



Published in final edited form as:

*Oncogene*. 2013 February 28; 32(9): 1135–1143. doi:10.1038/onc.2012.135.

## The NF2 Tumor Suppressor Regulates Microtubule-Based Vesicle Trafficking via a Novel Rac, MLK and p38<sup>SAPK</sup> Pathway

Robert F. Hennigan<sup>1</sup>, Chandra A. Moon<sup>1</sup>, Linda M. Parysek<sup>2</sup>, Kelly R. Monk<sup>3</sup>, Gerardo Morfini<sup>4,5</sup>, Sarah Berth<sup>4,5</sup>, Scott Brady<sup>4,5,\*</sup>, and Nancy Ratner<sup>1,5,\*</sup>

<sup>1</sup>Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, 240 Albert Sabin Way, Cincinnati, OH 45229

<sup>2</sup>Dept. of Cell and Cancer Biology, University of Cincinnati School of Medicine, 3333 Burnet Ave., Cincinnati, OH, 45229

<sup>3</sup>Dept. Genetics, Washington University School of Medicine, St. Louis, MO

<sup>4</sup>Dept. Anatomy and Cell Biology, University of Illinois at Chicago, 808 S Wood St., Chicago, IL., 60612-7308

<sup>5</sup>Marine Biological Laboratory, Woods Hole, MA

### Abstract

Neurofibromatosis Type 2 patients develop schwannomas, meningiomas and ependymomas resulting from mutations in the tumor suppressor gene, *NF2*, encoding a membrane-cytoskeleton adaptor protein called merlin. Merlin regulates contact inhibition of growth and controls the availability of growth factor receptors at the cell surface. We tested if microtubule-based vesicular trafficking might be a mechanism by which merlin acts. We found that schwannoma cells, containing merlin mutations and constitutive activation of the Rho/Rac family of GTPases, had decreased intracellular vesicular trafficking relative to normal human Schwann cells. In *Nf2*<sup>-/-</sup> mouse Schwann (SC4) cells, re-expression of merlin as well as inhibition of Rac or its effector kinases, MLK and p38<sup>SAPK</sup>, each increased the velocity of Rab6 positive exocytic vesicles. Conversely, an activated Rac mutant decreased Rab6 vesicle velocity. Vesicle motility assays in isolated squid axoplasm further demonstrated that both mutant merlin and active Rac specifically reduce anterograde microtubule-based transport of vesicles dependent upon the activity of p38<sup>SAPK</sup> kinase. Taken together, our data suggest loss of merlin results in the Rac dependent decrease of anterograde trafficking of exocytic vesicles, representing a possible mechanism controlling the concentration of growth factor receptors at the cell surface.

### Keywords

Merlin; NF2; Rac; Trafficking; Exocytosis

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\*Contributed equally

## INTRODUCTION

Neurofibromatosis Type 2 is an inherited autosomal dominant disease characterized by bilateral schwannomas of the 8th cranial nerve (1–3). The tumor suppressor gene responsible for this disorder, *NF2*, is also inactivated in spontaneously arising tumors, including schwannoma, meningioma and malignant mesothelioma, thus implicating it in a range of human cancers (4). Targeted deletion of *Nf2* in Schwann cells leads to schwannoma formation in the mouse (5). The *NF2* gene encodes merlin, a 70 kDa member of the ezrin, radixin, moesin (ERM) family of membrane-cytoskeleton adaptor proteins. The precise mechanisms by which merlin functions as a tumor suppressor are poorly understood.

Merlin shares a conserved secondary structure with other members of the ERM family consisting of an N-terminal FERM domain, followed by a central  $\alpha$ -helical (CH) region and a C-tail domain (CTD) (6). Transition between the open, FERM-accessible conformation and the closed, FERM-inaccessible conformation controls merlin tumor suppressor function and is modulated by phosphorylation of serine 518 (7). Phosphorylation of S518 correlates with a growth permissive state and is a key point of integration of merlin activity with signal transduction pathways (8, 9). Under growth suppressive conditions, merlin is activated upon dephosphorylation of S518 by cellular phosphatases such as MYPT1-PP1 $\delta$  (10). Inactivation of merlin is achieved via the action of the small GTPase, Rac, via its effector kinase, PAK, resulting in phosphorylation of merlin at S518 (11, 12). Merlin in turn antagonizes Rac activity by an unknown mechanism, forming a negative feedback loop of mutual inhibition (13). This antagonism appears to be lost in human schwannomas because these merlin-deficient cells are characterized by constitutive activation of Rac (14–16). *Nf2*-null cells grow to abnormally high densities *in vitro*, and it has been suggested that this loss of contact inhibition is the biological basis for schwannoma formation (17, 18). Merlin is thought to control contact inhibition by regulating the cell surface availability of growth factor receptors (19, 20). However, the mechanisms by which merlin, and perhaps activated Rac, affects growth factor receptor abundance are unclear.

We found that merlin-deficient tumor cells show decreased intracellular vesicular trafficking relative to normal human Schwann cells. Significantly, this effect was dependent upon the activity of Rac and the MAP kinase p38<sup>SAPK</sup>. Inhibition of Rac and the effector kinase MLK3 also impaired growth in merlin null cells at high density. In *Nf2*<sup>-/-</sup> SC4 Schwann cells, re-expression of merlin or inhibition of Rac, MLK, or p38<sup>SAPK</sup> all resulted in increased velocity of exocytic vesicles. In a squid axoplasm system, open conformation mutants of merlin and active Rac each specifically reduced fast anterograde axonal vesicle transport. This effect was independent of the plasma membrane and dependent upon the activity of p38<sup>SAPK</sup>. Together these data show that the loss of merlin reduces microtubule-based exocytic vesicle velocity in a Rac-MLK-p38<sup>SAPK</sup> dependent manner. We propose that merlin-Rac signaling may normally modulate vesicle release from microtubules, influencing concentrations of growth factor receptors at the cell surface.

