Scyl1 scaffolds class II Arfs to specific subcomplexes of coatamer through the γ-COP appendage domain

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ABSTRACT

Coatomer (COPI)-coated vesicles mediate membrane trafficking in the early secretory pathway. There are at least three subclasses of COPI coats and two classes of Arf GTPases that couple COPI coat proteins to membranes. Whether mechanisms exist to link specific Arfs to specific COPI subcomplexes is unknown. We now demonstrate that Scyl1-like protein 1 (Scyl1), a member of the Scyl1-like family of catalytically inactive protein kinases, oligomerizes through centrally located HEAT repeats and uses a C-terminal RKXX-CCR motif to interact directly with the appendage domain of coatomer subunit γ-2 (also known as COPG2 or γ2-COP). Through a distinct site, Scyl1 interacts selectively with class II Arfs, notably Arf4, thus linking class II Arfs to γ2-bearing COPI subcomplexes. Therefore, Scyl1 functions as a scaffold for key components of COPI coats, and disruption of the scaffolding function of Scyl1 causes tubulation of the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) and the cis-Golgi, similar to that observed following the loss of Arf and Arf-guanine-nucleotide-exchange factor (GEF) function. Our data reveal that Scyl1 is a key organizer of a subset of the COPI machinery.

KEY WORDS: Coatomer, Arf4, Scyl1, γ-COP, ERGIC-53, Tubulation, Early secretory pathway

INTRODUCTION

Coatomer (COPI)-coated vesicles mediate membrane trafficking in the early secretory pathway. There are at least three subclasses of COPI coats and two classes of Arf GTPases that couple COPI coat proteins to membranes. Whether mechanisms exist to link specific Arfs to specific COPI subcomplexes is unknown. We now demonstrate that Scyl1-like protein 1 (Scyl1), a member of the Scyl1-like family of catalytically inactive protein kinases, interacts selectively with class II Arfs, notably Arf4, thus linking class II Arfs to γ2-bearing COPI subcomplexes. Therefore, Scyl1 functions as a scaffold for key components of COPI coats, and disruption of the scaffolding function of Scyl1 causes tubulation of the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) and the cis-Golgi, similar to that observed following the loss of Arf and Arf-guanine-nucleotide-exchange factor (GEF) function. Our data reveal that Scyl1 is a key organizer of a subset of the COPI machinery.

KEY WORDS: Coatomer, Arf4, Scyl1, γ-COP, ERGIC-53, Tubulation, Early secretory pathway

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Arfs. Arf4 and COPI use non-overlapping binding sites on Scyl1; thus, Scyl1 is well placed to couple COPI and Arf4 on ERGIC membranes. Remarkably, Scyl1 uses a C-terminal RKXX-COO motif to interact directly with the appendage domain of γ2-COP, and it associates predominantly with γ2-bearing COPI subcomplexes. Therefore, Scyl1 scaffolds class II Arfs to a γ2-COP-positive population of COPI, and the disruption of this scaffolding function by Seyl1 overexpression or knockdown causes ERGIC tubulation, similar to what is seen following the disruption of Arf4 and Arf1 (Szul et al., 2007; Ben-Tekaya et al., 2010). Taken together, our studies suggest that Seyl1 plays a role in COPI-mediated retrograde transport in the early secretory pathway and implicate Scyl1 function in a system that is regulated by γ2-COP and Arf4.

RESULTS
Scyl1 interacts specifically with class II Arfs
In a screen to identify interaction partners for Scyl1, we used GST-tagged full-length Scyl1 in affinity-selection assays. As expected, mass spectrometry analysis of affinity-selected proteins revealed components of COPI coats. We also detected the small GTPase Arf4, which, along with Arf5, is member of the class II family of Arfs that were recently demonstrated to function in COPI-vesicle formation (Popoff et al., 2011). To further examine the specificity of the Arf4 interaction with Scyl1, we used affinity-selection assays with western blots. Owing to the lack of availability of antibodies against specific Arf isoforms, we used constructs encoding C-terminally tagged fluorescent Arf proteins, which are widely used for the study of Arfs (Chun et al., 2008; Duijsings et al., 2009; Jain et al., 2012; Kudelko et al., 2012). Scyl1 bound specifically to class II Arfs, with a preference for Arf4, but did not bind to class I Arfs (Fig. 1A,B). GFP-tagged forms of both Arf4 and Arf1 bound well to the GAT domain of the GGA3 protein (supplementary material Fig. S1), which is known to bind to both class I and class II Arfs (Dell’Angelica et al., 2000; Takatsu et al., 2002), indicating that the Arf-GFP constructs were not simply misfolded, and further highlighting the specificity of Scyl1 for class II Arfs.

