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Targeted Transgenic RNAi Knockdown of Cell Fate Determinants Induces Neoplastic Tumor Growth and Metastasis in a *Drosophila* Transplantation Model of Neural Stem Cell Derived Cancer

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Abstract

Genetic models such as *Drosophila* have sophisticated transgenic and molecular genetic tools available to investigate proliferation control in normal and tumorigenic neural stem cells. In this report, we adapted a targeted transgenic RNAi knockdown approach based on the Gal4/UAS expression system to the study of neoplastic tumor formation and metastatic growth in the *Drosophila* brain tissue transplantation model. Transgenic RNAi driven knockdown of *numb*, *brain tumor (brat)* and *prospero* (*pros*) in all neuroblasts (type I and type II) resulted in a high incidence of neoplastic tumor formation after transplantation that was comparable to that of loss-of-function mutations in these cell fate determinants. RNAi knockdown of *numb* and *brat* specifically restricted to type II neuroblast lineages also resulted in tumor formation after transplantation. A marked temperature dependence of tumor formation after transplantation was documented and quantified for RNAi-induced knockdown of *numb*, *brat* and *pros*. An *in vivo* assay for micrometastasis formation in ovarioles revealed significant metastatic potential of transplanted overproliferating brain tissue induced by RNAi knockdown of these cell fate determinants. These findings establish the foundation for RNAi-based investigations of the mechanisms which underlie the proliferation, invasion and metastatic potential of neural stem cell induced tumors in the *Drosophila* model.

Keywords: Neural Stem Cells; Neuroblasts; *Drosophila*; RNAi; Brain Tumors; *Brat; Numb; Prospero*

Abbreviations: INPs: Intermediate Neural Precursors; GMC: Ganglion Mother Cell; Brat: Brain tumor; Pros: Prospero; *wor-Gal4*, *ase-Gal80*: *worniu-Gal4*, *asense-Gal80; insc-Gal4*: *inscuteable-Gal4*

Introduction

A central feature of stem cells is their ability to generate copies of themselves while giving rise to more differentiated progeny. In consequence, stem cells must avoid cell cycle exit and differentiation while simultaneously avoiding uncontrolled proliferation and tumor formation. As errors in stem cell division rate or in the fine balance between self-renewal and differentiation can result in tumorigenesis, stem cells might be the cells of origin of certain human cancers ("tumor stem cells") [1-3]. The possible contribution of dysregulated stem cells to tumor formation is especially relevant for neural stem cells and the development of neural stem-cell based regenerative therapies for neurological diseases. Self-renewal and amplification of progeny number are known to be striking features of many neural stem cells in the mammalian brain [4-6]. Clearly, both features make neural stem cells promising for transplant-based regenerative therapy, however, they are also potentially hazardous and prone to cancerous dysregulation having devastating results in potential recipient patients [7]. Genetic model systems have been useful in determining the possible contribution of normal and abnormal stem cells to the initiation of cancer and in identifying the molecular events that might drive such transformation [8-11]. Moreover, in genetic model systems such as *Drosophila*, sophisticated transgenic and molecular genetic tools are now available to investigate the fundamental problem of proliferation control in normal and tumorigenic neural stem cells. Recent results obtained on *Drosophila* neural stem cells, also called neuroblasts, imply a causative link between impaired neural stem-cell division and brain tumor formation in this genetic model [10,12,13].

Drosophila neuroblasts can be broadly categorized as type I and type II, both of which have a distinct mode of proliferation. Type I neuroblasts proliferate through self-renewing divisions that also give rise to a smaller daughter cell called a Ganglion Mother Cell (GMC) which further only divides once to generate two neural progeny. Type II neuroblast lineages on the other hand proliferate through self-renewing divisions that give rise to intermediate neural precursors (INPs), each of which undergo limited rounds of self-renewing division that also generate a GMC. Recent work on both types of neuroblasts has provided an insight into the asymmetric cell division process, whereby the unequal distribution of cell fate determinants leads to the generation of daughter cells with different fates (for recent reviews see [9,11,14-16]). Three important asymmetrically segregated cell fate determinants have been analysed in greater detail. The first is Numb, a tissue-specific repressor of the Notch pathway [17]. The second is Prospero (Pros), a homeodomain transcription factor that can act as a transcriptional activator and repressor [17,18]. The third is Brain tumor (Brat), a member of the NHL domain family involved in translational regulation and cell growth inhibition [19-21]. Consistent with the functions of these genes in repressing growth and self-renewal, loss of either *pros*, *brat* or *numb* results in neuroblast lineages that escape differentiation. This causes overgrowth characterized by the

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unregulated overproduction of neuroblast-like cells at the expense of differentiated neurons [19-22].

