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Drosophila brain tumor metastases express both neuronal and glial cell type markers

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Summary

Loss of either *lgl* or *brat* gene activity in *Drosophila* larvae causes neoplastic brain tumors. Fragments of tumorous brains from either mutant transplanted into adult hosts over-proliferate, and kill their hosts within 2 weeks. We developed an *in vivo* assay for the metastatic potential of tumor cells by quantifying micrometastasis formation within the ovarioles of adult hosts after transplantation and determined that specific metastatic properties of *lgl* and *brat* tumor cells are different. We detected micrometastases in 15.8% of ovarioles from wild type host females 12 days after transplanting *lgl* tumor cells into their abdominal cavities. This frequency increased significantly with increased proliferation time. We detected micrometastases in 15% of ovarioles from wild type host females 10 days after transplanting *brat* tumor cells into their abdominal cavities. By contrast, this frequency did not change significantly with increased proliferation time. We found that nearly all *lgl* micrometastases co-express the neuronal cell marker, ELAV, and the glial cell marker, REPO. These markers are not co-expressed in normal brain cells nor in tumorous brain cells. This indicates deregulated gene expression in these metastatic cells. By contrast, most of the *brat* micrometastases expressed neither marker. While mutations in both *lgl* and *brat* cause neoplastic brain tumors, our results reveal that metastatic cells arising from these tumors have quite different properties. These data may have important implications for the treatment of tumor metastasis.

Introduction

Tumor metastasis is the leading cause of cancer morbidity. The journey for cells from a primary tumor to a distant metastatic location involves many steps integrating diverse cellular processes. First, the cells must migrate out of the primary tumor through adjacent extracellular matrix to enter either the blood or lymph circulatory system. As the cells travel in the circulatory system, they clump and adhere to the vascular wall, and must leave through the vessel wall at a new site in the body. Subsequently, angiogenesis occurs and the tumor cells proliferate forming a secondary tumor (reviewed in Woodhouse et al. 1997). To complete all of these steps of metastasis, cancer cells require a remarkable number of different abilities including degradation of extracellular matrix material, migratory behavior, intravasation, extravasation, and formation of new tumors in secondary locations.

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Human tumors are the result of gene mutations, gross chromosomal abnormalities, and epigenetic changes in gene expression profile (Weinstein 2002). While tumors are largely clonal in origin, the combination of genetic and epigenetic events as well as influence of the tumor microenvironment leads to heterogeneity of tumor cell populations (Macaluse et al. 2003). One view on the origin of metastatic cells holds that continuing changes in gene expression in the primary tumor create a subpopulation of cells that gain a survival advantage as well as metastatic ability. Despite advances in understanding the mechanisms involved in metastasis, the key factors involved in generating the metastatic cells remain unknown.

To better understand the factors that make tumor cells metastatic we examined tumors in *Drosophila melanogaster*. Single gene mutations exist that cause tumors in specific tissues with 100% penetrance. Many Drosophila tumor suppressor genes have been identified in which disruption of gene function causes hyperplasic tumors (organs overgrow but retain normal arrangement of cells). Far fewer Drosophila tumor suppressor genes have been identified that when mutated cause neoplastic tumors (organs overgrow in an abnormal arrangement of cells). Three of the most studied are *lethal giant larvae* (*lgl*), *discs large* (*dlg*), and *scribble* (*scrib*) (Gateff 1978, Woods and Bryant 1989, Bilder and Perrimon 2000). Genetic interactions and interdependence of these proteins for localization indicate that they function in the same pathway (Bilder et al. 2000). Mutations in any of the three result in neoplastic overgrowth of the brain and imaginal discs during an extended third instar larval period. LGL protein is cortically localized and functions with DLG and SCRIB to maintain cell polarity in epithelial cells (Manfruelli et al., 1996; Tanentzapf and Tepass 2003; Hutterer et al. 2004) and neuroblasts (Peng et al. 2000; Ohshiro et al. 2000; Albertson and Doe 2003). Neuroblasts in *lgl* mutant larval brains undergo symmetric division to produce two neuroblasts rather than the normal asymmetric division producing a neuroblast and a ganglion mother cell (Lee et al. 2006a). Phosphorylation by atypical Protein Kinase C in the PAR complex regulates LGL function (Betschinger et al. 2003 ; 2005, Rolls et al. 2003). Unlike other genes involved in maintaining apical/basal polarity, LGL, DLG, and SCRIB have been shown to negatively regulate cell proliferation. All three were found to affect CyclinE activity in a genetic screen (Brumby et al. 2004).

Larvae mutant for the tumor suppressor *brain tumor (brat)* also show neoplastic overgrowth of larval brains (Arama et al. 2000). During normal development, BRAT is a negative regulator of rRNA synthesis. Loss of function results in an increase in cell growth and the amount of rRNA in the nucleoli, possibly causing one aspect of the tumor phenotype (Frank et al. 2002). BRAT also functions in embryos as a translational repressor of hunchback mRNA when complexed with Nanos and Pumilio (Sonada and Wharton 2001). Additional targets of repression are currently unknown. Recent studies have shown that BRAT is asymmetrically localized to ganglion mother cells in larval brains and functions to promote neuronal differentiation and inhibit self-renewal (Betschinger et al.2006, Lee et al. 2006b).

Drosophila is a powerful model to study the mechanisms of tumor metastasis because of the ability to manipulate the tissue *in vivo* and do forward genetics. Homozygous mutant clones of tumor suppressors have been generated specifically in eye discs and were found to be nonmetastatic in this system. Expression of activated RAS in conjunction with the loss of *lgl*, *dlg*, or *scrib* function resulted in cells migrating out of the discs into the brain lobes and ventral ganglia of the larva (Pagliarini and Xu 2003). Another *in situ* approach to studying metastasis involved studying ovarian tumors generated by temperature sensitive alleles of *dlg*. Mutant follicle cells in ovaries lost apical/basal polarity and migrated towards developing oocytes. This method was used to study the spatial and temporal patterns of epithelial tumor cell invasions (Goode et al. 2005).

Our lab analyzed the proliferative and metastatic abilities of Drosophila brain tumors using a transplantation assay. Fragments of tumorous brains from *lgl*, *dlg*, or *brat* mutant larvae were transplanted into the abdomens of adult hosts. Cells from these fragments were able to proliferate and travel to distant sites of the body including the leg, wing, and head (Woodhouse et al. 1997). Woodhouse et al. (2003) continued this work by using this assay as the basis of a screen to find genes involved in tumorigenesis and metastasis of *lgl*. They found that mutations in either of two genes, *apontic* or *pointed*, affect metastasis. They also found that *semaphorin 5c* gene activity was required for tumorigenesis and activation of the DPP (TGF-β) pathway in *lgl* tumors.

