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An intrinsically disordered region in OSBP acts as an entropic barrier to control protein dynamics and orientation at membrane contact sites

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Abstract

Lipid transfer proteins (LTPs) acting at membrane contact sites (MCS) between the ER and other organelles contain domains involved in heterotypic (e.g. ER to Golgi) membrane tethering as well as domains involved in lipid transfer. Here, we show that a long \approx 90 aa intrinsically unfolded sequence at the N-terminus of oxysterol binding protein (OSBP) controls OSBP orientation and dynamics at MCS. This Gly-Pro-Ala-rich sequence, whose hydrodynamic radius is twice as that of folded domains, prevents the two PH domains of the OSBP dimer from homotypically tethering two Golgi-like membranes and considerably facilitates OSBP in-plane diffusion and recycling at MCS. Although quite distant in sequence, the N-terminus of OSBPrelated protein-4 (ORP4) has similar effects. We propose that N-terminal sequences of low complexity in ORPs form an entropic barrier that restrains protein orientation, limits protein density and facilitates protein mobility in the narrow and crowded MCS environment.

Keywords: Intrinsically disordered protein (IDP); low complexity sequence; Membrane Contact Site (MCS); OSBP-related protein (ORP), Lipid transfer protein (LTP).

1 Introduction

2 Oxysterol binding protein (OSBP) and OSBP-related proteins (ORPs) constitute a 3 conserved family of lipid transfer proteins (LTPs) that transport key lipids such as cholesterol 4 or phosphatidylserine between cellular membranes (Kim et al., 2013; Olkkonen and Li, 2013; 5 Pietrangelo and Ridgway, 2018). LTPs contribute to the establishment of lipid gradients 6 between organelles and counterbalance the homogenization in lipid composition that 7 accompanies vesicular traffic or lipid signaling reactions (Kim et al., 2015; Saheki et al., 2016; 8 Yadav et al., 2015). ORPs share an OSBP-related domain (ORD), which is responsible for the 9 lipid transfer activity (Im et al., 2005) and functions in a directional manner by counter 10 exchanging a specific lipid for the phosphoinositide PI(4)P (Chung et al., 2015; de Saint-Jean et 11 al., 2011; Mesmin et al., 2013; Moser von Filseck et al., 2015a). Thus, OSBP transports 12 cholesterol from the endoplasmic reticulum (ER) to the trans Golgi network (TGN) owing to 13 back transfer of PI(4)P, which is synthesized in the TGN and hydrolyzed at the ER (Mesmin et 14 al., 2013; 2017; Zewe et al., 2018).

15 In addition to the ORD, many ORPs carry targeting determinants for the ER and other 16 organelles. These determinants allow ORPs to tether two membranes (e.g. ER and PM or ER and 17 TGN), hence restricting the lipid exchange to membrane contact sites (MCSs), which are regions 18 of close apposition between organelles (intermembrane distance \approx 10-50 nm)(Levine, 2004; 19 Mesmin et al., 2013). OSBP contains a N-terminal pleckstrin homology (PH) domain, which 20 interacts with the TGN or endosomal phosphoinositide PI(4)P and a central FFAT (two 21 phenylalanines in an acidic tract) motif, which interacts with the general ER receptor VAP-A/B 22 (Furuita et al., 2010; Kaiser et al., 2005; Levine and Loewen, 2006; Levine and Munro, 2002; 23 Loewen et al., 2003). Cellular observations as well as *in vitro* reconstitution experiments suggest 24 that the tripartite PH-FFAT-ORD architecture of OSBP allows this protein to function as a ferry-25 bridge, bridging the TGN and the ER through its PH domain and FFAT motif, respectively, and ferrying cholesterol between the ER and TGN through its ORD (Antonny et al., 2018; Mesmin et
al., 2013).

Although the tripartite PH-FFAT-ORD architecture of OSBP explains its membrane 28 29 tethering and lipid transfer activities, many mechanistic questions remain. The very nature of 30 MCSs where two parallel membranes sandwich a thin layer of cytosol should create unique 31 constraints. For example, it is not known whether the linker regions connecting the PH domain, 32 FFAT motif, and ORD are long enough to allow simultaneous membrane tethering and ORD 33 movement between membranes (Antonny et al., 2018; Dittman and Menon, 2017). The 34 compatibility between the movements of folded domains and the linker length is a general issue 35 in MCS, also applying to proteins such as the PI phosphatase Sac1, which hydrolyzes PI(4)P 36 (Stefan et al., 2011; Zewe et al., 2018), and the extended synaptotagmins (Schauder et al., 2014). 37 A second question is the degree of cooperativity between proteins at MCS. The establishment of 38 MCS by some 'pioneered' tethers should indirectly favor the subsequent recruitment of other 39 tethers of the same length. However, having too many proteins in MCS might create some steric 40 hindrance. A recent study using artificial tethers made of fluorescent proteins held by 41 elementary blocks of defined height has started uncovering the complex behavior of proteins at 42 MCS, revealing the possibility of an exclusion mechanism based on the incompatibility between 43 proteins of different lengths or *via* crowding effects (Schmid et al., 2016).

44 In this study, we focus on an overlooked feature of ORPs: the presence of a long N-45 terminal region (hereafter abbreviated as N-ter) of low amino-acid complexity upstream of the 46 PH domain. Using OSBP and ORP4 as models, we show that the N-ter has two functions. First, it 47 imposes the geometry of membrane tethering, favoring heterotypic membrane tethering 48 between a VAP-containing membrane and a PI(4)P-containing membrane at the expense of the 49 homotypic configuration where two PI(4)P-rich membranes are held by the two PH domains of 50 OSBP or ORP4, which are dimers. Second, the N-ter prevents OSBP from being too concentrated 51 at MCS, thereby facilitating protein diffusion within MCS and protein recycling between MCS.

- 52 Our work uncovers a new function of intrinsically unfolded regions in the functioning of multi-
- 53 domain proteins.

55 **Results**

56 Bioinformatic analysis of the N-terminal region of OSBP and related proteins

57 The mammalian ORP family includes 12 members, many of which display a similar 58 domain organization (Figure 1A, S1A). The N-terminal half (including PH domain and FFAT 59 motif) is involved in membrane tethering while the C-terminal half (ORD) transfers lipids 60 (Mesmin et al., 2013). For ORP5 and ORP8, which lack FFAT motif, ER tethering depends on a C-61 ter transmembrane segment (Du et al., 2011; Yan et al., 2008). Intriguingly, most ORPs (OSBP, 62 ORP3 - ORP8, ORP10 and ORP11) contain a long sequence upstream of their PH domain 63 (Figures 1A, S1 and S2). The exceptions are ORP2, which only consists of the FFAT motif and ORD, and ORP9, which does not contain a sequence upstream of its PH domain. Phylogenetic 64 65 analysis indicates that the N-ter of OSBP as well as the coiled-coils between the PH domain and 66 FFAT motif appeared later and showed less sequence conservation than the PH domain and 67 ORD (Figure S2B).

68 We assessed the order/disorder distribution along the ORP sequences using Predictor of 69 Naturally Disordered Regions (PONDR®) (Figure S1A). We employed two algorithms, VL3 and 70 VSL2, trained on a set of hundreds of disordered and ordered protein regions. Both predictors 71 are based on attributes including amino acid frequencies, sequence complexity and averaged 72 flexibility. The N-ter of OSBP, ORP3–8, ORP10 and ORP11 have a high disorder score (> 0.8), in 73 contrast to the low disorder score of the PH domain and ORD. The low disorder score of the N-74 ter of ORP1 correlates with the presence of three ankyrin repeats that bind the small GTPase 75 Rab7 on late endosomes (Johansson et al., 2003). The second region of high disorder was found 76 in the linker between the PH domain and ORD. Thus, most ORPs contain long 60-140 aa N-77 terminal sequences that are predicted to be disordered.

We compared the aa composition of the N-ter of human ORPs using the PH domain and
ORD as references for well-folded domains (Figures 1A and S1B; exact aa range of each region

80 is shown in **Figure S2A**). Pie charts show that the aa composition of the PH domain and ORD is 81 well distributed between the 20 aa, as generally observed for folded domains, whereas the N-82 terminal sequences display contrasting features (Figure S1B). Some N-ter are very rich in 83 proline (black), the strongest disorder-promoting residue (Theillet et al., 2013). Others are 84 enriched in glycine (dark grey), alanine (light grey) and/or in charged residues (red: negative; 85 blue: positive). Common features for all N-ter are the paucity of large hydrophobic residues 86 (Phe, Trp, Ile and Leu; yellow and green) and the abundance of serine (shown in light pink), 87 which is after proline and glutamic acid the third most disorder-promoting residue (Theillet et 88 al., 2013) (Figure S1B). Because sequences enriched in proline and in polar and charged 89 residues at the expense of hydrophobic amino acids are not prone to fold, we hypothesized that 90 most N-terminal sequences in ORPs are intrinsically disordered.

91 The N-terminal regions of OSBP/ORP4 strongly increase their hydrodynamic radii

92 In the following, we focused on the N-ter of OSBP and ORP4. This choice was motivated 93 by two reasons. First, numerous *in vitro* and cellular assays have been developed for OSBP, 94 thereby facilitating the analysis (Mesmin et al., 2013). Second, ORP4 is the closest OSBP 95 orthologue, but its N-ter is quite different (**Figure 1A** and **S1B**). The N-ter of OSBP is almost 96 entirely composed of Gly, Pro and Ala residues (GPA), which account for 75% of the sequence, 97 whereas ORP4 N-ter is significantly longer and also rich in Ser and Glu. The comparison 98 between ORP4 and OSBP could help defining the general *vs* peculiar properties of their N-ter.

99 Unstructured sequences occupy a larger volume than structured sequences of the same 100 aa length. To estimate the contribution of N-ter to protein size, we performed gel filtration 101 chromatography on purified constructs. The analysis was performed on the full-length protein 102 (OSBP *vs* Δ N-OSBP) as well as on shorter constructs (**Figure 1B-D**). N-PH-FFAT and PH-FFAT 103 correspond to the first half of OSBP and can be used to recapitulate its tethering activity, 104 independently of lipid transfer (Mesmin et al., 2013). N-PH- Δ CC-FFAT and PH- Δ CC-FFAT lack

105 the predicted coiled-coil regions between the PH domain and FFAT motif and behave as 106 monomers rather than as dimers in the gel-filtration column. In all cases, we observed that N-107 ter had a large impact on protein size, increasing the Stoke's radius by ~ 0.5 to 1 nm for OSBP 108 constructs (OSBP vs Δ N-OSBP, N-PH-FFAT vs PH-FFAT, N-PH- Δ CC-FFAT vs PH- Δ CC-FFAT) and 109 by ~1.3 nm for ORP4 constructs (N-PH-FFAT vs PH-FFAT) (Figure 1D). This increase was about 110 2-fold higher than what was expected from the slope of the calibration curve, which was 111 established with folded globular standards. Because denaturation increases the hydrodynamic radius of well-folded proteins by ca. two-fold (Dutta and Bhattacharyya, 2001), the analysis 112 113 suggests that the N-ter of both OSBP and ORP4 are intrinsically unfolded. In addition, the PH-114 FATT region appeared more elongated than ΔN -OSBP, which eluted as a globular protein 115 (Figure 1D). This difference suggests that the presence of the second intrinsically disordered 116 sequence and of coiled-coils in PH-FATT makes this tether region elongated, whereas the 117 presence of the ORD promotes a compact quaternary structure.

