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Drosophila Photoreceptor Axon Guidance and Targeting Requires the Dreadlocks SH2/SH3 Adapter Protein

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Summary

Mutations in the Drosophila gene *dreadlocks* (*dock*) disrupt photoreceptor cell (R cell) axon guidance and targeting. Genetic mosaic analysis and cell-type-specific expression of *dock* transgenes demonstrate *dock* is required in R cells for proper innervation. Dock protein contains one SH2 and three SH3 domains, implicating it in tyrosine kinase signaling, and is highly related to the human proto-oncogene *Nck*. Dock expression is detected in R cell growth cones in the target region. We propose Dock transmits signals in the growth cone in response to guidance and targeting cues. These findings provide an important step for dissection of signaling pathways regulating growth cone motility.

Introduction

Nervous system function relies on the establishment of appropriate neuronal connections. Despite the enormous set of connections possible in both vertebrate and invertebrate nervous systems, patterns of neuronal connectivity are remarkably precise. The formation of neuronal connections occurs in a stepwise fashion (Goodman and Shatz, 1993). First, a neuron extends a projection, an axon or dendrite, from the cell body which navigates toward its target region (Keynes and Cook, 1995). Projections can interact with one another and are frequently arranged in bundles or fascicles that can influence targeting. Upon reaching its target region, a target cell is selected (Garrity and Zipursky, 1995) and synaptic connections are established (Burns and Augustine, 1995). Some systems, such as the vertebrate visual and somatosensory systems, have an additional level of complexity in which projections maintain their relative spatial relationships as they innervate their target region, thereby elaborating a topographic map.

The growth cone, a sensorimotor structure at the leading edge of neuronal projections, plays a key role in establishing precise patterns of neuronal connectivity (Letourneau et al., 1992). The growth cone guides neuronal processes by sensing cues present in its environment and converting them into cytoskeletal changes that control directed movement (Tanaka and Sabry, 1995). Remarkably, growth cones can function relatively autonomously; they can navigate normally in vivo for several hours after being severed from the cell body (Harris et al., 1987). Thus, the growth cone must contain cell-surface receptors to receive guidance cues, signal transduction machinery to transmit and integrate the information received, and regulators of cytoskeletal structure that translate guidance decisions into changes in cell shape and movement.

Several recent studies have led to the view that tyrosine phosphorylation plays an important role in guidance and target recognition. In Drosophila, the Derailed receptor tyrosine kinase (RTK) regulates axonal guidance of a subset of embryonic neurons (Callahan et al., 1995) and mutations in receptor tyrosine phosphatases (Desai et al., 1996; Krueger et al., 1996) and cytoplasmic tyrosine kinases (Gertler et al., 1989) have been shown to disrupt neuronal connectivity patterns in the embryonic central nervous system. In vertebrates, members of the Eph family of RTKs and their ligands have received particular attention; in vitro studies with the rat REK7 receptor and its ligand AL-1 have provided evidence for their role in controlling the fasciculation of cortical axons (Winslow et al., 1995). The distribution of Eph receptors and their ligands in the developing vertebrate visual system has led to the speculation that members of this family regulate the formation of precise retinotopic maps (Cheng et al., 1995; Drescher et al., 1995). Pharmacological studies also support a role for tyrosine kinases in growth cone guidance (McFarlane et al., 1995) and immunohistological studies show a concentration of phosphotyrosine in filopodia, at the leading edge of the growth cone (Wu and Goldberg, 1993).

Little is known about the mechanisms that regulate changes in the growth cone's actin cytoskeleton in response to guidance cues. It seems likely that the basic strategy will be similar to that used by other systems in which the cytoskeleton is modulated by extracellular signals. Studies in fibroblasts have demonstrated the importance of Rho-family GTPases (i.e., Rho, Rac, and CDC42) in modifying cytoskeletal structures in response to extracellular signals (Nobes and Hall, 1995) and expression of dominant interfering mutants of Rac and CDC42 in the Drosophila central nervous system lead to marked defects in the organization of neuronal pathways (Luo et al., 1994). In Saccharomyces cerevisiae, cytoskeletal reorganization in response to mating pheromone also utilizes Rho-family GTPases (i.e., CDC42) (Chenevert, 1994), alluding to a highly conserved mechanism for remodeling the actin-based cytoskeleton in response to extracellular signals (reviewed in Chant and

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Stowers, 1995). The signaling mechanisms that link receptors for extracellular cues to such potential modulators of the cytoskeleton are poorly understood. In this paper, we describe an essential component of the tyrosine kinase signaling pathways controlling growth cone motility in the Drosophila visual system.

We have taken a genetic approach to dissecting guidance and target recognition. Our studies have focused on the development of photoreceptor (R cell) axons. The adult eye is composed of ~750 repeated units, each containing eight R cells (R1–R8). Each R cell type has a stereotyped projection pattern (Meinertzhagen and Hanson, 1993): R1–R6 axons terminate in the superficial lamina layer of the optic lobe, while R7 and R8 axons project through the lamina and terminate in separate layers of the underlying medulla. In addition, R cells elaborate topographic maps in both the lamina and the medulla. This pattern of R cell projections forms during late larval and pupal development. Differentiating R cells in the retinal primordium, the eye imaginal disc, project axons into the developing optic lobes of the larval brain. R cell axons from the posterior region of the disc project into the brain first, followed by axons from more anterior regions. Both the retinotopic maps and the targeting of different R cell axons to the lamina and medulla are easily visualized, facilitating the identification of mutations disrupting connectivity (Martin et al., 1995).

Genetic screens for R cell connectivity defects have led to the identification of a large number of mutants (Martin et al., 1995; P.A.G. and Y. R., unpublished data). Critical analysis of cell fate determination and pattern formation both in the retina and the target revealed that only a small fraction of these are likely to affect the process directly by disrupting specific functions in the growth cone. In this paper, we describe the isolation and characterization of one of these genes, *dreadlocks* (*dock*), which plays a direct role in regulating growth cone function. *dock* encodes an evolutionarily conserved adapter protein comprising three SH3 and a single SH2 domain and is expressed in growth cones. We propose that it links tyrosine kinase signaling to changes in the actin cytoskeleton underlying growth cone guidance and target recognition.

Results

Identification of the *dock* mutation

We screened 535 late larval and pupal lethal P element lines for R cell projection defects by staining third instar eye brain complexes with MAb24B10, which recognizes R cells and their axons (Fujita et al., 1982). In all, 91 mutations were identified that disrupted the pattern. All but 3 mutations were likely to affect this pattern indirectly, as they displayed patterning and cell fate defects in the eye or defects in the optic lobe prior to R cell innervation, and were not analyzed further. One mutant did not show a genetic requirement in R cells (data not shown), and hence is unlikely to encode a protein that functions in the growth cone. The remaining two recessive mutations, alleles of the same gene called *dreadlocks* (*dock*) (named for the appearance of the projection pattern in the mutant), were shown to be required in the eye specifically for R cell growth cone guidance and targeting (see below). $dock^{P1}$, $dock^{P2}$ and $dock^{3}$ (derived by imprecise excision of $dock^{P1}$; see Experimental Procedures) mutant phenotypes were completely penetrant and indistinguishable from one another. The phenotypes of these alleles were not enhanced in trans to a deficiency for the region, consistent with these alleles being strong loss-of-function mutations. Although all three alleles are largely pupal-lethal, some homozygous mutant animals survived to adulthood. These animals were sluggish and uncoordinated, dying within a few days after eclosion. Precise excision of the $dock^{P1}$ P element reverted both lethality and the R cell connectivity defects (unpublished data).

dock Disrupts R Cell Projections

Multiple defects in R cell projection patterns were observed in *dock* mutants. R cell cluster formation (see below) and the initial stages of axon outgrowth appeared normal. Upon entering the optic lobe, wild-type R cell bundles fan out, maintaining their neighbor relations with R cell growth cones elaborating a smooth retinotopic array in the lamina and medulla (Figure 1A). The R1–R6 neurons terminate between layers of glia in the lamina (Winberg et al., 1992), while R7 and R8 neurons project through the lamina and into the medulla neuropil. In wild-type animals stained with MAb24B10, the array of expanded R1–R6 growth cones appears as a continuous line of immunoreactivity while the R7/R8 terminals form an array in the medulla neuropil.

