The TRIM-NHL Protein Brat Promotes Axon Maintenance by Repressing *src64B* Expression

Giovanni Marchetti,¹ Ilka Reichardt,² Juergen A. Knoblich,² and Florence Besse¹

¹Institute of Biology Valrose, University of Nice Sophia Antipolis, CNRS UMR7277, INSERM U1091, 06108 Nice Cedex 2, France and ²Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), 1030 Vienna, Austria

The morphology and the connectivity of neuronal structures formed during early development must be actively maintained as the brain matures. Although impaired axon stability is associated with the progression of various neurological diseases, relatively little is known about the factors controlling this process. We identified Brain tumor (Brat), a conserved member of the TRIM-NHL family of proteins, as a new regulator of axon maintenance in *Drosophila* CNS. Brat function is dispensable for the initial growth of Mushroom Body axons, but is required for the stabilization of axon bundles. We found that Brat represses the translation of *src64B*, an upstream regulator of a conserved Rho-dependent pathway previously shown to promote axon retraction. Furthermore, *brat* phenotypes are phenocopied by *src64B* overexpression, and partially suppressed by reducing the levels of *src64B* or components of the Rho pathway, suggesting that *brat* promotes axon maintenance by downregulating the levels of Src64B. Finally, Brat regulates brain connectivity via its NHL domain, but independently of its previously described partners Nanos, Pumilio, and d4EHP. Thus, our results uncover a novel post-transcriptional regulatory mechanism that controls the maintenance of neuronal architecture by tuning the levels of a conserved rho-dependent signaling pathway.

Key words: axon maintenance; *Drosophila*; mushroom body; post-transcriptional

Introduction

Neuronal circuitries established during early development face several challenges during brain maturation (Bénard and Hobert, 2009). First, they maintain their morphology and connectivity in an environment undergoing extensive changes in its mechanical and molecular properties. Second, they keep a certain flexibility to support structural plasticity in response to stimulation, a process essential for the formation and storage of memory traces. Although the mechanisms underlying the maintenance of neuronal circuits have received much less attention than those involved in the establishment of circuits, their alterations appear to be crucial for the progression of neurodegenerative diseases (Luo and O'Leary, 2005).

To identify new genes involved in the formation and/or maintenance of axonal processes, we have used *Drosophila* Mushroom Body (MB) as a model system. MBs are symmetric structures essential for high-order functions (Heisenberg, 2003). MB neu-

ron cell bodies are located in the posterior cortex, and extend their axons anteroventrally, forming a fiber called peduncle. Anteriorly, MB axons bifurcate to form a dorsal and a medial lobe. Each MB is composed of three types of neurons ($\alpha\beta$, $\alpha'\beta'$, and γ) generated sequentially during development: γ neurons are generated during embryogenesis and early larval stages, $\alpha'\beta'$ neurons during late larval stages, and $\alpha\beta$ during early metamorphosis (Lee et al., 1999). γ neurons undergo a specific remodeling program, as γ larval medial and dorsal axonal branches are pruned during early metamorphosis, and replaced by a main branch extending medially (Lee et al., 1999; Watts et al., 2003).

In this system, we have identified the tumor-suppressor gene *brain tumor (brat)* as a novel regulator of axon maintenance. Brat belongs to the tripartite motif (TRIM)-NHL family of proteins, which have been implicated in the regulation of various biological processes (Wulczyn et al., 2011). Similarly to its mammalian ortholog, TRIM32, Brat has, for example, been shown to control the proliferation and differentiation of specific neural precursor lineages during early neuronal development (Bowman et al., 2008; Schwamborn et al., 2009). Here, we show that *brat* function does not regulate the specification or the initial growth of MB axons, but is required for the maintenance of MB axons. Interestingly, *brat* phenotypes are partially suppressed by reducing the dose of components of a conserved Rho-dependent pathway shown to promote axon retraction (Billuart et al., 2001). Furthermore, elevated levels of Src64B, an upstream regulator of this pathway, are not only observed in *brat* mutant context, but also sufficient to trigger axon retraction, suggesting that Brat prevents axon retraction by keeping the levels of Src64B low. Finally, we genetically demonstrate that Brat is acting independently of its previously described partners Nanos, Pumilio, and d4EHP to

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Correspondence should be addressed to Florence Besse, Institute of Biology Valrose, University of Nice Sophia Antipolis, CNRS UMR 7277, INSERM U1091, Parc Valrose, 06108 Nice Cedex 2, France. E-mail: besse@unice.fr.

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Figure 1. *brat* downregulation disrupts MB axon morphology. **A–D'**, Adult MB lobes from control (**A, C**) and *brat* RNAi (**B, D**) brains stained with anti-FasII (**A–B'**) or anti-Trio (**C–D'**) antibodies (red in the overlay). Scale bar, 50 μ m. **E–F'**, Cell bodies of control (**E, E'**) and *brat* RNAi (**F, F'**) adult MB neurons stained with anti-Brat antibodies (red in the overlay). Scale bar, 50 μ m. **G–H'**, Cross sections of control (**G, G'**) and *brat* RNAi (**H, H'**) adult MB peduncles stained with anti-FasII antibodies (red in the overlay). Arrows point to the three different subpopulations of axons organized in concentric layers. Scale bar, 10 μ m. Green in **A–H'**: OK107-GAL4-driven mCD8::GFP. **I**, Left, Percentages of adult MBs exhibiting wild-type, intermediate, or strong axonal defects in four different *brat* RNAi lines. Numbers correspond to the total number of scored MBs. Right, Representative images of the three phenotypic categories (based on FasII staining). Statistical comparisons to the control: *** p < 0.001 (Fisher's exact test). Scale bar, 20 μ m.

