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Host Response to Malignant Tumors in *Drosophila melanogaster*

by

Alejandra Figueroa-Clarevega

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

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Committee in charge:

Professor David Bilder, Chair
Professor Iswar Hariharan
Professor Kunxin Luo
Professor Lewis Feldman

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ABSTRACT

Host Response to Malignant Tumors in *Drosophila melanogaster*

by

Alejandra Figueroa-Clarevega
Doctor of Philosophy in Molecular and Cell Biology
University of California, Berkeley
Professor David Bilder, Chair

Cancer is the abnormal growth of cells. This growth can kill by disrupting the normal function of its organ of residence or through long-range pathophysiological interactions with distant tissues. Despite the morbidity and mortality associated with cancer, the mechanisms underlying these lethal interactions have remained elusive.

We have established a Drosophila tumor model to explore the poorly-understood mechanisms of tumor-host interactions. Using a classic technique, we transplanted larval tumors into adult flies and investigated the effects imposed on host tissues. We find that only 5 days post-transplantation, malignant fly tumors induce robust wasting of muscle, adipose and gonadal tissues. Interestingly, these wasting phenotypes recapitulate key characteristics of the enigmatic cancer cachexia suffered by about half of human cancer patients. We have identified the Insulin Growth Factor Binding Protein (IGFBP) homolog ImpL2 as key mediator of these cachexia-like phenotypes. This factor is secreted specifically by malignant tumors and is both necessary and sufficient for tissue wasting. Consistent with its role as an insulin antagonist, tumor-secreted ImpL2 interrupts systemic insulin signaling to induce insulin resistance, resulting in the cachexia-like wasting of peripheral host tissues.

Our work demonstrates that this Drosophila model lends itself to the dissection of complex tumor-host interactions, such as cachexia. We have also used this model to investigate other important features of tumor-host interactions, including metastasis, bloating, immune response and lethality.

The genetic manipulability of this system has facilitated the identification of a critical factor mediating the observed cachexia-like phenotypes and can now be used to explore other contributors to the tumor-induced systemic wasting or the molecular mechanisms underlying other important tumor-host interactions.

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CHAPTER 1

Introduction

CANCER

Tumor Formation

Cells are the building blocks of life. Their growth, division, differentiation and death are tightly regulated processes that enable organisms to develop and maintain homeostasis throughout a lifetime. Although complex mechanisms ensure the spatio-temporal execution of these processes, a combination of genetic and environmental factors can give cells the ability to activate these plans outside of the normal developmental or homeostatic context. This abnormal hijacking of a cell's intrinsic capabilities remains one of the most important enigmas in the biological and medical fields.

Deciphering the etiology of cancer has been an arduous task. About 90% of tumors are epithelial in origin, yet every tumor is different. Analyzing a plethora of tumor types and models has revealed a set of common capabilities that are required for tumorigenesis. These "hallmarks of human cancer" include an acquired ability to grow, proliferate, vascularize and invade, while evading apoptosis and anti-growth signals [1]. These capabilities are developed through genetic and epigenetic changes in the genome as a result of inherited or newly acquired mutations; they usually involve the dominant gain of function of an oncogene and/or the loss of function of a tumor suppressor gene. These initial mutations can also generate genetic instability, which in turn amplifies the number and variety of mutations. As tumors evolve, they select for mutations that result in the advantageous, adaptive traits described above. This multi-step process of selection transforms normal cells into malignant cancers.

Lethality

An official war on cancer was declared in the 1970's. Sadly, despite enormous research efforts to better understand and treat this disease, cancer remains a major health problem around the world; a longer life expectancy and changes in life-style will only further aggravate the prognosis. Cancer is currently the second leading cause of death in industrialized nations, but an increasing number of diagnosed cases in developing countries each year, with figures predicted to continue to rise, has converted this disease into a global health crisis. The impact of cancer is not only measured in its associated disability, morbidity and mortality but also on its incredibly high financial costs to both the patient and society as a whole. To develop better, more cost-effective and life-saving treatments, it is not only necessary to know how tumors form and grow, but more importantly, we must understand the mechanisms by which a tumor kills its host.

Over 90% of cancer deaths are attributed to metastasis, the formation of secondary tumors at a distant site from the primary tumor mass [2]. Cancer is best treated when detected early, as a single tumor can be surgically removed or therapeutically eliminated. Once the tumor has metastasized, the burden imposed by additional tumors on multiple organ systems becomes increasingly difficult to manage as compensating mechanisms are overwhelmed, jeopardizing

patient's survival. The lethal effects of tumors can be local, physically compromising the function of the organ where they reside, or distant, causing systemic changes that impact multiple organs.

TUMOR-HOST INTERACTIONS

Local Tumor Effects

In some instances, local tumor growth itself is responsible for the death of its host. As previously described, a series of cell-intrinsic changes give rise to the capabilities that drive robust tumor growth. As this growth continues, the tumor occupies a larger area, creating physical pressure in the organ of its origin or the associated blood vessels and nerves. This is one of the reasons brain tumors are so dangerous [3]. In the brain, cerebrospinal fluid (CSF) is responsible for bringing in nutrients, removing waste and controlling intracranial pressure. Tumor growth can obstruct the flow of CSF. As this fluid builds-up, pressure continues to increase, displacing brain tissue and blood vessels from their normal position (herniation). Once the damage compromises the normal function of this vital organ, patients enter a coma and die. This deadly, physical disruption also occurs with tumors in other vital organs. Intestinal tumors prevent proper food digestion and absorption of essential nutrients, essentially starving the patient. Tumor growth in the lungs reduces oxygen absorption capability; limited oxygen compromises the function of other vital organs.

While life-threatening physical disruption is caused by cell-intrinsic capabilities that accelerate tumor growth, host death can also be induced by cell-extrinsic mechanisms. These mechanisms involve heterotypic interactions between the tumor cells and their local microenvironment [4]. Tumor growth beyond its tissue boundaries can often result in the breaking of the barrier that protects the organ from external insults, essentially creating a “wound that does not heal” [5]. The tissue is now susceptible to foreign bodies that cause infection and severe damage, including necrosis. In response, the host elicits a wound-repair, inflammatory response, activating the tumor stroma [6]. This increases the availability of growth factors, facilitates tissue rearrangement, allows hematopoietic inflammatory cell infiltration and supports the formation and recruitment of blood vessels. The tumor takes advantage of these extrinsic mechanisms to promote its own growth, claiming oxygen and nutrients from the surrounding tissue. Together, the tissue damage, infection, inflammation and resource starvation the tumor causes on its local microenvironment compromises organ function and can claim the life of the host.

Distant Tumor Effects

In other instances, a tumor can exert detrimental effects beyond its local microenvironment. Since they occur at a distance from the primary and secondary tumors, these remote effects can’t be explained by tumor compression or invasion. In the 1940’s these tumor non-autonomous influences were classified as “paraneoplastic syndromes” [7]. They include physiological alterations to a wide variety of organ systems, including neurological, hematologic and endocrine, and result in high levels of morbidity and mortality. Some of these tumor-induced symptoms serve to diagnose early malignancies, while others are associated with late stage cancers and a poor prognosis. Recognizing these symptoms and understanding their underlying causes would

enable us to treat early-stage cancers in some cases, develop effective therapeutics, improve quality of life and response to therapy, and importantly, increase patient survival.

Paraneoplastic Syndromes

Paraneoplastic syndromes usually arise from tumor-produced hormones, peptides, antibodies, cytokines and metabolic waste, as well as through inappropriate immune crosstalk between the tumor and normal host cells [7, 8]. For example, tumor-secreted adrenocorticotropic hormone or corticotropin-releasing factor induces production and release of cortisol into the bloodstream. Known as Cushing Syndrome, this condition results in hypertension, muscle weakness and edema. On the other hand, tumor-secreted cytokines like IL-6 elevate platelet counts. This thrombocytosis is associated with advanced cancer and worsens clinical outcomes. In the case of impaired immune cross-reactivity, the body produces antibodies to target the tumor, but antigenic similarity of these neural antibodies results in an attack to the host's nervous system instead.

In some cases, there is a strong association between the type of tumor and a particular paraneoplastic effect. This is the case for two types of lung cancer [9]. The incidence of humoral hypercalcemia is highest among patients with squamous cell carcinoma, in which it can double that observed in other lung cancers. Here, high levels of tumor-secreted parathyroid hormone and parathyroid-related proteins induce bone resorption and imbalances in calcium and phosphate levels, resulting in renal failure and coma. Hence, hypercalcemia is usually indicative of poor prognosis. On the other hand, the Syndrome of Inappropriate Antidiuretic Hormone secretion (SIADH) is most common in small cell lung cancers. As the name suggests, tumor production of this hormone cause abnormal sodium levels and this hypo-osmotic condition can lead to adrenal insufficiency and heart failure, having a detrimental effect on patient survival.

Other paraneoplastic syndromes are broadly associated with various types of cancers. For example, Trousseau's Syndrome is a frequent paraneoplastic coagulopathy observed in many advanced cancers [10]. The roots of the migratory thromboses have been attributed to mucin or tissue factor (TF) produced by tumor cells. While these products give the tumor aggressive growth and angiogenic properties, they significantly decrease host survival. Indeed, soon after diagnosing himself with thromboembolism, Trousseau himself died of gastric cancer.

From the examples above, it is clear that paraneoplastic syndromes kill the host, regardless of differences in manifestation or organs affected. Moreover, all these syndromes cause enormous pain and discomfort, debilitating patients and making them less responsive to therapy. Current treatment is limited to elimination of the tumor mass, but a better understanding of how the tumor causes these lethal effects would significantly improve patient morbidity and survival.

CANCER CACHEXIA

Wasting Syndrome

A particularly devastating paraneoplastic syndrome is cancer cachexia [11, 12]. This metabolic syndrome is characterized by an involuntary decrease in body weight, specifically through the depletion of skeletal muscle and adipose reserves. Symptoms worsen as pre-cachexia develops into cachexia and become difficult to reverse at the refractory cachexia stage. Whether cachectogenic symptoms are detected when the tumor is still occult or evident only after a cancer diagnosis, this wasting syndrome correlates to a decreased survival. The progressive loss of weight heightens toxicity from chemotherapy and dampers the patient's response to this treatment and surgical interventions. When patients near a 30% weight loss, the functional impairment of essential organs, such as the respiratory muscle, leads to their death. Cachexia affects between 50-80% of cancer patients and contributes to 20% of their deaths. It is prevalent in only some types of cancer and its incidence varies within these: 80% in gastric and pancreatic cancers, 50% with lung and prostrate tumors and 40% in breast cancer and leukemias [13]. Cachexia is also associated with other chronic, end-stage diseases, including cancer, tuberculosis, AIDS and cystic fibrosis, but whether they share a common wasting mechanism remains unknown.

Nutritional Intake

Wasting phenotypes are indicative of an energy imbalance, which can result from its decreased intake or increased expenditure. Decreased food intake (anorexia) affects about half of cancer patients and is exacerbated as chemotherapy alters food taste and smell [13]. Although tempting to simply attribute the detrimental effects of cachexia to anorexia, important differences exist. During anorexia adipose reserves are used before muscle; in cachexia, these tissues are depleted simultaneously. Moreover anorexia affects both skeletal and visceral muscle, while cachexia results in the loss of skeletal muscle only [14]. Cases where the loss of skeletal and fat tissues precedes decreased food intake are further evidence that anorexia alone is not responsible for the wasting phenotypes in cancer patients. Nutritional supplementation and appetite stimulation are not sufficient to reverse cachectic wasting and the degree of malnutrition is not indicative of the stage of cachexia [12].

Energy Expenditure

In addition to a decreased nutritional intake, the energy imbalance in cachexia is also caused by increased energy expenditure [12, 13, 15]. One reason patients suffering from some cachectogenic cancers exhibit a higher Resting Energy Expenditure (REE) is that tumor growth itself is extremely energetically demanding. This growth relies on glucose; lactate recycling, which uses large amounts of energy, ensures the availability of this source of fuel. In the absence of sufficient oxygen for the Krebs Cycle, the tumor produces lactate instead of carbon dioxide, which can be converted back to glucose in the liver.

This process involves the Cori Cycle and results in an energy deficit as it uses more energy than that generated by the tumor when it converts glucose to lactate. Higher energy expenditure is also caused by tumor products or host-secreted cytokines that increase respiration and activate mitochondrial uncoupling proteins (UCPs) by altering the expression of genes controlling energy metabolism. Mitochondrial uncoupling of oxidative phosphorylation is associated with decreased ATP synthesis in skeletal muscle. Together, these mechanisms deplete the host's energy stores, resulting in a negative energy balance that contributes to cachectic wasting.

Muscle Wasting

In cachexia, the wasting of skeletal muscle is mediated by increased protein breakdown and decreased protein synthesis [11, 13, 15]. Proteolysis is a known ubiquitin-dependent process and the up-regulation of two E3 ligases, MAFBX (muscle atrophy F-box protein) and MURF1 (muscle RING finger-containing protein 1) are strongly associated with cachexia. Protein degradation is exacerbated by increased Myostatin and aberrant activation of the apoptotic p38 and JAK MAPK cascades.

Decreased protein synthesis is a result of limited amino acid availability, mediated by the acute-phase response (APR) and reduced insulin sensitivity[12, 16]. The APR is triggered to reduce and repair damage caused by infection, inflammation or tumor presence. It involves multiple local and systemic changes, including muscle protein depletion. Insulin insensitivity is a common characteristic of many cancer patients . Since proper insulin signaling is necessary for protein synthesis, nutritional supplementation alone is not sufficient to restore normal protein anabolic rates. Reduction of PAX7 and other regeneration proteins impedes restoration of the lost muscle mass [13].

Together, elevated protein catabolism and reduced anabolism are responsible for decreased ATP synthesis, muscle weakness and extreme fatigue.

Adipose Wasting

The other important hallmark of cancer cachexia is the loss of white adipose tissue (WAT). This process is mainly mediated by increased lipolysis and fat oxidation [12, 13]. The increased breakdown of adipose tissue into glycerol and fatty acids is induced by both increased activity of the hormone-sensitive lipase (HSL) and decreased sensitivity to the anti-lipolytic effect of insulin. Low activity of LPL, the lipoprotein lipase that is normally responsible for the uptake of fatty acids into WAT, also contributes to adipose wasting. WAT can also undergo "browning", a conversion into brown adipose tissue associated with uncoupling proteins that induces lipid mobilization and energy expenditure. Together the breaking down of WAT, its impaired uptake and conversion into BAT, depletes the host of these lipid reserves and results in a severe energy imbalance. Interestingly, inter-communication between muscle and fat tissue in response to tumor presence could further feed the response from the reciprocal tissue and result in greater tissue wasting.

Inflammation and Metabolic Alterations

Cachexia is reminiscent of wasting induced by infection, sepsis and inflammation [11, 13]. Similarly to these conditions, a tumor triggers systemic inflammation, releasing cytokines and chemokines that can activate lipolysis, muscle proteolysis and a systemic stress response. The stress response is mediated in part by glucocorticoids, which further contributes to muscle wasting. In many cases, the cytokines and chemokines can be secreted by the tumor itself.

Hypermetabolism is another consequence of systemic inflammation. Tumor growth demands large amounts of glucose and amino acids. These resources are further reduced by decreases in neuropeptide Y (NPY) that limit food intake [13]. Inflammation-induced metabolic alterations and other compensatory mechanisms, such as insulin resistance and diabetes mellitus, protect energy homeostasis despite these resource imbalances. Insulin resistance prevents protein synthesis despite available resources and increases risk of mortality. This systemic reduction in insulin sensitivity is caused by cytokines and the associated inflammatory response, increased expression of TNFα, decreased GLUT4 levels and certain cancer drugs, like corticosteroids [11, 14]. The resulting decreased glucose-uptake capacity and reduced PI3K activity lead to increased expression of Atrogin-1, MURF-1 and other E3 ligases involved in ubiquitin-dependent proteolysis of skeletal muscle.

Wasting Mediators

Mediators are classified by their source: those produced by the tumor and those that can be produced by either tumor or host cells. Tumor catabolic products include lipid mobilizing factor (LMF) and proteolysis-inducing factor (PIF), which induces adipose tissue breakdown and protein degradation, respectively [13]. These factors have no effect on appetite and hence are directly associated with cachexia. Certain tumors also induce high levels of myostatin, a key regulator of muscle homeostasis.

Cytokines, TNFα, Interleukin-1 and 6 and Interferon-γ can be produced by the tumor or by the host as a response to tumor presence [12]. These factors affect appetite, involve multiple organ systems and are commonly associated with a systemic inflammation. Given its essential role in inflammation, many cachexia studies have focused on TNFα or “cachectin” [17]. This pro-inflammatory cytokine can induce insulin resistance and increase the activity of E3 ligases, making it a strong candidate to mediate the wasting phenotypes. Levels of IL-6, another driver of systemic inflammation are correlated with weight loss and survival of cancer patients.

While initial identification of these factors was promising, over time, the validity of these proposed molecular mediators has been questioned. TNFα and Interleukin-6 in particular have received much attention as top drug target candidates, but the evidence supporting their role in human cachexia is mostly derived from rodent models [18]. In humans, the potential source of TNFα has

not been identified. Serum TNFα levels are not indicative of the progression of the condition and the expression of these genes in cachectic patients is no different than in other cancer patients. In the few trials testing the body weight benefits of therapies against the action or production of TNFα, it is still unclear whether weight improvements are simply due to water retention in combination with a minimal rescue of adipose tissue [19]. Effective therapies would need to demonstrate improvement of lean body mass. Antibodies against IL-6 receptor are more promising, but in rodent models this factor can only induce cachexia wasting when supplied in doses well beyond the normal physiological range [20]. Together these data suggest that while these mediators might be playing a role, we still do not fully understand the underlying molecular mechanisms linking them to wasting in human cancer patients. Moreover, the inability of these factors to fully recapitulate the human condition suggests it is very likely that other factors are also involved.

Therapeutic Treatments

For cancer patients suffering from cachexia, therapeutic options are currently limited. At the time of diagnosis, efforts to remove or reduce the size and spread of primary and secondary tumors are the main focus and little attention is given to the associated wasting condition. If any, cachexia treatment is given in the late stages, when reversing its detrimental effects becomes extremely difficult.

To treat the accompanying anorexia and restore the depleted energy reserves, nutritional supplementation is a common treatment for cancer patients. Fish oil in particular has been shown to decrease IL-6 serum levels, cortisol and PIF, while increasing insulin [21]. In addition to dietary supplementation, resistance exercises antagonize the muscle atrophy associated with wasting and aggravated by bed rest due to weakening and extreme fatigue [11].

With the identification of molecular targets, novel therapies are being developed, particularly against inflammatory agents [11]. Thalidomide, for example, blocks TNFα production by macrophages. Unfortunately, this drug has severe adverse effects, including thromboembolism and renal insufficiency. An IL-6 receptor antibody can restore body weight and reverse fatigue. It acts primarily by inhibiting muscle proteolysis, without affecting protein synthesis. Mice treated with antibodies against the myostatin and Activin receptor have higher survival rates despite no change in tumor growth.

Insulin administration is another promising venue. This therapy can counteract the increased protein breakdown and decreased synthesis that result from cachexia-induced insulin resistance, ameliorating symptoms and increasing survival rates [11]. Metformin, commonly used to treat type 2 diabetes is now considered a potential anti-cachexia treatment as it acts to increase insulin sensitivity.

Complexity

The presentation of cachexia depends on many factors, making its diagnosis and study of underlying mechanisms challenging [11]. Its onset and

severity can vary depending on the type of tumor, its location and size. Genetic heterogeneities in the host can greatly affect the elicited immune and inflammatory responses that contribute to wasting. Sexual dysmorphism can also influence the diagnosis and prognosis. Moreover, this wasting condition can be difficult to distinguish from accompanying comorbidities, including anorexia and chemotherapy toxicity, which affect each patient differently. In other instances, the signature weight loss associated with cachexia can be masked by weight gained from ascite and peripheral oedema formation or difficult to detect in the background of an increasing obesity epidemic. The additional adipose reserve of overweight patients can serve as a protective mechanism against the cancer-induced wasting.

Cancer therapy is another confounding factor. While aiming to inhibit tumor growth, chemotherapies usually target regulatory pathways common to tumor and host cells alike. Blocking anabolic process in normal tissues contributes to their wasting.

Overview

Cachexia is a complex, multi-factorial condition so its investigation and treatment require a multimodal approach. Cachectic patients benefit from early intervention. Identifying which cancer types are associated with cancer and developing biomarkers that can detect these at their earliest stages would provide a significant advantage. In addition, more sensitive measurements of changes in body weight, particularly muscle mass would enable physicians to treat wasting at as soon as the cancer is diagnosed.

Therapies against the few known cachexia mediators have been ineffective and those targeting systemic effects, like inflammation, can result in adverse side effects. A better understanding of the molecular mechanisms underlying this wasting would provide novel therapeutic agents and limit toxicity in normal tissues. In the era of personalized medicine, treatment options should be individualized to the wasting symptoms of each patient as the mix and severity of these varies.

The complexities mentioned above, regarding patient and tumor heterogeneity, comorbidities, accompanying cancer therapy and the questioning of results from rodent studies, have limited our understanding of the underlying factors mediating cancer cachexia and prevented the development of effective therapies for this wasting condition. Future investigations would benefit from a fresh approach and simpler perspective to gain insight into the molecular mechanisms of cachexia amidst its underlying complexities.