118 The N-ter accelerates OSBP cellular dynamics

119 To assess the role of N-ter on OSBP subcellular localization, we transfected RPE1 and 120 HeLa cells with the following C-terminally mCherry-tagged constructs: OSBP, ΔN-OSBP, N-PH-121 FFAT and PH-FFAT (Figure 1B). In both cell types, full-length OSBP localized mainly to the Golgi 122 complex (as evidenced by co-localization with the marker BFP-GalT, Figures 2A and S3A) and, 123 to a lesser extent, to late endosomes (colocalized with EGFP-Rab7, Figure S3A). However, a 124 large fraction of OSBP was also found in the cytosol. Compared to OSBP, ΔN-OSBP displayed a 2-125 fold higher Golgi partitioning ratio, whereas both N-PH-FFAT and PH-FFAT localized almost 126 completely to the Golgi complex with a very minor cytosolic fraction (Figure 2A). The large 127 decrease in the apparent Golgi partitioning of the constructs containing the ORD (OSBP and ΔN -128 OSBP) as compared to the constructs lacking the ORD (N-PH-FFAT and PH-FFAT) is due to a 129 negative feedback effect of the ORD, which by transferring PI(4)P destabilizes the interaction of OSBP with membranes (Mesmin et al., 2013). Of note, constructs containing only the N-ter of
OSBP fused to mCherry or GST were essentially soluble (Figure S3B-C).

In agreement with a previous report (Wang et al., 2002), ORP4 constructs containing the
ORD (ORP4 and ΔN-ORP4) co-localized with intermediate filaments (Figure S3D and E) and
only modestly stained the Golgi making their analysis complicated. We thus focused on ORP4 NPH-FFAT and ORP4 PH-FFAT, which only partitioned between the cytosol and the Golgi. ORP4
PH-FFAT showed significantly higher Golgi partitioning than ORP4 N-PH-FFAT (Figure 2B).
Thus, the N-ter of OSBP and ORP4 reduce the Golgi membrane partitioning of these proteins.

We assessed the dynamics of the OSBP and ORP4 constructs at the Golgi by fluorescence 138 139 recovery after photobleaching (FRAP) experiments. As shown in Figure 2C, the rate of recovery 140 inversely correlated with the Golgi partitioning of the constructs: full-length (FL) > Δ N > N-PH-141 FFAT > PH-FFAT. In addition, the recovery kinetics of the OSBP constructs were systematically 142 slower than that of the corresponding ORP4 constructs, in good agreement with the higher Golgi 143 partitioning of OSBP vs ORP4 (Figure 2A and B). Importantly, all N-ter-harboring constructs 144 manifested much faster (\approx 3-5-fold) recovery than the corresponding Δ N-constructs, suggesting 145 that the N-ter facilitates protein lateral motility at the membrane surface and/or exchange with 146 the cytosolic pool (Figure 2C). This effect depended on N-ter length: a construct of ORP4 147 harboring half of the original N-ter (N/2-PH-FFAT) recovered faster than PH-FFAT but slower 148 than N-PH-FFAT (Figure S3F).

149 *N-ter facilitates OSBP recycling under conditions of restricted PI(4)P synthesis*

To further assess the role of N-ter in OSBP dynamics, we performed experiments in which we artificially forced OSBP to move within the TGN (Mesmin et al., 2017). For this, we inhibited the Golgi-associated kinase PI4KIIIβ, which co-localizes with OSBP and locally provides PI(4)P to the OSBP cycle. When PI4KIIIβ is inhibited by the specific inhibitor PIK93, OSBP exhibits travelling waves, which probably corresponds to movements of OSBP contact sites towards PI(4)P-rich regions produced by remote PI4-kinases (Mesmin et al., 2017). These waves are very regular, making them adapted to precise dynamics measurements. The experiments were performed in cells stably expressing GFP-PH_{OSBP}, which acts as a PI(4)P/Arf1 reporter at the Golgi. After silencing endogenous OSBP using specific siRNA, PIK93 no longer induced travelling waves of PH_{OSBP} indicating that OSBP is largely responsible for PI(4)P turnover at the Golgi (**Figure 3A-C**).

161 We performed rescue experiments by expressing siRNA-resistant forms of either full-162 length OSBP or ΔN-OSBP (OSBP-res-mCherry and ΔN-OSBP-res-mCherry, respectively). ΔN-163 OSBP-res-mCherry was more abundant at the TGN than OSBP-res-mCherry (Figure 3D-E) in 164 agreement with other localization experiments (Figure 2A-B). Upon PIK93 addition, OSBP-res-165 mCherry restored the formation of large PH_{OSBP} travelling waves (green signal) and showed 166 itself (red signal) travelling waves in phase with that of PH_{OSBP} (Figure 3F). In contrast, ΔN -167 OSBP-res-mCherry did not rescue the siRNA phenotype. Upon PIK93 addition, we observed a 168 sharp decrease in GFP-PH_{OSBP} at the TGN suggesting that Δ N-OSBP consumed PI(4)P (compare 169 the black trace in Figure 3C to the green trace in Figure 3F lower panel). However, this 170 decrease was followed by waves of much smaller amplitude than that observed with full-length 171 OSBP (Figure 3F-G).

Altogether, the experiments presented in **Figures 2** and **3** suggest that OSBP N-ter facilitates protein turnover within or between cellular membranes without affecting its PI(4)P transfer activity.

175 The N-ter of OSBP has no effect on its lipid transfer activity

176 The fact that the N-ter is not involved in PI(4)P transfer was confirmed by steady-state 177 experiments where we analyzed the consequence of overexpressing OSBP or Δ N-OSBP on the 178 Golgi/cytosol ratio of GFP-PH_{OSBP}. Both forms strongly diminished the level of the PI(4)P probe 179 at the Golgi (**Figure S4A-B**).

To directly address the influence of the N-ter of OSBP on lipid transfer, we performed 180 181 liposome-based reconstitution experiments. We followed the transfer of the naturally 182 fluorescent analog of cholesterol (DHE) from ER-like to Golgi-like liposomes (Figure S4C). 183 Conversely, we followed the relocation of PI(4)P from Golgi-like liposomes (4% PI(4)P) to ER-184 like liposomes by monitoring the quenching of a PI(4)P probe (Figure S4D). OSBP was used in 185 catalytic amount compared to PI(4)P (1 OSBP for 30 accessible PI(4)P molecules) but in large 186 excess over the liposomes (≈ 60 OSBP molecules for one liposome). Therefore, equilibration of 187 PI(4)P between the donor and acceptor liposomes required OSBP to undergo multiple rounds of 188 lipid exchange on the same liposomes without the need for OSBP to translocate between 189 liposomes. As shown in **Figure S4C-D**, OSBP and Δ N-OSBP were indistinguishable in their sterol 190 and PI(4)P exchange activity. This similarity also applied when cholesterol and PI(4)P were 191 initially present in the ER and Golgi liposomes, respectively (Figure S4D). This condition 192 considerably accelerates the rate of PI(4)P transfer due to coupling between forward 193 cholesterol transfer and backward PI(4)P transfer (Mesmin et al., 2017; Moser von Filseck et al., 194 2015b). We concluded that the N-ter is not involved in the mechanism of sterol/PI(4)P 195 exchange by OSBP.

196

The N-ter limits the membrane recruitment of the PH domain by a crowding effect

197 The above analysis suggests that the N-ter of OSBP is an inert appendage that does not 198 interfere with the molecular mechanism of lipid transfer but controls OSBP dynamics on 199 membranes. Given its low complexity sequence, its position in the protein and its effect on the 200 hydrodynamic volume of the protein, we hypothesized that the N-ter could have a simple steric 201 effect, controlling OSBP density on membranes by limiting the surface concentration of the 202 downstream PH domain. To test this hypothesis, we compared the membrane-binding 203 properties of N-PH- Δ CC-FFAT and PH- Δ CC-FFAT.

204 We first labeled N-PH- Δ CC-FFAT and PH- Δ CC-FFAT with the fluorophore Alexa488 and 205 tested their binding to giant unilamellar vesicles (GUVs) of Golgi-like lipid composition 206 containing 0.1% Atto390-DOPE and 4% PI(4)P. We incubated these GUVs with increasing 207 concentrations of N-PH- Δ CC-FFAT or PH- Δ CC-FFAT (Figure 4A). N-PH- Δ CC-FFAT displayed 208 lower recruitment on GUVs and saturated the membrane at lower protein concentration, as 209 indicated by the smaller increase in signal intensity with concentration, and stagnation of 210 fluorescence signal above 400nM. On the contrary, PH-ΔCC-FFAT displayed a higher density on 211 GUVs, and continued binding to GUVs at higher concentrations (Figure 4A). This observation 212 suggested that the N-ter limits the surface density of OSBP on PI(4)P-containing membrane.

213 To further analyze this density effect, we performed liposome-binding experiments. We 214 first noticed that the N-ter of OSBP fused to GST showed no significant binding to PI(4)P-215 containing liposomes (Figure S3C). Next, we increased the surface density of PI(4)P in 216 liposomes from 1, 2, 5, 8, to 15 mol% while reciprocally decreasing the liposome concentration 217 from 2, 1, 0.4, 0.25, to 0.13 mM, thereby keeping the total PI(4)P concentration constant (20 μM) 218 (Figure 4B). After centrifugation and quantification of bound protein, we observed that N-PH-219 Δ CC-FFAT and PH- Δ CC-FFAT were equally recruited on liposomes with the lowest (1%) PI(4)P 220 density. However, as the density of PI(4)P increased, N-PH- Δ CC-FFAT showed lower binding as 221 compared to PH- Δ CC-FFAT. These experiments suggest that the N-ter does not affect the affinity 222 of the PH domain for PI(4)P but limits protein density at the membrane by a crowding effect.

The PH domain of OSBP and of the cognate proteins CERT and FAPP2 contain a hydrophobic loop that wedges into lipid membranes upon interaction with PI(4)P (Lenoir et al., 2010; 2015). This loop contains tryptophans offering the possibility of FRET measurements with fluorescent lipids such as diphenyl-hexatriene-PC (DPH-PC) (Antonny et al., 1997). We used this approach to further analyze the interaction of OSBP constructs with PI(4)P-containing liposomes by equilibrium and kinetics measurements.

229 Figure 4C shows the results of a titration experiment in which we added increasing 230 amounts of PH- Δ CC-FFAT or N-PH- Δ CC-FFAT to liposomes ([L] = 18 μ M) containing DPH-PC and 231 a high density of PI(4)P (10 %). The FRET signal upon the first addition of PH- Δ CC-FFAT or N-232 PH- Δ CC-FFAT was similar, suggesting that the two constructs had comparable affinity for 233 PI(4)P-containing membranes. The N-ter has no tryptophan implying that the FRET signal for 234 each bound N-PH-ACC-FFAT or PH-ACC-FFAT molecule should be the same. However, the N-PH-235 Δ CC-FFAT signal saturated at lower protein concentration suggesting that the liposome surface 236 can host a lower number of N-PH- Δ CC-FFAT molecules compared to PH- Δ CC-FFAT molecules. 237 Fitting the dose-response curves with a quadratic equation indicated that PH- Δ CC-FFAT and N-PH- Δ CC-FFAT had a similar affinity for the liposomes (K_d = 0.230 ± 0.035 μ M and 0.225 ± 0.036 238 239 µM, respectively) but that the liposomes had a lower binding capacity (expressed in 240 concentration of apparent binding sites, BS) for N-PH- Δ CC-FFAT than for PH- Δ CC-FFAT (BS = 241 $0.70 \pm 0.06 \mu$ M and $0.37 \pm 0.05 \mu$ M, respectively). The membrane area (A) occupied by the 242 protein and expressed in number of lipids is the ratio between the concentration of accessible 243 lipids (0.5[L]) and the concentration of binding site: $A = 0.5[L]/BS = 13.1 \pm 1$ and 26.5 \pm 3.5 244 lipids for PH- Δ CC-FFAT and N-PH- Δ CC-FFAT, respectively (**Figure 4C**). This two-fold difference 245 in the membrane 'footprint' of the proteins matched the difference in the maximum FRET 246 change observed at saturating protein concentration. Altogether, these titration experiments 247 provide an independent line of evidence for the role of the N-ter in limiting protein density at 248 the membrane surface.

