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Sbf/MTMR13 coordinates PI(3)P and Rab21 regulation in endocytic control of cellular remodeling

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ABSTRACT Cells rely on the coordinated regulation of lipid phosphoinositides and Rab GTPases to define membrane compartment fates along distinct trafficking routes. The family of disease-related myotubularin (MTM) phosphoinositide phosphatases includes catalytically inactive members, or pseudophosphatases, with poorly understood functions. We found that Drosophila MTM pseudophosphatase Sbf coordinates both phosphatidylinositol 3-phosphate (PI(3)P) turnover and Rab21 GTPase activation in an endosomal pathway that controls macrophage remodeling. Sbf dynamically interacts with class II phosphatidylinositol 3-kinase and stably recruits Mtm to promote turnover of a PI(3)P subpool essential for endosomal trafficking. Sbf also functions as a guanine nucleotide exchange factor that promotes Rab21 GTPase activation associated with PI(3)P endosomes. Of importance, Sbf, Mtm, and Rab21 function together, along with Rab11-mediated endosomal trafficking, to control macrophage protrusion formation. This identifies Sbf as a critical coordinator of PI(3)P and Rab21 regulation, which specifies an endosomal pathway and cortical control.

INTRODUCTION Remodeling of the cell surface through membrane trafficking is important for changes in cell shape and function. The correct combinations of phosphoinositides, phosphorylated forms of phosphatidylinositol, and members of the Rab GTPase family are crucial to control dynamic membrane identities between intersecting trafficking pathways in the cell (Di Paolo and De Camilli, 2006; Grosshans et al., 2006). Early endosomal membranes under the direction of phosphatidylinositol 3-phosphate (PI(3)P) and Rab5 GTPase are sites of protein sorting that can lead to cargo recycling back to the plasma membrane or to lysosomal degradation. Thus endocytosis provides a means for rapid changes in cell shape and surface protein composition, activity, and organization (Grant and Donaldson, 2009).

PI(3)P mediates membrane recruitment of effectors with specific roles in endocytosis or autophagy (Lindmo and Stenmark, 2006), making the coordinated regulation of PI(3)P critical. PI(3)P regulation is best understood through its synthesis by two classes of phosphatidylinositol 3-kinases (PI3-kinases). The conserved class III PI3-kinase Vps34 is recruited to the Vps15/p150 adaptor within distinct protein complexes (Backer, 2008). The metazoan-restricted class II PI3-kinase (PI3KC2; MacDougall et al., 1995) is implicated in cortical functions (MacDougall et al., 1995; Maffucci et al., 2005; Falasca et al., 2007; Srivastava et al., 2009; Velichkova et al., 2010) and can interact with clathrin and signaling adaptors at the plasma membrane (Gaidarov et al., 2001; Wheeler and Domin, 2001), although mechanisms of PI3KC2 functions are unclear. Conversely, PI(3)P down-regulation occurs with endosomal maturation, both by conversion to phosphatidylinositol 3,5 bisphosphate (PI(3,5)P$_2$; Gary et al., 1998) and by depletion upon endosomal membrane efflux (efflux), potentially through kinase inactivation, sorting, and/or dephosphorylation (Egami and Araki, 2008; Velichkova et al., 2010). However, the cellular demands and mechanisms that govern down-regulation of PI(3)P subpools are less well understood.
The large family of disease-relevant myotubularin (MTM) phosphoinositide phosphatases are selective for Ptd(3)P and Ptd(3,5)P₂ (Laporte et al., 1996; Bolino et al., 2000; Taylor et al., 2000; Berger et al., 2002; Kim et al., 2002). MTMs have been identified with endosomal roles that impact the trafficking of specific surface receptors and adhesion proteins, or cortical remodeling (Kim et al., 2002; Tsuchiya et al., 2004; Cao et al., 2008; Lee et al., 2010; Silhankova et al., 2010; Velichkova et al., 2010; Ribeiro et al., 2011). MTM-specific functions suggest a need for differential regulation toward distinct phosphoinositide pools. Half of the MTM family members encode for catalytically inactive phosphatases (“pseudophosphatases”; Laporte et al., 1998; Robinson and Dixon, 2006). MTM pseudophosphatases have been found to share mutant phenotypes and form direct protein interactions with MTM catalytic phosphatases (Firestein et al., 2002; Azzedine et al., 2003; Kim et al., 2003; Senderek et al., 2003; Dang et al., 2004; Robinson and Dixon, 2005; Robinson et al., 2008; Berger et al., 2006; Lorenzo et al., 2006; Tersar et al., 2007; Silhankova et al., 2010). Although speculated to serve as phosphatase adaptors, the contributions of MTM pseudophosphatases to phosphoinositide homeostasis in cell functions have not been tested.

Like phosphoinositides, Rab GTPases are under dynamic regulation to mediate membrane trafficking. Opposing functions of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) direct localized Rab activity (Behnia and Munro, 2005). The importance of Rab GTPase and phosphoinositide combinations is illustrated at endosomes, where codependent Rab5 and Vps34 activities coordinate the recruitment of effectors via coincident detection of Rab5-GTP and Ptd(3)P (Behnia and Munro, 2005; Di Paolo and De Camilli, 2006). Although phosphoinositides and Rab are often functionally coupled, surprisingly few mechanisms for their coordinated regulation are established. Of interest, differentially expressed in neoplastic versus normal cells (DENN)-domain-containing proteins, including MTM55/MTMR1, were recently shown to exhibit Rab GEF activity (Yoshimura et al., 2010), raising the possibility of pseudophosphatase roles in simultaneous phosphoinositide and Rab regulation.

Immune cell surveillance requires rapid changes in cell shape, including extension–retraction of cell protrusions. Previously, we showed that Drosophila Mtm phosphatase, the single homologue of human MTM1/MTMR2/MTMR1, regulates a PI3K-dependent Ptd(3)P pool to mediate macrophage protrusion formation and recruitment to wounds (Velichkova et al., 2010). Here we show that the mechanism of cellular remodeling relies on Sbf, an MTMR13/MTMR5 pseudophosphatase, and its dual roles in phosphoinositide and Rab GTPase regulation.

RESULTS
Sbf and mtm are codependent in macrophages for cell protrusions and animal viability
We recently described specific immune cell roles for mtm phosphatase in hemocytes, or insect macrophages, for cell remodeling, recruitment to wounds, and animal viability (Velichkova et al., 2010), highlighting the importance of a Ptd(3)P pool balance for normal macrophage function. To explore unknown roles for MTM pseudophosphatases in Ptd(3)P regulation and functions, we investigated a macrophage requirement for any of the three coexpressed Drosophila MTM pseudophosphatases (Sbf, CG14411, CG5026; Supplemental Figure S1A). We found that only Sbf, encoding the single homologue of human MTM55/MTMR13 (Figure 1, A and B), was required for fly adult viability when depleted in macrophages (Supplemental Figure S1, B–I; with either of two RNA interference [RNAi] hairpins). Fly viability was rescued with coexpression of a wild-type (WT) mCherry:Sbf cDNA (Supplemental Figure S1C), indicating the specificity of Sbf knockdown. The lack of a complete rescue can be explained by a high efficiency of RNAi, as suggested by endogenous Sbf depletion and undetectable mCherry:Sbf fluorescence despite restored essential Sbf function (Supplemental Figure S1, F–I). These results suggest that Sbf may act specifically in the same processes as mtm that are important for macrophage function.

Macrophages exhibit a dynamic cell cortex that is important for immune surveillance functions. When isolated from the animal, larval macrophages spread flat and extend and retract prominent radial cell protrusions rich in filamentous actin (F-actin; Figure 1C). Given an mtm role that promotes cell protrusion formation to mediate immune cell recruitment, we examined whether Sbf shares a similar cortical function. Sbf-depleted macrophages spread but failed to extend F-actin–rich protrusions (Figure 1, D–F, Supplemental Figure S1, J–M, and Supplemental Video S1), similar in severity to the mtm-null allele (Velichkova et al., 2010). In contrast, overexpression of Sbf exhibited an opposite phenotype of an increased number of protrusions per cell when expressed alone or in combination with mtm (Figure 1, G–I, and Supplemental Figure S1, N–Q). Thus both Sbf and mtm are necessary and sufficient to establish the appropriate balance of F-actin protrusions in macrophages.