## RESULTS

### VAMP-2 Vesicle Mobility is Reduced in Schwannoma Cells in a Rac and p38<sup>SAPK</sup>-Dependent Manner

To determine if loss of merlin expression affects intracellular vesicular trafficking, we designed an assay to measure the mobility of a subset of membrane bounded organelles in live primary normal human Schwann cells relative to live, patient-derived primary human schwannoma cells. To visualize internal vesicle motion by time lapse imaging, we marked transfected cells with a plasmid expressing GFP fused to the ubiquitously expressed v-SNARE protein VAMP2/synaptobrevin 2 (21–24). The relative mobility of VAMP2-GFP positive vesicles was used as a measure of general intracellular trafficking (Figure 1). Primary cultures transfected with plasmids expressing a VAMP2-GFP fusion protein and general mobility was quantified by measuring the percentage of VAMP2-GFP positive vesicles that changed position between successive 3-second intervals over 180 seconds. Normal human Schwann cells showed highly motile VAMP2-positive vesicles with a broad range of values, (Figure 1C) with a mean and SEM of  $4.2 \pm 0.1\%$ . In contrast, primary human schwannoma cells had a more restricted range of values (Figure 1D), with a mean and SEM of  $2.0 \pm 0.1\%$ , suggesting an inhibition of intracellular membrane traffic in tumor relative to normal cells. Since loss of merlin expression results in activation of Rac (13), we measured VAMP-2 in schwannoma cells treated with the specific Rac inhibitor NSC23766 (25). Rac inhibition significantly increased VAMP-2 mobility (Figure 1E), mean and SEM of  $6.0\% \pm 0.1\%$ . The MAP kinase, p38<sup>SAPK</sup> functions downstream of Rac and has been shown to phosphorylate and inhibit kinesin heavy chain, thereby implicating it in the regulation of trafficking (26). Treatment of schwannoma cells with the p38<sup>SAPK</sup> inhibitor, SB203580, significantly increased VAMP-2 mobility (Figure 1F), mean and SEM of  $5.8\% \pm 0.1\%$ . These data implicated a Rac-p38<sup>SAPK</sup> pathway as regulating intracellular trafficking in schwannoma cells.

### Rac Regulates High Density Growth in Merlin Null Cells

The results of the VAMP2 experiments suggested that Rac controls a p38<sup>SAPK</sup> dependent pathway that regulates intracellular vesicle motility. We next tested if this pathway was required to mediate the major biological consequence of merlin mutation, loss of contact inhibition of growth (27). *Nf2*<sup>-/-</sup> merlin deficient fibroblasts grow to much higher densities than *Nf2*<sup>+/+</sup> cells, demonstrating merlin dependent contact inhibition of growth (Figure 2). Treatment with the Rac inhibitor NSC23766 did not alter growth rates at low density but did significantly inhibit growth of *Nf2*<sup>-/-</sup> cells once they achieved high density (Figure 2A). Growth rates of *Nf2*<sup>+/+</sup> cells were unaffected by NSC23766 (Figure 2A). Similarly, CEP11004, an inhibitor of the Mixed Lineage Kinases family (MLK), a Rac effector kinase that regulates p38<sup>SAPK</sup> activity (28) also specifically inhibited growth once *Nf2*<sup>-/-</sup> cells achieve high density in but did not affect *Nf2*<sup>+/+</sup> cells (Figure 2B). The p38<sup>SAPK</sup> inhibitor SB203580 slowed growth in *Nf2*<sup>-/-</sup> at high density but also suppressed the growth rate *Nf2*<sup>+/+</sup> cells (Figure 2C). These data suggest that the same signal transduction pathways that affect trafficking are involved in merlin-dependent contact inhibition of growth.

## Rab6 is a Marker for Exocytic Vesicles in Merlin Deficient Schwann Cells

The VAMP2-GFP experiments suggested that merlin may regulate intracellular trafficking via Rac in a p38<sup>SAPK</sup> dependent manner, possibly implicating constitutive exocytosis (29). We therefore developed a system to specifically measure constitutive exocytosis in an immortalized, transformed merlin-null Schwann cell line, SC4. We used plasmids expressing Rab6 fused to the red fluorescent protein variant mStrawberry as a marker for constitutive exocytosis (30). To confirm that Rab6 exocytic vesicles carry a cargo relevant to Schwann cells we co-transfected SC4 cells with plasmids expressing Rab6-mStrawberry and an ErbB2-GFP construct (31). Images were acquired from live SC4 cells maintained at 37°C six hours after transfection, before the bulk of the newly synthesized ErbB2-GFP establishes itself in the plasma membrane. Under these conditions Rab6 was localized primarily to the Golgi with a minority expressed in multiple small vesicles dispersed throughout the cytoplasm (Figure 3A). ErbB2 had plasma membrane localization and a significant amount of nascent ErbB2-GFP in the Golgi but was also localized to small vesicles dispersed within the cytoplasm (Figure 3A, B). A subpopulation of ErbB2 expressing vesicles co-localized with Rab6 vesicles (Figure 3B, C). Time lapse images of live cells also identified double positive vesicles moving towards the plasma membrane (Supplemental Movie 1). This indicates that a subset of Rab6 positive, post-Golgi vesicles carry the relevant ErbB2 cargo, and confirm Rab6 as a marker of constitutive exocytosis in this system.

## Regulation of Rab6 Vesicle Velocity by the Merlin-Rac-MLK3-p38<sup>SAPK</sup> Pathway

To measure the motion of exocytic Rab6 vesicles we performed live-cell time lapse experiments in SC4 cells, maintained at 37°C (Supplemental Movie 2). A single frame of a representative time lapse shows intense signal in the Golgi with numerous small vesicles dispersed within the cytoplasm (Figure 4A). To visualize motion, all images of the time lapse (60 seconds) were collated into a single image. This procedure revealed a network of vesicle tracks extending from the Golgi to the plasma membrane, a pattern consistent with vesicles moving along the microtubule network (Figure 4B). To aid in tracking specific vesicles and establish their anterograde direction, time-lapse stacks were color coded to display three successive frames in red, green and blue, then the velocity of individual vesicles was measured (Figure 4C, Supplemental Movie 2).

To test whether merlin loss affects Rab6 vesicle velocity, *Nf2*<sup>-/-</sup> SC4 cells were co-transfected with plasmids expressing Rab6-mStrawberry and either merlin or empty vector then velocities were measured as described above. The average velocities of 200 to 500 vesicle tracks per condition were calculated. Re-expression of merlin caused a reproducible shift to a faster moving population of Rab6 vesicles compared to empty vector (Figure 5A). Inhibition of Rac using 100  $\mu$ M NSC23766 increased vesicle velocity to a similar degree that merlin transfection did (Figure 5B). This suggests that merlin expression in live cells suppresses Rac activity, thereby controlling Rab6 vesicle velocity. Conversely, transfection with a hyperactive fast cycling Rac mutant, F28L, caused a significant decrease in Rab6 vesicle velocity (Figure 5C), consistent with Rac activity acting as a brake on exocytic transport. Interestingly, a constitutively GTP-bound, active Rac mutant, Q61L, did not affect Rab6 velocity (Figure 5D), suggesting that the ability of Rac to cycle from the GDP to the

GTP bound states is critical to this regulation. Interestingly, we found that Rac-GTP co-localized with microtubules in SC4 cells (Supplemental Figure 1), consistent with previous reports that Rac-GTP binds to tubulin polymers (32). These data place active Rac in a position to influence exocytic vesicle transport.

