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by

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Professor David Bilder, Chair
Professor Lin He
Professor Iswar Hariharan
Professor Daniel Nomura

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Abstract

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At its simplest, cancer can be defined as a disease of uncontrolled cell growth and proliferation. The genetic changes and consequences of these changes are now well-defined, owing to modern advances in molecular biology. These changes within a tumor cell extend beyond just the tumor itself. Tumor cells release a plethora of signals that alter the behavior of their cellular neighbors as well as the physiology of their host. Unlike the better-understood genetic changes that drive malignant transformation, the mechanisms that govern the myriad of ways tumors influence the behavior and physiology of distant host tissues remains opaque.

We have developed a novel *Drosophila* adult ovarian tumor model that overcomes the drawbacks of existing tumor models and creates a unique opportunity to investigate the molecular mechanisms underlying tumor-host interactions. We analyzed the transcriptome of ovarian tumors and found to our surprise that ovarian tumors upregulate several clotting factors, leading us to hypothesize that tumors may interfere with host coagulation, reminiscent of a human paraneoplastic syndrome called disseminated intravascular coagulation. We found that the overproduction of Fondue, one of the clotting factors identified in our transcriptome, drives systemic coagulopathy in tumor-bearing flies, and knockdown of Fondue rescued multiple clotting defects. Importantly, Fondue knockdown significantly improved the survival of tumor-bearing flies compared to controls, indicating that Fondue-driven coagulopathy contributes to host mortality.

We took advantage of the ovarian tumor model to perform an unbiased, forward genetic screen to search for novel mediators regulating tumor-host interactions. This screen made use of a library of deletions available to fly biologists, and we identified two potential candidate genes that may play a role in tumor-host interactions – *NKCC* and *tartan*. Although we were unable to identify a specific mechanism, our current results suggest that *NKCC* may regulate paraneoplastic dysregulation of ion homeostasis, while the mechanism through which *tartan* acts requires further investigation. Additional work using the ovarian tumor model suggested the existence of a previously unidentified paraneoplastic syndrome that manifests as defects in heart physiology. Thus, our work builds upon previous work on tumor-host interactions in *Drosophila* and demonstrates the power of the ovarian model to study paraneoplastic syndromes.

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CHAPTER 1

Introduction

The evolution of our understanding of cancer biology

With the rise of multicellularity, cells began working in concert, coordinating their behaviors and decisions to improve their survival in an unforgiving environment. However, inherent in this way of life lies the possibility that one of these cells may shun its responsibilities, placing its own growth and survival above that of the whole organism. For as long as humans have existed, these rogue cells have given rise to malignant tumors whose existence confounded physicians. The original copy of the Edwin Smith Papyrus, written in Egypt circa 3000 B.C.E., describes one of the earliest written accounts of a breast tumor for which there was no treatment [1,2]. Additional Egyptian texts from approximately 1500 B.C.E. detail the case of an unknown soft-tissue tumor, as well as descriptions of carcinomas of the skin and gastrointestinal tract [2]. During the Age of Pericles in Greece, Hippocrates argued that the underlying cause of cancer was natural, rather than some product of the supernatural. To Hippocrates, his patient's malignant tumors and their associated blood vessels appeared reminiscent of a crab, leading him to call these growths καρκίνος (*karkinos*), from which the term "cancer" is derived.

Although these mysterious growths were at one point primarily thought to arise due to an imbalance of humors, other causes were eventually proposed. In *Recherches sur la Nature et la Guerison des Cancers* (*Research on the Nature and Treatment of Cancers*), the French surgeon Deshaies Gendron proposed that cancers arose from the transformation of otherwise normal tissues [3]. External or environmental factors were also proposed to play a critical role in the formation of cancerous lesions. Herman Boerhaave and Jean Astruc argued that inflammation which blocked excretory ducts was key to the induction of cancer [3]. Their proposal was not completely without merit. In the mid-19th century, Rudolph Virchow noted the presence of immune cells within tumors, providing one of the earliest accounts of a connection between tumors and inflammation[4]. Moreover, the idea that irritation or inflammation contributes to cancer development formed the basis for Katsusaburo Yamagiwa and Koichi Ichikawa's well-known experiments that employed continuous application of coal-tar to induce tumors on rabbit ears [5], and it is now understood that inflammation and cancer are closely intertwined with either process fueling the other [6–8].

As new tools used to study the world around us developed, scientists turned these tools towards unraveling the mysteries of cancer. Novel advances in microscopy during the 19th century paved the way for the identification of our own cells as the origin of cancer. Johannes Müller detailed cancers as a mass of unusual cells and supporting tissue in his 1838 treatise, *Über den feineren Bau und die Formen der krankhaften Geschwülste* (*About the Finer Structure and Forms of Morbid Tumors*) [9], and his student, Virchow, became the first to propose that cancer cells arise from otherwise normal cells, and in doing so, he provided a cellular basis for Gendron's original proposal that transformed tissues gave rise to cancer, although he was stumped by what could cause this abnormal transformation [10]. This shift towards a greater appreciation and understanding of the cellular origins of cancer became critical for the identification and diagnosis of carcinomas, sarcomas, and melanomas. However, the changes within our cells that turn them against us remained unknown.

Model systems to study cancer

Among the tools developed to study biology, *in vitro* models and animal models created an opportunity for researchers to dissect cancer's inner workings in systems more amenable to manipulation and investigation. Cell culture has been critical in this regard. Cells from a cancer patient can be isolated and grown in a petri dish, thereby allowing researchers to manipulate cell behavior, test novel drug treatments, and study cancer progression. One of the first recorded cancer cell lines, known as HeLa cells, contributed to numerous scientific breakthroughs that advanced our understanding of not only cancer but also many basic cellular processes [11]. The key advantage to cell culture lies in its minimalism. Its simplicity facilitates reductionist approaches towards studying biology, and as such, once opaque cellular processes become readily manipulated and interrogated. This advantage has proven to be a disadvantage as well, since the minimalist environment of *in vitro* systems provides only a limited picture of what cells can do.

In vivo animal models trade the simplicity and minimalism of cell culture for a more comprehensive picture of biological processes. *Mus musculus*, the house mouse, is the most common animal model currently used in laboratories worldwide, and their use in cancer research has greatly advanced our understanding of tumorigenesis and the constellation of interactions that exist between a tumor and its host. *Mus's* close evolutionary relationship to humans means that discoveries in mice often inform us of our own biology, and mouse models of cancer have had significant clinical impacts. Cancer immunotherapies arose from the pioneering research performed by James P. Allison's lab here at UC Berkeley who sought to understand the regulation of the T cell response [12,13]. The mouse model enabled them to parse through the intricate interactions between cancer and the many cells of the immune system, something that would otherwise be incredibly difficult to do *in vitro* at the time. Mouse models however do have their own set of disadvantages. Ethical issues concerning mammals, low numbers of experimental cohorts due to logistics and cost, and their relatively longer lifespan for a model organism preclude detailed survival studies.

Drosophila melanogaster, the humble fruit fly, exists in a kind of sweet spot between cell culture and mouse models, where it has both the complexity of a multicellular organism and the simplicity of a more minimalist model, owing to fewer genetic redundancies. Although visibly different from humans, flies share many key developmental pathways and have contributed greatly towards our understanding of cancer biology. Tumors were discovered in flies as early as 1916, and over a hundred years of research in *Drosophila* have elucidated conserved genes involved in cancer formation, tumor suppression, and tumor-host interactions [14–16]. Thus, both *in vitro* and *in vivo* models have facilitated more sophisticated, mechanistic studies into cancer biology that cannot be done in patients, making them indispensable systems for advancing our understanding of human cancers.

Genetic changes transform a single cell into a malignant tumor

Hints into the molecular changes that cause cells to become malignantly transformed could be found by looking at the association between the emergence of cancerous lesions and exposures to different environmental factors. In his 1775 text, Percivall Pott described the association of scrotal cancer and chimney-sweeps and argued that their cancer was due to chronic exposure to soot [9]. Yamagiwa and Ichikawa's experiments into the induction of carcinomas by regular application of coal-tar also lent support to the idea that cancers arise due to a transformation of otherwise normal cells [5]. Importantly, the observations of dividing cancer cells by David von Hansemann and Theodor Boveri provided one of the earliest insights into the possibility that chromosomal alterations may underlie malignant transformation [17]. Coupled with the later finding that DNA is the unit of inheritance [18] and the association between defined changes within our DNA and specific types of cancer [19–21], scientists reasoned that changes within a cell's genome were the major driving force for malignant transformation. However, a single mutation is insufficient to induce the formation of cancer in vertebrates; a normal cell usually accumulates a number of genetic changes before it can give rise to an over-proliferating mass of cells called a tumor. These series of genetic changes are now understood to disrupt a cell's ability to work in concert with its neighbors, resulting in the dysregulation of homeostatic growth and proliferation.

Growth factor signaling, organ size control, and cell polarity in tumorigenesis

Normally, the production of growth factors and integration of their downstream signals are tightly regulated, providing cells with the necessary instructions to grow or maintain tissue homeostasis. During an animal's life, genes involved in growth factor signaling have the potential to mutate and drive oncogenic transformation, and as such, are called "proto-oncogenes". Once mutated, oncogenes drive hyperactivation of normal growth factor signaling, disrupting the concerted actions of cells and pushing them towards malignancy [22–24]. From *Caenorhabditis elegans* to *Drosophila* to mammals, one critical signaling axis regulating cell proliferation and differentiation is the receptor tyrosine kinase (RTK)/mitogen-activated protein kinase (MAPK) signaling cascade. This pathway integrates signals from key growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) [25,26], facilitating coordinated cellular decision making. Multiple constituents of this pathway have been implicated in numerous cancers. Most notably is the GTPase, Ras, originally discovered as the transforming factor in the Harvey and Kirsten murine sarcoma viruses and later as mammalian H-ras and K-ras [27–29]. In a normal cell, Ras GTPases function as a binary switch regulating the transduction of growth factor signaling within the receiving cell. It does so through its binding to GTP and its intrinsic enzymatic activity to cleave GTP into GDP. In its GTP-bound state, Ras is active, mediating signal transduction from the receptor into the nucleus, and when bound to GDP, Ras is turned off, shutting down signal transduction. Oncogenic mutations affecting Ras enzymatic activity render it permanently in its active state [30], leading to hyperactive growth factor signaling even in the absence of any ligands. Therefore, cells containing constitutively active Ras lose their ability to coordinate their actions with their neighbors, driving them towards malignancy.

Whereas growth factor signaling plays a key role in promoting cell survival and proliferation, a second pathway called the Hippo pathway regulates organ size. Normally, signals

from neighboring cells restrict cell proliferation once the optimal tissue size has been reached [31–34]. Conserved from the fruit fly to mammals, the Hippo pathway is a key regulator controlling organ size determination and development that was originally discovered through *Drosophila* genetic screens looking into the genes responsible for controlling an organ's final size [31,32,34–36]. To ensure that organ growth is properly controlled, multiple important tumor suppressors within the Hippo pathway are responsible for regulating the activity of a key transcription factor, Yki [37–39]. Mutations in these critical tumor suppressors lead to the dysregulation of coordinated tissue growth and loss of organ size control [35,40–47], resulting in visible malformations in the adult fly. Demonstrating the conserved importance of Hippo signaling in tumor suppression, mutations in the mammalian Hippo pathway also disrupt cellular coordination of organ size, resulting in significant overgrowth of mutant tissues [41,46,48–50]. Thus, through the course of normal development, cells coordinate closely with one another to regulate organ size, and loss of this coordination via mutations in the Hippo pathway lead to tumor formation.

Although not typically thought of as regulators of cell division, mutations in key players that regulate cell polarity can also lead to tissue overgrowth and neoplastic transformation [51]. Cell polarity is crucial for our cells to carry out their designated functions. Nerve cells are organized to transmit electrical signals from dendrites to the cell body and out towards the axon terminals. Epithelial cells exhibit apico-basal polarity, where the apical surface faces the external environment or luminal cavities, and the basal surface contacts the basement membrane. Even migrating cells exhibit polarity, with the leading edge functionally differentiated from the trailing edge. However, when cell polarity is compromised, cells cannot organize themselves appropriately, and as a result, they may fail to differentiate and may become malignantly transformed [52–54]. Oncogenic mutations in epithelial cells have been found to affect both apical proteins as well as basolateral proteins, and in either case, the affected cell takes another step closer towards malignancy. The apically localized protein, atypical protein kinase C (aPKC), helps partition the cell into apical and basolateral domains through the phosophorylation of the Scrib module protein, Lethal giant larvae (Lgl), thereby restricting Lgl localization and activity to the basolateral membrane [55–58]. Transforming mutations in *aPKC* alter its expression or subcellular localization and have been found in various human cancers, particularly ovarian cancer [59,60]. Similarly, mutations affecting the localization of proteins in the Scrib module drive neoplastic overgrowth as well [51]. Although these mutations affecting polarity proteins do not directly affect proliferation, their mislocalization promotes the dysregulation of Hippo signaling, and affected cells begin expressing genes promoting tissue overgrowth [54,61]. Moreover, activating mutations in aPKC have also been shown to cooperate with other oncogenic proteins to drive tissue overgrowth [62], reinforcing the link between cell polarity and uncontrolled cell proliferation. Thus, whether it affects growth factor signaling, Hippo signaling, or cell polarity, each genetic change that impairs the ability of cells to coordinate their collective actions push them step by step down a path towards malignant transformation.

Transformed cells manipulate their surrounding environment to their advantage

Tumors can have a profound impact on their immediate environment (microenvironment), which is comprised of a heterogeneous population of transformed and non-transformed cells, as well as non-cellular constituents such as extracellular matrix (ECM) [63,64]. Changes within the tumor microenvironment can sometimes arise from mutations acquired by the tumor that change protein activity or gene expression. In other cases, changes around the tumor are brought about as a direct consequence of uncontrolled tumor cell proliferation or physiological constraints. Regardless of its cause, the intimate relationship between the tumor and its microenvironment often promotes the growth and survival of the tumor itself. As we shall see, many of these interactions are common to fly tumors as well as mammalian tumors.

Cellular crosstalk facilitates tumor growth and proliferation

Reciprocal signaling interactions between cells normally ensures proper coordination and tissue homeostasis; however, tumors can co-opt these pre-existing signaling pathways to fuel their own growth and survival. In mammals, stromal cells normally support the survival and proliferation of overlying epithelial cells through the production of growth factors and other ligands [65]. However, cancer cells alter the behavior of these supporting stromal cells, transforming them into cancer-associated fibroblasts (CAFs). There exists a diverse number of mechanisms through which tumors can induce the activation of CAFs in the microenvironment, ranging from inflammatory signals to growth factor signaling [64,66]. CAFs in turn will signal back to the tumor via the upregulation of its own set of growth factors, such as transforming growth factor-β (TGF-β), fibroblast growth factor, and hepatocyte growth factor, thereby promoting tumor cell proliferation [66,67]. *Drosophila* tumor cells similarly hijack developmental signaling axes to accelerate their growth. In fruit flies, tumors arising from intestinal stem cells will physically displace enterocytes from the basement membrane, triggering the stem cell niche to release signals that normally govern the homeostatic replacement of enterocytes [68]. The abnormal overactivation of this process leads to a feedforward loop, wherein the tumor's unrestrained growth promotes the production of signals that will further fuel cell proliferation. Importantly, the ability of tumors to leverage a range of homeostatic signaling axes both in flies and in mammals to fuel its growth would suggest that this phenomenon is a fundamental characteristic of malignancy between species.

Hypoxia drives tumors to remodel their microenvironment to survive

As the tumor continues to grow larger, the ability of oxygen to passively diffuse into the center of the tumor will become the limiting factor constraining its size. This places selective pressure on the tumor to initiate an angiogenic switch and encourage blood vessel infiltration into the tumor center; otherwise, it will become dormant [69]. In mice, tumor cells experiencing hypoxia will exhibit increased levels of HIF1-α protein, which promotes the recruitment of bone marrow-derived myeloid cells through upregulation of stromal-derived factor 1α (SDF1α) [70]. These cells express matrix metalloprotease-9 (MMP-9), whose enzymatic activity remodels the extracellular matrix, and this induces the release of sequestered vascular endothelial growth factor (VEGF),

which promotes neovascularization. VEGF can also be produced directly from the tumor and other cells within the tumor microenvironment. HIF-1α stabilization within tumor cells is associated with increased VEGF levels [70], and mammalian tumors can induce CAFs to secrete VEGF as well [71]. In response to increased VEGF, new blood vessels begin infiltrating the tumor, alleviating its hypoxia and allowing it to continue growing unrestrained. Although flies lack a closed circulatory system like mammals, they have evolved analogous structures called trachea to help facilitate oxygen diffusion to their tissues [72]. Fruit fly tumors similarly exhibit elevated HIF-1α transcriptional activity and increased *branchless* (*bnl*) expression, thereby promoting neotracheation in response to tumor hypoxia [16,73]. Critically, fly gut tumors that cannot upregulate *bnl* are smaller than ones that can, indicating that the angiogenic switch is a key limiting factor restricting tumor growth, much like mammalian tumors. Thus, the crucial need for tumors to secure an oxygen supply to sustain their proliferation established this phenomenon as a hallmark of cancer [74].

Tumor-associated immune cells influence tumor growth, survival, and invasion

Immune cells recruited to the tumor microenvironment also interact extensively with tumor cells, and depending on the context, their interactions may promote or inhibit tumor growth. In mammals, tumors exploit the functional plasticity of macrophages to fuel their proliferation [75,76], and an increased density of macrophages within the tumor microenvironment is correlated with poorer prognoses in cancer patients [77,78]. A pro-tumorigenic polarization of tumor-associated macrophages (TAMs) arises out of the milieu of signals from the tumor, as well as the immunosuppressive activity of regulatory T cells, which normally facilitate tissue repair following an inflammatory response [79–81]. Tumor-secreted growth factors and cytokines, such as colony stimulating factor-1 (CSF-1) and VEGF, lure immune cells to the tumor microenvironment [82]. Once there, TAMs have a range of effects on tumor growth. Accumulation of TAMs within hypoxic pockets is associated with significant vascularization of the tumor [70,83], and inflammatory cytokines and growth factors produced by TAMs promote tumor initiation and facilitate progression. In the context of hepatocellular carcinoma, TAMs secrete interleukin-6 (IL-6), an inflammatory cytokine, that induces STAT3 activity within hepatocytes to drive tumor initiation and progression, with mice lacking IL-6 exhibiting reduced tumor incidence and burden [84]. By altering its microenvironment to be more immune suppressive, tumor cells exploit key homeostatic processes to promote its own growth and survival.

Other immune cells can, however, mount an antitumor immune response. Natural killer (NK) cells are a type of innate immune cell involved in viral and antitumor immunity [85,86]. All nucleated cells of the human body, with the exception of immune privileged cells, express the major histocompatibility complex (MHC) class I protein on their surface, facilitating the differentiation of "self" from "non-self" via presentation of peptide fragments recognized as "self" [87]. Because tumor cells frequently mutate and create neo-antigens that will be recognized as "non-self", selective pressure exists for tumors to downregulate MHC class I protein expression in an attempt to evade NK cell-mediated elimination [88]. However, the lack of MHC class I proteins on the tumor cell surface may fail to hide the tumor from immune detection since NK cells initiate an antitumor response through multiple mechanisms [86,89–91].

Fly tumors, like mammalian tumors, elicit a cellular immune response, wherein the consequences largely depend on the context of malignant transformation. However, it is important to note that flies lack an adaptive immune system, which comprises a significant portion of the mammalian antitumor response. Nonetheless, flies have much to contribute towards our elucidating evolutionarily conserved immune responses to cancer. Even without an adaptive immune system, *Drosophila* evolved their own distinct mechanisms to differentiate "self" from "non-self". Basement membrane and cell integrity play a key role in this mechanism, and disruption in either of these by tumor cells elicits an innate immune response from the fly [92,93]. Like mammals, fly macrophages are recruited to transformed tissues, where they initiate an antitumor response to kill tumor cells and restrict tumor growth [93–95]; however, fly tumors can hijack antitumor immunity to fuel their own growth and invasion, reminiscent of how mammalian tumors hijack normal immune processes. Tumors carrying an oncogenic mutation in *Ras* divert the antitumor signals secreted by fly macrophages into pro-tumorigenic ones [94], thereby fueling their proliferation and invasiveness and reinforcing how the context of malignant transformation plays an influential role in antitumor immunity.

Tumors remodel their extracellular environment to facilitate proliferation and invasion

A growing tumor has the ability not only to change the behavior of the cells around it but also to manipulate the surrounding extracellular matrix (ECM), which comprises the non-cellular components of the tumor microenvironment. Consequently, rather than remaining a static structure, the ECM changes dynamically throughout the course of tumorigenesis, playing a crucial role in influencing cancer cell behavior. In mammalian tumors, the ECM can contribute up to 60% towards the tumor's mass [96], owing to the overproduction of ECM proteins from the tumor and CAFs [97,98]. These proteins include collagens, laminins, and the enzymes necessary for their proper incorporation into the ECM [97–99]. Fly tumors likewise remodel their ECM extensively. Upregulation of JNK pathway activity in tumor cells induces the expression of matrix metalloproteases (MMPs) [94,100–102], enzymes that remodel the basement membrane [103]. Critically, the lethality and propensity to metastasize in both mammalian and fly tumors is closely associated with the degree of ECM remodeling. When breast cancer samples were categorized into groups based on their ECM expression profile, a significant correlation emerged between the expression of ECM proteins and enzymes and their invasiveness and clinical outcome, with tumors expressing high levels of collagens, laminins, and MMPs correlated with the poorest prognosis [104,105]. In flies, a similar trend emerges, wherein the invasiveness of imaginal disc tumors requires MMP production by tumor cells [106]. Whether through cell autonomous or non-cell autonomous means, ECM remodeling is a dynamic process that contributes not only to tumor progression but also to invasiveness, literally paving the way for tumors to spread beyond their site of origin.

A tumor's sphere of influence extends beyond its primary site of origin

Studies in the past largely focused on the causes of cancer and how its microenvironment shapes malignancy, and through these foundational studies, we now have a keen understanding of the molecular changes that drive malignant transformation. Moreover, these studies have facilitated the critical development of a large repertoire of drugs that have improved modern cancer therapies by targeting key oncogenic mutations and signaling pathways. While historical observations of cancer patients have suggested that cancer's sphere of influence is not just restricted to its primary site of origin, much less attention has been paid to the ability of tumors to influence distant tissues and organs. Ranging from metastases to metabolic disruption, these distant effects can have profound impacts on patient outcomes and overall physiology.

Metastasis

A tumor's ability to invade and colonize distant organs is perhaps cancer's most infamous consequence, and this notoriety is not unwarranted. Metastases are estimated to be responsible for up to 90% of cancer-related deaths [107]. Approximately a quarter of colorectal cancer patients will already present with metastases during their initial diagnosis and eventually half will develop metastasis at some point [108]. Locations of these secondary tumors can be diverse even for a single cancer type. Breast cancer metastasizes frequently to the lungs, bone, and lymph nodes, whereas melanomas will often colonize the brain, bone, lymph nodes, and lungs [109]. The frequency, diversity, and lethality of secondary sites highlight one consequential way through which a tumor can extend its influence beyond its original location.

For a metastasis to successfully colonize a secondary site, a specific sequence of events called the metastatic cascade must occur so that the new lesion can survive distant from the primary tumor. The primary tumor first needs to acquire the ability to invade the surrounding tissue, which requires several changes to the tumor cell itself, as well as to its ECM. Breast cancers, which commonly metastasize, remodel their extracellular environment and degrade the underlying basement membrane [110]. This remodeling creates an environment favorable to invasion through the removal of physical barriers, as well as the release of sequestered growth factors that promote proliferation and invasion [111–114]. Coupled with changes to cell-cell adhesion and cell-ECM adhesion, tumors transition from an epithelial state to a mesenchymal state and begin invading into the surrounding tissue [115]. Progression of the metastatic cascade continues when tumors acquire the ability to intravasate and enter circulation. Here, the few tumor cells that can survive in circulation will eventually extravasate and embed themselves in a new location [116]. New lesions do not immediately outgrow following colonization; instead, they enter a period of dormancy, dictated by microenvironmental factors, immune surveillance, and even distant signals sent by the primary tumor [117,118]. Once these inhibitions are removed, growth at the secondary site commences, and the tumor's sphere of influence expands.

Paraneoplastic syndromes

Whereas metastases are a clear physical mechanism through which tumors can influence a distant site, tumors can also release signals that influence a patient's physiology and metabolism systemically. These systemic effects, called paraneoplastic syndromes, are not directly attributable to the tumor's mass and can affect a diverse range of organs, from the skeletal muscle to the kidneys and even the brain [119]. Although paraneoplastic syndromes were readily observed by physicians in the past, deeper understanding of the mechanisms governing them remained wanting. Importantly, since some paraneoplasias can affect up to half of cancer patients and their effects can complicate treatment, uncovering their root causes may provide novel avenues for cancer therapies and improving patient outcomes.

One of the most prominent paraneoplastic syndromes is cancer-associated cachexia, which is characterized by a dramatic wasting of skeletal muscle and adipose tissue. In testament to its prominence, it was well understood even by the 19th century that if cancer was left untreated, a fatal nutritional disruption will occur [9], and an estimated half of all cancer patients experience some degree of atrophy, wherein supplemental nutrition fails to reverse weight loss [120,121]. In some cancers, patients can lose greater than 10% of their initial body weight [122], and post-mortem analyses of cancer patients have suggested that as much as 10-20% of cancer patients will die as a direct result of cachexia [121]. Modern studies in both mammals and fruit flies have shed much needed light on the mechanisms that underlie this debilitating paraneoplastic syndrome. Inflammatory mediators such as IL-6 and tumor necrosis factor-α (TNF-α) can induce systemic wasting in mammals by interfering with lipid metabolism in adipose tissue and protein homeostasis in skeletal muscles [123–127]. In flies, disruption of normal insulin signaling due to tumor-secretion of the insulin signaling antagonist, ImpL2, a functional ortholog of mammalian insulin growth factor binding proteins, results in systemic wasting [128,129]. Malignant tumors release ImpL2 into circulation where it can bind to insulin and prevent it from activating its receptor on target cells [130,131], leading to systemic insulin resistance.

Other paraneoplasias are less overtly noticeable as the atrophy caused by cancer-associated cachexia; however, they still have profound impacts on patient physiology and outcomes, with different cancer types giving rise to different paraneoplastic syndromes. Up to 45% of small cell lung cancer patients go on to develop a paraneoplasia called the syndrome of inappropriate antidiuretic hormone secretion (SIADH) [132]. In these patients, osmotic homeostasis breaks down due to the overproduction of the antidiuretic hormone vasopressin, which causes the kidneys to reabsorb excessive amounts of water, and the cells of the patient experience significant osmotic stress, requiring careful restoration of normal blood salt levels [132]. Hypercalcemia resulting from malignancy is another paraneoplastic syndrome affecting systemic ion levels, wherein the secretion of parathyroid hormone related protein by tumor cells disrupts calcium ion homeostasis [133]. As its name suggests, patients with paraneoplastic hypercalcemia exhibit elevated serum calcium levels, putting them at risk of coma and kidney failure. Other syndromes affecting the endocrine system include paraneoplastic Cushing syndrome and hypoglycemia, all of which complicate patient care. The list of paraneoplastic syndromes continues with nearly all organs and organ systems impacted by malignant transformation – neurologic, dermatologic, rheumatologic, and hematologic paraneoplasias among them [119].

The existence of all these paraneoplasias further demonstrate how tumors can hijack and disrupt not only local but also systemic signaling axes, oftentimes to the detriment of the host. Given all the above local and systemic changes caused by tumors, it is critical that we gain a deeper understanding of the underlying mechanisms that govern the plethora of ways a tumor interacts with host tissues and in turn, how the host responds to malignant overgrowth.

Flies as a model to study tumor-host interactions

The fly's unique power as a model system to study tumor-host interactions emerges out of its over hundred years of history in scientific research. *Drosophila* biologists have a wide range of genetic tools facilitating tissue-specific manipulations with near surgical precision. The Gal4-UAS system employs a yeast-specific transcription factor, Gal4, whose expression is spatially restricted via tissue-specific promoters. Together with the upstream activation sequence (UAS), researchers can drive the expression of specific genes, fluorescent reporters, and interfering RNAs [134,135]. More complicated genetics in the fly builds upon the foundation set by the Gal4-UAS system. By using a temperature sensitive Gal80, which inhibits Gal4 activity at permissive temperatures, researchers can temporally restrict gene expression to achieve precise control of where and when a genetic construct is expressed [135,136]. Clonal analysis can be performed by triggering mitotic recombination through heat-shock driven expression of FLP recombinase. The resulting site-specific recombination removes an intervening insertion, thereby allowing individual cells to express genetic constructs of interest [136]. Fly biologists also have with them libraries of different fly lines with genetic deficiencies, unique interfering RNAs, and mutations, creating avenues for more discovery-oriented approaches, such as forward genetic screens.

The characteristics inherent to *Drosophila* that have made it an effective model organism also make it an attractive model to untangle the web of interactions between a tumor and its host. Flies are easily and cheaply reared in the lab in large numbers, lending statistical power to scientific inquiries. Unlike mouse models, the short lifespan and lack of ethical concerns in flies opens the way for survival studies. Although anatomically different from mammals, *Drosophila* have several analogous organs, whose development is regulated by the same handful of evolutionarily conserved genes. Critically, flies lack the genetic redundancy present in mammals; therefore, they are more amenable to genetic perturbations than mouse models. Thus, the vast genetic toolkit combined with the characteristics inherent to *Drosophila* provides unique opportunities to explore the complexities that underlie tumor-host interactions and to tackle the question, "How does a tumor kill its host?".

The fruit fly has had an indelible impact on our understanding of the origins of cancer, its influence on the tumor microenvironment, and even paraneoplastic diseases. Peppered throughout this introduction are only a few of the many studies using fruit flies that have shed light on the mysteries of cancer biology. The Hippo signaling pathway was first discovered in flies [32], and since then, its importance in normal development and disease is difficult to overstate. Foundational studies in flies dissected the molecular makeup and genetic logic of the RTK/MAPK signaling axis, whose constituents are frequently mutated in many human cancers. The elegance and power of *Drosophila* genetics facilitated research into the complex web of cell-cell interactions that occur between malignantly transformed cells and its neighbors, and cell competition is just one such example [16]. Although flies lack an adaptive immune system, studies investigating tumor-immune interactions have shed light on the antitumor response of innate immune cells, as well as that of the humoral immune system. Recent studies in flies have turned towards the mechanisms behind various paraneoplastic syndromes. Using flies as a model for cancer-associated cachexia, researchers identified that tumors disrupt systemic insulin signaling to induce tissue wasting throughout the host fly [128,129]. Studies from our lab have also identified a novel paraneoplastic syndrome, wherein systemic inflammation due to factors released by the tumor cause the failure

of normal blood brain barrier function [137]. Intriguingly, tumor-bearing flies exhibit excessive fluid buildup within their body cavity, manifesting as abdominal bloating in adults, and the mechanism regulating this enigmatic paraneoplasia remain unknown. With its history of foundational discoveries in cancer biology, flies present an opportunity to unravel the evolutionarily conserved ways through which the tumor interacts with its host.

Here, I present my thesis work where I used the power and elegance of *Drosophila* genetics to study tumor-host interactions with the aim of providing some clarity into the myriad of ways through which cancer may kill its host. In Chapters 2 and 3, I describe new systems and approaches that I and my labmates have set up to study tumor-host interactions in the fly. In Chapter 2, I detail how my work and that of my collaborators has uncovered a systemic coagulopathy in tumor-bearing flies that drives host lethality, with potential implications for a greater understanding of the causes of mortality of human cancer patients. In Chapter 3, I establish a novel forward genetic screen through which I identified two new candidate genes potentially involved in regulating tumor-host interactions. Finally, Chapter 4 presents a review article that I co-authored, comprehensively laying out the significant contributions flies have made to our understanding of tumor-host interactions, which was only briefly touched upon in this introduction.

CHAPTER 2

Systemic coagulopathy drives host mortality in a new Drosophila tumor model

This chapter is a reproduction of the paper by the same name that is currently available as a preprint on bioRxiv and to be submitted to Current Biology. For this paper, Katy Ong, David Bilder, and I designed the study and wrote the manuscript. Katy Ong and I performed most of the experiments and created the figures. Jorian J. Sepers obtained ovarian tumor transcriptome data, which I managed. Jung Kim and David Bilder assisted with developing the ovarian tumor fly model. Jung Kim also carried out experiments testing blood-brain barrier permeability and gut integrity.

SUMMARY/INTRODUCTION

Malignant tumors trigger a complex network of inflammatory and wound repair responses, prompting Dvorak's characterization of tumors as 'wounds that never heal'[138]. Some of these responses lead to profound defects in blood clotting, such as Disseminated Intravascular Coagulopathy (DIC), which correlate with poor prognoses [139–141]. Here, we demonstrate that a new tumor model in Drosophila provokes phenotypes that recapitulate coagulopathies observed in patients. Fly ovarian tumors overproduce multiple secreted components of the clotting cascade and trigger hypercoagulation of fly blood (hemolymph). Hypercoagulation occurs shortly after tumor induction and is transient; it is followed by a hypocoagulative state that is defective in wound healing. Cellular clotting regulators accumulate on the tumor over time and are depleted from the body, suggesting that hypocoagulation is caused by malignant growth exhaustion of host clotting components. Interestingly, clinical studies have suggested that lethality in patients with high serum levels of clotting components can be independent of thrombotic events [142,143]. We show that rescuing coagulopathy improves survival of tumor-bearing flies, despite the fact that flies have an open circulatory system. Our work establishes a platform for identifying alternative mechanisms by which tumor-driven coagulopathy triggers early mortality, as well as exploring other conserved mechanisms of host responses to chronic wounds.

