The type V myosin-containing complex HUM is a RAB11 effector powering movement of secretory vesicles
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SUMMARY

In the apex-directed RAB11 exocytic pathway of Aspergillus nidulans, kinesin-1/ KinA conveys secretory vesicles (SVs) to the hyphal tip, where they are transferred to the type V myosin MyoE. MyoE concentrates SVs at an apical store located underneath the PM resembling the presynaptic active zone. A rod-shaped RAB11 effector, UDS1, and the intrinsically disordered and coiled-coil HMSV associate with MyoE in a stable HUM (HMSV-UDS1-MyoE) complex recruited by RAB11 to SVs through an interaction network involving RAB11 and HUM components, with the MyoE globular tail domain (GTD) binding both HMSV and RAB11-GTP and RAB11-GTP binding both the MyoE-GTD and UDS1. UDS1 bridges RAB11-GTP to HMSV, an avid interactor of the MyoE-GTD. The interaction between the UDS1-HMSV subcomplex and RAB11-GTP can be reconstituted in vitro. Ablating UDS1 or HMSV impairs actomyosin-mediated transport of SVs to the apex, resulting in spreading of RAB11 SVs across the apical dome as KinA/microtubule-dependent transport gains prominence.

INTRODUCTION

How the multiple functions of molecular motors are implemented within the crowded cytosol of a cell without causing an intracellular traffic jam constitutes a fundamental question of cell biology. Across the eukaryotic realm, type V myosins play a key role in the transport of membranous cargoes, often acting in concert with microtubule-dependent motors (Hammer and Sellers, 2012). For example, in the RAB11 pathway of the filamentous fungus Aspergillus nidulans, a single type V myosin (denoted MyoE) and a kinesin-1 (KinA) cooperate to transport RAB11 secretory vesicles (SVs) originating at the Golgi to the Spitzenkörper (SPK) (Pantazopoulou et al., 2014; Peñalva et al., 2017; Zhang et al., 2011). The SPK is a membraneless organelle adjacent to the apical plasma membrane (PM), characteristic of hyphal fungi. It acts as a vesicle supply center where SVs gather before being delivered to the growing tip’s PM, with involvement of a second RAB GTPase, Sec4 (Riquelme et al., 2014; Steinberg et al., 2017). The SPK contains an F-actin organizing center (Sharpless and Harris, 2002), such that actin cables span the region of the tip spreading out from the apex like the ribs of an umbrella (Bergs et al., 2016; Pantazopoulou et al., 2014; Pearson et al., 2004; Taheri-Talesh et al., 2008). In contrast, microtubules (MTs) make contacts with their plus-ends at a broad, crescent-shaped region of the tip PM, which we denote here as “the apical dome.”

A division of roles underlies cooperation between actomyosin and MT transport of A. nidulans RAB11 SVs (Pantazopoulou et al., 2014; Peñalva et al., 2017; Pinar et al., 2015; Pinar and Peñalva, 2020; Schuchardt et al., 2005; Zhang et al., 2011): KinA (kinesin-1) conveys RAB11 SVs to the hyphal tips, whereas MyoE concentrates them at the SPK (Figure 1A). The partially redundant role played by kinesin-1 makes MyoE nonessential, although its absence slows down growth markedly and causes morphological abnormalities resulting from inability to focus exocytosis at the apex. Cooperation between the microtubule and the actin cytoskeletons is not uncommon in tip-growing cells of organisms that are evolutionary distant from fungi. Another notable example of this cooperation occurs in the prothenea of the moss Physcomitrella patens, which contains a cluster of F-actin at the apex that governs the directionality of growth and that strikingly resembles the fungal SPK/vesicle supply center (Wu and Bezania, 2018).
The key role that myosin-V-mediated transport plays in eukaryotic cells has prompted intensive research on the molecular mechanisms by which vesicles are recruited to the motor. Membranous cargoes attach to the globular C-terminal domain (GTD) of myosin V via “receptors” that are cargo-specific adaptors (Hammer and Sellers, 2012; Pashkova et al., 2006; Wu et al., 2002). In the case of SVs, these adaptors can be a RAB GTPase, be it RAB11, Sec4/RAB8, or both (Wong and Weisman, 2021). In Saccharomyces cerevisiae, Ypt31/32 (yeast RAB11s) and Sec4 (yeast Rab8) bind directly and without involvement of any other proteinaceous co-adaptor to the GTD of the type V myosin Myo2p (Jin et al., 2011; Lipatova et al., 2008; Santiago-Tirado et al., 2011), with additional involvement of PtdIns4P present in SVs in their association with the motor (Santiago-Tirado et al., 2011). However, in the particular case of RAB11, this paradigm of the RAB as the only component of the myosin V adaptor to vesicles is far from being universal. For example, in mammalian cells the RAB11a effector RAB11-FIP2 (RAB11 family interacting protein 2) acts as co-adaptor cooperating with the GTPase to recruit MyoVb to recycling endosome vesicles (Hales et al., 2002; Schafer et al., 2014; Wang et al., 2008), the actin nucleator SPIR-2 acts as co-adaptor between RAB11 and MyoVa (Pylypenko et al., 2011), with additional involvement of PtdIns4P present in SVs in their association with the motor (Santiago-Tirado et al., 2011). It has been suggested that SVs are handed over from kinesin-1 to myosin V in the region of the tip, hypothetically by switching from MT to actin cables, yet the mechanism by which myosin V prevails over kinesin-1 in the tip region is not understood. In view of the crucial role that myosin V plays in the lifestyle of hyphal fungi, we hypothesized that accessory proteins might help this motor to engage RAB11 SVs robustly, to ensure the efficiency of the latest step in their transport. Here we report the molecular composition of a novel myosin-V-containing complex that engages SVs. This complex, denoted HUM, also contains UDS1 and HMSV, two proteins whose orthologues in Neurospora crassa have been recently identified as components of the SPK (Zheng et al., 2020). Trafficking of RAB11 SVs to the SPK/vesicle supply center is impaired if the HUM complex is disrupted.
RESULTS

MyoE is key for the delivery of RAB11 secretory vesicles to the hyphal apex

The efficiency of MyoE transport is reflected in the distribution of RAB11 SVs accumulating in the tips before fusing with the PM. Although in the wild type these SVs gather at the SPK and its environs, in myoEΔ cells lacking type V myosin SVs cannot be focused at the apex, yet they still arrive at the tip by kinesin-1/ microtubule-mediated transport (Pantazopoulou et al., 2014; Peñalva et al., 2017) (Figures 1A and 1B); this results in relocalization of RAB11 vesicles to a tip crescent whose shape reflects the steady-state distribution of the microtubules’ plus-ends contacting the apical dome cortex (Figure 1A). myoEΔ-dependent delocalization of RAB11 to this crescent is paralleled by a conspicuous reduction of RAB11 in the tip (Figure 1B), strongly suggesting that MyoE is a major contributor to the transport of RAB11. Consistent with a secretory defect, loss of MyoE markedly reduces growth (Figure 1C) and alters hyphal morphogenesis, leading to abnormally wide cells (Figures 1B and 1F; note that exocytosis determines the shape of the cell wall) (Hernández-González et al., 2014, 2018a; Pinar et al., 2013a, 2013b).

In budding yeast, the RAB GTPase Sec4 (metazoan RAB8) acts downstream of RAB11 during transport between the trans-Golgi network (TGN) and the PM and ultimately mediates fusion of SVs with the membrane (Donovan and Bretscher, 2015a; 2015b; Jin et al., 2011; Santiago-Tirado et al., 2011). In A. nidulans Sec4 localizes to the hyphal tips, which suggests a similar role. Therefore, Sec4 was an obvious candidate to adapt MyoE to SVs; this could be tested directly because Sec4 is not essential in A. nidulans, although its absence is as debilitating as that of MyoE (Figure 1C). However, contrasting to myoEΔ mutants, sec4Δ mutants are still capable of gathering RAB11 SVs at the tip (Figure 1D), indicating that Sec4 is not essential for transport. Instead, we observed that the cluster of SVs accumulating in sec4Δ tips was twice as broad as in the wild type (1.68 ± 0.5 SD versus 0.74 ± 0.2 μm², p=0.0001 in a Mann-Whitney t test), while still displaying a similar average intensity per pixel, which suggests that the number of vesicles accumulating in the mutant was consistently higher than the wild type (Puerner et al., 2021). This cluster of RAB11 SVs at the tip, less tightly packed in the sec4Δ mutant than in the wild type, appears to be submitted to the opposing forces exerted by anterograde and retrograde motor teams, often causing the detachment of RAB11 SV ‘lumps’ away from the apex (Video S1). Therefore, all these data suggest that rather than mediating the myosin V-mediated delivery of SVs to the tip, the main role of Sec4 is participating in the consumption of SVs. Yet, Sec4 detectably contributes to transport, as although sec4Δ cells still concentrated MyoE at the SPK, they did so with lesser efficiency than the wild type (Figure 1E and Video S2; note that MyoE is activated by cargo loading). Altogether, these data established that there must be another adaptor sharing with Sec4 the task of engaging SVs to MyoE.

