf-38, [62]], members of the cell death pathways [e.g. apical and effector caspases, [63]], along with other genes whose exact contribution remains to be dissected. This experimental model has the potential to inform us about IR-induced cell fate plasticity in tumors.

13.7 Drug Screens for Radiation Modulators

IR doses that kill about half of the cells in larval imaginal discs still allow regeneration of these tissues to the extent that viable fertile flies will eclose, albeit after a developmental delay [17, 21]. The extent of delay is IR dose-dependent [17, 21]. IR-induced developmental delay is exacerbated by mutations in DNA Damage Response signaling such as *mei-41* (*Drosophila* ATR) and *grp* (*Drosophila* Chk1) and is dependent on p53 and retinoic acid signaling [16, 68]. The delay in pupariation means that irradiated larvae spent more time feeding than their unirradiated controls, before crawling up the side of the culture vial to initiate the pupa stage. These observations led us to suspect that the delay in pupariation reflects a need to continue food uptake, which in turns allow cellular growth and proliferation needed for regeneration. In support, inhibition of food uptake by switching larvae to poor nutrition after irradiation decreased the survival of larvae into adulthood [20]. Similarly, reduction in the dosage of genes encoding components of growth regulation, using heterozygous mutants in Insulin-like Growth Factor Receptor substrate *chico*, cdk4 and Myc, also reduced the survival of irradiated larvae into adults [20]. *chico*, cdk4 and Myc heterozygotes are viable without irradiation. In other words, (partial) inhibition of growth and regeneration was synthetically lethal with radiation. These findings led us to design a screen for chemical modulators of growth and regeneration that was synthetically lethal with radiation [12, 13, 20]. Such chemicals have the potential for use in combination with radiation therapy.

In the screen, 3rd instar larvae were irradiated with doses that allowed 50% of larvae to reach adulthood. Those that produced viable adults 'eclosed' from the pupa case, leaving it empty while those that failed to do so left a 'full' pupa case. Thus, counting full vs. empty pupae produced a quantitative measure of radiation sensitivity [12, 13, 20]. Irradiated larvae were placed in culture vials each of which contained a chemical of interest in the screen. Chemicals that reduced survival in a statistically significant manner were identified. Exploiting *Drosophila* genetics, an additional layer was added to the screen. Chemical libraries were screened using p53 or *grp* (*Drosophila* Chk1) mutant larvae and the hits were counter screened against wild type larvae

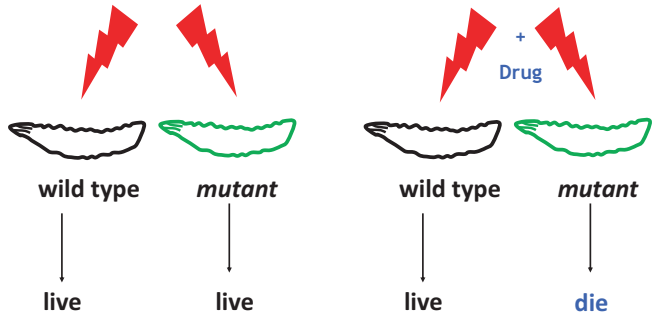

Fig. 13.4 The design of a screen to identify drugs that are synthetic lethal with radiation on mutant larvae. In the absence of the drug, wild type (black) and *grp*/Chk1 mutant (green) larvae are equally sensitive to X-rays. The screen is designed to identify drugs, that when present, allow irradiated wild type larvae to survive but kill irradiated mutant larvae. Thus the drug is synthetically lethal with radiation, with greater effect on *grp* mutants than on wild type

(Fig. 13.4). Those that showed greater effect on larvae with cancer-relevant mutations compared to wild type were further selected for study. Thus, the screen aimed to identify molecules with a potential therapeutic index (greater efficacy on mutant cancer cells over normal tissues).

Screens through chemical libraries identified drugs approved for use in combination with radiation such as camptothecin, a topoisomerase I inhibitor, providing proof of concept data that a *Drosophila* screen can identify drugs that are applicable to human cancer [11, 20]. The screens yielded an interesting group of three chemical scaffolds, all of which to act by inhibiting translation elongation [11]. This is of interest because stimulation of translation elongation, by degradation of the inhibitor EF2 Kinase, has been shown to be critical during recovery from radiation damage in human osteosarcoma cells [29]. Thus, inhibition of translation elongation, with chemical hits found in the *Drosophila* screen, was expected to interfere with recovery after IR damage, thereby increasing the effect of IR. In support of this idea, one of the inhibitors of translation elongation found in the *Drosophila* screen, bouvardin (NSC259968), was subsequently found to enhance the effect of IR in human cancer models [57]. Of more interest, the ability of bouvardin as a radiation enhancer was greater on cancer cells than on non-transformed cells, mirroring how the *Drosophila* screen was designed to identify chemicals that differentiated between p53/chk1 mutants and wild type.

