The cytoskeletal regulator Genghis khan is required for columnar target specificity in the *Drosophila* visual system

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SUMMARY

A defining characteristic of neuronal cell type is the growth of axons and dendrites into specific layers and columns of the brain. Although differences in cell surface receptors and adhesion molecules are known to cause differences in synaptic specificity, differences in downstream signaling mechanisms that determine cell type-appropriate targeting patterns are unknown. Using a forward genetic screen in *Drosophila*, we identify the GTPase effector Genghis khan (Gek) as playing a crucial role in the ability of a subset of photoreceptor (R cell) axons to innervate appropriate target columns. In particular, single-cell mosaic analyses demonstrate that R cell growth cones lacking Gek function grow to the appropriate ganglion, but frequently fail to innervate the correct target column. Further studies reveal that R cell axons lacking the activity of the small GTPase Cdc42 display similar defects, providing evidence that these proteins regulate a common set of processes. Gek is expressed in all R cells, and a detailed structure-function analysis reveals a set of regulatory domains with activities that restrict Gek function to the growth cone. Although Gek does not normally regulate layer-specific targeting, ectopic expression of Gek is sufficient to alter the targeting choices made by another R cell type, the targeting of which is normally Gek independent. Thus, specific regulation of cytoskeletal responses to targeting cues is necessary for cell type-appropriate synaptic specificity.

KEY WORDS: *Drosophila*, Axon targeting, Photoreceptor

INTRODUCTION

Sequences of genetically programmed developmental decisions play an important role in determining the complex architecture of the adult brain. At the level of single neurons, these decisions produce cellular morphologies that are characteristic of each cell type and reflect specific guidance and targeting choices made by both axons and dendrites. Although many molecules have been identified that affect synaptic specificity (Sanes and Yamagata, 2009), the molecular mechanisms that allow closely related cell types to make distinct targeting decisions in vivo are almost completely unknown. Here, we examine these mechanisms in the *Drosophila* visual system, and demonstrate that cell type-specific utilization of a cytoskeletal regulatory pathway plays a crucial role in the targeting choices made by a subset of R cells.

The visual systems of many animals are characterized by a broad organization of axons and dendrites into columns and layers (Sanes and Zipursky, 2010). Columns are organized into retinotopic arrays to allow the brain to process, in parallel, information from different points of visual space. Within each column, layers process different qualities of light and reflect different combinations of pre- and post-synaptic inputs. Closely related cell types make different choices with respect to these architectural features. How axons can be genetically programmed to innervate both the correct column and the appropriate layer, making cell type-specific targeting decisions, remains an open question.

Photoreceptor axons in *Drosophila* display both columnar and layer-specific targeting. The *Drosophila* visual system comprises the compound eye and four optic ganglia: the lamina, the medulla, the lobula and the lobula plate (Meinertzhagen and Hanson, 1993). Each facet of the eye, or ommatidium, contains eight R cells, which can be divided into three distinct types. R1-R6 cells, the outer photoreceptors in each ommatidium, project their axons into the lamina. Each of these axons innervates a specific column of post-synaptic target neurons, reconstructing a topographic map of the visual world in the brain. By contrast, the inner R7 and R8 cells project axons that terminate in two distinct layers in the medulla, making synaptic connections across multiple layers (Takemura et al., 2008). Cell ablation studies and genetic manipulations demonstrate that the targeting of R1-R6 axons to specific targets is both genetically hard-wired and dependent on interactions amongst afferent axons, rather than on specific interactions between afferents and their particular targets (Clandinin and Zipursky, 2000; Hiesinger et al., 2006). The layer-specific targeting of R7 and R8 cells, however, develops in a precise temporal sequence and is dependent upon specific interactions with presumed post-synaptic cells and intermediate targets (Senti et al., 2003; Shinza-Kameda et al., 2006; Ting et al., 2005; Tomasi et al., 2008). Thus, the cellular mechanisms that underlie columnar versus layer-specific targeting in this system are at least partially distinct.

Both histological and behavioral screens have identified many genes involved in the targeting of R1-R6 axons to appropriate columns within the lamina and in the targeting of R7 and R8 axons to appropriate medulla layers (Berger et al., 2008; Mast et al., 2006; Ting and Lee, 2007). For example, the classical cadherin N-cadherin mediates interactions between pre- and post-synaptic cells that are required for the targeting choices made by R1-R6 cells, and by R7 cells (Ting et al., 2005; Lee et al., 2001; Prakash et al., 2005; Yonekura et al., 2007). The receptor tyrosine phosphatases Lar and Ptp69D, as well as the Lar-interacting protein Liprin-α, stabilize these connections (Newsome et al., 2000a; Clandinin et al., 2001;

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Maurel-Zaffran et al., 2001; Choe et al., 2006; Hofmeyer et al., 2006; Hofmeyer et al., 2009; Prakash et al., 2009). Dynamic regulation of the expression of N-cadherin and Lar is also crucial to the layer-specific targeting decisions of R7 and R8 (Petrovic and Hummel, 2008). The non-classical cadherin Flamingo (Starry night – FlyBase) is required for appropriate topographic mapping, the layer-specific targeting of R8, and the columnar targeting choices made by R1-R6 (Chen and Clandinin, 2008; Lee et al., 2003; Senti et al., 2003). Intriguingly, for all genes where mutant phenotypes have been assessed in R1-R6, R7 and R8 photoreceptors, both columnar targeting by R1-R6, as well as layer-specific targeting by R7 and/or R8, were affected, even though the selection criteria under which they were initially identified were specific to only one of these phenotypes. Thus, these two different types of targeting decisions clearly use many of the same molecular components. However, it remains unclear whether additional components are required specifically for either the columnar targeting decisions of R1-R6 or the layer-specific targeting of R7 and R8.

Rho family GTPases regulate many stages of neuronal growth, including growth cone motility, axonal migration and dendritic spine morphogenesis (Govek et al., 2005). In the *Drosophila* nervous system, these GTPases regulate axon growth and guidance (Luo et al., 1996; Ng and Luo, 2004; Ng et al., 2002; Scott et al., 2003). In the *Drosophila* visual system, Rac is an upstream activator of the Dock-Pak signaling pathway, affecting R cell axon targeting to the lamina and the medulla (Hakeda-Suzuki et al., 2001; Hing et al., 1999; Newsome et al., 2000b). However, our understanding of the links between small GTPases and specific axonal targeting decisions remains incomplete, and only a limited set of GTPase effector proteins have been identified (Koh et al., 2006). One of these, Genghis khan (Gek), the *Drosophila* homolog of mammalian Cdc42-binding protein kinase alpha [CDC42BPα (or MRCKα); myotonic dystrophy-related Cdc42-binding kinase (MRCK-1) in *C. elegans*], was originally identified using biochemical approaches to identify effectors of the small GTPase Cdc42 (Leung et al., 1998; Luo et al., 1997). These studies demonstrated that Gek binds specifically to activated Cdc42, that this interaction increases the kinase activity of Gek in vitro, and that Gek activity alters actin cytoskeletal organization (Luo et al., 1997). Finally, genetic studies of asymmetric cell division and epithelial morphogenesis in *C. elegans* have demonstrated that MRCK-1 functions downstream of Cdc42 in vivo (Gally et al., 2009; Kumfer et al., 2010). However, the function of Gek in neurons is unknown. Here, we examine the role of Gek in controlling axon outgrowth and target selection in all subtypes of R cells in the *Drosophila* eye.

MATERIALS AND METHODS
Animal husbandry and genetics
Flies were maintained at 25°C on standard medium. Chemical mutagenesis was performed using ethylmethanesulfonate (EMS) under standard conditions (Clandinin et al., 2001). Eye-specific mosaic animals were generated using the FLP/FRT system, in which the expression of FLP is controlled by a fragment of the *eyeless* promoter in eyFLP or ey3.5FLP (Newsome et al., 2000a; Chotard et al., 2005). We used R11, a recessive cell-lethal mutation, to reduce the size of the twin spot (Chotard et al., 2005). MARCM studies were performed by expressing Gal4 under the control of the pan-neural *elav* promoter, and FLP was expressed under the control of the heat-shock promoter, as previously described (Prakash et al., 2005). The following alleles were used: *gek^{NP5192}* (this work), *gek^{omb1080}* (this work), *flamingo^{e59}*, *N-cadherin^{Δ14}*, *Lar^{2127}* and *Cdc42^4* (Prakash et al., 2005; Maurel-Zaffran et al., 2001; Fehon et al., 1997; Usui et al., 1999).

Molecular genetics
A *gek* cDNA was obtained from EST clone LD24220 (Berkeley Drosophila Genome Project). Domain deletions were generated by PCR. All constructs were expressed using pUAST (Brand and Perrimon, 1993).

Generation of a Gek antibody
Anti-Gek antiserum was obtained for a rabbit immunized with a 6-His fusion protein containing 406 amino acids (433-838) of the coiled-coil domain region of Gek. The antiserum was purified against nitrocellulose containing the full-length protein and used at a dilution of 1:25.

Immunohistochemistry
Larval, pupal and adult brains were dissected and labeled as previously described (Clandinin et al., 2001). For structure-function studies of Gek, two independent insertions of each construct were scored for all constructs except *Gek-ΔCC*, where only a single insert was obtained. Between 10 and 37 larval brains of each genotype were examined. The following antibodies were used: mouse anti-Chaoptin mAb24B10 [1:25; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Repo (1:100; DSHB), goat anti-HRP FITC (1:100; Jackson), rat anti-mouse CD8 (1:100; Invitrogen), rabbit anti-β-galactosidase (1:100; Kappel), goat anti-mouse IgG Alexa Fluor 594 (1:200; Invitrogen), goat anti-rabbit IgG Alexa Fluor 488 (1:200; Invitrogen) and goat anti-rat IgG Alexa Fluor 488 (1:200; Invitrogen). The phospho-MRLC antibody was obtained from Cell Signaling Technology (Beverly, MA, USA) and used at 1:10.

