Cell, Vol. 107, 195–207, October 19, 2001, Copyright ©2001 by Cell Press

Regulating Axon Branch Stability: The Role of p190 RhoGAP in Repressing a Retraction Signaling Pathway

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Summary

Mechanisms that regulate axon branch stability are largely unknown. Genome-wide analyses of Rho GTPase activating protein (RhoGAP) function in *Drosophila* using RNA interference identified p190 RhoGAP as essential for axon stability in mushroom body neurons, the olfactory learning and memory center. p190 inactivation leads to axon branch retraction, a phenotype mimicked by activation of GTPase RhoA and its effector kinase Drok and modulated by the level and phosphorylation of myosin regulatory light chain. Thus, there exists a retraction pathway from RhoA to myosin in maturing neurons, which is normally repressed by p190. Local regulation of p190 could control the structural plasticity of neurons. Indeed, genetic evidence supports negative regulation of p190 by integrin and Src, both implicated in neural plasticity.

Introduction

Experience can cause rapid changes in the connection patterns of maturing neurons (e.g., Trachtenberg and Stryker, 2001). While much progress has been made in our understanding of the molecular mechanisms of axon guidance and dendritic elaboration during the initial wiring of the nervous system (reviewed in Tessier-Lavigne and Goodman, 1996; Scott and Luo, 2001), little is known about the regulation of axon and dendrite branch stability in maturing neurons. Rho family of small GTPases act as molecular switches that transduce extracellular signals to changes in the actin cytoskeleton (Hall, 1998). Rho GTPase signaling pathways play important roles in regulating multiple processes in the morphological development of neurons, including axon growth, guidance, and dendritic elaboration (reviewed in Luo, 2000; Dickson, 2001; Scott and Luo, 2001). Recent studies suggest that Rho GTPases also regulate the stability of dendritic branches and spines in relatively mature neurons (Nakayama et al., 2000; Li et al., 2000; Wong et al., 2000; Tashiro et al., 2000).

The activities of Rho GTPases are regulated positively by guanine nucleotide exchange factors (GEFs) and negatively by GTPase activating proteins (GAPs). In turn, RhoGEFs and RhoGAPs can be regulated by upstream cell surface receptors for guidance cues or adhesion proteins (Luo, 2000; Dickson, 2001). RhoGEFs and RhoGAPs far outnumber Rho GTPases. The *Drosophila* genome contains six Rho GTPases, but at least 20 predicted RhoGEFs and as many RhoGAPs (Rubin et al., 2000). The human genome is predicted to contain 59 to 77 RhoGAPs (I.H.G.S., 2001; Venter et al., 2001). While it is interesting to speculate on why so many Rho regulators are in the genome, their importance in the function of the human nervous system is highlighted by recent findings that mutations in a RhoGAP and a RhoGEF cause X-linked nonsyndromic mental retardation (Billuart et al., 1998; Kutsche et al., 2000).

Several RhoGEFs have been shown to be important for axon guidance (Steven et al., 1998; Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000; Shamah et al., 2001). Less is known about the cellular function of RhoGAPs in the development and function of the nervous system. One RhoGAP whose function has been investigated is mammalian p190 RhoGAP (p190). Originally identified as a binding partner for p120 RasGAP in Src-transformed cells (Ellis et al., 1990; Settleman et al., 1992), p190 preferentially regulates the GTPase RhoA (Ridley et al., 1993) and is a substrate for Src tyrosine kinase both in vitro (Ellis et al., 1990) and in vivo (Brouns et al., 2001). Upon growth factor stimulation or integrin activation, p190 is recruited to the actin cytoskeleton (Burbelo et al., 1995; Chang et al., 1995; Sharma, 1998). p190A is highly expressed in the developing and adult mammalian CNS, and knockout mice exhibit defects in a number of developmental processes, including neural tube closure, axon outgrowth, guidance, and fasciculation (Brouns et al., 2000, 2001).

To understand the functions of RhoGAPs in the nervous system, we undertook a comprehensive loss-of-function analysis of this class of proteins using transgenic double-stranded RNA interference (RNAi), focusing on the *Drosophila* mushroom body (MB) neurons. The MBs are the insect center for olfactory learning and memory (Heisenberg, 1998). MB neurons have complex axonal and dendritic developmental programs (Lee et al., 1999), allowing us to study various aspects of neuronal development. Of the 18 RhoGAPs we studied, three show distinct loss-of-function phenotypes in MB neurons. In particular, inactivation of the *Drosophila* homolog of mammalian p190 results in retraction of axonal branches. This phenotype is mimicked by activation of RhoA or of its effector kinase Drok, and is modified by the level and phosphorylation state of myosin regulatory light chain. These experiments indicate that a previously established pathway from RhoA to Drok to the regulation of myosin (Winter et al., 2001) also functions in maturing neurons to cause axon branch retraction. Under physiological conditions, this pathway is repressed by p190 to maintain axon branch stability. We also present evidence suggesting that p190 is negatively regulated by integrin and Src, both implicated in learning and memory.

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Results

Genome-wide Analysis of RhoGAP Function via Transgenic RNAi

We defined 20 RhoGAPs in the *Drosophila* genome (see Experimental Procedures) by the presence of a putative catalytic GAP domain with three characteristic motifs (Boguski and McCormick, 1993). Sequence analysis of the 20 RhoGAP domains showed a low degree of similarity amongst pairs, except for RhoGAP-84C8 and RhoGAP-50C14, which share 48% amino acid identity (Figure 1A). To systematically study the function of all RhoGAPs in neuronal morphogenesis, we used the transgenic RNAi approach (Figure 1B) (Tavernarakis et al., 2000), making use of the GAL4-UAS binary expression system (Brand and Perrimon, 1993). To test if MB neurons are sensitive to transgenic RNAi perturbation, we generated *UAS-RhoA* inverted repeat transgenic flies capable of expressing double-stranded RNA (hereafter refer to as *UAS-dsRNA*, see Figure 1B). We then introduced into these flies *GAL4-OK107*, which is highly expressed in all MB neurons and their neuroblasts (Lee et al., 1999). Previous work indicated that *RhoA* is required for MB neuroblast proliferation and cytokinesis (Lee et al., 2000). Loss-of-function *RhoA* clones in the MB result in reduction in neuronal number produced by mutant neuroblasts and the presence of large multinucleated cells (Figure 1D, compared to Figure 1C) (Lee et al., 2000). This phenotype is mimicked by *RhoA* dsRNA expression (Figure 1F, compared to Figure 1E). The cytokinesis defect is evident from the double labeling of spectrin (marking the cell cortex) and mCD8-GFP (marking the internal and cytoplasmic membrane) (Lee et al., 2000) (Figure 1F, inset). Phenotypes induced by RNAi are insertion-dependent. Lines with strong dsRNA expression consistently cause severe loss-of-function phenotypes (Figure 1F, see legend). We therefore applied this strategy to inactivate the RhoGAP genes.

We analyzed >3 independent insertions of *UAS-RhoGAP dsRNA* for 17 of the 20 predicted *Drosophila* RhoGAPs (Table 1). We excluded: (1) a RhoGAP from the *rotund*(*rn*) region (RhoGAP-84C8) not expressed in the nervous system (Agnel et al., 1992); (2) the fly homolog of vertebrate RLIP (RhoGAP-93B3), a putative effector of Ral GTPase involved in receptor-mediated endocytosis (Jullien-Flores et al., 2000); and (3) RhoGAP-71E1, which has P element insertions in its 5′ UTR that allowed us to analyze the loss-of-function phenotypes using the MARCM system (Lee and Luo, 1999). Positively labeled MB neuroblast clones for *RhoGAP-71E1* exhibited reduction of cell number and misguidance of MB axons (Table 1 and our unpublished data). Of the 17 different RhoGAPs subjected to RNAi analysis, six resulted in lethality when expressed ubiquitously using *tubulin-GAL4* (Table 1), suggesting that they correspond to genes essential for viability. Three exhibited visible phenotypes or lethality when dsRNA was expressed in the imaginal discs using *GAL4-T80* (Table 1). Only two genes gave significant and distinct defects in adult MBs as a result of strong dsRNA expression in MB neurons with *GAL4-OK107*. *DRacGAP* (*RhoGAP-50C14*) (Sotillos and Campuzano, 2000) dsRNA expression resulted in reduction of neuronal number, abnormally large cells, and overextension of the axons (Table 1 and our unpublished data). The second gene, *RhoGAP-16B12*, is the focus of the remainder of this study.

