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Metastatic ability of *Drosophila* tumors depends on MMP activity

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Abstract

We analyzed how cells from tumors caused by mutations in either *lgl* or *brat* use matrix metalloproteinases (MMPs) to facilitate metastasis in *Drosophila*. MMP1 accumulation is dramatically increased in *lgl* larval imaginal discs compared to both wild type and *brat* mutants. Removal of *Mmp1* gene activity in *lgl* brain tumor cells reduced their frequency of ovarian micro-metastases after transplantation; whereas, removal of *Mmp1* gene activity in *brat* tumor cells had no such effect. Host ovaries showed increased *Mmp1* gene expression in response to transplantation of *brat* tumors but not of *lgl* tumors. Reduction of MMP activity in host ovaries by ectopic expression of TIMP significantly reduced both *lgl* and *brat* metastases in that organ. These results highlight the mechanisms that *lgl* and *brat* tumor cells use to metastasize. Our interpretation of these data is that secretion of MMP1 from *lgl* tumor cells facilitates their metastasis, while secretion of MMP1 from host ovaries facilitates *brat* tumor metastasis. This study is the first demonstration that *Drosophila* tumors utilize MMP activity to metastasize.

Keywords: *Drosophila*; Matrix metalloproteinase; *Lethal giant larvae*; *Brain tumor*; Metastasis

Introduction

Metastasis is the formation of secondary tumors in distant sites of the body, leading to poor prognosis and poor chance of patient survival. The process of metastasis consists of several steps. First, tumor cells leave the primary tumor, move through the basement membrane and migrate into the surrounding stroma. The tumor cells then enter the vasculature, travel to distant sites, leave the vasculature, migrate into new tissues, and form secondary tumors (Geho et al., 2005; Woodhouse et al, 1997).

Understanding the factors involved in tumor cell metastasis is complicated by contributions from host stroma, extracellular matrix, and angiogenesis. Primary tumors recruit normal cells into the tumor and stimulate angiogenesis to support the tumor mass. Tumor cells also interact with the host environment by releasing signals to the host cells that stimulate the surrounding stroma to release pro-migratory factors that facilitate metastasis (Allinen et al., 2004; Bowden et al., 1999; Fidler, 2002; Liotta and Kohn, 2001). Knowing how host responses affect tumor metastasis is important as a potential target for cancer therapy. The host tissue is not under the same selective pressures as tumor cells and less prone to genetic instability. This is potentially advantageous as a therapy target because there is less chance of adaptation to the therapy.

Proteases are released by both host cells and cancer cells, altering the microenvironment of tumor cells (Airola and Fusenig, 2000; Werb, 1997). Proteases facilitate migration by degrading extracellular matrix and by cleaving signaling molecules, other proteases, and proteins allowing for activation of pro-migratory signals and inactivation of anti-migratory signals (Andreason et al., 1997; Nakahara et al., 1997; Xu et al., 2001). Matrix metalloproteinases (MMPs) are one class of proteases that have been implicated in tumor progression. There are over 24 MMPs identified in mammals; these fall into a number of subgroups based on substrate specificity and protein domains. Broadly, MMPs fall into two groups, soluble-secreted proteases and membrane-bound proteases (reviewed by Sternlicht and Werb, 2001). Increased expression of MMPs within tumors has been positively correlated with the malignancy of

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many different cancer types such as advanced melanoma, colon cancer, and squamous cell carcinomas (MacDougall et al., 1995; Murray et al., 1996; Yamamoto et al., 2004).

MMPs have been implicated in several stages of tumor progression such as angiogenesis, proliferation, and metastasis. Numerous studies have highlighted the importance of MMP expression in both tumor cells and in host cells for tumor progression (examples: Coussens et al., 2000; Hiratsuka et al., 2002; Lynch and Matrisian, 2002; Sabeh et al., 2004; Sounni et al., 2002). For example, host expression of MMP-9 has been correlated with increased angiogenesis and proliferation of transplanted tumors (Huang et al., 2002). Mice deficient for MMP-7 as well as carrying a germline mutation in APC show reduced benign intestinal tumors compared to mice carrying the mutant APC alone (Wilson et al., 1997). Tumor cells expressing MT1-MMP showed increased ability to grow and migrate in a 3D matrigel assay compared to cells deficient for MT1-MMP (Hotary et al., 2003). Some MMPs have been shown to have conflicting effects on tumor progression. Expression of MMP-12 in squamous cell carcinoma tumors correlates with invasiveness while expression of MMP-12 in macrophages generates angiostatin causing an inhibition of angiogenesis in tumors (Kerkela et al., 2002; Dong et al., 1997; Cornelius et al., 1998). The large number of MMPs and their involvement in many stages of tumor progression make it difficult to evaluate separate contributions of individual MMPs at specific stages in tumor progression. Additionally, MMPs can have different functions when expressed in tumor cells or in host tissues.

Tissue Inhibitors of Matrix Metalloproteinases, TIMPs, are endogenous inhibitors of MMP activity. In mammals, four TIMPs have been identified that are capable of reversibly binding to the catalytic site of MMPs and inhibiting activity (Sternlicht and Werb, 2001). Each TIMP has distinct functions and expression patterns in the body (Chirco et al., 2006). *Drosophila* has only one TIMP; it has been shown to inhibit MMP activity *in vivo* (Page-McCaw et al., 2003).

Drosophila has just two MMPs; they both contain all of the hallmark domains of MMPs but do not appear orthologous to specific mammalian MMPs. MMP1 is a soluble protease that is required for larval trachea development and metamorphosis during pupation (Page-McCaw et al., 2003). MMP1 is expressed at low levels throughout development with increased expression at the end of embryogenesis and during the pupal stage (Llano et al., 2000; Page-McCaw et al., 2003). MMP2 is membrane bound by a putative GPI anchor; it is required in tissue histolysis and metamorphosis in pupae (Llano et al., 2002; Page-McCaw et al., 2003). The lack of redundancy in *Drosophila Mmp* genes potentially allows for examination of their specific roles in normal development and tumor progression.