DROSOPHILA AS A MODEL SYSTEM

Drosophila as a Human Disease Model

Drosophila is a powerful model to begin to dissect the complexity of cachexia and other tumor-host interactions. Having been studied for over 100 years, the fruit fly is perhaps the best characterized organism across a wide range of developmental, growth and behavioral processes as well as the disease states that result when these processes go awry. Flies have given us a better understanding of the intrinsic cellular and molecular mediators as well as the extrinsic environmental factors that regulate the formation and progression of various disease states including neurodegenerative disorders and cancer [22]. These studies have benefited from the comprehensive genetic toolbox that the fly offers and the high degree of evolutionary conservation of shared molecular signaling pathways. Indeed, 50-75% of genes involved in human diseases have a corresponding match in Drosophila and functional studies of these genes are made easier in fly because of its lower genetic redundancy [23, 24].

Their small size, short life cycle and the low cost of obtaining large numbers has made Drosophila the subject of large-scale, high-throughput screens to identify genes underlying essential developmental processes or diseases. Recently, the fly is becoming a new platform to streamline the process of drug discovery [25, 26]. It can be used to identify novel targets or test libraries of approved chemical compounds individually or in combination. Carried out in whole organisms, this approach can be more informative than previous cell culture-based screens and can quickly determine the efficacy and toxicity of drugs.

Drosophila as a Cancer Model

Although spontaneous tumors are not observed in the fly, certain genetic lesions can recapitulate key hallmarks of mammalian tumors, such as tissue overgrowth, loss-of-polarity, uncontrolled proliferation, failure to differentiate and in some cases, lethality to the host [27]. In *Drosophila*, these can be generated as genetic mosaics or clones within a wild-type tissue, recreating the situation in which most human tumors arise and allowing the investigation of cell autonomous and environment-dependent effects. The fact that some of these genetic lesions are in genes that are also mutated in human tumors and most importantly, the similarity in the molecular mechanisms underpinning the cancer-like phenotypes, have served to validate *Drosophila* as a model for certain aspects of cancer.

Drosophila has pioneered the identification of several growth-regulation pathways and tumor progression mechanisms that have deeply impacted our understanding of key factors driving human cancer. Recent examples include the identification of the Hippo pathway and the phenomena of cell competition and apoptosis-induced compensatory proliferation.

The discovery of the Hippo pathway and its role in growth control and regeneration has identified new cancer mediators [28, 29]. Studies prompted by

fly research have demonstrated that components of this pathway are mis-regulated in human carcinomas and indicate poor prognosis.

Cell competition was described more than 30 years ago in flies. This mechanism that selects for the most advantageous cells to maximize tissue fitness is akin to “field cancerization” in human tumors, where the most malignant cells expand at the expense of their neighbors [25, 30]. Activation of Myc or inactivation of the Hippo pathways, which give fly tumor cells a competitive advantage, are also deregulated in human cancers and could serve to give clonal populations a competitive advantage during early tumor development [31].

Compensatory proliferation, another well-studied phenomenon in flies, has informed our understanding of the role of non-autonomous signals that stimulate tumor growth. In flies, apoptosis-resistance cells, “undead cells” secrete mitogens to induce proliferation of their neighbors [32, 33]. This has motivated investigations to determine whether a similar mechanism drives tumor growth in humans and whether these mitogens could be derived from similar undead cells or from surrounding cells as a tumor-induced inflammatory response.

TUMOR GROWTH IN DROSOPHILA

Tissue Growth Regulation

One of the key contributions of Drosophila to our understanding of human cancer has been the identification of the autonomous mechanisms that regulate tissue growth. In Drosophila, growth and development have been predominantly studied in imaginal discs, groups of proliferating epithelial cells in the larvae that give rise to adult structures. Transplantation of these discs into adult flies is a classic technique initially used to study their regeneration and differentiation. In addition, the nutrient rich medium of the adult female abdomen serves a tissue culture disc, enabling us to use transplantation to also investigate the intrinsic and extrinsic factors mediating imaginal disc growth.

Genetic screens searching for regulators of growth and proliferation have predominantly identified recessive mutations in tumor suppressor genes (TSG). TSGs normally act to inhibit tumor formation, but when inactive, result in tumorigenesis. Loss-of function mutations in tumor suppressor genes can transform an imaginal disc with organized polarity and architecture into a tumorous mass [34]. These immortal tumors can be maintained through serial transplantations [35], allowing the investigation of factors involved in tumor progression. Importantly, these “fly tumors” recapitulate key features of human solid tumors. They are self-sufficient in growth and proliferation and capable of evading apoptosis and anti-proliferation signals; they also fail to differentiate [27]. Some tumors are even capable of invading into neighboring tissues and metastasizing to distant sites [36, 37]. TSG mutations can generate two types of tumors with different characteristics: malignant neoplastic and benign hyperplastic tumors.

Neoplastic Tumors

Interestingly, it was Drosophila where the first tumor suppressor gene was revealed: the *lethal (2) giant larvae, lgl*, gene was discovered in 1930’s by Bridges and studied over a decade by Hadorn and colleagues regarding its developmental role. It was not until 1967 that Gateff et al. demonstrated that mutations in this gene result in abnormal growth, inability to differentiate and lethality [38, 39]. Lgl forms part of what is now known as the “Scribble Polarity Module”, along with discs large (dlg) and scribble (scrib) [40, 41]. Mutations in any of the components of this polarity complex induce the formation of neoplastic tumors in larval brains and imaginal discs.

While uncontrolled growth and proliferation is common to neoplastic and hyperplastic tumors alike, neoplastic tumors have key distinguishing features [34]. First, growth is accompanied by a loss of polarity and tissue organization that disrupts the architecture of the epithelial monolayer. The rate of tumor growth is slower than that of wild-type cells, making tumor cells susceptible to elimination by cell competition. The tumorous mass is also unable to differentiate into an adult structure. Finally, neoplastic tumors have invasive capabilities and can form secondary tumors at distant sites. Given their malignant nature, these tumors eventually kill the host.

Hyperplastic Tumors

Hyperplastic mutations produce very large tumors. One of the main regulators of cell growth and proliferation is the Hippo pathway; mutations in components of this signaling cascade give rise to extensive, hyperplastic tumors, allowing us to use it as a model to understand the key characteristics and mechanisms of this tumor type [34, 42]. Alterations in the Hippo pathway give cells a growth advantage and resistance to apoptosis. Despite an increased growth rate and enhanced proliferation potential, the polarity and organization of these hyperplastic tumors are maintained. Cells remain within the epithelial monolayer and overall architecture is unaltered. Hyperplastic tumors are still capable of differentiation and can give rise to adult structures. Importantly, tumor growth remains confined within the basement membrane, so these benign tumors have a less severe interaction with host.

Genetic Cooperation

Genetic mosaics combining loss of function of neoplastic tumor suppressor genes and oncogenic activation reproduce the heterogeneity, the clonal nature and multi-step formation of human cancers and provide tumors with additional advantages. *Scrib* mutations produce a small neoplastic mass that proliferates uncontrollably, but grows at a slow rate due to elimination of cells undergoing JNK-mediated apoptosis [43, 44]. The activated prototypical human oncogene Ras, Ras^{V12}, induces hyperplastic growth, but is insufficient for these cells to invade other tissues. Genetic cooperation between *scrib* and Ras^{V12} inhibits JNK-mediated cell death, giving rise to a much larger neoplastic mass and results in aggressive invasive capabilities, not only to adjacent tissues, but also to secondary, distant sites [36]. Similar robust neoplastic progression and metastasis is observed in *scrib NotchACT* tumors cooperation models [37]. The proliferative and survival advantages are induced whether these genetic alterations occur in the same cell or between neighbors, indicating malignant cooperation at the cell-autonomous and non-autonomous levels.

Limitations

Key features of human tumors, including loss of polarity, uncontrolled growth, inability to differentiate and aggressive invasion, can be recapitulated in Drosophila, but to correctly use the fly as a model of human cancers, the limitations of this system also need to be considered. Flies do not normally develop cancer, but a single mutation in a TSG can transform an epithelial, imaginal disc into a tumorous mass [43]. This single mutation transformation is very different from the numerous genetic changes required in the multi-step process of mammalian tumor formation. Moreover, the imaginal discs of origin have no true human counterpart.

It is also worth noting that some of the genetic alterations capable of generating a fly tumor might not play an essential role in humans and vice-versa. *Scrib* is a major neoplastic tumor suppressor gene in Drosophila; mutations in this gene induce robust tumor growth [43]. *Scrib* deregulation can also induce

dysplasia in mammary epithelia and it is downregulated or mislocalized in transgenic mammary tumors and human breast carcinomas, but *scrib* is not frequently mutated in human cancers [45, 46]. Similarly, some mutations that are common in human tumors do not contribute to fly tumorigenesis: mutations in fly p53 do not result in tissue over-proliferation [40].

Another important aspect to consider is invasion and metastasis. The *scrib* Ras^{V12} genetic cooperation tumor model can induce migration and invasion of tumor cells to distant sites, but this process is very different from the human metastatic cascade [47]. The open circulatory system of the fly impedes the study of intravasation and extravasation from blood and lymph vessels and the recruitment of tracheal branches to the tumors is reminiscent, but not equivalent to angiogenesis.

Bearing in mind these and other differences, Drosophila remains an excellent model to decipher the molecular basis of conserved biological processes, like growth and proliferation. Studies in the flies enable the rapid analysis of the mechanism of action of key regulators of these evolutionary conserved processes. Understanding the function of TSG and oncogenes under normal physiological conditions provides invaluable insight into their abnormal contribution during disease states like cancer.

TUMOR-HOST INTERACTIONS IN DROSOPHILA

While autonomous tumor features have been well-described in Drosophila, the fly has recently gained attention as a model for understanding distant tumor-host interactions. These long-range effects in Drosophila recapitulates those of human cancers in the systems and organs they involve and in the role they play in tumor progression and host survival.

Developmental Delay

One of the most obvious effects of tumor growth in Drosophila is the “giant larvae” phenotype, which gave name to the first neoplastic tumor suppressor gene, *lethal (2) giant larvae* [38]. This is the result of an extended larval phase in which the tumor mass continues to grow uncontrollably, 3-5 days beyond the normal transition into the pupal phase. In some cases, tumorous growth can completely block metamorphosis. This major developmental transition is a tightly regulated process that involves multiple signals coordinated by the endocrine system, particularly ecdysone [48-50]. Production of prothoracicotropic hormone (PTTH) in the brain regulates the production and secretion of this hormone, increasing levels significantly before metamorphosis. Ecdysone activation induces gene expression changes that result in molting and pupariation. At the time of this transition, the organism must ensure that the growth and development of all organs, despite their different proliferation rates, are matched to the same stage, in order to achieve the right symmetry and proportionality among them. Certain circumstances, including tissue damage, growth abnormalities or bacterial infections, can prevent an organ from reaching the right stage at the right time. To cope with these events, the transition can be delayed; the extent of delay is proportional to the severity of these factors on growth and development. This delay provides an opportunity for damaged or abnormal tissue to repair or regenerate to its expected size and shape, essentially giving the organ enough time to “catch-up” before transitioning to the next stage.

Fly tumors can delay the larvae-to-pupae transition via long-range interactions with the endocrine system [51, 52]. Dilp8, a tumor produced and secreted factor, acts at a distance to delay expression of *disembodied* and *phantom*, regulators of ecdysone biosynthesis. This ecdysone inhibition delays metamorphosis. *Dilp8* also mediates the developmental delay resulting from X-ray-irradiation and genetic- and chemical-induced tissue damage, providing further evidence that tumors act as “wounds that never heal” and elicit a similar repair response from the host.

Tumor Immune Surveillance

Deciphering the role of the immune system in human cancer has been a difficult task because, depending on the context, it can protect against tumor growth or accelerate its progression. Drosophila also displays these confounding roles in the presence of a tumor, but its simple innate immune system has allowed for an easier dissection of this complex interaction [53]. In Drosophila, distant interactions with the host’s immune system can not only detect, but also

counter tumor growth [54]. In their quest to metastasize malignant neoplastic tumors will break their underlying basement membrane, allowing a route for tumor cells to exit. Tumor immune surveillance detects this loss of tissue integrity and recruits hemocytes, the fly's blood cells, to the site of damage to initiate tissue repair and constrain tumor growth. A similar response is observed in the presence of a wound, providing evidence that tumors are equivalent to chronic wounds. Tumor expression of the cytokine *upd3* activates JAK/STAT signaling in the recruited hemocytes, inducing their proliferation. Additional cytokine expression in hemocytes and the distant fat body encourages further proliferation. This increased number of circulating hemocytes strengthens the immune response and further restricts tumor growth.

At the same time, this interaction with the host immune system can also promote tumorigenesis and metastasis [55]. Hemocytes recruited to the tumor also express high levels of TNFα. While TNFα induces apoptosis in polarity deficient tumor clones as part of the cell-competition response, in combination with the activated oncogene RasV12, these tumors now evade cell death. In this genetic cooperation model, hemocyte-expressed TNFα now promotes growth of the local tumor and the invasion of tumor cells in distant sites.

Invasion and Metastasis

Metastasis accounts for the majority of human cancer deaths as treatment options at this stage are limited. The study of conserved morphogenesis and developmental plans in Drosophila has provided insights into the mechanistic programs that are hijacked by tumor cells to acquire migratory and invasive capabilities [56-58]. Moreover, several fly models recapitulate in situ and distant metastasis and can be subjected to genetic screening to identify factors mediating metastatic progression.

Transplantations of larval tumors into the abdomen of an adult fly is a technique akin to mouse tail vein injections of tumor cells and has been extensively used to asses metastatic potential in Drosophila [59]. Back in 1978, Gateff and colleagues first documented the proliferative and invasive capabilities of transplanted *lgl* tumors [60]. In the fly, tumor metastasis after transplantation is a rare event; the frequency of metastatic events varies greatly depending on the tumor's genetic composition and tissue of origin, as well as time after transplantation and site of secondary tumor formation [61].

Importantly, the metastatic spread of neoplastic tumors share similarities with human tumors, including increased collagenase type IV and dMMP1 [62, 63]. These two factors enable the active crossing of basement membranes by tumor cells. Indeed, *lgl* tumors are able to exit the primary tumor mass, cross the epithelial sheath that surrounds the host's ovarioles and form micrometastasis at this distant site. Genetic screens have served to identify other factors involved, such as semaphrin-5c [64].

The *scrib* Ras^{V12} genetic cooperation tumor model has also served to model certain aspects of metastasis [36]. These tumor cells are capable of aggressive invasion to nearby tissue as well as to remote location in the larvae. Like in invasive human tumors, loss of E-cadherin in *scrib* Ras^{V12} cells is

important for their mobilization and Mmp1 is necessary for extracellular matrix degradation and tumor exit.

Lethality

A wide variety of in situ and transplanted tumor models claim the life of their host, but we still do not understand how this lethal tumor-host interaction is mediated. While the contribution of other tumor-host interactions needs to be evaluated, insight into death mechanisms can also be gained by understanding how aging flies die. Few investigations have focused on the underlying pathophysiology of aging, but intestinal permeability and cardiac stress have been proposed.

In aging flies, fat body immunosenescence induces a strong systemic inflammation response, promoting gut hyperplasia that induces intestinal permeability [65, 66]. This intestinal barrier dysfunction is associated with increased antimicrobial peptides and altered metabolism and is correlated with a fly's lifespan. Loss of cardiac function is another strong predictor of death in flies [67]. As in elderly humans, aging flies exhibit increased stress-induced heart failure and a significant reduction in resting heart rate, inducing rhythmic disturbances that result in poor cardiac performance. As tumors also induce a systemic inflammation response and can affect organs at a distance, it would be interesting to test whether tumor-bearing hosts exhibit intestinal permeability or declining heart function.

Since the discovery of the first TSG, it has been reported that neoplastic tumors induce bloating in the larvae or of the abdominal cavity upon transplantation [68]. While this is one of the earliest and most evident effects of tumor presence, its contribution to host death remains unknown. Determining whether the accumulation of excess hemolymph is a result of changes in osmolarity/metabolism, abnormal function of the malphigian tubules, aberrant hemolymph production or other factors could shed light into the contribution of this poorly understood tumor-host interaction.

SUMMARY

Despite our knowledge of the molecular mechanisms driving tumor growth, proliferation, survival and metastasis, the effects of this growth on peripheral tissues remain enigmatic. These distant tumor-host interactions are the major culprits in the death of cancer patients, yet our limited understanding of how these kill remains a major obstacle to the development of effective therapeutic treatments.

In this thesis I present a Drosophila model that serves to investigate these important questions in cancer biology. In Chapter 1, I review our current understanding of cancer lethality and the paraneoplastic syndromes that are responsible for the lives of so many cancer patients. I focus on cancer cachexia, a particularly detrimental tumor-host interaction, its known effects and molecular mediators. I also critically analyze the evidence supporting these proposed mediators in an attempt to highlight the key aspects that demand further research. Finally, I discuss the strengths and weaknesses of Drosophila as a model for cancer and complex tumor-host interactions.

In Chapter 2, I use our Drosophila model to dissect the molecular mechanisms underlying cancer cachexia. I use a combination of molecular and genetic approaches to identify ImpL2 as a specific tumor-secreted cachectic mediator. I show that ImpL2, the homolog of the human Insulin Growth Factor Protein (IGFBP), is necessary and sufficient to induce robust wasting of peripheral muscle, adipose and gonadal tissues. My results demonstrate that consistent with its role as an insulin antagonist, tumor-secreted ImpL2 interrupts insulin signaling in host tissues. We propose this induces systemic insulin resistance and results in the observed wasting phenotypes.

In Chapter 3, I use this same model to further investigate cancer cachexia as well as other aspects of tumor-host interactions. I take a closer look into the wasting of muscle and gonadal tissues and further characterize the apoptotic phenotypes. I test other candidate factors that might be involved in mediating the wasting response. I also examine metastasis in this model. While metastatic events are rare and unpredictable, I find their frequency increases with time and correlates with host death. I also record my findings on tumor-induced abdominal bloating and death curves.

Together, my thesis work establishes a simple, but powerful Drosophila model to dissect the complexity underlying distant tumor host interactions, including cancer cachexia and metastasis. Importantly, the observed effects on peripheral tissues of Drosophila recapitulate key characteristics of human cancer conditions and this model revealed a novel mediator of cachexia wasting.

CHAPTER 2

Malignant Drosophila tumors interrupt insulin signaling to induce cachexia-like wasting

This chapter is a reproduction of the paper by the same name published in Developmental Cell April 2015. For this paper, I performed all the experiments, made all the figures and wrote the manuscript with D.B.

SUMMARY

Tumors kill patients not only through well-characterized perturbations to their local environment, but also through poorly understood pathophysiological interactions with distant tissues. Here we use a Drosophila tumor model to investigate the elusive mechanisms underlying such long-range interactions. Transplantation of tumors into adults induces robust wasting of adipose, muscle and gonadal tissues that are distant from the tumor, phenotypes that resemble the cancer cachexia seen in human patients. Interestingly, malignant but not benign tumors induce peripheral wasting. We identify the Insulin Growth Factor Binding Protein (IGFBP) homolog ImpL2, an antagonist of insulin signaling, as a secreted factor mediating wasting. ImpL2 is sufficient to drive tissue loss, and insulin activity is reduced in peripheral tissues of tumor-bearing hosts. Importantly, knocking downs *ImpL2* specifically in the tumor ameliorates wasting phenotypes. We propose that the tumor-secreted IGFBP creates insulin resistance in distant tissues and thus drives a systemic wasting response.

INTRODUCTION

Cancer is a leading cause of death in industrialized societies, yet the mechanisms by which a tumor claims the life of its host are not always clear. In some cases the growth of primary or secondary tumors disrupts the function of essential organs, but in other instances lethality is caused by physiological alterations at a distance from the tumor site. These distant influences, sometimes grouped as 'paraneoplastic syndromes', are major contributors to the morbidity and mortality of cancer patients.

A particularly debilitating distant tumor-host interaction is cancer cachexia, which is estimated to occur in >80% of patients with advanced cancers and to account for >20% of cancer deaths [11, 12]. Cancer cachexia is a metabolic disorder that produces progressive tissue wasting, most evident in loss of adipose and muscle tissue. Cachectic patients show heightened risk of respiratory failure, increased susceptibility to chemotherapeutic toxicity, and other lethal sequelae. Unlike anorexia, in which caloric intake is reduced, wasting induced by cachexia is not reversed by supplemental nutrition. Available therapies for this clinically critical condition are notably limited in scope and effect.

Mediators of cancer cachexia remain mysterious. Cachexia is seen frequently with certain types of tumors and only rarely with others. Patient studies are complicated by heterogeneities in patient population, presentation, tumor pathology, comorbidities, and accompanying therapeutic regimes. Most experimental investigations of cachexia rely on transplants of tumor cell lines into rodents [18]. These studies have implicated several factors such as IL-6, TNF-α, and metabolic products, but these reflect only a subset of human cachectic conditions [15]. Overall, the dearth of knowledge of mechanisms by which tumors induce cachexia has prompted the National Cancer Institute to highlight it as a 'Provocative Question' limiting progress in cancer treatment (provocativequestions.nci.nih.gov).

In recent years, studies in Drosophila have contributed significant insight into genetic factors driving human cancer. Analysis of fly 'oncogenes and tumor suppressor genes' have led for instance to the identification of the Hippo pathway [29, 42] and uncovered the cancer-relevant phenomenon of cell competition [25, 30]; flies have also been used to develop new cancer therapeutics [26]. While most fly cancer models focus on autonomous tumor growth, fly tumors also show interactions with their host, including invasion of local tissues [36, 61] and recruitment of innate immune cells [54, 55]. The recent appreciation of parallels in physiological regulation between flies and humans [69] creates an opportunity to use Drosophila as a simple system to study mechanisms by which cancer can perturb this homeostasis. Here we use a Drosophila model to demonstrate that fly tumors can induce cachexia-like phenotypes, and identify a tumor-secreted factor that drives wasting by preventing insulin signaling reception in peripheral tissues.