In most *dock* mutants, axon bundles fan out unevenly as they exit the optic stalk en route to the developing lamina (Figure 1B). Fibers pathfind abnormally in this region with evidence of crossing over, abnormal fasciculation, and gross alterations in retinotopy (Figures 1C and 1D). Clumps of R cell growth cones terminating in the lamina separated by gaps are frequently observed. Thicker bundles project through these clumps into the medulla, resulting in hyperinnervated regions of the medulla separated by uninnervated regions. In many cases, R cell axons terminate at different levels within the lamina, giving rise to an uneven lamina neuropil. In addition, some R1–R6 growth cones fail to terminate in the lamina and innervate the medulla terminal field instead (see below and Figure 3). Thus *dock* mutants show defects in R cell fasciculation, targeting and retinotopy.

Defects in the organization of axons in the optic stalk were frequently observed in the light microscope (Figure 1B). In contrast to wild type, bundles of stained axons were often separated by gaps. Ultrathin sections were examined by electron microscopy to explore these defects in more detail. A cross sectional view of a wild-type optic stalk revealed a regular array of axon bundles separated by fine glial processes (Figures 2A and 2B). Each fascicle contained eight R cell axons, with a central fiber surrounded by seven. The youngest ingrowing axons found near the perimeter of the optic stalk are not yet sorted into fascicles of eight fibers (Meinertzhagen and Hanson, 1993). In *dock*, the sorting process occurs normally; nearly all fascicles contain eight axons surrounded by glial processes as in wild type (fascicles with 1 or 2 supernumerary axons were rarely observed:

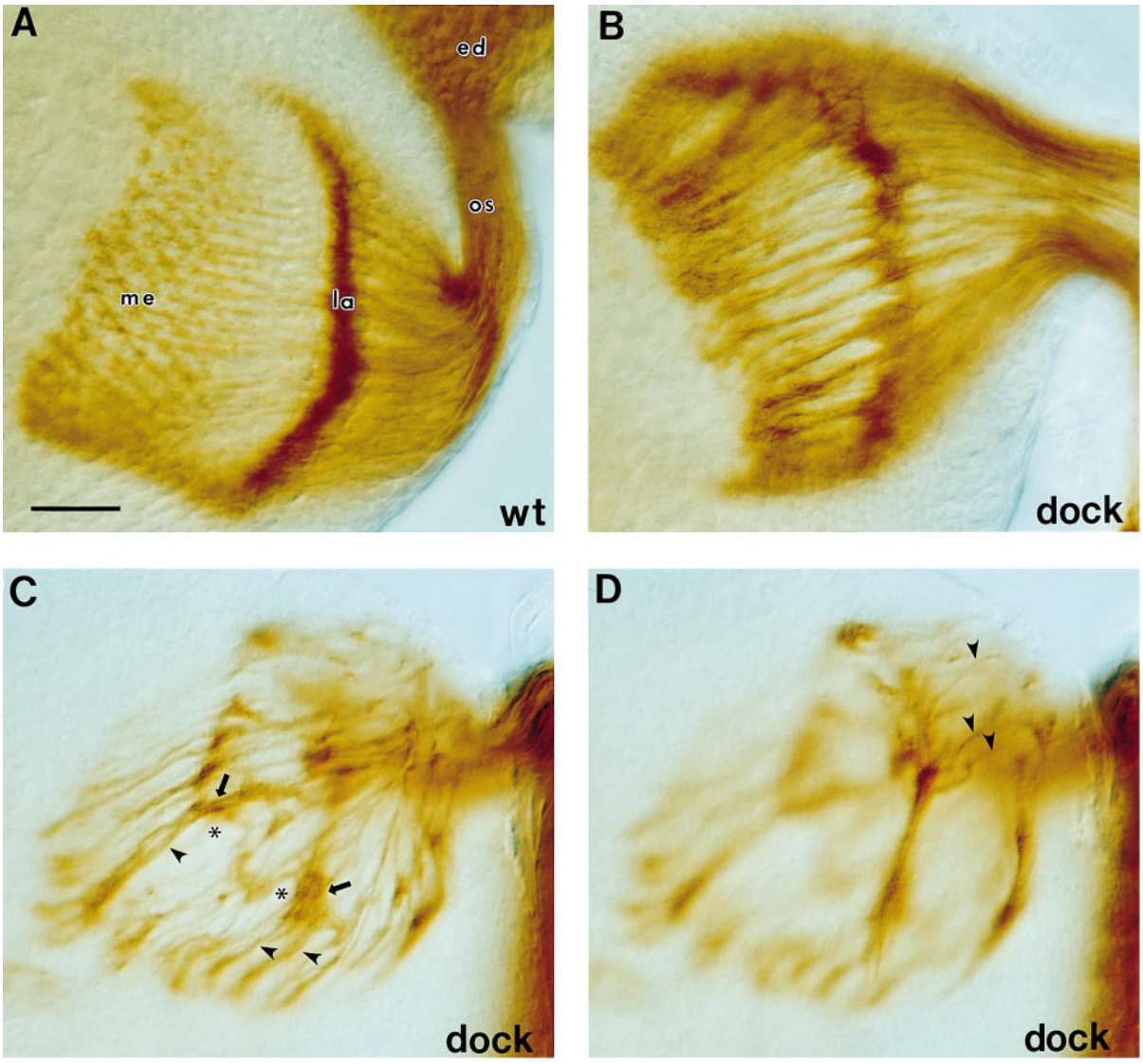

Figure 1. Developing R Cell Projections in Wild Type and *dock*

R cell projections in third instar larvae visualized using MAb24B10. (A) Projection pattern in wild-type. R cells located in the developing eye disc (ed) project axons through the optic stalk (os) to reach their targets in the developing optic lobes. R1–R6 axons project to the lamina (la), while R7 and R8 axons project to the underlying medulla (me). Each class of axons establishes a topographic map in its target region. Note the even plexus of R cell terminals in the lamina and the precise array of terminals in the medulla. (B–D) Projection patterns in *dock* mutants. $dock^{P1}$, $dock^{P2}$, and $dock^{3}$ mutants are completely penetrant and show variable expressivity with a similar range of defects. These examples represent the range of observed phenotypes. (B) A $dock^{P1}$ homozygote. The plexus of R cell terminals in the lamina is uneven. Thicker bundles are seen projecting to the medulla where they establish an uneven array of terminals. The grouping of axons in the optic stalk is also aberrant (see Figure 2). (C and D) A $dock^{P2}$ homozygote viewed at two focal planes. In (C), the R cell axons terminate at different depths in the developing lamina and form clumps of terminals instead of an even array. Asterisks mark gaps in the lamina adjacent to clumps of terminals (arrowheads). Thicker bundles of axons (arrows) project through these regions into the medulla. In (D), axons (arrowheads) from different regions of the stalk converge to form a large bundle. This bundle projects along an abnormal path. Scale bar, 20 μm.