13.8 Conclusions

From revealing the mutagenic effect of X-rays to dissecting the molecular basis for Apoptosis-induced Proliferation, *Drosophila melanogaster* has been a proven experimental model to study radiation responses and regenerative mechanisms that are conserved to human. Additional uses of the *Drosophila* model to address other aspects of radiation biology such as the cross-talk with the immune system, IR-induced cell fate plasticity, and identification of chemical radiation-modulators hold promise. With powerful genetic tools, *Drosophila* remains the premier model for gene discovery. It is through innovative use of forward genetic screens, combined with the power of reverse genetics to illuminate mechanism, that we will uncover new mechanisms in *Drosophila* towards improving radiation therapy of human cancers.

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Conflict of Interest The author owns equity in SuviCa, Inc.

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A Drosophila Based Cancer Drug Discovery Framework

14

Erdem Bangi

Abstract

In recent years, there has been growing interest in using Drosophila for drug discovery as it provides a unique opportunity to screen small molecules against complex disease phenotypes in a whole animal setting. Furthermore, gene-compound interaction experiments that combine compound feeding with complex genetic manipulations enable exploration of compound mechanisms of response and resistance to an extent that is difficult to achieve in other experimental models. Here, I discuss how compound screening and testing approaches reported in Drosophila fit into the current cancer drug discovery pipeline. I then propose a framework for a Drosophila-based cancer drug discovery strategy which would allow the Drosophila research community to effectively leverage the power of Drosophila to identify candidate therapeutics and push our discoveries into the clinic.

Keywords

Drosophila · Cancer drug discovery · Compound screening

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14.1 Introduction

With its sophisticated genetic tools and practical advantages, Drosophila has been a favorite model organism for developmental biology and genetics research for decades. Genetic screens and epistasis studies in Drosophila have opened the door to understanding fundamental aspects of development, cell biology and signal transduction by uncovering novel genes and signaling networks involved in key developmental and molecular pathways. Drosophila also has a strong track record as a useful disease model; many complex disease states have been successfully modeled in flies [1–4]. In the last two decades, a number of pioneering studies have demonstrated the potential of Drosophila as a powerful drug discovery platform [5–12], paving the way for future studies where its genetic power can be leveraged to discover new candidate therapeutics as well as to explore mechanisms of action and resistance of drugs already in the clinic or in clinical development.

Briefly, this early body of work demonstrated that (1) compounds can be introduced into flies by feeding or culturing dissected tissues in the presence of compounds, (2) developmental phenotypes and pathway specific target gene expression can be used as read-outs to monitor compound activity, and (3) compounds can be tested for their ability to modify disease phenotypes generated by genetic manipulations of

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disease relevant genes. Overall, these studies demonstrated that many compounds identified in mammalian assays also effectively modulate the activity of Drosophila orthologs of their targets, indicating a high degree of conservation of compound activity in Drosophila. These studies have been extensively reviewed elsewhere [1, 13–18] and will not be discussed in detail. Here, I will discuss how drug screening and testing approaches reported in Drosophila fit into the current cancer drug discovery pipeline. I then propose a Drosophila-based drug discovery framework where sophisticated genetic tools and practical advantages of flies can be effectively leveraged to develop novel candidate therapies that can address the genetic complexity of cancer.

14.2 Compound Screening

For the past 20 years, the prominent drug discovery approach in oncology has been a target-based one [19–22]. The underlying rationale for this approach is that pharmacological modulation of the activity of a target with a key role in driving and/or maintaining a tumor phenotype will lead to a clinically relevant response. This "target-first" approach starts with the identification of a potential genetic vulnerability through functional studies or mining of large "omics" datasets. Chemical modifiers of the activity of the target are then identified using a variety of approaches including high throughput compound screening, *in silico* methods and rational design. There have been a few spectacularly successful examples of this approach, such as imatinib (GLEEVEC) [23] and gefitinib (IRESSA) [24]. However, target-based drug discovery approaches have had low overall success rates for most solid tumors in clinical trials [19, 25, 26]. At least part of the difficulty comes from the complex and diverse nature of tumor genome landscapes. The presence of highly redundant signaling networks and multiple compensatory feedback mechanisms make it particularly challenging to find an individual target whose pharmacological modulation leads to a therapeutically relevant response.

