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WASp is required for the correct temporal morphogenesis of rhabdomere microvilli

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Microvilli are actin-based fingerlike membrane projections that form the basis of the brush border of enterocytes and the *Drosophila melanogaster* photoreceptor rhabdomere. Although many microvillar cytoskeletal components have been identified, the molecular basis of microvillus formation is largely undefined. Here, we report that the Wiskott-Aldrich syndrome protein (WASp) is necessary for rhabdomere microvillus morphogenesis. We show that WASp accumulates on the photoreceptor apical surface before microvillus formation, and at the time of microvillus initiation WASp colocalizes with amphiphysin and moesin. The loss of WASp delays the enrichment of F-actin on the apical photoreceptor surface, delays the appearance of the primordial microvillar projections, and subsequently leads to malformed rhabdomeres.

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Introduction

Microvilli evolved as a strategy to expand the apical cell surface for various critical biological functions including nutrient uptake, the chemical sensing of the environment, and phototransduction. Morphological and structural characterization of the brush border microvilli of enterocytes (intestinal epithelial cells) and invertebrate photoreceptor rhabdomeres have demonstrated that microvilli contain two distinct domains, the microvillus projection and the terminal web domain (Louvard, 1989; Heintzelman and Mooseker, 1992). The projection domain contains an actin-based cytoskeleton as well as the specific receptors and channels required for function. The terminal web, located at the interface between the microvillus projection and the cell cytoplasm, serves as a transition zone for the constant delivery of proteins needed for the maintenance and function of the microvilli and as a scaffold for the support of the microvillus projection.

Biochemical analyses of brush border microvilli have provided key insights into the protein contents and organizational arrangement of the molecular components of each domain. In particular, the core of each microvillus projection consists of bundles of actin filaments (Tilney and Mooseker, 1971), which not only provide the scaffold for the overlying membrane but also create the protruding force for the generation of each microvillus. The brush border microvillus core also consists of myosin I and the actin bundling proteins villin and fimbrin (Bretscher and Weber, 1979, 1980; Coluccio and Bretscher, 1989; Mooseker and Coleman, 1989). Based on the identified components, it is not clear how or what initiates the construction of the actin cytoskeleton. For instance, villin is one of the earliest proteins to be enriched on the apical surface of enterocytes and has the ability to nucleate and collect F-actin into bundles (Friederich et al., 1990; Fath and Burgess, 1995). However, genetic knockouts of *villin* resulted in no discernible disruptions of microvilli ultrastructure (Pinson et al., 1998; Ferrary et al., 1999).

One set of candidates for initiating actin nucleation during microvillus formation is the ezrin/radixin/moesin family of proteins (Bretscher, 1989; Berryman et al., 1993). In the retinal pigment epithelium, the temporal and spatial expression of Ezrin corresponds with the creation of the apical microvilli, and in primary retinal pigment epithelium cultures, Ezrin antisense treatment eliminates microvillus formation (Bonilha et al., 1999). Nonetheless, how Ezrin may be coordinating microvillus biogenesis is unknown. A second possibility is that the molecular mechanisms of microvillus initiation may be similar to the formation of filopodia and lamellipodia membrane protrusions. In both cases, a signaling cue activates a small Rho GTPase upon which the rapid rearrangement of actin proceeds through the activation of Wiskott-Aldrich syndrome protein (WASp) and WAVE family members (Takenawa and Miki, 2001; Thrasher, 2002). WASp and WAVE family members can directly

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Key words: chaoptin; photoreceptor; *Drosophila*; amphiphysin; moesin

Abbreviations used in this paper: Amph, amphiphysin; APF, after puparium formation; Mtl, Mig2-like; PAK, p21-activated kinase; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; WASp, Wiskott-Aldrich syndrome protein.

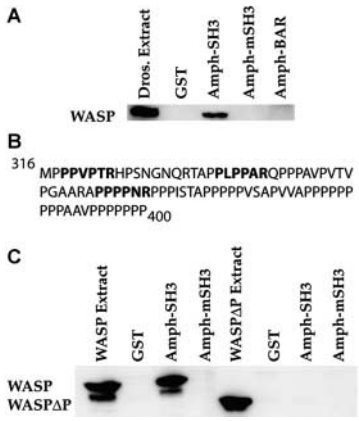

Figure 1. **WASp physically interacts with amphiphysin (Amph).** (A) The SH3 domain and not the BAR domain of Amph is capable of binding WASp from *Drosophila* extracts. Mutation of the SH3 domain (mSH3) eliminates WASp binding. (B) Consensus Amph binding sites in *Drosophila* WASp. (C) Deletion of the proline-rich region of WASp eliminates binding to Amph in cellular extracts.

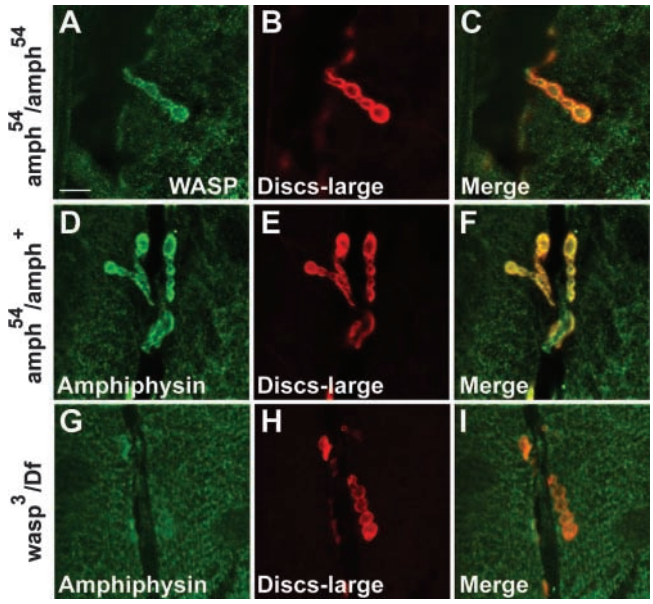

Figure 2. **WASp and Amph colocalize at the *Drosophila* neuromuscular junction.** (A–F) WASp and Amph localize to the postsynaptic portion of the third instar larval neuromuscular junction synaptic bouton, and WASp localization (D–F) is not dependent on the presence of Amph. (G–I) The localization of Amph to the synaptic bouton is dependent on WASp. Bar, 5 μm.

bind actin as well as activate the actin nucleating complex Arp 2/3 (Mullins, 2000).

