Lim kinase regulates the development of olfactory and neuromuscular synapses

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Abstract

Lim Kinase (Limk) belongs to a phylogenetically conserved family of serine/threonine kinases, which have been shown to be potent regulators of the actin cytoskeleton. Despite accumulating evidence of its biochemical actions, its in vivo function has remained poorly understood. The association of the Limk1 gene with Williams Syndrome indicates that proteins of this family play a role in the nervous system. To unravel the cellular and molecular functions of Limk, we have either knocked out or activated the Limk gene in Drosophila. At the neuromuscular junction, loss of Limk leads to enlarged terminals, while increasing the activity of Limk leads to stunted terminals with fewer synaptic boutons. In the antennal lobe, loss of Limk abolishes the ability of p21-activated kinase (Pak) to alter glomerular development. In contrast, increase in Limk function leads to ectopic glomeruli, a phenotype suppressible by the coexpression of a hyperactive Cofilin gene. These results establish Limk as a critical regulator of Cofilin function and synapse development, and a downstream effector of Pak in vivo.

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Introduction

Neuronal pathfinding and synaptogenesis are critical events in the formation of the intricate pattern of connectivity in the brain. At the heart of these events is the precise regulation of the axonal actin cytoskeleton by signals from the environment and from inside the cell. In recent years, the Lim-kinase (Limk) protein has emerged as an important link between regulatory cues and the actin cytoskeleton. Limk belongs to a novel, evolutionarily conserved family of serine/threonine kinases, characterized by two N-terminal LIM motifs, a central PDZ motif, and a C-terminal Kinase domain (Fig. 1A). Numerous experiments, in which members of this family were exogenously expressed in cultured cells, showed that Limks are potent inducers of the actin cytoskeleton (Nagata et al., 1999; Ohashi et al., 2000; Sumi et al., 1999). Associated biochemical studies have also begun to unravel the molecular mechanisms by which Limk directs actin cytoskeletal assembly. In vitro kinase assays using the mammalian Limk1 protein show that it directly phosphorylates and inactivates Cofilin, a major actin-depolymerizing factor (Arber et al., 1998; Lappalainen and Drubin, 1997; Rosenblatt et al., 1997; Yang et al., 1998). Consistent with the notion that Cofilin inactivation is a principal mechanism by which Limk1 promotes actin polymerization, coexpression of Cofilin strongly curtailed Limk1’s ability to induce actin polymerization (Arber et al., 1998; Yang et al., 1998). A recent biochemical study also shows that human Limk1 is directly phosphorylated and activated by the human p21-activated kinase (Pak) (Edwards et al., 1999), an enzyme that is stimulated by Rac and Cdc42 (Daniels and Bokoch, 1999; Manser et al., 1994). Taken together, the results have led...
Fig. 1. The fly Limk gene is mutated by imprecise transposon excision. (A) The Limk protein consists of an N-terminal regulatory domain with two LIM and a PDZ motif, a kinase domain and a long C-terminal tail. (B) The fly Limk gene consists of 5 exons that span 6 kb. By mobilizing a P element, EP(X)1313 located 2.8 kb downstream of Limk, 5 deletion alleles of the Limk gene were generated. The extents of the deletions in each of the alleles are indicated above the Limk gene. (C) Southern blot of the agarose gel (right). Genomic DNA of Limk mutants and control animals were digested with BamHI and BglII and probed with the Limk cDNA. The 3.6 kb band is more intense than the 4.3 kb band because it contains more of the Limk cDNA. The gene is deleted in the Limk1, Limk3, and Limk5 alleles. (D) When probed with promoter sequences, alleles Limk3 and Limk5 also show the loss of 5′ regulatory sequences. (E) RT-PCR of mRNA from Limk5 and wild type (using primers against Limk or a control gene, Akap200) showed that the mutant does not express any Limk transcripts.
to the speculation that Pak, Limk1, and Cofilin form a regulatory cascade that links the actin cytoskeleton with regulatory signals (Lawler, 1999). It is not known, however, if such a pathway exists in vivo, or what specific cellular events it regulates.

The observation that Limk1 is strongly expressed in the brain indicates that it may function in the nervous system (Frangiskakis et al., 1996; Mizuno et al., 1994; Mori et al., 1997). This notion is reinforced by a recent finding that Limk1 heterozygosity is associated with Williams syndrome (WS) (Frangiskakis et al., 1996). WS is a developmental disorder characterized by a distinctive array of learning and behavioral disabilities (Morris and Mervis, 2000). WS children exhibit relatively low intelligence quotients and severe deficits in visuospatial constructive cognition. Genetic mapping showed that WS is caused by a hemizygous deletion of approximately 25 genes in the 7q11.23 region of the genome (Lowery et al., 1995; Nickerson et al., 1995). These genes include Syntaxin1A, frizzled3, and Limk1. Using microdeletions that do not affect either Syntaxin1A or frizzled3, the WS cognitive defect was specifically linked to Limk1 (Frangiskakis et al., 1996). The linkage between Limk1 and WS has been challenged however (Tassabehji et al., 1999). To date, it is unclear whether or how the loss of Limk1 provokes the neuropathology of WS.

