The WASH complex, an endosomal Arp2/3 activator, interacts with the Hermansky–Pudlak syndrome complex BLOC-1 and its cargo phosphatidylinositol-4-kinase type IIα

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ABSTRACT Vesicle biogenesis machinery components such as coat proteins can interact with the actin cytoskeleton for cargo sorting into multiple pathways. It is unknown, however, whether these interactions are a general requirement for the diverse endosome traffic routes. In this study, we identify actin cytoskeleton regulators as previously unrecognized interactors of complexes associated with the Hermansky–Pudlak syndrome. Two complexes mutated in the Hermansky–Pudlak syndrome, adaptor protein complex-3 and biogenesis of lysosome-related organelles complex-1 (BLOC-1), interact with and are regulated by the lipid kinase phosphatidylinositol-4-kinase type IIα (PI4KIIα). We therefore hypothesized that PI4KIIα interacts with novel regulators of these complexes. To test this hypothesis, we immunoaffinity purified PI4KIIα from isotope-labeled cell lysates to quantitatively identify interactors. Strikingly, PI4KIIα isolation preferentially coenriched proteins that regulate the actin cytoskeleton, including guanine exchange factors for Rho family GTPases such as RhoGEF1 and several subunits of the WASH complex. We biochemically confirmed several of these PI4KIIα interactions. Of importance, BLOC-1 complex, WASH complex, RhoGEF1, or PI4KIIα depletions altered the content and/or subcellular distribution of the BLOC-1–sensitive cargoes PI4KIIα, ATP7A, and VAMP7. We conclude that the Hermansky–Pudlak syndrome complex BLOC-1 and its cargo PI4KIIα interact with regulators of the actin cytoskeleton.

INTRODUCTION Vesicular trafficking is a general cellular mechanism by which secretory and endocytic pathway organelles selectively exchange components. This exchange mechanism requires coordinated steps, which include sorting and concentration of membrane protein cargo into nascent vesicles, membrane deformation and scission, directional movement through the cytosol, and fusion at the target organelle (Bonifacino and Glick, 2004). Many of these steps require mechanical force, which is generated within vesicular trafficking pathways by association of specialized molecular machines. These molecular machines include coat proteins that sort membrane protein cargo, BAR-domain proteins to sense or induce membrane deformation, the dynamin GTPase for membrane scission, and tethers and soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complexes for membrane fusion (Kaksonen et al.,...
molecules and functional mechanisms of these complexes both
Dell’Angelica, 2009; Wei and Li, 2013). Therefore exploring the
Recruitment and platelet dysfunction, pulmonary fibrosis, and, in some cases,
plexes in mammals and invertebrates lead to the hypopigmenta-
phenotypes (Bultema and Di Pietro, 2013). Defects in these com-
and regulatory enzymes trigger Hermansky–Pudlak syndrome–like
-3 (BLOC-1 to BLOC-3). In addition, genetic defects in specific Rabs
and the biogenesis of lysosome-related organelles complexes-1 to

A fundamental unanswered question concerns the extent to
which this cytoskeletal interaction is a general principle for coat-pro-
tein function. One of the best-characterized interactions between
vesicle trafficking machinery and the cytoskeleton is the formation
of vesicles at the plasma membrane by the clathrin and AP-2 adap-
tor coats. In this vesicle biogenesis pathway, the coat and coat-asso-
ciated factors, such as BAR-domain proteins, locally regulate the
polymerization and organization of actin filaments. Actin polymer-
ization is nucleated in large part by the Arp2/3 complex and its acti-
vator, the nucleation-promoting factor neural Wiskott–Aldrich syn-
drome protein (N-WASP; Conner and Schmid, 2003; Kaksonen
et al., 2005, 2006; Schmid and McMahon, 2007; McMahon and
Boucrot, 2011; Taylor et al., 2011; Mooren et al., 2012). The recent
identification of a nucleation-promoting factor that specifically local-
izes to endosomes, the WASP and SCAR homologue (WASH; Derivery et al., 2009; Gomez and Billadeau, 2009) suggests that
coat association with the actin cytoskeleton may be a general prin-
ciple operating in the generation of vesicles. Supporting this possi-
bility, depletion of WASH leads to functional defects in recycling, endosome-to-Golgi, and endosome-to-lysosome trafficking path-
ways (Derivery et al., 2009; Gomez and Billadeau, 2009; Gomez
et al., 2012; Duleh and Welch, 2010; Carnell et al., 2011; Zech et al.,
2011; Harbour et al., 2012; Hao et al., 2013; Pietrowski et al., 2013).
To date, however, WASH has been only been described to interact
with one endosomal coat complex, the retromer complex, which
primarily sorts cargo into the endosome-to-Golgi pathway (Gomez
and Billadeau, 2009; Gomez et al., 2012; Harbour et al., 2012; Jia
et al., 2012; Seaman, 2012; Hao et al., 2013; Helfer et al., 2013).
Therefore whether endosomal coats coordinate with the actin
cytoskeleton as a general principle has been untested.
We provide evidence supporting this hypothesis through the un-
biased identification of an interaction between the WASH complex
and sorting complexes associated with the Hermansky–Pudlak syn-
drome. This syndrome is genetically defined by defects in four
molecular complexes: the adaptor protein complex-3 (AP-3) coat
and the biogenesis of lysosome-related organelles complexes-1 to
-3 (BLOC-1 to BLOC-3). In addition, genetic defects in specific Rabs
and regulatory enzymes trigger Hermansky–Pudlak syndrome–like
phenotypes (Bultema and Di Pietro, 2013). Defects in these com-
plexes in mammals and invertebrates lead to the hypopigmenta-
platelet dysfunction, pulmonary fibrosis, and, in some cases,
immune dysfunction and neurologic phenotypes (Newell-Litwa
et al., 2007; Raposo and Marks, 2007; Raposo et al., 2007; Huizing
et al., 2008; Dell’Angelica, 2009; Wei and Li, 2013). These pheno-
types are a consequence of impaired cargo trafficking between
early endosomes and target organelles such as lysosome-related
organelles and synaptic vesicles (Newell-Litwa et al., 2007; Raposo
and Marks, 2007; Raposo et al., 2007; Huizing et al., 2008;
Dell’Angelica, 2009; Wei and Li, 2013). Therefore exploring the
molecules and functional mechanisms of these complexes both
increases our understanding of the molecular basis of the Herman-
sky–Pudlak syndrome and allows us to test fundamental questions
concerning principles of vesicular trafficking processes.
To identify functional mechanisms of complexes associated with
Hermansky–Pudlak syndrome, we focus on a membrane-anchored
and endosome-localized lipid kinase, phosphatidylinositol-4-kinase
(type IIa (PI4KIIα; Balla et al., 2002; Guo et al., 2003; Minogue et
al., 2006; Craigie et al., 2008). Biochemical and genetic evidence indi-
cates that PI4KIIα regulates and binds two complexes mutated in
Hermansky–Pudlak syndrome, AP-3 and BLOC-1 (Craigie et al.,
2008; Salazar et al., 2009; Larimore et al., 2011; Gokhale et
al., 2012a). We therefore hypothesized that PI4KIIα would interact with
novel proteins modulating the function of BLOC-1 and AP-3. To test
this hypothesis, we isolated PI4KIIα and its interactors by quantita-
tive immunopurification from in vivo cross-linked cell lysates.
PI4KIIα preferentially coisolated with proteins that regulate actin
cytoskeleton polymerization, including the WASH complex and
RhoGTPase 1. Depletion of PI4KIIα, BLOC-1 subunits, RhoGTPase 1, or the
WASH complex modified the content of other components in the
PI4KIIα interactome, thereby establishing genetic interactions in addi-
tion to physical interactions. Further, depletion of the WASH com-
plex resulted in changes in PI4KIIα endosomal morphology and
mislocalization of two BLOC-1 cargoes, the Menkes disease copper
transporter ATP7A and a lysosomal SNARE, VAMP7. We propose that
diverse coats such as clathrin–AP-2, retromer, and AP-3–BLOC-1
use common principles to garner force or create discrete membrane
domains by localized actin polymer assembly.