Like other GTPases, Arfs cycle between active GTP-bound configurations and inactive GDP-bound configurations. In the active form, Arfs bind to effectors such as coat proteins and lipid-modifying enzymes, bringing them to the membrane. We sought to determine whether Scyl1 is an Arf4 effector; that is, whether it would preferentially bind to GTP-loaded Arf4. Cells were transfected with wild-type Arf4–GFP, Arf4-T31N–GFP (inactive) (Chun et al., 2008), Arf4-T48N–GFP and Arf4-Q71L–GFP (GTP-locked) (Lowery et al., 2010). Taken together, our studies suggest that Seyl1 plays a role in COPI-mediated retrograde transport in the early secretory pathway and implicate Scyl1 function in a system that is regulated by γ2-COP and Arf4.

Scyl1 colocalizes with Arf4 and COPI at the ERGIC
We next sought to better define the compartment where Scyl1 and Arf4 colocalize. The peripheral Scyl1-positive Arf4 punctae were also positive for ERGIC-53 (also known as LMAN1, a marker of the ERGIC) and for β-COP (Fig. 2A,B). Thus, Scyl1, Arf4 and COPI interact biochemically and colocalize at the ERGIC. We next used super-resolution microscopy to better characterize the nature of the peripheral Scyl1 punctae. Interestingly, an analysis of Scyl1 by using stimulated emission depletion (STED) microscopy revealed that it localized to a subset of COPI structures, decorating a large portion of the roughly 250–500-nm structures (Fig. 2C, arrowheads), which were slightly larger in size than ERGIC structures (Klumperman et al., 1998). However, Scyl1 was absent from other COPI-positive structures (Fig. 2C, arrows). Therefore, Scyl1 interacts with a subset of COPI coats in structures that are morphologically similar to the ERGIC.

Residues on the opposite side of the nucleotide-binding pocket of Arf4 mediate Scyl1 interaction
Despite the high degree of amino acid conservation between human Arf4 and Arf5 (Fig. 3A; only 18 non-identical residues, shown in bold), the binding of Scyl1 to Arf4 is approximately three times greater than to Arf5 (Fig. 1B). Thus, surface residues on Arf4 that are unique to this isoform are likely to contribute to the interaction. Using the available Arf5 crystal structure (PDB ID 2B6H), we identified non-identical residues that are surface exposed (Fig. 3A,B, bold and shaded). We then mutated Arf4 to the Arf5 residue in these positions, generating Arf4-LV112QE–GFP, Arf4-AI135PV–GFP and Arf4-N150S–GFP. GST–Scyl1 was incubated with cell lysates that contained Arf4–GFP, Arf5–GFP or the Arf4–GFP mutants, and proteins that interacted specifically with Scyl1 were identified by western blotting (Fig. 3C). The interaction of GST–Scyl1 with Arf4-LV112QE and Arf4-AI135PV was reduced to a level similar to that seen with Arf5, whereas the Arf4-N150S mutant interacted with GST–Scyl1 at a level that was not significantly different from the Arf4 wild type (Fig. 3D). Thus, select residues on the surface of Arf4 that are opposite to the nucleotide-binding pocket are crucial for the interaction of the GTPase with Scyl1 (Fig. 3B). These data are consistent with the observation that Arf4 interacts with Scyl1 in a manner that is independent of the nucleotide-bound state (Fig. 1C,D).