Our previous work demonstrated that Drosophila tumor cells are able to proliferate and travel throughout host's bodies. Because of the open circulatory system in Drosophila, the tumor cells could have been passively carried to distant sites. Their presence at these distant sites was not critical evidence of metastasis. In this study, we addressed this limitation by altering our assay to more effectively analyze invasion of host tissue by *lgl* and *brat* tumor cells. We also developed a method of quantifying the rate of metastasis that allowed for more critical evaluation of the invasive properties of these tumor cells. We refined our criteria for invasion by specifically measuring micrometastasis formation in host ovarioles. We found that both *lgl* and *brat* tumor cells were able to cross cell layers and basement membrane to form micrometastases in host ovarioles. Quantification of metastatic frequency revealed differences in the invasive abilities of *lgl* and *brat* tumor cells after extended proliferation. We discovered additional differences in *lgl* and *brat* tumor cells by analyzing cell type marker expression in the micrometastases. Our critical evaluation of *Drosophila* tumor cell metastasis demonstrated that these two tumor suppressor mutants that were previously believed to be metastatically similar in fact have distinct properties. This implies that the process by which tumor cells become metastatic in these mutants are different despite both tumors originating from the same organ and appearing superficially similar.

Materials and Methods

Fly Stocks

Drosophila stocks were maintained at 25°C on standard cornmeal, molasses, yeast, and agar food containing tegosept and proprionic acid as mold inhibitors.

Stocks used for donors in invasion assay

yw^{67} armadillo-lacZ; lgl^4/y^+CyO, yw^{67}; Df(2L)net^{62}/y^+CyO, yw^{67} armadillo-lacZ; $brat^{14}$/ y^+CyO, yw^{67}; $brat^{18}$/y^+CyO All *lgl* mutant larvae were progeny of crosses with the genotype of , *yw^{67} armadillo-lacZ; lgl^4/net^{62}* and they were identified by the *y* mutant phenotype. *Brat* mutant larvae were the progeny of a cross with the genotype of , *yw^{67} armadillo-lacZ; $brat^{14}$/$brat^{18}$* and they were identified by the *y* mutant phenotype.

Stocks used for hosts in invasion assay

Canton-S, *yw^{67}, VikingGFP/VikingGFP, $ovo^{D1}v^{24}$/C(1)DX,$y^1w^1f^1w^{11}$* Wild type hosts were generated by crossing Canton-S females with *yw* males. *Ovo^{D1}* mutant hosts were generated by crossing Canton-S females with *ovo^{D1}* males.

Transplantation of larval brain fragments

Transplantations of larval brain fragments were performed as previously described (Woodhouse, et al. 1998). *lgl* brain fragments were cultured in wild type hosts for 12 days at 25°C. *brat* brain fragments were cultured for 10 days in wild type hosts at 25°C. Both *lgl* and

brat fragments were cultured in *ovo*^{D1} hosts for 7 days at 25°C. All of the *lgl* and *brat* mutant cells were marked with armadillo-LacZ and detected in the host using an anti-βGal antibody.

Detection of micrometastases within host ovaries

After tumor culturing, the adult abdomens were opened ventrally to expose ovaries to solution. Ovaries were kept within the abdomen to prevent damage to the ovaries and ovariole loss. Host abdomens were fixed for 30 minutes in 3.7% formaldehyde in PBS, rinsed in PBS, washed 3×30minutes PBS, then 3×30minutes PBS + 0.6% Triton-x-100 (PBT), then 30 minutes in antibody incubation buffer consisting of PBT, 0.3% BSA, and 0.5% sheep serum. Samples were incubated overnight at 4°C on a rocker with primary antibodies diluted in incubation buffer. Samples were washed 3×30 minutes in PBT at RT. Secondary antibody incubation was performed overnight at 4°C then 3×30 min washes at RT. Samples were then incubated with 5U/mL Phalloidin (Molecular probes) and DAPI in incubation buffer for 1 hour at RT. Ovaries were dissected and ovarioles were separated onto a slide in VectaShield mounting medium with ovarioles from 1 host/slide. Tumor presence within an ovariole was detected using a Zeiss LSM 510 Meta microscope.

Primary antibodies used—chicken anti-□Gal 1:50 (Immunology Consultants Laboratory), mouse anti-Repo 1:150 (8D12 Developmental Studies Hybridoma Bank), rat anti-Elav 1:200 (Developmental Studies Hybridoma Bank). Anti-laminin B antibody 8E6 1:1 (D. Montell lab)

Secondary antibodies used—All secondary antibodies were used at a concentration of 1:200. FITC conjugated goat anti-chicken (Immunology consultants laboratory), TRITC conjugated rabbit anti-chicken (Sigma), Alexa Fluor 546 goat anti-mouse (Molecular Probes), Alexa Fluor 633 goat anti-Rat (Molecular Probes), Rhodamine Phalloidin (Molecular Probes).

Statistical Analysis

All statistics were computed using the G-test of independence from Sokal and Rohlf (1969).

Immunofluorescence of larval brains

Third instar larvae were inverted and fixed for 15 minutes in 3.7% formaldehyde in PBS, rinsed in PBS, washed 3 × 30minutes PBS. Samples were then washed 3×30minutes in PBT then 30 minutes in antibody incubation buffer described above. Samples were incubated in primary antibody in incubation buffer overnight at 4 degrees on a shaker. Samples were rinsed in PBT then washed 3 × 30 minutes then incubated in secondary antibody for 1hr at room temperature on a shaker. Samples were washed 3× 30 minutes in PBT then mounted in VectaShield mounting medium.

RESULTS

Drosophila model for tumor invasion

Previous work in our lab demonstrated that brain cells from tumor suppressor mutant larvae transplanted into adult hosts' abdomens traveled to distant sites within the hosts such as the head, wing, and leg. However, it did not provide critical evidence for invasion of host tissue by donor tumor cells. In this study, such evidence was provided by assaying micrometastasis formation within the ovarioles that make up the Drosophila ovary. For tumor cells to be found within the ovarioles, they must pass through cell layers and extracellular matrix.

The Drosophila ovary consists of 15-20 individual ovarioles surrounded by a peritoneal sheath of cells. Each ovariole is an egg assembly line with a germarium at the anterior tip containing the stem cells and progressively more developed egg chambers towards the posterior. Each egg chamber contains a developing oocyte and nurse cells surrounded by a follicular

epithelium. Each ovariole is surrounded by an epithelial sheath which consists of three layers: a layer of muscle cells that is sandwiched between two acellular layers of basement membrane (Fig 1 A,B). The inner layer of the epithelial sheath rests against a basement membrane surrounding the egg chambers providing support for the follicle cells (Fig 1 A, Cummings 1974). For a micrometastasis to be found within an ovariole, tumor cells must actively pass through the cell layers and basement membranes that constitute the peritoneal and epithelial sheaths (Fig1 A arrow).

Tumor suppressor mutant lines were marked with armadillo-LacZ to allow for detection of tumor cells within the host, as previously described (Woodhouse et al. 1997). Larval brain lobes were quartered and transplanted into abdomens of adult-female hosts. The tissue proliferated for 10 to 12 days depending on the mutant to allow for maximum tumor growth with minimum host death (Fig. 1 C). After this culture period, hosts' abdomens were opened ventrally to reveal the ovaries. We detected tumor cells within the host ovary by immunofluorescence of the reporter gene product.