Next, we examined the role of MLK and p38<sup>SAPK</sup> on exocytic vesicle mobility. Treatment of SC4 cells with the MLK inhibitor CEP11004 increased Rab6 vesicle velocity (Figure 5E), indicating that a member of the MLK family participates in this regulatory pathway. Inhibition of p38<sup>SAPK</sup> with 20  $\mu$ M SB203580 also increased Rab6 vesicle velocity (Figure 5F). Taken together these data provide compelling evidence that merlin regulates constitutive exocytosis via a Rac, MLK, p38<sup>SAPK</sup> mediated pathway.

### Open Conformation Merlin Mutants Slow Anterograde Fast Axonal Transport

To directly assess the effect of Merlin and Rac on microtubule-dependent vesicle traffic, we used vesicle motility assays in isolated squid axoplasm. This experimental system allows for quantitative analysis of fast axonal transport in both anterograde (conventional kinesin-dependent) and retrograde (cytoplasmic dynein-dependent) in the absence of the plasma membrane (33). Vesicle motility assays in isolated axoplasm were instrumental in the original discovery of conventional kinesin (34) and novel regulatory pathways for microtubule-based vesicle transport (35, 36)

Perfusion of squid axoplasm with 1  $\mu$ M purified recombinant wild type merlin protein had little or no inhibitory effect on the velocity of membrane bound organelles in the anterograde direction over the 50 minute assay period (Figure 6A). We then tested FERM-Helix, a C-terminal deletion mutant of merlin containing amino acids 1-502, that was shown to be FERM accessible relative to wild type by virtue of increased interaction with NHERF(7). Perfusion with the FERM-Helix mutant significantly reduced anterograde vesicle velocity (Figure 6B). Inhibition of p38<sup>SAPK</sup> activity by co-perfusion with the SB203580 inhibitor rescued anterograde vesicle inhibition by the FERM-Helix mutant (Figure 6C). Perfusing a nonphosphorylatable mutant, S518A, had little or no effect on vesicle velocity in this system (Figure 6D), a similar response to wild type. A phosphomimetic mutant S518D, which is predicted to have an accessible FERM domain and fails to suppress cell growth (13), also reduced anterograde vesicle transport (Figure 6E). This effect was not rescued by SB203580, suggesting different mechanisms of inhibition between FERM-Helix and S518D (Figure 6F). Retrograde velocity was essentially unchanged in all proteins tested. These data suggest that the open, FERM accessible isoforms of merlin, FERM-Helix and S518D can specifically regulate kinesin mediated anterograde vesicle transport. This inhibition occurs in the cell-free squid axoplasm system and is thus independent of the plasma membrane, where the bulk of merlin is localized, and of the cell nucleus, another reported site of merlin action (37). The dependence of FERM-Helix inhibition of anterograde vesicle transport on p38<sup>SAPK</sup> activity indicates that suppression is mediated by a kinase chain initiated by merlin, leading to a reduction in anterograde vesicle velocity.

## Rac Regulates Anterograde Fast Axonal Transport via p38<sup>SAPK</sup>

It is striking that the merlin mutants which inhibit anterograde vesicle transport are predicted to have an open, FERM accessible conformation and a growth permissive phenotype. The growth permissive phenotype correlates with the higher levels of active Rac-GTP, seen in schwannoma cells. In contrast, isoforms that do not affect vesicle transport are predicted to have a closed, FERM-blocked conformation. Growth suppression phenotype and correlates with low levels of Rac-GTP. We therefore tested the effect of Rac on anterograde vesicle transport. Perfusion of squid axoplasm with 1  $\mu$ M active Q61LRac caused a significant and specific reduction in anterograde vesicle transport (Figure 7A). Retrograde transport was unaffected. As with the merlin proteins, inhibition of p38<sup>SAPK</sup> reversed the effect of Q61L Rac (Figure 7B) suggesting that it functions in the same pathway as merlin to regulate anterograde transport. This effect was specific for active Rac since perfusion with dominant negative N17Rac mutant protein failed to affect either anterograde or retrograde transport (Figure 7C). The effect was also specific for Rac, since perfusion with recombinant active V12 Ras did not affect vesicle transport in either direction (Figure 7C). These results suggest that merlin regulates Rac to control a p38<sup>SAPK</sup> dependent pathway that specifically regulates anterograde trafficking (Figure 8). Collectively, results from our experiments indicate that loss of merlin increases Rac activity which, in turn, promotes activation of the MLK/p38 kinase pathway, ultimately resulting in inhibition of anterograde vesicle motility.

## DISCUSSION

At the cellular level, merlin function is critical for contact inhibition of growth (8, 17), as illustrated by the continued proliferation of *Nf2* null fibroblasts at high cell density (27), yet the specific biochemical functions that merlin performs remain uncertain. Growing evidence suggests that merlin may control some aspect of intracellular vesicle trafficking (29, 38, 39). We hypothesized that merlin regulates anterograde vesicle trafficking and designed a set of experiments to test this hypothesis in the context of live mammalian cells as well as in a plasma membrane-free axoplasm system. Our data suggest that in the absence of merlin active Rac slows kinesin motor-driven transport of vesicles along microtubules (Figure 9). Consistent with this activity, Rac was recently reported to antagonize the function of the mitotic kinesin, Eg5, at microtubule asters during metaphase (40). This, together with our data, suggests that Rac-mediated signaling pathways may regulate multiple members of the kinesin family.

Our finding that decreased intracellular vesicular trafficking in primary human *NF2* schwannoma cells relative to primary normal human Schwann cells was dependent on Rac and p38<sup>SAPK</sup> identifies a novel pathway regulating transport. Notably, the active inhibition of trafficking in merlin deficient cells by a Rac and p38<sup>SAPK</sup> mediated signaling pathway correlated with restored contact inhibition of growth in merlin null fibroblasts, suggesting that this pathway is critical to merlin function. Trafficking experiments in primary human schwannoma cells ensured that the trafficking phenotype is relevant to tumor pathobiology. However, since tumors are the end result of a multi-hit progression we could not unambiguously attribute changes in vesicle motility to the loss of merlin. Also, because VAMP-2 does not discriminate among different types of intracellular vesicle trafficking (41)



we could not identify the specific molecular systems responsible changes in vesicle mobility. To address these issues, we designed an experimental system to directly test the effect of merlin on constitutive exocytosis, one of the trafficking modalities implicated in NF2 (29).