13/805 fascicles in *dock* optic stalks, n = 8; 0/304 fascicles in wild-type stalks, n = 2). Furthermore, the proportion of axons segregated into fascicles in *dock* and wild-type optic stalks was the same (data not shown). However, fascicles were less densely packed (compare Figures 2B and 2C), with glial cells showing larger cellular profiles in all *dock* animals. This loose packing may explain the gaps between axon bundles observed in the light microscope and could reflect disruptions in neuron–glia interactions (see below).

dock Is Required in the Eye for R Cell Guidance and Targeting

The projection defects observed in the third instar may be a result of defects in the eye, the path of outgrowth, or the optic lobe. To determine whether the *dock* gene is required in the eye, we performed a genetic mosaic analysis. Patches of retinal tissue homozygous for the $dock^{P1}$ mutation were generated in heterozygous animals by X-ray-induced mitotic recombination. R cell projections were analyzed in cryostat sections of the adult

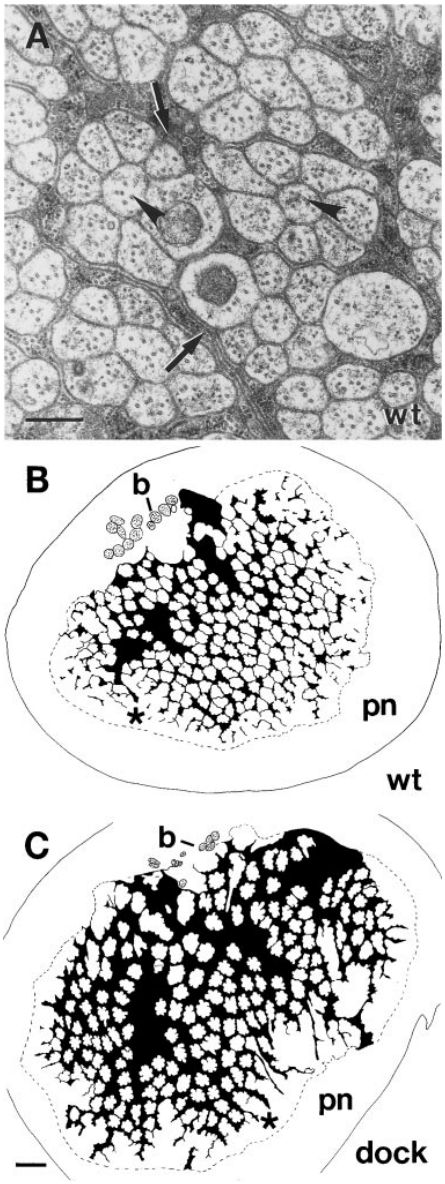

Figure 2. Optic Stalk in Wild Type and *dock*

(A) Fine structure of a wild-type optic stalk. R cell axons are grouped in fascicles, with one fiber in the middle surrounded by seven (arrowheads). Each fascicle contains axons from R cells in the same ommatidium and is enwrapped by fine glial processes (arrows). Glial processes can be distinguished from neuronal profiles by their higher ribosome content and their darker cytoplasm. Scale bar, 0.5 μm.

(B and C) Tracings of glial–neuronal interfaces in cross sections of a wild-type and a mutant optic stalk. Optic stalks shown in (B) and (C) have about the same number of axons (~2100). Glial cell bodies and their processes are shown in black. Fascicles in the *dock* mutant stalk are more separated from each other than in wild type. Asterisks, border of the optic stalk containing R cell axons not yet segregated into fascicles enwrapped by glial processes; b, Bolwigs nerve; pn, perineurial glial cells ensheathing the whole optic stalk. Scale bar, 2 μm.

head stained with MAb24B10, which reveals the precise columnar organization of R cell projections in the medulla (Figure 3A). This allows us to detect defects in the medulla terminal field. Due to the density of R cell fibers and the diffuse staining of MAb24B10, we cannot distinguish individual fibers in the lamina, precluding detailed analysis of innervation patterns in this region. As in the larva, R1–R6 terminate in the lamina and R7/R8 in the medulla, and topographic organization is retained in the adult. The adult structure differs from the larval due to morphogenetic changes during pupal development.

Larvae were X-irradiated after the separation of the eye and optic ganglion precursor cells (Kankel and Hall, 1976) and mutant clones were observed in 2% of the flies. Hence, it is highly unlikely that *dock* mutant clones were generated in the eye and optic lobe of the same hemisphere. The gross morphology of the brain innervated by patches of $dock^{P1}/dock^{P1}$ mutant R cells appeared wild-type. However, examination of the R cell projection pattern revealed defects in the medulla terminal field innervated by mutant R cells (15 of 18 mutant patches had observable defects) (Figure 3B). Defects included gaps in the array, hyperinnervation, and crossing of fibers from adjacent columns. In a few cases, fibers were observed in deeper layers of the medulla not normally innervated by R cells (data not shown). In all cases, the positions of mutant R cell projection defects in the medulla were consistent with the location of the mutant patch in the retina; for instance, anterior patches resulted in defects in the posterior medulla neuropil. In large part, then, the gross retinotopic order of the fibers is maintained in these mosaic animals. These results provide strong evidence that the *dock* gene is required in the eye for normal connectivity.

To definitively address the effect of *dock* on R cell target choice, the axons of different R cell subclasses (R1–R6, R7, and R8) must be distinguished. There are no larval markers specific to subsets of R cell axons, but such markers are available in the adult. $dock^{P1}$ mutant patches were produced in a genetic background carrying the adult R1–R6 marker, Rh1-*lacZ* (Mismer and Rubin, 1987). In sections of a wild-type visual system, LacZ staining was restricted to the retina (R1–R6 cell bodies) and the lamina (R1–R6 axon terminals) (Figure 3C; $n = 12$). In sections of the $dock^{P1}$ mosaic flies, many LacZ-positive fibers underlying the $dock^{P1}$ mutant patch passed through the lamina and penetrated into the medulla (Figures 3D and 3E; 7 of 8 hemispheres examined), demonstrating that the *dock* gene is required in the eye for proper R1–R6 targeting.

Several lines of evidence indicate that R cell fate determination and differentiation occur normally in *dock* mutant flies and thus that defects in pathfinding and targeting are not due to alterations in earlier steps of R cell development. No defects in R cell differentiation or organization were seen in developing eye discs stained with various markers (data not shown). R cell morphology was examined in sections of the adult mosaic eyes generated using mitotic recombination and in adult escapers (Figure 3F). Each R cell can be uniquely identified by its position and morphology in either sectioned material or using the pseudopupil technique to assess the position and morphology of the rhabdomere, the photosensitive structure of the R cells. Analysis of ommatidia in mosaic patches and in surviving homozygous adults

