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The *Drosophila melanogaster* gene *brain tumor* negatively regulates cell growth and ribosomal RNA synthesis

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SUMMARY

The regulation of ribosome synthesis is likely to play an important role in the regulation of cell growth. Previously, we have shown that the *ncl-1* gene in *Caenorhabditis elegans* functions as an inhibitor of cell growth and ribosome synthesis. We now indicate that the *Drosophila melanogaster* tumor suppressor *brain tumor* (*brat*) is an inhibitor of cell growth and is a functional homolog of the *C. elegans* gene *ncl-1*. The *brat* gene is able to rescue the large nucleolus phenotype of *ncl-1* mutants. We also show that *brat* mutant cells are larger, have larger nucleoli, and have more ribosomal RNA than wild-type cells. Furthermore, *brat* overexpressing cells contain less ribosomal RNA than control cells. These results suggest that the tumorous phenotype of *brat* mutants may be due to excess cell growth and ribosome synthesis.

Key words: *Drosophila*, cell growth, ribosome, *brain tumor*

INTRODUCTION

The overall size of a metazoan is controlled at the cellular level by the coordinate regulation of cell division and cell growth. Although it has long been established that inappropriate cell division can lead to cancer, it is becoming increasingly clear that cell growth, or increase in cell mass, is of equal importance. For example, the oncogenes *Myc*, *Ras* and *Cyclin D* (Prober and Edgar, 2001) and the tumor suppressors *retinoblastoma* (White, 1997) and *Pten* (Gao et al., 2000; Goberdhan et al., 1999; Huang et al., 1999) have all been shown to regulate cell growth. Although the factors that regulate cell division have been extensively studied (Sherr and Roberts, 1999), the processes that control cell growth are just beginning to be elucidated (Stocker and Hafen, 2000).

Given the dependence of cell growth on protein synthesis, regulation of translation is likely to play an important role in growth control. In fact, recent studies have shown that one mechanism of cell growth regulation is achieved through an insulin receptor signaling pathway, one of the most downstream targets of which is the ribosomal protein S6 (Weinkove and Leevers, 2000). Phosphorylation of this protein appears to lead to stimulation of translation of mRNAs that contain 5′-terminal oligopyrimidine tracts. It has been proposed that the coordination between cell growth and division might be achieved through a balance of translation of messages that contain this sequence and those that lack it. Translation of the former, which include translation factors and ribosomal proteins, would favor growth, while translation of the latter, which include genes involved in cell cycle progression, would favor division (Thomas, 2000).

An additional way in which cells can regulate protein synthesis, and therefore growth, is through control of ribosome synthesis. For example, *Myc* overexpression in mice can induce the transcription of multiple genes involved in ribosome synthesis (Kim et al., 2000). The fruitfly *D. melanogaster* genes *minifly* (Giordano et al., 1999) and *pitchoune* (Zaffran et al., 1998) have been found to be required for ribosomal RNA (rRNA) processing and are also important for organism growth. Notably, *pitchoune* appears to be a target of *Myc* in flies (Zaffran et al., 1998). In contrast to genes that are required for ribosome synthesis, *Rb* appears to function to negatively regulate ribosome synthesis through its ability to inhibit both RNA polymerase I (Cavanaugh et al., 1995) and III (White et al., 1996) transcription.

The only other gene that has so far been shown to negatively regulate RNA polymerase I and III transcription is *ncl-1* (for abnormal nucleolus) from the soil nematode *Caenorhabditis elegans*. Previously, we have demonstrated that *ncl-1* functions not only to negatively regulate rRNA synthesis, but also to inhibit cell growth (Frank and Roth, 1998). *ncl-1* mutant worms are larger than wild-type worms and have larger cells. Furthermore, they have enlarged nucleoli in almost all of their cells (Hedgecock and Herman, 1995), which is indicative of a higher rate of rRNA synthesis, that results in a higher steady state level of rRNA. Additionally, they have a higher rate of 5S RNA synthesis and probably contain more ribosomes as they contain more protein than do wild-type worms (Frank and

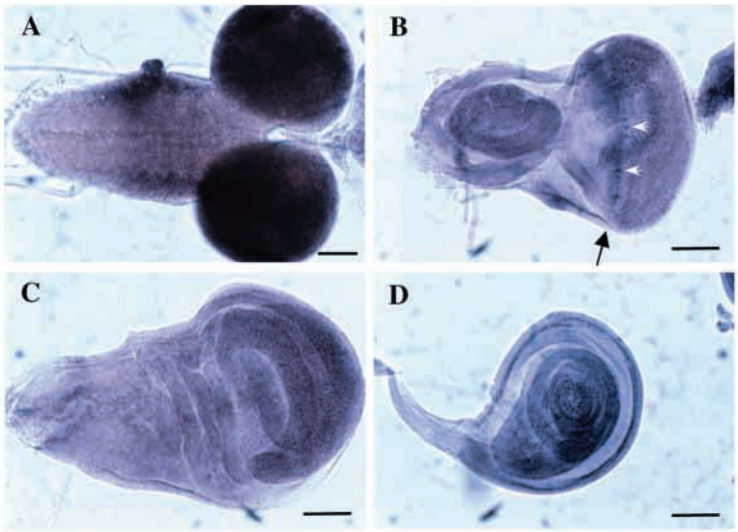

Fig. 1. *brat* expression pattern in brain and imaginal discs. Brains and imaginal discs were dissected from third instar larvae and hybridized in situ using a *brat* antisense RNA probe. (A) Brain with the ventral nerve chord to the left and the brain hemispheres to the right. (B) Antenna (left) and eye (right) disc. Black arrow, morphogenetic furrow; white arrows, neuronal preclusters. (C) Wing disc. (D) Leg disc. No signal was detected when the sense probe was used (not shown). Scale bars: 50 μm in A; 100 μm in B-D.

Roth, 1998). NCL-1 protein is predominantly cytoplasmic and its levels of expression in cells of the embryo, gonad (Frank and Roth, 1998) and adult somatic tissue (D. J. Frank, PhD thesis, University of Washington: Seattle, 2000) are inversely related to the size of nucleoli: cells with small nucleoli have high level expression of NCL-1, whereas cells with large nucleoli have low level expression.

