RESEARCH ARTICLE

The NAV2 homolog Sickie regulates F-actin-mediated axonal growth in Drosophila mushroom body neurons via the non-canonical Rac-Cofilin pathway

Takashi Abe1,2, Daisuke Yamazaki1, Satoshi Murakami1, Makoto Hiroi1, Yohei Nitta1, Yuko Maeyama1 and Tetsuya Tabata1,2,*

ABSTRACT

The Rac-Cofilin pathway is essential for cytoskeletal remodeling to control axonal development. Rac signals through the canonical Rac-Pak-LIMK pathway to suppress Cofilin-dependent axonal growth and through a Pak-independent non-canonical pathway to promote outgrowth. Whether this non-canonical pathway converges to promote Cofilin-dependent F-actin reorganization in axonal growth remains elusive. We demonstrate that Sickie, a homolog of the human microtubule-associated protein neuron navigator 2, cell-autonomously regulates axonal growth of Drosophila mushroom body (MB) neurons via the non-canonical pathway. Sickie was prominently expressed in the newborn F-actin-rich axons of MB neurons. A sickie mutant exhibited axonal growth defects, and its phenotypes were rescued by exogenous expression of Sickie. We observed phenotypic similarities and genetic interactions among sickie and Rac-Cofilin signaling components. Using the MARCM technique, distinct F-actin and phospho-Cofilin patterns were detected in developing axons mutant for sickie and Rac-Cofilin signaling regulators. The upregulation of Cofilin function alleviated the axonal defect of the sickie mutant. Epistasis analyses revealed that Sickie suppresses the LIMK overexpression phenotype and is required for Pak-independent Rac1 and Slingshot phosphatase to counteract LIMK. We propose that Sickie regulates F-actin-mediated axonal growth via the non-canonical Rac-Cofilin pathway in a Slingshot-dependent manner.

KEY WORDS: Drosophila mushroom body, Axon development, F-actin, Microtubule, Neuron navigator/UNC-53, Rac-Cofilin pathway, Slingshot LIMK

INTRODUCTION

During brain development, neurons undergo multiple morphological changes to form an elaborate neural network. The Drosophila mushroom body (MB), which forms bilaterally symmetric and dorsomedially bifurcated axonal lobe structures in the central brain, has been well studied as a model of neuronal development (Awasaki et al., 2011; Ito et al., 1997; Lee et al., 1999; Miura et al., 2013; Zhu et al., 2006). Among various regulators of neuronal morphogenesis, ADF/Cofilin and Rac GTPase (Rac) are key molecules in controlling cytoskeletal remodeling in axonal development (Bernstein and Bamburg, 2010; Hakeda-Suzuki et al., 2002; Hall and Lalli, 2010; Ono, 2007). Cofilin [Twinstar (Tsr) in Drosophila] plays an essential role as a regulator of axonal growth by severing and depolymerizing F-actin. Because Cofilin is activated by dephosphorylation by the Slingshot (Ssh) phosphatase and is inactivated by phosphorylation by LIMK, the loss of Ssh or excessive activation of LIMK results in an axonal growth defect (Mizuno, 2013; Ng and Luo, 2004). In Drosophila, Rac has been proposed to act as a bidirectional switch for signaling cascades. One signaling event is the canonical Rac-Pak-LIMK pathway to suppress Cofilin-dependent axonal growth. The overexpression of Pak, a downstream effector of Rac, induces axonal growth defects similar to those observed with LIMK overexpression. In addition, introducing one mutant copy of Rac or Pak suppresses the axonal defect induced by LIMK overexpression. Another signaling event is the Pak-independent non-canonical pathway to positively regulate axonal growth. Rac mutant animals show multiple MB axonal defects, but the axonal growth defect is alleviated by the exogenous expression of Rac1Y40C (Ng et al., 2002), which lacks the ability to activate Pak but does not affect lamellipodia formation (Joneson et al., 1996; Lamarche et al., 1996). Furthermore, Rac1Y40C overexpression remarkably suppressed the LIMK overexpression phenotype (Ng and Luo, 2004).