The formation of cellular protrusions provides a clear and relevant cellular read-out of Sbf and mtm function. To assess whether Sbf and mtm act in the same pathway, we performed epistasis analysis by testing whether depletion of one could block the formation of excess protrusions driven by expression of the other. Sbf depletion completely blocked mtm-induced protrusions, and, moreover, most cells lacked any protrusions (Figure 1, H, H’, H”, and J), indicating that ectopic mtm could not overcome the requirement for Sbf. The corequirement for Sbf is unlikely to be simply due to effects on Mtm protein level, since mCherry:Mtm fluorescence was still detectable when expressed in affected Sbf RNAi cells yet was undetectable despite an ability to rescue mtm RNAi cells (Figure 1, K and L). Likewise, mtm was required for Sbf-induced excess protrusions (Figure 1, I’, I”, and M), although the macrophages exhibited a new phenotype of unextended F-actin fibers (Figure 1I), suggesting that Sbf might possess additional functions independent of mtm (see later discussion). Taken together, these results show that Sbf and mtm functions are codependent in cell protrusion formation.

Sbf pseudophosphatase recruits Mtm into a stable interaction
The foregoing results identified a corequirement for Sbf and mtm in the control of cell shape. To test whether Sbf and mtm share functions mediated through their protein interactions, we performed coimmunoprecipitation (coIP) and phosphatase activity assays. Sbf and Mtm could be isolated in a protein complex (Figure 2A and Supplemental Figure S1A), suggesting that Sbf shares a similar cortical function. To test whether Sbf and mtm are necessary and sufficient to establish the appropriate balance of F-actin protrusions in macrophages.
Sbf and mtm are corequired for macrophage protrusions. (A) Drosophila Mtm phosphatase and Sbf pseudophosphatase. (B) Mtm and Sbf human homologues and associated diseases. (C–E, H–H′′) F-Actin in primary macrophages. (C) Normal number and extent of radial protrusions in control cell. Protrusions are missing upon Pxn-GAL4 macrophage–targeted (D) mtm RNAi or (E) Sbf RNAi. (F) Percentage of macrophages without cell protrusions upon RNAi. (G) Percentage of cells with excess protrusions upon wild-type cDNA expression. (H) mtm or (I) Sbf induces excess macrophage protrusions, suppressed by (H′, I′) mtm or (H″, I″) Sbf coRNAi. (J) Percentage of control and mtm-overexpressing cells with excess (black) and loss (white) of protrusions. (K) mCherry:Mtm (red) in control, (K′) mCherry:mtm RNAi cells. (L) mCherry:Mtm fluorescence intensity per cell. (M) Percentage of control and Sbf-overexpressing cells with excess protrusions, +SEM. Bars, 5 μm.

(Supplemental Video S2). In contrast to the cortical Sbf localization, green fluorescent protein (GFP):Mtm when expressed alone was mainly cytoplasmic and weakly detectable on intracellular rings (Figure 2G). However, coexpression of mCherry:Sbf with GFP:Mtm (Figure 2, H–H″″) dramatically shifted Mtm from a mostly diffuse to a striking cortical and vesicular distribution that colocalized with Sbf (Figure 2H″; 0.56 ± 0.02 Pearson’s correlation), suggesting that Sbf recruits Mtm into a stable protein complex at membranes.

To explore the ability of Sbf to control Mtm localization, we performed fluorescence recovery after photobleaching (FRAP). Recovery curves for bleached regions of GFP:Mtm were rapidly restored...
to near prebleach levels (85.2 ± 2.0% mean intensity at 46 s), indicating that Mtm is highly mobile and diffuses rapidly in cells (Figure 2, I and J). In contrast, mCherry:Sbf exhibited delayed and only partial recovery (43.5 ± 3.2% mean intensity at 46 s), revealing that more than half of the Sbf was in an immobile or more stabilized fraction (Figure 2I). In agreement with Sbf recruitment of Mtm protein, there was a marked decrease in Mtm FRAP recovery when coexpressed with Sbf (Figure 2, I and K). There was also a slight decrease in Sbf recovery curves when coexpressed with Mtm (Figure 2, I and K'). These FRAP results clearly demonstrate that Sbf, an MTM pseudo-phosphatase, modifies and stabilizes the localization of Mtm phosphatase within a shared protein complex. Given that Mtm catalytic activity is required for macrophage remodeling, we propose that Sbf acts to membrane recruit an Mtm phosphatase activity required for protrusion extension.

### FIGURE 2: Sbf recruits Mtm into a stable complex at membranes.

(A) Mtm interacts with Sbf in Kc167 cells. IP of 3xFLAG:Mtm and GFP immunoblot of GFP:Sbf. (B, C) Phosphatase activity toward PI(3)P and PI(3,5)P2. (B) FLAG (white) and 3xFLAG:Sbf (gray) IP-released phosphate concentration normalized to protein level. (C) 3xFLAG:Mtm IP, with or without Sbf coexpression. Fold activity normalized to condition with Mtm expressed alone, +SEM. Sypro Red stain shows a similar amount of IP. (D–K) Primary macrophages. (D) Endogenous Sbf localization at the plasma membrane of the cell body (arrows) and internal puncta. (E) Sbf immunostaining in Sbf RNAi-depleted cells; note the absence of membrane staining. (F) Live cell imaging of mCherry:Sbf, with a similar pattern as endogenous protein. (G) GFP:Mtm localizes mainly to cytoplasm and few internal foci. (H–H') When coexpressed, (H) mCherry:Sbf recruits (H') GFP:Mtm to the cell cortex and internal rings, and both exhibit stabilized levels. (H') Merge and (H'') zoom of box. (I) Time course of recovery shown as normalized fluorescence intensity for GFP:Mtm (red) or mCherry:Sbf (yellow) expressed alone or in combination (Mtm, green; Sbf, blue), ±SEM. (J–K') Representative bleached regions at 0 s for data in I. Cg-GAL4. Bars, 5 µm, except H'', 1.25 µm.

An Sbf role in cellular remodeling depends on class II PI3K68D kinase activity

The functional codependence between Sbf and mtm suggests that Sbf might control phosphoinositide regulation and function. To test this, we first explored a relationship between Sbf and the class II PI3-kinase PI3K68D in the formation of cell protrusions. Codepletion of Sbf and PI3K68D restored normal protrusion number (Figure 3, A–C). This suggests that PI3K68D is responsible for synthesis of a phosphoinositide pool that is either the target of the Sbf–Mtm phosphatase complex or has an antagonistic function. To further test this significance, we looked at the in vivo distribution of a population of adherent macrophages normally dispersed evenly along the larval body wall (Supplemental Figure S2, A and B), correlating with an mtm-dependent ability for macrophage recruitment to epidermal wounds (Velichkova et al., 2010). In accordance with the loss of cell...
**FIGURE 3:** PI3K68D-dependent Sbf functions in PI(3)P turnover, endosomal membrane exit, and macrophage protrusions. (A, B, D, E, G) F-Actin in primary macrophages. (A) Lack of protrusions with macrophage-targeted Sbf RNAi. (B) Rescue of normal radial protrusions with Sbf, PI3K68D codepletion. (C) Percentage of cells without protrusions in Sbf RNAi or PI3K68D codepleted cells; +SEM. (D) Sbf-induced excess protrusions, suppressed by (E) PI3K68D codepletion. (F) Percentage of macrophages with excess protrusions upon Sbf or mtm cDNA expression, suppressed by PI3K68D RNAi; +SEM. (G) Loss of cell protrusions with severe PI3K68D depletion, using two copies of RNAi hairpin and strong Cg-GAL4 driver. (H) Percentage of macrophages without cell protrusions upon PI3K68D knockdown; +SEM. (I–L, O–P) mCherry:2xFYVE detection of PI(3)P in single macrophages. (I) Normal PI(3)P distribution on several rings. (J) Accumulation of PI(3)P-containing rings with Sbf or (K) mtm RNAi, (L) enhanced by Sbf and mtm coRNAi. (M) Cartoon of PI(3)P-containing rings observed (rings and vesicles) and quantified in control and knockdown conditions. (N) Number of PI(3)P-containing rings with visible lumen per cell; +SEM; Pxn-GAL4. (O) Partial depletion of PI(3)P with PI3K68D RNAi. (P) Suppressed accumulation of PI(3)P with Sbf and PI3K68D coRNAi. (Q–S) Still images of mCherry:2xFYVE dynamics from Supplemental Video S3. Bars, 5 μm, except 2.5 μm in crops.
protrusions, Sbf and mtm knockdown resulted in a clumped and uneven distribution of macrophages in intact larvae (Supplementary Figure S2, C–E). Codepletion of PI3K68D and Sbf restored the normal macrophage distribution and, of importance, suppressed fly lethality (Supplementary Figure S2, F–I). Macrophage number and phagocytic ability were unaffected in all conditions (Supplementary Figure S2, I and J). Taken together, these results demonstrate an interaction between Sbf and PI3K68D in macrophage remodeling that is significant to macrophage distribution in vivo and likely impacts immune surveillance or developmental tissue remodeling roles essential for animal viability.