The gene most similar to *ncl-1* is *brat* (brain tumor) from *D. melanogaster* (Arama et al., 2000; G. R. Hankins, PhD thesis, University of Virginia: Charlottesville, 1991). Both genes contain two B-box zinc fingers, a coiled-coil domain and multiple NHL (NCL-1, HT2A and LIN-41) (Slack and Ruvkun, 1998) repeats. The NCL-1 and BRAT proteins are 38% identical overall and 80% identical in the most C-terminal 280 amino acids, the region that contains the NHL repeats (Arama et al., 2000). Homozygous *brat* mutants die before eclosion and have greatly enlarged brains, up to eight times the normal size (G. R. Hankins, PhD thesis, University of Virginia: Charlottesville, 1991). The brain tumor phenotype of *brat* mutants is primarily due to expansion of the optic neuroblasts (Kurzik-Dumke et al., 1992). Imaginal discs from third instar *brat* mutant larvae, although appearing normal in situ, are able to metastasize and form secondary tumors when injected into the abdomen of a wild-type host fly (Woodhouse et al., 1998), thus indicating that *brat* also has a function in the imaginal discs. Superficially, *brat* and *ncl-1* mutants do not appear to have analogous phenotypes at the organismal level. We now address whether or not they have similar phenotypes at the cellular level. We show that, in addition to being structurally related, *ncl-1* and *brat* are functionally homologous. Therefore, this mechanism for controlling growth through repression of rRNA synthesis is conserved between worms and flies. We propose that excess ribosome synthesis and cell growth may be important aspects of the tumorous phenotype of *brat* mutants.

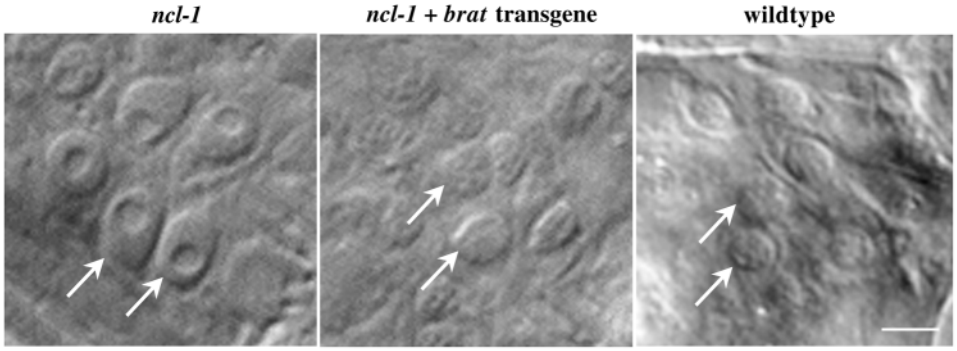

Fig. 2. Rescue of *ncl-1* with a *brat* transgene. Worms mutant for *ncl-1*, those carrying a *brat* transgene, and wild-type worms were visualized using Nomarski optics. The left image shows neurons anterior to the posterior bulb of the pharynx in a *ncl-1(e1942)* worm. The middle image shows neurons from the same region in a *ncl-1(e1942)* worm carrying an array containing KS+ncl5′&3′/brat. The right image shows the same region from a wild-type worm. In each panel, two nuclei are indicated with white arrows. Note that large nucleoli are visible in nuclei in the left image only. Scale bar: 5 μm.

MATERIALS AND METHODS

In situ hybridization and antibody staining

Digoxigenin-labeled (Boehringer Mannheim) *brat* RNA probes were made by transcribing plasmid LD28374, linearized with *Eco*RI or *Bam*HI, with SP6 polymerase (antisense probe) or with T7 polymerase (sense probe), respectively. Tissue fixation and hybridization were performed as described previously (Kozopas et al., 1998).

For anti-Nop60B staining, dissected larvae were fixed for 30 minutes in 4% paraformaldehyde in PBS containing 0.1% Tween-20. Antibody was used at a 1:500 dilution and detected using a rhodamine-labeled anti-rabbit secondary antibody (Jackson Laboratories). Images of 0.2 μm optical sections were acquired using a Delta Vision microscope and processed using deconvolution software (Applied Precision). Projections of 20 or 30 sections were generated and Adobe Photoshop was used to measure nucleolar areas.

Transgenic rescue of *ncl-1*

The construct KS+/ncl5′&3′/ncl, which consists of the *ncl-1* cDNA flanked by *ncl-1* promoter region (8 kb of genomic DNA upstream of the *ncl-1* transcription start site) and 1.4 kb of genomic sequence downstream of the *ncl-1* polyA site, was able to rescue the Ncl phenotype of *ncl-1(e1942)* mutant worms (data not shown). The *ncl-1* cDNA sequence was replaced by the coding region of *brat* cDNA LD28374 to create the plasmid KS+/ncl5′&3′/brat. This plasmid was injected at a concentration of 80 ng/μl with the dominant marker Rol (Mello et al., 1991) at 80 ng/μl into the syncytial gonad of