Previously, our lab demonstrated differences in metastatic properties of *lgl* and *brat* tumors (Beaucher et al., 2006). Loss of either gene function results in morphologically similar neoplastic brain tumors. We transplanted fragments of mutant brains into adult hosts where the tumor cells proliferated and filled the abdomen. This method allowed for independent manipulation of the host and tumor genotypes. We assayed for micro-metastasis formation in the ovariole. The *Drosophila* ovary consists of approximately 15 individual ovarioles that consist of germ line stem cells at the apical tip and a series of developing egg chambers, each containing an oocyte and supporting nurse cells surrounded by follicle cells. The entire ovary is surrounded by a peritoneal sheath of cells and each ovariole is surrounded by an epithelial sheath of cells as well as basement membranes that underlie the epithelial sheath and also surround each egg chamber. Tumor cells must actively cross two cell layers (peritoneal and epithelial sheaths) as well as basement membranes to form micro-metastases in ovarioles.

We determined the metastatic frequency as the percentage of ovarioles containing micro-metastases. By this method, we found that both *lgl* and *brat* tumor cells had similar frequencies of micro-metastases. Differences became clear with serial transplantation of tumor cells into hosts. Increased time in hosts led to a significant increase in the frequency of *lgl* micro-metastases while the frequency of *brat* micro-metastases remained constant. Additional differences were shown in expression of cell fate markers in the micro-metastases. Brain tumors in *lgl* and *brat* mutant larvae arise through disruption of asymmetric division in neuroblasts (Lee et al., 2006a; Betschinger et al., 2006; Lee et al., 2006b). Despite their seemingly similar origins, *lgl* and *brat* tumors have remarkably different metastatic properties.

In this study, we continued our exploration of the differences between *lgl* and *brat* metastatic properties. To start understanding the mechanisms utilized by the tumor cells to alter their microenvironment, we examined the contribution of MMPs to metastasis. Our study demonstrates that MMP1 facilitates the metastasis of both *lgl* and *brat* tumors. We found that *Mmp1* expression in *lgl* tumor cells but not in *brat* tumor cells facilitates metastasis. We reduced MMP activity in host ovaries by ectopic expression of TIMP. This resulted in a decreased frequency of micro-metastases by both *lgl* and *brat* tumors. Host ovaries increase their level of *Mmp1* expression in response to *brat* but not *lgl* tumors. This suggests that *brat* tumor cells stimulate host ovaries to secrete MMP1. We infer that MMP1 facilitates metastasis both by *lgl* and *brat* tumor cells but they utilize it from different sources. This study is the first demonstration of any MMP involvement in *Drosophila* tumor metastasis.

Experimental procedures

Fly stocks

Drosophila stocks were maintained at 25° 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*; lgl^{4} *Mmp1*^{2}/y^{+}*CyO*, yw^{67}; *Df (2L)net*^{62}*Mmp1*^{101}/y^{+}*CyO*. All *lgl* mutant and *lglMmp1* double mutant larvae were the progeny of crosses between these stocks and were identified by the *y* mutant phenotype.

yw^{67} *armadillo-lacZ; brat*^{14}/y^{+}*CyO*, yw^{67}; *brat*^{18}/y^{+}*CyO*, yw^{67} *armadillo-lacZ; brat*^{14}*Mmp1*^{101}/y^{+}*CyO*, yw^{67}; *brat*^{18}*Mmp1*^{2}/y^{+}*CyO*. All *brat* mutant and *bratMmp1* double mutant larvae were the progeny of crosses between these stocks and were identified by the *y* mutant phenotype.

Stocks used for hosts in invasion assay: *Canton-S*, yw^{67}, $ovo^{D1}v^{24}$/*C(1)DX*, $y^1w^1f^1$.

Wild type hosts were the progeny of a cross between *Canton-S* females and yw^{67} males. Female sterile hosts were the progeny of a cross between *Canton-S* females and $ovo^{D1}v^{24}$ males.

Stocks used for ectopic TIMP expression: yw^{67}; *UAS-TIMP/ UAS-TIMP* (transgene on third chromosome), yw^{67}; *UAS-GFP/UAS-GFP* (transgene on third chromosome), Gal4 driver lines: yw^{67}; *c323a/* y^+ *CyO,* yw^{67}*; btlGAL4/*y^+ *TM3 Ser,* yw^{67}*;ElavGAL4/ElavGal4*.

Transplantation of larval brain fragments

Transplantations of larval brain fragments were performed as previously described (Woodhouse et al., 1994). Due to the small size of *lgl Mmp1* and *brat Mmp1* brains, whole brain lobes were injected into hosts unlike *lgl* and *brat* brain lobes that were quartered first. *LglMmp1* brain fragments were cultured in wild type hosts for 12 days at 25°. *BratMmp1* brain fragments were cultured for 10 days in wild type hosts at 25°. All brain fragments were cultured for 7 days at 25° when *ovoD* hosts were used.

Detection of micro-metastases within host ovaries

All of the mutant cells were marked with *arm-LacZ* to allow for detection within adult hosts using an anti-βGal antibody. After tumor culturing, the adult abdomens were opened ventrally to expose ovaries. Ovaries were kept within the abdomen to prevent damage to the ovaries and ovariole loss. Host abdomens were fixed for 30 min in 3.7% formaldehyde in PBS, rinsed in PBS, washed 3×30 min PBS, then 3×30 min PBS+0.6% Triton-X-100 (PBT), then 30 min 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 min 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 h at RT. Ovaries were dissected and ovarioles were separated onto a slide in VectaShield mounting medium. All of the ovarioles from each host were mounted together on single slides. Tumor cell presence within ovarioles was detected using a Zeiss LSM 510 Meta microscope.

Primary antibody used: chicken anti-βGal 1:50 (Immunology Consultants Laboratory). 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), Rhodamine Phalloidin (Molecular Probes).

Statistical analysis

The G-test of independence as described in Sokal and Rohlf (1969) was computed for the analysis of metastasis data. Student's *t*-test was performed on all real-time RT PCR data, unpaired and with 4 degrees of freedom.