Figure 2. Brat is required cell autonomously for MB $\alpha'\beta'$ and γ axon morphogenesis. **A, B**, Control (**A**) and *brat*¹⁹² (**B**) adult MB neuroblast clones labeled with the OK107-GAL4 driver. Insets, Cross sections of MB peduncles. **C**, Adult *brat*¹⁹² MB neuroblast clone expressing a wild-type copy of *brat*. **D**, Quantification of *brat*¹⁹² axonal defects in control, *brat*¹⁹², and *brat*¹⁹²; UAS-*brat* MB neuroblast clones. ****p* < 0.001 or n.s. (Fisher's exact test). **E, F**, Control (**E**) and *brat*¹⁹² (**F**) adult neuroblast clones labeled with the 201Y-GAL4 driver. **G, H**, Control (**G**) and *brat*¹⁹² (**H**) adult γ single-cell clones labeled with the OK107-GAL4 driver. **I, J**, Control (**I**) and *brat*¹⁹² (**J**) adult neuroblast clones labeled with the GMR35B12-GAL4 driver. **K, L**, Control (**K**) and *brat*¹⁹² (**L**) adult $\alpha'\beta'$ two-cell clones labeled with the OK107-GAL4 driver. Green: mCD8::GFP. Red: FasII. Scale bar, 20 μ m. Numbers in the upper right corners indicate the numbers of scored clones that exhibited morphologies similar to those shown in the images. Complete genotypes: (**A, G, K**) *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A; OK107-GAL4/+ (control); (**B, H, L**) *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A *brat*¹⁹²; OK107-GAL4/+ (*brat*¹⁹²); (**C**) *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A *brat*¹⁹²; UAS-*brat*+/+; OK107-GAL4/+ (*brat*¹⁹²; UAS-*brat*); (**E**) *hsp-flp*, UAS-mCD8::GFP; 201Y-GAL4 FRT40A tub-GAL80/FRT40A (control); (**F**) *hsp-flp*, UAS-mCD8::GFP; 201Y-GAL4 FRT40A tub-GAL80/FRT40A *brat*¹⁹² (*brat*¹⁹²); (**I**) *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A; GMR35B12-GAL4/+ (control); (**J**) *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A *brat*¹⁹²; GMR35B12-GAL4/+ (*brat*¹⁹²).

promote axon stability, and that its NHL domain is necessary and sufficient for axon maintenance. These results reveal that Brat is a novel regulator of brain connectivity that controls the stability of neuronal circuits by modulating the levels of the conserved kinase Src.

Materials and Methods

Fly strains. Fly stocks used were as follows: UAS-*brat* RNAi lines (GD-31333 and KK-105054 from the VDRC; HM05078 and HMS01121 from the Bloomington stock center); OK107-GAL4, GMR35B12-GAL4, tub-Gal80^{ts}, UAS-*Src64B*, *mys*¹, *Df(3L)Exel7208*, *rho1*^{72F}, *sqh*^{AX3}, *Drok*¹, *Drok*² (Bloomington stock center); *Df(2R)ED2457*, *Src64B*^{CPI000567} (DGRC); *FRT40A*, *brat*¹⁹² (Betschinger et al., 2006); *hs-FLP*, UAS-mCD8::GFP; FRT40A tub-GAL80/Cy0; OK107-GAL4 (Wu and Luo, 2006); *hs-FLP*, UAS-mCD8::GFP; 201Y-GAL4 FRT40A tub-GAL80/Cy0 (Yaniv et al., 2012); UASp-*Brat*, UASp-*Brat*^{G774D}, UASp-*Brat*^{R837D} (Harris et al., 2011); UAS-*Brat* ^{Δ 724–1037} (Δ CT), UAS-*Brat* ^{Δ 63–721} (Δ NT Sonoda and Wharton, 2001); UAS-*Wld⁸* (Hoopfer et al., 2006); *Src64B*^{KO} (O'Reilly et al., 2006); *FRT82B pumilio*^{ET1} (Ye et al., 2004); *nanos*^{BN} (Bier et al., 1989); *nanos*^{RC} (Lehmann and Nusslein-Volhard, 1991). Unless specified, KK-105054 line was used for *brat* RNAi. Flies were raised at 29°C for all RNAi experiments, and at 25°C for all MARCM experiments. For the *src64B* mild overexpression experiment, flies were

raised at 18°C. For the temperature-shift experiments, 0–24 h embryos were raised at 25°C (or 29°C), shifted to 29°C (or 25°C) after 4 d, 5 d, 6 d, or 7 d of development, and dissected 3–4 d after eclosion.

Immunostaining and imaging. Brains of either sex were dissected in cold PBS, fixed, and immunostained as previously described (Wu and Luo, 2006). The following primary antibodies were used: mouse anti-FascinII 1D4 (1/15; Developmental Studies Hybridoma Bank, DSHB), mouse anti-Trio 9.4A (1/10; DSHB), rabbit anti-GFP A11122 (1/1000; Invitrogen), and rabbit anti-Brat (1/200; Betschinger et al., 2006). Secondary antibodies coupled to Cy3 or Cy5 were used (1/1000; Jackson ImmunoResearch). Immunofluorescent signals were collected with a Zeiss LSM 510 Meta confocal microscope using a 40 $\times$ 1.3 NA oil-immersion objective. Fluorescence intensities were measured on peduncle cross sections (Fig. 6B–E) or cell bodies (Fig. 6F–I), using the Fiji software (<http://fiji.sc/Fiji>). In Figure 6D, signal intensities were measured throughout entire peduncle sections. In Figure 6E, regions of interest were defined based on the FascinII staining. In both cases, background signals observed in the GFP channel (i.e., signal detected outside the peduncle) were subtracted. Recovered intensities were normalized to the control condition. In Figure 6, F–I, regions of interest were defined based on GFP staining, and intensities were normalized to control values.