RESULTS
The PI4KIIα interactome enriches actin-regulatory proteins
PI4KIIα binds the AP-3 and BLOC-1 complexes, and these interac-
tions regulate its subcellular distribution (Craigie et al., 2008;
Salazar et al., 2009; Larimore et al., 2011; Gokhale et al., 2012a).
In addition, the lipid kinase activity of PI4KIIα regulates the recruit-
ment of the AP-3 and BLOC-1 coat complex to endosomal mem-
branes (Craigie et al., 2008; Salazar et al., 2009). Therefore we
hypothesized that interactors of PI4KIIα are previously unrecog-
nized regulators of the Hermansky–Pudlak syndrome–associated
AP-3 and BLOC-1 complexes. We tested this hypothesis by isola-
tion of endogenous PI4KIIα and interacting proteins. To isolate
PI4KIIα, we raised an antibody to a peptide containing the dleu-
icine sorting motif that is required for interaction of PI4KIIα with
the AP-3 and BLOC-1 coat complex to endosomal mem-
branes (Craigie et al., 2008). Mutation of the dileucine sorting motif abrogates the
recognition of PI4KIIα by this antibody by immunoblotting
(Supplemental Figure S1A). Therefore we premised that this anti-
body would compete with AP-3 for binding to PI4KIIα and thereby
preferentially enrich for PI4KIIα that is not bound to AP-3. This
design strategy allowed for the enrichment of PI4KIIα-interacting proteins that are upstream or downstream of AP-3 binding. After
establishing that this antibody specifically recognizes PI4KIIα, we
immunoprecipitated PI4KIIα from minimally cross-linked SHSY-5Y
neuroblastoma cell lysates (Supplemental Figure S1A and Figure 1A).
As we extensively characterized, this cross-linking strategy 1)
stabilizes protein interactions to allow rigorous biochemical isla-
aton strategies, as described later; 2) uses a cell-permeable and
chemically reducible cross-linker, dithiobis succinimidyl propionate
(DSP; Lomant and Fairbanks, 1976), that allows for in vivo cross-
linking but is amenable to protein identification by mass spectrom-
etry and immunoblot; and 3) is nonsaturating to minimize stabiliza-
tion of unrelated protein complexes (Salazar et al., 2009; Zlatic
et al., 2010; Gokhale et al., 2012a,b; Perez-Cornejo et al., 2012).
genic peptide outcompeted the endogenous protein for binding to the immunoprecipitation antibody (Figure 1A, lane 4). However, eluting protein complexes from antibody-coated beads with Laemmli sample buffer released background polypeptides that Silver stain of immunoprecipitated protein complexes revealed several polypeptides that coisolate with PI4KII\(\alpha\) (Figure 1A, lane 5). As expected, several of these coisolated polypeptides were not detected in control reactions where an excess of antigenic PI4KII\(\alpha\) peptide was included (lane 4). Bead elution was performed with Laemmli sample buffer (lanes 3–5). Lanes 6 and 7 depict antigenic peptide eluates from beads similar to those in lanes 4 and 5. Immunofluorescence purification allowed for the selective elution of PI4KII\(\alpha\)-coisolating proteins against low background (compare lanes 6 and 7; lanes 6’ and 7’ depict contrast-enhanced lanes 6 and 7). An arrow marks the band containing PI4KII\(\alpha\). Silver stain is a representative image of two independent experiments. Bottom, immunoblot of PI4KII\(\alpha\) in a parallel set of assays as those in the silver-stained SDS–PAGE. Immunoglobulin G chains are marked by asterisks. Input represents 1 and 0.33% for immunoprecipitation and immunofluorescence purifications, respectively. (B) Plots represent PI4KII\(\alpha\) and copurifying proteins (see Supplemental Table S1 for details). The x- and y-axes show SILAC fold of enrichment and the total number of spectral counts used for protein identification, respectively. Reference dots highlight PI4KII\(\alpha\) (green) and Nedd4-1. (C) Teal and red dots highlight actin-related proteins coisolated with PI4KII\(\alpha\). These include GDP exchange factors RhoGEF1, DOCK7, and GEF-H1. Red dots indicate the actin-related proteins that are subunits or interactors of the WASH complex: strumpellin (KIAA0196), SWIP (KIAA1033), Fam21B, FKBP15, capping protein \(\alpha\), and capping protein \(\beta\). (D) Blue dots highlight membrane-trafficking related proteins coisolated with PI4KII\(\alpha\), including clathrin heavy chain (CLTC), dynamin-2 (DNM2), and \(\beta\)3A subunit of the AP-3 complex (AP3B1). (E) Functional annotation analysis of the identified proteins using Gene Ontology annotations revealed a preponderance of actin-related proteins. Enrichment of categories such as cytoskeleton (30/124 interactors) and actin cytoskeleton (13/124) were significant at \(p = 1.62 \times 10^{-7}\) and \(7.67 \times 10^{-7}\), respectively. See Supplemental Table S2 for details. (F) Network analysis of putative PI4KII\(\alpha\) interactome components and BLOC-1 subunits. PI4KII\(\alpha\) (green), the BLOC-1 subunits (blue), and WASH complex subunits (red) are highlighted.
precluded the identification of PI4KIIα and interacting proteins by silver stain (Figure 1A, lanes 3–5). For this reason, we eluted protein complexes after immunoprecipitation by incubating the washed beads with an excess of the PI4KIIα antigenic peptide (Figure 1A, lane 7). This immunofinity purification strategy greatly reduced background contaminants and immunoglobulins, as revealed by silver stain and immunoblot (Figure 1A, compare lanes 4 and 5 to lanes 6 and 7). Finally, immunoblot demonstrated that our immunofinity purification approach highly enriched for PI4KIIα (Figure 1A, compare lanes 2 and 7).