Although several pieces of evidence have suggested the importance of the non-canonical pathway and predicted the existence of its mediator, whether this pathway finally converges upon the downstream Cofilin pathway and subsequent F-actin reorganization remains unclear (Kligys et al., 2007; Nagata-Ohashi et al., 2004; Ng and Luo, 2004). Moreover, many biochemical studies have assessed the regulation of Cofilin function and F-actin states using in vitro systems; the endogenous changes in F-actin and Cofilin phosphorylation appear not to have been analyzed simultaneously with an internal control in developing brain. To address these issues, we searched for a novel factor that interacts with Rac-Cofilin signaling components and positively regulates MB axonal growth. We observed that Sickie, which has a calponin homology (CH) actin-binding domain and shares structural similarities with the human microtubule-associated protein (MAP) neuron navigator 2 (NAV2), showed prominent expression in F-actin-rich newborn MB axons and genetically interacted with Rac-Cofilin signaling regulators. Although Sickie was originally identified by genome-wide RNAi screening in Drosophila S2 cells and the report proposed the involvement of Sickie in the innate immune response (Foley and O’Farrell, 2004), in this report we focused on the function of Sickie in the regulation of Cofilin-mediated F-actin remodeling and propose an expanded model of regulatory mechanisms during axonal development (Ng and Luo, 2004).
RESULTS
Characterization of Sickie
To identify novel key factors that regulate MB axonal development through cytoskeletal remodeling we used pBGay, a piggyBac and P-element hybrid vector (gift of Claude Desplan, New York University, USA), to obtain a larger number of genomic insertion lines. Among the 2000 putative enhancer trap lines, we found that the 163pBGay-gal4 line expressed the GFP reporter in developing MB α/β axons at ~36 h after puparium formation (APF) (Fig. 1A). In this line, the gal4 vector was inserted in the sixth intron of sickie (Fig. 1B). To examine the endogenous expression pattern of the Sickie protein, we generated a polyclonal antibody (Fig. 1C). We found that Sickie was broadly expressed in the neuropile of the developing brain (Fig. 1A′), and overlapped with the GFP signal in MB axons (Fig. 1A″). As described in FlyBase, Sickie is a homolog of the human NAV2 MAP and shares several conserved domains and motifs: a CH actin-binding domain, coiled-coil (CC) motifs and an AAA ATPase (AAA) domain (Fig. 1C,D) (Akhmanova and Steinmetz, 2010; Banuelos et al., 1998; Erzberger and Berger, 2006; Galjart, 2010; Klein et al., 2011; Martinez-Lopez et al., 2005; Schmidt et al., 2009). SxIP EB1-binding motifs were identified with GENETYX.

To test the requirement of Sickie in axonal development, we generated several deletion mutant alleles by FLP-FRT-based mutagenesis (Fig. 1B) (Parks et al., 2004). sickie^A and sickie^L-2 are sickie locus-specific deletion alleles. The sequence that encodes 510 amino acids corresponding to the third proline-rich region and the first CC motif was deleted in these sickie mutants. sickie^L-2 lacked the coding sequence corresponding to the CH domain, the first and second proline-rich regions, and the first SxIP motif. The sickie^L-1 and sickie^L-3 alleles lacked all domains deleted in the former three alleles. We also found a piggyBac transposon insertion allele, sickie^384, by complementation tests with sickie^A and Df(2L)ED1303. Df(2L)ED1303 is a large deficiency line that is lacking the entire coding sequence of sickie and the neighboring genes.

We found that the sickie^A^384 mutant displayed short axonal lobes compared with those of the control animal (Fig. 1E,F), although the morphology of the peduncle appeared normal. Because the axonal defect was most clearly distinguished in Fasciclin 2 (FasII)-labeled α/β axons (Crittenden et al., 1998), two categories were defined for the α/β lobe phenotype. If either the α or β axonal lobe failed to extend its axons beyond the half-line of a normally formed α or β lobe, the phenotype was classified as Class I. If both α and β lobes failed to extend their axons beyond the half-line of normal α/β lobes, the phenotype was classified as Class II (Fig. 1H). We compared the phenotypic severity among several sickie mutants and found that the penetrance of the sickie^A^384 mutant was nearly identical to that of the sickie^L-1/Df(2L)ED1303 mutant, an allelic combination of large deletions lacking the coding sequence including a start coding for Sickie (Fig. 1C,M). In addition, the sickie^A^384 mutants displayed defective ellipsoid body (EB) ring structures in each hemisphere of the central brain, presumably resulting from the axonal growth defect (Fig. 1J,J). We also detected a prominent Sickie signal in the core region of the peduncle, an area occupied by axons of newborn α/β neurons and marked by a reduced FasII signal (Fig. 1K−K′). By contrast, prominent Sickie expression was not detected in the sickie^A^384 mutants (Fig. 1L−L′). These results suggest that sickie^A and sickie^384 are loss-of-function alleles. In summary, we conclude that Sickie is strongly expressed in the axons of newborn α/β MB neurons, and mutants show axonal lobe extension defects suggesting that Sickie is required for the proper formation of MB axonal lobe structure.

Sickie is cell-autonomously required for the axonal growth of newborn MB neurons
To address whether Sickie regulates axonal development cell-autonomously, we performed single-cell MARCM analysis (Wu and Luo, 2006). Compared with the axons of wild-type α/β neurons (Fig. 2A), sickie^A mutant neurons failed to extend their branched axons to the lobe terminus (Fig. 2B). Next, we conducted rescue experiments to test the sufficiency of Sickie in MB axonal growth. First, we used elav-gal4, a pan-neuronal driver, to induce the expression of wild-type Sickie (UAS-sickieWT). Sickie was broadly detected throughout neuronal structures, and the axonal growth defect was nearly fully suppressed, except for the β-lobe fusion phenotypes (Fig. 2C−C′). This phenotype appears to be due to sickie overexpression because the phenotype was observed when sickie was expressed with elav-gal4 in an otherwise wild-type background. Second, we introduced the OK107-gal4 MB-expressing driver and observed the morphological rescue of MB axons (Fig. 2D−D′). Third, to further dissect the spatiotemporal timing at which Sickie is required, we used NP7175-gal4, which drives gene expression in the α/β core neurons of adult MBs (Aso et al., 2009; Tanaka et al., 2008). We examined the gal4 expression patterns of this driver line from the onset of puparium formation to the adult stage because α/β neurons are born at the beginning of the pupal stage (Lee et al., 1999). GFP expression was detected in the inner regions where young neurons reside in the FasII-labeled α/β lobes, although expression was weak, most likely owing to the delay of GFP expression (supplementary material Fig. S1). We found a significant rescue of the axonal defects with this driver (Fig. 2E, rightmost column), suggesting that Sickie expression in newborn α/β neurons is important for axonal growth. Similar rescue results were obtained using UAS-mCherry::sickie^WT (Fig. 2F). Finally, to examine whether the function of the AAA domain is required, we introduced a sickie^K1881A mutant form (UAS-mCherry::sickie^K1881A) and did not find significant rescue effects in either the OK107-gal4-based or NP7175-gal4-based experiments (Fig. 2G−H). Taken together, these results suggest that Sickie is cell-autonomously required in newborn MB neurons to positively regulate axonal growth through the function of the AAA domain.