In situ hybridizations. Fluorescent *in situ* hybridization experiments were performed as follows: brains were fixed for 1 h in 4% formaldehyde/PBS at 4°C, washed in PBS/0.1% Triton (PBT), gradually transferred into methanol, and stored at –20°C for at least one night. Brains were then gradually transferred to PBT, incubated for 1 h in PBT/0.3% H₂O₂, washed first in PBT, then in Hybe (formamide 50%; SSC 5 $\times$; Tween 0.1%/PBT solution). Brains were subsequently incubated for 1 h at 55°C in hybridization buffer (formamide 50%, SSC 5 $\times$, Tween 0.1%, heparin 50 μ g/ml; salmon sperm 200 μ g/ml, tRNA 50 μ g/ml) and hybridized overnight at 55°C in the same solution supplemented with DIG-labeled probes (0.5 μ g/ml final concentration). Samples were then washed 1 h in hybridization buffer at 55°C and extensively in PBT. Next, they were incubated for 1 h in blocking solution (1% blocking reagent/PBT; Roche #11096176001) and overnight in the presence of HRP-coupled anti-DIG antibodies (1:500; Roche) and anti-GFP antibodies. After extensive washes in PBT, brains were incubated for 25 min in a Cy3 TSA plus solution (PerkinElmer), washed again extensively with PBT, and incubated with fluorescently coupled secondary antibodies. The *src64B* probes were cloned into the pSC-B vector using the StrataClone blunt PCR kit (Stratagene).

Cell sorting and qRT-PCR analysis. qRT-PCR experiments were performed on total RNA extracted from FACS-purified larval MB neurons, largely as described previously (Berger et al., 2012). Briefly, control (UAS-mCD8::GFP/+; OK107-GAL4/+) or *brat* RNAi (UAS-mCD8::GFP/UAS-*brat*-RNAi; OK107-GAL4/+) third instar larval brains were dissected in Rinaldini's solution (Ceron et al., 2006), enzymatically digested in papain and collagenase I (1 mg/ml) for 60 min, and then gently disrupted by pipetting in supplemented Schneider's medium. GFP⁺ cells were isolated from the resulting cell suspension by FACS, and total RNA TRIzol-extracted. qRT-PCR experiments were performed as described previously (Berger et al., 2012), using the following primers: *brat*_{fwd}

TCACGCGTTGCGGCAGTAC/*brat*_{rev} GATGCCCGAGTTGCCGCC; *src64B*_{fwd} GGGCAGTCTGCTGGACTTCT/*src64B*_{rev} CTCCGATCAGCACATTACGG; *RpS8*_{fwd} CTTGGTGAA GAACAGCATCGTG/*RpS8*_{rev} GTCGTTCTC GTCCTCTTCTGG. Six technical replicates from two independent biological samples were performed.

Results

Brat is required for proper MB axon morphology

To identify new regulators of axon morphogenesis, we performed a selective RNAi screen using *Drosophila* MBs as a model system. Inducible UAS-RNAi constructs were expressed in all MB neurons using the OK107-GAL4 driver, and axons visualized with a membrane-tagged GFP (mCD8::GFP). Four independent UAS-RNAi lines, all targeting the *brat* gene, induced strong axonal morphology defects. In wild-type adults, MB axons exhibit specific branching patterns: $\alpha\beta$ and $\alpha'\beta'$ neuron axons bifurcate to produce a dorsal and a medial branch, while γ neuron axons only project medially. As a result, two dorsal lobes (α' and α) and three medial lobes (γ , β' , and β) can be observed (Fig. 1A). In contrast, adult *brat* RNAi MBs showed absence or truncation of dorsal lobes, as well as shortened medial lobes (Fig. 1B,D,I). These defects correlated with an accumulation of axonal material at the basis of MB lobes.

To test the efficiency of *brat* knock-down, we stained control and *brat* RNAi brains with anti-Brat antibodies (Betschinger et al., 2006). While a ubiquitous cytoplasmic signal was observed in the cell bodies of control larval and adult brains (Fig. 1E,E'; data not shown), this signal was dramatically reduced in MB cell bodies upon *brat* RNAi (Fig. 1F,F'; data not shown).

Brat was originally identified as a tumor suppressor, since *brat* mutations lead to neoplastic overgrowth characterized by enlargement of larval brain hemispheres (Arama et al., 2000). To test if *brat* regulates MB neuroblast proliferation, we first counted the number of MB neurons produced upon *brat* RNAi. Consistent with previous reports (Bello et al., 2006; Betschinger et al., 2006; Bowman et al., 2008), we did not find any significant difference in the number of adult MB neurons between control and *brat* RNAi conditions (data not shown). To then test if *brat* downregulation is associated with cell fate changes, we used two markers: FasciclinII (FasII), which is strongly expressed in $\alpha\beta$ neurons and weakly in γ neurons, and Trio, which is expressed in $\alpha'\beta'$ and γ neurons (Fig. 1A,A',C,C'). Both markers are detected in *brat* RNAi MB lobes (Fig. 1B,B',D,D'). As better visualized in optical sections across MB peduncles, *brat* RNAi axons segregate into typical concentric layers, with early born γ axons in the outer layer, $\alpha'\beta'$ axons in the intermediate layer, and later born $\alpha\beta$ axons in the core layer (Fig. 1G,H). Thus, the three populations of MB