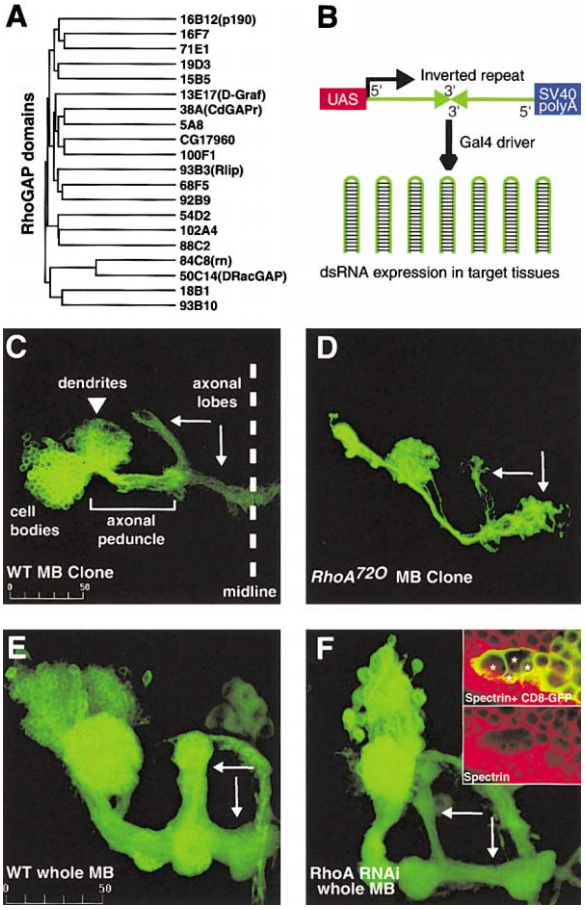

Figure 1. Tissue-Specific RNAi Strategy to Systematically Study RhoGAP Function in Neurons

(A) Cluster analysis of the GAP domains of 20 predicted *Drosophila* RhoGAPs using the Clustal W program. RhoGAP genes are named according to their cytological locations.

(B) Schematic of using the GAL4-UAS system to express dsRNA derived from inverted repeat sequences in a cell type-specific manner.

(C–F) *RhoA* mushroom body phenotypes generated either in MARCM neuroblast clones (D) or by RNAi (F) compared with wild-type (WT) controls (C and E). All brains are from wandering 3rd instar larvae. MBs are visualized using mCD8-GFP staining either in positively labeled clones (C and D) or in whole organisms using MB *GAL4-OK107* (E and F). Inset in (F) shows a single confocal section double labeled with mCD8-GFP (in green, outlining the nuclei) and spectrin (in red, outlining the entire cell). Asterisks (*) label the four nuclei present within a single cell. There are four MB neuroblasts in each hemisphere (Ito et al., 1997), so if the phenotype induced by RNAi is as severe as a *RhoA* null mutation, we expect four times as many MB neurons in dsRNA-expressing brains as compared to the MARCM clone.

In these and all subsequent images, MBs are oriented such that dorsal is up and the midline is toward the right. Vertical and horizonal arrows point to the medial and dorsal lobes, respectively. The unit for the scale bar is μm.

Sequence analysis revealed that RhoGAP-16B12 is the *Drosophila* homolog of mammalian p190 RhoGAP (see Introduction). In addition to C-terminal GAP do-

Table 1. Analysis of RhoGAP Gene Function by Tissue-Specific RNAi

Map Positions of the RhoGAP Genes	Gene Symbols According to GadFly (Gene name)	Other Predicted Domains	References from Previous Studies	Phenotypes when UAS-dsRNA Transgenes Are Crossed to:			
				Tubulin GAL4	*GAL4-T80*	*eyeless GAL4*	*GAL4-OK107*
X; 5A8	CG3208	C1/SH2					
X; 13E17-18	CG8948 (D-Graf)	PH/ADH-Short/ NLS BP/SH3					
X; 15B5	CG4937	RA					
X; 16B12	**CG8267 + CG8240 (D-p190)**	**GTP binding domain**		**lethal (6/6)**			**Axon branch retraction (12/15)**
X; 16F7	CG7122						
X; 18B1	CG7481						
X; 19D3	CG1412	PDZ/NLS BP Antifreezing					
2L; 38A	CG10538 (CdGAPr)	SH3	Sagnier et al., 2000				
2R; 50C14	**CG13345 (DRacGAP)**	**C1**	**Sotillos and Campuzano, 2000**	**lethal (7/7)**	**semi-lethal (6/7)**	**rough eyes (2/7)**	**Cell number reduction, big cells, axon overextension (4/7)**
2R; 54D2	CG6477						
3L; 68F5	CG6811	CRAL/TRIO		lethal (2/5)			
3L; 71E1	**CG16980 + CG7396**			**nd**	**nd**	**nd**	**Cell number reduction (100%), axon misguidance (20%) (MARCM analysis)**
3R; 84C8	CG2595 (rn)	C1	Agnel et al., 1992	nd	nd	nd	nd
3R; 88C2	CG8480	NLS BP/START		lethal (5/5)	Reduction or absence of wing L2 vein (4/5)		
3R; 92B9	CG4755			lethal (4/4)			
3R; 93B3	CG11622 (Rlip)	NLS BP	Julien-Flores et al., 2000	nd	nd	nd	nd
3R; 93B10-11	CG3421	WW/ MyTH4		lethal (4/5)	lehtal (4/5)	reduction of eye size (2/5)	
3R; 100F1-2	CG1976	PDZ					
4; 102A4	CG1748						
Unknown	CG17960	PH/DH					

UAS-dsRNA transgenes corresponding to each RhoGAP gene (symbol according to GadFly) have been tested using the following GAL4 drivers: tubulin (ubiquitous), T80 (imaginal discs), eyeless (eye-antennal disc), and OK107 (MB). The phenotypes and their penetrance (number of lines presenting the phenotype/number of lines tested) are summarized. Blanks indicate no obvious phenotype. nd: not determined. Bold: 3 RhoGAPs with a loss-of-function phenotype in MB. C1: Phorbol esters/diacylglcerol binding domain. SH2 and SH3: Src homology domains 2 and 3. PH: Pleckstrin homology. ADH-short: Short-chain dehydrogenases/reductases family signature. NLS BP: Bipartite nuclear localization signal. RA: Ras-associated. PDZ: PSD95/Dlg/ZO1 homology domain. Antifreezing: Type I antifreeze protein signature. CRAL/TRIO: Cellular retinaldehyde-binding protien/Triple function domain. START: lipid-binding domain in StAR,HD-ZIP. WW: Domain with 2 conserved Trp (W) residues, also known as the WWP or rsp5 domain, binds proline-rich polypeptides. MyTH4: Core domain in kinesin and myosin motors. DH: Dbl-homologous.

mains, *Drosophila* and rat p190A (Figure 2A) contain near their N termini a predicted GTP binding domain most similar to the Rab subfamily of small GTPases (Figure 2B). Specific tyrosine residues of R-p190A are phosphorylated by the nonreceptor tyrosine kinase Src, including two residues (Y1087 and Y1105) involved in the binding of p120 RasGAP (Hu and Settleman, 1997; Roof et al., 1998). Those sites are not conserved in *Drosophila*. However, two tyrosine residues in the GTP binding domain of R-p190A are conserved in the *Drosophila* protein (Figure 2B). Phosphorylation of these residues in R-p190A by c-Src abolishes its capability to bind GTP (Roof et al., 2000), which in turn is necessary for the GAP activity (Tatsis et al., 1998).