Both RAB11 and Sec4 interact directly with MyoE

Previous studies with fungal and metazoan cells pointed to RAB11 as the most likely candidate (Goldenring, 2015; Hales et al., 2002; Lipatova et al., 2008; Roland et al., 2011). In the intensively studied transport of SVs to the growing bud of S. cerevisiae, the RAB11 homologues Ypt31/32 and Sec4 recruit the MyoE orthologue Myo2p through direct binding to the highly conserved globular tail domain (GTD) of the latter (Jin et al., 2011; Lipatova et al., 2008). If this mechanism were conserved in A. nidulans, a polypeptide consisting solely of the MyoE GTD domain should bind the RABs present on SVs, being passively transported along with them to the tips. To test this prediction, we expressed a construct consisting of the GFP-tagged MyoE GTD domain in wild type, myoEΔ and sec4Δ hyphae. In the wild type, GFP-GTD, although cytosolic in part, concentrated at a tip cluster (Figure 1F), on which it strictly colocalized with RAB11, and to punctate structures barely noticeable over the cytosolic background (Figures 1F and 1G). The low signal-to-background ratio and the rapid movement of some of these cytosolic structures made colocalization studies with RAB11 challenging, but we managed to obtain informative time-lapse sequences with a temporal resolution of 2 fps using a beam splitter to film the two channels simultaneously, coupled to a CMOS camera that allowed us to set different acquisition times for each channel to compensate differences in brightness. These experiments showed that GFP-GTD puncta were RAB11 positive (Figures 1G and 1H and Video S3). This key in vivo observation strongly suggested that the GTD is indeed sufficient to localize to RAB11-containing SVs powered by resident KinA and MyoE. Figure 1I shows that the region at which GFP-GTD localizes is broader than the apical spot at which full-length MyoE-mCherry does, suggesting that the localization of GFP-GTD cannot be the result of an interaction of the GFP-GTD fusion protein with resident MyoE. Indeed, in myoEΔ cells GFP-GTD localized to the apical dome, recapitulating the distribution of RAB11 SVs in this mutant (Figures 1B and 1F) (Video S4) and indicating that GFP-GTD can still ride on SVs when MyoE is absent. The observation that MyoE GTD still concentrated at the apex of sec4Δ cells (Figure 1F)
Figure 2. UDS1, a novel, direct effector of RAB11

(A) Proteins retained by GTPyS- and GDP-loaded RAB11-GST columns were identified by shotgun proteomics. Spectral counts obtained for each protein and condition and the relative enrichment detected in one sample versus the other are listed. Note that markedly abundant GdiA (GDP dissociation factor) interacts preferentially with GDP-RAB11. AP-2α was used as negative control.

(B) Features of UDS1. The probability of forming coiled-coils (red graph) and disordered regions (gray area) is indicated, as are the positions of the UDS1 and SCOP superfamily domains.

(C) GST pull-down assays with the indicated baits, using a prey extract of A. nidulans-expressing UDS1-HA3 from the endogenously tagged gene. Pull-downs were analyzed by western blotting with α-HA3 antibody. GST-GFP was used as negative bait control.

(D) UDS1 is a dimer in vitro. Equilibrium ultracentrifugation of purified UDS1 at a concentration of 4 μM; (top) the concentration gradient obtained (empty circles) is shown together with the best-fit analysis assuming that the protein is a dimer. (Bottom plot) Differences between experimental data and estimated values for the dimer model (residuals). See also Figure S2E Negative-stain electron microscopy of purified UDS1. The proteins were stained with uranyl acetate and...
strongly indicated that Sec4 cannot be an exclusive receptor for MyoE. Thus, we concluded that the MyoE GTD is sufficient to engage the motor to at least two receptors present on SVs, with the obvious candidates being Sec4 and RAB11.

In view of this conclusion, we asked whether the type V myosin MyoE is a direct effector of these RABs. We purified His-tagged versions of Sec4, RAB11, and, as control, Rab5b acting in the endocytic pathway (Abenza et al., 2010). These RABs were loaded with GDP or GTPγS and used as preys in pull-down assays with MyoE GST-GTD or GST-GFP baits (GFP, known to be sticky, was used as negative control). Bound proteins were resolved by electrophoresis, and RABs retained by the baits were detected by western blotting, after reacting the membranes with α-His antibody. Both GTPγS-loaded RAB11 and Sec4 were captured by the GTD, but not by the GFP bait, whereas RAB11-GDP, Sec4-GDP, and Rab5b (whether GDP or GTPγS) were not (Figure 1J). Thus, Sec4 and RAB11 bind the MyoE GTD directly, specifically, and in nucleotide switch-dependent manner.

A double mutant in which sec4Δ was combined with hypA1ts, a mutation affecting the RAB11 GEF TRAPPII (Pinar et al., 2015), is virtually lethal (Figure S1A); this is consistent with the involvement of both Sec4 and RAB11 in exocytosis. However, the fact that sec4Δ can accumulate vesicles in the apical region (Figure 1D) indicates that, of these two RABs, RAB11 is by itself sufficient to mediate the MyoE-mediated clustering of SVs at the vesicle supply center.

The actomyosin pathway protein UDS1 is a novel RAB11 effector

As we were most interested in the effectors of RAB11, we investigated if, similar to the situation with mammalian RAB11 and myosin Vb (Hales et al., 2002; Schafer et al., 2014), other associates cooperate with RAB11 in the transport of secretory vesicles. We identified by liquid chromatography and tandem mass spectrometry (LC-MS/MS) the proteins retained by glutathione Sepharose beads containing RAB11-GST baits loaded with GDP or GTPγ-S. The resulting hits were ordered by abundance of peptide spectral matches (PSMs) in the GTPγS sample relative to the GDP one, which helped to identify potential physiological hits. The highly abundant GDP-dissociation inhibitor GdiA (Pinar et al., 2015) served as specific GDP-RAB binder control, the previously characterized and abundant RAB11-GTP effector BapH (Pinar and Penalva, 2017) served as positive control, and the unrelated AP-2 alpha-adaptin as negative one (Figure 2A). This analysis highlighted two potential actin-related hits. One was MyoE itself, which was exclusively retained by GTP-loaded, but not by GDP-loaded, GST-RAB11 beads, reinforcing the conclusion that MyoE is a RAB11 effector. The second was the relatively abundant and highly specific RAB11-GTP effector AN5595 (Figure 2A). The 941 residue AN5595 product has a strong tendency to form coiled-coils (Figure 2B). An N. crassa orthologue of AN5595 denoted JANUS-1 interacts with the polarisome component Spa2 and has been suggested to serve as an SPK scaffold (Zheng et al., 2020), yet A. nidulans Spa2 is not required for the establishment or maintenance of the SPK, nor for the localization of formin (Virag and Harris, 2006). However, AN5595 shows features of an actomyosin regulator (Figure 2B), as it contains a SCOP superfamilyp tropomyosin domain (SSF57997) suggestive of a parallel coiled-coil quaternary structure, and a UDS1 domain (PF15456). This domain was named after the as-yet uncharacterized AN5595 Schizosaccharomyces pombe homologue, whose name stands for “upregulated during septation,” and which localizes to the contractile actin ring in the fission yeast mitotic septum (Ikebe et al., 2011). Therefore, we denoted AN5595 as UDS1.

To confirm that UDS1 is a bona fide RAB11 effector, we HA3-tagged the protein endogenously and used UDS1-HA3 cell extracts in pull-down assays with purified GST-RAB baits, loaded with GTPγS or GDP, and with GST-GFP as negative control. UDS1-HA3 was pulled down solely by GTPγ5-RAB11 but not by GFP, GDP-RAB11, GTPγS-RAB5b, or GDP-RAB5b baits (Figure 2C), confirming that UDS1 is subordinated to RAB11.
Next, we purified UDS1-His6 from bacteria. By gel filtration chromatography UDS1 eluted at a position corresponding to >600 kDa (Figure S2), suggesting homo-oligomerization and/or a 3D structure substantially deviating from the globular shape. Sedimentation equilibrium ultracentrifugation of purified UDS1 (Mr 106,857 Da) revealed a buoyant mass of 57,002 ± 403 Da corresponding to a molar mass of 209,073 Da ± 1,612 Da, matching the molecular weight of a dimer (Figure 2D). Moreover, although the flexibility observed at the level of individual particles precluded us from obtaining 2D averages, individual EM images revealed that UDS1 presents a rod-shaped structure highly suggestive of an elongated coiled-coiled dimer, with an approximate length of ~500 Å (Figure 2E).

We used AlphaFold to gain insight into the architecture of UDS1, after imposing the restriction that the protein is a dimer. We obtained a structure that has notable resemblance to some of the EM pictures (Figures 2E and 2F; Figures S3A and S3B). The structure contains a 436 Å-long parallel coiled-coil, which fits reasonably well with the experimentally determined length of ~500 Å for the complete protein (Figure 2E histogram). This section of the protein, which is predicted with good pDLLT (per residue-estimate of confidence) values (Figure S3A), contains on its C-terminal side the SCOP tropomyosin domain. The V-shaped UDS1 domain is composed of two adjacent tri-helical units, with the two longest helices forming the two arms of the V (Figure 2G).

Lastly, as the above RAB pull-down experiments using cell extracts do not rule out the possibility that RAB11 and UDS1 interact by way of bridging protein(s), we used His-tagged UDS1 to repeat the GST-RAB pull-down assays with purified proteins. Figure 2H shows that UDS1-His behaves as the protein present in Aspergillus extracts, being pulled down by GTPγS-RAB11 but not by GDP-RAB11, nor by the inactive or active forms of RAB5b and Sec4. In summary, UDS1 is a coiled-coil dimer that binds directly to the (GTP) active form of RAB11. Importantly, UDS1 does not bind to Sec4, the other MyoE receptor present on SVs.