Imaging
Images were acquired on a Leica TCS SP2 AOBS confocal microscope using a 40× NA 1.25 lens or a 100× NA 1.4 lens and were rendered using Imaris (Bitplane) and mounted using Photoshop (Adobe). Axon images in Figs 5 and 6 were also deconvolved using Huygens (SVI).

RESULTS
A forward genetic screen identifies a gene specifically required for R1-R6 axon targeting
To identify mutations affecting photoreceptor axon targeting, we undertook a forward genetic screen based on a behavioral assay in which we assessed the ability of adult flies to see motion (Clandinin et al., 2001). In this approach, eye-specific somatic mosaic flies were made homozygous for EMS mutagenized chromosomes, while the rest of the fly remained heterozygous (and thus phenotypically wild type) (Newsome et al., 2000a; Stowers and Schwarz, 1999). These animals were then screened using an optomotor behavioral assay. Screening ~15,000 mutagenized copies of the right arm of chromosome 2 identified 215 optomotor defective (*omb*) lines. We then examined the ability of mutant R cell axons to reach their post-synaptic targets in each of these lines, and identified a single mutation, designated *omb1080*, that caused specific defects in target selection.

To investigate the targeting of photoreceptor subsets in adult animals, we first examined the columnar targeting of R1-R6 axons within the lamina using the R cell-specific antigen Chaoptin. In control animals, R1-R6 cells choose post-synaptic targets that are arranged in a characteristic pattern, forming a reiterated array of barrel-like structures designated cartridges (Fig. 1A-C). In most of the target field, each cartridge contains exactly six R1-R6 terminals, reflecting a remarkably precise pattern of target selection. By contrast, in animals in which R cells were homozygous for *omb1080*, or bore *omb1080* in trans to a deficiency, the overall array of cartridges was highly disorganized, with individual cartridges displaying variable numbers of axon terminals (Fig. 1D-F).

To examine the ganglion- and layer-specific targeting of all R cell axons we labeled R cells in eye-specific somatic mosaic animals using either the R1-R6-specific marker *Rh1-lacZ*, the R7-specific marker *Rh4-lacZ*, or the R8-specific marker *Rh6-lacZ* (Fig. 1G-N).
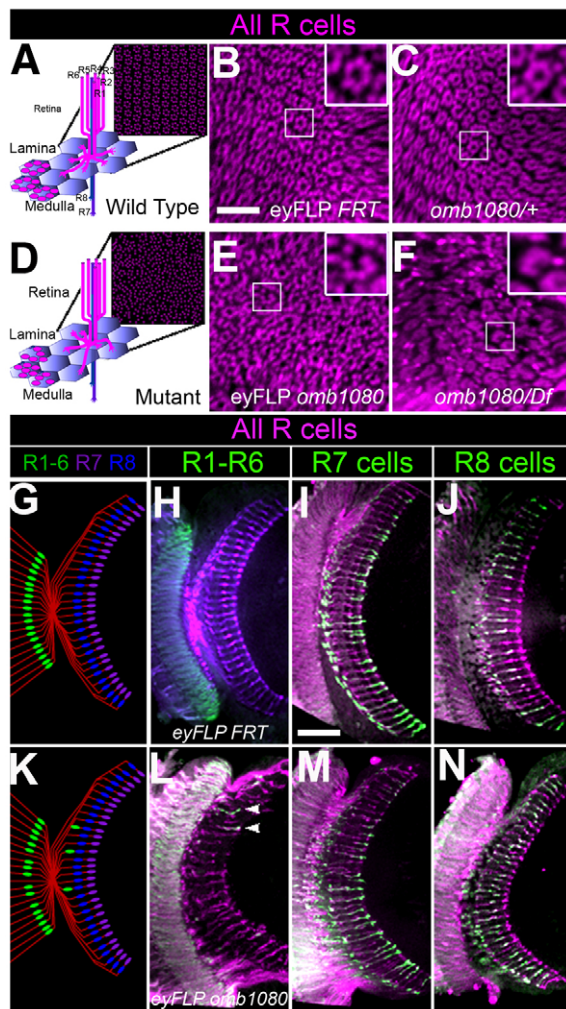
Fig. 1. Gek specifically affects the targeting of R1-R6 photoreceptors. (**A**) Wild-type *Drosophila* R cell axons from the retina project down into the brain in a common fascicle before extending laterally to innervate specific target columns, forming an array of barrel-like cartridges, each containing six terminals (stained with the R cell-specific mAB24B10, magenta). (**B**) Adult R cell axon terminals homozygous for a control chromosome. (**C**) *omb1080*/+ animals. (**D**) An *omb1080* homozygote. (**E**) Adult R cell axon terminals homozygous for *omb1080*. (**F**) R cell axon terminals in an *omb1080/Df* animal. Insets in B,C,E,F display single cartridges. (**G-J**) In adult control eye-specific mosaic animals, R1-R6 cells expressing *Rh1-lacZ* (green) targeted to the lamina (H), whereas R7 axons labeled with *Rh4-lacZ* (green) and R8 axons labeled with *Rh4-lacZ* targeted to single layers in the medulla (I,J). (**K-N**) Somatic mosaic animals homozygous for *omb1080* displayed rare instances in which R1-R6 cells projected into the medulla (L, arrowheads), whereas R7 (M) and R8 (N) targeting was unaffected. Scale bars: 30 μm.

R1-R6 axons homozygous for *omb1080* almost invariably targeted to the lamina, with only rare fibers extending into the medulla (Fig. 1H,I). Thus, ganglion-specific targeting of R1-R6 axons was only subtly affected by *omb1080*. More strikingly, layer-specific targeting of R7 and R8 axons was virtually unaffected (Fig. 1I,J,M,N). In particular, 96% (*n*=432) of mutant R7 axons and 99% (*n*=503) of mutant R8 axons innervated the correct target layers in the medulla, as compared with 100% of control R7 and R8 axons (*n*=396 and *n*=580, respectively). Thus, *omb1080* acts cell type specifically,

affecting only the ability of R1-R6 axons to choose appropriate targets within the lamina. As the optomotor response is specifically dependent upon the function of R1-R6 cells, these defects are sufficient to explain the behavioral phenotype of *omb1080*.

omb1080 affects the serine-threonine kinase *genghis khan*

To identify the gene affected by *omb1080* we used single nucleotide polymorphism (SNP) and deficiency mapping (supplementary material Fig. S1). As *omb1080* was homozygous viable, these studies used direct histological examination of the targeting phenotype, and revealed that *omb1080* corresponds to a tryptophan-to-stop amino acid change at position 374 in the *genghis khan* (*gek*) gene (Luo et al., 1997) (supplementary material Fig. S1). A second allele, *gek^{NP5192}*, was subsequently obtained and consists of a *Gal4* insert (in inverse orientation) just before the start codon (supplementary material Fig. S1). Both of these alleles were homozygous viable, caused highly penetrant R cell targeting phenotypes, and corresponded to strong reduction-of-function alleles (supplementary material Fig. S2; data not shown).

The predicted structure of Gek includes a serine/threonine kinase (STK) catalytic domain, a coiled-coil (CC) domain, a cysteine-rich region (CR), a pleckstrin homology (PH) domain, a citron homology (CH) domain and a Cdc42/Rac interactive binding (CRIB) domain (Luo et al., 1997) (supplementary material Fig. S1). This protein is highly similar in domain organization and primary sequence to the human CDC42BPα and CDC42BPβ (MRCKα and MRCKβ) isoforms (Luo et al., 1997) (supplementary material Fig. S1).

Gek is expressed on R cell axons and growth cones

To determine where Gek protein is expressed, we generated an affinity-purified polyclonal antibody directed against a bacterially expressed fragment of the coiled-coil domain. Using this antiserum, we examined Gek expression in the developing visual system at the third instar larval stage, at an early stage of pupal development [16 hours after puparium formation (APF)] when R7 and R8 axons project towards their intermediate targets, and at the mid-pupal stage when R1-R6 axons are extending to their targets (30 hours APF). In wild-type animals at the third larval stage, Gek expression was observed in R cell axons extending to the lamina, in growth cones within the lamina plexus, as well as within the medulla neuropil (Fig. 2A-D). Weak staining was seen in the R cell bodies in the eye disc (data not shown). No distinct staining pattern was detected in animals homozygous for *gek^{NP5192}*, and photoreceptor clones homozygous for *gek^{NP5192}* lost Gek expression within the clone, demonstrating that the immunoreactivity we observed reflected endogenous Gek protein (Fig. 2E-H; data not shown). Early in pupal development, Gek was strongly expressed in R cell axons in both the lamina and the medulla (Fig. 2I-L). Similarly, at 30 hours APF, Gek was highly expressed on R1-R6 growth cones within the lamina plexus, and was also expressed in many other neurons in other optic ganglia (Fig. 2M-P). Thus, Gek is expressed in R cell growth cones as target selection occurs.

