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The SH2/SH3 Adaptor Protein Dock Interacts with the Ste20-like Kinase Misshapen in Controlling Growth Cone Motility

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Summary

Recent studies suggest that the SH2/SH3 adaptor Dock/Nck transduces tyrosine phosphorylation signals to the actin cytoskeleton in regulating growth cone motility. The signaling cascade linking the action of Dock/Nck to the reorganization of cytoskeleton is poorly understood. We now demonstrate that Dock interacts with the Ste20-like kinase Misshapen (Msn) in the *Drosophila* photoreceptor (R cell) growth cones. Loss of *msn* causes a failure of growth cones to stop at the target, a phenotype similar to loss of *dock*, whereas overexpression of *msn* induces pretarget growth cone termination. Physical and genetic interactions between Msn and Dock indicate a role for Msn in the Dock signaling pathway. We propose that Msn functions as a key controller of growth cone cytoskeleton in response to Dock-mediated signals.

Introduction

Neuronal growth cone functions as a cellular antenna to receive guidance and targeting signals from the surrounding environment and to subsequently convert the signals into rearrangements of cytoskeletal structures to induce directed movement (guidance) or a cessation of movement (e.g., upon reaching the final target). The growth cone integrates the guidance information through the interactions between its cell surface receptors and extracellular attractive or repulsive factors (reviewed by Tessier-Lavigne and Goodman, 1996). Following an external stimulation, the growth cone is able to reorganize its actin-based cytoskeleton within seconds (reviewed by Tanaka and Sabry, 1995), underscoring the importance of intracellular signaling machinery in the control of growth cone motility.

There is increasing evidence for the involvement of protein tyrosine phosphorylation in growth cone signaling. Immunohistological studies demonstrate the localization of phosphotyrosine in filopodia at the leading edge of the growth cone (Wu and Goldberg, 1993). Pharmacological studies show that inhibition of protein tyrosine kinase and phosphatase can alter growth cone behavior (e.g., Bixby and Jhabvala, 1992; McFarlane et al., 1995). Furthermore, a number of tyrosine kinases and tyrosine phosphatases have been implicated in controlling growth cone guidance and targeting in vivo in both vertebrates and invertebrates (reviewed by Desai et al., 1997; Van Vactor, 1998). The signaling cascade linking protein tyrosine phosphorylation to the change in growth cone motility, however, remains largely undefined.

Recent studies suggest a role for the Dock/Nck adaptor protein in coupling downstream effectors to tyrosine-phosphorylated proteins to regulate growth cone motility. Dock/Nck protein is composed exclusively of three N-terminal SH3 domains and a single C-terminal SH2 domain (Lehmann et al., 1990; Garrity et al., 1996). It belongs to a growing family of SH2/SH3 adaptor proteins that are involved in signal transduction by mediating specific protein–protein interactions (reviewed by Downward, 1994; Schlessinger, 1994). Genetic analysis in *Drosophila* implicates a specific role for Dock in growth cone guidance and targeting (Garrity et al., 1996; Desai et al., 1999). Biochemical studies of its vertebrate homolog Nck demonstrate that Nck binds to phosphotyrosine on activated receptor tyrosine kinases (e.g., Li et al., 1992; Kochhar and Iyer, 1996; Stein et al., 1998) via its SH2 domain; Nck also binds to consensus proline-rich (PXXP) motifs on effector molecules via its SH3 domains (e.g., Bagrodia et al., 1995; Rivero-Lezcano et al., 1995; Bokoch et al., 1996; Galisteo et al., 1996; Quilliam et al., 1996; Su et al., 1997). Thus, Dock/Nck may transmit guidance and targeting signals into the changes in the activity of downstream effectors, which in turn modulate the dynamic rearrangements of actin-based cytoskeleton in controlling growth cone motility.

Nck-interacting kinase (NIK) is an attractive candidate that links Nck/Dock-mediated signals to growth cone cytoskeleton. NIK binds to Nck in cultured cells (Su et al., 1997). Although the biological function of NIK is still unclear, genetic analysis of its homolog Misshapen (Msn) in *Drosophila* and Mig-15 in *Caenorhabditis elegans* implicates a role for Msn/Mig-15 in regulating cell shape changes during development (Treisman et al. 1997; Su et al., 1998). NIK/Msn/Mig-15 belongs to the Ste20-like serine/threonine kinase family. Based on primary structure, the Ste20-like kinases can be divided into the Pak and GCK subfamilies (reviewed by Sells and Chernoff, 1997). Compared with GCK members, Paks are more closely related to the yeast Ste20 kinase, which consists of a C-terminal kinase domain and an N-terminal regulatory region containing binding sites for Nck (e.g., Bokoch et al., 1996; Galisteo et al., 1996), the small GTPases (e.g., Manser et al., 1994; Quilliam et al., 1996), and the guanine nucleotide exchange factor Pix (Manser et al., 1998). Like other GCK subfamily members, NIK is composed of an N-terminal kinase domain and a C-terminal regulatory region containing binding sites for Nck but not for small GTPases (Su et al., 1997). To gain insight into the mechanism underlying the regulation of growth cone cytoskeleton by Dock/Nck-mediated signals, we investigate the role of Msn in the control of photoreceptor (R cell) growth cone motility in the *Drosophila* visual system.

The *Drosophila* adult visual system consists of the

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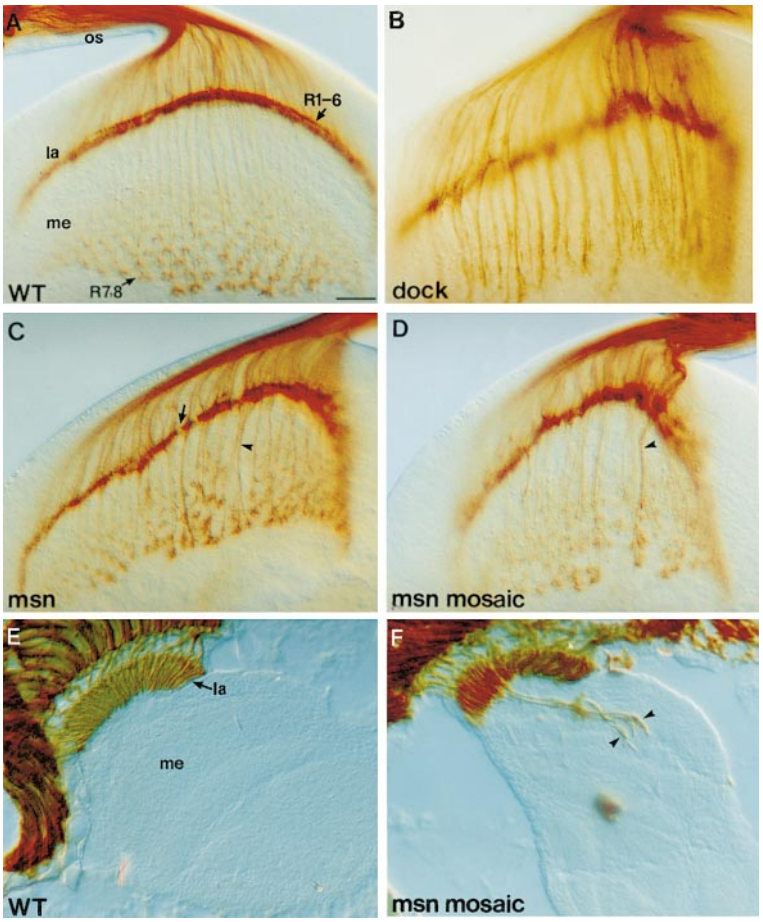

Figure 1. *msn* Is Required for R1–R6 Growth Cone Targeting

(A–D) Eye–brain complexes of third-instar larvae were stained with mAb 24B10.