Immunofluorescence of larval brains

Third instar larvae were inverted and fixed for 15 min in 3.7% formaldehyde in PBS, rinsed in PBS, washed 3×30 min PBS. Samples were then washed 3×30 min in PBT then 30 min in antibody incubation buffer described above. Samples were incubated in primary antibody in incubation buffer overnight at 4° on a shaker. Samples were rinsed in PBT then washed 3×30 min then incubated in secondary antibody for 1 h at room temperature on a shaker. Samples were washed 3×30 min in PBT then mounted in VectaShield mounting medium. Primary antibody: anti-MMP1 mouse monoclonal described in (Page-McCaw et al., 2003), secondary antibody: Alexa Fluor 488 goat anti-mouse (Molecular Probes).

RNA isolation and reverse transcription of tissue samples

Fresh tissue was harvested from either larvae or adult *Drosophila* and put into 250 μL of Trizol Reagent (Invitrogen 15596-026) in a sterile microfuge tube. Ovaries harvested from adult hosts after incubation with tumor cells were washed with PBS to remove loosely adhering tumor cells on the surface and then were put into the Trizol reagent. Samples were ground with a pestle and allowed to sit at room temperature (RT) for 5 min. 100 μL of chloroform was added and samples were hand shaken for 15 s, incubated at RT for 2 min, then centrifuged at top speed for 30 min at 4 °C. The aqueous phase was transferred to a clean tube and 125 μL of isopropanol was added then allowed to sit at RT for 10 min. The samples were spun at top speed for 30 min at RT to pellet the RNA. The supernatants were removed, 500 μL 75% ethanol was added to pellets then samples were centrifuged for 10 min at 4 °C. The pellets were air dried for 5–10 min, then resuspended in 10 μL of TE and incubated at 60 °C for 2 min. The amount of RNA was quantified by OD260 in a spectrophotometer and the concentration was adjusted to 1 μg/μL.

RNA was reverse transcribed into cDNA using the BioRad iScript cDNA synthesis kit (cat #170-8890) following the protocol included with the kit.

Real-time quantitative PCR of cDNA samples

All reactions were performed using a Bio-Rad iCycler with iQ Sybr green master mix (Bio-Rad), 2 μL of cDNA, and each primer at a concentration of 0.2 μM. The PCR protocol was 95 °C for 3 min, then 40 cycles of 95 °C for 5 s, 57 °C for 10sec, then 72 °C for 20 s. A melt-curve analysis of each reaction was performed starting at 55 °C and increasing temperature by 0.5 °C every 10 s. The melt-curve analysis verified that only one PCR product was made in each reaction. Primers used: *Mmp1*: GGCAGAGGCGGGTAGATAG, TTCAGTGTTCATAGTCGTAGGC, *Mmp2*: CGCAGAGCACCCGTTCTT, CTGTCCCTCCCACCCGAAG, *RpL11A*: CTTCATGGCATCCTCCTTGG, CGGTATCTATGGTCTGGACTTC, *GapDH*: GTCGGGCTTGTAGGCATCC, AGGCATCCACTCACTTGAAGG.

Calculation of relative gene expression

C^t is the cycle when the fluorescence is appreciably above background and is linear with the log of the starting copy number. To determine the relative expression of a gene of interest (GOI) we calculated the number of cycles between when the house keeping gene (HK) crossed the threshold (C^t) and when the GOI crossed. Because the expression of the HK gene should be unchanged in all samples, any difference in the number of cycles between HK^{Ct} and GOI^{Ct} should be due to a change in the starting amount of the GOI. The number of cycles between HK and GOI for wild type samples was normalized and had a relative expression of 1. The experimental samples were then normalized in relation to the wild type samples. We performed each experiment using 2 housekeeping genes Rpl11A and GapDH to ensure that there was no change in expression of the HK in *lgl* or *brat* samples compared to wild type. The results were from three independent experiments that were averaged prior to normalization.

Results

Mmp1 accumulation is altered in lgl but not in brat mutant larvae

We examined MMP1 accumulation in *lgl* and *brat* mutant larvae compared to wild type to determine if there was any misregulation of MMP1 in tumorous mutants. MMP1 protein expression in the brains and imaginal discs of wild type and *brat* mutant larvae is not detectable over background levels by immunofluorescence; any signal seen is from adjacent trachea which strongly expresses MMP1 during the larval stage (Figs. 1A, C). By contrast, *lgl* mutant larvae showed a dramatic increase in the amount of MMP1 protein present in the imaginal discs, although MMP1 in the brain was not detected above background (Fig. 1B).

We performed real-time RT PCR on the brains and eye discs of wild type, *lgl*, and *brat* mutants to allow for sensitive

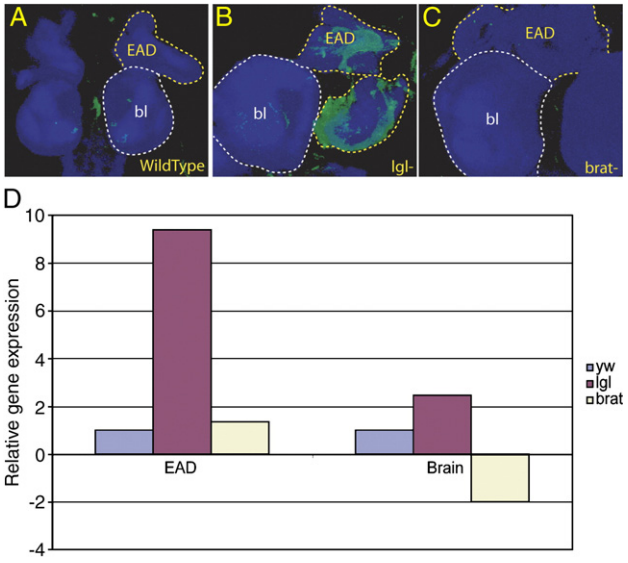

Fig. 1. MMP1 accumulation in larvae. Wild Type larval brain lobes (white dashed lines) and eye discs (yellow dashed lines) have no detectable MMP1 protein by immunofluorescence (A). MMP1 protein accumulates (green) in *lgl* eye discs (B) while MMP1 levels in *brat* brain lobes and discs are similar to wild type (C). *Mmp1* relative gene expression in eye discs and brains (D).

detection of *Mmp1* and *Mmp2* mRNA accumulation levels. The accumulation of *Mmp1* transcript in *lgl* brains was 2.46 fold higher than in wild type while accumulation in *lgl* mutant eye-antennal discs was increased 9.38 fold compared to wild type. *Brat* mutant brains and eye-antennal discs showed no significant change in *Mmp1* transcript accumulation compared to wild type samples (Fig. 1D, Table 1). The levels of *Mmp2* mRNA in *lgl* and *brat* mutant brains and eye-antennal discs were not significantly different than the levels in wild-type brains and eye-antennal discs as determined by Student's *t*-test (Table 1) so we focused our studies on *Mmp1*.