If Sbf and mtm functions reflect a requirement for a PI(3)P phosphohydrolysis–dephosphorylation sequence at successive sites, then PI3K68D function for synthesis of this pool also would be required for protrusion formation. Alternatively, if depletion of this PI(3)P pool is simply permissive, then loss of PI3K68D function would instead lead to formation of excess protrusions. We found that codepletion of PI3K68D was able to revert the formation of Sbf- or mtm-induced excess protrusions (Figure 3, D–F, and Supplementary Figure S2, K and L). Consistent with this suppression, a requirement for PI3K68D in cell protrusion formation was revealed with severe PI3K68D knockdown (Figure 3, G and H). Loss of PI3K68D function did not alter Sbf or Mtm protein levels and, moreover, stabilized their colocalization (Supplemental Figure S2, M–O), suggesting that the cell protrusion defect was unlikely simply due to loss of Sbf–Mtm function. These results support the former model, suggesting a mechanism in which a PI3K68D-dependent PI(3)P pool is subsequently a target of Sbf–Mtm. Taken together, these results demonstrate that both the normal and Sbf-induced protrusions depend on a phosphoinositide phosphohydrolysis–dephosphorylation sequence regulated by PI3K68D, Mtm and Sbf (see model, Supplemental Figure S3A).

**Sbf regulates a PI3K68D-dependent PI(3)P pool and endosomal membrane efflux**

To test Sbf function in PI(3)P regulation, we assessed effects of Sbf knockdown on 2xFYVE localization as an indicator of PI(3)P distribution, previously shown to correlate with mtm-dependent PI(3)P levels (Velichkova et al., 2010). In wild-type macrophages, PI(3)P is detected both enriched at endosomal membrane rings with obvious lumen and more weakly at small, motile vesicles (Velichkova et al., 2010). Consistent with a normal Sbf role to promote Mtm-mediated PI(3)P turnover, both Sbf and mtm RNAi led to a similar increase in the number of PI(3)P-enriched rings, as well as the amount of combined size and number of PI(3)P-containing membranes (Figure 3, I–N, and Supplemental Figure S3B). In contrast, codepletion of Sbf and PI3K68D restored the PI(3)P membranes to normal (Figure 3, M–P, and Supplemental Figure S3B), suggesting that PI3K68D kinase activity is required for the PI(3)P accumulation. Together these results show that Sbf function affects a PI3K68D/mtm-coregulated PI(3)P pool, with consequences both for amount and characteristics of PI(3)P-containing membranes.

PI(3)P is known to recruit effectors involved in endosomal sorting, maturation, and membrane fusion, whereas PI(3)P turnover promotes membrane exit. Using time-lapse microscopy to monitor the dynamics of PI(3)P-containing compartments, we found that tubulation events indicative of exiting membrane normally seen in control cells were infrequent in Sbf-depleted cells (Figure 3, Q and R). The PI(3)P membrane dynamics was rescued by PI3K68D and Sbf codepletion (Figure S3 and Supplemental Video S3). As the first demonstration of a requirement for an Mtm pseudophosphatase in phosphoinositide regulation, this shows that Sbf is critical for Mtm dephosphorylation of a PI(3)P pool that promotes endosomal membrane exit, or efflux.

**Sbf and PI3K68D interact on cell membranes**

The foregoing results indicate that Sbf functionally interacts with both mtm and PI3K68D. Mtm family members were shown to interact in a Vps34 kinase–MTM phosphatase complex through the adaptor Vps15 (Cao et al., 2007, 2008). We asked whether Sbf could harbor such an adaptor role to complex both PI3K68D and Mtm to promote coordinated functions. In coIP experiments from cell cultures with low levels of protein expression (see Materials and Methods), we found that Sbf interacts with both Mtm and PI3K68D (Figure 4A). To corroborate the novel Sbf/PI3K68D interaction, we performed coIP on endogenous proteins using newly generated specific antibodies (Figures S1E and S4A). Again, an Sbf/PI3K68D interaction was detected with coIP of both endogenous proteins (Figure 4, B and C), confirming that Sbf and PI3K68D are part of a common protein complex.

PI3K68D was brought down by Sbf whether Mtm was coexpressed or not (Figure 4A), and in the converse coIP, PI3K68D interacted with Mtm only when Sbf was coexpressed (Figure 4D, compare lanes 4 and 7). This suggests that the PI3K68D–Sbf interaction is not through Mtm but that a tripartite complex can exist. Mtm IP could bring down PI3K68D in the absence of Sbf coexpression (Supplemental Figure S4B), suggesting that Mtm can interact with the kinase, perhaps via endogenous Sbf. We were unable to map an interaction site between Sbf and PI3K68D using truncation constructs, pointing potentially to multiple sites of interaction or to indirect interactions in a multiprotein complex. Given the complex interactions, we asked whether interactions between Sbf and PI3K68D alter Mtm phosphatase activity. Mtm activity for PI(3)P showed only a minor decrease when coexpressed with PI3K68D (Supplemental Figure S4C), suggesting that PI3K68D expression does not interfere with Mtm phosphatase activity per se. Together these data suggest that Sbf can bridge PI3K68D and Mtm in an active tripartite complex.

To determine what might distinguish the different enzyme interactions, we fractionated cells to find where the Sbf/Mtm and Sbf/PI3K68D interactions occur. We identified that Sbf/Mtm interact both in membrane and cytoplasmic fractions. In contrast, the Sbf/PI3K68D binding was only observed in the membrane fraction (Figure 4E), suggesting a transient or weak Sbf and PI3K68D protein interaction at, or corecruitment to, a common membrane. In accordance with this, we observed by video microscopy under conditions of normal macrophage protrusions and cell function a transient colocalization between mCherry:Sbf and PI3K68D:GFP motile particles (Supplemental Figure S4D and Supplemental Video S4). Of interest, levels and localization of expressed PI3K68D were diminished in Sbf-depleted cells (Figure 4, F and G), consistent with an Sbf scaffolding role in PI3K68D regulation.

**Conserved MTMR13 and PI3KC2α interaction in mammals**

MTMR13 was shown in mammals to have essential functions in Schwann cells for proper neuronal conductance underlying mutant association with Charcot–Marie–Tooth disease (Azzedine et al., 2003; Senderek et al., 2003; Tersar et al., 2007; Robinson et al., 2008). To assess the broader relevance of Sbf and PI3K68D interactions discovered in flies, we asked whether MTMR13 also associates with class II PI3-kinase in mouse. Highlighting the importance of such an interaction, we found MTMR13 and PI3KC2α coIP from mouse brain (Figure 4H). Detection of PI3KC2α interaction with MTMR13, along with the previously described MTMR13–MTMR2 interaction (Robinson and Dixon, 2005), suggests conservation of a
Pi3K68D/Sbf/Mtm pathway in mammals and potential implication of Pi3KC2α in Charcot–Marie–Tooth disease.

Sbf interacts with Rab21 and Rab11 GTPases associated with endosomes

The foregoing results point to a role for an Sbf–Mtm complex in the regulation of PI(3)P and membrane dynamics, which in turn could affect cargo trafficking. We reasoned that a phosphatase activity could promote membrane exit from PI(3)P-containing endosomes, for example, in trafficking to recycling endosomes. PI(3)P is enriched on early endosomal membranes with Rab5 GTPase. The Rab5 subfamily includes Rab21 (66% similarity; Zhang et al., 2007a), which in mammals has been implicated in endocytic uptake and recycling functions (Simpson et al., 2004; Pellinen and Ivaska, 2006; Egami and Araki, 2008; Mai et al., 2011). To address sites of Sbf function, we investigated Sbf association with Rab GTPases that specify PI3K68D/Sbf/Mtm pathway in mammals and potential implication of Pi3KC2α in Charcot–Marie–Tooth disease.