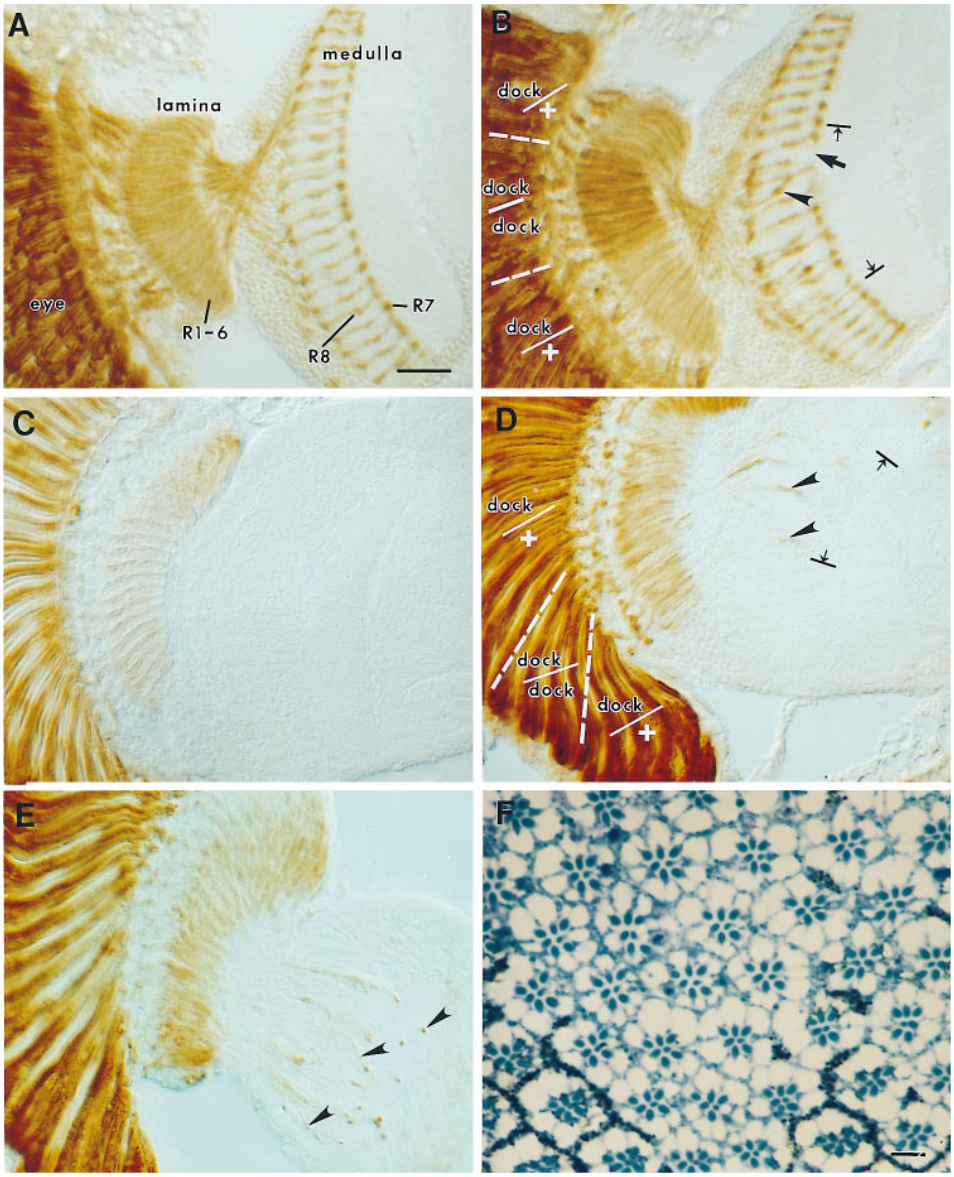

Figure 3. Genetic Mosaic Analysis

Patches of *dock*^{P1} mutant tissue were generated by X-ray irradiation. (A and B) Cryostat sections of adult wild-type (A) and mosaic (B) heads stained with the R cell–specific antibody MAb24B10. The projections of *dock*^{P1} mutant fibers in the medulla terminal field were abnormal. Gaps in R7 terminal field (arrow) and crossing of fibers between columns (arrowhead) are seen in this preparation. (C–E) Cryostat sections of adult heads carrying an R1–R6 specific marker, Rh1-*lacZ*, were stained with anti-LacZ antibody. In wild type, these axons all terminate in the lamina (C). In mosaic animals, some R1–R6 axons underlying mutant patches project into the medulla (arrowheads in [D] and [E] show R cell axons and terminals). In (B) and (D), the genotype of the retina is indicated; in (E), most of the R cells are mutant. The brain is heterozygous for *dock*. R cells project into the region of the lamina directly beneath them and through the chiasm into the opposite side of the medulla (see text). Based on the position of the mutant patch, we estimated the approximate boundaries of the regions innervated by the mutant R cells (indicated by black lines and small arrows). (F) Tangential section of a *dock*^{P1} mutant patch in an otherwise heterozygous or wild-type eye. The regions devoid of dense pigment granules contain *dock*^{P1} mutant R cells. Scale bars in (A)–(E), 20 μm; and for (F), 5 μm.

revealed that the vast majority of ommatidia was indistinguishable from wild type, with 28/1006 ommatidia lacking a single R cell. Since previous studies have shown that innervation of the optic lobe is necessary for R cell survival in the adult (Campos and Fischbach, 1992), the small number of R cells missing may reflect a weak defect in cell survival due to abnormal innervation.

The Development of the R Cell Target Region in *dock*

The optic lobe neuroblasts of the outer proliferation center (OPC) generate the lamina and outer medulla while those of the inner proliferation center (IPC) give rise to the inner medulla and lobula complex (Meinertzhagen and Hanson, 1993). R cells play a key role in inducing