Arfs interact directly with COPI (Zhao et al., 1997), and we therefore questioned whether Scyl1 interacts with Arf4 indirectly, through a complex with COPI. Scyl1 binds COPI primarily through a dibasic cargo-like motif (RKLD–COO−) at the extreme C-terminus (Ma and Goldberg, 2013), but a C-terminal coiled-coil domain also contributes to binding (Burman et al., 2008). We thus took advantage of the Scyl1 truncation mutant GST–Scyl1 isoforms (Arf1, Arf3, Arf4 or Arf5) for 18–22 h, and were then fixed and stained for endogenous Scyl1, which was visualized by using indirect immunofluorescence. Given the lack of antibodies against specific Arf isoforms, Arf–GFP constructs have been widely used to examine the localization of Arfs (Vasudevan et al., 1998; Chun et al., 2008; Manolea et al., 2010). Scyl1 colocalized with Arf4 and Arf5, especially in cytoplasmic punctae that were distributed throughout the cell (Fig. 1G,H). There was less colocalization with Arf1 and Arf3 at peripheral punctae, and these two GTPases were found primarily at the Golgi (Fig. 1E,F). Taken together, the data in Fig. 1 indicate that Scyl1 interacts with class II Arfs, notably Arf4.
1–750 (Fig. 3E), which does not bind COPI (Burman et al., 2008), to test the potential involvement of COPI in mediating the interaction between Scyl1 and Arf4. Whereas full-length GST–Scyl1 (1–806) interacted with both Arf4–GFP and endogenous β-COP in lysates from Arf4–GFP-transfected cells, GST–Scyl1 1–750 retained full Arf4 interaction but showed no β-COP binding (Fig. 3F). However, because it is known that Arfs interact with COPI, it is possible that COPI will show a small amount of binding to Scyl1 1–750 mediated by the bound Arf4. To test this possibility, we co-expressed Myc–γ2-COP with Arf4–GFP or GFP alone, and repeated the pull down with wild-type Scyl1 and the 1–750 mutant. Under these conditions, we detected the binding of γ2-COP to Scyl1 1–750 only in the presence of Arf4–GFP (supplementary material Fig. S2). These data indicate that the interaction of Scyl1 with Arf4 is not indirect through COPI, that COPI and Arf4 engage different sites on Scyl1, and that Arf4 can bind COPI when engaged with Scyl1. Coupled with the observation that Scyl1 binds to Arf4 on the opposite side of the nucleotide-binding pocket, and that the nucleotide-binding region of Arf interacts with coatomer (Yu et al., 2012), these data suggest that Scyl1, COPI and Arf4 form a tripartite complex that would help organize COPI coats on the ERGIC membrane.
Scyl1 oligomerizes through centrally located HEAT repeats

Epitope-tagged Scyl1 expressed in COS-7 cells runs at ~270 kDa on SDS-PAGE following cross-linking (Kato et al., 2002). Although Scyl1 might be cross-linked to other proteins in this experiment, given that it is overexpressed, and thus likely to be present at higher levels than its binding partners, these data are consistent with Scyl1 forming a trimer (Scyl1 has a molecular mass of ~90 kDa) (Kato et al., 2002). Gel-filtration chromatography on soluble extracts of HEK-293T cells overexpressing FLAG–Scyl1 revealed a peak of Scyl1 (Fig. 4A, asterisk) corresponding to a native molecular mass of a globular 412-kDa protein (calculated based on known globular markers, indicated by arrows). Thus, the migration of native Scyl1 is consistent with a pentamer, assuming that it has a fully globular structure and that additional interacting proteins are not contributing to the size of the native protein. The migration of Scyl1 is greater than that of the heterotetrameric AP-2 complex (Fig. 4A, γ-adaptin), which is composed of two ~110 kDa subunits and two subunits of ~50 kDa and ~20 kDa, but is less than that of clathrin triskelia (CHC, which comprises three ~170 kDa subunits with lengthy non-globular regions). We suggest that FLAG–Scyl1, with a globular N-terminal kinase domain and a partially unstructured C-terminal region, oligomerizes into structures ranging from trimers to pentamers.

HEAT repeats mediate protein–protein interactions and act as scaffolds in protein complexes (Groves and Barford, 1999). We therefore hypothesized that the HEAT repeats of Scyl1 are responsible for oligomerization. To test this, we performed a series of co-immunoprecipitation experiments using FLAG- and GFP-tagged Scyl1 constructs. Cells were transfected with GFP only, GFP–Scyl1 342–538 (GFP–HEAT) or full-length GFP–Scyl1, and were co-transfected with full-length FLAG–Scyl1, FLAG–Scyl1 349–806 or FLAG–Scyl1 547–806. Immunoprecipitation of full-length GFP–Scyl1 or GFP–HEAT with anti-GFP antibodies led to co-immunoprecipitation of FLAG–Scyl1 (Fig. 4B,C). The kinase-like deletion construct (FLAG–Scyl1 349–806) co-immunoprecipitated to the same extent as full-length FLAG–Scyl1, but a construct lacking the kinase-like domain and the HEAT repeats (FLAG–Scyl1 547–806) did not co-immunoprecipitate (Fig. 4B,C). These data demonstrate that the region of Scyl1 encoding the HEAT repeats mediates the co-immunoprecipitation and therefore the homo-oligomerization of Scyl1.