Sbf interacts with Rab21 and Rab11 GTPases associated with endosomes

The foregoing results point to a role for an Sbf–Mtm complex in the regulation of PI(3)P and membrane dynamics, which in turn could affect cargo trafficking. We reasoned that a phosphatase activity could promote membrane exit from PI(3)P-containing endosomes, for example, in trafficking to recycling endosomes. PI(3)P is enriched on early endosomal membranes with Rab5 GTPase. The Rab5 subfamily includes Rab21 (66% similarity; Zhang et al., 2007a), which in mammals has been implicated in endocytic uptake and recycling functions (Simpson et al., 2004; Pellinen and Ivaska, 2006; Egami and Araki, 2008; Mai et al., 2011). To address sites of Sbf function, we investigated Sbf association with Rab GTPases that specify
endosomal membrane identities. In cultured cells, Sbf was able to communoprecipitate Rab21 but not Rab5 (Figure 5A). A weaker physical interaction was also seen between Sbf and Rab11, an established component and regulator of recycling endosomes. In addition, Sbf colocalized to a similar extent with Rab21 and Rab5, which were found to be highly colocalized in mammalian cells (Simpson et al., 2004), and to a greater extent with Rab11 (Figure 5, B and C), but not with other organelle markers (Supplemental Figure S4E). Taken together, these results suggest an Sbf involvement in endosomal trafficking.

FIGURE 5: Sbf acts as a GEF for Rab21 activation and associates with Rab11-recycling endosomes. (A) Sbf interacts with Rab21 and weakly with Rab11. FLAG IP 3xFLAG:Sbf and immunoblot of coexpressed GFP-tagged Rab5, Rab11, and Rab21. (B–B′′′) Endogenous Sbf colocalization with GFP:Rab21 and (C–C′′′) mCherry:Sbf colocalization with GFP:Rab11 in macrophages. (D) Sbf DENN domain mediates interaction with Rab21. Rab:FLAG pull-downs of GFP:SbfDENN from Kc cell lysates, analyzed by anti-GFP immunoblot. Purified Rab proteins shown by Coomassie stain (bottom). (E, F) Sbf preferentially interacts with inactive Rab21. (E) FLAG IP 3xFLAG:Sbf and immunoblot of coexpressed GFP-tagged Rab21-WT, Rab21-CA, and Rab21-DN. (F) 35S-labeled, in vitro–translated Sbf interacts with bacterially expressed Rab21 preloaded with GDP vs. with non-hydrolyzable GTPγS (top). Purified Rab21 protein shown by Coomassie stain (bottom). Plot of trends seen in two experiments. (G, H) Sbf has GEF activity for Rab21. (G) Rab5 (red) or Rab21 (blue) alone or with SbfDENN (Rab5, green; Rab21, black); ±SEM. (H) SbfDENN can perform Rab21–GTP exchange. Counts per million (cpm) of 35S-labeled Rab21-GTPγS after 1 min of incubation of GDP-Rab21 alone (Control) or together with Sbf-DENN protein, normalized to cpm at time 0; ±SEM. Bar, 5 μm, except 2.5 μm in crops.
Sbf is a Rab21 GEF

The Sbf and MTMR5/MTMR13 subfamily of pseudophosphatase proteins contain an N-terminal DENN domain (Figure 1A). DENN domains have been shown to exhibit Rab GEF activity (Alaire et al., 2010; Yoshimura et al., 2010). If Sbf is a GEF for an endosomal Rab, we predicted that an Sbf/Rab interaction would be preferential and direct for the GDP-Rab form and support GDP release with GTP exchange. First, we tested the ability of the SbfDENN domain to interact with any of the endosomal RabS (Rab5, Rab7, Rab11, and Rab21). Of these four RabS tested, only bacterially purified Rab21:FLAG was able to pull down SbfDENN from cell lysates (Figure 5D), indicating a specific Rab21–Sbf interaction mediated by the DENN domain. In agreement with the possibility of Sbf as a Rab21 GEF, we found a strong interaction by coIP between Sbf and a constitutively inactive form, Rab21-DN (dominant negative; Figure 5E). The Sbf interaction with Rab21-DN was much stronger than that seen with Rab21-WT, whereas we did not detect any interaction with a constitutively GTP-bound form, Rab21-CA (constitutive active). Conversely, we observed colP of Sbf with both Rab11-WT and Rab11-CA and only weakly with Rab11-DN (Supplemental Figure S4F), further confirming an Sbf/Rab11 interaction independent of the Sbf DENN domain (Figure 5, A and D).

To address whether direct binding can occur between Sbf and Rab21, we assayed association between full-length, in vitro–translated Sbf with different nucleotide-loaded forms of recombiant Rab21. Radiolabeled Sbf showed a preferential interaction again—indicative here of direct binding—with purified GDP-loaded Rab21 but did not interact with nonhydrolyzable GTPγS-loaded Rab21 (Figure 5F). Finally, to address whether Sbf has Rab21 GEF activity, we monitored the ability for SbfDENN to promote Rab21 GDP release and GTP exchange in vitro. SbfDENN was sufficient to catalyze GDP-3H release from preloaded Rab21 but not from Rab5 (Figure 5G) and to transfer GTPγS to GDP-loaded Rab21 (Figure 5H). In contrast, GDP-Rab21 protein incubated alone was deficient in GDP release and GTP transfer activity (Figure 5, G and H). The preferential, direct, and stimulatory interaction between Sbf and inactive Rab21 (and exclusion of an interaction with Rab21-GTP) demonstrates that Sbf pseudophosphatase is a Rab21 GEF in addition to having a direct regulatory role required for Mtm phosphatase function.

Sbf coregulates Rab21 and PI(3)P required for macrophage cortical dynamics

We next asked whether the discovered Rab21 interactions also reflect a shared functional role in macrophage protrusion formation. We found that, similar to Sbf or mtm, expression of Rab21-WT was sufficient to induce excess F-actin-based cell protrusions (Figure 6, A–C). The inability of Rab21-CA to induce a similar phenotype may be due to an inability to bind Sbf (Figure 5E) or to properly localize or cycle. Conversely, macrophage-specific expression of Rab21-DN or knockdown of Rab21 by RNAi revealed a requirement for normal cell protrusion formation (Figure 6, C–E). The level of endogenous Rab21 protein depletion correlated with severity in the percentage of cells lacking protrusions (Supplemental Figure S5, A and B) and was further enhanced in combination with Sbf knockdown (Figure 6F). This shows that Rab21, like Sbf and mtm, is both necessary and sufficient for cell protrusion formation in macrophage remodeling.

To determine whether Rab21 is important for the Sbf and mtm roles in this process, we performed epistasis analysis of the induced cell protrusions. Both Sbf- and mtm-induced formation of excess cell protrusions required Rab21 function, as seen by the inhibition with either Rab21-DN or Rab21 depletion (Figure 6, G–K, and Supplemental Figure S5, C and D). Similarly, Rab21-induced formation of excess protrusions required both Sbf and mtm functions (Figure 6, L–N, and Supplemental Figure S5, E and F). In all cases, disruption of the endogenous functions not only reverted the induced excess protrusions but also compromised the formation of normal protrusions, showing that either enforced PI(3)P turnover or Rab21 activity alone is not sufficient to compensate for loss of the other.

We further addressed the sufficiency of Sbf roles in either Rab21 or Mtm regulation to mediate induction of excess cell protrusions. The expression of truncated Sbf constructs deleted for either the N-terminus DENN domains or the C-terminus coiled-coil and PH domains could no longer induce excess protrusions (Figure 6O), indicating that the full-length Sbf interactions with both Rab21 and Mtm, respectively, are corequired. Taken together, these results demonstrate that Sbf acts via protein interactions with both Rab21 and Mtm and that all three genes are codependent in promoting cell protrusion formation. Of importance, this establishes a functionally significant regulatory relationship for a DENN domain–containing Mtm pseudophosphatase toward a specific Rab.