Phenotypic similarities and genetic interaction among mutants for sickie and Rac-Cofilin signaling regulators
During development, newborn MB neurons extend their axons into the core region of the axonal bundle, an area in which the F-actin signal is prominent (Fig. 3A, asterisk) but the FasII signal is weak (Fig. 3A′) (Kurusu et al., 2002). We found that the prominent Sickie signal (Fig. 3A″) overlapped with F-actin at the core region in α/β axonal bundles (Fig. 3A″), and Sickie has a CH actin-binding domain (Fig. 1C). These findings raise the possibility that Sickie plays a role in regulating the actin cytoskeleton in growing MB axons.

In a current model, Rac canonically signals to the downstream effector Pak, which activates LIMK to negatively regulate Cofilin-dependent axonal growth (Fig. 3B, red arrows) (Ng and Luo, 2004). Rac also signals to a Pak-independent pathway to promote axonal growth (Fig. 3B, green arrow), although the downstream signaling mechanism remains unclear (Fig. 3B, dotted lines). We found that the expression of constitutively active (CA) forms of Rac1 (UAS-Rac1V12), Pak (UAS-PakMyr) and LIMK (UAS-LIMK60S) all resulted in axonal growth defects (Ang et al., 2006; Hing et al., 1999; Luo et al., 1994). The CA mutants of Rac1 or Pak failed to extend axons to form peduncles and lobe structures, and we defined this axonal growth defect as ‘posterior arrest’, a more severe phenotypic class than Class I or Class II (Fig. 3D,E). This axonal defect was also
observed in a cofilin-RNAi animal (Fig. 3G), suggesting that this phenotype reflects a significant impairment in Cofilin function. By contrast, the CA LIMK mutant frequently showed axonal growth defects at the lobe region but not the peduncle (Fig. 3F), and these defects were similar to those observed in the ssh1-63 or sickieΔ/Δ mutants (Fig. 3H,I). We also found that expression of dominant-negative Rac1 (UAS-Rac1N17) frequently induced axonal growth defects in the lobe region (Fig. 3J). Although sickie cofilin
heterozygous mutants did not show obvious axonal growth defects (Fig. 3K,K′) and Rac1 Rac2 Mtl heterozygous mutants showed the Class I phenotype most frequently (Fig. 3L,L′). Rac sickie cofilin triple-heterozygous mutants showed a dramatic increase in the penetrance of the posterior arrest, and this mutant always showed the split EB (Fig. 3M-N).

Taken together, these results suggest that sickie genetically interacts with the Rac-Cofilin pathway and demonstrates a possible involvement of Sickie in Cofilin-dependent F-actin regulation in MB axonal growth.

Distinct patterns of F-actin enrichment in developing axons mutant for sickie and Rac-Cofilin signaling regulators

If Sickie functions in Cofilin-dependent F-actin reorganization, the F-actin expression pattern would be altered in developing axons in the sickie mutant, as the ssh mutant shows increased F-actin signal in epithelial tissues, such as eye and wing discs, and in follicle cells in the egg chamber (Corrigall et al., 2007; Nagel et al., 2010; Niwa et al., 2002). As expected, a prominent increase in F-actin signal was detected in the core region of the defective α/β axonal lobe in the sickieΔA384 mutant (Fig. 4B′, asterisk) compared with the yw
control animals (Fig. 4A′, asterisk). We also examined F-actin levels using the MARCM technique (Lee and Luo, 1999), which enabled us to detect even slight changes in F-actin levels by comparing a lobe containing homozygously mutated axons with a contralateral heterozygous lobe as an internal control within the same brain (Fig. 4C′, white asterisk). A sickieΔ clone showed a slightly enhanced F-actin signal in the α/β lobe core region at 72 h APF (Fig. 4C′, yellow asterisk). The mean intensity of the endogenous F-actin signal was significantly elevated in the α/β lobe containing the sickieΔ clones compared with that of the contralateral control lobe (Fig. 4F′). These results suggest that Sickie is required for F-actin-mediated axonal growth of MB neurons.