(A) The normal pattern of R cell projections in wild type. R1–R6 axons terminate in the lamina (la). Their expanded growth cones form a smooth, continuously staining line at the lamina termination site. R7 and R8 axons pass through the lamina into the deeper medulla (me) layer and elaborate a precise array of expanded growth cones.

(B) Homozygous $dock^{P1}$ mutant. Many R1–R6 axons migrated ectopically into the medulla (Garrity et al., 1996). Abnormal, large bundles were seen in both the lamina and medulla. The *dock* phenotype is completely penetrant.

(C) Homozygous $msn^{l(3)03349}$ mutant. Gaps (arrow) were observed at the lamina R1–R6 termination site. Abnormal, large bundles (arrowhead) were seen in the medulla.

(D) *msn* mosaic larvae exhibited defects in R cell fasciculation and targeting. Eye-specific mitotic recombination using the FLIP-FRT system (Xu and Rubin, 1993) was induced by Flipase under control of the Eyeless promoter (*EyFLP*). Note the uneven lamina and the appearance of abnormal, large bundles in the medulla (arrowhead).

(E and F) Cryostat sections of wild-type (E) and *msn* mosaic (F) heads carrying an adult R1-R6-specific marker, Rh1-*lacZ*, were stained with anti-β-galactosidase antibody. In wild type (E), *lacZ* staining was only seen in the lamina since all R1–R6 axons terminate in this layer, whereas in *msn* mosaic head (F), R1–R6 axons (arrowheads) passed through the lamina into the medulla layer.

Scale bar, 20 μm.

compound eye and the optic lobe. The compound eye is composed of ~800 ommatidia. Each contains eight different neurons, called R1–R8. The formation of an adult R cell projection pattern begins at the third instar larval stage. R cell axons converge at the optic stalk and project retinotopically into the optic ganglia. R1–R6 growth cones terminate in the first optic ganglia layer, the lamina, whereas R7 and R8 axons extend further into the second optic ganglia layer, the medulla. Loss of *dock* function affects R cell pathfinding, target recognition, and topographic map but does not affect R cell growth cone extension (Garrity et al., 1996).

In this report, we provide molecular and genetic evidence for the in vivo interaction of Dock and Msn in R cell growth cones. We demonstrate that like Dock, Msn is localized to R cell axons and growth cones and is required for the proper targeting of R1–R6 growth cones. Msn associates with Dock in intact flies and interacts with Dock genetically. We propose that Msn functions downstream of Dock in controlling R cell growth cone motility.

Results

Loss of *msn* Function Disrupts R cell Growth Cone Termination Pattern

To investigate the potential role of *msn* in R cell growth cones, we assessed the effect of *msn* mutations on R cell projections. As strong loss-of-function alleles of *msn* are embryonic lethal, we examined R cell projections in third-instar larvae homozygous for a hypomorphic allele of *msn* [$msn^{l(3)03349}$]. While in *msn* mutants R cell growth cones were able to extend into the developing optic lobe, their innervation patterns within the lamina and the medulla were altered (compare Figure 1C with Figure 1A). The *msn* phenotype exhibited a certain similarity to that of *dock* loss-of-function mutants.

In *dock* mutants (Figure 1B), many R1–R6 growth cones passed over their normal target (i.e., lamina) and extended further into the medulla layer, generating gaps in the lamina R1–R6 termination site (a smooth continuous line of immunoreactivity in wild type; Figure 1A). In addition, *dock* affected R cell fasciculation and growth cone morphology. Similarly, we found that loss of *msn* function caused defects in R cell targeting and fasciculation (Figure 1C); gaps were observed in the R1–R6 termination site, coincident with projections of abnormal, large bundles into the medulla. R cell growth cone morphology was also altered in *msn* mutants. Unlike in *dock*, however, in *msn*, R cell growth cones were able to expand upon reaching the target. While all *msn* mutants examined ($n > 30$) exhibited defects in R cell innervation pattern, the severity of the phenotype varied from individual to individual.

msn Is Required in the Eye for the Termination of R1–R6 Growth Cones

To determine whether *msn* is required in the developing eye for R cell projections, we carried out genetic mosaic

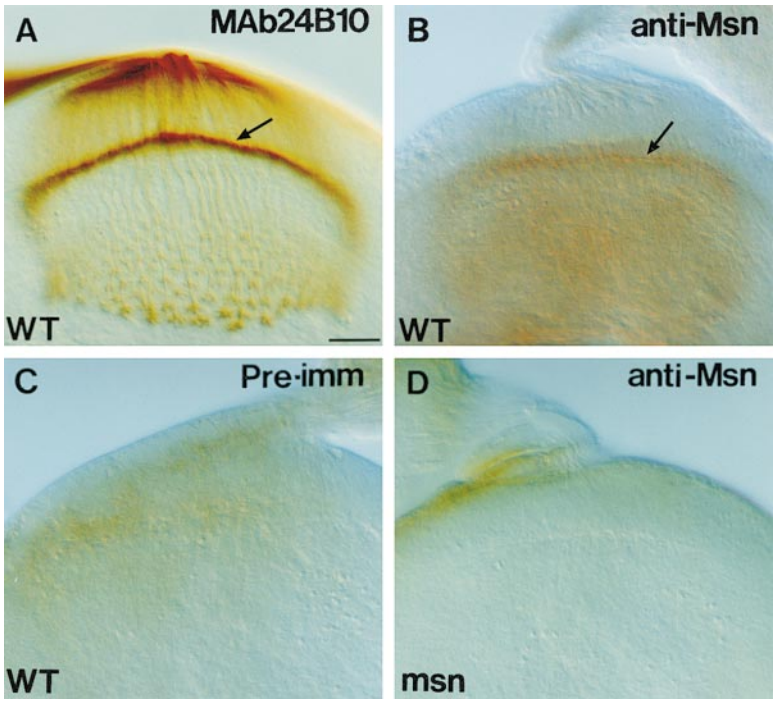

Figure 2. Msn Is Localized to R Cell Axons and Growth Cones

(A–C) Wild-type third-instar eye–brain complexes were stained with mAb 24B10 (A), anti-Msn serum (B), or preimmune serum (C). In (A), R cell axons were visualized with mAb 24B10. (B) shows an optic lobe stained with anti-Msn serum. Msn staining was detected in R cell axons from the eye disc, through the optic stalk, to the lamina. R1–R6 terminals at the lamina termination site were strongly stained (arrow). Msn staining was also seen in the medulla neuropil. Since the medulla neuropil was uniformly stained, we could not tell whether Msn is localized to R7 and R8 growth cones. No such staining pattern was seen in wild type stained with preimmune serum (C).