**Aspergillus UDS1 colocalizes with both MyoE and RAB11 on SVs**

In current models (Figure 1A), RAB11 SVs arrive at the tip using KinA (kinesin-1) and are concentrated at the SPK. Indeed, Video S7 shows how UDS1-GFP recurs in the apical dome of a fungus (Figure 3E), which strongly supports the notion that UDS1 travels with SVs rather than being a permanent resident of the SPK. Indeed, Video S7 shows how UDS1-GFP recurs in the apical dome of a myoE tip as SVs containing UDS1 arrive by MT transport to the PM. Thus, UDS1 is a RAB11 effector that is present in SVs and concentrates at the SPK.

**MyoE associates directly with HMSV, a further novel component of the RAB11 pathway**

To investigate the possibility that MyoE and UDS1 associate, we analyzed, by LC-MS/MS, GFP-Trap immunoprecipitates of MyoE-GFP and UDS1-GFP cell extracts, using immunoprecipitates of a strain expressing the unrelated bait Uso1-GFP as a negative control (Uso1/p115 acts as a tether in the ER/Golgi interface). UDS1 indeed pulled down MyoE, whereas MyoE pulled down UDS1 inefficiently, suggestive of weak or indirect interaction (Figure 4A). Remarkably, an as-yet uncharacterized protein, the product of AN1213, co-precipitated with MyoE-GFP quite efficiently. Conversely, MyoE coprecipitated efficiently with GFP-tagged AN1213. An orthologue of AN1213, denoted SPZ-1, has been investigated in N. crassa and proposed to serve as scaffold at the SPK (Zheng et al., 2020). However, for reasons that become clear below we denoted AN1213 as HMSV (hodking myosin to SVs). HMSV coprecipitated with UDS1-GFP as well, indicating that these proteins also interact (Figure 4A). Notably, the polarisome component SpaA/Spa2, which has been shown to associate with N. crassa SPZ-1, JANUS-1, and myosin V (Zheng et al., 2020), does not immunoprecipitate with the A. nidulans orthologues (Figure 4A) (see discussion).

HMSV is a 994 residue-long protein whose 350 N-terminal residues are predicted by COIL to be disordered, whereas the remaining ~650 residues have strong propensity to form coiled-coils (Figure 4B). We used
AlphaFold to predict the 3D organization, and secondary structure elements of HMSV (Figure 4C; Figures S3C and S3D) display the confidence tests for the prediction. AlphaFold identifies eight potential \( \alpha \)-helices, of which helices V, VII, and VIII (the latest containing the C-terminus) form a three-helical coiled-coil. The long helix II (Figure 4C) appears free to establish protein-protein interactions. As anticipated by COIL, the protein also contains two long disordered regions, one corresponding to the N-terminal 350-residue region and a second to residues 551–625 (Figure 4C). Proteins containing disordered regions associate spontaneously. These intrinsically disordered proteins are associated with a score of processes and are thought to promote liquid-liquid phase separation that could be the basis for the organization of membrane-less organelles (Musacchio, 2022). These observations were appealing, as HMSV, like UDS1,
Figure 4. HMSV, an uncharacterized interactor of the MyoE GTD

(A) Cell extracts expressing the indicated GFP-tagged baits by allelic replacement were immunoprecipitated with GFP-Trap. Pulled-down proteins were analyzed by LC-MS/MS. The table lists the spectral counts obtained for each of the indicated co-precipitating proteins. A Uso1-GFP-expressing strain was used as negative control.

(B) Prediction of coiled-coils (red graph) and disordered regions (gray area) along the primary sequence of HMSV. Roman numerals indicate α-helical regions (color-coded). Two large disordered regions are indicated as loop 1 and loop 2. See also Figures S3C and S3D.

(C) Spectral counts of HMSV detected in GFP-trap immunoprecipitates of cell extracts expressing the MyoE GTD or the analogue cargo-binding, C-terminal domain (CTD) of KinA/kinesin-1. Spectral counts of HMSV were confirmed with Coomassie-stained heavy chains.

(D) Growth tests at 37°C of indicated strains. See also Figure S1B.

(E) Endogenously tagged MyoE-mCh and HMSV-GFP strictly colocalize. MIPs of deconvolved Z-stacks.

(F) Kymograph derived from the 4D sequence shown in Video S8, mounted with MIPs of Z-stacks acquired with a beam splitter every 15 s for 15 min. The hypha was growing at 0.62 μm/min.

(G) Widths of wild-type and mutant hyphae stained with calcofluor to label the cell walls. Top, middle planes of representative tips. (Bottom) x,y images of septa alongside with the corresponding orthogonal views. (Right) Quantitation of cell width. Bars are the average value for ~20 hyphae per condition; error bars are SD. Significance was assessed with an ANOVA Kruskal-Wallis test with Dunn’s multiple comparison correction. n.s., not significant.

(H) MyoE and HMSV interact directly. MyoE and HMSV-HA3 obtained from TNT reactions were mixed with Protein A beads preloaded with polyclonal α-MyoE antiserum or with antiserum against the unrelated protein Uso1. Immunoprecipitates were analyzed by α-HA western blotting. Equal IgG loading was confirmed with Coomassie-stained heavy chains.

(I) Spectral counts of HMSV detected in GFP-trap immunoprecipitates of cell extracts expressing the MyoE GTD or the analogue cargo-binding, C-terminal domain (CTD) of KinA/kinesin-1.

(J) HMSV associates with MyoE through the GTD of the motor. Extracts of myoEΔ cells co-expressing HMSV-HA3 with GFP-tagged MyoE GTD or MyoEΔGTD (MyoE lacking the GTD) were immunoprecipitated with GFP-Trap nanobody. A Uso1-GFP-HMSV-HA3 strain was used as negative control. (Left) Pulled-down material was analyzed by α-HA western blotting. (Right) Relative levels of the preys by α-GFP western blotting.

(K) HMSV interacts directly with the MyoE GTD: pull-down assays with indicated GST baits. Preys were in vitro expressed (with TNT) HMSV-HA3 or, as control, UDS1-HA3. Blots were revealed with α-HA antibody.

localizes to the SPK, strictly colocalizing with MyoE during hyphal growth (Figures 4E, 4F and Video S8). To determine the consequences of removing UDS1 and HMSV, we constructed strains carrying null uds1Δ and hmsVΔ alleles. These are phenotypically indistinguishable, resulting in a radial colony growth defect (Figure 4D), and, at the cellular level, in abnormally wide hyphae (Figure 4G), both features indicative of defective exocytosis. Notably, the colony growth defect resulting from uds1Δ and hmsVΔ was markedly weaker than that caused by myoEΔ. Double uds1Δ hmsVΔ mutants behaved like the parental single mutants, consistent with the corresponding products being components of the same functional unit (Figure S1B). The fact that both uds1Δ and hmsVΔ are hypostatic to myoEΔ (Figure 4D) suggested that this hypothetical complex acts through MyoE, although neither UDS1 nor HMSV plays an essential role in MyoE function.

The high yields of HMSV and MyoE recovered with their respective GFP-trap immunoprecipitates suggested that MyoE and HMSV are direct interactors (Figure 4A). This prediction was confirmed by co-immunoprecipitation experiments using MyoE and HMSV-HA3 expressed by coupled transcription-translation reactions primed with their respective cDNAs. The two proteins were combined and immunoprecipitated with α-MyoE-specific IgGs or with IgGs raised against the unrelated protein Uso1 (acting in the ER/Golgi interface). α-MyoE IgGs, but not α-Uso1 IgGs, immunoprecipitated HMSV-HA3 (Figure 4H), establishing that HMSV and MyoE interact directly. GFP-trap co-immunoprecipitation coupled to MS/MS showed that the GFP-MyoE [GTD] construct discussed in Figure 1, but not a similar construct carrying the carboxy-terminal region of KinA (kinesin-1), efficiently pulled down HMSV, strongly indicating that MyoE uses its GTD domain to interact with HMSV (Figure 4I). Next, we determined, using GFP-TRAP immunoprecipitation experiments of cell extracts (of myoEΔ cells, to avoid heterodimerization) expressing either the GFP-MyoE [GTD] construct or the complementary GFP-tagged MyoE [ΔGTD] construct (i.e. the motor, IQ repeats and coiled-coil domains, without the GTD), that the GTD domain of MyoE is necessary and sufficient to interact with HMSV (Figure 4J). That this interaction is direct was further established after reconstructing the interaction in vitro using purified GST-GTD and in vitro synthesized HMSV. GST-GTD beads pulled down HMSV but not the unrelated prey Uso1, whereas neither prey was pulled down by GST-GFP, demonstrating specificity (Figure 4K). Thus, HMSV interacts directly with the GTD of MyoE, which together with data above suggested that HMSV acts as a connector between UDS1 and MyoE (to be reinforced below).

The MyoE-containing complex HUM is a RAB11 effector scaffolded by HMSV

Unlike UDS1, HMSV did not appear to interact with RAB11 in shotgun proteomic experiments (Figure 2A). To confirm this observation, we performed more sensitive GST-pull-down assays with whole cell extracts expressing HA3-tagged preys. Under conditions in which UDS1-HA3 strongly associated with RAB11-GST, HMSV-HA3 did not (Figures S5A and S8). We noted, however, that strong overexposure of the blots
Figure 5. UDS1 bridges its direct interactor HMSV to the active form of RAB11. HMSV does not interact directly with RAB11.