Next, we examined mutant clones of Ssh and LIMK. An sshΔ-63 clone showed a stronger increase in F-actin signal at and around the core region of the α/β lobe (Fig. 4D′) than that of the sickieΔ clone, and the mean F-actin level was significantly elevated (Fig. 4G). By contrast, in a LIMKΔ-63 CA clone, although the prominent F-actin signal was similarly detected in the α/β lobe branching point, an additional signal increase was observed toward the distal region of the medial axons (Fig. 4E′). The mean F-actin signal level was also elevated in the LIMKΔ-63 mutant clones (Fig. 4H). Unlike the sickieΔ, sshΔ-63 and LIMKΔ-63 mutants, the PakΔCA and Rac1V12 clones displayed the posterior arrest phenotype (Fig. 4I′, J′) with ectopic regions of increased F-actin (Fig. 4I′, J′). A cofilin-RNAi clone also displayed the posterior arrest growth defect (Fig. 4K′) with a greater increase in F-actin (Fig. 4K′).

**Distinct phospho-Cofilin expression patterns in developing axons mutant for sickie and Rac-Cofilin signaling regulators.** To address whether Sickie is essential for the Cofilin-mediated F-actin reorganization, we next focused on the regulation of Cofilin, the critical converging point of the signaling cascades. MARCM analysis was also conducted to simultaneously detect changes in Cofilin phosphorylation and F-actin states in mutant axons to estimate Cofilin activity. Because Ssh directly activates Cofilin through dephosphorylation, we first examined whether increased phospho-Cofilin (P-Cofilin) signal was detectable in the axons of the sshΔ-63 clone. We used a P-Cofilin antibody (Zhang et al., 2011) and detected a broad increase in the P-Cofilin signal (Fig. 5A′, red outline) in the sshΔ-63 mutant axons compared with the internal control axons (Fig. 5A′, white outline), with a moderate increase in the F-actin signal (Fig. 5A′, yellow asterisk) at ~90 h APF. If Sickie is also essential for Cofilin dephosphorylation and Cofilin function is impaired in the sickieΔ mutant axons, a similar increase in the P-Cofilin signal would be observed. However, we did not detect obvious increases in P-Cofilin (Fig. 5B′) and F-actin (Fig. 5B′). Considering the moderate F-actin elevation in the sickieΔ clone (Fig. 4C′) compared with sshΔ-63 (Fig. 4D′), we reasoned that increased P-Cofilin would not be sufficiently high to detect immunohistochemically; therefore, we used immunoblotting. We found that the P-Cofilin levels were increased ~1.8-fold (n = 3) in the pupal brains of sickieΔ-455 mutants compared with control animals, as opposed to the cases of Ssh overexpression in which the corresponding signal was decreased as expected (Fig. 5C), suggesting that Sickie is involved in Cofilin dephosphorylation in developing axons.

Next, we tested the LIMKΔ-63 CA mutant clone. Previous in vitro studies showed that LIMK inactivates Cofilin through phosphorylation (Aizawa et al., 2001; Bernard, 2007; Endo et al., 2007; Nishita et al., 2005), and we found greater increases in the F-actin signal (Fig. 4E′, H). Therefore, we predicted a higher elevation of the P-Cofilin signal in the LIMKΔ-63 CA mutant. Surprisingly, however, we did not detect an obvious increase in P-Cofilin in the mutant axons (Fig. 5D′) despite continuous detection of the strong F-actin signal (Fig. 5D′), as compared with the sshΔ-63 mutant showing a moderate F-actin increase at the late pupal stage (Fig. 5A′). We further induced a sickieΔ LIMKΔ-63 CA
double-mutant clone and detected elevated P-Cofilin levels in a part of the mutant axon bundle (Fig. 5E″, red outline). This P-Cofilin increase was modest yet higher than that of the sickieΔ or LIMKKD single mutant (Fig. 5B″, D″). A moderate F-actin increase was detected at and around the newborn αβ axons compared with the control internal region (C″). (D-D″) In an ssh1−63 clone, the F-actin signal was strongly detected at and around the αβ lobe branching point compared with the internal control (D′). (E-E″) A LIMKKD clone showed strong F-actin signals at and around the αβ lobe branching point (E′). The double asterisk indicates strong F-actin signal in the distal region of MB axons. (F-H) Quantification of increased F-actin signal in mutant axons. In all three genotypes, the mean F-actin signal intensities significantly increased in the αβ lobe containing mutant axons. Paired t-test, n=6 (represented by the different shapes; error bars indicate s.e.m.): sickieΔ, ***P=8.10×10−5; ssh1−63, ***P=3.26×10−4; LIMKKD, ***P=3.29×10−4. (I-I″) A PakMyr clone showing ectopic F-actin signal (I′) in the posteriorly arrested axons (I″). (J-J″) A Rac1V12 clone similarly showed ectopic F-actin signal (J′) in the posteriorly arrested axons (J″). (K-K″) A cofilin-RNAi clone showing strong F-actin signals (K′), with the posterior arrest axonal defect (K″). Scale bars: 20 μm.