We used a genetically encoded, fluorescent Rab6-mStrawberry fusion protein to record the trafficking of exocytic vesicles from the Golgi to the plasma membrane (30). This system focused our analysis on anterograde vesicle movement along microtubules, a direct measure of kinesin motor function in live mammalian cells. Re-expression of merlin or Rac inhibition in *Nf2*<sup>-/-</sup> SC4 Schwann cells increased Rab6 vesicle velocity. The surprising association of the Rac pathway with vesicle trafficking is strengthened by our finding that inhibition of downstream Rac effectors, MLK and p38<sup>SAPK</sup>, also significantly increased Rab6 vesicle velocity.

A fast-cycling mutant of Rac, F28L, decreased Rab6 vesicle velocity, providing compelling positive evidence that Rac acts to antagonize anterograde vesicular trafficking. The failure of the constitutive GTP bound Rac mutant Q61L to reduce vesicle velocity in mammalian cells implies that a specific guanine exchange factor (GEF) is required, most likely to bind and activate Rac at specific subcellular sites (42). In contrast, in the squid axoplasm the Rac Q61L did slow transport. In this case, perfusion of GTP-Rac directly into the axoplasm likely obviated a requirement for nucleotide exchange factors to localize Rac. These results 1) confirm experiments performed in primary schwannoma cells, 2) demonstrate that loss of merlin is sufficient to impair anterograde trafficking and 3) identify a pathway consisting of merlin, Rac, MLK and p38<sup>SAPK</sup> that modulates microtubule-based trafficking (Figure 9).

The kinesin motor protein heavy chain is a substrate for p38<sup>SAPK</sup> (26), and kinesin phosphorylation is the most likely mechanism responsible for changes in vesicle kinetics that we describe. A member of the MAP kinase family, p38<sup>SAPK</sup> is activated by the intermediary kinases, MKK4 and MKK6 which in turn may be activated by MLK (43, 44). MLK itself is activated by binding to Rac-GTP or cdc42-GTP (45). It is interesting to note that the MLK family member MLK2 can interact with microtubules and complex with the kinesin Kif3 (46), supporting the idea that a Rac-MLK-p38<sup>SAPK</sup> pathway may play a central role in regulating trafficking of multiple motors.

Use of a plasma membrane-free axoplasm isolated from the giant axon of the Atlantic squid *Loligo pealei* allowed us to distinguish between merlin exerting a direct effect at the level of the microtubules or vesicles and an indirect event caused by merlin regulating upstream signal transduction events originating at the plasma membrane or in the nucleus. Our finding that perfusion of merlin mutants specifically inhibited anterograde transport, argued for a direct regulation of trafficking at the level of microtubules. More significantly this data is consistent with recent reports in the literature suggesting that merlin interacts with microtubules and promotes tubulin polymerization (47). In *Drosophila* merlin is expressed in cytoplasmic particles that move along microtubules via interaction with kinesin and dynein motor proteins (48). Inhibition of anterograde trafficking was restricted to the open conformation mutants of merlin, the FERM-Helix domain and S518D. For the FERM-Helix mutant, treatment with the p38<sup>SAPK</sup> inhibitor SB203580 abrogated this inhibition,

confirming that merlin's effect was dependent on a kinase based signal transduction cascade, most likely targeting the kinesin motor protein complex. The SB203580 did not rescue inhibition by the S518D, suggesting some other mechanism action for this mutant. Regardless of the specific mechanism of action, these intriguing results suggest that presumably growth permissive forms of merlin may play an active role in orchestrating membrane trafficking during cell growth.

Microtubule based trafficking is critical for selective delivery of membrane proteins to specific cellular domains in normal cells and tissues. Changes in the regulation of microtubule based trafficking that alter localization of specific growth factor receptors may be one mechanism by which loss of merlin disrupts contact inhibition of growth. This is consistent with previous observations that merlin regulated growth factor availability (19, 39), endocytosis (38) and exocytosis (29). Microtubule based trafficking may play other critical roles in oncogenesis as well. This idea is supported by the fact that activation of kinase-mediated pathways is universal in tumor cells, and recent findings show that many of these same kinases can also affect trafficking. It is therefore of considerable interest to explore this novel relationship between microtubule based trafficking and cancer.

## Materials and Methods

### Inhibitors

The MLK inhibitor CEP11004 (Cephalon) was used at 200 nM final concentration from a 100  $\mu$ M stock in DMSO. The Rac inhibitor, NSC23766, was a gift from Dr. Yi Zheng (Cincinnati Children's Hospital, Cincinnati, OH USA) used at 100  $\mu$ M final concentration from a 100 mM aqueous stock. The p38<sup>SAPK</sup> inhibitor SB203580 (Calbiochem) was used at 100  $\mu$ M final concentration from a 100 mM stock in DMSO.

### Plasmids

A plasmid expressing the mStrawberry-Rab6 fusion protein (30) was a kind gift from Dr. Anna Akhmanova (Erasmus Medical Center, Rotterdam, The Netherlands). ErbB2 was visualized using pYEC, a gift from Dr. Bo van Deus (University of Copenhagen, Copenhagen, Denmark). pYEC expresses a YFP-ErbB2-CFP fusion protein (49) and is referred to in the text as ErbB2-GFP. Rac Q61L (Dr. Yi Zheng, Cincinnati Children's Hospital, Cincinnati, OH, USA), Rac-F28L (Dr. Yi Zheng, Cincinnati Children's Hospital, Cincinnati, OH, USA), pIRESpuro3 (Clontech), pIRES-Merlin (7), VAMP2-GFP was a kind gift from Dr. Martin ter Beest (University of Chicago, Chicago, IL).

### Recombinant proteins

Purified V12HRas-GST, N17Rac-GST and L61Rac-His proteins (Cytoskeleton) were each reconstituted at 20  $\mu$ M in ddH<sub>2</sub>O and aliquots stored at  $-80^{\circ}$ C. Recombinant merlin proteins were purified as previously described (7). Each was perfused into axoplasm in Buffer X/2 with the addition of 5 – 10 mM Hepes, pH7.2 and 5 mM ATP.



## Cell lines

MEF3flox2 and MEF3<sup>-/-</sup> cells were described previously (27). SC4 *Nf2*<sup>-/-</sup> Schwann cells were obtained from Dr. Helen Morrison (Leibniz Institute for Age Research). Cell lines were maintained in DMEM (Life Technologies) supplemented with 10% FBS (Gemini Bioproducts) and 1% Pen/Strep (HyClone) at 37°C, 7.5% CO<sub>2</sub>.