Importantly, transplantation of the corresponding mutant brain tissue into wild type hosts results in immortalized transformed cells as well as lethal malignant neoplastic overgrowth, and the resulting tumors can even be successively re-implanted into new hosts for many years [23]. Thus for these three determinants, the connection between self-renewing asymmetric stem cell division and tumorigenicity supports the hypothesis that impaired cell-fate determination is a major cause of cancerous overgrowth in neural stem cell lineages [24,25]. A current challenge is to understand the molecular mechanisms that link aberrant asymmetric neural stem cell divisions to the type of uncontrolled, immortalized cell proliferation that characterize these transplantation-induced tumors in the *Drosophila* model. This molecular understanding will be crucial for designing and testing novel strategies aimed at preventing neural stem cell-derived brain tumors, which can subsequently be assessed in comparative molecular genetic studies performed on mammalian neural stem cells *in vitro* and *in vivo* before translation to human stem cell transplantation therapy.

The recent establishment of transgenic RNA interference (RNAi) technology together with the availability of genome-wide UAS-RNAi libraries in *Drosophila* makes it possible to extensively and rapidly investigate gene function in complex developmental and pathological processes in a cell- as well as tissue-specific manner in intact organisms [26-28]. Moreover, since RNAi expression is under Gal4/UAS control, it can be targeted in a tissue-specific manner by any of the numerous available Gal4 strains, including an *inscuteable-Gal4* (*insc- Gal4*) line which drives expression in all of the brain neuroblasts and a *worniu-Gal4* driver combined with an *asense-Gal80* repressor (*wor-Gal4*, *ase-Gal80*) which drives expression specifically in type II neuroblast lineages [20,29,30]. The power of this type of transgenic RNAi approach for understanding neuroblast self-renewal and proliferation has recently been demonstrated in a landmark investigation of the network of functionally interacting genes that control cytokinesis, cell growth and differentiation in the *Drosophila* brain [30].

In this report, we apply the targeted transgenic RNAi knockdown approach used to investigate neuroblast overproliferation in the developing brain to the study of neoplastic tumor formation and metastatic growth in the *Drosophila* brain tissue transplantation model. We first confirm that RNAi knockdown of *numb*, *brat* and *pros* in all brain neuroblasts (using the *insc-Gal4* driver) or specifically in type II brain neuroblast lineages (using the *wor-Gal4*, *ase-Gal80* drivers) results in overproliferation in the intact larval brain. We then transplant the corresponding RNAi knockdown brain tissue into wild type hosts and assay for tumor formation. Our findings show that overproliferating brain tissue containing *insc-Gal4* driven RNAi knockdown of *numb*, *brat* or *pros* in all neuroblasts results in a remarkably high incidence of neoplastic tumor formation after transplantation, and we quantify the frequency of these tumor formations for each of the three cell fate determinants. We then show that overproliferating brain tissue containing *wor-Gal4*, *ase-Gal80* driven RNAi knockdown of *numb* or *brat* specifically in type II neuroblast lineages also results in tumor formation after transplantation. A marked temperature dependence of tumor formation after transplantation of overproliferating brain tissue is documented and quantified for RNAi-induced knockdown of *numb*, *brat* and *pros*. Subsequently we use an *in vivo* assay for micrometastasis formation in ovarioles to investigate the metastatic potential of transplanted overproliferating brain tissue induced by RNAi knockdown of *numb*, *brat* and *pros*. These studies form the basis for future RNAi-based investigations of the molecular mechanisms which underlie the immortalized proliferation, invasive behavior and metastatic potential of neural stem cell induced tumors as well as for exploring molecular genetic manipulations which might prevent tumorigenesis in the *Drosophila* model.

Materials and Methods

Fly stocks

All *Drosophila* stocks were maintained on standard yeast-cornmeal-agar medium at optimum temperature of 25°C and on a 12:12h light/dark cycle. Fly stocks carrying an inducible UAS-RNAi construct and both the driver lines *UAS-Dicer-2; insc-Gal4, UAS-CD8::GFP* or *UAS-Dicer-2; wor-Gal4, ase-Gal80; UAS-CD8::GFP* were obtained from the Vienna *Drosophila* RNAi Center (VDRC) [30]. $brat^{k06028}$ mutants were obtained from Bloomington Stock Center. w^{1118} flies were used as host flies as they provide a good contrast and enable easy visualization of GFP labeled transplanted brain tissue and subsequent tumor formation.

Dissection and visualization of larval brains

Third instar larval brains were dissected in ice-cold Phosphate Buffer Saline (PBS, Sigma 081M8207) and fixed in 2% paraformaldehyde (Riedel-de Haen, Sigma-Aldrich, CAS nr. 30525-89-4) for 1h at room temperature, washed several times in PBS/0.5% Triton X-100 (Sigma), and then several times in PBS before being incubated in Vectashield (Vector Laboratories, Inc. Reactolab S.A., H-1000) overnight at -20°C. Then larval brains were neatly dissected and were mounted onto a slide in Vectashield mounting medium. Pictures of GFP-positive larval brains (direct-GFP), both in control and overproliferating larval brains were taken using a Leica TCS SP5 confocal microscope and the images were processed using standard Fiji [31] or ImageJ 1.42a (NIH, USA).