RESULTS

Tumor Induces Cachexia-Like Wasting in Host Tissues

Transplantation of imaginal discs from larvae into the hemocoel, the open body cavity of adults, is a classical technique for evaluating tissue growth [35]. We transplanted GFP-labeled eye discs that were either WT or else mutant for the tumor suppressor gene *scrib* and overexpressing oncogenic Ras^{V12}, an established genetic cooperation system that induces malignant Drosophila tumors [36, 37]. As shown by Pagliarini and Xu (2003), initially ~70 mm^2 *scrib* Ras^{V12} tumor fragments grew continuously and induced a distinctive bloating of the abdomen before killing the host, when the tumor reached ~1,000 mm^2, whereas WT discs grew only a limited amount before ceasing and the transplanted host survived for weeks **(Fig. 1A,B; Fig. S1A-E)**. We observed small numbers of cells disseminating from the tumor and invading other tissues, but these events were rare. At 5 days after transplantation, when the tumor is ~ 600 mm^2 **(Fig. S1C,E)**, metastatic-like events were seen in <5% of the hosts.

While autonomous features of the tumor have been well-documented, we asked whether the tumor also had non-autonomous effects on the host. By contrast to the rare metastatic events, we discovered that on day 5, 100% of tumor-bearing hosts showed robust wasting phenotypes in tissues distant from the transplant. No phenotypes were seen in control hosts transplanted in parallel with WT disc fragments, indicating that wasting is not due to surgery nor any potential microbial infection. We first examined adipose tissues. Transplantation into hosts carrying an adipose reporter revealed a marked reduction in the fat body throughout the abdomen, irrespective of proximity to the tumor **(Fig. 1C,D)**. In addition to the reduction of tissue mass, analysis of individual fat body cells with the lipophilic dye Nile Red demonstrated enlargement of lipid droplets, a phenotype associated with resource depletion **(Fig. 1E,F)** [70]. We next analyzed muscle. Microscopic examination of mitochondrially-imported GFP reveals that whereas mitochondria are regularly spaced between indirect flight muscle fibers of WT adults, packing in tumor-bearing hosts is irregular with a distinctly abnormal morphology **(Fig. 1G, H)**. This phenotype is also seen in flies with degenerating muscle and mitochondrial fragmentation [71-73], and consistent with this interpretation, muscle ATP levels were strongly reduced in tumor-bearing hosts **(Fig. 1M)**. Functional tests revealed muscle weakness phenotypes specifically in tumor-bearing hosts. In climbing assays, both ability and speed **(Fig. 1K, L)** were strongly reduced, suggesting deteriorating muscle function [74]. Muscle defects were progressive, being evident at 3 days and increasing in severity with time after transplant.

The largest tissue in the adult female is the ovaries, where tumor-induced tissue loss was particularly evident. Female hosts transplanted with WT discs contained ovaries filling a substantial portion of the abdomen, but ovaries in tumor-bearing flies were almost rudimentary **(Fig. 1I,J)**. A fly ovary contains ~16-

20 ovarioles, each of which produces a sequential series of egg chambers that develop into mature eggs [75]. We quantitated ovarian reduction by evaluating the health of each ovariole, defined as its ability to produce a late-stage egg chamber. Tumor-bearing hosts showed an 85% reduction in ovarian health **(Fig. 1N)**; apoptosis of mid-stage egg chambers was evident **(Fig. 2E,F)**, and there was a complete absence of late-stage and mature eggs. As with fat and muscle, ovarian phenotypes were highly penetrant and did not depend on physical contact with the tumor. Transplantation into male hosts also induced similar tissue wasting (**Fig. S1F-O**), indicating that these phenotypes are not sex-specific. Together, these results demonstrate that *scrib Ras*^{V12} tumors influence distant tissues, including muscle, fat and gonads, to undergo a wasting-like phenotype reminiscent of human cachexia.

Tumor-Induced Wasting is not due to Starvation

Deterioration of adipose, gonadal and muscle tissue are also phenotypes seen in adult flies undergoing starvation **(Fig. 2D,H)** [76-78]. We therefore tested the possibility that tumor-bearing hosts were unable to consume normal amounts of food, leading to systemic malnutrition. We first used a qualitative test to measure ingestion [79], and found no difference between hosts transplanted with a tumor and control hosts transplanted with a WT disc fragment **(Fig. 2I)**. We then quantitated food consumption over a 24 hour period using the capillary feeding (CAFÉ) assay [80]. Again, no difference was found between tumor-transplanted and control transplanted hosts **(Fig. 2J)**. Finally, we used an acute consumption assay [81] that can distinguish feeding behavior: starved flies will consume larger amounts than fed flies in a short (5 minute) time period (Liming Wang, personal communication). In this feeding assay, as in the other two, tumor-bearing hosts did not differ from hosts transplanted with a WT disc fragment **(Fig. 2K)**. Some human patients suffering from cancer exhibit reduced appetite, and such patients can benefit from supplementary caloric intake. We found that raising tumor-bearing fly hosts on high-calorie food did not rescue tissue wasting (data not shown), demonstrating that altered food intake (anorexia) is not responsible for tissue deterioration.

ImpL2 is Secreted by Malignant Tumors and Sufficient to Induce Wasting

As tumor-bearing flies feed normally but show wasting, we hypothesized that the tumor might interfere with the host's normal physiological response to food intake. To investigate the mechanism, we began by considering whether all fly tumor genotypes were capable of eliciting a similar response. We first tested the two components of the genetic cooperation system individually. Despite their slower growth, transplanted *scrib* tumors recapitulated the ovarian wasting phenotypes induced by *scrib Ras*^{V12} tumors **(Fig. 3E,F)**, even when *scrib* tumors were ~3 fold smaller **(Fig. 3A,B)**. By contrast, transplanted *Ras*^{V12} tumors had no effect **(Fig. 3G)**, even though these tumors grew to be ~2 fold larger than *scrib* **(Fig. 3B,C)**. In Drosophila, *Ras*^{V12} expression alone induces benign 'hyperplastic' growth [82] that does not disturb epithelial architecture, remains confined by a basement membrane, and retains differentiation potential; these are all

characteristics distinct from the malignant 'neoplastic' growth of *scrib* tumors [40, 83]. To distinguish the effects of tumor burden from tumor character, we transplanted hyperplastic tumors induced by ectopic activation of the Hippo pathway transcription factor Yki (Yki^{SA}) [84]. *yki*^{SA} -expressing tumors grow in adult hosts in an epithelial fashion and become much larger (~4-6 fold) than *Ras*^{V12} as well as *scrib Ras*^{V12} tumors **(Fig. 3D)**. Nevertheless, even the largest such tumors did not induce ovarian degeneration, (**Fig. 3H),** nor defects in fat or climbing speed as did much smaller *scrib* tumors **(Fig. S2A-H)**. These findings demonstrate that it is not the size, but rather the nature of the tumor that defines its ability to induce wasting.

Since *scrib Ras*^{V12} and *scrib* tumors trigger wasting in tissues distant from their location, we hypothesized that the effect could be mediated by a secreted factor. We therefore searched transcriptome datasets [85] for candidate factors upregulated specifically in malignant tumors but not benign tumors or WT discs. Two secreted factors that are upregulated >10-fold in *scrib* and *scrib Ras*^{V12} discs are the IL-6-like cytokine Upd2 [86], and ImpL2 [87], a homolog of IGF-binding proteins; we also considered the insulin-like peptide dILP8 [51, 52] which is >100-fold upregulated in *scrib, scrib Ras*^{V12,} and *yki*^{SA} **(Fig. 3I)**. To test these candidates, we expressed each using a hindgut-specific GAL4 driver and examined whether wasting of gonadal and adipose tissue was induced. Interestingly, only *ImpL2* expression resulted in a substantial reduction in ovarian size, which was accompanied by apoptotic egg chambers, loss of mature eggs and reduced fecundity **(Fig. 3J-L)**. Hindgut-driven *ImpL2* expression also reduced abdominal fat body and induced lipid droplet enlargement **(Fig. S2O-Q)**. Ectopically-driven *ImpL2* levels in hindgut were comparable to those expressed by *scrib Ras*^{V12} tumors **(Fig. S2S),** and similar ovarian and adipose wasting phenotypes were observed when expressing *ImpL2* using a muscle-specific GAL4 driver **(Fig. S2I-N)**. Thus, excess ImpL2 production alone, independent of tumor growth, is sufficient to induce wasting in distant adult tissues.

Tumor Reduces Insulin Signaling Pathway Activity in Host Tissues

ImpL2 has been demonstrated to bind insulin in solution and to antagonize the insulin pathway *in vitro* [87] and *in vivo* [88]. If ImpL2 secreted from *scrib Ras*^{V12} tumors plays a role in peripheral wasting, then tumor-bearing host tissues should show reduced insulin signaling. To assess this, we transplanted into hosts expressing an insulin pathway reporter. *tGPH* produces a GFP protein fused to the PH domain of GRP1, which is recruited to the plasma membrane upon insulin-stimulated PI3-Kinase activity [89]. We found that in tumor-bearing but not control hosts, tGPH remains substantially cytoplasmic in both the fat body **(Fig. 4A,B)** and in egg chambers **(Fig. 4C,D)**, indicating defective insulin signaling reception. We also used qPCR to measure mRNA levels o*f 4E-BP*, a downstream FOXO target that is elevated when insulin signaling is low. In both muscle and ovary, *4E-BP* levels are increased in tumor-bearing as compared to control hosts **(Fig. 4E)**. Importantly, we compared levels of Drosophila Insulin-like Peptides (dILPs) in their neuroendocrine source cells between tumor-bearing

and control hosts (**Fig. S3A,B**) and did not detect the retention seen for instance in nutrient-depleted flies [90]. Metabolic assays revealed elevated circulating trehalose levels **(Fig. 4F)**, characteristic of hyperglycemia, but minor or no differences in triglycerides and glycogen, respectively **(Fig. S3C-E)**. As defects in insulin secretion are not evident, but peripheral tissues nevertheless experience reduced insulin signaling and high circulating sugar levels, this suggests that the tumor induces insulin resistance.

ImpL2 is Necessary for Robust Tumor-Induced Cachexia-Like Wasting

Finally, to test whether tumor-secreted ImpL2 in fact mediates the wasting phenotypes, we depleted its activity specifically within the malignant tumor. We knocked down *ImpL2* via RNAi in an *eyeless GAL4*-driven dlg^{RNAi} Ras^{V12} tumor model [91] that causes both autonomous growth and non-autonomous wasting comparable to *scrib* Ras^{V12} **(Fig. 5C,E,G,I, J)**. Importantly, dlg^{RNAi} Ras^{V12} $ImpL2^{RNAi}$ tissue formed a tumorous mass within five days of transplantation, dlg^{RNAi} Ras^{V12} **(Fig. 5A,B)**. Strikingly, however, reducing *ImpL2*within the tumor itself significantly ameliorated each of the peripheral tissue phenotypes. Hosts bearing dlg^{RNAi} Ras^{V12} $ImpL2^{RNAi}$ tumors showed increased abdominal fat body mass **(Fig. 5C,D)**; rescue of this tissue was also evident in the restoration of lipid droplet size **(Fig. 5E,F)**. Muscle function assays further revealed improvements in both climbing ability and speed **(Fig. 5I,J)**. Lastly, there is significant rescue of ovariole health, leading to a restoration of egg production **(Fig. 5G,H,K)**. Rescue was only observed when *ImpL2* was knocked down in the tumor; transplanting dlg^{RNAi} Ras^{V12} tumors into *ImpL2* null hosts did not ameliorate wasting (**Fig. S4**). The lack of full recovery with *ImpL2*-depleted tumors suggests that additional factors associated with malignancy may contribute. Nonetheless, the substantial rescue demonstrates that it is a central secreted factor driving tumor-induced tissue wasting.

DISCUSSION

Cachexia remains a major obstacle to cancer treatment, in part because the molecular mechanisms that drive it remain uncertain. Here, we describe a fly model that mimics certain aspects of human cachexia, and utilize this model to identify a specific cachectic mediator. The tumor-induced wasting that we describe in flies resembles cancer cachexia in its independence from food consumption, its target tissues, its progressive nature, and its induction by certain but not all types of tumors. The fly model does not parallel all features associated with the human condition; for instance, we detect only slight upregulation of putative fly orthologs of mammalian regulators implicated in muscle catabolism (**Fig. S3F**) [16, 92]. Human cancer cachexia is clearly a heterogeneous and multifactorial condition [15], and this complexity has impeded progress in its understanding. In this work we use a reductionist system to identify a single tumor-derived factor that can drive the robust deterioration of peripheral tissues.

Insulin signaling is a central regulator of tissue mass in both flies and humans. Our data demonstrate that ImpL2, a secreted insulin antagonist produced by malignant tumors, is a major mediator that is both necessary and sufficient for wasting. In an accompanying paper, [93] show that ImpL2 is also a systemic wasting factor in a different fly tumor model. Reduced insulin signaling is further responsible for wasting induced by mycobacterial infection of flies [94]; whether ImpL2 is the relevant mediator in this case is not known. ImpL2 is the single fly homolog of mammalian IGFBPs, and can bind to systemic insulin-like ligands to antagonize insulin signaling. By this mechanism, the tumor effectively induces insulin resistance in peripheral tissues.

Insulin resistance is a frequent feature of both cachectic patients and rodent cachexia models [14, 95]; indeed some evidence suggests that exogenous insulin can ameliorate tissue loss in these contexts. The seven mammalian IGFBPs are variously upregulated or downregulated in different tumors, but have been evaluated in cancer primarily with respect to their affects on tumor growth [96]. Our data motivate assessments of whether highly cachectogenic human tumors, such as pancreatic and gastric cancers, display elevated expression of IGFBPs, and how therapies designed to correct insulin resistance might be used to treat such tumors.

ImpL2 joins the list of effectors induced by neoplastic transformation in fly tumors, including mitogens and pro-invasive factors. Recent work from our lab shows that the Upd3 mitogen is upregulated by dual activity of JNK and Hippo signaling [85]. The ImpL2 regulatory region, like that of Upd3, contains evolutionarily conserved binding sites for AP-1 and Sd transcription factors, suggesting that it may also be synergistically regulated by these pathways that monitor epithelial integrity. Despite the reduced insulin signaling in neoplastic tumors themselves (e.g. *4EBP* levels are elevated ~21 fold [85]; they are hypersensitive to PI3K reduction [91]), the tumors nevertheless robustly proliferate. How ImpL2-upregulating tumors escape insulin resistance remains

an unanswered question, although metabolic changes suggested by transcriptome alterations may be a possible mechanism.

While tumor-specific inhibition of ImpL2 causes a significant amelioration of the wasting phenotype, rescue is not complete, suggesting that other aspects of tumor-host interaction remain to be uncovered. We found that a fly homolog of IL-6, a molecule implicated in several rodent cachexia models, was not sufficient to induce wasting, while partial ablation of host innate immune cells [97] did not qualitatively alter wasting phenotypes (data not shown); however, contributing roles for these factors have not been ruled out. Future work will analyze other tumor-produced factors, including metabolites generated by anabolic and catabolic alterations in the tumor, to evaluate their involvement as well. The manipulability of the simple model developed here, including the ability to rapidly assess fully-defined combinations of host and tumor genotypes, opens the door to candidate as well as forward genetic approaches to identify additional factors mediating tumor-host interactions.

EXPERIMENTAL PROCEDURES

Genetics and Transplantation

scrib^{1} *Ras*^{V12} and *Ras*^{V12} tumors were generated using the eyMARCM genetic system as in [36]. In knockdown experiments, *dlg*^{RNAi} *Ras*^{V12} [91] was used in combination with *white*^{RNAi} (Bloomington #28980) or *ImpL2*^{RNAi} (VDRC #30930); similar results were seen with an independent *ImpL2*^{RNAi} (NIG-FLY #1590-R3). *Yki* tumors were obtained from *MS1096-GAL4 UAS Yki*^{S168A} larvae and *scrib* tumors from *scrib*^{1} homozygotes. Transplantation was adapted from [61]: WT or tumorous imaginal discs were dissected from wandering third instar larvae, fragmented and introduced through a pulled glass capillary needle into the abdomen of one day old virgin females. Host females were kept on a high-yeast diet, in the presence of males at 25º C, except for starved females who were kept on water only. Hosts were either *OreR, Yolk GAL4 UAS-GFP, Mef2 GAL4 UAS-Mito-GFP, tGPH* [89] or *ImpL2*^{Def20} [87]. Ectopic expression experiments used *Mef2GAL4 GAL80ts* or *bynGAL4 GAL80ts*, raised at 18º and then shifted to 29º as adults to drive expression of *UAS-s.ImpL2* [87]*, UAS-dILP8* [51] and *UAS-Upd2* [90].

Feeding, Locomotive, and Ovarian Assays

Ingestion was scored using a blue dye feeding assay adapted from [79]. Briefly, FD & C Blue No.1 food dye (2.5% w/v) was incorporated into yeast paste in order to visualize and score the presence of food in the crop and intestinal areas. CAFÉ [80] and PER [81] assays were perfomed in triplicate to measure consumption and feeding behavior, respectively (Liming Wang, personal communication)**.** For CAFÉ, groups of 6 females were allowed to feed *ad libitum* for 24 hours on liquid food (5% yeast extract and 5% sucrose) dispensed from calibrated glass capillaries (World Precision Instruments); amounts consumed were measured and adjusted for evaporation. For PER, groups of 4 females were presented with a calibrated capillary (Drummond Scientific Company) containing liquid food and the amount consumed per feeding response was measured until fly was satiated. Presentation of water was used to control for thirst.

Climbing ability and speed assays were adapted from [98] and [73]. Groups of 10 females were placed in empty vials and after 1 hour of recovery were gently tapped to the bottom. Climbing ability was determined by the number of flies that reached an 8cm mark in 20 seconds; speed was calculated using the length of climbing time. For each group, 3 trials were recorded per assay; experiments were conducted in triplicate.

Ovarian health was quantified as the percentage of ovarioles that contained one or more non-apoptotic egg chamber at stage 9-10.

Microscopy and Image Analysis

All samples were fixed in 4% formaldehyde in phosphate buffered saline (PBS). Staining followed standard protocols, with anti-Dilp2 antibody (1:2,000; J. Veenstra), TRITC-phalloidin (1:400; Sigma), DAPI (1:1,000; Life Technologies) or

Nile Red (1:5,000; Sigma). Images were captured on a Zeiss 700 confocal microscope. Images of ovaries, tumors, fat body and testis were assembled from tiled confocal scans of single samples. Figures were assembled using Adobe Illustrator.

Quantitative RT-PCR

Total RNA was prepared from groups of 15 tumors, 20-30 ovaries (RNeasy Mini Kit; QIAGEN), 20-25 thoraces and 12-16 whole flies (TRIzol reagent; Invitrogen and Direct-zol RNA MiniPrep Kit; Zymo Research). qPCR experiments were performed in triplicate using SYBR GreenER qPCR SuperMix. Relative quantification of mRNA levels was determined using the Comparative C_T method and normalized to *alpha-tubulin* (thoraces), *rpl23* (ovaries) and *gapdh* (tumors and whole flies).

ATP Measurements

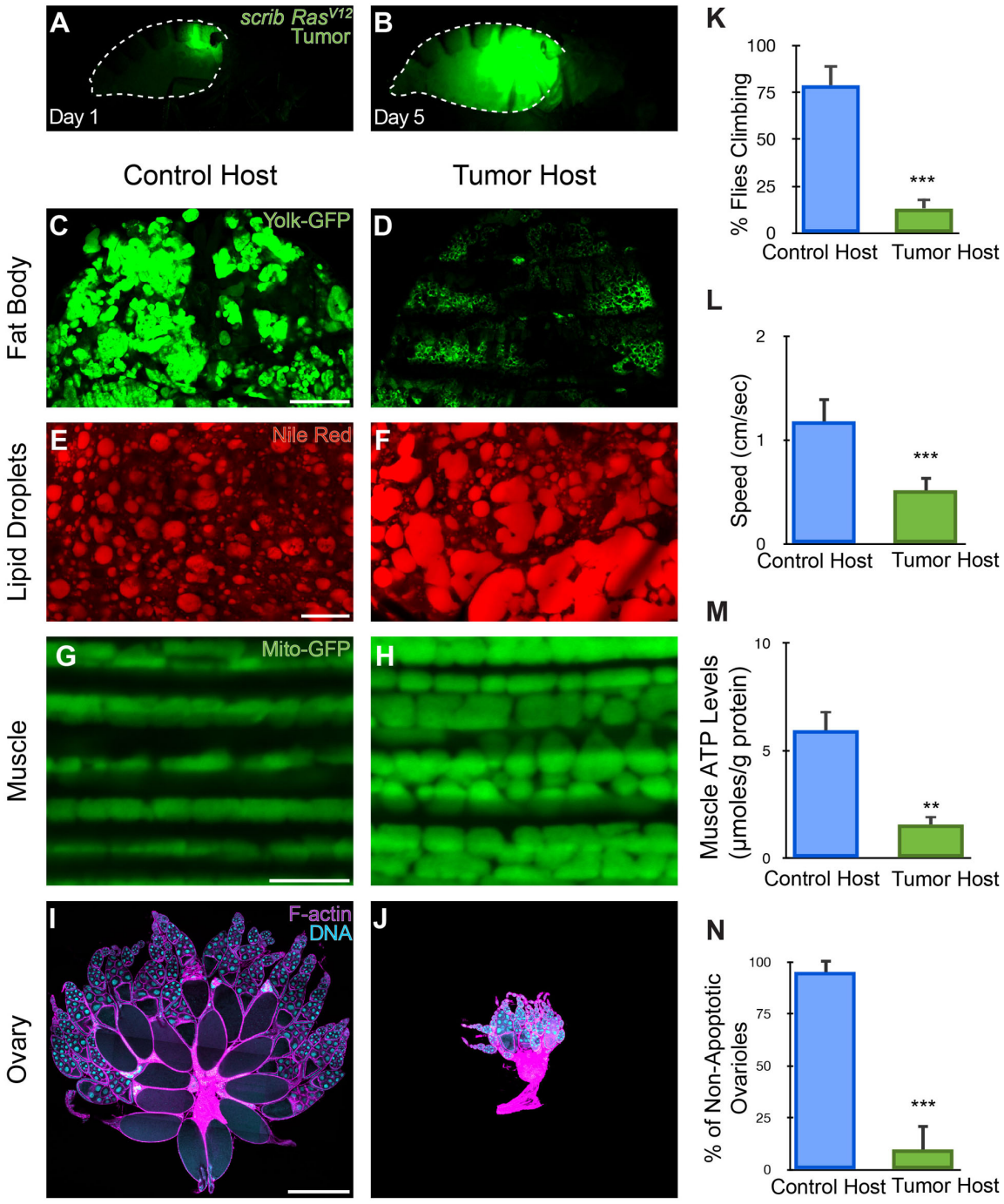
In triplicate, 5 thoraces were homogenized in 80 µl of extraction buffer (6 M Guanidine Hydrochloride, 4mM EDTA, 100mM Tris-HCl, pH 8.0), boiled for 5 minutes and centrifuged at 4º for 5 minutes. Supernatant was collected and diluted 1:50. ATP levels were quantified using an ATP Determination Kit (Life Technologies/Invitrogen) and normalized to total protein levels (Bradford method).