Figure 3. Brat is not required for the initial growth of γ and $\alpha'\beta'$ axons or for γ larval axonal branch remodeling. **A, B**, Control (**A**) and *brat*¹⁹² (**B**) third instar larval neuroblast clones labeled with the OK107-GAL4 driver. **C, D**, Eighteen hour APF control (**C**) and *brat*¹⁹² (**D**) neuroblast clones labeled with the OK107-GAL4 driver. **E, F**, Twenty-four to twenty-six hour APF control (**E**) and *brat*¹⁹² (**F**) neuroblast clones labeled with the 201Y-GAL4 driver. Green: mCD8::GFP. Red: FasII. Scale bar, 20 μ m. Numbers in the upper right corners indicate the numbers of scored clones that exhibited morphologies similar to those shown in the images. Complete genotypes: (**A, C**) hsp-flp, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A; OK107-GAL4/+ (control); (**B, D**) hsp-flp, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A *brat*¹⁹²; OK107-GAL4/+ (*brat*¹⁹²); (**E**) hsp-flp, UAS-mCD8::GFP; 201Y-GAL4 FRT40A tub-GAL80/FRT40A (control); (**F**) hsp-flp, UAS-mCD8::GFP; 201Y-GAL4 FRT40A tub-GAL80/FRT40A *brat*¹⁹² (*brat*¹⁹²).

neurons are correctly specified in *brat* RNAi context, but exhibit defective axonal projection patterns in adult.

Brat cell autonomously regulates the morphology of γ and $\alpha'\beta'$ axons

As the development of the different populations of MB neurons is interdependent (Wang et al., 2002), we used the MARCM technique to generate *brat* mutant neuroblast (Nb) clones in which the entire progeny of only one of the four MB neuroblasts is mutant (Lee et al., 1999). In control adult MB Nb clones, axonal branches of γ , $\alpha'\beta'$, and $\alpha\beta$ neurons project into distinct lobes (Fig. 2A; $n = 16$). In *brat*¹⁹² Nb clones ($n = 17$), in contrast, the majority of $\alpha'\beta'$ and γ axonal branches failed to project into their respective lobes. Although both γ and $\alpha'\beta'$ axons were detected in peduncle sections in this context (Fig. 2B, inset), they appeared to accumulate in the most proximal region of MB lobes (Fig. 2B). These defects were suppressed upon re-expression of a wild-type copy of Brat, confirming that *brat*

Figure 4. Brat prevents MB axon retraction. MB lobe morphology at third instar larval stage (**A, B**), 18 h APF (**C, D**), 27 h APF (**E, F**), and 36 h APF (**G, H**) in control (upper line) and *brat* RNAi (lower line) conditions. Numbers in the upper right corners indicate the numbers of scored MBs that exhibited morphologies similar to those shown in the images. Signal in **A–H**: mCD8::GFP. Scale bar, 20 μ m. Complete genotype: UAS-mCD8::GFP/+; OK107-GAL4/+ (control); UAS-mCD8::GFP/UAS-*brat*-RNAi; OK107-GAL4/+ (*brat* RNAi). **I**, Temperature-shift experiments. Graph showing the percentages of MBs with wild-type morphology as a function of temperature-shift timing. At 29°C (restrictive temperature), Gal80^{TS} is not active, enabling expression of the *brat* RNAi transgene. At 25°C (permissive temperature), in contrast, Gal80^{TS} is active, preventing expression of the *brat* RNAi transgene. The temporal resolution of these experiments is 24 h (see Materials and Methods). Note that developmental timing is different at 25 and 29°C: while pupariation occurs ~5 d after egg laying at 25°C, it occurs ~4 d after egg laying at 29°C. Complete genotype: UAS-mCD8::GFP/UAS-*brat*-RNAi; tub-Gal80^{TS}/+; OK107-GAL4/+.

Figure 5. *brat* genetically interacts with components of a conserved *rho*-dependent signaling pathway. **A**, *Rho*-dependent genetic pathway controlling axon retraction. Dashed arrows indicate that *Mys* and *Src* are not directly regulating *Rho1*. Adapted from Billuart et al., 2001. **B**, Percentages of MBs exhibiting wild-type, intermediate, and strong axonal defects (as defined in Fig. 1), after removal of one copy of *Rho*-signaling components in a *brat* RNAi context (UAS-*brat*-RNAi/+; OK107-GAL4/+). For technical reasons, KK-105054 *brat* RNAi line was used for group I experiments; GD-31333 line was used for group II experiments. Note that the proportion of strong phenotypes observed upon *brat* RNAi is lower in the absence of UAS-mCD8::GFP transgene (compare with Fig. 1). Numbers correspond to the total number of scored MBs. Statistical comparison to the *brat* RNAi control: **p* < 0.05; ***p* < 0.01, and ****p* < 0.001 (Fisher's exact test).

cell autonomously controls $\alpha'\beta'$ and γ axonal projection patterns (Fig. 2C,D).

Strikingly, *brat*¹⁹² mutant $\alpha\beta$ neurons showed normal dorsal and medial branches in this analysis (Fig. 2B), revealing that Brat regulates MB axon morphology in a population-specific manner. To better visualize the different populations of MB neurons in our MARCM analysis, we used two complementary GAL4 drivers, one expressed in γ neurons and core $\alpha\beta$ neurons (201Y-GAL4), the other in $\alpha'\beta'$ neurons (GMR35B12-GAL4). As shown in Figure 2, E,F,I, and J, axonal material accumulating proximally was observed when using either 201Y-GAL4 or GMR35B12-GAL4 to label *brat*¹⁹² Nb clones (*n* = 13 and 14, respectively), indicating that both γ and $\alpha'\beta'$ neurons mutant for *brat* exhibit defective axon elongation.