To elucidate the in vivo functions of Lim kinase and generate a model for understanding WS, we have either abolished or activated Link function in Drosophila. We find that Limk dysfunction impairs the development of the antennal lobe (AL) neuropil and the growth of the neuromuscular junction (NMJ). At the NMJ, loss of Limk leads to significant overgrowth of the synaptic terminal. Conversely, increase in Limk leads to petit synapses. During AL development, increase in Limk activity results in the displacement of endogenous glomeruli to ectopic positions. Limk acts downstream of Pak, but upstream of Cofilin in glomerular development. We hypothesize that Pak, Limk, and Cofilin are components of an evolutionarily conserved signaling pathway that regulates synapse development in animals.

Materials and methods

Experimental animals

Mutations in Limk were generated via imprecise transposon excision. EP(X) 1313, containing an EP insertion 2.8 kb downstream of the Link gene, was from the Drosophila Stock Center. Mobilization of the EP element resulted in the recovery of 667 lines in which the transposon is lost. PCR screening, followed by detailed Southern blot analyses, showed that the Link gene is fully or partially deleted in five lines, Link 1 to Link 5. The OK6-Gal4 line was kindly provided by B. McCabe and M. O’Connor. The Or22a-Nsyh::GFP line was a gift from L. Vosshall. All other fly lines are described in Ang et al. (2003) or were obtained from Drosophila Stock Center.

Transgenes

The Link cDNA (Ohashi et al., 2000) was first tagged by fusing two copies of the myc-epitope to the carboxyl terminus of the protein sequence. The entire amino-terminal regulatory domain (Met1 to Leu305) was then removed and replaced with an ATG codon, to create LinkΔ. Both the full-length Link and LinkΔ cDNAs were subcloned into the pUAST vector to create UAS-Link and UAS-LinkΔ transgenes, respectively. CofilinΔ was constructed by mutating the codon TCT, which codes for Ser3, to GCT, which codes for Ala3, by PCR-mediated mutagenesis. Both the wild-type Cofilin and the CofilinΔ cDNAs were subcloned into the pUAST vector to create UAS-Cofilin and UAS-CofilinΔ, respectively. The GH-mGFP transgene was created by ligating the 3.8 kb regulatory sequences from the CG17390 locus to the mCD8::GFP cDNA. The GH-wGFP transgene was subcloned into the pCasper-G vector. Transgenic animals were generated by standard transformation procedures.

Immunohistochemistry

Immunohistochemical procedures were carried out as described in Ang et al. (2003). Confocal optical sections were collected with the Zeiss 510. Antibodies used in this paper are as follows. nc82 (1:20, Hofbauer, 1991) was a generous gift from A. Hofbauer. Anti-Syt (1:1000; Littleton et al., 1993) was a gift from H. Bellen. Affinity-purified rabbit anti-GFP (1:100) was purchased from Molecular Probes.

ORN cell body counting

Adult antennae from animals expressing Or-Gal4/UAS-mCD8 GFP were dissected in phosphate-buffered saline (PBS) and mounted in PBS with 0.1% Triton-X-100. ORN cell bodies were examined by direct visualization of the GFP fluorescence using the Confocal microscope.

Quantification of AL phenotypes

Frontal optical sections of nc82-stained preparations were examined to quantify the AL anatomy of various mutants. To obtain AL dimensions, the widest part of the lobe (~20 μm from the anterior face of the AL) was measured. At least 20 lobes were measured per genotype. To estimate Syt:GFP accumulation (in the green channel), the pixel values specifically within the glomeruli were quantified using the NIH Image J software. At least 8 ALs were measured per genotype. Statistical analyses of the AL sizes and pixel values were carried out using the Prism statistical software. To quantify glomerular structures, we scored four representative glomeruli, VA1d, DA4, VA6, and DM6. VA1d and VA6 (located superficially) were scored in 3D projections of confocal stacks. DA4 and DM6 (located within the lobe) were scored from single optical sections. The criteria we used were the appearance of a distinct border devoid of nc82 staining that surrounds each glomerulus, and the shape, size, and positioning of the glomerulus. Only when all of these characteristics were present could a glomerulus be unambiguously identified and scored as being present.