Metabolic Assays

For Glucose, Glycogen and TAG assays, 5 flies, in triplicate, were homogenized in PBST, heated to 70º for 10 minutes and centrifuged; supernatant was collected. Samples were processed and levels measured using manufacturer's protocols: Glucose (HK) Assay Kit (Sigma), Glycogen Colorimetric Assay Kit (BioVision) and Triglycerides LiquiColor Test (Stanbio Laboratory), respectively. Protein levels were determined with the BCA Protein Assay Kit (Thermo Fisher Scientific) and used for normalization.

For circulating trehalose assays, 1 µl of hemolymph, in triplicate, was collected by centrifugation and diluted in Trehalase Buffer. Samples were heated at 70º for 10 minutes and treated with porcine trehalase (Sigma). Levels were measured using the Glucose (HK) Assay Kit (Sigma) following manufacturer's protocol. Total levels were calculated after subtracting free glucose and normalized per fly.

FIGURE 1


A
scrib Ras^V12
Tumor
Day 1
B
Day 5
Control Host
Tumor Host
Fat Body
C
Yolk-GFP
D
Lipid Droplets
E
Nile Red
F
Muscle
G
Mito-GFP
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Ovary
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F-actin
DNA
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Speed (cm/sec)
2
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Control Host
Tumor Host
M
Muscle ATP Levels
(µmoles/g protein)
10
5
0
**
Control Host
Tumor Host
N
% of Non-Apoptotic
Ovarioles
100
75
50
25
0
***
Control Host
Tumor Host

Figure 1. **Drosophila tumors can induce peripheral wasting**

(**A, B**). GFP-labeled *scrib Ras*^{V12} tumor transplanted into WT adult host, after 1 and 5 days. (**C-N**) Phenotypes of peripheral tissues in control and tumor-hosting females. Fat body-specific reporter indicates depletion of this tissue in the abdomen of hosts in the presence of the tumor (**C, D;** green=Yolk-GFP). Lipid droplets, which are the storage vesicles of the adipose tissue, mobilize and aggregate into enlarged units (**E, F**; red=Nile red). Mitochondria-localized reporter reveals abnormal structure in the thoracic muscle of tumor-bearing hosts (**G, H**; green=Mito-GFP). Reduced ATP levels (**M**) and defects in both climbing ability (**K**) and climbing speed (**L**) indicate compromised muscle function. Ovaries are severely shrunken (**I,J**; F-actin=magenta; nuclei=cyan) in tumor-bearing compared to control hosts. **N** quantifies ovarian health as the percentage of non-apoptotic stage 9-10 ovarioles (see Materials and Methods). Scale bars: C=250µm; E=25µm; G=5µm; I=500µm. **p<0.01, ***p<0.001, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE 2

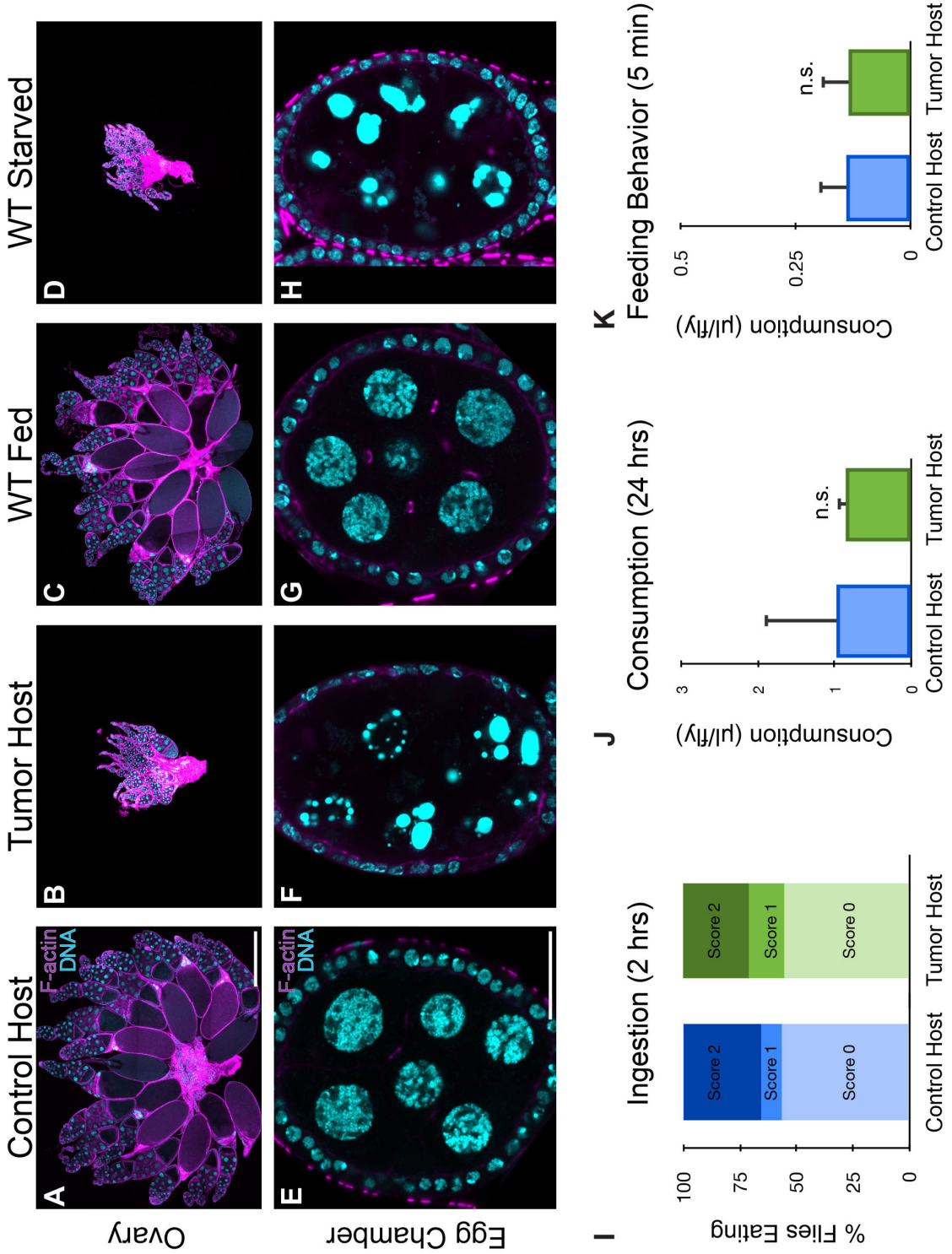

Control Host
Tumor Host
WT Fed
WT Starved
Ovary
Egg Chamber
A
B
C
D
E
F
G
H
F-actin
DNA
I
Ingestion (2 hrs)
% Flies Eating
100
75
50
25
0
Score 2
Score 1
Score 0
Control Host
Tumor Host
J
Consumption (24 hrs)
Consumption (µl/fly)
3
2
1
0
n.s.
Control Host
Tumor Host
K
Feeding Behavior (5 min)
Consumption (µl/fly)
0.5
0.25
0
n.s.
Control Host
Tumor Host

Figure 2. Tumor-bearing hosts are not starved

Whole ovaries (**A-D**) and mid-stage follicles (**E-H**) of control hosts and tumor-bearing hosts, compared to those of WT fed and starved flies. Ovary wasting and follicle apoptosis in tumor-bearing hosts resemble that seen in starved WT flies, as evidenced by shrunken ovarian size and nuclear fragmentation. Assays measuring food ingestion, by scoring the presence of dyed-food in the intestinal tract (**I;** Scoring: 0= no food in abdomen; 1= some food detected; 2= gut and crop are full), prolonged capillary food consumption (CAFÉ) (**J**), and short-term feeding behavior by proboscis extension (**K**) show no significant differences between control hosts and tumor-bearing hosts. Scale bars: A=500µm; E=25µm. ns= p> 0.05, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE 3

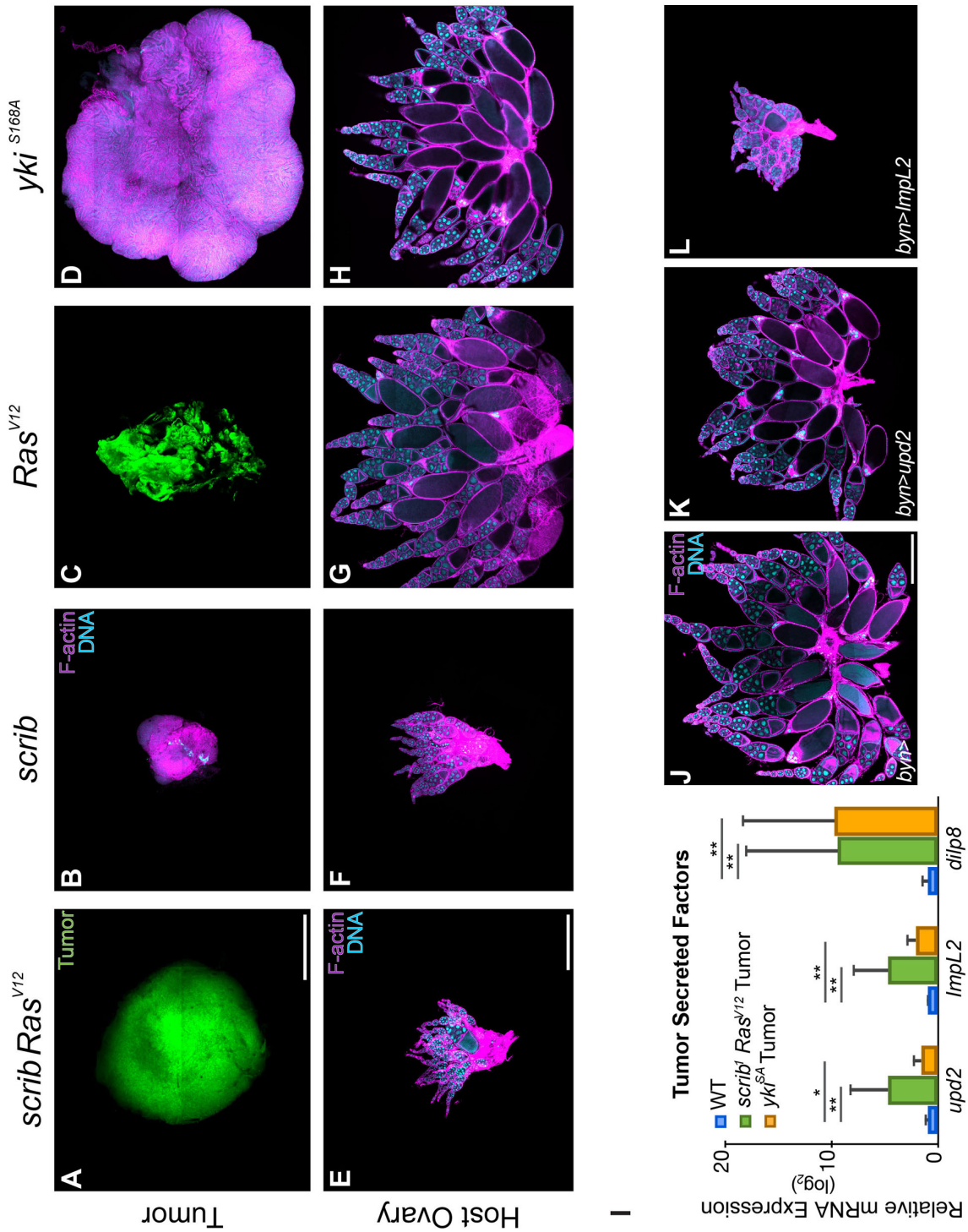

scrib Ras V12
scrib
Ras V12
yki S168A
Tumor
Host Ovary
Tumor
F-actin
DNA
byn>
byn>upd2
byn>ImpL2
Tumor Secreted Factors
WT
scrib1 RasV12 Tumor
ykiSA Tumor
Relative mRNA Expression
(log2)
20
10
0
upd2
ImpL2
dilp8
*
**

Figure 3. Neoplastic but not hyperplastic tumors induce wasting

Growth of tumors (**A-D**) of different genotypes at 5 days post-transplantation, along with associated ovarian phenotypes (**E-H**). *scrib Ras*^{V12} and *scrib* tumors induce wasting, while *Ras*^{V12} and *yki*^{SA} tumors do not; wasting is independent of tumor burden. (**I**) Quantitative RT-PCR measurement of levels of transcripts (log_2 scale) encoding candidate secreted factors in *scrib Ras*^{V12} vs. *yki*^{SA} tumors compared to controls. (**J-L**) Hindgut-driven ectopic expression demonstrates that ImpL2 but not Upd2 is sufficient to drive ovarian wasting. Scale bars: 500μm. *p<0.05, **p<0.01, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE 4

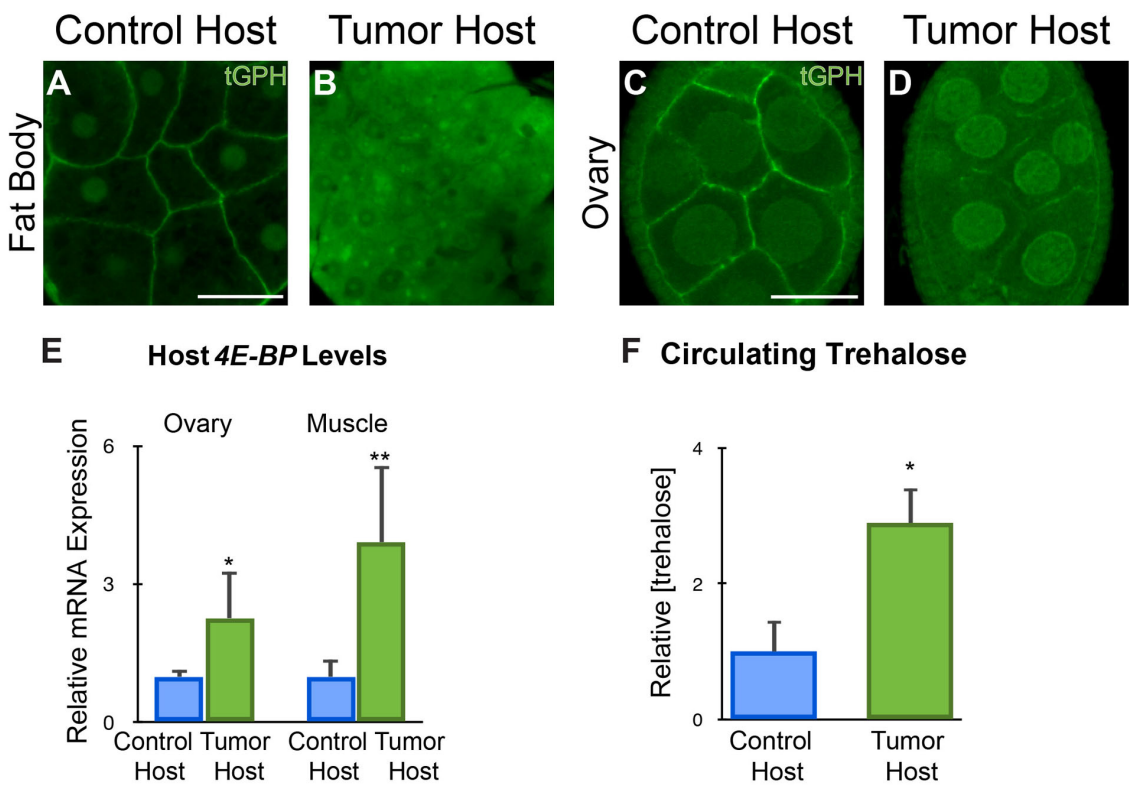

Control Host
Tumor Host
Control Host
Tumor Host
A
tGPH
B
C
tGPH
D
Fat Body
Ovary
E
Host 4E-BP Levels
Relative mRNA Expression
Ovary
Muscle
6
3
0
*
**
Control Host
Tumor Host
Control Host
Tumor Host
F
Circulating Trehalose
Relative [trehalose]
4
2
0
*
Control Host
Tumor Host

Figure 4. Tumor alters insulin signaling and metabolism in host

Compared to control, *scrib Ras*^{V12} tumor-bearing hosts show decreased plasma membrane recruitment of the tGPH reporter in fat body (**A, B**) and ovaries (**C, D**), illustrating decreased insulin signaling reception. (**E**) Increased transcription of the FoxO target *4E-BP* by quantitative RT-PCR measurement in ovary and thorax also reveals decreased insulin signaling activity. Metabolic analysis (**F**) reveals elevated circulating trehalose levels in tumor-bearing hosts relative to controls; absolute values: Table S2. Scale bars: A, C=25µm. ns= $p > 0.05$, *$p<0.05$, **$p<0.01$, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE 5

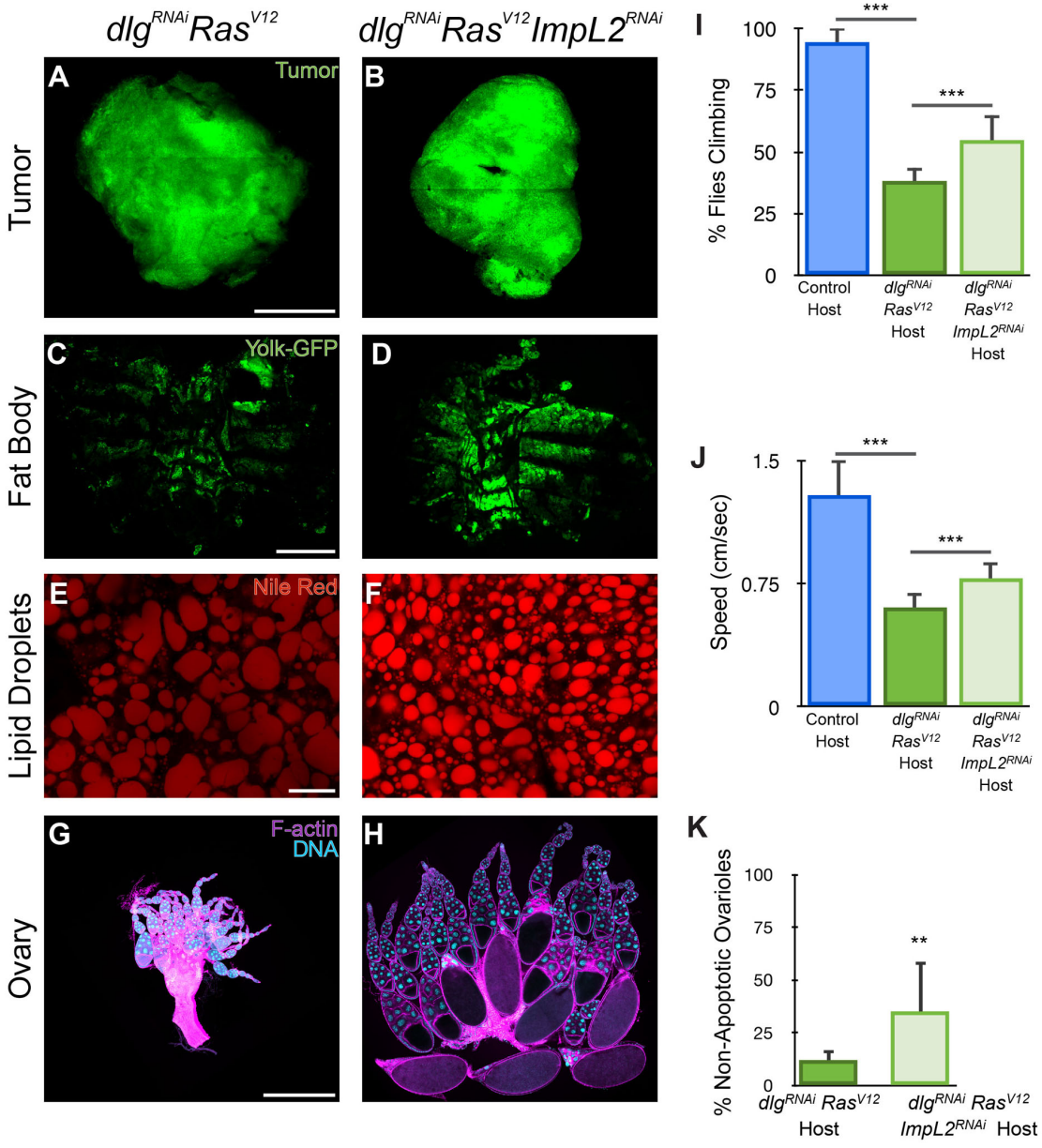

dlg^RNAi Ras^V12
dlg^RNAi Ras^V12 ImpL2^RNAi
Tumor
Fat Body
Lipid Droplets
Ovary
A
Tumor
B
C
Yolk-GFP
D
E
Nile Red
F
G
F-actin
DNA
H
I
% Flies Climbing
100
75
50
25
0
***
***
Control Host
dlg^RNAi Ras^V12 Host
dlg^RNAi Ras^V12 ImpL2^RNAi Host
J
Speed (cm/sec)
1.5
0.75
0
***
***
Control Host
dlg^RNAi Ras^V12 Host
dlg^RNAi Ras^V12 ImpL2^RNAi Host
K
% Non-Apoptotic Ovarioles
100
75
50
25
0
**
dlg^RNAi Ras^V12 Host
dlg^RNAi Ras^V12 ImpL2^RNAi Host

