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Article

Human VAPome Analysis Reveals MOSPD1 and MOSPD3 as Membrane Contact Site Proteins Interacting with FFAT-Related FFNT Motifs

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SUMMARY

Membrane contact sites (MCS) are intracellular regions where two organelles come closer to exchange information and material. The majority of the endoplasmic reticulum (ER) MCS are attributed to the ER-localized tether proteins VAPA, VAPB, and MOSPD2. These recruit other proteins to the ER by interacting with their FFAT motifs. Here, we describe MOSPD1 and MOSPD3 as ER-localized tethers interacting with FFAT motif-containing proteins. Using BioID, we identify proteins interacting with VAP and MOSPD proteins and find that MOSPD1 and MOSPD3 prefer unconventional FFAT-related FFNT (two phenylalanines [FF] in a neutral tract) motifs. Moreover, VAPA/VAPB/MOSPD2 and MOSPD1/MOSPD3 assemble into two separate ER-resident complexes to interact with FFAT and FFNT motifs, respectively. Because of their ability to interact with FFNT motifs, MOSPD1 and MOSPD3 could form MCS between the ER and other organelles. Collectively, these findings expand the VAP family of proteins and highlight two separate complexes in control of interactions between intracellular compartments.

INTRODUCTION

Eukaryotic cells are defined by the presence of membrane-bound organelles with specialized functions. As the compartmentalization of biochemical processes into distinct organelles is fundamental to complex cellular life, the benefits of this segregation come at the price of needing to exchange information and material between organelles. Membrane contact sites (MCS), domains where two or more organelles come into close proximity to interact, have emerged as a powerful example to address the issue of interorganellar communication (Scorrano et al., 2019). As MCS participate in a multitude of biological processes, including calcium storage, endosomal trafficking, and lipid transfer, aberrations in MCS proteins have demonstrated physiological consequences, such as VAPB in amyotrophic lateral sclerosis, ACBD5 in retinal dystrophy, and NPC1 in Niemann-Pick disease (Abu-Safieh et al., 2013; Höglinger et al., 2019; Holthuis and Menon, 2014; Neefjes et al., 2017; Nishimura et al., 2004; Scorrano et al., 2019).

Stretching from the nuclear envelope (NE) to the plasma membrane, the endoplasmic reticulum (ER) can form MCS with all membrane-bound organelles and the plasma membrane. The majority of ER contact sites are facilitated by the VAMP-associated proteins VAPA and VAPB (Phillips and Voeltz, 2016; Wu et al., 2018). VAPA and VAPB are localized throughout the ER

and function as hubs to recruit numerous proteins and their organelles to the ER (Loewen et al., 2003; Murphy and Levine, 2016). For example, the interaction of the lysosomal cholesterol sensor protein ORP1L with VAPA determines the intracellular positioning of lysosomes (Cabukusta and Neefjes, 2018; Rocha et al., 2009). VAPA and VAPB interact with so-called FFAT (two phenylalanines [FF] in an acidic tract) motifs in other proteins. The canonical FFAT motif has a core of seven residues, E-F-F-D-A-x-E, and an adjacent upstream region with multiple acidic or phosphorylated residues that additionally contribute to the motif (Loewen et al., 2003; Murphy and Levine, 2016; Slee and Levine, 2019). Although FFAT motif sequences are found in 0.8% of the human proteome, most verified FFAT motifs deviate from the canonical motif in their core and/or upstream residues (Slee and Levine, 2019). This flexibility therefore requires biochemical validation of the predicted motif for its interactions with VAPA and VAPB.

The interaction between a FFAT motif and VAPA/VAPB is mediated through its MSP domain (Figure 1B). This begins with the electrostatic binding of the negatively charged FFAT motif to the positive surface of an MSP domain followed by the motif fastened in the hydrophobic groove of the domain (Furuita et al., 2010). A recently described third FFAT-binding protein, MOSPD2 (abbreviated as MSPD2), hinted at other proteins with FFAT-binding MSP domains (Di Mattia et al., 2018). As the