A complementary approach to target-based drug discovery is a phenotype-based approach [19, 26]. This target agnostic, function-first approach aims to identify a chemical entity that can reverse a tumor phenotype or eliminate cells demonstrating such phenotypes through compound screens that use cancer specific phenotypes as read-outs. As most tumor phenotypes arise as emergent interactions between multiple genomic alterations in complex and diverse genetic contexts, this approach holds significant promise to identify candidate therapeutics that can address disease complexity.

Drosophila is a particularly useful platform for phenotype-based cancer drug discovery approaches as it provides an opportunity to screen compounds using sophisticated *in vivo* read-outs in a whole animal setting where compound efficacy and toxicity can be monitored simultaneously. Genetic modifier screens, a classical approach that has been a key strength of Drosophila in developmental biology and cell signaling, have been adapted by several groups to identify chemical modifiers of developmental or disease phenotypes [12, 27–32]. Rescue from lethality, which has been an effective read-out in genetic screens, has also proven to be a particularly useful high throughput compound screening read-out and is the most commonly used assay for compound screening.

Historically, a key limitation of the phenotype-based drug discovery approach has been the lack of diverse phenotypic read-outs for compound screening. Reliance on cell survival or proliferation as the primary read-out in high throughput screens lead to the identification of mostly cytotoxic or cytostatic compounds as candidate therapeutics. In recent years, many hallmarks of cancer—including proliferation, apoptosis, senescence, epithelial-mesenchymal transition, migration and dissemination—have been successfully captured in Drosophila cancer models [2, 33–36], making it possible to develop high throughput screening assays to discover compounds that modify these complex and disease relevant phenotypes. Drosophila compound screens that use imaging or luciferase-based screening read-outs have already been reported

[27, 28]. Compound screens that focus on hallmarks of cancer beyond proliferation and survival as primary assays hold great promise to identify novel classes of lead compounds with more complex target profiles and mechanisms of action, diversifying the portfolio of candidate therapeutics in clinical development.

Another key advantage of Drosophila as a cancer drug discovery platform is the ability to generate sophisticated cancer models that reflect the complexity and diversity of human tumor landscapes. Large scale tumor sequencing studies have revolutionized our understanding of tumor genome landscapes [37], leading to precision medicine approaches and sophisticated, biomarker-based clinical trial designs [38–41]. While this is a promising approach [42], focusing on individual genomic alterations can oversimplify the genetic diversity and complexity of tumor genome landscapes and has not always been sufficient to predict drug response [43–48]. Drosophila offers an opportunity establish large collections of tumor-genome based, genetically complex cancer models for compound screening in a cost and time-effective manner. For instance, using a panel of colorectal cancer models based on sequenced colon tumors, we have shown that genetically complex models are resistant to most compounds identified through target-based drug discovery approaches [33]. Compound screens using such models can lead to a new generation of candidate therapies that can address disease complexity and could lead to precision medicine approaches that use broader genomic landscapes rather than individual cancer driver alterations for patient stratification.

14.3 Exploring Compound Mechanisms of Action (MoA)

Identifying MoAs for hits identified in phenotype-based compound screens can be particularly challenging, as these are target- and mechanism-agnostic by design. Phenotypic screening can yield compound MoAs that include modulation of more than one target as well as non-autonomous effects on neighboring wild type cells and tissues. Several Drosophila studies have demonstrated how gene-compound interaction and epistasis studies can be used to identify compound MoA.

As an example of using flies to explore MoA, a compound screen against a Drosophila lung cancer model established by targeting oncogenic *ras* and *pten* loss to the tracheal system identified trametinib/fluvastatin as a candidate therapeutic combination [29]. The MoA for the combination included suppression of whole animal toxicity associated with trametinib by fluvastatin, at least in part through its activity on RAS pathway signaling. In other words, fluvastatin improved the efficacy of trametinib by allowing an otherwise toxic dose for trametinib to be used as part of the combination, in effect, broadening its therapeutic window. In another example, a study that used a stem cell derived intestinal tumor model established by targeting oncogenic *raf* to stem and progenitor cells of the adult intestine identified a class of chemotherapy agents that promoted the proliferation of wild type stem cells while inhibiting the growth of stem cell tumors [28]. This proved due to a non-autonomous effect mediated by the secretion of JAK-STAT ligands, which altered the immediate tissue microenvironment and promoted proliferation of neighboring wild type stem cells.