Scyl1 interacts preferentially with γ2-COP

There are multiple pools of COPI vesicles containing different heterotetrameric F-subcomplexes that are composed of different isoforms of γ- and ζ-COP – specifically γ1β1δ1ζ1, γ1β2δ1ζ1 and γ2βζ1ζ-COP variants (Wegmann et al., 2004). Because Scyl1 interacts specifically with class II Arfs (Fig. 1A), we wondered whether Scyl1 links the regulatory circuit of class II Arfs to an isoform-specific pool of coatamer vesicles. To test this hypothesis, we transfected HEK-293T cells with GFP–Scyl1, and then immunoprecipitated GFP (Fig. 5A). It was necessary to use...
the tagged protein because our antibody against endogenous Scyl1 was raised against the C-terminus containing the COPI-binding motif. As expected, the GFP–Scyl1 immunoprecipitate was positive for β-COP (Fig. 5B). Intriguingly, GFP–Scyl1 co-immunoprecipitated endogenous γ2-COP but γ1-COP was co-immunoprecipitated to a lesser degree (Fig. 5B). This suggests that Scyl1 interacts with the heterotetrameric γβζδ-COP F-subcomplex, and more specifically with the γ2βζδ-COP variant. The γβζδ-COP subcomplex is structurally similar to the AP-2 complex of clathrin-coated vesicles, with γ-COP most similar to α-adaptin (Hoffman et al., 2003). The appendage domain of α-adaptin interacts with various accessory proteins that regulate the formation of clathrin-coated vesicles. We hypothesized that the appendage domain of γ2-COP mediates the interaction of Scyl1 with COPI coats and, as such, confers specificity for the γ2βζδ-COP variant. We thus used GST-tagged γ1-appendage and γ2-appendage in affinity-selection assays with lysates from cells expressing FLAG–Scyl1 (Fig. 5C). Interestingly, Scyl1 bound to the γ-appendage domains with a preference for γ2 versus γ1 (Fig. 5C). The appendage domains and FLAG–Scyl1 are readily detectable by Ponceau S staining, with no other proteins at comparable levels, supporting the hypothesis that the interaction is direct (Fig. 5C). We next used FLAG–Scyl1 constructs to map the interaction with the γ2-appendage. HEK-293T cells were transfected with FLAG–Scyl1 349–806 (a kinase-like-domain deletion construct), FLAG–Scyl1 547–806 [which removes the kinase-like domain and the HEAT repeats necessary for oligomerization (Fig. 5E)] or FLAG–Scyl1 full-length RK→AA mutant [which reduces coatomer binding (Burman et al., 2008; Ma and Goldberg, 2013)]. GST–γ2-appendage bound the kinase-deletion protein with the same enrichment as full-length Scyl1 (Fig. 5D,E). FLAG–Scyl1
547–806 also bound, but with less affinity than that of full-length protein. The FLAG–Scyl1 full-length RK→AA mutant did not bind the GST–γ2 appendage (Fig. 5D,E). Thus, Scyl1 uses the RKXX-COO² motif to bind directly to the γ2-appendage domain with stronger binding when the protein contains HEAT repeats and is presumably oligomerized. It thus appears that Scyl1 links class II Arfs in an oligomeric form to a γ2-coatomer-positive pool of COPI.