In *Drosophila melanogaster*, the photoreceptor cells have a very extensive network of microvilli contained within the rhabdomere. The rhabdomere is a specialized organelle that forms on the photoreceptor apical surface. It is the functional equivalent of vertebrate rod and cone cell outer segments and both are responsible for housing the phototransduction proteins. Like the brush border, each rhabdomeric microvillus contains an actin cytoskeleton core but only has two parallel actin filaments (Arikawa et al., 1990). As for formation of the rhabdomere actin cytoskeleton core, only Bifocal has been implicated in its assembly. Bifocal is a novel protein that binds F-actin, and the loss of Bifocal results in irregular rhabdomere morphology (Bahri et al., 1997).

Clearly, the combination of structural and biochemical analyses have identified numerous proteins involved in the development of brush border or rhabdomeric microvilli. Nevertheless, little is known about how assembly of the actin cytoskeleton core is initiated, how the actin cytoskeleton interacts with the plasma membrane, and how the growth of each microvillus is regulated. Using *Drosophila* rhabdomere development as an experimental model system for understanding the biogenesis and maintenance of microvilli, we demonstrate that the *Drosophila* WASp is required for the correct temporal apical enrichment of F-actin and initiation of the primordial microvillar projections.

Results

WASp interacts with amphiphysin (Amph)

To investigate the molecular mechanisms underlying the creation of the microvillus cytoskeleton core, we asked what proteins interact with the *Drosophila* rhabdomeric protein Amph. Our previous work implicated *Drosophila* Amph as an accessory factor mediating an interaction between the actin cytoskeleton and changes in the plasma membrane during rhabdomere morphogenesis (Zelhof et al., 2001). Amph has an NH_2-terminal coiled-coil domain (BAR domain) that is capable of tubulating liposomes (Takei et al., 1999; Razzaq et al., 2001) as well as an SH3 domain for mediating protein–protein interactions. Even though the loss of Amph does not result in any detectable defects in rhabdomere development, overexpression of Amph demonstrated that it has the ability to disrupt the proper distribution of rhabdomeric F-actin; presumably, the SH3 domain of Amph can titrate away factors necessary for the organization of the F-actin cytoskeleton core (Zelhof et al., 2001).

To further address this hypothesis, we took a biochemical approach to identify proteins that bind the SH3 domain of Amph. GST pull-down experiments using *Drosophila* extracts indicated several proteins that specifically interact with the SH3 domain of Amph (unpublished data). Testing a library of antibodies, one protein was identified as the *Drosophila* homologue of WASp. WASp is a member of an evolutionarily conserved family of proteins that act as integrators of multiple signaling pathways to direct the specific subcellular localization and polymerization of actin. The binding of WASp to Amph is dependent on the SH3 domain of Amph, and mutation of SH3 domain results in the disruption of this interaction (Fig. 1). SH3 domains bind proline-rich sequences, and within WASp there are three peptides that loosely fit the characterized consensus-binding site for the vertebrate SH3 domain of Amph-2 (PXRPXR; Owen et al., 1998; Fig. 1 B). The removal of this proline-rich domain also eliminates binding to Amph (Fig. 1 C).

To further investigate this interaction, we asked whether WASp colocalizes with Amph. Besides colocalizing with Amph at the *Drosophila* neuromuscular junction (Fig. 2), we find WASp is expressed in *Drosophila* photoreceptor cells. Notably, WASp expression is first detected in photoreceptor

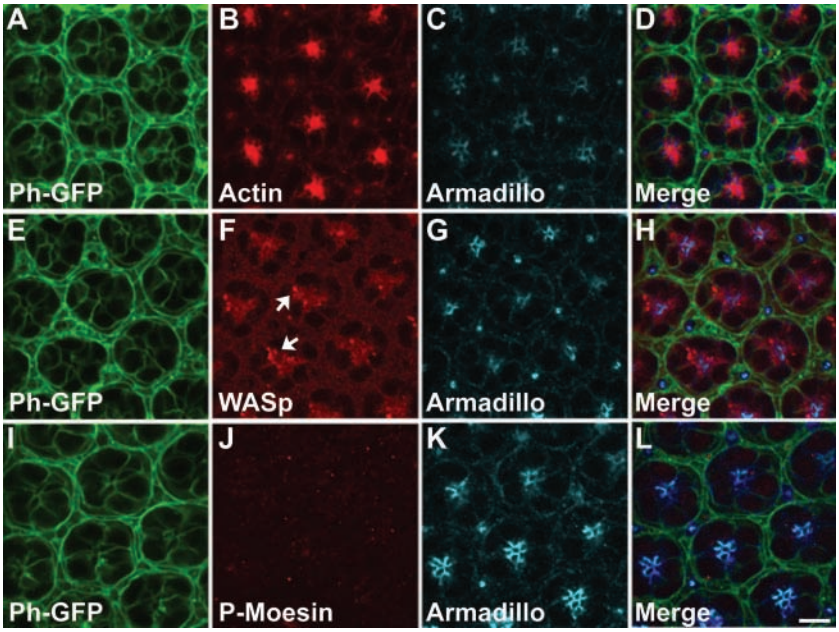

Figure 3. **Expression of photoreceptor and rhabdomeric proteins at 36 h APF.** (A–D) Expression and localization of cortical F-actin (red), phosphoinositide PI(4,5)P2 (green), and Armadillo (blue). (E–H) Expression and localization of WASp (red), phosphoinositide PI(4,5)P2 (green), and Armadillo (blue). WASp protein can be detected accumulating on the apical photoreceptor surface (arrows). (I–L) Expression and localization of activated moesin (red), phosphoinositide PI(4,5)P2 (green), and Armadillo (blue). Activated moesin cannot be detected at this time, and Armadillo localizes to the entire apical surface of the photoreceptor cells. Bar, 5 μm.

cells 36 h after puparium formation (APF), before the separation of the photoreceptor cell apical surfaces and the appearance of the first microvillar protrusions (Fig. 3 F). At 48 h APF, WASp concentrates on the apical surface when the first microvillar extensions are detected and coincident with the enrichment of F-actin, a prerequisite for the establishment of the cytoskeleton core of each rhabdomere microvillus (Fig. 4 F). At 72 h APF, when the microvillar projections are elongating, WASp expression is no longer detected (unpublished data).