Wild type third instar larval brain fragments transplanted into adult hosts and cultured for 12 days were able to survive but not proliferate (fig 2 A-C). Larvae mutant for *lgl* have an extended third instar period during which the brain and imaginal discs undergo neoplastic overgrowth. Brain fragments from 10 day old *lgl* larvae transplanted into adult hosts and incubated for 12 days proliferated extensively and filled hosts' abdomens (Fig 2 D-F). *Brat* tumors proliferate at a faster rate than *lgl* tumors (Woodhouse et. al. 1998), killing the hosts in a shorter span of time. Because of the reduced numbers of host recovered after 12 days, all hosts transplanted with *brat* tumors were harvested after 10 days. The *brat* tumors were able to proliferate and fill the host as effectively as *lgl* tumors (fig 2 G-I).

Lgl and *brat* tumors are metastatic

Our first goal in this study was to determine if the tumor cells gave rise to micrometastases in host ovaries. We assessed micrometastasis formation by immunofluorescence of the host ovaries. We first asked if tumor cells were able to invade past the cell layers that surround the ovarioles. The epithelial sheath contains a muscle layer that continuously surrounds the ovariole; there are no gaps between the cells that would allow for passive entry of tumor cells. We visualized the muscle layer of individual ovarioles by detecting cortical actin using phalloidin.

Micrometastases that formed were clearly contained within the muscle layer of the epithelial sheath, lying next to the germarium or more developed egg chambers (Fig3 A-C, E-G). Both *lgl* and *brat* tumor cells were able to pass through the muscle layer and form micrometastases. Previous work in *Drosophila* detected migration of tumor cells from the eye disc into the brain (Pagliarini and Xu 2003) as well as the migration of mutant follicle cells through nurse cells towards the developing oocyte (Goode et al. 2005). Neither instance demonstrates active metastasis involving crossing cell layers or extracellular matrix. Our study is the first critical demonstration of *Drosophila* metastasis formation in a location distant from the primary tumor. In our system, only cells that leave a primary tumor and actively pass through cell layers and extracellular matrix are classified as micrometastases.

Having established that tumor cells invaded through both the peritoneal sheath and the muscle layer of the epithelial sheath, we next examined whether they were able to invade past the dense extracellular matrix that constitutes basement membranes. To visualize basement membranes, we used a Drosophila line that has a non-lethal insertion of GFP into the *viking* gene. The *viking* gene product is a subunit of Type IV collagen, a primary component of the basement membrane. This reporter highlights both the basement membranes that are on either side of the muscle layer in the epithelial sheath as well as the basement membrane that surrounds the

follicular epithelium that protect the germ line. Mutant brain fragments were transplanted into VikingGFP hosts. Cells from both tumor types were able to pass the basement membranes that surrounds the muscle layer of epithelial sheath (fig 3 D,H), demonstrating that the tumor cells were able to completely pass through all three layers of the epithelial sheath. Each micrometastasis we found developed in the hemolymph filled space between the epithelial sheath and the basement membrane surrounding the follicular epithelium

Lgl and *Brat* tumors have similar rate of metastasis

After documenting the presence of micrometastases in host ovaries, we wanted to quantify the metastatic properties of these tumors cells. Our method of using ovarioles to examine invasions provided us with a means to quantify the frequency of metastasis. The frequency was determined by the percentage of ovaries with micrometastases. Ovarioles with multiple micrometastases were only counted once due to the difficulty in determining whether multiple micrometastases represented separate invasion events or one event that spread in the ovariole. Due to this method of quantification, the frequencies we determined are conservative estimates.

Lgl mutant cells transplanted into wild type hosts formed micrometastases in 15.8% of the ovarioles investigated after 12 days of proliferation (58 out of 367). All of the hosts examined had at least one ovariole with a micrometastasis. *Brat* tumor cells formed micrometastases in 15% of ovarioles assayed after 10 days of proliferation (61 of 406). Wild type brain tissue transplanted into adult hosts did not show any micrometastases after 12 days of culture (Table 1).

Lgl tumor metastatic rate increases with continuous passaging

Lgl and *brat* tumor cells formed micrometastases at approximately the same frequency. One current view of metastasis proposes that within a primary tumor a rare subpopulation of cells have a selective advantage and metastatic ability. If the metastatic cells have a selective advantage, then increased proliferation time would allow for an enrichment of this subpopulation in the primary tumor. We examined if there was a change in the frequency, the size, and/or location of micrometastases after extending the proliferation time of the tumor.

Hosts die within two weeks after transplantation because of primary tumor growth, prohibiting extended proliferation in one host. To extend the amount of culture time, we serially transplanted tumor masses into multiple hosts over time (Fig 4A). Brain fragments were transplanted into female-sterile ovo^{D} hosts. These hosts have rudimentary ovaries so there was more space for tumor proliferation and allowed for harvesting of the primary tumor without interference from host tissue. The tumor cells were cultured for 7 days in hosts' abdomens then the primary tumors were harvested from the hosts' abdomens based on the morphology of the cells. Cells from the primary tumor were then retransplanted into new, young hosts. Tumor cells can proliferate for an extended period of time with repeated transplantations. Because of the large amount of tumor cells that can be grown within a single host, many new hosts can be injected from the same primary tumor mass. We retransplanted the tumor cells into ovo^{D} hosts to continue proliferation of the primary tumor and retransplanted into wild type hosts to assess the frequency of metastasis.

As described above, *lgl* tumors transplanted directly into wild type hosts for 12 days formed micrometastases in 15.8% of the ovarioles examined (58 of 367). After transplanting the *lgl* brain tissue into ovo^{D} hosts for 7 days then retransplanting into wild type hosts for 12 days, micrometastases formed in 23.8% of the ovarioles analyzed (87/366). This was a statistically significant increase in metastatic rate compared to 12 days of tumor growth ($P<0.01$). Further incubation time again increased the rate of metastasis. *Lgl* brain fragments were transplanted into ovoD hosts for 7 days, then retransplanted into ovo^{D} hosts for 7 days, and finally

transplanted into wild type hosts for 12 days. *Lgl* tumor cells formed micrometastases in 41.3% of the ovarioles examined (92 of 223, table 1). While the frequency of invasion increased significantly, the micrometastases that formed in the hosts did not penetrate the follicular epithelium and appeared similar in size at each time point (Fig. 4 B-D).

Brat tumors maintain the same metastatic rate with continuous passaging

Brat tumor cells were transplanted into multiple hosts as described above, with growth of tumor cells in ovo^D hosts for 7 days, and growth in wild type hosts for 10 days. This allowed us to determine if *brat* tumors showed alterations in metastatic ability after 10, 17, or 24 days.