Having analytically established a selective approach to isolate PI4KIIα and interacting proteins, we performed a preparative immunofinity purification of cross-linked PI4KIIα complexes. We identified interactors by quantitative mass spectrometry using stable isotope labeling with amino acids in cell culture (SILAC; Ong et al., 2002). We identified interactors by quantitative mass spectrometry using stable isotope labeling with amino acids in cell culture (SILAC; Ong et al., 2002). SHSY-5Y neuroblastoma cells were grown to equilibrium in either a light-label medium in which the amino acids arginine and lysine contain 12C and 14N isotopes (R0K0 medium) or a heavy-label medium in which these amino acids contain 13C and 15N (R10K8 medium; Supplemental Figure S1B). After incorporation of these amino acid isotope labels, PI4KIIα was immunoprecipitated from light-labeled cell lysates. As a control, immunoprecipitations from heavy-labeled cell lysates were performed in the presence of an excess of PI4KIIα antigenic peptide (Supplemental Figure S1B). As described earlier, both samples were then competitively eluted from the beads with an excess of PI4KIIα antigenic peptide. Light- and heavy-labeled eluted polypeptides were mixed 1:1 for tandem mass spectrometry analysis. This purification enriched PI4KIIα by ~55-fold, and its relative abundance was represented by 220 spectral counts (Figure 1, B–D, green dot). In addition, we identified 701 coisolating proteins with two or more unique peptides. Of the total 702 proteins, 268 were enriched more than twofold by PI4KIIα and 5 to lanes 6 and 7). Finally, immunoblot demonstrated that our immunofinity purification approach highly enriched for PI4KIIα (Figure 1A, compare lanes 2 and 7).

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Biochemical and genetic confirmation of PI4KIIα-interacting proteins

We confirmed PI4KIIα interactions identified by SILAC mass spectrometry by independent immunofinity purification of PI4KIIα followed by immunoblotting to test for the presence of proteins of interest. We found that PI4KIIα interacts with vesicle-mediated transport proteins such as clathrin heavy chain and the pallidin subunit of BLOC-1 (Figure 2A, lanes 6 and 7). Interacting proteins were found only in PI4KIIα isolations and not in controls, where an excess of antigenic peptide was used to outcompete immunoprecipitation of PI4KIIα complexes (Figure 2A, lanes 4 and 5). Consistent with our previous reports, detection of interactions between PI4KIIα and vesicle-mediated transport proteins required treatment with the cross-linker DSP, presumably because these associations are labile and/or transient. Further, we found that PI4KIIα interacts with two known interactors of the BLOC-1 and AP-3 complexes, respectively—the antioxidant enzyme peroxiredoxin-1 (Prdx-1) and a ubiquitin ligase Nedd4-1 (Mossinger et al., 2012; Figure 2A, lanes 6 and 7; also see Salazar et al., 2009; Gokhale et al., 2012a). Nedd4-1 represents the lower boundaries of our significance thresholds in terms of spectral count and enrichment cut-offs for our SILAC quantification, thereby validating that these thresholds minimize inclusion of false negatives in the PI4KIIα interactome (three spectral counts, 3.69-fold enrichment; Figure 1B). Finally, we tested the interaction between PI4KIIα and proteins that regulate the actin cytoskeleton, since functional annotation and network analysis suggested that these interactions were highly significant. We confirmed the interaction between PI4KIIα and the stumpellin subunit of the WASH complex and two guanine exchange factors for small GTPases RhoGFE1 and Dock7. Of note, these copurifications did not require the DSP cross-linker, suggesting that PI4KIIα interacts with these proteins with either higher affinity or a longer half-life than with the membrane-trafficking related proteins (Figure 2A, lanes 6 and 7). To further test the specificity of these interactions, we isolated hemagglutinin (HA)-tagged PI4KIIα from HEK293T cells by HA immunoprecipitation. We confirmed the presence of RhoGFE1 in these protein complexes, demonstrating that these interactions can be detected regardless of purification strategy (Figure 2B, lane 3). Taken together, these results indicate that the PI4KIIα interactome enriches proteins whose gene ontology annotation significantly implicates them in actin function.

Our network analysis of the PI4KIIα interactome and BLOC-1 subunits suggested connections between PI4KIIα-interacting proteins and BLOC-1 subunits (Figure 1F). Moreover, BLOC-1 regulates the subcellular localization of PI4KIIα (Larimore et al., 2011). For these reasons, we predicted that these previously unrecognized actin-related PI4KIIα interactors would coisolate with BLOC-1. To
test this prediction, we exploited an SHSY-5Y cell line that stably expresses a FLAG-tagged dysbindin subunit of BLOC-1. This tagged subunit incorporates into functional BLOC-1 complexes (Larimore et al., 2011; Gokhale et al., 2012a). First, we confirmed that this BLOC-1 subunit could be detected in PI4KIIα immunoaffinity purifications (Figure 2C, lane 3). We next tested whether PI4KIIα interactors would be present in BLOC-1 protein complexes isolated by FLAG immunoaffinity purification (Figure 2D, lanes 4 and 5). As previously reported, PI4KIIα can be detected by this method (Figure 2D, lane 5; Larimore et al., 2011). In addition, two PI4KIIα interactors, RhoGEF1 and the WASH complex subunit strumpellin, coisolate with BLOC-1 complexes (Figure 2D, lane 5). Further, we also detected the presence of two known BLOC-1 cargos, the Menkes disease copper transporter ATP7A and the v-SNARE VAMP7 (Salazar et al., 2006; Setty et al., 2008; Newell-Litwa et al., 2009, 2012a). These interactions were not detected in controls in which an excess of FLAG peptide was included in the reaction to outcompete the endogenous PI4KIIα (lanes 1, 4, and 6) or presence (C, all lanes, and D, lanes 2, 5, and 7) of DSP cross-linker, and detergent-soluble extracts were immunoprecipitated with antibodies against PI4KIIα (Figure 2D, compare lanes 5 and 7). Our results indicate that isolation of PI4KIIα complexes was significantly reduced in response to the depletion of either muted or pallidin (Figure 3, B and D). The total cellular content of PI4KIIα, Rho-GEF1, and BLOC-1 subunits was significantly reduced in response to the depletion of either muted or pallidin (Figure 3, B and D). We then analyzed the content of two well-characterized BLOC-1 subunits and with changes in the total cellular content of pathway cargoes and associated factors (Peden et al., 2002; Kantheti et al., 2003; Li et al., 2003; Starcevic and Dell’Angelica, 2004; Jia et al., 2010). We used this principle to independently test components of the PI4KIIα interactome. We predicted that perturbation of either PI4KIIα or BLOC-1 would affect the content of PI4KIIα interactome components if these proteins were regulators of a BLOC-1–dependent vesicle biogenesis pathway. PI4KIIα and two BLOC-1 subunits, muted and pallidin, were independently depleted by short hairpin RNA (shRNA) in HEK293T cells (Figure 3, A, B, and D). Semiquantitative immunoblotting analysis demonstrated that PI4KIIα depletion up-regulated the content of RhoGEF1, as well as that of subunits of AP-3 and BLOC-1. The latter observation is consistent with the existence of a tripartite complex between PI4KIIα, AP-3, and BLOC-1 (Salazar et al., 2009; Figure 3, A and D). These relationships among components of the PI4KIIα interactome were also observed after depletion of the BLOC-1 complex (Figure 3, B and D).
Hsp90 (D, E) were used as controls for comparisons. The AP-3 subunit δ3 determination per replicate. Significant Gray shading represents 50–100% of control. All experiments were done in biological triplicates with at least one quantifications where the targeted protein is highlighted in blue and antigens significantly changed are labeled in red.