In summary, these results suggest that Cofilin function is impaired in the sickie mutant and raise the possibility that Sickie is involved in the pathway counteracting canonical Rac-Pak-LIMK signaling.

\[ \text{Upregulation of Cofilin function in young MB neurons alleviates the axonal defect of the sickie mutant} \]

To test the above hypothesis, epistatic studies were performed. We found significant rescue of the sickieΔ MARCM phenotype when wild-type (UAS-cofilinWT) or active state-mimicking (UAS-cofilinS3A) Cofilin was expressed in young αβ neurons (Fig. 6A,B,E), albeit less efficiently than Sickie itself (Fig. 2E). The overexpression of wild-type Ssh (UAS-HA::sshWT) but not the inactive form (UAS-HA::sshCS) also partially rescued the sickie phenotype (Fig. 6C,D,F). These results suggest that Sickie functions upstream of Ssh and Cofilin in MB axonal growth.

\[ \text{Sickie suppresses the axonal growth defect induced by LIMK overexpression} \]

We next focused on the interaction between Sickie and LIMK. A previous study demonstrated the counteracting effect of Ssh and Pak-independent Rac1 against LIMK. The axonal growth defect induced by LIMK overexpression is significantly suppressed by the co-expression of SshWT or Rac1Y40C (Ng and Luo, 2004). In addition, the expression of LIMKWTM6 (UAS-HA::LIMKWTM6), a stronger gain-of-function allele, frequently induces the Class II type...
axonal growth defect with a remarkable increase in F-actin level (Fig. 7A-A‴) (Ng, 2008). We found that SshWT or Rac1Y40C expression suppressed the strong defects in both the F-actin level and morphological phenotype induced by LIMKWTM6 (Fig. 7B-C‴).

If Sickie functions in the Pak-independent pathway to facilitate Cofilin function by similarly counteracting LIMK, the strong F-actin increase and axonal defects would be alleviated. We found this to be the case; SickieWT expression reduced the elevated F-actin signal and rescued the defect in medial axon growth, although complete morphological rescues were rarely observed in the dorsal axons (Fig. 7D-D‴, F). Such suppressions were not observed when SickieK1881A was expressed (Fig. 7E-F).

**Sickie is required for Pak-independent Rac1 and Ssh to counteract LIMK**

Based on the above results, we hypothesized that Sickie relays the non-canonical Rac pathway signal to Ssh to facilitate Cofilin function. To test this hypothesis, we examined whether Sickie is necessary for the counteracting effect of Pak-independent Rac1 or Ssh against LIMK (Fig. 7B,C). The counteracting effects by Rac1Y40C and SshWT would be diminished when Sickie function is lost. The results were consistent with this hypothesis; the counteracting effects of Rac1Y40C and SshWT were suppressed in the absence of Sickie, as the F-actin signals remained elevated (Fig. 7G″, I″, compared with 7C′, B′, respectively), and the axonal growth defect with a remarkable increase in F-actin level (Fig. 7A-A‴) (Ng, 2008). We found that SshWT or Rac1Y40C expression suppressed the strong defects in both the F-actin level and morphological phenotype induced by LIMKWTM6 (Fig. 7B-C‴). If Sickie functions in the Pak-independent pathway to facilitate Cofilin function by similarly counteracting LIMK, the strong F-actin increase and axonal defects would be alleviated. We found this to be the case; SickieWT expression reduced the elevated F-actin signal and rescued the defect in medial axon growth, although complete morphological rescues were rarely observed in the dorsal axons (Fig. 7D-D‴, F). Such suppressions were not observed when SickieK1881A was expressed (Fig. 7E-F).

**Sickie is required for Pak-independent Rac1 and Ssh to counteract LIMK**

Based on the above results, we hypothesized that Sickie relays the non-canonical Rac pathway signal to Ssh to facilitate Cofilin function. To test this hypothesis, we examined whether Sickie is necessary for the counteracting effect of Pak-independent Rac1 or Ssh against LIMK (Fig. 7B,C). The counteracting effects by Rac1Y40C and SshWT would be diminished when Sickie function is lost. The results were consistent with this hypothesis; the counteracting effects of Rac1Y40C and SshWT were suppressed in the absence of Sickie, as the F-actin signals remained elevated (Fig. 7G″, I″, compared with 7C′, B′, respectively), and the axonal growth defect with a remarkable increase in F-actin level (Fig. 7A-A‴) (Ng, 2008). We found that SshWT or Rac1Y40C expression suppressed the strong defects in both the F-actin level and morphological phenotype induced by LIMKWTM6 (Fig. 7B-C‴). If Sickie functions in the Pak-independent pathway to facilitate Cofilin function by similarly counteracting LIMK, the strong F-actin increase and axonal defects would be alleviated. We found this to be the case; SickieWT expression reduced the elevated F-actin signal and rescued the defect in medial axon growth, although complete morphological rescues were rarely observed in the dorsal axons (Fig. 7D-D‴, F). Such suppressions were not observed when SickieK1881A was expressed (Fig. 7E-F).