Figure 5. Tumor-secreted ImpL2 is necessary and sufficient for robust wasting

dlg^{RNAi} *Ras*^{V12} *ImpL2*^{RNAi} and *dlg*^{RNAi} *Ras*^{V12} tumors are comparable in size (**A, B**). Knockdown of *ImpL2* in *dlg*^{RNAi} *Ras*^{V12} tumors improves host abdominal fat body mass (**C, D**), reduces lipid droplet aggregation (**E, F**), and restores ovarian tissue size (**G, H**) and health (**K**) as compared to hosts bearing *dlg*^{RNAi} *Ras*^{V12} tumor alone. Knockdown of *ImpL2* in *dlg*^{RNAi} *Ras*^{V12} tumors also restores host muscle function, as measured by climbing ability (**I**) and speed (**J**). Scale bars: A, C, G=500µm; E=25µm. **p<0.01, ***p<0.001, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE S1

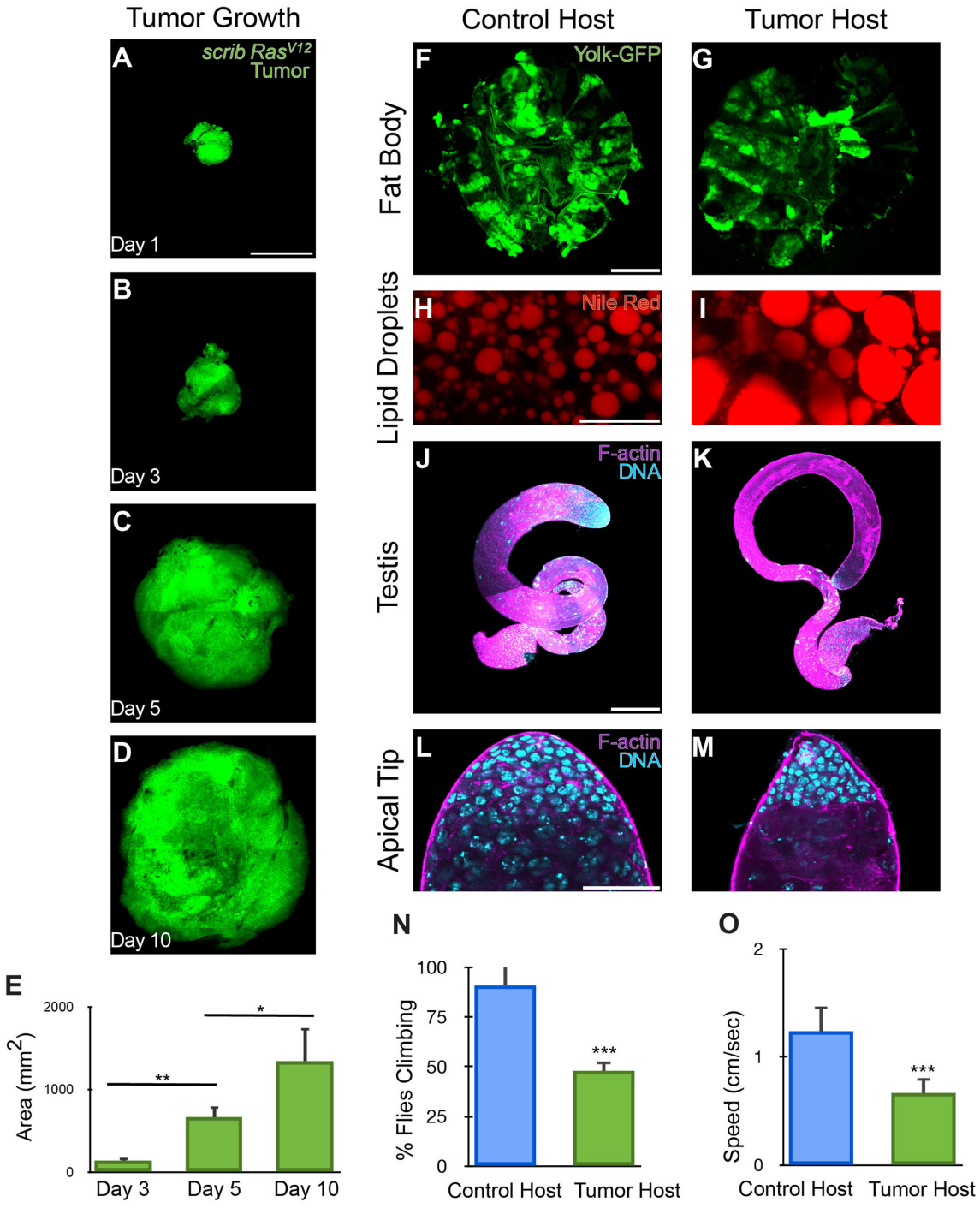

Tumor Growth
Control Host
Tumor Host
A
scrib RasV12
Tumor
Day 1
B
Day 3
C
Day 5
D
Day 10
F
Yolk-GFP
G
Fat Body
H
Nile Red
I
Lipid Droplets
J
F-actin
DNA
K
Testis
L
F-actin
DNA
M
Apical Tip
E
Area (mm2)
2000
1000
0
**
*
Day 3
Day 5
Day 10
N
% Flies Climbing
100
75
50
25
0
***
Control Host
Tumor Host
O
Speed (cm/sec)
2
1
0
***
Control Host
Tumor Host

Figure S1. (Related to Figure 1)

scrib^{1} *Ras*^{V12} tumor at 1 (**A**), 3 (**B**), 5 (**C**) and 10 (**D**) days post-transplantation with corresponding size quantitation (**E**). Phenotypes of peripheral tissues in male hosts: total abdominal fat body is reduced in tumor-bearing hosts (**F, G;** green=Yolk-GFP) with size abnormalities in lipid droplets (**H, I**; red=Nile red). Shrunken testis (**J,K**; F-actin=magenta; nuclei=cyan) and reduced proliferation in apical tip (**L, M**; F-actin=magenta; nuclei=cyan) indicate gonadal wasting in the presence of a tumor. Muscle function as assayed by climbing ability (**N**) and speed (**O**), is also compromised. Scale bars: A, F, J=500µm; H=25µm; L=50µm. *p<0.05, **p<0.01, ***p<0.001, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE S2

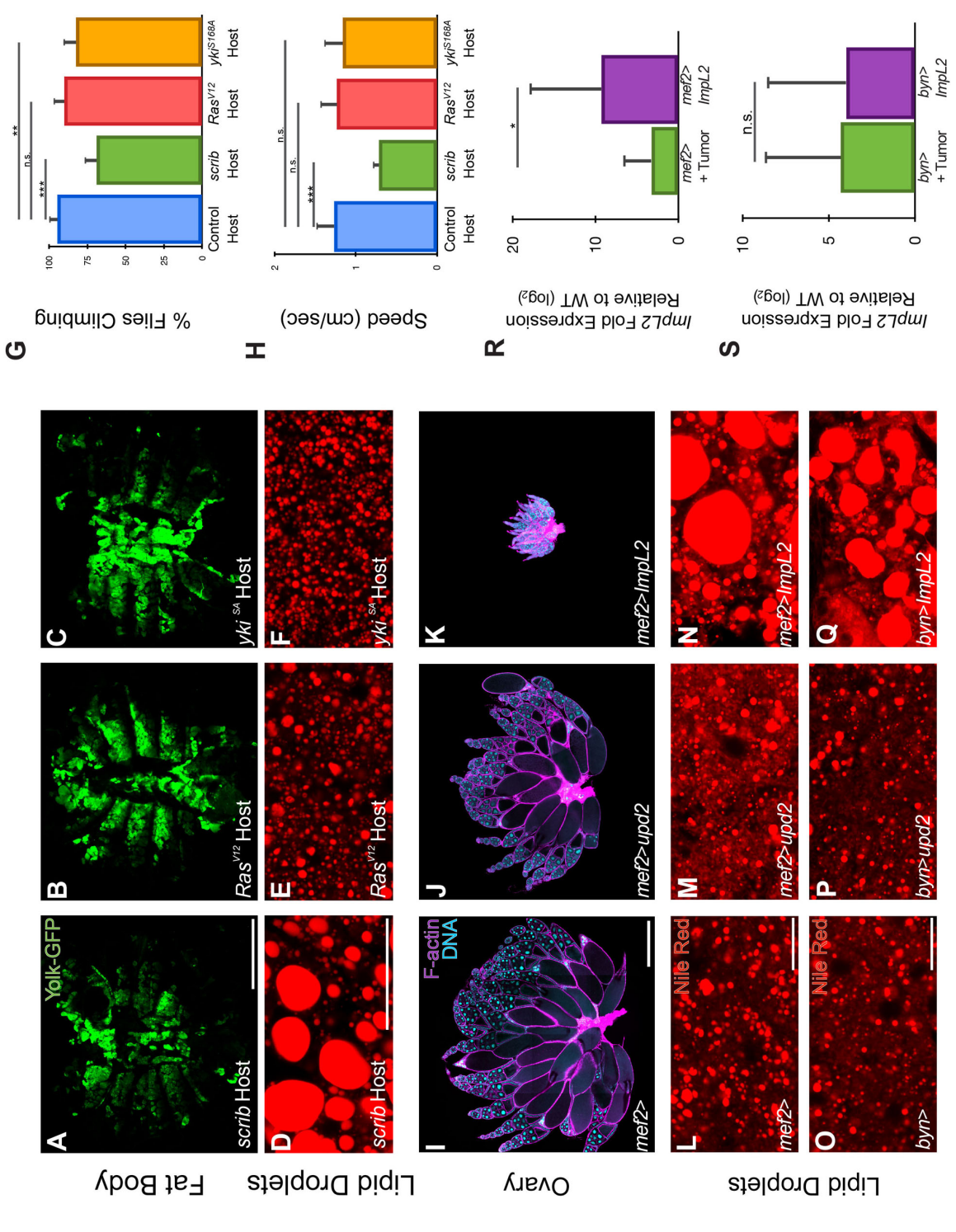

Fat Body
Lipid Droplets
Ovary
Lipid Droplets
A
Yolk-GFP
scrib Host
B
Ras^V12 Host
C
yki SA Host
D
scrib Host
E
Ras^V12 Host
F
yki SA Host
I
F-actin
DNA
mef2>
J
mef2>upd2
K
mef2>ImpL2
L
Nile Red
mef2>
M
mef2>upd2
N
mef2>ImpL2
O
Nile Red
byn>
P
byn>upd2
Q
byn>ImpL2
G
% Flies Climbing
Control Host
scrib Host
Ras^V12 Host
yki^S168A Host
H
Speed (cm/sec)
R
ImpL2 Fold Expression Relative to WT (log2)
mef2> + Tumor
mef2> ImpL2
S
ImpL2 Fold Expression Relative to WT (log2)
byn> + Tumor
byn> ImpL2
n.s.

Figure S2. (Related to Figure 3)

Compared to *Ras*^{V12} and *yki*^{SA} tumors, only *scrib*^{1} tumors robustly induce loss of adipose tissue (**A-C**), lipid droplet enlargement (**D-F**) and defects in muscle function, as assayed by climbing ability and speed (**G, H**). Muscle-driven ectopic expression of *ImpL2* or *Upd2* shows that only *ImpL2* is sufficient to induce ovarian wasting (**I-K**) and abnormal lipid droplet aggregation (**L-N**). Similar results are observed in adipose tissue when inducing ectopic expression with a hindgut driver (**O-Q**). Quantitative RT-PCR measurements (log_2 scale) indicate that *ImpL2* levels produced via the hindgut driver are comparable to tumor-produced levels, while muscle-driven expression is higher (**R,S**). Scale bars: A**,** **I**=500µm; D,L,O=25µm. n.s.=p>0.05, *p<0.05, **p<0.01, ***p<0.001, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE S3

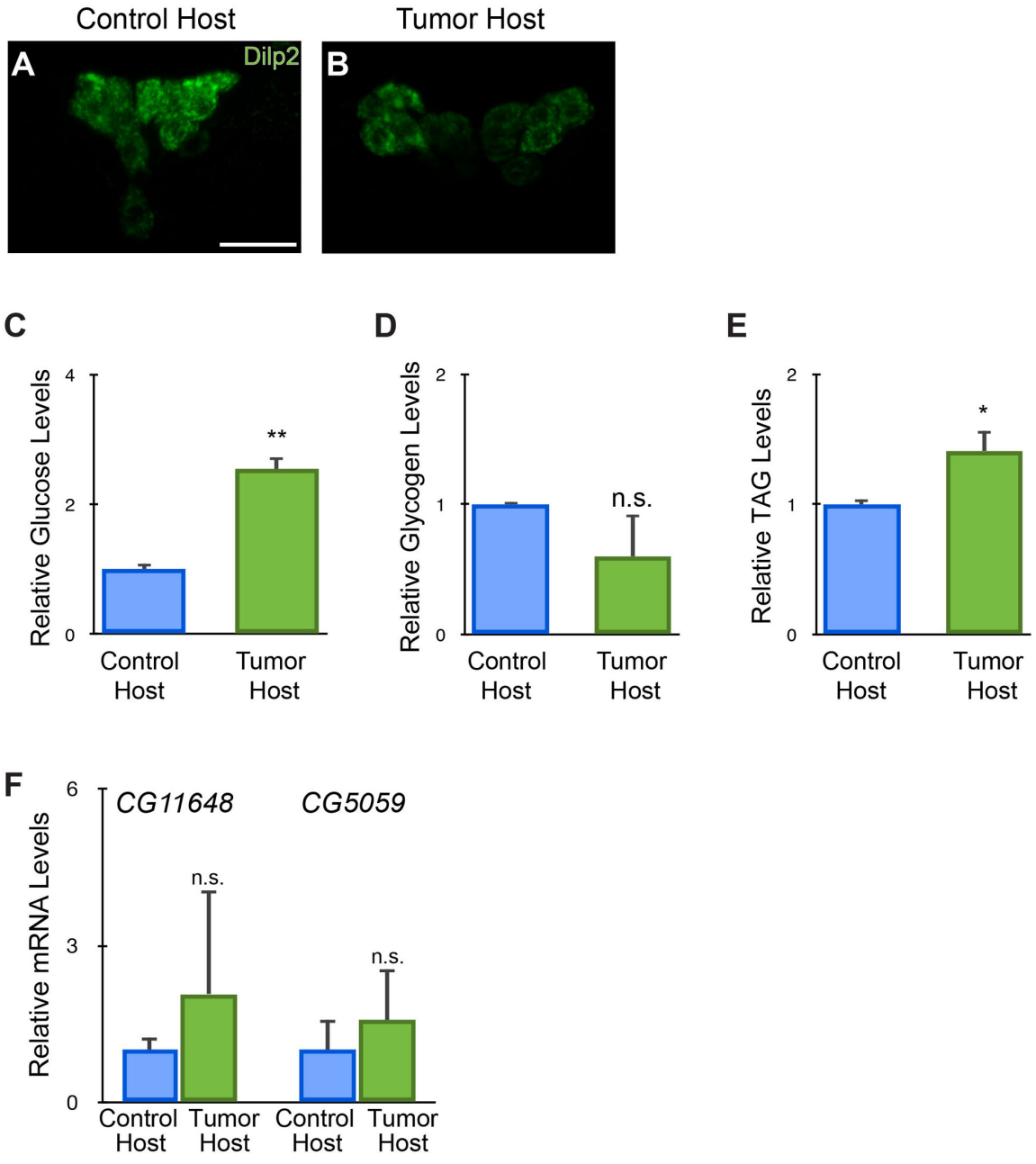

Control Host
Tumor Host
A
Dilp2
B
C
Relative Glucose Levels
4
2
0
**
Control Host
Tumor Host
D
Relative Glycogen Levels
2
1
0
n.s.
Control Host
Tumor Host
E
Relative TAG Levels
2
1
0
*
Control Host
Tumor Host
F
Relative mRNA Levels
6
3
0
CG11648
CG5059
n.s.
n.s.
Control Host
Tumor Host
Control Host
Tumor Host

Figure S3 (Related to Figure 4)

Maximum intensity projections of Insulin-producing cells (IPCs) reveal no accumulation of dILP2 levels in brains of tumor-bearing hosts compared to control (**A, B**). Elevated total glucose levels in tumor hosts relative to controls (**C**); small or no difference in glycogen and triglyceride measurements (**D, E**); absolute values: Table S2. Transcript levels of *CG11658* and *CG5059,* the putative *Drosophila* orthologs of regulators of human muscle catabolism (Atrogin-1/MAFbx and Bnip3, respectively), are not significantly upregulated in thoracic muscles of tumor-bearing hosts as measured by quantitative RT-PCR (**F**). Scale bars: A=25µm. n.s.=p>0.05, *p<0.05, **p<0.01, Student's *t*-test; standard deviation is indicated. p and N values: Table S1.

FIGURE S4

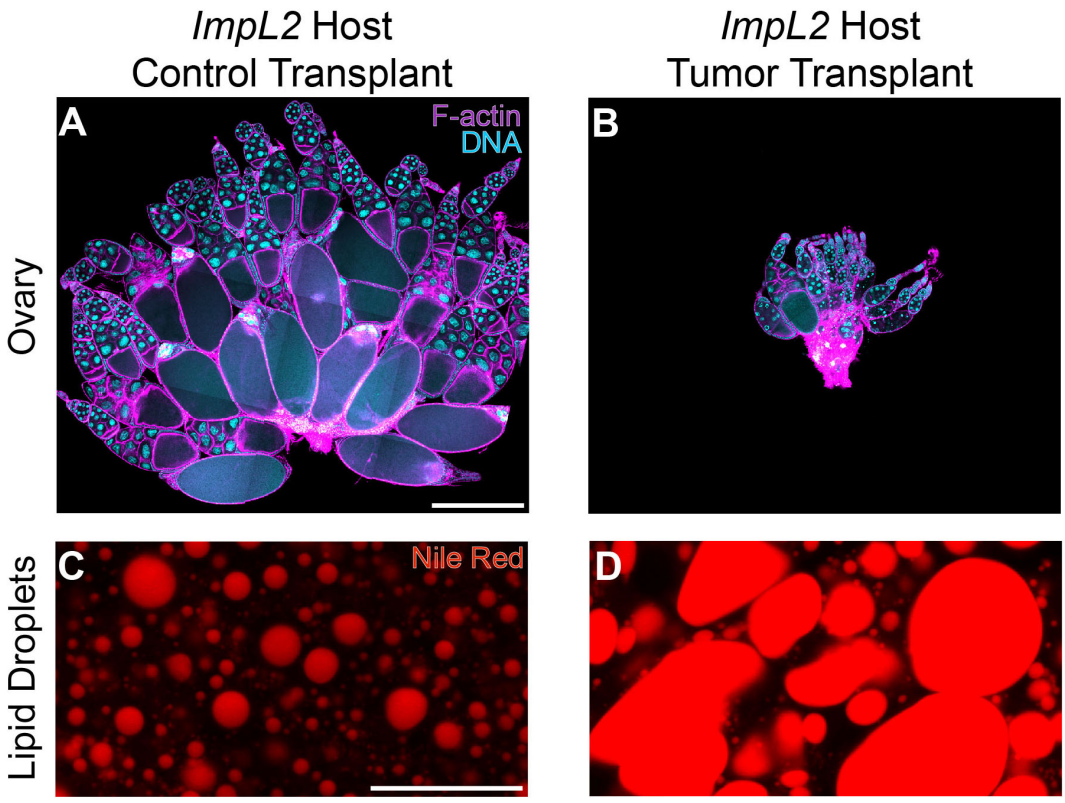

ImpL2 Host
Control Transplant
ImpL2 Host
Tumor Transplant
A
F-actin
DNA
B
Ovary
C
Nile Red
D
Lipid Droplets

Figure S4 (Related to Figure 5)

Phenotypes of peripheral tissues in *ImpL2*-null females. *scrib Ras*^{V12} tumor induces ovarian (**A, B**) and adipose (**C, D**) tissue wasting in the absence of host *ImpL2* expression, compared to control transplants. Scale bars: A=500µm; C=50µm.