Unlike *lgl* tumors, *brat* tumors show no significant increase in the frequency of micro-metastases when continuously passaged (Table1). As stated above, *brat* brain fragments transplanted into wild type hosts for 10 days formed micrometastases in 15% of the ovarioles examined (61 of 406). *Brat* brain fragments transplanted into ovo^D hosts for 7 days then wild type hosts for 10 days formed micrometastases in 20.5% of the ovarioles examined (80 of 391); this was not a statistically significant increase as determined by a G-test of independence. *Brat* tissue serially transplanted into *ovoD* hosts for two 7 day periods then into wild type hosts for 10 days, formed micrometastases in 20% of the ovarioles examined (102 of 508); again this was not a significant change. *Brat* tumor cells that were grown in multiple hosts also did not show any change in depth of penetration into the ovariole. All of the micrometastases had similar size and were found between the epithelial sheath and the follicular epithelium at each time point (Fig. 4 E-G).

Lgl tumors within ovarioles show cell fate determinants for both neuronal and glial cells

This analysis of metastatic frequencies after prolonged culturing demonstrated that *lgl* and *brat* invasive properties are different. To further explore the differences between *lgl* and *brat* tumor cells, we compared expression of cell type markers in the primary tumors as well as in the micro-metastases derived from them.

Neuronal and glial cells arise from the same progenitors called ganglion mother cells (GMCs) but have completely separate cell fates. During normal development ELAV, an RNA binding protein, is found only in neuronal cells (Soller and Whiter 2004); REPO, a transcription factor, is only present in glial cells (Jones 2005). We examined accumulation of these markers in larval brains to establish that they were present in the mutant brains and that they do not co-express in the same brain cells. Wild type larval brains show expression of ELAV and REPO in distinct cells and areas of the brain (Fig. 5 A). *Lgl* mutant larval brains are disorganized but still showed distinct expression of ELAV and REPO in separate cells (Fig. 5 B).

We performed immunofluorescence on *lgl* tumor cells that proliferated within hosts but did not invade. There were many ELAV positive cells and some REPO positive cells, but there was no accumulation of both markers in the same cell (Fig. 5 D). Examination of micrometastases showed a dramatically different expression pattern. Almost all of the micrometastases within ovarioles, 93%, were positive for both ELAV and REPO in all cells (Fig. 6 A-D, Table 2). A small number of micrometastases within ovarioles expressed REPO alone (7%) (Fig. 6 E-H).

Brat tumors within ovarioles show variable staining for neuronal and glial markers

Brat mutant larval brains also showed a loss of normal brain structure and organization. Overall, there was a reduced amount of ELAV and REPO expression in the brain. As with *lgl* mutants, there was no overlap of ELAV and REPO in any cells of the brain. (Fig. 5 C). When we examined tumor cells that grew within the host and remained in the primary tumor,

some had ELAV accumulation and a few had REPO accumulation but there were no cells with both markers (Fig. 5 E).

The micrometastases within the ovarioles showed variable expression of REPO and ELAV. Half of the micrometastases examined (51.6%) had neither ELAV nor REPO accumulation (Fig. 6 U-X, Table 2). Less than half of the micrometastases (34.9%) were positive for both ELAV and REPO (Fig. 6 I-L). The remainder of the micrometastases examined expressed either REPO alone, 4.7%, (Fig. 6 M-P) or ELAV alone, 9.3% (Fig. 6 Q-T). The analysis of ELAV and REPO expression show that multiple cell populations are metastatic in *brat* tumors. The expression patterns also demonstrate that the micrometastases are clonal in origin. There were no single micrometastases in which some cells expressed one marker and some expressed the other. Each micrometastasis has a uniform expression pattern indicating that a single cell invaded and proliferated.

Discussion

Drosophila Tumor metastasis

Our previous work established that *lgl* and *brat* mutant brain cells transplanted into wild type female hosts were capable of proliferating and traveling to distant sites in the host. Because flies have open circulatory systems, cells that disseminated from the primary tumor could have traveled to distant sites passively via the flow of hemolymph. In this study, we demonstrated that *lgl* and *brat* tumor cells transplanted into wild type hosts were capable of invading a specific host tissue and forming micrometastases after transplantation. This required the tumor cells to pass through the peritoneal sheath of cells surrounding the ovary and then the three layers of the epithelial sheath surrounding the ovariole. This is a more critical assay of metastasis than our previous work since both the peritoneal and epithelial sheath are continuous layers that would prevent any passive movement of cells into ovarioles.

Brat and *lgl* tumor cells have different metastatic properties

After establishing that *lgl* and *brat* tumors are metastatic, we determined the frequency of invasion. After transplantation into adult hosts, *lgl* and *brat* tumor cells invaded ovarioles at a similar frequency, 15.8% and 15% respectively. By extending the proliferation time for the tumor cells in hosts, *lgl* tumors invaded ovarioles with increased effectiveness. This result is consistent with the view that metastasis is caused by a subset of cells from primary tumors that have a survival advantage such as increased resistance to apoptosis or a more rapid cell cycle in addition to increased migratory behavior. The advantage in the metastatic subpopulation could be due to genetic or epigenetic alterations in the metastatic cells or be due to more primary tumor cells transforming into metastatic cells over time. The extended proliferation time increased the frequency of metastasis while the size and location of micrometastases within ovarioles were all the same. This suggests that the number of cells in the metastatic population increased over time but the characteristics of that subpopulation remained unchanged. The metastatic frequency of *brat* tumor cells did not increase with extended proliferation time. One possible explanation is that the metastatic cells do not have a selective advantage over the rest of the cells preventing enrichment of the metastatic subpopulation in the primary tumor.

Comparing the effects of extended culturing time on *lgl* and *brat* tumor cells clearly demonstrates differences in the metastatic cells of each tumor. While both *lgl* and *brat* mutations cause brain tumors in larvae, the mechanisms of that transformation are different. This difference must be explained by the different primary defects caused by the lack of LGL activity (Betschinger et al. 2006) compared to the lack of BRAT activity (Lee et al. 2006). Neuroblasts are stem cells; they divide asymmetrically to produce another neuroblast and a ganglion mother cell (GMC) The lack of LGL activity prevents asymmetric localization of

BRAT and other determinants required for the formation of GMCs. Consequently many of these divisions in *lgl* mutants are symmetric; they produce two neuroblasts. BRAT activity in presumptive GMCs is important for preventing self-renewal and promoting differentiation. In *brat* mutants, some presumptive GMCs revert to a neuroblast-like pattern of self renewal.

Recent studies suggest that tumors have cancer stem cells which are responsible for most of the tumor proliferation while most of the tumor bulk does not continue to divide indefinitely (Al-Hijj et al. 2003, Lapidot et al. 1994, Singh et al 2004). A recent review has speculated that the origin of cancer stem cells could arise in two ways (Clark and Fuller 2006): first the constraints on normal stem cells are altered or removed allowing for cancerous proliferation. This appears to be how *lgl* tumors arise. Second, cells that normally proliferate a few times before terminal differentiation revert to a more stem cell like pattern of proliferation. This appears to be how *brat* tumors arise. We have shown that *lgl* and *brat* tumor metastatic abilities are very different, one reason could be due to the different origins of the proliferating cells in the tumors. Understanding how the difference in the origin of cancer stem cells affects the behavior of the tumor has profound implications on the development of treatments specifically aimed at cancer stem cells rather than the bulk tumor.