Transplantation of larval brain pieces

Males carrying a Gal-4 inducible UAS-RNAi construct were crossed to virgin flies of the respective driver lines. Crosses were set up at 29°C and third-instar larvae were collected after 5-6 days and transplantations of GFP-positive, larval brain pieces were performed as previously described [23,32], with minor modifications. Freshly enclosed host flies were collected and allowed to age at 25°C such that they were 3-4 days old at the time of transplantation. These w^{1118} female adult hosts were anesthetized by CO_2 and immobilized on a metal plate kept on ice, with double-side sticky tape, ventral side up. Small pieces of GFP-positive larval brains were transplanted with a constructed glass capillary needle (needle puller- Narishige Japan model PN-30; needles made from Pasteur pipettes of length 230 mm- Fortuna Cat no. 3.326) tangentially into the mid-ventral abdomen of female host flies. Post recovery from anesthesia, the host flies were maintained at standard conditions at 29°C/ 25°C or 18°C depending on experimental needs.

Surviving flies were transferred to fresh food bottles every second day. Both the surviving and dead host flies were observed under a fluorescent scope once or twice a week (more frequent if required) to assay the formation of tumors. Pictures of transplanted host flies (with or without) tumors were taken with a Nikon Coolpix 4500 digital camera.

Dissection of ovarioles of transplanted flies and Detection of micrometastases

After tumor formation, the adult abdomens were dissected in Grace's insect medium (1 X, GIBCO, Invitrogen, 11605) at room temperature (RT) and the dissected ovaries were immediately fixed in 4% paraformaldehyde, 0.2% of Triton-X-100 dissolved in Grace's insect medium for 30 min without shaking. The fixative was then rinsed three times in Phosphate Buffer Saline (PBS)+0.5% Triton-X-100 (PBST), then washed three times for 10 min each in PBST. Samples were then incubated for 1h at RT with Phalloidin-alexa 568 (Molecular Probes, Invitrogen detection technologies) diluted 1:200 in PBST, followed by three washes with PBST for 10 minutes each and a second incubation with Toto-3 iodide (Molecular Probes, Invitrogen detection technologies) diluted 1:1000 in PBST for 1 h at RT. Samples were rinsed three times in PBST, and washed three times for 10 min each in PBST, rinsed two times in PBS, washed two times for 10 min each in PBS and then embedded in Vectashield overnight at -20°C. Then ovaries were dissected and the separated ovarioles were mounted onto a slide in Vectashield mounting medium. The presence of metastases within ovarioles was detected using a Leica TCS SP5 confocal microscope and the images were processed using standard Fiji [31] or ImageJ 1.42a (NIH, USA).

Results

Targeted transgenic knockdown of numb, pros and brat in neuroblasts causes overproliferation phenotypes in the larval central brain

Previous work has shown that mutational loss-of-function of the cell fate determinant-encoding genes *numb*, *brat* and *pros* in neuroblasts results in overproliferation in the developing central brain of *Drosophila* [19-22]. More recent work using *insc-Gal4* to drive the expression of transgenic RNAi in central brain neuroblasts indicates that knockdown of *numb*, *brat* or *pros* can replicate the corresponding mutant phenotypes [33]. To confirm this and to document the resulting knockdown phenotypes, we first carried out the corresponding targeted knockdowns for each of these three cell fate determinants in all central brain neuroblasts by using *insc-Gal4* to drive the corresponding UAS-knockdown transgene as well as the *UAS-CD8::GFP* label to visualize the affected cells and *UAS-Dicer2* to enhance the RNAi effect.

In the wild type control (without the UAS-knockdown transgene), all of the neuroblasts and a subset of their progeny are labeled as distinct and well defined cell clusters in the central brain and ventral nerve cord of the third instar larval stage (Figure 1A). (Some labeling is also seen in the developing optic lobes, however, this is not considered further in this report). In the *brat* knockdown, a dramatic overproliferation occurs in the central brain hemispheres, which is increased significantly in size at the third instar larval stage, and owing to this overproliferation, intense GFP labeling is seen throughout the brain hemispheres (Figure 1B). (This intense fluorescence resulting from the strong GFP labeling masks the normal label of unaffected neuroblasts in the ventral nerve cord). In the *numb* knockdown situation, a comparable overproliferation is seen in the central brain hemispheres, which are also increased in size at the third instar larval stage and are intensely labeled by GFP (Figure 1C). In the *pros* knockdown, significant overproliferation is observed in the central brain hemispheres, however, the extent of this overproliferation is markedly reduced as compared to the *numb* and *brat* knockdowns in the third instar larval stage (Figure 1D).