WASp mutant rhabdomeres are malformed

The temporal and spatial expression patterns of WASp correlate with the initial formation of the membrane folds and the organization of apical F-actin that generate the rhabdomere microvilli. As a first step to define the role of WASp in rhabdomere microvillus biogenesis, we examined the structure of rhabdomeres in adult *WASp* mutant photoreceptor cells. *WASp* mutant cells were created by two methods. First, mosaic patches of *WASp* mutant cells were induced using the $WASp^3$ allele and the FRT/FLP system (Xu and Rubin, 1993). Second, *WASp* mutant eyes were dissected from the transheterozygote of $WASp^3/WASp^1$. This genetic combination results in pharate adults and also serves as a control for any secondary mutations that may be present on either *WASp* mutant chromosomes. In both cases, many rhabdomeres do not obtain the normal characteristic oval shape, but rather are pointed and squared (Fig. 5, A–E). In

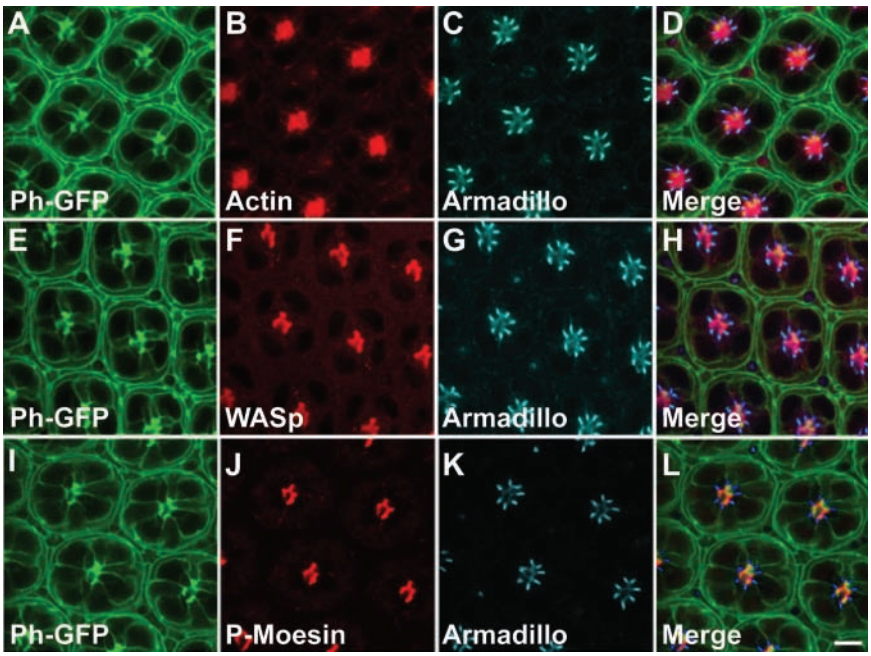

Figure 4. **Expression of photoreceptor and rhabdomeric proteins at 48 h APF.** (A–D) Expression and localization of cortical F-actin (red), phosphinositide PI(4,5)P2 (green), and Armadillo (blue). (E–H) Expression and localization of WASp (red), phosphinositide PI(4,5)P2 (green), and Armadillo (blue). (I–L) Expression and localization of activated moesin (red), phosphinositide PI(4,5)P2 (green), and Armadillo (blue). Both WASp and activated moesin colocalized with each other and with F-actin during microvillus formation. Armadillo staining shows a considerable decrease on the apical surfaces of photoreceptor cells but strong localization to the zonula adherens junctions. Bar, 5 μm.

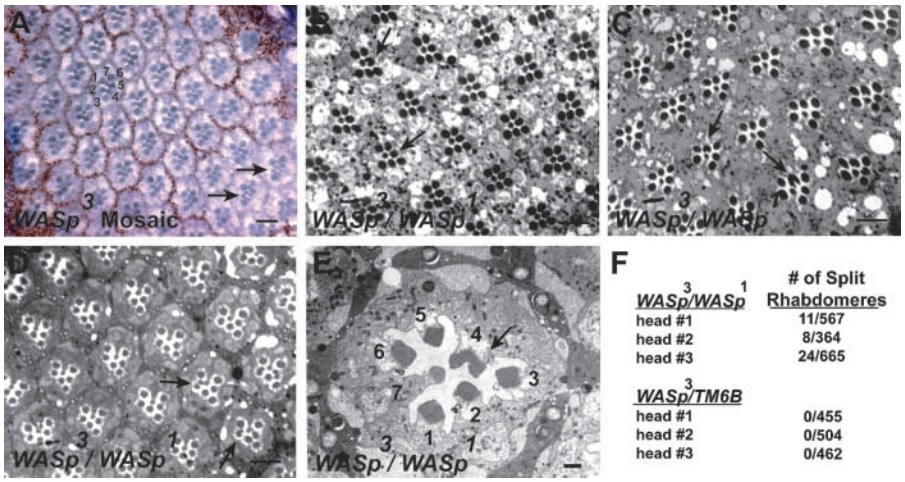

Figure 5. **WASp is required for rhabdomere morphogenesis.** (A) Phase-contrast image of 2-μm section of *WASp*^{3} adult eye mosaic clones. Mutant tissue can be identified by the lack of red pigment granules. In A and E, numbers correspond to the individual rhabdomeres. In some of the mutant ommatidia rhabdomeres, 2 and 4 are split (arrows). Bar, 5 μm. (B–D) 0.5-μm section of *WASp*^{3}/*WASp*^{1} adult eye ommatidia from three different heads. Many rhabdomeres are irregularly shaped and a few are split (arrows). Bars, 5 μm. (E) Higher magnification image of *WASp*^{3}/*WASp*^{1} ommatidium. Bar, 1 μm. (F) Quantitation and comparison of the number of split rhabdomeres in *WASp* homozygous and heterozygous ommatidia. Split rhabdomeres are not observed in wild-type ommatidia.

addition, 2–3% of the mutant rhabdomeres are split. Neither of these phenotypes is observed in control WASp heterozygous flies (Fig. 5 F). Our results reveal that WASp is required for overall rhabdomere morphology, but apparently WASp is not essential for individual microvillus formation. The microvillar protrusions are still present in each rhabdomere. Even though the rhabdomere structure is irregular, import and stabilization of the phototransduction machinery and the detection of light is normal in *WASp* mutant eyes (unpublished data).