(D) In homozygous $msn^{l(3)03349}$ mutants, no Msn staining was seen in R cell axons within the optic stalk, or in the lamina or medulla neuropils.

Scale bar, 20 μm.

analysis. Mutant eye clones homozygous for msn^{102}, a strong loss of function allele, were generated in an otherwise wild-type fly by eye-specific mitotic recombination (see Experimental Procedures). R cell projections in mosaic larvae were visualized with mAb 24B10. Similar defects in R cell innervation pattern were observed (Figure 1D). The percentage (~46%, n = 15) of larvae showing obvious defects was close to the percentage (~50%) of individuals with relatively large mutant eye patches (identified as white eye tissue in adult).

To specifically assess the role of Msn in R1–R6 growth cones, we generated *msn* mutant eye patches in *msn* heterozygous flies carrying the adult R1-R6-specific marker Rh1-*lacZ* (Mismer and Rubin, 1987). In wild-type adult flies, all R1–R6 axons terminated in the lamina, as assessed by *lacZ* staining (Figure 1E). In contrast, in all mosaic adults examined (n = 26), R1–R6 axons from *msn* mutant patches passed over the lamina and terminated abnormally in the medulla (Figure 1F). These results indicate that *msn*, like *dock*, is genetically required in the eye for R1–R6 growth cone targeting. Similarly, we detected no obvious defects in the differentiation of the R1–R6 targeting region (i.e., lamina) in *msn* mutants, as assessed by anti-Dachshund staining (data not shown). Moreover, eye-specific expression of a *msn* transgene rescued R cell projection defects in homozygous *msn* mutants (data not shown).

The effect of strong *msn* loss-of-function mutations (including the msn^{102} allele) on eye development has been critically assessed by Treisman et al. (1997). By sectioning adult mosaic eyes, they demonstrated that *msn* mutations affect the morphology of R cell bodies but that other aspects of R cell differentiation remain generally normal. Our results from analysis of msn^{102} mutant eye patches (data not shown) were consistent with their report. These observations indicate that *msn* also plays a role in coordinating cytoskeletal changes in R cell bodies.

Msn Localizes to R cell Axons and Growth Cones

It has been shown that Dock protein is enriched in R cell axons and growth cones (Garrity et al., 1996). If Msn has a functional relationship with Dock in R cell growth cones, we expect that Msn protein is expressed in R cells and is localized to growth cones. The expression pattern of Msn in third-instar larval eye–brain complexes was determined with a rabbit anti-Msn serum. Msn staining was seen in R cell axons along the path of projections (from the developing eye disc to the lamina) in wild-type whole-mount preparations (compare Figure 2B with Figure 2A; data not shown). The lamina plexus was strongly stained as a continuous layer of immunoreactivity, a pattern that is indistinguishable from that stained with anti-Dock antibody (Garrity et al., 1996). Since at this stage the vast majority of axonal processes in the lamina neuropil are expanded R1–R6 growth cones, the uniform staining of Msn and Dock in the lamina neuropil suggests strongly that Msn and Dock colocalize to R1–R6 growth cones. No such staining was seen in wild-type larvae stained with preimmune serum (Figure 2C) or in *msn* mutants stained with anti-Msn serum (Figure 2D), confirming the specificity of the anti-Msn serum. Like Dock, Msn was also detected in the medulla neuropil, consisting of R7 and R8 axons as well as non-R cell axons. In addition to strong staining in R cell axons, weak staining in R cell bodies was observed (data not shown). The localization of Msn in R1–R6 growth cones is consistent with a role for Msn in coordinating the response to target-derived signals.

Overexpression of *msn* Induces Pretarget Termination of R cell Growth Cones

That loss of *msn* caused the failure of R1–R6 growth cones to stop at their target lamina suggests a role for *msn* in the shutdown of growth cone motility when axons

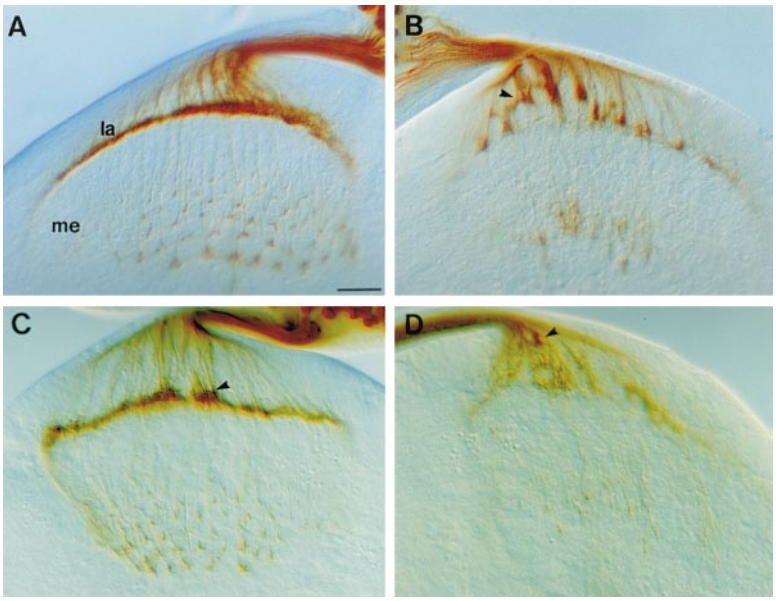

Figure 3. Overexpression of *msn* Induces Pretarget Growth Cone Termination

R cell projection pattern in third-instar larvae was visualized with mAb 24B10.
(A) R cell projections in wild type.
(B) The endogenous *msn* gene was overexpressed under control of the GMR promoter. Overexpression of Msn was confirmed by immunohistochemical analysis (data not shown). Many R1–R6 growth cones (arrowhead) terminated before they reached the normal lamina termination site. The medulla terminal field was also disorganized. The phenotype was observed in all larvae examined ($n > 40$). Genotype: GMR-*GAL4*/+;*EP(3)549*/+.
(C and D) R cell projections in third-instar larvae overexpressing a *msn* transgene. In (C), the larvae carried two copies of the GMR-*GAL4* transgene and one copy of the *UAS-msn* transgene. Some R1–R6 growth cones (arrowhead) terminated slightly earlier than others, causing an uneven termination pattern within the lamina (6 of 16 hemispheres examined). In (D), the larvae carried two copies of the GMR-*GAL4* transgene and two copies of the *UAS-msn* transgene. The increase in the dosage of *msn* transgene dramatically enhanced the phenotype. The phenotype became completely penetrant ($n = 10$). The severity of the phenotype was also enhanced. (D) is an example of the most severe phenotype (6 of 20 hemispheres examined). Note the termination of R cell growth cones (arrowhead) immediately after axons migrate from the optic stalk.
Scale bar, 20 μm.