Next, we addressed the role of the N-ter on the association and dissociation kinetics of the OSBP constructs with liposomes. In the first stage, we added a non-saturating concentration of protein to liposomes containing 10% PI(4)P and 5% DPH-PC to monitor the association phase (**Figure 4D and E**). Thereafter, we added an excess of liposomes containing PI(4)P but no DPH-PC to promote the dissociation of the protein from the fluorescent liposomes and its transfer to the unlabeled ones (**Figure 4D and E**). These experiments were performed at a lower temperature (25°C *vs* 37°C) and at a higher sampling rate (0.2 s *vs* 1 s) than the titration experiments to improve temporal resolution. Binding of PH-ΔCC-FFAT or N-PH-ΔCC-FFAT to liposomes could not be resolved (half-time ≈ 0.2 s, in the same range as the mixing time), indicating that $k_{on} > 2 \text{ s}^{-1}$ (**Figure 4D**). Although very rapid, the dissociation of PH-ΔCC-FFAT and N-PH-ΔCC-FFAT could be resolved and occurred at a similar rate ($k_{off} = 0.75 \pm 0.02 \text{ s}^{-1}$ and 1.05 ± 0.16 s⁻¹, respectively; **Figure 4D**).

261 Last, we determined the association and dissociation rates of PH-FFAT or N-PH-FFAT, 262 which by their dimeric nature could theoretically associate with the membrane with their two 263 PH domains (Figure 4E). In general, proteins having several membrane-binding regions display 264 a much higher avidity for membranes than the affinity of individual domains due to reduction of 265 dimensionality (Dumas et al., 2001). Association of PH-FFAT ($k_{on} = 0.67 \pm 0.07 \text{ s}^{-1}$) and N-PH-266 FFAT ($k_{on} = 0.28 \pm 0.02 \text{ s}^{-1}$) to liposomes was slower than that of the corresponding monomeric 267 constructs ($k_{on} > 2 \text{ s}^{-1}$) (**Figure 4E**). This drop in k_{on} might arise from a decrease in the number 268 of productive collisions between the protein and the membrane. Indeed, association kinetics are 269 very sensitive to geometrical factors (Schreiber et al., 2009) and the probability of having the 270 two PH domains of N-PH-FFAT correctly orientated towards the membranes and not shielded 271 by the N-ter might be low. However, these negative effects are compensated by a dramatic 272 decrease in the dissociation kinetics. N-PH-FFAT and PH-FFAT dissociated from the liposomes 273 about 200 times slower than the corresponding monomeric constructs. Moreover, the 274 dissociation kinetics of N-PH-FFAT and of PH-FFAT, were indistinguishable ($k_{off} = 0.0039 \pm$ 275 0.0013 and 0.0040 ± 0.0017 s⁻¹, respectively; **Figure 4E**). These observations indicate that both 276 PH domains of the dimeric constructs interact with the membrane regardless of the presence of 277 the N-ter, thereby resulting in a much longer dwell time than monomeric constructs (250 s and 278 1 s, respectively).

We concluded from these various GUV and liposome binding experiments that the N-ter of OSBP has no significant effect on the membrane affinity of the PH domain for PI(4)P and does not impede the protein from using its two PH domains to stably bind to PI(4)P containing
membranes. However, the N-ter limits protein density at the membrane surface.

283 **OSBP** N-ter controls the geometry of membrane tethering

284 OSBP interacts not only, via its PH domain, with PI(4)P on Golgi membrane but also, via 285 its FFAT motif, with the ER-resident protein VAP-A (Levine and Munro, 1998; Loewen et al., 2003). This dual interaction allows OSBP to act as a membrane tether. To determine whether 286 287 the N-ter influences OSBP and ORP4 tethering activities, we reconstituted membrane tethering 288 using purified components (Mesmin et al., 2013). We prepared two liposome populations with a 289 composition corresponding to that of the ER with 2% DGS-NTA-Ni and of the Golgi with 4% 290 PI(4)P. The presence of DGS-NTA-Ni allowed recruitment of a histidine-tagged form of VAP-A 291 onto the ER liposomes. Then, we mixed the two liposome populations, added purified N-PH-292 FFAT or PH-FFAT and followed liposome aggregation in real time by dynamic light scattering 293 (DLS).

In presence of all tethering determinants (Golgi + ER + VAP), we observed no difference in the tethering activity of N-PH-FFAT and PH-FFAT from both OSBP and ORP4 (**Figure 5A and S5A**). Surprisingly, when we removed VAP-A (**Figures 5B and S5B**) or when we replaced VAP-A with a mutant (VAP-A KM/DD) unable to interact with the FFAT motif (**Figure S5C**) we observed a striking difference between the two constructs: PH-FFAT caused liposome aggregation, whereas N-PH-FFAT did not.

We suspected that, in the absence of VAP-A, the ER-like liposomes might not be included in the aggregates promoted by PH-FFAT, which would instead promote homotypic tethering of Golgi liposomes. To distinguish homotypic (Golgi-Golgi) *vs* heterotypic (ER-Golgi) tethering, we labeled the Golgi-like liposomes with Texas Red-DOPE and the ER-like liposomes with Oregon Green-DOPE. As such, we could complement the DLS measurements with the visualization of liposome aggregates by confocal microscopy (Ho and Stroupe, 2015). When VAP-A was present, both N-PH-FFAT and PH-FFAT of OSBP or ORP4 promoted the tethering of ER with Golgi
liposomes as the aggregates displayed the color of the two lipid probes (Figure 5A and S5A).
When VAP-A was absent, N-PH-FFAT did not aggregate any liposome population, whereas PHFFAT selectively promoted the tethering of the Golgi liposomes (Figure 5B and S5B). Indeed,
under these conditions, we only observed red aggregates, revealing that N-ter-lacking
constructs gained an unusual capacity in tethering liposomes in a Golgi-Golgi manner.

312 To investigate how PH-FFAT promotes the homotypic tethering of Golgi liposomes, we performed additional experiments. First, we replaced PH-FFAT or N-PH-FFAT by equivalent 313 314 constructs lacking the coiled-coil region (PH-ΔCC-FFAT or N-PH-ΔCC-FFAT) (Figure 5C-D). 315 Second, we removed PI(4)P from the Golgi liposomes (Figure S6A-B). In both cases, we 316 observed no Golgi-Golgi homotypic tethering (Figure 5D and S6A), although PH- Δ CC-FFAT and 317 N-PH-ΔCC-FFAT kept the ability to tether ER liposomes to Golgi liposomes (**Figure 5C**). These 318 observations demonstrate that the two PH domains of the PH-FFAT construct from OSBP, which 319 forms homodimers, can simultaneously bind two PI(4)P-containing membranes, thereby 320 causing homotypic tethering. When the PH domains are flanked by the N-ter, homotypic 321 membrane tethering is prevented, whereas heterotypic membrane tethering is not affected.

322 CryoEM analysis of homotypic tethering by PH-FFAT

323 We visualized by cryoEM the homotypic membrane contacts induced by the PH-FFAT 324 region of OSBP on PI(4)P-containing liposomes. In incubations with PH-FFAT but not with N-325 PH-FFAT, we observed large regions of juxtaposition between the liposomes (Figures 5E and 326 **S6C**). In both cases, proteins as seen by dotted electron densities were present bound to the 327 external lipid leaflet of the membrane (see inserts in **Figure 5E**). The contact sites induced by 328 PH-FFAT had a well-defined intermembrane distance $(15 \pm 1 \text{ nm})$ and showed a thin band of 329 protein electron density right in the middle of the two juxtaposed membranes, suggesting that 330 PH-FFAT adopts a symmetric conformation to tether the two liposomes. Taking 2.5 nm as the thickness of the PH domain, this geometry would require a 2 x 5 nm linker between the two monomer, which fits with the length of the sequence between the PH domain and the first coiled-coil (n = 17 aa, corresponding to an average end-to-end distance of 51 Å considering the equation $l = \sqrt{150 \times n}$; (Creighton, 1993)).

335

OSBP N-ter regulates protein enrichment and diffusion at homotypic membrane interfaces

336 Next, we investigated the influence of the N-ter on the distribution and mobility of OSBP 337 constructs at membrane-membrane interfaces made by GUVs under the two main tethering 338 conditions identified in this study, i.e. homotypic Golgi-Golgi and heterotypic ER-Golgi tethering. 339 In all cases, the experiments consisted in adding GUVs of defined features (Golgi-like and/or ER-340 like) to solutions of fluorescent proteins (e.g. VAP-A, PH-FFAT, N-PH-FFAT, PH-ΔCC-FFAT, N-341 PH- Δ CC-FFAT). Two to three fluorescent dyes were used to visualize the GUVs and proteins by 342 confocal microscopy and to analyze protein dynamics by FRAP experiments (Figure 6). Note 343 that these assays were complementary to the bulk assays using LUVs and dynamic light 344 scattering. The GUV assay provided unique information on the distribution and dynamics of the 345 proteins at the membrane surface but was less quantitative in term of tethering activity than 346 bulk assays as it is difficult to master the GUV concentration and therefore the protein/lipid 347 ratio.

348 We first incubated Golgi-like GUVs (labelled with the blue probe Atto 390 and containing 349 2 mol% PI(4)P) with either N-PH-FFAT or PH-FFAT (labeled with Alexa 488 or Alexa568 350 probes) (Figure 6A). With PH-FFAT, we observed large GUV-GUV contacts, as manifested by the 351 mutual deformation of the tethered GUVs at the interfacial region. Surprisingly, we also 352 observed GUV-GUV contacts in the presence of N-PH-FFAT, which seemed at odds with the 353 results of the DLS experiments (Figure 6A). However, there was an obvious difference between 354 N-PH-FFAT and PH-FFAT. Whereas PH-FFAT was greatly enriched at the GUV-GUV contact, N-355 PH-FFAT decorated all membrane surface and showed only a slight enrichment at the interface

356 (Figure 6A). We also noticed that N-PH-FFAT was not perfectly pure. On SDS-PAGE, the protein 357 exhibited some contaminants with a lower molecular weight than the major band (Figures 1C 358 and S7A). An antibody against the C-terminal FFAT motif showed that all bands contained the 359 C-terminus indicating that N-PH-FFAT was contaminated at \approx 16% by fragments with a 360 truncated N-ter, which could explain the homotypic tethering in the GUV assay. In spite of this 361 contamination, the comparison between N-PH-FFAT and PH-FFAT indicated that the presence 362 of the N-ter favored a balanced protein distribution over all membrane surfaces, whereas the N-363 ter-lacking construct tended to accumulate at membrane interfaces (Figure 6A).

364 We compared the membrane diffusion rates of OSBP-derived constructs by FRAP on 365 tethered GUVs (Figure 6B). The bleaching area was located either on the free GUV surface or in 366 the center of the GUV-GUV contact site. For both constructs, the recovery on the free GUV 367 surface was instantaneous (k > 1 s⁻¹; Fig. S4B) (Figure S7B). In contrast, we could resolve 368 protein recovery at the GUV-GUV interface and observed that recovery of N-PH-FFAT was much 369 quicker than that of PH-FFAT (recovery time of ≈ 1 s and >> 100 s, respectively, **Figure 6B**). 370 Thus, the N-ter has a large impact on protein density and diffusion rate of the protein at the 371 **GUV-GUV** interface.

372 In studies with model proteins at the interface between GUVs, it has been reported that 373 small proteins able to transversally bind each other and establish a contact site can exclude 374 larger non-binding proteins (Schmid et al., 2016). This exclusion is size-dependent and also 375 influenced by protein crowding. Given the clear difference in membrane distribution and 376 diffusion rate between PH-FFAT and N-PH-FFAT, we wondered if the effect of N-ter would also 377 be evident under conditions where both proteins co-localize on the same GUV-GUV contact. We 378 thus added GUVs to a 1:1 protein mixture of N-PH-FFAT and PH-FFAT, which could be 379 distinguished from each other by different fluorescent dyes. After several hours of incubation, 380 we observed a clear segregation between the two proteins: PH-FFAT concentrated in the GUV-381 GUV contact sites, whereas N-PH-FFAT predominantly localized on the free GUV surface (Figure

382 6C). Thus, the presence of the N-ter upstream of the PH domain is an effective way of regulating383 protein crowding/exclusion at membrane interfaces.