WASp is required for the temporal appearance of microvillar F-actin

How does the loss of *WASp* lead to the irregular rhabdomere morphology? Given that WASp acts as a mediator of localized actin polymerization, we might expect that the abnormal morphology is the consequence of defects in F-actin assembly and creation of the microvillar cytoskeleton core. To examine the F-actin arrangement in *WASp* mutant photoreceptor cells, we used the MARCM system to create mosaic eyes containing GFP-marked *WASp* mutant tissue (Lee and Luo, 1999). Our clonal analysis revealed a striking defect in F-actin enrichment. Before the first primordial projections can be detected (36 h APF), a network of F-actin on the apical surface of photoreceptor cells is already present (Fig. 3 B; Speck et al., 2003). However, there is a pronounced increase and rearrangement of F-actin at the time of microvillus initiation (Fig. 4 B), corresponding to the creation of the actin cytoskeleton core of each microvillar projection. In *WASp* mutant cells, we observed that this enhancement of F-actin accumulation along the photoreceptor apical surface is delayed and disorganized. During *Drosophila* retinal development, each photoreceptor apical surface involutes such that they become juxtaposed. Upon involution, the apical surfaces expand downward from the overlying cone cells (proximal) toward the retinal basal lamina (distal; Longley and Ready, 1995). Concurrent with the downward extension of the apical surface, the primordial microvilli begin to extend outward into the future interrhabdomeral space. At 48 h in *WASp* mutant cells, examination of distal sections along the depth of the photoreceptor cells reveals regions of the photoreceptor cell in which the apical surface has not expanded and/or the primordial microvillar projections have not formed. These regions are characterized by little or no F-actin enrichment compared with neighboring wild-type cells (Fig. 6 B). The initiation of rhabdomere microvilli formation appears to be delayed. Even though the temporal delay is fully penetrant and is observed in every WASp mutant cell, assembly of the rhabdomere structure still occurs. WASp mutant photoreceptor cells do form microvilli and these microvilli have an actin cytoskeleton core present (Fig. 5 and not depicted). In addition, examination of developing mutant rhabdomeres at 72 h APF demonstrates that F-actin has accumulated and organized on the apical surface. Nevertheless, the developing mutant rhabdomeres are smaller in size or malformed compared with their wild-type counterparts (Fig. 6 F).

The enrichment of F-actin is not the only indication that there is a delay in microvillus initiation in WASp mutant cells. During rhabdomere morphogenesis, the cell–cell contacts between the juxtaposed photoreceptor apical surfaces within an ommatidium are abolished, whereas the zonula adherens junctions between neighboring photoreceptor cells are maintained. In addition, as the apical cell surface expands downward, the zonula adherens junctions also extend, maintaining the tight junction between adjacent photoreceptor cells. Armadillo, the *Drosophila* β-catenin, is an excellent guide for following the elimination of the apical cell–cell contacts (Sang and Ready, 2002) and a marker of the established adherens junctions. Before 48 h APF, Armadillo decorates the entire apical surface (Fig. 3), but with the appearance of the first microvillar projections, Armadillo localization is eliminated on the apical surface and redistributed to the zonula adherens junctions (Fig. 4). In WASp mutant cells, the lack of F-actin enrichment also corresponds to the abnormal maintenance of Armadillo on the entire apical surface (Fig. 6 C). This result would suggest that the photoreceptor cells, at this depth, have not separated from each other compared with their wild-type counterparts. Nevertheless, by 72 h APF, Armadillo staining in *WASp* mutant cells is indistinguishable from wild type, at any optical section, even though obvious differ-

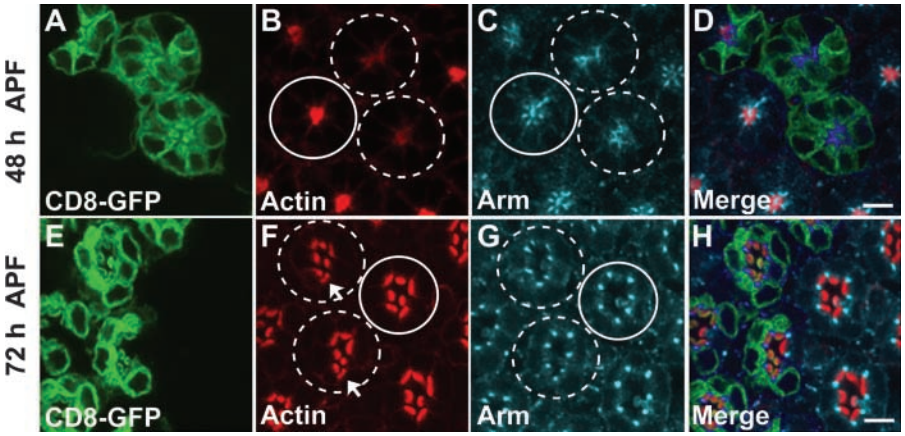

Figure. 6. **WASp is required for the enrichment/rearrangement of F-actin and redistribution of Armadillo during rhabdomere morphogenesis.** Homozygous mutant photoreceptor cells are marked with CD8-GFP. (B, C, F, and G) Dashed circles represent WASp mutant ommatidium and solid circles represent wild-type ommatidium. (A–D) *WASp*^{3} mosaic eye clones at 48 h APF. A single distal optical section of differentiating photoreceptor cells stained for F-actin (red) and Armadillo (blue). Little accumulation of F-actin is observed in mutant photoreceptor cells compared with wild-type cells. In addition, there is a considerable amount of Armadillo on the entire apical surface, and the zonula adherens junctions at this level are not well defined in mutant photoreceptor cells. (E–H) *WASp*^{3} mosaic eye clones at 72 h APF. A distal single optical section of differentiating photoreceptor cells stained for F-actin (red) and Armadillo (blue). Armadillo localization is indistinguishable in mutant cells compared with wild-type photoreceptor cells. In mutant cells, rhabdomeres have formed but appear smaller and split (arrows). Bars, 5 μm.

ences in rhabdomere morphology exist in mutant cells (Fig. 6 G).

Localization of rhabdomeric proteins depends on WASp function

Based on our initial findings, we now have a mutation that affects, in a cell-autonomous fashion, the F-actin enrichment associated with the formation of the primordial microvillar projections that give rise to the rhabdomere. Numerous components involved in microvillus development have been identified, but the method of assembly or the epistatic relationships of the various proteins remain largely undefined. We can now address the question of whether the formation of the F-actin core is critical for the subsequent recruitment or stabilization of other rhabdomere proteins. For our analysis, we investigated three proteins (Amph, moesin, and chaoptin) that have been implicated in the rearrangement of the actin cytoskeleton and the required morphogenetic changes in the plasma membrane to create the microvillus protrusion.