## Primary Cell Culture

Primary Schwann cells and schwannoma tumor cells were isolated from normal human nerves and patient-derived schwannomas isolated as previously described (50, 51). For analysis, cells at passage 3–5 were trypsinized and plated onto poly-L-lysine and laminin coated glass coverslips, and transfected with VAMP-GFP using Fugene-6. After 36 to 48 hours, cells were imaged directly or after 2 hr. incubation in designated inhibitors.

## Growth Curves

*Nf2*<sup>+/+</sup>MEF3flox2 and *Nf2*<sup>-/-</sup> MEF3<sup>-/-</sup> cells each were plated at 50,000 cells per well in replicate 6- well plates. Plates were incubated for six hours then inhibitor or vehicle in triplicate wells. Plates were incubated overnight then one set was trypsinized and cells were counted with a hemocytometer (Day1). Remaining wells were counted every 48 hours until the end of the assay.

## Live cell imaging

SC4 cells were plated into 35 mm dishes with a cover slip bottom (Mattek), phenol red free DMEM, 25 mM HEPES pH 7.4, 10% FBS, 1% Pen/Strep supplemented with 20mM Trolux (Sigma Aldrich) as an antifade reagent, then transfected with a mixture of 0.5 µg pRab6-mStrawberry and 1.5 µg either pIRESpuro 3 (Clontech) empty vector or expression vector (merlin, Rac or ErbB2) using Fugene-6 (GE BioSciences) at a 3:1 Fugene:DNA ratio, as per manufacturer's instructions. Images were acquired on a Zeiss Axiovert 200M microscope equipped with a stage incubator (In Vivo Scientific) maintaining 37°C using an Orca ER II camera (Hamamatsu), controlled by MicroManager v1.3 software (52).

## Image Analysis

VAMP2 mobility was measured by determining the percentage of VAMP2-GFP positive pixels that changed position relative to total fluorescence, between each 3 second interval the time lapse. Statistical analysis by Mann-Whitney non-parametric t-test and graph generation was performed using Prism (GraphPad).

To image Rab6 vesicle motility, a stack of 60 images were acquired at 1 second intervals with 200 msec exposure with the camera bin set to 2 x 2 for a total of 72 seconds. Image analysis was performed with Image J software (NIH). To visualize motion, the intensity of small vesicles was enhanced by subtracting a blurred copy of the image, and then all 60 images of the time lapse were collated into a single image. To measure the velocity of individual vesicles, time-lapse stacks were color coded to display successive frames in red, green and blue, the position of individual vesicles was lapse was logged and the distance traveled was measured. At least 21 cells were imaged per replicate with from 10 to 20 tracks

measured per cell for a total of 200 to 500 velocity measurements per experiment. Data was collated and analyzed with Excel (Microsoft). Statistical analysis by Mann-Whitney non-parametric t-test and graph generation was performed using Prism (GraphPad).

### Axoplasm and Video Microscopy

Axoplasm was extruded from giant axons dissected from squid (*Loligo pealei*) supplied by the Marine Biological Laboratory (Woods Hole, MA) and video microscopy of membrane bound organelles and perfusion with recombinant proteins was performed as previously described (53, 54) (34).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

This work was supported by NIH R01 CA118032 (to NR), and MBL research fellowships (to N.R and G.M.), NIH R01 NS23868 (to STB). We thank Atira Dudley Hardiman for assistance with growth and transfection of human cells, Jamie M. Walker for expert technical assistance and Dr. Yi Zheng for critical reading of the manuscript.