RESULTS

Generation of an ovarian carcinoma model to investigate tumor-host interactions

Tumor-host interactions, as well as autonomous growth of the tumor itself, play central roles in cancer progression, morbidity and mortality [74,144]. *Drosophila* has recently emerged as a valuable system to study these interactions, elucidating mechanisms that can be conserved with mammals [16,145]. However, current systems have key limitations. In larval models, tumor-associated pupation defects prevent lifespan analysis, whereas in adult allograft models, wounding from transplantation confounds the study of the response to the tumor alone. In adult transgenic gut tumor models, perturbation of this essential organ may directly impact host metabolism, physiology, and the microbiome. We therefore developed an alternative genetic model, wherein a tumor is induced to grow in the non-essential [146] ovarian epithelium of an adult female fly (**Figure 1A**). In the ovarian carcinoma (OC) model, tumorigenesis is driven by expression of the oncogenes Ras^{V12} and $aPKC^{\Delta N}$ [57], directed to the follicle epithelium via *traffic jam-Gal4* (*tj-GAL4*) and restricted to adulthood via a ubiquitously expressed temperature-sensitive GAL4 repressor (*tubGAL80ts*). This spatiotemporal control facilitates the study of both tumor and adult host from the initial stages of transformation to a fully formed malignancy.

Defining characteristics shared by mammalian and fly carcinomas include overproliferation, loss of cell polarity, and defective differentiation [51,74]. Phosphohistone H3 staining revealed elevated mitotic rates in OC cells (**Figure 1B, C**). Epithelial organization was strongly disrupted, as was the localization of apically and basolaterally polarized proteins (**Figure 1D, E, S2**). Tumor cells failed to upregulate Hindsight (Hnt), a marker of mature differentiated follicle cells, and exhibited prolonged expression of the early follicle cell marker FasIII (**Figure 1F, G**) [147]. Thus, fly OC cells are transformed into malignant epithelial tumors.

An additional characteristic of malignant tumors is their potential to kill hosts. Importantly, OC-bearing flies show dramatically accelerated mortality compared to controls, with median survival reduced by ~50% (**Figure 1H**). GFP labeling of oncogene-expressing cells revealed no dissemination, suggesting a lack of metastasis (**Figure S1**). Since female flies do not require ovaries to live, lethality appears to arise from systemic effects of the tumor on the host, often called paraneoplasias. Similar to other adult tumor models [128,129,148,149], OC-bearing flies exhibit bloating resulting from the accumulation of fluid in the hemocoel (**Figure 1I - K**). Over the course of tumorigenesis, OCs also induce changes in fat body lipid storage (**Figure 1L, M**) [128,129,148,150]. Finally, tumor-bearing hosts displayed breakdown of the blood-brain barrier (BBB) (**Figure 1N**) [137]. The OC model therefore readily recapitulates previously documented *Drosophila* paraneoplasias.

To better understand OC progression and its impact on host survival, we developed a system to grade OC tumors based on distinct morphological characteristics (**Figure S2**). Assessing grade was more appropriate than mass because OC tumors, particularly at early grades, contain non-transformed germline cells that are polyploid and large. We defined grade 1 tumors as exhibiting a loss of epithelial polarity and overproliferation, most evident at the poles of individual follicles. While grade 1 tumors do not disrupt follicle organization and transformed cells remain *in situ*, grade 2 tumors show fusion between individual follicles as well as germline cell death. At

grade 3, the muscle sheath and its associated basement membrane breaks down, allowing fusion between neighboring ovarioles. Further disruption of ovariole morphology resulting from organ-wide basement membrane breakdown characterizes grade 4 tumors. Using this system, we found that tumor grade reliably increased with time after induction, and that most dying animals displayed grade 3 or 4 tumors regardless of that individual's time of death (**Figure S3**). The accelerated host mortality driven by tumor progression in a non-essential organ in the absence of metastasis place the OC model in a unique position to study tumor-host interactions.

The OC transcriptome reveals candidate paraneoplasia mediators

To identify factors regulating these interactions, we performed bulk transcriptome analysis comparing wildtype (WT), pre-vitellogenic follicle cells to OC follicle cells 20 days after tumor induction (ATI). Expression of *matrix metalloproteinase 1* and *puckered* were increased (Figure 2A), indicating high levels of JNK activity, as were *midline fasciclin* and *SOCS36E*, suggesting increased STAT activity (**Figure 2A**) [151,152]. Fluorescent reporters validated the increased activity of both pathways (**Figure S4**), similar to other *Drosophila* epithelial tumors. *GstS1* and *Zfh*, markers of follicle stem cells and early prefollicle cells respectively [147], were upregulated alongside downregulation of *Hnt* which marks more mature follicle cells, suggesting that some OC follicle cells retain a progenitor-like identity (**Figure 2B**).

Since tumor-released peptides are likely mediators of paraneoplasias, we then focused on putative secreted proteins upregulated in the OC transcriptome. Of 3567 genes showing at least 2-fold increase overall, 246 are predicted to encode secreted factors (**Figure 2C**). Several of these encode known signals overproduced by adult gut tumors as well as larval disc tumors (reviewed in ref. 16), including the IL-6 like Unpaireds, PDGF/VEGF-related factors, and the Insulin Growth Factor Binding Protein-like ImpL2 (**Figure 2D**).

Pro-clotting factors are upregulated in OC cells

Strikingly, among the upregulated genes predicted to encode secreted factors, we noted multiple genes that participate in the *Drosophila* clotting cascade (**Figure 2E**). As in mammals, flies have an essential circulatory fluid whose loss following wounding must be prevented. In both mammals and flies, this happens through clotting, in which soluble factors are polymerized and crosslinked to restore hemostasis [153–155]. Genes whose products are found in larvae to form the initial 'soft clot' were strongly overexpressed in tumor cells, including *fondue* (*fon*), *hemolectin*, *Lsp1y*, *fat body protein 1*, and *Eig71Ee*, which can be considered functionally analogous to human fibrin [156–158]. Some of these gene products are substates of the fly *Transglutaminase* (*Tg*), which is homologous to mammalian clotting factor XIIIa [159], and is also upregulated in OCs. The clotting pathway in flies includes an additional insect-specific reaction, called the melanization cascade [160]. This reaction is responsible for clot maturation to form a 'hard clot' and is regulated by the activity of pro-phenoloxidases (PPOs), which are released by specialized blood cells called crystal cells (CCs) following injury [160–163]. Transcript levels of *PPO1* and *PPO2* were elevated in OC cells as well. Taken together, these data raise the possibility that fly tumors may interface with the host clotting cascade.

OC tumors induce coagulopathies in hosts

We therefore asked whether coagulation is altered by OC tumors. We first measured the capacity of hemolymph from tumor-bearing adult flies to form soft clots. Adapting an assay previously used for larvae [164,165], we incubated inert Dynabeads with extracted hemolymph *ex vivo*. Hemolymph from control adults at day 5 and 10 induced clumping of beads, albeit less strongly than seen in larvae (**Figure S5**). By contrast, hemolymph from OC flies 5-10 days ATI readily generated large, macroscopic bead aggregates, indicating that these animals are in a hypercoagulable state (**Figure 3A**). Aggregation activity subsequently decreases at days 15 and 20 ATI, demonstrating that the hypercoagulable state triggered by tumors is transient.

We then tested the ability of tumor-bearing hosts to create hard, melanized clots. We used two assays: first, measuring PO activity levels of hemolymph *ex vivo* on a colorimetric substrate [163,166], and second, by observing melanization of cuticular wounds *in vivo* [167]. At day 15 ATI, PO activity was decreased in tumor-bearing flies in comparison to control (**Figure 3B, C**), and this decrease became more severe at day 20 (**Figure 3D**). Tumor-bearing flies at days 15 and 20 ATI also showed a strong failure to melanize thoracic wounds compared to control flies (**Figure 3E - G**). Indeed, two hours after wounding, bleeding was staunched in control flies, but hemolymph remained on the wounds of tumor-bearing flies 20 days ATI (**Figure 3H**). The defects in hard clotting along with wound-healing are consistent with a hypocoagulable state that follows the hypercoagulable phase.

Tumor-induced clotting creates a sink for clot cascade components

Given that hard clotting deficiencies temporally follow a period of increased soft clotting activity, we hypothesized that, as with some human paraneoplastic coagulopathies [139], fly tumors overstimulate the clotting system, leading to an exhaustion of clot components within the host. We tested whether OC-induced ectopic clots might act as a sink for limiting factors in the clotting cascade. A good candidate for such a factor are CCs, which lyse to release PPOs following activation and are produced only in the larvae but persist into adulthood [168]. CCs, labelled by BCF2::GFP, were rarely detected on control ovaries (**Figure 3I, K**). However, at day 10, near the end of the hypercoagulable phase, multiple GFP-expressing cells were seen on OC tissue, and this became significantly higher than control at day 15 (**Figure 3J, K**). Lysis of these non-renewing CCs would be consistent with the observed depletion of hemolymph PO activity, contributing to clotting failure (**Figure 3B - G**). Indeed, counts of CCs in OC hosts revealed a decrease 15 days ATI compared to control (**Figure 3L**). These results are consistent with the OC model causing a coagulopathy that consumes essential components of the clotting cascade.

Coagulopathies driven by tumor upregulation of clotting components regulate host mortality

Finally, we asked whether coagulopathy plays a role in the premature lethality of OC flies. We tested this by using RNAi to deplete Fon, which is overproduced >25 fold in the tumor and is

required for clotting in larvae [169]. Remarkably, knockdown of Fon in OC tumors extended median survival by ~38% (**Figure 4K**). This extension was robust compared to carefully matched control constructs, and is of a magnitude comparable to the lifespan extension of tumor-bearing hosts seen when blood-brain barrier disruption is prevented in a different tumor model [137]. Increased lifespan correlated with reduced coagulopathy, since knocking down Fon attenuated early hypercoagulation as well as late hypocoagulation phenotypes. Quantitation revealed that hemolymph of flies bearing OC tumors depleted of Fon at day 5 ATI aggregated beads to a much lower extent (**Figure 4A - C**). At day 20 ATI these flies showed higher hemolymph PO activity and increased ability to melanize wounds compared to OC flies alone (**Figure 4D - J**). Thus Fon upregulation in the tumor regulates systemic coagulopathy and paraneoplastic lethality in the host.

One explanation for coagulopathy accelerating TBH mortality could be an autonomous effect on tumor progression. Yet quantitation of OC grade revealed no change when Fon was depleted (**Figure S6A, B**). A second possibility is that coagulopathy might reduce lifespan through enhancing BBB permeability, which has been recently shown to contribute to tumor-associated lethality [137]. BBB breakdown was present in OC flies even upon depletion of Fon (**Figure S6C**). We investigated a third possibility –that coagulopathy causes an elevation of ROS levels through CC release of PPOs. ROS has well-documented deleterious effects on lifespan, but also has been suggested to have cytoprotective effects [170,171]. We found that OC flies heterozygous for the *Bc* mutation, which causes premature rupture of many CCs during the larval stage, show no changes in lifespan, nor do OC flies carrying an engineered dominant PPO1 allele with similar effects on CCs (**Figure S7A, B**) [172,173]. Furthermore, treatment with antioxidant or pro-oxidant compounds under different regimes did not significantly change OC mortality (**Figure S7C, D**). Finally, dying OC flies did not show increased intestinal permeability as detected by the 'smurf' assay (**Figure S7E**) [174]. Overall, these data suggest that coagulopathy contributes to tumor-driven death via a currently unknown paraneoplastic mechanism.

DISCUSSION

Our data reveal a remarkable and unexpected parallel between cancer patients and tumor-bearing flies: both can show widespread clotting defects that contribute to lethality. Systemic coagulopathies are common in cancer patients and have been studied since Trousseau's syndrome was described in 1865 [139,141]. One coagulopathogenic mechanism of many human tumors is abnormal upregulation of Tissue Factor, which can trigger the clotting cascade [175]. Although insects do not have a similar single coagulant-initiating factor, the broad cocktail of clot-regulating proteins secreted by OC tissue suggests an analogous response in fly tumors. Indeed, to our knowledge, the tumor data shown here reveal the first hypercoagulative phenotype documented in the fly. This adds to compelling evidence that the similar physiological reaction of the host to wounds and tumors has a quite ancient origin with a deeper level of conservation than previously appreciated.

Clotting complications are the second leading cause of death for cancer patients, primarily through venous thromboembolisms (VTEs) which are detected in 10-15% of all malignancies [141]. However, some evidence suggests that coagulopathy may also impact mortality through non-thrombotic pathways as well. Elevated markers of clotting factors in circulation are strongly correlated with poor survival, yet thrombosis is often not observed in patients [142,143]. There is also some evidence that prophylactic treatment with anticoagulants can improve cancer patient outcomes beyond prevention of thrombosis [176]. Thus, there may be unappreciated mechanisms through which altered clotting behavior contributes to morbidity and mortality. The fly, with its open circulatory system, is unlikely to be dying from tumor-induced thrombosis, and thus may be used as a discovery system for potential alternative mechanisms. In this work, we have not determined the exact reason why coagulopathy promotes the death of tumor-bearing flies, but our data argue against several *prima facie* feasible possibilities.

The initial hypercoagulative phenotype of OC flies followed by hypocoagulation echoes features of patient conditions such as Disseminated Intravascular Coagulopathy (DIC). DIC is considered a consumptive coagulopathy, where ectopic activation of pro-coagulation pathways can paradoxically lead to excessive bleeding through local depletion of hemostatic components [140]. Our data point to fly CCs, which lyse to provide PO clot-hardening activity, as one consumed regulator, but hint that the dramatic hypercoagulation, rather than hypocoagulation, of tumor bearing flies may drive the detrimental consequences for mortality. Future studies will explore how this simple fly system reacts to the tumor-induced danger response, perhaps by triggering inflammatory, immune, or metabolic responses that have a negative impact on host survival.

EXPERIMENTAL PROCEDURES

Fly husbandry and stocks

Flies were maintained on cornmeal, molasses, and yeast food at 21ºC in wide vials unless otherwise noted. A complete list of stocks used is given in **Table S1**. The *fon* RNAi construct was validated by confirming that it can recapitulate previously described larval soft clotting and pupariation defects [169].

Ovarian tumor induction and lifespan assays

After eclosion, adult flies were kept at 21ºC on food with yeast powder for two days. No more than 25 flies were kept in each wide vial, a density that was previously determined did not impact longevity. Flies were then put on new food and shifted to 29ºC to initiate tumor induction. Food was changed every two days and the number of dead flies was counted. For each lifespan assay, at least 50 flies were used for each sample group and all assays were repeated twice, with control and experimental groups run in parallel. For RNAi lines used in lifespan assays, the lines were backcrossed at least four generations to w^{1118} (BL#5905) to minimize variation in genetic background between stocks.

Tumor transcriptome sequencing and data analysis

Twenty days after shifting to restrictive temperature, around two hundred *tj-GAL4 tubulinGal80ts* and *tj-GAL4 tubulinGa80ts; UAS-aPKCΔN UAS-RasV12* ovaries were dissected in Schneider's *Drosophila* Media (Life Technologies, 21720024) on ice for each biological replicate. At least two biological replicates were sequenced per genotype. Follicles ~stage 6 and older of control ovaries were removed by cutting the distal regions. Ovaries were washed three times in DPBS (Dulbecco's Phosphate-buffered saline; Life Technologies, 14190144) followed by 10 minute incubation in 10mg/mL *Bacillus licheniformis* protease (Sigma, P5380) prepared in DPBS at 6°C while vigorously agitating. Protease activity was quenched by adding a half volume of 10% fetal bovine serum (FBS) in Schneider's *Drosophila* Media. Cell suspension was then filtered using a 50µm filter (Sysmex Partec, 04-004-2327) to remove germ cells, and the filtered suspension was subsequently spun down for 7 minutes at 1000g. RNA of pelleted cells was isolated using RNeasy mini kit (QIAGEN, 74104), and RNA quantity and quality was analyzed by the Functional Genomics Laboratory at the California Institute for Quantitative Biosciences at UC Berkeley (QB3-Berkeley).

Libraries were sequenced by 50bp single-end reads on HiSeq4000 platform (Illumina, San Diego, CA), and sequencing was performed by the Vincent J. Coates Genomics Sequencing Lab at QB3-Berkeley. Sequences were aligned to the *Drosophila melanogaster* reference genome (version 6.26) using Kallisto under default parameters for single-end reads [177]. Lowly expressed genes were removed prior to differential expression analysis using DESeq2 [178].

Immunofluorescence

Ovarian tumors, thoracic muscle, brains and intestines were dissected in PBS and fixed in 4% PFA-PBS for one hour at room temperature without agitation. Samples were washed three times with PBS-TX (0.1% Triton-X in 1X PBS) before proceeding to dye treatment. Samples were blocked in 2% BSA or 4% NGS/1% BSA blocking solution for 30-60 minutes. Primary antibodies were incubated overnight at 4ºC and used at the following concentrations: anti-Dlg (1:100), anti-

phospho-histone H3 (1:250), and anti-Hindsight (1:100). Samples were washed three times with PBS-TX and incubated with AlexaFluor-conjugated secondary antibodies for 1 hour at room temperature. Tissues were again washed three times with PBS-TX prior to additional chemical staining. To stain actin, fixed tissues were incubated for 30-60 minutes at room temperature with Rhodamine-Phalloidin at a concentration of 1:500. To stain nuclei, a 1:1000 DAPI solution was applied for 10 minutes. After all incubations, samples were washed three times with PBS and incubated in the final wash for at least 20 minutes. Samples were mounted in Diamond Antifade Mountant before imaging.

Visualizing fat body wasting

Dorsal cuticles were dissected from adult flies in PBS and fixed in 4% PFA for 30 minutes. Samples were washed once in PBS-TX and three times in PBS. Dorsal cuticles were incubated in Nile Red staining solution (0.5 µg/mL in 1X PBS) for 10 minutes followed by three washes in PBS. Samples were mounted in Diamond antifade mountant and stored at 4ºC. Images were taken at multiple focal planes on Zeiss AxioImager M2 using Zeiss Zen (blue edition 2.3) pro imaging software, and multifocus image was created using Helicon Focus 7.

PO activity measurements

We used a modified protocol from ref. 166. To measure PO activity of hemolymph *ex vivo*, individual flies were bled onto small squares of Whatman filter paper (1cm^2; Whatman 1001 – Grade 1). Immediately following each bleeding, 20µL of 20mM L-DOPA in PBS was applied to each blot. Samples were then covered to prevent evaporation and incubated for 30 minutes at 25°C. The blots were rapidly dried by heating in a microwave for 10 seconds and then allowed to completely air-dry for 30 minutes. Following drying, each blot was sealed in clear Scotch tape and scanned using an Epson Perfection 4490 Photo Scanner. Intensity of each blot was quantified in FIJI, and the mean background intensity was subtracted from all samples.

Thoracic wound healing assay

Flies were anesthetized with CO_2, and one side of the thorax was punctured using a blunt 0.005 inch diameter tungsten needle (Ted Pella #27-11). Flies were then returned to vials, and two hours later counted for melanized thoracic wounds using a dissecting microscope. To determine wound-healing failure, a glass micropipette tip (World Precision Instruments #TIP30TW1) was gently brought into contact with the thoracic wound two hours after wounding, and assessed for whether hemolymph was drawn up by capillary action.

Bead aggregation assay

We used a modified protocol from ref. 165. Dynabeads were washed with 10X PBS twice and blocked in a 0.1% BSA-PBS solution overnight on a rotator at 4ºC. The beads were washed three times with 0.1X PBS and reconstituted in Ringer's-PTU (130mM NaCl, 5mM KCl, 1.5mM $CaCl_2$ x $2H_2O$, 2mM Na_2HPO_4, 0.37mM KH_2PO_4, 0.01% PTU) buffer at a final concentration of 50%. The blocked beads were stored up to two weeks on a rotator at 4ºC. To extract hemolymph, we used a modified protocol from ref. [179]. A Qiagen spin column was disassembled and the filter paper removed. All other components were rinsed in MilliQ purified water. The column was reassembled without the filter and centrifuged at 13.2 x 1000g for 10 minutes at room temperature. The tube that the column was nested in was replaced with a fresh 1.5mL Eppendorf tube. Flies were anesthetized and shallowly cut across the dorsal thorax with a 33G needle. The flies were

transferred to the modified column stored on ice. All flies were wounded in less than 10 minutes to avoid loss of clotting activity. The column was spun at 5000g for 5min at 4ºC. 2µL of hemolymph was combined with 2uL of blocked beads in a well of a 15-well glass slide by pipetting up and down ten times. The slide was incubated in a humid chamber at 25ºC for 30 minutes. Bead aggregates were revealed by swirling the solution with a 10µL micropipette tip for 30 seconds. The well was photographed with an iPhone 12 camera mounted on a dissecting microscope. To measure bead aggregation, we used the Bernsen Adaptive Local Thresholding Method available in FIJI to generate a binary image. A polygon was drawn around the liquid droplet and the percent area unoccupied by beads (i.e. the white area) was measured. The final measurement was generated after subtracting the average white area percent measured across five wells of control reactions with 2µL of Ringer's-PTU buffer instead of hemolymph.

Confocal microscopy

Fixed samples were imaged on the Zeiss LSM700 Scanning Confocal Microscope with a Plan-APOCHROMAT 20x/0,8 objective. The microscope was controlled with Zeiss Zen 2010 imaging software. Images were processed and analyzed with FIJI software.

Blood brain barrier permeability assay

Assays were done as described in ref. 137. Approximately 100nL of 25 mg/ml 10,000 MW TR-dextran was injected in the abdomen of adult females using a fine glass needle. If tumor-bearing hosts excreted hemolymph upon injection due to bloating, excess hemolymph was removed. 15 hours after injection, flies were fixed in 4% PFA-PBS for 80 minutes and washed in 0.1% PBS-TX before brain dissection. Brains were imaged on the same day of fixation to minimize diffusion of dextran. Average intensity was measured in a cross-section at the center of the brain at two regions using FIJI software.

Drug Treatments

Paraquat was used at either 2mM or 5mM. N-Acetyl Cysteine (NAC) was administered at 20mM. All drugs were dissolved in MilliQ-filtered water. 0.74g of 4-24 Carolina Instant Blue Food was mixed with 2mL of water or drug cocktail. Drugged food was always made fresh the day of use. Paraquat treatment was started 6 days after tumor induction. After two days, flies were transferred to food reconstituted with water, and thereafter consistently alternated every two days between Paraquat food and water only food. This alternating treatment was done to avoid Paraquat-driven mortality [180,181]. NAC treatment was begun 10 days after tumor induction to avoid potential delays in early tumorigenesis from the antioxidant treatment.

Water weight measurements

Measurements were done as described in ref. 182. Briefly, four to five flies were placed into a 1.5mL centrifuge tube, and wet weight was measured on Mettler Toledo AG104 scale. Flies were then dried at 65°C overnight with the centrifuge tube opened to allow for evaporation. Dry weight was then measured. Wet-dry weight was obtained by subtracting the dry weight measurement from the wet weight measurement, and the result was divided by the number of flies in the respective tube.

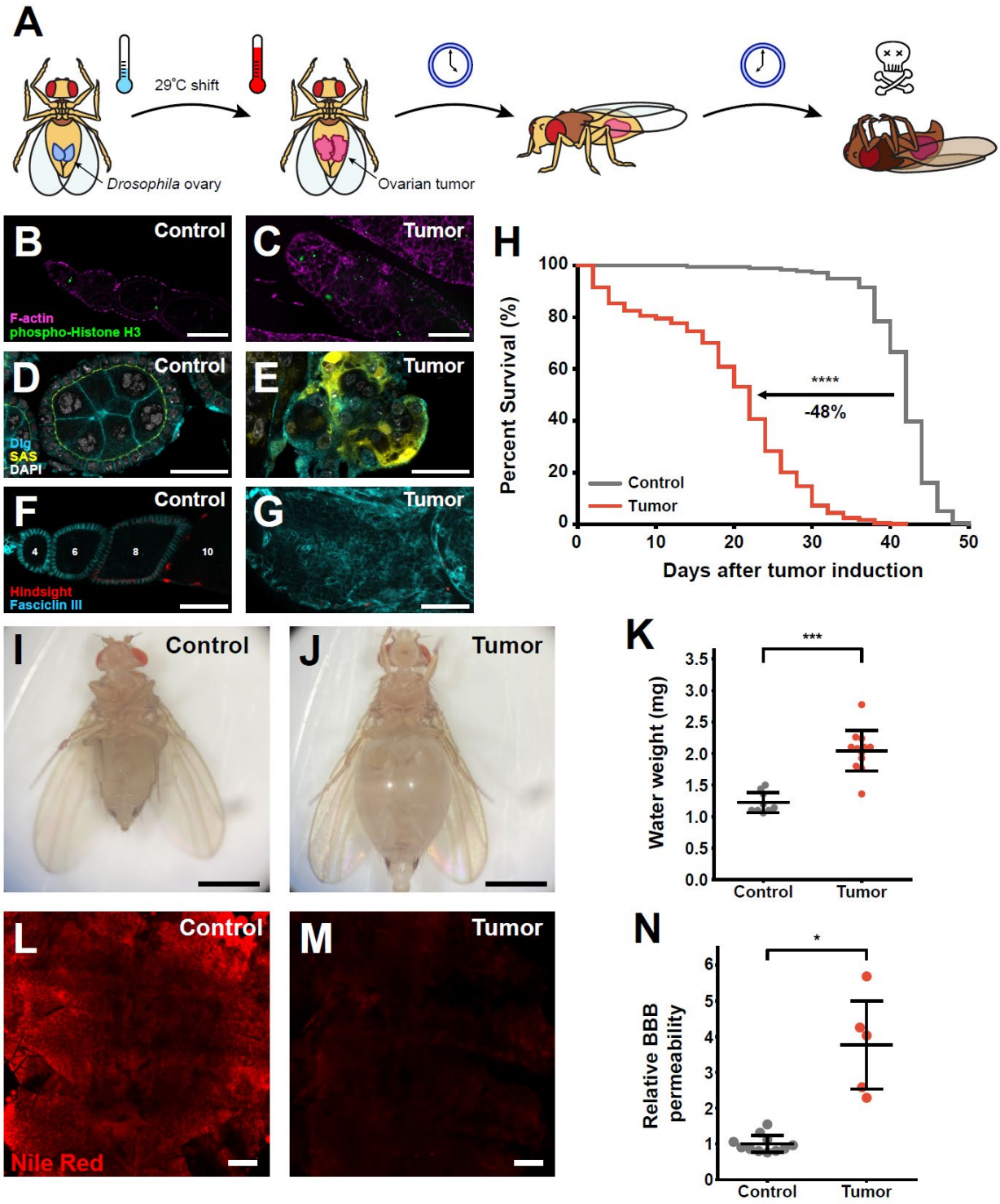
Smurfing Assay

The smurfing assay was carried out as described in ref. 174. In brief, flies were moved onto food containing 2.5% wt/vol Blue Dye No. 1. After 9 hours, the flies with dye present outside of the digestive tract were counted.

Statistics

A one-way ANOVA test or Student's T-test was used for all parametric data (crystal cell area, crystal cell counts, PO activity, bead aggregation, water weight and BBB permeability). The Kruskal-Wallis test or Fisher's exact test was used for all non-parametric data (wound melanization, tumor grading, bleeding defects and smurf assay). The Log-Rank test was used to determine significant differences in survival curves. Graphpad Prism and Python were used to perform statistical testing.

FIGURE 1


A
29°C shift
Drosophila ovary
Ovarian tumor
B
Control
F-actin
phospho-Histone H3
C
Tumor
D
Control
Dlg
SAS
DAPI
E
Tumor
F
Control
4
6
8
10
Hindsight
Fasciclin III
G
Tumor
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Days after tumor induction
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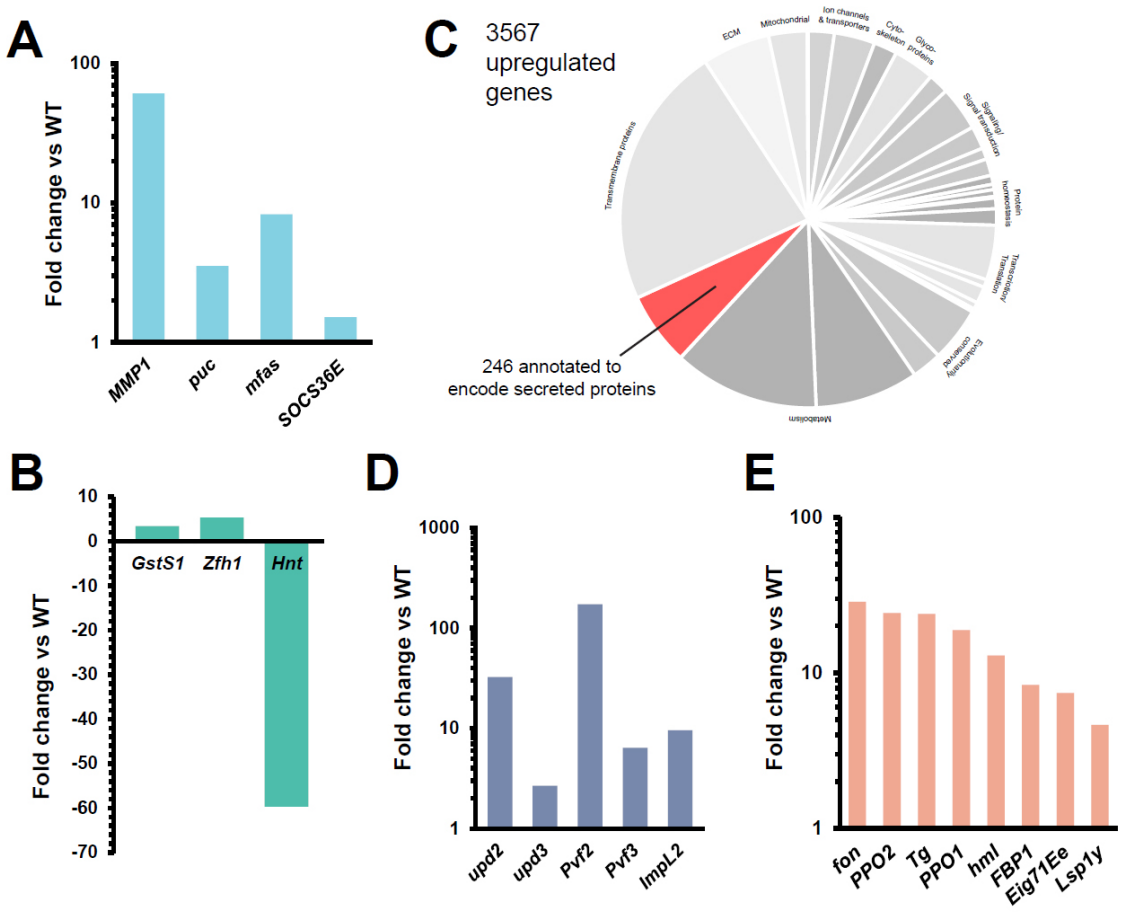
Figure 1. A novel ovarian carcinoma model to study paraneoplasias in *Drosophila*.
(A) Schematic of Drosophila ovarian carcinoma (OC) induction in adult flies. (B, C) OC tumors exhibit increased anti-phospho-histone H3 staining (green). (D, E) SAS-Venus (yellow) and anti-Dlg staining (cyan) reveal disruption of cellular organization in transformed follicle epithelial cells. (F, G) OC tumors fail to differentiate into the mature Hnt-positive (red), FasIII-negative (cyan) follicles seen in control. (H) Flies carrying OCs have greatly reduced survival compared to control, non-tumor-bearing flies. Tumor-bearing flies indicates substantial fluid accumulation in their hemocoel as indicated by macroscopic abdomen distention (I, J) and quantification of water weight (K). (L, M) Lipid staining (red) shows decreased fat tissue in OC flies. (N) Flies carrying OCs exhibit increased permeability of the blood-brain barrier. Scale Bars = 50µm (B,C,F,G), 25µm (D,E), 1mm (I,J), 100µm (L,M); Error bars = S.D.; *$p < .05$, ***$p < .0005$

FIGURE 2


A
Fold change vs WT
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10
1
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puc
mfas
SOCS36E
C
3567
upregulated
genes
246 annotated to
encode secreted proteins
ECM
Mitochondrial
Ion channels & transporters
Cyto-skeleton proteins
Glycop-
Signaling/ Signal transduction
Protein homeostasis
Transcription/ Translation
Extracellular conserved
Metabolism
Transmembrane proteins
B
Fold change vs WT
10
0
-10
-20
-30
-40
-50
-60
-70
GstS1
Zfh1
Hnt
D
Fold change vs WT
1000
100
10
1
upd2
upd3
Pvf2
Pvf3
ImpL2
E
Fold change vs WT
100
10
1
fon
PPO2
Tg
PPO1
hml
FBP1
Eig71Ee
Lsp1y

Figure 2. OCs differentially express clotting factors and regulators, among a number of other secreted proteins.