p190 RhoGAP Inhibition Disrupts the Axon Branches of MB Neurons

The adult mushroom body is composed of γ, α′/β′, and α/β neurons, totaling about 2500 per brain hemisphere. The axons of later-born α′/β′ and α/β neurons are bifurcated, each projecting one branch dorsally and the other medially. The axons of the early-born γ neurons project only medially in adult (Lee et al., 1999; Figures 2C and 3A). MB neurons expressing *p190* dsRNA exhibited truncation or loss of dorsal branches (Figures 2D–2G, compared with Figure 2C). No obvious defect was observed in cell number or morphology of the calyx where the dendrites are confined (data not shown). The phenotype is dependent on the insertion site of the transgenes. Of the fifteen *p190* dsRNA lines analyzed, three showed very weak or no phenotypes. Seven (e.g., line 14.1, Figure 2H) showed intermediate phenotypes, including a mixture of normal, pointed dorsal lobes ("weak"; Figure 2D, left side), shortened dorsal lobes ("medium"; Figure 2D, right side), or a complete lack of dorsal lobes ("strong"; Figure 2E–2G) (see Experimental Procedures for definitions of these categories). Five lines (e.g., line 5.2, Figure 2J) gave reproducibly strong phenotypes (~70% belonging to the "medium" or "strong" categories). We made use of the dorsal axon branch phenotype to study the genetic interaction of *p190* with other genes. The presence of two transgene copies of an intermediate line markedly enhanced the RNAi-induced phenotype (Figure 2H), confirming our interpretation of phenotypic strengths among different categories and supporting that the RNAi-induced phenotype can serve as a sensitive assay for genetic interactions.

Although inhibition of p190 in MB neurons preferentially affects the dorsal branch, the specificity is not absolute. As the truncation of the dorsal lobe became more severe, defects were observed in medial lobe fasciculation. Anti-Fasciclin II (Fas II) staining—which strongly labels α/β neurons, weakly labels γ neurons, and does not label α′/β′ neurons (Crittenden et al., 1998) in wild-type organisms (Figure 2C)—revealed that α/β neurons contribute to most fasciculation defects in the medial bundles (Figures 2E and 2F, red). In extreme cases, we started to observe truncated β lobes (Figures 2F–2G, arrows).

Several lines of evidence support the specificity of the *p190* phenotype. First, although we used the nucleotide sequence of the RhoGAP region for the original *UAS-p190 dsRNA* construct, the RhoGAP domain shares only 31% amino acid sequence identity with the closest RhoGAP, far below the threshold of nucleotide identity (80%) between two genes believed to be required for cross-inhibition to occur (Parrish et al., 2000). Second, the fact that expression of the 16 other RhoGAP dsRNAs does not generate a similar phenotype argues against the possibility that the p190 phenotype is caused by inhibition of another RhoGAP. Third, we have subsequently generated independent *UAS-p190 dsRNA* transgenic flies targeting an mRNA sequence unique to *p190*, and found similar phenotypes (Figures 2E–2G). Fourth, the *p190* RNAi-induced phenotypes from two independent lines tested were both enhanced by heterozygosity of a deletion that uncovers the 16B12 region where *p190* is located (*Df(1)BK10*) (Figure 2I). This experiment also suggests that the dorsal lobe phenotypes we observed are due to reduction, rather than elimination, of *p190* function. Lastly, the *p190* phenotype is suppressed by simultaneous overexpression of *p190* cDNA (*Myc-Dp190**) producing a Myc-tagged truncated protein, but not by analogous expression of Myc-tubulin (Liu et al., 2000), in MB neurons (Figure 2J). These experiments demonstrate that the specific phenotype we observed is due specifically to reduction of p190 function.

To test if mammalian *p190* could complement the RNAi-induced phenotype of *Drosophila p190*, we generated transgenic flies expressing rat p190A under the control of the UAS promoter. Expression of R-p190A in MB neurons almost completely rescued the *p190* dorsal lobe truncation phenotype (Figures 2J and 6A), demonstrating the functional conservation between fly and mammalian p190.

p190 RhoGAP Is Essential for the Stability of Dorsal Branches of MB Neurons

The spectrum of the *p190* RNAi-induced phenotypes (Figures 2D–2G) suggests that they are caused by MB axon retraction. To confirm this interpretation, we analyzed the *p190* phenotypes at different stages of development using a strong RNAi line (Figures 3E–3I). At the end of the third instar larval stage, MBs are composed of γ and α′/β′ neurons; each forms a dorsal and a medial branch (Lee et al., 1999) (Figures 3A and 3B). We did not find any RNAi-induced phenotypes at this stage (Figure 3E). At 18 hr after puparium formation (APF), wild-type MB γ neurons have pruned their axons in the dorsal and medial lobes, but α′/β′ neurons retain their axon branches in the lobes (Figures 3A and 3C) (Lee et al., 1999). A large proportion of MB neurons expressing *p190* dsRNA also possessed the dorsal α′ lobes, indicating that most, if not all, dorsal branches of α′/β′ neurons were originally present (Figures 3F and 3I). At this stage we observed pointed dorsal lobes and very thin processes punctuated by dots at the tip of these lobes (Figures 3F and 3H), resembling aspects of axon retraction (Bernstein and Lichtman, 1999). By 36 hr APF, most *p190* dsRNA-expressing MB neurons exhibit medium or strong phenotypes (Figure 3G, compared to Figure 3D). Quantification (Figure 3I) indicated a time-dependent reduction of dorsal branches in *p190* dsRNA-expressing MB neurons. These experiments indicate that p190 is required for the stability of the dorsal axon branches of at least α′/β′, and possibly α/β, neurons during development.

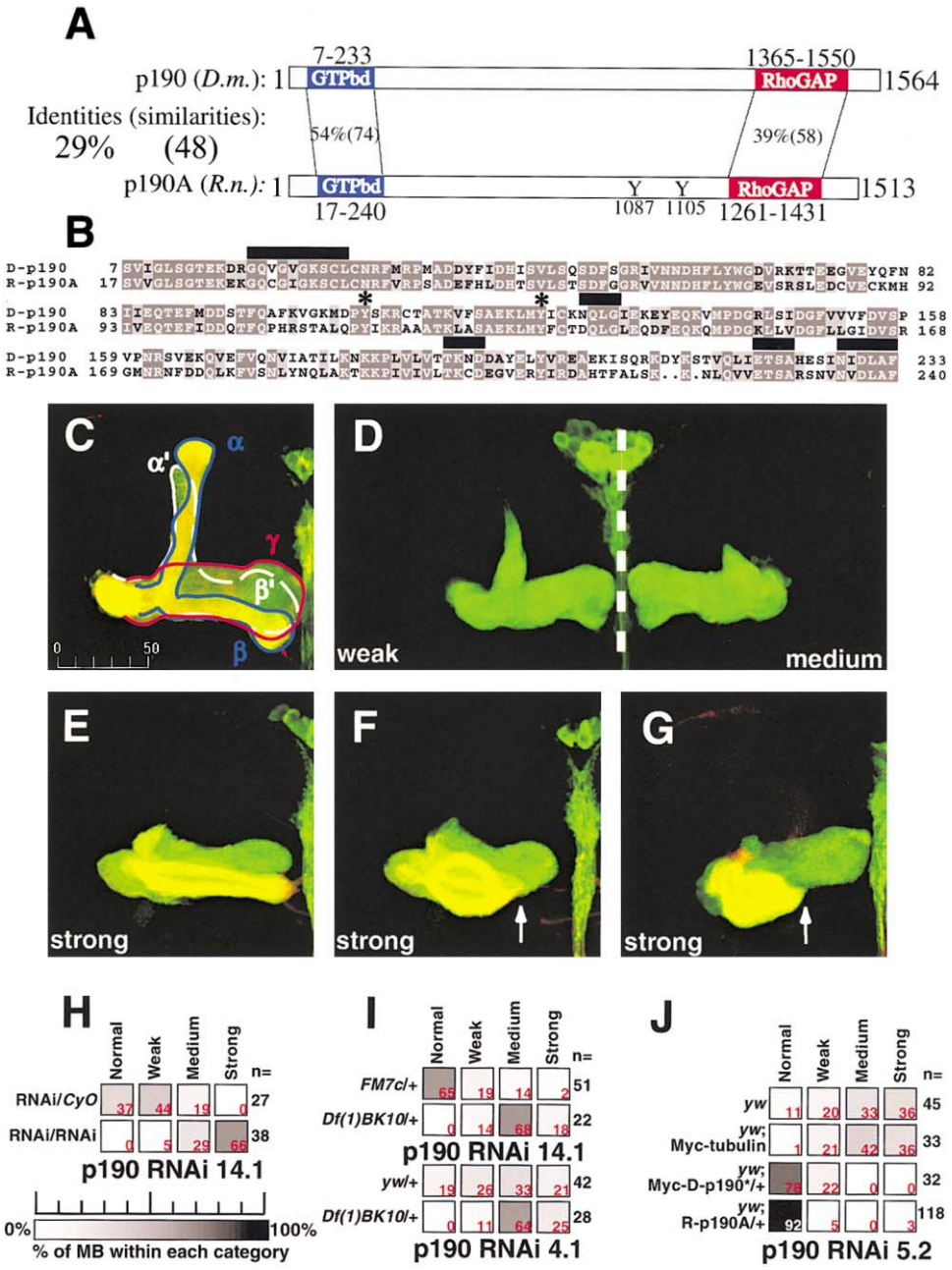

Figure 2. p190 RhoGAP Inhibition Disrupts MB Axon Branches

(A) Schematic diagram of the domain structures of *Drosophila* (*D.m.*) p190 and rat (*R.n.*) p190A.