384 To confirm the effect of the N-ter on protein exclusion, we compared the ability of N-PH-385 FFAT or PH-FFAT to invade a membrane interface that was already occupied by the other 386 protein. We first pre-incubated GUVs with either N-PH-FFAT or PH-FFAT labelled with one color 387 and then added an excess of the other protein (PH-FFAT or N-PH-FFAT, respectively) labeled 388 with a different fluorophore. Whereas N-PH-FFAT did not invade PH-FFAT-tethered membrane 389 interfaces, PH-FFAT was able to invade and even replace N-PH-FFAT after a certain time 390 (Figure S7C). This observation further underlines the impact of the N-ter on protein 391 distribution and density at membrane interfaces.

392 **OSBP** N-ter regulates protein diffusion at heterotypic membrane interfaces

393 Last, we moved to conditions of heterotypic tethering by mixing the OSBP constructs 394 with two populations of GUVs: ER-like GUVs containing 3% DOGS-NiNTA to promote the 395 binding of histidine tagged VAP-A and Golgi-like GUVs containing 2% PI(4)P (Figure 6D). The 396 former contained no probe and thus were visible only when a fluorescent protein was bound to 397 them, whereas the latter were labelled with a blue lipid probe. In pilot experiments, we noticed 398 that incubations with N-PH-FFAT of PH-FFAT constructs gave complex assemblies where the 399 GUVs underwent both homotypic and heterotypic contacts, making the analysis complicated. To 400 simplify the system and focus only on the role of the N-ter, we compared N-PH- Δ CC-FFAT and 401 PH- Δ CC-FFAT. The lack of the coiled-coil domain made these constructs monomeric and thus 402 only able to drive heterotypic tethering (see Figures 1D and 5C-D). Furthermore, their 403 monomeric nature necessarily decreased the number of putative interactions with the GUVs, 404 thereby shortening their dwell time at the membrane (see Figure 4D-E), which should favor 405 protein recycling and therefore FRAP analysis. Figure 6D presents confocal microscopy 406 pictures of heterotypic ER-Golgi GUV-GUV contacts promoted by N-PH-ACC-FFAT or PH-ACC-

407 FFAT. The heterotypic nature of these GUV-GUV contacts was manifested by the blue 408 fluorescence distribution, which was present only on the Golgi-like GUVs. In all cases, we 409 noticed a dramatic enrichment of both VAP-A and N-PH-ΔCC-FFAT or PH-ΔCC-FFAT at the ER-410 like Golgi-like GUV interface (Figure 6E-F). However, FRAP analysis showed that N-PH-FFAT 411 recovered within seconds in the GUV-GUV contact region, whereas PH-FFAT showed very slow 412 recovery (Figure 6E). This striking difference in recovery rate was also observed for VAP-A in 413 the GUV-GUV contact zone (Figure 6F). Thus, the N-ter also controls OSBP density and mobility 414 under heterotypic tethering conditions, i.e. when the protein is engaged in a dual interaction 415 with VAP-A on one membrane and PI(4)P on the other membranes.

417 **Discussion**

418 The geometry of MCS creates a confined (≈ 20 nm-thick) environment, which could 419 impede protein dynamics. However, the function of MCS as privileged zones for material 420 exchange between organelles suggests that proteins in MCS should keep some mobility. This is 421 especially true for LTPs, which are responsible for massive lipid flows in the cell (Hanada et al., 422 2003; Mesmin et al., 2017). Our work reveals a key role for the long N-terminal tail of OSBP in 423 its dynamics at MCS. The N-ter considerably accelerates OSBP mobility within the narrow MCS 424 environment without affecting the affinity of the adjacent PH domain for PI(4)P and the 425 mechanism of lipid transfer. In addition, the N-ter restricts the orientation of OSBP by favoring 426 heterotypic over homotypic membrane tethering. These two observations suggest a model in 427 which the N-ter acts as an entropic barrier to prevent OSBP mis-orientation and crowding at 428 MCS (Figure 7).

429 As determined by FRAP experiments, the N-ter accelerates the apparent mobility of 430 OSBP or of its PH-FFAT membrane tethering region by 3 to 10-fold, both in cells and in 431 reconstituted lipid membrane-based systems. This effect is striking by its trend and by its 432 amplitude. Increasing the size of a protein generally decreases its mobility due to higher friction 433 with the milieu; the cytoplasm in the case of a soluble protein, the surrounding lipid bilayer in 434 the case of a transmembrane protein (Lippincott-Schwartz et al., 2001). However, the effect of 435 protein size is generally modest: for spherical proteins, doubling the molecular weight 436 decreases the diffusion coefficient by 1.25-fold given the cubic relationship between MW and radius (Lippincott-Schwartz et al., 2001). For transmembrane proteins, reconstitution 437 438 experiments in model liposomes indicate that the diffusion of large multipass membrane 439 proteins is only 1.5 slower than that of a single transmembrane helix (Ramadurai et al., 2009). These comparisons underline the remarkable effect of OSBP N-ter: it constitutes only one tenth 440 441 (80 aa) of OSBP sequence (807 aa) and yet it increases the apparent mobility of OSBP and ORP4 442 derived constructs in MCS by almost an order of magnitude (Figures 2C, 6B and 6E).

443 The complex arrangements of membranes at the Golgi makes the interpretation of FRAP 444 recovery curves in this region difficult (Lippincott-Schwartz et al., 2001). OSBP recovery could 445 occur by lateral diffusion within MCS as well as by exchange with the cytosol pool. Furthermore, 446 the lipid transfer activity of OSBP creates a negative feedback loop whereby PI(4)P transfer 447 weakens in turn OSBP membrane association (Mesmin et al., 2013). In contrast, the large and 448 simple geometry of reconstituted GUV-GUV contacts promoted by tethering constructs that 449 have no lipid exchange activity ((N)-PH-FFAT and (N)-PH-ΔCC-FFAT) facilitates the analysis. 450 Akin to what happens in cell-cell contacts (Bell, 1978; Wu et al., 2008), fluorescence recovery in 451 a central spot within a MCS probably occurs via a mixture of diffusive motions and 452 association/dissociation events. Fitting the recovery curves for N-PH-ΔCC-FFAT suggests an 453 apparent diffusion coefficient in the range of 0.15 μ m² s⁻¹, whereas no significant recovery was 454 observed when the construct lacked the N-ter. In addition, OSBP N-ter imposes the mobility of 455 VAP-A, suggesting a general effect on MCS dynamics.

456 When a tether molecule starts bridging two membranes, other similar molecules get 457 enriched in the forming MCS as their size matches the intermembrane distance (Schmid et al., 458 2016). Although this cooperativity is advantageous for MCS formation, it could lead to an excess 459 of molecules in the MCS, which would in turn prevent their motion. Protein diffusion under 460 crowding conditions is no longer dominated by friction with the solvent but by protein-protein 461 collisions (Frick et al., 2007; Ramadurai et al., 2009). Akin to what has been described in neurofilaments (Brown and Hoh, 1997), our experiments suggest that OSBP N-ter acts as an 462 463 entropic barrier whose thermal motion sets the limit for OSBP density at the membrane surface, 464 thereby preventing OSBP immobilization by crowding. Indeed, protein disorder has a striking 465 effect on the diffusion of model proteins (Wang et al., 2012). In the dilute regime, an intrinsically 466 unfolded protein diffuses slower than a folded protein of similar MW because the former 467 experiences higher friction with the solvent than the latter. Under high crowding regime, the 468 unfolded protein diffuses faster owing to higher intrinsic flexibility.

469 The second effect of the N-ter is to prevent the two PH domains of OSBP from bridging 470 two PI(4)P-containing membranes. This tethering geometry should be avoided as it diverts 471 OSBP from productive ER to Golgi sterol transfer. Thus, the N-ter could prevent the binding of 472 OSBP to PI(4)P transiently present in the ER before SAC1 hydrolysis. However, the possibility of 473 homotypic tethering pauses the question of the utility of OSBP dimerization. The coiled-coils 474 between the PH domain and the FFAT motif are probably dispensable for membrane tethering 475 and lipid transfer. For example, Osh3 displays the same triad organization as OSBP (PH domain 476 - FFAT motif – ORD), but the linker between the PH domain and the FFAT domain is predicted to 477 form a 37 nm-long monomeric unstructured region (Tong et al., 2013). Similarly, phylogenetic 478 analysis of OSBP indicates that the N-ter and coiled-coil regions are recent features as compared 479 to the PH domain and ORD (Figure S2B). We suggest that the appearance of the N-ter and of the 480 coiled-coils has allowed OSBP to finely adapt to the complex membrane organization of the 481 Golgi apparatus in higher organisms.

482 Compared to flexible monomeric linkers, dimeric coiled-coils should precisely set the 483 intermembrane distance, which might be advantageous for the movement of the adjacent ORD 484 during lipid exchange as well as coordination with other LTPs displaying a tether of similar 485 length. However, the switch from a monomer to a dimer is accompanied by a dramatic increase 486 in the dwell time of the protein at the membrane surface because the probability of concomitant 487 dissociation of two PH domains is much lower than that of a single one. Consequently, a dimeric 488 LTP should not readily move by spontaneous dissociation and should instead rely on lateral 489 diffusion. By facilitating diffusion without decreasing the stability of the PH domain at the 490 membrane surface, the N-ter provides an original way to promote protein dynamics under the 491 conditions of high protein crowding in MCS.

492 Many LTPs acting at MCS contain a low complexity N-ter region upstream their PH 493 domain. These include not only ORPs but also members of the Lam family of sterol transporters 494 (Ysp3p, Lam4P, Lam5p, Lam6p in yeast, GRAMD1A-C in human) (Gatta et al., 2015). These 495 sequences are different, which might reflect either additional functions or the weak 496 evolutionary pressure for sequence conservation in intrinsically disordered regions.
497 Nevertheless, the fact that two different N-ter (in OSBP and in ORP4) have similar effects on
498 protein mobility in a cellular context suggest than the mere control of protein orientation and
499 density is a plausible unifying role.

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509 Author Contributions

510 Conceptualization, D.J., J.B., and B.A.; Methodology, D.J., J.P., M.D., B.M. and J.B; Investigation, D.J.,

511 J.P., B.M., M.D. and J.B; Writing - Original Draft, D.J and B.A.; Writing – Review & Editing, B.A.;

512 Funding Acquisition, B.M., D.L. and B.A.; Supervision, J.B., D.L. and B.A.

513

514 **Declaration of Interests**

515 None

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659 Figure legends

660 Figure 1. Low complexity N-terminal sequences increase the hydrodynamic radius of661 OSBP and ORP4

- (A) Scheme of various domains of OSBP and ORP4. Amino acid sequences of the N terminal
 regions are indicated.
- 664 (B) Constructs used in this study.
- 665 (C) SDS-PAGE analysis of the purified constructs. Staining: Sypro Orange.
- (D) Stoke's radius *vs* MW of OSBP- and ORP4-derived constructs (colored symbols) and
 folded standards (black circles) as determined by gel-filtration. The N-ter increases the
 Stoke's radius by a factor twice as large as that expected for a folded globular domain.

669 Figure 2 OSBP and ORP4 N-ter influence protein subcellular localization and dynamics

- 670 (A and B) Localization of OSBP-mCherry and ORP4-mCherry constructs overexpressed in
 671 HeLa cells. Left: representative confocal images. Bar = 20 μm. Right: ratio between mean
 672 Golgi signal and mean total cell signal. All localization experiments were also performed
 673 on RPE1 cells with similar results. Each point corresponds to one cell. At least two
 674 experiments were done for each cell type.
- 675 (C) FRAP recovery curves of OSBP-mCherry (left) and ORP4-mCherry (right) in HeLa cells
 676 after bleaching a circular area (3 μm diameter) at the Golgi. At least 5 measurements of
 677 each construct from one representative experiment among three independent
 678 experiments are shown.