Mmp1 mutant larvae have small brains and imaginal discs due to hypoxia

MMP research in mammals has indicated that MMPs are involved in several stages of tumor progression. We wanted to study MMP contribution specifically in metastasis. *Mmp1* mutant larvae survive through third instar but are extremely small compared to wild type raising the possibility that MMP1 affects cell proliferation (Figs. 2A, B). Any effect on cell proliferation would hinder our ability to focus on MMP1 involvement in metastasis. Therefore we evaluated the cause of the reduced size seen in *Mmp1* mutant larvae. These mutant larvae have severe tracheal defects and small imaginal discs and brain lobes. Previous analysis of *Mmp1* mutants indicated that the larvae were under hypoxic conditions due to the tracheal defect (Page-McCaw et al., 2003).

To determine whether the small brains and discs were due to hypoxia-induced cell cycle arrest we used tissue specific expression of TIMP, an inhibitor of MMP activity. Previous work showed that ubiquitous TIMP misexpression phenocopies the loss of *Mmp1* function phenotype (Page-McCaw et al., 2003). Larvae expressing TIMP in the trachea using a breathless GAL4 driver should have reduced MMP activity in the trachea but normal levels of MMP activity in the brains and imaginal discs. We observed that expression of TIMP with the breathless GAL4 driver caused tracheal defects like those seen in *Mmp1* mutants resulting in larvae smaller than sibling controls. Significantly, the brains and discs were reduced in size as well (Fig. 2C). As a negative control for this experiment, we used ElavGAL4 to express TIMP in brains and discs to reduce the level of MMP activity and assay for brain and disc size. ElavGAL4 would be expected to accumulate in differentiating neuronal cell of brains and discs but not in trachea. Indeed, expression of TIMP with ElavGAL4 did not affect larval size or tracheal development. Dissection of the larvae revealed that the brains and discs were the same size as wild type (Fig. 2D). A caveat to this control, however, is that ElavGAL4 accumulates in differentiating neuronal cell of brains and discs and may not have accumulated early enough in development to affect brain and disc cell proliferation.

Nevertheless, we interpret these data as indicating that the reduced organ size seen in *Mmp1* mutants was a secondary result of the tracheal defects and not due to a direct affect of *Mmp1* on brain and disc growth. This interpretation is supported by experiments described below showing that both *lglMmp1* and *bratMmp1* double mutant brains grow like *lgl* and *brat* mutant brains when transplanted into adult hosts.

Mmp1 is required in the tumor cell for metastasis of lgl tumors

We generated fly lines that were mutant for both *lgl* and *Mmp1*. As were *Mmp1* larvae, the *lglMmp1* larvae were extremely small and had reduced brain lobes and discs. We transplanted fragments of mutant brains into adult hosts that had rudimentary ovaries (*ovoD*) and allowed them 12 days for proliferation. Qualitatively, *lgl Mmp1* tumor cells proliferated as well as *lgl* tumor cells in host abdomens. Dissection of the abdomens revealed the host's guts and an abundance of tumor cells (Figs. 3A, B). Previously, we demonstrated these cells

Table 1
Relative expression of *Mmps* in larval eye-antennal discs and brains

Tissue sample	Gene analyzed	Cycles between *Mmp* C^t and HK C^t	Relative expression
Wild type EAD	*Mmp1*	8.93±0.42	1
lgl EAD	*Mmp1*	5.70±1.04**	9.38
brat EAD	*Mmp1*	8.50±0.46	1.35
Wild type brain	*Mmp1*	8.93±0.06	1
lgl brain	*Mmp1*	7.63±0.75*	2.46
brat brain	*Mmp1*	9.93±1.86	−2
Wild type EAD	*Mmp2*	7.8±0.98	1
lgl EAD	*Mmp2*	6.3±0.98	2.8
brat EAD	*Mmp2*	9.04±1.01	0.4
Wild type brain	*Mmp2*	7.02±0.79	1
lgl brain	*Mmp2*	7.28±0.59	0.84
brat brain	*Mmp2*	8.76±0.76	0.3

*=significant ($p \leq 0.05$); **=highly significant ($p \leq 0.01$) as determined by Student's *t*-test. All other values were not significantly different from wild type as determined by Student's *t*-test.
EAD=eye-antennal disc, C^t is the threshold cycle.