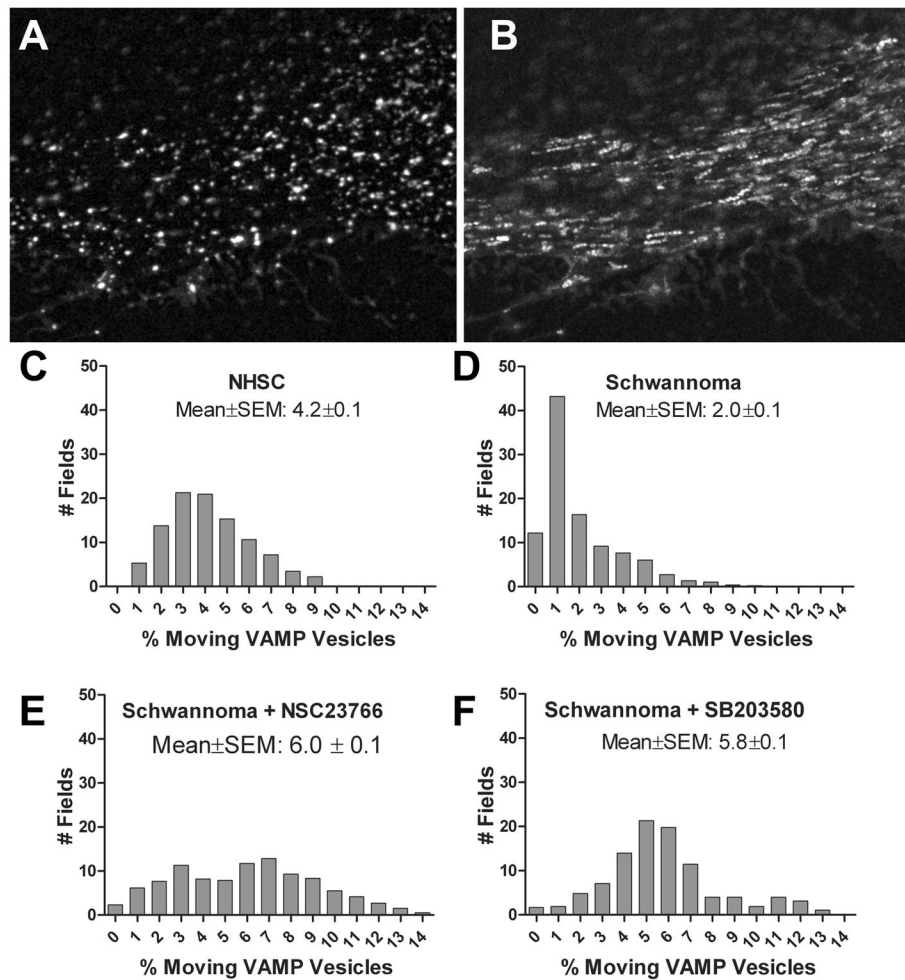
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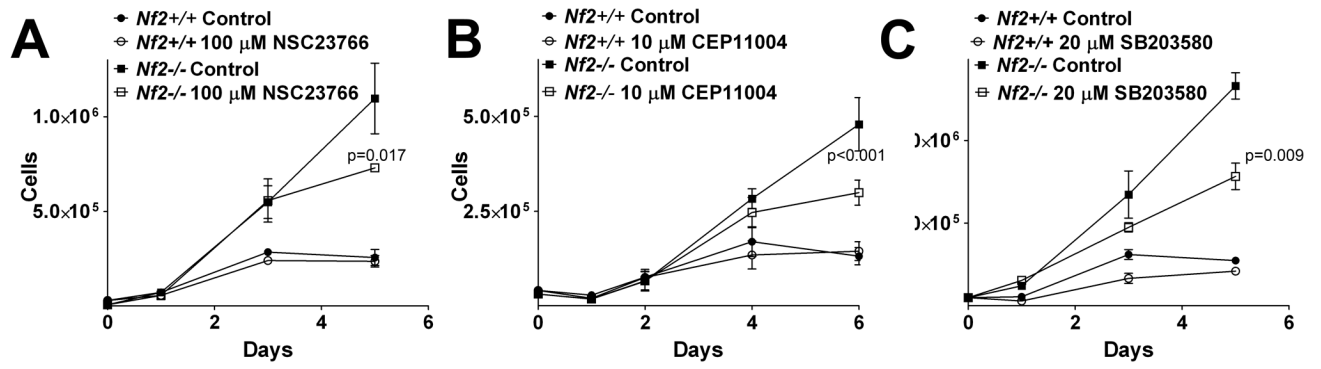
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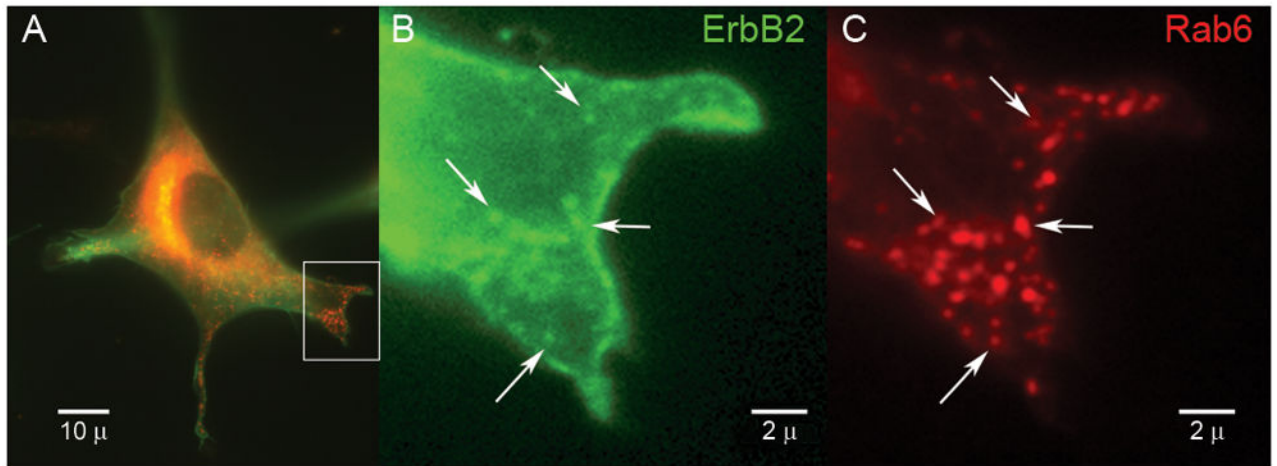
**Figure 1. Inhibition of Rac and p38 increases VAMP2 positive vesicle mobility in merlin-null schwannoma**

Human schwannoma cells transfected with VAMP-2-GFP expressing plasmid display a large number of discrete intracellular vesicles (A). The cumulative vesicle motility is depicted by combining all images of the time lapse from live cells that were imaged at 3 second intervals for a total of 3 minutes (B). Frequency histograms show the mobile fraction of VAMP2-GFP vesicles per 3 second interval. C) NHSC, mean  $\pm$  SEM:  $4.2 \pm 0.1$ . D) schwannoma, mean  $\pm$  SEM:  $2.0 \pm 0.1$ . E) schwannoma + NSC23766, mean  $\pm$  SEM:  $6.0 \pm 0.1$ . F) schwannoma + SB203580, mean  $\pm$  SEM:  $5.8 \pm 0.1$ .



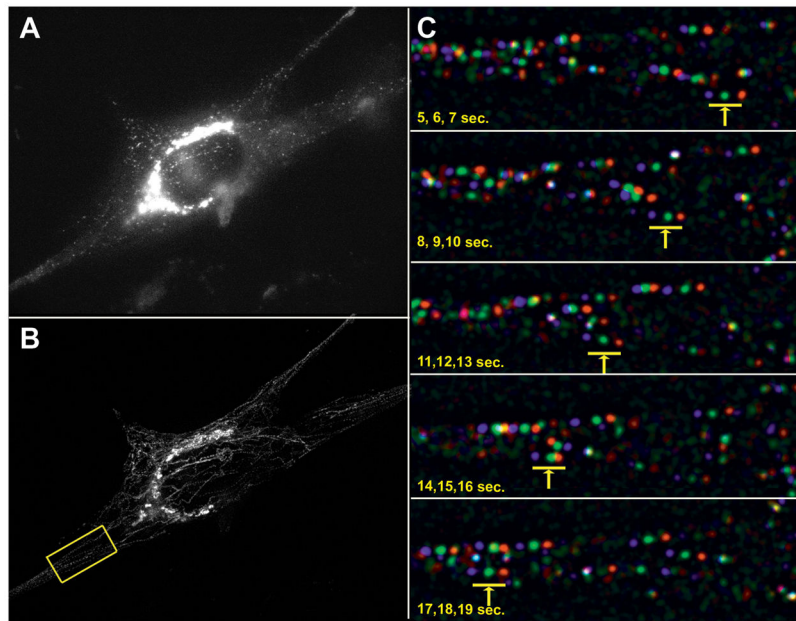


**Figure 2. Rac activity specifically regulates high density growth in NF2 null fibroblasts**  
 Immortalized *Nf2*<sup>+/+</sup> and *Nf2*<sup>-/-</sup> fibroblasts were plated at low density and allowed to grow to saturation density for 5–6 days in the presence or absence of inhibitors to A) Rac, 100  $\mu$ M NSC23766 B) MLK, 10  $\mu$ M CEP11004 and C) p38<sup>SAPK</sup>, 10  $\mu$ M SB203580.