reach their target. Target-derived stop signals may activate Msn, which in turn coordinates cytoskeletal reorganization in decelerating growth cone motility. If this model is correct, one may predict that ectopic activation of Msn should induce abnormal termination of R cell growth cones. To test this, we overexpressed the endogenous *msn* gene in differentiating R cells using the eye-specific promoter GMR (Brand and Perrimon, 1993; Hay et al., 1994; Rorth et al., 1998). Overexpression of Msn in R cell axons was confirmed by immunohistochemical staining (data not shown). Compared with wild-type (Figure 3A), overexpression of *msn* caused a large number of R1–R6 growth cones to stop before they reached their normal target lamina (Figure 3B). Overexpression of *msn* also caused defects in the medulla terminal field. In contrast, neither the shape of R cells nor their localization on the developing eye disc was affected (data not shown). Overexpression of *msn* from a transgene containing *msn* cDNA under control of the GMR promoter caused a similar early stop phenotype (Figures 3C and 3D). The severity of the phenotype was dose dependent, as the increase in the copies of *msn* transgene enhanced the phenotype.

One possible explanation for the gain-of-function phenotype is that overexpression of Msn activates the Msn pathway prematurely, which sends a terminating signal to growth cone cytoskeleton to induce pretarget termination. Alternatively, the hyperactivation of the Msn pathway may cause some general defects in the reorganization of growth cone cytoskeleton, leading to the arrest of growth cones before they reach the target. We favor the former interpretation—that Msn plays an instructive role in terminating R1–R6 growth cones due to the following reasons. First, in *msn* gain-of-function mutants, the early stop growth cones expand, similar to growth cones that terminate correctly in the lamina. In wild type, R cell growth cones expand only when they terminate in the target. Second, the fact that the early stop R cell growth cones are still able to expand in the lamina argues against a general defect in the reorganization of growth cone cytoskeleton.

Msn Associates with Dock In Vitro and In Vivo

To examine whether Msn interacts with Dock physically, we generated a glutathione S-transferase (GST) fusion protein containing a fragment of Msn that encompasses multiple consensus PXXP motifs for SH3 domain–binding (Feng et al., 1994; Lim et al., 1994) (Figure 4A). The immobilized GST–Msn fusion protein precipitated Dock from adult fly lysates in a dose-dependent manner (Figure 4B), indicating the direct association of Msn with Dock. To test whether Msn associates with Dock in intact flies, we carried out coimmunoprecipitation experiments. Fly lysates were prepared from third-instar larval eye–brain complexes or adult heads. Anti-Dock antibody was used to precipitate Dock and its interacting proteins from the lysates. Msn protein was detected in anti-Dock precipitates but not in control serum precipitates (Figure 4C), indicating an in vivo association of Msn with Dock in flies at both developmental and adult stages.

To define the domains of Dock and Msn that mediate the binding, we employed the yeast two-hybrid system to analyze their interactions using either different portions of Msn or mutant Docks in which the ligand-binding capacity of individual SH2 and SH3 domains was abolished (Rao and Zipursky, 1998). Consistent with binding experiments using GST–Msn fusion protein, the PXXP fragment of Msn bound to Dock in yeast (Table 1). Neither the N-terminal kinase domain nor the C-terminal

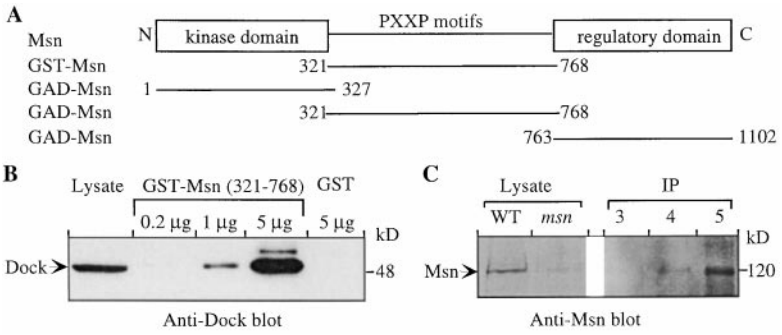

Figure 4. Physical Association of Dock with Msn

(A) Fragments of Msn polypeptide used in binding experiments are represented schematically. Msn is composed of an N-terminal serine/threonine kinase domain, a middle region containing multiple consensus PXXP motifs for SH3-binding, and a C-terminal regulatory domain. The kinase domain and regulatory domain are highly conserved. Numbers indicate amino acids present in the fragments. Abbreviations: GST, glutathione S-transferase; and GAD, *GAL4* activation domain.

(B) Dock protein was precipitated from adult fly lysates with GST–Msn (321–768) fusion protein. Precipitates were blotted with anti-Dock antibody. Lanes (from left to right): lane 1, adult fly lysate alone; lanes 2–4, precipites produced, using different amounts of GST–Msn (0.2 μg, 1 μg, or 5 μg); and lane 5, precipites produced, using 5 μg of GST.

(C) Coimmunoprecipitation of Msn with Dock. Msn protein was detected in lysate from 20 wild-type larval eye–brain complexes (lane 1). The amount of Msn protein was dramatically reduced in *msn*$^{l(3)03349}$ mutants (lane 2). This reduction was not due to the difference in sample loading, as similar amounts of Dock protein were detected in lanes 1 and 2 (data not shown). Msn coimmunoprecipitated with anti-Dock antibody in lysate from 20 larval eye–brain complexes (lane 4) or 20 adult heads (lane 5) but not with control antiserum (lane 3).

regulatory domain displayed Dock-binding activity. The binding of Dock to Msn is mediated mainly by its SH3-1 and SH3-2 domains. Mutations in either SH3-1 or SH3-2 inhibited the association of Dock with Msn, indicating that a stable association requires the simultaneous binding of SH3-1 and SH3-2 to the PXXP sequence in the polypeptide of Msn, whereas SH3-3 is less necessary for the binding.