**Disruption of Scyl1 scaffolding function induces tubulation of the ERGIC**

Overexpression of Scyl1 should alter the stoichiometry of the scaffolding complex, with the potential to alter membrane budding at the cis-Golgi and/or ERGIC. We used live-cell imaging to study the effect of the expression of Scyl1 on ERGIC dynamics. In HeLa cells transfected with YFP–ERGIC-53 and low levels of mCherry, the YFP signal was seen in punctae. Highly dynamic YFP-positive tubules extended from the punctae and moved towards the center of the cell, towards the periphery or laterally between YFP punctae that were roughly the same distance from the nucleus (Fig. 6A; supplementary material Movie 1). The observed behavior of YFP–ERGIC-53 is similar to previous descriptions of tagged ERGIC-53 (Appenzeller-Herzog and Hauri, 2006). By contrast, in cells expressing mCherry–Scyl1, the YFP–ERGIC-53 signal was significantly disrupted (Fig. 6B; supplementary material Movie 2). The expression of fluorescently tagged Scyl1 often caused the formation of fluorescent cytoplasmic inclusions, in addition to the fainter fluorescent signal similar to the distribution of fixed endogenous Scyl1. YFP–ERGIC-53-positive tubules connected juxta-nuclear and peripheral punctae. These tubules were mostly static, remaining connected between punctae over several minutes and occasionally breaking off or forming new tubules. In addition, we observed that mCherry–Scyl1 appeared to be absent from tubules and was associated predominantly with the punctae.

We performed quantification on fixed cells, revealing that there were more and longer ERGIC-53 tubules in GFP–Scyl1 expressing cells than in cells expressing only GFP (Fig. 6C,D). Scyl1 knockdown also induced tubulation, as determined by staining for endogenous ERGIC-53 (supplementary material Fig. S3). This corroborates a screen in which disruption of the early secretory pathway was examined following the...
knockdown of putative kinases. The study revealed that Scy1 knockdown causes tubulation of the ERGIC (Farhan et al., 2010). Thus, disruption of the scaffolding function of Scy1 by the overexpression or loss of function of Scy1 leads to alterations in vesicle budding at the ERGIC and the cis-Golgi, with the formation of abnormally long and static tubules.

**DISCUSSION**

We originally identified the pseudo-kinase Scy1 as a COPI-interacting protein that functions in COPI-mediated retrograde trafficking (Burman et al., 2008). Scy1 interacts with COPI through a C-terminal RKXX-COO⁻ dibasic motif, similar to many other COPI-binding proteins (Nilsson et al., 1989; Jackson et al., 1993). Pseudo-kinases containing multiple protein domains often act as scaffolds. Here, we demonstrate that Scy1 uses the dibasic motif to bind directly to the appendage domain of γ-COP, with an isoform preference for γ2-COP. Moreover, Scy1 forms oligomers and, through a site distinct from the COPI-binding motif, interacts with Arf4 but not with the class I Arfs. Thus, Scy1 links an Arf4-regulated pathway to a specific class of COPI vesicles.

The specificity of Scy1 for class II Arfs is interesting given the high degree of sequence similarity between class II Arfs and other Arf isoforms. Most studies of Arf function have focused on Arf1 and Arf6, and much less is known regarding the function of class II Arfs. Homozygous Arf4 deletion in mice is embryonic lethal, whereas Arf4 heterozygotes have a neurological phenotype that is characterized by a reduction in the number of dendritic spines in the dentate gyrus, with consequent deficiencies in related behavioral tasks (Jain et al., 2012). The role that Arf4 plays in development or neuronal survival has not been tested, but because mdf mice also have a severe neurological deficit with motor neuron degeneration (Blot et al., 1995), and Arf4 and Scy1 interact biochemically, we speculate that there is an underlying relationship between the two phenotypes.

GDP-bound Arfs are cytosolic and the myristoylated N-terminal amphipathic helix is necessarily shielded from the aqueous environment by packing against the globular protein (Liet et al., 2010). It is thought that Arfs are first recruited to membranes in a GDP-bound form by a membrane-associated receptor, and that they are subsequently activated by Arf-GEFs.
For example, Arf1–GDP is recruited to membranes by the cytosolic domain of p23 (also known as TMED10) (Gommel et al., 2001) or by the ER-Golgi SNARE membrin (Honda et al., 2005). A class II Arf receptor has not been described (Chun et al., 2008), but is predicted to remain associated with ERGIC and Golgi membranes in the presence of brefeldin A (BFA), and to interact with class II Arfs in their GDP-bound form. Although Scyl1 has these properties (Burman et al., 2008; this study), and thus fits many criteria of a class II Arf receptor, we have been unable to directly demonstrate an influence of Scyl1 knockdown on the membrane recruitment of Arf4–GFP, and therefore the full understanding of this potential role awaits further study.