(A) OCs exhibit heightened transcription of JNK pathway targets *MMP1* (60.97X) and *puc* (3.53X), as well as STAT pathway targets *mfas* (8.28X). (B) Increased proportion of OC tumor cells in early differentiation is indicated by enhanced transcript levels of *GstS1* (3.45X) and *Zfh1* (5.35X), and the failure to upregulate *hnt* (-59.71X). (C) 246 of 3567 transcripts upregulated in OC tumors vs control follicles are predicted to encode secreted proteins. (D) Like other *Drosophila* tumors, OCs upregulated *upd2* (32.58X) and *upd*3 (2.68X), *Pvf2* (173.90X), *Pvf3* (6.41X), and *ImpL2* (9.54X). (E) Expression of many factors associated with hemolymph clotting was increased in OCs compared to wild-type follicle cells: *fon* (28.58X), *hml* (12.89X), *Lsp1γ* (4.62X), *Tg* (23.92X), *PPO1* (18.84X), *PPO2* (24.22X), *FBP1* (8.35X) and *Eig71Ee* (7.44X).

FIGURE 3

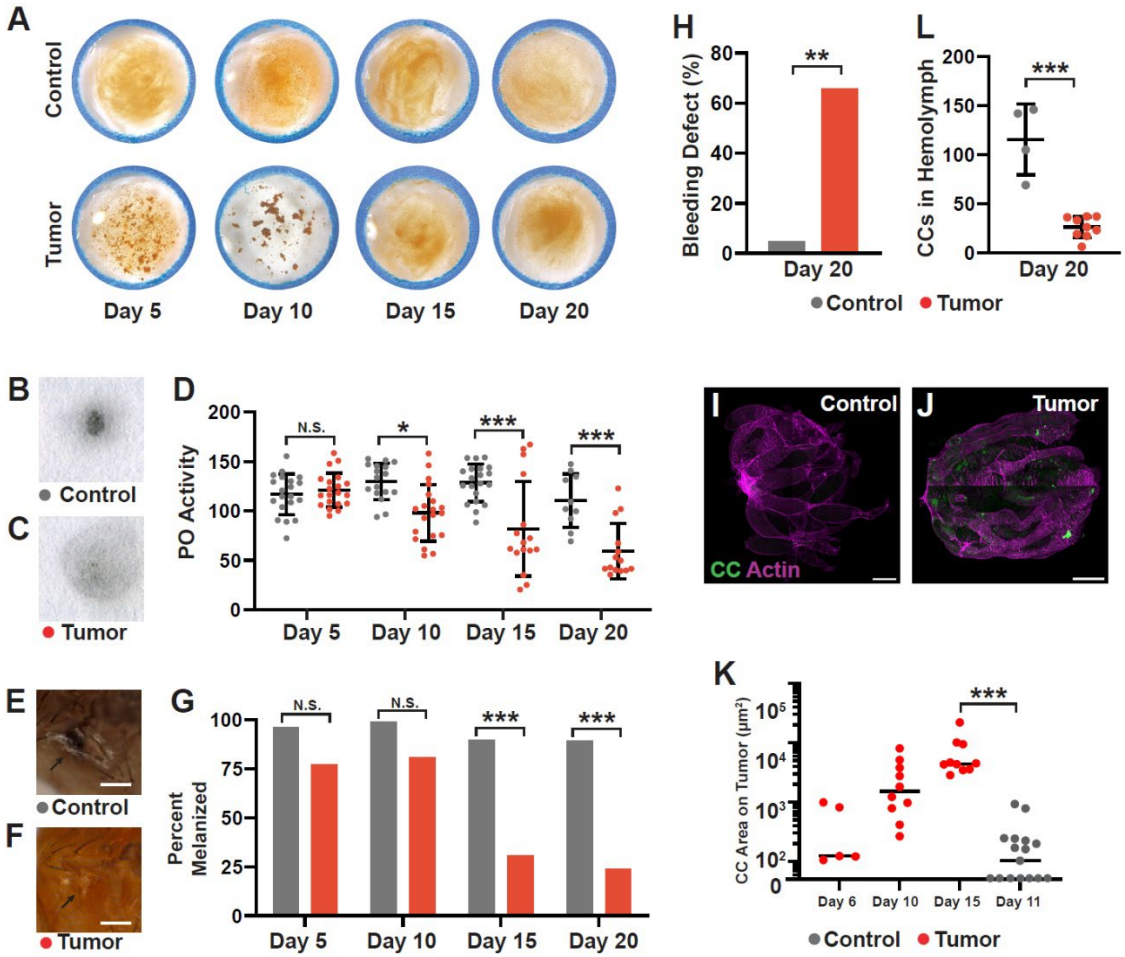

A
Control
Tumor
Day 5
Day 10
Day 15
Day 20
H
Bleeding Defect (%)
80
60
40
20
0
**
Day 20
L
CCs in Hemolymph
200
150
100
50
0
***
Day 20
Control
Tumor
B
Control
C
Tumor
D
PO Activity
200
150
100
50
0
N.S.
*
***
***
Day 5
Day 10
Day 15
Day 20
I
Control
CC Actin
J
Tumor
E
Control
F
Tumor
G
Percent Melanized
100
75
50
25
0
N.S.
N.S.
***
***
Day 5
Day 10
Day 15
Day 20
K
CC Area on Tumor (μm²)
10⁵
10⁴
10³
10²
0
***
Day 6
Day 10
Day 15
Day 11
Control
Tumor

Figure 3. Drosophila ovarian tumors induce multiple defects in clotting.

(A) Bead aggregation assay shows that soft clotting activity of control adult hemolymph (top) is dramatically increased in OC adult hemolymph (bottom) through day 10 ATI. Images are representative of multiple experiments and do not come from a single time course. PO activity in control (B) and OC flies (C) measured by L-DOPA blot reactivity. (D) Quantification of PO activity reveals decreases in OC flies following 10 days ATI. Thoracic wound response in control (E) and OC flies (F). (G) Percentage of flies able to melanize wounds reveals strong differences on days 15 and 20 ATI. (H) Hypocoagulation of OC flies, demonstrated by ability to measure hemolymph on throracic wounds after 2 hours. Crystal cells are rarely found on control ovaries (I) and accumulate on day 15 ovarian tumors (J). (K) Crystal cell area on tumors increases over time. (L) Crystal cell counts in hemolymph decrease in tumor-bearing flies at day 20 ATI. Scale Bars = 250μm; Error bars = S.D.; *$p < .05$, ***$p < .0005$

FIGURE 4

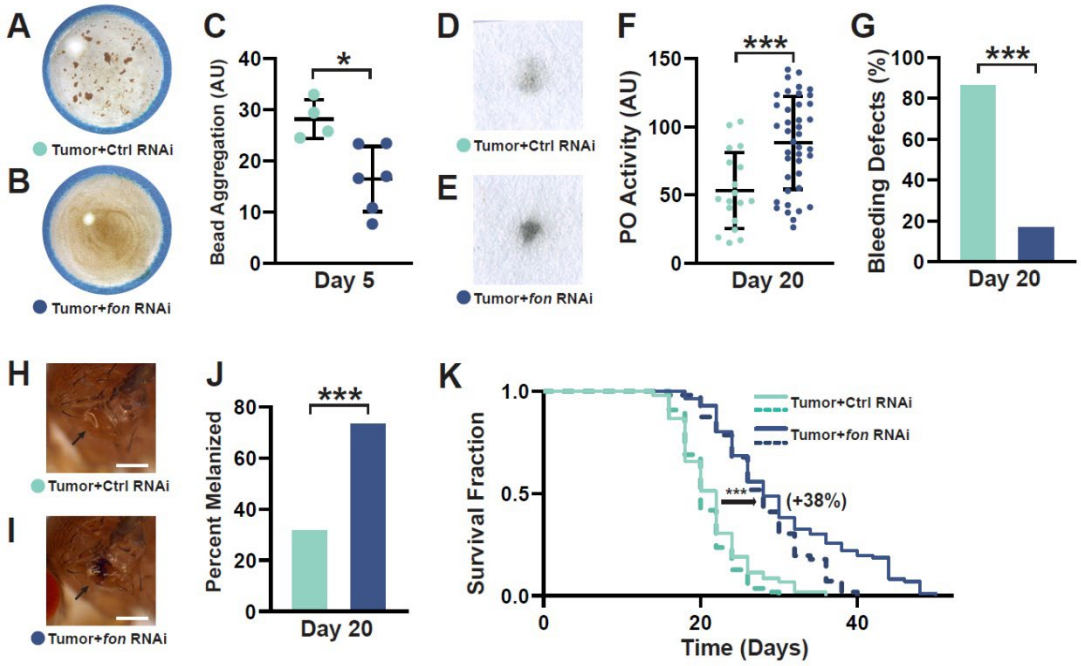

A
Tumor+Ctrl RNAi
B
Tumor+fon RNAi
C
Bead Aggregation (AU)
Day 5
D
Tumor+Ctrl RNAi
E
Tumor+fon RNAi
F
PO Activity (AU)
Day 20
G
Bleeding Defects (%)
Day 20
H
Tumor+Ctrl RNAi
I
Tumor+fon RNAi
J
Percent Melanized
Day 20
K
Survival Fraction
Time (Days)
Tumor+Ctrl RNAi
Tumor+fon RNAi
(+38%)

Figure 4. Tumor-secreted Fondue drives coagulopathies and early mortality.
Compared to control RNAi depletion in OC cells (A), Fon depletion in OC cells (B) rescues the strong hypercoagulation displayed by tumor-bearing flies, revealed by the bead aggregation assay quantitated in (C). Compared to control RNAi depletion in OC cells (D), Fon depletion in OC cells (E) also ameliorates the loss of PO activity, quantitated in (F), and defects in bleeding (G) and wound melanization (H-J). (K) Early mortality induced by OC tumors is significantly reduced upon Fon depletion. Solid and dashed lines represent different replicates of the experiment. Scale Bars = 250μm; Error bars = S.D.; *$p < .05$, ***$p < .0005$

FIGURE S1

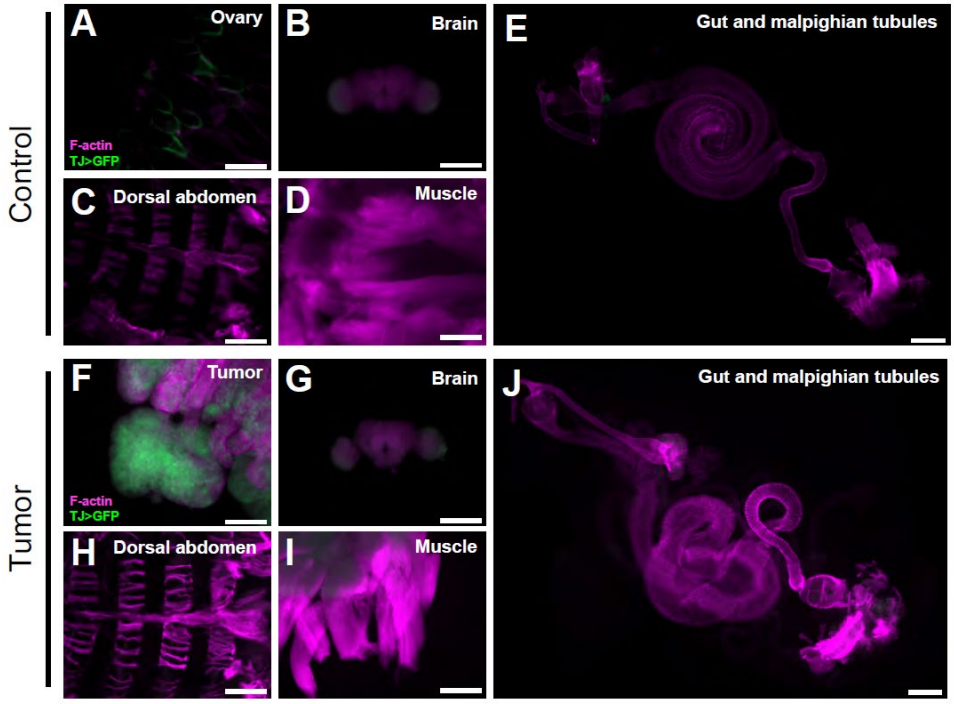
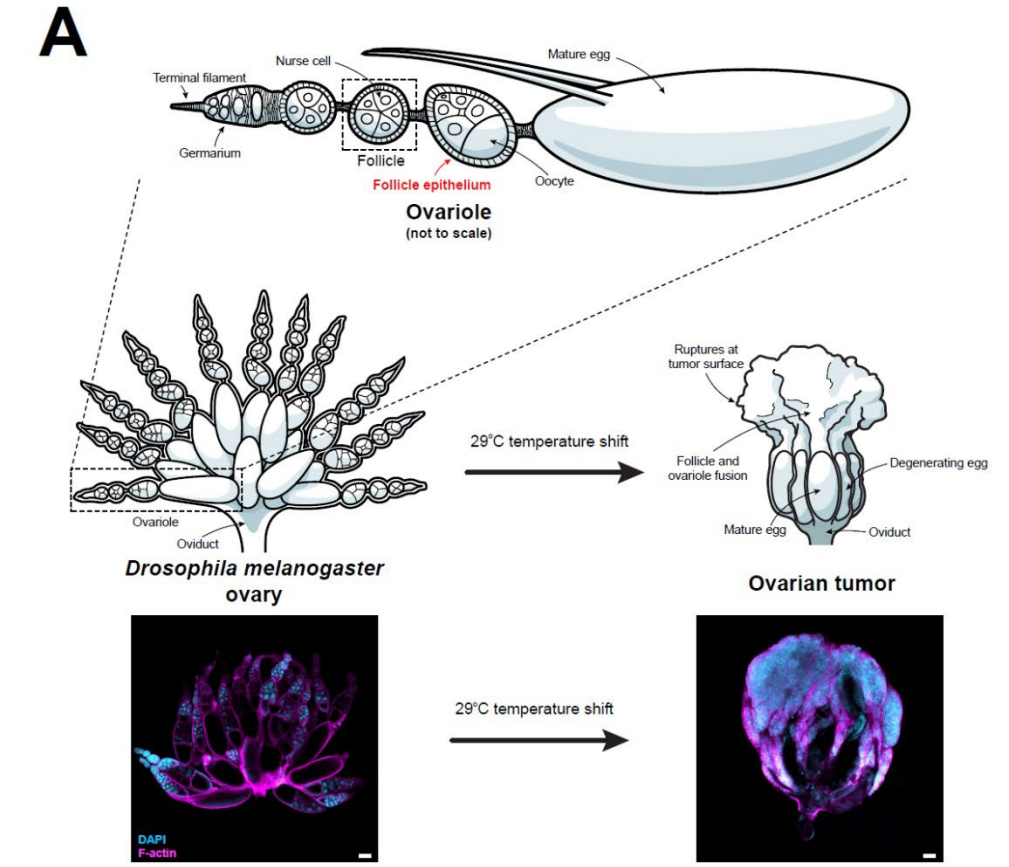

Control
Tumor
A
Ovary
F-actin
TJ>GFP
B
Brain
E
Gut and malpighian tubules
C
Dorsal abdomen
D
Muscle
F
Tumor
F-actin
TJ>GFP
G
Brain
J
Gut and malpighian tubules
H
Dorsal abdomen
I
Muscle

Figure S1: OC model lacks metastasis.

Images from adult tissues of control (A-E) vs OC flies (F-J) expressing *UAS-NLS-GFP* 20 days ATI. Green fluorescence clearly marks cells in the control ovary (A) that show morphological transformation in OC flies (F). *tj-GAL4* shows some expression in the optic lobes but these structures do not obviously change upon coexpression of the OC oncogenes (B, G). No green cells are seen in muscle, abdomen, or digestive tract (C-E, H-J) with the exception of small GFP-labeled cells by the crop that are also present in OC flies. Scale bars = 250μm.

FIGURE S2


A
Terminal filament
Nurse cell
Mature egg
Germarium
Follicle
Follicle epithelium
Oocyte
Ovariole
(not to scale)
Ovariole
Oviduct
Drosophila melanogaster
ovary
29°C temperature shift
Ruptures at
tumor surface
Follicle and
ovariole fusion
Degenerating egg
Mature egg
Oviduct
Ovarian tumor
DAPI
F-actin
29°C temperature shift

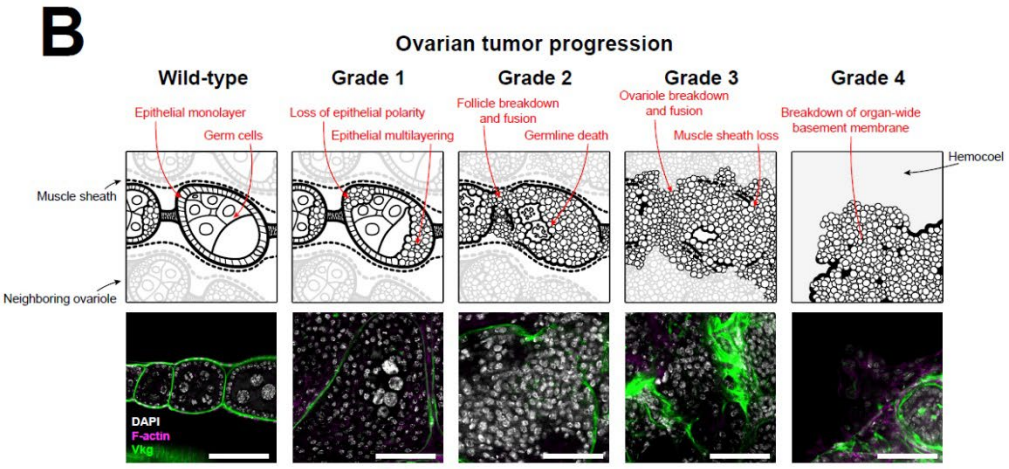

B
Ovarian tumor progression
Wild-type
Grade 1
Grade 2
Grade 3
Grade 4
Epithelial monolayer
Germ cells
Loss of epithelial polarity
Epithelial multilayering
Follicle breakdown
and fusion
Germline death
Ovariole breakdown
and fusion
Muscle sheath loss
Breakdown of organ-wide
basement membrane
Hemocoel
Muscle sheath
Neighboring ovariole
DAPI
F-actin
Vkg

Figure S2. Adult Drosophila ovarian tumor characteristics and progression.
(A) Schematic of the *Drosophila* ovarian carcinoma (OC) model illustrates the architecture of normal ovaries and the progression of malignant transformation of follicle epithelial cells. Representative confocal micrographs are shown below illustrations of ovaries. (B) Illustrations of distinguishing features of tumor grade stages 1-4 with corresponding representative micrographs below. Nuclear (white) and Actin (magenta) stains show dramatic changes in cellular and tissue organization. Vkg::GFP (green) reveals basement membrane breakdown. Scale bars = 100 μm (A), 50μm (B).

FIGURE S3

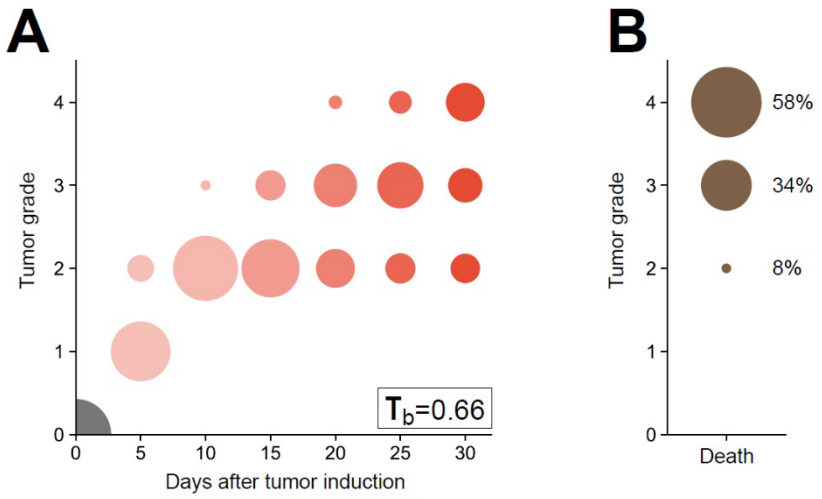

A
Tumor grade
Days after tumor induction
T_b=0.66
B
Tumor grade
Death
58%
34%
8%

Figure S3. Higher tumor grade associated with lethality.
(A) Tumor grade is well correlated with time after tumor induction, with a greater proportion of grade 3 and 4 tumors in later time points. Size of circle represents proportion of sample in each class. Correlation determined by calculating Kendall's τ_b correlation coefficient; τ_b=0.66. (B) The majority of flies necropsied following death between 10 and 28 days ATI had tumors of grades 3 or 4, indicating a strong association between tumor grade and its lethality.

FIGURE S4

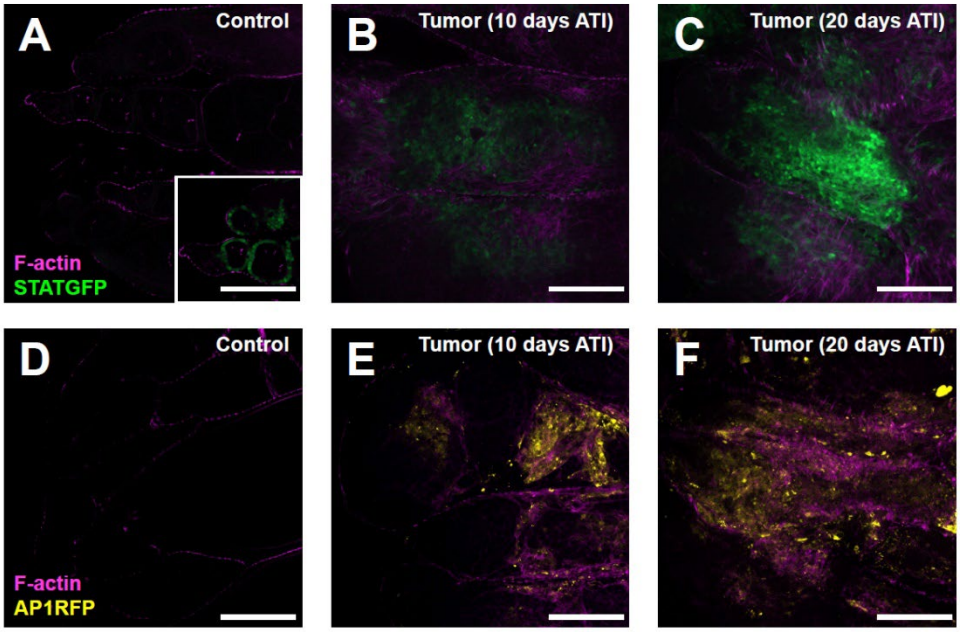

A
Control
F-actin
STATGFP
B
Tumor (10 days ATI)
C
Tumor (20 days ATI)
D
Control
F-actin
AP1RFP
E
Tumor (10 days ATI)
F
Tumor (20 days ATI)

Figure S4. Jnk and Jak/STAT signaling in OC tumors.

(A) Control ovaries show expected low-level Jak/STAT signaling (STATGFP activity reporter, green) in the follicle epithelium induced by Upd from polar cells. These signals are highlighted in inset using lower fluorescence threshold values. (B) Tumor at 10 days ATI shows elevated STAT signaling throughout (threshold values matched to main panel of A). (C) Tumor at 20 days ATI shows greatly increased STAT signaling (threshold values matched to main panel of A). (D) AP1 activity reporter (yellow) shows minimal Jnk signaling in control ovaries. (E) 10 days PTI and (F) 20 days ATI, Jnk signaling is strongly activated. Scale bars = 100μm

FIGURE S5

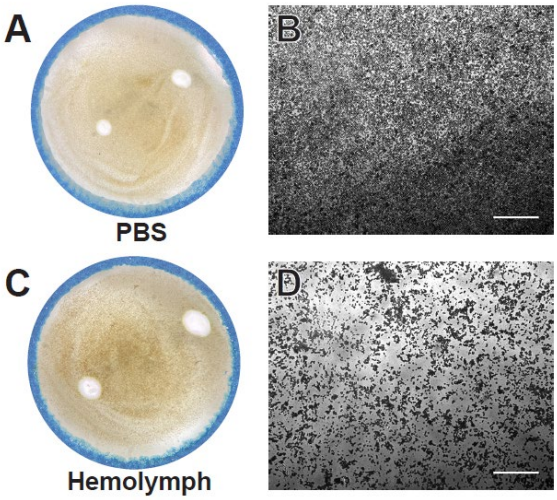
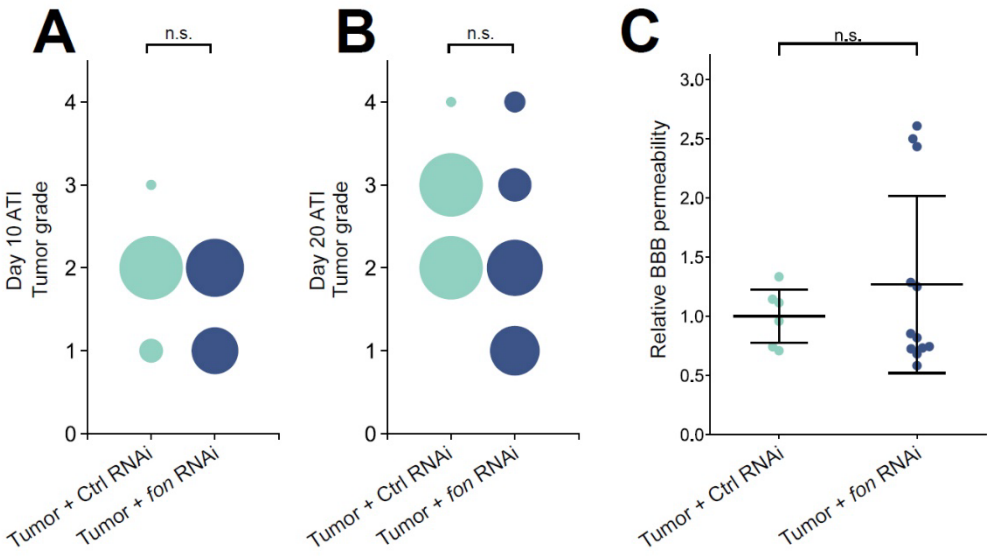


Figure S5. Clotting activity in adult fly hemolymph.
4μL of either PBS (A, B) or hemolymph from 14 day-old w^{1118} adult flies (C, D) mixed with bead solution reveals aggregation activity in adult Drosophila. The fine clot formed by adult fly hemolymph is more evident when beads are imaged microscopically rather than macroscopically. Scale bars = 100μm.

FIGURE S6


A
n.s.
Day 10 ATI
Tumor grade
0
1
2
3
4
Tumor + Ctrl RNAi
Tumor + fon RNAi
B
n.s.
Day 20 ATI
Tumor grade
0
1
2
3
4
Tumor + Ctrl RNAi
Tumor + fon RNAi
C
n.s.
Relative BBB permeability
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Tumor + Ctrl RNAi
Tumor + fon RNAi

Supplemental Figure 6. *Fon* depletion in OC tumors does not reduce tumor grade nor BBB permeability.

Knockdown of *fon* via RNAi in OC tumors does not significantly change tumor grade at day 10 (A) or 20 ATI (B). Size of circle represents proportion of sample in each class. Knockdown of fon via RNAi in OC tumors does not significantly change BBB permeability at day 20 ATI relative to control RNAi (C).

FIGURE S7

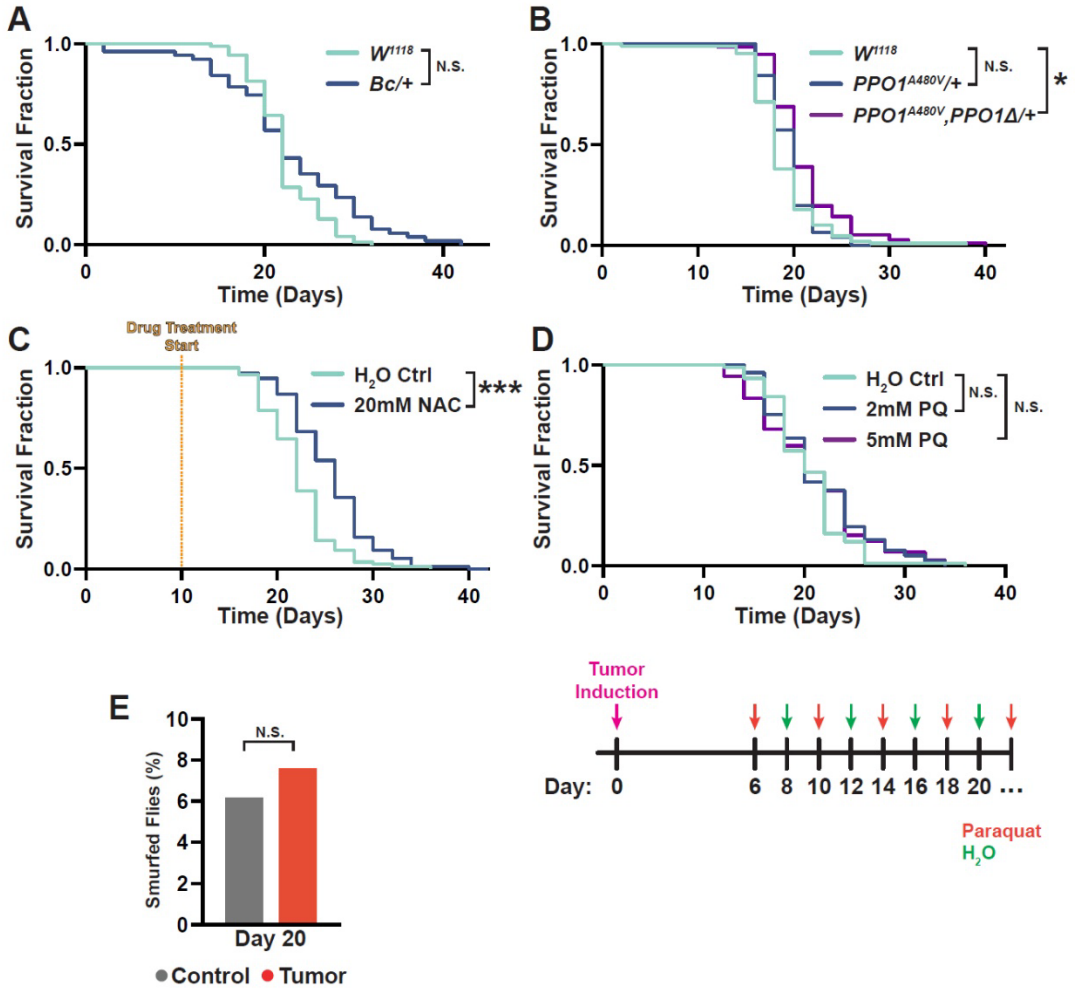

A
Survival Fraction
1.0
0.5
0.0
0
20
40
Time (Days)
W1118
Bc/+
N.S.
B
Survival Fraction
1.0
0.5
0.0
0
10
20
30
40
Time (Days)
W1118
PPO1A480V/+
PPO1A480V,PPO1Δ/+
N.S.
*
C
Drug Treatment Start
Survival Fraction
1.0
0.5
0.0
0
10
20
30
40
Time (Days)
H2O Ctrl
20mM NAC
***
D
Survival Fraction
1.0
0.5
0.0
0
10
20
30
40
Time (Days)
H2O Ctrl
2mM PQ
5mM PQ
N.S.
N.S.
E
Smurfed Flies (%)
10
8
6
4
2
0
N.S.
Day 20
Control
Tumor
Tumor Induction
Day: 0 6 8 10 12 14 16 18 20 …
Paraquat
H2O

Figure S7. PO, ROS, and intestinal permeability do not account for early lethality in OC flies.

(A) Tumor-bearing flies heterozygous for a dominant mutation in PPO1 (*Bc*/+) do not live longer than flies with normal PO levels. (B) Flies carrying a copy of an independently-generated dominant mutation in PPO1 ($PPO1^{A480V}$/+) do not die significantly later than respective controls. The additional loss of a copy of PPO1 ($PPO1^{A480V}$, $PPO1^{\Delta}$/+) only extends the mean survival by 1 day. (C) Reducing systemic ROS by treating tumor-bearing flies with the antioxidant N-acetyl Cysteine (NAC) only improved mean survival by 3 days. (D) Feeding tumor-bearing flies with paraquat (PQ), which increases ROS, did not significantly alter lifespan. Below, the PQ-feeding schedule with respect to tumor induction is shown. (E) OC flies at day 20 (median lifespan) do not show increased intestinal permeability, as assessed by the smurf assay. *$p < .05$, ***$p < .0005$.