Rab21 associates with PI(3)P endosomes and interacts with Mtm and Pi3K68D

To address a Rab21 relationship to the Sbf complex, we first investigated the Rab21 relationship to PI(3)P. Using the 2xFYVE biosensor, we found that Rab21, like Rab5, colocalized with PI(3)P but also to non–PI(3)P-containing membranes (Figure 7, A–D). Of interest, the expression of Rab21 or Rab5 altered the morphology of PI(3)P-containing membranes from rings to more collapsed and reticulated structures (Figure 7, A and B). In contrast, constitutively active forms did not (Figure 7, E and F), and moreover, only Rab5-CA led to swollen endosomes. The effects of Rab21 or Rab5 knockdown on PI(3)P-containing membranes were also distinct, exhibiting the known Rab5 function in Vps34-synthesized endosomal PI(3)P versus an apparent Rab21 effect on PI(3)P-membrane ring size and distribution (Figure 7, G and H).

We next investigated a Rab21 relationship to the Sbf complex through possible interactions with Pi3K68D or Mtm. Similar to Sbf, we detected colP of the Rab21-DN or Rab21-WT forms with Mtm but only with Sbf coexpression (Supplemental Figure S5G). This suggests that Mtm and Rab21 interact indirectly through Sbf and that Sbf can exist in a co-complex with both Mtm and Rab21. Similarly, we detected colP of Pi3K68D preferentially with the Rab21-DN form, even in the absence of Sbf coexpression (Figure 7, I–K). In contrast, Pi3K68D showed weak to no interaction with Rab5-DN, indicating a preferential association between class II Pi3K and Rab21 (Figure 7, I and K). Thus Sbf, Pi3K68D, and Mtm each preferentially interacts with inactive Rab21 as well as influences regulation of a PI(3)P pool, further indicating coordination between PI(3)P and Rab21 regulation with shared consequences for cortical functions.

Endocytic recycling and Rac GTPase are important for Sbf complex role in protrusion formation

The complex of protein interactions and PI(3)P-related phenotypes point to an important Sbf role in endosomal membrane dynamics, suggesting that Sbf may promote membrane trafficking involved in endocytic recycling. This idea therefore also predicts a requirement for additional steps in endocytosis for normal cell protrusion formation. We found that inhibition of shibire, the single Drosophila dynamin GTPase involved in membrane scission, also led to lack of cell protrusions, implicating a requirement for internalization at the plasma membrane (Figure 8, A–C). Rab5 GTPase has widely established roles at early endosomes in the regulation of Vps34 kinase...
function and corecruitment of PI(3)P effectors (Christoforidis et al., 1999). Unlike Rab21, we found that Rab5 knockdown, as indicated by severe depletion of endosomal PI(3)P and dramatic increase in macrophage cell number (Figure 7G and Supplemental Figure S5, H and I), did not overtly affect cell protrusion formation (Figure 8, C and D). This suggests that Rab21 can function independent of Rab5 and may act on distinct endosomal compartments or microdomains or that low levels of Rab5 protein are sufficient for this role. Late steps in endocytosis represented by Rab7 function also were not required in primary macrophages (Figure 8E), suggesting that an imbalance in degradation pathways likely is not central to the cortical phenotypes.

One trafficking route upon endosomal exit is through recycling endosomes marked by Rab11 GTPase, which broadly controls endocytic recycling back to the plasma membrane. Consistent with the identified Sbf–Rab11 protein interaction and colocalization (Figure 5, A–C, and Supplemental Figure S4, E and F), we found a requirement for Rab11 in cell protrusion formation (Figure 8, C and F) and for mtm-induced formation of excess cell protrusions (Figure 8, G and H).

The specific Sbf-related cortical defects are typical of those described from perturbations of Rho GTPases that are central regulators of F-actin organization and cell protrusions. Accordingly, we found that expression of activated Rac-CA or Rho-CA was sufficient...
to induce excess protrusions (Figure 8, I–L), similar to Sbf-complex gain-of-function conditions (Figures 1, 3, and 6). To address a role for specific Rho GTPases in Sbf-complex cortical functions, we performed epistasis studies. Expression of Rac-CA, but not Rho-CA, was able to rescue the loss of cell protrusions with Sbf knockdown (Figure 8, M–P). This suggests that Rac, either as a functionally important cargo or effector (Palamidessi et al., 2008), is under endosomal regulation of the Sbf complex for normal GTPase activity.

Sbf complex controls endosomal membrane cargo exit

We next asked whether turnover of specific cargo at the plasma membrane is affected by Sbf function. Transmembrane integrin receptor adhesion complexes are important for mammalian and fly macrophage spreading. Of interest, Rab21 was shown to regulate both integrin endocytic uptake and turnover in mammalian cells (Pellinen et al., 2006, 2008; Mai et al., 2011), and we showed that mtn and P3K68D coregulate integrin localization with turnover in fly and human muscle (Ribeiro et al., 2011). To address a role for the Sbf pathway in integrin turnover in fly macrophages, we assayed endocytic uptake and trafficking of surface-labeled βPS-integrin using a specific antibody in a two-step staining method (Supplemental Figure S5, J and K). After 5- to 10-min pulses in uptake of surface-labeled βPS-integrin, no significant differences in internalized βPS-integrin were seen with Sbf and mtn depletion as compared with control cells (Figure 9, A and B). However, after a 30-min pulse in uptake, an accumulation of internalized βPS-integrin was nearly tripled with Sbf and mtn depletion versus in control cells (Figure 9, B–D), pointing to an Sbf role in promoting postendosomal trafficking. In contrast, Rab21 RNAi led to a block in integrin endocytic uptake (Figure 9, B and E), consistent with reports in mammalian cells.
(Pellinen et al., 2006; Mai et al., 2011), and confounding interpretations of Rab21 function at a later step. The distinct phenotypes seen here and the known integrin independence for cell protrusion formation (Kadandale et al., 2010) suggest that Sbf/mtm and Rab21 roles in integrin trafficking do not entirely account for Sbf complex–related roles in cell protrusion formation and that Rab21 may function at distinct Sbf-independent and Sbf-dependent endocytic steps.

Specific cortical functions for Sbf-coordinated PI(3)P and Rab21 endosomal regulation

We have shown that endocytic internalization and Rab21/Rab11-mediated endosomal trafficking is important for controlling the balance in the number of macrophage cell protrusions. In this pathway, the Sbf pseudophosphatase provides the mechanism for coordination between PI(3)P and Rab21 regulatory machinery involved in endocytic membrane dynamics and trafficking (Figure 10).

DISCUSSION

Using macrophage cell protrusions as a relevant in vivo model to study membrane regulation, we uncovered an endocytic trafficking mechanism controlled by an MTM pseudophosphatase. Our data show that the Sbf pseudophosphatase directly scaffolds Rab21 and Mtm, coordinating GTPase activation and PI(3)P dephosphorylation responsible for specification of an endocytic step with key cell regulatory roles (Figure 10).

In the simplest model, we envision that class II PI3-kinase either at the cell cortex or on distinct early endosomal vesicles or microdomains synthesizes a PI(3)P subpool that is the eventual target of Sbf–Mtm function. We speculate that the class II PI3-kinase may be distinguished from class III Vps34 endosomal function by being localized or stimulated for PI(3)P synthesis, for example, in conjunction with particular cargo selection or Sbf–complex interactions. This PI3P pool could serve to recruit effectors involved in protein sorting, such as specific sorting nexins, motor proteins, or endosome maturation. From our data, we favor a model in which subsequently Sbf promotes membrane traffic exiting from early to Rab11

**FIGURE 8:** Sbf functions with a dynamin- and Rab11-mediated endocytic pathway important for cell protrusions.

(A, B, D–K, M–O) F-Actin in primary macrophages. (A, I) Control. (B) Dominant-negative dynamin, shi-K44A. (C) Percentage of macrophages without cell protrusions; ±SEM; Pxn-GAL4. (D) Rab5 RNAi. (E) Rab7 RNAi. (F) Rab11 RNAi. (G) Excess protrusions with mtm expression, (H) suppressed by Rab11 depletion. (J) Rac-CA. (K) Rho-CA. (L) Percentage of macrophages with excess cell protrusions; ±SEM. (M) Lack of protrusions with Sbf RNAi, (N) suppressed by coexpressed Rac-CA, (O) but not Rho-CA. (P) Percentage of cells with lack of protrusions; ±SEM. Bars, 5 μm.