To visualize the morphology of individual axons, we then generated single-cell clones using a previously established protocol (Lee et al., 1999). Single-cell γ neuron clones were induced at early larval stages and observed in adults. While adult γ axons normally exhibit axonal branches reaching the tip of MB medial lobe, *brat*¹⁹² γ neurons showed markedly shorter axonal branches that typically failed to extend beyond the proximal half of the medial lobe (Fig. 2G,H). In parallel, two-cell $\alpha'\beta'$ neuron clones were induced in late larval stages and observed in adults. *brat*¹⁹² $\alpha'\beta'$ neurons exhibiting axon projection defects could be observed (Fig. 2L), although at a low frequency (1/26). Such a low penetrance was not observed when generating $\alpha'\beta'$ neuroblast mutant clones (Fig. 2J), and thus likely results from the perdurance of wild-type Brat proteins in two-cell clones induced late during development (Zhu et al., 2005).

Altogether, these results demonstrate that Brat regulates the morphology of specific subpopulations of MB axons: *brat* function is required cell autonomously in γ and $\alpha'\beta'$ MB neurons, but not in $\alpha\beta$ neurons.

Brat is required for the maintenance of MB axon branches

brat mutant adult phenotypes can be interpreted either as a failure of MB axons to elongate or as defective axon maintenance. To distinguish between these two possibilities, we first analyzed the morphology of MB lobes at larval and early pupal stages. At the end of the third instar larval stage, wild-type MBs are composed of γ and $\alpha'\beta'$ neurons, both projecting axonal branches into a dorsal and a medial lobe (Fig. 3A). At this stage, no morpho-

Figure 6. Brat post-transcriptionally represses *src64B* expression. **A**, Alignment between *hunchback* (*hb*) NREs and the NRE-like sequences found in the 3' UTR of *Drosophila melanogaster* (*m*) and *virilis* (*v*) *src64B* mRNA. **B–C''**, Cross sections of control (**B–B''**) and *brat* RNAi (**C–C''**) adult MB peduncles expressing Src64B::GFP protein-trap fusions and stained with anti-GFP (green in the overlay) and anti-FasII antibodies (red in the overlay). Note that Src64B-GFP signal intensities in **B** and **C** were color coded using the ImageJ Rainbow RGB look-up table (see intensity level scale in **B**). Scale bar, 10 μ m. **D, E**, Quantification of Src64B::GFP signal intensities in control and *brat* RNAi contexts for all MB axons (**D**) or $\alpha'\beta'$ axons only (**E**); $n = 20$ for each genotype. Statistical comparison to the control: *** $p < 0.001$; ** $p < 0.01$ (Mann–Whitney tests). **F–H'**, Cell bodies of control (**F–G'**) and *brat* RNAi (**H, H'**) larval MB neurons hybridized with sense (**F, F'**) or antisense (**G–H'**) *src64B* probes, and labeled with anti-GFP antibodies (green in the overlay) and DAPI (blue in the overlay). The RNA signal is shown in white in **F–H** and in red in **F'–H'**. Scale bar, 20 μ m. **I**, Quantification of *src64B* mRNA levels in control and *brat* RNAi contexts. Signal intensities were measured within GFP⁺ cells and normalized to the control. AS, antisense probes; S, sense probes; $n = 28$ and 30 for control brains hybridized with sense and antisense probes, respectively, and $n = 40$ for *brat* RNAi brains hybridized with antisense probes. Statistical comparison to the control: *** $p < 0.001$ or n.s. (Mann–Whitney tests). **J**, Normalized expression of *brat* (left) and *src64B* (right) mRNA in control (dark gray) and *brat* RNAi (red) MB neurons. qRT-PCR was performed on RNA extracted from FACS-sorted larval neurons (see Materials and Methods). Error bars indicate STD. Complete genotypes: UAS-mCD8::GFP/+; OK107-GAL4/+ (control) and UAS-mCD8::GFP/UAS-*brat*-RNAi; OK107-GAL4/+ (*brat* RNAi).

logical defects were observed in *brat*¹⁹² Nb clones (Fig. 3B; $n = 11$) or *brat* RNAi (Fig. 4A, B; $n = 15$) conditions, suggesting that *brat* is not required for the initial growth of early born MB neuron axons. During early metamorphosis, the larval axonal branches

of γ axons undergo controlled pruning, a process that eliminates both the dorsal and the medial terminal branches and is completed 18 h after puparium formation (APF; Lee et al., 1999; Watts et al., 2003). This process is specific for γ neurons, as $\alpha'\beta'$

Figure 7. *src64B* overexpression phenocopies *brat* downregulation. Adult MB lobes from control (**A**, **A'**), *brat* RNAi (**B**, **B'**), and *src64B*-overexpressing (**C**, **C'**) brains. Green in **A–C**: OK107-GAL4-driven mCD8::GFP. Red in **A–C** and white in **A'–C'**: FasII. Scale bar, 20 μ m. Complete genotypes: UAS-mCD8::GFP/+; OK107-GAL4/+ (control); UAS-mCD8::GFP/UAS-*brat*-RNAi; OK107-GAL4/+ (*brat* RNAi), and UAS-mCD8::GFP/+; UAS-*src64B*/+; OK107-GAL4/+ (*src64B* overexpression).

axonal branches are maintained throughout metamorphosis. As shown in Figure 3, *C* and *D*, *brat*¹⁹² Nb clones exhibited a morphology similar to that of wild-type Nb clones at 18 h APF ($n = 12$ and 10 for wild-type and *brat*¹⁹² Nb clones, respectively). Larval γ axonal branches were pruned normally, and $\alpha'\beta'$ neurons with extended dorsal and medial branches were observed. Shortly after pruning, MB γ axons initiate a regrowth program characterized by the extension of medial adult-specific projections (Yaniv et al., 2012). At 24–26 h APF, *brat*¹⁹² γ axons had regrown similarly to wild-type axons (Fig. 3*E,F*; $n = 13$ and 11 for wild-type and *brat*¹⁹² Nb clones, respectively), indicating that *brat* phenotypes do not result from a failure to initiate the γ axon regrowth program. Altogether, these results indicate that *brat* is not required for larval and early pupal axon morphogenesis.