**Sickie is required for Pak-independent Rac1 and Ssh to counteract LIMK**

Based on the above results, we hypothesized that Sickie relays the non-canonical Rac pathway signal to Ssh to facilitate Cofilin function. To test this hypothesis, we examined whether Sickie is necessary for the counteracting effect of Pak-independent Rac1 or Ssh against LIMK (Fig. 7B,C). The counteracting effects by Rac1Y40C and SshWT would be diminished when Sickie function is lost. The results were consistent with this hypothesis; the counteracting effects of Rac1Y40C and SshWT were suppressed in the absence of Sickie, as the F-actin signals remained elevated (Fig. 7G″, I″, compared with 7C′, B′, respectively), and the axonal growth defect with a remarkable increase in F-actin level (Fig. 7A-A‴) (Ng, 2008). We found that SshWT or Rac1Y40C expression suppressed the strong defects in both the F-actin level and morphological phenotype induced by LIMKWTM6 (Fig. 7B-C‴). If Sickie functions in the Pak-independent pathway to facilitate Cofilin function by similarly counteracting LIMK, the strong F-actin increase and axonal defects would be alleviated. We found this to be the case; SickieWT expression reduced the elevated F-actin signal and rescued the defect in medial axon growth, although complete morphological rescues were rarely observed in the dorsal axons (Fig. 7D-D‴, F). Such suppressions were not observed when SickieK1881A was expressed (Fig. 7E-F).
defects were not rescued (Fig. 7G,I, compared with 7C,B, and 7H,J, respectively). These results suggest that Sickie is required for both Pak-independent Rac1 and Ssh to counteract LIMK in MB axonal growth.

**Sickie regulates Cofilin-mediated axonal growth in an Ssh-dependent manner**

As shown previously in the simple Gal4-UAS system (Fig. 7A,A′,D,D′), increased F-actin and axonal defects caused by the overexpression of LIMKWTM6 (Fig. 8A,A′) were also suppressed by the co-expression of SickieWT in the MARCM system (Fig. 8B,B′). We examined whether this suppression by Sickie was dependent on Ssh by introducing an sshL−Δ−63 mutation into the MARCM system. LIMKWTM6 sickieWT sshL−Δ−63 triple-mutant clones showed highly elevated F-actin (Fig. 8C′, asterisk) and P-Cofilin (Fig. 8D′, red outline) signals with the posterior arrest axonal defect (Fig. 8C″), and the phenotypes were indistinguishable from those of a LIMKWTM6 sshL−Δ−63 double-mutant clone (Fig. 8E′′,E″,E‴), suggesting that Sickie functions upstream of Ssh in counteracting LIMK. This posterior arrest axonal defect of the LIMKWTM6 sshL−Δ−63 mutant (Fig. 8E‴′) was stronger than that of the LIMKWTM6 (Fig. 8A) or sshL−Δ−63 (Fig. 3H) single-mutant clones and reminiscent of that of the cofillin-RNAi clone (Fig. 3G), indicating that Cofilin was nearly fully inactivated in the LIMKWTM6 sshL−Δ−63 double mutant.

**DISCUSSION**

**Role of the non-canonical Rac-Cofilin pathway in F-actin-mediated axonal growth**

By combining the MARCM technique with epistatic analysis, we demonstrated that Sickie regulates the axonal growth of *Drosophila* MB neurons via the non-canonical Rac-Cofilin pathway. We propose the following model (Fig. 9). In wild type, Sickie relays the non-canonical pathway signal to Ssh (green arrows) to facilitate F-actin-mediated axonal growth by counteracting the canonical signal (red arrows). In a sickie mutant, mediation of the non-canonical pathway is defective, which causes an imbalance in the regulation of Cofilin activity. Because neurons are morphologically polarized and the amount of actin is limited in each cell, the growing axons may efficiently control actin recycling by facilitating F-actin turnover (Buggy and Carlier, 2010) by balancing between the non-canonical and canonical pathways. Consistently, we have found a stronger axonal growth defect with increased P-Cofilin in the LIMKWTM6 sshL−Δ−63 and sickieΔ LIMK−Δ− double-mutant animals (Fig. 8E′ and Fig. 5E′) than in the single mutants sshL−Δ−, sickieΔ and LIMK−Δ− (Fig. 5A′,B′,D′). Cofilin activity might be decreased in the developing axons of these double mutants by the preponderance of the canonical pathway. If so, these results highlight an essential role of the non-canonical pathway to balance Cofilin activity in axonal growth.

**Simultaneous detection of P-Cofilin and F-actin changes in developing axons**

Unlike the clear elevation of P-Cofilin levels in the sshL−Δ−63 mutant (Fig. 5A′), constitutive activation of LIMK did not result in a similar increase in P-Cofilin (Fig. 5D′) despite F-actin elevation (Fig. 5D′). This apparent paradox might be explained by considering the positive regulation of Ssh by F-actin. The phosphatase activity of SSH-1L is F-actin dependent, and the addition of F-actin dramatically increases its phosphatase activity (Nagata-Ohashi et al., 2004; Yamamoto et al., 2006). In the LIMK−Δ− mutant axons, endogenous Ssh may be activated by a large amount of F-actin and subsequently dephosphorylates Cofilin. Consistently, highly elevated signals of both F-actin and P-Cofilin