Loss of *gek* function disrupts R cell growth cone targeting to the lamina

To determine when Gek acts during R cell axon targeting, we first examined third instar larvae. In wild-type animals, R cell axon fascicles project through the optic stalk, forming a retinotopic array
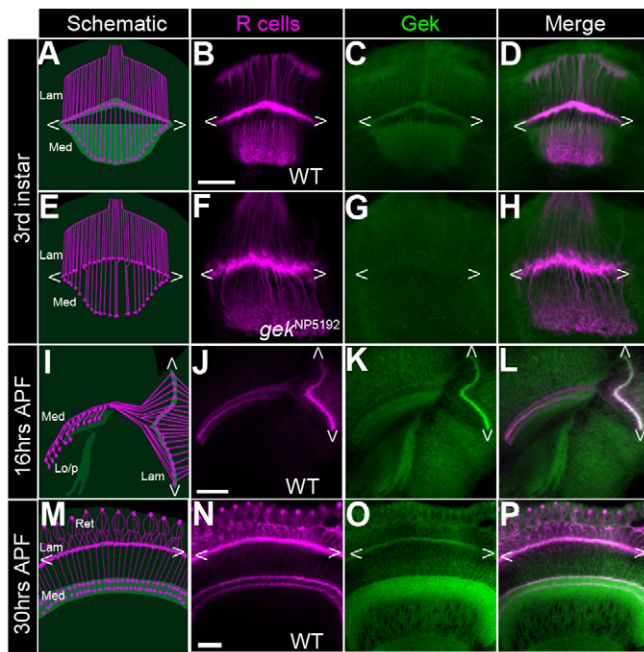
Fig. 2. Gek is enriched in photoreceptor growth cones. (**A-D**) At the third larval stage in wild type, Gek protein (green) colocalized with R cell axons (mAb24B10, magenta) in both lamina and medulla. (**E-H**) No specific pattern of Gek expression was detected in a mutant *gek^NP5192*/*Df* brain. (**I-L**) Gek expression in wild type 16 hours after puparium formation (APF). (**M-P**) Gek expression in wild type at 30 hours APF. Ret, retina; Lam, lamina; Med, medulla; Lo/p, lobula and lobula plate. Chevrons delineate the lamina plexus Scale bars: 20 µm.

(Fig. 3A,B). At this stage, R1-R6 axons stop in the first optic ganglion, the lamina, whereas R7 and R8 axons project through the lamina plexus to terminate in the medulla. Within the lamina, R cell axon fascicles above and below the plexus are evenly spaced, and within the plexus R1-R6 cell growth cones form a uniform band of staining. By contrast, in transheterozygous animals bearing either *gek^NP5192* or *gek^omb1080* in trans to a small deficiency (Fig. 3C-E; data not shown), R cell axon fascicles above the lamina plexus were occasionally clumped and the lamina plexus appeared broken and varied in thickness. This phenotype was observed in all brains examined. Additionally, we occasionally observed bundles of axons extending into the medulla that were thicker than normal. Animals homozygous for either *gek^omb1080* or *gek^NP5192*, or transheterozygous for both alleles, displayed indistinguishable

phenotypes (data not shown). Similar, but weaker, phenotypes were also observed in somatic mosaic animals in which only R cells were made homozygous mutant using ey3.5FLP (Fig. 3E). These weaker phenotypes are consistent with Gek playing a specific role in R cell targeting during pupal development (see below), but might also reflect some perdurance of Gek protein in these mosaic clones.

As thickened bundles of axons extending into the medulla can be indicative of R1-R6 cell axons failing to stop in the lamina plexus, we directly labeled R2-R5 axons using *ro-lacZ^tau* (Callahan and Thomas, 1994; Heberlein and Rubin, 1990; Garrity et al., 1999). Consistent with previous observations, in control flies and *gek* heterozygotes bearing this construct, only rare R2-R5 cells extended into the medulla (Fig. 3F-I). In *gek* transheterozygous animals, we observed slightly increased numbers of R2-R5 cell axons projecting into the medulla (Fig. 3J; data not shown). However, overall, R2-R5 axons extending through into the medulla were rare, even in the most severely affected brains, indicating that virtually all R1-R6 axons stopped in the lamina in *gek* mutant animals.

To exclude the possibility that Gek indirectly affects targeting by regulating cell fate decisions, we stained mutant eye discs for the R7-specific Prospero protein or the R8-specific protein Senseless (Kauffmann et al., 1996; Frankfort et al., 2001). No transformations of R1-R6 neurons into R7 or R8 neurons were observed (supplementary material Fig. S3). We also examined the organization of lamina neurons in mutant flies using the pan-neural marker Elav, and found no differences between control and mutant animals (supplementary material Fig. S4). Finally, we assessed the organization of glial cells using Repo. In *gek* homozygous mutant animals, glia migrated normally into the lamina and were only subtly disorganized (supplementary material Fig. S4), which is likely to be a secondary consequence of the clumping of R1-R6 axon terminals in the lamina plexus. Thus, Gek does not cause errors in R cell axon targeting through indirect effects on target neuron or glial development.

Gek kinase activity is required for Gek function in R cell axons

To examine the function of each Gek domain, we attempted to rescue the *gek* mutant phenotype seen at the third larval stage using UAS-Gek constructs bearing either deletions of, or alterations in, individual domains (Fig. 4A). As controls, we expressed a full-length *gek* transgene (*GekFL*) specifically in the eye, under the control of the *glass* promoter, in both wild-type animals and in animals lacking Gek activity. We observed that overexpression of full-length Gek had no effect on axon
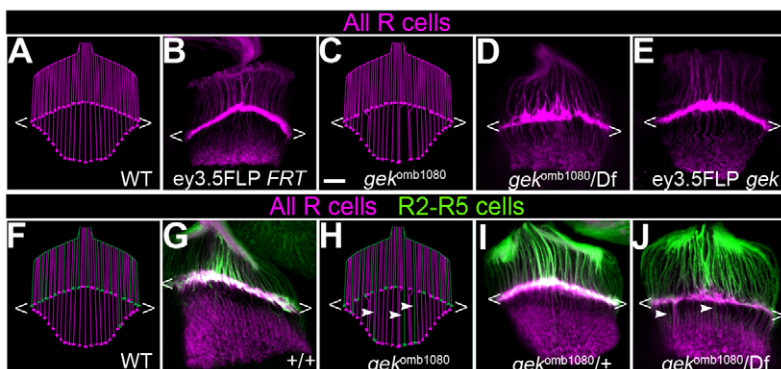

Fig. 3. *gek* mutant axons display targeting phenotypes during *Drosophila* larval development. (**A-J**) Third instar larval optic lobes, labeling R cells (mAB24B10, magenta) and (F-J) R2-R5 axons (green). (A,C,F,H) Schematics illustrating R cell axon projections into the lamina. Whereas control brains were invariably normal (B,G,I), somatic mosaic animals in which R cells were homozygous for *gek^omb1080* (E), or transheterozygous for *gek^omb1080*/*Df* (D,J) displayed axon bundling phenotypes, breaks in the lamina plexus and occasional axons entering the medulla (arrowheads). Chevrons delineate the lamina plexus. Scale bar: 20 µm.
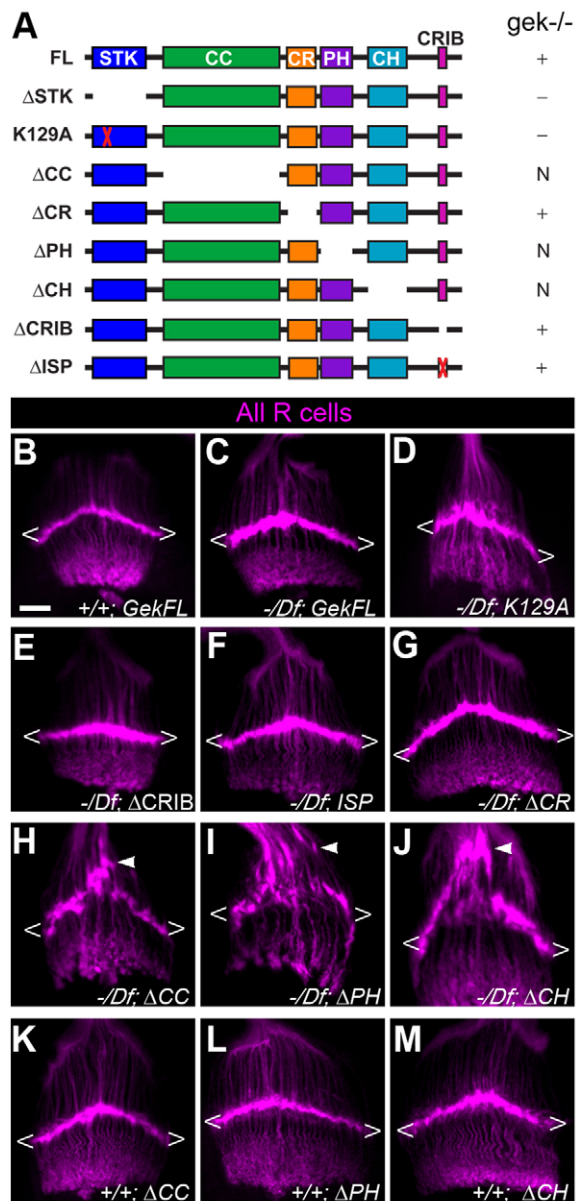
Fig. 4. Structure-function analysis of Gek. (**A**) Deletion and point mutation constructs. Plus sign denotes constructs that rescue and minus sign denotes constructs that were inactive; N, constructs that caused a novel phenotype. Red cross indicates deletion of the activation (ΔSTK) or binding (ΔCRIB) domain. STK, serine/threonine kinase catalytic domain; CC, coiled-coil domain; CR, cysteine-rich region; PH, pleckstrin homology domain; CH, citron homology domain; CRIB, Cdc42/Rac interactive binding domain. (**B-M**) Third instar larval optic lobes stained with mAb24B10 (magenta). (B) Full-length *gek* transgene (*GekFL*) expression in wild type. (C-J) *gek^omb1080^/Df* brains expressing (C) *GekFL*, (D) *Gek-K129A*, (E) *Gek-ΔCRIB*, (F) *Gek-ΔISP*, (G) *Gek-ΔCR*, (H) *Gek-ΔCC*, (I) *Gek-ΔPH* and (J) *Gek-ΔCH*. Premature stopping of R cells above the lamina is denoted by arrowheads. (K-M) Expression of *Gek-ΔCC*, *Gek-ΔPH* or *Gek-ΔCH* in wild type. Chevrons delineate the lamina plexus. Scale bar: 20 μm.

targeting in wild-type animals, and that retinal expression was sufficient to strongly rescue the mutant phenotype (Fig. 4B,C; *n*=11/11 for wild type; *n*=23/28 indistinguishable from wild type in the Gek rescue).