679 Figure 3. OSBP N-ter facilitates protein turnover under conditions of remote PI(4)P 680 synthesis

- 681(A-B) RPE1 cells stably expressing the PI(4)P probe GFP-PH_{OSBP} were treated with control682siRNA (siNT, A) or with siRNA against OSBP (siOSBP, B). After 24 hours, widefield time-683lapse imaging was performed. When indicated, PIK93 (500 nM) was added into the cell684medium. Top: snapshots of cells taken at t=0 using an inverted grayscale lookup table685(fluorescence in black). Scale bar = 20 μ m. Bottom: kymographs taken from a686rectangular TGN region ("K", $\approx 20 \ge 3 \mu$ m).
- 687 (C) Temporal analysis showing normalized mean fluorescence intensity of GFP-PH_{OSBP} from
 688 a circular TGN region (see "T" on cell images in A-B; diameter 8 μm) over time. Silencing
 689 of endogenous OSBP abolished GFP-PH_{OSBP} oscillations (black line), in contrast to control
 690 siNT-treated cells (green line).

- (D-E) RPE1 cells stably expressing GFP-PH_{OSBP} and treated with siOSBP were transfected
 with siRNA-resistant OSBP (OSBP-res-mCherry) or ΔN-OSBP (ΔN-OSBP-res-mCherry)
 for 24 h before imaging. The signal from both GFP-PH_{OSBP} and the mCherry construct
 was monitored by time-lapse microscopy.
- (F) Temporal analysis. OSBP-res-mCherry rescued GFP-PH_{OSBP} oscillations, whereas ΔN OSBP-res-mCherry did not.
- 697 698

(G) Quantification of GFP-PH_{OSBP} oscillations at TGN from recordings similar to that shown in (C) and (F).

Figure 4. N-ter limits protein density on PI(4)P-containing membranes without affecting the affinity

- (A) Visualization of N-PH-ΔCC-FFAT or PH-ΔCC-FFAT (400 nM) on Golgi-like GUVs (2%
 PI(4)P) and quantification of protein recruitment as a function of protein concentration
 from the fluorescence level on the GUV surface. Each point corresponds to one GUV.
- 704(B) Liposome sedimentation assay at increasing PI(4)P density. PI(4)P concentration was705held constant (20 μ M) but PI(4)P was present at increasing surface concentration while706the liposome concentration was decreased. The plot shows the results (mean ± SEM) of707the liposome sedimentation assay from 5 independent experiments. PH- Δ CC-FFAT and708N-PH- Δ CC-FFAT showed similar binding to liposomes at low PI(4)P density, but not at709high PI(4)P density where PH- Δ CC-FFAT associated to the liposomes more significantly710than N-PH- Δ CC-FFAT.
- 711 (C) Binding of N-PH- Δ CC-FFAT and PH- Δ CC-FFAT to liposomes measured by FRET. The 712 sample initially contained liposomes with 10 % PI(4)P and 5 % DPH-PC (total lipid 713 concentration $[L] = 18 \,\mu\text{M}$). Thereafter, increasing amount of protein was added (*n* x 0.2 714 μ M). The FRET change as a function of protein concentration was fitted by a quadratic 715 equation (see methods), which gives the equilibrium constant (K_d) and the 716 concentration of protein binding sites on the liposomes (BS). BS was used to determine 717 the elementary area (in lipid number) occupied by the proteins: A = 0.5[L]/BS. The bar 718 plot shows the mean \pm SEM of K_d and A from 5 independent experiments using two sets 719 of liposomes.

720(D) Association and dissociation kinetics of PH- Δ CC-FFAT and N-PH- Δ CC-FFAT. At t = 50 s,7210.2 μ M of PH- Δ CC-FFAT (blue trace) or N-PH- Δ CC-FFAT (green trace) was added to the722liposomes (15 μ M lipid, 10 % PI(4)P, 5 % DPH-PC). At t = 100 s, unlabeled liposomes (75723 μ M lipid, 10 % PI(4)P) were added. The association kinetics could not be resolved. The724dissociation kinetics were fitted with an exponential decay. The scheme illustrates the

- reactions and gives the kinetic constants (mean ± SEM from 4 independent experiments
 with two sets of liposomes).
- (E) Same as in (D) with PH-FFAT and N-PH-FFAT. The kinetics constants are mean ± range
 from two independent experiments with two sets of liposomes.

729 Figure 5. Control of liposome tethering geometry by OSBP N-ter

- 730(A-B) The samples initially contained 15 μ M of ER-like liposomes (2% DGS-NTA-Ni) labelled731with Oregon Green-DHPE, 15 μ M of Golgi like liposomes (2% PI(4)P) labelled with Texas732Red-DHPE, and either 300 nM VAP-A (which binds to ER-like liposomes, A) or no VAP-A733(B). At t = 100 s, 600 nM of the indicated tethering construct (green: N-PH-FFAT, blue734PH-FFAT) was added and liposome aggregation was followed over time by DLS. After735kinetics completion, the DLS samples were visualized by confocal microscopy to736determine the nature of the liposome aggregates. Bar =20 μ m.
- 737 (C-D) Same as in (A-B) with N-PH- Δ CC-FFAT or PH- Δ CC-FFAT.
- (E) Cryo EM analysis of Golgi-like liposomes (5% PI(4)P) after incubation with N-PH-FFAT or
 PH-FFAT. In both cases, liposomes were decorated with proteins (inset). With N-PHFFAT, no liposome-liposome contact formed. With PH-FFAT, liposome-liposome
 contacts, often associated with membrane remodeling, formed with an intermembrane
 distance of 15 ± 1 nm and a characteristic central dense layer of protein between them
 (white arrow head). Bar = 100 nm. Larger EM fields are shown in Figure S6C.

Figure 6. The N-ter controls OSBP distribution and lateral motility under homotypic and heterotypic tethering conditions

- (A) GUVs containing 2% PI(4)P and labeled with Atto390-DOPE were incubated with 100
 nM N-PH-FFAT or PH-FFAT (labelled with Alexa488 or Alexa568) as schematized.
 Enrichment index was calculated from line scans across the GUV-GUV interface and free
 GUV surface (see star methods and(Schmid et al., 2016)). Each point corresponds to one
 interface. Bar = 5 μm.
- (B) FRAP measurements of N-PH-FFAT (green trace) and PH-FFAT (blue trace).
 Photobleaching was performed on a circular area (diameter 2 μm) at the center of the
 GUV-GUV interface (see A). Measurements performed with proteins labelled with
 Alexa488 or Alexa568 gave similar results. Means ± SD of one representative
 experiment is shown (n: numbers of recordings per condition).
- (C) 50 nM N-PH-FFAT labeled with Alexa488 (green) and 50 nM PH-FFAT labeled with
 Alexa568 (red) were incubated with Golgi-like GUVs (2% PI(4)P, Atto390-DOPE). After

- four hours, the suspension was visualized by confocal microscopy. Experiment was also
 performed with inverse color combination with similar results. Bar = 5 μm.
- 760(D) 50 nM N-PH- Δ CC-FFAT or PH- Δ CC-FFAT labeled with Alexa568 (red) was mixed with76150 nM VAP-A-His labeled with Alexa488 (green). Golgi-like GUVs (2% PI(4)P, Atto390-762DOPE) and ER-like GUVs (2% DGS-NTA-Ni, no color) were added and the sample was763very gently mixed. After 30 min, tethered GUVs were observed by confocal microscopy.764Bar = 5 μ m.
- 765(E) Protein enrichment index and FRAP measurements at 568 nm of N-PH-ΔCC-FFAT or766PH-ΔCC-FFAT from GUV-GUV contacts similar to that shown in (D). Photobleaching was767performed on a circular area (diameter 2 μ m) in the middle of the interface. Means ± SD768of one representative experiment is shown (n = number of recordings per condition).
- (F) Same as in (E) but protein enrichment index and FRAP were conducted on VAP-A-Hislabelled with Alexa-488.

Figure 7. Model for N-ter mediated regulation of OSBP tethering geometry and dynamics at MCS

773By being intrinsically disordered, the N-ter of OSBP acts as an entropic barrier. It774prevents the two PH domains of OSBP from simultaneously bridging two PI(4)P-775containing membranes. It limits OSBP density under heterotypic tethering conditions,776thereby facilitating protein in plane diffusion.

STAR methods

KEY RESOURCES TABLE

Antibodies Cat# HPA039227, RRID:AB_2676401 Mouse monoclonal anti V9 Vimentin Sigma-Aldrich Cat# HPA039227, RRID:AB_2676401 Secondary Alexa Fluor-conjugated Thermo Fisher Scientific Cat# A32723, RRID:AB_263275 Secondary HRP-conjugated Jackson ImmunoResearch Cat# A32723, RRID:AB_263275 Secondary HRP-conjugated Jackson ImmunoResearch Cat# 111-035-047, RRID:AB_2737940 Bacterial and Virus Strains Escherichia coli BL12(DE3) IPMC resources N/A Escherichia coli BL2(DE3)pRIL-TEV Kapust et al., 2001 N/A Biological Samples Cat# 40051C Biological Samples Chemicals, Peptides, and Recombinant Proteins Egg PC Avanti Polar Lipids Cat# 840051C Brain PS Avanti Polar Lipids Cat# 840045X Liver PE Avanti Polar Lipids Cat# 840045C Liver PE Avanti Polar Lipids Cat# 840042C Dansyl-PE Avanti Polar Lipids Cat# 840042C Dansyl-PE Avanti Polar Lipids Cat# 840042C Dansyl-PE Avanti Polar Lipids Cat# 840042C Dansyl-PE Avanti Polar Lipids Cat# 840042C Dansyl-PE Av	REAGENT or RESOURCE	SOURCE	IDENTIFIER
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Deposited Data	GeneArt I M Seamless Cloning and Assembly	Invitrogen	Cat# A14606
	Enzyme MIX		
	Deposited Data		