(A) Control showing that a Uds1-HA3 is efficiently pulled down from extracts by GTPγS RAB11 but not by GDP-RAB11. Pull-downs analyzed by α-HA western blotting.

(B) As in (A), but using HMSV-HA3 extracts as preys. Uds1-HA3 and HMSV-HA3 were expressed from allelic replacements. Pull-downs analyzed by α-HA western blotting.

(C) HMSV and UDS1 interact directly: pull-down assays with GST-UDS1 as bait and HMSV-HA3 or, as negative control, Uso1-HA3 as preys, which were obtained by TNT expression. Pull-downs analyzed by α-HA western blotting.
revealed a very faint signal in the GTPγS-RAB11 lane, arguing against HMSV being a direct interactor of RAB11 and suggesting instead that another factor(s)/component(s) of the HMSV-MyoE complex present in the reaction mixtures (note that total cell extracts—not purified proteins—were used as preys in this experiment) might bridge HMSV to RAB11, albeit inefficiently (see discussion). While an indirect linker of HMSV to RAB11 was MyoE, shotgun proteomic experiments with GFP traps (Figure 4A) suggested that UDS1 contributes to this bridging role. Figure 5C shows that GST-UDS1, but not the unrelated bait GST-GFP, pulled-down in vitro synthesized HMSV-HA3. In contrast, neither bait pulled-down Uso1-HA3, confirming specificity and establishing that UDS1 and HMSV interact directly. Therefore, through its capacity to bind directly to both MyoE and UDS1, HMSV would act as scaffold of a heteromeric complex that would be recruited by RAB11 to SVs by contacting both UDS1 and MyoE.

This model was tested with two sets of experiments. First, we demonstrated in vitro that HMSV is recruited to active RAB11 only if UDS1 is present to bridge the interaction (Figure 5D). To this end, we performed GST-RAB pull-downs in the presence of bacterially expressed UDS1, in vitro synthesized HMSV-HA3 or both. HMSV was recruited by GTPγS-RAB11, but did so only when UDS1 was present in the reaction mix. Neither conformation of RAB5b nor GDP-RAB11 pulled-down HMSV even when UDS1 was present. We conclude that the presence of UDS1 is sufficient for the efficient recruitment of HMSV by the active form of RAB11, establishing that HMSV is an indirect effector of the latter.

Secondly, we demonstrated that a stable complex consisting of MyoE, HMSV and UDS1 is present in cellular lysates, and that this complex, that we denoted HUM (for HMSV-UDS1-MyoE) is scaffolded by HMSV. As determined by anti-MyoE Western blotting of GFP-Trap immunoprecipitates of whole-cell extracts, MyoE strongly associates with UDS1-GFP and with HMSV-GFP, but not with the unrelated bait Uso1-GFP (Figure 6A). Indeed, MyoE association with UDS1 and HMSV is so efficient that co-immunoprecipitated MyoE could be visualized directly by silver-staining of SDS-PAGE gels (Figure 6A, right). Despite HMSV appearing to be the less abundant of the three baits (anti-GFP western blot, Figure 6A, right), HMSV pulled down MyoE markedly more efficiently than UDS1 did, in agreement with the conclusion that MyoE and UDS1 interact indirectly by way of HMSV. Consistently, the interaction between MyoE and UDS1 was undetectable with hmsVΔ extracts (i.e., was completely dependent on the presence of HMSV) (Figure 6B), whereas that between MyoE and HMSV was completely independent of UDS1, taking place irrespectively of whether wild-type or uds1Δ extracts were used (Figure 6C). Lastly, the interaction between UDS1-GFP and HMSV-HA3 was completely independent of MyoE (Figure 6D), as predicted by in vitro reconstitution experiments above.

Taken together these data show that these proteins form a complex in the order MyoE/HMSV/UDS1 that has the dual ability to interact with the active form of RAB11 through UDS1- and MyoE-mediated contacts.

Evidence that UDS1 and HMSV assist RAB11 to recruit MyoE to SVs

A diagnostic readout of MyoE transport is the focusing of SVs at the SPK. Consistent with UDS1 and HMSV acting in a complex regulating myosin V transport, both uds1Δ and hmsVΔ affected RAB11 SVs similarly, reallocating them from the SPK to a crescent-shaped distribution in the apical dome typical of impaired MyoE function (Figure 7A). This effect was markedly less conspicuous than that caused by myoEΔ, which resulted in a broader crescent and, as discussed above, in a marked reduction of the signal of SVs docked at the tip cortex (Figure 1B). Therefore, these data strongly indicate that myosin V transport is depleted in uds1Δ and hmsVΔ mutants, such that although this transport is not abolished, MT-mediated transport gains prominence, which results in targeting SVs to a broad surface determined by the sites at which MTs’ plus ends reach the apical dome. Impairment of actomyosin transport in these mutants explains the partial exocytic deficit that growth tests and hyphal morphologies indicate (Figures 4D and 4G).

The above experiments suggested that the UDS1 and HMSV subunits of HUM might play the role of a coreceptor reinforcing the RAB11-mediated recruitment of MyoE to SVs. Myosin V dwells in an inactive conformation that is shifted to the active conformation by cargo (Donovan and Bretscher, 2015a). Thus, a deficit in cargo loading would be translated into a drop in MyoE activity, which should in turn result in
a reduction in the levels of MyoE at the SPK. Figure 7A shows that both uds1Δ and hmsVΔ reduce the SPK MyoE signal by 5- to 6-fold, supporting the contention that in these mutant backgrounds the engagement of SVs with MyoE is compromised. Video S9 comparing the wild-type with an hmsVΔ strain depicts the impaired delivery of MyoE to the SPK in the mutant. MyoE SVs are visible in these movies.
We next investigated the dependence of UDS1 and HMSV on each other. In hmsVΔ cells UDS1 delocalized from the SPK to an apical crescent remarkably similar to that observed with RAB11 in hmsVΔ and myoEΔ backgrounds (Figures 7A and 3E). The finding that UDS1 ‘goes with’ RAB11 is consistent with the prediction that the connection of UDS1/RAB11 SVs with MyoE should be impaired by hmsVΔ (a broader distribution indicates that the balance between actomyosin and MT transport has been shifted toward the latter). In

Figure 7. HUM complex components cooperate with RAB11 to recruit MyoE to SVs. A model
(A) (Top) Localization of the HUM complex components in different genetic backgrounds. Images are MIPs of deconvolved Z-stacks. As GFP reporters were endogenously tagged, the corresponding null background images are empty. (Bottom) Quantitation (arbitrary units, A.U.) of the MyoE-GFP signal in the SPK of uds1Δ and hmsVΔ cells compared with the wild type. Means (±SD) were: left, wild-type 2,842 ± 227 versus uds1Δ 558 ± 159 (p < 0.0001 in unpaired t test). (Right) Wild type, 3496 ± 245 versus 654 ± 149 in the hmsVΔ mutant (p < 0.0001 in unpaired t test). See also Videos S9 and S10.

(B) A prototypic cargo of the RAB11 recycling pathway is delocalized from the SPK by uds1Δ and hmsvΔ. The scheme depicts endocytic recycling followed by the chitin-synthase ChsB. ChsB and RAB11 are similarly delocalized from the SPK, indicated by red arrowheads.

(C) Model for the engagement of HUM with RAB11 SVs. In the wild type, RAB11 is recruited to SVs during the Golgi-to-post-Golgi transition. RAB11 interacts both with the GTD of MyoE and with UDS1 in the HUM complex. UDS1 bridges active RAB11 to the HMSV scaffold. HMSV bridges RAB11/UDS1 to MyoE by direct interaction with the motor’s GTD. MyoE transport is most efficient in the context of the whole complex. However, in the absence of UDS1 or HMSV, MyoE-mediated SV transport remains partially operative due to the direct interaction between RAB11 and the MyoE GTD, albeit this transport is less efficient, accumulation of SVs in the SPK is impaired and MT-dependent transport becomes more prominent, leading to the characteristic apical dome distribution of SVs in these mutants.

We next investigated the dependence of UDS1 and HMSV on each other. In hmsVΔ cells UDS1 delocalized from the SPK to an apical crescent remarkably similar to that observed with RAB11 in hmsVΔ and myoEΔ backgrounds (Figures 7A and 3E). The finding that UDS1 ‘goes with’ RAB11 is consistent with the prediction that the connection of UDS1/RAB11 SVs with MyoE should be impaired by hmsVΔ (a broader distribution indicates that the balance between actomyosin and MT transport has been shifted toward the latter). In
sheer contrast, HMSV is not delocalized from the SPK in uds1Δ cells, but the signal was reduced to an extent roughly commensurate with the reduction in MyoE signal (Figure 7A), indicating that HMSV goes with the proportion of MyoE that is (less efficiently) loaded with cargo by way of the direct interaction of RAB11 with the motor, and therefore that HMSV binds to RAB11 by way of UDS1. Notably, the localization of HMSV in myoΔ cells is remarkably similar to that of RAB11 and UDS1 (Figure 7A) (Video S10 for HMSV). Thus, without MyoE the other HUM complex components associate with RAB11 and are delivered to the PM by MT transport, which results in their localization to the apical dome. This observation further demonstrates that UDS1 and HMSV are components of RAB11 SVs rather than structural constituents of the SPK.