FIGURE 3: Genetic confirmation of PI4KIIα-interacting proteins. (A, D) HEK293T cells were treated with scramble siRNA control or siRNA targeting PI4KIIα. (B, D) HEK293T cells were treated with lentiviruses carrying scramble shRNA control or shRNA targeting the pallidin or muted subunits of the BLOC-1 complex. (C, E) MNT-1 human melanoma cells were treated with lentiviruses carrying scramble shRNA control or siRNA targeting PI4KIIα. (A, D) HEK293T cells were treated with lentiviruses carrying scramble shRNA control or shRNA targeting the pallidin or muted subunits of the BLOC-1 complex. (C, E) MNT-1 human melanoma cells were treated with lentiviruses carrying scramble shRNA control or siRNA targeting PI4KIIα.

Next we tested whether shRNA-mediated depletion of BLOC-1, strumpellin, or RhoGEF1 would affect the total cellular content of other PI4KIIα interactome components. First, we explored the consequences of BLOC-1 depletion in the melanoma cell line MNT-1, a cell type in which localization of BLOC-1 to tubular endosomes has been defined at the subcellular level by electron microscopy (Di Pietro et al., 2006). Consistent with our finding in HEK293T cells, the total cellular content of the PI4KIIα interactome components RhoGEF1 and strumpellin did not modify the total cellular content of BLOC-1 subunits (Figure 3, C and E). However, the BLOC-1 cargo VAMP7 and ATP7A were significantly changed (Figure 3, C and E). The total cellular content of ATP7A increased in response to BLOC-1, RhoGEF1, or strumpellin depletion in MNT1 cells (Figure 3E; 2.3 ± 0.12–, 2.2 ± 0.46–, and 1.6 ± 0.13-fold increase compared with controls, respectively). In addition, VAMP7 levels were significantly decreased in BLOC-1 depletions, significantly increased in RhoGEF1 depletions, and unchanged in strumpellin depletions. These data suggest a disruption to the regulation of steady-state levels of these BLOC-1 cargoes upon depletion of PI4KIIα interactome components and thereby establish genetic interactions between PI4KIIα interactome components.

The WASH complex and filamentous actin reside on PI4KIIα-positive early endosomes
The WASH complex is an actin-regulatory complex that localizes to early endosomes (Derivery et al., 2009; Gomez and Billadeau, 2009; Gomez et al., 2012; Duleh and Welch, 2010; Carnell et al., 2011; Zech et al., 2011; Harbour et al., 2012). Similarly, PI4KIIα localizes to early endosomes, where it binds to BLOC-1 (Balla et al., 2002; Guo et al., 2003; Minogue et al., 2006; Craige et al., 2008; Salazar et al., 2009). Therefore we predicted that PI4KIIα-positive endosomes contain BLOC-1, the WASH complex, and actin. To test this prediction, we first separated endosomal compartments from HEK293T cell homogenates by sucrose velocity sedimentation (Clift-O’Grady et al., 1998; Clift-O’Grady et al., 1998; Lichtenstein et al., 1998). As expected, PI4KIIα cosedimented to 35% sucrose with transferrin receptor, an endosomal marker (Figure 4, A and B). In addition, the WASH complex subunit strumpellin, the BLOC-1 complex subunit pallidin, and actin were present in these fractions (Figure 4, A and B). Pallidin and actin were predominantly found at the top of gradients where free cytosolic proteins sediment (Clift-O’Grady et al., 1998; Lichtenstein et al., 1998). The presence of filamentous actin on PI4KIIα-containing endosomes was confirmed by live-cell imaging of fluorescently tagged EGFP-PI4KIIα with the LifeAct fluorescent probe (Figure 4C; Riedl et al., 2008). We tracked fluorescently labeled FLAG antibodies bound to an exofacial tag engineered in the β2-adrenergic receptor (SSF-B2AR; Figure 4D). We stimulated internalization of receptor-FLAG antibody complexes by addition of the adrenergic receptor agonist isoproterenol to functionally define early endosomes (Puthenveedu et al., 2010). In these
The WASH binds HPS complexes to early endosomes. Pi4KIIα, the WASH complex subunit strumpellin, and actin cytoskeleton components coreside at early endosomes. (A, B) HEK293T cells were homogenized, and low-speed supernatants were resolved by sucrose velocity sedimentation on a 10–45% sucrose gradient. Pi4KIIα and the WASH complex subunit strumpellin cosedimented to 35% sucrose with endosomal organelles, as identified by the transferrin receptor (TrfR). Cytosolic fractions sediment at the top of the gradient (10% sucrose). (B) Relative distribution of the indicated proteins for the fractionation shown in A. Images and distribution profile are representative of two independent experiments. (C) Live-cell imaging of fluorescently tagged Pi4KIIα and LifeAct to visualize filamentous actin in HEK293T cells confirmed the presence of actin at Pi4KIIα-positive organelles (n = 12 cells). (D) HEK293T cells stably expressing Pi4KIIα-GFP and Signal-Sequence FLAG-tagged β2-adrenergic receptors (SSF-B2AR) were transiently transfected with mCortactin-dsRed and imaged 10 min after addition of 10 μM isoproterenol. A single confocal stack was captured in three channels at 1-s intervals. (E) Expanded image of endosome indicated by an arrow in A over a 10-s period. (F) Diagram of the oval profile used to generate traces of fluorescence intensity around endosomes. (G) Representative trace of normalized fluorescence intensity for each protein in the endosome above.
PI4KIIα colocalizes with the WASH complex. (A, C) HEK293T cells were either transiently transfected with EGFP-tagged subunits of the WASH complex or stained with MitoTracker green, fixed, and processed for indirect immunofluorescence microscopy. Images were acquired by high-resolution deconvolution microscopy. The z-stack projections are shown. (B, D) Isosurface renderings of the z-stack data created using Imaris software. (E) Calculation of Pearson’s correlation coefficient between signal intensities of strumpellin-EGFP, WASH-EGFP, or MitoTracker and endogenous PI4KIIα. Correlation was lost when one channel was rotated by 15°. Here n = 6 for WASH-EGFP, n = 7 for strumpellin-EGFP, and n = 9 for MitoTracker-A488. Statistical comparisons were performed by nonparametric analysis using a group Kruskal–Wallis rank sum test followed by individual Wilcoxon–Mann–Whitney rank sum tests.