Electrophysiology

Wandering third instar w1118 and Link5 larvae were dissected and mounted on glass slides as previously described (White et al., 2001). The low Ca2+ recording physiological saline contained (in mM) NaCl 140, KCl 5, CaCl2 1, NaHCO3 4, MgCl2 6, TES 5, trehalose 5, sucrose 50 (pH 7.2). The bodywall preparation was imaged using water immersion objectives and digital optical microscopy with Nomarski optics. The SNb nerve was stimulated using suction electrodes with 2–3 μm openings, as previously described (Lennicka and Keshishian, 2000). The ventral longitudinal abdominal muscle fibers 7, 6, 13, and 12 in segments A2–A4 were examined. Evoked EJPs were recorded with sharp (25 MΩ) electrodes using a Dagan 8500 amplifier in current clamp mode (Dagan Corp., Minneapolis, MN), a Digidata 1320A analog-digital converter, and pClamp 8.0 software (Axon Instruments, Foster City, CA). Single electrode impalements of muscle fibers were used to measure spontaneous PSPs. Muscles with resting membrane voltages less than 50 mV were rejected.

Results

Link regulates the development of the neuromuscular junction

The Drosophila Link gene (hereafter referred to as Link) was cloned by screening the fly EST database with the rat Link1.
sequence (Ohashi et al., 2000). A search of the genome database showed that Limk is the only fly orthologue and maps to the cytological region 11B2 on the X chromosome. Using a P element, EP(X)1313, located 2.8 kb downstream of Limk, we generated 5 deletion alleles of the gene that we named Limk1 to Limk5 (Figs. 1B–E). Three of the alleles, Limk2, Limk3, and Limk5, remove all or most of the gene. Limk5 was used in most of our analyses. Limk hemizygotes are viable, fertile, and morphologically normal externally.

Mutation in the mouse Limk-1 gene resulted in the aberrant development of synapses (Meng et al., 2002), which raises the possibility that synapse development may be impaired in the fly Limk mutant. To address this possibility, we examine the neuromuscular structures of the third instar larva. At this stage, each abdominal hemisegment contains a stereotyped pattern of 30 muscles, which are innervated by ~35 motoneurons. Each motoneuron makes connections with specific muscles through NMJs that are highly stereotyped in terms of the number of synaptic boutons and the complexity of arborization (Keshishian et al., 1996). Third instar larval fillet of wild type and the mutant were stained with an antibody against Syntagmin (Syt) (Littleton et al., 1993) to visualize the morphology of the NMJ. Limk mutants have slightly smaller muscle fibers (e.g. muscle 13 = 18.32 ± 0.97 × 10³ μm², n = 6)

![Image of NMJ morphology](image-url)

**Fig. 2.** Limk negatively regulates NMJ growth. Photomicrographs of muscle 12 NMJs from abdominal segment A4 stained with anti-Syt (A–D). The simple morphology of the wild-type NMJ (A) is expanded in the Limk mutant (B). (C) Expression of the Limk cDNA with the motoneuron-specific OK6-Gal4 driver rescued the Limk NMJ-overgrowth phenotype. (D) Expression of the Limknull cDNA with OK6-Gal4 in the wild-type background repressed NMJ growth. (E) Staining of the wild-type muscle fiber 12 NMJ with anti-HRP (green) and the nc82 Mab (red) reveals the active zones (enlarged view of boutons in inset). (F) The number and distribution of active zones in the Limknull mutant are similar to those of wild type. (G) Quantification of bouton number in wild-type, Limknull/Y; OK6-Gal4/+; UAS-Limk/+; and OK6-Gal4/UAS-Limknull animals. The number of boutons per μm² of muscle surface on muscle 6/7, 12, and 13 multiplied by 10³ is plotted. We did not discriminate between type 1s and Ib boutons.
than wild type (23.25 ± 0.87 × 10³ μm², n = 6, P = 0.0037), and their NMJs exhibit increased bouton number (Figs. 2A, B). We quantified the NMJ overgrowth in the Limk mutant by counting the number of Type Ib and Is boutons per μm² of muscle area on muscles 6/7, 12, and 13. In the Limk mutants, there are increases in bouton numbers to 73–112% greater than those of wild type, with the largest increase shown by muscle 12 (n = 15 animals, 29 hemisegments, P < 0.0001; Fig. 2G). To determine whether Limk functions pre- or postsynaptically to regulate NMJ development, we attempted to rescue the mutant phenotype by cell type-specific cDNA expression. When wild-type Limk cDNA was expressed using the motoneuron-specific OK6-Gal4 driver (Aberle et al., 2002), the bouton numbers are reduced to 18–41% greater than those of wild type (n = 4 animals, 8 hemisegments, P < 0.0001; Figs. 2C, G). In contrast, expression of the cDNA using the muscle-specific 24B-Gal4 driver did not significantly alter the mutant NMJ phenotype (58–82% > wild type, n = 6 animals, 12 hemisegments, P > 0.27). We conclude from these results that Limk functions in the presynaptic cell to regulate the structural development of the synapse. To assess axonal projections in the Limk mutant, we stained mutant embryos with antibodies (anti-HRP, BP102, and nc82 antibody (Hofbauer, 1991) and determine the number active zones have a decreased probability of release. To distinguish between these possibilities, we stained the NMJs with the nc82 antibody (Hofbauer, 1991) and determine the number active zones at the Limk NMJs (Figs. 2E, F). We found that the Limk mutant has slightly less active zones per bouton (8.33 ± 0.63, n = 30 boutons, 4 animals) compared with the control (10.93 ± 0.68, n = 30 boutons, 4 animals, P = 0.0074). It is possible that the slight decrease in active zone number helps to compensate for the increase in bouton number, resulting in normal synaptic function in the Limk mutant.