TABLE S1: Reagents, chemicals, fly stocks and programs used

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-Dlg	Developmental Studies Hybridoma Bank	4F3
Anti-phospho-Histone H3	Cell Signaling	9701
Mouse Anti-Hindsight	Developmental Studies Hybridoma Bank	1G9
Chemicals, peptides, and recombinant proteins		
TRITC-Phalloidin	Sigma-Aldrich	P1951
DAPI	ThermoFisher Scientific	D1306
Nile Red	Sigma-Aldrich	N3013
Paraformaldehyde 16%	Electron Microscopy Sciences	15710
Phosphate Buffered Solution	Sigma-Aldrich	P4417
Triton-X	Sigma-Aldrich	T8787
Phenylthiourea	Sigma-Aldrich	P7629
L-DOPA	Sigma-Aldrich	D9628
Dynabeads M-280 Tosylactivated	Invitrogen	14203
Formula 4-24 Instant Drosophila Medium	Carolina Biological Supply Company	173210
Bovine Serum Albumin	Sigma Aldrich	A3912
10 kDa Texas red-Dextran	ThermoFisher Scientific	D1863
SlowFade Diamond Antifade Mount	Invitrogen	S36963
TWEEN 20	Sigma-Aldrich	P9416
NaCl	Fisher Chemical	S271
KCl	Fisher Chemical	P217
$CaCl_2$ x $2H_2O$	Fisher Chemical	C79
Na_2HPO_4	Fisher Chemical	S374
KH_2PO_4	Fisher Chemical	P284
Paraquat	Sigma-Aldrich	36541
N-Acetyl-L-Cysteine	Sigma-Aldrich	A7250
Alexa Fluor Phalloidin 647	Invitrogen	A22287
Schneider's *Drosophila* Medium	Gibco	21720-024
Dulbecco's Phosphate-buffered saline	Gibco	14190144
Protease from *Bacillus licheniformis*	Sigma-Aldrich	P5380
Fetal bovine serum	Cytiva Life Sciences	SH30070.03
RNeasy mini kit	QIAGEN	74104
Experimental models: Organisms/strains		
w^{1118}	Bloomington Drosophila Stock Center	#5905
BcF2-GFP	Gajewski et al 2007	FBal0244649
BcF6-GFP	Gajewski et al 2007	FBal0244650

UAS-Fondue shRNA	Vienna Drosophila Resource Center	330748
UAS-GFP shRNA	Vienna Drosophila Resource Center	#60201
10XSTAT-GFP	Bloomington Drosophila Stock Center	#26198
AP1-RFP	*Chatterjee & Bohmann 2012*	FBal0268835
Vkg::GFP	Vienna Drosophila Resource Center	v318167
TJ-Gal4	Kyoto Stock Center	#104055
UAS-aPKC$^{\Delta N}$	Bloomington Drosophila Stock Center	#51673
UAS-Ras^{V12}	Bloomington Drosophila Stock Center	#4847
Fasciclin III::GFP trap line	Bloomington Drosophila Stock Center	#59809
Tub-Gal80^{ts}		
UAS-SAS::Venus	*Firmino et al 2013*	FBtp0084565
UAS-NLS-GFP		
Software and algorithms		
FIJI		ImageJ.net
Zen 2010	Zeiss	
Zen (blue edition 2.3) pro	Zeiss	
Kallisto Alignment Software	Pachter Lab	https://pachterlab.github.io/kallisto/
R Statistical Software	The R Foundation	n/a
Graphpad	Prism	Ver 9.3.1
Gene List Annotation for Drosophila	DRSC/TRiP Functional Genomics Resources	https://www.flyrnai.org/tools/glad/web/
Python		Ver 3.9.7
Other		
15-well slides	MP Biomedicals	096041505
Pre-pulled glass pipette -- 30 µm diameter	WPI	TIP30TW1
50 µm filter	Sysmex Partec	04-004-2327
Qiaprep 2.0 Spin Columns	QIAGEN	27115

CHAPTER 3

A forward genetic screen in *Drosophila melanogaster* to identify novel mediators of tumor-host interactions

SUMMARY

Past studies of cancer biology have focused primarily on understanding the genetic and cellular changes that push cancer cells towards malignant transformation, and these studies have significantly advanced our understanding of cancer biology and treatment. Recent work has also spotlighted the importance of the tumor microenvironment for malignant overgrowth. However, much remains unknown about the more long-range, systemic impacts that a tumor can exert on its host and how these mechanisms contribute to lethality from the cancer itself. To tackle this question, we took advantage of the advanced genetics of *Drosophila melanogaster* and a new transgenic adult cancer model to devise an unbiased, forward genetic screen with the goal of identifying novel mediators regulating the interactions occurring between a tumor and its host. We used a dominant modifier strategy and screened through 92 fly lines that each contain a distinct deletion within the third chromosome and followed up on four candidate regions that were able to significantly suppress lethality of tumor-bearing hosts. Out of the haploinsufficient regions that improved the survival of tumor-bearing flies, we identified two candidate genes whose specific functions in tumor-host interactions are unknown: *NKCC* and *tartan*. Our preliminary results suggest that *NKCC* may regulate paraneoplastic dysregulation of ion homeostasis, while *tartan*'s function requires further study to uncover. Thus, these findings demonstrate the power of our approach to uncover new molecular players that regulate tumor-host interactions and open two potentially interesting avenues for future investigation.

INTRODUCTION

A continuous dialogue exists between a growing tumor and the cells of its host, as the tumor seeks to exploit homeostatic signaling axes and the host seeks to control the unrestrained growth of the tumor. From the plethora of signals exchanged between the malignant neoplasm and its host, a complex web of interactions emerges with stromal cells, epithelial cells, immune cells, and even distant organs embedded in it. The consequences of these interactions have profound impacts not only on the cells neighboring the lesion but also on overall host physiology and survival [64,119]. Locally, tumor cells can change the behavior of supporting stromal cells to fuel malignant overgrowth. Transformed cells degrade and restructure the extracellular matrix, releasing sequestered growth factors and creating an environment favorable to invasion. At the organism-level, tumors can colonize secondary sites and also disrupt homeostatic processes leading to dysregulated ion balance, tissue wasting, and compromised organ integrity [120,133]. Thus, unraveling the complexity of tumor-host interactions can shed much needed light on the ways in which tumors alter patient physiology and ultimately kill their hosts.

We have taken advantage of the powerful genetics of *Drosophila melanogaster* to identify novel mediators of tumor-host interactions. A wealth of precise genetic tools, including tissue-specific manipulation, are available to *Drosophila* biologists, facilitating studies into the complex signaling interplay between a tumor and its host [16,135]. Multiple aspects of the fly are conserved with mammals, including their organs, physiology, and key developmental pathways. Therefore, studies in the fly can provide novel insights into our understanding of human cancer. Importantly, fruit flies live relatively short lives compared to mammalian models and do not carry the same ethical concerns that preclude lifespan analyses in tumor-bearing mice. Thus, *Drosophila* is well-positioned as a model to study the mechanisms that underlie tumor-host interactions, especially those that impact lethality since survival can be used as a functional readout. Previous investigations, discussed in the Introduction and Chapter 4, have identified the molecular regulators involved in tissue wasting in tumor-bearing flies and even uncovered a novel paraneoplastic syndrome – blood-brain barrier dysfunction, highlighting the power of *Drosophila* as a model to study cancer biology [128,129,137,148,150]. Another paraneoplasia – abdominal bloating due to circulatory fluid accumulation — has been known for fifty years, but its mechanisms remain undetermined [149]. Thus, there are certainly molecular mediators of tumor-host interactions that have yet to be identified, as well as novel paraneoplastic syndromes to be discovered.

To pursue these goals, we devised a forward genetic screen to unbiasedly uncover previously unknown regulators of tumor-host interactions. We developed a genetic ovarian tumor model, detailed in Chapter I, wherein a simple temperature shift induces the malignant transformation of follicle epithelial cells in adult female flies. Whereas previous tumor models either require transplantation of tissue into the abdomen of the adult fly or induce tumors in essential organs, the ovarian carcinoma (OC) model induces malignant transformation in a non-essential organ and circumvents the physical trauma caused by transplantation. Moreover, the ease with which OC tumors are induced overcomes a logistical limitation of transplantations, which are time-intensive and low-throughput. We set out to use the OC model in a high-throughput genetic screen with the goal of uncovering novel mediators regulating tumor-host interactions, specifically using changes to host lifespan as the functional readout. Using a dominant modifier strategy, we found multiple regions on the third chromosome that improve the survival of tumor-bearing flies

compared to controls, as well as several that enhance lethality caused by the tumor. Two candidate regions were each narrowed down to a handful of genes, although the roles of individuals genes therein with regard to tumor-host interactions remain elusive.

RESULTS

Forward genetic screen to identify modifiers of host survival

To identify previously unknown mediators of tumor-host interactions, we took advantage of the genetic tools available to fly biologists and devised a forward genetic screen strategy wherein the OC model is crossed to a collection of fly lines harboring overlapping genomic deletions that together span >90% of the Drosophila genome (**Figure 1A**) [183,184]. F1 progeny carry only one copy of the deleted region; therefore, we sought to identify regions containing genes whose deletion renders them haploinsufficient with respect to the survival of tumor-bearing hosts. In this screen, genes that normally dose-sensitively suppress lethality caused by the tumor will shorten lifespan, and those that enhance lethality will extend it. Thus, large regions of the genome containing key genes involved in tumor-host interactions can be rapidly isolated. Once a candidate region is identified, secondary screens employing overlapping deletions help narrow down this region to a handful of genes by mapping out the shared regions that recapitulate the survival of the original candidate region (**Figure 1B**). Subsequent screens employing single-gene mutations can pinpoint key players regulating the survival of tumor-bearing hosts, and these can be followed up to uncover the mechanism by which they modify host lifespan. With this dominant modifier screen, we sought to rapidly screen through the fly genome as a first effort to identify regions that contain essential regulators of the host response to tumors, which can then be narrowed down to specific genes for further study.

We chose 92 fly lines from the Bloomington Deficiency kit that consisted of molecularly defined deficiencies generated in a common background [183]. These constituted three collections – BSC, DrosDel, and Exel – and we compared phenotypes only between these collections. We took F1 progeny heterozygous for a deficiency and carrying the OC genotype, shifted them to the restrictive temperature to induce a tumor, and carried out lifespan analyses. Cohort sizes averaged approximately 90 flies per deletion for the primary screen. After comparing the range of lifespans seen, we determined that significant hits would be larger than two standard deviations from the mean lifespan of each cohort's control since this cutoff would identify the strongest modifiers in our screen, which would be less susceptible to experimental variation. Standard deviation was calculated based on the standard deviation in mean lifespan of all control cohorts for the primary screen. We identified 29 deletions that suppressed tumor lethality and 48 that enhanced it (**Figure 1C, Table 1**). In thirteen cases, we repeated the primary assay with a second replicate and found nine that continued to significantly change lifespan compared to controls. Unfortunately, the tumor-host field is not yet developed enough to have a positive control to test sensitivity of the screen. Nevertheless, we conclude that this convenient assay is reasonably replicable to detect genomic regions haploinsufficient for survival of tumor-bearing hosts.

Identification of NKCC as a suppressor of tumor-lethality

Among the deletions that improved mean host survival by at least 30%, one deletion, *Df(3R)BSC549*, spanned approximately 120 kb and significantly suppressed tumor lethality, increasing the mean lifespan of tumor-bearing flies by ~40% compared to controls (**Figure 2A**). Secondary screens using four overlapping deletions narrowed the candidate region to 5 kb, which

partially contained a single gene – *NKCC*, which encodes a sodium potassium chloride transporter (**Figure 2B - E**). Importantly, loss of a single copy of *NKCC* also improved host survival (**Figure 2F**), suggesting that this gene warrants further investigation as a previously unidentified mediator of tumor-host interactions.

Because our primary screen does not differentiate between tumor-specific and host-specific impacts of each deletion (i.e. both tumor and host are haploinsufficient for the region), we employed both RNAi-driven knockdowns and transplantations to determine the relative impacts of NKCC loss in the host and in the tumor. Knockdown of NKCC specifically in the tumor resulted in a 15 day increase in median survival of tumor-bearing flies compared to that of controls (**Figure 2G**). Loss of NKCC specifically in the host also improved the survival of tumor-bearing flies, with an 8 day increase in median survival when both copies are affected, as shown through transplantation experiments (**Figure 2H**). Importantly, loss of NKCC alone does not extend lifespan; rather, flies with either one or two lost copies of NKCC exhibit shorter lifespans compared to flies with two normal copies, even without a tumor (**Figure 2I, J**). This would suggest that the loss of NKCC may be detrimental to otherwise normal flies but may somehow benefit tumor-bearing flies.

Identifying the specific tissue in which NKCC loss may be acting to suppress lethality

Because our primary interest lies with understanding host factors whose loss suppresses tumor lethality, we focused on the host-specific impacts of NKCC loss and sought to pinpoint the specific host tissues in which it acts. *NKCC*-promoter driven expression of β-galactosidase revealed broad expression, consistent with its role as a sodium potassium chloride transporter. Assaying β-galactosidase activity via X-gal staining of control flies showed *NKCC* expression in the brain, midgut, hindgut, malpighian tubules, and trachea (**Figure 3A-F**), which is in line with available expression data from FlyAtlas. Intriguingly, NKCC exhibited the same expression pattern in tumor-bearing flies compared to healthy flies (**Figure 3G-L**), suggesting that although NKCC loss suppresses lethality, tumors do not induce changes in NKCC expression in host tissues.

To determine the specific tissue in which NKCC loss suppresses tumor lethality, we transplanted larval imaginal disc tumors into female flies expressing RNAi against *NKCC* under tissue-specific control. Knockdown of NKCC globally or specifically within the host glia did not extend lifespan following disc tumor transplantation (**Figure 4A-D**). NKCC knockdown in the malpighian tubules, which are functionally equivalent to mammalian kidneys [185,186], initially appeared to extend lifespan following transplantation; however, mock transplantations seemed to suggest that this may not be the case given that host lifespan was also extended (**Figure 4E, F**). Follow-up experiments knocking down NKCC in malpighian tubules of OC tumor-bearing flies reinforced this, with tumor-bearing flies exhibiting no significant change in lifespan (**Figure 4G**). Thus, while NKCC loss in the host extended survival of tumor-bearing flies compared to controls, its loss in either malpighian tubules or glia failed to recapitulate the results from broad haploinsufficiency.

Investigations into two other deletions that suppressed tumor lethality

Among the deletions that extended host lifespan, two other candidate regions were particularly interesting. Loss of a 230 kb region covered by deletion *Df(3R)BSC47* extended the mean survival of tumor-bearing flies by ~40% (**Figure 5A**). Secondary screens using overlapping deletions helped narrow down the candidate region to 30 kb, containing 6 genes of interest (**Figure 5B-F**). These genes include: *castor*, *MLF1-adaptor molecule*, *Symplekin*, *CG1239*, *CG2100*, and *CG1236*.

Investigations into deletions that extended lifespan below the original cutoff of 30% extension in mean lifespan led to following up on a deletion, *Df(3L)BSC12*, which spanned 185 kb and extended the mean lifespan of tumor-bearing flies by ~15% (**Figure 6A**). Although there were no overlapping deletions with *Df(3L)BSC12*, only a small handful of genes were affected by its deletion: *Syntaxin13* (*Syx13*), *tartan* (*trn*), and *sneaky* (*snky*). Whereas *snky* is required in sperm for proper plasma membrane breakdown during fertilization [187], *Syx13* and *trn* warranted further investigation owing to their expression in somatic tissue. Lifespan analysis of tumor-bearing flies carrying a single mutant copy of *Syx13* ($Syx13^{01470}$) failed to suppress tumor lethality compared to control tumor-bearing flies (**Figure 6B**). Heterozygosity for *trn* improved the survival of tumor-bearing flies compared to controls (**Figure 6C**), identifying it as a potential candidate gene. However, the closely related gene *capricious* (*caps*) [188] did not significantly change survival (**Figure 6D**). Complementation tests with a known null mutation, $trn^{28.4}$ [189], and *Df(3L)BSC12* or $trn^{SO64117}$ confirmed that the deletion and original mutation indeed affects *trn* (**Figure 6E, F**). A reporter under the control of the *trn* promoter revealed increased *trn* expression within the tumor but limited expression in wild-type ovaries and other tissues (**Figure 7A - H**), raising the possibility that *trn* loss was acting at the level of the tumor rather than the host. This was partially supported by the survival of flies with KD of Trn in the tumor and tumor-specific overexpression of *trn* (**Figure 8A - C**). Follow-up experiments using independently generated trn null mutants and hypomorphs complicated this picture. The *trn* null mutant, $trn^{28.4}$, failed to suppress tumor lethality unlike the original *trn* allele (**Figure 8D**), and other *trn* alleles exhibited mixed results in their ability to suppress tumor-lethality (**Figure 8E, F**).

DISCUSSION

Prior studies from our lab and others have pioneered the study of paraneoplastic syndromes in flies, yielding molecular mechanisms that may inform the condition of human cancer patients. However, the traditional strength of forward genetic screening has not yet been applied. We describe here a discovery-based approach using a *Drosophila* screen to uncover new players within the complex tumor-host signaling network. We made use of a library of flies harboring various genomic deletions to screen the fly genome for previously unknown mediators of tumor-host interactions and found four haploinsufficient candidate regions exhibiting a significant suppression of tumor lethality (Figure 1). Secondary screens using overlapping deletions narrowed down two large genomic deletions to a handful of candidate genes, demonstrating how this approach can be used to identify putative regulators of tumor-host interactions.

We decided on a dominant modifier screen as our approach since the ease of F1 crosses to the OC genetic model allowed us to rapidly assess large regions of the genome, while still using the time-demanding assay of adult lifespans as a readout. Our experience highlighted several limitations of this approach, however. First and most obvious is that only a subset of genes may exhibit haploinsufficiency for a given phenotype, even if they play an important role. Dominant modifier screens have been successful in the past – for instance, in eye developmental pathways or cell competition phenotypes [190–192] – but we are not aware of a precedent for the type of adult survival screen we embarked on. Although the deletion screening stocks share a common genetic background, we suspect that the combined effects of heterozygosity for many genes within an individual deficiency may have often led to difficulty in tracing down individual genes within a given deficiency. Confirming hits with single-gene mutations faces its own set of problems, as suggested by our analysis of *trn*, where alleles from varying genetic backgrounds gave differing results. In the future, it may be necessary to backcross well-justified candidates into a common genetic background for a proper comparison, or perhaps generate new alleles on a defined background via CRISPR. When we first embarked on this screen, we hoped that allograft transplantation could allow us to rapidly distinguish between tumor and host impacts of a candidate gene. However, subsequent assays from this screen and other lab members' work suggest that the two tumor models show some discrepancies in tumor-host interactions, such as blood-brain barrier permeability, which exhibits different kinetics in the OC model compared to allografts. These discrepancies complicate interpretation, especially of negative results with allograft assays – for instance, transplanting into a host deficient for a candidate gene, as in the case of *NKCC* depletion in glia.

NKCC emerged from the forward genetic screen as a potentially interesting candidate gene, and complementary transplantation experiments suggested that NKCC loss suppresses tumor lethality through its actions in host tissues. NKCC is a sodium potassium chloride transporter found in both mammals and flies that is broadly expressed in multiple tissues (Figure 3) [186,193,194]. It functions to move sodium, potassium, and chloride together into the cell while maintaining electrical neutrality. In mammals, NKCC plays a role in secretion in exocrine glands and in the maintenance of the potassium-rich fluid that surrounds the cochlea [195,196]. In flies, NKCC is among the 50 most enriched genes in surface glia of the fly blood-brain barrier and is involved in regulating circadian rhythm [197,198]. Mammalian tumors disrupt normal ion homeostasis through several mechanisms from overproduction of antidiuretic hormone to dysregulation of bone

homeostasis [119], and the protective effects of NKCC loss within host tissues would suggest that fly tumors may likewise disrupt ion homeostasis. Future studies into bloating or hemolymph composition could potentially shed light on this matter.

Efforts looking to identify the specific host tissue in which NKCC is acting has yielded mixed results. Initially given the implication of NKCC in ion homeostasis and its enrichment in surface glia, we suspected that NKCC loss may be suppressing tumor lethality through its effects in glia or malpighian tubules. However, survival of tumor-bearing flies following transplantation of larval imaginal disc tumors suggested that the glia and the malpighian tubules may not necessarily be the tissues in which NKCC loss exerts its suppression. NKCC loss may potentially act in multiple tissues as well, but the failure of tubulin-driven NKCC knockdown to recapitulate lifespan extension following transplantation may suggest otherwise. Although the RNAi was previously validated [198], our experiment was not done with optimal RNAi controls from a similar genetic background. Therefore, confounding effects from differing genetic backgrounds could potentially complicate interpretation of these results, a consideration which is explored in the Appendix. The broad expression pattern of NKCC still leaves open other potential tissues to target, such as the gut and trachea. Although a role for NKCC in the gut and trachea are currently unknown, investigations into their involvement in tumor-host interactions may provide insight into their physiological functions in a healthy fly as well.

Another gene, *trn*, also emerged from the primary screen as a candidate potentially playing a role in regulating tumor-host interactions. Trn and a closely related protein, Caps, are transmembrane proteins containing leucine rich repeats [188]. Together they regulate axon guidance during embryonic development, as well as compartment boundary formation in the *Drosophila* wing and leg imaginal discs [188,199]. Interestingly, *trn* and *caps* are also involved in tracheal morphogenesis, although they each have distinct roles. While *caps* regulates tracheal branch outgrowth, *trn* provides a permissive substrate for tracheal cells to migrate as the network develops [200]. A key characteristic of all tumors is the angiogenic switch, wherein tumors must acquire a sufficient oxygen supply upon reaching a certain size threshold. Mammalian tumors accomplish this through the release of VEGF from the ECM, which induces vascularization of the tumor [70]. Fly tumors upregulate *bnl* which drives neo-tracheation, securing an oxygen supply for the rapidly growing tumor [73]. The upregulation of *trn* by tumor cells, revealed by a *trn* reporter (**Figure 5**) and our OC tumor transcriptome (**Chapter 2**), raise the intriguing possibility that tumor cells may upregulate *trn* as part of its angiogenic switch to create a permissive environment for tracheal growth. Loss of *trn* may make the tumor surface less supportive of neo-tracheation, which would in turn restrict tumor size. This could potentially explain why *trn* heterozygosity improved host survival, since these flies would theoretically have a lower tumor burden. Thus, tumors may reactivate an embryonic developmental pathway involving *trn* and *caps* to create a permissive environment for neo-tracheation. Future experiments could investigate tumor size and progression, as well as the tracheal network of tumors lacking *trn*.

A complex web of signals exchanged between a growing tumor and its host emerges out of the array of differentially expressed genes within the tumor. These signals alter the behavior of neighboring cells, disrupt physiological homeostasis, and complicate cancer treatment. Therefore, untangling this web and identifying key regulators are key to advancing our understanding of cancer biology and addressing the question, “How does cancer kill?”. Using our OC model, we

performed an unbiased forward genetic screen of the third chromosome of the fly and uncovered two candidate mediators of tumor-host interactions, demonstrating the potential of this approach. This approach does however have significant limitations to consider. Two limitations to keep in mind are the effects of genetic background on lifespan and the broad question of which organ is specifically impacted. Moreover, these two limitations come to a head when identifying a specific organ after the primary screen. Interpretation of lifespan results becomes complicated by the disparate genetic backgrounds in which different Gal4 lines, RNAi lines, and mutant lines are generated, creating a significant obstacle to subsequent analyses into molecular mechanisms. An alternative approach that could address these considerations may be instead to assess how haploinsufficient regions impact a phenotype other than survival since cellular changes caused by the tumor may be less sensitive to genetic background differences, potentially creating less ambiguity in interpreting results. Tumors appear to compromise the function or integrity of nearly every fly organ. The ovaries, brain, malpighian tubules, gut, and even the heart are all impacted in some form (impacts on heart function are explored more in the Appendix). Therefore, selecting a specific organ to study will allow the primary screen to focus on genes involved in mediating the interaction between the tumor and organ of interest, streamlining experimental questions that follow.

EXPERIMENTAL PROCEDURES

Fly stocks and husbandry

Flies were reared on cornmeal, molasses, yeast food, and crosses were maintained at room temperature. Ovarian tumors were generated using *traffic jam-Gal4* (*TJ-Gal4*) to drive the expression of *UAS-Ras*^{V12} and *UAS-aPKC*$^{\Delta N}$, and *tubulin-Gal80*^{ts} was used to control GAL4 activity. Wing disc tumors for transplantations were generated via *nubbin-Gal4* (*nub-Gal4*) driving the expression of *UAS-Ras*^{V12} and *UAS-aPKC*$^{\Delta N}$ in the wing pouch.

Deficiency lines used for the primary and secondary screens were obtained from the Bloomington Stock Center Deficiency Kit. See Table 1 for list of deficiencies screened. Mutant lines from Bloomington Drosophila Stock Center used were *yw* (as control), *NKCC*^{MI13864} (Bloomington #59219), *trn*^{SO64117} (Bloomington #4550), *Syx13*^{01470} (Bloomington #11536), and *caps*^{029370} (Bloomington #11579). Independently generated *trn* mutants, *trn*$^{28.4}$, *trn*^{11626}, and *trn*^{hypo} were generous gifts from the Zinn lab. RNAi lines used include *mcherry*^{RNAi} (Bloomington #35787), *NKCC*^{RNAi} (DGRC #2509-2R), and *trn*^{RNAi} (Bloomington #28525 and #50520). Tumor-specific overexpression of *trn* used *UAS-trn*$^{full\ length}$ from the Zinn lab. Lines used for X-gal staining of tissues were *NKCC*^{MI13864} and *trn*^{SO64117}.

Tissue-specific NKCC knockdowns used the following Gal4 drivers: *tubulin-Gal4*, *repo-Gal4*, and *ctb-Gal4*. Temporal control for global and glial knockdowns was achieved with a temperature-sensitive *Gal80*.

Dominant modifier screen and lifespan analysis

For OC model lifespan analysis, 20 virgin females carrying the OC constructs were crossed to 10 males carrying the appropriate genotype. Crosses were maintained at room temperature on cornmeal, molasses, yeast food that was supplemented with ground instant yeast (Fleischman's Yeast #2139). Crosses were flipped every other day for a total of six vials per cross. Water was added when food appeared dry. Two days after the first F1 progeny eclose, F1 females carrying the desired genotype were sorted on CO_2 and placed into fresh vials supplemented with yeast powder. A max of 25 flies were kept in a single vial for lifespan analysis. Every other day, F1 progeny were sorted out for a max of three times per vial. After two days on yeast, sorted F1 progeny were flipped into a fresh vial without yeast and shifted to 29°C. Vials were then flipped into fresh food every other day, and the number of dead flies were counted each flip. Lifespan data was analyzed in Excel, and log-rank test was used to determine significance.

Transplantation was adapted from ref. 137. Wing discs were dissected out of donor larvae in 1X PBS. A single intact wing disc was transplanted into the abdomen of one day old adult female flies using a fine glass needle [201]. Flies were placed into a fresh vial supplemented with yeast powder and allowed to recover at room temperature overnight. Tumors were induced by shifting flies to 29°C, and flies were flipped onto fresh food every other day. Each flip, the number of dead flies were counted. Lifespan data was analyzed in Excel, and log-rank test was used to determine significance.

X-gal staining and imaging

X-gal staining solution was prepared by diluting 8% X-gal stock solution 1:30 in pre-warmed X-gal staining buffer (7.2mM Na_2HPO_4, 2.8mM NaH_2PO_4, 150mM NaCl, 1mM $MgCl_2$, 3.05mM $K_3Fe(CN)_6$, 3.05mM $K_4Fe(CN)_6$ in H_2O) Samples were dissected in 1X PBS and then transferred to pre-warmed X-gal staining solution for incubation. After 30 minutes, staining progress was checked, and once staining was complete, samples were washed with 1X PBS and fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature. Following fixation, samples were washed three times in 1X PBS and mounted in 80% glycerol solution. Images were taken on a Leica Z16 APO mounted with a Leica DFC300 FX camera.

Complementation tests

Virgin females of the appropriate genotype and carrying a balancer chromosome were crossed to the desired males. Crosses were maintained at room temperature on cornmeal, molasses, yeast food that was supplemented with yeast powder. Crosses flipped to prevent crowding. F1 flies of each genotype was scored to determine complementation of mutant alleles and deletions.

TABLE 1

Deletion	BDSC #	Percent change in mean lifespan (%)	p-value
Df(3L)6B-29+Df(3R)6B-29	2596	-13	1.24×10^{-8}
Df(3L)BSC362	24386	25	0.0305
Df(3L)ED4196	8050	-9	1.25×10^{-7}
Df(3L)ED202	8051	-23	2.94×10^{-11}
Df(3L)ED4238	8052	-34	7.52×10^{-12}
Df(3L)ED207	8053	-16	1.65×10^{-13}
Df(3L)Exel6086	7565	-16	6.97×10^{-8}
Df(3L)BSC799	27371	-8	0.000309
Df(3L)BSC250	23150	10	0.0202
Df(3L)BSC178	9609	-9	0.00162
Df(3L)ED4256	8054	-2	0.24
Df(3L)BSC800	27372	-4	0.0614
Df(3L)ED4287	8096	-12	6.48×10^{-5}
Df(3L)BSC23	6755	20	0.00059
Df(3L)Exel6092	7571	9	0.617
Df(3L)BSC671	26523	-7	7.4×10^{-7}
Df(3L)ED4293	8058	25	0.00141
Df(3L)ED208	8059	33	1.42×10^{-6}
Df(3L)ED4341	8060	16	0.280
Df(3L)Exel9000	7921	55	5.91×10^{-17}
Df(3L)BSC371	24395	-18	8.88×10^{-7}
Df(3L)ZN47	3096	-36	1.35×10^{-21}
Df(3L)BSC410	24914	33	2.67×10^{-5}
Df(3L)BSC437	24941	-2	0.288
Df(3L)BSC411	24915	36	4.54×10^{-14}
Df(3L)BSC27	6867	16	0.000651
Df(3L)BSC375	24399	-11	$1.24 \times 10x^{-9}$
Df(3L)BSC388	24412	20	0.000723
Df(3L)BSC157	9544	4	0.124
Df(3L)ED4413	9070	3	0.972
Df(3L)ED4421	8066	11	0.0275
Df(3L)BSC816	27577	-17	1.99×10^{-6}
Df(3L)BSC391	24415	3	0.839
Df(3L)BSC392	24416	-13	0.00773
Df(3L)BSC393	24417	5	0.349
Df(3L)BSC673	26525	12	0.00593
Df(3L)ED4470	8068	-23	3.02×10^{-6}
Df(3L)ED4475	8069	4	0.844
Df(3L)ED4486	8072	-9	6.814×10^{-5}
Df(3L)BSC12	6457	13	0.0223
Df(3L)BSC845	27888	-15	1.56×10^{-9}

TABLE 1 (continued)

Deletion	BDSC #	Percent change in mean lifespan (%)	p-value
Df(3L)BSC775	27347	-15	0.00150
Df(3L)ED225	8081	-19	4.51×10^{-9}
Df(3L)BSC20	6646	-27	0.172
Df(3L)ED4858	8088	-19	2.94×10^{-13}
Df(3L)BSC797	27369	-18	4.81×10^{-6}
Unknown	23714	16	0.237
Df(3R)BSC549	25077	41	1×10^{-5}
Df(3R)ED5177	8103	-17	4.07×10^{-16}
Df(3R)BSC47	7443	24	0.00270
Df(3R)BSC738	268362	-13	5.35×10^{-6}
Df(3R)BSC633	25724	-13	0.0372
Df(3R)ED5230	8682	0.3	0.0134
Df(3R)BSC666	26518	-18	2.18×10^{-8}
Df(3R)BSC476	24980	16	0.327
Df(3R)ED5339	9204	-27	1.53×10^{-7}
Df(3R)BSC507	25011	-9	0.00917
Df(3R)BSC526	25054	-8	0.000126
Df(3R)BSC469	24973	15	0.211
Df(3R)ED5642	9279	19	0.447
Df(3R)ED5664	24137	-10	0.00164
Df(3R)BSC750	26848	14	0.223
Df(3R)BSC741	26839	11	0.387
Df(3R)BSC515	25019	15	0.760
Df(3R)ED10639	9481	1	0.717
Df(3R)ED10642	9482	-15	0.00326
Df(3R)P115	1467	-6	0.00148
Df(3R)ED5780	8104	-4	0.000548
Df(3R)BSC748	26846	19	0.167
Df(3R)BSC792	27364	-24	2.45×10^{-10}
Df(3R)ED5815	9208	10	0.0219
Df(3R)ED5938	24139	-0.1	0.169
Df(3R)BSC818	27579	-8	1.53×10^{-5}
Df(3R)BSC809	27380	-17	5.31×10^{-10}
Df(3R)BSC517	25021	-13	3.87×10^{-12}
Df(3R)BSC819	27580	-39	8.78×10^{-34}
Df(3R)ED10845	9487	-54	2.35×10^{-42}
Df(3R)BSC677	26529	-15	0.635
Df(3R)BSC137	9497	-7	0.00764
Df(3R)BSC489	24993	13	0.0240
Df(3R)ED6220	9211	-1	0.00246
Df(3R)BSC461	24965	4	0.915

TABLE 1 (continued)

Deletion	BDSC #	Percent change in mean lifespan (%)	p-value
Df(3R)FDD-0317950	27404	6	0.272
Df(3R)BSC140	9500	-3	0.223
Df(3R)BSC497	25001	-37	1.45×10^{-5}
Df(3R)ED6280	29667	-4	0.000306
Df(3R)BSC620	25695	-12	0.00814
Df(3R)Exel6214	7692	-35	2.49×10^{-6}
Df(3R)BSC503	25007	36	1.06×10^{-13}
Df(3R)BSC504	25008	15	0.00332
Df(3R)ED6346	24142	5	0.452
Df(3R)Exel7379	7919	-18	0.00950

Table 1. Dominant modifier screen of deletions covering the third chromosome.