In a third example of using Drosophila to explore drug MoA, we used a genetically complex '4-hit' model of colorectal cancer to identify the proteasome inhibitor bortezomib plus PI3K pathway inhibitor BEZ235 as an effective drug combination with a novel and unique MoA for reducing dissemination of tumor cells into the abdominal cavity [33]. Our fly studies demonstrated that bortezomib promoted dependence on BEZ235 by altering the output of the latter's target signaling pathway. This unique mechanism required sequential, alternating treatment with the drugs for the combination to be effective. These studies demonstrate how traditional approaches commonly used in a typical Drosophila laboratory to address basic science questions can be adapted to explore compound MoA in the context of a whole animal, a particularly challenging step in drug discovery.

Studies in Drosophila demonstrated that lead compounds identified through target-based approaches can also benefit from detailed mechanistic studies to clarify their mechanisms of action [8, 49]. A key goal of target-based drug discovery is to identify lead compounds with exquisite specificity and selectivity with the underlying assumption that such compounds are more likely to be therapeutically relevant [50, 51]. Despite these efforts, *in vitro* target profiling studies have shown that most compounds identified by this approach nevertheless have additional direct targets [52]. Genetic modifier screens and gene-compound interaction studies in Drosophila have been used to explore the functional relevance of direct targets identified by target profiling studies [8, 49]. One of the earliest examples of this approach has been reported using ZD6474 (vandetanib), originally identified as a selective inhibitor of the receptor tyrosine kinase VEGFR2 and subsequently shown to inhibit the activity of additional receptor tyrosine kinases (EGFR, PDGFR, RET) to a lesser extent [53–55]. Gene-compound interaction experiments in Drosophila have revealed an *in vivo* preference of ZD6474 for oncogenic RET [8]. ZD6474 was subsequently FDA approved for metastatic medullary thyroid cancer (MTC), a rare type of thyroid cancer mediated by oncogenic RET [56].

14.4 Exploring Compound Mechanisms of Resistance

Oncology has one of the lowest clinical trial success rates among all disease areas [57]. Most lead compounds with promising targets, MoAs and abundance of preclinical data fail to demonstrate a clinically relevant response. Drosophila models have been successfully used to explore mechanisms of resistance for such compounds by identifying genetic modifiers of drug resistance [30–33]. These studies have led to rational drug combinations specifically designed to overcome resistance, many of which have also been shown to be effective in preclinical mammalian cancer models. This approach provides a unique opportunity to repurpose lead compounds that failed in clinical trials either as single agents specifically targeting genomic landscapes most likely to respond or as part of drug combinations designed to overcome resistance.

Unfavorable responses in clinical studies are often attributed to the lack of selectivity and specificity of lead compounds. However, even in cases where a clean pharmacological inhibition of a target or signaling node is achieved, it is not always sufficient to elicit a clinical response [43–48]. Importantly, actionable genes targeted in these studies are almost always found in the context of other mutated genes that can alter drug response in unpredictable ways; this suggests that lack of response is an emergent feature of complex interactions within the disease network. Drosophila has also emerged as a useful platform to correlate response and resistance with broader genomic landscapes by testing lead compounds against large panels of genetically complex and diverse models. For instance, we have used this approach to identify specific genomic landscapes that correlate with response and resistance to inhibitors of the PI3K pathway [33], which have shown modest efficacy as single agents in clinical trials for most solid tumors [43, 58]. These findings provide opportunities for more sophisticated biomarker based clinical trials where patients can be stratified using broader genomic landscapes.

14.5 Structure-Activity Relationship (SAR) Studies and Rational Synthetic Tailoring

A key component of the target-based drug discovery pipeline is Structure-Activity Relationship (SAR) studies, in which a series of structurally related compounds are generated and tested to identify chemical groups responsible for the observed biological activity. This information can then be used to generate lead compounds with improved specificity, selectivity and favorable pharmacokinetic and pharmacodynamic (PK/PD) properties by chemical synthesis [59]. Perhaps the most innovative application of Drosophila in cancer drug development has been

the use of a phenotype-based SAR approach where compounds in a SAR series are tested for their ability to modify a complex disease phenotype rather than to specifically and selectively inhibit a single target [49, 60]. This rational synthetic tailoring approach uses an iterative process of chemical synthesis, functional studies in Drosophila and *in vitro* target profiling to identify and eliminate undesirable activities (anti-targets) from a chemical structure while retaining desirable activities (pro-targets); optimizing a compound's pro-target/anti-target profile can lead to an excellent therapeutic index, the ratio of efficacy to toxicity. The underlying assumption of this strategy is that pharmacologically complex compounds—selected for optimal pro-target/anti-target profiles rather than single target specificity—would be more effective in addressing genetic complexity of human tumors and less prone to acquired resistance.