Targeted transgenic knockdown can be limited to the type II neuroblast lineages in the central brain by using the *worniu-Gal4* driver combined with an *asense-Gal80* repressor [33]. To investigate the phenotypes caused by targeted knockdown of *numb*, *brat* or *pros* exclusively in type II central brain neuroblast lineages, we used *wor-Gal4*, *ase-Gal80* to drive the corresponding UAS-knockdown transgene (with *UAS-CD8::GFP* and *UAS-Dicer2*). In the wild type control (without the UAS-knockdown transgene), the 8 type II neuroblasts and a subset of their progeny are labeled in each brain hemisphere of the third instar larval stage as distinct cell clusters with corresponding axon tracts (Figure 1E). (Specific labeling is also seen in the developing optic lobes; this is not considered further). In the *brat* knockdown, a marked overproliferation occurs in the central brain hemispheres of the third instar larval brain (Figure 1F). This overproliferation is characterized by an increased number of labeled cell clusters which contain both large, neuroblast-like cells and numerous smaller cells

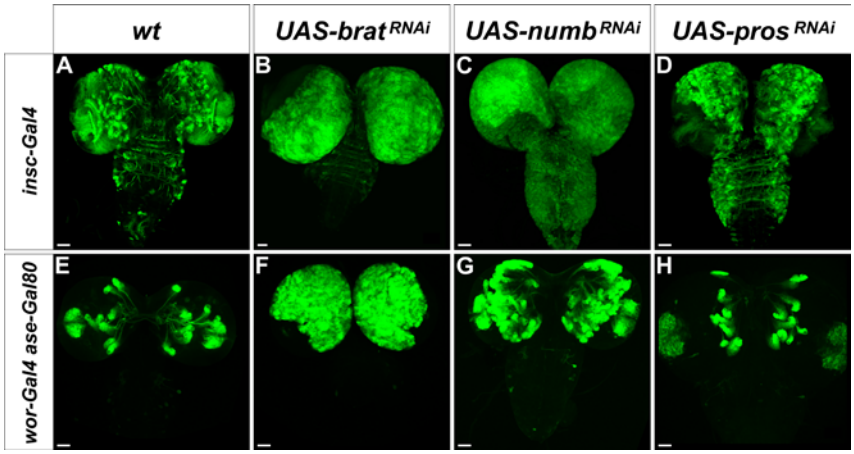

Figure 1: Targeted knockdown of *brat*, *numb*, or *pros* in neuroblasts causes overproliferation in the larval brain. Confocal images of larval brains at late third instar stage labeled with mCD8::GFP (green) of wild type (A and E), *brat* knockdown (B and F), *numb* knockdown (C and G), and *pros* knockdown (D and H). Targeted knockdown was carried out by using *insc-Gal4* driver (A-D) in all neuroblasts or by *wor-Gal4 ase-Gal80* driver in only the type II neuroblast lineages (E-H). Scale bars are 50 µm.

which may correspond to dysregulated INPs. Similarly, in the *numb* knockdown a significant overproliferation is seen in the central brain hemispheres of the third instar larval brain, and this overproliferation is also characterized by an increased number of labeled cell clusters which contain large, neuroblast-like cells and numerous smaller cells (Figure 1G). In contrast, the *pros* knockdown shows no overproliferation in the central brain hemispheres; the 8 type II neuroblasts and a subset of their progeny are labeled in each brain hemisphere of the third instar larval stage as distinct cell clusters which correspond to those observed in the wild type control (Figure 1H). This result is expected for the *pros* knockdown as, in type II neuroblast lineages, unlike in type I neuroblasts, the cell fate marker, Prospero is not expressed until the mature INPs stage [34].

These results confirm earlier work and indicate that targeted knockdown of *numb*, *brat* or *pros* in all brain neuroblasts and targeted knockdown of *numb* or *brat* in type II neuroblast lineages result in overproliferation phenotypes. Moreover, at least for the knockdown of *numb*, *brat* or *pros* in all brain neuroblasts, the resulting knockdown phenotypes in the late larval brain are comparable to those achieved by the corresponding loss-of-function mutations [19,23].

Tumor formation after transplantation of brain tissue containing RNAi knockdown induced overproliferating brain neuroblasts

The transplantation of brain tissue that is mutant for *numb*, *pros* or *brat* into the abdomen of wild type hosts can result in the formation of tumors that have the potential to grow enormously in size and eventually kill the host [23]. However, it is unknown if brain tissue that overproliferates due to RNAi-induced targeted knockdown of *numb*, *pros* or *brat* in brain neuroblasts can also induce tumors after transplantation. To investigate this, we targeted the RNAi knockdowns to all neuroblasts using *insc-Gal4* as well as restricted to type II neuroblast lineages using *wor-Gal4, ase-Gal80* and then transplanted the (GFP-labeled) central brain tissue from the corresponding third instar larval stages into the abdomen of adult wild type hosts and assayed for tumor formation. The results of these transplantation experiments are shown in Figure 2.