Chaoptin is an integral membrane protein and decorates the entire photoreceptor membrane and axon (Van Vactor et al., 1988). Chaoptin is expressed as early as 24 h APF, but enrichment to the apical membrane is first observed at 48 h APF and coincident with the appearance of an organized actin cytoskeleton. Chaoptin is essential for the cross-linking of the individual microvillus projections. In *WASp* mutant photoreceptor cells, chaoptin is expressed and shows equal distribution along the plasma membrane and axons of both wild-type and mutant photoreceptor cells (Fig. 7 C and not depicted). In addition, using chaoptin as a marker, we do not detect any defects in axon outgrowth or synaptic pruning of the photoreceptor connections in the optic lobe in *WASp* mutant cells (unpublished data). Nevertheless, if the

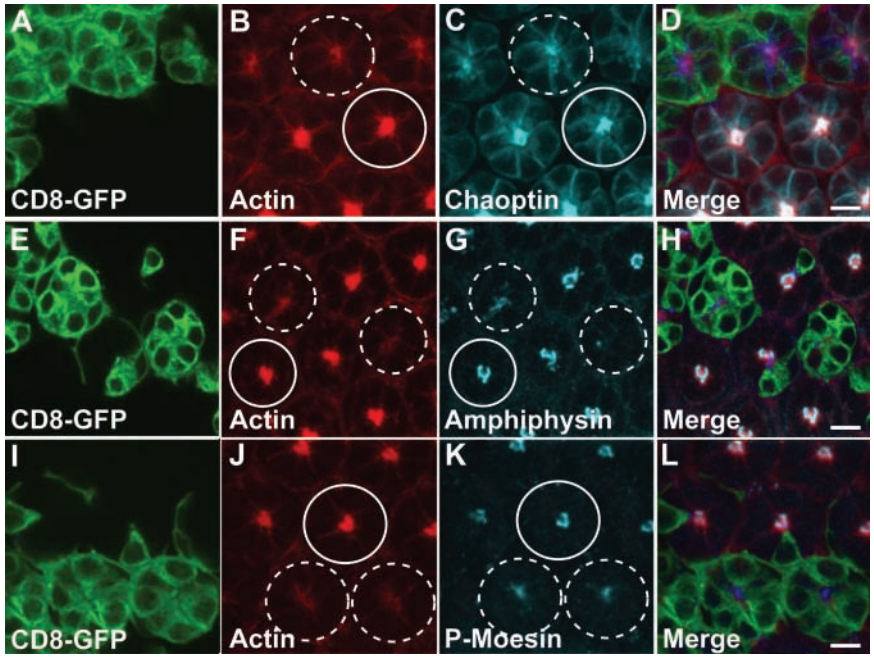

Figure 7. **Chaoptin, Amph, and moesin localization in *WASp* mutant photoreceptor cells.** *WASp*^{3} mosaic eye clones at 48 h APF. Homozygous mutant photoreceptor cells are marked with CD8-GFP. (B, C, F, G, J, and K) Dashed circles represent WASp mutant ommatidium and solid circles represent wild-type ommatidium. (A–D) A single optical section of differentiating photoreceptor cells stained for F-actin (red) and chaoptin (blue). Chaoptin is detected on the plasma membrane, but enrichment on the apical surface is impaired in WASp mutant ommatidium compared with wild type. (E–H) A single optical section of differentiating photoreceptor cells stained for F-actin (red) and Amph (blue). No accumulation of F-actin and Amph is detected in mutant photoreceptor cells compared with wild-type cells. (I–L) A single distal optical section of differentiating photoreceptor cells stained for F-actin (red) and activated moesin (blue). Like Amph, little or no accumulation of activated moesin is observed in mutant photoreceptor cells. Bars, 5 μm.

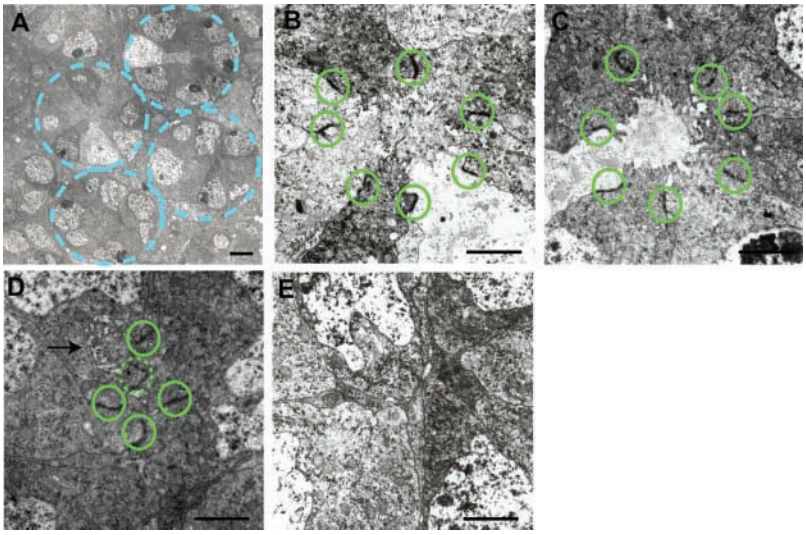

Figure 8. **Transmission EM analysis of *WASp*^{3} mosaic clones at 48 h APF.** (A) Low magnification of a 0.1-μm section containing putative *WASp* mutant and wild-type cells. Four individual ommatidia are outlined (blue dashed circles). Bar, 2 μm. (B–E) Higher magnification of the four ommatidia outlined in A. Bars, 1 μm. (B and C) Two ommatidia containing all wild-type photoreceptor cells. Seven zonula adherens junctions can be identified (green circles). Primordial microvillar projections can be seen as well as the creation of the interrhabdomeral space. (D) An ommatidium that contains a mixture of wild-type and mutant cells. Only four to five (green dashed and solid circles) of the seven zonula adherens junctions are identifiable, and primordial microvillar projections can only be seen on one of the photoreceptor cells (arrow). (E) An ommatidium that putatively contains all mutant photoreceptor cells. No zonula adherens junctions are identifiable, and neither the interrhabdomeral space nor primordial microvillar projections are visible.

apical accumulation of chaoptin is dependent on the initiation of the cytoskeleton core, we would expect to see an impairment in chaoptin accumulation at the apical surface in mutant cells. Indeed, just as we observe for F-actin, chaoptin enrichment on the apical surface is delayed (Fig. 7 C). Importantly, the examination of chaoptin expression in photoreceptor cells demonstrates that the defects observed in *WASp* mutant cells are not due to the possibility that the entire photoreceptor cell is delayed in developmental time but rather more likely due to only a temporal failure in microvillus formation.

In *Drosophila*, moesin, the only ezrin/radixin/moesin family member present, has a role in apical actin assembly and in cell polarity (Polesello et al., 2002; Speck et al., 2003). Moreover, moesin is expressed in and localizes to developing rhabdomeres (Chang and Ready, 2000). Using an antibody against the activated form of moesin, we find that in wild-type cells, phosphorylated moesin is detected on the apical surface of photoreceptor cells at 48 h APF but is not observed at 36 h APF (Fig. 3 J and Fig. 4 J). When examined in *WASp* mutant cells, we find the activation of moesin, like the accumulation of F-actin, to be inhibited (Fig. 7 K). We only distinguish activated moesin in *WASp* mutant cells when the initial microvillar projections are present, as suggested by the enrichment of F-actin on the apical surface. Our results indicate that moesin is downstream of F-actin formation and its function would be more consistent as a linker between the cytoskeleton core and the plasma membrane rather than required for the initiation of the actin cytoskeleton microvillar core.