Table of results for the primary screen detailing the change in the mean lifespan compared to control tumor-bearing flies for 92 deficiencies covering the third chromosome of the *Drosophila* genome. Statistical test used for lifespan analysis was the log-rank test.

FIGURE 1

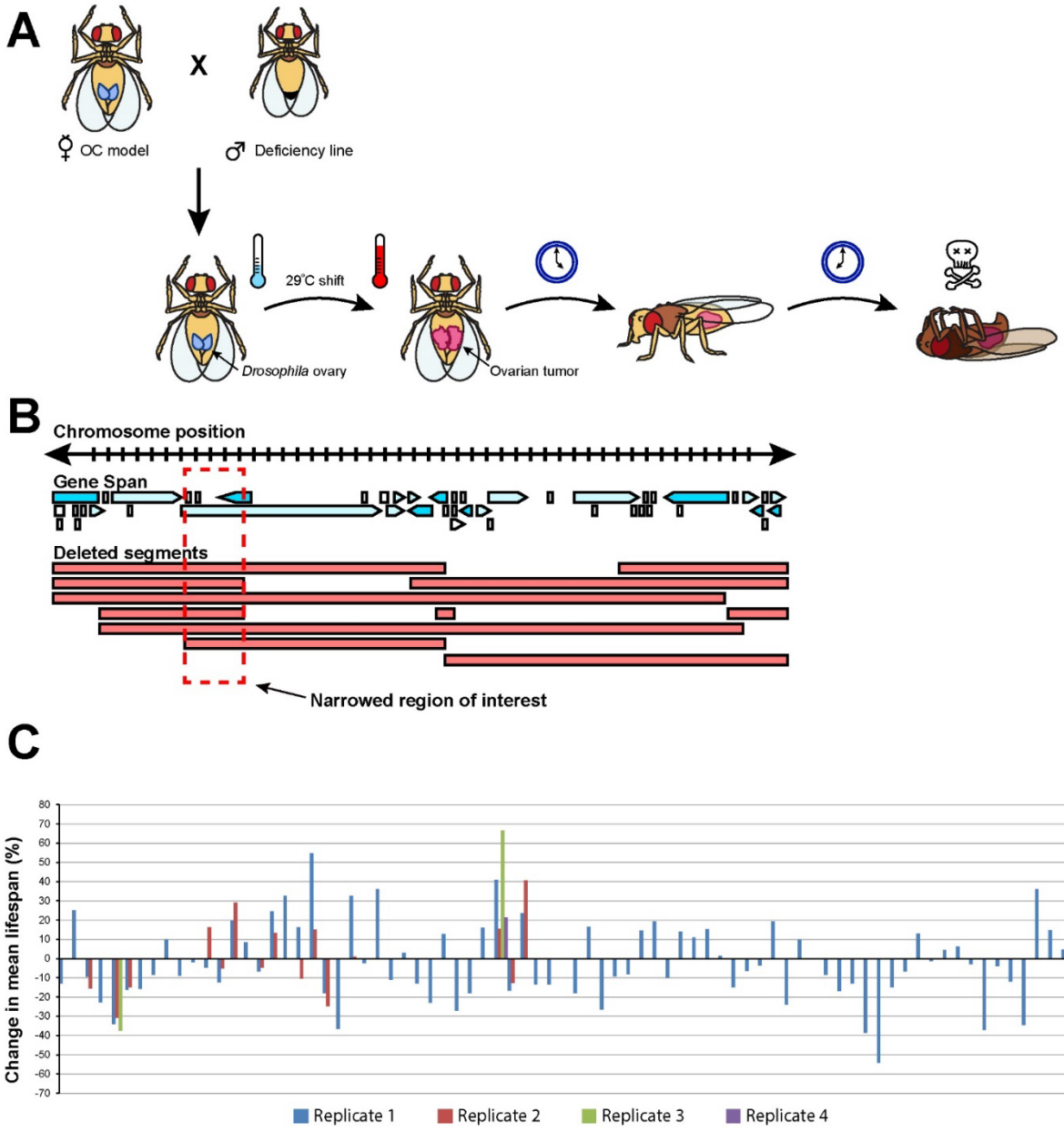

A
X
♀ OC model
♂ Deficiency line
29°C shift
Drosophila ovary
Ovarian tumor
B
Chromosome position
Gene Span
Deleted segments
Narrowed region of interest
C
Change in mean lifespan (%)
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
Replicate 1
Replicate 2
Replicate 3
Replicate 4

Figure 1. Dominant modifier screen to identify novel tumor-host interactors

Schematic of the experimental set-up for the dominant modifier screen using the ovarian carcinoma (OC) model (A). Secondary screens using overlapping deletions narrowed down a specific region of interest to a handful of genes as illustrated in a theoretical schematic (B). The primary screen identified multiple regions that show a significant difference in mean lifespan of tumor-bearing flies compared to controls. Similar change in mean lifespan between experimental replicates, indicated by the different colors, suggest the reproducibility of the results of the primary screen.

FIGURE 2

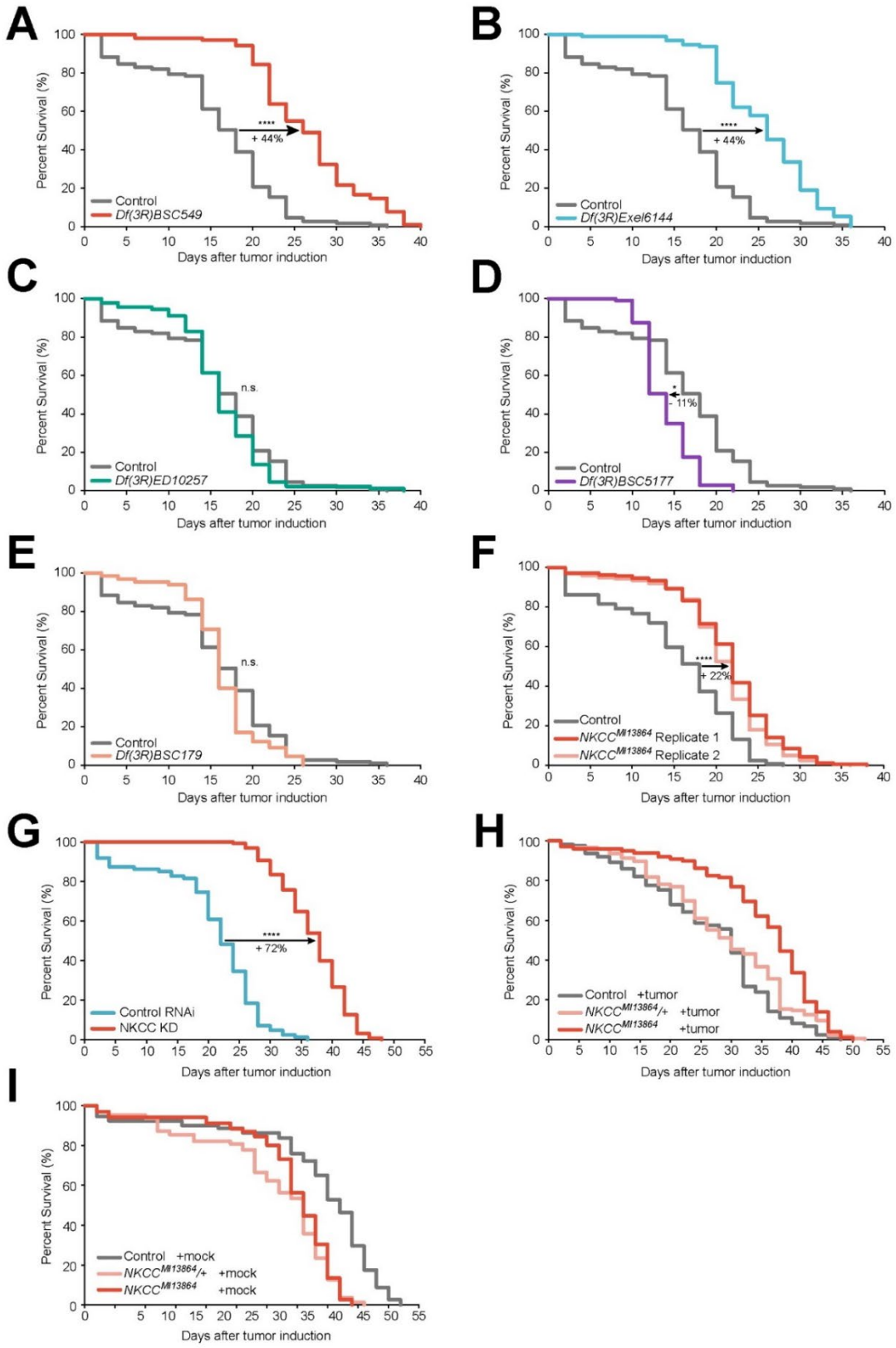

A
Percent Survival (%)
Days after tumor induction
****
+ 44%
Control
Df(3R)BSC549
B
Percent Survival (%)
Days after tumor induction
****
+ 44%
Control
Df(3R)Exel6144
C
Percent Survival (%)
Days after tumor induction
n.s.
Control
Df(3R)ED10257
D
Percent Survival (%)
Days after tumor induction
*
- 11%
Control
Df(3R)BSC5177
E
Percent Survival (%)
Days after tumor induction
n.s.
Control
Df(3R)BSC179
F
Percent Survival (%)
Days after tumor induction
****
+ 22%
Control
NKCC^MI13864 Replicate 1
NKCC^MI13864 Replicate 2
G
Percent Survival (%)
Days after tumor induction
****
+ 72%
Control RNAi
NKCC KD
H
Percent Survival (%)
Days after tumor induction
Control +tumor
NKCC^MI13864/+ +tumor
NKCC^MI13864 +tumor
I
Percent Survival (%)
Days after tumor induction
Control +mock
NKCC^MI13864/+ +mock
NKCC^MI13864 +mock

Figure 2. *NKCC* identified as a putative gene regulating tumor-host interactions.
Tumor-bearing flies lacking a 120 kb region of the third chromosome covered by *Df(3R)BSC549* survive significantly longer than controls (A). Overlapping deletions show variable survival compared to controls, with *Df(3R)Exel6144* recapitulating the survival seen in the primary screen (B-E). Mutation disrupting *NKCC*, a candidate gene identified through the screen, improves the survival of tumor-bearing flies in two biological replicates (F). Knockdown of NKCC specifically in the tumor reduces the lethality of tumors compared to control RNAi (G). Two copies of a mutant allele of *NKCC* improves the survival of flies transplanted with wing disc tumors compared to either a single mutant copy and controls (H). Mock transplants show that one or two copies of a mutant *NKCC* allele does not extend lifespan of flies compared to controls (I). Statistical test used for lifespan analysis was the log-rank test (* $p<0.05$, **** $p<0.00005$).

FIGURE 3

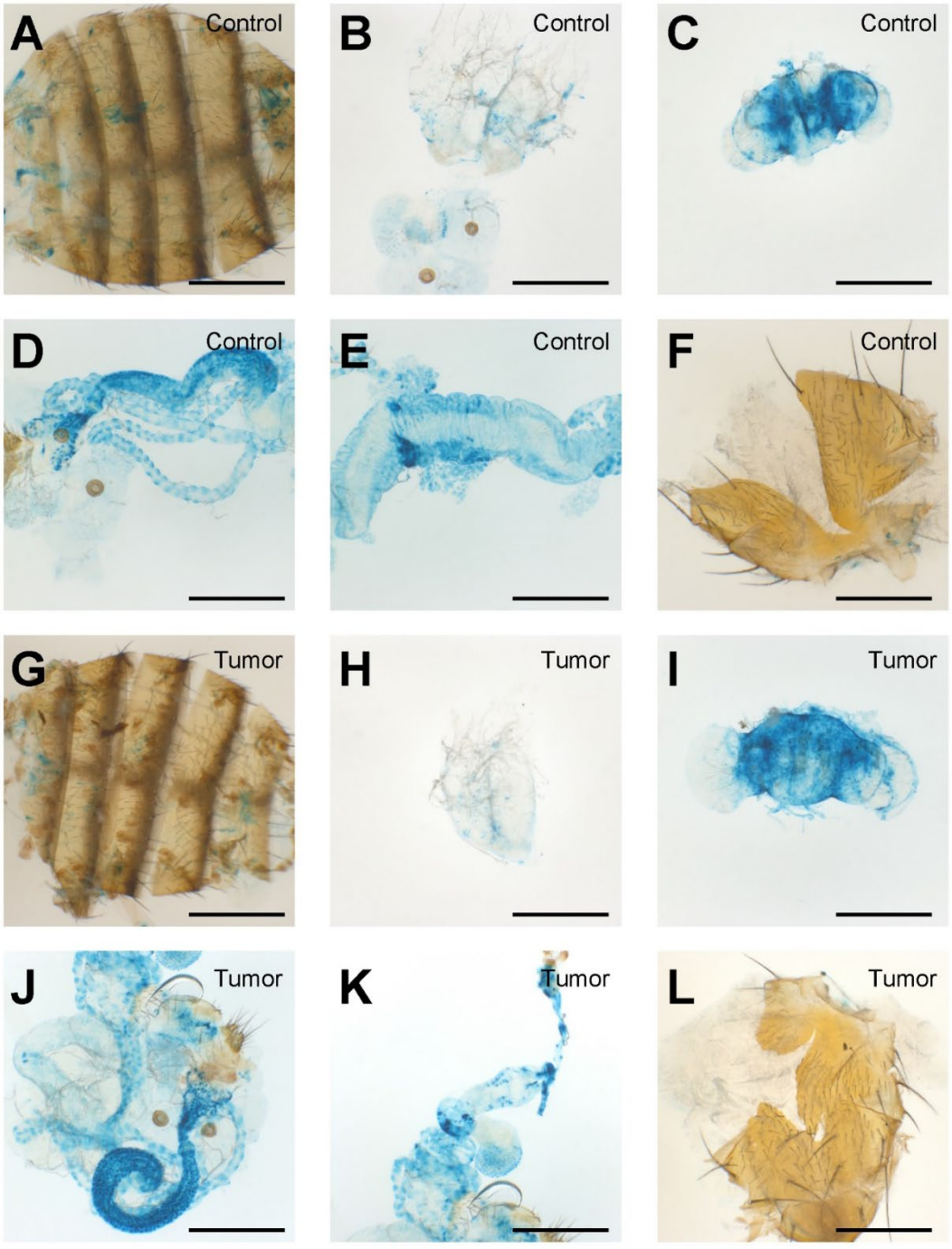

A
Control
B
Control
C
Control
D
Control
E
Control
F
Control
G
Tumor
H
Tumor
I
Tumor
J
Tumor
K
Tumor
L
Tumor

Figure 3. NKCC is broadly expressed in healthy and tumor-bearing flies.
X-gal staining of dorsal abdomen (A), ovary (B), brain (C), hindgut (D), midgut (E), malpighian tubules (D, E), and thoracic muscle (F) of control, healthy flies reveals a broad expression of *NKCC*. A similar pattern of X-gal staining in dorsal abdomen (G), ovary (H), brain (I), hindgut (J), midgut (K), malpighian tubules (J, K), and thoracic muscle (L) was observed in tumor-bearing flies as well. Scale bars indicate 0.5mm.

FIGURE 4

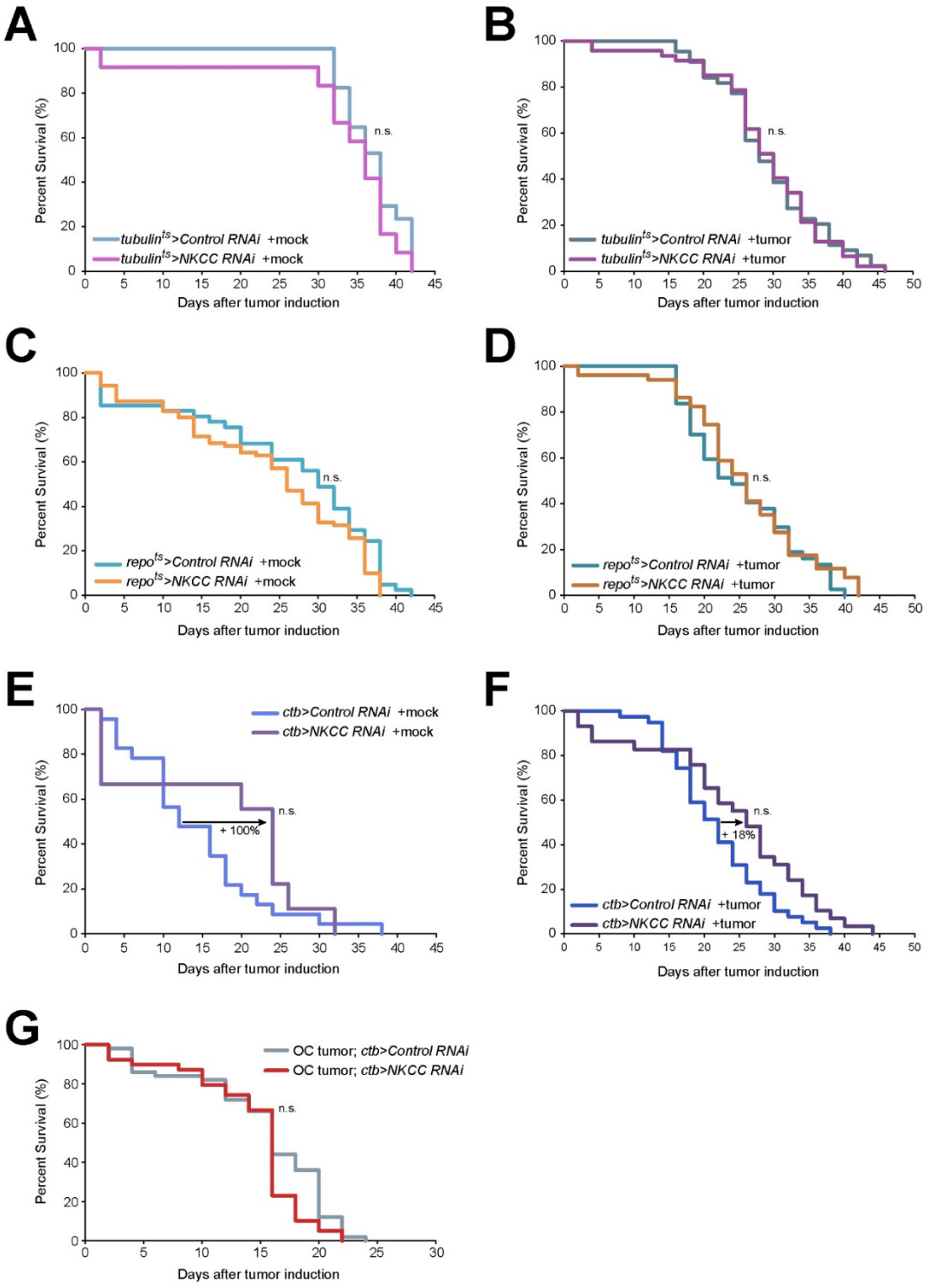

A
Percent Survival (%)
Days after tumor induction
n.s.
tubulin^ts>Control RNAi +mock
tubulin^ts>NKCC RNAi +mock
B
Percent Survival (%)
Days after tumor induction
n.s.
tubulin^ts>Control RNAi +tumor
tubulin^ts>NKCC RNAi +tumor
C
Percent Survival (%)
Days after tumor induction
n.s.
repo^ts>Control RNAi +mock
repo^ts>NKCC RNAi +mock
D
Percent Survival (%)
Days after tumor induction
n.s.
repo^ts>Control RNAi +tumor
repo^ts>NKCC RNAi +tumor
E
Percent Survival (%)
Days after tumor induction
ctb>Control RNAi +mock
ctb>NKCC RNAi +mock
n.s.
+ 100%
F
Percent Survival (%)
Days after tumor induction
n.s.
+ 18%
ctb>Control RNAi +tumor
ctb>NKCC RNAi +tumor
G
Percent Survival (%)
Days after tumor induction
OC tumor; ctb>Control RNAi
OC tumor; ctb>NKCC RNAi
n.s.

Figure 4. Tissue-specific KD of NKCC does not indicate a specific host tissue in which it acts. Global KD of NKCC does not change the lifespan of flies following mock transplantation (A) nor after wing disc tumor transplantation (B). Similarly, KD of NKCC in the glia does not change the survival of flies after mock transplants (C) nor after wing disc tumor transplants (D). Malpighian tubule-specific KD of NKCC does not significantly improve survival of mock transplants (E), wing disc tumor transplants (F), nor OC tumor-bearing flies (G). Statistical test used for lifespan analysis was the log-rank test (n.s. denotes not significant).

FIGURE 5

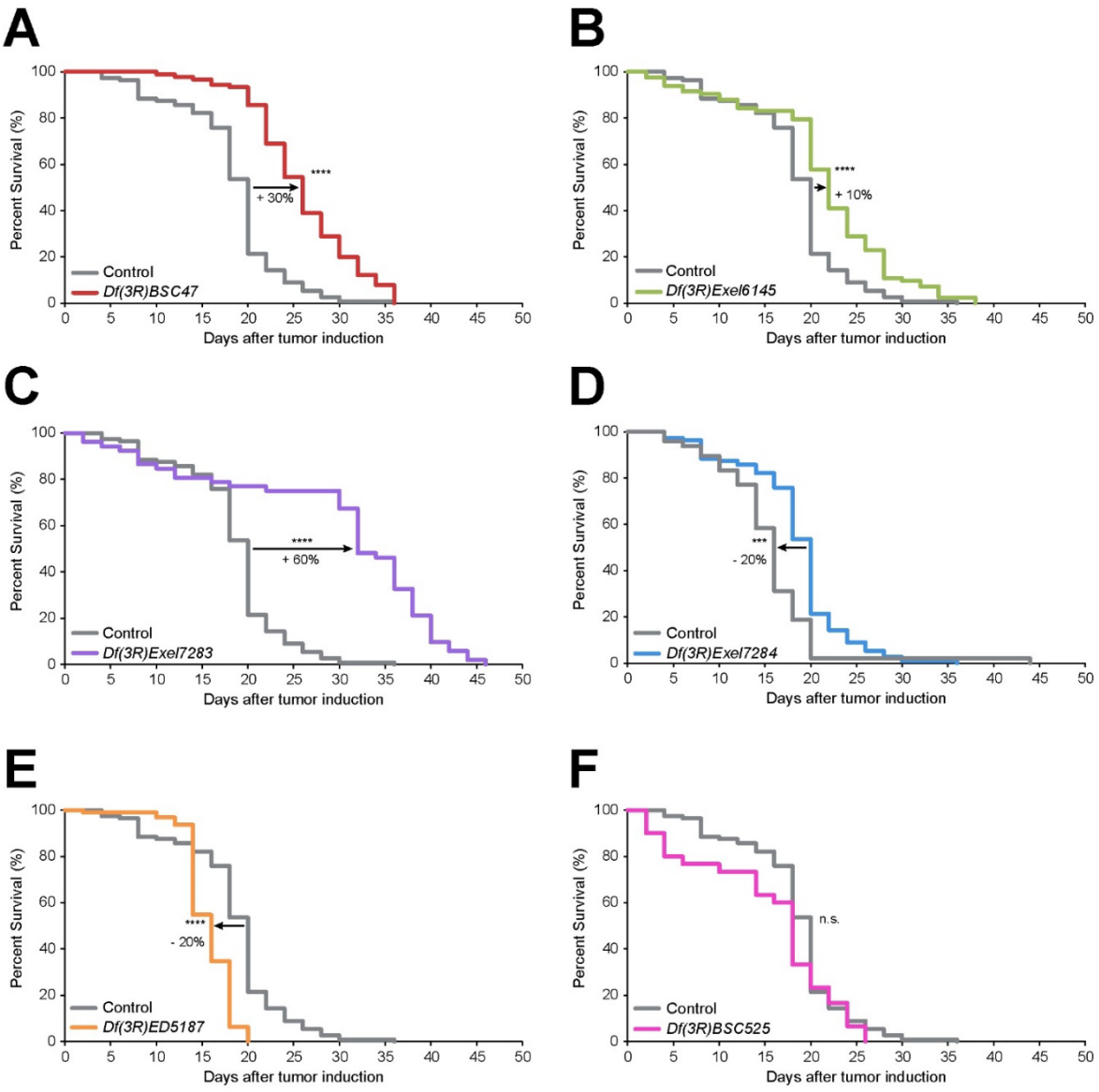
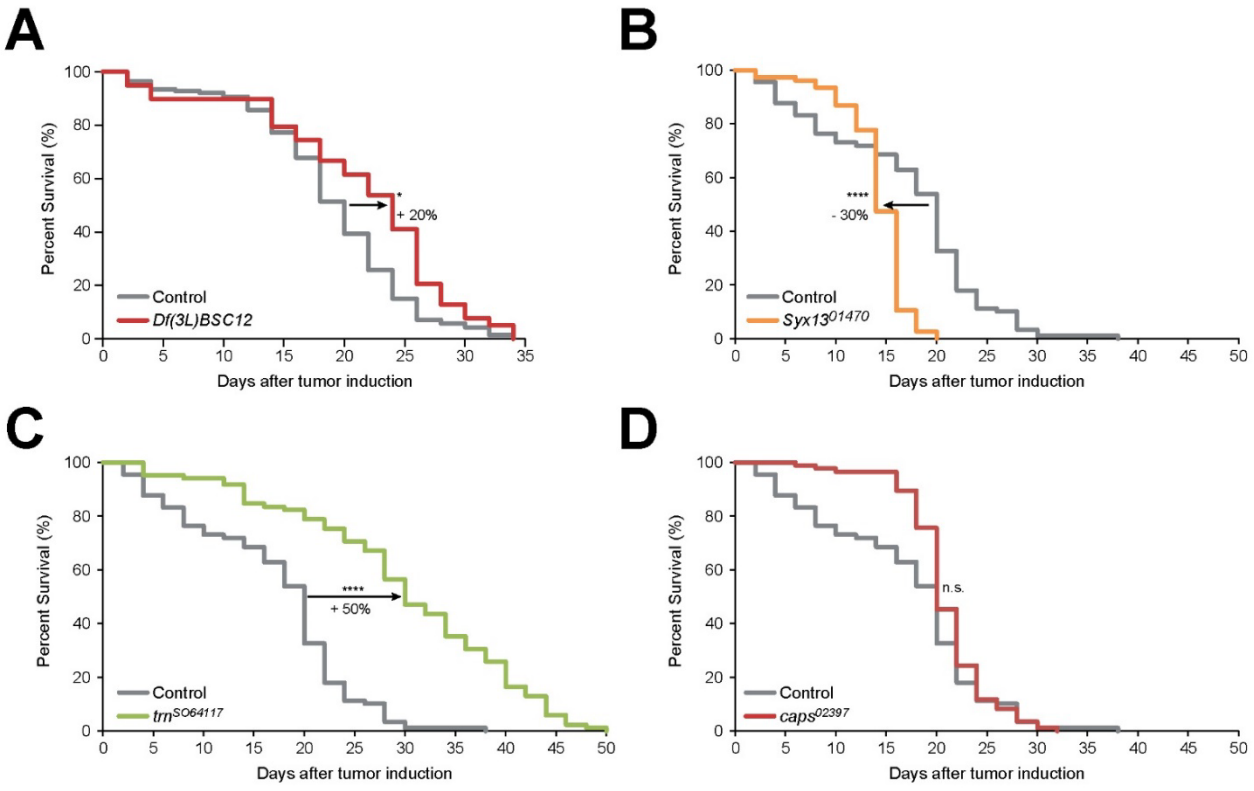

A
Percent Survival (%)
Days after tumor induction
Control
Df(3R)BSC47
****
+ 30%
B
Control
Df(3R)Exel6145
****
+ 10%
C
Control
Df(3R)Exel7283
****
+ 60%
D
Control
Df(3R)Exel7284
***
- 20%
E
Control
Df(3R)ED5187
****
- 20%
F
Control
Df(3R)BSC525
n.s.

Figure 5. Follow-up of another candidate region identified by primary screen.
A 230 kb region covered by *Df(3R)BSC47* significantly suppresses lethality caused by the tumor (A). Overlapping deficiencies exhibit variable extension of host lifespan, with *Df(3R)Exel7283* showing the greatest extension compared to control (B-F). Statistical test used for lifespan analysis was the log-rank test (n.s. denotes not significant, **** $p<0.00005$).

FIGURE 6



E

♂ \ ♀	*Df(3L)BSC12*	*TM6B*
trn$^{28.4}$	*Df(3L)BSC12/trn*$^{28.4}$ **0 flies**	*trn*$^{28.4}$*/TM6B* **74 flies**
TM3	*Df(3L)BSC12/TM3* **154 flies**	*TM6B/TM3* **0 flies**

F

♂ \ ♀	*trn*^{SO64117}	*TM3*
trn$^{28.4}$	*trn*^{SO64117}*/trn*$^{28.4}$ **0 flies**	*trn*$^{28.4}$*/TM3* **12 flies**
TM3	*trn*^{SO64117}*/TM3* **34 flies**	*TM3/TM3* **0 flies**

Figure 6. Another region of interest covers a potential mediator of tumor-host interactions. A 185 kb region covered by *Df(3L)BSC12* improves the survival of tumor-bearing flies compared to control (A). Within this region, a mutant allele for *Syx13* reduces survival of tumor-bearing flies (B), whereas a mutant allele for *trn* significantly extends lifespan compared to control (E). A mutant allele for *caps* does not significantly change survival of tumor-bearing flies compared to controls even though it is closely related to *trn* (D). Complementation tests with a known null mutation, $trn^{28.4}$, indicate that the original deletion from the primary screen includes the *trn* loci, and the original *trn* mutant tested is indeed null (E, F). Statistical test used for lifespan analysis was the log-rank test (n.s. denotes not significant, * $p<0.05$, **** $p<0.00005$).

FIGURE 7

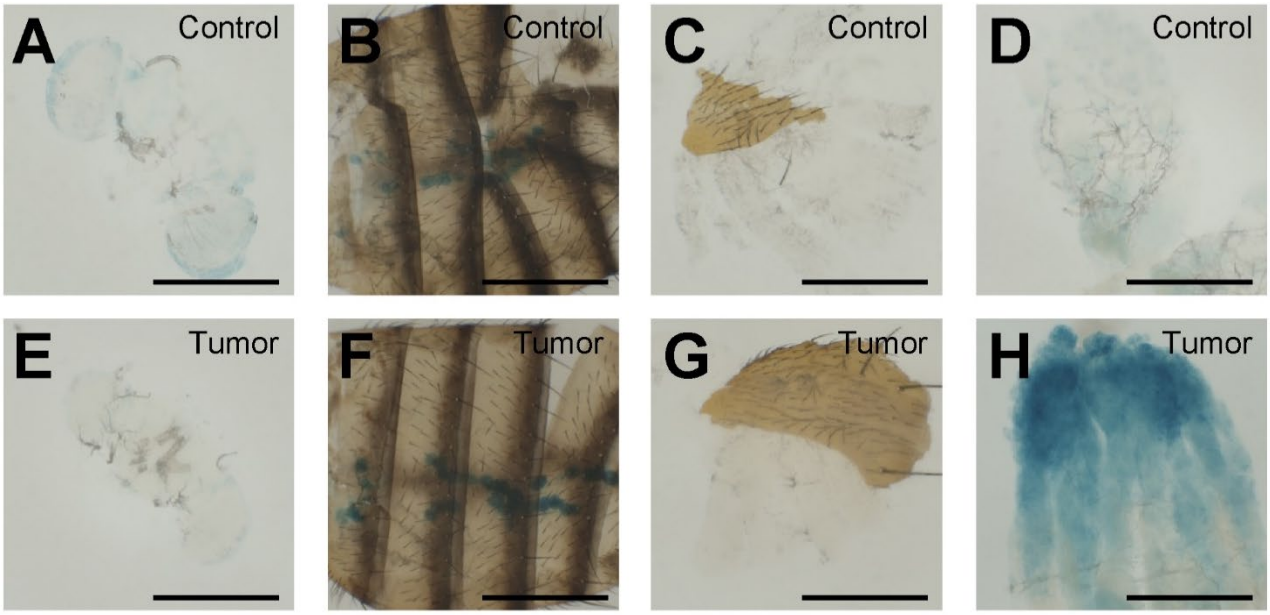

A
Control
B
Control
C
Control
D
Control
E
Tumor
F
Tumor
G
Tumor
H
Tumor

Figure 7. *trn* is expressed in the OC tumor but minimally in wild-type ovaries.
X-gal staining of brain (A), dorsal abdomen (B), thoracic muscle (C), and ovaries (D) of healthy control flies shows minimal expression of *trn* throughout the fly. Tumor-bearing flies do not show *trn* expression in brain (E), dorsal abdomen (F), and thoracic muscle (G), but ovarian tumors show significant upregulation of *trn* (H). Pericardial cells appear to show expression of *trn* regardless of whether or not a tumor is present (B, F). Scale bars indicate 0.5mm.