To determine the stage at which axonal defects start being observed, we analyzed the morphology of *brat* RNAi MB lobes across metamorphosis. Consistent with our MARCM analysis, *brat* RNAi lobes were indistinguishable from wild-type at larval stage and at 18 h APF (Fig. 4*C,D*; $n = 19$ and 15 for wild-type and *brat* RNAi larval MBs; $n = 16$ and 13 for wild-type and *brat* RNAi 18 h APF MBs). At 27 h APF, however, *brat* RNAi MBs lacked the most distal part of their dorsal lobe (Fig. 4*E,F*; $n = 18$). At 36 h APF, *brat* RNAi MBs lacked their dorsal lobes, and exhibited severely reduced medial lobes (Fig. 4*G,H*; $n = 13$). Notably, the distal to proximal progressive loss of MB lobes correlated with an accumulation of axonal material at the basis of the peduncle, suggesting that *brat* mutant axons undergo retraction rather than

degeneration. To validate this hypothesis, we expressed in a *brat* RNAi context the Wallerian degeneration slow (Wld^s) protein, which has been shown in different contexts to inhibit neurite degeneration (Luo and O'Leary, 2005; Hoopfer et al., 2006; Schoenmann et al., 2010). OK107-GAL4-driven expression of the Wld^s protein did not rescue the *brat* RNAi phenotypes (data not shown; $n = 60$), confirming that *brat* mutant axons are not undergoing Wallerian degeneration.

To determine when the function of *brat* is required during development, we used the TARGET system to temporally control the expression of the UAS-*brat* RNAi transgene (McGuire et al., 2004). Two complementary temperature-shift experiments were performed. In the first one, flies were raised at 25°C (no expression of *brat* RNAi) and shifted to 29°C (expression of *brat* RNAi) at different developmental time points (Fig. 4*I*, red curve). In the second one, flies were raised at 29°C and shifted to 25°C (Fig. 4*I*, blue curve). These experiments revealed that the function of Brat is necessary in a restricted window of time, at pupariation.

Thus, *brat* is neither required for MB axon initial growth, nor for the remodeling of MB γ neurons. Rather, its function is required at the onset of metamorphosis to prevent the retraction of γ and $\alpha'\beta'$ axons.

Brat regulates a Rho-dependent axon retraction pathway by repressing *src64B* post transcriptionally

Maintenance of MB axon lobes is an active process that requires inhibition of a Rho-dependent axon retraction pathway (Billuart et al., 2001). Indeed, expression of a constitutively active form of *Rho1* or of *Drosophila* Rho-associated kinase (*Drok*) leads to truncated dorsal lobes, a phenotype that can be partially suppressed by removing one copy of *spaghetti squash* (*sqh*), which encodes the *Drosophila* myosin regulatory light chain (Billuart et al., 2001). *src64B*, the *Drosophila* *src* homolog, and *myospheroid* (*mys*), the β PS integrin encoding gene, have both been proposed to act upstream of these components to prevent excessive axon extension (Fig. 5*A*) (Billuart et al., 2001). To test whether *brat* genetically interacts with components of this retraction pathway, we removed one copy of *mys*, *src64B*, *rho1*, *sqh*, or *Drok* in a *brat* RNAi context. As shown in Figure 5*B*, this significantly suppressed the *brat* mutant phenotypes, suggesting that Brat acts as a negative regulator of the Rho-dependent axon retraction pathway.

In early embryo, Brat represses the translation of *hunchback* mRNA by forming a tripartite complex containing Nanos (Nos) and Pumilio (Pum; Sonoda and Wharton, 2001). Although it had initially been proposed that the RNA binding protein Pum serves as a platform to recruit Brat and Nos to target RNAs (Sonoda and Wharton, 2001), it was recently demonstrated that Brat directly binds to the Nanos Response element (NRE) of *hunchback* 3'UTR (Loedige et al., 2014). Notably, Brat recognizes a sequence (BoxA motif) that flanks the Pum binding site (Fig. 6*A*). Furthermore, the recruitment of Brat is facilitated by Pum binding within the *hunchback* NRE (Loedige et al., 2014). To identify potential Brat targets, we analyzed the sequence of mRNAs encoding positive regulators of the previously described Rho-dependent pathway. *src64B* mRNA was the only transcript containing in its 3'UTR a BoxA-like sequence flanked by a canonical UGUA(A/U/C)AUA Pum-binding motif (Fig. 6*A*; Gerber et al., 2006). This sequence is conserved throughout evolution and closely matches that of *hunchback* NREs.

To test if *src64B* is a target of Brat *in vivo* in MB neurons, we used a protein trap line in which Src64B::GFP fusions are expressed from the endogenous locus (Knowles-Barley et al.,

2010). In control adult brains, low levels of Src64B::GFP are detected in γ and $\alpha\beta$ MB axons (Fig. 6B–B', cross section of the peduncle). Strikingly, a significant increase in Src64B::GFP levels was observed in MB axons upon *brat* inactivation (Fig. 6C–C', D). This increase was particularly clear in $\alpha'\beta'$ axons where Src64B::GFP was not detected in control MBs (Fig. 6E). To test if the observed increase in Src64B protein levels was associated with increased levels of *src64B* mRNA, we performed *in situ* hybridizations on *brat* RNAi and control brains. No significant change in *src64B* mRNA levels was observed upon *brat* inactivation in MB neurons (Fig. 6F–I). To more quantitatively estimate the levels of *src64B* mRNA, we isolated neuronal cells expressing GFP under the control of the OK107-GAL4 driver, and performed qRT-PCR on RNA extracted from GFP⁺ cells. Similar amounts of *src64B* mRNA were found in control and *brat* RNAi brains (Fig. 6J), indicating that Brat regulate *src64B* mRNA at the post-transcriptional level.