Table 1 (Related to Fig. 1-5 and Fig. S1-3)

Figure Ref	P VALUE	N VALUE
1K	1.99E-09	30
1L	1.99E-09	30
1M	7.15E-03	15
1N	1.74E-32	473(control host); 281(tumor host)
2J	8.43E-01	18
2K	8.04E-01	12(control host); 12(tumor host)
3I	3.87E-03; 2.66E-02; 1.85E-03; 5.90E-03; 9.72E-03; 4.09E-03;	3 replicates
4E	2.61E-02; 6.58E-03	3 replicates
4F	1.94E-02	3 replicates
5I	2.84E-17; 9.86E-05	30(control host); 36(tumor hosts)
5J	1.13E-20; 4.1E-11	30(control host); 36(tumor hosts)
5K	4.23E-03	166(tumor host); 194(ImpL2 KD tumor host)
S1E	3.32E-03; 3.77E-02	4
S1N	3.01E-09	30
S1O	1.94E-15	30
S2G	1.12E-06; 1.19E-01; 2.00E-03	30
S2H	9.53E-14; 5.12E-01; 1.06E-01	30
S2R	1.50E-02	3 replicates
S2S	0.79	3 replicates
S3C	4.26E-03	3 replicates
S3D	1.51E-01	3 replicates
S3E	1.85E-02	3 replicates
S3F	2.37E-01; 2.36E-01	3 replicates

Table 2 (Related to Fig. 4 and Fig. S3)

Figure Ref	Absolute Value Control Host	Absolute Value Tumor Host
4F	3.39 (µg /fly)	9.79 (µg /fly)
S3C	0.009 (µg /µg protein)	0.023 (µg /µg protein)
S3D	0.079 (µg /µg protein)	0.048 (µg /µg protein)
S3E	0.31 (µg /µg protein)	0.44 (µg /µg protein)

CHAPTER 3

Further Analysis of Tumor-Host Interactions in Drosophila

SUMMARY

While we have gained much insight regarding interactions between tumors and their local microenvironment, the majority of cancers deaths result from tumor interactions with distant tissues. Despite the morbidity and mortality induced by these long-range tumor effects, their underlying mechanisms remain poorly understood. Here we use a classic transplantation technique to evaluate how Drosophila tumors induce metastatic colonization, tissue wasting and a systemic immune response at a distance. We evaluated metastatic frequency across different tumor types and host backgrounds and found that it increases with time after transplantation, being highest close to the day of death and more common in the ovaries, compared to the gut. Independently of metastatic invasion, tumor presence induces apoptosis and tissue wasting in the ovaries and a systemic disruption of insulin signaling, evidenced in several peripheral tissues. We tested multiple tumor-secreted factors to determine whether they are individually capable of recapitulating these phenotypes. Finally, we analyzed the role of the immune system in tumor progression and its contribution to tumor-induced ovarian wasting. We propose that these three different tumor-host interactions have different effects and each contributes to host death.

INTRODUCTION

As much as we have learned regarding the genetic changes that can lead to the deregulation of cell cycle, apoptosis, and growth control in the nascent tumor and throughout cancer progression, little is known about how these changes in the tumor can induce systemic alterations in the host. These distant tumor-host interactions are largely responsible for the morbidity and mortality associated with human cancers, but the wide variety of responses they elicit and tissues affected, has complicated the study of their underlying mechanisms.

In Chapter 2, I have discussed cachexia wasting and identified a key tumor-secreted cachectic mediator. Other important features of tumor-host interactions, including metastasis, bloating, immune response and lethality, will be discussed in this chapter.

Metastasis is a particularly fatal tumor-host interaction, accounting for over 90% of cancer deaths [99, 100]. The complex metastatic cascade involves tumor growth, intravasation, homing, extravasation and colonization. Although each of these events contributes to the progression of the metastatic cascade, it is not well understood to what extent these are autonomously regulated or dependent on interactions with the host, particularly in terms of colonization. Deciphering the influence that the host has on the secondary tumor and vice versa will provide insight into the molecular mechanisms underlying these relationships. The indispensability of these interactions for the progression of the metastatic cascade and the establishment of the secondary tumor makes the study of tumor-host interactions of uppermost interest.

The immune system also plays an essential role in mediating tumor-host interactions. Bearing similarities to a “wound that never heals”, the presence of a tumor evokes a robust immune response in the host [5]. While this response can detect and inhibit tumor progression, there is also evidence that it can also support and accelerate tumor growth [101]. A better understanding of the factors mediating these aspects of the host’s immune response could shed light on how to harness the power of this response to halt the detrimental effects of cancer.

Surprisingly, despite our awareness these other distant tumor-host interactions, the factors mediating these effects remain largely unknown. The investigation of these long-range effects is crucial for deciphering the molecular mechanisms underlying cancer-associated morbidity and mortality.

To begin to elucidate the mechanisms that enable tumors to metastasize, induce tissue wasting and evoke a systemic immune response, we use Drosophila to model some aspects of these tumor-host interactions. Amenable to genetic manipulations, a short generation time and ease of dissection, among other advantages, have made Drosophila an important system for modeling diseases, including cancer [27, 53, 57]. Loss-of-function of neoplastic tumor suppressors, such as the junctional scaffold proteins (lethal giant larvae- lgl, discs large-dlg and scribble- scrib) results in abnormal epithelial architecture, massive over-proliferation and host death, resembling many of the phenotypes associated with human tumors [40, 41]. Transplantation of *lgl* tissue into the

abdomen of a female host will not only result in the continuous growth of such tumor, but may in addition allow a subset of these cells to migrate to distant tissues within the host [61]. Similarly, when *scrib* is combined with the constitutively active oncogene Ras^{V12}, the resulting tumor cells can also exist the primary tumor and migrate into distant host tissues upon transplantation [36]. In Drosophila, these metastatic events are uncommon and high variable. The incidence of metastasis depends on the genetic composition and tissue of origin of the transplanted tumor, as well as the site of secondary tumor formation examined. The effect that the secondary tumor has on its host and on the tissues where it metastasizes to, as well as other potential responses of the host to the presence of a tumor, remain unknown. Here we use the Drosophila transplantation model to investigate the factors that drive metastatic colonization, tissue wasting and provoke a systemic immune response and their role in mediating host-lethality.

RESULTS

Tumor Cells Metastasize to Distant Host Tissues

Tumor transplantation into the abdomen of a female host has been a classic technique for investigating metastasis in Drosophila. While invading tumor cells have been reported in the wing and thorax [61], the ovaries appear to be the preferred metastatic site, but given the unpredictability and variable frequency, these metastatic events have been poorly quantified. To explore this question in detail, we first generated *scrib* Ras^{V12} tumors in the eye imaginal disc, harvested the tumorous mass from the larvae and implanted a small fragment into the abdomen of an adult female host [36]. We then examined host ovaries 6, 8, 10 and 12 days after transplantation. Metastatic events were scored by the presence of GFP^{+} tumor cells that trespassed a basement membrane within this tissue, including the surrounding muscle sheath **(Fig. 1A-D)**. Metastatic frequency was determined by the percentage of females that had at least one metastatic event in the ovaries. On Day 6, only 1% of tumor-bearing females were found to have infiltrating tumor cells, suggesting that invasion before this time is rare or unlikely. Surprisingly, only a few days after, metastatic frequency increases substantially to 11% (Day 8) and 13% (Day 10) and up to 20% (Day 12) **(Fig. 1G)**. Consistent with our understanding of metastasis as a key culprit in host death, this incremental metastatic ability is paralleled with decreased survival rates: 67%, 67%, 59%, 30%, respectively. In some instances, tumor cells were also found to surround the gut and invade the basement membrane of this organ **(Fig. 1E,F)**. As in the ovaries, the frequency of metastatic events in the gut was also progressive **(Fig. 1H)**.

Curious about the prevalence of tumor metastasis to the ovary, we speculated that growth and survival factors from ovarian stem cells could facilitate secondary tumor formation at this site. One such factor is the growth morphogen decapentaplegic (Dpp) [102-104]. Mammalian literature has shown that the vertebrate homolog, BMP, becomes accessible upon basement membrane degradation at the colonization site and contributes to the establishment of the secondary tumor [105, 106]. To assess whether Dpp and other candidate factors prompted ovarian metastasis, we expressed this factor using a somatic, epithelial *GAL4* driver expressed throughout the ovary and assessed whether metastatic frequency to this tissue was increased. Ectopic *dpp* expression resulted in ovarian apoptosis and egg chamber fusion without increasing metastatic frequency **(Fig. 1I,J)**. We also tested whether promoting basement membrane degradation in the ovary would increase the availability of Dpp and other growth and survival factors, but using the same driver to express the matrix metalloproteinase, Mmp1, was lethal. These data suggest the levels and spatio-temporal expression of ovarian stem cell factors is tightly regulated and underlies the complexity of dissecting the individual contributors of metastasis to this tissue.

Since transplanted tumors predominantly metastasized to the ovary, we asked whether *in situ* ovarian tumors would invade other distant host tissues. In the larvae, dlg^{RNAi} Ras^{V12} tumors in the eye imaginal disc invade the neighboring optic lobes and ventral nerve cord. When driven in the somatic epithelium of the adult ovary, this genetic cooperation system resulted in a tumor-like multi-layering and invasion of tumor cells to the adjacent germline **(Fig. 1K,L)**. We did not find GFP^+ tumor cells beyond the ovary this tumor model, indicating metastatic frequency of ovarian tumors to other peripheral tissues is low. Regardless of this low metastatic frequency, ovarian dlg^{RNAi} Ras^{V12} tumor host displayed extremely bloated abdomens and died within 3 weeks. Abdominal bloating was first reported in *lgl* tumor transplants [68] and is associated with other tumor models, including the transplanted *scrib* Ras^{V12} tumors. Driving other tumorigenic genetic cooperation models in the ovaries recapitulated the bloating and death phenotypes of ovarian dlg^{RNAi} Ras^{V12} tumors **(Fig. 9A)**, suggesting these features might be characteristic of various malignant tumors.

Tumor Induces Wasting in Peripheral Tissues

As described in Chapter 2, only five days after transplantation, local growth of the *scrib* Ras^{V12} tumor robustly induced wasting of the distant host ovaries. Interestingly, this wasting occurs independently of the presence of invading tumor cells **(Fig. 2A,B)**. We quantified ovarian wasting by the percentage of dying ovarioles and classified these into two types: degenerating, identified by DNA fragmentation and nuclear condensation of the germline cells, and peas-without-pod, " pwop ", referring to germline cells without its surrounding follicle cell epithelial layer [107]**(Fig. 2C-E)**. While degenerating phenotypes resembled those associated with starvation, "pwop" phenotypes were exclusively detected in tumor-bearing females **(Fig. 2F)**. Detailed analysis revealed that the wasting was progressive **(Fig. 2G)** and occurred most frequently during the mid-stages of oogenesis, which are normally subjected to a nutritional-sensitive checkpoint [108]**(Fig. 2H)**. Consistent with these findings, the production of mature eggs is drastically reduced in tumor hosts compared to controls **(Fig. 2I)**. These results demonstrate that independently of metastasis, *scrib* Ras^{V12} tumors can also induce wasting of distant tissues, potentially contributing to host death.

Previously, pwop phenotypes have been ascribed to insulin pathway mutants [107], so we hypothesized that the tumor might be mediating the wasting phenotypes by disrupting the relay insulin activity in the host. qPCR analysis revealed a slight decrease in the activity of the neurosecretory cells that release the Drosophila insulin-like peptides (dILPs) in the brain. In the gut, *dILP* levels were comparable to control hosts, but pathway activation was altered: increased levels of *4E-BP*, the downstream FOXO target, indicated low insulin reception. Expression of the glucagon analog, *tobi* [109], was also elevated compared to control guts **(Fig. 3A).**

If decreased insulin production is responsible for the ovarian apoptosis phenotypes observed in the presence of a tumor, we speculated that increasing

insulin availability might rescue the wasting phenotypes. First, we doubled the amount of sucrose and protein in the food of transplanted females, but this nutritional boost did not improve the ovarian or adipose phenotypes induced by *scrib Ras*^{V12} tumors, suggesting the effect is independent of diet **(Fig. 3B-E)**. Transplanting tumors into hosts expressing the predominant insulin peptide in Drosophila, *dILP2*, upon heat shock, showed that increased insulin levels can promote tumor growth, but are insufficient to prevent wasting in the ovaries of tumor-bearing or starving females **(Fig. 3F-K)**. Adipose-driven *dILP2* expression was also unable to restore ovarian health of tumor-transplanted females **(Fig. 3N,O)**. Interestingly, *dILP2* over-expression alone can also induce apoptosis, suggesting that proper regulation of insulin is required for ovarian health **(Fig. 3L,M)**. Surprisingly, *dILP2* mutants exhibited healthy ovaries, but had significantly fewer ovarioles per ovary; similar results were observed in other *dILP* mutants **(Fig. 4A-E)**. Here, the action of the remaining dILPs can compensate for the loss of the individual mutant.

In addition to the ovaries, the thoracic muscle is another energetically demanding tissue, so we investigated whether tumors also altered insulin signaling in this peripheral organ. Compared to controls, in tumor hosts *4E-BP* levels were over six-fold higher, indicating low insulin activity in this tissue **(Fig. 5A)**. We then asked whether wasting phenotypes were also evident. While tumor transplantation into an actinin-reporter host failed to reveal gross structural differences in this tissue **(Fig. 5B,C)**, further microscopic analysis of this tissue showed DNA fragmentation and irregularities in mitochondrial spacing and morphology in the muscle of tumor-bearing hosts **(Fig. 5D,E)**. These data demonstrate that *scrib Ras*^{V12} tumors alter insulin activity in the brain neurosecretory cells and gut and induce wasting in distant tissues including the ovaries and thoracic muscle, reminiscent of tissue wasting in human cancer cachexia.

Candidate Tumor-Secreted Factors are Sufficient but not Necessary to Induce Wasting

Given the long-range effects of *scrib Ras*^{V12} tumors on host tissues, we hypothesized that the phenotypes might be mediated by secreted factors. We considered a variety of secreted candidates, including the JAK-STAT ligand Unpaired (Upd) [110], the insulin-like peptide dILP8 [51, 52], the TNF ligand Eiger [111] and the matrix metalloproteinase Mmp1 [112]. We expressed each factor individually in the fat body or in transplanted imaginal discs and assessed whether these factors could induce the apoptotic phenotypes observed in ovaries of tumor hosts. Adipose-driven *upd*, *eiger* and *dilp8*, as well as transplanted imaginal discs expressing *upd*, *eiger* and *Mmp1* recapitulated the ovarian phenotypes observed in tumor-bearing hosts **(Fig. 6A-H)**. Interestingly, tumor transplantation into hosts expressing the JAK/STAT reporter [113] showed increased pathway activation throughout the ovary, demonstrating that this tissue receives Upd signals from the tumor **(Fig. 6I,J)**.

To test whether expression of these secreted factors in the tumor is

responsible for the apoptotic phenotypes in distant host tissues, we depleted their activity specifically in the tumor. As with *scrib* Ras^{V12} tumors, transplantation of dlg^{RNAi} Ras^{V12} tumors also induced apoptosis in host ovaries **(Fig. 7E)**. In combination with either the inhibitor of matrix metalloproteinases, *TIMP* [114], or RNAi knockdown of *dilp8*, dlg^{RNAi} Ras^{V12} tumor size was not affected, but dlg^{RNAi} Ras^{V12} $upd2^{RNAi}$ tumors were smaller **(Fig. 7A-D)**. Regardless of their effect on tumor size, reduction of these secreted factors in the tumor did not ameliorate ovarian wasting **(Fig. 7E-I)**. Similarly, knockdown of the pro-apoptotic factor *eiger* in *scrib* tumors, resulted in a slightly larger tumor mass, and actually exacerbated ovarian apoptosis compared to *scrib* tumors alone **(Fig. 7J-N)**. Together these results indicate that the secreted factors tested here are sufficient, but not necessary for the wasting observed in tumor-bearing hosts.

Tumor Induces Wasting Independently of Host's Immune Response

The distant interactions between the tumor and the affected host tissues could be direct or via an intermediary, such as the host's immune system. To distinguish between these possibilities we partially ablated host immune cells by driving the pro-apototic gene *hid* in a large number of the host's hemocytes [97]. Transplanting *scrib* Ras^{V12} tumors into these immuno-compromised hosts, did not affect tumor growth and ovarian apoptosis was comparable to that of control hosts **(Fig. 8A-E)**. Although these results were unexpected given the previously documented role of hemocytes in regulating tumor growth, hemocyte ablation was only partial and hence the contribution of the remaining hemocytes cannot be ruled out.

DISCUSSION

Metastasis is one of the deadliest tumor-host interactions, but its treatment remains limited due to its complexity. As much as we have learned about the factors that enable tumor cells to exit the primary tumor and infiltrate into the vasculature, the mechanisms that facilitate the formation of colonizing tumors at the secondary site remain unknown. Here, we used a simple Drosophila model to quantitate metastatic events in detail and explore the factors that contribute to make some organs preferred metastatic sites. While the lack of certain physiological features, such as vasculature and macrophages, are important to keep in mind while modeling metastasis in Drosophila, the recreation of secondary tumors described above indicates that a phenomenon akin to colonization may occur in the fly, making this invertebrate a valuable system in which to investigate this process. Indeed, we find that metastatic frequency in the fly increases with time and is associated with decreased survival. Infiltrating tumor cells in the ovary were found as single cells or as a group of cells. In the latter, we have yet to determine whether single cells proliferated to form the group or if the group of cells invaded as a single unit. Distinguishing between these possibilities would provide insight into the proliferative potential of secondary tumors and determine whether a state of dormancy comparable to that observed in colonizing tumor cells in human patients exists in the fly [115].

The tumor-induced wasting of host ovaries described in Chapter 2 prompted further analysis of the apoptotic phenotypes in this tissue. Despite the phenotypic similarities with females under starvation [78, 116], we did not detect differences in the expression of key regulators of autophagy, suggesting that other mechanisms might be mediating ovarian death in tumor-bearing hosts **(Fig. 2J)**. We speculate that alterations in the activity of insulin producing cells and reception in peripheral tissues could in part be responsible. Moreover, the tumor also affected muscle tissue, indicating these phenotypes might be part of a systemic tumor-induced effect.

Drosophila can serve as a platform for the identification of the molecular mechanisms underlying distant tumor-host interactions, such as cachexia. In addition to the cachectic factor identified in Chapter 2, here we tested other candidate tumor-secreted factors that could also be playing a role in the wasting of peripheral tissues. Expressing these factors in fat body was sufficient to recapitulate tumor-induced wasting in the distant ovaries. Future studies will evaluate whether these factors directly induce distant tissue wasting or whether these results are an indirect consequence of their local effect on the fat body. This could be the case for *eiger*, which can induce ovarian wasting both when ectopically driven as well as when depleted in *scrib* tumors, yet interestingly, this factor was not found to be upregulated in malignant tumors [85]. The other tested factors were also insufficient to rescue wasting phenotypes in the ovaries. Although suggestive that these factors are not necessary for the tumor-induced effects, these data require further analysis. For example, Drosophila has multiple

Upds [86]. This could explain why depletion of *upd2* alone in dlg^{RNAi} Ras^{V12} tumors is insufficient to ameliorate tissue wasting. If *upd2* knockdown reduced growth of dlg^{RNAi} Ras^{V12} tumors, in combination with depletion of other upds, tumor growth could be completely inhibited, making it difficult to assess its effect on host tissues. It is also possible that the expression levels of these factors were not sufficiently reduced in the tumor and the observed ovarian wasting is a result of their residual activity. While here we have just tested a few candidate factors, a similar approach can now be used to screen for other potential factors in an unbiased manner.

Despite our awareness that tumors kill, the underlying mechanisms remain mostly enigmatic. Future work will analyze the relationship between the tumor-induced ovarian and muscle phenotypes and host death. Interestingly, we found that transplantation of tumors of different genetic composition resulted in variable death curves, both in terms of their length and acuteness **(Fig. 9B)**. We have previously identified ImpL2 as a key regulator of the tumor-induced systemic wasting response, but depletion of this tumor-secreted factor did not increase host lifespan, suggesting that multiple tumor factors might contribute to host lethality **(Fig. 9C)**. Distinguishing the secreted factors of these different tumors will provide insight into those key contributors to their corresponding death curve.

In addition to the observed tumor effects on peripheral tissues, other tumor-host interactions could also be implicated in host lethality. For example, it is known that the presence of a tumor can elicit a strong immune response in the fly, but whether this triggers host death remains to be determined. Although we attempted to manipulate the host immune response, hemocyte depletion was only partial. For these experiments, we transplanted into *hml>hid* hosts. While this system depletes hemocytes, without compromising viability, only approximately 60% of circulating hemocytes are removed [97, 117]. *He GAL4* is another hemocyte driver, but unlike *hml GAL4*, it also has fat body and salivary gland expression. To make our results conclusive, other hemocyte ablation methods will be needed as well a proper count of remaining hemocyte numbers.

While abdominal bloating continues to be poorly understood, its association with multiple malignant tumors makes this phenomenon a promising area of tumor-host investigation. Finally, intestinal permeability in aging flies has been proven to be a reliable indicator of death [65, 67]. It will be of interest to test whether commonalities between death of tumor-bearing hosts and those of aging or infected flies exist.

The assay established here lends itself to scrutinizing the cellular and molecular mechanisms that regulate the observed phenotypes and how these are mediated by tumor-host interactions. Future work will use this model to decipher other tumor-secreted factors as well as the molecular basis of host responses contributing to tumor-induced host death.