(B) Sequence alignment of the GTP binding domain (GTPbd) showing the five conserved motifs (black boxes) commonly found in the Rab subfamily of small GTPases and two conserved tyrosine residues (asterisks) phosphorylated by c-Src in mammalian p190.

(C) WT axon patterns of adult MB. The dorsal α and α' lobes are composed of the dorsal branches of axons from α/β (blue outline) and α'/β' (white outline) neurons; the medial lobes are composed of axon branches from all three types of MB neurons.

(D–G) Representative adult MB phenotypes induced by p190 RNAi. (D) and (E–G) were from RNAi targeting the GAP and the N-terminal domain, respectively.

(H)–(J) show the effects of double the dose of the dsRNA transgene (H) or of the heterozygosity of the *p190* gene (I) on the severity of *p190* phenotype and the rescue of this phenotype by overexpression of Myc-D-p190* or rat p190A (R-p190A) (J). The rescue by R-p190A combined identical results from three independent insertion lines.

In this and all subsequent figures, unless otherwise mentioned, green staining represents *UAS-mCD8-GFP* driven by *GAL4-OK107*; red staining represents FasII immunoreactivity. The percentages of MBs belonging to a particular category are represented on a gray scale bar and the values indicated in the lower right corners of the corresponding category.

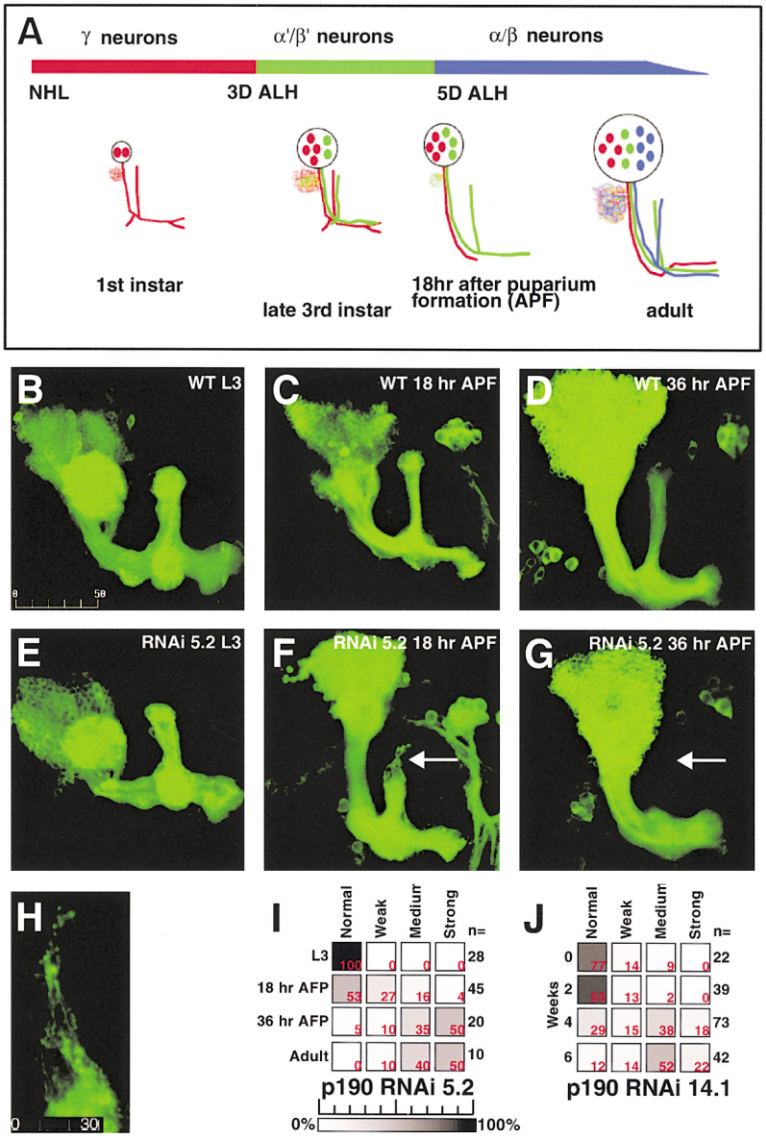

Figure 3. *p190 RhoGAP* RNAi-Induced Phenotype during MB Development and in Adults

(A) Schematic diagram of MB development (Lee et al., 1999). NHL: newly hatched larva. ALH: after larval hatching.

(B–H): Wild-type (B–D) and *p190* dsRNA (a strong line)-expressing (E–H) MB neurons in wandering third instar larvae (B and E), 18 hr after puparium formation (APF) (C and F) or at 36 hr APF (D and G). (H) Higher magnification of pointed dorsal lobe as seen in (F). Arrows in (F) and (G) represent retracting or missing dorsal lobes.

(I) Quantification of *p190* phenotypes during MB development.

(J) p190 RNAi phenotypes (an intermediate line) during adult life. At all ages examined, MBs from control flies are 100% normal with regard to the dorsal lobe phenotypes.

To test if axon retraction persists in adult life, we quantified the *p190* phenotype of an intermediate line from 0 to 6 weeks after adult eclosion and found that reduction in the dorsal lobe is progressive (Figure 3J). This result indicates that p190 is also required for axon stability throughout adult life.

The Primary Target for p190 RhoGAP Is a RhoA-Mediated Cytoskeletal Pathway

Microinjection of the GAP domain of p190 into fibroblasts resulted in actin stress fiber disassembly, suggesting that it primarily acts on RhoA (Ridley et al., 1993). Consistent with this observation, we found that overexpression of RhoA in MB neurons significantly enhanced the *p190* phenotype (Figure 4A). If p190 acts on RhoA, one would further predict that activation of the RhoA pathway would mimic *p190* loss-of-function phenotypes. To explore this possibility, we expressed constitutively active RhoA (RhoA V14) in MB neurons. We found that OK107-driven RhoA V14 expression results in adult lethality. When reared at 18°C, a few escapers were recovered which exhibited complex MB axon defects (Figure 4B). It was difficult to determine if the escapers share qualitatively similar phenotypes to those caused by *p190* RNAi. However, in pupae, we often observed a selective dorsal lobe reduction similar to the *p190* RNAi phenotype (data not shown).