Finally, to test if the elevated levels of Src64B were responsible for the axon retraction defects observed upon *brat* inactivation, we overexpressed *src64B* in MB neurons using OK107-GAL4. As shown in Figure 7, A–C, mild overexpression of *src64B* phenocopied the *brat* RNAi defects and induced a shrinkage of MB lobes ($n = 18$).

Altogether, these results indicate that Brat prevents MB axon retraction by repressing the translation of *src64B*.

Brat controls MB axon maintenance independently of the Nanos/Pumilio complex and of d4EHP

In embryo and larval peripheral nervous systems, Brat cooperates with Nos and Pum to repress the translation of target mRNAs (Sonoda and Wharton, 2001; Cho et al., 2006; Muraro et al., 2008; Oleśnicki et al., 2012). Therefore, we investigated whether Brat is acting together with Nos and Pum to inhibit axon retraction. First, we tried to phenocopy *brat* mutant phenotypes by inactivating *nos* or *pum* expression. None of the *nos* ($n = 3$) or *pum* ($n = 3$) RNAi transgenic lines we tested disrupted axon morphology when combined with OK107-GAL4 (data not shown). Furthermore, *nos^{RC}/nos^{BN}* mutant flies exhibited normal MB lobes ($n = 25$; Bier et al., 1989; Lehmann and Nusslein-Volhard, 1991), and *pum^{ET1}* mutant γ neurons (Forbes and Lehmann, 1998) were indistinguishable from control neurons when analyzed using the MARCM technique ($n = 21$; data not shown). Second, we tried to rescue *brat* phenotypes by expressing a form of Brat that cannot associate with the Pum/Nos/*hunchback* NRE complex (Brat^{G774D}; Sonoda and Wharton, 2001; Harris et al., 2011). Although the G774D mutation was initially thought to disrupt a putative Pum–Brat

Figure 8. Brat acts independently of the Nanos/Pumilio complex and of d4EHP. **A**, Schematic representation of Brat protein domains. Top, Full-length protein with the G774D and R837D point mutations indicated with arrows. Bottom, Brat^{ΔCT} and Brat^{ΔNT} truncated versions. **B**, Percentages of axonal defects in MB neuroblast clones from control, *brat¹⁹²*, and *brat¹⁹²; brat^{wt}*, *brat¹⁹²; brat^{G774D}*, or *brat¹⁹²; brat^{R837D}* rescue contexts. Statistical comparison to the *brat¹⁹²* mutant condition: *** $p < 0.001$ (Fisher's exact test). Numbers correspond to the total number of scored clones. **C**, Percentages of MBs with intermediate or strong phenotypes (as defined in Fig. 1I) in *brat* RNAi and Brat^{ΔCT} or Brat^{ΔNT} rescue contexts. Numbers correspond to the total number of scored MBs. *** $p < 0.001$, ** $p < 0.01$ or n.s. (Fisher's exact tests). Complete genotypes: (**B**) *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A; OK107-GAL4/+ (control); *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A *brat¹⁹²*; OK107-GAL4/+ (*brat¹⁹²*); *hsp-flp*, UAS-mCD8::GFP; FRT40A tub-GAL80/FRT40A *brat¹⁹²*, UAS-*brat* or *brat^{G774D}* or *brat^{R837D}*/+; OK107-GAL4/+ (*brat¹⁹²*; UAS-*brat* or UAS-*brat^{G774D}* or UAS-*brat^{R837D}*, rescue). **C**, UAS-mCD8::GFP/+; UAS-*brat*-RNAi/+; OK107-GAL4/+ (*brat* RNAi); UAS-mCD8::GFP/+; UAS-*brat*-RNAi/+; OK107-GAL4/+ (*brat* RNAi; UAS-*brat* or *brat^{ΔCT}* or *brat^{ΔNT}*, rescue).

interaction (Sonoda and Wharton, 2001), it was recently proposed to rather prevent the Pum-enhanced association of Brat to RNA (Loedige et al., 2014). Strikingly, expression of Brat^{G774D} was able to rescue *brat¹⁹²* Nb clone phenotype (Fig. 8A, B). Altogether, these results indicate that Brat acts independently of Nos and Pum to control MB axon maintenance.

Previous work has shown that Brat represses the translation of *hunchback* mRNA in embryo by interacting with d4EHP, an eIF4E-like cap binding protein (Cho et al., 2006). As shown in Figure 8B, however, a mutant form of Brat protein that cannot interact with d4EHP (Brat^{R837D}; Cho et al., 2006; Harris et al., 2011) rescued *brat¹⁹²* Nb clone phenotypes similarly to wild-type Brat protein, further indicating that Brat function in MB neurons is independent of its previously described partners.

Drosophila Brat contains conserved domains characteristic of members of the TRIM-NHL family: two B-Box motifs and Coiled-coil regions at the N terminus, and an NHL domain at the C terminus (Fig. 8A). To determine which domain of Brat is important for axon maintenance *in vivo*, we expressed in a *brat* RNAi context a Δ C-ter form of Brat lacking the NHL domain, or a Δ N-ter form of Brat lacking both B-Box and Coiled-coil domains. Expression of Brat ^{Δ NT}, but not Brat ^{Δ CT}, was able to rescue *brat* phenotypes (Fig. 8C), revealing that Brat NHL domain is both necessary and sufficient *in vivo* for axon maintenance.