**Limk regulates the development of the antennal lobe glomeruli**

Our previous observation that the AL structure is aberrant in the Pak mutant (Ang et al., 2003), together with our current finding, led us to wonder whether Limk also regulates the development of AL synapses. The AL is the first relay station of the olfactory pathway (Stocker et al., 1999), where olfactory receptor neuron (ORN) axons synapse on dendrites of the projection neurons (PNs). The synapses are not evenly distributed through the AL neuropil, but are concentrated in anatomically distinct structures, the glomeruli, each of which is unique based on its characteristic size, shape, and position in the AL neuropil. Axons from ORNs expressing a given odorant receptor terminate in a specific glomerulus (Gao et al., 2000; Vosshall et al., 2000).

We examined the morphology of the ALs of the Limk mutants by staining with the nc82 mAb, which stains the AL neuropil revealing the glomeruli. Glomerular development is assessed by several criteria. First, is whether each glomerulus is surrounded by a distinct boundary, a margin devoid of nc82 staining. Second, the size, shape, and location of the glomeruli (Couto et al., 2005; Laissue et al., 1999) are taken into account. Glomerular development is quantified by determining the frequencies with which four representative glomeruli (VA1d, DA4, VA6, DM6) can be unambiguously identified based on the above criteria. In the wild type, the AL is 72.7 ± 1.17 μm in diameter (n = 22) and is partitioned into distinct glomeruli (Fig. 4A). The indicator glomeruli can be identified in 100% of the ALs (Fig. 4G). In the Limk3 mutant, the ALs are 70.68 ± 1.10 μm in diameter (n = 22, P = 0.2116), a value similar to that of the wild type. The indicator glomeruli can be also discerned in all the ALs (Figs. 4B, G).

We also assessed synaptic development by examining Synaptotagmin:GFP (Zhang et al., 2002) accumulation in the DM2, DM3, and VA1v glomeruli using the Or22a-Gal4, Or47a-Gal4, and Or47b-Gal4 drivers, respectively. In the wild type, these glomeruli have relatively smooth boundaries and accumulate Syt:GFP (e.g. DM2 pixel value = 59.6 ± 3.2 units, n = 8; Figs. 4A, C, E). When examined under constant laser power, the level of Syt:GFP accumulation in the Limk3

**Limk does not regulate the function of the neuromuscular junction**

To ascertain if Limk plays a role in synaptic function, we examine the synaptic physiology of the Limk mutant neuromuscular synapses. We performed intracellular recordings from muscle 6/7, 12, and 13 from segments A3 or A4 of the third instar larva to measure the postsynaptic responses to spontaneous and evoked transmitter release. The amplitude of evoked postsynaptic potentials (EPSPs) at the various muscles in the Limk mutant is not significantly different from that of the wild type (Figs. 3A, C, Table 1). Both the size and amplitude distribution of the unitary potentials are also similar between Limk (0.731 ± 0.068 mV, n = 343 events, 4 animals) and the control (0.774 ± 0.053 mV, n = 301 events, 5 animals, P > 0.2) (Figs. 3B, D). These results indicate that the synaptic function of the NMJ is not altered in the Limk mutant, despite the expansion in NMJ size. The similarity in synaptic functions implies that the extra boutons are either less active or each has the same number of actives zones as normal, but the active zones have a decreased probability of release. To distinguish between these possibilities, we stained the NMJs with the nc82 antibody (Hofbauer, 1991) and determine the number active zones at the Limk NMJs (Figs. 2E, F).
mutant is not significantly different from that of the wild type (DM2 = 64.6 ± 1.5 units, n = 8, P = 0.18; Figs. 4B, D, F).