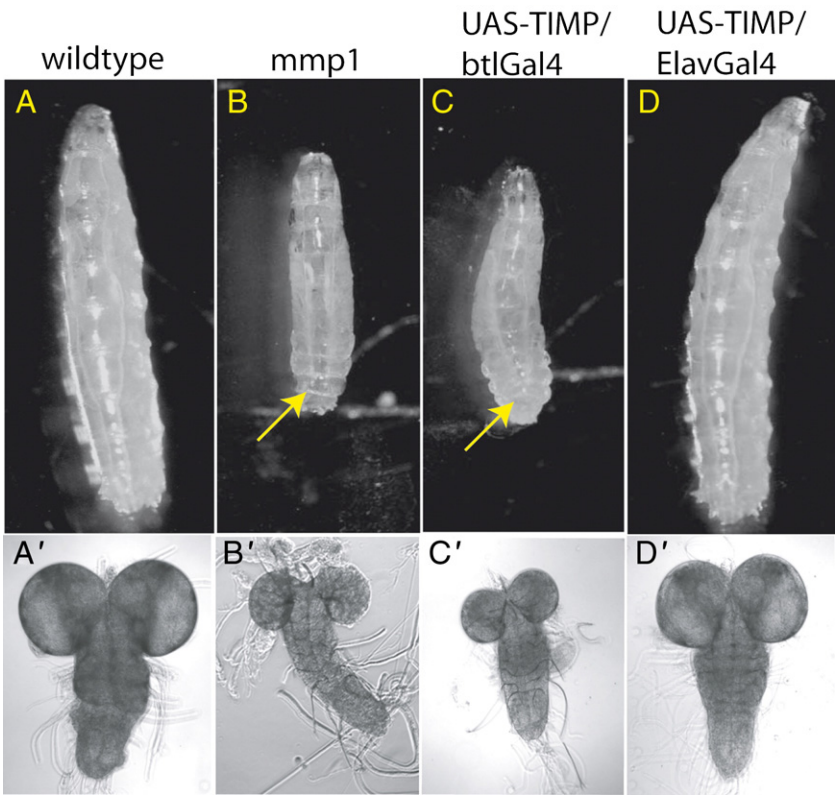

Fig. 2. Removal of MMP1 activity causes larval hypoxia resulting in reduced brain lobe size. Wild type third instar larva and dissected brain (A) compared to *Mmp1* mutant larva with tracheal defects (arrow) resulting in small body and brain size (B). TIMP expression in trachea results in tracheal defect (arrow) as well as small body and brain size (C). Expression of TIMP in the brain resulted in normal sized larva and brain with no tracheal defect (D).

present in the abdomen were tumor cells by detection of a reporter gene (Woodhouse et al., 1994).

To study the affect of removing *Mmp1* activity on metastasis, mutant larvae were marked with *arm-LacZ* that is expressed in all cells. This allows for detection of micro-metastases within host ovaries by immunofluorescence using an anti-βGal antibody. Metastatic frequency was determined by the percent of ovarioles with at least one micro-metastasis. We previously showed that *lgl* tumor cells are able to invade 15.8% of the ovarioles analyzed (58/367) after 12 days of proliferation in the hosts (Beaucher et al., 2006). Here, we transplanted brain fragments from *lglMmp1* larvae into wild type hosts. After 12 days of culturing, only 4% of the ovarioles had micro-metastases (7/175), a dramatic reduction compared to *lgl* tumors (Fig. 4C, Table 2). This demonstrates that *Mmp1* gene activity in tumor cells facilitates *lgl* metastasis. The *lglMmp1* micro-metastases that did form were similar in size and shape to *lgl* micro-metastases establishing that the reduced metastatic frequency was not a result of a failure to proliferate in ovarioles (Figs. 4A, B).

LglMmp1 brains were significantly smaller than *lgl* brains resulting in smaller brain fragments being injected. To ensure

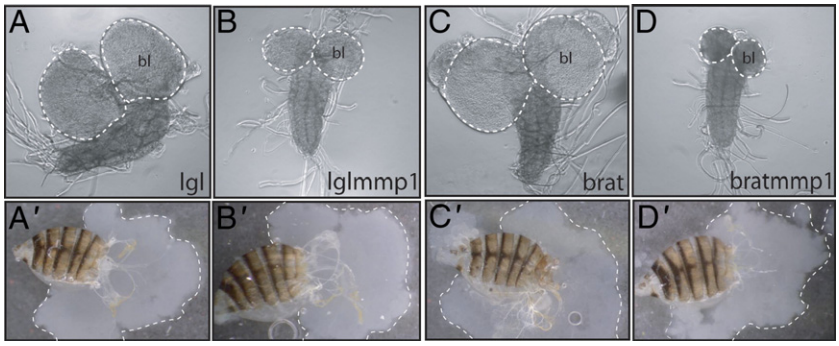

Fig. 3. *lgl Mmp1* and *brat Mmp1* tumor cells proliferate as well as *lgl* and *brat* cells in a transplantation assay. 10-day-old *lgl* larval brain lobes overgrew (A) and proliferated when transplanted into an adult ovoD female. The abdomen was dissected and tumor cells outlined in white dashed lines (A′). *lgl Mmp1* brain lobes were reduced in size (B) but proliferated when transplanted into an adult ovoD host (B′). *Brat* larval brain lobes were overgrown (C) and proliferated when transplanted into an adult ovoD host (C′). *Brat Mmp1* larval brain lobes were reduced (D) in size but proliferated when transplanted into an adult ovoD host (D′).