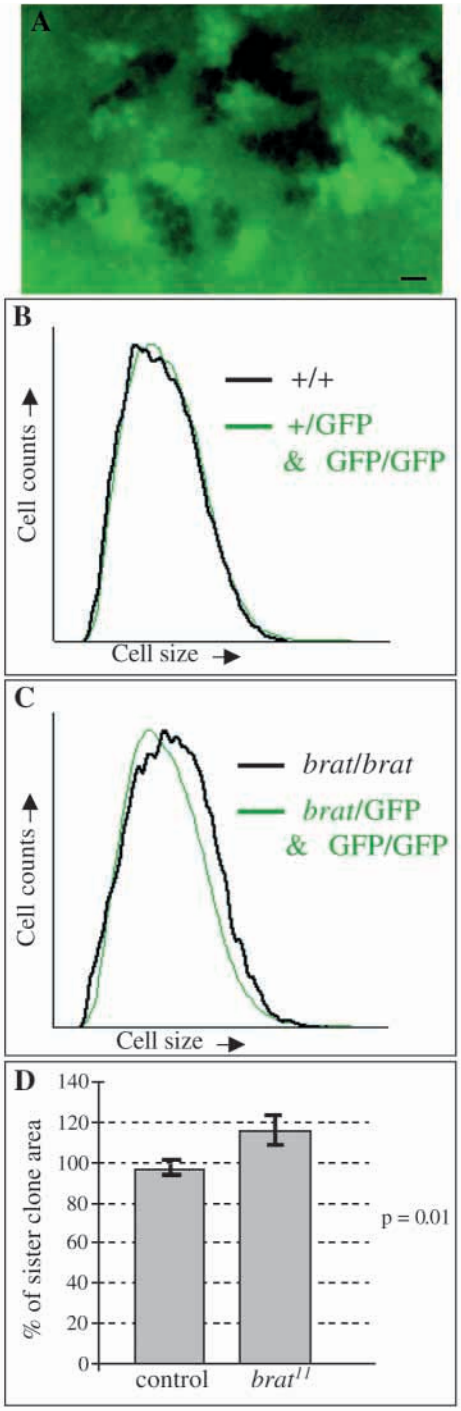

Fig. 3. *brat* inhibits cell growth. (A) Part of a wing imaginal disc in which mitotic recombination was induced with a 2 hour heat shock at 72 hour AED to generate clones of cells that are $brat^{11}/brat^{11}$ (black areas) and GFP/GFP (bright green areas). Clones are surrounded by unrecombined $brat^{11}$/GFP cells (light green areas). Scale bar: 10 μm. (B,C) Flow cytometric analysis of dissociated wing imaginal discs containing control clones (B) or $brat^{11}$ clones (C). The *x*-axis shows forward light scatter, a relative measure of cell size, and the *y*-axis shows cell counts. Representative data from one experiment in which a 2 hour heat shock was applied at 72 hours AED is shown. (D) +/+ (control) or $brat^{11}/brat^{11}$ clone areas were compared with the corresponding GFP/GFP sister clones. A 45 minute heat shock was applied at 48 hours AED and clones were measured at 115 hours AED. Mean area ratio is plotted with standard error indicated by error bars. Data from two independent experiments was compiled. Genotypes are: *hs-FLP*^{122}*; Ub-GFP FRT40A/*$brat^{11}$ *FRT40A* (A, C and '$brat^{11}$' in D) and *hs-FLP*^{122}*; Ub-GFP FRT40A/FRT40A* (B and 'control' in D).

ncl-1(e1942) worms. Adult Rol progeny were picked and their progeny were assayed by Nomarski microscopy for any effect on nucleolar size. Five transgenic rescued lines were identified.

Mitotic recombination

The FLP/FRT system (Xu and Rubin, 1993) was used to generate control and $brat^{11}$ mutant clones. The $brat^{11}$ allele has a nonsense mutation that creates a truncated protein (Arama et al., 2000). Although it is not known if $brat^{11}$ is a null allele, it is one of the strongest alleles of *brat* (G. R. Hankins, PhD thesis, University of Virginia: Charlottesville, 1991) (Woodhouse et al., 1998). Flow cytometry analysis of approximately 20 dissociated wing discs was performed as described (Neufeld et al., 1998). Similar results were obtained from five independent experiments in which mitotic recombination was induced by 37°C heat shock for 1 hour at 48 hours after egg deposition (AED) or for 1.5-2 hours at 72 hours AED. In all cases, discs were analyzed at 115 hours AED. For clone area measurements, a 45 minute heat shock was applied at 48 hours AED and discs were dissected and fixed at 115 hours AED. Areas were measured using Adobe Photoshop and data was analyzed using Microsoft Excel. The chromosome *Ub-GFP FRT40A* is from C. Martín-Castellanos and B. Edgar (unpublished).

Overexpression

UAS-brat lines were generated by P element-mediated transformation using the pUAST vector (Brand and Perrimon, 1993). The flip-out technique (Neufeld et al., 1998; Pignoni and Zipursky, 1997; Struhl and Basler, 1993) was used to generate clones overexpressing Gal4 in *HS-FLP*^{122}*; UAS-P35; Act>CD2>Gal4, UAS-GFP*$_{NLS}$*S65T* (± additional UAS transgenes) animals. Larvae were heat shocked for 45 minutes at 37°C at 72 hours AED. Similar results were obtained in two experiments without P35 using line *UAS-brat(5)* and seven experiments with P35 using lines *UAS-brat(1A)* and *UAS-brat(5)*.

Proliferation analysis

Induction of *brat* overexpressing clones was achieved with 18 to 25 minute heat shocks at 72 hours AED to generate approximately 5-10 clones per disc. Wing discs were dissected from wandering third instar larvae and fixed in 4% paraformaldehyde/phosphate-buffered saline. The number of GFP^+ cells per clone was counted on a Zeiss Axioplan microscope. Cell doubling times were determined using the formula (log2/logN)hr, where N=median number of cells/clone and hr=time between heat shock and disc fixation.

RESULTS

brat and *ncl-1* are functionally homologous

To address if any functional homology exists between *ncl-1* and *brat*, we first asked whether the two genes have analogous expression patterns. Because NCL-1 protein is most highly expressed in cells with low rates of rRNA and protein synthesis, such as cells of the early embryo (Frank and Roth, 1998) and neurons in the adult (D. J. Frank, PhD thesis, University of Washington: Seattle, 2000), we predicted that *brat* expression would be highest in cells with low levels of biosynthetic activity. We examined the *brat* expression pattern in *D. melanogaster* larvae using RNA in situ hybridization.