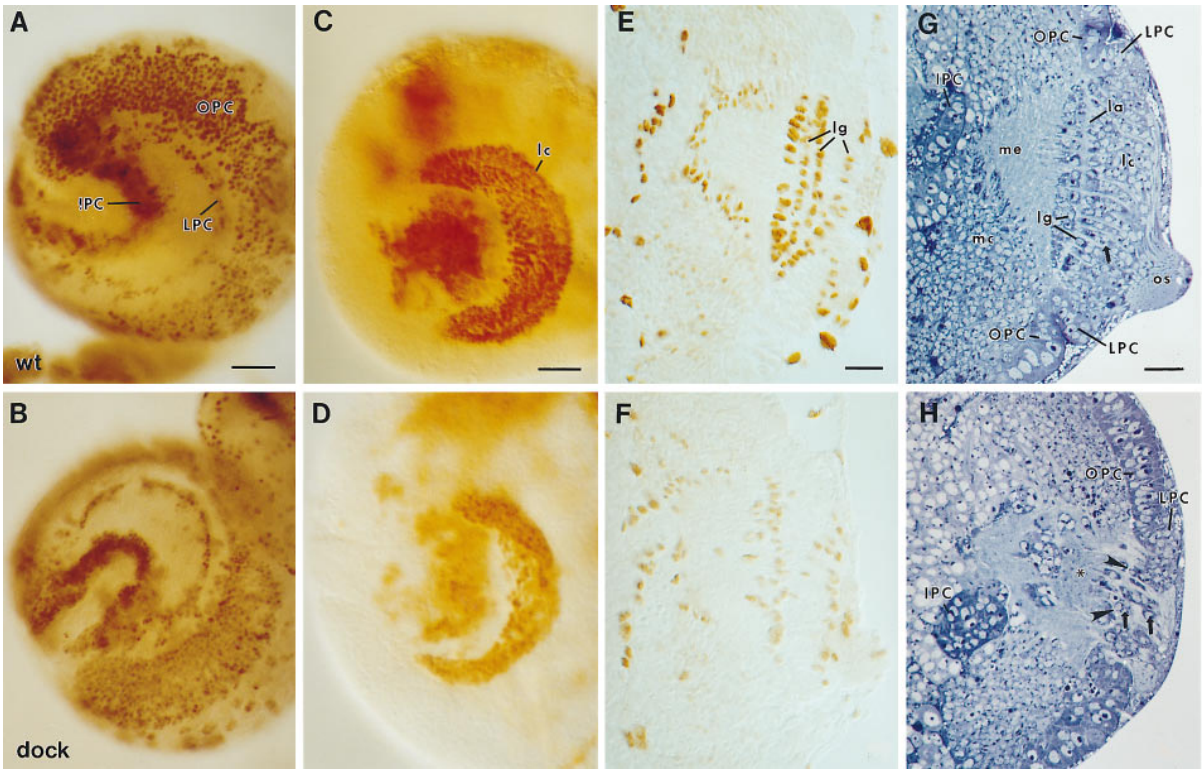

Figure 4. Patterning in Wild-Type and *dock*^{P1} Developing Optic Lobes

Patterning in wild-type (A, C, E, and G) and *dock*^{P1} mutant (B, D, F, and H) third instar larval optic lobes. (A and B) Cells in S-phase were visualized using BrdU staining. Three domains of cells in S phase were observed: outer proliferation center, lamina precursor cells, and inner proliferation center. The proliferation pattern of *dock* is indistinguishable from wild type. (C and D) Developing neurons in the lamina cortex stained with anti-Dachshund antibody, a nuclear marker. In *dock*, Dachshund protein was expressed but the organization of the cells was somewhat aberrant (see [H]). Owing to the disorganization of stained cells in [D], many neurons are out of the focal plane. (E and F) Frontal cryostat sections stained with the RK2 antibody recognizing the Repo protein in the nuclei of glial cells. In wild type, R1–R6 growth cones terminate between rows of RK2-positive glial cells. In *dock*, glial cells are disorganized. (G and H) Frontal plastic sections stained with toluidine blue. In *dock*, defects in the lamina and the medulla are seen. The columnar organization of the developing lamina cortex is disrupted (arrows) and gaps in the lamina neuropil (arrowheads) are observed. The structure of the medulla neuropil is altered (asterisk) either by the invasion of the neuropil by cortical regions or by fusion with the lobula complex neuropil. ipc, inner proliferation center; la, lamina neuropil; lc, lamina cortex; lg, lamina glia; lpc, lamina precursor cells; mc, medulla cortex; me, medulla neuropil; opc, outer proliferation center; os, optic stalk. Scale bar, 30 μm for (A) and (B); 20 μm for (C) and (D); and 20 μm for (E)–(H).

the development of the lamina and medulla. R cell innervation drives lamina precursor cells through their final division in a region called the LPC (Selleck and Steller, 1991). Both neuronal and glial cell differentiation markers are dependent upon retinal innervation for their expression (Selleck and Steller, 1991; Winberg et al., 1992).

The pattern of proliferation of optic lobe neuroblasts and lamina precursors as assessed using BrdU-labeling in *dock* animals was indistinguishable from wild-type (Figure 4A and 4B). As in wild type, *dock* lamina neurons expressed the neuronal marker Dachshund (Mardon et al., 1994) (Figures 4C and 4D) and lamina glia expressed the Repo protein (Campbell et al., 1994) (Figures 4E and 4F). Lamina neurons and glia were disorganized in many preparations. This is likely a consequence of defects in R cell innervation rather than an intrinsic defect in neurons or glia (see below). Abnormalities in the structure of the medulla neuropil also were seen in toluidine blue–stained sections in all preparations examined (n = 5; see Figures 4G and 4H). More centrally located regions of the optic lobe which form independently of R cell innervation (i.e., lobula complex) (Meinertzhagen and Hanson, 1993) also require *dock* function. Massive defects are seen in the organization of the neuropil in these regions in homozygous *dock* flies that survive to adulthood (data not shown). Whether these defects reflect abnormalities in the formation of connectivity patterns in more centrally located regions of the visual system or whether they reflect an additional developmental role for *dock* in these regions has not been established.

dock Encodes an Adapter Protein Homologous to Human Nck

DNA flanking the *dock*^{P1} insertion site was isolated by plasmid rescue and used to isolate ~30 kb of genomic DNA. A 9 kb genomic fragment spanning the insertion site identified dock cDNA from both eye imaginal disc and 0–24 hr embryo cDNA libraries. The longest cDNA was 3.7 kb and spanned the *dock*^{P1} and *dock*^{P2} P element

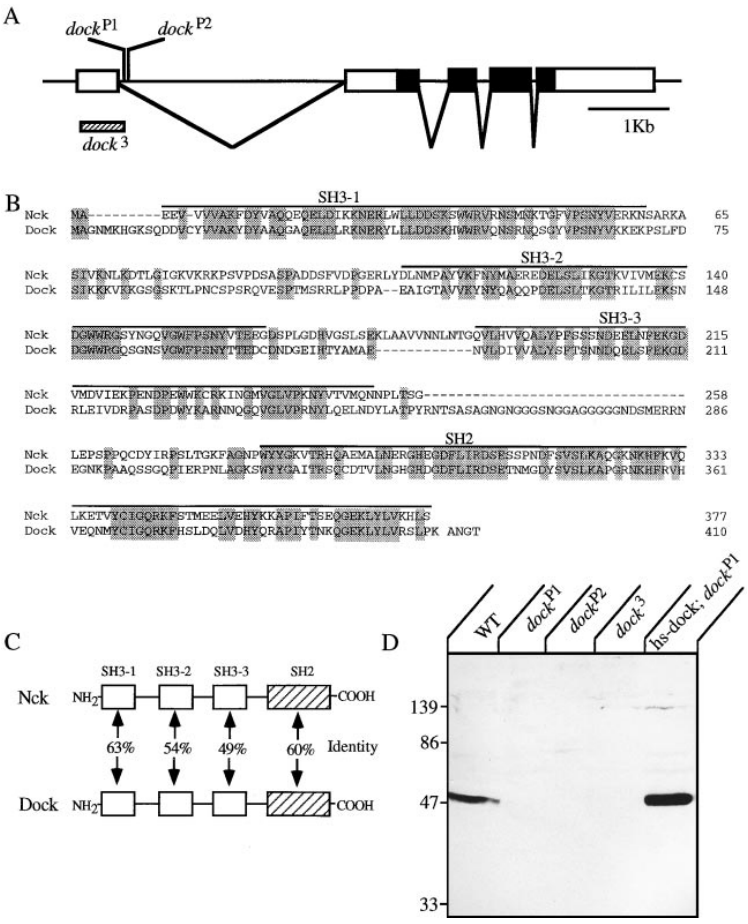

Figure 5. Molecular Characterization of the *dock* Gene

(A) Genomic structure of the *dock* gene. The exon–intron structure was determined by restriction mapping of cDNAs and genomic clones, polymerase chain reaction, complete cDNA sequence, and limited genomic sequence. The P element insertion sites for *dock*^{P1} and *dock*^{P2} are located 18 bp and 37 bp downstream of its first exon–intron boundary, respectively. The region deleted in *dock3* is shown in the hatched box. Closed boxes, coding regions; open boxes, noncoding regions.

(B) Comparison of Dock and human Nck amino acid sequences. Two closely spaced methionine codons could be used as translation initiation sites. Dock is closely related to human cytoplasmic protein Nck (see text). Identical amino acids are stippled.

(C) A comparison between the domain structure of Nck and Dock.

(D) Western blot of third instar larval extracts (equivalent to one larva per lane) probed with anti-human Nck antibody. A single band was detected at ~47 kDa in wild type, but not in homozygous *dock*^{P1}, *dock*^{P2}, and *dock*^{3}. Heat shock of hs-Dock flies in *dock*^{P1} background induced expression of the 47 kDa protein.

insertion sites (see Figure 5A). Rescue experiments established that this cDNA encodes the Dock protein. A transgene containing a heat-shock promoter driving the cDNA that encodes Dock was introduced into *dock*^{P1} mutants, and *dock* flies carrying the hs-Dock transgene were subjected to brief heat pulses throughout development. Rescue of both the R cell projection defects (data not shown; for rescue of R cell projection defects see below), and adult lethality (see Experimental Procedures for data) was observed and was heat shock dependent.