Drosophila brain tumor metastases have both neuronal and glial fate

We used expression of a neuronal and a glial cell marker to examine cell fate determination in micrometastases. We first established that both the neuronal marker ELAV and the glial marker REPO were expressed in the mutant brains. We showed that while both *lgl* and *brat* brains were overgrown and disorganized, they still contained distinct populations of neurons and glia as shown by ELAV and REPO expression; these markers are normally present only in differentiating cells and not in precursors. The *lgl* brains contain an equal if not greater number of ELAV and REPO positive cells as compared to wild type brains. Not all of the divisions are abnormal in *lgl* brains, some GMCs are produced and these cells differentiate normally producing the ELAV and REPO positive cells visible in the *lgl* brain. The *brat* brains exhibit a decrease in cells expressing REPO and ELAV which would be expected due to the necessity of BRAT function in the GMC for differentiation.

Tumor masses proliferating in hosts contain cells positive for either neuronal or glial cell type markers. Since wild type tissue does not proliferate in hosts, all of these cells must be transformed despite the presence of these cell type markers. Cells accumulating either marker remained in a morphologically less differentiated state; for example they do not have axon projections (unpublished result). Almost all of the *lgl* micrometastases in ovarioles expressed both neuronal and glial markers. The expression of ELAV and REPO could be a indicator of the changes necessary that allow a cell to become metastatic. If there were pro-metastatic cells co-expressing both markers within primary tumors, they must have been an extremely small population within the primary tumor because we could not detect them by confocal microscopy. Expression of these cell type markers does not necessarily indicate the differentiation state of the metastatic tumor cells. More likely, the expression of multiple cell type markers in a single tumor cell reveals deregulation of gene expression within that cell.

Brat primary tumors had very few REPO positive cells and some ELAV positive cells. Unlike *lgl* micrometastases which showed a consistent expression pattern, brat micrometastases were heterogeneous with regard to marker expression. Based on the multiple cell type marker expression patterns, *brat* tumors appear to contain multiple subpopulations that are able to invade that host ovary. The lack of increase in metastatic frequency of *brat* tumors after proliferation could be attributed to all of the cells in the tumor having equal survival ability. Therefore, increased proliferation time would not allow for an enrichment of the metastatic subpopulation.

This study showed the power and adaptability of using Drosophila for tumor metastasis study. *Lgl* and *brat* cause brain tumors initially appeared similar in ability to form micrometastases in a transplant host. In exploring how tumor cells invade it became apparent that the process of metastasis is not the same for these cells. The cells in each tumor mutant follow a different path to the same result. Such analysis should be applied to other tumor suppressor mutants to better understand their metastatic characteristics. *Dlg* and *scrib* are tumor suppressor mutants that interact with *lgl* and form similar tumors of the brain and imaginal discs. While all three proteins have been shown to interact, there are differences in localization and protein interactions. Do these mutants utilize the same pathway for metastasis as *lgl* or is each tumor different? Understanding the differences in metastatic properties will lead to uncovering the underlying molecular mechanisms.

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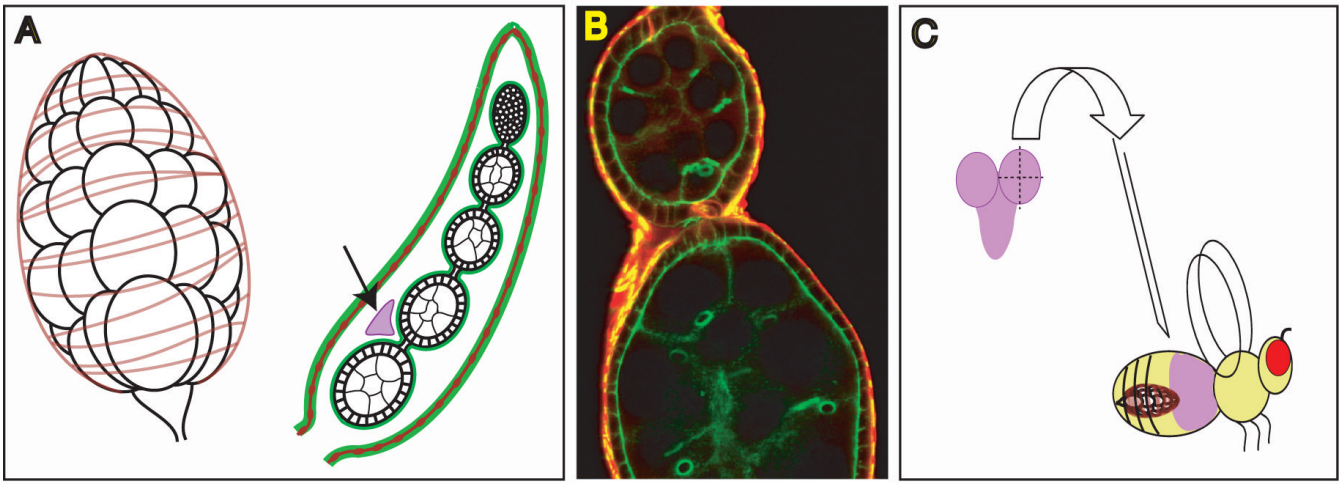

Figure 1.
Drosophila model for tumor metastasis
(A) A *Drosophila* ovary consists of 15-20 individual ovarioles surrounded by a peritoneal sheath of cells (pink). Diagram of a single ovariole highlighting the three layers of the epithelial sheath: muscle layer (red) between two layers of extracellular matrix (green). The individual egg chambers are surrounded by another basement membrane (green) that supports the follicular epithelium. In purple, an example of a micrometastasis that has passed the epithelial sheath (arrow). (B) Confocal section of an ovarioles demonstrating the continuous nature of the epithelial sheath. The basement membrane is highlighted in red (laminin) and the muscle layer in green (phalloidin). (C) Transplantation assay: Larval brain lobes marked with a reporter gene (purple) are quartered and injected into the abdomen of an adult host. Tumor cells proliferate and fill the abdomen. The host abdomens are dissected and immunofluorescence is performed on the ovaries to detect the reporter protein from tumor cells that have metastasized.