**FIGURE 9:** Sbf/Mtm promote endosomal membrane exit for cargo trafficking. (A, C–E) βPS-integrin uptake and trafficking assay, indicating βPS at surface (red) and cell interior (green). (A, C) Control cells at t = 0 and 30 min. (B) Fluorescence intensity ratio, βPS at cell surface:central with subtracted t = 0; ±SEM. (D, E) Integrin uptake at t = 30 min in (D) mtm, Sbf coRNAi and (E) Rab21 RNAi cells. Bars, 5 μm.
endosomes. Sbf function for cell protrusions is likely required on endosomes, where Sbf simultaneously recruits Mtm phosphatase and activates Rab21 GTPase. The localized turnover of PI(3)P in conjunction with recruitment of unknown Rab21 effectors could drive membrane exit and/or phosphoinositide conversion from PI(3)P-containing endosomes. Sbf is also present at sites along the plasma membrane, suggesting that Sbf may remain on membranes throughout an endocytic and redelivery cycle or serve additional functions.

Our data demonstrate the importance of an MTM pseudophosphatase for the membrane recruitment of a specific MTM catalytic phosphatase function. We identified a novel and conserved interaction between Sbf/MTMR13 and PI3K68D/PI3KC2 that is associated with cell membranes. This physical interaction further supports the demonstrated PI3K68D functional relationship to Sbf–Mtm and points to Sbf as a mechanism to efficiently bridge specific sequential kinase and phosphatase functions. A central role for a common scaffold that is shared between kinase–phosphatase pairs is emerging as an important theme in phosphoinositide regulation with broader significance in human disease (Duex et al., 2006; Chow et al., 2007; Zhang et al., 2007b; Chagar et al., 2010; Taguchi-Atarashi et al., 2010). Of importance, our work raises the potential significance of class II PI3-kinase activity in treatment strategies for human MTMR2- or MTMR13-associated CMT4B neuropathies.

We showed that Sbf is a Rab21 GEF. The shared loss- and gain-of-function phenotypes for Sbf and Rab21 are consistent with Sbf serving a positive role to promote Rab21 function. As a GEF, Sbf may recruit and/or amplify Rab21 activity at a specific endosomal compartment, as described for cascading roles of different Rab5 GEFs (Horiiuchi et al., 1997; Sato et al., 2005; Lodhi et al., 2007; Zhu et al., 2007). Rab21 shares sequence homology, endosomal localization, and trafficking functions with Rab5-related GTPases (Simpson et al., 2004; Pellinen et al., 2006; Zhang et al., 2006; Egami and Araki, 2008), and here we show that Rab21 also colocalizes with PI(3)P and interacts with PI(3)P regulators. The shared interactions and functions with Sbf, Mtm, and PI3K68D point to a specific early endosomal role for Rab21 in macrophage protrusion formation. Previously, Rab21 was shown to mark dynamic membrane tubules induced by PI(3)P depletion with PI3K inhibitors (Egami and Araki, 2008). We observed that Rab21 function altered PI(3)P membrane morphology, suggesting that Rab21 may act in parallel with or as an effector of PI(3)P. Rab21 was previously shown to directly bind to and promote integrin turnover in cell migration (Pellinen et al., 2006) and to be required for endosomal to plasma membrane cargo delivery (Hooper et al., 2010; Tower-Gilchrist et al., 2011), and we recently described a role for PI3K68D-Mtm in integrin trafficking during muscle remodeling (Ribeiro et al., 2011). Here we show that Sbf-Mtm functions are also important for integrin trafficking in macrophages. Taken together, these results point to a mechanism in which Sbf-Mtm dephosphorylation of PI(3)P is specifically coupled to Rab21 activation and possible roles in selection or sorting of cargo(s) and/or membrane tubulation in endocytic recycling.

DENN-domain proteins recently were shown to act as Rab GEFs with restricted specificity (Wada et al., 1997; Allaire et al., 2010; Yoshimura et al., 2010). The DENN domains from MTMR5/MTMR13 were shown to exhibit GEF activity toward Rab28 and weakly Rab5 (Yoshimura et al., 2010). We demonstrate that the Drosofila Sbf DENN domain specifically interacts with Rab21—and not Rab11, Rab5, or Rab7—to promote Rab21-GDP to GTP exchange. Given that there is no obvious homologue of mammalian Rab28 in flies, evolutionary divergence is one explanation for the different MTMR13 and Sbf GEF specificities. Our work may point to a broader specificity for the DENN domain in the Sbf/MTMR subfamily. In addition, Rab21 has been shown to be a target of the Varp GEF (Zhang et al., 2006). Therefore it is possible that Sbf Rab21 GEF activity and shared functions represent a subset of Sbf GEF targets, and conversely, Rab21 regulators. This is in keeping with examples of both the Rab5 and yeast Ypt1 GTPases, which can each be regulated by multiple GEFs within different protein complexes (Barrowman et al., 2010). Of interest, an Sbf–Rab11 interaction that is independent of the Sbf DENN domain raises the possibility of a cascade of Sbf–Rab GTPase interactions in the progression of endosomal traffic.

Sbf serves dual roles in both phosphoinositide and Rab regulation. The ability of Sbf to associate with Mtm and Rab21 permits a mechanism for the coordination of PI(3)P turnover and Rab21 activation. A few examples are known for coupling PI(3)P kinase–phosphatase regulators and Rab GTPases (Cao et al., 2007, 2008), phosphoinositide pools with the regulation of Rab GEF recruitment and Rab activity (Mizuno-Yamasaki et al., 2010) and coincidence detection of joint phosphoinositide and Rab effectors (Di Paolo and De Camilli, 2006). One other example of coordination of specific Rab and phosphoinositide regulators, as we demonstrate here, was shown for p85 interactions as a scaffold for p110 and Pten (Chagar et al., 2010) and as a Rab5 GAP (Chamberlain et al., 2004). Of importance, MTMR13 mutations within either the DENN or myotubularin-related domains are similarly associated with Charcot–Marie–Tooth 4B2 (Azzedine et al., 2003; Senderek et al., 2003), signifying the likely importance of both Rab GEF– and MTM phosphatase–related functions in humans as well.

We demonstrate here the identity and mechanisms of endosomal trafficking machinery essential for cortical remodeling and cell protrusion formation. What remains to be determined is the identity of the protein cargo or lipids that mediate the underlying
structural remodeling and cell protrusion formation. We envision that trafficking of either a transmembrane receptor or Rac GTPase (Palamidessi et al., 2008) could localize or activate signaling that controls F-actin regulators at the cortex. An alternative possibility is that the Sbf complex may have a more direct role in the recruitment of F-actin regulators. The assembly of Sbf/MTMR13 protein complexes with different catalytic proteins raises the possibility for a network of interactions affecting Rab and phosphoinositide functions, with implications for the balance of membrane trafficking in both normal and disease states. The expansion of the MTM family in metazoa, including the emergence of MTM pseudophosphatases, points to their likely roles in the regulation of different phosphoinositide pools that meets the membrane-trafficking demands for dynamic cell regulation.

MATERIALS AND METHODS

Cell staining and microscopy

Macrophages were bled from four or five wandering third-instar larvae 4 d after egg laying into 100 μl of complete medium (for live cell imaging) or phosphate-buffered saline (PBS) and allowed to attach to a glass coverslip for 1 h at 25°C. For F-actin visualization and immunofluorescence, macrophages were fixed 15 min in 3.7% formaldehyde, 1× PBS, washed twice for 5 min in PBS with 0.1% Triton X-100, and blocked for 60 min in PBSTB (PBS with 0.05% Tween 20 and 3% bovine serum albumin [BSA]). Cells were stained overnight at 4°C with Alexa Fluor 647 phallolidin (1:100; Invitrogen, Carlsbad, CA), with or without primary antibodies of affinity-purified rabbit anti-Sbf (1:400). Cells were washed five times with PBST, incubated for 1 h with anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen) in PBSTB, stained 5 min with 4’,6-diamidino-2-phenylindole (1:1000; Invitrogen), and washed five times with PBST. Digital imaging was performed on a point-scanning microscope controlled by a FluoView program (FV1000; Olympus, Tokyo, Japan) using a 60x/1.2 numerical aperture (NA) Plan Apo objective with a 1.5x or 2x zoom. ImageJ (National Institutes of Health, Bethesda, MD) was used to export tagged image file formats and three-dimensional reconstruction. Photoshop (Adobe, San Jose, CA) was used to adjust the levels and curves and to crop and resize images. For live cell imaging, spilled macrophages were kept in complete SM media containing 1:1000 Hoechst 33342 to stain nuclei.