Experimental Models: Cell Lines		
hTERT-RPE1	ATCC	RRID:CVCL_4388
HeLa	Gift from Thierry Coppola,	N/A
	Institut de Pharmacologie	
	Moléculaire et Cellulaire,	
	Valbonne	
SF9	From lab	N/A
Experimental Models: Organisms/Strains		
Oligonucleotides		
ON-TARGETplus Human OSBP (5007) siRNA	Dharmacon (GE	Cat# J-009747-06-
Target sequence : GCAAUGACUUGAUAGCUAA	Healthcare)	0020
ON-TARGETplus Non-targeting siRNA	Dharmacon (GE	Cat# D-001810-02-05
Target sequence : UGGUUUACAUGUUGUGUGA	Healthcare)	
siRNA-resistant OSBP-For : CTT GAG CAC GTG	This study	N/A
CAA TGA TCT TAT AGC TAA GCA TGG C		
siRNA-resistant OSBP-Rev : GCC ATG CTT AGC	This study	N/A
TAT AAG ATC ATT GCA CGT GCT CAA G	,	
pFastBac ⁻ HTA-mod1-For: CTG TAT TTT CAG GGC	This study	N/A
GCC TAA TAG CCG GAA TTC AAA GGC CTA		
pFastBac ⁻ HTA-mod2-For : TCT CGG TCC GAA	This study	N/A
TAC CAT CAC CAT CAC CAT C		
Recombinant DNA		
pmCherry-N1-OSBP PH-FFAT (76-408)	(Mesmin et al., 2013)	N/A
pmCherry-N1-OSBP FL (1-807)	This study	N/A
pmCherry-N1-OSBP Δ N (86-807)	This study	N/A
pmCherry-N1-OSBP N-PH-FFAT (1-408)	This study	N/A
pmCherry-N1-ORP4 FL (1-878)	This study	N/A
pmCherry-N1-ORP4 ∆N (144-878)	This study	N/A
pmCherry-N1-ORP4 N-PH-FFAT (1-474)	This study	N/A
pmCherry-N1-ORP4 PH-FFAT (144-474)	This study	N/A
pmCherry-N1-res-OSBP FL (1-807)	This study	N/A
pmCherry-N1- <i>res</i> -OSBP ∆N (86-807)	This study	N/A
pmCherry-C1-N-ter (1 - 90)	This study	N/A
pENTR/D-OSBP FL (1-807)-SThr-6His	Mesmin et al., 2013	N/A
pFast-Bac-6His Cter (intermediate plasmide)	This study	N/A
pFB- OSBP Δ N (88-807)-SThr-6His	This study	N/A
pET.His6.StrepII.TEV.LIC (2HR-T)	gift from Scott Gradia,	Addgene # 29718
	University of California,	
	Berkeley	
pET.His10.TEV.LIC (2B-T-10)	gift from Scott Gradia	Addgene # 78173
	University of California,	
	Berkeley	
pET16b. StrepII.TEV.OSBP N-PH-FFAT (1-408)	This study	N/A
pET16b. StrepII.TEV.OSBP PH-FFAT (76-408)	This study	N/A
pET16b.His10.TEV.LIC (intermediate plasmide)	This study	N/A
pET16b.His10.TEV. ORP4 N-PH-FFAT (1-475)	This study	N/A
pET16b.His10.TEV. ORP4 PH-FFAT (128-475)	This study	N/A

pGEX-4T3-PH _{FAPP1} (C37S/C94S/T13C/T100S)	(Mesmin et al., 2013)	N/A
pET-21b-VAP-A	(Mesmin et al., 2013)	N/A
pET-21b-VAP-A (K94D/M96D)	(Mesmin et al., 2013)	N/A
pEGFP-C1-PH _{OSBP}	(Mesmin et al., 2013)	N/A
pTagBFP-N-β-1,4-Galactosyltransferase-1 (1-82)	(Mesmin et al., 2013)	N/A
pEGFP-Rab7	Gift from Sylvain	N/A
	Feliciangeli, Institut de	
	Pharmacologie Moléculaire	
	et Cellulaire, Valbonne	
Software and Algorithms		
Predictor of Natural Disordered Regions	Molecular Kinetics, Inc.	http://www.pondr.co
(PONDR®)		<u>m/</u>
ProtParam	ExPASy Bioinformatics	https://web.expasy.or
	Resource Portal, SIB	g/protparam/
Phylogeny.fr	Sebastien Santini -	http://www.phylogen
	CNRS/AMU IGS UMR7256	y.fr/
Clustal Omega	(Sievers et al., 2011)	https://www.ebi.ac.uk
		/Tools/msa/clustalo/
NPS@ web server	(Lupas et al., 1991)	https://npsa-
		prabi.ibcp.fr/cgi-
		bin/npsa_automat.pl?
		page=/NPSA/npsa_lup
		as.html
ImageJ 1.51w	NIH, USA	
Zen 2011	Carl Zeiss SAS	
MetaMorph Version 7.8.9.0	Molecular Devices LLC	
Dynamics v6.1	Protein Solutions	
SigmaPlot 14	Systat Software Inc.	
Canvas X	ACD System	
Other		

782 CONTACT FOR REAGENT AND RESOURCE SHARING

- Further information and requests for resources and reagents should be directed to and will befulfilled by the Lead Contact, B. Antonny (antonny@ipmc.cnrs.fr).
- 785

786 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

787

788 Cell culture

Human, female HeLa cells were cultured in DMEM medium with GlutaMAX (Gibco)
supplemented with 10% fetal calf serum, 1% antibiotics (Zell Shield, Minerva Biolabs) and were
incubated at 37°C in a 5% CO₂ humidified atmosphere.

- 792 Human, female hTERT-RPE1 cells (ATCC Cat# CRL-4000, RRID:CVCL 4388; hereafter RPE1 793 cells), were cultured in DMEM/F12 (Gibco) supplemented with 10% fetal calf serum and 1% 794 antibiotics (Zell Shield) and were incubated at 37°C in a 5% CO₂ humidified atmosphere. RPE1 795 cells stably expressing EGFP-PH_{OSBP} were selected using G418 (Sigma). Surviving colonies were 796 isolated using cloning cylinders (Bel-Art), expanded and further sorted by FACS (FACSAria III, 797 BD Biosciences). RPE1 cells stably expressing EGFP-PH_{OSBP}, EGFP-P4M_{SidM} were cultured in 798 medium supplemented with G418 (500 μ g/ml). For microscopy, cells were seeded at suitable 799 density to reach 50-90% confluence on the day of imaging. 800 Insect (Spodoptera frugiperda) SF9 cells were cultured at 27°C in SF-900 II media supplemented
- with 1,5% FCS in absence of antibiotic. For protein expression, SF9 cells were infected at 10⁶ cells/ml and MOI of 0.1 in 0.5 l CELLSPIN Spinner. After 72h, cells were collected by centrifugation at 300xg for 15 min, washed in PBS and stored at -20°C.
- 804

805 **METHOD DETAILS**

806

807 Reagents

Rabbit polyclonal antibody against OSBP (Atlas Antibodies Cat# HPA039227,
RRID:AB_2676401)) and mouse monoclonal V9 Vimentin antibody (Sigma-Aldrich Cat# V6389,
RRID:AB_609914) were from Sigma-Aldrich. Secondary Alexa Fluor-conjugated antibody
(Thermo Fisher Scientific Cat# A32723, RRID:AB_2633275) were from Invitrogen and
secondary HRP-conjugated antibody were from Jackson ImmunoResearch (Jackson
ImmunoResearch Labs Cat# 111-035-047, RRID:AB_2337940).

814 Egg PC, brain PS, brain PI(4)P, liver PI, liver PE, dansyl-PE (1,2-dioleoyl-sn-glycero-3-

815 phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl)), rhodamine-PE [1,2-

- 816 dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)], and DGS-
- 817 NTA(Ni) [1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid)

- 818 succinyl] (nickel salt)] were from Avanti Polar Lipids. 2-(3-(diphenylhexatrienyl)propanoyl)-1-
- 819 hexadecanoyl-sn-glycero-3-phosphocholine (DPH-PC) was from Molecular Probes Europe.
- 820 $\,$ Cholesterol, dehydroergosterol (DHE), Methyl- β -cyclodextrin (MCD) and G418 were from
- 821 Sigma-Aldrich. Texas Red-DHPE, Oregon Green-DHPE and Atto390-DHPE were from Invitrogen.
- 822

823 Plasmids and cell transfection

- 824 OSBP PH-FFAT (residues 76-408) was sub-cloned into a pGEX-4T3 vector (GE Healthcare) for
 825 expression as a GST-fused protein in *E. coli* (Mesmin et al., 2013).
- 826 Human OSBP sequences [full-length (residues 1-807), △N (residues 86-807), N-PH-FFAT 827 (residues 1-408) and human ORP4 sequences [FL (residues 1-878), ∆N (residues 144-878), N-828 PH-FFAT (residues 1-474), PH-FFAT (residues 144-474)] were cloned into the BamHI site of 829 pmCherry-N1 vector using the GeneArt[™] Seamless Cloning and Assembly Kit (Invitrogen). 830 mCherry-OSBP N-ter construct was prepared by introducing two stop codons at the beginning 831 of the PH domain sequence in OSBP FL pmCherry-C1 vector. The N/2-PH-FFAT ORP4 construct 832 was prepared by PCR using the ORP4 N-PH-FFAT in pmCherry-N1 as template with primers: F: 833 GCT TCG AAT TCT GCA GTC ACC ATG CCA TCG GAA CTG CTG CAG G and R: CCT GCA GCA GTT
- 834 CCG ARG GCA TGG TGA CTG CAG AAT TCG AAG C.
- 835 The siRNA-resistant OSBP constructs (full-length and ΔN) were prepared by PCR using the 836 corresponding pmCherry-N1 plasmids as template with primers F: CTT GAG CAC GTG CAA TGA 837 TCT TAT AGC TAA GCA TGG C and R: GCC ATG CTT AGC TAT AAG ATC ATT GCA CGT GCT CAA G. 838 For protein expression, cells were transfected with Lipofectamine 2000 reagent (Invitrogen) or 839 by electroporation with Nucleofector Solution (Lonza) using the Amaxa Nucleofector device 840 (Lonza), for 18-24 hrs.
- 841

842 **Purification of OSBP and ΔN-OSBP**

843 Full-length (1-807) human OSBP and Δ N-OSBP (88-807) were purified from baculovirus-844 infected Sf9 cells. The construct of full-length (1-807) human OSBP was created by subcloning 845 PCR-amplified DNA into pENTR/D-Topo vector (Invitrogen), followed by insertion of a 846 thrombin site at the end of the OSBP sequence. The PCR fragment was then inserted by 847 recombination into linearized BaculoDirect vector (C-terminal V5 and His-tagged), and 848 transfected into Sf9 cells (Mesmin et al., 2013). For △N-OSBP, the pFastBac™HTA vector 849 (Invitrogen) was modified by successive mutations to allow the insertion of a PCR amplified 850 sequence upstream and in frame with the 6His tag. These modifications were: 1) transformation 851 of the original BamHI site into 2 stop codons (F oligo sequence: CTG TAT TTT CAG GGC GCC TAA 852 TAG CCG GAA TTC AAA GGC CTA); 2) insertion of a new BamHI site upstream of the His tag (F 853 oligo sequence: TCT CGG TCC GAA TAC CAT CAC CAT CAC CAT C). [OSBP ΔN (88-807) +

thrombin site] DNA sequence was PCR amplified using the pENTR/D-(OSBP-FL-thrombin site)
as matrix and cloned into the BamHI-digested pFastBac HTA modified vector using the
GeneArt[™] Seamless Cloning and Assembly Kit (Invitrogen). Recombinant vectors were then
transformed into DH10 Bac *E.coli*. Recombinant bacmides were selected as described in Bac to
Bac^R Expression System user manual (Invitrogen) and used to produce recombinant
Baculovirus.

860 Full-length OSBP and Δ N-OSBP with a C-terminal 6His tag were produced in Sf9 cells, which 861 were infected with baculovirus at MOI of 0.1 for 1h at room temperature, suspended in 500 – 862 1000 ml of Sf-900 II media supplemented with 1.5% FCS and incubated with mixing at 27°C for 863 72 hours. Thereafter, cells were collected by centrifugation at 300 x g for 10 min and pellets 864 were stored at -20°C. Cell pellets were resuspended in lysis buffer (20 mM Tris pH 7.5, 300 mM 865 NaCl, 20 mM imidazole, EDTA-free protease inhibitors and phosphatases inhibitors) and lysed 866 with Dounce homogenizer. After ultracentrifugation (125 000 g), OSBP or Δ N-OSBP from the 867 supernatant was adsorbed on an HisPur[™] Cobalt Resin (Thermo Scientific), submitted to 3 868 washes with lysis buffer supplemented with 800, 550, and 300 mM NaCl, respectively, and then 869 eluted with 250 mM imidazole-containing buffer. OSBP fractions were pooled, concentrated on 870 Amicon Ultra (cut-off 30 kDa), and submitted to thrombin cleavage for 1 hr at 25°C to eliminate 871 the His tag, then purified on a Sephacryl S300 HK16/70 column (GE Healthcare) using an AKTÄ 872 purifier system (GE Healthcare). All steps were performed at 4°C. The purified protein fractions 873 were pooled, concentrated, supplemented with 10 % glycerol, flash-frozen in liquid nitrogen 874 and stored at -80°C.