In the wild type control (without the UAS-knockdown transgene), transplanted brain tissue never resulted in tumor formation when assayed up to five weeks after transplantation. This confirms the fact that the transplantation procedure of wild type GFP-labeled tissue itself is not responsible for the tumorigenesis. In contrast to wild type transplantations and in accordance with reports from previous studies, when *brat* mutant (loss-of-function) brain tissue was transplanted, tumors resulted in majority of the host flies. In experiments involving targeted *insc-Gal4* driven knockdown of *numb*, *brat* or *pros*, transplanted brain tissue also resulted in tumor formation with high frequency. In all three cases GFP-labeled tumors that filled the abdomen of the host were observed in majority of the flies within one week after transplantation, and after two weeks most of these host flies had died. In experiments involving targeted *wor-Gal4, ase-Gal80* driven knockdown of *numb* or *brat*, transplanted brain tissue also resulted in tumor formation. However, there was a marked delay in the time needed for tumors to become visible; tumors were only seen in *brat*-knockdown transplantation experiments after 2 weeks and in *numb*-knockdown experiments after 3 weeks. Thus, knockdown of *numb* or *brat* specifically in type II neuroblast lineages not only results in a more restricted overproliferation of brain tissue, but also results in tumor formation after transplantation that is more delayed in time and/or reduced in frequency of occurrence. (Transplantation of brain tissue from *wor-Gal4, ase-Gal80* targeted knockdown of *pros* did not result in tumor formation; data not shown).

Taken together, these findings demonstrate that targeted knockdown of *numb*, *brat* or *pros* in all central brain neuroblasts results

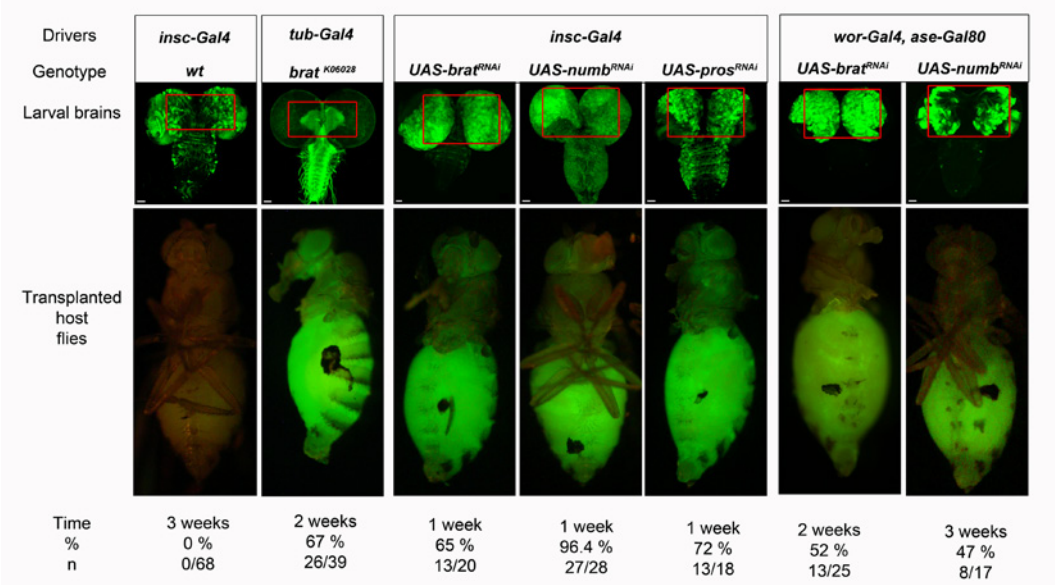

Figure 2: Tumor formation in the abdomen of w^{1118} host flies, after transplantation of overproliferating brain tissue of *brat*, *numb* or *pros* RNAi knockdown. Top panel: confocal images of third instar larval brains labeled with mCD8::GFP (green) with respective genotypes and drivers (scale bars are 50 µm). *tub-Gal4* (tubulin) drives UAS mCD8::GFP (green) in $brat^{k06028}$ mutant. Bottom panel: w^{1118} host flies transplanted with pieces of central brain (shown by red outline) of the corresponding genotypes. (Black spot on the abdomen is the scar of the point of injection). The transplantation of $brat^{k06028}$ mutant; *brat*, *numb* or *pros* RNAi knockdowns, induce tumor formation (green abdomen) in contrast to wild type (wt) control in host flies. 'n' represents the number of tumor-positive host flies out of the total number of transplanted host flies and the percentage of tumor formation is shown at the respective time point.

in overproliferating brain tissue and that transplantation of this brain tissue can cause a high frequency of tumor formation in host flies which is comparable to that obtained with loss-of-function mutant brain tissue. In view of these findings, we conclude that a targeted transgenic RNAi approach is well suited for investigations of neuroblast-derived tumor formation in the *Drosophila* transplantation model.

Temperature dependence of tumor formation after transplantation of overproliferating neuroblast tissue in transgenic RNAi-knockdown experiments

The targeted transgenic knockdown of *numb*, *brat* or *pros* in brain neuroblasts is mediated by the Gal4/UAS binary expression system, and this system manifests a small but significant variation of expression as a function of temperature [29]. Moreover, the rate of the cellular overproliferation that characterizes tumors in *Drosophila* is likely to be temperature dependent as well, although this has not been investigated in a quantitative manner before. Both considerations suggest that the rate and/or frequency of tumor formation after transplantation of overproliferating brain tissue in experiments involving targeted Gal4 driven knockdown of *numb*, *brat* or *pros*, might be temperature dependent. To investigate this in more detail, we determined the frequency of tumors formed in transplantation experiments at 18°C, 25°C and 29°C for *insc-Gal4* driven knockdown of *numb*, *pros* and *brat*.