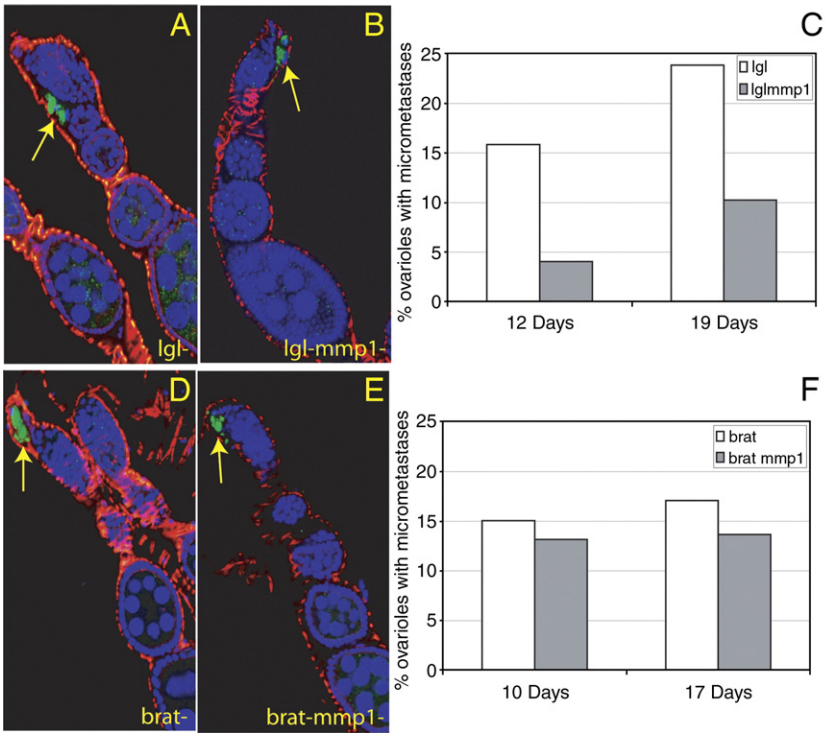

Fig. 4. Removal of *Mmp1* gene activity from tumor cells reduces *lgl* metastasis but not *brat* metastasis. *Lgl* micro-metastases (arrow) within the host ovariole (A) are similar in size to *lgl Mmp1* micro-metastases (B). *Lgl Mmp1* tumor metastatic frequency was significantly reduced compared to *lgl* tumors (C). *Brat* tumor cells form micro-metastases (D) that are of similar size as *brat Mmp1* (E) and there is no difference in metastatic frequency (F). Tumor cells were marked with arm-LacZ and detected with an anti-βGal antibody in green, Ovariole Epithelial sheath visualized with Rhodamine Phalloidin in red, DAPI in blue. *$p \leq 0.05$, **$p \leq 0.01$ †Metastatic frequencies of *lgl* and *brat* tumors were previously reported (submitted).

that this was not responsible for the decreased metastasis seen, we endeavored to standardize the amount of tissue injected. We transplanted *lglMmp1* brain fragments into female sterile hosts that had rudimentary ovaries (*ovoD* hosts) and cultured the tumor cells for 7 days. The lack of ovaries in these hosts allowed for easy harvesting of tumorous tissue from the hosts' abdomens. Equal amounts of *lgl* and *lglMmp1* tumor cells were retransplanted into wild type hosts for analysis of metastatic frequency. The tumor cells proliferated for 12 days in the secondary hosts and then the ovaries were analyzed for micro-metastasis formation. As we previously reported, *lgl* tumors showed an increase in metastasis after the prolonged incubation with 23.8% of the ovarioles containing micro-metastases (87/366) compared to 15.8% after incubation in one host. The *lglMmp1* tumors also showed an increase in metastasis after prolonged incubation with 10.2% of the ovarioles containing metastases (38/372) compared to 4% after incubation in one host. While the frequency of ovarioles with *lglMmp1* micro-metastases increased with time, it is still significantly lower than the metastatic frequency of *lgl* tumors after the same amount of incubation time (Fig. 4C, Table 2).

Table 2
Ovarioles with micrometastases after incubation with tumor cells

Donor genotype	Host genotype	Days of incubation	Invaded/ total	% invasion
lgl	Wildtype	12	58/367	15.8
	Wt	19	87/366	23.8
	UAS-GFP/c323a	19	11/100	11
	UAS-TIMP/c323a	19	1/121	0.8
lglmmp1	Wt	12	7/175	4
	Wt	19	38/372	10.2
brat	Wt	10	61/406	15
	Wt	17	80/391	20.5
	UAS-GFP/c323a	17	29/142	20.4
	UAS-TIMP/c323a	17	3/87	3.4
bratmmp1	Wt	10	26/195	13.3
	Wt	17	24/176	13.6

Mmp1 is not required in the tumor cell for brat metastasis

As with *lgl*, we generated fly lines that were mutant for *brat* and *Mmp1*. The tracheal defect and reduced brain size were also present in these double mutant larvae. After transplantation, the *bratMmp1* tumor cells proliferated as well as *brat* tumor cells in the adult hosts (Figs. 3C, D). We then examined the effect of removing *Mmp1* gene activity on *brat* tumor metastasis. We previously reported (Beaucher et al., 2006) that *brat* tumor cells form micro-metastases in 15% of the ovarioles examined after 10 days of incubation (61/406). *BratMmp1* brain fragments transplanted into hosts formed micro-metastases in 13.3% of the ovarioles examined (Fig. 4F, Table 2). This was not a significant reduction in metastatic frequency. The *bratMmp1* micro-metastases (Fig. 4E) were similar in size and shape compared

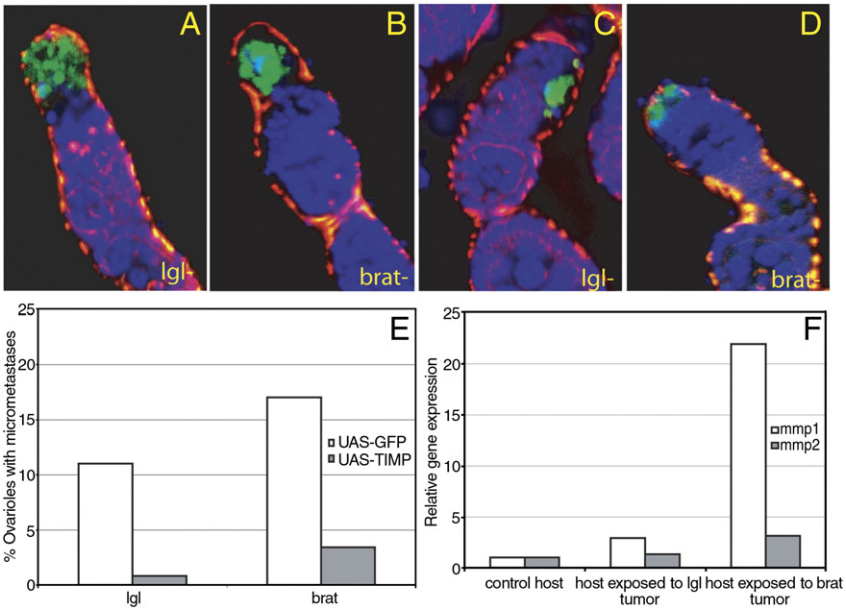

Fig. 5. TIMP expression in the host ovary dramatically reduces both *lgl* and *brat* micrometastasis formation. *Lgl* micro-metastases (A) and *brat* micro-metastases (B) develop in ovarioles expressing GFP by an ovary specific driver. The *lgl* (C) and *brat* (D) micro-metastases in TIMP expressing ovaries are able to form within the epithelial sheath. The expression of TIMP in the ovary significantly reduced the rate of metastasis for both *lgl* and *brat* tumor cells compared to hosts expressing GFP (E). Tumor in green, Epithelial sheath in red, DAPI in blue. Relative *Mmp1* expression in host ovaries exposed to *brat* tumors is significantly higher than uninjected hosts and hosts exposed to *lgl* tumors (F). The expression of *mmp2* is slightly increased in host ovaries exposed to *brat* tumors compared to uninjected or *lgl* injected hosts. $*p \geq 0.05$.

to *brat* micro-metastases (Fig. 4D) that formed in hosts' ovarioles.