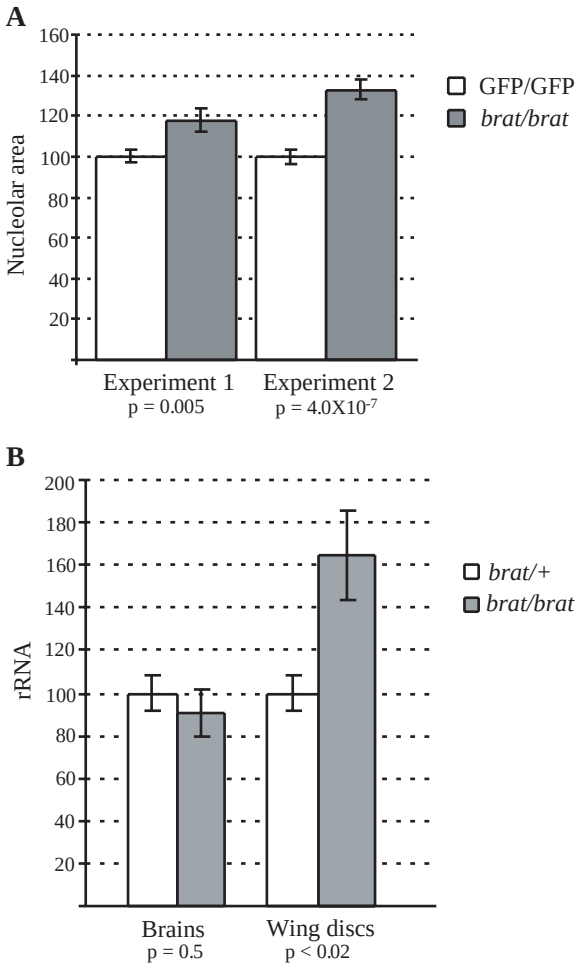

Fig. 4. Increased nucleolar size and rRNA level in *brat/brat* cells. (A) Mitotic recombination was induced with a 90 minute heat shock at 48 hours AED. At 115 hours AED, wing imaginal discs were fixed and stained with anti-Nop60B antibody. Data from two independent experiments are shown with the area of nucleoli in GFP/GFP cells set to 100 for simplicity. Mean nucleolar area is plotted with standard error indicated by error bars. The numbers of nucleoli analyzed are as follows: Experiment 1 GFP/GFP, 83; *brat/brat*, 47; Experiment 2 GFP/GFP, 74; *brat/brat*, 63. The genotype is *hs-FLP^{122}; Ub-GFP FRT40A/brat^{11} FRT40A*. (B) Larvae from the cross, *y; brat^{14}/CyOY^{+}* X *y w; brat^{11}/CyOY^{+}* were separated based on mouth hook color at 24 hours AED. When they became wandering third instars, the genotypes were confirmed and wing discs and brains were removed and processed for RNA and DNA isolation using TRIzol Reagent (Gibco BRL). Isolated RNA (standardized to isolated DNA) was applied to nitrocellulose using a slot-blot apparatus and probed with radiolabeled *D. melanogaster* rDNA PCR product. Quantitation was performed using a BioRad Molecular Imager FX. rRNA level in control *brat/+* is set to 100 for simplicity. Average rRNA level and standard error are indicated. Ten samples of each genotype were obtained on five subsequent days; each sample consisted of 9-16 brains or 14-24 wing discs.

Using a *brat* RNA probe, we observed high level *brat* expression in brains from wild-type third instar larvae (Fig. 1A). This expression was quite uniform throughout the entirety of the brain hemispheres, including the optic lobe. Weaker but fairly uniform expression was also seen in virtually all cells of the imaginal discs. Examples of eye-antennal, wing and leg discs from third instar larvae are shown in Fig. 1B-D, respectively. In the eye disc, higher *brat* expression levels were observed in small clusters of cells along the morphogenetic furrow (Fig. 1B). These are likely the neuronal preclusters, the first cells in the eye disc to exit the cell cycle and differentiate into relatively metabolically inactive cells. This expression pattern is consistent with a hypothesis that *brat*, like *ncl-1*, functions as an inhibitor of cell growth.

To determine whether *brat* and *ncl-1* are functionally homologous, we asked whether the *brat* gene could rescue the large nucleolus phenotype of *ncl-1* mutant worms. The *brat*-coding region was inserted into a vector such that it was flanked by the *ncl-1* promoter region and downstream genomic sequence. This construct was injected into *ncl-1 (e1942)* worms and nucleolar sizes of transformants were observed using Nomarski optics. While *ncl-1* mutants have large nucleoli, *ncl-1* worms expressing the *brat* transgene have small nucleoli that are indistinguishable from those seen in wild type (Fig. 2). Therefore, the *brat* gene is able to functionally replace the *ncl-1* gene in *C. elegans*, indicating that these two genes not only have similar sequence and are expressed in similar types of cells, but are indeed functionally homologous.

brat mutant cells are larger than wild-type cells

As *brat* is able to functionally replace *ncl-1* in *C. elegans*, we wanted to learn whether *brat* mutants show the same cellular phenotypes as *ncl-1* mutant worms, such as enlarged cells. To analyze *brat* mutant and wild-type cells within the same tissue, we used FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993) in larvae heterozygous for *brat^{11}* to generate paired clonal populations of cells: those that are homozygous *brat^{11}* and those that are homozygous wild type. In this system, the wild-type chromosome is marked with GFP. Thus, *brat^{11}/brat^{11}* cells can be differentiated by the fact that they do not express GFP. Unfortunately, this system does not allow us to separate unrecombined *brat^{11}*/+ cells from +/+ cells (Fig. 3A). Thus, any cell size difference we observe could be an underestimate of the actual effect if *brat^{11}*/+ cells are larger than +/+ cells. We expect that cells might be sensitive to *brat* dose, as we previously found that *ncl-1* heterozygous worms have larger nucleoli than wild-type worms (though smaller than *ncl-1* homozygotes) (D. J. Frank and M. B. Roth, unpublished). Nevertheless, flow cytometric analysis revealed that *brat^{11}/brat^{11}* cells from third instar wing imaginal discs were consistently larger than the internal control *brat^{11}*/+ and +/+ cells (Fig. 3B,C). Furthermore, area measurements showed that clones of *brat^{11}* cells were larger than their corresponding sister clones in wing imaginal discs (Fig. 3D). The increased size of *brat^{11}/brat^{11}* cells and clones suggests that, similar to *ncl-1*, loss of function mutations in *brat* lead to excess cell growth.

brat mutant cells have enlarged nucleoli and excess rRNA

To further characterize any functional relationship between *brat* and *ncl-1*, we next asked whether *brat* affects nucleolar size. We used mitotic recombination as described above to generate *brat^{11}/brat^{11}* mutant clones and +/+ control clones in wing imaginal discs. These were stained with an anti-Nop60B antibody (Phillips et al., 1998) to visualize nucleoli. We found that *brat* mutant cells have nucleoli that are 18-33% larger than