We next examined whether the kinase domain was required for Gek function using two transgenes: *Gek-ΔSTK*, in which the kinase domain had been deleted; and *Gek-K129A*, in which the kinase domain had been inactivated using a point mutation demonstrated to eliminate kinase activity in vitro (Luo et al., 1997). Consistent with this domain being essential for Gek function, we observed little rescue of the R cell targeting phenotype when either of these transgenes was expressed in a *gek* mutant background (Fig. 4D; data not shown; 2/31 and 2/20 brains appeared indistinguishable from wild type for *Gek-ΔSTK* and *Gek-K129A*, respectively). Indeed, animals overexpressing the *Gek-K129A* transgene frequently displayed a severe phenotype, suggesting that this mutation might disrupt additional aspects of targeting. By contrast, *gek* transgenes that either deleted the Cdc42-binding domain (*Gek-ΔCRIB*), or which bore point mutations in the CRIB domain that prevent binding to activated Cdc42 in vitro (*Gek-ISP*) (Luo et al., 1997), fully rescued the *gek* mutant phenotype (Fig. 4E,F; *n*=17/18 and 21/27 indistinguishable from wild type for *Gek-ΔCRIB* and *Gek-ISP*, respectively). Thus, at least under conditions in which Gek protein is likely to be expressed at high levels, direct interactions between Cdc42 and Gek are not essential to Gek function. Similarly, expression of a Gek form that lacks the cysteine-rich domain, *Gek-ΔCR*, was also capable of fully rescuing the phenotype (Fig. 4G; *n*=18/22 indistinguishable from wild type). In vitro studies of mammalian CDC42BP demonstrate that this domain binds phorbol ester (Tan et al., 2001a). However, our data demonstrate that this interaction is not essential to Gek function.

Deletion of a subset of Gek domains causes gain-of-function phenotypes

We observed a very different phenotype when we expressed *gek* transgenes that lacked the coiled-coil domain (*Gek-ΔCC*), the pleckstrin homology domain (*Gek-ΔPH*) or the citron homology domain (*Gek-ΔCH*) (Fig. 4H-J). Expression of *gek* transgenes bearing any of these deletions in a mutant background caused early termination of many R cell growth cones, with large clumps appearing in the optic stalk and the distal lamina (10/11 *Gek-ΔCC*, 9/9 *Gek-ΔPH* and 21/22 *Gek-ΔCH* brains). These transgenes do not simply activate Gek. If this were the case, increasing Gek activity by restoring the wild-type *gek* locus should enhance these phenotypes. However, we observed that R cell axon targeting was normal when any of these three transgenes was expressed in wild-type backgrounds (Fig. 4K-M; *n*=16/16 *Gek-ΔCC*, 7/7 *Gek-ΔPH* and 4/4 *Gek-ΔCH* brains). Thus, deleting these domains did not simply constitutively activate Gek, nor are they strictly neomorphic (since neomorphic mutations cannot be competed by the presence of wild-type protein).

Gek is required cell-autonomously for R1-R6 cell axons to choose appropriate targets

To examine *gek* mutant phenotypes in individual growth cones, we used mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 2001). To generate sparse somatic mosaic animals, we expressed FLP recombinase under the control of the heat-shock promoter and generated single R cells that were homozygous for either a control chromosome or a *gek* mutant chromosome in an otherwise heterozygous animal. These cells were labeled with mouse (m) CD8GFP under the control of the pan-neuronal promoter *elavGal4*, and thus their projections could be traced from the retina into the brain during mid-pupal development (Fig. 5). As the cell bodies of R1-R6 have characteristic positions and morphologies, each could be uniquely identified (Fig. 5A,A′,G,G′,M,M′,S,S′).
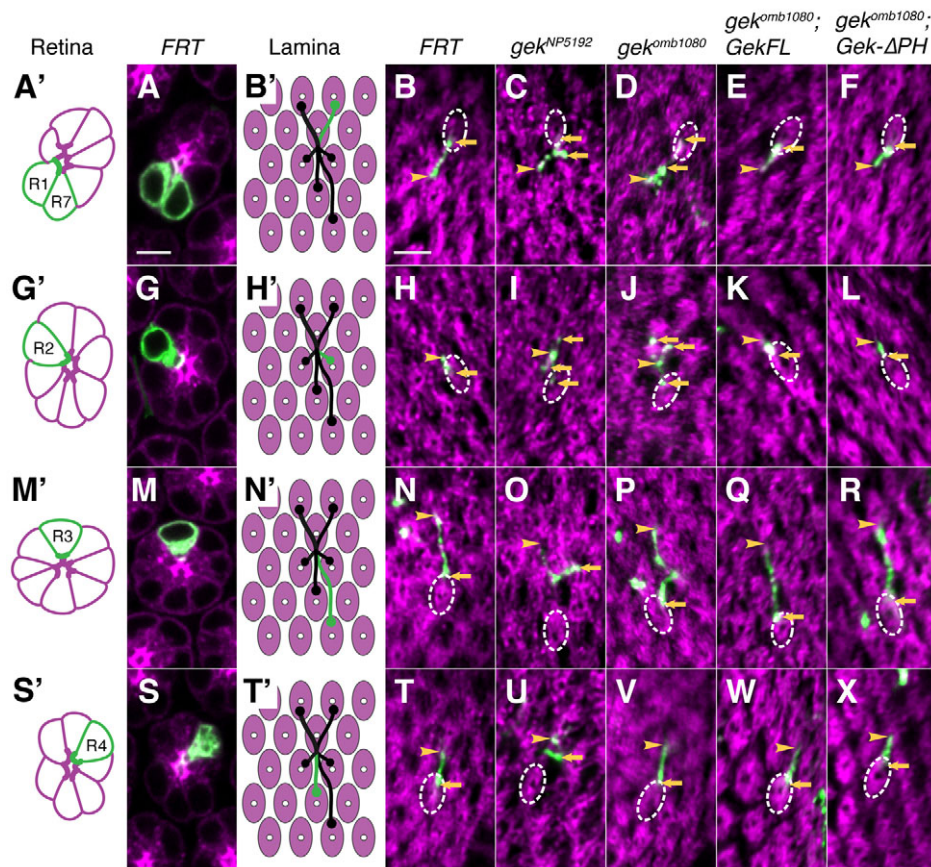
Fig. 5. Gek is necessary for columnar targeting of R1-R6 axons. (**A',G',M',S'**) Schematics of individual ommatidia (magenta) with individual R cell subtypes (green). (**A,G,M,S**) Confocal images of individual ommatidia (labeled with mAb24B10, magenta) and the control R cell (green). (**B',H',N',T'**) Schematic projection patterns of one ommatidium. (**B,H,N,T**) Control R cell axons. (**C,I,O,U**) *gekNP5192* R cell axons. (**D,J,P,V**) *gekomb1080* R cell axons. (**E,K,Q,W**) *gekomb1080* R cell axons that expressed *GekFL*. (**F,L,R,X**) *gekomb1080* R cell axons that expressed *Gek-ΔPH*. Shown are R cell clones (mCD8GFP, green) and target cartridges (mAb24B10, magenta). Arrowheads denote the start of the axon extension and arrow(s) mark the end(s). A dashed line delimits the correct target cartridge. Scale bars: 5 μm.

Each R cell subtype chooses a single columnar target located in an invariant position with respect to the ommatidial axon bundle (Fig. 5B',H',N',T'). When R cells are made homozygous for a control chromosome, they invariably made subtype-appropriate projections to the correct target (*n*=72/72 cells; Fig. 5B,H,N,T; supplementary material Fig. S5). Photoreceptors homozygous for either *gekNP5192* or *gekomb1080* invariably extended out of the retina into the brain, fasciculated normally, and stopped in the lamina (data not shown). However, once within the lamina, mutant photoreceptors often made errors in columnar targeting, making projections to one or more incorrect target cartridges in addition to the correct target, or failing to extend at all (Fig. 5C,D,I,J,O,P,U,V; supplementary material Fig. S5). In particular, for *gekomb1080*, only 35% (*n*=18/51) of R cells made the correct extension, whereas 35% (*n*=18/51) mistargeted and 30% (*n*=29/51) failed to extend (supplementary material Fig. S5). Targeting errors were observed in all R1-R6 cells, demonstrating that Gek is broadly required for R cell columnar targeting decisions (supplementary material Fig. S5). Expressing a full-length *gek* transgene in R cells homozygous mutant for *gekomb1080* strongly rescued all targeting defects, as 91% (*n*=61/67) of axons of this genotype extended correctly, only 3% (*n*=2/67) mistargeted and 6% (*n*=4/67) did not extend (Fig. 5E,K,Q,W; supplementary material Fig. S5). Thus, Gek is cell-autonomously required for R1-R6 axons to choose appropriate targets.