**Ablation of UDS1 or HMSV impairs the delivery of an exocytic cargo to the SPK**

A well characterized cargo of RAB11 SVs is the chitin synthase ChsB (Hernández-González et al., 2018a). This integral membrane protein is exocytosed to the apical PM by way of the SPK, diffuses away from the tip and it is taken up by a highly active endocytic collar that transports it to a sorting endosome. From this compartment ChsB returns to the TGN where it is incorporated into RAB11 SVs delivered to the SPK (Figure 7B). In the wild-type, a proportion of ChsB is present in the SPK. In uds1Δ and hmsVΔ cells this accumulation of ChsB in the SPK is no longer seen, resembling the situation with RAB11, which is included in Figure 7B for comparison. We interpret that the absence of the UDS1/HMSV co-receptor role affects transport of a RAB11 cargo from the TGN to the SPK.

In summary, our data strongly support a model in which HMSV and UDS1 are components of a MyoE-containing complex that we denoted HUM and that is recruited by RAB11 to SVs through direct interactions with both UDS1 and MyoE. HMSV/UDS1 serves as a co-adaptor between these vesicles and the motor (Figure 7C). When this co-adaptor is disorganized by ablation of either of its two components, actomyosin transport of these SVs still occurs, albeit less efficiently, due to the direct interaction between RAB11 and MyoE.

**DISCUSSION**

The ability of type V myosins to transport cargo is crucial for the biogenesis and distribution of membranous compartments (Hammer and Sellers, 2012; Wong and Weisman, 2021). *A. nidulans* has a single type V myosin, MyoE (Taheri-Talesh et al., 2012), implying that specificity for different cargoes must be mediated by different adaptors (Cross and Dodding, 2019; Wong and Weisman, 2021). Adaptors often involve a RAB family member, as individual RABs display a high selectivity for their cognate membrane compartment (Pfeffer, 2013; Pinar and Peñalva, 2021). RABs can interact directly with myosin V or, indirectly, by means of intermediate proteins that bridge the activated RAB and the motor (Hammer and Sellers, 2012; Wong and Weisman, 2021). A well understood co-adaptor is melanophilin bridging RAB27 on melanosomes to MyoVa (Wu et al., 2002).

Even if the binding of the RAB to the type V myosin is direct, it might involve co-adaptors that help stabilizing the complex. This is the case of metazoan RAB11, FIP2 and MyoVb (FIP2 is a direct effector of RAB11), which form a tripartite complex required for traffic between recycling endosomes and the PM (Hales et al., 2002; Li et al., 2007; Schafer et al., 2014; Wang et al., 2008). In addition to complex stabilization, co-adaptors play additional roles. The C-terminal region of melanophilin binds F-actin, dramatically increasing the processivity of MyoVa (Sckolnick et al., 2013). Moreover, melanophilin tracks, by hitchhiking on EB1, the plus-ends of MTs, recruiting MyoVa to them, which might ensure the efficient transfer of melanosomes from MTs to actin cables (Wu et al., 2005). Another example of additional functions of RAB-containing type V myosin adaptors occurs in mouse oocytes, where MyoVa is recruited to RAB11 vesicles by cooperative interactions with both the GTPase and the actin nucleator SPIR-2, which help to coordinate MyoVa vesicle transport with actin nucleation (Pylypenko et al., 2016). Adaptors may also play roles unrelated to transport. For example, phosphorylation and ubiquitin-mediated degradation of the yeast vacuolar adaptor Vps17 is required to release the organelle from Myo2 (Wong et al., 2020).

In *A. nidulans*, the biogenesis of SVs dispatched to the PM by way of the SPK (a vesicle supply center located underneath the apical plasma membrane) is mediated by RAB11. These SVs are loaded with the type V myosin MyoE, kinesin-1 (KinA) and dynein (Pantazopoulou et al., 2014; Peñalva et al., 2017), yet the adaptors linking these molecular motors to SVs remain uncharacterized. One current model proposes that, resembling melanosome transport, kinesin-1 hauls SVs to tip-proximal regions before transferring them to MyoE, which concentrates them at the SPK (arguably the analogue of the cell periphery in melanocytes) (Pantazopoulou et al., 2014). This two-step mechanism would involve the transfer of SVs from
MT-to F-actin-mediated transport, a relay that would be compromised by the high density of cytoskeletal tracks and organelles populating the hyphal tip. This model accounts for the synthetic lethal phenotype displayed by kinAD myoED double mutants (Peñalva et al., 2017; Zhang et al., 2011) and is supported by the finding that SVs are loaded with both MT-dependent (kinesin-1, dynein) and F-actin-dependent motors (myosin V) (Pantazopoulou et al., 2014), which would enable them to switch from MTs to F-actin directly. However, an alternative model would account for the lethality resulting from kinAD myoED, namely that myosin V transport acts in parallel to kinesin-1, such that instead of receiving SVs from the latter, myosin V would be able to attach and transport them directly to the tip, at least from the apicalmost proximal TGN cisternae. This second model is supported by the observation that the area occupied by RAB11 at the tips extends beyond that occupied by MyoE, which is restricted to the SPK. Thus, at least some MT-dependent transport reaches the PM directly by MTs, without involving actomyosin transport.

Here we characterized HUM, a heteromeric complex minimally containing MyoE and two coiled-coil proteins, HMSV and UDS1, but almost certainly including other factors such as calmodulin and myosin light chains. HUM is recruited to SVs through direct interactions between both MyoE and UDS1 and the active GTP conformer of RAB11, the GTPase governing traffic between the TGN and the SPK (Pantazopoulou et al., 2014; Peñalva et al., 2017; Pinar et al., 2015). RAB11 interacts both with the GTD domain of MyoE and with UDS1, the MyoE GTD interacts both with RAB11 and with HMSV, and HMSV scaffolds the complex by interacting with both UDS1 and the MyoE GTD, but not with RAB11 (RAB11 cannot possibly interact with more than two effectors simultaneously, see (Burke et al., 2014; Vetter et al., 2015)). The MyoE GTD domain binding RAB11 is also necessary and sufficient to recruit HMSV, emphasizing the role of this domain as an interaction hub. Amino acid sequences of the mammalian MyoVa,b,c and fungal Myo2 and MyoE GTDs are conserved (Pashkova et al., 2006; Pylipenko et al., 2013).

In hmsVΔ cells, UDS1-GFP distributes like RAB11 whereas in uds1Δ cells HMSV-GFP distributes like myosin V, suggesting that the HUM complex can be split in two stable subcomplexes, nucleated by RAB11-GTP and by MyoE-GTD, respectively. Therefore, both UDS1 and HMSV are necessary for the assembly of a HUM, whose absence debilitates F-actin-mediated transport, as reflected in the spreading of RAB11 SVs across the apical dome. Inefficient F-actin transport of RAB11 resulting from ablation of UDS1 or HMSV correlates with slower colony growth, and spreading of RAB11 SVs across the hyphal tip dome correlates with delocalization of its cargo, ChsB, from the SPK. Of note, myosin V transport is not abolished in the absence of HUM, possibly because RAB11 is able to bind MyoE directly, which makes the phenotypic consequences of ablating UDS1 or HMSV less severe than those resulting from removing MyoE. That MyoE recruitment by Sec4 does not appear to involve HUM (Figure 2H) in all likelihood also contributes to the relatively weak growth phenotypes of uds1Δ and hmsVΔ.

Cargo adaptors for myosin V are difficult to identify by primary sequence- or domain composition-based searches (Wong and Weisman, 2021). Both UDS1 and HMSV are coiled-coil proteins, which frequently serve as adaptors of molecular motors, including myosin V (Cross and Dodding, 2019). A well-understood example is the coiled-coil melanosome protein RILPL2 (RAB interacting lysosomal protein-like 2) bridging RAB36 with the MyoVa GTD (Matsui et al., 2012; Wei et al., 2013).

While our data clearly implicate the HUM complex in adapting MyoE to RAB11 SVs, we cannot rule out that, in addition, UDS1 and HMSV play non-receptor roles, such as retaining SVs in the SPK, either directly or through its regulation of F-actin microfilaments at the core of the SPK. This F-actin regulatory role would be supported by the presence of the polarisome component Spa2 as an associate of the N. crassa MYO-5/JANUS-1/SPZ-1 complex (Zheng et al., 2020). However, A. nidulans SpaA (=Spa2) does not copurify with MyoE/UDS1/HMSV, and SepA, the only formin of Aspergillus, localizes to the SPK in a SpaA-independent manner (Virag and Harris, 2006). These two facts, together with the absence of a Pea2 equivalent in Aspergillus, strongly argue against equivalent roles of the polarisome in A. nidulans and S. cerevisiae. Of note, Zheng et al. (2020) concluded that MYO-5/JANUS-1/SPZ-1 is not involved in vesicle trafficking, unlike the Aspergillus HUM complex.