RhoA transduces signals to both the nucleus and the cytoskeleton (Figure 4C). To address which downstream signaling pathway mediates axon retraction, we used activated RhoA mutants (RhoA V14) with additional "effector domain" mutations. The F39V mutation blocks RhoA's function in inducing stress fiber formation without affecting nuclear signaling, whereas the E40L mutation allows both nuclear and cytoskeletal pathways weakly (Sahai et al., 1998) (Figure 4C). When RhoA V14(E40L) (Fanto et al., 2000) was expressed in MB neurons, we found a dorsal lobe phenotype similar to the *p190* phenotype (Figures 4D and 4F). However, RhoA V14(F39V) (Fanto et al., 2000) expression had almost no

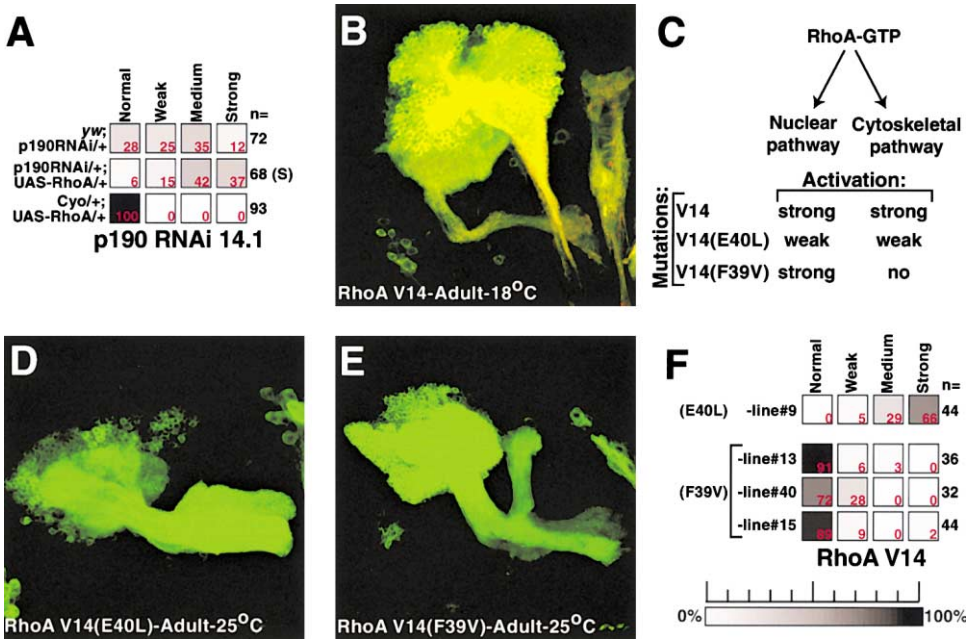

Figure 4. *p190* RhoGAP Primarily Acts on a RhoA Cytoskeletal Pathway

(A) Enhancement of the *p190* phenotypes by MB expression of RhoA (S: χ^2 test, $p < 0.001$).

(B) Axon phenotypes of an adult escaper caused by MB expression of RhoA V14.

(C–F) Effects of two RhoA V14 variants with additional mutations in the effector domains on the RhoA nuclear and cytoskeletal pathways (C) and representative images showing their axon phenotypes in MBs upon overexpression (D and E), quantified in (F).

phenotype (Figures 4E–4F), suggesting that a cytoskeletal pathway is responsible for RhoA's effect on axon branch retraction.

Drok and Myosin Regulatory Light Chain Phosphorylation in Axon Retraction

Drosophila Rho-associated kinase (Drok), an effector of RhoA, is essential for transducing signals to the actin cytoskeleton in wing cells (Winter et al., 2001). Since the effector domain mutant analysis of RhoA suggests that a cytoskeletal pathway is important for axon retraction (Figure 4), we tested if the Drok pathway is involved. Carboxy-terminal truncation of mammalian Rho-kinase/ROCK results in its constitutive activation (Amano et al., 1997). Expression of an analogous activated form (Drok-CAT; Winter et al., 2001) in MB neurons led to truncated dorsal lobes (Figures 5A and 5C) similar to the phenotypes of *p190* RNAi (Figure 2D) and weak RhoA activation (Figures 4D and 4F). A presumptive kinase-dead point mutation (Drok-CAT.KG; Winter et al., 2001) had no effect (Figures 5B and 5C), indicating that Drok signaling is dependent on its kinase activity. Developmental studies (data not shown) indicated that the Drok-CAT phenotypes also resulted from axon retraction, as does the *p190* RNAi phenotype (Figures 3E to 3I).

Biochemical and genetic evidence indicates that a key output for Drok signaling in vivo is the regulation of phosphorylation of myosin regulatory light chain (MRLC) encoded by *spaghetti squash* (*sqh*) (Winter et al., 2001). To test if endogenous MRLC is part of the axon retraction pathway regulated by p190, we performed genetic interaction experiments by reducing the dose of endogenous *sqh* in the context of the p190 dsRNA expression. We observed marked suppression of the phenotype in flies heterozygous for a null mutation of *sqh* (*sqh*^{AX3}) (Figure 5E). In contrast, expression of a phosphomimetic mutant, Sqh-E20E21, markedly enhanced the p190 phenotype (Figures 5D–5E), whereas analogous expression of a nonphosphorylable form (Sqh-A21) had no effect (Figure 5E). Further, we frequently observed truncation of the medial lobe when Sqh-E20E21 was expressed with the intermediate *p190* RNAi line. This is evident from the FasII staining, showing that the medial β axons (strongly FasII positive) only extend a fraction of the length of the medial lobe (Figure 5D). This phenotype was only observed in the strongest *p190* RNAi lines (Figures 2F–2G), never in the intermediate line alone. Taken together, these results strongly suggest that Drok and phosphorylation of *Drosophila* MRLC participate in mediating axon retraction as a result of p190 inactivation.

Overexpression of p190 RhoGAP Results in Axon Overextension

The results presented so far implicate an axon branch retraction pathway in MB neurons involving RhoA, Drok, and MRLC; this pathway is repressed by p190 to maintain axon branch stability. What are the consequences of preventing its activation? Insight first came from analyzing MB neurons overexpressing rat p190A, which almost completely rescued the *Drosophila p190* RNAi phenotype (Figures 2J and 6A). Moreover, MB neurons overexpressing R-p190A alone (data not shown) or in the *p190* RNAi background exhibit other phenotypes including overextension of dorsal axon branches (Figures 6A and 6E), the opposite of the *p190* RNAi pheno-

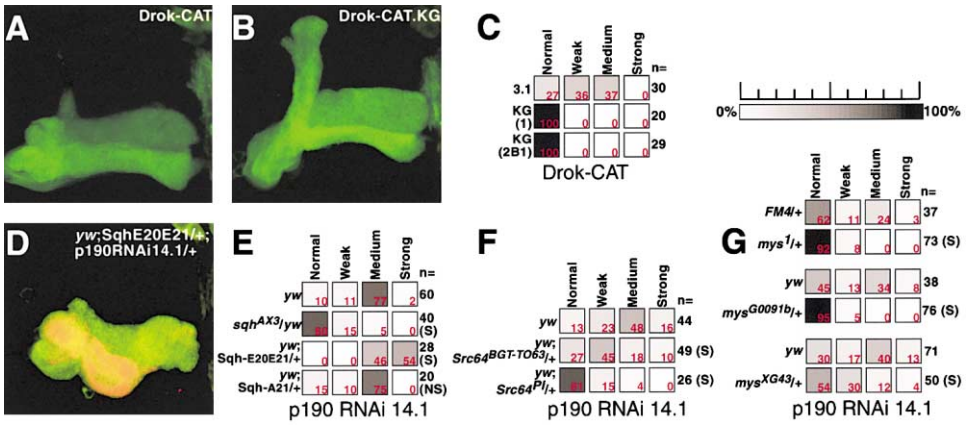

Figure 5. Downstream Effectors and Upstream Regulators of RhoA-Mediated Retraction Pathway

(A–C) Effects of overexpressing constitutively active forms of Drok (Drok-CAT) in the MBs (A) and Drok-CAT with a second kinase dead mutation (KG) (B), quantified in (C).

(D) FasII staining (red) reveals severe medial β lobe retraction when the phosphomimetic Sqh mutant is expressed in conjunction with an intermediate *p190* RNAi line.

(E) Genetic interactions between *p190* and *sqh*. sqh^{AX3}: null allele; *Sqh-E20E21*: phosphomimetic mutant, *Sqh-A21*: nonphosphorylable mutant (Jordan and Karess, 1997; Winter et al., 2001).

(F) Dosage sensitive genetic interactions between *p190* and *Src64*. $Src64^{BGT-TO63}$: weak allele; $Src64^{PI}$: strong allele.

(G) Dosage sensitive genetic interactions between *p190* and *myospheroid* (*mys*). mys^{1}: 10 kb deletion including most of the ORF; mys^{G0091b}: P element insertion in the first exon; mys^{XG43}: EMS-generated mutant whose molecular identity is unknown (Bunch et al., 1992; our unpublished data).