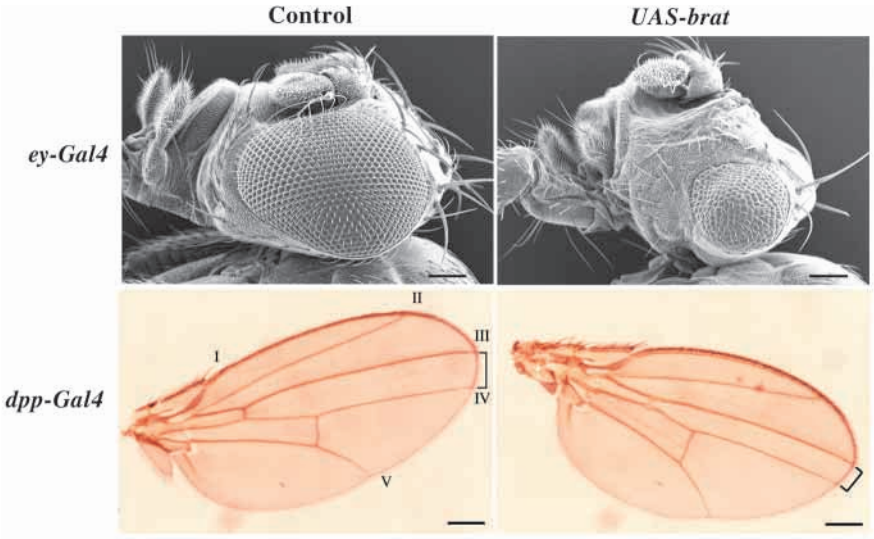

Fig. 5. Effect of *brat* overexpression in the eye and wing. The top left image shows a scanning electron micrograph of the head of a control fly. The top right image is the head of a fly in which *brat* is overexpressed in the eye. The bottom left image is of a wing from a fly carrying the *dpp-Gal4* driver alone. The bottom right image is of a wing overexpressing *brat* under the control of *dpp-Gal4*, which expresses Gal4 between veins LIII and LIV (Staehling-Hampton and Hoffmann, 1994). Positions of veins are indicated in the bottom left image (I-V). Note the decrease in area between veins LIII and LIV as indicated by the bracket. All flies are female. Genotypes are *w; P[w$^+$; ey-Gal4]/+; +/TM6B* (top left), *w; P[w$^+$; ey-Gal4]/+; P[w$^+$; UAS-brat(5)]/+* (top right), *w; P[w$^+$;UAS-brat(1A)]/Sp; P[w$^+$; dpp-Gal4]/+* (bottom left) and *w; Sp/+; P[w$^+$; dpp-Gal4]/+* (bottom right). Scale bars: 100 μm (top) and 250 μm (bottom).

wild-type cells (Fig. 4A). Because the size of the nucleolus is indicative of the level of rRNA synthesis (Altmann and Leblond, 1982; Kurata et al., 1978; Moss and Stefanovsky, 1995), this result suggests that *brat* mutant cells may have a higher level of rRNA synthesis than wild-type cells. To test this hypothesis, we compared rRNA levels in brains and wing imaginal discs from homozygous *brat/brat* mutants and control *brat/+* heterozygotes. We found that while there was no effect on rRNA levels in brains, homozygous *brat/brat* wing disc cells contained 1.6 times more rRNA than control cells (Fig. 4B). Because rRNA is very stable (Liebhaber et al., 1978) reported a rRNA half-life of at least 700 hours in primary human fibroblasts), this increase in steady state rRNA level is probably due to an increased level of rRNA synthesis in *brat* mutant cells.

brat overexpression inhibits organ growth

Given that *brat* mutant cells are larger than wild-type cells, we hypothesized that *brat* functions to inhibit cell growth, such that overexpression of *brat* would be expected to lead to a decrease in cell and organ size. Because ubiquitous overexpression of *brat* resulted in lethality (not shown), the *Gal4-UAS* system (Brand and Perrimon, 1993) was used to overexpress a wild-type *brat* cDNA specifically in the developing eye using the *eyeless-Gal4* line (*ey-Gal4*). The *eyeless* enhancer directs expression in actively proliferating cells of the eye disc (Halder et al., 1998). Expression of *brat* in the developing eye, using two different *UAS-brat* lines, resulted in a dramatic decrease in organ size (Fig. 5).

We next used the *Gal4-UAS* system to overexpress *brat* in the developing wing using the *decapentaplegic-Gal4* line (*dpp-Gal4*). In this line, Gal4 is expressed between wing veins LIII and LIV (Staehling-Hampton and Hoffmann, 1994). Overexpression of *brat* led to an obvious decrease in the size of this intervein region using two different *UAS-brat* lines (Fig. 5). To quantitate this growth inhibition, we measured the wing blade area bounded by veins LIII and LIV and compared it with the area bound by veins LII and LIII (Table 1) which served as an internal control as it was affected only slightly. We found that *brat* overexpression resulted in a 36% decrease in wing area relative to the control. The decrease in eye and wing size caused by *brat* overexpression is probably due to a combination of cell growth inhibition and cell death. To determine if there was an effect on cell size in the wing, we counted the number of bristles in a defined area. As each cell in the wing is associated with a single bristle, the inverse of the number of bristles in a region of a defined area gives a relative estimate of cell size. Surprisingly, the *UAS-brat* line appeared to have increased cell sizes in the wing (Table 1).

brat overexpression inhibits cell growth and slows cell division

Because overexpression of *brat* in the wing appeared to cause an increase in cell size while inhibiting organ growth (Table 1; Fig. 5), we wanted to examine the effect of *brat* overexpression in clones of cells, thus allowing us to compare overexpressing and control cells directly in the same tissue. The flip-out technique (Struhl and Basler, 1993) was used to overexpress *brat* and GFP in clones of cells. We dissociated wing discs from staged larvae in which overexpression was induced and analyzed them by flow cytometry. We found that overexpression of *brat* resulted in a slight increase in cell size (Fig. 6A,B) with no effect on cell cycle phasing (not shown). Microscopic examination of clones revealed that overexpression of *brat* led to cell death as evidenced by