An appealing yet highly speculative possibility is that RAB11 co-adaptors contribute to the organization of the SPK by promoting liquid-liquid phase partition to form a membraneless organelle. Alpha-Fold prediction indicates that HMSV is composed of coiled-coils and long unstructured regions, of which the most conspicuous corresponds to the N-terminal 350 amino acids. Intrinsically disordered proteins are frequent dwellers of membraneless compartments supposedly mediating liquid-liquid demixing that results in phase separation (Musacchio, 2022).
UDS1 and/or HMSV might also contribute to the efficiency of myosin V transport. In the absence of MyoE, RAB11 SVs decorate the array of tip actin cables radiating from the SPK (Pantazopoulou et al., 2014), suggesting that these vesicles contain an F-actin-binder. It is tempting to speculate that UDS1 or HMSV resemble melanophilin (Scolnick et al., 2013) or the Sec4p-Myo2p accessory factor Smy1p (Hodges et al., 2009; Lwin et al., 2016) in that they interact with actin cables to increase the processivity of the motor. Alpha-Fold prediction of UDS1 buttressed by analytical ultracentrifugation and EM studies strongly suggested that this protein is an elongated (circa 500 Å-long) dimer formed by a long coiled-coil core. Each UDS1 domain is formed by three small helices associating with those of the second domain to form a V-shaped ‘wing’ that protrudes from the long axis. We speculate that this long structure could play a tethering role, for example by providing an ATPase cycle-independent hold of the motor to F-actin filaments. Even more suggestive is the hypothetical possibility that F-actin binding by the MyoE co-adaptors facilitates the switch between MT and F-actin transport. It should be noted that Sec4 cannot recruit UDS1, which is consistent with the view that A. nidulans Sec4 acts downstream of the RAB11-mediated transport of SVs to the SPK, mediating the ultimate step of exocytosis (Pinar and Penaña, 2021).

In summary, we have identified HUM, a novel myosin-V-containing complex required for the efficient coupling of RAB11 SVs to MyoE. Proof of concept that a motor-cargo interface can be targeted by a small chemical has been recently provided (Randall et al., 2017). Although speculative, the possibility of interfering with fungal growth by diminishing the efficiency of myosin-V-mediated transport is appealing.

Limitations of the study
Structures of the RAB11 accessory factors should inspire experiments addressing their mechanistic roles. Our approaches to the UDS1 structure including size exclusion chromatography, analytical ultracentrifugation, and negative-staining EM of the purified protein strongly supports the AlphaFold model. However, the intrinsic flexibility of UDS1 precluded any attempt to average molecule shapes. Moreover, the 3D structure of HMSV relies solely on AlphaFold, because the protein is insoluble when expressed in bacteria. Our inability to obtain HMSV in quantities required for its structural characterization and for reconstituting the whole transport process by bottom-up synthetic approaches is currently a bottleneck. Secondly, as already mentioned, the HUM complex in all likelihood has other partners that cannot be reliably identified by GFP-trapping and MS/MS, perhaps due to the very transient interactions in which they are engaged. A third caveat is that our current data do not discriminate between a “relay model” for the cooperation between actin and MTs and a “parallel pathways model” in which myosin V would deliver SVs to the vesicle supply center (SPK), whereas kinesin-1 would deliver SVs directly to the cell cortex at the sites of MT plus-end contacts (A hybrid model is also possible).

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:
○ RAB-GST pull-downs with purified proteins
○ Pull-down of the UDS1-HMSV complex with RAB11-GST
○ Pull-down experiments of TNT-expressed proteins with GST fusion protein baits
○ Pull-down experiments of RAB preys with GST-GTD
○ ProA immunoprecipitations
○ GFP-trap and western blotting
○ Shotgun proteomic analysis of RAB11-GST effectors
○ Analytical ultracentrifugation
○ Negative staining electron microscopy
○ Fluorescence microscopy
○ Alpha-Fold predictions

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104514.

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AUTHOR CONTRIBUTIONS
MP, AA, and IB-P carried out biochemical and genetic experiments. VdR conducted MS/MS analyses; EA-P and AdG conducted electron microscopy experiments, AG conducted AlphaFold predictions, and MAP carried out fluorescence protein localization analyses, supervised the project, and, with MP, wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-HA rat mAb     | Merck  | Cat# 11867423001, RRID:AB_390918 |
| Anti-HIS            | Clontech | Cat# 631212, RRID:AB_2721905 |
| Anti-MyoE           | This study | Custom made/custom c |
| Anti-GFP            | Merck  | Cat# 11814460001, RRID:AB_390913 |
| Anti-Rat Ig, Mouse ads-HRP | Southern Biotechnology | Cat# 3010-05, RRID:AB_2795801 |
| Anti-Mouse IgG (H + L) HRP-conjugated | Jackson Immunoresearch | Cat# 115-035-003, RRID:AB_10015289 |
| Anti-Rabbit IgG, HRP-linked whole Ab (from donkey) | Cytiva | NA934, RRID:AB_772206v |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Hi-TRAP NHS columns | Cytiva | Cat# 17-0716-01 |
| Glutathione Sepharose 4B | Sigma-Aldrich | Cat# 17-0756-01 |
| nProtein A Sepharose 4 Fast Flow | Cytiva | Cat# 17-5280-01 |
| Ni-Sepharose High performance | Sigma-Aldrich | Cat# 17-5268-01 |
| GFP-TRAP magnetic agarose | Chromotek | Cat# gtma-20 |
| PD-10 desalting columns | Cytiva | Cat# 17-0851-01 |
| PD midiTrap G25 columns | Cytiva | Cat# 28918008 |
| HiLoad 16/600 superdex column | Sigma-Aldrich | Cat# GE28-9893-35 |
| GDP                 | Jena Bioscience | Cat# UN-1172 |
| GTP-S               | Jena Bioscience | Cat# UN-412 |
| Complete, EDTA-free Protease Inhibitor Cocktail | Sigma-Aldrich | Cat# 11873580001 |
| Complete ULTRA Tablets, EDTA-free | Sigma-Aldrich | Cat# 05892953001 |
| Pierce Centrifuge Columns, 0.8 mL | ThermoFisher | Cat# 89869 |
| Clarity Western ECL Substrate | Biorad | Cat#1705061 |
| **Critical commercial assays** |        |            |
| TNT SP6 Quick Transcription/Translation System | Promega | Cat# L2080 |
| NucleoBond Xtra Midi columns | Macherey Nagel | Cat# 740412 |
| **Experimental models: Organisms/strains** |        |            |
| Please refer to Table S1 | N/A |
| Oligonucleotides | N/A |
| Please refer to Table S2 | N/A |
| Recombinant DNA | N/A |
| Plasmid: pET21b | Fisher Scientific | Cat# 69-741-3 |
| Plasmid: pGEX2T | Merck | Cat# GE28-9546-53 |
| Plasmid: pSP64 poly(A) | Promega | Cat# P1241 |
| Plasmid: pET21b-RAB11-GST | This study | N/A |
| Plasmid: pET21b-Sec4-GST | This study | N/A |
| Plasmid: pET21b-Rab5b-GST | This study | N/A |
| Plasmid: pGEX2T-UDS1 | This study | N/A |
| Plasmid: pGEX2T-GTDMyoE | This study | N/A |
| Plasmid: pSP64 MyoE | This study | N/A |
| Plasmid: pSP64 HMSV-HA | This study | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for reagents should be directed to and will be fulfilled by the Lead Contact: Mario Pinar (mps@cib.csic.es). Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas Margarita Salas, Ramiro de MAeztu, 9, Madrid, 28040, Spain.

Materials availability
All materials generated in this study are available from the lead contact without restriction.

Data and code availability

- All data central to supporting the main claims of the paper are included with the text. Raw microscopy series will be available from the lead contact, mps@cib.csic.es.
- This paper does not report any original code.
- All additional information required to re-analyze the data reported in this paper is available from the lead contact, mps@cib.csic.es.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Aspergillus techniques
Standard A. nidulans media were used for strain propagation and conidiospore production (Cove, 1966). GFP and epitope-tagged alleles were introduced in the different genetic backgrounds by meiotic recombination (Todd et al., 2007) and/or transformation (Tilburn et al., 1983), which used recipient nkuΔ strains deficient in the non-homologous end joining pathway (Nayak et al., 2005). Complete strain genotypes are listed in Table S1.

Null mutant strains and protein tagging
uds1Δ, hmsVΔ, sec4Δ (Pantazopoulou et al., 2014) and myoEΔ (Taheri-Talesh et al., 2012) were constructed by transformation-mediated gene replacement with cassettes made by fusion PCR carrying appropriate selectable markers (Szewczyk et al., 2006). Integration events were confirmed by PCR with external primers.
The following proteins were C-terminally tagged endogenously, using cassettes constructed by fusion PCR (Nayak et al., 2005; Szewczyk et al., 2006): UDS1-GFP, UDS1-HA3, UDS1-tdTomato, HMSV-GFP, HMSV-HA3, MyoE-GFP (Taheri-Talesh et al., 2012), MyoE-mCherry and ChsB-GFP (Hernández-González et al., 2018a). GFP-RAB11 (Pantazopoulou et al., 2014) and mCherry-RAB11 (Pinar and Peñalva, 2020) were expressed from its own promoter. Primers used in strain construction are listed in Table S2.

**METHOD DETAILS**

**GFP-MyoE GTD and GFP-MyoE ΔGTD transgenes driven by the inuA promoter**

**GFP-MyoE GTD**

A transforming cassette consisting of, from 5’ to 3’, the sucrose-inducible promoter of the inulinase gene (*inuA*) (Hernández-González et al., 2018b), the GFP-coding sequence translationally fused to the coding sequence for residues 1082 through 1569 of MyoE, the *Aspergillus fumigatus* pyrG gene and the *inuA* gene 3’-flanking region was constructed by 5-way fusion PCR (Taheri-Talesh et al., 2008), using the following primers (underlined sequences indicate regions of overlap used for fusion PCRs):

1. : *inuA* promoter region, PCR-amplified with primers:

   - 5’-GTGGAGGCCACTCTCGGAAAC-3’
   - 5’-CAGTGAAAAGTTCTTCTCTTTACTCATTTTGGTGATGTCGCTGACC-3’

   (the underlined overlapped with GFP-coding region)

2. : GFP-(Gly-Ala)₆

   - 5’-ATGAGTTAAGGAGAAGAATTTTC-3’
   - 5’-GGCACCGGCTCCAGCGCCTGC-3’

3. : MyoE-GTD

   - 5’-CTGGTGCAAGGCCTGGAGCGGTGCCGCCCTGTTGAAAGACGAAGACGCAC-3’: [the underlined overlapping with the GFP-(Gly-Ala)₆] coding region.