"S" in (E–G): $p < 0.001$; "NS" in (E), $p = 0.83$; χ^2 tests.

type. Double labeling of MB axons and R-p190A revealed that R-p190A is preferentially located at the tip of axon terminals (Figure 6A, red arrowheads) and is concentrated in overextended axons.

Overexpression of *Drosophila* p190 (D-p190) in MB neurons also caused axon overextension (Figures 6B, 6C, and 6E). A single point mutation (R1389L) in the GAP domain of D-p190 predicted to interfere with the GAP activity (Li et al., 1997) largely abolished this phenotype (Figures 6D and 6E), demonstrating that axon overextension is dependent on the GAP activity of p190.

Axon Overextension in *Drok* Mutant MB Neurons

The simplest interpretation for the overextension phenotype is that overexpression of p190 inhibits normal RhoA signaling, suggesting that RhoA signaling is required for

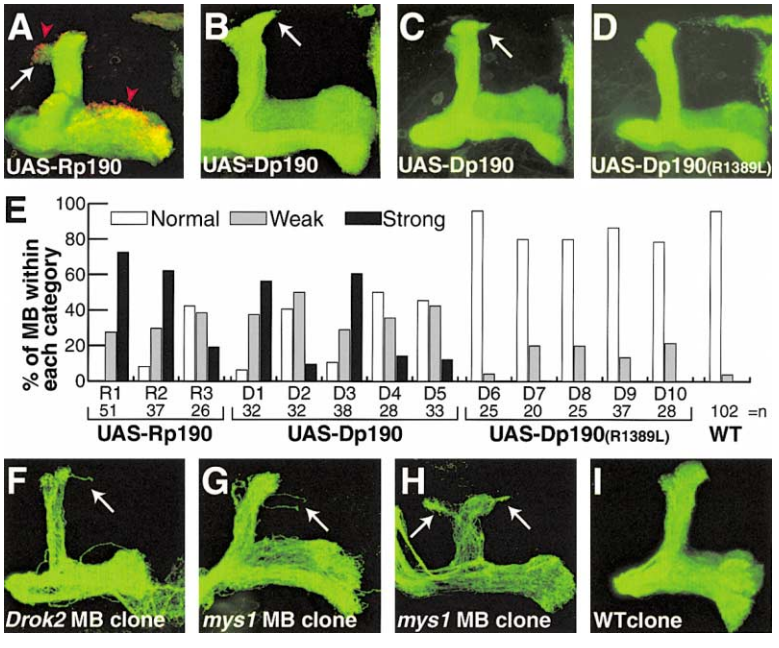

Figure 6. Dorsal Lobe Overextension Caused by Overexpression of p190 RhoGAP, or in *Drok* and *mys* Mutants

(A) MB neurons coexpressing Rat p190A and *Drosophila p190* dsRNA are stained for mCD8-GFP (green) and an antibody against R-p190 (red). The arrow indicates overextended axon processes at the tip of the dorsal lobe. Arrowheads show the enriched R-p190 distribution at the tip of axon terminals.

(B–D) MB neurons overexpressing wild-type *Drosophila* p190 (B and C) or p190 R1389L (D, GAP mutant). Strong (B) or weak (C) dorsal lobe overextensions are indicated by the arrows.

(E) Quantification of overextension phenotypes in (A–D). Normal, weak, or strong dorsal lobe axon overextensions were scored by an experimenter blind to the genotypes. "WT" is comprised of pooled data of the same blind experiment from brains that do not carry any *D-p190* transgenes, as retrospectively verified by the lack of myc staining. D1–D10 are independent transgenic lines that show indistinguishably high levels of myc staining.

(F–I) MB neuroblast clones generated by MARCM that are homozygous for $Drok^{2}$ FRT^{19A} (F), mys^{1} FRT^{19A} (G–H), or FRT^{19A} alone (I). Arrows indicate overextended axons from the dorsal lobes.

preventing axon overextension. Loss-of-function mutants in the RhoA pathway would be predicted to lead to axon overextension. Using the MARCM system (Lee and Luo, 1999), we generated MB neuroblast clones homozygous for *RhoA*, *Drok*, and *sqh* to critically test this hypothesis. Loss of RhoA activity leads to severe neuroblast proliferation defects (Lee et al., 2000; Figures 1D and 1F) such that neurons contributing to the adult dorsal lobes are never born (Figure 3A). We found similar proliferation defects in sqh^{AX3} neuroblast clones (data not shown). Thus, we could not evaluate the effects of RhoA and MRLC directly using loss-of-function mutants.

Neuroblast clones homozygous for $Drok^{2}$ (Winter et al., 2001) did not show apparent defects in cell proliferation, as the adult clones contain dorsal axon lobes contributed by later born neurons. Close examination of $Drok^{2}$ neuroblast clones revealed that 10 of 17 contain at least one axon that extends significantly further than the heterozygous neurons within the same MB (Figure 6F). Although this phenotype is subtle, it is not seen in 19 control clones (homozygous for FRT^{19A}; Figure 6I), the parental chromosome for the $Drok^{2}$ mutant, nor in many other genotypes we have studied (data not shown). Thus, we conclude that Drok is required to limit dorsal axon extension.

Evidence that p190 RhoGAP Is Negatively Regulated by Src and Integrin

Since rat p190 can rescue the *Drosophila p190* loss-of-function phenotypes, we tested if upstream regulators of mammalian p190 could interact with *Drosophila* p190 to regulate MB axon morphogenesis. The Src family of tyrosine kinases phosphorylate mammalian p190. We tested if the *Drosophila* Src homolog, Src64 (Dodson et al., 1998), regulates p190 activity. We found that heterozygosity for two *Src64* alleles (Dodson et al., 1998) significantly suppressed the *p190* RNAi phenotype (Figure 5F), with the strength of suppression correlating with the strength of the alleles used. This result is consistent with the notion that Src64 negatively regulates p190.

We also tested if integrin could regulate p190 activity in MB neurons, since mammalian studies suggest potential links between integrin and p190, as well as integrin and Src (Arthur et al., 2000; Burbelo et al., 1995; Klinghoffer et al., 1999; Nakahara et al., 1998; Sharma, 1998). Integrins function as heterodimers of one α and one β subunit. *Drosophila* has five genes for integrin α subunits (Hynes and Zhao, 2000), including one, *volado* (*vol*), which is preferentially expressed in MB neurons and, when mutated, results in a short-term memory defect (Grotewiel et al., 1998). We did not observe significant modification of the *p190* RNAi phenotype in flies heterozygous for a null mutation of *vol* (vol^{4}) (Rohrbough et al., 2000) (data not shown). This may be because *vol* is not dosage sensitive or because it functions redundantly with other integrin α subunits in regulating p190 activity. Only two genes encode integrin β subunits in the fly, βPS and $\beta\upsilon$; $\beta\upsilon$ may not associate with an α subunit (Hynes and Zhao, 2000). The *myospheroid* (*mys*) gene, encoding βPS, showed robust genetic interaction with p190. In flies with one wild-type copy of *mys*, the p190 RNAi phenotype was markedly suppressed for each of the three *mys* alleles tested (Figure 5G), suggesting that p190 is negatively regulated by integrin.

Finally, we examined MB neuroblast clones homozygous for mys^{1} using the MARCM system (Lee and Luo, 1999). We found 12 of 52 neuroblast clones exhibited obvious dorsal lobe axon overextension not seen in control flies (FRT^{19A}, Figure 6I). These include overextension of thin axon bundles near the tip of dorsal lobe similar to those seen in $Drok^{2}$ clones (compare Figure 6G with Figure 6F), or overextension of a large portion of the dorsal axons similar to those seen in MB neurons overexpressing p190 (compare Figure 6H to Figures 6A–6C). This experiment indicates that integrins are essential negative regulators of axon extension in MB neurons.