To assess projections of the ORN axons, we expressed a membrane-anchored GFP (encoded by UAS-mCD8∷GFP) under the control of the Or22a-Gal4, Or47a-Gal4, and Or47b-Gal4 drivers. In the mutant antennae, the cell bodies of Or22a, Or47a, and Or47b neurons are found in numbers (Table 2) and distribution similar to those of wild type (Figs. S2A–D). The Or22a and Or47a axons also converge precisely to their cognate glomeruli (Figs. S2E–H). We conclude from these observations that Limk is not required for the differentiation of the ORNs or the targeting of their axons.

The lack of obvious synaptic defects in the ALs of the loss-of-function Limk mutant suggests that a redundant gene, acting in parallel with Limk may partially compensate for its role in AL development. To further examine Limk’s function in the ORNs, we increased Limk activity in the ORNs during glomerular development. We expressed the N-terminally truncated, hyperactive Limkkd molecule (encoded by the UAS-Limkkd transgene) under the control of the SG18.1-Gal4 driver, which is preferentially expressed in a large subset of ORNs (Ang et al., 2003; Shyamala and Chopra, 1999). In the Limkkd over-expressing animal, the AL is elongated dorsoventrally (86.13 ± 1.23 μm, n = 16, compared with wild type, 72.7 ± 1.17 μm, n = 22, P < 0.0001; Figs. 5A, B). The structures of many glomeruli are disrupted. Inspection of the marker glomeruli shows that while those located at the lateral position (VA1d and DA4) are easily identifiable, those located more medially (VA6, DM6) can only be identified in 0 to 64% of the time (n = 22, Fig. 4G). In addition to the disruption in glomerular structures, nc82-stained structures appear at the midline, a situation not seen in the wild type (14/14 brains; Figs. 5C, D). These midline structures accumulate synaptobrelin∷GFP (Nsyb∷GFP), indicating that they contain synapses.

Table 1

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<tr>
<th>Muscle fiber</th>
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<td>7</td>
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<td>21.35 ± 5.65 (n = 12)</td>
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<td>12</td>
<td>27.98 ± 5.59 (n = 6)</td>
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<tr>
<td>w1118</td>
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<tr>
<td>7</td>
<td>21.47 ± 5.66 (n = 3)</td>
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<td></td>
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<tr>
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<td>22.24 ± 9.10 (n = 5)</td>
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<td>hemisegments, 3 animals</td>
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<td>21.47 ± 5.66 (n = 3)</td>
<td>P &gt; 0.08</td>
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Fig. 3. Limk is not required for NMJ function. (A) The evoked Excitatory Junction Potentials (EJP) recorded from mutant ventral longitudinal muscle fibers (Limk5; gray bars) show no significant difference from those of control larvae (w1118; white bars). (B) Comparison of the amplitude distributions of spontaneous miniature EJPs. White bars = w1118, mean amplitude 0.774 ± 0.053 mV, n = 301 events. Gray bars = Limk5; mean amplitude 0.731 ± 0.068 mV, n = 343 events. (C) Sample evoked EJPs from muscle fiber 13 of control (w1118) and mutant (Limk5) larvae, showing similar amplitude and time course. (D) Sample spontaneous miniature EJPs from muscle fiber 13 of control (w1118) and mutant (Limk5) larvae, showing similar amplitude and time course. Scale bars = 5 mV and 100 ms (C), and 1 mV and 100 ms (D).
Labeling of the ORN axons with a membrane-anchored GFP indicates the midline structures reside within the antennal commissure, which normally contains only axons (Figs. 5E, F). Globose structures also appear at the midline when the full-length \( \text{Limk} \) gene is expressed in ORNs (data not shown), indicating that this effect is not due to a neomorphic function caused by truncation of the Limk protein. These results show that \( \text{Limk} \) has a powerful effect on the glomeruli, supporting a role for \( \text{Limk} \) in AL development.