875

876 **Purification of OSBP and ORP4 fragments**

N-PH-FFAT and PH-FFAT fragments of OSBP and ORP4 were expressed in *E. Coli* BL21 (DE3).
The corresponding constructs were prepared using pET.His6.StrepII.TEV.LIC (2HR-T, Addgene
plasmid # 29718) and pET.His10.TEV.LIC (2B-T-10, Addgene plasmid # 78173) cloning vectors

880 (gift from Scott Gradia).

OSBP N-PH-FFAT (1-408) and OSBP PH-FFAT (76-408) fragments were first inserted into
 pET.His6.StrepII.TEV.LIC vector, subcloned into pET16b, and then expressed as N-terminal 6His
 tag- StrepII-TEV site constructs.

ORP4 DNA sequences [ORP4(1-475)=N-PH-FFAT, ORP4(128-475)=PH-FFAT] were PCR
amplified using the pmCherry-ORP4 FL as matrix. The host plasmid pET16b.His10.TEV.LIC was
prepared from the pET16b.His6.StrepII.TEV.LIC OSBP(76-408) by replacing the fragment
His6.StrepII.TEV.LIC OSBP(76-408) by a His10.TEV.LIC fragment. In addition, the SspI site of
pET16b was mutated (AATATT into AATAGC). Last, the ORP4 N-PH-FFAT or PH-FFAT PCR

- 889 fragments were inserted into the SspI digested pET16b.His10.TEV.LIC vector using GeneArtTM
- 890 Seamless Cloning and Assembly Enzyme Mix (Invitrogen).
- 891 After protein expression, bacteria were lysed with a French Press (SLM AMINCO) and incubated
- for 30 min on ice with DNAse and MgCl₂ (5mM) before ultracentrifugation (125 000 g). His
- tagged proteins were purified using HisPur[™] Cobalt Resin (Thermo Scientific). Protein fractions
- 894 were pooled and submitted to TEV protease cleavage overnight at 4°C. Digested proteins were
- purified on a SourceQ HR 10/10 column (GE Healthcare) with a 0-1M NaCl gradient in 25mM
 Tris pH7.5 followed by a Sephacryl S200 HK16/70 column (GE Healthcare) equilibrated in
 25mM Tris pH7.5, 120 mM NaCl, 2mM DTT. Purified proteins were pooled, concentrated,
- supplemented with 10% glycerol, flash-frozen in liquid nitrogen and stored at -80°C.
- 899 N-PH-ΔCC-FFAT and PH-ΔCC-FFAT constructs were prepared from pGEX4.T1 (GE Healthcare)
- 900 plasmids expressing the OSBP (1-408) or (76-408) sequence. A NaeI restriction was introduced
- 901 by site directed mutagenesis to remove the coiled-coil (207-329) region by digestion / ligation
- 902 taking advantage of another Nael site. Proteins were expressed as N-terminal (GST-thrombin-
- site) constructs in *E. coli* BL21(DE3). Purification was performed using Glutathione Sepharose
 beads (GE Healthcare). GST was removed by thrombin cleavage. The subsequent purification
- 905 steps were the same as that used for N-PH-FFAT / PH-FFAT.
- GST-N-ter (1-90) from OSBP was prepared from the pGEX4.T1 plasmid of N-PH-ΔCC-FFAT by
 introducing a stop codon at position 91. The GST-N-ter protein was purified using Glutathione
 Sepharose beads.
- 909 All new constructs were confirmed by sequencing. All purification steps were analyzed by SDS-
- 910 PAGE using Sypro-Orange staining and a Fusion FX imager (Vilber Lourmat).
- 911

912 **Other protein constructs**

913 The PH domain of FAPP1 was kindly provided by Franck Perez (Institut Curie, Paris). PHFAPP1 914 sequence was subcloned into pGEX-4T3 and modified by point mutagenesis 915 (C37S/C94S/T13C/T100S) for specific NBD labeling of C13. A linker (NGNLSSLSA) was inserted 916 upstream of the PH sequence to allow thrombin cleavage of the GST tag. PH_{FAPP1} was expressed 917 in *E. coli* and purified on glutathione Sepharose beads (GE Healthcare) and cleaved by thrombin. 918 Human VAP-A fragment (residues 8-212) was subcloned into pET-21b vector for expression as 919 C-terminally His-tagged protein in *E. coli*. The C-terminal His tag corresponds to the beginning 920 of the C-terminal transmembrane region and, therefore, the purified protein should be 921 positioned on DGS-NTA(Ni) containing liposomes in orientation similar to the authentic 922 transmembrane protein. VAP-A was purified on Ni-NTA beads (QIAGEN).(de Saint-Jean et al., 923 2011; Mesmin et al., 2013).

- 924 The TEV protease plasmid (gift from D. Waugh) (Kapust et al., 2001) was used to expressed the
- 925 protein in Bl21(DE3)pRIL *E.coli* which was purified as described (Tropea et al., 2009).
- 926

927 **Protein Labeling**

928 We removed DTT from the purified constructs by applying them to a buffer exchange NAP-5 929 Column (GE Healthcare) using freshly degassed buffer (Hepes 50 mM pH 7.5, KAcetate 250 930 mM). The eluted protein peak was incubated with Alexa fluor 488 C5 maleimide or Alexa fluor 931 568 C5 maleimide (Invitrogen)(stock solution in DMSO) at an equimolar Dye/Cystein ratio for 932 10 min at room temperature in the dark. The free dye was then quenched with a 10-fold excess 933 of L-cystein on ice for 15 min. After the further addition 2 mM DTT, the free dye was eliminated 934 by applying the sample to a NAP-5 Column. After elution, the extent of protein labeling was 935 estimated from the visible spectrum of the protein fraction, which was also analyzed by SDS-936 PAGE. The gel was directly visualized on a Fusion Imager (Vilber Lourmat) using filters adapted 937 for Alexa 488 and 568 dyes.

938 **RNA interference**

For endogenous OSBP silencing and simultaneous expression of siRNA resistant OSBP, RPE-1
cells stably expressing GFP-PH^{OSBP} were electroporated with 90 pmol siRNA and 1 μg siRNAresistant OSBP plasmid using RNAiMAX (Invitrogen) and plated on 6-well plate or on μ-Dish^{35mm}
(Ibidi). ON-TARGETplus Human OSBP siRNA was from Dharmacon (GE Healthcare, target
sequence: GCAAUGACUUGAUAGCUAA). After 18-24 hrs cells were used for live-cell imaging or
western blotting.

945

946 Liposome preparation

947 Lipids in chloroform or in chloroform: methanol (2:1) in the case of mixtures containing PI(4)P) 948 were mixed at the desired molar ratio and the solvent was removed in a rotary evaporator. For 949 most assays, the lipid films were hydrated in 50 mM HEPES pH 7.2 and 120 mM potassium 950 acetate (HK buffer, which was degassed before use) to give a suspension of large multilamellar 951 liposomes (lipid concentration: 2-5 mM). In the case of sedimentation assay, the lipid films were 952 hydrated (lipid concentration: 2mM) in 50 mM HEPES pH 7.2, sucrose 210 mM. The suspensions 953 were then frozen in liquid nitrogen and thawed in a water bath (40°C) four times. Liposomes 954 were extruded through 0.1 µm pore size polycarbonate filters using hand extruder (Avanti Polar 955 Lipids) and were used within 1-2 days.

956

957 **GUV Preparation**

Giant unilamellar vesicles were generated by electro-formation. Lipids mixtures of the chosencomposition (0.5 mg/ml) in chloroform or in 2:1 chloroform:methanol in the case of mixtures

containing PI(4)P were deposited on indium tin oxide coated glass slides and dried at RT for 45
min to remove all solvents. Lipids were then hydrated in 250 mM sucrose osmotically
equilibrated with buffers. GUVs were electroformed using Vesicle Prep Pro (Nanion
Technologies) by applying an AC electric field (3 V and 5 Hz), at 37 °C for 60 min.

964

965 Liposomes aggregation measurements

966 Liposome aggregation induced by OSBP or ORP4 fragments was followed in real time by 967 dynamic light scattering (DLS) using a DynaPro instrument (Protein Solutions). Initially, Golgi-968 like liposomes (15 µM) containing 0 - 4% PI(4)P and ER-like liposomes containing 2% DGS-969 NTA(Ni) (15 μ M) were mixed in HK buffer supplemented with 1 mM MgCl₂ and 1 mM DTT 970 (HKMD buffer) and with VAP-A-His or VAP-A(KM-DD)-His (300 - 600 nM), as indicated 971 (temperature 30° C). Ten DLS autocorrelation curves (= 10×10 seconds) were acquired to 972 determine the initial size distribution of liposome suspension. Thereafter, 300 - 600 nM 973 OSBP/ORP4 fragment was manually injected, gently mixed, and liposome aggregation was 974 followed in real time by acquiring one autocorrelation curve every 10 s. The total DLS sample 975 volume was 40 µl. Data were analyzed using the Dynamics v6.1 software (Protein Solutions).

976

977 Microscopy, FRAP assays

978 Confocal microscopy of fixed cells was performed with a Zeiss LSM780 microscope run by ZEN
979 software using a Plan-Apochromat 63X/1.4 Oil objective (Carl Zeiss). Confocal microscopy of
980 liposomes or GUVs was performed using the same microscope. The liposome or GUV suspension
981 in HKM buffer (50mM HEPES, 120mM potassium acetate and 1mM MgCl₂) was placed in 8-well
982 Dish (Ibidi) coated by casein (Sigma Aldrich); imaging was performed at room temperature.

983 Fluorescence recovery after photobleaching recordings were performed with a Zeiss LSM780 984 microscope or with a Nicon Eclipse Ti microscope equipped with an UltraVIEW VoX spinning 985 disc imaging system (PerkinElmer) operated by Volocity software, and using a CFI Plan Apo 986 100X/1.4 Oil objective (Nikon). Cells were placed in phenol red-free medium supplemented 987 with HEPES (Gibco) and FRAP assays were carried out at 37°C. Photobleaching was performed 988 on circular areas of 3 µm within perinuclear regions positive for BFP-GalT signal. FRAP assays 989 with liposomes/GUVs were performed in HKM buffer at room temperature. Photobleaching was 990 performed on circular areas of 2 µm diameter in the middle of GUV-GUV interface.

991 Time-lapse widefield microscopy was performed using an Olympus IX83 inverted microscope
 992 equipped with a Z-drift compensator, a scanning stage SCAN IM (Märzhäuser) and an iXon3
 993 camera (Andor). Cells plated in μ-Dish^{35mm} (Ibidi) were put into a stage chamber set at 37°C
 994 (Okolab). Tag BFP, EGFP and mCherry signal were detected using Chroma fluorescence filter

sets (ref. 49000, 39002, 39010). Multidimensional acquisition and analysis was performed with
MetaMorph software (Molecular Devices).

997

998 Analytical gel filtration

999 Purified proteins (100 µl, 5 µM) were applied on a Superose 12[™]column (GE Healthcare) and 1000 eluted at a flow rate of 0.5 ml.min⁻¹ in 25 mM Tris pH 7.5, 120 mM NaCl and 1mM DTT. The 1001 column was calibrated using the following standards (MW/Stokes radius): Apoferritin (443 1002 kDa/6.1 nm), Alcohol dehydrogenase (150 kDa/4.6 nm), Bovine serum albumin (67 kDa/3.5 1003 nm), Carbonic anhydrase (25 kDa/2.1 nm) and Cytochrome C (12.4 kDa/ 1.7 nm). The elution 1004 volume and Stoke's radius of the standards were used to establish a first calibration curve, from 1005 which the Stoke's radius of the OSBP/ORP4 constructs were determined. Thereafter, we plotted 1006 the Stoke's radius as a function of MW for both protein standards and for OSBP/ORP4 1007 constructs.

1008

1009 In vitro lipid transfer assays

DHE and PI(4)P-transfer assays were performed as described previously using purified
recombinant proteins and 0.2μm filter-extruded liposomes mimicking ER and Golgi membranes
(de Saint-Jean et al., 2011; Mesmin et al., 2017; 2013). All measurements were carried out in a
Jasco FP-8300 spectrofluorimeter using a cylindrical quartz cuvette (600 μl) equilibrated at
37°C and equipped with a magnetic bar for continuous stirring.