We next examined the effect of deleting the regulatory domains of Gek. Surprisingly, expressing either *Gek-ΔCC* or *Gek-ΔPH* in R cells homozygous for *gekomb1080* was almost as effective as expressing the full-length *gek* transgene at rescuing the axon targeting phenotypes associated with loss of Gek activity. With *Gek-ΔPH*, 83% (*n*=34/41) of axons extended normally, whereas 12% (*n*=5/41) mistargeted and 5% did not extend (*n*=2/41; Fig.

5F,L,R,X; supplementary material Fig. S5). Similarly, with *Gek-ΔCC*, 84% (*n*=41/49) of axons extended normally, whereas 4% (*n*=2/49) mistargeted and 12% did not extend (*n*=6/49) (supplementary material Fig. S5; data not shown). Thus, these *gek* transgenes retain their normal function in the growth cone. Consistent with the fact that generating single-cell clones using the pan-neural ElavGal4 driver causes much lower levels of transgene expression that that seen using GlassGal4, we did not observe premature stopping of R1-R6 axons above the lamina plexus.

Cdc42 is required for columnar targeting of R1-R6 axons

As Gek was originally identified based on its biochemical association with the small GTPase Cdc42, we tested whether Cdc42 plays a similar role to Gek in R cell axons. Using *Cdc424*, a loss-of-function allele, we generated R1-R6 cells that were homozygous mutant using the MARCM method, and scored their axonal targeting decisions (Fig. 6; supplementary material Fig. S5). Compared with control R cells, which invariably targeted normally (*n*=68/68; Fig. 6A-B',D-E',G-H',J-K'; supplementary material Fig. S5), only 26% (*n*=21/82) of *Cdc42* mutant R cells targeted normally, whereas 23% (*n*=19/82) selected inappropriate target columns and 51% (*n*=42/82) failed to extend (Fig. 6C,F,I,L; supplementary material Fig. S5). These defects were thus quantitatively and qualitatively indistinguishable from those associated with mutations in *gek*. Intriguingly, expression of the *Gek-ISP* transgene fully rescued the *gek* mutant phenotype (supplementary material Fig. S5). We infer that although physical association between Gek and Cdc42 occurs in a strictly CRIB-dependent fashion in vitro, either other proteins that are present in vivo allow them to interact independently of this domain, or their

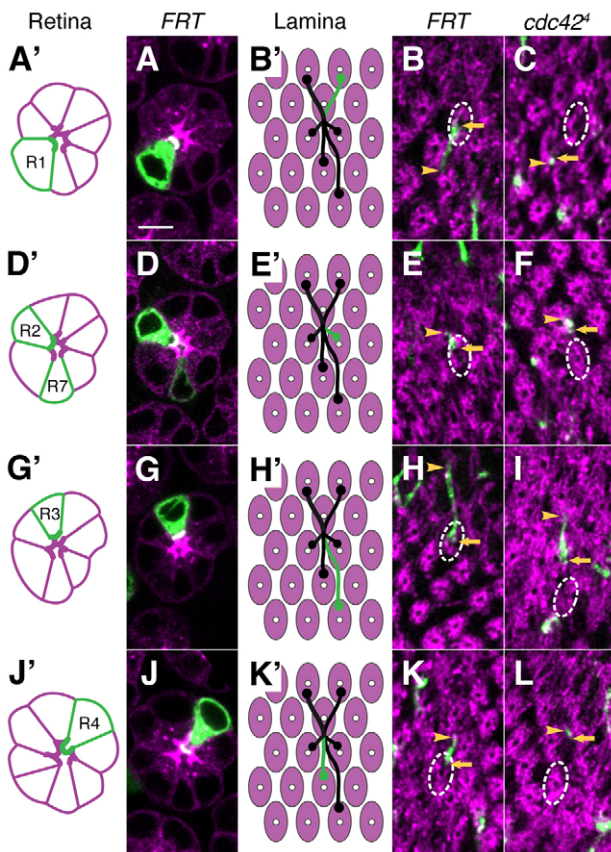**Fig. 6. Cdc42 is required for columnar targeting.** Single-cell MARCM analysis of Cdc42. (**A′,D′,G′,J′**) Schematics of individual ommatidia (magenta) with individual R cells (green). (**A,D,G,J**) Individual ommatidia (mAb2410, magenta) and R cells (mCD8GFP, green). (**B′,E′,H′,K′**) Schematic of the axon projection pattern of one ommatidium. (**B,E,H,K**) Control R cell axons. (**C,F,I,L**) *Cdc42⁴* R cell axons. Labels are as in Fig. 5.

direct association is not required for their function in R cell growth cones. Moreover, Gek is not the only downstream target of Cdc42, as overexpression of a wild-type *gek* transgene did not rescue the *Cdc42* mutant phenotype (supplementary material Fig. S5). Finally, although the targeting phenotype observed in R cell axons mutant for *Cdc42* was specific, these mutant photoreceptors sometimes displayed morphological defects in the retina (data not shown), consistent with Cdc42 playing a broader role in R cell development. These defects were not, however, correlated with the axon targeting phenotype (data not shown).

Cdc42 and Gek regulate a common target in R cell growth cones

Homologs of Cdc42 and Gek can regulate the phosphorylation of the regulatory subunit of myosin light chain (MRLC), affecting actin-myosin contractility in non-neuronal cells (Dong et al., 2002; Wilkinson et al., 2005). Using an antibody that specifically recognizes a phosphorylated form of MRLC in *Drosophila* (Lee and Treisman, 2004), we tested whether Gek phosphorylates MRLC in R cell growth cones. Phosphorylated MRLC could be detected in growth cones in the lamina, displaying strongly punctate staining above a relatively uniform pattern. Clones homozygous mutant for *gek^omb1080* displayed strongly reduced punctate staining, while
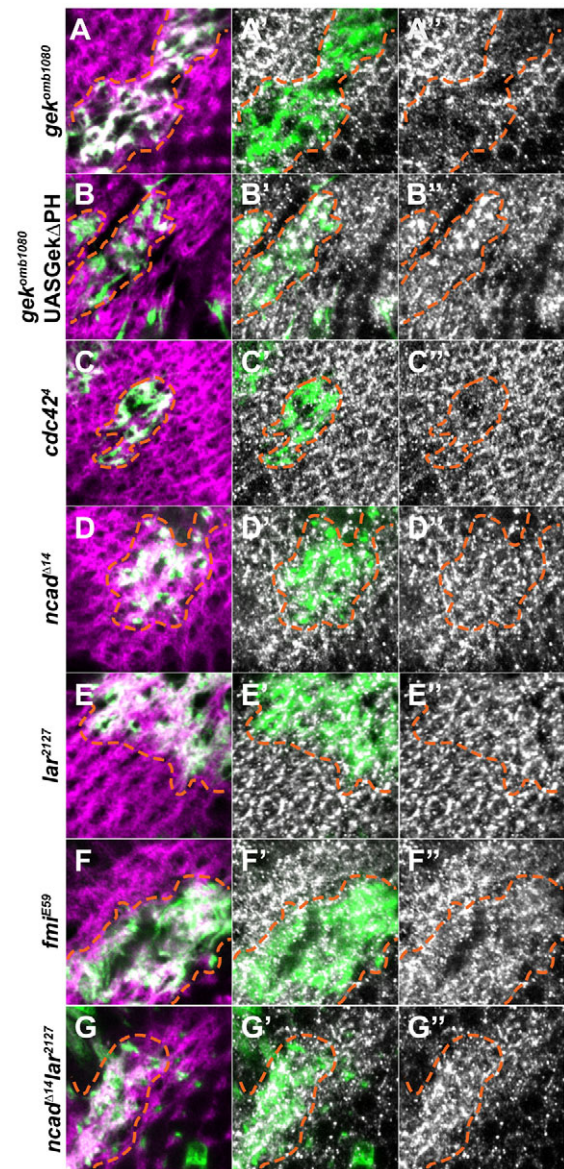

Fig. 7. Gek and Cdc42 share a common molecular target in R cell growth cones. Phospho-MRLC staining (white) in axon projections of R-cell clones. The clone is labeled in green and axonal projections are labeled with mAb24B10 (magenta). Orange dashed lines indicate the clone border. (**A-A″**) A *gek^omb1080* clone. (**B-B″**) A *gek^omb1080* clone expressing *Gek-ΔPH*. (**C-C″**) A *Cdc42⁴* clone. (**D-D″**) An *Ncad^Δ14* clone. (**E-E″**) A *Lar^2127* clone. (**F-F″**) A *flamingo^E59* clone. (**G-G″**) An *Ncad^Δ14 Lar^2127* clone.

retaining the uniform background (Fig. 7A-A″). Consistent with the apparent gain-of-function effect of deleting the PH domain, expression of *Gek-ΔPH* in *gek* null cells increased phospho-MRLC staining beyond that in wild-type cells (Fig. 7B-B″).

We also observed that clones homozygous for *Cdc42⁴* displayed a subtle reduction in phospho-MRLC staining, consistent with the notion that Gek and Cdc42 regulate this common target (Fig. 7C-C″). We emphasize, however, that *gek* mutant clones displayed stronger defects in phospho-MRLC staining than *Cdc42* mutant clones, consistent with other upstream mechanisms regulating Gek.