FIGURE 8

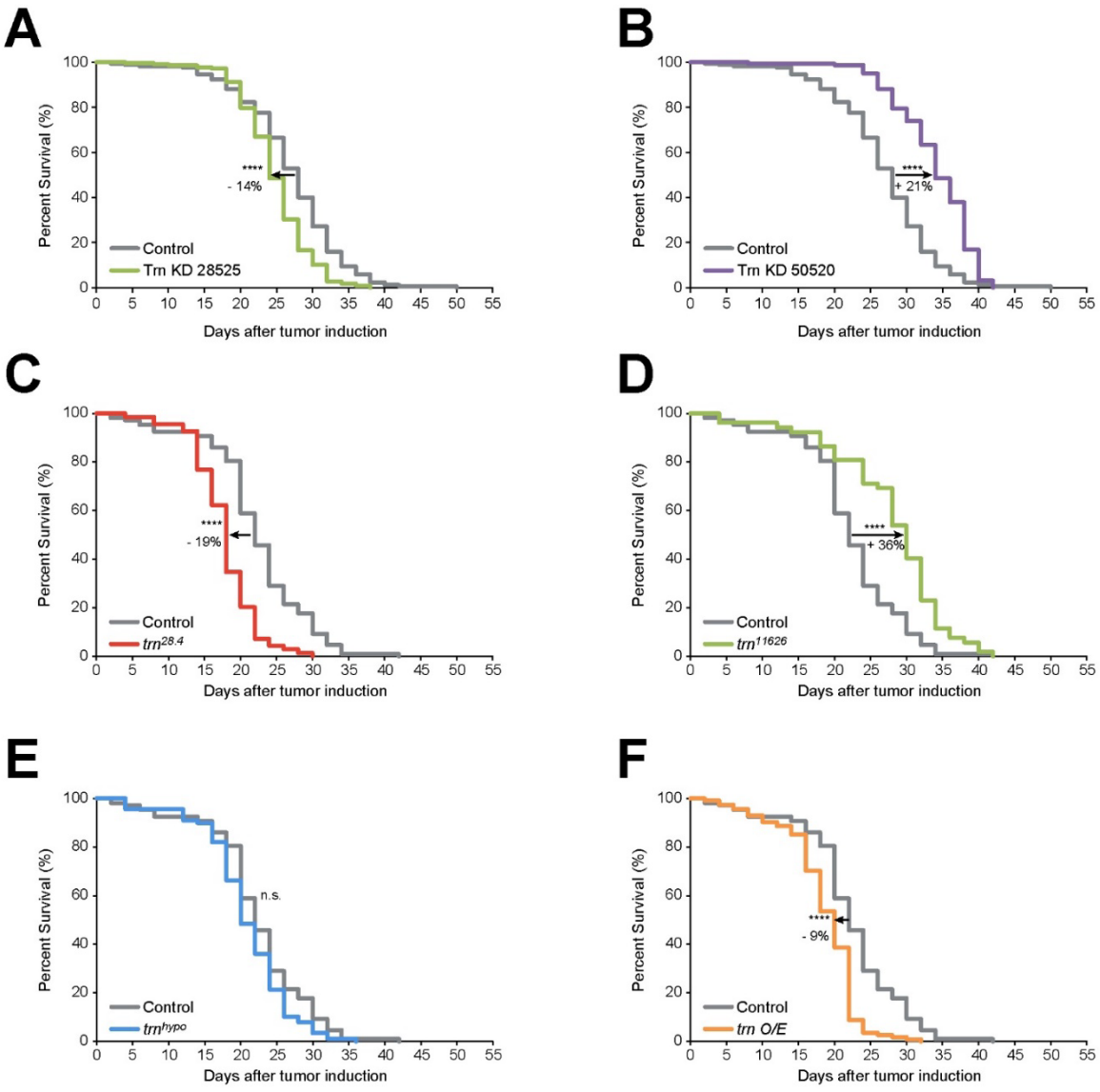

A
Percent Survival (%)
Days after tumor induction
****
- 14%
Control
Trn KD 28525
B
Percent Survival (%)
Days after tumor induction
****
+ 21%
Control
Trn KD 50520
C
Percent Survival (%)
Days after tumor induction
****
- 19%
Control
trn28.4
D
Percent Survival (%)
Days after tumor induction
****
+ 36%
Control
trn11626
E
Percent Survival (%)
Days after tumor induction
n.s.
Control
trnhypo
F
Percent Survival (%)
Days after tumor induction
****
- 9%
Control
trn O/E

Figure 8. Interpretation of lifespans for *trn* results are complicated by tumor-specific KD and independently generated alleles.

RNAi targeting *trn* within the tumor shows variable suppression of lethality depending on the RNAi line used (A, B). Similarly, depending on the *trn* allele, the survival of tumor-bearing flies varies from significantly reduced to significantly extended versus control tumor-bearing flies (C-E). Overexpression of full-length *trn* in OC tumors significantly shortens the lifespan of tumor-bearing flies compared to control (F). Statistical test used for lifespan analysis was the log-rank test (n.s. denotes not significant, **** $p<0.00005$).

CHAPTER 4

Tumor-host interactions through the lens of *Drosophila*

This chapter is a reproduction of the paper by the same name published in Nature Reviews Cancer in August 2021 with minor formatting changes. For this paper, David Bilder wrote the manuscript, and Jung Kim, Katy Ong, Kavya Adiga, and I made revisions. Kavya Adiga put together the tables, and Katy Ong and I designed the figures, which were then created by illustrators at Nature Reviews Cancer.

SUMMARY

There is a large gap between the deep understanding of mechanisms driving tumor growth and the reasons why patients ultimately die of cancer. It is now appreciated that interactions between the tumor and surrounding non-tumor (sometimes referred to as host) cells play critical roles in mortality as well as tumor progression, but much remains unknown about the underlying molecular mechanisms, especially those that act beyond the tumor microenvironment. *Drosophila* has a track record of high-impact discoveries about cell-autonomous growth regulation, and is well-suited to now probe mysteries of tumor–host interactions. Here we review current knowledge about how fly tumors interact with microenvironmental stroma, circulating innate immune cells, and distant organs to influence disease progression. We also discuss reciprocal regulation between tumors and host physiology, with a particular focus on paraneoplasias. The fly's simplicity along with the ability to study lethality directly provide an opportunity to shed new light on how cancer actually kills.

INTRODUCTION

A signature triumph of modern biology is deciphering the causes of cancer. From the isolation of tumour viruses to the discovery of oncogenes and tumour suppressor genes to contemporary cancer genomics, we now have a detailed understanding of the genetic, epigenetic and signalling changes that drive the malignant growth of mutant cells. Although these insights have strongly improved diagnosis and enabled development of rational therapies, their impact on patient mortality has been less than could be wished. Difficulties in drugging major oncogenic drivers, along with tumour heterogeneity and the development of chemotherapeutic resistance, all impede treatment of patients through strategies that seek to prevent or reverse tumour growth.

Progress has also been limited by a historical focus on cell-autonomous characteristics of tumours. It is now clear that cancer, as a disease, emerges as a cellular and molecular dialogue between genetically altered tumour cells and genetically wild-type surrounding cells in a patient's body. For example, four of the ten features highlighted in the 2011 update to the seminal review 'Hallmarks of Cancer' explicitly concern interactions between tumour and normal tissue [74]. These hallmarks encompass activation of invasion and metastasis, induction of angiogenesis, tumour-promoting inflammation and avoidance of immune destruction. They reflect critical mechanisms through which local interactions in the tumour microenvironment (TME) promote or restrain growth and dissemination.

A hallmark still awaiting recognition is cancer's ability to induce systemic shifts of patient physiology. Such effects, in which the tumour can cause strong alterations in the activity of distant organs, can be grouped together as 'paraneoplastic syndromes' [119]. Although underappreciated, paraneoplasias have severe pathological consequences and are important drivers of mortality for numerous cancers. The best-known paraneoplasia is cancer cachexia, the wasting of muscle and adipose tissue that accompanies most advanced cancer cases, which is thought to drive more than 20% of patient deaths [120,202,203]. The mode of action of cachexia and many other paraneoplastic syndromes remains opaque, so there is much to be learned about the mechanisms through which tumours compromise the organ systems of patients to induce lethality.

The challenges of studying interactions between the tumour and the many tissues of a patient are exponentially more complex than those of studying tumour-autonomous growth regulation alone. Yet the pay-off in terms of therapeutic strategies that could avoid obstacles faced by tumour-targeted approaches may be immense. To achieve this goal, simple model organisms have great potential to reveal underlying conceptual principles as well as specific mediators. Here, we review current knowledge of tumour–host interactions in *Drosophila*, discuss how the fly system can uncover conserved molecular mechanisms and consider what these reveal about how animal bodies cope with the presence of malignant growth.

We begin by laying out the case for using the fly to investigate this important question. In the following sections, for key tumour–host interactions seen in human patients, we describe what is known about the comparable interaction in *Drosophila*. We adopt vertebrate terminology for fly genes, proteins and cell types where fair analogies can be made. We first discuss the tumour's juxtracrine and paracrine interactions with its immediate neighbours. We then consider fly models of metastasis, before moving on to systemic changes induced by long-distance tumour–host

signalling and reflecting on the mechanisms by which tumours kill hosts. We close with a perspective on how the tumour–host studies possible in the fly can complement and enhance knowledge from traditional mammalian systems, particularly by highlighting the breadth of host responses to cancer.

Investigating fly tumor-host interactions

To probe mysteries about how tumours interact with normal tissues to promote disease progression and kill patients, we will need to study animal models. Vertebrates, with nearly identical tissue repertoire, endocrine signalling and metabolic networks, have obvious advantages for these studies. But there are also disadvantages. For mice, high animal care costs create a barrier to large-scale population cohorts as well as genetic screens. An average lifespan of ~28 months discourages longitudinal experiments that reflect the course of human disease. Additionally, ethical and regulatory considerations usually prevent analysis of mortality as a study end point; instead, tumour size is taken as a proxy, resulting in a focus on tumour growth rather than lethality. Zebrafish share regulatory constraints around vertebrate animal suffering with mice, and have a comparably long lifespan. However, fish often tolerate tumours better than mice, and form an appealing substitute for several other reasons; some work has begun to leverage these advantages [204,205].

In this Review, we consider a simple alternative for investigating how tumours kill animal hosts: using the fruit fly *Drosophila*. Although it lacks central features of mammalian anatomy relevant to cancer, such as a closed circulatory system and an adaptive immune system, the fly shows remarkable conservation with humans not just of genes and molecular mechanisms but also of functional organs and organismal physiology (**Figure 1**) [206,207]. With an average lifespan of ~7 weeks and no concerns that necessitate pre-mortem sacrifice, *Drosophila* is well suited for explicit studies of mortality itself. *Drosophila* also has an unparalleled genetic toolkit that can be utilized in rapid experiments with high numbers of animals for low cost. This includes techniques to simultaneously and independently manipulate both tumour and host tissue genetics, leveraging publicly available reagents that allow analysis of both loss and gain of function on a genome-wide scale. Moreover, the wealth of knowledge about normal *Drosophila* physiology, metabolism, immunity and inter-organ communication provides a solid basis for investigations of pathological conditions. The fly is thus poised to face the challenge of studying the entire suite of local and systemic interactions that a tumour can induce throughout the body.

Drosophila has a long track record of seminal contributions to cancer biology, including elucidating the mechanisms of oncogenic signalling pathways such as epidermal growth factor receptor (EGFR), WNT, Hedgehog (HH), Notch and Hippo. These pathways are among those dysregulated in fly tumours, about which more information is provided in **Box 1**. A bioinformatic search reveals that 90% of genes identified by The Cancer Genome Atlas to drive human cancer have an orthologue in the fly (**Supplementary Table 1**) [208,209]. Although the precise genetic constituency of fly tumours generally differs from those of humans, the transformed phenotype — unrestrained and invasive growth of disorganized, immortalized cells — can be quite similar [51,210–212]. Using genetically engineered fly models (GEFMs), tumours can be generated in vivo in epithelial tissues of the larva (imaginal discs) or adult stage. Tumours can also be easily transplanted into the abdomen of adult hosts [149,201]; we will refer to this as the allograft model (**Box 2**). Most studies of fly tumour–host interactions analyse ‘neoplastic’ tumours that arise in epithelial organs, which are roughly analogous to mammalian malignant carcinomas. A popular variation co-expresses oncogenic Ras with mutation of a fly neoplastic tumour suppressor gene such as *scribble* (*scrib*) to enhance malignancy [213], which can be termed a ‘cooperative neoplastic’ model. For simplicity, we will refer to neoplastic and cooperative neoplastic models as ‘tumours’,

acknowledging that there are many different tumour models in the fly (for example, Supplementary Table 2) and that results with one may not generalize to all.

Local interactions regulating fly tumors

As the transformed mutant cells that form a tumour overproliferate, they engage in a bevy of signalling exchanges with surrounding normal cells, altering the functions of the normal cells in ways that fuel tumour growth and progression. These local interactions take place in the TME, a specialized area containing a plethora of genotypically wild-type cell types from the tissue in which the cancer arose. The physical and signalling characteristics of the TME differ substantially from those seen in healthy tissue; it is now appreciated that the TME is not a passive bystander but, instead, an active contributor to malignancy through intercommunication with tumour cells and more distant organs [63,214].

In human patients with cancer, beyond various infiltrating adaptive immune cells, several TME cell types are prominent in supporting tumour growth[215,216]. One type is the normal epithelial cells that neighbour the nascent tumour. A second cell type is cancer-associated fibroblasts, which can enhance the proliferative properties of transformed cells and create pro-metastatic or immunosuppressive environments as well. A third type is endothelial cells, which are recruited to transformed cells in the process of neovascularization to induce a tumour-directed blood supply that also provides a route to tumour dissemination. Each of these heterotypic interactions has an analogy in *Drosophila* tumour progression (**Figure 2**). The influence of the non-cellular TME — the extracellular matrix — on fly tumour progression has yet to be investigated, and interactions with innate immune cells in the stroma are treated separately below.

Neighboring epithelial cells

The most immediate interaction that transformed cells have in their microenvironment is with the surrounding non-transformed epithelium, which can profoundly influence tumour progression. In fly cancer models where adult intestinal stem cells (ISCs) are transformed, these interactions induce mitogenic paracrine signalling loops akin to those that occur between tumour and stroma [68,217]. As an initially hyperplastic ISC-derived tumour grows, it physically displaces differentiated gut cells from the basement membrane, triggering a damage response from wild-type tissue. This response invokes signalling through interleukin 6 (IL-6)-like cytokines (the Unpaired (Upd) family in fly) to replace the displaced cells, which would normally be a self-limited process that ceases when homeostasis is restored. However, transformed ISCs make this process chronic, initiating a feedforward loop that accelerates tumour growth.

Tumours can also signal one-way to accelerate growth of neighbouring wild-type cells. In one class of neoplastic disc tumours, defects in endocytosis trigger inappropriate Notch cleavage; this ligand-independent Notch activation then drives production of Upd/IL-6 to stimulate proliferation of surrounding host cells (**Figure 2a**) [218,219]. A similar circuit is triggered by tumorigenic loss of epigenetic silencing mediated by the Polycomb repressive complex [220–222]. In all these cases, heterotypic signalling between genetically normal and transformed epithelia promote proliferative accumulation of tissue mass.

Importantly, local interactions with wild-type neighbours can prevent tumour growth instead of stimulating it. Fly imaginal epithelia display a remarkable ability to detect and then

eliminate small groups of cells with malignant genotypes, particularly those bearing mutations in scrib-class genes (that is, genes with functions similar to *scrib* such as *lethal giant larvae* (*lgl*) and *discs-large* (*dlg*)). This tumour-suppressive ability is part of the larger phenomenon of cell competition through which cells with one genotype influence the proliferation of their immediate neighbours. This fascinating aspect of tumour–host interactions has been amply reviewed recently [223–226], and is recognized to play an important role in mammalian epithelial homeostasis and tumour development.

The mechanisms by which neoplastic *scrib* mutant cells are eliminated are molecularly distinct from those underlying competitive removal of cells with altered levels of growth regulators such as Myc or ribosomal proteins. The cue for the former is the disruption of cell polarity within the epithelium, which triggers JUN amino-terminal kinase (JNK) signalling and a resultant apoptotic programme [225]. Host cell factors required for elimination of neoplastic neighbours include the tumour necrosis factor (TNF) homologue (Eiger (Egr) in the fly) and a juxtracrine signalling molecule called Sas; both are latent in normal epithelia but appear to be activated by the architectural changes of mutant cells alone (**Figure 2b**) [94,227–229]. TNF signalling through JNK is part of a normal process that can restore epithelial homeostasis after physical damage and is also used to eliminate potentially malignant cells upon their onset. If this process fails, or if additional mutations in the mutant cells (for example, activated *Ras* in the cooperative models) allow them to evade elimination, sustained tumour growth can ensue.

Mesenchymal stroma

Flies do not have fibroblasts or connective tissue but they do contain mesenchymal cells that develop in close association with epithelia. An example is the larval myoblasts, muscle precursors that lie over regions of the wing imaginal disc. In a cooperative tumour model that generates neoplastic wing tissue through EGFR overexpression and depletion of the chromatin regulator Pipsqueak, a significant portion of the growing mass was composed of genetically normal myoblasts [230]. The transformed epithelial cells induced proliferation of adjacent myoblasts via transforming growth factor-β (TGFβ) and Notch signalling (**Figure 2c**) [230,231]. Interestingly, the overproliferating mesenchymal cells were reciprocally required to drive epithelial growth and tumour progression, in a paracrine signalling loop reminiscent of that seen with mammalian cancer-associated fibroblasts [63]. However, myoblasts are not required for epithelial growth in several other neoplastic fly models[232], so cancer-associated fibroblast-like interactions may not be a general feature of fly tumours.

Oxygen-supplying tubules

Flies are small creatures without thick tissues, and their cellular need for oxygen is met by diffusion within an open circulatory system rather than blood cells carrying oxygen through vasculature. Internal oxygenation in flies is passively supplied through epithelial tubes called trachea (**Figure 1**). Trachea form an elaborate network whose branches can ramify directly on cells but also transfer oxygen into the circulatory fluid called haemolymph [72]. The normal pattern

of tracheal branching is strongly influenced by fibroblast growth factor (FGF) homologue signalling via a process equivalent to mammalian angiogenesis [233].

Similar to growing human tumours, large neoplastic cell masses in the fly can show signatures of hypoxia and its associated response, stabilizing hypoxia-inducible factor 1α (HIF1α) homologues that induce metabolic reprogramming (discussed below). Downstream of HIF1α signalling, the fly FGF ligand Branchless (Bnl) is upregulated within tumours, inducing three changes in the host that echo vertebrate neovascularization [234–238]. First, endogenous trachea show ectopic branching towards tumour cells (**Figure 2d**). Second, some tumour cells themselves develop tube-like architecture and tracheal gene expression, associating with normal tracheal cells in a phenomenon reminiscent of vascular mimicry [239]. Last, tumour cells in several models have been visualized migrating along trachea, perhaps using them as a route to dissemination. Although the requirement of neo-tracheation in tumour growth remains to be tested, these phenotypes are suggestive of thematic interactions between the fly tumour and the host oxygen supply that parallel those seen in mammals [240].

Fly tumors and the immune system

In addition to sessile cells in the stroma, tumours engage in extensive interactions with circulating immune cells. We will highlight here the role of macrophages, innate immune cells that play critical roles in tissue repair as well as the initial response to pathogens. In human patients, some tumour-associated macrophages (TAMs) can have tumour-promoting activity, including facilitating invasion and angiogenesis, whereas others are tumour-suppressive, assisting the immune recognition and subsequent execution of transformed cells [241]. The revolution in tumour immunology and recognition of the immune system's importance in shaping cancer progression raises the interesting question of whether these phenomena have a deep evolutionary origin. Flies do not have an adaptive immune system but have a well-developed innate immune system that has been central to understanding conserved mechanisms of pathogen detection [242]. Exciting recent studies identify pro-oncogenic effects of fly innate immune cells that involve inflammatory signalling, as well as tumour-restricting activity by signalling to systemic immune effectors.

Cellular immune system

The cellular arm of the *Drosophila* immune system is composed of haemocytes. Haemocytes share functional properties with the vertebrate myeloid lineage, and may have a common evolutionary origin [168,243]. The bulk of haemocytes (95%) are a cell type called plasmatocytes, which are analogous to mammalian macrophages; we will refer to them hereafter as 'macrophages'. In the fly, macrophages are attracted to wounds, where they actively phagocytose dying cells and microbial invaders. In the immune response, they release antimicrobial peptides as well as cytokines including fly homologues of IL-6, TNF and ligands for the Toll innate immune pathway, which is homologous to mammalian TLR signalling.

Haematopoiesis takes place in the embryo and the larvae, but is absent in the adult[244]. As in mammals, many macrophages are sessile and reside in specific tissues, whereas others circulate, pumped through the haemolymph by the fly heart. Macrophages actively migrate to or are passively captured by sites of tissue damage, regulated by cues that include H_2O_2 production or basement membrane destruction. There, in addition to phagocytosis, macrophages secrete factors that form an initial 'soft' clot; the clotting cascade also generates reactive oxygen species (ROS) [155]. Together, cytokines and ROS signal to remote tissues such as the fat body and trachea, activating the humoral arm of the immune system to secrete antimicrobial peptides in a systemic defence response. Thus, as in mammalian innate immunity, fly macrophages survey and defend the body from microbial threats and tissue damage. As we now describe, they can also detect and respond to the 'altered self' of malignantly transformed cells.

Interactions between fly tumours and macrophages (**Figure 2f**) were first documented in 2008. In a pioneering paper, Pastor-Pareja et al. [93] demonstrated that macrophages are recruited to neoplastic imaginal discs in larvae. The presence of fly TAMs is due to their association with sites of basement membrane degradation, as well as systemic stimulation of macrophage proliferation by Upd/IL-6 signals from the tumour and, perhaps, other tissues. Strikingly, either elimination of all macrophages or blocking Upd/IL-6 signalling within them resulted in a significant increase in

tumour burden within the animal. This provided the first evidence for immune restraint of tumours in invertebrates akin to that known in vertebrates.

Building on this study, Cordero et al. found that TAMs in disc tumours upregulated expression of the fly TNF, Egr [94]. Egr/TNF is a potent activator of JNK signalling in recipient cells, and when released from TAMs it binds directly to transformed but not wild-type disc tissue. In simple neoplastic tumours, it induces apoptosis, limiting the tumour burden. On the other hand, when neoplastic cells co-express oncogenic Ras^{V12}, cell death is blocked and Egr/TNF instead promotes phenotypes such as upregulation of matrix metalloproteinases (MMPs) that drive invasion. A further consequence of TNF signalling in both tumour types is the upregulation of Pvf1, homologous to vascular endothelial growth factor (VEGF), which elevates macrophage numbers in circulation. Whether due to mobilization of sessile cells or stimulated proliferation, this increase of Egr/TNF-expressing TAMs creates a feedforward loop that enhances the tumour's response. Together, these data show that fly TAMs, like their mammalian counterparts, can suppress tumours in some contexts but in others promote tumour growth. It should be noted that a second study did not find a role for either TAMs or TNF signalling in growth of a comparable tumour type [232]; the reasons for this discrepancy remain to be determined.

Humoral immune system

The number of macrophages that associate with fly tumours is not large, suggesting that they might restrict tumour growth by calling on additional partners. Interestingly, TAMs not only upregulate Egr/TNF, but also Spaetzle (Spz), the major ligand for the fly Toll signalling pathway [95]. Spz secretion into circulation then triggers activation of the humoral arm of the immune system. Both Toll and the IMD pathway, a second innate immune signalling pathway driven by nuclear factor-κB (NF-κB) homologues, are activated in peripheral tissues of tumour-bearing larvae, and manipulations that block Toll or IMD signalling prevent the tumour-suppressive activity of macrophages.

A further paper revealed that this fly immune response kills tumour cells by an unexpected mechanism [245] (**Figure 3a**). Death is not due to direct induction of apoptotic signalling by TAM-derived Egr/TNF; instead, TNF signalling promotes tumour cell exposure of the lipid phosphatidylserine. Phosphatidylserine generally marks cells for death in several ways, including serving as a recognition signal for macrophages. In this case, it enhances binding of a circulating antimicrobial peptide called Defensin. Defensin is normally secreted during the Toll-dependent humoral response to microbial infection. In tumour-bearing larvae, TAM stimulation of immune-responsive organs produces Defensin and other antimicrobial peptides, although only Defensin seems to bind specifically to tumour cells. Interestingly, the authors provide evidence that Defensin exhibits direct killing activity on tumour cells. This coordinated link between the cellular and humoral arms of the innate immune system allows a microbial defence effector to eliminate the 'altered self' of transformed cells. Interestingly, humans produce Defensin orthologues that also bind phosphatidylserine-enriched tumour cells and exhibit oncolytic properties on several cancer cell lines [246].

Key to all of these responses is the ability of TAMs to be recruited to a tumour. Evidence for fly macrophage adhesive receptor roles comparable with mammalian innate immune recognition of tumours [247] does not yet exist, but several recruitment mechanisms lie downstream of JNK signalling. JNK transcriptionally upregulates *Mmp1* in all neoplastic fly tumour models [152], and basement membrane damage by MMPs may be the proximate cue detected by haemocytes, as it is sufficient to recruit them to imaginal discs [93,248]. JNK in tumour cells also stimulates ROS production through the plasma membrane-localized NADPH oxidase; this process is dependent on activation of initiator caspases, and is enhanced if activated Ras is expressed in tumours to block the execution of the apoptotic programme [249]. ROS is required alongside JNK for haemocyte recruitment, perhaps by stimulating MMP enzymatic activity. Finally, JNK activation may also trigger cells to produce other secreted signals that attract haemocytes. Once attracted to the tumour, the transformation into TAMs also seems to involve ROS, which induce acquisition of a distinct morphology and upregulation of Egr/TNF. Such fly studies may shed light on the important roles that ROS play in mammalian antitumour immunity [250].

The extensive interactions described above raise the question of whether paraneoplastic impacts on other immune cell functions exist. Macrophages and a second cell type, called crystal cells, play critical roles in the clotting cascade [155], but whether fly tumours affect haemostasis, as in human paraneoplastic coagulopathies such as Trousseau's syndrome [251], has not been investigated. Similarly, macrophages are important mediators of the immune response, but it is unknown whether tumour-initiated and microbial-initiated activation of these pathways synergize or interfere with each other. Both of these questions are ripe for future study.

Metastasis in *Drosophila*

In humans, the tumour–host interaction most strongly associated with lethality is metastasis. Metastasis in vertebrates is a multistep process that involves not only exit from the primary tumour but both entry into and exit from the vasculature, as well as survival and proliferation at a secondary site. The open circulatory system of *Drosophila* is not optimal for studying vascular intravasation and extravasation, although fly immune cells do traverse other vessel-like structures [252]. Nevertheless, fly tumour cells show clear invasive behaviours [253,254]. Tumour cells lose polarized architecture, expand their actin cytoskeletal network and acquire mesenchymal morphology with pro-migratory characteristics. As mentioned above, concomitant upregulation of MMPs degrades the basement membrane (**Figure 2e**) to allow exit from the tissue of origin. For the purposes of this Review, we refer to movement into neighbouring tissues as invasion, and reserve metastasis for the presence of tumour-derived cells at a site that is not contiguous with the tissue of origin.

Invasive behaviour of fly tumours was evident in early allograft studies, where neural-derived tumours were noted to envelop and penetrate adult host organs [255]. Epithelial-derived tumours, by contrast, were less invasive and predominantly formed compact independent growths. Nevertheless, in GEFM larvae containing cooperative neoplastic eye discs, tumour cells consistently move into the neighbouring ventral nerve cord [213]. The dependence of this phenotype on MMP activity [100,101] is consistent with it being true invasion.

Histological tracing clearly documents cell dispersal beyond the primary tumour. In neural tumours transplanted into adult abdomens, genetically labelled cells can be later found in distant organs, including sites that required crossing a host basement membrane [256]. Dispersal required MMP activity and involved a handful of cells [106]; these 'micrometastases' of 2–100 cells are typical of several types of neural tumour [201]. The small size of such secondary tumours compared with the robust growth of the primary may suggest tumour dormancy. Long-distance dissemination can also be seen in larvae bearing cooperative neoplastic eye discs [213]. Although this phenotype can be confounded by 'leaky' labelling [257], a more recent study indicated that bona fide cellular migration from the primary tumour occurs and shows organotropic characteristics, as tumour cells travel to certain tissues while avoiding others in a manner dependent on a host-derived signal[236]. However, allografts of cooperative neoplastic discs into adults result only in very rare metastases (J.K., D.B. and A. Figueroa-Clarevega, unpublished data).

A robust system to study invasion and dispersal is emerging in GEFMs that manipulate the adult gut. When targeted to the differentiating epithelial cells of the hindgut, transgenic models that mimic multi-hit genetic constituencies of human colorectal cancers yield cells that frequently disseminate into the body cavity, driven by oncogenic Ras [237]. In a model using midgut ISCs, Ras activation alone is sufficient to drive invasion and dissemination [258]. A recent paper shows that ISC fly tumours carrying three genetic changes — activation of Ras, mutation of the colorectal tumour suppressor gene orthologue Apc and overexpression of the epithelial–mesenchymal transition regulator *snail* — can form large metastases at secondary sites [259]. Although rare (~1%), these metastases are amenable to analysis; the animals also frequently generate circulating tumour cells in the haemolymph. Advances in both short-term and long-term in vivo live imaging [260,261] promise to shed further light on metastasis in these systems.

To summarize, invasion into the local microenvironment is a prominent feature of fly neoplastic tumours. Dispersal is more variable and depends on the tissue origin and context; it is high in neural tumour allografts, intermediate in cooperative neoplastic larvae and low but consistent in adult gut GEFMs. In allografted disc tumours, metastasis is sufficiently rare that effects remote from the primary tumour are much more likely to be mediated by mobile cells or secreted bioactive molecules. We now turn to these long-distance tumour–host interactions.

Physiological changes induced by fly tumours

Beyond the TME, cancers can induce profound systemic shifts that manifest in distant tissues throughout the organism. These changes are collectively called paraneoplastic syndromes, and although different tumours have propensities for particular effects, there is a set that is commonly induced irrespective of the tissue of origin or genetic constituency. Paraneoplastic syndromes are not thought to contribute to oncogenic growth or metastasis per se, and are thus not specifically selected for during tumour evolution [119,144]. Instead, they are regarded as epiphenomena that are caused by tumour secretion of diffusible molecules with the ability to impact remote organs. Such secreted factors have profound effects in flies as well as humans [144], and we coin the term 'oncokine' to refer to these as well as signalling molecules that act locally in the TME. Table 1 lists currently known fly oncokines, their human orthologues, and their influence on host physiology; in the following sections, we describe several examples of systemic oncokine signalling.

Many paraneoplastic syndromes have been documented in human patients with cancer. Some of these, such as hypercalcaemia, the syndrome of inappropriate antidiuretic hormone secretion, Cushing's syndrome (involving excess cortisol production) and paraneoplastic autoimmune reactions, involve signalling axes or organ systems that are specific to mammals and will not be considered here. Several others that impact tissues with fly analogues, such as paraneoplastic coagulopathies and remote immunosuppressive effects, have yet to be investigated in *Drosophila*. **Figure 3** provides a summary of fly paraneoplasias. We first discuss fly studies of cachexia, anorexia and related research linking tumour growth to host metabolic changes. We then consider other fly paraneoplastic syndromes whose relation to human morbidity is not yet clear.

Tissue wasting

Recent years have seen the development of several fly models of cancer cachexia. Cachexia is a complex phenomenon, but a defining criterion is that it involves tumour-induced systemic tissue wasting that is not due to insufficient nutritional intake. Despite its high prevalence and lethal impact, cachexia is a poorly understood syndrome with little available therapeutic amelioration [120,202,203]. A wealth of experimental studies in rodents have identified secreted factors that have cachectogenic properties, but there is often poor correlation with factors detectable in patients with the symptoms of cachexia. This gap increases the appeal of novel models for study.

Cachectic phenotypes in the fly were first demonstrated in two adult tumour models: in allografts of neoplastic discs and in a GEFM when ISCs overexpress an active form of the Hippo pathway transcription factor Yorkie (Yki), orthologous to mammalian YAP and TAZ [128,129]. As the tumours grow, several host tissues display clear wasting (**Figure 3b, c**). These include the fat body, in which triglyceride levels are reduced, and muscle, in which mitochondria degenerate with associated reductions in energy production and motor activity. Such phenotypes are also seen when animals are starved, but careful analysis showed that — even in the presence of gut tumours — the host flies feed normally and do not display markers of starvation. Thus, the wasting is not due to anorexia (reduced nutrient consumption) but, instead, cachexia (failure of ingested nutrients to support metabolic homeostasis).