The *msn* Loss-of-Function Phenotype Is Sensitive to the Amount of *dock*

To determine the biological relevance of the physical association of Msn with Dock, we tested whether *dock* and *msn* interact genetically. The dosage of *dock* gene was reduced in larvae homozygous for the hypomorphic *msn* allele [*msn*$^{l(3)03349}$]. Interestingly, we found that the reduction by half of *dock* gene dosage dramatically enhanced the *msn* phenotype (compare Figure 5B with Figures 1C and 5A). The R1–R6 termination site at the lamina became more disorganized. R cell growth cones were much less expanded and appeared more similar to those of *dock* mutants (Figures 1B and 5D). This enhanced phenotype was completely penetrant (n = 20). We estimated that in each hemisphere, ~70%–100% of growth cones were less expanded compared with those in controls. In *dock* and *msn* double mutants (Figure 5C), R cell projections were indistinguishable from those in *dock* mutants (Figure 5D). These results, together with

Table 1. Interaction of Msn and Dock in Yeast

Lex A fusion	GAD fusion	β-galactosidase activity
LexA	Msn (321-768)	-
Dock (SH3 1 2 3, SH2)	GAD	-
Dock	Msn (1-327)	-
Dock	Msn (321-768)	++
Dock	Msn (763-1102)	-
W48K	Msn (321-768)	-
W151K	Msn (321-768)	-
W225K	Msn (321-768)	+
R336Q	Msn (321-768)	++

Docks and fragments of Msn were fused to the *LexA* DNA–binding domain (LexA) or the transcription activation domain of *GAL4* (GAD), respectively. The relative binding activity was indicated by the relative level of β-galactosidase activity. ++, strong interaction (appearance of blue color within 20 min); +, intermediate interaction (blue color appeared within 20 min but was less intense than ++); and −, no detectable interaction (no trace of blue color within 2 hr).

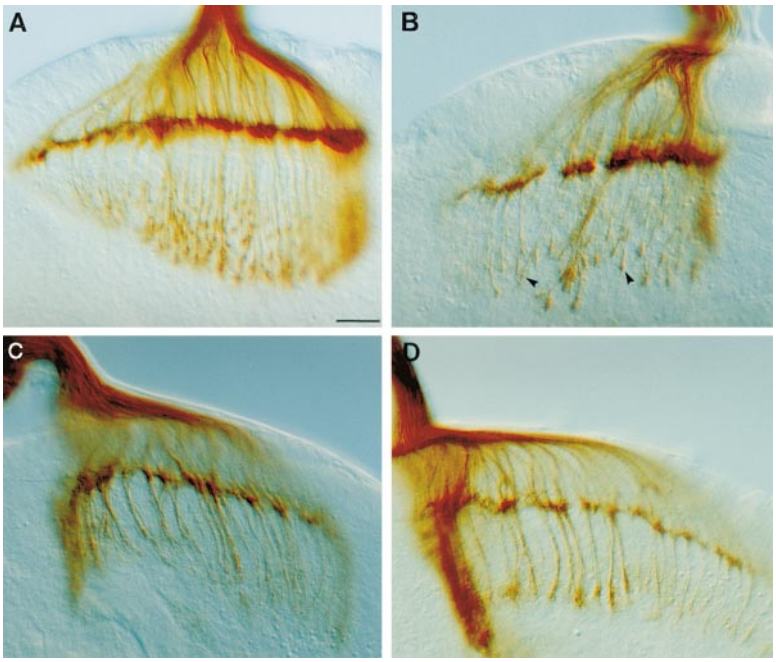

Figure 5. The *msn* Hypomorphic Phenotype Is Enhanced by Reducing the Dosage of *dock*

Third-instar eye–brain complexes were stained with mAb 24B10.
(A) R cell projections in $msn^{l(3)03349}$ mutants.
(B) R cell projections in $msn^{l(3)03349}$ mutants carrying only one copy of the endogenous *dock* gene. The R1–R6 nonstop phenotype became more severe. The size of R cell growth cones (arrowheads) was reduced significantly and became more similar to that of *dock* mutants.
(C) R cell projections in $dock^{P2}$ and $msn^{l(3)03349}$ double mutants (16 of 16 hemispheres examined) were indistinguishable from those in $dock^{P1}$ mutants.
(D) R cell projections in $dock^{P1}$ mutants.
Scale bar, 20 μm.

the physical association of Msn with Dock (Figure 4), strongly suggest that Msn and Dock function in the same signaling pathway controlling R cell projections.

Overexpression of Msn in *dock* Null Mutants Prevents R1–R6 Growth Cones from Migrating into the Medulla

That Dock/Nck is capable of binding activated receptor tyrosine kinases via its SH2 domain (e.g., Li et al., 1992; Galisteo et al., 1996; Stein et al., 1998), together with the above phenotypic analysis of *dock* and *msn* mutants, suggests that Msn is activated by Dock-mediated stop signals in terminating R1–R6 growth cones in the lamina. This model makes the simple prediction that gain of function in *msn* should suppress the R1–R6 nonstop phenotype in *dock* mutants. To assess this possibility, we overexpressed the endogenous *msn* gene in homozygous *dock* mutants. In *dock* mutants (Figure 6A), the medulla layer was hyperinnervated, as many R1–R6 axons failed to stop at the lamina termination site. Overexpression of Msn in *dock* mutants largely suppressed the R1–R6 nonstop phenotype (Figures 6B and 6C); R cell axons in the medulla were dramatically reduced in all larvae examined (n = 16). That gain of function in *msn* is capable of terminating R1–R6 growth cones in *dock* null mutants is consistent with the prediction that Dock functions upstream of Msn activation in decelerating R1–R6 growth cone motility.

Surprisingly, overexpression of *msn* in the absence of *dock* also caused the premature termination of many R cell growth cones within the optic stalk (Figures 6B and 6C), a phenotype that was not observed in wild-

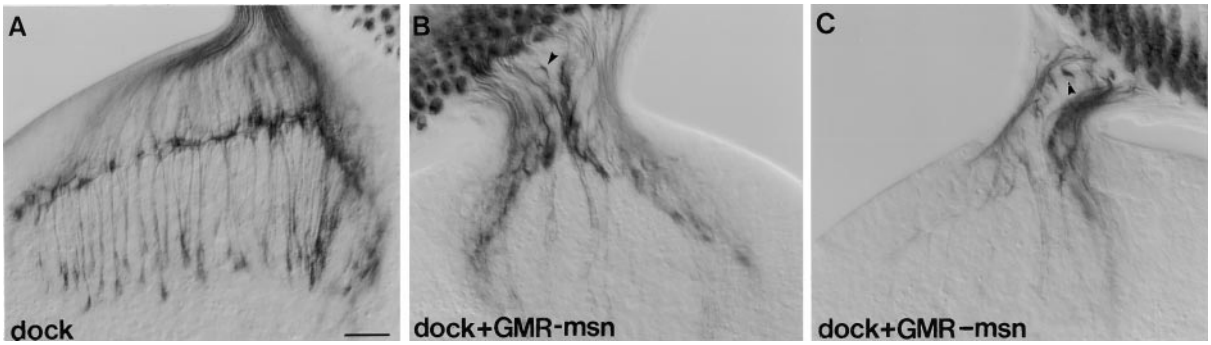

Figure 6. Overexpression of *msn* in *dock* Mutants Suppresses the R1–R6 Nonstop Phenotype

Third-instar eye–brain complexes were stained with mAb 24B10.
(A) R cell projection pattern in $dock^{P1}$ mutants.
(B and C) The endogenous *msn* gene was overexpressed in homozygous $dock^{P1}$ mutants. The number of axons in the medulla was dramatically reduced compared with that measured in $dock^{P1}$ mutants. Many R cell growth cones terminated within the optic stalk, with the consequence that the optic stalk expanded significantly in size. Arrowheads indicate expanded R cell growth cones within the optic stalk. Scale bar, 20 μm.

type flies overexpressing *msn* (Figure 3B). This result raises the intriguing possibility that Dock is also able to negatively regulate the function of Msn at certain stages of axonal projections.