Figure 3 shows the temperature dependence of tumor formation after transplantation of brain tissue in which *insc-Gal4* driven knockdown of *numb* was targeted to all neuroblasts. Transplantation of overproliferating brain tissue from third instar larval stages into the abdomen of adult hosts maintained at 29°C resulted in large (GFP-labeled) tumors in 27 out of 28 flies (96.4%) after one week. In contrast, when overproliferating brain tissue was transplanted into adult hosts kept at 25°C, only 1 out of 26 (3.8%) flies had visible tumors after one week. This rate of tumor formation increased to 50% of the flies after 2 weeks and 70% of the flies after 3 weeks. When overproliferating brain tissue was transplanted into host flies kept at 18°C, no tumors were visible after one or two weeks, and after 3 weeks tumors were visible in only 1 out of 27 (3.4%) flies. This low rate of tumor formation increased slightly to 7.7% at four weeks and a substantial number of tumors (41.5%) were only visible after five weeks at this lower temperature. These results indicate that the frequency of tumor formation is markedly temperature dependent for *numb* knockdown brain tissue transplants. At 29°C virtually all hosts develop visible tumors after one week; at 18°C less than 10% of the hosts develop visible tumors after four weeks.

The corresponding temperature dependence of tumor formation after transplantation of *brat* or *pros* knockdown tissue (*insc-Gal4* driver) is summarized in Table 1 (*pros* knockdown) and Table 2 (*brat* knockdown). In both cases the percentage of visible tumors formed at a given time point after transplantation was highest at 29°C, intermediate at 25°C and lowest at 18°C. For example, after two weeks in the case of *pros* knockdown, 80% of flies had visible tumors at 29°C, 70% at 25°C, and 8% at 18°C. Similarly, after two weeks in the case of *brat* knockdown, 83% of flies had visible tumors at 29°C, 60% at 25°C, and 16% at 18°C.

These experiments demonstrate that the rate of visible tumor formation after transplantation has a marked temperature dependence in experiments involving targeted Gal4 driven knockdown of *numb*, *brat* or *pros* in brain neuroblasts. Highest levels of tumor formation are achieved in shortest time period at 29°C indicating that this temperature is well suited for induction of tumors after transplantation in host flies. Nevertheless, even at 18°C a relatively high percentage of visible tumors are formed after 4-5 weeks in the *numb*, *pros* and *brat* knockdown transplant experiments. This is of interest for future experiments on mechanisms of tumor formation since 18°C is generally the permissive temperature for the use of temperature sensitive genetic control elements such as *Gal80^{ts}*.

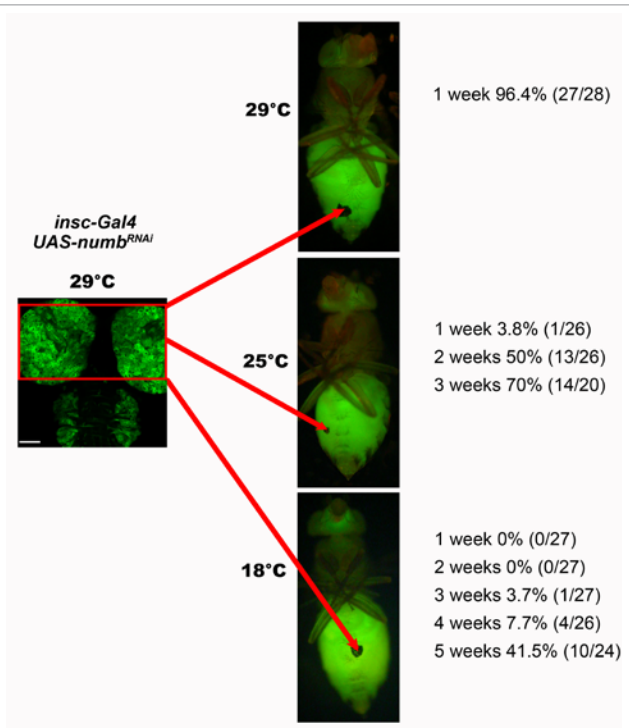

Figure 3: Temperature dependence of tumor formation after transplantation of overproliferating brain tissue of *numb*-RNAi knockdown. The frequency of tumor formation at different time points (after transplantation) is recorded and displays a marked temperature dependence effect, with maximum frequency of tumor formation seen at 29°C, intermediate at 25°C and minimum at 18°C.