Structurally related compounds with well-characterized direct target profiles generated for SAR studies represent a valuable resource for lead selection and optimization in different tumor types or genetic contexts. It is reasonable to hypothesize that tumors with different genomic landscapes would respond to different pro-target/anti-target profiles. For instance, a target that represents a key vulnerability in one genomic landscape can be a significant anti-target in another genetic context. The unique pro-target/anti-target profile required to effectively target a specific tumor genomic landscape can be identified in genetic experiments in Drosophila. Previously established SAR series can then be repurposed to identify lead compounds with the appropriate profile to target a new tumor type or genomic landscape.

14.6 Drosophila as a Personalized Cancer Drug Discovery Platform

Perhaps the most direct test of the utility of Drosophila as a clinically relevant cancer drug discovery platform is a personalized "fly-to-bedside" clinical study currently ongoing at the Icahn School of Medicine at Mount Sinai. In this experimental study, personalized fly models are generated for each cancer patient; these models are then used to screen a library of FDA approved drugs to identify a drug combination specifically tailored to each patient.

The study begins with a comprehensive analysis of each patient's genomic landscape including tumor and normal (germline) whole exome sequencing and copy number analysis. A personalized Drosophila model is then generated for each patient that reflects their tumor's genomic landscape. This model is used in iterative screens of FDA approved drug libraries that use rescue-from-lethality as a read-out to identify a drug combination for each patient. After findings are reviewed by a multidisciplinary tumor board that includes oncologists, pharmacologists as well as scientists with expertise in Drosophila genetics, cancer genomics and clinical trial design, a personalized treatment plan is identified for each patient.

Early results from this study are promising: We have identified a 2-drug cocktail that led to a strong partial response followed by several months of stable disease for a KRAS mutant patient with metastatic colorectal cancer who had previously progressed on multiple FDA approved therapies (in press). I would like to include it as a proper citation). Outcome data from additional patients will be necessary to determine the viability of this approach as a personalized treatment option for cancer and feasibility of integrating it into clinical practice.

14.7 How Not to Get Lost in Translation: Bringing Discoveries to the Bedside

Clinical development of candidate therapeutics is a complex, multi-step effort and can be a challenging prospect. Clinical trials are expensive and often require investment from the private sector or foundations; intellectual property (IP) protection is key to generate interest for clinical development of lead compounds [61]. Validation studies using mammalian preclinical cancer

models are required to confirm the mechanism of action and efficacy of hits from Drosophila screens. Additional studies to explore PK/PD properties of hits are essential to evaluate their potential for clinical development. Many of these steps are outside the expertise of a typical Drosophila laboratory, and the idea of pushing candidate therapeutics identified using Drosophila into the clinic can be daunting for many of us. In this section, I propose a Drosophila-based cancer drug discovery framework that incorporates feedback from and collaborations with chemists, clinicians, pharmacologists and scientists with expertise in mammalian cancer models and discuss some important features of this approach (Fig. 14.1).

14.7.1 Choosing a Cancer Model

Perhaps the most important consideration for choosing a model for cancer drug discovery is to ensure that it addresses a clinical need for development of new therapies. While cancer in general represents a key area of unmet medical need, tumor types with multiple FDA approved therapies and/or lead compounds with promising clinical data may be less attractive to investors. The performance of standard of care in the clinic is also a key factor. For instance, some combination therapies FDA approved for multiple myeloma can provide overall survival ranges of 7–10 years [62]. Clinical trials designed to identify candidate therapies that outperform current standards in this case would be very long and expensive, and companies may be reluctant to support them. Choosing a tumor type based on a careful analysis of the availability and performance of FDA approved therapies and lead compounds in clinical development can make it easier to establish partnerships for clinical development of lead compounds identified through Drosophila screens.