Time-lapse microscopy

Macrophages were imaged every 15 s (11 z-planes spaced by 0.5 μm acquired per time point) for 5 min using a 60x oil/1.42 NA Plan Apo objective on a spinning-disk fluorescence microscope (Ultraview VoX; PerkinElmer, Waltham, MA). Images were exported using Velocity software (PerkinElmer), and exported files were cropped and thresholded in Photoshop. Images were exported in ImageJ, in which they were cropped and thresholded.

FRAP analysis

Macrophages spilled into complete media were imaged at 5x zoom on scanning confocal microscope (FV1000). Five frames were acquired before photobleaching. Photobleaching was performed on equally sized rectangular regions of interest (ROI) with 20 repetitive scans using a 488-nm laser at 100% power. Images of the entire cell in each channel were immediately acquired after photobleaching at a 2.4-s interval for 110 s. ROI mean intensities were measured using FluoView software. FRAP analysis to determine normalized fluorescence intensity was performed as described (Goodwin and Kenworthy, 2005).

Integrin trafficking assay

Macrophages were isolated as described and incubated for 30 min at room temperature (RT). Cells were washed 2× in ice-cold media and incubated on ice for 60 min in media containing 5 μg/ml BPS-integrin CG.6G11 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Cells were washed 3× in ice-cold media and 2× in RT media and incubated for 0, 5, 10, and 30 min at 25°C to permit BPS uptake and trafficking. Macrophages were then washed 2× in ice-cold PBS, fixed in 4% paraformaldehyde for 30 min at RT, and washed 2× in PBS. Noninternalized integrin was labeled for 1 h at RT with 1:200 goat anti-mouse Alexa Fluor 546. Excess antibody was washed away with 5× PBS with 0.025% Tween 20. Remaining BPS-integrin was blocked for 1 h at RT using excess unconjugated goat anti-mouse antibody in PBS (1:7.5; Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were then washed 5× in PBS–TWEEN 20 and subsequently permeabilized and blocked for 30 min at RT in blocking buffer (PBS containing 30% goat serum and 0.3% Triton X-100). Internalized BPS was labeled with goat anti-mouse Alexa Fluor 488 for 1 h in blocking buffer. Cells were washed 6× in PBS–TWEEN 20 and 2× in PBS. Images were acquired in PBS at four different z-positions on a Zeiss LSM700 microscope (Carl Zeiss, Jena, Germany) using a 63/1.40 NA Plan Apochromat objective. Image analysis and fluorescence quantification were carried out using CellProfiler. Integrin uptake was plotted as the mean internalized BPS subtracted by t = 0 from at least two independent experiments.

Cell culture, transfections, and RNAi

Drosophila Kc167 and S2R+ cells were cultured in Schneider’s Drosophila medium (Invitrogen) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) at 24°C. Cells were transfected by calcium phosphate transfection (Invitrogen) or by FuGENE HD (GE Healthcare, Piscataway, NJ). pUAST constructs were transfected along with a metallothionein–GAL4 plasmid. Transfected cells were incubated with 0.75 mM CuSO4 in complete media for 6–8 h before immunoprecipitation. Generation of double-stranded RNAs and RNAi in cells were performed as previously described (Kiger et al., 2003). RNAi efficiency after 3 d was determined by reverse transcriptase-PCR or Western blot analysis, as described (Velichkova et al., 2010).

Antibody generation

Sbf protein fragment (amino acids 1866–1973), Pi3Kδ68D protein fragment (2–236 amino acids), and Rab21PC (amino acids 1–222) were expressed in Escherichia coli BL21. Glutathione S-transferase (GST) fusion proteins were purified on glutathione–Sepharose 4B beads (GE Healthcare). For Sbf and Rab21PC, the GST moieties were cleaved with Factor Xa (New England BioLabs, Ipswich, MA), and Factor Xa was removed with the Factor Xa removal resin (Qiagen, Valencia, CA). For Pi3Kδ68D, the GST:Pi3Kδ68D fusion protein was eluted with 25 mM reduced glutathione, 50 mM Tris-HCl, pH 8.8, 100 mM NaCl, and 0.1 mM dithiothreitol (DTT). Purified peptides were injected into rabbits (Robert Sargeant Antibody Services, Ramona, CA). For affinity purification of the Sbf antibody, 2 ml of serum was purified on an Sbf amino-linked column (Pierce, Rockford, IL).

Immunoprecipitations and immunoblots

Immunoprecipitations were carried out as described for the phosphatase assay, with these modifications. A total of 2.25 × 10⁶ Kc167 cells or 5 × 10⁶ S2R+ cells was plated per well of a six-well plate. Cells were lysed in 300 μl of lysis buffer, and 200 μl was used per...
immunoprecipitation. After the fourth wash, proteins were eluted from beads in 2× SDS–PAGE loading buffer and heated at 95°C for 5 min. Antibodies used were mouse anti-GFP (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-α-tubulin (1:5000; Sigma-Aldrich), mouse anti-FLAG (1:2500; Sigma-Aldrich), mouse anti–Myc 9E10 (1:5000; Sigma-Aldrich) or rabbit anti-hemagglutinin (HA; 1:2500; Abcam, Cambridge, MA), followed by anti-mouse or anti-rabbit horseradish peroxidase (1:10,000; Invitrogen). Some Western blot scans were processed to realign lanes in the final figures. When such manipulations were needed, all adjustments on levels and contrasts were performed before realigning the lanes, thus preserving intensity differences between bands exactly as the raw scan before alignments. For assessment of protein extracts from primary tissues, we dissected 15–20 fat bodies from third-instar larvae with spiked macrophages in PBS. Tissue was homogenized in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM KCl, 6.5% glycerol, 0.5 mM DTT, 0.1% Triton X-100, and 1× protease inhibitors and incubated on ice for 20 min. Extracts were spun at 13,200 rpm for 10 min at 4°C. Mouse MMTVR/3iP3K2ε immunoprecipitations were done as previously described (Robinson et al., 2008) using anti-3iP3K2ε (611046; BD Biosciences, San Diego, CA) and anti-MTMR13 (116-AP) antibodies (Robinson and Dixon, 2005).

**Phosphatase activity assay**

Kc167 cells transfected with 3xFLAG:mtm and additional appropriate constructs were processed for immunoprecipitation and malachite green assay (Echelon Biosciences, Salt Lake City, UT), as previously described (Velichkova et al., 2010). Because the in vitro phosphate release assay is conducted in the absence of ATP, experiments with colP of Pi3K68D are not confounded by kinase activity. Briefly, transfected cells were washed twice with ice-cold PBS, lysed in 500 μl of lysis buffer, and spun at 13,000 rpm for 10 min; the supernatant was collected for IP using anti-FLAG M2 agarose beads (Sigma-Aldrich) and heated at 4°C, washed thrice with lysis buffer, once with lysis buffer without NP40, and twice in reaction buffer and then split into four tubes (two used for PI(3)P; two used for PI(3,5)P3). Reaction mix (reaction buffer plus 50 μM phosphoinositides) was added to each tube, incubated for 20 min at 25°C, and then stopped by addition of 7 μl of 0.05 mM Na2VO4 and heated at 95°C for 2 min. Free phosphate in solution was measured by plate reader. Beads were loaded onto a 4–12% SDS–polyacrylamide gel (Invitrogen) and then stained with Sypro Ruby red stain to estimate the amount of protein. No ATP was present in the reaction buffer, preventing any contribution from Pi3K68D activity when present to phosphate release.

**Cell fractionation**

Cell fractionations were carried out as in Stegman and Robbins (2007), with these modifications. A total of 10 × 107 KC167 cells plated in 2× 100-mm dishes per condition was lysed in 400 μl of GNE buffer (50 mM glycophosphate, 10 mM NaF, 1.5 mM EGTA, and pH 7.6 with HCl). HSS1 fractions (cytosol) and HSP1 fractions (membrane) were used for immunoprecipitations. HSS1 was supplemented with 25 mM Tris-HCl, 1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid, 5 mM MgCl2, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM DTT, and 1× protease inhibitor (Sigma-Aldrich) to normalize to HSP1, which was resuspended in full lysis buffer. Immunoprecipitations were carried out as described, except that 750 μl was used as starting material.