Table 1. Effect of *brat* overexpression in wings

	Area III-IV	Area II-III	Cell size	*n*
Control	100±1	100±2	100±2	6
UAS-brat	64±3 (P<0.001)	96±1 (P<0.05)	108±1 (P<0.005)	6

Pictures were taken of wings of control and *brat* overexpressing flies. The area of the wing blade encompassed by wing veins III and IV (area III-IV) and by veins II and III (area II-III) was measured using Photoshop. For simplicity, measurements in the control line were set to 100. Standard deviations are indicated. *P* values were obtained by comparing experimental values with control values. Cell sizes were determined by taking the inverse of the number of bristles counted in a rectangle placed between veins III and IV. *n* indicates the number of wings of each genotype that were analyzed. All wings were from female flies raised at room temperature. Genotypes are *Sp/+; dpp-Gal4/+* (control) and *UAS-brat(1A)/Sp; dpp-Gal4/+*.

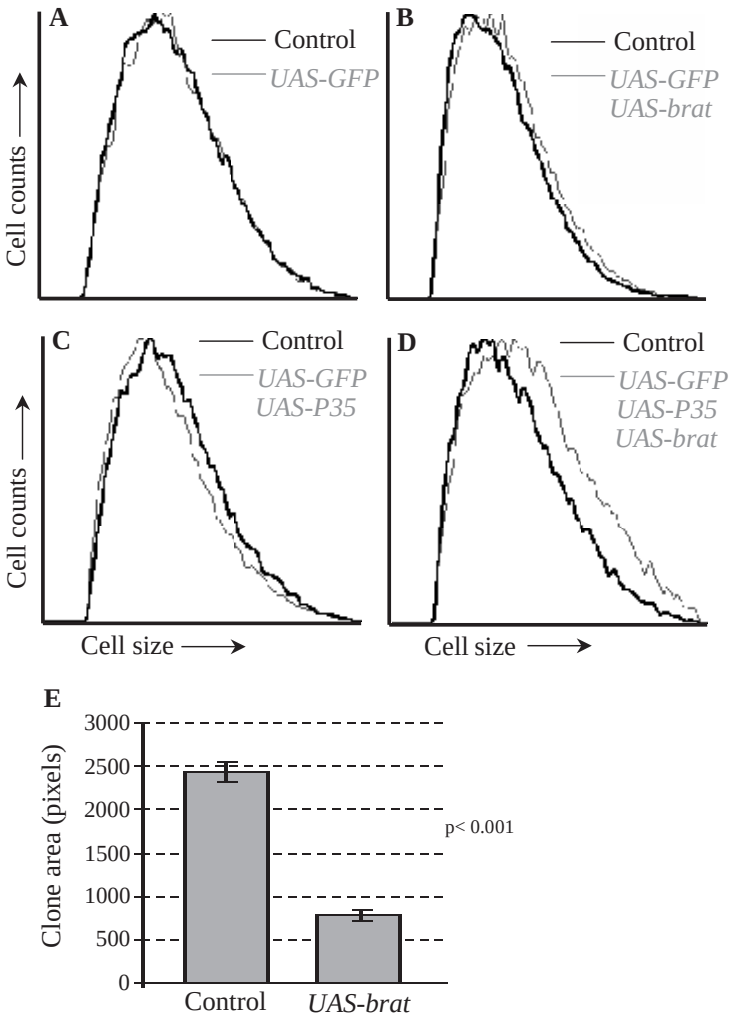

Fig. 6. *brat* overexpression increases cell size but inhibits clone growth. (A-D) Flow cytometric analysis of dissociated wing imaginal disc cells containing clones overexpressing the indicated genes. In all graphs, the *x*-axis shows forward light scatter, an indicator of relative cell size, and the *y*-axis shows cell counts. Overexpression of P35 regularly results in a small decrease in cell size relative to controls that is probably due to a deleterious effect on cell metabolism. (E) Control or *UAS-brat* clones were induced at 48 hours AED and analyzed 67 hours later. Clone areas were measured using Adobe Photoshop and the average pixel area is plotted with standard error. Eighty-eight control and 77 UAS-brat clones were measured. Genotypes are *y w hs-FLP^{122}; Sp/+; Act>CD2>Gal4 UAS-GFP/+* (A), *y w hs-FLP^{122}; Act>CD2>Gal4 UAS-GFP/UAS-brat(5)* (B), *y w hs-FLP^{122}; Sp/UAS-P35; Act>CD2>Gal4 UAS-GFP/+* (C and 'control' in E), and *y w hs-FLP^{122}; +/UAS-P35; Act>CD2>Gal4 UAS-GFP/UAS-brat(5)* (D and 'UAS-brat' in E).

pycnotic nuclei visualized by DAPI staining of clones (data not shown). To overcome this effect, we also co-expressed the cell death inhibitor P35 (Hay et al., 1994) with *brat* in the clones and observed an even larger increase in cell size (Fig. 6C,D). P35 expression appears to be somewhat deleterious to cells, as on its own it caused a small but reproducible decrease in cell size. The increased cell size in the presence of P35 is probably due to the fact that P35 expression inhibited the cell death caused by *brat* overexpression, thus allowing a greater proportion of the *brat* overexpressing cells to be analyzed.

Although *brat* overexpression results in enlarged cells, analysis of clone areas showed that *brat* overexpression actually inhibits total clone growth. We compared the areas of wing imaginal disc clones expressing *brat*, GFP and P35 with control clones expressing only GFP and P35, and found that *brat* overexpression led to a significant decrease in clone area (Fig. 6E).