A related issue to consider is whether to establish a Drosophila model of a specific tumor type, focus on a genomic landscape common to multiple tumor types or a specific hallmark of tumorigenesis such as invasion. While Drosophila models of a specific tumor type represent the most straightfor-

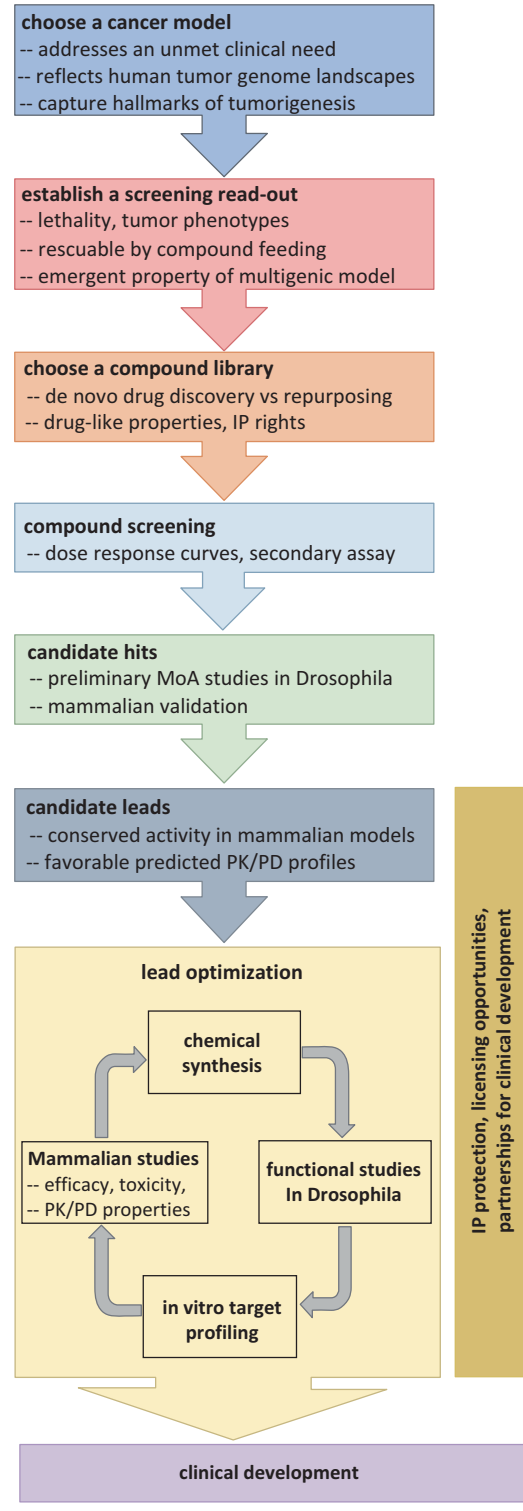

Fig. 14.1 A Drosophila based cancer drug discovery pipeline

ward approach to address an unmet clinical need, the latter two approaches can lead to commercially viable lead compounds with broader potential relevance for cancer therapy. However, identifying patient populations for clinical development and mammalian preclinical models for validation studies can be challenging in these cases and should be considered prior to screening.

Another important factor to consider is how well the Drosophila model captures the genomic landscape of the tumor type of interest. Rather than focusing on a single genetic model, use of a panel of genetically complex models that better capture the overall genomic landscape of a tumor type can better leverage the advantages of Drosophila as a model system and increase the clinical relevance of the model. Compound response profiles of different genomic landscapes can be explored by screening multiple models in parallel or testing lead compounds identified from one screen against additional models. The ability to correlate compound response with tumor genotype is a powerful tool to identify the most appropriate preclinical model for validation studies and define a target patient population for clinical development of candidate therapies. Of note, while most cancer relevant genes and signaling pathways are highly conserved, not all tumor types or recurrent genomic alterations can be modeled in flies. For instance, modeling hormone dependent cancer types such as breast and prostate cancer may be challenging in flies, as flies do not have clear estrogen or androgen orthologs. Focusing on tumor types with highly conserved cancer driver genes and demonstrating that the Drosophila model captures key aspects of tumorigenesis prior to screening will be essential to establish its clinical relevance.

High throughput screens are open ended by design; it is not easy to estimate the likelihood of success. Therefore, whether one is designing a new Drosophila cancer model or evaluating the potential of an existing model for cancer drug discovery, considering these issues prior to screening is critical to ensure that there will be a path forward for the clinical development of lead compounds if they are identified.

14.7.2 Choosing a Screening Assay

The most commonly used compound screening read-out in Drosophila is rescue of a lethal phenotype typically generated by inducing genetic alterations during development. More disease relevant read-outs can also be used for screening; for instance, imaging and luciferase-based Drosophila compound screens have been reported [27, 28]. Assays for additional hallmarks of cancer such as invasion, apoptosis, senescence or tumor metabolism can also be adapted as primary screening read-outs; however careful pilot studies to evaluate, cost, time and effort would be advisable to ensure that a useful throughput can be achieved. If multigenic cancer models are being used, relative contributions of individual genetic alterations in the model to the screening read-out should also be determined. A phenotype that arises as an emergent property of the particular multigenic combination being used would be more likely to identify hits that can address genetic complexity.