DEVELOPMENT

We next reasoned that if Gek or Cdc42 were regulated by the cell surface molecules known to be required for R1-R6 targeting, phosphorylation of MRLC should also be altered in these mutants. However, staining R cell clones homozygous for mutations in the classical cadherin *N-cadherin* (*Ncad*), the receptor tyrosine phosphatase *Lar*, the atypical cadherin *flamingo*, or both *N-cadherin* and *Lar*, did not indicate reductions in phospho-MRLC staining during mid-pupal development (Fig. 7D-G″). As a further test of the idea that Gek might function downstream of these molecules, we expressed a *Gek-ΔCC* transgene in R cells homozygous for mutations in *N-cadherin* or *Lar*, and observed no phenotypic rescue (data not shown). Thus, although Cdc42 and Gek regulate MRLC in R cell growth cones, the cell surface proteins known to be important for R cell target selection are not individually necessary for Cdc42 and Gek to do so. As mutations in *spaghetti squash*, the homolog of MRLC, cause severe defects in early R cell morphogenesis (Lee and Treisman, 2004), precluding analysis of axon targeting, functional tests of this regulation await temporally targeted manipulations of gene activity.

Mutations in *gek* affect R cell growth cone morphology

Given the phenotypic similarities between *gek* and *Cdc42* mutant clones, as well as the effects on phosphorylation of MRLC, a cytoskeletal regulator, we examined whether Gek regulates growth cone morphology. We examined single growth cones in control and *gek* mutant R cells at a stage prior to target selection in the lamina, and observed that although *gek* mutant growth cones appear qualitatively similar to control growth cones, they are quantitatively different (supplementary material Fig. S6). In particular, *gek* mutant growth cones displayed 20% fewer filopodia and were ~30% smaller than corresponding control growth cones (supplementary material Fig. S6). These data are consistent with Gek regulating cytoskeletal dynamics in R cell growth cones, affecting their ability to respond to targeting cues.

Gain-of-function mutations in *gek* alter its subcellular activity

Each R cell body has a unique cross-sectional profile, defined by a developing rhabdome located at a central position within the ommatidium. In photoreceptors homozygous for *gek^omb1080*, these cell type-specific morphologies were invariably normal (*n*=61/61; Fig. 8A,B,I). Similarly, in *gek^omb1080* cells expressing a full-length *gek* transgene, virtually all R cell bodies displayed a normal morphology (99%, *n*=83/84), with a single cell (*n*=1/84) displaying an abnormal cross-sectional profile (Fig. 8C,D,I). Thus, Gek activity is not required for photoreceptor cell body morphogenesis, and overexpression of an intact Gek protein does not disrupt this process. However, in R cells that were homozygous for *gek^omb1080* and expressed either *Gek-ΔPH* or *Gek-ΔCC*, we observed two types of defects. First, in both cases, ~18% of R cells (*n*=21/88 for *Gek-ΔPH* and *n*=16/86 for *Gek-ΔCC*) displayed abnormal morphologies in their cross-sectional profile, with apical domains extending either too far towards the center of the ommatidium or being located basally (Fig. 8E-I). In addition, ~6% (*n*=5/88 for *Gek-ΔPH* and *n*=6/86 for *Gek-ΔCC*) of all R cell bodies had dropped below the plane of the ommatidium (Fig. 8G,I). All remaining R cells in both genotypes were normal. Photoreceptor morphogenesis phenotypes could not be detected in third instar eye discs using GlassGal4 to overexpress *Gek-ΔCC*, *Gek-ΔPH* or *Gek-ΔCH*, although adult animals of these genotypes had rough eyes
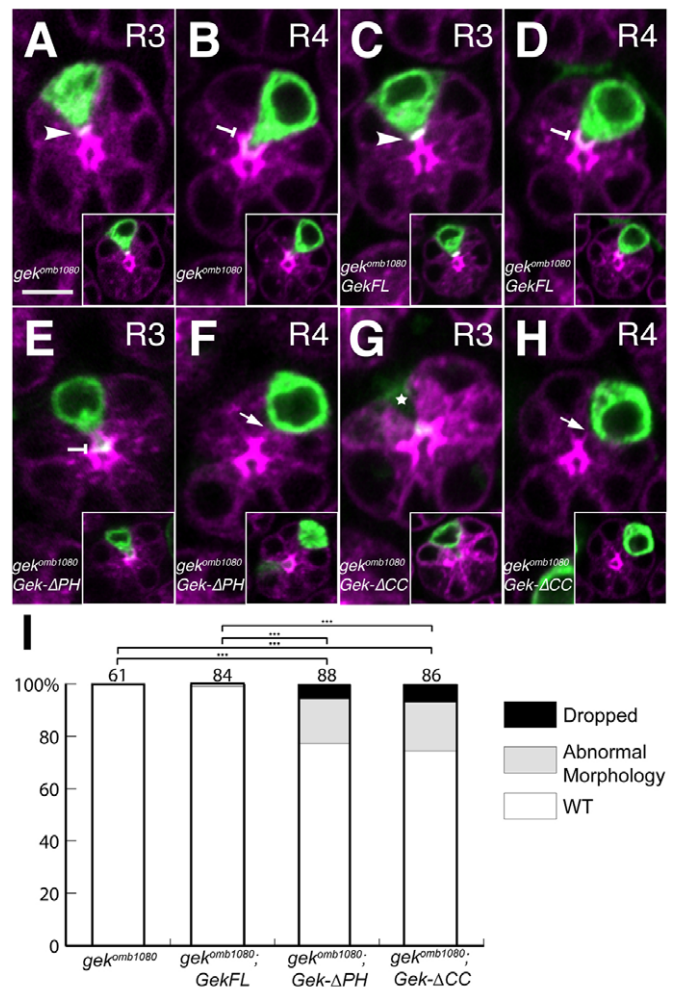

Fig. 8. Gek autoinhibitory function restricts Gek activity to growth cones. (**A-H**) Ommatidia from *gek^omb1080* (A,B), *gek^omb1080*; *GekFL* (C,D), *gek^omb1080*; *Gek-ΔPH* (E,F) and *gek^omb1080*; *Gek-ΔCC* (G,H) flies. Homozygous R cells are green and the ommatidium magenta (mAb24B10). The bright magenta regions demark developing rhabdomes. (A,C,E,G) R3 cells; (B,D,F,H) R4 cells. Insets display the same cell at a more basal section plane. Normal R3 cells have short apical domains (arrowheads), whereas R4 apical domains protrude centrally (flat-ended arrow). *gek* mutant R cells expressing these transgenes displayed either abnormally protrusive apical domains (in R3), or lacked apical domains (arrows), or dropped basally within the retina (star). Scale bar: 5 μm. (**I**) Quantification of mutant phenotypes. Cell bodies were classified as wild-type (WT), abnormal in morphology, or dropped basally. ***, *P*<0.001 (Fisher's exact test).

(data not shown). We infer that these regulatory domains normally prevent Gek from interacting with the molecular machinery that drives R cell morphogenesis in the pupal retina.

Overexpression of full-length Gek induces early termination of R7 axons

To test whether high levels of wild-type Gek expression are sufficient to affect the targeting decisions of other R cell subtypes, we overexpressed full-length Gek in all R cell axons and visualized R7 and R8 cells in adult animals using the opsin transgenes *Rh4-lacZ* and *Rh6-lacZ*, respectively. In control experiments in which mCD8GFP was overexpressed using GlassGal4, 1.5% (*n*=393) of
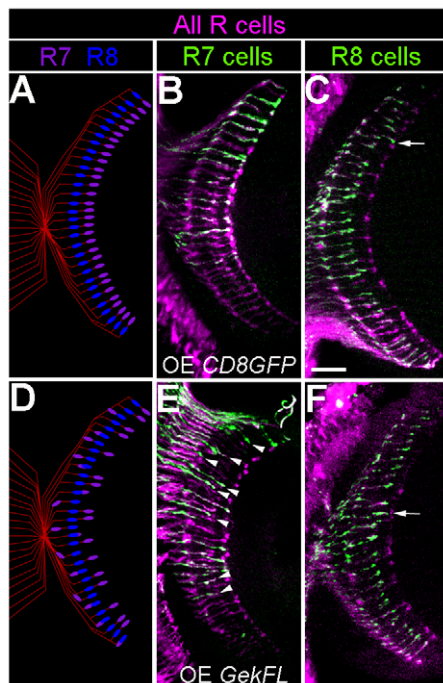
Fig. 9. Overexpression of wild-type Gek alters layer-specific targeting of R7 axons. Transverse sections of the adult medulla stained with mAb24B10 (magenta) and anti-β-galactosidase (green). (**A-C**) Overexpression of mCD8GFP in all R cells using GlassGal4. (**D-F**) Overexpression of *GekFL* using GlassGal4. R7 and R8 cells were labeled with *Rh4-lacZ* and *Rh6-lacZ,* respectively. Arrows indicate prematurely terminating axons. Scale bar: 20 μm.

R7 cells targeted to the R8 layer, whereas 5.4% (*n*=1178) of R8 axons extended aberrantly (Fig. 9A-C). By contrast, 28.3% (*n*=1278) of R7 cells expressing full-length Gek targeted either to the R8 layer or to an even more superficial layer in the outer medulla, or occasionally to the lamina (Fig. 9D,E). R8 axon targeting was unaffected in this genetic background (4.9%, *n*=857; Fig. 9F). Thus, increasing Gek activity can cause premature termination of axon extension, leading to targeting errors in R7, but not R8, cells.