4. : pyrGAF

   - 5’-ATTCAGACSACTGGGGGCCTCAGTTTACTTCTCCATCCCCTATTCTC-3’: (the underlined overlapping with *pyrGAF*).

5. : *inuA* 3’-UTR

   - 5’-ACGCATCAGTGCCTCCTCTCGAGATCGATGCTGTTTGTG-3’

   (the underlined overlapping with *pyrGAF*).

**GFP-MyoE ΔGTD**

Constructed as above, but using as primers (3) to amplify the coding sequence for residues 1-1081 of MyoE the following oligonucleotides:

5’-CTGGTGCAAGGCCTGGAGCGGTGCCGCTCAGCATAATTATGAGTGTCGACGC-3’
(the underlined overlapping with the GFP-(Gly-Ala)6 coding region.

5’-ATCCAGCACACTGGGCCGTATATTAAGGGTAATGCTCCTTTGCG-3’:

(the underlined overlapping with pyrGa).

The cassettes were used to replace the inuA gene, considered to be a safe haven, by homologous recombination. inuA does not affect growth on carbon sources other than inulin (glucose is used as standard carbon source in A. nidulans media). The inuA is inducible by sucrose, and results in moderate levels of expression (Hernández-González et al., 2018b)

**Plasmids for protein expression**

**GST constructs**

pET21b-RAB11-GST: carries cDNA encoding cysteine-less RAB11 with GST C-terminally attached. Ndel/BamHI insert in pET21b.

pET21b-Sec4-GST: carries cDNA encoding cysteine-less Sec4 with GST C-terminally attached. Ndel/Xhol insert in pET21b.

pET21b-RAB5b-GST: carries cDNA encoding cysteine-less RAB5b with GST C-terminally attached. NheI/NotI insert in pET21b.

Note that in all three constructs GST is attached to the C-termini of the RABs, and that they all include a stop codon after the GST coding region to interrupt translation before the His tag.

pGEX2T-GFP: pGEX-2T derivative encoding a GST-sGFP fusion as a BamHI-EcoRI.

pGEX2T-UDS1: UDS1 cDNA cloned as BamHI in pGEX-2T (N-terminal GST).

pGEX2T-GTDMyoE includes coding sequence for residues 1082 through 1569 (the C.terminus) of MyoE, cloned in phase with GST as BamHI-Xmal.

**TNT expression constructs**

pSP64 MyoE: MyoE cDNA cloned as a BamHI fragment in Promega’s #P1241 pSP64 poly (A).

pSP64 HMSV-HA: C-terminally HA3-tagged cDNA encoding HMSV cloned as PstI/Xmal in pSP64 poly (A).

pSP64 UsO1-HA: C-terminally HA3-tagged cDNA encoding UsO1 cloned as PstI/Xmal in pSP64 poly (A).

**His6-tagged constructs**

pET21b-UDS1: UDS1 cDNA, cloned as Nhel/NotI in pET21b.

pET21b-RAB11: RAB11 cDNA sequence without the two C-terminal Cys residues, cloned as Ndel/Xhol in pET21b.

pET21b-RAB5b: Rab5b cDNA sequence without the two C-terminal Cys residues, cloned as NheI/Xhol in pET21b.

pET21b-Sec4: Sec4 cDNA sequence without the two C-terminal Cys residues, cloned as Ndel/Xhol in pET21b.

**In vitro transcription/translation**

Proteins were synthesized using TNT® SP6 Quick Coupled Transcription/Translation system (Promega #L2080) using the standard reaction mix (rabbit reticulocyte lysate plus amino acids) supplemented with 20 μM methionine. Reactions were primed with 1 μg of purified, circular pSP64 derivatives, which were purified using NucleoBond Xtra-Midi columns (Macherey Nagel, #740412).
Antibodies and western blotting

Antisera against MyoE and Usol were raised in rabbits by Davids Biotechnology (https://www.davids-bio.com). Animals were immunized with His6-tagged polypeptides containing residues 1082-1569 of MyoE (the GTD) or residues 1-659 of USO1. These polypeptides were purified by Ni2+ affinity chromatography after expression in E. coli BLB21 as described (Pinar et al., 2015). Antibodies against the target proteins were purified from raw antisera (40 mL) by affinity chromatography with the respective antigens, previously coupled to 1 mL HI-TRAP NHS columns (Cytiva #17-0716-01) packed with Sepharose pre-activated for covalent coupling of ligands containing primary amino groups, following instructions of the manufacturer. Antibodies were eluted with 100 mM glycine, pH 3.0, neutralized with 2M Tris, pH 7.5 and stored at –20°C.

Western blots were reacted with the following antibodies

For HA3-tagged proteins
α-HA rat mAb (1/1,000) (Merck #11867423001) as primary antibody, and HRP-conjugated α-rat IgM+IgG, as secondary antibodies (Southern Biotechnology #3010-05; 1:4,000).

For His6-tagged UDS1 and RABs
α-His primary antibody (1/10,000; Clontech #631212) and HRP-conjugated goat anti-mouse IgG (H + L) secondary antibodies (Jackson Immunoresearch #115-035-003, 1/5000).

For MyoE: MyoE was detected with a custom-made α-MyoE-GTD antiserum (1/4000; see above) and donkey HRP-coupled α-rabbit IgG (Cytiva NA-934) as secondary antibodies.

For α-GFP western blotting
we used Merck #11814460001 mixture of two mouse mAbs (1/5000) as primary antibodies and HRP-conjugated AffiniPure goat anti-mouse IgG (H + L) secondary antibodies (Jackson Immunoresearch #115-035-003, 1/5000). In all cases reacting bands were detected with Clarity western ECL substrate (Biorad Laboratories #1705061).

RAB-GST purification and nucleotide loading

500 mL bacterial cultures in LB plus antibiotics as appropriate were incubated at 37°C until reaching a O.D. of 0.6–0.8 at 600 nm. These primary cultures were induced with 0.1 mM IPTG, transferred to a 15°C incubator and shaken for an additional 20 h. Cells were collected by centrifugation and stored at –80°C. A pellet corresponding to 250 mL of the culture was resuspended in PBS containing cOmplete™ protease inhibitor cocktail (Sigma-Aldrich #11873580001), 0.2 mg/mL lysozyme and 1 µg/mL of DNase I (Abenza et al., 2010) and lysed in a French Press. After centrifugation at 30,000 × g and 4°C for 30 min, the supernatant was mixed with 300 µL of glutathione Sepharose 4B (Sigma-Aldrich #17-0756-01) and incubated at 4°C for 1 h in a rotating wheel. Sepharose-bound RABs were resuspended in a buffer consisting of 25 mM HEPES PH 7.5, 110 mM KCl, 1 mM DTT, 10 mM EDTA and 125 µM GDP or GTPγS (Jena Bioscience UN-1172 and UN-412, respectively).

UDS1-His6 expression and purification from bacteria

E. coli cells (BLB21 pRIL) carrying pET21b-UDS1 were cultured at 37°C in LB containing ampicillin and chloramphenicol until reaching and OD<sub>660</sub> of 0.5. At this point cultures were induced with 0.1 M IPTG, transferred to 15°C and incubated overnight before collecting cells by centrifugation and storing pellets at –80°C. Bacterial pellets were thawed, resuspended in 25 mL of lysis buffer (as for RAB-GST proteins), incubated for 30 min in ice, and lysed with a French Press. Lysates were clarified by centrifugation (30,000 × g at 30 min at 4°C) and purified in a Ni-Sepharose High Performance column (Sigma-Aldrich #17-5268-01). Imidazole (0.5 M) present in the eluted fraction was removed with a PD-10 desalting columns (Cytiva #17-0851-01) equilibrated in PBS, pH 7.4 containing 5% glycerol and 1 mM DTT. The eluate (3.5 mL) was loaded onto a HiLoad 16/600 Superdex column (Sigma-Aldrich #GE28-9893-35) that was run at 1 mL/min. Fractions containing protein were analyzed by SDS-PAGE, stained with Coomassie and pooled as appropriate.
RAB-His6 purification and nucleotide loading

Expression and Ni\(^{2+}\) Sepharose affinity purification were carried out as above. Imidazole was removed with PD midiTrap G25 columns (Cytiva #28918008) in 75 mM HEPES, pH 7.5, 150 mM KCl and 1 mM DTT. Eluted RABs were incubated with 1 mM GDP or GTP\(_\gamma\)S and 10 mM EDTA for 30 min at 30°C. Then, 20 mM MgCl\(_2\) was added, and the solutions were aliquoted, flash-frozen and stored at −80°C.