For most chemical genetic studies in Drosophila, compounds are orally introduced by mixing compounds in the food. As developing animals have access to food only for approximately 3 days during larval development, the specific developmental stage of the phenotype is an important consideration when choosing a screening assay. If compounds that can serve as positive controls are available, they should be used to validate and calibrate the screening assay. If this is not possible, the best strategy is to choose larval lethal phenotypes. Avoiding genetic manipulations during embryogenesis is a good strategy to eliminate embryonic lethality and later stage lethal phenotypes that may arise as a result of irreversible defects occurred during embryogenesis. Pupal lethal read-outs have been successfully used for compound studies and can also serve as useful read-outs especially if lethality is due to defects during larval development. However, this can be difficult to empirically determine. Pupal lethal read-outs could also bias screening results by selecting for compounds that are particularly stable in Drosophila and persist through pupal development.

14.7.3 Choosing a Compound Library

Compound libraries for *de novo* drug discovery can be obtained from a wide number of sources, including commercial libraries and compound collections through collaborations with chemists or pharmaceutical companies. Engaging chemists as well as expertise in intellectual property (IP) protection during the library selection process helps ensure that a library of compounds with drug-like properties in patentable chemical space is identified. Most academic institutions have offices that deal with IP, technology transfer and commercialization issues that can be engaged to leverage their expertise. If compound libraries are obtained through external collaborations, how IP rights for potential discoveries would be assigned should be clarified prior to screening. For collaborations with the private sector, publication rights and timelines should also be discussed in advance as most projects in academic laboratories are carried out by postdocs and graduate students for whom the ability to publish in a timely manner is an important consideration.

Historically, natural products have been a useful source for drug discovery, yet in recent years drug development efforts from natural products have declined significantly [63, 64]. This is mostly due to IP concerns and difficulty in obtaining sufficient quantities of material for clinical studies. Reproducibility of findings can also be an issue with natural products due to significant batch-to-batch variability as well as seasonal and environmental changes in content and composition. For these reasons, if natural product libraries are used for compound screening, identifying active components responsible for the desired biological activity and demonstrating that commercially viable leads can be generated by chemical synthesis will be necessary to successfully bring initial hits into the clinic.

de novo drug discovery requires a significant amount of effort for lead optimization, not only for improving efficacy but also to develop leads with favorable pharmacokinetic and pharmacodynamic (PK/PD) properties. For this reason, drug repurposing—in which libraries of FDA approved drugs or those in clinical development are screened in an effort to identify novel indications—is a particularly attractive alternative approach. The existence of significant preclinical and clinical safety data and the availability of compounds already formulated for human use can significantly shorten the clinical development of these drugs for new indications [65, 66].

Despite these advantages, there are still significant regulatory and logistical challenges associated with drug repurposing [67]. First, if the mechanism of action for the new indication is different from the original indication or involves a previously uncharacterized target/activity, additional safety and dosing studies may be necessary, increasing the cost of clinical development. Second, off label use of generics is difficult to prevent in practice, reducing the profitability of repurposing a drug for a new indication. As a result, companies may have less financial incentive for repurposing drugs that are off-patent or soon to be off-patent. In principal, "regulatory data exclusivity" can be obtained for a new indication, which in theory prevents other parties from relying on clinical data generated for the new indication in regulatory applications for generic versions [67]. However, this is very difficult to enforce in practice, as the cheapest version of the drug (i.e. the generic) is usually prescribed or dispensed regardless of indication.

For these reasons, availability of a path forward for clinical development of potential hits should be carefully weighed when considering a drug repurposing approach. One possibility is to prioritize hits that are either recently FDA approved with a long period of protection from generics or those that are still in clinical development. Another approach is to use compounds whose clinical development has been discontinued due to lack of efficacy. Finding a novel indication for such compounds as single agents or as part of drug combinations provides a path towards achieving a return on the investment already placed in their clinical development. Importantly, compounds whose development was halted due to significant toxicity or unfavorable PK/PD

properties would not be ideal candidates for such an approach unless chemical expertise to optimize PK/PD profiles through SAR studies is available.

14.7.4 Hit Selection and Preliminary Mechanism of Action Studies in Drosophila

Once hits from the primary screen are confirmed, preliminary follow-up studies in Drosophila should be conducted to obtain insights into their potential MoAs and choose appropriate preclinical models for mammalian validation. These typically include dose response studies to identify the best dose for future experiments in Drosophila, use of a panel of secondary assays to identify which hallmarks of tumorigenesis are targeted by the hit and testing efficacy in different genetic contexts to identify sensitive and resistant genomic profiles. More detailed mechanistic studies can be performed for hits with confirmed efficacy in mammalian models.