Overexpression of Dock Suppresses the *msn* Gain-of-Function Phenotype

To further investigate the relationship between *msn* and *dock* in the control of growth cone motility, we examined the effect of overexpressing Dock on the *msn* gain-of-function phenotype. Dock was overproduced in R cells under control of the GMR promoter. Overexpression of Dock in R cell axons was confirmed by immunohistochemical analysis (data not shown). In wild type, overexpression of Dock has no effect on R cell projections (data not shown), suggesting that Dock is not rate limiting in the termination of growth cones. Overexpression of Dock in *msn* gain-of-function mutants largely suppressed the pretarget termination phenotype (Figure 7B), confirming that Dock also negatively regulates the function of *msn*.

To define the domains of Dock responsible for negative regulation of Msn, we examined the ability of mutant Docks to suppress the *msn* gain-of-function phenotype. SH3 mutants incapable of binding Msn in yeast either completely failed to suppress the phenotype (SH3-2 mutant) (Figure 7D) or only weakly suppressed the phenotype (SH3-1 mutant) (Figure 7C). In contrast, the SH3-3 mutant, displaying Msn-binding activity, suppressed the phenotype as efficiently as wild-type Dock (Figure 7E). These results argue that the physical association of Dock with Msn is essential for the regulation of Msn by Dock. Interestingly, although the R336Q mutation (eliminating phosphotyrosine-binding activity of the SH2 domain) does not affect the binding of Dock to Msn (Table 1), it completely abolished the ability of Dock to suppress the *msn* gain-of-function phenotype (Figure 7F). These data suggest that the negative regulation of Msn function by Dock involves an SH2-dependent tyrosine phosphorylation signal.

Discussion

Precise control of growth cone motility is essential for the proper guidance and targeting of axons during embryonic development. While the directed extension of growth cones leads axons toward their destination, deceleration of growth cone motility is required for the formation of synaptic connections at the final stage of axonal projections. Upon reaching the target region, growth cones must recognize specific signals and convert them into the rearrangements of cytoskeletal structures, which then leads to the cessation of growth cone movement. Ultimate understanding of this process requires the identification of key players and the elucidation of the signaling pathway in which they are involved.

Previous studies demonstrated that the *Drosophila* SH2/SH3 adaptor protein Dock is required for R cell growth cone targeting (Garrity et al., 1996). While *dock*-defective R1–R6 growth cones can enter the target region (i.e., lamina), many of them fail to stop there and extend further into the deeper layer instead (i.e., medulla), suggesting a role for Dock in transducing target-derived stop signals into the shutdown of growth cone motility. In the present paper, we provide molecular and genetic evidence that suggests that Msn is a functional partner of Dock in R cell growth cones. We demonstrate that Msn, like Dock, is required for the proper termination of R1–R6 growth cones. This is not likely due to a general requirement of Msn in the control of cytoskeletal structures, as the outgrowth of *msn*-defective axons appears normal. Similarly, we show that like Dock, Msn protein preferentially localizes to R cell axons and growth cones. Moreover, overexpression of Msn is sufficient to induce the termination of growth cones. That Msn has a direct functional relationship with Dock is further supported by the physical association of Msn with Dock, the dose-dependent genetic interaction between them, and the fact that gain-of-function in *msn* suppressed the R1–R6 nonstop phenotype in *dock* null mutants. While the above observations support a model in which Dock-mediated stop signals activate Msn in terminating R1–R6 growth cones, we further demonstrate that Dock can also negatively regulate the function of Msn.

How is the function of Msn regulated by Dock, an adaptor protein without catalytic activity? Studies on the well-known adaptor protein Grb2/Drk/Sem-5 provide a prototype for the function of SH2/SH3 adaptors. Grb2 binds simultaneously to activated receptor tyrosine kinases via its SH2 domain, and the guanine nucleotide exchange factor Sos via its SH3 domains, which positions Sos close to Ras and subsequently leads to the activation of Ras (reviewed by Downward, 1994; Schlessinger, 1994). By analogy to Grb2, we propose two possible mechanisms for the positive regulation of Msn function by Dock. In one model, Dock-mediated terminating signals recruit Msn into multiple signaling protein complexes in specific regions within the growth cone, thus positioning Msn close to its substrate. In an alternative model, signals directly stimulate the enzymatic activity of Msn by regulating its subcellular localization. For instance, the recruitment of another Ste20-like kinase, Pak, to the plasma membrane by Nck, the vertebrate homolog of Dock, leads to the activation of Pak (Lu et al., 1997).

That Dock also negatively regulates the function of Msn underscores the complexity of the regulation of Msn. Our results show that Dock requires both its Msn-binding activity and phosphotyrosine-binding activity to efficiently inhibit the function of Msn, suggesting that negative regulation of Msn by Dock is also dependent on upstream signals. One likely explanation for this is that Dock is a common target of multiple signals. Indeed, it has been shown that Nck, the vertebrate homolog of Dock, is capable of binding to a variety of activated receptor tyrosine kinases (e.g., Li et al., 1992; Galisteo et al., 1996; Stein et al., 1998). Similarly, in addition to its role in R cell growth cones, Dock is required for motor neuron projections (Desai et al., 1999) and for the formation of precise fiber pattern in the inner optic ganglia (Rao and Zipursky, 1998).