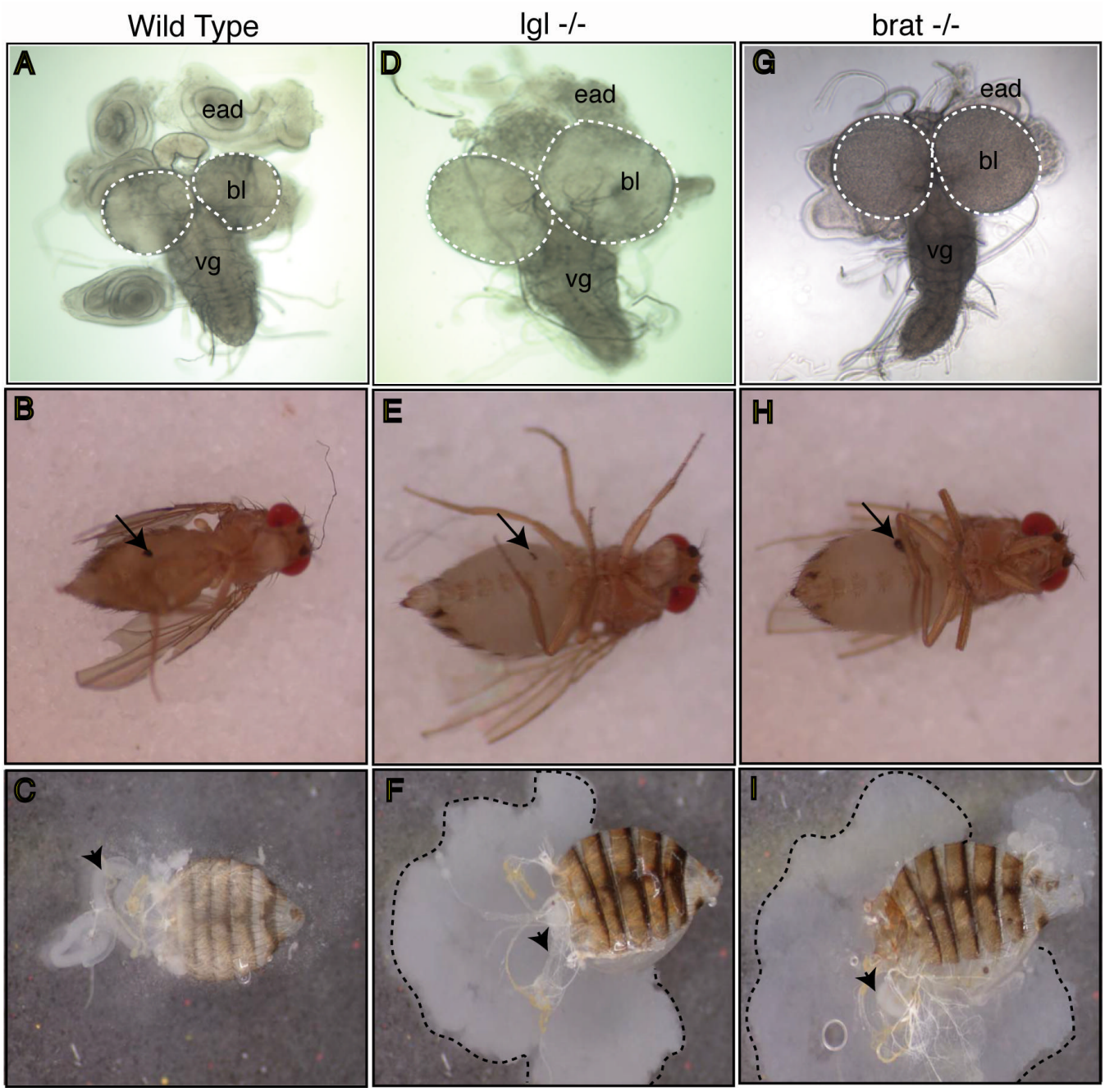

Figure 2.

Fragments of tumorous brains proliferate in adult hosts after transplantation

Wild type larval brain (A) was injected into an adult host and did not proliferate (B). Arrow indicates the injection scar. (C) The dissected abdomen did not contain any overgrown tissue. Arrowhead indicates the gut of the host. *Lgl* brain lobes (D) were quartered and injected into adult host. (E) Adult host 12 days post transplantation. The abdomen was distended compared to (F). Arrow indicates injection scar. (G) The dissection of an adult host 12 days after *lgl* tumor transplantation. Dashed line marks tumor cells that were contained within the host abdomen. Arrowhead indicates host gut. *Brat* brain lobes (H) transplanted into adult hosts overproliferated and distended the host abdomen after 10 days of culturing (I). The dissected

abdomen was filled with tumor cells outlined by the dashed line (J) bl: brain lobe, ead: eye-antennal disc, vg: ventral ganglia

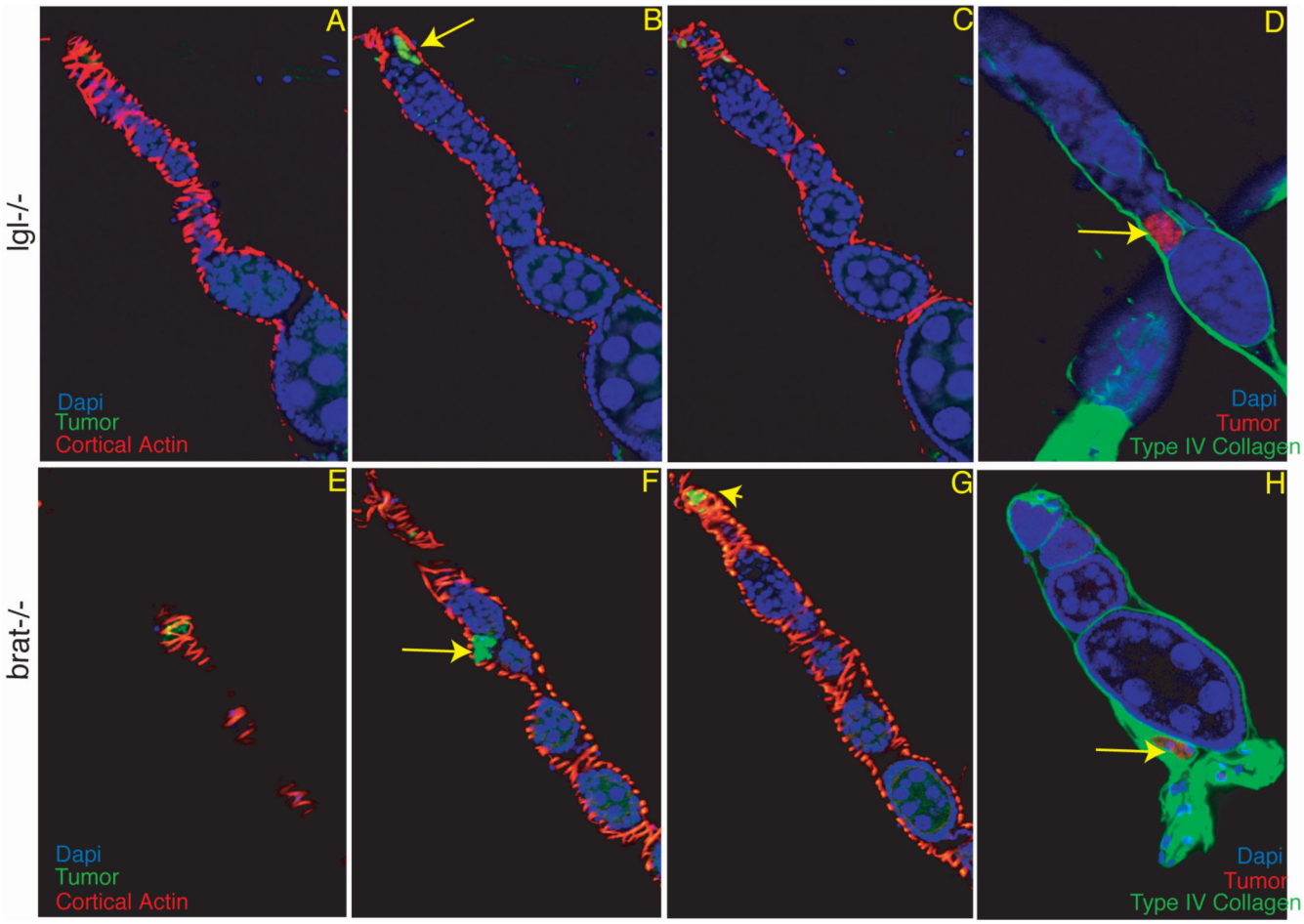

Figure 3.