Presynaptic \( \text{Limk}^{kd} \) expression induces ectopic glomeruli

To probe the nature of the midline structures in the \( \text{Limk}^{kd} \)-expressing animal, we labeled the DM2 glomerulus using the \( \text{Or22a-Nsyb}:\text{GFP} \) transgene. In the wild type, DM2 is located at the dorsomedial corner in all ALs (Fig. 6A). No nc82-stained structures are seen at the midline. In the \( \text{Limk}^{kd} \)-expressing animal, DM2 is located entirely at the midline in 60% of the ALs (\( n = 20 \); Figs. 6C, D). In the remaining ALs, DM2 shows...
only minor medial displacement (Fig. 6B). Thus, the ectopic structures at the midline of the \textit{Limk}\textsuperscript{kd}-expressing animals are innervated by ORN axons. We wanted to know if the midline structures are also innervated by PN dendrites. We created the GH-mGFP transgene (Material and methods), which is expressed in a subset of the PNs. In the GH-mGFP transgenic animal, a stereotyped subset of PN dendrites is labeled creating a characteristic patchwork pattern in the AL neuropil (data not shown). Careful examination of the confocal sections showed that a pair of dorsomedial arbors extend posteriorly (Fig. 6E), but was never found at the level of the ellipsoid body (0/20 ALs; Fig. 6G). In the \textit{Limk}\textsuperscript{kd}-expressing animal, GH-mGFP labels the same stereotyped set of dendritic arbors. However, the pair of dorsomedial arbors extend to the level of the ellipsoid body where they converge on the midline (26/32 ALs; Figs. 6F, H). The PN dendrite arbors overlap with nc82-stained midline structures. These results indicate that the midline structures are innervated by ORN axons and PN dendrites. Together, the observations suggest that the midline structures of the \textit{Limk}\textsuperscript{kd}-expressing animals are displaced endogenous glomeruli.

\textbf{Limk is necessary for Pak’s ability to direct glomerular development}

In vitro experiments with the human proteins show that Pak1 phosphorylates and activates Limk1 (Edwards et al., 1999). We hypothesize that, in the fly, Pak acts upstream of Limk to regulate glomerular development in the ALs. In the Pak\textsuperscript{4}/Pak\textsuperscript{6} null mutant, the ALs are smaller compared with those of wild type. ORNs differentiate normally in \textit{Limk} mutants

<table>
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<th>ORN subtype</th>
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<tr>
<td>Or22a</td>
<td>19.4 ± 1.3 (n = 10)</td>
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<tr>
<td>Or47a</td>
<td>28.5 ± 2.3 (n = 13)</td>
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<tr>
<td>Or47b</td>
<td>58.4 ± 4.6 (n = 12)</td>
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<td>Limk\textsuperscript{5}</td>
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<td>31.2 ± 2.3 (n = 13)</td>
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<tr>
<td>58.1 ± 3.6 (n = 12)</td>
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Fig. 5. Constitutively active \textit{Limk}\textsuperscript{kd} induces abnormal midline structures. Preparations from animals in which SG18.1-Gal4 drives the expression of UAS-Nsyb∷GFP (C, D), UAS-mCD8∷GFP (E, F) and UAS-Limkk\textsuperscript{kd} (B, D, F). The ALs were stained with nc82 (red) and anti-GFP (green) antibodies. (A) A 3-D projection of a pair of wild-type ALs, showing well-defined glomeruli that are arranged in a stereotyped pattern. The marker glomeruli, VA1d, DA4, VA6, and DM6, are indicated. (B) A 3-D projection of ALs from the \textit{Limk}\textsuperscript{kd}-expressing animal. The marker glomeruli cannot be reliably identified due to strong disruption in the glomerular structures. (C) A deep section of a pair of wild-type ALs showing the absence of nc82-stained structures at the midline. The approximate position of the commissure is outlined. (D) A comparable section as panel C of a pair of ALs from the \textit{Limk}\textsuperscript{kd}-expressing animal showing nc82-stained structures at the midline. The structures accumulate Nsyb∷GFP. (E) An optical section of a pair of wild-type ALs showing the antennal commissure (outlined), which contains only ORN axons (labeled by mCD8∷GFP). (F) A comparable section as panel E of a pair of ALs from a \textit{Limk}\textsuperscript{kd}-expressing animal. The commissure is enlarged due to the presence of abnormal nc82-stained structures. Scale bar = 25 μm in panel F.
type (63.71 ± 1.39 μm, n = 22, P < 0.0001; Figs. 7A, B). Staining with the nc82 mAb shows that the boundaries surrounding individual glomeruli are frequently missing, resulting in loss in distinctiveness of most of the marker glomeruli (Fig. 4G, glomeruli identifiable in only 0% to 36% of the ALs, n = 22). Thus, Pak activity is necessary for glomerular development. To determine if elevated Pak is sufficient to alter AL development, we expressed the hyperactive Pak\textsuperscript{myr} molecule (Ang et al., 2003; Hing et al., 1999) under the control of SG18.1-Gal4. Expression of Pak\textsuperscript{myr} in the ORNs results in strong disruption in the anatomy of the ALs. Although the neuropil is partitioned, the compartments correspond poorly to known glomeruli (Figs. 7C, D). Consequently, the marker glomeruli are identifiable in only 0 to 79% of ALs (n = 14; Fig. 4G). The abnormal structures of the glomeruli are reflected in the DM2 glomerulus (labeled with the Or22a-Nsyb::GFP transgene). Unlike the wild type where it is distinct and symmetrically positioned (Fig. 7C), in the Pak\textsuperscript{myr} animal, it is poorly demarcated from the surrounding neuropil and asymmetrically positioned (Fig. 7D). It is possible that adjacent glomeruli are fused to form larger structures in the Pak\textsuperscript{myr}-expressing animal. Thus, increased presynaptic Pak activity is
sufficient to disrupt glomerular morphogenesis. To determine if Limk mediates Pak function, we expressed UAS-Pakmyr in the Limk5 null mutant background. In the Limk5/Y; SG18.1-Gal4/UAS-Pakmyr animal, the AL neuropil is partitioned into compartments that correspond well to known glomeruli (Figs. 7E, F). Marker glomeruli can now be identified in 79% to 100% of the ALs (n = 14; Fig. 4G). The DM2 glomeruli are morphologically distinct and symmetrically positioned (Figs. 7E, F). Thus, mutation in Limk blocks the ability of Pakmyr to interfere with glomerular development. This result supports the idea that Limk functions downstream of Pak to regulate glomerular development.