The Scyl1 C-terminal RKLD–COO sequence was recently co-crystalized with b9-COP (Ma and Goldberg, 2013), a subunit of the heterotrimeric ab9e-COP B-subcomplex that forms the outer shell of COPI vesicles. Here, we find that Scyl1 interacts with the appendage of c2-COP, a component of the heterotetrameric γ2βcδ-COP F-subcomplex, and that this interaction is also mediated by the RKLD–COO sequence, because mutation to AALD–COO abolishes the interaction. The multiple binding sites for Scyl1 on COPI were predicted previously, as a Scyl1 C-terminal peptide competitively inhibits an interaction between p24a2 (also known as TMED9) and COPI, but a p24a2 dibasic peptide does not interfere with the interaction between Scyl1 and COPI (Burman et al., 2008). Scyl1 oligomerizes through the centrally located HEAT repeats. This creates a scaffold with three to five RKLD–COO sequences and, as such, Scyl1 might not simply organize COPI coats on the membrane but could also contribute to biochemically linking ab9e- and c2bf1d-COP subcomplexes and/or linking the γ2βcδ-COP subcomplex to other γ2βcδ-COP subcomplexes, through the γ2-appendage. The ability to rapidly polymerize and depolymerize is a central feature of vesicle-coat proteins, and Scyl1 oligomers likely have an important role in promoting COPI-coat polymerization, perhaps in different configurations (Faini et al., 2012).

Knockdown of Scyl1 has been shown previously to cause tubulation of the ERGIC (Farhan et al., 2010). Here, we confirm that Scyl1 knockdown induces ERGIC tubulation. Moreover, we demonstrate that the overexpression of fluorescently tagged Scyl1 (which generates Scyl1-positive inclusions that likely sequester binding partners including endogenous Scyl1, Arf4 and coatomer) induces robust tubulation of both the ERGIC and the cis-Golgi. Considering that Arf4 is sufficient for Arf-dependent COPI-vesicle formation (Popoff et al., 2011), we speculate that the tubulation that is observed upon disruption of Scyl1 function results from an impairment in the ability of COPI cargo carriers to Fig. 6. Overexpression of Scyl1 tubulates ERGIC-53-containing membranes in living cells. (A) HeLa cells were plated onto coverslips on 35-mm dishes (MatTek) and were transfected with mCherry and YFP–ERGIC-53 for 18–22 h. Cells were imaged over the course of 5 min and a representative field is shown. The two upper panels are the first frames of the movie (supplementary material Movie 1) and the lower panels are a 5× magnified view of the box in the upper panel of the YFP–ERGIC-53 channel at the indicated time points. (B) HeLa cells were plated onto coverslips on 35-mm dishes (MatTek) and transfected with mCherry–Scyl1 and YFP–ERGIC-53 for 18–22 h. Cells were imaged over the course of 5 min and a representative field is shown. The two upper panels are the first frames of the movie (supplementary material Movie 2) and the lower panels are a 5× magnified view of the box in the upper panel of the YFP–ERGIC-53 channel at the indicated time points. Scale bars: 10 μm (upper panels), 2 μm (lower panels). (C) The mean (± s.e.m.) number of endogenous ERGIC-53 tubules per fixed cell expressing GFP only (n=15) or GFP–Scyl1 (n=16) was quantified by using the ImageJ (NIH) ‘measure’ function. (D) The mean (± s.e.m.) ERGIC-53 tubule length was quantified as in C. (E) The mean (± s.e.m.) number of endogenous GM130 tubules per cell expressing GFP only (n=14) or GFP–Scyl1 (n=15) was quantified as in C. (F) The mean (± s.e.m.) GM130 tubule length was quantified as in C.
vesiculate and bud from the membrane. We observed previously that the knockdown of Scyl1 alters Golgi morphology (Burman et al., 2010), and the phenotype is therefore most likely due to Golgi tubulation.

Taken together, our data biochemically link multiple early-secretory-tubulation phenotypes to Scyl1, and further our understanding of COP1 regulation. As Scyl1 is the gene product that is lost in the mdf mouse, these findings expand our knowledge of the pathways and potential mechanisms underlying selective motor neuron death.

MATERIALS AND METHODS

Cell culture

Mammalian cells were cultured using standard procedures. Cells were grown at 37°C under 5% CO2. Cells were grown in DME (High Glucose, L-Glutamine, Phenol Red) liquid medium plus 10% bovine calf serum (Gibco). Cells were grown on poly-L-lysine cell culture plates or coverslips.