The *dock* cDNA contains an open reading frame that is predicted to encode a 410 amino acid polypeptide (Figure 5B). Dock contains three Src homology 3 (SH3) domains and one Src homology 2 (SH2) domain, and has extensive sequence similarity throughout to the human cytoplasmic protein Nck (Figure 5B) (Lehmann et al., 1990). The overall identity between Dock and human Nck is 43%. The identity within the SH3 and SH2 domains was higher: SH3-1 (63%), SH3-2 (54%), SH3-3 (49%), and SH2 (60%) (Figure 5C).

The structure and ligand-binding specificity of a number of SH2 and SH3 domains have been investigated in detail (e.g., Eck et al., 1993; Waksman et al., 1993; Songyang et al., 1994; Feng et al., 1994). These domains appear to adopt generalizable structures that permit structural predictions to be made about other SH2 and SH3 domains. The seven amino acids predicted to form the ligand-binding pocket of each Nck SH3 domain are identical in Dock except for a single phenylalanine to tyrosine change in both SH3-1 and SH3-2. In the SH2 domain, Dock and Nck contain the two key arginine residues that contact the phosphotyrosine, and the six amino acids predicted to confer much of the ligand-binding specificity. All residues are identical except for the substitution of histidine for glutamine at one position. In Nck glutamine confers a preference for an acidic amino acid in the ligand two amino acids C-terminal to its phosphotyrosine. Since histidine at the equivalent position in the N1 SH2 domain of PLC-γ also selects an acidic amino acid, the glutamine to histidine change is likely to be conservative. On the basis of the high overall sequence identity and the conservation of key residues between their SH3 and SH2 domains, we propose that Dock and Nck will show related ligand-binding specificities and functions.

A rabbit polyclonal antibody raised against the three SH3 domains of human Nck recognizes a single band of ~47 kDa, the predicted size for Dock, in wild-type larval extracts (Figure 5D). This band was missing in extracts of *dock* mutants and restored by the hs-Dock transgene in response to heat shock treatment, establishing the 47 kDa band as the product of the *dock*

locus. Dock protein was also detected by Western blot in embryos, larval eye/brain complexes, and adult heads and bodies (data not shown).

dock Is Required in Developing R Cells

Genetic mosaic analysis indicated that the *dock* gene functions in the eye. However, this analysis did not allow us to distinguish between a requirement in R cells and subretinal glial cells since both cell types are generated in the retinal primordium. To explore whether *dock* is required in R cells, we expressed *dock* cDNA under the glass-responsive promoter pGMR (Hay et al., 1994), which is expressed in R cells but not glia. This promoter also drives expression in other undifferentiated cells in the columnar epithelium of the disc, which are unlikely to play a role in R cell connectivity. pGMR-dock rescued *dock* R cell projection defects (Figure 6). R1–R6 growth cone termination sites in the lamina were largely indistinguishable from wild type, indicating that expression of Dock protein in R cells was sufficient for the proper targeting of R1–R6 axons. The R7 and R8 terminal field was substantially rescued as well. Minor defects in the array were occasionally observed and may reflect an independent requirement for *dock* in optic lobe neurons. pGMR-dock also rescued the defects in packing of R cell axon fascicles in the *dock* optic stalk (data not shown). This suggests that the packing defects are not due to a requirement for *dock* in the glia, but may reflect inappropriate interactions between glia and mutant axons. In addition, expression of Dock in postmitotic neurons under the control of the neuron-specific *elav* promoter (Yao and White, 1994) completely rescued both *dock* R cell projection defects and lethality (data not shown), further establishing the role of *dock* in neurons.

dock Is Expressed in R Cell Growth Cones

The P element in $dock^{P1}$ is an enhancer trap containing the E. coli gene encoding LacZ, under the control of a weak promoter. Hence, its expression may reflect the pattern of the endogenous gene. Consistent with the genetic mosaic studies and the pGMR transgene rescue experiments, LacZ expression was observed in differentiating R cells but not glia (data not shown). The expression pattern and subcellular localization of Dock protein was determined in cryostat sections of third instar eye/brain complexes stained with the human anti-Nck antibody used for Western blots shown in Figure 5D. In wild-type sections (Figure 7), uniform staining was seen in the lamina neuropil sandwiched between layers of glial cells (cf. Figures 7A and 7C). At this stage of development, the lamina neuropil largely consists of a plexus of R1–R6 growth cones as lamina interneurons have just begun to extend axons that will form a punctate array of thin processes. Thus, the uniform staining in the lamina neuropil observed corresponds largely, if not exclusively, to R cell growth cones (see Figure 7B). Immunoreactivity also was observed in a uniform pattern in the medulla neuropil. Weak staining also is seen in R cell bodies and medulla neurons, making it likely that, in addition to R7 and R8, medulla neurons contribute to staining in the medulla neuropil. No staining was seen in $dock^{P1}$ mutants (Figure 7D) indicating that the distribution of immunoreactivity reflects Dock protein expression. We conclude that Dock is expressed in R cells and localizes to R cell growth cones.

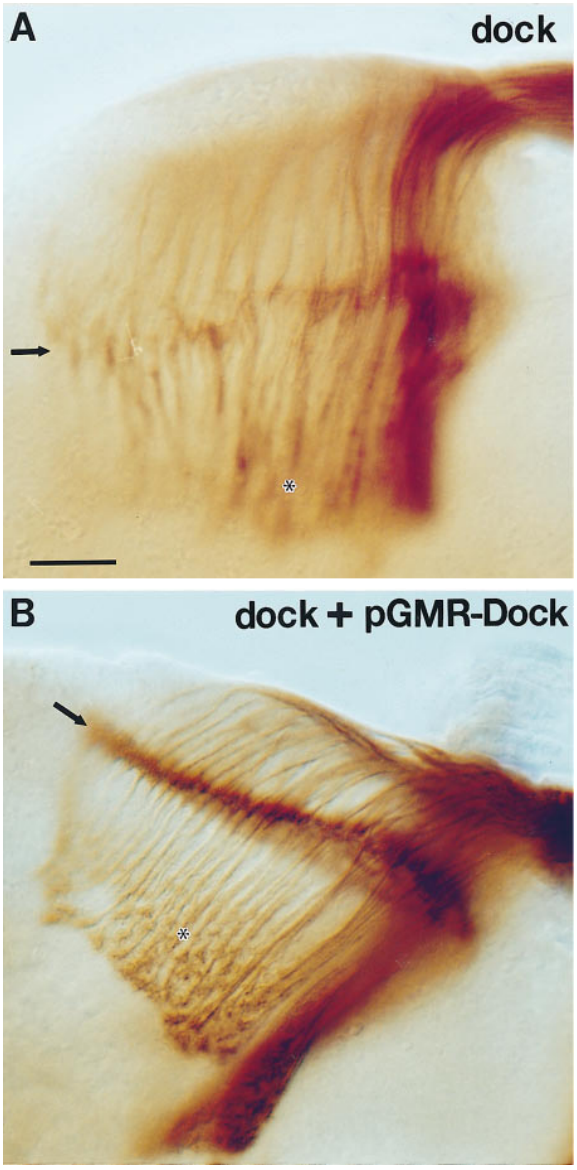

Figure 6. Expression of Dock in R Cells Rescues *dock* R Cell Projection Defects

R cell projections in whole-mount preparations of third instar larvae visualized using MAb24B10. (A) $dock^{P1}$ homozygote. (B) A $dock^{P1}$ homozygote (a sibling of the animal in [A]) containing a transgene that drives expression of Dock cDNA in the developing R cells but not in glia or optic lobe neurons. Note that an even plexus of terminals in the lamina (arrow) is restored, and the array of projections in the underlying medulla (asterisk) is nearly wild type (cf. Figure 1A). The dense staining of fibers at right in both preparations is a result of the perspective shown here in which the curved lamina and medulla project out of the plane of the page. Scale bar, 20 μm.