Cofilin functions downstream of Limk to regulate AL innervation

Biochemical experiments have shown that the Drosophila Limk specifically phosphorylates the serine 3 amino acid of Cofilin, a key regulator of actin turnover (Ohashi et al., 2000). In human, phosphorylation of serine 3 of Cofilin has been shown to shut down its actin depolymerizing activity (Agniew et al., 1995). Indeed, mutation of the serine 3 amino acid of Cofilin to an alanine leads to a constitutively activated Cofilin protein. We therefore hypothesize that Limk down-regulates Cofilin function during glomerular development. To test this hypothesis, we expressed Limkkd either alone, with wild-type Cofilin, or with CofilinS3A in ORNs. Expression of Limkkd results in disruption of glomerular structures and ectopic glomeruli at the midline in 100% of the ALs (n = 14; Figs. 8A, C). Coexpression with wild-type Cofilin did not modify the Limkkd phenotype (data not shown). In contrast, coexpression with CofilinS3A leads to the reappearance of distinct glomerular structures and the loss of ectopic midline structures in 83% of the ALs (n = 12; Figs. 8B, D). These results support the idea that Limk negatively regulates Cofilin function by acting through the serine-3 amino acid during glomerular development.

Discussion

In recent years, the Limk protein has emerged as a key regulator of the actin cytoskeleton. Numerous in vitro experiments showed that Limk strongly directs the assembly
of the actin cytoskeleton. However, what Limk does in the context of the living animal has remained mysterious. The discovery that human Limk1 is expressed in the brain and its association with WS has further spurred interests in ascertaining its in vivo functions. To elucidate its in vivo functions, we have either knocked out or activated the Limk gene in the fly. Our results show that Limk dysfunction results in defects in synapse development. Limk plays a role in synapse development

By either knocking out or activating Limk, we observed that Limk regulates synapse structures at the NMJ and in the AL of Drosophila. At the NMJ, the loss of Limk leads to overgrowth of the synaptic terminal. Conversely, activation of Limk leads to stunted terminals. In the AL, the loss of Limk does not induce dramatic structural changes. However, overexpression of Limk results in ORN axons establishing glomeruli at ectopic locations. These results support the idea that Limk regulates structural development of synapses in vivo. Our findings are in accord with the study of Meng et al. (2002), which showed that the loss of mouse Limk1 gene results in abnormal dendritic spines morphology in the hippocampus. Interestingly, hippocampal slices from the Limk1 knockout mouse also exhibit changes in synaptic functions, an effect not seen at the fly Limk mutant. Despite the structural defects, Limk mutant NMJs display normal synaptic physiology. Collectively, these observations across different systems indicate that proteins of the Limk family play a conserved role in synapse development. Disruption in synapse development may underlie the behavioral and developmental symptoms of WS patients.