How do fly tumours induce wasting of distant organs? Remarkably, although the models involve different driver genes that transform different cell types, wasting in both depends on a shared target gene that is strongly upregulated in the tumour tissue. This target is *ImpL2*, which encodes a secreted antagonist of insulin signalling with functions analogous to the insulin growth factor binding protein (IGFBP) family in vertebrates [130]. Insulin signalling is a major endocrine regulator of biosynthesis, metabolism and cell survival in all animals, and is activated by ligands that in flies are called insulin-like peptides (ILPs). ImpL2/IGFBP is released into the haemolymph, where it binds to and sequesters circulating ILPs; expression of ImpL2/IGFBP alone in the absence of a tumour is sufficient to cause peripheral wasting. Accordingly, tissues that waste when a tumour is present show reduced insulin signalling, with a hyperglycaemic state throughout the animal. Most importantly, depletion of ImpL2/IGFBP in the tumour itself leads to a rescue of wasting, with associated improvements in peripheral organ function. This rescue is not complete, and a recent study identified Pvf1, a VEGF family ligand produced in the ISC model, as an additional, independent cachetogenic factor [148]. Another group using a larval cooperative tumour model enhanced by a high-sugar diet (see below) identified Bnl/FGF, rather than ImpL2/IGFBP or Pvf1/VEGF, which promotes muscle wasting in this context [262]. As with human patients, fly cancer cachexia can be driven by several different endocrine signalling axes.

How do the mechanisms that drive tumour-induced wasting in flies compare with human cancer cachexia? Cachexia is a heterogeneous condition, likely induced in different cancer types by multiple distinct pathways. One mechanism frequently implicated in mammals is upregulation of E3 ubiquitin ligases to drive protein degradation [202]; this has not been documented in fly models. Nevertheless, systemic insulin resistance, akin to that induced by ImpL2/IGFBP in the fly models, is seen in some patients with cancer, and evidence supports a role for reduced insulin signalling in several rodent cancer cachexia models [263,264]. Moreover, certain human tumours induce IGFBP3, and IGFBP3 can directly induce wasting of cultured muscle cells [265]. Finally, in a mouse cancer cachexia model, muscle-specific ERK signalling akin to that demonstrated in tumour-bearing flies [148] was shown to drive atrophy associated with increased proteolysis [266]. Thus, discovery-based approaches in the fly can provide new leads for effectors of cachexia and other paraneoplasias.

Anorexia

Weight loss in patients with cancer is often driven not only by cachexia but also by accompanying anorexia [267]. Although clinically intertwined, anorexia is distinct from cachexia as it results from reduced food intake due to behavioural changes in appetite. Our understanding of human cancer-associated anorexia is complicated by the impacts of concomitant therapies, although rodent models implicate imbalances of appetite-regulating neuropeptides as well as changes in central neural regions such as the hypothalamus [268]. A recent paper has used *Drosophila* to identify an oncokine that acts directly on neurons to reduce feeding behaviour (**Figure 3e**) [269]. In the fly model, hyperactivation of Yki in adult eye cells induces a secreted protein called insulin-like peptide 8 (Ilp8), previously discovered as the oncokine responsible for developmental delay of tumour-bearing larvae (see below). Despite its name, Ilp8 resembles relaxins more closely than insulin-like growth factors, and signals through Lgr3, a homologue of LGR7 and LGR8 receptors (also known as relaxin receptors 1 and 2, respectively) found in a small set of central brain neurons

[270–273]. Ilp8 binding to Lgr3 suppresses the fly's food intake by cell-autonomously increasing production of an anorexigenic hormone homologous to mammalian NUCB2 (also known as Nesfatin) while decreasing production of the orexigenic neuropeptide Y (NPY) hormone. The authors show that the same signalling axis exists in mammalian hypothalamic cells: relaxin-like peptide INSL3 binds to LGR8 to upregulate NUCB2 and downregulate NPY. Moreover, they provide strong evidence that several implanted mouse tumours induce anorexia using this INSL3-dependent circuit, and show a correlation between anorectic severity and INSL3 serum levels in a small cohort of human patients with pancreatic cancer. Thus, by leveraging simple experiments in the fly, this work revealed a mechanism for paraneoplastic anorexia through altering appetite-regulating brain hormones.

Autophagy and amino acid import

All tumours require substantial anabolic input to enable their inappropriate growth, arousing debate around whether transformed cells actively solicit nutrients from normal tissues [202,203,274]. Fly tumour models have revealed that, indeed, some metabolic building blocks come not from ingestion but instead from catabolic processes in the host. Prominent among these is autophagy. Tumour cells in cooperative neoplastic larvae induce strong autophagic processes in wild-type neighbouring epithelial cells as well as distant tissues (**Figure 3d**), and blocking autophagy in these cells suppresses growth of the tumour itself [275]. The signalling pathway responsible for non-autonomous autophagic induction is not yet defined, but requires autocrine Upd/IL-6 signalling in tumour cells and, perhaps, local generation of ROS. Pharmacological blockade of autophagy, as well as depletion of a specific amino acid transporter in tumour cells, is sufficient to block tumour growth, although neither manipulation impacts the growth of wild-type cells. These results are consistent with a model in which tumour cells signal to drive autophagy in the TME, importing the liberated amino acids to fuel their own growth. Thus, autophagy in near or distant host cells, alongside the well-documented role for tumour-intrinsic autophagy [276], can generate nutrients used for tumour growth.

A requirement for host tissue-derived nutrients has been further demonstrated in a second fly tumour model, in which modest benign growth of larval imaginal disc cells expressing activated *Ras* and *Src* oncogenes is enhanced by a high-sugar diet, promoting full cooperative neoplastic transformation [235]. As mentioned above, these tumours induce wasting of host muscles in a Bnl/FGF-dependent manner, and the authors find that free circulating amino acids are elevated coordinately with muscle breakdown [262]. Intriguingly, in the high-sugar diet, tumour cells upregulate several amino acid transporters, one of which increases import of proline. Blocking this transporter activity either genetically or pharmacologically reduced tumour growth, whereas feeding the larva extra dietary proline was sufficient to increase tumour size and malignancy in the absence of the high-sugar diet. Together, these studies support the idea that, in some cases, tumour-induced host catabolic processes can be not just epiphenomena but, instead, active contributors to tumour growth. They also illustrate how the fly system, with sophisticated genetic manipulation of different tissues, can resolve questions that are challenging to approach in vertebrate models.

Other fly paraneoplasias

Fly tumour models display several paraneoplasias that do not yet have clear parallels in humans. Indeed, the first tumour suppressor mutant was identified because of its unusual systemic phenotype. Animals lacking the *lgl* gene are incapable of entering pupation [277,278]. During the subsequently prolonged larval stage, the animal becomes strikingly enlarged and filled with fluid. *lgl* mutant larvae were later found to develop neoplastic imaginal discs and brains [279], raising the question of whether developmental delay and fluid retention were due to cell-autonomous activities of the *lgl* gene product or, instead, to tumour–host interactions. Genetically, mosaic larvae demonstrate that the latter is the case: a single pair of neoplastic discs in an otherwise wild-type animal is sufficient to induce both phenotypes [280]. Interestingly, the oedema-like 'bloating syndrome' is also seen in allograft as well as GEFM adult tumour models, demonstrating a consistent and dramatic systemic perturbation in fluid balance (**Figure 3f**) [129,149,281].

Developmental delay and bloating are separable paraneoplasias. One signalling molecule responsible for the former is Ilp8, discussed above as a regulator of cancer-associated anorexia. *Ilp8* is the most highly upregulated gene in neoplastic fly tumours [282,283], and tumour-derived Ilp8 binds to Lgr3 in neurons to regulate a circuit that releases hormones triggering the larva to pupa transition (**Figure 3e**). When produced and secreted into the haemolymph, Ilp8 binds to Lgr3 in the brain and causes neuroendocrine changes that delay pupation until Ilp8 levels drop. Ilp8 expression in imaginal discs declines as the animal enters the L3 larval stage, but can be strongly upregulated by JNK and Hippo signalling, for instance in response to wounding or cell stress. This signalling axis thus normally couples tissue damage to the neuroendocrine system in order to allow time for tissue repair before metamorphosis. However, like an unhealing wound, tumours with chronic activation of JNK and Hippo signalling hijack this pathway and prevent normal maturation. Interestingly, Upd/IL-6 production in the imaginal disc shows similar JNK and Hippo-dependent regulation [54], and a recent paper suggests that tumour-derived Upd/IL-6 acts directly on neuroendocrine cells to promote developmental delay, enhancing the effect of Ilp8 [284].

In contrast to developmental delay, the mechanism by which tumours induce fluid retention is not yet understood. Bloating correlates with wasting phenotypes in ISC tumour models, and one proposal is that fluid retention results from osmotic compensation for elevated haemolymph sugar levels, induced by systemic insulin resistance (see below) [129,148]. Alternative possibilities also exist. Both solute balance and fluid secretion are ultimately regulated by the Malpighian tubules, the *Drosophila* kidney analogue [285]. In tumour-free adults, bloating phenotypes can result from developmental defects of the tubules [182,286], from defective signalling of a diuretic hormone that regulates tubule function [287] or from defects in ion channels in the tubule cells themselves [288]. Whether tumours interrupt any of these endogenous fluid-balancing circuits merits investigation.

Host impacts on tumour progression

So far, we have discussed how the tumour impacts host tissues. Conversely, systemic physiology is a critical regulator of disease progression in cancer. Indeed, amongst the many challenges of studying human patients with cancer, the complex interactions between individual environment, comorbid conditions and tumour genotype loom large. Obesity, in particular, is a major risk factor for cancer, through mechanisms that include inflammation; diet may also have more direct interfaces with tumour metabolism [289]. We now describe what is known in the fly about the impact of these host environmental parameters on tumour progression.

The influence of diet on *Drosophila* tumours differs widely with the model used. For example, the mild overgrowth of mitotic clones mutant for the tumour suppressors *Pten, Tsc1* or *Tsc2* in imaginal discs is strongly enhanced in nutrient-deprived conditions [290]. However, in *Pten* mutants this is due to increased cell proliferation, whereas with *Tsc1* or *Tsc2* mutants it is due to cell hypertrophy. Diet can also affect the stability of the fly homeodomain interacting protein kinase (Hipk), which has oncogenic properties [291]. In a study mentioned earlier, diet had a dramatic impact in a cooperative oncogenesis model combining *Ras* and *Src* hyperactivation [235]. Under standard conditions, most eye disc cells with these genetic changes die. When the host larvae are fed a high-sugar diet, mutant cells undergo full neoplastic transformation including dispersal to secondary sites. In wild-type host tissue, a high-sugar diet induces hyperglycaemia, hyperinsulinemia and insulin resistance. In the transformed cells, it creates a feedforward loop involving Wnt signalling that results in upregulation of the insulin receptor [292], allowing the cells to evade insulin resistance and express the full malignant phenotype.

Evasion of insulin resistance may be a frequent feature of *Drosophila* tumours. For instance, neoplastic clones can escape competitive elimination and form tumours if systemic insulin levels are elevated [293]. When reviewing fly cachexia above, we described how allografted and GEFM flies upregulate ImpL2/IGFBP, which blunts insulin signalling throughout the animal [128,129]. Tumours in these models nevertheless proliferate aggressively, although they remain dependent on PI3K activity [294]. How such cells decrease reliance on insulin for growth is not known, but fly tumours have a distinct metabolism, with aspects of the glycolytic Warburg effect seen in many human tumours [295–297]. Many fly tumours upregulate lactate dehydrogenase (LDH), perhaps through oncogenic ERK and PI3K signalling pathways as well as hypoxia. Moreover, in one model, LDH expression has been shown to drive the transition from hyperplasia to neoplasia [297]. Thus, diet and, particularly, metabolic dysfunction can enhance tumour progression in the fly.

To date, few studies have looked deeply at the effects of obesity on fly cancer models [298]. Our group has found that tumour-bearing flies on a high-fat diet show accelerated death (J.K. and D.B., unpublished data). Flies fed a high-fat diet are known to upregulate Upd/IL-6, a central inflammatory player implicated in many fly tumour phenotypes, suggesting one possible mechanism [299]. It is known that dysbiosis of commensal bacteria can fuel gut tumour production in flies [300,301], but there are few studies on the interface between infection and fly tumour progression [302,303] and none on the impact of the changing physiology of aged animals. All of these are feasible in the *Drosophila* system, where approaches described in sections above can untangle the role of tumour-autonomous, local and paraneoplastic effects.

Perspectives

We feel that the general field of tumour–host interactions is well poised to benefit from work in the reductionist system of *Drosophila*. Beyond molecules and mechanisms, fly studies are providing general insight into how an animal reacts to the growth of a pathological 'neo-organ'. For instance, the anti-neoplastic activities of neighbouring wild-type epithelial cells, circulating macrophages, humoral immune-induced factors and systemic cytokines make it clear that even in this short-lived invertebrate, evolution has selected for multiple potent mechanisms to prevent tumour formation. Moreover, fly data support the idea that tumour defence was an early evolved role for the innate immune system, rather than a later co-option of adaptive pathogen-fighting cells. Finally, similarities between the fly responses to injury and to cancer demonstrate that Dvorak's formulation of a tumour as a 'wound that does not heal', first used to describe the composition of tumour stroma [138], can also serve as an insightful guide to the evolutionary origin of the host response.

The emphasis in this Review on paraneoplasia reflects our belief that the growing interest in tumour–host interactions should expand well beyond the TME and embrace a view of cancer as a disease not just of mutant cells but of interacting physiological systems throughout the body. Approaching cancer as a multi-organ disease brings with it an intimidating complexity. Yet *Drosophila* research has a long and impactful track record in the 'discovery' phase of complex biological questions, when organizational principles have yet to emerge. Flies' superb experimental approaches, including forward genetic screens, have untangled phenomena that appeared unapproachably intricate, such as the molecular bases of development and pathways mediating intracellular communication [304–306]. Analogous genetic screens are conceivable for the tumour–host interactions described above, and high-throughput therapeutic screening as well as follow-up mechanism of action studies are also possible [307,308]. Whereas the example of cachexia shows the value of the fly for deciphering mechanisms of recognized paraneoplasias, this simple system also can be used to identify new and potentially conserved tumour–host interactions.

We have discussed mechanisms of short-range and long-range tumour–host interactions, yet the impacts of these interactions on host survival per se are seldom explicitly studied. Tumour growth and lethality are often treated as synonymous, but patients can also die with a relatively small tumour burden that does not impede an essential organ. Moreover, the ultimate cause of cancer death can often be unclear. In these situations, paraneoplastic alterations might play an important role, highlighting the value of a better understanding of how they actually promote lethality. It is not currently known why flies bearing tumours die (**Figure 3g**). Ageing flies, like other model organisms, often present with defects in intestinal permeability shortly before death [309]. However, comparable defects are not regularly seen in flies bearing tumour allografts (J.K. and D.B., unpublished data). Flies suffering from infection may die not only from pathogenic effects of the microorganism itself but also from the deleterious impact of the host immune response, such as prolonged inflammation [310]. The organ(s) whose failure is responsible for death of such infected flies, like tumour-bearing flies, remains unknown. Answering 'how cancer kills flies' appears achievable using existing tools and knowledge, and should shed light on what is truly the ultimate tumour–host interaction.

Just as fundamental cancer research focuses on mechanisms of tumour growth, most current therapies focus on limiting or reversing this growth. Radiotherapy, chemotherapy and immunotherapy all place a selective pressure directly on cancer cells. Given the genetic instability of human tumours, this pressure can lead to the emergence of resistant clones, which account for the high frequency of cancer recurrence after initial treatment success. Appreciating the scope of paraneoplastic syndromes and their fatal impacts suggests an alternative tactic. Mechanistic understanding of these syndromes could permit targeted therapeutics that interfere with the host side of the tumour–host dialogue. These are less likely to select for resistant variants; they would also — given that widely different cancers elicit a common handful of paraneoplastic syndromes — reduce the need to customize therapy to an exact tumour genotype.

An important paradigm in microbial pathogenesis makes the distinction between resistance and tolerance: in the former, the patient fights disease by actively reducing their microbial burden, whereas in the latter the patient instead endures by ameliorating the pathological effects of the infection [311]. This paradigm has recently been ported to cancer, and explored in *Drosophila* [312,313]. Given the success of fly models in revealing functionally relevant host responses to tumours, it is appealing to consider that the lessons learned could inspire host-directed therapeutic strategies for human patients. With molecular knowledge of paraneoplastic morbidity mechanisms, one can envision developing cancer therapies that focus on ‘tolerance’, promoting longer health and life even in the presence of a tumour. Such an approach would provide a distinct and appealing complement to the standard tumoricidal strategies being pursued today.

FIGURE 1

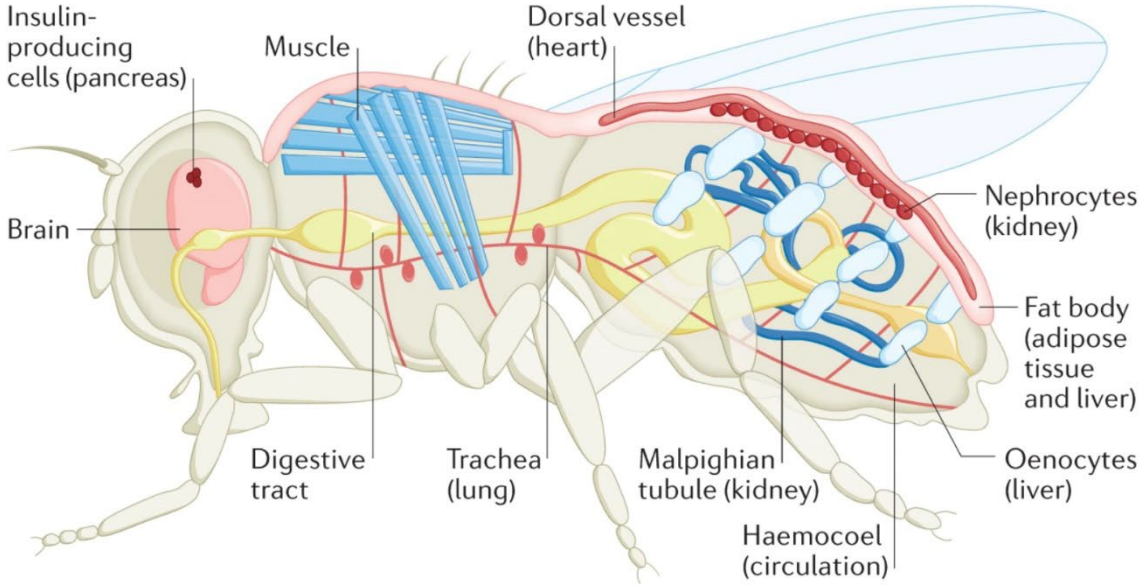

Insulin-producing cells (pancreas)
Muscle
Dorsal vessel (heart)
Brain
Nephrocytes (kidney)
Fat body (adipose tissue and liver)
Digestive tract
Trachea (lung)
Malpighian tubule (kidney)
Oenocytes (liver)
Haemocoel (circulation)

Figure 1. *Drosophila* organ systems and their human analogues.
Many fly organs have straightforward structural and functional homologues in vertebrates: brain, muscle, digestive tract and dorsal vessel (*Drosophila* heart). Neuroendocrine cells in the fly brain secrete glucagon-like and insulin-like peptides (ILPs) similarly to pancreatic α-cells and β-cells, respectively. The fly fat body stores lipids and carbohydrates, akin to adipose tissue and the liver in humans; it is the metabolic hub as well as a major secretory organ. Fly oenocytes also play a hepatocyte-like role in lipid processing and mobilization. The fly Malpighian tubule serves the excretory and diuretic (water and ion homeostasis) function of human kidneys, whereas nephrocytes serve the glomeruli and podocyte role in filtering circulatory fluid. Oxygenation takes place through passive transport through tracheal tubules, whose complete network of air sacs and extensively ramifying branches are not shown in full. The haemocoel is the open body cavity of the fly; it is filled with circulatory fluid that transports oxygen, nutrients, waste and immune cells analogous to human blood.

FIGURE 2

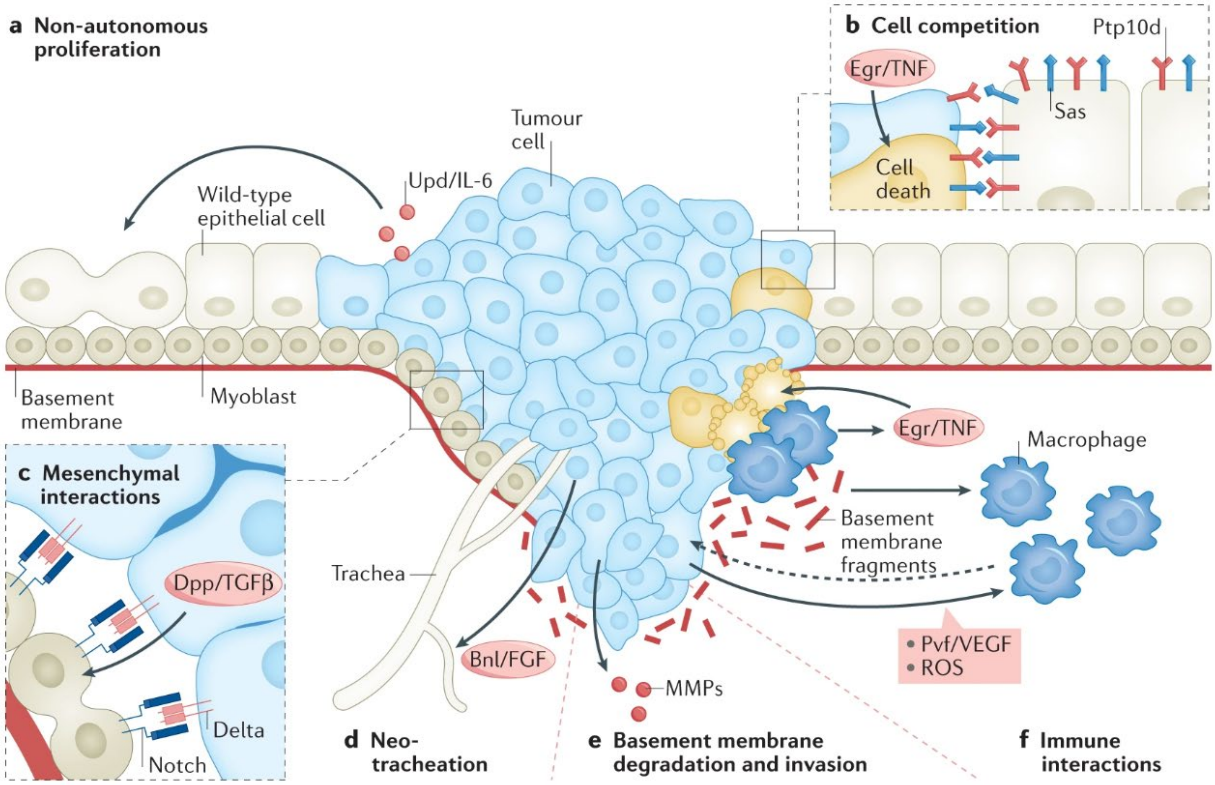

a Non-autonomous proliferation
b Cell competition
Ptp10d
Egr/TNF
Sas
Cell death
Tumour cell
Upd/IL-6
Wild-type epithelial cell
Basement membrane
Myoblast
Egr/TNF
Macrophage
Basement membrane fragments
c Mesenchymal interactions
Dpp/TGFβ
Delta
Notch
Trachea
Bnl/FGF
MMPs
• Pvf/VEGF
• ROS
d Neo-tracheation
e Basement membrane degradation and invasion
f Immune interactions

Figure 2. Interactions in the fly tumour microenvironment (TME).

Tumour-produced signals alter the surrounding stroma. (a) Upregulation of Unpaired (Upd)/interleukin-6 (IL-6) in tumour cells can stimulate proliferation of neighbouring wild-type cells. (b) Mispolarization of Sas and Ptp10D at the interface between a tumour clone and wild-type epithelium promotes death of the tumour cells, triggered by Eiger (Egr)/tumour necrosis factor (TNF). The source of Egr/TNF is not yet clear. (c) Paracrine Dpp/transforming growth factor-β (TGFβ) and juxtacrine Delta produced by tumour cells can promote proliferation of underlying mesenchymal myoblasts. (d) Branchless (Bnl)/fibroblast growth factor (FGF) production from hypoxic tumours can attract new tracheal branches. (e) Tumour production of matrix metalloproteinases (MMPs) degrades basement membrane and promotes invasion. (f) Tumours attract macrophages (dashed arrow) that detect tumour-produced Pvf/vascular endothelial growth factor (VEGF), extracellular reactive oxygen species (ROS) and basement membrane damage. Macrophages then upregulate Egr/TNF, which binds to tumour cells and promotes their death.

FIGURE 3

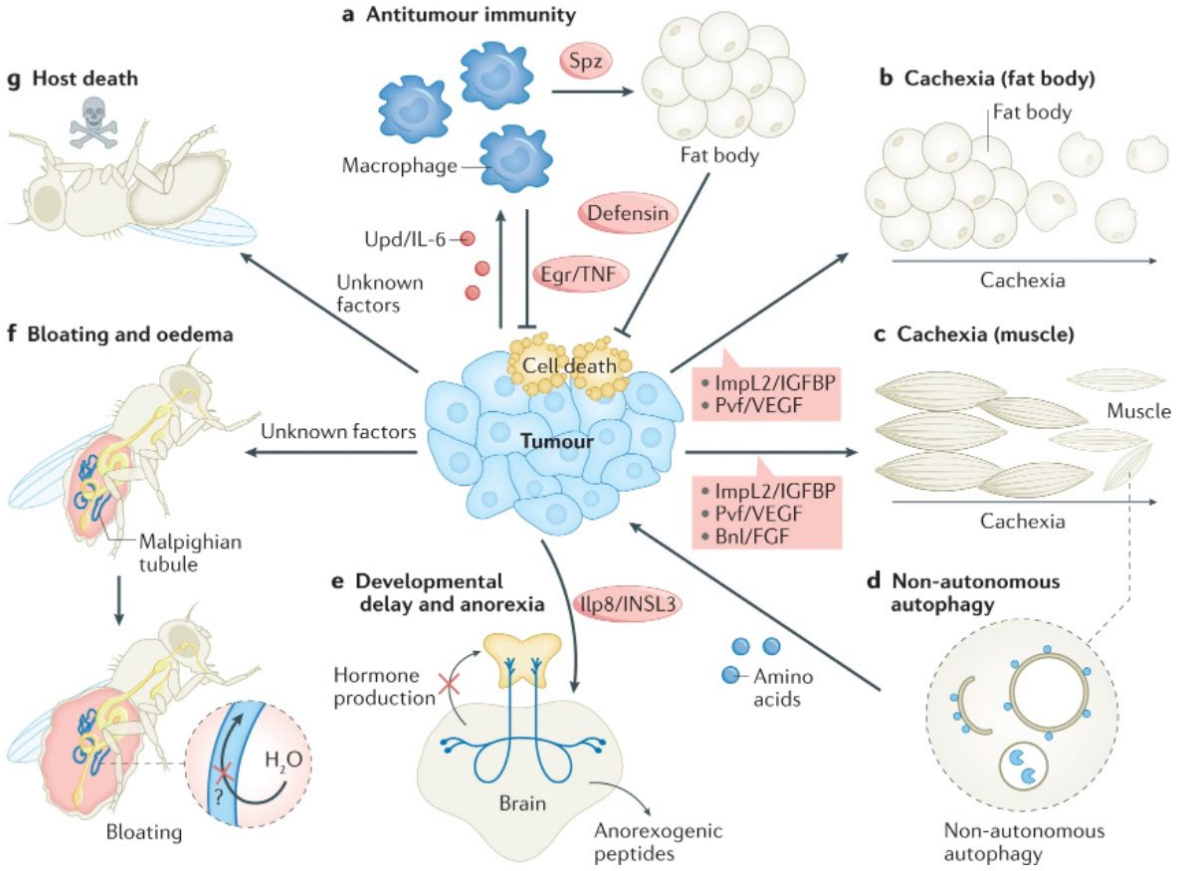

a Antitumour immunity
Spz
Macrophage
Fat body
g Host death
b Cachexia (fat body)
Fat body
Cachexia
Defensin
Upd/IL-6
Unknown factors
Egr/TNF
f Bloating and oedema
Cell death
• ImpL2/IGFBP
• Pvf/VEGF
c Cachexia (muscle)
Muscle
Unknown factors
Tumour
• ImpL2/IGFBP
• Pvf/VEGF
• Bnl/FGF
Cachexia
Malpighian tubule
e Developmental delay and anorexia
Ilp8/INSL3
d Non-autonomous autophagy
Hormone production
Amino acids
H_2O
?
Brain
Bloating
Anorexogenic peptides
Non-autonomous autophagy

Figure 3. Paraneoplastic effects of fly tumours.

Endocrine signals from tumours cause pathologies in distant organs. Some effects have been demonstrated in larvae and others in the adult; see text for details. (a) Tumour-associated macrophages (TAMs), stimulated by Unpaired (Upd)/interleukin-6 (IL-6), secrete Spaetzle (Spz), which activates Toll signalling in the fat body to trigger production of the antimicrobial peptide Defensin. Defensin, working along with macrophage-produced Eiger (Egr)/tumour necrosis factor (TNF), binds to and kills tumour cells. (b,c) ImpL2/insulin growth factor binding protein (IGFBP) induces systemic insulin resistance, leading to cachexia-like wasting and degeneration of fat body (part b) and muscle (part c). Reception of tumour-produced Pvf/vascular endothelial growth factor (VEGF) in adult fat body and muscle, and Branchless (Bnl)/fibroblast growth factor (FGF) in larval muscle, also induces wasting. (d) Wasting muscles supply amino acids via autophagy that are taken up by the tumour and promote tumour growth. (e) Insulin-like peptide 8 (Ilp8)/INSL3 acts on brain neurons to inhibit the production and release of pupation-promoting hormones in the larvae; and also acts in the adult to stimulate brain production of anorexigenic peptides. (f) Unknown tumour-dependent factors cause the host to retain excess fluids; this may be due to actions on the Malpighian tubules. (g) Some combination of these pathologies, along with other currently unrecognized effects, kills hosts prematurely.

Box 1. *Drosophila* tumour genetics.

As in mammals, tumours in *Drosophila* are defined as groups of cells that overproliferate due to a loss of normal growth-restraining mechanisms. Although relatively short-lived, *Drosophila* and other insects can develop tumours spontaneously, for instance in the gut [314–316]. In the laboratory, tumours can be readily induced through genetic manipulation of various epithelial, neural, haematopoietic and germline tissues. Homologues of some fly tumour suppressor genes and oncogenes (for example, RAS family genes, *TSC1, TSC2, NF2* and the Hippo pathway) are frequently mutated in human cancer; others (for example, *scribble* (*SCRIB*)) are not but have, nevertheless, been experimentally implicated in mammalian oncogenesis [317,318]. A striking feature of Drosophila tumours is that they can be induced by altered activity of single genes, and do not require multiple genetic lesions or loss of p53. The resultant tumours are grossly genetically stable and therefore relatively homogeneous [319], simplifying analyses.

Drosophila tumours are divided into two classes, called hyperplastic and neoplastic [51,317]. These are roughly comparable with benign and malignant mammalian tumours, respectively. Hyperplastic tumours preserve the fate as well as cell architecture of their tissue of origin, and undergo accelerated mitoses. Neoplastic tumours, by contrast, often grow more slowly than wild-type cells, but never stop proliferating. Neoplastic cells show signs of dedifferentiation, lose cell polarity and show invasive behaviours including degradation of basement membranes; they also become functionally immortal. Whereas flies can often tolerate extensively overgrown hyperplastic tissue, the presence of a neoplastic tumour causes more rapid death. Comprehensive reviews on the biology of fly tumours are available for readers seeking more detail [51,210–212,308,317–319].

Box 2. Fly models for tumour-host interaction studies.

Although transformation can occur in other cell types, epithelial tumours have been best characterized for tumour–host interactions (**Supplementary Table 2**). Neoplastic carcinomas are most readily induced by mutation of *scribble* (*scrib*)-class genes [51,318,320]. A popular model combines *scrib* mutants with overexpression of oncogenic Ras [213]. This cooperativity creates a more aggressive and rapidly malignant tumour.

Neoplastic transformation can also occur through other means, involving, for example, activation of Notch and Src or chromosomal instability [319]. Some models mimic the genetic constituency and tissue of origin of human cancers, with a focus towards therapeutic screening [308,321]. Despite differing driver genes, many neoplasms act through a common set of signalling pathways — JUN amino-terminal kinase (JNK), Hippo, STAT and ERK — that regulate the shared phenotypic hallmarks. In this Review, we treat these tumours collectively as providing a new and malignant source of growth that host physiology must react to, and also acknowledge that there may be significant differences in the details of the host response.

Although the larva is excellent for studying autonomous tumour growth, it presents disadvantages for studying tumour–host interactions. The accessible third larval instar lasts only 48 h, terminating when metamorphosis creates a pupa. Such insect-specific events impair analysis of long-term tumour–host interactions familiar in mammals. Studying cancer in adult *Drosophila* avoids these limitations. The earliest model uses 'allograft' transplantation of imaginal discs or brains from the larva [149]. Flies have an open circulatory system and no transplant histoincompatibility, so simple injection of tissue allows growth in the permissive environment of the adult abdomen [201]. Transplanted wild-type tissue does not undergo excess growth, but tumorous tissue can increase ~200-fold in size over 1–2 weeks before the host dies. Whereas neural tissue disseminates throughout the body cavity, epithelial disc tissue remains intact, growing as a compact mass. Remarkably, tumours can be propagated to successive hosts apparently indefinitely, supporting an 'immortal' transformation of the original tissue.