As with the *lgl* and *lglMmp1* mutants, we determined if the unequal brain size between the mutants affected metastatic rate in any way. We transplanted *brat* and *bratMmp1* brains into female sterile hosts and incubated for 7 days then retransplanted the tumor cells into wild type hosts and incubated for 10 days. We previously found that *brat* tumor cells transplanted into secondary hosts formed micro-metastases in 20.5% of ovarioles examined (80/391) which is not a significant increase in metastasis compared to the frequency of 15% seen after 10 days of culturing in primary hosts. *BratMmp1* tumor cells formed micro-metastases in 13.6% of the ovarioles examined (Fig. 4, Table 2) after prolonged culture which was not significantly different from the metastasis frequency of 13.3% seen after 10 days of culturing in one host. Even with prolonged culturing in two hosts, there was not a significant difference in metastatic rate between *brat* and *brat Mmp1* tumors. Therefore, *Mmp1* activity in the tumor cell does not facilitate *brat* tumor metastasis.

Host expression of MMP1 is required for metastasis

Tumor cells can act directly on the microenvironment to facilitate metastasis and indirectly by inducing host cell responses. We determined if metastasis was facilitated by MMPs secreted by host ovaries in response to tumor cells. Because *Mmp1* mutants die as late larvae/early pupae we could not use these flies as hosts. To remove MMP activity in hosts, we used an ovary specific GAL4 driver, c323a, to express UAS-TIMP in the host ovaries. The driver expresses strongly in the late stage follicle cells and weakly throughout the ovary. This was determined by the pattern of UAS-GFP accumulation in response to this driver (data not shown). In addition to driving expression in ovaries, c323a also drives expression in the gut during larval development. The larval expression of TIMP by the c323a driver is lethal in larvae raised at 25° (unpublished observation). To prevent larval lethality, UAS-TIMP/c323a larvae and control reporter UAS-GFP/c323a larvae were raised at 17° to reduce GAL4 expression. Once the flies enclosed, the UAS-TIMP/c323a and UAS-GFP/c323a flies were shifted to 25° 2 days prior to transplantation of tumor cells. This allowed us to express TIMP or GFP in the adult ovary without the larval tracheal defect and lethality seen with expression of TIMP by the c323a driver at room temperature.

We transplanted brain tissue into female sterile hosts for 7 days then retransplanted into either reporter or TIMP expressing hosts. This ensured that the same tumor cell population was being injected into both host types. Transplanted *lgl* tumor cells formed micro-metastases in reporter expressing hosts in 11% of the ovarioles analyzed (Figs. 5A, E, Table 2). The *lgl* tumor cells transplanted in TIMP expressing hosts only formed micro-metastases in 0.8% of the ovarioles analyzed (Figs. 5C, E, Table 2). This was a significant reduction in the metastatic rate of *lgl* tumors.

Brat tumor cells transplanted into reporter hosts were able to form micro-metastases in 17% of the ovarioles analyzed (Figs. 5B, E, Table 2). The rate of metastasis was significantly lower in TIMP expressing hosts with 3.4% of the ovarioles containing micro-metastases (Figs. 5D, E, Table 2). Thus, expression of TIMP in host ovaries reduced the metastatic frequency of both *lgl* and *brat* tumors.

Host ovaries exposed to brat tumors but not lgl tumors show an increase in Mmp1 gene expression

We showed above that inhibiting MMP activity in the host caused a drastic reduction in both *lgl* and *brat* metastatic ability. Research in mammals has shown that host stroma can increase expression of MMPs in response to tumors, facilitating invasion. We looked to see if a similar induction of MMPs is seen in host ovaries after exposure to tumors. We performed real-time RT PCR on ovaries dissected from wild type hosts that had been transplanted with either *lgl* or *brat* tissue and compared to ovaries from untransplanted hosts. We looked for changes in expression of both *Mmp1* and *Mmp2* (Table 3). *Mmp1* expression was not significantly changed in ovaries exposed to *lgl* compared to untransplanted hosts. Ovaries exposed to *brat* tissue showed a 21.9 fold increase in *Mmp1* expression. Expression of *Mmp2* was unchanged in response to *lgl* tumors and increased 3.1 fold in response to *brat* tumor cells. Although, the ovaries dissected from these hosts contained micro-metastases, we do not believe that the presence of these tumor cells contributed significantly to these data. Only 15–20% of the ovarioles in these ovaries contained micro-metastases; we estimate that each micro-metastasis contains on average 20 cells. If 20% of the 15 ovarioles in an ovary have micro-metastases, that would be 3 micro-metastases per ovary. Each ovary on average would only have 60 tumor cells (3 micrometastases ×20 cells) compared to the tens of thousands of cells that make up an ovary. It is unlikely that the level of *Mmp1* or *Mmp2* transcript in these 60 cells, even if dramatically elevated, could significantly affect the level of transcript we measure in total ovaries. However, further investigation is required to determine which cells in the ovary are upregulating *Mmp1* in response to exposure to *brat* tumors.

Discussion

MMP1 requirement in tumor cells

Upregulation of MMP1 is part of the *lgl* larval tumor phenotype. *Lgl* mutant eye-antennal discs and brains showed an increase in *Mmp1* transcription as well as protein expression compared to wild type. Removal of *Mmp1* gene expression from *lgl* mutants dramatically decreased the metastatic ability of the tumor cells demonstrating a role for *mmp1* in facilitating the metastasis of *lgl* tumor cells. The decrease was not due to the difference in size of the brain fragments transplanted since a significant decrease was still observed after standardizing the amount of tissue transplanted by serial transplantation. Our previous results showed that the rate of *lgl* tumor metastasis increased with serial passaging. We observed the same result here with *lglMmp1* tumors indicating that the factors involved in the increased metastasis with serial passaging were not *Mmp1* dependent.