Drosophila brain tumor cells formed micrometastases within host ovarioles

(A-C) Confocal sections through a host ovariole containing an *lgl* mutant micrometastasis (green, arrow) that has crossed the muscle layer of the epithelial sheath (red) (E-G) Confocal sections through a host ovariole containing a *brat* mutant micrometastasis (green, arrow) that has formed past the muscle layer of the epithelial sheath (red). Arrowhead indicates a second micrometastasis within the ovariole. (D,H) *lgl* and *brat* micrometastases (red) that have formed past the basement membrane (green) of the epithelial sheath in host ovarioles. The micrometastases did not pass the basement membrane that surrounds the follicular epithelium. Tumor cells were detected by the expression of a lacZ reporter construct driven by an armadillo promoter.

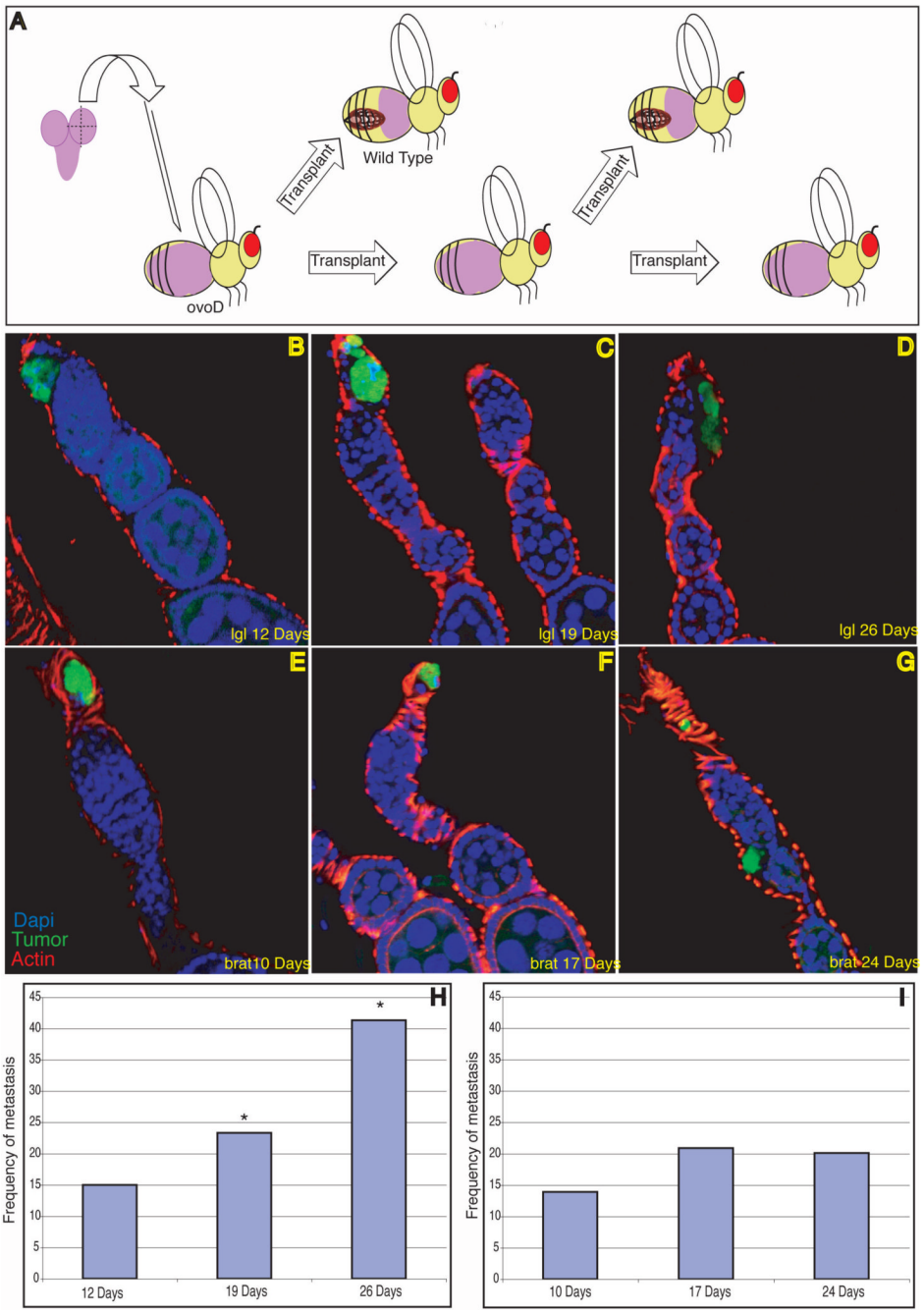

Figure 4.

Serial transplantation of tumor cells increases *lgl* metastatic frequency but not *brat* metastatic frequency.

(A) Diagram of serial transplantation assay. Fragments of tumorous brains were injected into an *ovo*^{D} host and allowed to proliferate (purple). The primary tumor mass was harvested from the host and cells from the primary tumor were retransplanted into new wild type hosts for analysis as well as new *ovo*^{D} hosts for extended proliferation time of the primary tumor cells. (B-D) *lgl* micrometastasis formation after extended proliferation. Micrometastases were able to pass the epithelial sheath and one layer of basement membrane. Additionally, the micrometastases were similar in size and shape. (E-G) *Brat* micrometastasis formation after

extended proliferation. Micrometastases were similar in size and shape. (H) Frequency of *lgl* tumor cell metastasis significantly increases with extended proliferation (* $p < 0.01$). (I)The frequency of *brat* metastasis does not increase with extended proliferation.