The WASH complex modulates the targeting of BLOC-1 cargoes

WASH complex depletion is characterized by the appearance of enlarged cargo-laden tubules from endosomes and cargo missorting (Gomez and Billadeau, 2009; Gomez et al., 2012; Harbour et al., 2012; Jia et al., 2012; Seaman, 2012; Helfer et al., 2013). Therefore we hypothesized that PI4KIIα is present in tubular structures whose length is regulated by the WASH complex. To test this hypothesis, we expressed PI4KIIα-EGFP in HEK293T cells stably expressing FLAG-tagged β2-adrenergic receptor and imaged cells by live-cell microscopy. Endosomes were unequivocally identified by ligand-induced internalization of fluorescent FLAG antibodies bound to the exofacial domain of the β2-adrenergic receptor. Tubules containing PI4KIIα and/or β2-adrenergic receptor emerged from early endosomes, and their size increased in WASH complex-depleted cells (Figure 6, A and C). Overall the size of early endosomes containing PI4KIIα and β2-adrenergic receptor was unaffected by WASH complex deficiency (Figure 6A). In contrast, BLOC-1 depletion by shRNA targeting of pallidin was characterized by an enlargement of these early endosomes without enlargement of tubules (Figure 6, A and B). The changes in the architecture of early endosomes containing PI4KIIα and β2-adrenergic receptor by depletion of either BLOC-1 or WASH complexes support a model in which WASH complexes in concert
with BLOC-1 regulate the targeting of BLOC-1 cargoes from early endosomes. We predicted that WASH complex deficiencies would alter the subcellular distribution of BLOC-1 cargoes if these complexes act on the same pathway. To test this prediction, we treated MNT1 melanoma cells with shRNA targeting the strumpellin subunit of the WASH complex or scramble control. We assessed the cell surface level of two BLOC-1 cargoes: the Menkes copper transporter ATP7A and the lysosomal SNARE protein VAMP7 (Salazar et al., 2006; Setty et al., 2008; Newell-Litwa et al., 2009, 2010). As previously described, we observed an ∼1.5-fold increase in the total cellular content of ATP7A in WASH complex depletion (Figure 7A, lanes 1–4). Cell surface levels of ATP7A, however, increased 3.3 ± 0.8-fold in WASH complex depletion (Figure 7A, lanes 5 and 6, and 7C; average ± SEM). Similarly, cell surface levels of VAMP7 increased 2.5 ± 0.6–fold in WASH complex depletions as compared with control, whereas the total cellular levels of VAMP7 were moderately but not significantly affected (Figure 7A, lanes 5 and 6, and Figure 3, C and D). These cell surface increases were specific to BLOC-1 cargoes rather than global perturbations, as the total cellular content and cell surface levels of transferrin receptor were unaffected in WASH complex–depleted cells (Figure 7A, lanes 5 and 6, and Figure 3, B and C). Moreover, silver stain of total biotinylated cell surface protein showed no global increases in cell surface proteins from WASH complex depletions (Figure 7B). Thus BLOC-1 cargoes are missorted to the cell surface in WASH complex depletion. These data support a model in which WASH complexes modify the traffic of BLOC-1 cargoes.

DISCUSSION

In this study we identified previously unrecognized components of a membrane protein sorting pathway affected in the Hermansky–Pudlak syndrome by isolating the membrane-anchored lipid kinase PI4KIIα. PI4KIIα binds and regulates two complexes mutated in the Hermansky–Pudlak syndrome, the AP-3 and BLOC-1 complexes, by means of a dileucine sorting motif and its kinase activity (Craige et al., 2008; Salazar et al., 2009; Larimore et al., 2011; Gokhale et al., 2012a). We therefore hypothesized that PI4KIIα interacts with unrecognized components of a vesicle transport pathway requiring the BLOC-1–AP-3–PI4KIIα complex. PI4KIIα could interact with these components either concurrently with binding to AP-3 and BLOC-1 and/or at steps either preceding or following the binding to these complexes. We therefore designed a strategy to enrich PI4KIIα interactors upstream and downstream of the PI4KIIα dileucine sorting motif recognition event. To achieve this goal, we raised an antibody against a 20-residue peptide of PI4KIIα that contains the ERQPLL sorting motif. Mutation of this motif to ERQPAA is sufficient to prevent recognition of PI4KIIα by this antibody by immunoblot. Although the ERQPLL motif is present in five other proteins encoded in the human genome, none of these proteins is enriched in the PI4KIIα interactome, ensuring

FIGURE 6: Depletion of pallidin and strumpellin alter endosomal morphology. (A) HEK293T cells stably expressing Signal-Sequence FLAG-tagged β2-adrenergic receptors (SSF-B2AR) and PI4KIIα-EGFP were transduced with scrambled, pallidin, or strumpellin shRNA lentiviruses. Pallidin-depleted cells were imaged 8 d from initiation of transduction, and strumpellin-depleted cells were imaged 5 d after transduction. Cells were grown under selection after day 2 of transduction. All knockdowns were compared with parallel transduction with scrambled shRNA. Images are maximum projections of three confocal slices taken with 0.3-μm intervals. (B) Frequency histogram of endosomal circumference from cells treated with either scrambled (blue) or pallidin (red) shRNA. Insert to the left depicts a probability plot of the same data. The p value determined by nonparametric Kolmogorov–Smirnov test. Here n = 150 for control cells and n = 117 for pallidin-depleted cells. (C) Percentage of cells expressing scrambled or strumpellin shRNA displaying endosomal tubulation. Cell selection was performed in the SSF-B2AR channel and blinded for PI4KIIα before FLAG antibody internalization, then imaged 15 min after addition of 10 μM isoproterenol. The p value was determined by nonparametric Fisher exact test. Here n = 112 for control cells and n = 101 for pallidin-depleted cells.
that this antibody specifically recognizes PI4KIIα (www.mrc-lmb.cam.ac.uk/ genomes/madanm/harvey/). We used this antibody to immunopurify PI4KIIα from isotope-labeled cell lysates (SILAC), allowing for the quantitative identification of interacting proteins over nonspecific contaminants and immunoglobulins (Ong et al., 2002; Mann, 2006; Zlatic et al., 2010; Gokhale et al., 2012a). Strikingly, PI4KIIα isolation preferentially coenriched proteins that regulate the actin cytoskeleton, including guanine exchange factors for Rho family GTPases, RhoGEF1, DOCK7, and GEF-H1, and several subunits of the WASH complex. In addition, PI4KIIα coisolated with membrane trafficking protein complexes, such as clathrin heavy chain and the β3A subunit of the AP-3 complex. These membrane-trafficking complexes coisolated with PI4KIIα at a lower stoichiometry as compared with actin regulators, as intended with our isolation strategy. We biochemically confirmed several of these PI4KIIα interactions. Further, we predicted that if PI4KIIα interactome components interact, then perturbing these proteins would change the total cellular content of other interactome components. With this guiding rationale, we established that genetic depletion of PI4KIIα itself, the BLOC-1 complex, RhoGEF1, or the WASH complex altered the total cellular content of either PI4KIIα interactome components or BLOC-1 cargoes, the Menkes disease copper transporter ATP7A and the lysosomal SNARE VAMP7 (Salazar et al., 2006; Setty et al., 2008; Newell-Litwa et al., 2009, 2010). We conclude that these novel PI4KIIα interactors biochemically and genetically interact with components of the actin cytoskeleton. We favor a model in which membrane protein cargoes are sorted by a BLOC-1–dependent mechanism into tubular carriers whose size is controlled by the WASH complex, possibly by generating force for membrane scission.