---

**Fig. 6. Upregulation of Cofilin function in newborn MB neurons alleviates the axonal defect of the sickie mutant.** (A,B) Forced expression of CofilinWT (A) or CofilinΔ3A (B) driven by NP7175-gal4, an u/I core driver, partially rescued the axonal growth defect in a sickieΔ3A mutant. (C,D) The sickieΔ3A mutant was also partially rescued by SshWT expression (C) but not by SshCS expression (D; yellow arrowheads). (E) Quantification of rescue effects of upregulation of Cofilin function. Expression of CofilinWT or CofilinΔ3A resulted in a significant reduction in the penetrance of the sickieΔ3A mutant phenotype. CofilinΔ3A, ***P=2.87×10−4; CofilinWT, **P=2.22×10−3; CofilinΔ3A, P=0.228; Fisher’s exact test. (F) Quantification of the rescue effect of Ssh overexpression on the sickieΔ3A mutant phenotype. SshWT expression significantly rescued the defect: NP7175/SNP7175>sshWT, ***P=1.50×10−5; NP7175/SNP7175>sshCS, P=0.295; sshWT/NP7175>sshWT, ***P=1.45×10−4; NP7175>sshWT, NP7175>sshCS, **P=1.59×10−3; Fisher’s exact test. Scale bars: 20 μm.
were detected in the LIMKWTM6 ssh1-63 double-mutant clones (Fig. 8E′, E″). In this mutant, Cofilin activity was severely reduced by high phosphorylation levels due to constitutive LIMK activation and a lack of Ssh phosphatase activity, resulting in the posterior arrest severe axonal defect, similar to the cofilin knockdown mutant (Fig. 8E‴ and Fig. 3G). In addition, relatively moderate increases in P-Cofilin signal were detected in the developing axons of the sickieΔLIMKKD double mutant (Fig. 5E″). These results also support the model that Sickie functions in the same pathway as Ssh to positively regulate Cofilin function by counteracting the canonical Rac-Pak-LIMK pathway. Ssh might be downregulated in the sickie mutant axons due to defects in the mediation of Pak-independent Rac1 expression.
function or in the interaction among Ssh and F-actin by the loss of Sickie. The similar increases in the P-Cofilin and F-actin signals and the similar posterior arrest phenotype in the \( LIMK^{WT} ssh^{1-63} \) double-mutant clone and those of the \( Pak^{Myr} \) mutant clone are also consistent with results of in vitro studies that showed that SSH-1L is inactivated by Pak4 (Soosairajah et al., 2005; Van Troys et al., 2008). Thus, in our model, Pak concurrently inactivates Ssh and activates LIMK in axonal growth (Fig. 9).

**Possible function of Sickie**

Whereas \( ssh \) or \( cofilin \) mutants are embryonic lethal and their mutant clones display developmental defects in non-neuronal tissues, sickie...
mutants are not embryonic lethal, and conspicuous phenotypes are found only in the substructures of the central brain, such as MB and EB, implying that more elaborate mechanisms involving Sickie function are required for ensuring their proper development. Given that MB neurons exhibit a densely bundled axonal morphology, the growing MB axons might require Sickie to smoothly extend their neurites within the lobe core region by coordinating the dynamics of actin and microtubules (MTs). Sickie and human neuron navigator proteins (NAVs) have conserved EB1-binding motifs (Fig. 1D) (van Haren et al., 2009), and Sickie shows a genetic interaction with MT components (supplementary material Fig. S3). We found that double RNAi of sickie and EB1 or β-tubulin both resulted in synergistic increases in the axonal defects. In addition, a recent cell biological study demonstrated a functional link between Cofilin and MTs (Flynn et al., 2012). Through its interaction with EB1 (Fig. 9, blue dotted arrow), Sickie might act as a navigator for the plus-end of MTs to link to the F-actin complex and thereby ensure elaborate neuronal wiring. To further elucidate the signaling mechanism, the relationships with other components of the Sh-dependant Cofilin pathway need to be studied. Recent studies have revealed that PKD, 14-3-3 protein and Pak4 play key roles in suppressing Ssh function (Eiseler et al., 2009; Kligys et al., 2009; Spratley et al., 2011).

Identification and generation of sickie mutant alleles

The following insertion lines were used for the generation of the FLP-FRT deletion mutants, sickieΔ1; sickie007725 and sickie009531, sickieΔ2; sickie006853 and sickie02238, sickieΔ3; sickie007728 and sickie004819, sickieΔ4; sickie004735 and sickie006853, sickieΔ5; sickie004737 and sickie002238. The parental lines were obtained from the Exelixis Collection at Harvard Medical School. The A384 piggyBac element was inserted into the sixth intron of the sickie locus (Beelen, 2004; Ring and Garza, 2003) and the revertant allele, sickie11874 revertant, was obtained through precise excision.

Generation of transgenic constructs

Total mRNA was extracted from dissected brains using the RNeasy Purification Kit (Qiagen), and reverse transcription was performed using the PrimeScript II High Fidelity RT-PCR Kit (Takara Bio). sickie cDNA was amplified using PrimeSTAR DNA polymerase (Takara Bio). The alignment of homologous sequences was performed using GENETYX (GENETYX Corporation). The mCherry (Clontech) coding sequence was inserted upstream of the sickieWT or sickieΔ1881A coding sequences using EcoRI/SpeI sites. An alanine mutation was introduced at the K1881 residue in the consensus ATP/GTP-binding (GxxxxGKS/T) motif in the AAA domain. These constructs were inserted into the pUAST (Brand and Perrimon, 1993) vector using EcoRI/XhoI sites. These plasmids were injected into yw embryos (BestGene).