How does Limk regulate synaptic growth? The inverse correlation of NMJ synapse size with the dose of Limk activity indicates that the function of Limk at the NMJ is to inhibit synapse expansion. We propose that, by phosphorylating Cofilin and stabilizing the actin cytoskeleton, Limk prevents synapse remodeling and sprouting. Since the motoneuron synapse grows 10-fold in size between the first and the third larval instar stages (Zito et al., 1999), expression of Limkkd early during larval development (with the OK6-Gal4 driver) would arrest the NMJ in a premature state. Although we do not precisely understand the role of Limk in the olfactory system, the model of Limk function that we have proposed above could also account for the ability of Limkkd to cause ORNs to form glomeruli in aberrant positions. During pupal development, terminal arbors from adjacent glomeruli initially overlap but subsequently draw apart to form discrete glomeruli (Jefferis et al., 2004; Jhaveri et al., 2000). Expression of Limkkd early in development (with the SG18.1-Gal4 driver) would be expected to inhibit the retraction of neighboring arbors from each other.

**Fig. 8. Cofilin functions downstream of Limk to regulate glomerular development.** AL from Limkkd-expressing animals is stained with nc82 (magenta) and anti-GFP (green) antibodies to probe glomerular development. (A, C) In the wild-type background, Limkkd directs the assembly of ectopic glomeruli in the commissure (arrowhead). (B, D) Coexpression of CofilinS3A leads to the reappearance of distinct glomerular structures and suppression of ectopic glomeruli in the commissure (arrow head). (E) Model for the regulation of synapse remodeling by Pak, Limk, and Cofilin. Pak is proposed to phosphorylate and activate Limk and a presently unknown molecule (X). Both Limk and X then function to repress the activity of Cofilin. Our results indicate that Limk phosphorylates Cofilin on serine 3 thus shutting down its actin depolymerizing activity. The resultant actin cytoskeletal assembly promotes synapse stability and inhibits synapse remodeling and sprouting. Scale bar = 25 μm in panel D.
Subsequent maturation of the overlapping terminal arbors may then result in the formation of aberrant glomeruli. Another possibility is that Link directly induces glomerular expansion, a role that is opposite to that postulated above for NMJ development. How Linkkd instructs the formation of ectopic glomeruli is currently being investigated in our laboratory.

While we were revising this paper, Eaton and Davis reported that Link functions downstream of the BMP receptor, wit, to increase NMJ stability and stimulate NMJ growth (Eaton and Davis, 2005). That Link promotes synapse stability is compatible with our findings that Link restricts synaptic remodeling. Eaton and Davis also reported, however, that NMJs have normal sizes in the Linkkd allele, which contrast with our finding of enlarged NMJs in the Linkkd allele. Furthermore, they found that expression of a wild-type Link transgene restored NMJ growth in the wit mutant, leading to the proposal that Link stimulates NMJ growth. In contrast, we found that expression of a gain-of-function Link transgene produced stunted NMJs, leading to our conclusion that Link represses NMJ growth. These divergent results can be reconciled by the idea that Link can both restrict and promote NMJ growth, and that the dose of Link determines its effects. It is likely that the synaptic stability induced by Link is critical not only to prevent unregulated growth, but also for growth elicited by stimulatory signals by inhibiting retraction of newly formed boutons. In the wild type, Link would be present at a “threshold” level where dynamic remodeling and synaptic stability coexist. In this scenario, the complete loss of Link, as in the Linkkd null mutant, would cause excessive NMJ sprouting. A residual level of activity, as in the hypomorphic Linkkd mutant (Eaton and Davis, 2005), would be sufficient for a normal NMJ size. Finally, a high level of activity, as in the Linkkd-expressing animal, would prevent NMJ growth. This model suggests that Link may play a pivotal and dosage-sensitive role in specifying NMJ development.

Pak, Link, and Cofilin are components of a signaling pathway in synaptic growth

In vitro studies have implicated molecules functioning both up- and downstream of Link. For example in human, Pak1 phosphorylates and activates Limk1 (Edwards et al., 1999). In the fly, Link phosphorylates Cofilin in vitro (Ohashi et al., 2000). It is not known if these proteins function in a signaling pathway in vivo. We present evidence that Pak, Link, and Cofilin are components of a signaling cascade that govern glomerular development in the olfactory system. First, loss of Pak results in an aglomerular phenotype whereas its activation disrupts glomerular structure, indicating that Pak plays a critical role in glomerular development. Second, a functional Link is needed for Pak to disrupt glomerular structure, consistent with the idea that Pak activates Link. Third, the glomerular-inducing activity of Linkkd is suppressed by coexpression with CofilinS3A, showing that Link represses Cofilin. Our genetic results thus show that Pak activates Link, which in turn down-regulates Cofilin during glomerular development (Fig. 8E). Our results also hint at the presence of a gene that acts in parallel with Link: whereas elevation of Link in the ORNs leads to profound glomerular assembly, loss of Link only slightly impairs olfactory synapses. We hypothesize that a redundant gene masks the requirement for Link in glomerular development (Fig. 8E).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.01.030.

References