Discussion

We have shown that Dock function is required for R cell axon guidance and targeting. The structure of Dock

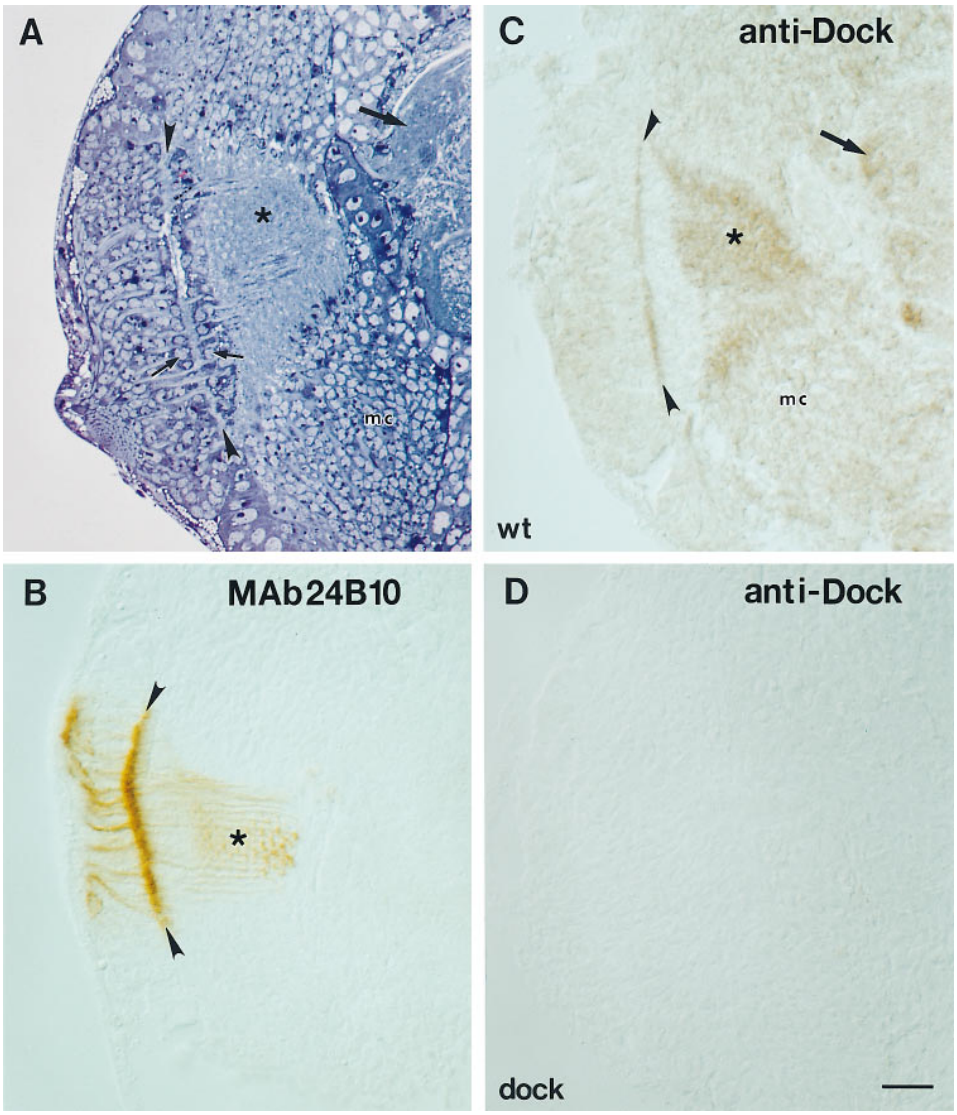

Figure 7. Dock Distribution in Developing Optic Lobes

Photomicrographs of four frontal sections located in approximately the same plane of the third-instar optic lobes. (A) Semithin section of a wild-type optic lobe stained with toluidine blue. (B) Cryostat section of a wild-type optic lobe stained with MAb24B10. R1–R6 cell growth cones form the darkly stained stripe in the lamina neuropil, and the R7 and R8 neurons terminate in regularly spaced rows within the medulla neuropil. (C) Cryostat section of a wild-type optic lobe stained with anti-human Nck antiserum to detect Dock expression (here designated as anti-Dock; see Figure 5D). Comparison of (B) and (C) reveals that strongest Dock expression is detected in the neuropils of the lamina and medulla, where the growth cones of photoreceptor axons terminate. Labeling is visible at the edges of the central neuropil (arrow), and weak staining of medulla neurons (mc, medulla cortex) is also seen. (D) No Dock expression is detected in cryostat sections of *dock* mutant optic lobes. Arrowheads, lamina; asterisk, medulla neuropil; large arrow, central brain neuropil; small arrow, lamina glial cells. Scale bar, 20 μm.

suggests that it functions as an adapter protein linking tyrosine kinases to intracellular signaling pathways (Pawson et al., 1993). Dock is homologous to the human Nck protein, comprised of 3 SH3 domains and 1 SH2 domain, and colocalizes with R cell terminals in the developing target region. Although its function in mammalian cells is not known, human Nck binds through its SH2 domain to a number of receptor tyrosine kinases (Li et al., 1992, Nishimura et al., 1993) and tyrosine kinases that function downstream of receptors, such as the focal adhesion kinase (FAK) associated with integrins (Schlaepfer et al., 1994). Recent evidence has implicated receptor tyrosine kinases as guidance cue receptors in a number of systems (see Introduction). The identification of Dock as a regulator of R cell growth cone guidance indicates that tyrosine kinase signaling also is involved in R cell axon guidance.

The signal transduction machinery that links RTKs and other receptors using tyrosine kinases to the cytoskeletal changes underlying guidance are unknown. The localization of the Dock adapter protein makes it an attractive candidate for functioning in such a signaling pathway. Dock protein is concentrated in the R cell growth cone, where guidance cues are sensed leading to changes in the cytoskeleton. Genetic mosaic analysis and rescue by R cell expression of Dock demonstrate

that *dock* functions in these axons. Although the effector pathways involved in the cytoskeletal changes underlying growth cone guidance are not well understood, Nck has been shown to physically interact through its SH3 domains with potential downstream components of such pathways. These include Sos (Hu et al., 1995), an activator of Ras, and mPAK3 (Bagrodia et al., 1995), a serine/threonine kinase activated by murine CDC42 and Rac, rho-family GTPases involved in regulating cytoskeletal organization (see Introduction).

dock mutants show defects in pathfinding, fasciculation, target selection, and topographic mapping. This may reflect a role for Dock in responding to a single cue, disruption of which leads to a cascade of defects. Alternatively, Dock may function in the response to multiple cues. Studies on GRB-2 demonstrate how adapter proteins can couple multiple signals to a single cellular process. In mammalian cells, GRB-2 links different growth factor receptors to a common Ras pathway regulating cellular proliferation (Downward, 1994). By analogy, Dock could couple different guidance cue receptors to a limited set of common signaling pathways regulating cytoskeletal changes in the growth cone.