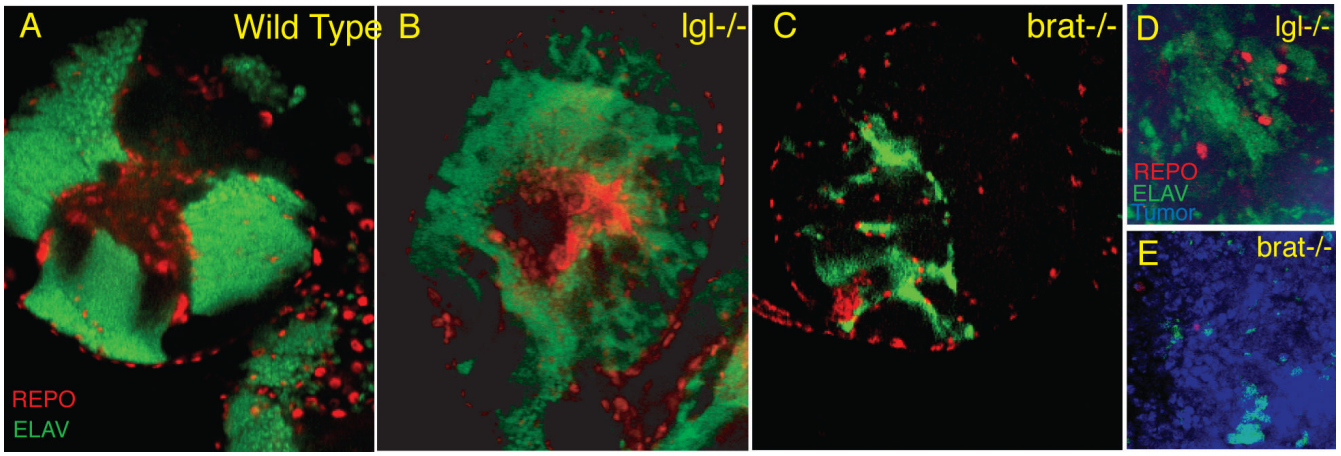

Figure 5.

Brain cells and primary tumor cells do not co-express ELAV and REPO cell type markers. Confocal sections of a wild type larval brain lobe (A), *lgl* brain lobe (B), and *brat* brain lobe (C) contain individual cells expressing ELAV (green) and REPO (red) with no cells expressing both cell type markers. (D) *lgl* primary tumor mass contains cells expressing either ELAV or REPO but no cells co-expressing both markers. (E) *brat* primary tumor mass contains fewer cells expressing either ELAV or REPO.

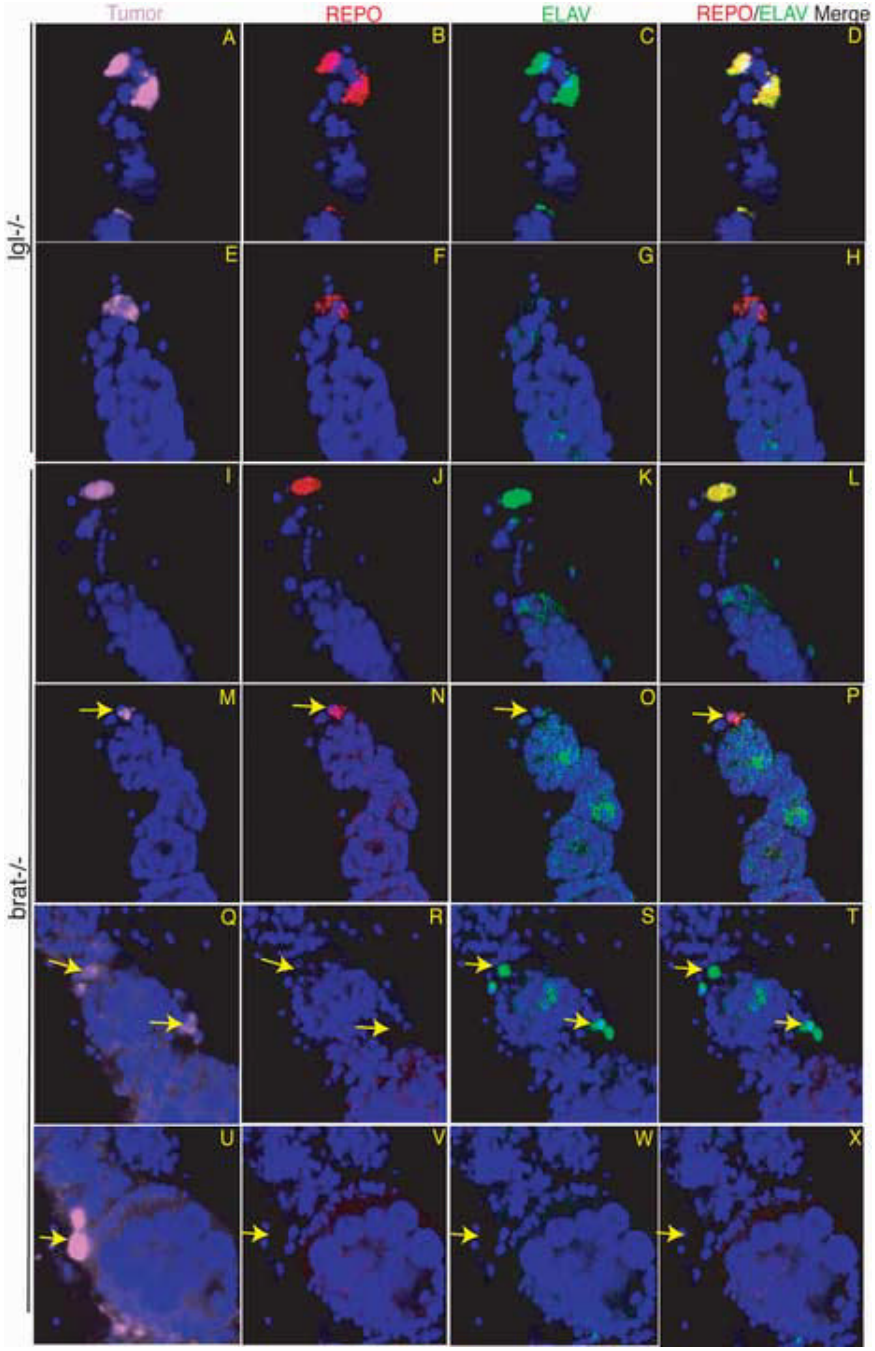

Figure 6.

Expression of ELAV and REPO in micrometastases

(A-D) *Lgl* micrometastasis (pink) expressing both REPO (red) and ELAV (green) in all of the cells. The ovariole is highlighted by DAPI (blue). (E-H) *lgl* micrometastasis expressing REPO but not ELAV. (I-L) *Brat* micrometastasis expressing both REPO and ELAV in all cells. (M-P) *Brat* micrometastasis expressing REPO in cells but not ELAV. Background ELAV expression is visible within the ovariole. (Q-T) *Brat* micrometastasis expressing ELAV alone. (U-X) *Brat* micrometastasis without expression of REPO or ELAV in the tumor cells. Tumor in pink, REPO in red, ELAV in green, DAPI in blue.

Table 1

Ovarioles with micrometastases after incubation with tumor cells

Injected Tissue	Incubation Time in Host		
	10/12Days	17/19 Days	22/24 Days
Wild Type	0/276 (0%)	ND	ND
lgl −/−	58/367 (15.8%)	87/366 (23.5%)*	92/223 (41.3%)*
brat−/−	61/406 (15%)	80/391 (20.5%)	102/508 (20%)

* = Number of ovarioles with invasions is significantly increased ($p < 0.01$) compared to previous time point according to G-test of independence

Table 2

Neuronal and glial cell type marker expression in micrometastases

Tumor	Total	REPO	ELAV	REPO+ELAV	None
lgl −/−	58	4	0	54	0
brat −/−	43	2	4	15	22