Amph, like activated moesin, is not expressed or enriched on the apical surface until 48 h APF in wild-type photoreceptor cells (Zelhof et al., 2001). At the neuromuscular junction Amph is completely dependent on WASp for postsynaptic localization but not localization to the muscle T-tubule system (Fig. 2). In *WASp* mutant photoreceptor cells, Amph localization is severely hindered, whereas WASp accumulation is not affected by the loss of Amph (unpublished data). At 48 h APF, Amph accumulation is absent in distal optical sections and when present, appears discontinuous and blotchy (Fig. 7 G). In addition, the loss of one copy of *amph* in a *WASp* mutant background does not enhance the temporal delay seen in *WASp* mutant cells, but double mutants rarely survive to the stage in which rhabdomere initiation can be scored. Surprisingly, Amph recruitment to the apical surface, like moesin, is not completely abolished. By 72 h APF, Amph and moesin do assemble onto the extending microvilli in the absence of WASp (unpublished data). Together, our results suggest that both the function and physical presence of WASp contribute to the proper recruitment of microvillar proteins.

WASp mutant photoreceptor cells are delayed in the appearance of the primordial microvillar projections

Our immunofluorescence data imply that with the loss of WASp there is a temporal delay in the formation of the primordial microvillar projections. To corroborate this possibility, we examined the progression of microvillus formation in *WASp* mutant photoreceptor cells via an EM analysis. If WASp is required for the correct temporal enrichment of F-actin (initiation of the microvillar primordial projections) and the coincident removal of Armadillo from the juxtaposed apical surfaces of each photoreceptor cell, then examination of *WASp* mosaic clones should reveal defects in these processes. As such, we would predict, based on our immunofluorescence data, that ommatidia containing mutant cells should be recognizable by the inadequate separation of photoreceptor membranes, the lack of clear definable adherens junctions, and, finally, the lack of microvillar projections. Our analysis reveals that such deficiencies are present in our *WASp* mosaic clones and not present in *WASp*^{3} heterozygote ommatidia (Fig. 8 and not depicted). In an ommatidium in which all photoreceptor cells are wild type, we observe seven adherens junctions, the primordial microvillar projections are present on each photoreceptor cell, and the interrhabdomeral space is forming (Fig. 8, B and C). In adjacent ommatidia that putatively contain a mixture of wild-type and mutant cells, we cannot define all seven adherens junctions

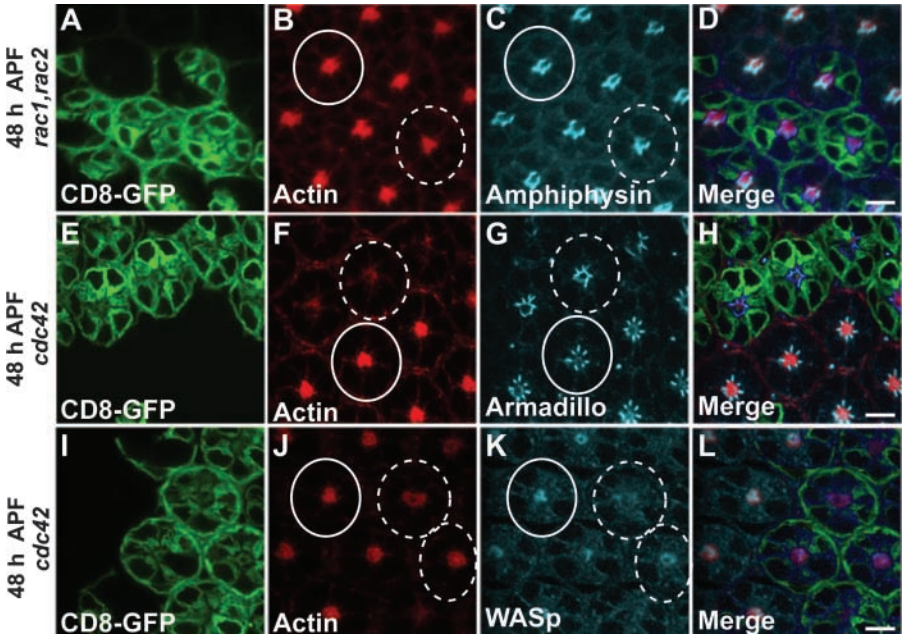

Figure 9. **Mutation of *cdc42* and not *rac* mimics the *WASp* mutant phenotype.** Homozygous mutant photoreceptor cells are marked with CD8-GFP. In all panels, dashed circles represent WASp mutant ommatidium and solid circles represent wild-type ommatidium. (A–D) *rac1, rac2* double mutant mosaic eye clones at 48 h APF. There is no discernible difference in F-actin (red) accumulation or Amph (blue) enrichment on the apical surface of mutant photoreceptor cells. (E–H) *cdc42* mosaic eye clones at 48 h APF. F-actin (red) and Armadillo (blue) accumulation is reduced in mutant cells (dashed circles) compared with their wild-type counterparts (solid circles). (I–L) *cdc42* mosaic eye clones at 48 h APF. F-actin (red) and WASp (blue) accumulation is reduced/abnormal in mutant cells. Bars, 5 μm.

because the interrhabdomeral space is not equally distributed but, more importantly, there is a lack of primordial microvillar projections on all the photoreceptor cells (Fig. 8 D). In the case in which all cells of the ommatidium are mutant, we do not observe any adherens junctions, no separation or elimination of cell–cell contacts between photoreceptor cells, and no presence of the microvillar projections (Fig. 8 E). The combination of our immunofluorescence and EM structural analysis strongly indicates that WASp is mediating a signal for the correct temporal initiation of microvillus formation in photoreceptor cells.

Upstream components of WASp signaling

It is thought that the recruitment and activation of WASp to the plasma membrane is accomplished through an interaction with small Rho GTPases. As for the relationship between WASp and small Rho GTPases, a previous paper demonstrated that *Drosophila* WASp binds Cdc42 and Rac1 but not Rho (Ben-Yaacov et al., 2001). The role of either Rac or Cdc42 in rhabdomere morphogenesis is not clearly defined. In *Drosophila*, there are three Rac molecules, Rac1, Rac2, and Mig2-like (Mtl), and only one Cdc42 homologue. Rac1 is expressed in photoreceptor cells and has been implicated in the formation of the rhabdomere terminal web (Chang and Ready, 2000). To test whether any Rac molecule is required for F-actin formation, we examined F-actin accumulation in double mutant clones of Rac1 and Rac2 as well as single mutant clones of Mtl. In both cases, we find F-actin enrichment is not delayed and accumulation of rhabdomeric proteins proceeds normally at 48 h APF (Fig. 9, A–D, and not depicted).