Hits with the best efficacy or biological activity in Drosophila may not necessarily be the ones with the highest potential for commercial development. Therefore, it is crucial to prioritize hits based on consultations with chemists, pharmacologists and IP experts to explore patentability, PK/PD properties, and the ability to modify the chemical structure for SAR studies for lead optimization. Pursuing more than one hit in subsequent mammalian validation studies may also increase the likelihood that a commercially viable lead is identified.

14.7.5 Mammalian Validation

Most common cancer driver genes and signaling pathways are highly conserved in Drosophila. As a result, hits from a Drosophila-based screening platform typically have conserved biological activity. However, Drosophila models may not capture all aspects of human cancer; for instance, lack of adaptive immunity and the absence of an extensive stroma make it difficult to capture the complexity of tumor microenvironment and tumor-stromal interactions. Mammalian validation studies using multiple preclinical models if possible should be an essential and early component of the lead selection process.

Use of genetically complex Drosophila cancer models and *in vivo* screening read-outs has the potential to identify drug candidates with sophisticated mechanisms that may be missed in traditional cell-based drug discovery pipelines. This very same feature that makes Drosophila such an attractive platform for drug screening can also make it difficult to find an appropriate preclinical model for mammalian validation, particularly for mechanistic studies that require sophisticated genetic manipulations. Therefore, it is useful to start thinking about appropriate mammalian models for validation studies early on in the process and seek collaborations to establish new ones if necessary.

Once hits with conserved efficacy are identified, additional mammalian studies may be required to validate their MoAs. Time, cost and effort associated with these studies can be minimized by performing large scale exploratory studies and experiments that require sophisticated genetic manipulations in Drosophila in an effort to identify more specific hypotheses to be tested in mammalian models. For instance, *in vitro* target profiling is a powerful tool to identify direct targets for candidate leads. These studies typically identify multiple targets; Drosophila is an ideal platform to explore their *in vivo* relevance and relative contributions to efficacy and toxicity. Unbiased genetic screens designed to identify modifiers of drug response can also clarify mechanisms of action and provide more direct hypotheses that can be tested in mammalian models.

In addition to demonstrating conserved biological activity, PK/PD properties of candidate leads also need to be explored, particularly for *de novo* drug discovery. PK/PD modeling and simulation studies can be conducted early on to prioritize hits with favorable predicted PK/PD profiles [68, 69]. However, efficacy, safety,

metabolism and PK/PD profiles will eventually need to be determined experimentally using animal models [70, 71]; these experiments can be conducted in collaboration or outsourced to contract research organizations that specialize in these types of studies.

14.7.6 Lead Optimization

Lead optimization studies are typically conducted in an effort to further improve efficacy, PK/PD properties and reduce toxicity of candidate leads. This is a costly and time-consuming process which often requires multiple rounds of chemical synthesis, *in vitro* target profiling and mammalian validation studies. A possible strategy to minimize the cost and effort associated with lead optimization is to start exploring partnership opportunities with the biotech sector early on in the process, as soon as a lead with conserved biological activity and favorable predicted PK/PD profile is identified and IP protection is obtained. Focusing on smaller biotech companies at this stage would be a better approach as partnerships with Big Pharma typically require more extensive preclinical development and may be more appropriate for leads at later stages of development [71].

14.8 Final Words

A large and growing body of published work has demonstrated the utility of Drosophila to identify new candidate therapeutics, explore mechanisms of action and resistance for drugs that are currently in development and identify novel vulnerabilities that can be targeted in future studies. The broader cancer research community is also becoming aware of this work and recognizing the value of Drosophila as a drug discovery platform, which makes the collaborative effort required to push our discoveries into the clinic possible. Another key advantage we often overlook and perhaps even undervalue as Drosophila researchers is the unique perspective we bring to cancer drug discovery through our genetic expertise and experience with *in vivo* research. This, combined with sophisticated genetic tools and practical advantages of Drosophila, can open the door to the development of a new class of candidate therapeutics that can address disease complexity and contribute to the idea of personalized medicine.

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Conflicts of Interest The author declares no potential conflicts of interest.

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Correction to: Autophagy and Tumorigenesis in *Drosophila*

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Correction to:
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The chapter was inadvertently published with one of the co-author's name incorrectly spelled as "Royjar" instead of "Rojyar". This error has now been corrected to read as "Rojyar Khezri".

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