Constructs and proteins

Bacterially expressed GST–Scyl1 constructs were cloned into pGEX-4T-1 (Clontech) and were expressed in Escherichia coli BL21. Mammalian-expressed tagged-Scyl1 constructs were PCR amplified from mouse Scyl1 DNA (Burman et al., 2008) into N-terminally tagged pCMV-Tag2B, pEGFP-C2 or mCherry-C2 (mCherry inserted into AgeI and BsrGI digested pEGFP2C). Arf1–GFP, Arf3–GFP, Arf4–GFP and Arf5–GFP in pEGFP-N1 were as described previously (Chun et al., 2008). Arf4 mutants were generated from wild-type human Arf4 using PCR mutagenesis and were cloned into pEGFP-N1. Human GST–γ1/COP and GST–γ2/COP (Moelelen et al., 2007) were expressed in E.coli BL21. Mouse Myc–γ2-COP was expressed in pcDNA3.1. All plasmids were verified by sequencing.

Antibodies and reagents

Affinity-purified rabbit polyclonal antibody was raised against the C-terminal amino acids (KTFTKGPMKLGLKGLD–COO−) of mouse Scyl1. Polyclonal antibodies against γ1-COP and γ2-COP were kindly provided by Felix Wieland (Heidelberg University, Heidelberg, Germany). Affinity-purified rabbit polyclonal antibody was raised against human clathrin heavy chain (amino acids QEHLQQLNGLINPANIGFS). Polyclonal antibodies against GFP and monoclonal antibody against FLAG epitope (M2) were from Invitrogen (Carlsbad, CA) and Sigma (St Louis, MO), respectively. Monoclonal antibodies against β-COP and GFP (which were used to verify polyclonal GFP immunoprecipitation) were obtained from Abcam (Cambridge, MA). Monoclonal antibodies against GAPDH, ERGIC-53 and GM130 were from Santa Cruz Biotechnology (Santa Cruz, CA), ENZO Life Sciences (Farmingdale, NY) and BD Labs (Franklin Lakes, NJ), respectively. HeLa cells were transfected for localization and tubulation assays with JetPrime reagent (Polyplus Transfection; Illkirch, France). Mouse and rabbit antibodies conjugated to Alexa Fluor 488, 568 or 647 that were used for indirect immunofluorescence were obtained from Abcam.

Affinity selection and co-immunoprecipitation assays

For GST fusion proteins, bacteria were lysed in PBS containing 1 mM DTT, 0.83 mM benzanidine, 0.23 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin (protease inhibitors), pH 7.4. For GST–GGA3 pulldowns, bacteria were lysed in 50 mM Tri-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, 0.1% SDS, 0.5% deoxycholate (DOC), 1% Triton X-100, 5% glycerol and protease inhibitors as above. For affinity-selection assays, HEK-293T cells were collected in lysis buffer (20 mM HEPES pH 7.4, protease inhibitors, 1 mM MgCl2, 30 nM okadaic acid, 5 mM sodium pyrophosphate and 1 mM DTT) and Triton X-100 was added to a final concentration of 1%. Following rocking for 10 min at 4°C, the extracts were spun at 205,000 g for 15 min to remove insoluble material. Aliquots of 1 ml were incubated for 1 h at room temperature with GST or GST–Scyl1 fusion proteins that were pre-coupled to glutathione–Sepharose beads (GE Healthcare). The beads were subsequently washed three times in lysis buffer with 1% Triton X-100 and were analyzed by SDS-PAGE. For nucleotide-specificity assays, 50 μM aluminium fluoride or 50 μM GMPP(NH)P was added to cell lysis buffer as indicated. Bands specifically interacting with GST–Scyl1 fusion proteins were excised and analyzed by tandem mass spectrometry (MS) or were processed for western blotting.

Gel-filtration chromatography

HEK-293T cells were transfected with FLAG–Scyl1 by the calcium phosphate method and were incubated for 48 h. Plates of cells (2×15 cm) were collected in 5 ml of lysis buffer and the lysates were immediately run on a pre-calibrated HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare). Fractions were collected, run on SDS-PAGE gel and analyzed by western blot for anti-FLAG and the indicated proteins as internal controls.