We propose that Dock couples different signals to Msn at different stages of axonal projection. At an early stage, signals promoting growth cone extension may induce tyrosine phosphorylation on specific proteins (e.g., docking protein), which then recruit Msn through Dock (via the SH2 domain) to specific regions within the

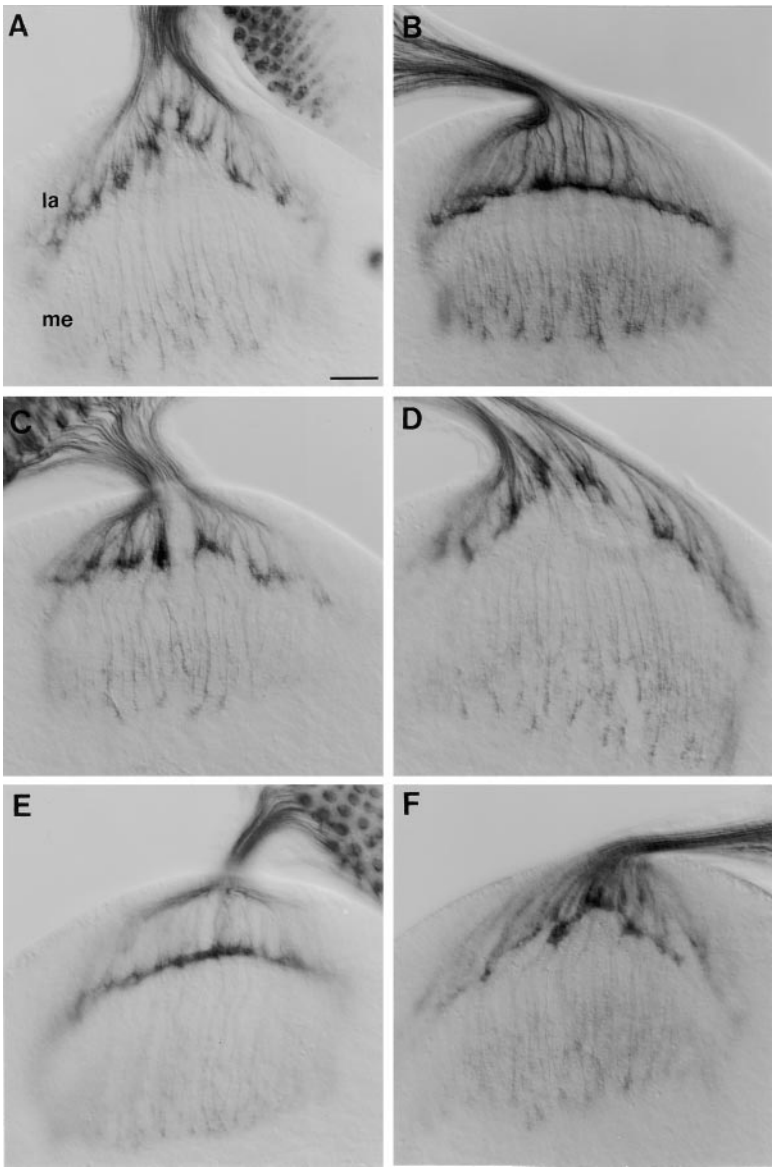

Figure 7. Overexpression of Dock in *msn* Gain-of-Function Mutants Suppresses the Pretarget Termination Phenotype

Third-instar eye–brain complexes were stained with mAb 24B10.

(A) R cell projection pattern in larvae overexpressing the endogenous *msn* gene.

(B–F) Overexpression of wild-type and mutant Docks in *msn* gain-of-function mutants. For each *dock* transgene, at least two independent lines were examined. Overexpression of wild-type and mutant Dock proteins was confirmed by immunohistochemical analysis (data not shown).

(B) Overexpression of wild-type Dock largely restored R1–R6 termination pattern (20 of 22 hemispheres examined).

(C) Overexpression of the SH3-1 mutant. Among four independent lines examined, only two lines showed weak suppression. In those lines, R1–R6 growth cones terminated closer to their normal targets (~43%, n = 21) than those without SH3-1 mutant, but the lamina terminal field was still largely disorganized.

(D) Overexpression of the SH3-2 mutant. R cell projection pattern in all larvae examined (n = 22) was indistinguishable from that of *msn* gain-of-function mutants.

(E) Overexpression of the SH3-3 mutant. R1–R6 termination pattern was largely restored (40 of 40 hemispheres examined).

(F) Overexpression of the SH2 mutant. No suppression of the *msn* gain-of-function phenotype was observed (n = 20).

Genotypes: in (A), GMR-*GAL4*/+;*EP(3)549*/+; in (B) through (F), *msn* gain-of-function larvae (A) carrying one copy of the following *UAS* transgene constructs: *dock* (B); W48K (C); W151K (D); W225K (E); and R336Q (F). Scale bar, 20 μm.

growth cone. Consequently, this may segregate Msn from its substrates, thus preventing the premature activation of the Msn pathway. In growth cones overexpressing Msn, however, excessive Msn that cannot be recruited by a limited amount of endogenous Dock may diffuse freely into certain regions to activate its substrates, which then induce pretarget growth cone termination. Similarly, we showed that the pretarget termination phenotype was enhanced by loss of *dock* and was suppressed by overexpression of *dock*. Once the growth cone reaches the target, upregulation of Msn may be accomplished in two steps through the combination of reducing the extension signal and increasing the stop signal. First, the Dock–Msn complex needs to be released from those docking sites, which would be achieved by dephosphorylation through the activation of some protein tyrosine phosphatases. One such candidate is the receptor tyrosine phosphatase PTP69D, which has recently been shown to be required for the proper targeting of R1–R6 growth cones (Garrity et al., 1999). Second, the stop signal activates the function of Msn through Dock by either positioning Msn close to its substrate or directly stimulating its activity (see above), leading to the termination of the growth cone in the target. In the absence of Dock, endogenous Msn may not reach a threshold local concentration or activity required for growth cone termination. Our observation that reduction of *dock* gene dosage enhanced the hypomorphic *msn* loss-of-function phenotype is consistent with this view. While the above model fits with our results, understanding of the exact biochemical mechanism underlying the regulation of Msn by Dock awaits identification of upstream regulators of Dock in R cell growth cones.

In cultured cells, overexpression of NIK (the vertebrate homolog of Msn) leads to the activation of the c-Jun N-terminal kinase (JNK) (Su et al., 1997). Similarly, Msn has been shown to be upstream of JNK activation in

the control of dorsal closure during embryonic development (Su et al., 1998). Recent work demonstrates that the JNK pathway is involved in regulating cell shape changes during development (Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996). These studies lead us to test the possibility that Msn activates JNK in terminating R cell growth cones. We examined the potential genetic interaction between *msn* and *bsk*, the *Drosophila* homolog of JNK. Our results showing that the function of *msn* in R cell growth cones is not sensitive to the dosage of *bsk* (data not shown), however, argue against this model. We suggest that the function of Msn in R cell growth cones is independent of JNK activation.

Msn may regulate the rearrangements of cytoskeletal structures within the growth cone by phosphorylating components of cytoskeleton. In *Dictyostelium*, a Ste20-like kinase has been shown to phosphorylate the actin-binding protein severin (Eichinger et al., 1998). Severin binds to actin and fragments actin filaments in a Ca^{2+}-dependent manner (Yamamoto et al., 1982). Pak1, another Ste20-like kinase family member, phosphorylates myosin light chain kinase (MLCK) (Sanders et al., 1999). The decrease in the activity of MLCK by phosphorylation is correlated with the changes in cytoskeletal structures. It will be interesting to determine whether Msn phosphorylates similar molecules in vivo.