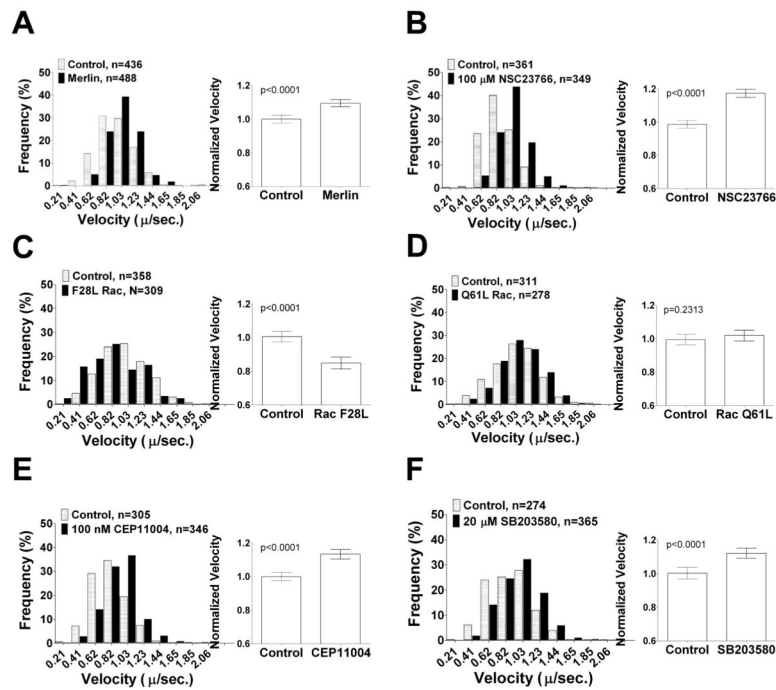
**Figure 3. Rab6 vesicles carry ErbB2 cargo**

A) Live SC4 cells co-transfected with ErbB2-GFP (green) and Rab6-mStrawberry (red) expressing plasmids. The box identifies the region shown at high magnification for ErbB2-GFP (B) and Rab6-mStrawberry (C). The arrows indicate a subset of Rab6 positive intracellular vesicles that contain ErbB2-GFP.



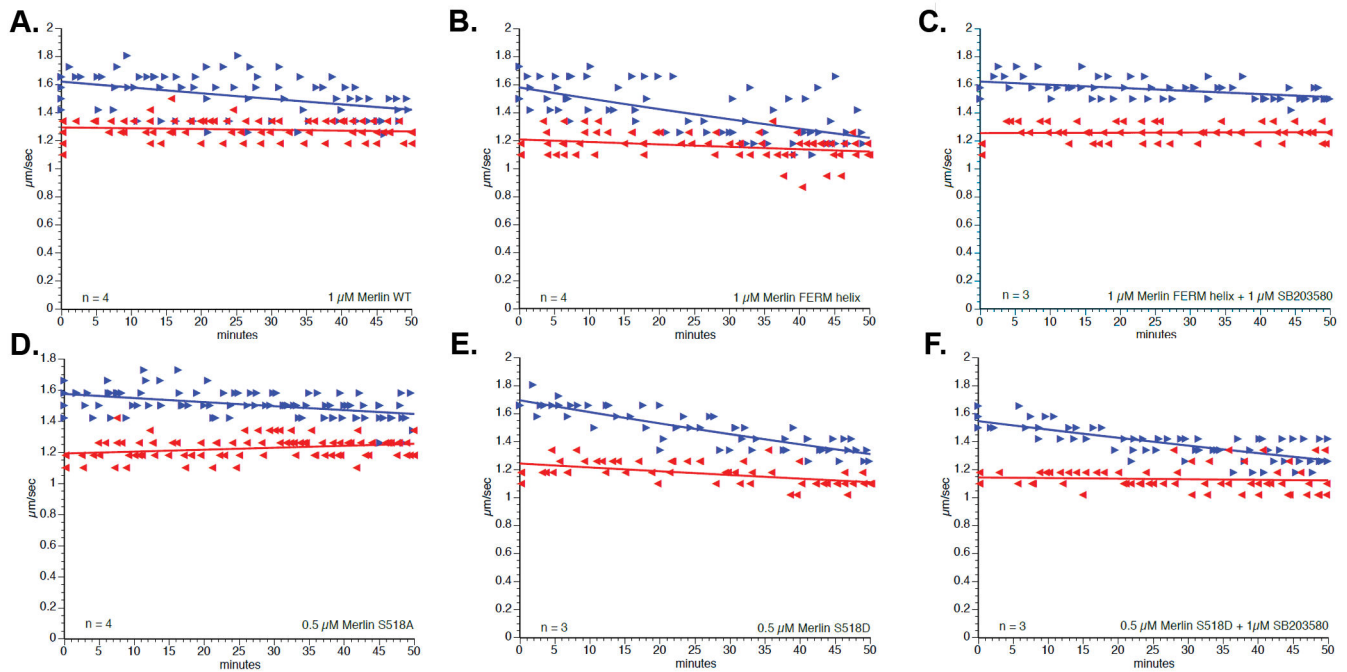
**Figure 4. Rab6 exocytic vesicle velocity measurement in live SC4 cells**

A) A single frame from a time lapse SC4 cells transfected with plasmid expressing Rab6-mStrawberry, imaged at 1 second intervals for 1 minute. B) Total mobile vesicles depicted by subtracting the minimum pixel projection from the maximum pixel projection. C) Five frames of the time lapse from the region of interest in B, processed to facilitate tracking individual vesicles, are shown with successive frames in red, green and blue. The arrow and bar indicates the progress of an individual vesicle over a ten second time frame.