Total cell extracts

These were carried out as described (Pinar et al., 2019). 70 mg of lyophilized mycelia were ground to a fine powder in 2 mL tubes containing a ceramic bead and a 20 s pulse of a FastPrep set at power 4. The powder was suspended in 1.5 mL of ‘low KCl buffer’ (25 mM HEPES, pH 7.5, 175 mM KCl, 5mM MgCl\(_2\), 1 mM DTT and 0.1% Triton X-100) before bound material was eluted with 20 µL of Laemmli loading buffer. 15 µL aliquots were analyzed by western blotting using α-His antibody (for UDS1-His6) or α-HA antibody (for HMSV-HA3) and 7.5% polyacrylamide gels, or α-MyoE antibodies and Biorad’s pre-casted 4–15% polyacrylamide gels (for MyoE).

Pull-down of the UDS1-HMSV complex with RAB11-GST

10 µL of glutathione Sepharose beads loaded with RAB11-GST GTP\(_\gamma\)S or GDP were incubated in Pierce microcolumns for 2 h at 4°C with 2.5 µg of UDS1-His6 and 10 µL of TNT-synthesized HMSV-HA3 in 400 µL of ‘low KCl’ buffer containing 10% glycerol. Beads were washed four times with ‘medium KCl’ buffer. Equal amounts of bound material were analyzed by western blotting using α-HA and α-His antibodies.

Pull-down experiments of TNT-expressed proteins with GST fusion protein baits

GST-UDS1, GST-GTD and GST-GFP baits were purified as described for RAB-GST proteins. 15 µL of glutathione Sepharose beads containing bait protein fusions were mixed with 10 µL of TNT-synthesized HMSV-HA3 or Uso1-HA3 preys (10 µL of each reaction mix) in 0.4 mL of 25 mM HEPES pH 7.5, 300 mM KCl, 0.5% Triton, 0.5 mM EDTA and 1 mM DTT, using Pierce microcolumns, which were incubated for 2 h at 4°C in a rotating wheel. Beads were washed four times with the same buffer and eluted with 20 µL of Laemmli buffer. 5 µL aliquots were analyzed by western blotting using α-HA and α-His antibodies.

Pull-down experiments of RAB preys with GST-GTD

Sepharose beads (10 µL slurry) loaded with either GST-GTD or, as negative control, GST-GFP fusion proteins were mixed with the different RABs (final concentration 50 nM) in 50 mM HEPES pH 7.5, 110 mM KCl, 5 mM MgCl\(_2\), 0.1% Triton X-100 (v/v) and 10% (v/v) glycerol (500 µL final volume) in 0.8 mL Pierce microcolumns. The reaction mixtures were rotated overnight at 4°C before beads were recovered by centrifugation and washed four times in 50 mM HEPES pH 7.5, 250 mM KCl, 5 mM MgCl\(_2\) and 0.1% Triton X-100 (v/v) for
10 min at 4°C. Bound proteins were eluted with 20 µL of Laemmli loading buffer. 15 µL samples were analyzed by α-His western blotting using 12% polyacrylamide gels. A gel run in parallel was stained with Coomassie blue to assess equal loading of GST baits.

**ProtA immunoprecipitations**

For α-MyoE co-immunoprecipitation experiments of HMSV-HA3, 5 µL samples of Protein A-Sepharose (Cytiva #17-5280-01) were preincubated with 10 µL each of purified α-MyoE or α-Uso1 antibodies for 3 h at room temperature. Antibody-loaded beads were mixed with 25 µL of TNT-synthesized MyoE and 25 µL of TNT-synthesized HMSV-HA3 in 0.4 mL of 25 mM HEPES pH 7.5, 500 mM NaCl, 0.5% Triton, 0.5 mM EDTA and 2% BSA, using 0.8 mL Pierce microcolumns. Beads were recovered by microcentrifugation, washed four times in the same buffer (without BSA) and eluted with 20 µL of Laemmli loading buffer. 5 µL of each sample were analyzed by western blot (7.5% polyacrylamide gel) using α-HA mAb. A gel run in parallel was stained with Coomassie blue to assess equal loading of Protein A beads with IgG heavy chains.

**GFP-trap and western blotting**

Cell extracts [strains, MyoE-GFP (MAD4406), UDS1-GFP (MAD6379), HMSV-GFP (MAD7326) and Uso1-GFP (MAD6358)] were prepared as described above, but using the lysis buffer recommended by the manufacturer, which containing 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40 and Complete protease inhibitors. Approximately 100 mg of total protein (4 mL of extract) were immunoprecipitated with 25 µL of GFP-Trap magnetic agarose beads (Chromotek #gtma-20) following incubation for 2 h at 4°C in a rotating wheel. Beads were washed four times with the same buffer before eluting the immunoprecipitated material with 60 µL of Laemmli buffer. 10 µL aliquots were analyzed by α-HA3 western blotting (7.5% polyacrylamide gels) or α-MyoE western blotting. 2 µL were analyzed by α-GFP western blotting to determine levels of immunoprecipitated baits. Lastly 8 µL were analyzed by SDS-PAGE and silver staining.

**Shotgun proteomic analysis of RAB11-GST effectors**

Large scale purification of proteins interacting with the GDP and GTP forms of RAB11-GST was carried out as described previously for GST-RAB11 (Pinar and Penalva, 2017).

Bound proteins were loaded onto a 10% polyacrylamide gel, which was run until proteins moved 1 cm into the gel. The protein mixture band was detected by colloidal Coomassie staining, excised and processed for tryptic digestion and subsequent analysis by MS/MS essentially as described (Pinar et al., 2019). For MS/MS analyses of GFP-tagged bait associates, proteins were digested using the ‘on-bead digest protocol for mass spectrometry following immunoprecipitation with Nano-Traps’ recommended by Chromotek. In both cases mass spectra .raw files were used to search the *A. nidulans* FGSC A4 version_s10m02-r03_orf_trans_all-MODI proteome database (8,223 protein entries) using Mascot search engine version 2.6 (Matrix Science). Peptides were filtered using Percolator (Kall et al., 2007), with a q-value threshold set to 0.01.

**Analytical ultracentrifugation**

Sedimentation equilibrium analysis of UDS1-His was carried out in the Molecular Interactions Facility of the Centro de Investigaciones Biológicas using an XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with a UV-VIS detector set at 237 nm. Centrifugation was carried out in short (95 µL) columns at speeds ranging from 6,000 to 9000 rpm, with a last high-speed (48,000 rpm) run to deplete the protein from the meniscus and obtain the corresponding baseline offsets. Weight-average buoyant molecular weights were determined by fitting, using HeteroAnalysis software (Cole, 2004), a single-species model to the experimental data (corrected for temperature and solvent composition with SEDNTERP software (Laue et al., 1992).

**Negative staining electron microscopy**

Purified UDS1 was diluted to 0.2 µM in 150 mM NaCl, 25 mM HEPES pH 7.5 and 5% glycerol, and stained with 2% (w/v) uranyl acetate. Specimens were examined under a JEOL 12300 electron microscope equipped with a TVIPS CMOS 4kx4k camera and operated at 100 kV. Data were collected at a nominal magnification of 40,000 x, which corresponds to 2.84 Å/pixel at the micrograph level. The length of 71 representative particles selected from multiple micrographs was measured using ImageJ (https://imagej.nih.gov/ij/).
Fluorescence microscopy
Hyphae were cultured in watch minimal medium (WMM) (Peñalva, 2005). Microscopy chambers, hardware, software and image acquisition procedures have been thoroughly documented (Pinar and Peñalva, 2020), with the sole exception that some of the experiments using the Hamamatsu Gemini beam splitter were carried out in a Leica DMi8 inverted microscope instead of a Leica DMi6000. Z-stacks were deconvolved using Huygens Professional software (Hilversum, Holland), version 20.04.0p5 64 bits. Images (usually MIPs unless otherwise indicated) were contrasted with Metamorph (Molecular Devices) and annotated using Corel Draw. Movies were assembled with Metamorph and compressed using QuickTime (Apple Inc.). Quantitation of average MyoE-GFP signals in the SPK was made using MIPs of raw images. Datasets were analyzed with GraphPad Prism 7.03 (GraphPad).

To estimate the areas occupied by the RAB11 cluster in wild-type and sec4Δ cells (Figure 1D), regions were automatically drawn after thresholding the image and the areas were calculated with Metamorph. A similar procedure was used to determine total intensities of MyoE-GFP in the wild-type and sec4Δ SPKs (Figure 1E). Linescans of hyphal tips used for Figure 1I correspond to average values of 3 px-wide ROIs. To determine the widths of wild-type and mutant cells, hyphae were cultured overnight as above before adding calcofluor at 1 µg/mL to label cell walls. Fluorescence pictures (middle planes or, for 3D reconstructions of the septae, z-stacks; 100× magnification optics, 1 px = 0.06 µm) were taken and used to draw linescans perpendicular to the growth axis, which were used to measure the distances between the two sharp peaks corresponding to intersections with the cell wall.

Alpha-Fold predictions
The UDS1 and HSMV AlphaFold2 (Jumper et al., 2021) predictions were run using versions of the program installed locally and on Colab-Fold (Mirdita et al., 2022) with the MMseqs2 MSA option. The input UDS1 sequence (AN5595, 941 residues) was submitted as two separate chains (using a 1:1 homo-oligomer setting) guided by the experimental characterization of the protein.

QUANTIFICATION AND STATISTICAL ANALYSIS
Details are described in the legends to Figures 1, 4 and 7. Analysis was carried out with GraphPad Prism software (v. 7.03).