Because *brat* overexpression inhibited clone growth yet resulted in enlarged cells, we hypothesized that *brat* might be causing a slowing of cell division. To address this possibility, we induced clones to express *brat*, P35 and GFP at 72 hours AED, and counted the number of cells per clone 43 hours later. Clones expressing *brat* had significantly fewer cells than control clones expressing only P35 and GFP (Fig. 7). We calculated that these cells had 50% longer doubling times than control cells (Fig. 7). Thus, overexpression of *brat* resulted in a slowing of cell division. As cell size is controlled by the rates of both cell growth and cell division, we interpret the fact that *brat* overexpressing cells are larger than control cells to mean that the inhibition of cell division rate is more severe than the inhibition of cell growth.

brat inhibits ribosomal RNA accumulation

Because *brat* mutant wing imaginal disc cells contained more rRNA than control cells (Fig. 4B), we wished to determine if *brat* overexpression would inhibit rRNA accumulation. We used the flip-out method to generate clones overexpressing GFP and P35 as a control, or GFP, P35 and *brat* in larvae. We then isolated wing imaginal discs from third instar larvae, dissociated the cells and used fluorescence activated cell sorting (FACS) to isolate GFP-expressing and non-expressing cells. RNA was isolated from equivalent numbers of cells and the relative amount of rRNA was determined. While GFP + P35 expression led to an increase in total rRNA, cells overexpressing *brat*, GFP and P35 had approximately half as much rRNA as control cells (Table 2). This decrease in rRNA per cell occurs even though *brat* overexpressing cells are larger than control cells (Fig. 6D). Given that rRNA is very stable (Liebhaber et al., 1978),

Table 2. *brat* overexpression inhibits ribosomal RNA production

	rRNA in GFP pos/neg cells	*n*
Control	1.45±0.56	5
UAS-brat	0.53±0.22 (*P*=0.02)	4

RNA was isolated from GFP-positive and -negative cells after fluorescence activated cell sorting. Equivalent cell number quantities of RNA were applied to a slot blot and probed with a radiolabeled PCR fragment corresponding to *D. melanogaster* rDNA. Blots were quantitated using a phospor-imager and the mean ratio of signal from GFP-positive to GFP-negative cells is given (±s.d.). *n* is the number of samples sorted. For each sample, a minimum of 18 third instar wing discs were dissociated and sorted. The P value is the statistical significance of the difference between control and *UAS-brat*. Genotypes are *y w hs-FLP^{122}; Sp/UAS-P35; Act>CD2>Gal4 UAS-GFP/+* (control) and *y w hs-FLP^{122}; +/UAS-P35; Act>CD2>Gal4 UAS-GFP/UAS-brat(5)*.

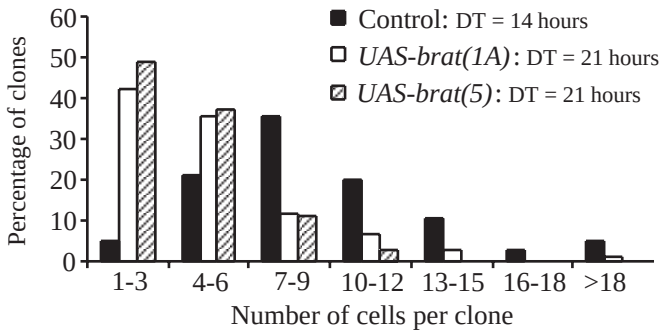

Fig. 7. *brat* overexpression inhibits cell division. Control, *UAS-brat(1A)* or *UAS-brat(5)* clones were induced at 72 hours AED and analyzed 43 hours later. The number of cells per clone was counted and plotted as a percentage of the total number of clones analyzed for each genotype. Doubling time (DT) is indicated next to each label. The statistical significances of the differences between experiments and control are: $P=8\times10^{-10}$ for *UAS-brat(1A)* and $p=2\times10^{-13}$ for *UAS-brat(5)*. Number of clones analyzed was 76 for control, 104 for *UAS-brat(1A)* and 116 for *UAS-brat(5)*. Genotypes are *y w hs-FLP*^{122}*; Sp/UAS-P35; Act>CD2>Gal4 UAS-GFP/+* (black bars), *y w hs-FLP*^{122}*; UAS-brat(1A)/UAS-P35; Act>CD2>Gal4 UAS-GFP/+* (white bars) and *y w hs-FLP*^{122}*; +/UAS-P35; Act>CD2>Gal4 UAS-GFP/UAS-brat(5)* (striped bars). Similar results were obtained in two independent experiments.

this decrease in the steady state level of rRNA in cells overexpressing *brat* probably represents a significant downregulation of rRNA synthesis. We conclude that *brat* can negatively regulate the level of cellular rRNA.

DISCUSSION

Our results demonstrate that *brat* functions to repress ribosomal RNA synthesis and cell growth. We found that *brat* mutant cells are larger than control cells, have enlarged nucleoli and contain excess rRNA. Furthermore, *brat* overexpression inhibits clone and organ growth, and leads to a decreased level of rRNA per cell. Excess cell growth may be a requisite precursor to the excess cell division that is observed in *brat* mutant brains (G. R. Hankins, PhD thesis, University of Virginia: Charlottesville, 1991). There is growing evidence to suggest that this model of hyperplasia being preceded by hypertrophy may be an important mechanism of tumor formation. For example, excess cell growth is seen before transformation in mice in which *Myc* is overexpressed (Iritani and Eisenman, 1999), and many of the transcriptional targets of *Myc* are genes involved in cell growth (Coller et al., 2000). Additionally, tumor promoting agents such as phorbol esters cause rapid increases in ribosomal RNA transcription (Allo et al., 1991; Garber et al., 1991; Vallett et al., 1993), suggesting that excess ribosome synthesis may also be an important early step in transformation. In future, it will be important to try to understand how it is that excess cell growth and ribosome synthesis can trigger excess cell division.

Why do *brat* mutants get only brain tumors?