As for Cdc42, as reported previously, null or strong hypomorphic alleles result in photoreceptor lethality before microvillus initiation can be assayed (Genova et al., 2000; unpublished data). When a weak hypomorph of *cdc42* is examined, we do observe a similar delay in F-actin enrichment, the redistribution of Armadillo from the apical surface, as well as the accumulation of rhabdomeric proteins including WASp (Fig. 9, E–L, and not depicted). Furthermore, we do observe some variability in the penetrance of these phenotypes in mutant cells but this simply could be the result of the nature of the *cdc42* allele that we are using (Genova et al., 2000). We have attempted to detect a genetic interaction between *cdc42* and *WASp*. However, we do not observe any significant enhancement of the $cdc42^2$ phenotype observed in adult ommatidia with the removal of one copy of *WASp* (unpublished data). Nevertheless, these results suggest that Cdc42 and WASp may be acting together for the proper temporal initiation of microvillus formation in *Drosophila* photoreceptor cells.

Discussion

The identification of the major constituents and the description of the ultrastructure of microvilli have been known for numerous years. However, how this structure is initiated and regulated remains a mystery. Our phenotypic analysis of WASp function in *Drosophila* photoreceptor cells has now provided several insights into the molecular mechanisms responsible for the formation of the microvillus core. Knowing that the primary constituent of the microvillus projection is F-actin, we searched for molecules that could be candidates for coordinating the formation of the actin filaments. Using the SH3 domain of Amph as bait, we were able to isolate another rhabdomeric protein WASp. This interaction is also conserved in *Saccharomyces cerevisiae*. The homologues of Amph (Rvs167p) and WASp (Las17p/Bee1p) also interact (Lila and Drubin, 1997; Colwill et al., 1999; Tong et al., 2002). Furthermore, WASp is an excellent candidate for initiating microvillus formation. *WASp* is the causative gene product of Wiskott-Aldrich syndrome and lymphocytes from Wiskott-Aldrich syndrome patients have a reduction in cell surface microvilli (Derry et al., 1994).

Our results demonstrate that WASp is expressed and localizes to the apical surface before the appearance of microvillar apical folds and before the enrichment/rearrangement of F-actin in photoreceptor cells. More importantly, the loss of WASp function results in malformed rhabdomeres. Typically, *WASp* mutant rhabdomeres are misshapen and a small percentage are split, which are phenotypes not observed in wild-type photoreceptor cells. Knowing WASp has been identified as a key component in the specific subcellular localization and polymerization of actin, we speculated that these phenotypes were a result of defects in establishing the F-actin cytoskeleton core.

The transformation of the apical surface into the rhabdomere is a highly coordinated event. After each apical surface involutes inward, there is an expansion of the apical surface down toward the retinal floor. As such, the examination of tangential sections through the depth of the photoreceptor cell represents a temporal profile of the photoreceptor apical surface as it is transformed into a rhabdomere. Inspection of markers for the initiation of microvillus formation (F-actin) and the separation and delimitation of each photoreceptor apical surface (Armadillo) in *WASp* mutant cells clearly demonstrated a temporal delay in rhabdomere formation compared with their neighboring wild-type counterparts. Furthermore, molecules that are implicated in the stabilization of the actin cytoskeleton core (moesin), the cross-linking of each microvillus (chaoptin), or the deformation of the overlying plasma membrane (Amph) are dependent on the presence of the primordial microvilli—confirmed from our EM analysis—for their recruitment/stabilization to the apical surface, and thus, are epistatic to WASp function. In the case of Amph, the direct association with WASp may aid in the recruitment of Amph to the apical surface. Even though all of our observations are consistent with the idea that WASp is coordinating the signal required for the transformation of the apical surface, surprisingly, WASp is not essential for the formation or growth of microvilli.

If WASp is responsible for integrating the signal for microvillus formation, what is the signal? Numerous studies have implicated a combination of upstream factors for the recruitment and activation of WASp, such as small Rho GTPases, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) phosphoinositide, and SH3-containing proteins that bind the proline-rich domain of WASp (Miki et al., 1996; Symons et al., 1996; Prehoda et al., 2000; Fukuoka et al., 2001; Takenawa and Miki, 2001). Our data are suggestive, but not conclusive, that Cdc42 may play a role in the activation of WASp. A similar delay in F-actin enrichment and subsequent recruitment of rhabdomeric proteins is observed in *cdc42* mutant cells. This idea directly conflicts with the result that the Rho GTPase binding domain of WASp is not required for the rescue of viability in *WASp* mutants (Tal et al., 2002). However, the binding site for PI(4,5)P2 is also dispensable, and our own data demonstrate that the proline-rich domain is not necessary to rescue viability (Tal et al., 2002; unpublished data). Given that each individual domain in vivo is expendable, may be indicative that any one or a combination of the three domains can be sufficient for the recruitment and activation of WASp. In rhabdomere biogenesis, all three potential ways of activating WASp are present. The SH3 domain of Amph binds WASp. Mutation of Cdc42 results in defects in rhabdomere morphogenesis, and there is a coincident accrual of PI(4,5)P2 on the photoreceptor apical surface during the initiation of microvilli formation (Figs. 3 and 4). Only the combination of in vitro activation studies, further in vivo WASp structure–function studies, and deciphering the role of PI(4,5)P2 metabolism in microvillus biogenesis will we be able to clarify the contribution of each regulatory domain of WASp in microvillus initiation.

Finally, it is evident from our results that a second pathway for initiating F-actin formation is present. WASp is not essential for microvillus formation, and clearly, F-actin accumulates and functional microvilli do form in *WASp* mutant cells. The molecular basis of this second pathway is unknown. One possible mechanism could involve a p21-activated kinase (PAK). Besides a role in cell differentiation and proliferation, PAK proteins have been implicated in the regulation of the cytoskeleton (Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999). A recent paper has implicated Mbt (mushroom bodies tiny PAK) in photoreceptor morphogenesis (Schneeberger and Raabe, 2003). *mbt* mutant photoreceptor cells have malformed rhabdomeres, and Cdc42 is required for its correct localization in photoreceptor cells. That Cdc42 may be responsible for the activation of more than one pathway would be consistent with the fact that photoreceptor cell differentiation, and in particular rhabdomere formation, is more severely affected in $cdc42^2$ mutant cells compared with *WASp* mutant cells (unpublished data). In addition to a broader role of Cdc42, a second member of the WASp/WAVE family, SCAR, exists in *Drosophila*. *SCAR* is required for patterning of the eye ommatidium and external morphology (Zallen et al., 2002). Thus, SCAR activity could account for the assembly of actin observed in *WASp* mutant cells. However, it will be necessary to genetically separate the early requirement of *SCAR* in ommatidial patterning before examining its contribution to microvillus initiation. Overall, our work has now defined a budding network of molecules required for microvillus initiation and provided a framework in which we can further elucidate the mechanisms of microvillus biogenesis and regulation.