DISCUSSION

Our data demonstrate that Gek and Cdc42 function as regulators of the actin cytoskeleton within R1-R6 cell growth cones. R1-R6 growth cones lacking Gek or Cdc42 activity display two prevalent targeting phenotypes, as they can either fail to extend to their terminal targets or can extend to one or more inappropriate targets within the lamina. Taken together with the biochemical studies of MRCK, we propose that localized activation of Gek by Cdc42 within subdomains of R1-R6 growth cones is a necessary step in making a directed extension towards a specific target. In this view, Gek acts downstream of adhesive cues and/or targeting signals generated on the cell surface, integrating these cues and altering the organization of the actin cytoskeleton so as to promote lateral axon extension. *gek* mutant growth cones that fail to extend presumably fail to define an extension domain; those that mistarget or that extend to multiple targets must initiate extensions in multiple, spatially inappropriate domains. These latter cases might reflect perduring Gek or Cdc42 protein in our single-cell clones, or might result from the activities of other regulators of protrusion. Our data

also demonstrate that the known regulators of R cell axon targeting, including the cadherins Flamingo and N-cadherin as well as the tyrosine phosphatase Lar, are not individually required to regulate Gek or Cdc42. Thus, either these regulators act redundantly to control Gek, or an as yet unknown pathway is required for columnar targeting of R1-R6 growth cones.

Our structure-function analyses of Gek, combined with biochemical studies examining MRCK regulation in vitro, provide insight into the mechanism of Gek regulation. Work in both contexts demonstrates that the kinase domain is the crucial effector of the protein, as deletion or inactivation of this domain removes all detectable activity both in vivo and in vitro (this work) (Luo et al., 1997; Leung et al., 1998). Previous studies of MRCK uncovered multiple negative regulatory mechanisms (Tan et al., 2001a; Tan et al., 2001b; Dong et al., 2002). In particular, the coiled-coil domain drives oligomerization and contains a kinase-inhibitory motif (KIM) that sequesters the kinase domain when the protein is inactive (Tan et al., 2001a; Dong et al., 2002; Garcia et al., 2006). Inhibitory activity has also been associated with the pleckstrin homology domain and the citron homology domain (Chen et al., 1999). Consistent with these observations, when we delete any one of these three domains from Gek and express these constructs in *gek* mutant R cells, we observe premature stopping of R cell axons above the lamina or defects in cell morphogenesis, depending on the level and timing of transgene expression. However, these mutant forms of Gek do not correspond to simple 'hyperactivated' proteins, as adding additional wild-type Gek protein eliminates, rather than enhances, the premature axon termination phenotype. Thus, the presence of wild-type protein restores normal inhibition to these mutant forms of Gek. These results are consistent with biochemical studies of MRCK showing that a conserved N-terminal domain can mediate dimerization, and that kinase activation is critically dependent on transautophosphorylation (Tan et al., 2001a). In this model, activation of a Gek dimer requires that both monomers are independently disinhibited, making them competent to be transphosphorylated by their partner. Thus, in a heterodimer comprising one wild-type subunit and one subunit lacking an autoinhibitory domain (Gek-ΔCC, Gek-ΔPH or Gek-ΔCH), the truncated monomer cannot phosphorylate its inactive wild-type partner (which remains autoinhibited) and does not get phosphorylated by it in turn. As a result, the presence of wild-type protein will suppress an otherwise gain-of-function mutant protein. Conversely, in the absence of wild-type protein, homodimers comprising only truncated subunits will transactivate throughout the cell, making growth cones competent to extend laterally, but also disrupting morphogenesis. Of course, because we cannot directly measure Gek activity within R cell growth cones, we cannot exclude the possibility that the gain-of-function *gek* mutations that we have generated act differently from the inhibitory activities that have been associated with these domains in MRCK in vitro.

Of the many molecules known to be involved in R cell axon target selection, Gek is the first to be specifically required for R1-R6 axons, but not R7 or R8 axons, to reach their targets. A simple mechanism to achieve such functional specificity would be cell type-specific expression of Gek. However, Gek is strongly expressed in all photoreceptor axons at developmentally appropriate stages. Thus, cell type-specific function must emerge through post-translational regulation of Gek activity. We propose that the cell type-specific requirement for Gek emerges from

differences in how upstream signaling pathways control common regulators of the actin cytoskeleton to achieve columnar versus laminar targeting.

MRCK function has been linked to two important regulators of the actin cytoskeleton, LIMK and MRLC, the latter through both direct and indirect pathways Gally et al., 2009; Kumfer et al., 2010; Wilkinson et al., 2005; Dong et al., 2002; Sumi et al., 2001; Tan et al., 2001b). Our data are consistent with the notion that at least one of these, MRLC, is a regulatory target of both Gek and Cdc42 in R cell growth cones, albeit one of as yet untested functional significance. We propose that the cell type-specific requirement for Gek in R1-R6 axons reflects the fact that an as yet unknown upstream signaling pathway requires a specific pattern of modulation of MRLC (achieved via Gek and Cdc42) for R1-R6 axons to be competent to achieve columnar target selection. In this view, the various cell surface interactions that are required for layer-specific targeting of R7 do not signal through Gek, but do use downstream effectors that can be modulated by Gek. When Gek is overexpressed in R7, mistargeting is caused by high levels of Gek bypassing this upstream regulation, causing abnormal activation of relatively 'generic' effector proteins in R7 growth cones. Thus, the columnar targeting of R1-R6 axons and the layer-specific targeting of R7 axons reflect the modulation of at least some of the same effector genes, but the upstream signals that direct columnar targeting are uniquely dependent on Gek function. More broadly, given that axon targeting in most systems is generally dependent on multiple, partially redundant cell surface interactions, whereas many of the direct cytoskeletal regulators (such as MRLC) are essential and pleiotropic, our results demonstrate that one way by which cell type-appropriate differences in targeting can be achieved is through the use of specific cytoskeletal modulators like Gek. In this way, subtle differences in the control of the cytoskeleton can cause significant differences in the overall pattern of target selection, making one group of cells competent to target in a columnar fashion. Thus, cell type-specific morphologies can emerge both through differences in cell surface interactions and through differences in cytoplasmic signaling mechanisms.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
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References
Berger, J., Senti, K. A., Senti, G., Newsome, T. P., Asling, B., Dickson, B. J. and Suzuki, T. (2008). Systematic identification of genes that regulate neuronal wiring in the *Drosophila* visual system. *PLoS Genet.* **4**, e1000085.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Callahan, C. A. and Thomas, J. B. (1994). Tau-beta-galactosidase, an axon-targeted fusion protein. *Proc. Natl. Acad. Sci. USA* **91**, 5972-5976.

Chen, P. L. and Clandinin, T. R. (2008). The cadherin Flamingo mediates level-dependent interactions that guide photoreceptor target choice in *Drosophila*. *Neuron* **58**, 26-33.

Chen, X. Q., Tan, I., Leung, T. and Lim, L. (1999). The myotonic dystrophy kinase-related Cdc42-binding kinase is involved in the regulation of neurite outgrowth in PC12 cells. *J. Biol. Chem.* **274**, 19901-19905.

Choe, K. M., Prakash, S., Bright, A. and Clandinin, T. R. (2006). Liprin-alpha is required for photoreceptor target selection in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **103**, 11601-11606.

Chotard, C., Leung, W. and Salecker, I. (2005). glial cells missing and gcm2 cell autonomously regulate both glial and neuronal development in the visual system of *Drosophila*. *Neuron* **48**, 237-251.

Clandinin, T. R. and Zipursky, S. L. (2000). Afferent growth cone interactions control synaptic specificity in the *Drosophila* visual system. *Neuron* **28**, 427-436.

Clandinin, T. R., Lee, C.-H., Herman, T., Lee, R. C., Yang, A. Y., Ovasapyan, S. and Zipursky, S. L. (2001). *Drosophila* LAR regulates R1-R6 and R7 target specificity in the visual system. *Neuron* **32**, 237-248.

Dong, J. M., Leung, T., Manser, E. and Lim, L. (2002). Cdc42 antagonizes inductive action of cAMP on cell shape, via effects of the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) on myosin light chain phosphorylation. *Eur. J. Cell Biol.* **81**, 231-242.

Fehon, R. G., Oren, T., Lajeunesse, D. R., Melby, T. E. and McCartney, B. M. (1997). Isolation of mutations in the *Drosophila* homologues of the human Neurofibromatosis 2 and yeast CDC42 genes using a simple and efficient reverse-genetic method. *Genetics* **146**, 245-252.

Frankfort, B. J., Nolo, R., Zhang, Z., Bellen, H. and Mardon, G. (2001). Senseless repression of rough is required for R8 photoreceptor differentiation in the developing *Drosophila* eye. *Neuron* **32**, 403-414.

Gally, C., Wissler, F., Zahreddine, H., Quintin, S., Landmann, F. and Labouesse, M. (2009). Myosin II regulation during C. elegans embryonic elongation: LET-502/ROCK, MRCK-1 and PAK-1, three kinases with different roles. *Development* **136**, 3109-3119.

Garcia, P., Ucurum, Z., Bucher, R., Svergun, D. I., Huber, T., Lustig, A., Konarev, P. V., Marino, M. and Mayans, O. (2006). Molecular insights into the self-assembly mechanism of dystrophia myotonica kinase. *FASEB J.* **20**, 1142-1151.