Generation of Sickie antibody

A rat polyclonal anti-Sickie antibody was generated by Takara Bio. The antibody was raised against the 464 amino acids corresponding to residues 1734 to 2197 of the full-length Sickie protein. Anti-Sickie antibody was used at 1:1000.

Statistical test for genetic interaction

For the statistical analyses, two-sided Fisher’s exact tests were used (Cyrus and Nitin, 1983). We made a 2×2 cross table that was composed of genotype A/B and the sum of the number of phenotypic samples (Class 1+Class 2) posterior arrest/number of non-phenotypic samples, and compared the difference in the percentages between the two groups. For multiple comparisons, Bonferroni correction was applied by the dividing significance levels by the number of genotypes in comparison.

Quantification of F-actin levels

F-actin signals in the α/β lobe region were combined to obtain the total F-actin intensity. The region of interest was extracted by the Surface program in Imaris software (Bitplane). In the mutant axons, the region of interest was further restricted by the GFP signal. The differences in the means of the average F-actin signal intensities were compared by a paired t-test.

Biochemistry

For the preparation of loading sample, pupal brains were dissected in TBS-T solution [Tris-buffered saline with Tween 20 tablets (pH 7.6), #T9142, TaKaRa] and collected in lysis buffer at −80°C. The following reagents were used for western blotting: rabbit anti-P-Cofilin (as above; 1:100), rabbit anti-Cofilin (rabbit polyclonal antisera to Tsr; a gift from T. Uemura; TaKaRa) and collected in lysis buffer at

Histochemistry

Flies were dissected in a cold PBS solution and fixed in 4% formaldehyde for 40 min at room temperature. Immunostaining was performed as described previously (Huang and Kunes, 1996; Sato et al., 2006). The following antibodies were used: rabbit anti-GFP Alexa Fluor 488 conjugate (Molecular Probes; 1:2000); mouse anti-FasII (DSHB; 1:50); rabbit anti-DrRed (Takara #632496; 1:1000); mouse anti-HA (Covance; 1:500); rabbit anti-HA (Abcam #ab9110; 1:1000); rat anti-Slingshot (a gift from T. Uemura, Kyoto University, Japan; 1:1000); mouse anti-c-Myc (9B11, Cell Signaling #2276S; 1:10,000); rabbit anti-P-Cofilin (Signalway Antibody #11139-1; 1:200); anti-mouse Cy3, anti-rabbit Cy3, anti-rat Cy3, anti-mouse Cy5 and anti-mouse DyLight649 (all Jackson ImmunoResearch; 1:200); anti-rat Alexa Fluor 488, anti-rabbit Alexa Fluor 660 and anti-rat Alexa Fluor 647 (all Molecular Probes; 1:200). Alexa Fluor 488 and 568 phalloidin were from Molecular Probes (#A12379, #A22283; 1:200). Images were captured and analyzed on a Zeiss LSM 710 confocal microscope and processed using Zeiss LSM Image Browser and Photoshop (Adobe Systems).


Fig. S1. The expression pattern of NP7175-gal4 at pupal stage.

Throughout development, GFP signals were detected in the inner regions of FasII-labeled α/β axonal bundles. From 12 to 84 h APF, Sickie expression was prominently detected in the core regions of the axonal bundles (arrowheads) in which FasII signals were weak. We did not detect strong GFP signals in this exact core region of the peduncle. This result might be due to a delay in the Gal4/UAS system. Scale bar, 20 µm.
Fig. S2. Duplicated Fig. 5 images free of markings.
Fig. S3. Sickie genetically interacts with microtubule components.

(A-A’) Any obvious axonal growth defect was not detected when sickie-RNAi knockdown was singly induced at 25°C. (B-B’) Some RNAi-treated flies show axonal defects when RNAi was induced with dicer-2 co-expression at 29°C. (C) The penetrance of the axonal defect was significantly increased when dicer-2 was co-expressed and the flies were reared at 29°C. ***p=1.50×10⁻¹¹. (D-D’,E-E’) RNAi of either EB1 or β-tubulin induced lobe
formation defects with \textit{dicer}-2 co-expression at 29°C. (F,G) Either \textit{EB1}- or \textit{β-tubulin}-RNAi also showed low penetrance of the axonal lobe formation defects at 25°C. However, compared with the single knockdown of \textit{sickie}, the penetrance of the axonal defects synergistically increased in both RNAi treatments by combining with \textit{sickie-RNAi}, even at 25°C. \textit{sickie}/\textit{sickie-EB1-RNAi}: ***p=1.54\times10^{-9}, \textit{EB1}/\textit{sickie-EB1-RNAi}: ***p=3.63\times10^{-5}, \textit{sickie}/\textit{sickie-β-tubulin-RNAi}: ***p=4.88\times10^{-7}, and \textit{β-tubulin}/\textit{sickie-β-tubulin-RNAi}: ***p=3.25\times10^{-12}. Scale bar, 20 µm.