Table 3
Relative expression of *Mmp1* and *Mmp2* in host ovaries

Tissue sample	GOI	Cycles between GOI C^t and HK C^t	Relative expression
Uninjected host ovary	*Mmp1*	11.64±1.83	1
lgl injected host ovary	*Mmp1*	10.10±1.28	2.9
brat injected host ovary	*Mmp1*	7.19±1.86*	21.9
Uninjected host ovary	*Mmp2*	9.31±0.78	1
lgl injected host ovary	*Mmp2*	8.92±0.80	1.3
brat injected host ovary	*Mmp2*	7.66±0.91*	3.1

*$p \leq 0.05$ as determined by Student's *t*-test of independence, all other values were not significantly different from uninjected ovary values.
GOI = gene of interest; HK = housekeeping gene; C^t = threshold cycle.

Brat mutant larvae had no alterations in *Mmp1* transcript accumulation in the brain or eye disc compared to wild type. Removal of *Mmp1* from *brat* tumors had no significant effect on metastasis. Serial transplantation did not significantly alter the metastatic rate of either *brat* or *bratMmp1* tumor cells. These results demonstrate that in *brat* tumors *Mmp1* expression in the tumor cells themselves does not facilitate metastasis.

Our previous work found dramatic differences in metastatic properties between *lgl* and *brat* tumors. The results presented here are further examples of the different mechanisms used by these tumors to undergo metastasis. Our data suggest that metastasis is a phenotype that can be achieved by multiple mechanisms. *Lgl* tumor cells require *Mmp1* activity from the tumor cell to facilitate metastasis while *brat* tumor cells do not. How MMP1 alters the microenvironment to facilitate *lgl* metastasis is unknown. MMP1 is capable of degrading type IV collagen *in vitro* but *in vivo* specificity for MMP1 activity is currently unknown. Type IV collagen is a primary component of the extracellular matrix in *Drosophila* and MMP1 from the tumor cell may facilitate metastasis by cleaving ECM and allowing tumor cells to pass. Alternatively, MMP1 may facilitate metastasis by activating pro-migratory factors or inhibiting anti-migratory factors in the tumor microenvironment. Mammalian work found that MMPs cleave receptors on cell surfaces, other proteases, factors contained within the ECM, and other proteins (reviewed by Sternlicht and Werb, 2001).

MMP1 expression in the host

Work in mammalian metastasis has highlighted the importance of the tumor microenvironment. Tumor cells use similar mechanisms and signaling pathways as are utilized by cells during normal physiological invasion. They stimulate the surrounding host stroma to release promigratory signals. One response of host cells to tumor signals is the secretion of MMPs and other proteases that break down the ECM, facilitating the invasion of metastatic cells.

We removed MMP activity from the tumor microenvironment by expressing the inhibitor TIMP in the host ovaries. *lgl* tumor metastasis was almost completely inhibited with the removal of MMP activity in the host ovary. TIMP is a secreted protein, so TIMP expression in the ovary would be expected to inhibit MMPs secreted both by host tissue and tumor cells. This makes it difficult to determine if the reduced metastasis was due to inhibition of host or tumor cell MMPs. Most likely, the reduction in *lgl* metastasis seen is due to the removal of MMP activity derived from the tumor cells. This inference is based on

the lack of increase in *Mmp1* or *Mmp2* gene expression in host ovaries after exposure to *lgl* tumors as well as the dramatic results seen with the removal of *Mmp1* from the *lgl* tumor cells.

Removal of *Mmp1* from *brat* tumor cells did not alter metastatic ability. However, TIMP expression in host ovaries did significantly reduce *brat* micro-metastasis formation. These data coupled with the data showing a 21.9 fold increase in *Mmp1* mRNA accumulation in host ovaries after exposure to *brat* tumor cells suggest that *brat* utilizes MMP1 from the host to facilitate metastasis. How *brat* tumor cells induce the expression of *Mmp1* in the host ovary is unknown. The requirement for MMP1 activity from the host could explain the lack of increase in frequency of *brat* micro-metastases with serial passaging of tumor cells. The ovaries in each host transplanted with *brat* tumors would start out with a low level of MMP1 secretion. That level would increase with time. However, the new host transplanted with tumor cells from the previous host would again start out with a low level of MMP1 secretion.

Mutations in *lgl* and *brat* cause tumors that are superficially similar, yet the metastatic characteristics of each are very different. In wild type larval brains, neuroblasts divide asymmetrically to produce a neuroblast and a ganglion mother cell (GMC) which will then divide once more to produce 2 daughter cells. Recently, it was shown that *lgl* mutant brains overgrow due to symmetric division of neuroblasts (Lee et al., 2006a). Some of the neuroblasts in *lgl* mutant brains do divide asymmetrically, the resulting GMC appears to differentiate normally. *Brat* mutant larval brains also have overproliferation of neuroblasts (Betschinger et al., 2006; Lee et al., 2006b). In *brat* brains, neuroblasts divide asymmetrically but the resulting GMCs do not down regulate neuroblast markers. Some of these aberrant GMCs differentiate but most expand in size and reenter the cell cycle producing more neuroblasts. The differences in how these tumors originate must account for their different metastatic properties. We show in this study that *lgl* tumors rely primarily on secreted MMP1 from the tumor cells while *brat* tumors appear to induce MMP1 expression from the host to facilitate metastasis. The tumors require the same protein but from different sources and for potentially different purposes. Alternative mechanisms of metastasis could explain why histologically similar human tumors might respond differently to radiation and drug treatments. We have started to understand the underlying mechanisms in two morphologically similar *Drosophila* tumors. There are mutations in other genes that affect asymmetric neuroblast division and cause brain tissue to overproliferate after transplantation in adult hosts (Caussinus and Gonzalez, 2005). Analysis of metastatic characteristics from tumors generated by these mutations will allow for a better understanding of the different possible mechanisms used.

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