The *brat* gene has been shown to function in both the brain and imaginal discs of *D. melanogaster* larvae. *brat* mutants have enlarged brains, and imaginal discs from *brat* mutants can form tumors when transplanted into a wild-type host. Furthermore, we have shown that *brat* is expressed in imaginal discs and that mutant wing imaginal disc cells are larger than control cells and contain more ribosomal RNA. However, brains do not contain excess rRNA. So why do *brat* mutants get brain tumors but not tumorous discs? This may be due to the plasticity of the imaginal discs. For example, experimental manipulations that affect cell division rates are compensated for by changes in cell growth so that the disc always ends up the same size, regardless of its number of cells (Neufeld et al., 1998). Therefore, if *brat* mutant cells in wing discs are larger than wild-type cells, there may be compensation so that the wing does not overgrow. In the brain, such compensation might not exist; thus, excess cell growth might stimulate excess cell division and result in an overgrown brain containing normal sized cells with normal rRNA levels.

ncl-1 mutants do not develop tumors

If *brat* and *ncl-1* are functional homologs, then why do mutations in these two genes not result in the same phenotype? The answer to this probably lies in the fact that flies and worms have very different patterns of development. While worms have determinate lineages in which every cell division and cell fate decision is absolutely identical from one worm to the next (Sulston et al., 1983), this is not the case for flies. Studies of clonal populations of cells have shown that cell proliferation patterns in *D. melanogaster* imaginal discs differ from one fly to the next (Bryant, 1970; Bryant and Schneiderman, 1969). Furthermore, in *D. melanogaster* development, proliferation is often temporally separate from differentiation. For example, the wing disc starts as an embryonic primordium of about 50 cells that grow and proliferate during the four days of larval development to result in 50,000 cells that finally differentiate to form the adult wing (Cohen, 1993). By contrast, the only tissue type in *C. elegans* in which a stem cell population divides throughout life is the germline. In fact, the germline is the only tissue in worms in which tumorous phenotypes have been clearly observed (Schedl, 1997). This tissue does not become tumorous in *ncl-1* mutants, most probably because *ncl-1* does not function in the germline. In support of this, the germline nuclei have very large nucleoli that do not appear to enlarge in *ncl-1* mutants, possibly indicating that these nuclei are synthesizing ribosomes at maximum capacity even in wild-type worms.

Why does *brat* overexpression result in larger cells?

Given that *brat* mutant cells are larger than wild-type cells, it seems surprising that overexpression of *brat* should also result in larger cells. *brat* overexpression also resulted in a slowing down of cell division; the doubling time for *brat* overexpressing cells was 21 hours compared with 14 hours for wild type. A possible model to explain these results comes from recent work showing that mouse liver cells in which the 40S ribosomal protein S6 was conditionally knocked out were able to grow but not proliferate in the absence of nascent ribosome synthesis (Volarevic et al., 2000). These authors suggest that cells will not divide unless there is a sufficient level of nascent ribosome synthesis. Applying this model here, because overexpression of *brat* leads to a dramatic decrease in rRNA synthesis (and therefore decreased ribosome synthesis), cell division is slowed. Cell growth also is slowed, as evidenced

by the small *brat* overexpressing clones. Growth is not completely inhibited, however, as cells are able to use ribosomes synthesized before the onset of *brat* overexpression. The end result, therefore, is large, slowly dividing cells.

brat represses cell growth and ribosome synthesis

Previous studies have demonstrated that activation of the insulin receptor and its downstream targets affects the activity of ribosomes and ultimately regulates cell, organ and organism size in *D. melanogaster* (Weinkove and Leevers, 2000). For example, the phosphoinositide 3-kinase (PI3K) *Dp110* is a positive regulator of growth; its overexpression leads to increased cell growth (Leevers et al., 1996; Weinkove et al., 1999) while $Dp110^{-/-}$ cells are smaller than wild-type cells (Weinkove et al., 1999). Conversely, *D. melanogaster Pten* is a negative regulator of the insulin receptor/PI3K pathway. $Pten^{-/-}$ cells are bigger than wild-type cells, while overexpression leads to decreased cell growth (Gao et al., 2000; Goberdhan et al., 1999; Huang et al., 1999). One of the final downstream targets of this pathway is ribosomal protein S6. Phosphorylation of ribosomal protein S6 results in increased translation of a set of mRNAs that contain a unique 5′ sequence; these include mRNAs for ribosomal proteins and translation factors (Thomas, 2000).

In contrast to the cell growth regulation pathway of the insulin receptor and its effectors, our results indicate that *brat* affects cell growth not through the activity of ribosomes, but rather through the regulation of their synthesis. We have shown that, similar to *Pten*, *brat* is a negative regulator of cell growth in that $brat^{-/-}$ cells are bigger than wild-type cells. Unlike *Pten*, however, *brat* overexpression results in an increase in cell size, presumably because *brat* inhibits cell division yet allows some cell growth to continue. Furthermore, *Pten* is not known to inhibit nascent ribosome synthesis. We therefore propose that *brat* does not function in the insulin receptor/PI3K pathway, but instead functions in a unique pathway to regulate the synthesis of ribosomes.

The cellular phenotype caused by overexpression of *brat* is similar to the effect of other genetic perturbations that affect ribosome synthesis. Heterozygous mutations in any one of a large class of genes termed *Minutes*, most of which encode ribosomal proteins, result in slow growing flies (Lambertsson, 1998). Clones of heterozygous *Minute* cells are also slow growing (Neufeld et al., 1998) and, in the one case that has been examined, consist of cells that are larger than wild type (Martín-Castellanos and Edgar, 2002). The tumor suppressor Retinoblastoma (Rb) has also been shown to control ribosome synthesis through its ability to inhibit rRNA and 5S RNA synthesis (Cavanaugh et al., 1995; White, 1997). Similar to our results with *brat*, overexpression of the *D. melanogaster Rb* homolog *Rbf* has been shown to slow cell division by 50% while increasing cell size (Neufeld et al., 1998). It is not yet known whether *Rbf* functions to control ribosome synthesis in flies.

How might *brat* and *ncl-1* affect ribosome synthesis? It has been proposed that in *E. coli* (Nomura et al., 1984) and in *D. melanogaster* (Yamamoto and Pellegrini, 1990) rRNA synthesis is regulated by the polysome to free ribosomal subunit ratio. When this ratio is high, rRNA synthesis is upregulated. Conversely, when translation, and therefore this ratio, are low, rRNA synthesis is inhibited. As NCL-1 (Frank and Roth, 1998) and BRAT (Sonoda and Wharton, 2001) are both cytoplasmic proteins, one possibility is that *brat* and *ncl-1* serve as sensors of this ratio. Alternatively, they could directly affect this ratio by serving as translational repressors. Interestingly, recent work has shown that *brat* functions in the translational regulation of at least one mRNA (Sonoda and Wharton, 2001). Future work should provide insight into the specific mechanism of *brat* and *ncl-1* action.

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