EXPERIMENTAL PROCEDURES

Genetics and Transplantation

scrib^{1} *Ras*^{V12} and *Ras*^{V12} tumors were generated using the eyMARCM genetic system as in [36] and *dlg*^{RNAi} *Ras*^{V12} tumors used the eyFLP system as in [91]. *yki* tumors were obtained from *MS1096 GAL4 UAS Yki*^{S168A} larvae. *scrib* tumors were generated from *scrib*^{1} homozygotes alone or in combination with *eiger*^{3}. In knockdown experiments, *dlg*^{RNAi} *Ras*^{V12} was used in combination with *white*^{RNAi} (Bloomington #28980), *TIMP* [114], *dilp8*^{RNAi} (VDRC #102604), *upd2*^{RNAi} (Bloomington #33988) or *ImpL2*^{RNAi} (VDRC #30930). Ectopic disc expression experiments used *MS1096 GAL4* in combination with *UAS-Upd*, *UAS-Eiger* or *UAS-Mmp1*.

Transplantation was adapted from [61]: WT or tumorous imaginal discs were dissected from wandering third instar larvae, fragmented and introduced through a pulled glass capillary needle into the abdomen of one day old virgin females. Host females were kept on a high-yeast diet, in the presence of males at 25º C, except for starved females who were kept on water only and females on a high-calorie diet, where sucrose and protein content was doubled. Hosts were either *OreR, Yolk GAL4 UAS-dILP2, hs GAL4 UAS-dILP2* (Bloomington #37472)*, hml GAL4 UAS-RFP, hml GAL4 UAS-hid*^{ala5}*, Mef2 GAL4 UAS-Mito-GFP, Actn*^{CC01961} (Bloomington #51573) or *STAT-GFP* [113]. *Hs GAL4 UAS-dILP2* females were heat-shocked at 18º for 1hr/day, for 5 days post-transplantation. Ectopic expression experiments used *yolk GAL4* to drive expression of *UAS-dILP2* [118]*, UAS-Eiger, UAS-Upd* or *UAS-dILP8* [51] at RT or *tj GAL4GAL80ts*, raised at 18º and then shifted to 29º as adults to drive expression of *UAS-dlg*^{RNAi} *Ras*^{V12}[91]*, UAS-Crb*^{Intra} *Ras*^{V12}*, UAS-dpp* or *UAS-Mmp1*.

Microscopy and Image Analysis

All samples were fixed in 4% formaldehyde in phosphate buffered saline (PBS). Staining followed standard protocols, with TRITC-phalloidin (1:400; Sigma), DAPI (1:1,000; Life Technologies) or Nile Red (1:5,000; Sigma). Images were captured on a Zeiss 700 confocal microscope. Figures were assembled using Adobe Illustrator.

Quantitative RT-PCR

Total RNA was prepared from groups of 24 guts, 20-30 ovaries (RNeasy Mini Kit; QIAGEN), 20-25 thoraces and 25 heads (TRIzol reagent; Invitrogen and Direct-zol RNA MiniPrep Kit; Zymo Research). qPCR experiments were performed in triplicate using SYBR GreenER qPCR SuperMix. Relative quantification of mRNA levels was determined using the Comparative C_T method and normalized to *mef2* (guts), *rpl23* (ovaries), *alpha-tubulin* (thoraces), and *su(var)* (heads).

FIGURE 1

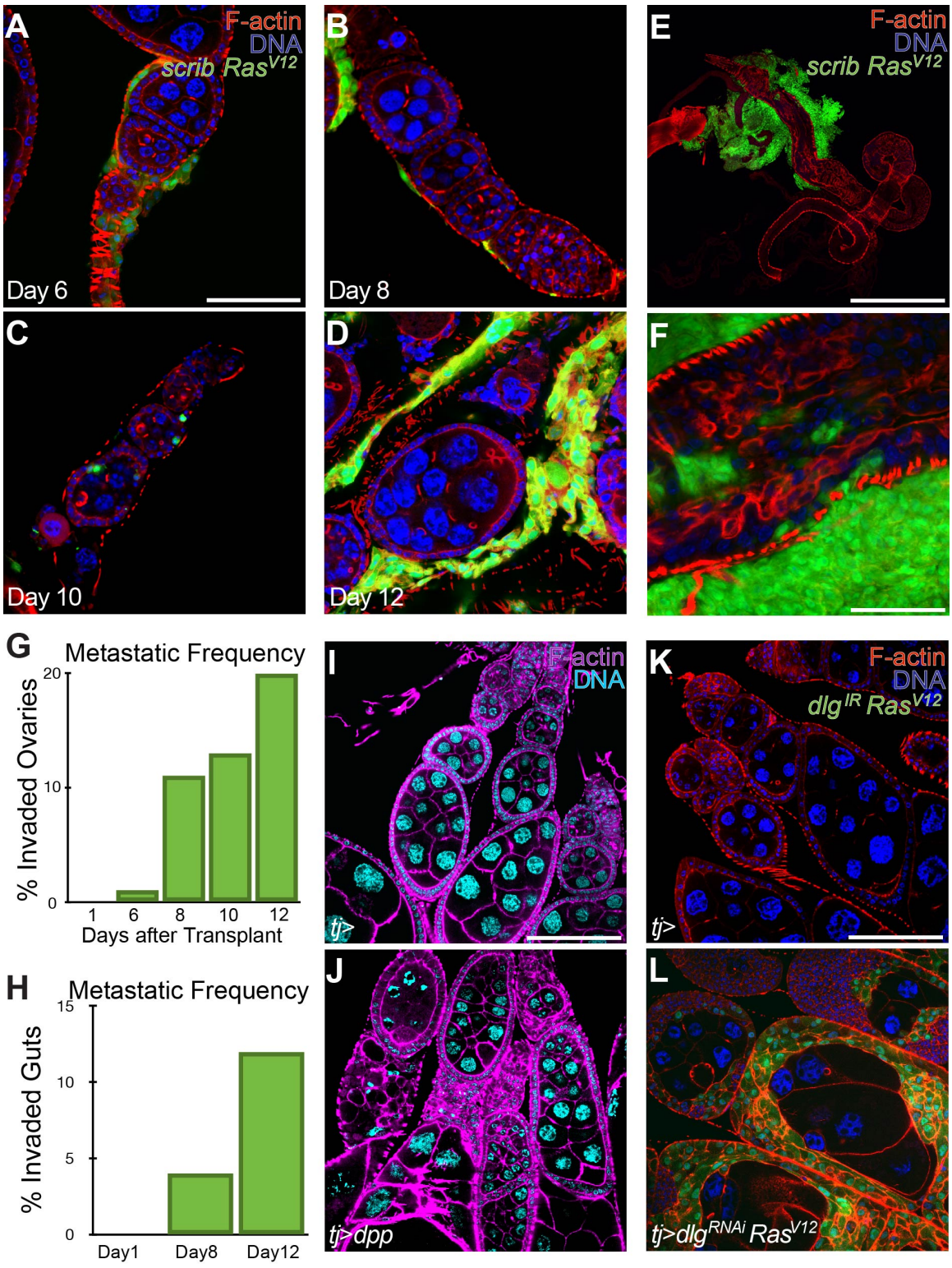

A
F-actin
DNA
scrib Ras^V12
Day 6
B
Day 8
E
F-actin
DNA
scrib Ras^V12
C
Day 10
D
Day 12
F
G
Metastatic Frequency
% Invaded Ovaries
20
10
0
1 6 8 10 12
Days after Transplant
I
F-actin
DNA
tj>
K
F-actin
DNA
dlg^IR Ras^V12
tj>
H
Metastatic Frequency
% Invaded Guts
15
10
5
0
Day1 Day8 Day12
J
tj>dpp
L
tj>dlg^RNAi Ras^V12

Figure 1. **Drosophila tumors can invade locally and metastasize to distant tissues**

GFP-labeled *scrib* Ras^{V12} tumor transplanted into WT adult host metastasizes to the ovaries and gut at distance. Tumor cells trespass the muscle sheath surrounding the ovary and infiltrate into the egg chambers, 6, 8, 10 and 12 days after transplantation (**A-D**; F-actin=red; nuclei=blue; green=*scrib* Ras^{V12} tumor). Tumor wraps around and invades the gut 8 days post-transplantation (**E,F**; F-actin=red; nuclei=blue; green=*scrib* Ras^{V12} tumor). In both tissues, metastatic frequency increases with time after tumor transplantation (**G,H**). Ovarian-driven ectopic expression of Dpp results in egg chamber fusion and apoptosis, without increasing metastatic frequency (**I,J**; F-actin=magenta; nuclei=cyan). In an *in situ* tumor model, GFP-labeled dlg^{IR} Ras^{V12} somatic tumor cells invade neighboring germline and disrupt egg chamber morphology (**K,L**; F-actin=red; nuclei=blue; green=dlg^{RNAi} Ras^{V12} tumor). Scale bars: A,F=50µm; E=500µm; I,K=100µm. N values: G=87(Day6), 90(Day8), 82(Day10), 40(Day12); H=90(Day8), 40(Day12).

FIGURE 2

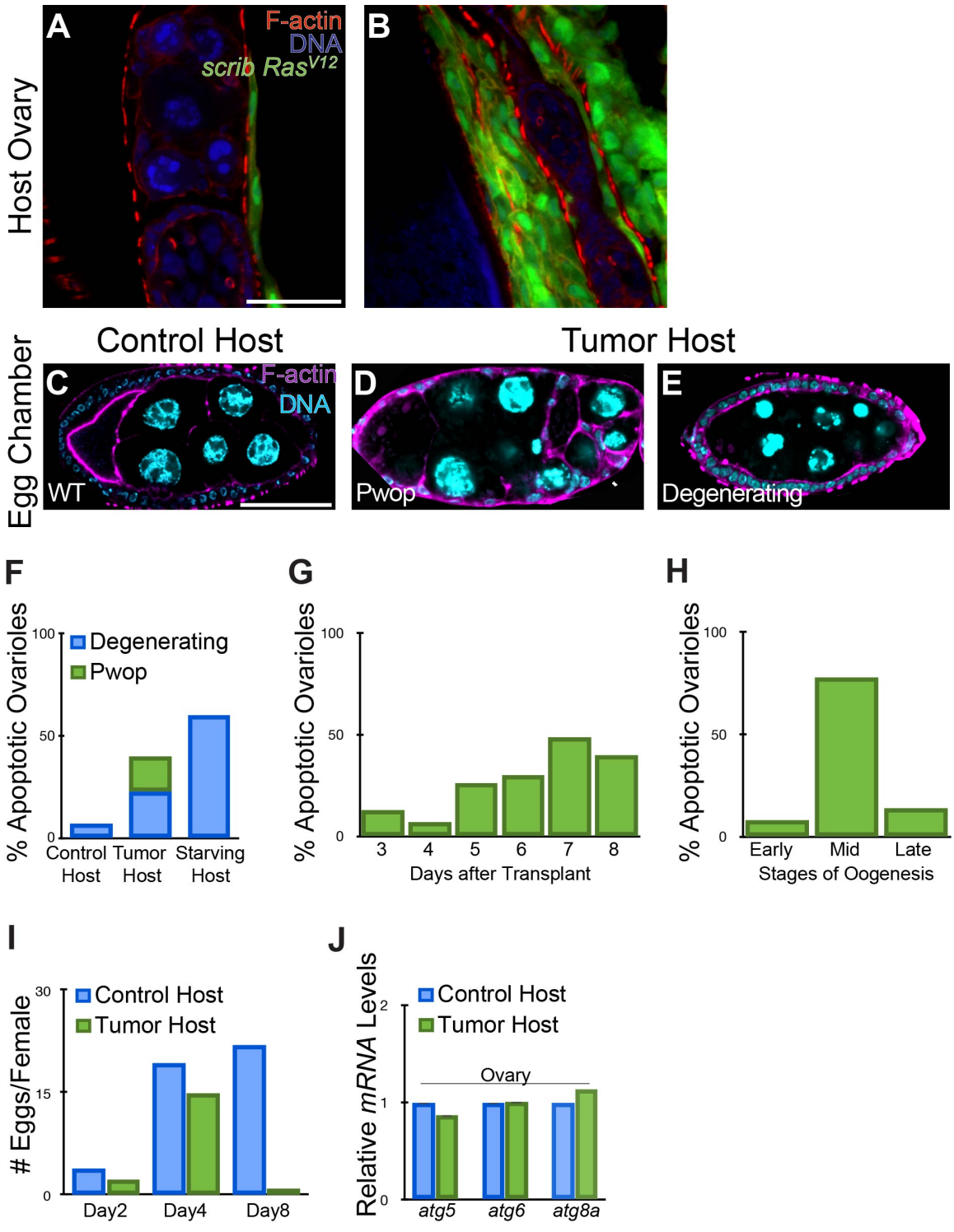

A
F-actin
DNA
scrib Ras^{V12}
B
Host Ovary
Control Host
Tumor Host
Egg Chamber
C
F-actin
DNA
WT
D
Pwop
E
Degenerating
F
% Apoptotic Ovarioles
100
50
0
Degenerating
Pwop
Control Host
Tumor Host
Starving Host
G
% Apoptotic Ovarioles
100
50
0
3
4
5
6
7
8
Days after Transplant
H
% Apoptotic Ovarioles
100
50
0
Early
Mid
Late
Stages of Oogenesis
I
Eggs/Female
30
15
0
Control Host
Tumor Host
Day2
Day4
Day8
J
Relative mRNA Levels
2
1
0
Control Host
Tumor Host
Ovary
atg5
atg6
atg8a

Figure 2. **Drosophila tumors induce wasting in host ovaries**

Ovarian wasting is independent of metastasis: GFP-labeled *scrib Ras*^{V12} tumors can induce ovarian apoptosis in the presence or absence of metastatic events (**A,B**; F-actin=red; nuclei=blue; green=*scrib Ras*^{V12} tumor). In tumor hosts, pwop and degenerating phenotypes are observed (**C-E**; F-actin=magenta; nuclei=cyan), while in control and starving hosts, all apoptotic ovarioles are degenerating (**F**). Wasting in the ovaries increases with time after tumor transplantation (**G**), affects predominantly the checkpoint-sensitive, mid-stage egg chambers (**H**) and results in a decreasing egg lay (**I**). Expression of autophagy regulators in the ovaries of tumor-bearing host is similar to controls (**J**). Scale bars: A,C=25µm. N values: G=160(Day3), 158(Day4), 177(Day5), 145(Day6), 134(Day7), 115(Day8); I=25(Day2), 21(Day4) and 19(Day8) in control females and 61(Day2), 43(Day4) and 7(Day8) in tumor females.

FIGURE 3

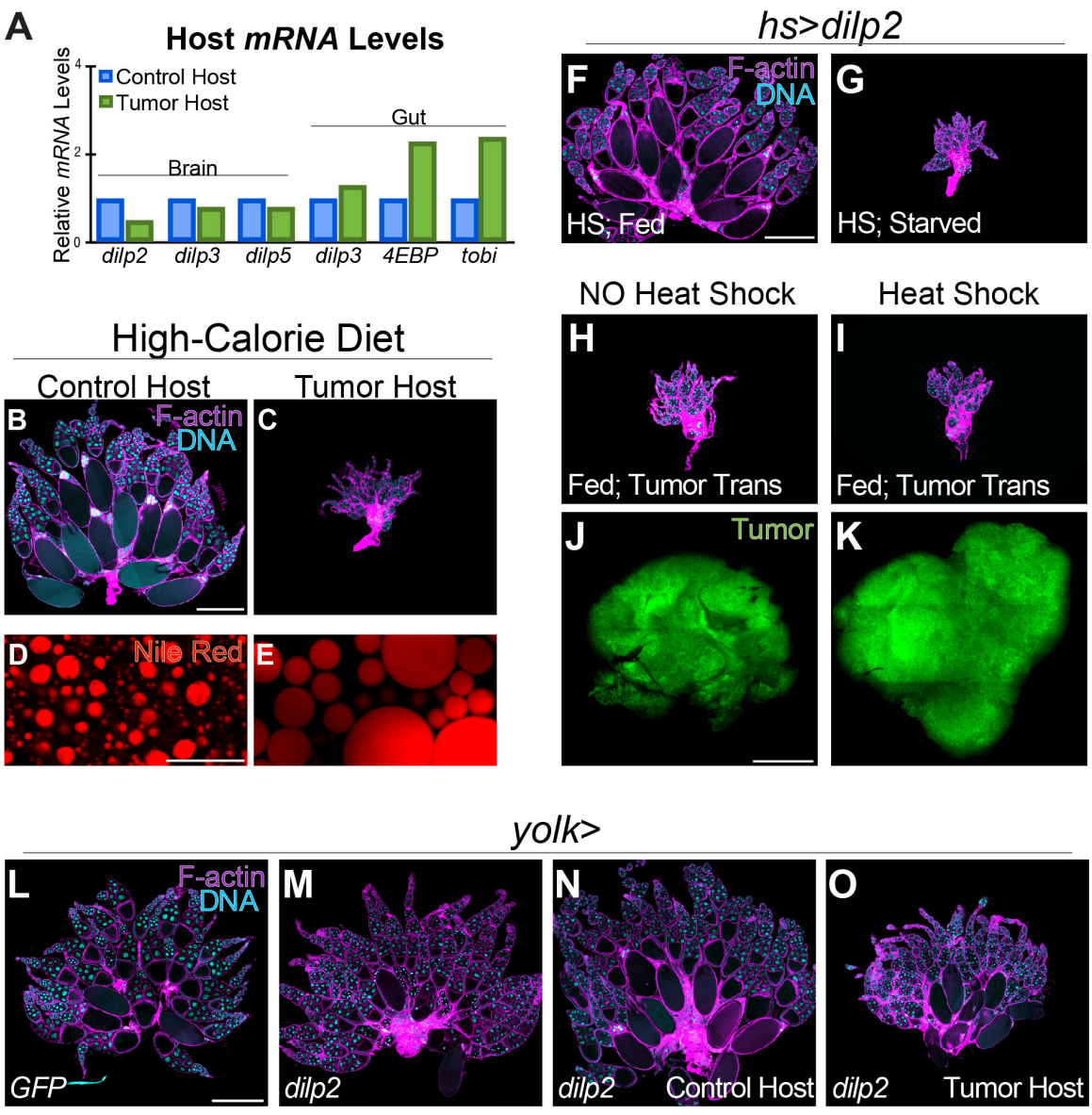

A
Host mRNA Levels
Relative mRNA Levels
Control Host
Tumor Host
Brain
Gut
dilp2
dilp3
dilp5
dilp3
4EBP
tobi
High-Calorie Diet
Control Host
Tumor Host
B
F-actin
DNA
C
D
Nile Red
E
hs>dilp2
F
F-actin
DNA
HS; Fed
G
HS; Starved
NO Heat Shock
Heat Shock
H
Fed; Tumor Trans
I
Fed; Tumor Trans
J
Tumor
K
yolk>
L
F-actin
DNA
GFP
M
dilp2
N
dilp2
Control Host
O
dilp2
Tumor Host

Figure 3. **Tumor alters insulin signaling in hosts**

In tumor bearing hosts, activity of the insulin producing cells in the brain is slightly decreased and insulin signaling in the gut is altered compared to control hosts (**A**). Increasing dietary sugar and protein does not rescue tumor-induced ovarian wasting (**B,C**; F-actin=magenta; nuclei=cyan) or abnormal lipid aggregation (**D,E**; Nile Red=red). Heat-shock induced *dILP2* expression drives growth of the transplanted tumor (**J,K**; green=dlg^{IR} Ras^{V12} tumor), but is not sufficient to restore ovarian health in starving (**F,G**; F-actin=magenta; nuclei=cyan) or tumor bearing hosts (**H,I**). Adipose-driven ectopic *dILP2* expression alone induces apoptosis (**L,M**; F-actin=magenta; nuclei=cyan), but partially restores ovarian health in tumor-bearing hosts (**N,O**). Scale bars: B,F,J,L=500μm; D=25μm.