Cellular imaging

For fixed-cell confocal imaging, cells were grown on poly-L-lysine-coated coverslips, were washed in PBS and then fixed for 20 min in 4% paraformaldehyde (PFA) at 4°C. After fixation, cells were permeabilized with 0.2% Triton X-100 in PBS for 2 min and were processed for immunofluorescence with the appropriate primary and secondary antibodies in PBS plus 0.01% BSA. Images were obtained by using a Zeiss (Thornwood, NY) 710 laser scanning confocal microscope. Single-slice confocal projections were obtained for each channel in the same focal plane and saved as LSM files before processing.

For STED imaging, normal rat kidney epithelial (NRK) cells were fixed in 4% PFA and permeabilized with 0.3% NP40 plus 0.1% Triton X-100. After immunolabeling, cells were post-fixed in 3% PFA plus 0.1% glutaraldehyde and then embedded using Ultra Bed Low Viscosity Epoxy Kit (EMS). Cells were then cut into 80-nm thin sections using a Leica Microsystems DiATOME Ultra 45 Diamond Knife. The sections were dried onto coverglass of thickness 1.5, mounted in Mowiol and imaged using a Leica TCS STED microscope featuring a puised diode laser (PDL 800-B, PicoQuant) emitting at 640 nm (~8–65 μW) for excitation and a Ti:Sapphire laser (Mai Tai, Spectra Physics) for depletion (~130 mW). ATTO-647N-labeled samples were depleted at 770 nm.

For live-cell imaging, HeLa cells were plated at 30% confluence on 35-mm plates (MatTek Corporation; Ashland, MA) with glass coverslips in 2 ml of DME plus 10% serum. Cells were transfected with mCherry constructs and YFP–ERGIC-53, and the plates were incubated for 20–22 h. Live-cell imaging was performed by using an Axio Observer Z1 microscope equipped with an epifluorescent plan-apochromat ×40 NA 1.4 oil objective, Definite Focus system and an AxioCam MR3 camera (Zeiss). Cells were kept at 37°C under 5% CO2 using the Incubation System S (Pecom, Germany). YFP and mCherry were illuminated using 470-nm and 591-nm laser lines, respectively, from a CoLiBr.2 illumination source (Zeiss). Acquisition and analysis was performed using the ZEN 11.0 software (Zeiss), and movies were made using ImageJ v1.43m.

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Competing interests

The authors declare no competing interests.

Author contributions

J.N.R.H., P.M. and P.S.M. conceived and designed the experiments. J.N.R.H., P.M. and P.S.M. wrote the paper.

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Supplementary material
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References
**Fig. S1. Arf-GFP constructs interact with GST-GGA3-GAT domain.** GST-tagged full-length GGA3-GAT domain or GST alone were incubated with HEK-293T cell lysates expressing Arf1-GFP or Arf4-GFP and specifically bound proteins were processed for Western blot with antibodies against GFP. An aliquot of the cell lysate (Input) equal to 1/20th of that added to the beads was processed in parallel.
Fig. S2. Scyl1-Arf4-COPI form a tri-partite complex. GST-tagged full-length Scyl1, GST-Scyl1 (1–750) or GST alone were incubated with HEK-293T cell lysates co-expressing GFP and myc-γ2-COP or Arf4-GFP and myc-γ2-COP and specifically bound proteins were processed for Western blot with antibodies against the indicated proteins. An aliquot of the cell lysate (Input) equal to 1/20th of that added to the beads was processed in parallel.
Fig. S3. Knock down of Scyl1 causes tubulation of the ERGIC. (A) HeLa cells were transfected with siRNA targeting Scyl1 or with non-specific (mock) siRNA for 72 h. The cells were mixed and reseeded onto the same coverslips for 24 h, fixed and processed for indirect immunofluorescence with antibodies against the indicated proteins. Bottom panel magnification of boxed region. Arrowheads indicate tubulation of endogenous ERGIC-53. Scale bars, 20 μm, 5 μm (magnification). (B) Cells analyzed in A were harvested in parallel and processed for western blot with an antibody against Scyl1 and Clathrin heavy chain (CHC).
Movie 1. Live cell imaging of YFP-ERGIC-53 in cells expressing mCherry alone. Movie corresponding to Fig. 6A.

Movie 2. Live cell imaging of YFP-ERGIC-53 in cells expressing mCherry-Scyl1. Movie corresponding to Fig. 6B.