Discussion

In this article, we demonstrated that the conserved TRIM-NHL protein Brat is a novel regulator of neuronal structure maintenance that post-transcriptionally tunes the levels of the kinase Src, an upstream regulator of a conserved Rho signaling pathway preventing axon retraction.

Brat post-transcriptionally represses *src64B* expression

In *Drosophila* early embryo and larval peripheral nervous systems, Brat cooperates with Nanos, Pumilio, and d4EHP to repress the translation of target mRNAs (Sonoda and Wharton, 2001; Cho et al., 2006; Muraro et al., 2008; Olesnicki et al., 2012). We have shown that, in centrally located MB neurons, Brat promotes axon maintenance by repressing the translation of *src64B* mRNA. Surprisingly, our genetic analysis has revealed that Brat prevents axon retraction independently of its previously described partners. Although *src64B* mRNA contains a Pum-binding motif, its regulation by Brat is thus likely independent of Pum activity. This is consistent with recent work indicating that translation repression by Brat can occur independently of Pum in *Drosophila* S2 cells, and that Brat binds RNA independently of Pum, via its NHL domain (Loedige et al., 2014). Notably, similar conclusions were drawn with Brat mammalian ortholog TRIM71 (Loedige et al., 2013). This, together with modeling analyses (Loedige et al., 2014) and large-scale mRNA interactome studies (Kwon et al., 2013) led to an emerging model in which TRIM-NHL proteins are direct RNA binding proteins that recognize target mRNAs via their NHL domain.

Here, we have shown that Brat NHL domain is both necessary and sufficient for MB axon maintenance *in vivo*, and have identified *src64B* mRNA as a target of Brat in MB neurons. How Brat represses *src64B* at the molecular level remains to be addressed. Three closely related members of the TRIM-NHL family of proteins (*Drosophila* Mei-P26 and mammalian TRIM-32 and TRIM71) have been shown to physically interact with the RISC component Ago1 or Ago2 (Neumüller et al., 2008; Schwamborn et al., 2009; Wulczyn et al., 2011; Chang et al., 2012). Furthermore, these proteins regulate microRNA activity, either positively (Schwamborn et al., 2009; Wulczyn et al., 2011; Chang et al., 2012) or negatively (Neumüller et al., 2008). Although Brat coimmunoprecipitates with Ago1 in embryo extracts (Neumüller et al., 2008), a role of Brat in the regulation of the microRNA machinery has not been demonstrated so far. In MBs, the observed *brat* mutant phenotype does not appear to result from a downregulation of microRNA levels and/or activity, as no axon retraction defects were detected in neurons mutant for core components of the microRNA pathway (data not shown). In addition, *brat* mutant phenotypes could not be suppressed by removing one dose of miRNA pathway components, further supporting a miRNA-independent function of Brat in this system.

Brat, a novel regulator of axon maintenance

Our study has uncovered that *brat* function, while dispensable for the larval growth of *Drosophila* MB axon bundles, is essential to prevent MB axon retraction during metamorphosis. Interestingly, our clonal analysis has revealed that *brat* cell autonomously promotes axon maintenance in early born γ and $\alpha'\beta'$ neurons, but that its function is not intrinsically required in $\alpha\beta$ neurons. Such a cell specificity may reflect the use of $\alpha\beta$ -specific pathways preventing axon retraction or alternatively a differential stability of late born $\alpha\beta$ axons.

Although the mechanisms regulating axon maintenance are still largely unknown, previous work has shown that downregulating the activity of the small GTPase Rho is essential for the stabilization of axons (Jalink et al., 1994; Billuart et al., 2001; Govek et al., 2005). In a variety of neuronal types, ectopic activation of Rho and its downstream effector Rho-kinase (Rock) causes axon retraction, largely by increasing the contractility and the stability of the actomyosin cytoskeleton (Govek et al., 2005). In *Drosophila* MB neurons, both the activation and the expression levels of components of a Rho-dependent signaling pathway controlling axon retraction must be tightly controlled. Ectopic activation of Rho1 or Drok leads to MB lobe retraction, a phenotype suppressed by reducing the dosage of other components of the pathway including *mys*, *src*, *sqh*, or *drok* (Billuart et al., 2001). Conversely, axon overextension phenotypes are observed upon *mys* or *drok* loss of function. Several lines of evidence obtained both *in vivo* and *in vitro* have indicated that the conserved kinase Src regulates the activity of Rho by phosphorylating the Rho-GAP inhibitory protein p190 (Billuart et al., 2001; Brouns et al., 2001). Consistent with this model, reducing the dosage of *src64B* significantly suppresses the *p190* loss-of-function retraction phenotype observed in MB neurons (Billuart et al., 2001). Here, we identified Brat as a novel upstream regulator of the Rho-dependent pathway controlling axon retraction, and showed that it is required *in vivo* to keep the levels of Src64B low during development. This discovery identifies a versatile mechanism that controls neuronal structure maintenance, and demonstrates the importance of post-transcriptional regulations in the modulation of signaling pathway activation.

Tightly controlling the levels of Src and Rho-GTPase is not only important during neuronal development, but also in the context of neural plasticity (Lu et al., 1998; Tolia et al., 2011). Thus, it is tempting to speculate that Brat may help in controlling the balance between stabilization of connectivity patterns and neuronal remodeling in response to experience and learning. To address this possibility, it will be interesting in the future to determine whether Brat is required for the learning and memory processes implicating MB neurons.

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