**In vitro protein binding**

Direct GTPase binding assays were performed as in Christoforidis and Zerial (2000) with modifications. Using a scaled-down protocol, we loaded 200 μl of GST:Rab21PC beads with either GDP or GTPγS. Sbf was in vitro translated and labeled with 35S-methionine using TNT T7 Quick Coupled Transcription/Translation from Promega (Madison, WI) according to instructions. To avoid GDP/GTP contamination, we purified 100 μl of TNT reaction through G-25 Sephadex in exchange buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 100 mM NaCl, and 0.5 mg/ml BSA) and collected 150-μl fractions. The 35S-Sbf–containing fractions (as determined by scintillation counting) were pooled. We added 75 μl of purified 35S-Sbf to 25 μl of GST, GST:Rab21–GDP, or GST:Rab21–GTPγS in 500-μl nucleotide stabilization buffer (NS) containing 100 μM of either GDP or GTPγS and incubated it on a rotating wheel for 4 h. Beads were washed 4× in NS buffer containing 10 μM of the proper nucleotide, and proteins were eluted in 2× SDS–PAGE sample buffer. Proteins were loaded on an SDS–PAGE gel, transferred on a polyvinylidene fluoride (PVDF) membrane, and exposed on a Typhoon PhosphorImager (GE Healthcare). For Rab pull-downs, 5 μg of bacterially purified Rab:FLAG (Rab5, Rab7, Rab11, Rab21) immobilized on FLAG-M2 affinity gel was incubated with Kc cell lysate (as described for IPs) expressing GFP:SbfDENN (amino acids 1–508). Lysates in 400 μl were adjusted to a final GDP concentration of 100 μM and added to Rab beads. Beads were incubated for 4 h at 4°C on a nutator. After incubation, beads were washed 4× in 1 ml of NS containing 100 μM GDP. Protein elution, SDS–PAGE, transfer to PVDF membrane, and immunoblotting were carried out as described.

**GDP release assay**

Bacterially purified Rab5:FLAG and Rab21:FLAG (2 μg) immobilized on FLAG-M2 affinity gel were used for GDP release assay. Rab5 were immobilized in GDP loading buffer (10 μM GDP, 5 μCi GDP-3-H [PerkinElmer], 5 mM EDTA, 100 mM NaCl, and 20 mM Tris-HCl, pH 7.5) at 30°C for 15 min. GDP–Rab5 were stabilized by addition of 10 mM MgCl2 and centrifuged at 13,000 rpm, and the supernatant was removed. GDP–Rab5 beads were resuspended in 200 μl of GEF buffer (0.5 mg/ml BSA, 5 μM GTPγS, 0.5 mM DTT, 5 mM MgCl2, 100 mM NaCl, and 20 mM Tris-HCl, pH 7.5). Each GDP-loaded Rab was split into two tubes and brought to 130 μl with GEF buffer or with −100 nM SbfDENN (amino acids 1–508) and incubated at room temperature. At indicated time points, 20 μl was removed from the bead suspension and added to 1 ml of ice-cold wash buffer. Beads were washed 3× in 1 ml of wash buffer and after the last wash transferred to 5 ml of scintillation liquid and quantified in a scintillation counter. Values were adjusted as percentage of GDP released compared with time 0.

**GTP exchange assay**

HEK293T cells (10×, 100 mm) were transfected by calcium phosphate precipitation (Jean et al., 2010) for expression of tagged Drosophila genes, FLAG:Rab21, or FLAG:SbfDENN domain (amino acids 1–508). At 24 h posttransfection, cells were lysed in 400 μl of lysis buffer (as described for IPs) per plate, and supernatants were pooled and added to 100 μl of FLAG-M2 affinity gel (Sigma-Aldrich) and incubated 2 h at 4°C on a rotating wheel. FLAG beads were washed 3× with complete lysis buffer, 1× with lysis buffer lacking NP-40, 3× with high-salt TBS (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 1 mM DTT), and 3× with TBS (20 mM Tris-HCl, pH 7, 100 mM NaCl, and 1 mM DTT). FLAG-tagged proteins were eluted 5× with 100 μl of elution buffer (TBS containing 250 μg/ml 3xFLAG peptide) and dialyzed to remove the 3x peptide. Protein concentrations were estimated by comparing Coomassie staining to known control on a 10% SDS–PAGE gel. Proteins were flash frozen prior to GEF assay. GTP exchange assays were carried out as described in Allaire et al.
Generation of DNA constructs

Full-length Drosophila Sbf cDNA or deletion mutants (DENN/GRAM nucleotides 1–2953; ΔDENN nucleotides 1359–5922 and ΔC-PP 1–5079) were PCR amplified, cloned into the pENTR/D-TOPO entry vector (Invitrogen), subcloned by an LR recombination reaction into Gateway destination vectors pTGW-1075 (upstream-activating sequence [UAS]-eGFP:Sbf, UAS-eGFP:SbfDENN/GRAM, UAS-eGFP:SbfαDEC Ph), pUAST-mcherry-cd8β (UAS-mcherry:mtm), pTFW-1115 (UAS-3xFLAG:Sbf), pTMW-1107 (UAS-6xMyc:Sbf), pTHW-1099 (UAS-3xHA:Sbf), and pDEST17 (T7promoter-Sbf). The 3xFLAG-, 6xMyc-, and 3xHA-tagged forms of Pi3Kδ6δ cDNA were created from pENTr1a-Pi3Kδ6δ-WT, as described for Sbf. GST:Sbf-PH and GST:Pi3Kδ6δ-N-terminal were cloned into pGEX5X-3 using the restriction enzymes BamH1 and NotI. Rab21 constructs were created by PCR amplification from Kc167 cDNA. The Rab21-RC transcript was amplified and cloned into pENTR/D-TOPO gateway vector. GFP- and FLAG-tagged constructs were made as described for Sbf. Rab21-Q73L (constitutively active, referred to as Rab21-CA) or Rab21-T27N (dominant negative, referred to as Rab21-DN) constructs were created at the pENTR/D-TOPO-Rab21PC vector with gene-specific mutagenic primers using the same method as for Pi3Kδ6δ-D1457A. GST-Rab21PC was obtained by PCR amplification from the pENTR/D-TOPO-Rab21PC vector with primers harboring the restriction enzyme sites. The PCR fragment was digested by BglII/NotI and ligated into pGEX5X-3 digested with BamH1/NotI. All DNA constructs were validated by DNA sequencing and protein expression.

Statistical methods

CellProfiler software (www.cellprofiler.org/) was used to determine the ratio of PI(3)P-membrane area to cytoplasmic area per cell. Briefly, cell edges were first identified to measure total cell area. A mask of thresholded GFP:2xFYVE signal was subtracted from the total cell area, giving the cytoplasmic area unoccupied by the GFP:2xFYVE signal (masked cytoplasmic area). In Excel (Microsoft, Redmond, WA), 1 – (masked cytoplasmic area/total cell area) was used to determine the ratio of GFP:2xFYVE/cytoplasmic area. This measurement accounts for both the number and size of the GFP:2xFYVE-identified membrane while also correcting for differences in cell size. In addition, GFP:2xFYVE rings were manually counted from the same experimental sets. Prism software (GraphPad Software, La Jolla, CA) was used to calculate the mean-normalized GFP:2xFYVE area from ≥108 cells across two independent experiments, SE, and Student’s t test. Cell morphology of F-actin–labeled spread macrophages was assessed manually and categorized as normal, with excess, or with no protrusions. Cells with excess protrusions were identified as those exhibiting tightly spaced protrusions, with approximately >20 protrusions. Cells were scored from within entire image fields from three or more experiments, and results were plotted as the mean percentage of cells in each category. Prism was used to calculate SEM and Student’s t test. Colocalization was quantified using Pearson’s r with CellProfiler. For phosphatase assays, a Student’s t test was used in Figure 2C and Supplemental 5AC, and a one-sample t test was used in Figure 2B.
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