Discussion

Genome-wide Analysis of RhoGAP Functions

RhoGAPs outnumber the Rho GTPases they regulate by 3- to 5-fold in human, fly, and worm (I.H.G.S., 2001; Venter et al., 2001). Given that most RhoGAPs exhibit GAP activity on multiple Rho GTPases, at least in vitro (Lamarche and Hall, 1994), why are so many RhoGAPs in multicellular organisms, and how redundant are they? Our genome-wide analysis provides insight. Of the 17 *Drosophila* RhoGAPs analyzed by RNAi, six caused lethality when their expression was ubiquitously disrupted, a subset of which gave distinct morphological defects in particular tissues (Table 1). These observations support the notion that each RhoGAP has specific functions. The specificity could come from their unique temporal and spatial expression patterns, subcellular localizations, and/or ability to form specific signaling complexes. The latter two possibilities are supported by the fact that interfering with the functions of three different RhoGAPs gave qualitatively different phenotypes in MB neurons (Table 1). Future studies in different biological processes using these *UAS-dsRNA* lines and different GAL4 lines will provide more insight into the specific functions of individual RhoGAPs.

Analyzing specific functions of Rho GTPases has been complicated by their pleiotropic functions. If individual RhoGAPs control Rho GTPase signaling in discrete processes, then disrupting the function of specific RhoGAPs may help dissect the numerous roles of Rho GTPase. For instance, previous studies using *RhoA* null mutants failed to reveal its function in MB axon morphogenesis due to a severe cell proliferation defect (Lee et al., 2000). Expression of a strong RhoA gain-of-function mutant leads to lethality and profound defects in MB axon development (Figure 4B). However, perturbing the function of one particular regulator, p190, revealed one specific function of RhoA.

A "Retraction Signaling Pathway" Involving RhoA-Drok-MRLC that Is Repressed by p190 RhoGAP

By inhibiting the activity of p190, we uncovered a "retraction signaling pathway" from RhoA and Drok to the regulation of myosin II activity via modulation of myosin regulatory light chain (MRLC) phosphorylation (Figure 7A). This pathway is largely dormant, such that loss-of-function *RhoA* did not cause detectable phenotypes (Lee et al., 2000) and loss-of-function *Drok* gave rise to a subtle axon overextension phenotype (Figure 6F). Yet, this pathway is extant, as activation of RhoA or Drok or

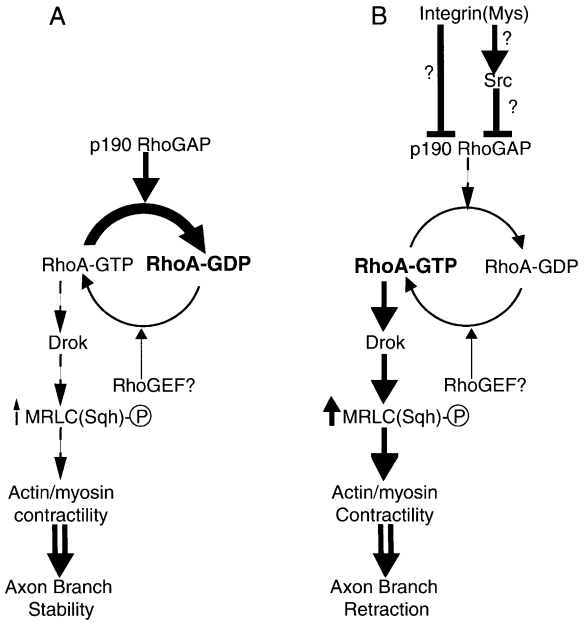

Figure 7. A Model for RhoGAP Repression of a RhoA-Mediated Retraction Signaling Pathway

(A) A retraction pathway from RhoA to Drok to regulation of myosin regulatory light chain (MRLC) phosphorylation, although extant, is largely repressed by p190, thus allowing for the stability of axon branches.

(B) Inactivation of p190 causes activation of RhoA, Drok, and MRLC phosphorylation, leading to retraction of axon branches. We provide genetic evidence that integrin and Src serve as negative regulators of p190. The question marks reflect that the biochemical links are inferred from studies of mammalian homologs. In principle, an unidentified RhoGEF(s) activates RhoA to allow the RhoA GTPase cycle.

inactivation of p190 by RNAi resulted in robust axon branch retraction phenotypes. Further, the *p190* phenotype was modulated by changing the endogenous level or the phosphorylation state of MRLC, a critical output of Drok signaling in vivo (Winter et al., 2001). Thus, it appears that all components in the pathway are present in MB neurons and participate in mediating the axon retraction when p190 function is inhibited.

Our previous studies of mammalian hippocampal pyramidal neurons provide an analogous example of an extant signaling pathway maintained in a dormant state. While activation of RhoA or ROCK (mammalian homolog of Drok) in maturing pyramidal neurons resulted in dendritic branch retraction and elimination, inhibition of RhoA or ROCK activity did not lead to detectable phenotypes. However, the effect of RhoA activation could be completely suppressed by ROCK inhibition, indicating that endogenous ROCK is used to mediate the action of the activated RhoA (Nakayama et al., 2000). In light of the current study, it is possible that under physiological conditions, the RhoA pathway in pyramidal neurons is actively repressed by mammalian p190 RhoGAP to stabilize dendritic branches. Regulation of a repressor of RhoA signaling is likely to be a widely used mechanism to regulate stability of neuronal processes.

Selective Stability of Axon Branches

Why are the dorsal and medial axon branches of the same neurons differentially sensitive to the retraction pathway? It is possible that some signaling components are differentially distributed between these two branches. Since p190 inhibition and activation of RhoA or Drok all selectively affect dorsal branches, differential distribution of signaling components downstream of Drok, such as MRLC, MyoII, or MRLC phosphatase could account for this difference.

An alternative explanation is that the relative levels of RhoA and Rac activity may differ in dorsal and medial branches. This hypothesis is based on the general scheme in which Rac plays a positive role in process outgrowth, whereas RhoA acts negatively (Luo, 2000; Dickson, 2001). If Rac activity were higher in the medial branches, then medial branches would be expected to be more resistant to retraction upon activation of RhoA. Two lines of evidence support this Rac/RhoA antagonism hypothesis. First, the MB γ neurons, which only send medial branches after metamorphosis, appear to resist the retraction under all experimental conditions tested, even though *GAL4-OK107*-driven dsRNAs is highly expressed in γ neurons. Of the three types of MB neurons, γ neurons are the only ones that express Trio, a RacGEF (Newsome et al., 2000), during the critical period for axon retraction of MB development (Figure 3I) (Awasaki et al., 2000). Second, RNAi-induced *DRacGAP* loss-of-function resulted in overextension of the axons at the tip of the dorsal lobe (Table 1), a phenotype similar to overexpression of p190 (Figure 6A–6C) but opposite to the axon branch retraction resulting from *p190* RNAi. These opposite loss-of-function phenotypes of *p190* and *DRacGAP* suggest that the relative balance of RhoA and Rac activation regulates axon branch stability.

Recently, Shamah et al. (2001) showed that the axon guidance molecule, Ephrin, conveys its repulsive signal within the growth cone by elevating the GEF activity of Ephexin toward RhoA, while reducing its activity toward Rac. Hu et al. (2001) demonstrated that another repulsive axon guidance receptor, Plexin B, works by inhibiting Rac and activating RhoA signaling. Together, these studies suggest mechanistic similarities between repulsive axon guidance and axon retraction.

Regulation of p190 RhoGAP Activity by Src and Integrin

Identifying upstream signals that regulate p190 activity may provide further insight into the physiological signals that derepress the RhoA-mediated retraction pathway. Two upstream regulators of mammalian p190 are Src family protein tyrosine kinases and integrin family extracellular matrix adhesion molecules. Whether p190 is positively or negatively regulated by integrin and Src remains controversial; indeed, a biphasic regulation of RhoA by integrin through Src and p190 has been proposed (Arthur et al., 2000; Ren et al., 1999). Given the functional similarities of *Drosophila* and mammalian p190 by both genetic complementation and overexpression assays, it seems likely that *Drosophila* p190 and Src or integrin work in the same pathway, as mammalian biochemical studies have suggested. If that is the case, then our genetic results (Figures 5F and 5G) support the notion that p190 is negatively regulated by both Src and integrin (Figure 7B). However, genetic interaction studies alone cannot rule out the possibility that integrin and Src positively regulate RhoA through an as-of-yet

unidentified mechanism independent of p190, or that signals are integrated downstream of RhoA.