We focused our efforts to characterize the consequences of these interactions on the WASH complex. Wash1 is a type I nucleation-promoting factor that binds the Arp2/3 complex to locally nucleate and organize the polymerization of branched actin filaments (Derivery et al., 2009; Jia et al., 2010; Veltman and Insall, 2010; Rotty et al., 2012). The WASH complex localizes to tubular domains of early endosomes, where actin polymerization is proposed to create localized membrane domains for cargo sorting and/or regulate tubule morphology by membrane scission (Gomez and Billadeau, 2009; Gomez and Billadeau, 2009; Gomez et al., 2012; Duleh and Welch, 2010, 2012; Harbour et al., 2012; Helfer et al., 2013). Our work identifies the WASH complex as a previously unrecognized interactor of BLOC-1 and PI4KIIα. This assertion is founded on the following findings and rationale. We found that WASH complex subunits localize to PI4KIIα-containing organelles and coprecipitate with BLOC-1 complex subunits, which is consistent with a previous report that Wash1 interacts with the BLOS2 subunit of the BLOC-1 complex by yeast two-hybrid analysis and immunoprecipitation of tagged subunits (Monfregola et al., 2010). Further, we found that PI4KIIα is contained in small tubular structures that emanate from early endosomes, which were functionally labeled by internalized β-adrenergic receptors. If BLOC-1 and the WASH complex coordinate to regulate early endosomal tubule morphology and/or cargo sorting, then we predicted morphological and missorting phenotypes would arise upon perturbation of protein complexes. First, we predicted early endosomal morphological changes in BLOC-1 and WASH complex deletions, leading to either enlarged endosomes or endosomal tubules. Indeed, we found that BLOC-1 depletion increases the size of functionally identified early endosomes without increasing tubule size. This phenotype is consistent with our previous findings that endosomes increase in size after AP-3, BLOC-1, or PI4KIIα depletion (Salazar et al., 2009). Further, in WASH complex depletion we found elongation of early endosomal PI4KIIα-containing tubules, which is consistent with previous reports demonstrating increases in endosomal tubule length upon Wash1 depletion (Derivery et al., 2009; Gomez and Billadeau, 2009). Second, we predicted missorting of
BLOC-1 cargo in WASH complex depletions, which would suggest an impaired flow of cargoes or membrane out of endosomes. We concentrated on the missorting of specific endolysosomal cargos to the cell surface, which is a characteristic phenotype in AP-3, BLOC-1, and PI4KIIα deficiencies (Dell’Angelica et al., 1999; Peden et al., 2002; Di Pietro et al., 2006; Salazar et al., 2006; Setty et al., 2007; Baust et al., 2008). Two BLOC-1 cargoes, ATP7A and VAMP7, mislocalize to the cell surface in WASH complex depletions. This phenotype was specific to endolysosomal cargo, as cell surface levels of a receptor that undergoes constitutive recycling, the transferrin receptor, were unchanged.

How do BLOC-1 and the WASH complex mechanistically coordinate to regulate endosomal morphology and cargo sorting? Actin polymerization fulfills many roles in membrane protein sorting and trafficking, including creating domains to spatially organize sorting machinery, providing mechanical support for membrane deformation during vesicle budding, generating force to aid in membrane scission of vesicles, and propelling carriers after budding is completed (Qualmann et al., 2000). The BLOC-1 cargo missorting phenotype observed after WASH complex depletion suggests a role for Wash1 in steps occurring at or upstream of the membrane scission of vesicles. Actin polymerization by the WASH complex is proposed to generate force required for membrane scission, a hypothesis that awaits testing by in vitro biochemical reconstitution (Bear, 2009; Derivery et al., 2009; Gomez and Billadeau, 2009; Rotty et al., 2012).

However, this model is consistent with the binary biochemical association of WASH complex subunits, PI4KIIα, BLOC-1, and the BLOC-1 cargoes ATP7A and VAMP7 documented here and in our previous work (Salazar et al., 2009; Larimore et al., 2011). In addition, this model predicts that these components interact concurrently, which remains to be documented. An alternative hypothesis is that PI4KIIα and the BLOC-1 complex independently associate with the WASH complex to regulate its activity. Irrespective of whether BLOC-1 and PI4KIIα act in concert or as independent factors, we speculate that the WASH complex acts as a generic mechanism to nucleate branched actin polymerization in endosomes, with spatial or cargo specificity dictated by different upstream accessory factors such as coats and/or lipid modifications.

PI4KIIα is an interesting candidate upstream activator of the WASH complex because of its capacity to produce PI4P (Balla et al., 2002; Barylko et al., 2002). The WASH complex Fam21 subunit, a component of the PI4KIIα interactome, is proposed to localize WASH to endosomal membranes by binding phosphoinositides and membrane protein sorting complex subunits, such as the Vps35 subunit of the retromer complex (Gomez and Billadeau, 2009; Harbour et al., 2012; Helfer et al., 2013). Fam21 binds PI4P in vitro, the lipid product of PI4KIIα (Jia et al., 2010). PI4KIIα and PI4P may thereby either recruit the WASH complex to early endosomal membranes or stabilize the complex at these membranes for function within the AP-3–BLOC-1 or multiple sorting pathways. As discussed later, genetic disruption of a WASH complex subunit and PI4K2A cause common neurodegenerative phenotypes in human patients and mouse models, supporting a regulatory role for PI4KIIα in WASH localization (Valdmanis et al., 2007; Simons et al., 2009; Clemen et al., 2010). In addition, PI4KIIα interactome components may regulate WASH complex activation. A common feature of nucleation-promoting factors such as Wash1 is their regulation and activation by small GTPases. The regulatory GTPase for the WASH complex has remained elusive in mammalian cells. In Drosophila melanogaster genetic interactions between Rho and Wash1 have been reported (Liu et al., 2009). The high-stoichiometry association of a RhoA guanine exchange factor, RhoGEF1, with PI4KIIα and the similarities between strumpellin depletion and RhoGEF1 depletion in MNT1 cells suggest a regulatory mechanism between these two PI4KIIα interactors. Future experiments are needed to test precise molecular and functional relationships between PI4KIIα, RhoGEF1, the WASH complex, and the BLOC-1 complex.

In addition to expanding our conception of the vesicular trafficking pathway controlled by the Hermansky–Pudlák syndrome complex BLOC-1, our findings have novel implications for the understanding of neurodegenerative diseases that affect central or peripheral motor neuron axons, such as hereditary spastic paraplegia (HSP). Point mutations in the WASH complex subunit strumpellin cause autosomal-dominant HSP (OMIM 610657), which is characterized by the degeneration of axons projecting to the spinal cord from the brain motor cortex (Valdmanis et al., 2007; Clemen et al., 2010). More than 50 genes have been linked to HSP in humans (Finsterer et al., 2012). The common theme of this disease is degeneration of central neurons with long axons, presumably due to the high burden of maintaining a synapse distant from the corresponding cell body. Consistent with this theme, many of the 50 identified genes are endosomal membrane trafficking complexes (Blackstone, 2012). No interactions between strumpellin and other HSP-causative genes have been described, making it difficult to place strumpellin and the WASH complex within a cellular framework to understand how mutations in the strumpellin protein lead to disease. A recent study found that strumpellin subunits containing HSP disease–causing mutations assemble into WASH complexes that localize to early endosomes in neurons and have no apparent consequence for retromer complex–mediated recycling of the β-adrenergic receptor (Freeman et al., 2013). Our data suggest that PI4KIIα interactome components could be an alternative pathway disrupted by these mutations. This hypothesis is supported by the HSP-like phenotype that results after targeted disruption of PI4KIIα in mice (Simons et al., 2009). In addition to a role of the PI4KIIα interactome in degeneration of central motor neurons, our data suggest connections with disease mechanisms in peripheral motor neuron axonal degeneration. Point mutations to the BLOC-1 cargo ATP7A in human patients cause a degeneration of peripheral motor axons (Kennerson et al., 2010; Yi et al., 2012). In this article, we found that ATP7A is mislocalized in strumpellin-depleted cells. In addition, PI4KIIα itself is a BLOC-1 cargo, suggesting that it too might mislocalize in strumpellin perturbations that cause HSP in humans. Therefore we propose that disruption to PI4KIIα interactome components might contribute to the pathophysiology of central and peripheral motor neuron axon degeneration.