The existence of chemoaffinity molecules, in the form of guidance cues and receptors, has recently been demonstrated (reviewed by Keynes and Cook, 1995; Garrity and Zipursky, 1995). Elucidating their roles in axon guidance and targeting will require identifying the signal transduction cascades through which they control growth cone motility. We have identified a growth cone–localized adapter protein, Dock, that plays an important role in axon guidance and targeting and is a candidate for transducing information from guidance cue receptors that cause cytoskeletal changes. Dock provides a powerful tool for biochemical and genetic dissection of the signal transduction machinery that allows axons to reach their specific targets. Given the extensive sequence conservation between Dock and Nck, we propose that Nck plays a similar role in forming precise patterns of neuronal connections in vertebrates.

Experimental Procedures

Genetics

Genetic markers and chromosomes are described in Lindsley and Zimm (1992). P element–induced lethal mutations on the second chromosome (Karpen and Spradling, 1992; Torok et al., 1993) were provided by the Berkeley Drosophila Genome Project (BDGP) and Bloomington Drosophila Stock Center and maintained over In(2-LR)GlaBcElp. In the BDGP mutant collections, $dock^{P1}$ corresponds to *l(2)04723* and $dock^{P2}$ to *l(2)13421*. $dock^{P1}$ was mapped to bands 21D3-D4 by the BDGP. Deficiency $(2L)ast^{2}$ uncovers *dock*. Mobilization of the $dock^{P1}$ [ry^{+}] P element was performed as described (Ebens et al., 1993). DNA from [ry^{-}] revertants was examined by polymerase chain reaction as described (Cheyette et al., 1994). The sequence surrounding the $dock^{3}$ deletion was amplified by polymerase chain reaction and sequenced. In $dock^{3}$ a 510 bp region extending from bp 48 in the first exon to the $dock^{P1}$ insertion site is deleted and is replaced by a 15 bp sequence not found in wild-type *dock*.

Genetic Mosaic Analysis

Genetic mosaics were induced by X-irradiation of first instar larvae (Ashburner, 1989). To analyze the projection of all R cells, *yw;* $dock^{P1}$/*CyO* females were mated to *w* males carrying a P[w^{+}] transgene inserted at 30C (Xu and Rubin, 1993). Mosaic clones were identified as patches of white eye tissue in the straight-winged progeny. Cryostat sections were prepared, mutant patches were photographed to record their location in the eye, and sections were stained with MAb24B10. To assess R1–R6 projection specifically, *w;* $dock^{P1}$/*CyO* females were mated to *w; P[*w^{+}*]30C/+; P[neo, Rh1-LacZ]/+* males. Mosaic clones were identified as above and cryostat sections were stained with anti-LacZ antibody to detect the expression of Rh1-LacZ, a marker specific for R1–R6 cells. Genetic mosaics to assess R cell fate were induced using both X-irradiation and FLP-mediated recombination (Xu and Rubin, 1993). Eye sections were performed as described (Van Vactor et al., 1991).

Rescue

Germ-line transformation of Drosophila used standard methods (Spradling and Rubin, 1982). Heat shock rescue of lethality was performed by mating *yw;* $dock^{P1}$/*CyO* females to P[*w*+, hs-*dock*] line 1/Y; $dock^{P1}$/*In(2LR)GlaBcElp* males. Male progeny from this cross lacked the P[*w*+, hs-*dock*] transgene; female progeny carried one copy. Progeny were maintained at 25°C. Heat shocks were 37°C for 30 min every 12 hr. Rescue was assessed by comparing males and females of each genotype raised with and without heat shock. The genotypes of adult females were as follows: (without heat shock) *Gla*/*CyO*, 2 (1%); *Gla*/$dock^{P1}$, 59 (43%); *CyO*/$dock^{P1}$, 66 (49%); and $dock^{P1}$/$dock^{P1}$, 9 (7%); and (with heat shock) *Gla*/*CyO*, 5 (2%); *Gla*/$dock^{P1}$, 129 (39%); *CyO*/$dock^{P1}$, 113 (34%); $dock^{P1}$/$dock^{P1}$, 81 (25%). The genotypes of adult males were as follows: (without heat shock) *Gla*/*CyO*, 11 (7%); *Gla*/$dock^{P1}$, 71 (46%); *CyO*/$dock^{P1}$, 68 (44%); $dock^{P1}$/$dock^{P1}$, 3 (2%); and (with heat shock) *Gla*/*CyO*, 23 (9%); *Gla*/$dock^{P1}$, 135 (50%); *CyO*/$dock^{P1}$, 112 (41%); and $dock^{P1}$/$dock^{P1}$, 1 (<1%). P[*w*+, hs-*dock*] line 1 also rescued lethality when present in males; a second independent P[*w*+, hs-*dock*] insertion behaved similarly. Rescue of photoreceptor projections in the larvae by P*[w+, hs-dock]* line 1 was performed by mating *yw;* $dock^{P1}$/*In(2LR)GlaBcElp* females to P[*w*+, hs-Dock]/Y; $dock^{P1}$/*In(2LR)GlaBcElp* males. P*[w+,* p*GMR-dock]* rescue of larval projections was performed using *yw;* $dock^{P1}$/*In(2LR)GlaBcElp*; P[*w*+, p*GMR-Dock*(line 1)]/+ flies and *yw;* $dock^{P1}$ P[*w*+, p*GMR-Dock* (line 2)]/*GlaBcElp* flies. Larvae carrying the line 1 transgene were identified by immunostaining with anti-human Nck to detect the pGMR-driven expression of Dock prior to staining with MAb24B10.

Histology

Immunostaining of whole-mount preparation (Van Vactor et al., 1991) and adult and larval cryostat sections (Fujita et al., 1982) was essentially as described. BrdU labeling was a modified version of Ito and Hotta (1992). The preparation of toluidine blue–stained semithin sections (1 and 2 μm) for light microscopy and of ultrathin sections for electron microscopy (Zeiss EM 10) was as described (Salecker and Boeckh, 1995). Details of these protocols are available upon request.

Molecular Biology

Genomic DNA next to the P element insertion site in $dock^{P1}$ was isolated by plasmid rescue and subsequently used to screen a Drosophila genomic library in λEMBL3 (Tamkun et al., 1992). Five independent clones were isolated that cover an ~30 kb region. A 9 kb SalI-BamHI genomic fragment spanning the P element insertion site was used to screen a λgt 10 eye disc-specific cDNA library (a gift from G. Rubin) and a 0–24 hr embryo cDNA library in λEXLX(+) (Palazzolo et al., 1990). Dock cDNAs were isolated from both libraries. Two other classes of cDNAs were also isolated. Type A cDNA was mapped about 300 bp 5′-upstream of the 5′ end of the *dock* transcript, and type B cDNA was located within the first intron of the *dock* gene. Recombinant DNA techniques used were as described (Sambrook et al., 1989). Sequencing was done using the Sequenase kit (United States Biochemical). DNA and protein databases were searched for homologous sequences using the BLAST program (Altschul et al., 1990). Protein sequences were aligned using GeneWorks 2.1 (IntelliGenetics). Rescue constructs were made by insertion of the longest 3.7 kb dock cDNA into the pGMR and CaspeR-hs transformation vectors.

Western Blot Analysis

Western blot analysis was performed as described (Biggs and Zipursky, 1992). Details of anti-human Nck antibody are available upon request.

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GenBank Accession Number

The GenBank accession number for Drosophila *dock* is U57816.

Note Added in Proof

Clemens et al. (personal communication) have independently identified Dock as a protein that interacts with a Drosophila tyrosine phosphatase, dPTP61F, in a yeast two-hybrid screen.