Temperature dependence of tumor formation in w^{1118} host flies after transplantation of third instar larval brain pieces of *pros-RNAi* knockdown using *insc-Gal4* driver			
Temperature	**Time after Transplantation**	**Percentage of Tumor**	**Number of tumor-positive flies/total number of transplanted flies**
29 °C	1 week	72%	13/18
	2 weeks	**80%**	**12/15**
25 °C	1 week	67%	8/12
	2 weeks	**70%**	**7/10**
18 °C	**2 weeks**	**8%**	**1/12**
	3 weeks	16%	2/12
	4 weeks	72%	5/7

Table 1: *pros-RNAi* driven by *insc-Gal4* in all brain neuroblasts results in an overproliferation, which results in tumor formation in host flies upon transplantation. The frequency of tumor formation at different time points (after transplantation) is recorded and displays a marked temperature dependence effect, with maximum frequency of tumor formation seen at 29°C, intermediate at 25°C and minimum at 18°C.

Temperature dependence of tumor formation in w^{1118} host flies after transplantation of third instar larval brain pieces of *brat-RNAi* knockdown using *insc-Gal4* driver			
Temperature	**Time after Transplantation**	**Percentage of Tumor**	**Number of tumor-positive flies/ total number of transplanted flies**
29 °C	1 week	65%	13/20
	2 weeks	**83%**	**10/12**
25 °C	1 week	42%	5/12
	2 weeks	**60%**	**6/10**
18 °C	**2 weeks**	**16%**	**2/12**
	3 weeks	16%	2/12
	4 weeks	40%	4/10
	5 weeks	78%	7/9

Table 2: *brat-RNAi* driven by *insc-Gal4* in all brain neuroblasts results in an overproliferation, which results in tumor formation in host flies upon transplantation. The frequency of tumor formation at different time points (after transplantation) is recorded and displays a marked temperature dependence effect, with maximum frequency of tumor formation seen at 29°C, intermediate at 25°C and minimum at 18°C.

Tumor invasion and metastasis after transplantation of overproliferating neuroblast tissue in transgenic RNAi-knockdown experiments

In the *Drosophila* tumor transplantation model it is possible to distinguish between non-invasive benign or hyperplastic overgrowth and malignant neoplastic overgrowth which results in invasion and metatstases [25]. Previous work has established an *in vivo* assay system for metastasis formation by tumor cells which is based on analyzing micrometastases within the ovarioles of adult hosts after transplantation [36]. Since the ovary is surrounded by an epithelial sheet and muscle layers surround the ovarioles, micrometastases can appear in this assay system only if tumorigenic cells leave the transplanted neoplastic tumor mass and actively invade theses cell layers and colonize the host ovarioles [36]. To determine if the tumors caused by transplantation of brain tissue that overproliferates due to RNAi-induced targeted knockdown of *numb*, *pros* or *brat* can lead to micrometastases, we analysed the ovarioles of the transplanted host flies.

Transplantation of wild type control brain tissue (without the UAS-knockdown transgene) into host flies did not result in visible tumors; ovarioles isolated from these host flies never showed GFP-labeled cells indicating that invasion and metastasis formation did not occur (Figure 4A). In contrast, and as described above, transplantation of brain tissue in which the *insc-Gal4* driver was used to knockdown *numb*, *brat* or *pros* in all neuroblasts resulted in visible tumor formation at high frequency in the host. Ovaries isolated from these host flies regularly showed clear micrometastases formation manifest as groups of GFP-labeled cells which invaded and colonized the ovarioles (Figure 4B). Similar findings were obtained for transplantation of brain tissue in which *wor-Gal4, ase-Gal80* driver was used to knockdown *numb* or *brat* in type II neuroblast lineages; large visible tumors formed and micrometastases developed in the ovarioles of the host flies (Figure 4C). The rate of micrometastasis formation, like the rate of tumor formation, was markedly temperature dependent. The percentage of ovarioles that contained GFP-labeled micrometastases at a given time point after transplantation was highest at 29°C, intermediate at 25°C and lowest at 18°C (data not shown).

Taken together, these experiments indicate that the tumors, which are formed after transplantation of overproliferating neuroblast tissue in targeted transgenic knockdown experiments, are neoplastic, invasive and give rise to metastases in the host. This, in turn, implies that the powerful transgenic RNAi approach can be applied to the study of the uncontrolled immortalized proliferation, tumor invasion and metastatic potential of tumors that derive from transplanted dysregulated neuroblasts in the *Drosophila* model.

Discussion

For several decades, the *Drosophila* model system has been successfully applied to the investigation of the molecular basis of cancer, notably due to the ability to carry out large scale genetic screens [13]. These screens have led to identification of key regulatory genes which, when mutated, contribute to tumor-like overproliferation in the intact organism and to transplantable tumors with invasive and metastatic properties in host flies [12,23,25]. A remarkable finding obtained in the last decade is that mutational inactivation of key regulators of asymmetric cell division of the neural stem cell-like neuroblasts of *Drosophila* is causal in brain tumor formation implying that defects in neural stem cell asymmetric division can result in tumorigenesis [10,33]. For brain neuroblasts, this has been particularly well documented for mutations in the cell fate determinants *numb*, *brat* or *pros* both *in vivo* and in the transplantation model of neoplastic tumor formation and metastasis formation [19-22,36]. Thus, at least for these