Garrity, P. A., Lee, C.-H., Salecker, I., Robertson, H. C., Desai, C. J., Zinn, K. and Zipursky, S. L. (1999). Retinal axon target selection in *Drosophila* is regulated by a receptor tyrosine phosphatase. *Neuron* **22**, 707-717.

Govek, E. E., Newey, S. E. and Van Aelst, L. (2005). The role of the Rho GTPases in neuronal development. *Genes Dev.* **19**, 1-49.

Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L. and Dickson, B. J. (2001). Rac function and regulation during *Drosophila* development. *Nature* **416**, 438-442.

Heberlein, U. and Rubin, G. M. (1990). Structural and functional comparisons of the *Drosophila* virilis and *Drosophila* melanogaster rough genes. *Proc. Natl. Acad. Sci. USA* **87**, 5916-5920.

Hiesinger, P. R., Zhai, R. G., Zhou, Y., Koh, T. W., Mehta, S. Q., Schulze, K. L., Cao, Y., Verstreken, P., Clandinin, T. R., Fischbach, K. F. et al. (2006). Activity-independent prespecification of synaptic partners in the visual map of *Drosophila*. *Curr. Biol.* **16**, 1835-1843.

Hing, H., Xiao, J., Harden, N., Lim, L. and Zipursky, S. L. (1999). Pak functions downstream of Dock to regulate photoreceptor axon guidance in *Drosophila*. *Cell* **97**, 853-863.

Hofmeyer, K. and Treisman, J. E. (2009). The receptor protein tyrosine phosphatase LAR promotes R7 photoreceptor axon targeting by a phosphatase-independent signaling mechanism. *Proc. Natl. Acad. Sci. USA* **106**, 19399-19404.

Hofmeyer, K., Maurel-Zaffran, C., Sink, H. and Treisman, J. E. (2006). Liprin-alpha has LAR-independent functions in R7 photoreceptor axon targeting. *Proc. Natl. Acad. Sci. USA* **103**, 11595-11600.

Kauffmann, R. C., Li, S., Gallagher, P. A., Zhang, J. and Carthew, R. W. (1996). Ras1 signaling and transcriptional competence in the R7 cell of *Drosophila*. *Genes Dev.* **10**, 2167-2178.

Koh, C. G. (2006). Rho GTPases and their regulators in neuronal functions and development. *Neuro-Signals* **15**, 228-237.

Kumfer, K. T., Cook, S. J., Squirell J. M., Eliceiri K. W., Peel N., O'Connell K. F. and White, J. G. (2010). CGEF-1 and CHIN-1 regulate CDC-42 activity during asymmetric division in the Caenorhabditis elegans embryo. *Mol. Biol. Cell* **21**, 266-277.

Lee, A. and Treisman, J. E. (2004). Excessive myosin activity in mbs mutants causes photoreceptor movement out of the *Drosophila* eye disc epithelium. *Mol. Biol. Cell* **15**, 3285-3295.

Lee, C. H., Herman, T., Clandinin, T. R., Lee, R. and Zipursky, S. L. (2001). N-cadherin regulates target specificity in the *Drosophila* visual system. *Neuron* **30**, 437-450.

Lee, R. C., Clandinin, T. R., Lee, C.-H., Chen, P.-L., Meinertzhagen, I. A. and Zipursky, S. L. (2003). The protocadherin Flamingo is required for axon target selection in the *Drosophila* visual system. *Nat. Neurosci.* **6**, 557-563.

Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. *Trends Neurosci.* **24**, 251-254.

Leung, T., Chen, X. Q., Tan, I., Manser, E. and Lim, L. (1998). Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol. Cell. Biol.* **18**, 130-140.

Luo, L., Jan, L. and Jan, Y. N. (1996). Small GTPases in axon outgrowth. *Perspect. Dev. Neurobiol.* **4**, 199-204.

Luo, L., Lee, T., Tsai, L., Tang, G., Jan, L. Y. and Jan, Y. N. (1997). Genghis Khan (Gek) as a putative effector for *Drosophila* Cdc42 and regulator of actin polymerization. *Proc. Natl. Acad. Sci. USA* **94**, 12963-12968.

Mast, J. D., Prakash, S., Chen, P. L. and Clandinin, T. R. (2006). The mechanisms and molecules that connect photoreceptor axons to their targets in *Drosophila*. *Semin. Cell Dev. Biol.* **17**, 42-49.

Maurel-Zaffran, C., Suzuki, T., Gahmon, G., Treisman, J. E. and Dickson, B. J. (2001). Cell-autonomous and -nonautonomous functions of LAR in R7 photoreceptor axon targeting. *Neuron* **32**, 225-235.

Meinertzhagen, I. A. and Hanson, T. E. (1993). In *The Development of the Optic Lobe*, Vol. 2 (ed. M. Bate and A. Martinez-Arias), pp. 1363-1491. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Newsome, T. P., Asling, B. and Dickson, B. J. (2000a). Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development* **127**, 851-860.

Newsome, T. P., Schmidt, S., Dietzl, G., Keleman, K., Asling, B., Debant, A. and Dickson, B. J. (2000b). Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in *Drosophila*. *Cell* **101**, 283-294.

Ng, J. and Luo, L. (2004). Rho GTPases regulate axon growth through convergent and divergent signaling pathways. *Neuron* **44**, 779-793.

Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., Sun, Y., Dietzl, G., Dickson, B. J. and Luo, L. (2002). Rac GTPases control axon growth, guidance and branching. *Nature* **416**, 442-447.

Petrovic, M. and Hummel, T. (2008). Temporal identity in axonal target layer recognition. *Nature* **456**, 800-803.

Prakash, S., Caldwell, J. C., Eberl, D. F. and Clandinin, T. R. (2005). *Drosophila* N-cadherin mediates an attractive interaction between photoreceptor axons and their targets. *Nat. Neurosci.* **8**, 443-450.

Prakash, S., McLendon, H. M., Dubreuil, C. I., Ghose, A., Hwa, J., Dennehy, K. A., Tomalty, K. M., Clark, K. L., Van Vactor, D. and Clandinin, T. R. (2009). Complex interactions amongst N-cadherin, DLAR, and Liprin-alpha regulate *Drosophila* photoreceptor axon targeting. *Dev. Biol.* **336**, 10-19.

Sanes, J. R. and Yamagata, M. (2009). Many paths to synaptic specificity. *Annu. Rev. Cell Dev. Biol.* **25**, 161-195.

Sanes, J. R. and Zipursky, S. L. (2010). Design principles of insect and vertebrate visual systems. *Neuron* **66**, 15-36.

Scott, E. K., Reuter, J. E. and Luo, L. (2003). Small GTPase Cdc42 is required for multiple aspects of dendritic morphogenesis. *J. Neurosci.* **23**, 3118-3123.

Senti, K. A., Usui, T., Boucke, K., Greber, U., Uemura, T. and Dickson, B. J. (2003). Flamingo regulates R8 axon-axon and axon-target interactions in the *Drosophila* visual system. *Curr. Biol.* **13**, 828-832.

Shinza-Kameda, M., Takasu, E., Sakurai, K., Hayashi, S. and Nose, A. (2006). Regulation of layer-specific targeting by reciprocal expression of a cell adhesion molecule, capricious. *Neuron* **49**, 205-213.

Stowers, R. S. and Schwarz, T. L. (1999). A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics* **152**, 1631-1639.

Sumi, T., Matsumoto, K., Shibuya, A. and Nakamura, T. (2001). Activation of LIM kinases by myotonic dystrophy kinase-related Cdc42-binding kinase alpha. *J. Biol. Chem.* **276**, 23092-23096.

Takemura, S. Y., Lu, Z. and Meinertzhagen, I. A. (2008). Synaptic circuits of the *Drosophila* optic lobe: the input terminals to the medulla. *J. Comp. Neurol.* **509**, 493-513.

Tan, I., Seow, K. T., Lim, L. and Leung, T. (2001a). Intermolecular and intramolecular interactions regulate catalytic activity of myotonic dystrophy kinase-related Cdc42-binding kinase alpha. *Mol. Cell. Biol.* **21**, 2767-2778.

Tan, I., Ng, C. H., Lim, L. and Leung, T. (2001b). Phosphorylation of a novel myosin binding subunit of protein phosphatase 1 reveals a conserved mechanism in the regulation of actin cytoskeleton. *J. Biol. Chem.* **276**, 21209-21216.

Ting, C. Y. and Lee, C. H. (2007). Visual circuit development in *Drosophila*. *Curr. Opin. Neurobiol.* **17**, 65-72.

Ting, C. Y., Yonekura, S., Chung, P., Hsu, S. N., Robertson, H. M., Chiba, A. and Lee, C.-H. (2005). Drosophila N-cadherin functions in the first stage of the two-stage layer-selection process of R7 photoreceptor afferents. *Development* **132**, 953-963.

Tomasi, T., Hakeda-Suzuki, S., Ohler, S., Schleiffer, A. and Suzuki, T. (2008). The transmembrane protein Golden goal regulates R8 photoreceptor axon-axon and axon-target interactions. *Neuron* **57**, 691-704.

Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R. W., Schwarz, T. L., Takeichi, M. and Uemura, T. (1999). Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. *Cell* **98**, 585-595.

Wilkinson, S., Paterson, H. F. and Marshall, C. J. (2005). Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. *Nat. Cell Biol.* **7**, 255-261.

Yonekura, S., Xu, L., Ting, C. Y. and Lee, C.-H. (2007). Adhesive but not signaling activity of Drosophila N-cadherin is essential for target selection of photoreceptor afferents. *Dev. Biol.* **304**, 759-770.