**MATERIALS AND METHODS**

**Antibodies and cell culture**

Antibodies used in this study are listed in Supplemental Table S3. The PI4KIIα antibody used here for immunoprecipitation, Western blot, and immunofluorescence microscopy experiments was raised against the sequence 51-PGHDREOROPPLDRARGAAAQ-70 and was described in Larimore et al. (2011). We obtained this antigenic peptide by custom peptide synthesis (Bio-Synthesis, Lewisville, TX). The antigenic peptide was diluted to a 20 mM stock in 0.5 M MOPS, pH 7.4, and stored at −80°C.

HEK293T and SHSY-5Y cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM with 4.5 g/l glucose, −pH 7.4, and stored at −80°C.

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inactivated at 65°C for 60 min), and 100 μg/ml penicillin/streptomycin at 37°C and 5% CO2.

For SILAC labeling, cells were grown in either a light medium containing only 12C and 14N in amino acids arginine and lysine (R0K0) or a heavy medium containing 13C and 15N in these amino acids (R10K8). All reagents for SILAC labeling were obtained from Dundee Cell Products (Dundee, United Kingdom). Cells were grown for seven passages to ensure maximal incorporation of these amino acids. Our previous work found that these conditions lead to >97.5% incorporation of these amino acids into the total cellular pool (Ong and Mann, 2006; Gokhale et al., 2012a; Perez-Cornejo et al., 2012).

DNA expression constructs
The following PI4KIIα expression constructs were used: PI4KIIα-EGFP WT, PI4KIIα-EGFP HA WT, PI4KIIα-EGFP D308A, and PI4KIIα-LL60,61AA (Craige et al., 2008). The FLAG-tagged dysbindin construct is described in previous work (Gokhale et al., 2012a). Wash1-EGFP, Strumpellin-EGFP, KIAA1033-EGFP, and Fam21-EGFP were described previously (Harbour et al., 2010). Cortactin-dsRed and Signal-Sequence FLAG tagged β2-adrenergic receptors (SSF-B2AR) were previously described (Kaksonen et al., 2000; Puthenveedu et al., 2010).

Immunoprecipitation and immunofluorescence microscopy

HEK293T cells were transfected in six-well plates with 1 μg of shRNA construct DNA construct by Lipofectamine 2000 for 4 h and then switched into growth medium. After 24 h, cells were seeded on glass coverslips coated with Matrigel. The next day, coverslips were moved to ice, washed two times in ice-cold PBS, and then fixed with 4% paraformaldehyde. After fixation, all coverslips were washed two times in PBS and then incubated with 0.1% Triton X-100 supplemented with Complete antiprotease (Roche, Indianapolis, IN). After lysis, cell debris were scraped from the plates, and lysates were spun at 16,100 ×g for 15 min. The supernatant was recovered and diluted to 1 mg/ml. For immunoprecipitation, 500 μg of this clarified cell lysate was applied to 30 μl of Dynal magnetic beads (Invitrogen, Carlsbad, CA) coated with the immunoprecipitation antibody of interest. These immunoprecipitation reactions were incubated for 2 h with end-over-end rotation at 4°C. For PI4KIIα peptide competition controls, the PI4KIIα antigenic peptide was included in the immunoprecipitation reaction at a concentration of 40 μM. For FLAG competition controls, the 3X FLAG peptide was included at 340 μM, as previously described (Gokhale et al., 2012a; Perez-Cornejo et al., 2012). For HA competition controls, the HA peptide was included at 10 μM. After the 2-h incubation, beads were washed five times in buffer A with 0.1% Triton X-100. Proteins were then eluted from the beads either by boiling in Laemmli sample buffer at 75°C for 5 min or incubating with the antigenic peptide for 2 h on ice. Peptides were diluted in lysis buffer to a concentration of 40 μM. For FLAG competition controls, the 3X FLAG peptide, 200 μM, 3X FLAG peptide, 340 μM. Samples were resolved by SDS–PAGE electrophoresis, followed by either silver stain or immunoblotting to detect individual proteins. For SILAC analysis, 18 immunoafluorescence chromatography and 18 control reactions were conducted. Eluates from these reactions were combined and trichloroacetic acid precipitated to concentrate the samples for SDS–PAGE and mass spectrometry.

Analysis samples were analyzed for SILAC protein identification by MS Bioworks (Ann Arbor, MI) and the Emory CND Proteomics Facility (Atlanta, GA). SILAC-labeled samples were separated on a 4–12% Bis-Tris Novex minigel (Invitrogen) using the MOPS buffer system and stained with Coomassie, and the lane was excised into 20 equal segments using a grid. Gel pieces were processed using a robot (ProGest; Digilab, Marlborough, MA) as described (Gokhale et al., 2012a; Perez-Cornejo et al., 2012).

Transient protein knockdown and immunoblot analysis
For shRNA-mediated protein knockdown, constructs in the pLKO.1 vector were obtained from Open Biosystems (Huntsville, AL). Clone IDs for the targeting constructs are as follows: muted (Bloc1s5), TRCN0000129117; palladin (Bloc1s6), TRCN0000122781; strumpellin, TRCN0000128018; and RhoGEF1, TRCN0000035367. A scramble control vector was obtained from Addgene (Cambridge, MA; Vector 1864). HEK293T cells seeded in six-well plates were transfected with 1 μg of shRNA construct DNA overnight by Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. MNT1 and SHSY-5Y cells were seeded in six-well plates and treated with 1–2 μl of high-titer lentiviral particles containing the appropriate lentiviral construct. The Emory NINDS Viral Vector Core prepared all high-titer lentiviruses. Two days after delivery of shRNA construct, transfected cells were selected by treatment with 2 μg/ml puromycin (Invitrogen) in the appropriate growth media as described. The total length of treatment was 5–10 d to allow for efficient knockdown of the proteins of interest. Small interfering RNA (siRNA) oligonucleotide sequences against PI4KIIα and controls, as well as procedures, were described previously (Craige et al., 2008; Salazar et al., 2009).

For immunoblot analysis of protein levels, cells were grown to confluence in six-well plates. Cells were treated with lentiviruses for 7 d. Plates were rapidly moved to an ice bath and immediately washed two times in ice-cold PBS. Cells were then lifted in buffer A and spun at 16,100 ×g for 1 min to pellet. MNT1 cells were incubated for 10 min in buffer A before lifting to facilitate release from the tissue culture plate. Cell pellets were resuspended in 100 μl of buffer A supplemented with Complete antiprotease. Cells were then lysed by sonication (three rounds of five 1-s bursts). Lysates were diluted to 1 mg/ml and resolved by SDS–PAGE and immunoblotting. From 5 to 15 μg of cell lysate was loaded per well.