In addition to transplants, autochthonous tumours can be induced in the adult via genetically engineered fly models (GEFMs) [212,322]. The adult fly has only a few active zygotic stem cell populations, but transgenic manipulation of these can induce tumorous growth. Activation of Hippo or Wnt, or reduction of Notch in intestinal stem cells (ISCs), creates malignant tumours, as does Ras overexpression in normally quiescent renal stem cells. Adult blood and neural cells are postmitotic, but oncogenic expression in neural or glial progenitors in the larva can yield viable adults with continuously proliferating tumours. Finally, our laboratory has achieved neoplastic transformation of adult ovarian epithelial cells (D.B., T.-C.H. and J.K. unpublished data).

TABLE 1 AND SUPPLEMENTAL TABLES

Table 1, Supplemental Table 1, and Supplemental Table 2 are available on Nature Reviews Cancer at https://doi.org/10.1038/s41568-021-00387-5

APPENDIX

Miscellaneous Considerations and Analyses in *Drosophila*

Considerations for the impact of genetic background on lifespan analyses

Careful consideration of genetic background should be taken to ensure that lifespan analysis of tumor-bearing flies reflect true biological phenomena and are reproducible between individuals and labs. Here, I provide an example of a mutant fly that in its uncleaned or "dirty" background appeared to extend the survival of tumor-bearing flies; however, after cleaning up the genetic background via backcrossing for ten generations, the original extension appeared to be due to the confounding effect of genetic background.

The *hop*$^{Tumorous\text{-}lethal}$ (*hop*^{TumL}) mutation was originally generated back in 1976 by Corwin and Hanratty [323]. This mutation is a gain-of-function, point mutation that results in the inappropriate activation of the fruit fly Janus kinase, hopscotch (hop) [324]. Larvae carrying a single copy of this mutation develop melanotic tumors due to the ectopic differentiation and activation of immune cells [325]. Adult flies with this mutation have shorter lifespans than wild-type flies [326]. Interestingly, when *hop*^{TumL} flies were crossed to the OC model, tumor-bearing flies with a single copy of *hop*^{TumL} exhibited dramatically reduced mortality compared to control tumor-bearing flies (**Figure 1A**). Taking advantage of the Gal4-UAS system, we expressed *hop*^{TumL} specifically within the OC tumor and found that it did not significantly change the survival of tumor-bearing flies, suggesting that the mutation may be acting within host tissues (**Figure 1B**). Transplantation experiments gave mixed results depending on the type of tumor transplanted (**Figure 1C, D**). We were unsuccessful in our attempts to narrow down the specific tissue in which this mutation could be acting (**Figure 1E - J**). Subsequent lifespan analyses of an independently generated *hop*^{TumL} mutation given to us by the Bach lab suggested that the genetic background of our line may be confounding our lifespan analyses (**Figure 1K**). Cleaning the genetic background of our "dirty" *hop*^{TumL} line largely abolished the original extension of lifespan (**Figure 1L, M**), suggesting that background was indeed having a profound effect on the survival of tumor-bearing flies. Healthy, non-tumor-bearing flies appeared to have minimal change in survival in the "dirty" background (**Figure 1N**).

The impacts of genetic background were further supported by lifespan analyses into the survival of tumor-bearing flies from different control backgrounds. Whereas *w1118* and *CantonS* appeared to track similarly with each other in terms of survival, *yw* appeared to have slightly increased survival and *OreR* more so (**Figure 1O**). Thus, careful consideration of the genetic background of each line must be taken to ensure that true biological phenomena are studied. This has implications for future investigations that employ different combinations of Gal4, UAS, RNAi, and other constructs and for the forward genetic screen because the mixing and complicated status of genetic backgrounds can confound results.

FIGURE 1

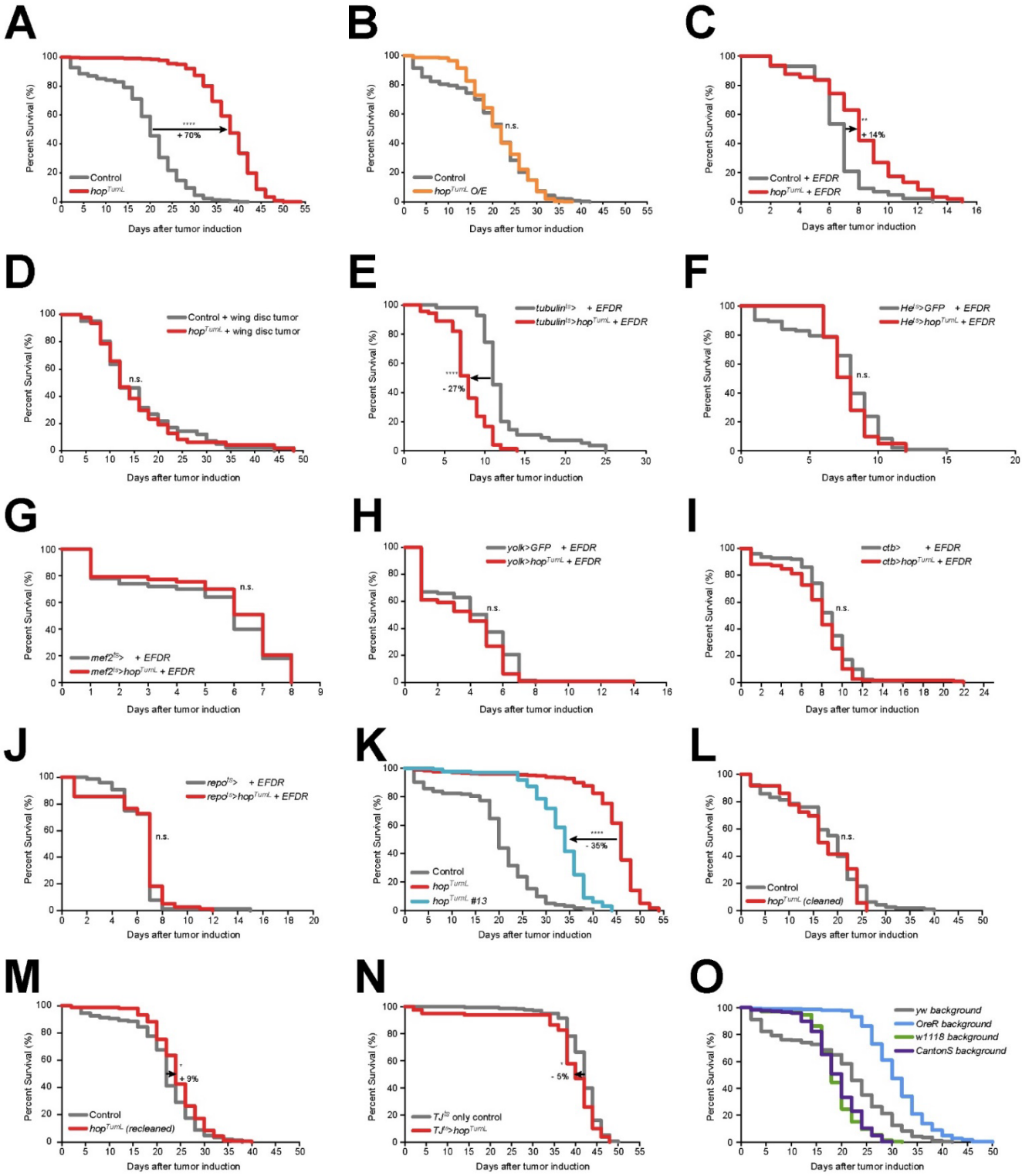

A
Percent Survival (%)
Days after tumor induction
Control
hopTumL
****
+ 70%
B
Control
hopTumL O/E
n.s.
C
Control + EFDR
hopTumL + EFDR
**
+ 14%
D
Control + wing disc tumor
hopTumL + wing disc tumor
n.s.
E
tubulints> + EFDR
tubulints>hopTumL + EFDR
****
- 27%
F
Hels>GFP + EFDR
Hels>hopTumL + EFDR
n.s.
G
mef2ts> + EFDR
mef2ts>hopTumL + EFDR
n.s.
H
yolk>GFP + EFDR
yolk>hopTumL + EFDR
n.s.
I
ctb> + EFDR
ctb>hopTumL + EFDR
n.s.
J
repots> + EFDR
repots>hopTumL + EFDR
n.s.
K
Control
hopTumL
hopTumL #13
****
- 35%
L
Control
hopTumL (cleaned)
n.s.
M
Control
hopTumL (recleaned)
*
+ 9%
N
TJts only control
TJts>hopTumL
*
- 5%
O
yw background
OreR background
w1118 background
CantonS background

Figure 1. Considerations into genetic background impact on survival assays in *Drosophila*.
OC tumor-bearing flies carrying a single copy of the hop^{TumL} gain-of-function mutation survive significantly longer than control tumor-bearing flies (A). Tumor-specific overexpression of hop^{TumL} does not significantly suppress lethality caused by the tumor, suggesting that it is acting in host tissues (B). However, transplantations either with eye disc tumors (C) or wing disc tumors (D) gave conflicting results. Using *tubulin-Gal4* to drive hop^{TumL} expression throughout the host fly following eye disc tumor transplantation failed to recapitulate the survival seen in the OC model (E). Hemocyte-specific (F), muscle-specific (G), fat body-specific (H), malpighian tubule-specific (I), and glia-specific (J) overexpression of hop^{TumL} failed to suppress lethality caused by eye disc tumors, precluding the identification of a specific host tissue in which hop^{TumL} is acting. A cleaned line provided by the Bach lab (cyan line) exhibited significantly reduced survival compared to the "dirty" hop^{TumL} line (red line) (K). Two independently cleaned lines failed to suppress lethality caused by the tumor compared to controls (L, M), suggesting a confounding effect of genetic background in the original line. The lifespan of non-tumor-bearing flies is not affected by the hop^{TumL} mutation (N). Examination of multiple control backgrounds reveals a range of lifespans for tumor-bearing flies (O). Statistical test used for lifespan analysis was the log-rank test (*p<0.05, ** p<0.005, **** p<0.00005).

Number of flies used for each panel are as follows:
(A) Control: N=642, hop^{TumL}: N=749
(B) Control: N=338, hop^{TumL} *O/E*: N=341
(C) Control + *EFDR*: N=43, hop^{TumL} + *EFDR*: N=97
(D) Control + wing disc tumor: N=41, hop^{TumL} + wing disc tumor: N=47
(E) $tubulin^{ts}$> + *EFDR*: N=55, $tubulin^{ts}$>hop^{TumL} + *EFDR*: N=72
(F) He^{ts}>*GFP* + *EFDR*: N=93, He^{ts}>hop^{TumL} + *EFDR*: N=61
(G) $mef2^{ts}$> + *EFDR*: N=50, $mef2^{ts}$>hop^{TumL} + *EFDR*: N=53
(H) *yolk>GFP* + *EFDR:* N=121, *yolk*> hop^{TumL} + *EFDR*: N=108
(I) *ctb*> + *EFDR*: N=123, *ctb*>hop^{TumL} + *EFDR*: N=118
(J) $repo^{ts}$> + *EFDR*: N:76, $repo^{ts}$>hop^{TumL} + *EFDR*: N=77
(K) Control: N=203, hop^{TumL}: N=239, hop^{TumL} *#13*: N=135
(L) Control: N=113, hop^{TumL} *(cleaned)*: N=36
(M) Control: N=148, hop^{TumL} *(recleaned)*: N=162
(N) TJ^{ts} only control: N=176, TJ^{ts}>hop^{TumL}: N=81
(O) *yw background*: N=194, *OreR background*: N=226, *w1118 background*: N=212, *CantonS background*: N=316

Systemic impacts of tumor include observable heart defects

As discussed in previous chapters, many organ systems are affected by malignancy. Here, I present preliminary data suggesting a novel *Drosophila* paraneoplastic syndrome manifests as observable differences in heart physiology (**Figure 2**). The dorsal vessel, which is analogous to mammalian hearts, is located within the abdomen of an adult fly, and it can be readily visualized through the dorsal cuticle of a live fly with a fluorescent reporter, facilitating *in vivo* studies of the *Drosophila* heart (**Figure 2A**). In a healthy fly, the dorsal vessel beats at a rate of 4-6 Hz, and this declines as the fly ages [327,328]. Interestingly, we discovered that tumor-bearing flies exhibit marked increase heart rate compared to control flies (**Figure 2B**), but arrhythmia is unchanged (**Figure 2C**). Because temperature and CO_2 anesthesia has been shown to affect heart rate, with the former elevating it and the latter halting it [329], our experimental set-up was designed to allow flies to return to room temperature and recover from CO_2. Comparing the heart rate of flies kept at room temperature and 29°C indicated that temperature was likely not a confounding factor in our experimental set-up (**Figure 2D, E**). Moreover, in our hands, the heart rate of control flies matched that of published literature for healthy flies, indicating that our set-up allowed sufficient time for flies to recover from anesthesia. Given all this, our preliminary exploration into heart physiology of tumor-bearing flies suggests that there may be a novel paraneoplastic syndrome that affects heart function. Future experiments could make use of high-speed cameras to perform more sophisticated analyses that are the standard for the field, and bloating should be considered as a potential confounding factor to be investigated in more detail.

FIGURE 2

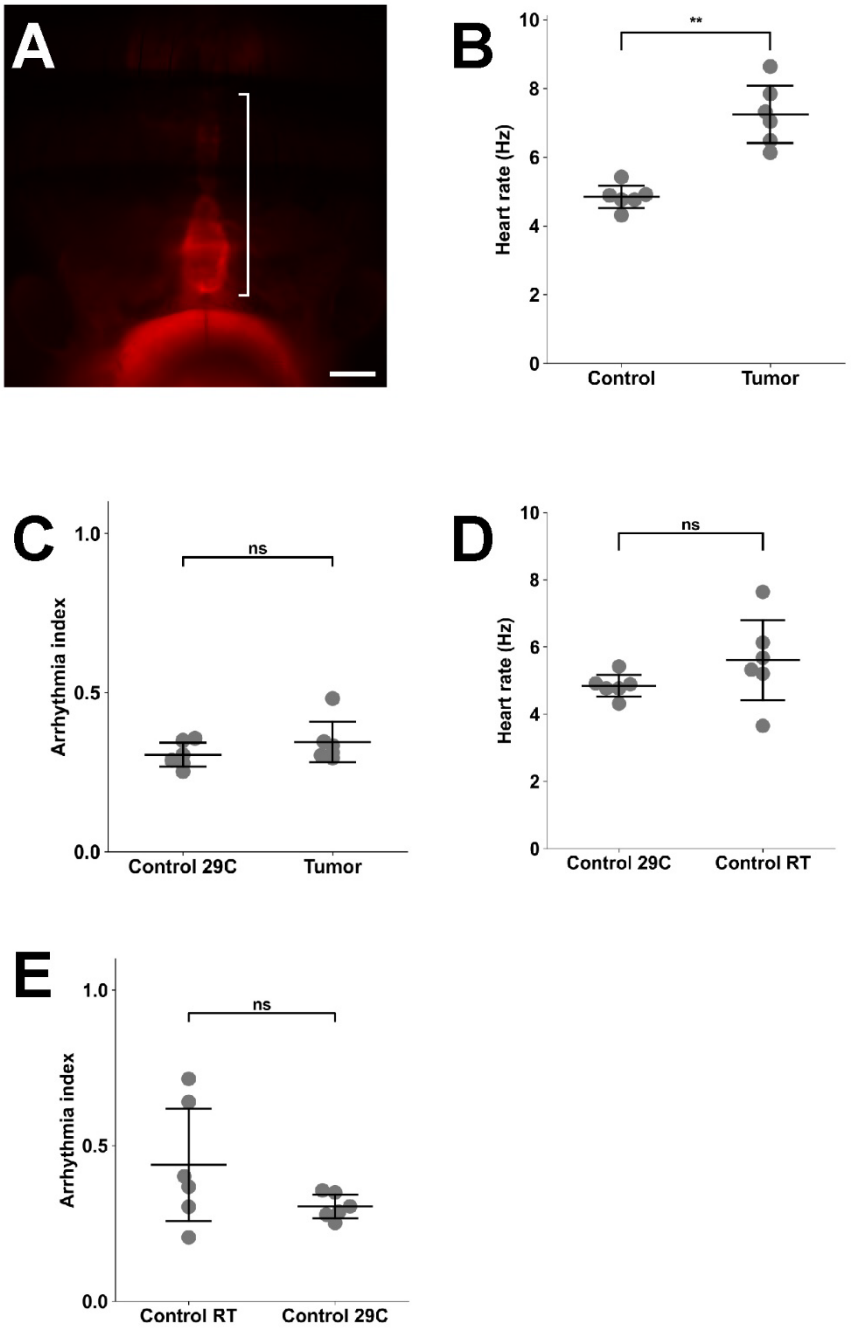

A
B
**
Heart rate (Hz)
10
8
6
4
2
0
Control
Tumor
C
ns
Arrhythmia index
1.0
0.5
0.0
Control 29C
Tumor
D
ns
Heart rate (Hz)
10
8
6
4
2
0
Control 29C
Control RT
E
ns
Arrhythmia index
1.0
0.5
0.0
Control RT
Control 29C

Figure 2. Tumors disrupt heart physiology.

Dorsal vessel (white bracket) of Drosophila can be visualized through the dorsal abdomen with the use of a fluorescent reporter (A). Flies carrying OC tumors exhibited significantly elevated heart rates compared to control (B), but the regularity of their heartbeats were unchanged, as measured by the arrhythmia index (C). Our experimental set-up minimizes the confounding effect of temperature on heart rate measurements (D, E). Scale bar denotes 100μm. Statistical test used was two-tailed t-test (n.s. indicates not significant, ** $p<0.005$).

Analysis of immune cell dynamics

Pioneering studies by Tian Xu's lab demonstrated that *Drosophila* immune cells are recruited to a growing tumor, suggesting common mechanisms between the innate immune response to wound and malignant transformation [93]. Here, I describe some exploratory experiments into the relationship between the fly's innate immune system and different tumor models, as well as some findings in healthy flies.

As would be expected, transplantation of either wild-type imaginal discs or tumorous discs induced plasmatocyte recruitment to the surface of the transplanted tissue, indicative of an innate immune response to foreign tissue (**Figure 3A - C**). Likewise, induction of OC tumors also elicited an innate immune response (**Figure 3D, E**). During investigations into hop^{TumL}, we found that this mutation appeared to increase the number of plasmatocytes recruited to the tumor surface (**Figure 4A, B**), and even promote the activation of recruited plasmatocytes to wild-type tissue (**Figure 4C, D**). Tumor size appeared to be mildly reduced in response to increased immune activity, consistent with published literature (**Figure 4E - G**) [93]. Compared to transplanted wing discs, the elevated immune response to wing disc tumors in hop^{TumL} flies appears to be a specific reaction to the tumor rather than to foreign tissue because the number of plasmatocytes recruited to wing discs is not significantly different between control and hop^{TumL} flies (**Figure 4F**). Whole fly imaging of hop^{TumL} flies supported the results in Figure 4C and D, wherein plasmatocyte activation is systemic and due to the mutation itself, instead of a specific response to the tumor (**Figure 4H, I**).

Hemocyte loss negatively impacts the immune response to infection and wounding [330]. In larvae lacking hemocytes, tumors are increased in volume, suggesting a lack of an anti-tumor response. We found that similarly, tumor-bearing adults lacking hemocytes also exhibited significantly reduced survival, suggesting that tumorigenesis is uninhibited when flies lack immune cells (**Figure 5A**). Whole fly imaging confirmed the lack of hemocytes in adult flies compared to control (**Figure 5B, C**).

FIGURE 3

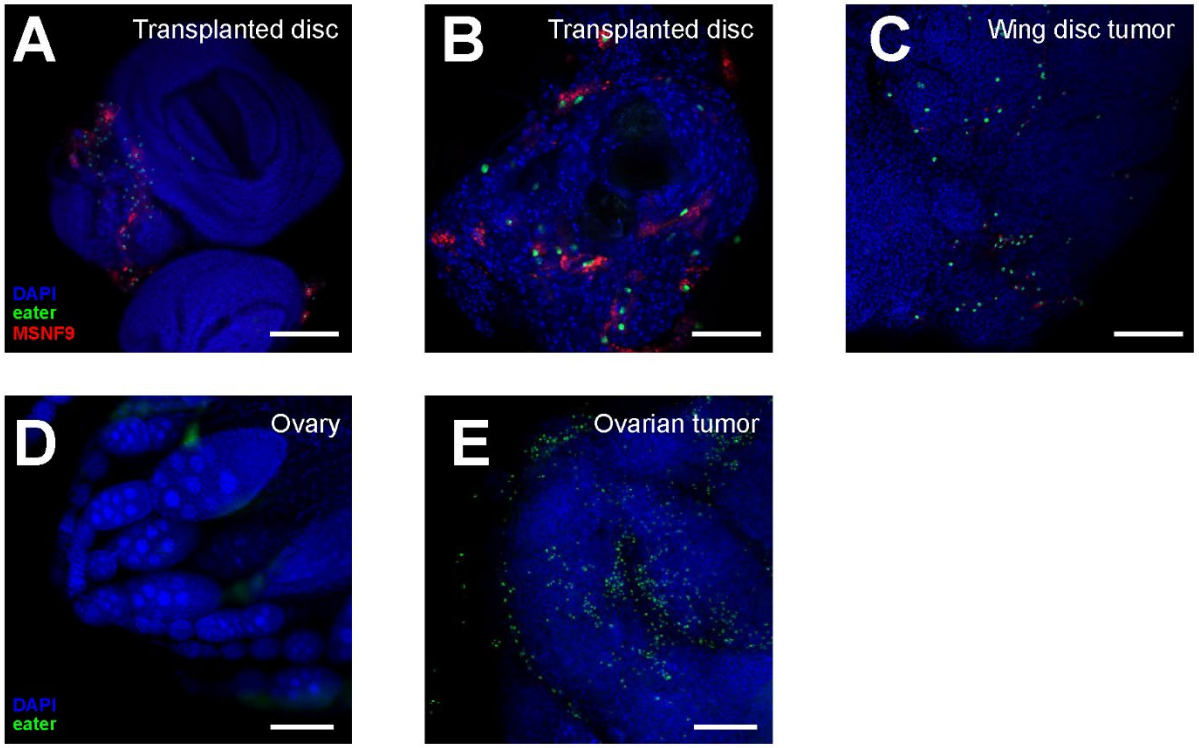

A
Transplanted disc
DAPI
eater
MSNF9
B
Transplanted disc
C
Wing disc tumor
D
Ovary
DAPI
eater
E
Ovarian tumor

Figure 3. Hemocyte recruitment to transplanted tissues and tumors.
Transplantation of undamaged discs (A), damaged discs (B), and wing disc tumors (C) into the abdomen of adult flies induced the recruitment of plasmatocytes to the tissue surface (green, red). Healthy ovaries do not cause immune cells to be recruited (D), whereas ovarian tumors recruit plasmatocytes to their surface (green) (E). Scale bars in A-C denote 50μm, and in D, E, they denote 100μm.

FIGURE 4

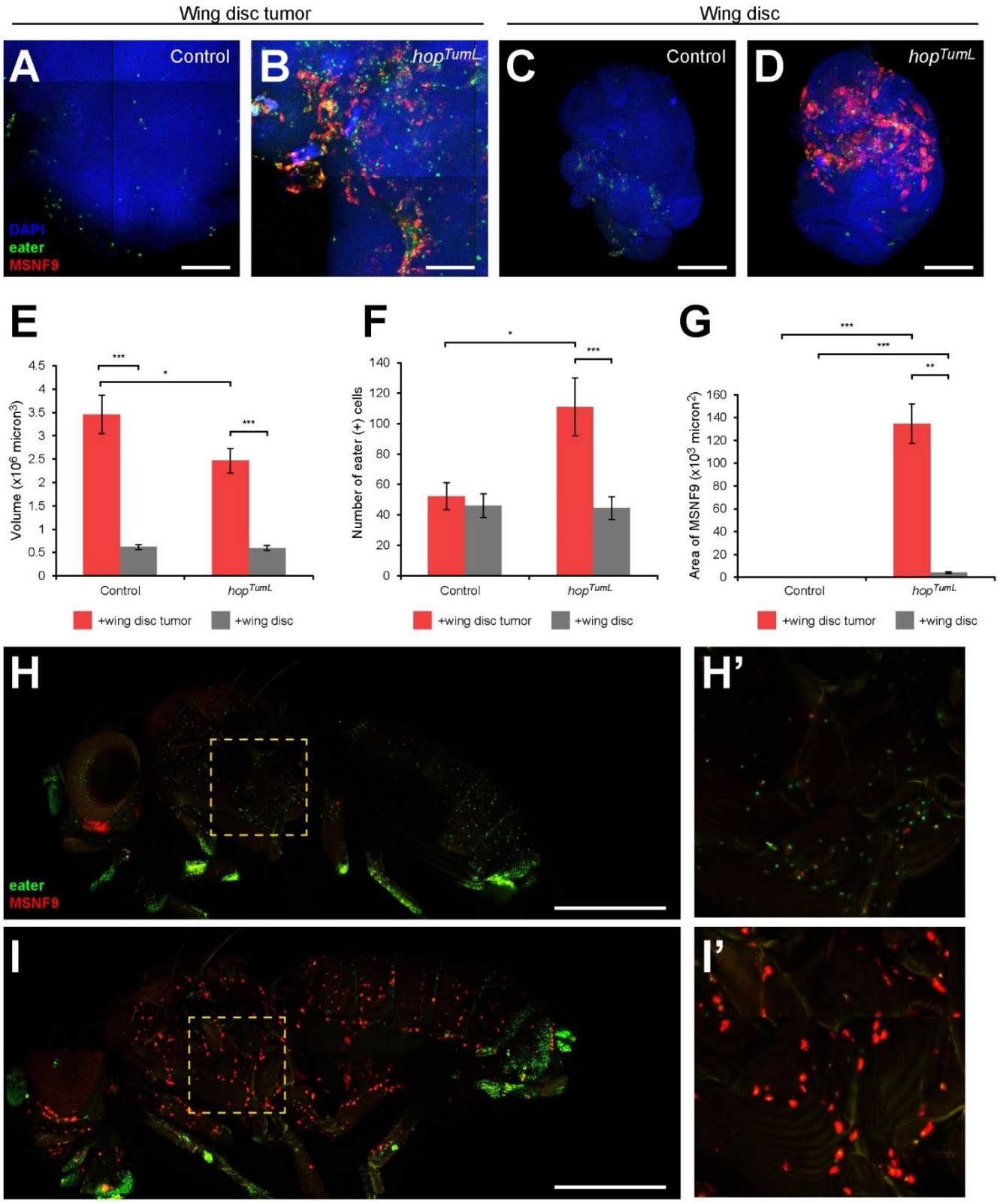

Wing disc tumor
Wing disc
A
Control
B
hopTumL
C
Control
D
hopTumL
DAPI
eater
MSNF9
E
Volume (x10^6 micron^3)
Control
hopTumL
+wing disc tumor
+wing disc
F
Number of eater (+) cells
Control
hopTumL
+wing disc tumor
+wing disc
G
Area of MSNF9 (x10^3 micron^2)
Control
hopTumL
+wing disc tumor
+wing disc
H
H'
eater
MSNF9
I
I'

Figure 4. *hop^{TumL}* impacts adult plasmatocyte behvior.

The gain-of-function *hop^{TumL}* mutation induces ectopic activation of plasmatocytes in adult flies compared to controls, seen in recruited plasmatocytes on both a wing disc tumor (A, B) and transplanted wing discs (C, D). Plasmatocyte activation from the *hop^{TumL}* mutation leads to an increased antitumor response and smaller tumors (E). More plasmatocytes are recruited to the surface of transplanted wing disc tumors (F), and a greater number of plasmatocytes are activated (G). Max Z-projections of whole fly images taken of cold anesthetized flies reveal a systemic change in plasmatocyte activation in *hop^{TumL}* flies compared to control flies (Figure H, I). The dotted yellow box indicates H' and I', respectively. Scale bars in A-D denote 100μm. Scale bars in H and I denote 500μm. For E-G, at least 30 samples were quantified for each group. Error bars denote standard error. Statistical test used was two-tailed t-test (* $p<0.05$, ** $p<0.005$, ***$p<0.0005$).

FIGURE 5

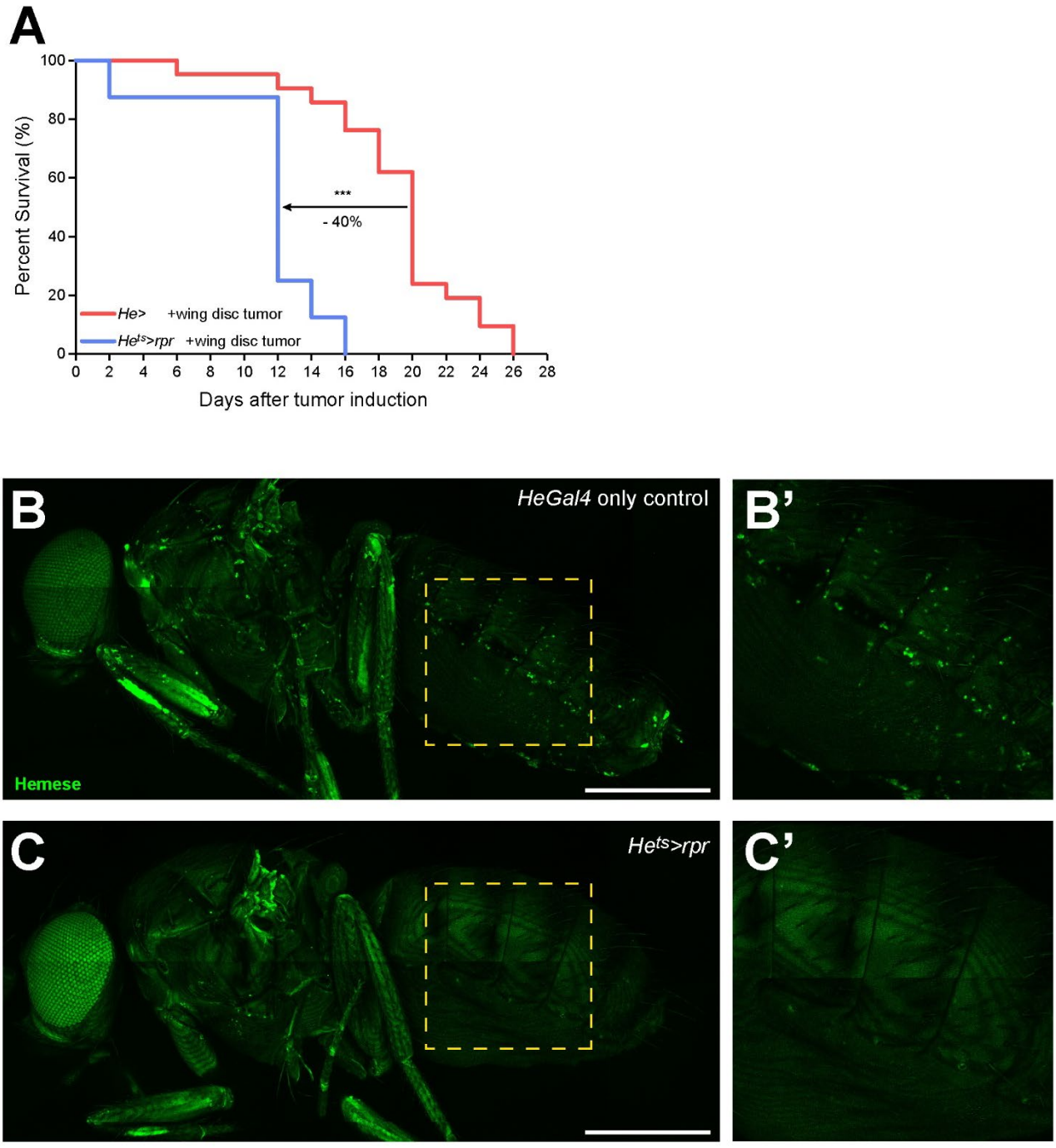

A
100
80
60
40
20
0
Percent Survival (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days after tumor induction
***
- 40%
He> +wing disc tumor
Hets>rpr +wing disc tumor
B
HeGal4 only control
Hemese
B'
C
Hets>rpr
C'

Figure 5. Tumor-bearing flies lacking hemocytes exhibited heightened mortality.
Flies with hemocytes ablated using *Hemese-Gal4* driving *UAS-rpr* in hemocytes show significantly reduced survival following wing disc tumor transplantation (A). Max Z-projections of whole fly images confirm hemocyte ablation (B, C). B' and C' are indicated by the dotted yellow squares. Statistical test used for lifespan analysis was the log-rank test (***$p<0.0005$). Number of flies used for panel A were as follows: *He>* +wing disc tumor: N=21, He^{ts}>*rpr* +wing disc tumor: N=8.

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by a desoxyribonucleic acid fraction isolated from pneumococcus type III. Journal of Experimental Medicine *79*, 137–158.

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