Negative regulation of p190 by Src could result from the fact that there is partial conservation of Src phosphorylation sites between mammalian and *Drosophila* p190. Src phosphorylation of mammalian p190 at Y1105 appears to be responsible for p190 activation (Haskell et al., 2001). Src phosphorylation of tyrosines in the GTP binding domain of mammalian p190 results in the loss of its GTP binding activity (Roof et al., 2000), and hence a disruption of the RhoGAP activity in vivo (Tatsis et al., 1998; Brouns et al., 2000). Thus, phosphorylation of the GTP binding domain is predicted to negatively regulate p190. Since only Src-phosphorylation sites in the GTP binding domain of mammalian p190 are conserved in *Drosophila* (Figure 2B), Src phosphorylation of D-p190 likely negatively regulates its activity.

The negative regulation of p190, and hence positive regulation of RhoA, by integrins is further supported by mutant phenotypes in *Drosophila* neurons. Previously studies have shown that loss of *Drosophila* βPS integrin (*myospheroid)* or αPS3 (*volado*) causes excessive synaptic sprouting and morphological growth in the neuromuscular junction (Beumer et al., 1999; Rohrbough et al., 2000). We show that homozygous loss of βPS integrin in MB neurons results in axon overextension phenotypes strikingly similar to those caused by p190 overexpression and Drok inactivation, but are opposite to phenotypes caused by loss-of-function p190 or activation of RhoA and Drok. Taken together with the genetic interaction data (Figure 5G), these experiments support a model that integrin derepresses the retraction pathway by negatively regulating p190 RhoGAP (Figure 7B). Future biochemical studies are required to elucidate the mechanisms by which integrin and Src regulate p190 in *Drosophila*.

Structural Plasticity for Learning and Memory?

Our findings suggest a link between a molecular pathway that regulates axon branch stability to Src and integrin, both implicated in neural plasticity and memory formation. Mice lacking the Src family kinase Fyn have impaired long-term potentiation (LTP) and spatial learning (Grant et al., 1992), phenotypes that are separable from developmental defects (Kojima et al., 1997). Src has also been implicated in regulating LTP induction (Lu et al., 1998). Likewise, inhibiting integrin function results in LTP defects in rats (Stäubli et al., 1998), synaptic plasticity in the *Drosophila* neuromuscular junction (Beumer et al., 1999; Rohrbough et al., 2000), and MB-mediated short-term memory in *Drosophila* (Grotewiel et al., 1998). The molecular mechanisms by which Src and integrin regulate plasticity are largely unknown. Mammalian p190 is highly expressed in the adult brain (Brouns et al., 2001). We show that in *p190* dsRNA-expressing MB neurons, axon branch retraction continues over the course of adult life (Figure 3J), suggesting that p190 RhoGAP continues to function in regulating axon stability in this olfactory learning and memory center in adult brain. Taken together, our study raises the intriguing possibility that regulation of p190 RhoGAP activity, and hence the structural changes of subcellular compartments of neurons, may contribute to the morphological plasticity essential for learning and memory.

Experimental Procedures

Bioinformatic Analysis

To identify all *Drosophila* RhoGAP genes, we searched the *Drosophila* genome with a 150 amino acid sequence covering the RhoGAP domain of D-Graf with the program "tblastn". We found 20 RhoGAP genes, 19 identified by GadFly (Genome Annotation Database of *Drosophila*), plus one located in 100F1-2 not previously reported. Of the three RhoGAP genes that showed MB phenotypes, sequence analysis of the corresponding EST clones (Research Genetics) revealed that two are composed of two adjacent predicted genes (Table 1). Due to these gene prediction errors, we named the RhoGAP genes according to their cytological locations.

Molecular Biology

UAS-dsRNA RhoGAP

Modified primers containing unique restriction sites (X and Y) at their 5′ ends were used to amplify cDNAs by RT-PCR using total RNA from 0–17 hr embryos. Each PCR product, from 300 to 700 base pairs in length, was subcloned into pAmp1 (GIBCO). X/Y double digested products were used in a trimolecular ligation with alkaline phosphatase-treated pUAST vector linearized with X. X: EcoRI or XbaI, Y: NotI. Ligation products were transformed into the *E. coli* SURE strain (Stratagene).

UAS-dsRNA RhoA

A EcoRI/XbaI cDNA fragment containing the entire *RhoA* ORF was subcloned in a trimolecular ligation into alkaline phosphatase-treated EcoRI digested pUAST vector.

UAS-myc-Dp190*

Two copies of the Myc tags were fused to the 5′ of p190 ORF from the LD39422 EST clone (Research Genetics) and subcloned into pUAST. After generating transgenic flies, we found by immunoblot that the Myc tagged protein is smaller than expected. We sequenced the original EST clone and found a single base deletion, leading to a truncated protein at the amino acid 1028 so that the Myc-Dp190* fusion protein lacks the RhoGAP domain. The suppression of the *p190* RNAi-induced phenotype by *UAS-myc-Dp190** (Figure 2J) is most likely due to titration of the dsRNA by the *myc-Dp190** mRNA.

UAS-rat-p190A

The rat cDNA of p190A was subcloned into alkaline phosphatase-treated EcoRI digested pUAST vector.

UAS-myc-Dp190 and UAS-myc-Dp190R1389L

Oligo-directed mutagenesis was used to add back the single base deletion in the original *Dp190* cDNA and to generate the R1389L point mutation. The DNA fragments were sequence verified and subcloned into pUAST for production of transgenic flies.

Fly Strains

MB *GAL4-OK107* (Lee et al., 1999), ubiquitous *tubP-GAL4* (Lee and Luo, 1999), pan-imaginal discs *T80-GAL4* (Wilder and Perrimon, 1995), and *eyeless-GAL4* (Bonini et al., 1997) drivers were used to express dsRNA in different tissues. Fly stocks were obtained from the Bloomington Stock Center, except mys^{XG43} (from K. Broadie), *Src64* alleles (from M. Simon), and *UAS-RhoA V14* and effector mutant flies (from M. Mlodzik). Other stocks have previously been described.

MARCM Analysis

Neuroblast clones homozygous for $Drok^{2}$ FRT^{19A}, mys^{1} FRT^{19A}, or FRT^{19A} were generated in newly hatched larvae as described (Lee et al., 1999). Axons were scored as overextended if they extended beyond those of the heterozygous neurons (visualized with anti-FasII antibody) within the same MB as the clone.

Immunohistochemistry and Microscopy

Brains were dissected, fixed, stained, and imaged as described (Lee et al., 2000; Liu et al., 2000). Mouse anti-human p190 was obtained from Transduction Laboratories and used at 1:100.

Quantification of Dorsal Lobe Retraction

We categorized the *p190* dorsal lobe retraction phenotypes (scored under a Nikon E600 fluorescence microscope) into four classes based on the dorsal lobe length: normal, weak (more than half the normal length), medium (less than half the normal length) and strong

(no visible dorsal lobe). The frequency of each category was expressed as a percentage of total number of MBs analyzed. Even though the RNAi-induced phenotype is rather stable for the same transgenic insertion line, we did observe variations from experiment to experiment, perhaps due to slight temperature variations. Therefore, for each experiment, we used an external control set up on the same day and raised with the experimental flies until brain dissection.

Acknowledgments

Special thanks to A. O'Reilly and M. Simon for their advice and mutants. We thank M. Mlodzik, K. Brodie, and the Bloomington Stock Center for mutant stocks, J. Settleman for a rat p190 RhoGAP cDNA clone, C. Goodman for anti-FasII antibodies, J. Reuter and T. Nardine for technical assistance, M. Feldman for advice on statistical analysis, and H. Hu, M. Scott, M. Simon, and members of the Luo Lab, especially J. Reuter, for comments on the manuscript. We thank H. Hu and C. Goodman for communicating results before publication. P.B. was supported by postdoctoral fellowships from EMBO and HFSP. This work was supported by an NIH grant (R01-NS 36632) to L.L.

Received May 23, 2001; revised September 4, 2001.

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Accession Numbers

Drosophila p190 RhoGAP cDNA sequence has been deposited in GenBank under the accession number AF387518.