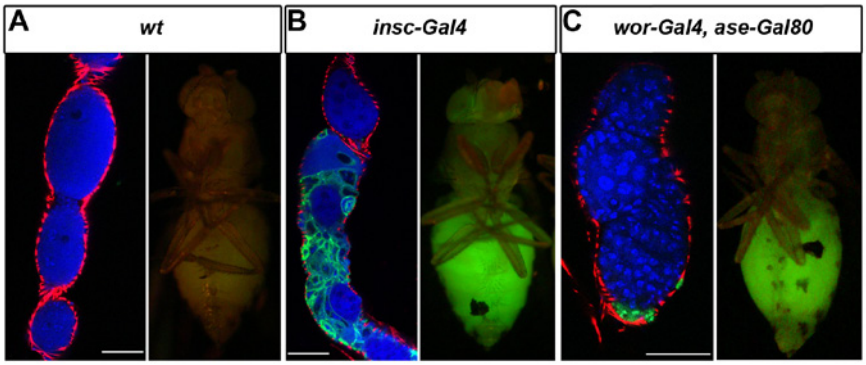

Figure 4: Micrometastasis formation in the ovarioles of host flies, after transplantation of overproliferating brain tissue of *numb* RNAi knockdown. Confocal images of ovarioles of corresponding w^{1118} transplanted host flies: (A) *insc-Gal4* driver control, (B) *insc-Gal4* driven *numb* RNAi and (C) *wor-Gal4*, *ase-Gal80* driven *numb* RNAi. In (B and C) the presence of GFP-labeled cells (in green) in the ovarioles indicates the presence of metastasis. DNA is labeled with Toto-3 iodide (in blue) and muscle layers by phalloidin (in red). Scale bars are 30 µm.

three genes, a solid molecular link between dysregulated neural stem cell proliferation and tumorigenesis has been established in *Drosophila*.

During the last decade, the arsenal of genetic tools available for investigating the *Drosophila* model has increased considerably and sophisticated transgenic methods for visualization and manipulation of gene expression have been developed, many of which are based on binary expression systems such as the Gal4/UAS system [38]. Importantly, genome-wide transgenic RNAi libraries for conditional gene inactivation of fly genes, and that can be targeted by the Gal4 system have become available and have been used successfully to identify near-complete sets of genes involved in key cellular processes *in vivo* [26,27]. Recently, a genome-wide analysis based on transgenic RNAi has been applied to the analysis of the molecular mechanisms of neural stem cell proliferation in *Drosophila* [30]. In this study, over 600 genes were identified that operate in fly neuroblast proliferation, and the network of key members of this gene population was established, thus documenting the power of a genome-wide analysis of normal and genetically dysregulated neural stem cell proliferation in the intact developing brain.

Here we have applied the same targeted transgenic RNAi approach used by Neumüller and colleagues [30] to the transplantation model of neuroblast-derived neoplastic tumorigenesis and metastasis in *Drosophila*. Our experiments with targeted knockdown of *numb*, *brat* or *pros* in brain neuroblasts demonstrate for the first time that transgenic RNAi gives rise to phenotypically similar phenomena of neoplastic tumor formation, tissue invasion and metastasis after transplantation as the loss-of-function null mutations of these genes. This implies that a manipulative RNAi-based knockdown approach should be very well adaptable to the mechanistic molecular analysis of neural stem cell-based tumor and metastasis formation in the fly transplantation model. Due to the targeted nature of the knockdown, our experiments already uncover a difference in the potential for transplantable tumorigenesis if the RNAi knockdown is targeted to all neuroblasts or only to type II neuroblast lineages. This demonstrates the potential of a binary Gal4/UAS-based transgenic knockdown approach for analysing cell- and tissue-specific mechanisms of tumor formation. In addition, our experiments document the prominent temperature dependence effect on the rate of knockdown-induced tumor formation after transplantation that is characteristic of this novel transgenic RNAi approach. This is an important prerequisite for the use of conditional temperature-sensitive binary expression system in this neural stem cell cancer model.

Taken together, our findings provide essential basic information for a subsequent large scale genome-wide analysis of the molecular basis of tumor and metastasis formation in *Drosophila*. As a first step, this will involve the RNAi-based analysis of the numerous new candidate genes recently identified as players in neuroblast proliferation, in tumor and metastasis formation [30]. Moreover, transgenic RNAi knockdown in the transplantation model will make it possible to perform targeted conditional genetic rescue experiments that are aimed at discovering candidate genes and genetic mechanisms for potential abrogation of neuroblast-derived tumors. This type of approach should also allow the combination of several different RNAi lines to test for interactive effects of new candidate genes. In addition, it will be possible to knockdown specific candidate genes in brain tissue transplanted into different mutant host backgrounds to assay for the mechanisms that underlie differential susceptibility of cancer in host organisms. Once the fundamental molecular elements and mechanisms that underlie neural stem cell-derived cancer have been analysed in *Drosophila*, these approaches should be useful for directing experiments in the field of tumorigenesis and metastases formation in mammalian models.

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