Materials and methods

Genetics

The $wasp^1$/TM6B, $wasp^3$/TM6B, and the FRT^{82B}, $wasp^3$/TM6B stocks were obtained from E. Schejter (Weizmann Institute of Science, Rehovot, Israel). $cdc42^2$ was obtained from R. Fehon (Duke University, Durham, North Carolina) and recombined onto the FRT^{19A} chromosome. The $Rac1^{J11}$, $Rac2^{\Delta}$, Mtl^{Δ}, and Moe^{G0323} mutants were obtained from the Bloomington Drosophila Stock Center. The following crosses were performed to produce the various mutant mosaic eyes. *WASp* clones: *ey-flp/ey-flp*; +/+; FRT^{82B}, *tub-GAL80* X *w*/Y; *GMR-GAL4, UAS-CD8-GFP/GMR-GAL4, UAS-CD8-GFP*; FRT^{82B}, $WASp^3$, *e*/TM6B. *Cdc42* clones: $Cdc42^2$, FRT^{19A}/FM6; *GMR-GAL4, UAS-CD8-GFP/GMR-GAL4, UAS-CD8-GFP*; +/+ X *tub-GAL80*, FRT^{19A}/Y; +/+; *ey-flp/ey-flp*. *Mtl* clones: *w*/Y; *GMR-GAL4, UAS-CD8-GFP*/+; FRT^{82B}, Mtl^{Δ}/TM6B X *ey-flp/ey-flp*; +/+; FRT^{82B}, *tub-GAL80*. $Rac1^{J11}$ clones: *hs-flp*/Y; *GMR-GAL4, UAS-CD8-GFP/OK107-GAL4*; *tub-GAL80*, FRT^{2A}/TM6B X *w/w*; +/+; Rac^{J11}, FRT^{2A}/TM6B. $Rac1^{J11}$, $Rac2^{\Delta}$ clones: *hs-flp*/Y; *GMR-GAL4, UAS-CD8-GFP/OK107-GAL4*; *tub-GAL80*, FRT^{2A}/TM6B X *w/w*; +/+; $Rac1^{J11}$, $Rac2^{\Delta}$ FRT^{2A}/TM6B. Pupae were aged at 22°C.

Staining of pupal photoreceptor cells

Developing whole retinas were dissected at the appropriate time and fixed in PEMFA (100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) for 15–60 min. The tissue was blocked in PBTB (PBS and 0.1% Triton X-100 with 1% BSA) for 30 min, incubated in primary antibody (in PBTB) overnight at 4°C, washed in PBTB, incubated in secondary antibody for 2 h at 22°C, washed in PBT (PBS and 0.1% Triton X-100), and mounted in polyvinyl alcohol/DABCO mounting media (Sigma-Aldrich). The primary antibodies used were rabbit anti-Amph (Zelhof et al., 2001), antiphosphorylated moesin (Cell Signaling), mouse anti-chaoptin (Zipursky et al., 1984), and mouse anti-Armadillo (DSHB). Rhodamine-conjugated Phalloidin (Molecular Probes) was used for the detection of F-actin. FITC-, Red-X–, and Cy5-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. All images were captured on a confocal microscope (model Diaphot 200 [Nikon]; model MRC1024 [Bio-Rad Laboratories]) with a 60× (1.4) oil lens (with an additional 5× zoom). The confocal Z-series were processed using Confocal Assistant software (version 4.02) and Adobe Photoshop.

Transmission EM analysis

Drosophila heads were fixed (4% formaldehyde, 3.5% glutaraldehyde, 100 mM cacodylate buffer, and 2 mM CaCl) for 3 h at 22°C, and then fixed (4% formaldehyde, 3.5% glutaraldehyde, 100 mM cacodylate buffer, 2 mM CaCl, and 1% tannic acid) overnight at 4°C. Heads were rinsed in 100 mM cacodylate buffer and postfixed in 2% osmium in 100 mm cacodylate buffer for 1 h at 22°C. The heads were dehydrated through an ethanol series and rinsed three times with propylene oxide. The heads were embedded in Spurrs for sectioning. Images of sections were captured on an electron microscope (model 1200 EX II; JEM) and photo-prints were scanned into Adobe Photoshop.

GST binding assays

Drosophila tissue was placed in extraction/binding buffer (100 mM KCl, 20 mM Hepes, 5% glycerol, 10 mM EDTA, and 0.1% Triton X-100 with the proteinase inhibitors [protease inhibitor cocktail; Roche]), homogenized, and sonicated. Transfected cells were placed in cell lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 1% NP-40, and 0.5% (wt/wt) sodium deoxycholate with proteinase inhibitors). In each case, the supernatant was collected and incubated with the appropriate GST fusion protein (Zelhof et al., 2001). The bound proteins were pelleted with glutathione Sepharose beads (Amersham Biosciences) and washed three times with extraction/binding buffer. The pellets were resuspended and equal volume of 2× sample/loading buffer was added. All extracts were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore). Protein detection was done as described previously (Baker et al., 1994). Rabbit anti-WASp antibody was used at a concentration of 1:1,000 (obtained from E. Schejter).

DNA constructs and transfections

The full-length *WASp* cDNA (RE12101) was obtained from Research Genetics/Invitrogen. The full-length cDNA and the cDNA representing the internal deletion of amino acids (316–400) were cloned into pcDNA3 (Invitrogen) and pUAST and transformed into flies. Modified HEK-293 cells ($PEAK^{RAPID}$ cells; Edge BioSystems) were transfected with GenePorter 2 (Gene Therapy Systems) and were harvested 48 h after transfection. The pleckstrin homology domain of $PLC\delta_1$ (Lemmon et al., 1995; Raucher et al., 2000) was cloned upstream and in frame with GFP in pUAST. The construct was transformed into flies and several transgenic lines were established. Expression was visualized by crossing the transgenic lines to GMR-GAL4.

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