1015 For DHE transfer experiments, the default lipid composition of the ER and Golgi liposomes was 1016 egg PC/brain PS/DGS-NTA(Ni) (93/5/2 mol%) supplemented with 10 % mol/mol of DHE and 1017 egg PC/liver PE/brain PS/liver PI/brain PI(4)P/ DNS-PE (63/17.5/5/2/10/2.5 mol%), 1018 respectively. FRET signal between DHE and DNS-PE was detected at 525 (bandwidth 5) nm 1019 upon excitation at 310 (bandwidth 1) nm. Golgi and ER liposomes (65 µM each) were incubated 1020 with Vap-A (1 μ M) and Sac1 (0.2 μ M). After 120s of baseline measurement, OSBP or Δ N-OSBP 1021 $(0.1 \mu M)$ was added and FRET signal was followed for 1000s. The maximal amplitude of the 1022 DHE transfer signal was determined by adding 1mM of methyl-β-cyclodextrin.

1023 For PI(4)P transfer experiments, the default lipid composition of the ER and Golgi liposomes 1024 was egg PC/brain PS/DGS-NTA(Ni) (93/5/2/0-15 mol%) supplemented with 0 to 15 % 1025 mol/mol of cholesterol and egg PC/liver PE/brain PS/liver PI/brain PI(4)P/ rhodamine-PE 1026 (64/17/5/10/2/2mol%), respectively. The sample initially contained NBD-PH_{FAPP1} (300 nM) 1027 and VAP-A-His (3 μ M) in HKM buffer. NBD emission was measured at 510 (bandwidth 10) nm 1028 upon excitation (bandwidth 1) at 460 nm. Golgi liposomes (300 µM lipid supplemented with 4% PI(4)P and 2% rhodamine-PE), ER liposomes (300 μ M lipid, ± 15% cholesterol) and OSBP (0.1 1029 1030 μ M) were then sequentially added at the indicated times.

1032 Liposome flotation and sedimentation assays

1033 For flotation assays comparing the binding properties of GST, GST-N-Ter, PH-FFAT and N-PH-1034 FFAT, the proteins (2 µM) were incubated with 0.75 mM Golgi-like liposomes containing 1035 (mol%) egg PC (66), liver PE (17), brain PS (5), liver PI (10), Rhodamine-PE (2) and PI(4)P (2) 1036 in HKM buffer (total volume 150 μ l). The sample was then supplemented with 100 μ l HKM 2.8 M 1037 sucrose and vortex and then overlaid with 200 µl HKM + 0.9 M sucrose and 50 µl HKM. After 1038 centrifugation (1 hr, 55000 rpm and a TL55 Beckman swing rotor), the bottom, medium and top 1039 fractions were collected with a Hamilton syringe for SDS-PAGE analysis with Sypro-Orange 1040 staining.

1041 For sedimentation assays comparing the binding properties of N-PH- Δ CC-FFAT and PH- Δ CC-1042 FFAT, we used sucrose-loaded Golgi-like liposomes containing (mol%) egg PC (61), liver PE 1043 (17), brain PS (5), cholesterol (10), Rhodamine-PE (2) and increasing amount of brain PI(4)P(0,1044 1, 2, 5, 8 or 15 mol%) at the expense of liver PI (15, 14, 13, 10, 7 or 0 mol%). Proteins (3 μM) 1045 and liposomes (up to 20 µM PI(4)P) were incubated in 50 mM Hepes (pH 7.2), 120 mM 1046 potassium acetate, and 1 mM MgCl₂ (HKM buffer) at room temperature for 30 min in a total 1047 volume of 50 μ L. The samples were centrifuged at 240 000*g* in a TLA 120.1 (Beckman) rotor for 1048 1 h. The pellets resuspended in 50µl HKM buffer before analysis on 13% SDS-PAGE by Sypro 1049 orange staining.

1050

1051 Liposome binding experiments by FRET

1052 Binding of OSBP constructs to Golgi-like liposomes was monitored by FRET between tryptophan 1053 and the fluorescent lipid DPH-PC (Antonny et al., 1997). The liposomes contained 5 mol % of 1054 DPH-PC, 10 mol % of PI(4)P and were used at a final concentration of 18 µM (total lipid) in KHM 1055 buffer in a 600 µl quartz cuvette. Thereafter, we added increasing amounts of the indicated 1056 OSBP constructs from stock concentrated solutions. The sample was continuously stirred with a 1057 small magnetic bar and fluorescence emission was monitored at 460 nm upon excitation at 280 1058 nm (optimal wavelengths for FRET measurements between tryptophan groups and DPH-PC). All 1059 experiments were performed with a Jasco fluorimeter. Equilibrium experiments were 1060 performed at 37°C with a sampling rate of 1 measurement/s. Kinetics experiments were 1061 performed at 25°C with a sampling rate of 1 measurement/0.2 s.

Because the concentrations of protein ([*P*]) and of PI(4)P were within the same range (0.75 μ M for PI(4)P; 0 - 2 μ M for the protein), [*P*]_{free} cannot be approximated by the total concentration of protein [*P*] and therefore we could not fit the titration curves with a simple hyperbolic curve (e.g. $\Delta F = \Delta F_{max}([P]_{free}/(K + [P]_{free}))$). Instead, we fitted the fluorescence change (ΔF) with the quadratic equation:

$$\Delta F = \Delta F_{max} \left(\frac{[P] + K_d + BS - \sqrt{([P] + K_d + BS)^2 - 4[P]BS}}{2} \right)$$

Where ΔF_{max} is the fluorescence change at saturating protein concentration, [P] is the total 1069 1070 protein concentration, K_d is the equilibrium dissociation constant and BS is the apparent 1071 concentration of protein binding sites on the liposomes. These binding sites should correspond 1072 to the concentration of PI(4)P available for protein interaction provided that there is no steric 1073 hindrance between the bound proteins at the liposome surface. Thus, BS_{max} could theoretically 1074 equal half the total concentration of PI(4)P (= 0.9 μ M considering the bilayer geometry). In 1075 effect, *BS* was significantly lower than *BS*_{max} and was used to estimate the impact of the N-ter on 1076 surface occupancy. For example, $BS = 0.2 \mu M$ implies that the bound protein occupies an elementary lipid area of $0.5 \times \frac{[L]}{RS} = 0.5 \times 18/0.2 \ \mu\text{M} = 45 \ \text{lipids}.$ 1077

1078

1079 To monitor the association and dissociation kinetics of the OSBP constructs with the DPH-PC-1080 containing liposomes, we used a two-stage protocol. First, we added a non-saturating 1081 concentration of protein to the PI(4)P and DPH-containing liposomes to obtain the association 1082 kinetics. Thereafter, we added a 5-fold excess of unlabeled liposomes to trigger the dissociation 1083 kinetics. As determined from control experiments, the half-time of sample mixing in the cuvette 1084 was 0.2 s. Therefore, only time courses slower than this time range were used to estimate 1085 kinetics constants (k_{on} and k_{off}). Exponential fits were used to determine the apparent values of 1086 $k_{\rm on}$ and $k_{\rm off}$.

- For association kinetics: $\frac{\Delta F}{\Delta F_{max}} = 1 e^{-k_{on}t}$ 1087 For dissociation kinetics: $\frac{\Delta F}{\Delta F_{max}} = e^{-k_{off}t}$ 1088

1089

1090 **Cryo-EM experiments**

1091 Lipid mixture in CHCl₃ composed of Egg PC/brain PS/brain PI(4)P (85/15/5 molar ratio) were 1092 dried under a nitrogen flux for 5 minutes and further dried under vacuum for 60 minutes. Lipid 1093 film was rehydrated at 1 mM in 50 mM Hepes pH 7, 120 mM K-acetate and liposomes were 1094 formed by 2 minutes vortex. Liposomes were diluted at 30 µM with 600 nM N-PH-FFAT or PH-1095 FFAT. After 5 minutes incubation, a 5 µL drop of the solution was deposited on a glow 1096 discharged lacey carbon electron microscopy grid (Ted Pella, USA). Blotting was carried out on 1097 the opposite side from the liquid drop and plunge frozen in liquid ethane (EMGP, Leica, 1098 Germany). Samples were imaged using a Tecnai G2 (Thermofisher, USA) microscope operated at 1099 200 kV and equipped with a 4k x 4k CMOS camera (F416, TVIPS). Image acquisition was performed under low dose conditions of 10 e⁻/Å² at a magnification of 50,000 or 29,500 with a 1100 pixel size of 2.13 Å and 3. Å, respectively. 1101

1104

1103 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1105 **Bioinformatic analysis**

1106 For disorder prediction, we used the following sequences of human ORPs (Uniprot access 1107 number): OSBP1 (P22059), OSBP2/ORP4 (Q969R2), ORP5 (Q9H0X9), ORP8L (Q9BZF1), ORP10 1108 (09BXB5), 0RP11 (09BXB4), 0RP6 (09BZF3), 0RP7 (09BZF2), 0RP1 (09BXW6), 0RP3 1109 (Q9H4L5), ORP9 (Q96SU4). Of note, our ORP4 sequence (gift from N. Ridgway) started at M39 1110 (as referred to UNIPROT Q969R2:ORP4-OSBP2 sequence), therefore, in all this study M1 1111 corresponds to M39 of the UNIPROT reference sequence. Order/disorder composition of 1112 different ORPs was determined with "Predictor of Natural Disordered Regions (PONDR®)" web 1113 server (http://www.pondr.com/) using VL3-BA and VSL2 predictors. Amino acids composition 1114 of selected domains was determined using Expasy/protparam web server 1115 (https://web.expasy.org/protparam/) and plotted as pie charts to highlight similarities and 1116 divergences between domains (N-terminus, PH domain, ORD). Limits of selected domains are 1117 detailed in Figure S2A.

1118 For phylogenetic analysis, protein sequences similar to human OSBP (P22059) were obtained 1119 from different species of higher eukaryotes in the UniProt database. To identify a single 1120 sequence corresponding to respective OSBP homolog, all sequences from each species were 1121 aligned to that of the human OSBP using Clustal Omega (Sievers et al., 2011). Only sequences 1122 that contained complete PH and ORD domains were used for phylogenetic analysis. The 1123 phylogenetic tree was created with the Phylogeny.fr server (Dereeper et al., 2008). From the 1124 dataset of OSBP homologs involved in the phylogenetic analysis, the N-termini, PH and ORD 1125 domains were separately aligned to that of corresponding human OSBP domains using Clustal 1126 Omega to obtain a percent identity matrix as indicator of domain conservation. Results were 1127 depicted as length of respective color fields in the graph next to the phylogenetic tree. The 1128 presence or absence of N-terminus was assessed with cut-off limit of 20 amino acids. Shorter N-1129 ter sequences were considered as "no N-ter" and were not included in the N-ter % identity 1130 analysis (blank fields in the green column on the graph). The prediction of coiled-coils was done 1131 using the NPS@ web server (Lupas et al., 1991). Only sequences located between the PH domain 1132 and the FFAT motif were evaluated for coiled-coil probability.

1133

1134 Image analysis

For TGN/cytosol ratio, two ROIs of the same area were applied in the TGN and in the cytosol. The average fluorescence was determined for each ROI and the ratio was then calculated. Kymographs were generated using the Metamorph software (Molecular Devices) from a line drawn on the image stack and projected across time of the complete time series. The lines were

- 1139 72 pixels long (ca. 20 μ m) with a width of 10 pixels (ca. 3 μ m), from which pixel values were
- 1140 averaged. Scan lines quantification on GUVs were generated using Image J software.



Molecular Weight (kDa)

4 OSBP PH-FFAT

7 ORP4 N-PH-FFAT 8 ORP4 PH-FFAT













Tethering geometry



MCS dynamics