Immunofluorescence microscopy

HEK293T cells were transfected in six-well plates with 1 μg of the DNA construct by Lipofectamine 2000 for 4 h and then switched into growth medium. After 24 h, cells were seeded on glass coverslips coated with Matrigel. The next day, coverslips were moved to ice, washed two times in ice-cold PBS, and then fixed with 4% paraformaldehyde in PBS for 20 min. For cells stained with the A488 MitoTracker dye (Invitrogen), cells were incubated with 500 nM MitoTracker dye diluted in growth medium for 30 min. After this incubation, cells were immediately fixed at 37°C in 4% paraformaldehyde. After fixation, all coverslips were washed two times in PBS and then blocked and permeabilized for 30 min at room temperature in a solution of 0.02% saponin (Sigma-Aldrich, St. Louis, MO), 15% horse serum (HyClone), 2% bovine serum albumin (Roche), and 1% fish skin gelatin (Sigma-Aldrich) in PBS. Primary antibodies (Supplemental Table S3) were diluted in blocking solution and applied for 30 min at 37°C. After primary antibody incubation, coverslips were washed three times in blocking solution. Coverslips were incubated for 30 min at 37°C with fluorophore-conjugated secondary antibodies (Supplemental Table S3) diluted in blocking solution. Coverslips
were washed two times in blocking solution, one time in PBS, and one time in ultrapure water and then mounted on glass slides in Gelvatol mounting medium (Sigma-Aldrich).

For fixed-cell deconvolution microscopy, images were collected on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany) with a Sedat filter set (Chroma Technology, Brattleboro, VT). Slidebook 4.0 OSX software was used to run a multilayered, wide-field, three-dimensional microscopy system (Intelligent Imaging Innovations, Denver, CO). Images were visualized with a 100x oil differential interference contrast objective with numerical aperture 1.4 and captured using a scientific-grade cooled, charge-coupled Cool-Snap HQ camera (Photometrics, Tucson, AZ) with an ORCA-ER chip (Hamamatsu Photonics, Hamamatsu, Japan). Images were captured with 200 nm between z-planes. A nearest-neighbor, constrained iterative deconvolution algorithm with Gaussian smoothing was used to remove out-of-focus light (Swedlow et al., 1997, 2002). Images were exported from the Slidebook 4.0 software as 8-bit TIF series. For colocalization analysis, in-focus image planes were background subtracted with a 10-pixel rolling-ball algorithm in Fiji (ImageJ; National Institutes of Health, Bethesda, MD). A region of interest immediately surrounding individual cells was selected, and the Pearson coefficient was calculated by the Coloc 2 plug-in. Three-dimensional reconstructions were created from deconvoluted 8-bit TIF series images imported into Imaris 6.3.1 software using manual thresholding and a minimal volume limit of 4 voxels (Bitplane Scientific Software, Zurich, Switzerland).

For live-cell imaging of EGFP-tagged PI4KIIα and mCherry-tagged LifeAct peptide, HEK293T cells expressing fluorescently tagged protein constructs were seeded in Matrigel-coated glass-bottom culture dishes (Matek Corporation, Ashland, MA). Before imaging, cells were maintained in HEK293T growth media as described at 37°C and 10% CO2. Immediately before imaging, an imaging medium consisting of Hank’s balanced salt solution minus phenol red and NaHCO3 (Sigma-Aldrich) supplemented with 10% FBS (HyClone) was applied. Cells were imaged using a Nikon TE2000 inverted microscope (Nikon Instruments, Melville, NY) equipped with a hybrid scanner, Perfect Focus, and an environmental chamber for temperature regulation to 37°C and 5% CO2. Images were captured with a 100x, 1.49 numerical aperture oil objective and 500–550/570–620 filter sets using NIS-Elements AR 3.1 (Nikon) software. Cells were alternately excited with 488- and 568-nm-wavelength lasers every 4.25 s, and a single confocal plane image was captured. Images were exported as an 8-bit TIF series in ImageJ (National Institutes of Health, Bethesda, MD). A region of interest immediately surrounding individual cells was selected, and back-projection analysis was performed. Images were exported as an 8-bit TIF series images imported into Imaris 6.3.1 software using manual thresholding and a minimal volume limit of 4 voxels (Bitplane Scientific Software, Zurich, Switzerland).

Surface labeling and streptavidin pull-downs
After 7 d treatment with scramble or strumpellin-targeted lentiviruses, plates of confluent MNT1 cells were moved to an ice bath and washed two times with ice-cold PBS-Ca-Mg (0.1 mM CaCl2 and 1 mM MgCl2 in PBS). All solutions used were ice-cold when applied to cells, and cells were maintained on an ice bath throughout. After washes, cells were incubated in a 1 mM sodium periodate (Sigma-Aldrich) solution prepared in PBS-Ca-Mg for 30 min, after which the periodate solution was removed and a quenching solution of 1 mM glycerol in PBS-Ca-Mg was added (Zeng et al., 2009). Cells were then washed two times with PBS-Ca-Mg, and a biotin ligation solution of 100 μM aminooxy biotin (Biotium, Hayward, CA) and 10 mM aniline (Sigma-Aldrich) was applied for 90 min. After biotin ligation, cells were washed two times in PBS-Ca-Mg and lysed for 30 min in buffer A with 0.5% Triton X-100 supplemented with Complete antiprotease. This biotinylation procedure is based on a periodate-generated aldehyde on sialic acids. This process is catalyzed by aniline-dependent ligation with an appropriate tag. Aniline catalysis accelerates ligation, facilitating the reaction with low concentrations of aminooxy-biotin at neutral pH to label the most cell-surface sialylated glycoproteins (Zeng et al., 2009). After lysis, cell debris were scraped from the plates, and lysates were spun at 16,100 × g for 15 min. The supernatant was recovered and diluted to 0.2 mg/ml. A small volume (50 μl) of NeutrAvidin-coated agarose beads (Thermo Scientific, Waltham, MA) was prewashed two times in buffer A with 0.1% Triton X-100, and 100 μg of cell lysate was incubated with the beads for 2 h with end-over-end rotation at 4°C. Beads were then washed five times in buffer A with 0.1% Triton X-100 for 5 min with end-over-end rotation at 4°C. Proteins were eluted from the beads by boiling in Laemmli sample buffer at 75°C for 5 min, and samples were analyzed by SDS-PAGE followed by silver stain or immunoblot.
Computational and statistical analysis

Functional gene annotation for the set of PI4KIIx-interacting proteins (Supplemental Table S2) was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.7) and gene ontology (GO) terms (Dennis et al., 2003; Huang da et al., 2007). The Cytoscape plug-in Enrichment Map was used to display overlap between gene ontology terms within Cytoscape (version 2.8.3; Merico et al., 2010). Node size was mapped to number of genes within a GO category, and node color was mapped to the p value for enrichment of a given category. For network analysis, a network interaction map was built using GeneGo Metacore (version 6.11, build 41105) for PI4KIIx-interacting proteins (Supplemental Table S1) and BIOC-1 subunits. The network was visualized in Cytoscape, and node color was mapped to proteins of interest.

Experimental conditions were compared using KaleidaGraph, version 4.1.3 (Synergy Software, Reading, PA), or StatPlus Mac Built6.0.pre/Universal (AnalystSoft, Vancouver, Canada). Data are represented as box plots displaying the four quartiles of the data, with the “box” comprising the two middle quartiles, separated by the median. The upper and lower quartiles are represented by the single lines extending from the box.

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