FIGURE 4

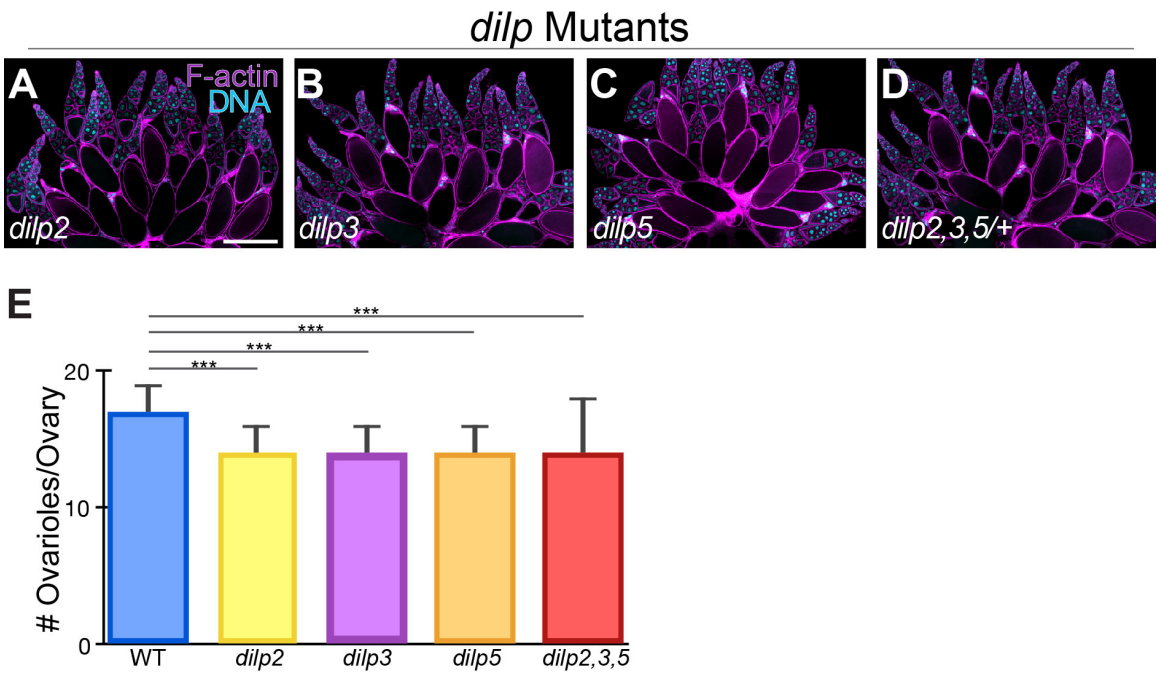

dilp Mutants
A
F-actin
DNA
dilp2
B
dilp3
C
dilp5
D
dilp2,3,5/+
E
***
***
***
***
20
10
0
Ovarioles/Ovary
WT
dilp2
dilp3
dilp5
dilp2,3,5

Figure 4. **dILP mutants have healthy ovaries**

Ovarian phenotypes of insulin mutants are distinct from those observed in tumor-bearing hosts: individual *dILP* mutants do not exhibit ovarian wasting (**A-D**; F-actin=magenta; nuclei=cyan), but have the number of ovarioles per ovary is reduced (**E**). Scale bars: A=500µm. ***p<0.001, Student's *t*-test; standard deviation is indicated. p values: E=3.88E-05(dilp2), 6.66E-05(dilp3), 7.55E-05(dilp5), 6.32E-05(dilp2,3,5). N values: E= 28(WT), 27(dilp2), 28(dilp3), 31(dilp5) and 30(dilp2,3,5).

FIGURE 5

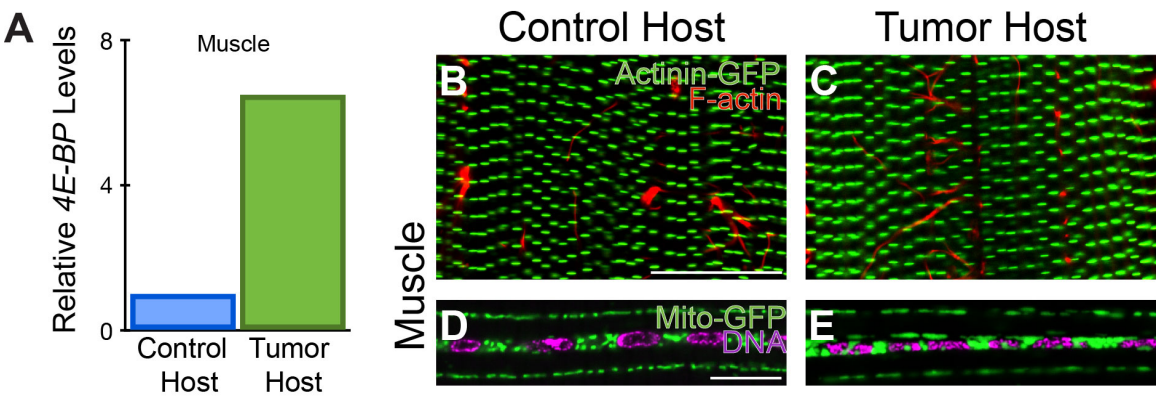

A
Muscle
Relative 4E-BP Levels
8
4
0
Control Host
Tumor Host
Control Host
Tumor Host
Muscle
B
Actinin-GFP
F-actin
C
D
Mito-GFP
DNA
E

Figure 5. **Drosophila tumors induce wasting in host muscle**

Increased transcription of the FoxO target *4E-BP* by quantitative RT-PCR measurement in thorax shows decreased insulin signaling activity in this tissue 8 days after tumor transplantation (**A**). While the actinin reporter indicates no obvious structural defects (**B,C**; Actinin-GFP=green; F-actin=red), the mitochondria-localized reporter reveals abnormal morphology and DNA fragmentation in the thoracic muscle of tumor-bearing hosts (**D,E**; Mito-GFP=green; nuclei=magenta). Scale bars: B=25µm; D=10µm.

FIGURE 6

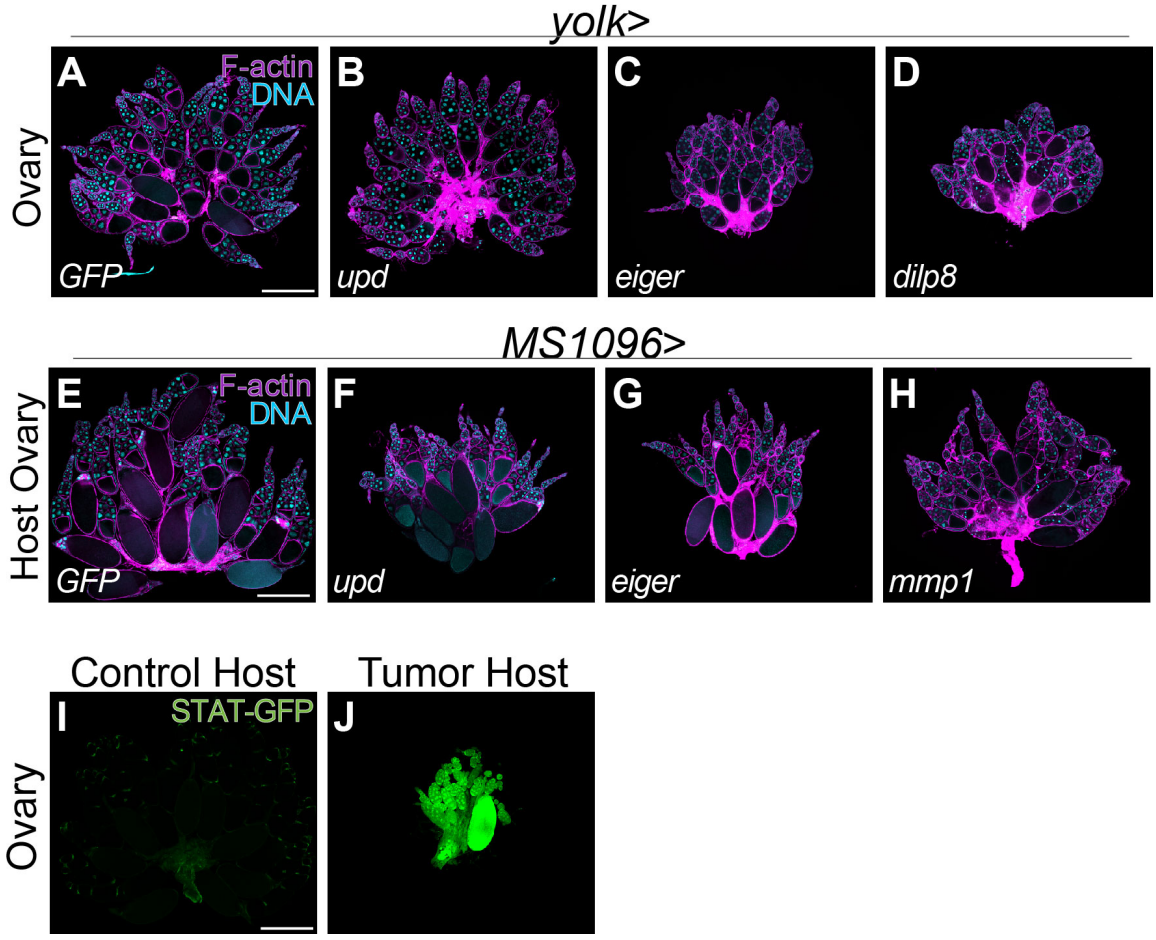

yolk>
Ovary
A
F-actin
DNA
GFP
B
upd
C
eiger
D
dilp8
MS1096>
Host Ovary
E
F-actin
DNA
GFP
F
upd
G
eiger
H
mmp1
Control Host
Tumor Host
Ovary
I
STAT-GFP
J

Figure 6. **Tumor-secreted factors are sufficient for wasting**

Adipose-driven ectopic expression of *upd*, *eiger* and *dILP8* is sufficient to induce ovarian wasting (**A-D**; F-actin=magenta; nuclei=cyan). Similarly, transplantation of discs expressing *upd*, *eiger* or *mmp1* can also induce wasting in ovaries at a distance (**E-H**; F-actin=magenta; nuclei=cyan). Transplantation of *scrib Ras*^{V12} tumors activates the JAK-STAT pathway in host ovaries (**I,J**; STAT-GFP=green). Scale bars: 500µm.

FIGURE 7

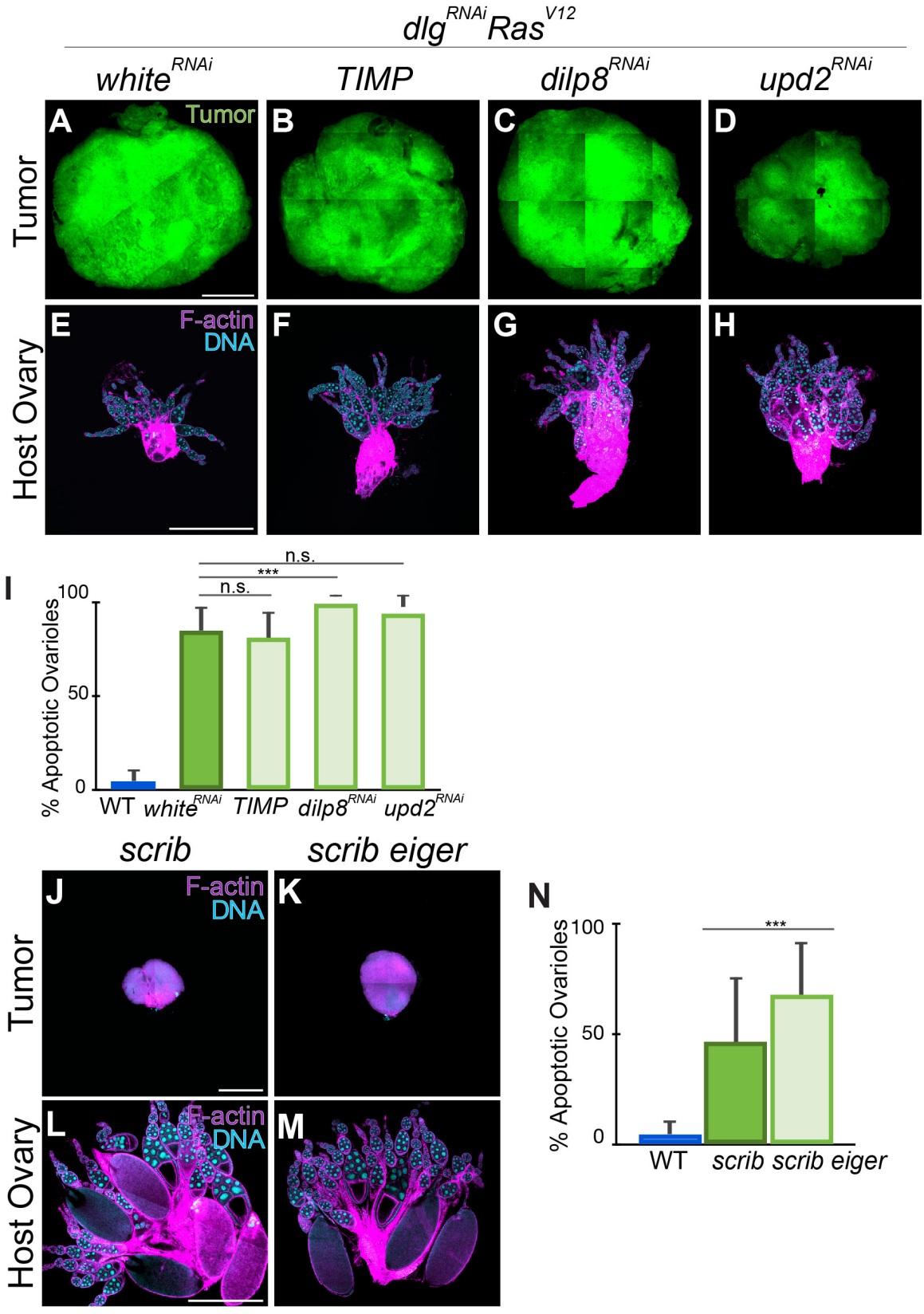

dlg RNAi Ras V12
white RNAi
TIMP
dilp8 RNAi
upd2 RNAi
Tumor
Host Ovary
A
B
C
D
E
F
G
H
Tumor
F-actin
DNA
I
% Apoptotic Ovarioles
100
50
0
n.s.
***
n.s.
WT
white RNAi
TIMP
dilp8 RNAi
upd2 RNAi
scrib
scrib eiger
J
K
L
M
N
% Apoptotic Ovarioles
100
50
0
***
WT
scrib
scrib eiger

Figure 7. **Tumor-secreted factors are not necessary for wasting**

Mmp1 inhibition or knockdown of *dilp8* in dlg^{RNAi} Ras^{V12} tumors does not affect size, but dlg^{RNAi} Ras^{V12} $upd2^{RNAi}$ tumors are smaller (**A-D**; green=dlg^{IR} Ras^{V12} tumor). Reduction of these factors in dlg^{RNAi} Ras^{V12} tumors does not improve ovarian health (**E-I**; F-actin=magenta; nuclei=cyan). Similarly, knockdown of the TNF ligand *eiger* in *scrib* tumors does not rescue ovarian wasting (**J-N**; F-actin=magenta; nuclei=cyan). Scale bars: 500µm. ***p<0.001, ns=p> 0.05, Student's *t*-test; standard deviation is indicated. p values: I=4.2E-01(TIMP), 5.9E-05(dilp8^{RNAi}), 3.06E-02(upd2^{RNAi}); N=8.8E-04. N values: I=245(white^{RNAi}), 345(TIMP), 315(dilp8^{RNAi}), 253(upd2^{RNAi}); N=510(*scrib*), 708(*scrib eiger*).

FIGURE 8

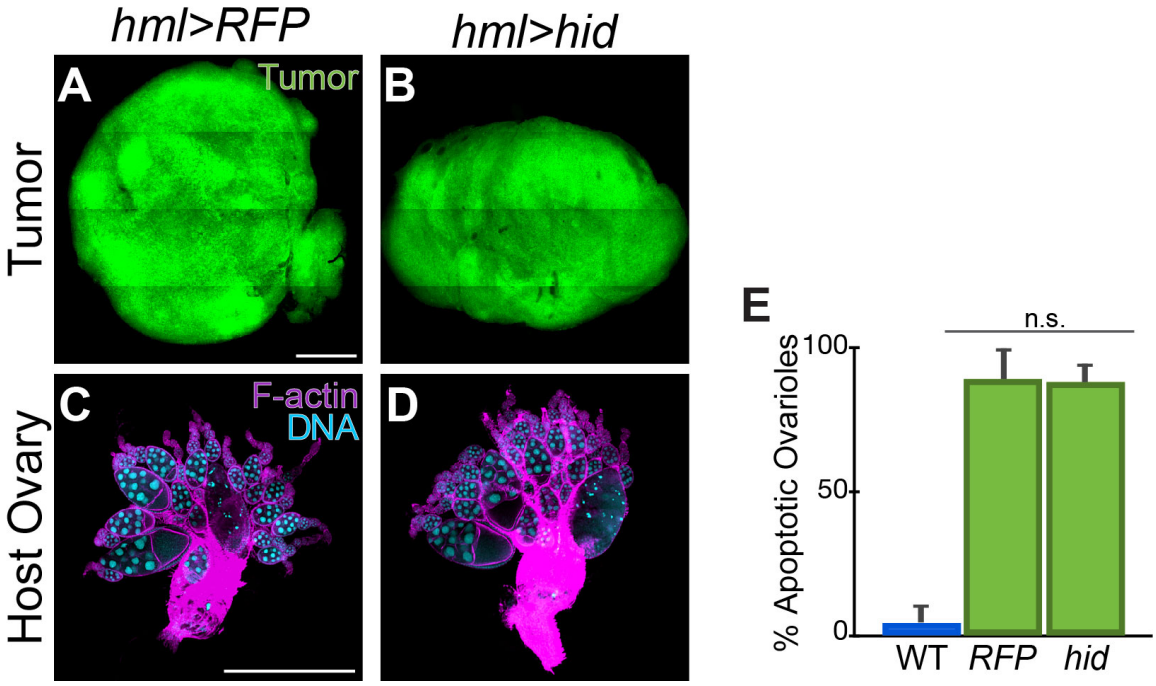

hml>RFP
hml>hid
A
Tumor
B
Tumor
C
F-actin
DNA
D
Host Ovary
E
n.s.
% Apoptotic Ovarioles
100
50
0
WT
RFP
hid

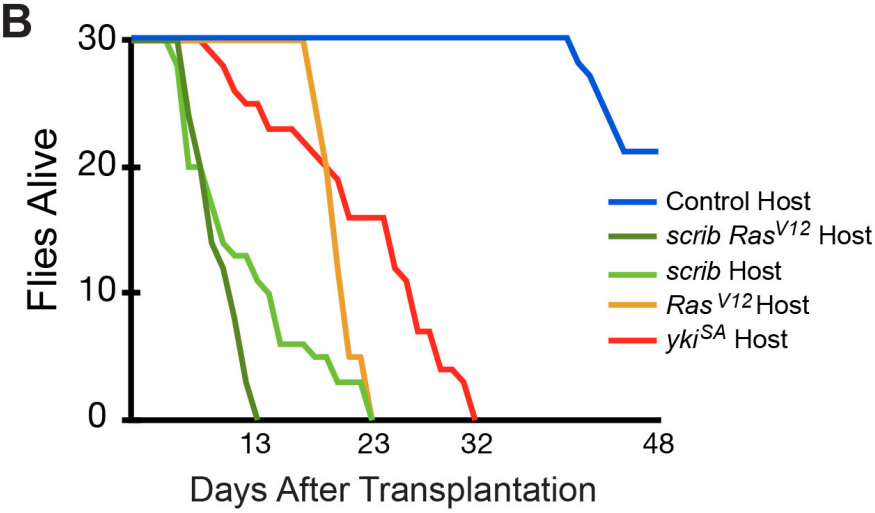
Figure 8. **Host hemocytes are not necessary for tumor-induced wasting**
Transplantation into *hml>hid*^{ala5} hosts does not impact tumor growth (**A,B**; green=*scrib Ras*^{V12} tumor) or restore ovarian health (**C-E**; F-actin=magenta; nuclei=cyan). Scale bars: 500µm. ns=p> 0.05, Student's *t*-test; standard deviation is indicated. p values: E=6.9E-01. N values: E=221(RFP), 138(*hid*^{ala5}).

FIGURE 9

A

Genotype	Bloating	Death
TJ^{TS}> Crb^{Intra} Ras^{V12}	YES	3 Weeks
TJ> Crb^{Intra} Ras^{V12}	YES	3 Weeks
esg^{TS}> yki^{S3A}	YES	3 Weeks
$esg^{TS\ F/O}$> yki^{S3A}	YES	-

B



C

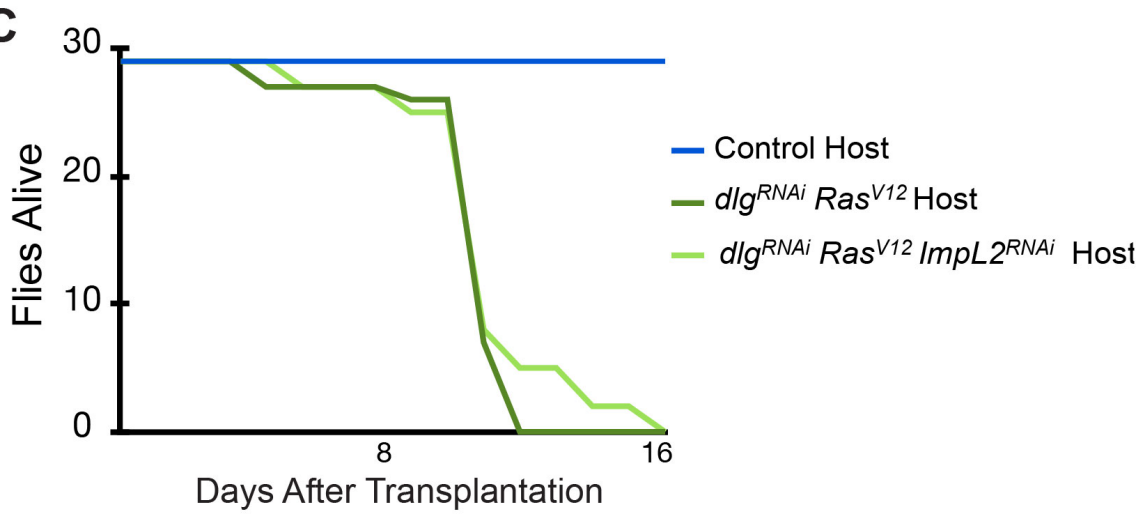


Figure 9. **Tumors induce bloating and kill host**

Similar to transplantation of *scrib* Ras^{V12} and dlg^{RNAi} Ras^{V12} tumors, *in situ* ovarian tumors induce abdominal bloating and result in host death (**A**). *scrib* Ras^{V12} tumors kill host after only 13 days, while other tumors kill more slowly. *scrib* and yki^{SA} tumors induce host death progressively, in contrast to the sharp death curve of Ras^{V12} tumors (**B**). Knockdown of *ImpL2* in dlg^{RNAi} Ras^{V12} tumors does not increase life-span (**C**).

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