While strong *dock* and *msn* loss-of-function mutants both exhibited completely penetrant R1–R6 nonstop phenotype, loss of *dock* function caused more severe defects in R cell fasciculation and growth cone expansion. These observations raise the possibility that Dock also interacts with other effector molecules in R cell growth cones. In this context, it is notable that in addition to its association with Msn, Dock binds to cytoplasmic protein tyrosine phosphatase PTP61F (Clemens et al., 1996) and Pak (Hing et al., 1999). Pak binds to small GTPases Cdc42 and Rac (Harden et al., 1997), important regulators of cytoskeleton (reviewed by Luo et al., 1997; Tapon and Hall, 1997). While we were preparing this paper, Zipursky and colleagues (Hing et al., 1999) demonstrated that Pak is required for R cell projections. Thus, it is highly possible that Msn cooperates with these molecules in some or all events regulated by Dock.

Recent studies suggest that Dock/Nck plays a highly conserved role in growth cone signaling. Nck can be recruited into signaling complexes in response to the activation of the vertebrate guidance receptors EphB1 (Stein et al., 1998) and EphB2 (Holland et al., 1997), two Eph receptor tyrosine kinase family members. Moreover, Nck can functionally replace Dock in R cell growth cones (Rao and Zipursky, 1998). Furthermore, Dock, like Nck, is capable of binding ligand-activated EphB1 (Stein et al., 1998). Given the extraordinary sequence conservation between Msn and NIK, it is highly likely that in vertebrate growth cones, NIK plays a similar role in response to Nck-mediated signals. Hence, the interaction between Dock/Nck and Msn/NIK may represent an evolutionarily conserved mechanism linking tyrosine phosphorylation to changes in growth cone behavior.

Experimental Procedures

Drosophila Stocks and Genetic Crosses

bsk^{1}, *bsk*^{2}, *msn*$^{l(3)03349}$, and *w*;70C[W^{+}],*FRT*80 lines were provided by the Bloomington *Drosophila* Stock Center. *w*;*msn*^{102},*FRT*80 and *UAS-msn* lines were provided by J. Treisman. The *EP(3)549* line was provided by the Berkeley *Drosophila* Genome Project. The transgenic line carrying *EyFLP* was from B. Dickson. The GMR-*GAL4* line was provided by S. L. Zipursky. *msn*$^{l(3)03349}$ was maintained over *TM6HuTb* for larval phenotypic analysis. *msn*^{102} mutant eye tissues were generated by crossing *w*;Rhl-*lacZ*, *msn*^{102}, *FRT*80/*TM6HuTb* males to *EyFLP*;70C[W^{+}],*FRT*80 females. To overexpress the endogenous *msn* gene in differentiating R cells, GMR-*GAL4*/GMR-*GAL4* males were crossed to *EP(3)549*/*TM6HuTb* females. In the *EP(3)549* line, a *UAS* element is inserted in the 5′ promoter region of *msn* (Rorth et al., 1998). R cell projections in GMR-*GAL4*/+;*EP(3)549*/+ larvae were compared with those in GMR-*GAL4*/+;*TM6HuTb*/+ or in *EP(3)549*/+ larvae. The *msn* transgene was overexpressed by generating the GMR-*GAL4*/*In (2LR)Gla Bc Elp*;*UAS-msn*/*TM6HuTb* line. To overexpress the endogenous *msn* gene in homozygous *dock*^{P1} mutants, *dock*^{P1}/*In (2LR)Gla Bc Elp*;*EP(3)549*/+ males were crossed to *dock*^{P1},GMR-*GAL4*/*In (2LR)Gla Bc Elp* females. Non-*Bc* larvae were double stained with mAb 24B10 and anti-Msn serum. All individuals overexpressing Msn showed the suppression of the R1–R6 nonstop phenotype (n = 16). R cell projections in individuals without overexpression of Msn were indistinguishable from those in *dock*^{P1} mutants (100%, n = 14). To investigate the genetic interaction between *dock* and *msn*, the *dock*^{P2}/*In (2LR)Gla Bc Elp*;*msn*$^{l(3)03349}$/*TM6HuTb* line was generated. To overexpress wild-type and mutant Docks in *msn* gain-of-function mutants, lines carrying *UAS-dock* transgenes were crossed with the GMR-*GAL4*/*In (2LR)Gla Bc Elp*;*EP(3)549*/*TM6HuTb* line.

Plasmid Construction

DNA fragments encoding different domains of Msn (Figure 4) were amplified by polymerase chain reaction (PCR) and subcloned into the pGAD424 vector. DNA fragments encoding the entire polypeptide (amino acids 1–411) of wild-type and mutant Docks (Table 1) were amplified by PCR and subcloned into the pBTM116 vector.

In Vitro Binding Assay

Adult flies were homogenized in the lysis buffer (0.5% Triton X-100, 5 mM EDTA, 250 mM NaCl, and 25 mM Tris-HCl [pH 7.5]). Insoluble materials were removed by centrifugation (13,000 × g for 10 min at 4°C). The supernatant was then incubated with GST-bound or GST-Msn-bound glutathione agarose beads for 1 hr at 4°C. The beads were washed four times with HNTG buffer (20 mM HEPES [pH 7.5], 10% glycerol, 0.1% Triton X-100, and 150 mM NaCl). Precipitates were eluted from the beads by boiling in SDS sample buffer and subjected to electrophoresis. Western blot analysis was performed as described (Biggs and Zipursky, 1992). Anti-Dock antibody was used at a dilution of 1:4,000. GST–Msn fusion protein was prepared as described (Smith, 1983) and used to raise a rabbit antiserum for Western blot and immunohistochemistry.

Coimmunoprecipitation

Lysates from third-instar larval eye–brain complexes and adult heads were prepared as above. G protein beads preincubated with anti-Dock antibody or control serum were incubated with fly lysates for 2 hr at 4°C, washed three times with HNTG buffer as above, resuspended in SDS-loading buffer, and analyzed by gel electrophoresis and Western blot.

Yeast Two-Hybrid Assay

Yeast strain L40 (Hollenberg et al., 1995) was cotransformed with pGAD424 and pBTM116 constructs. Yeast transformants containing both plasmids were selected on *trp*$^{-}$, *leu*$^{-}$, *ura*$^{-}$, and *lys*$^{-}$ plates. Activation of β-galactosidase expression was determined by filter assay as described (Bartel and Fields, 1995).

Immunohistochemistry

R cell axons in third-instar larvae were stained with mAb 24B10 as described (Van Vactor et al., 1991). The expression pattern of Msn protein in third-instar larvae was determined with the rabbit anti-Msn serum as described (Van Vactor et al., 1991). Cryostat sections of adult mosaic heads were stained with a rabbit anti-β-galactosidase antibody as described (Garrity et al., 1996).

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