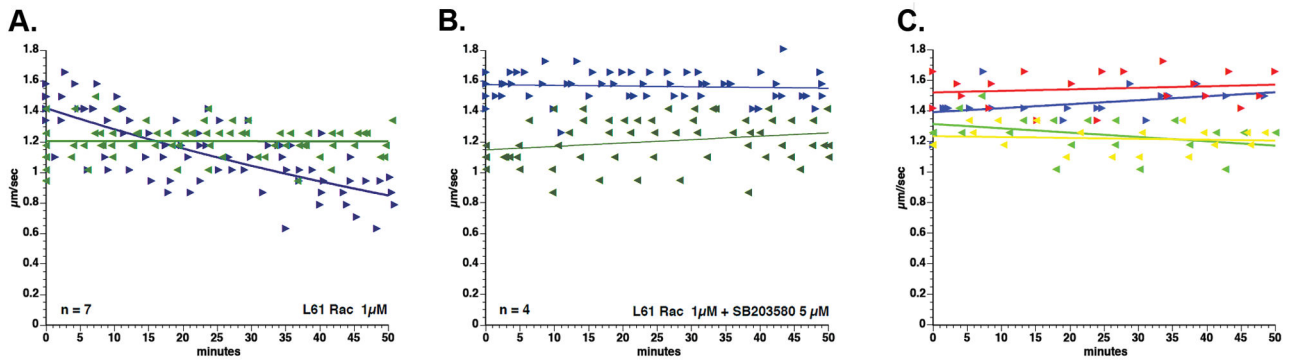


### Figure 5. Rab6 vesicle velocity is regulated by a Merlin-Rac pathway

The velocities of anterograde Rab6-mStrawberry exocytic vesicles in transfected SC4 cells displayed as both frequency histograms (left) and a bar graph of mean velocities with the 95% confidence interval (right). A) SC4 cells co-transfected with Rab6-mStrawberry and either a merlin expressing plasmid or empty vector. B) SC4 cells treated for 2–3 hours with 100  $\mu\text{M}$  of the Rac inhibitor NSC23766. C) SC4 cells co-transfected with Rab6-mStrawberry and either an active, fast cycling F28L-Rac mutant expressing plasmid or empty vector. D) SC4 cells co-transfected with Rab6-mStrawberry and either a constitutively GTP-bound Q61L-Rac mutant expressing plasmid or empty vector. E) SC4 cells treated for 2–3 hours with 100 nM of the MLK inhibitor CEP11004. F) SC4 cells treated for 2–3 hours with 20  $\mu\text{M}$  of the p38<sup>SAPK</sup> inhibitor SB203580.



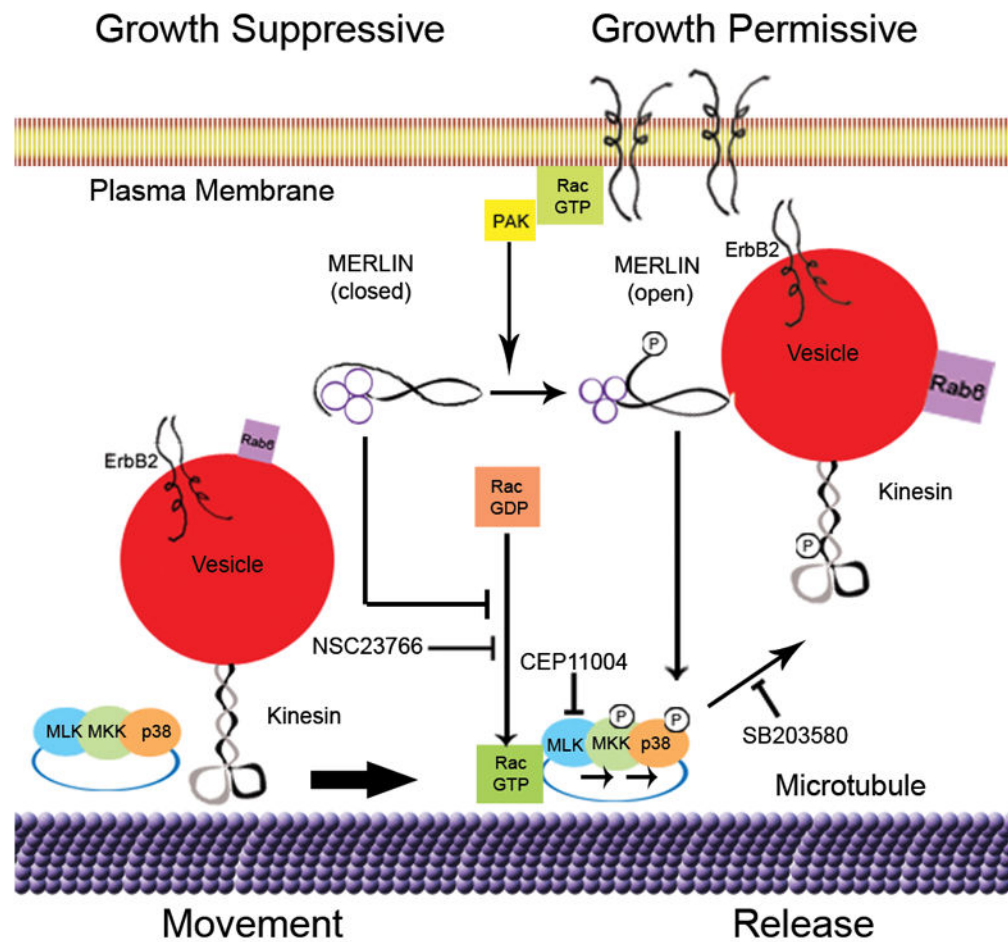
**Figure 6. Merlin Inhibits Anterograde Axonal Transport in Isolated Squid Axoplasm**  
 Membrane bound organelle velocities were measured after perfusion of axoplasm with buffer supplemented with 0.5 to  $1 \mu\text{M}$  purified recombinant merlin, or recombinant merlin plus the p38<sup>SAPK</sup> inhibitor, SB203580. Anterograde (blue) and retrograde (red) velocity measurements taken between 25 and 45 minutes were plotted as points. Curves were fitted for anterograde movement and retrograde movement using an exponential curve fit. A) Full length, wild-type merlin. B) C-terminal tail domain deletion mutant, FERM-Helix. C) FERM-Helix plus SB203580. D) The phosphorylation site mutant S518A. E) The phosphomimetic mutant S518D. F) The S518D mutant plus SB203580.



**Figure 7. Active Rac Inhibits Anterograde Axonal Transport in Isolated Squid Axoplasm**

A)  $1\mu\text{M}$  purified recombinant active Q61L Rac. B) Q61L Rac, treated with the p38<sup>SAPK</sup> inhibitor, SB203580. C) Dominant negative N17 Rac failed to inhibit anterograde (blue) or retrograde (green). Recombinant active V12 Ha-Ras also failed to inhibit anterograde (red) or retrograde (yellow) transport.





**Figure 8. Model for Merlin-Rac-p38<sup>SAPK</sup> Regulation of Kinesin**

A schematic diagram depicting the molecular pathways influenced by merlin and activated Rac that regulate anterograde exocytic vesicle movement by phosphorylation of kinesin heavy chain mediated by the MLK-p38<sup>SAPK</sup> kinase module.