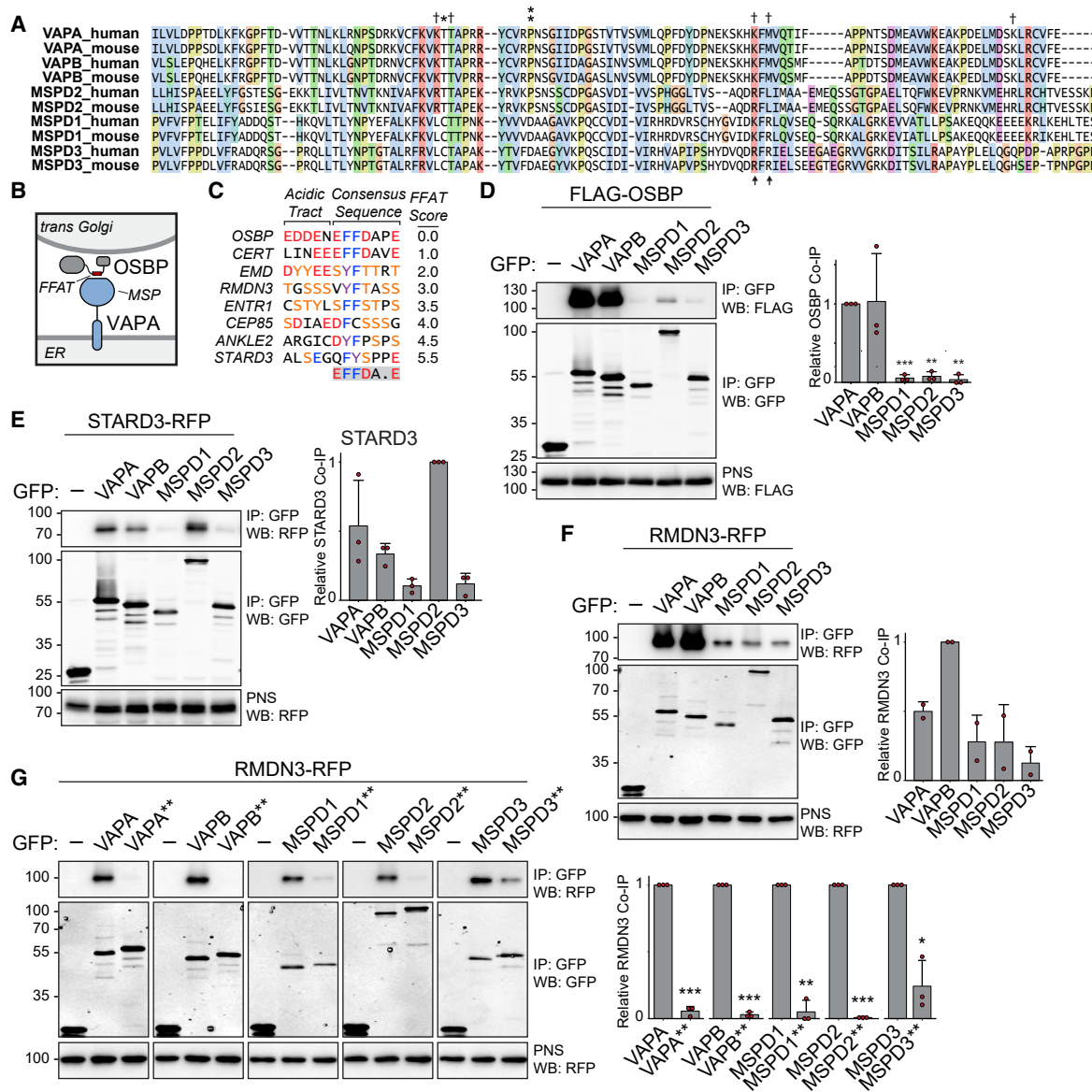


Figure 1. MSPD1 and MSPD3 Contain Functional MSP Domains

(A) Sequence alignment of human and mouse MSP domains. Residues that create the hydrophobic pocket accommodating the position 2 phenylalanine are indicated (dagger). Residues mutated in this study are marked with arrows. T46I (asterisk) and P56S (double asterisk) mutations in VAPB are associated with ALS8 and spinal muscular atrophy, respectively (Chen et al., 2010; Kanekura et al., 2006).

(B) Schematic representation of the FFAT motif-containing OSBP interacting with the ER protein VAPA to form MCS.

(C) FFAT score table of proteins studied. Colors indicate the different classes of amino acids: blue/violet, core aromatic residues; red, acidic; orange, can be phosphorylated.

(D–F) Co-immunoprecipitation (Co-IP) of GFP-tagged VAP proteins with FFAT motif proteins and quantifications: FLAG-OSBP (n = 3), STARD3-RFP (n = 3), and RMDN3-RFP (n = 2). Independent experiments were quantified, and the signal was normalized to highest obtained signal.

(G) Co-IP of GFP-tagged VAPA, VAPA(K94D/M96D), VAPB, VAPB(K87D/M89D), MSPD1, MSPD1(K94D/R96D), MSPD2, MSPD2(R404D/L406D), MSPD3, and MSPD3(R111D/R113D) with RMDN3-RFP and quantifications (n = 3). Mutant VAP proteins are denoted by double asterisks. All error bars indicate standard deviation.

human genome encodes two other MSP domain-containing proteins, MOSPD1 and MOSPD3, their ability to interact with FFAT motifs remains to be investigated.

Here, we characterize MOSPD1 and MOSPD3 (abbreviated respectively as MSPD1 and MSPD3) as ER-localized tether proteins interacting with FFAT motif-containing proteins. Using the

BioID (proximity-based biotin ligation followed by mass spectrometry identification) method, we identified proteins interacting with VAP and MOSPD proteins and found that MSPD1 and MSPD3 do not interact with classical FFAT motifs preferred by VAPA, VAPB, and MSPD2. Instead, MSPD1 and MSPD3 are selective for unconventional FFAT motifs disfavored by VAPA,

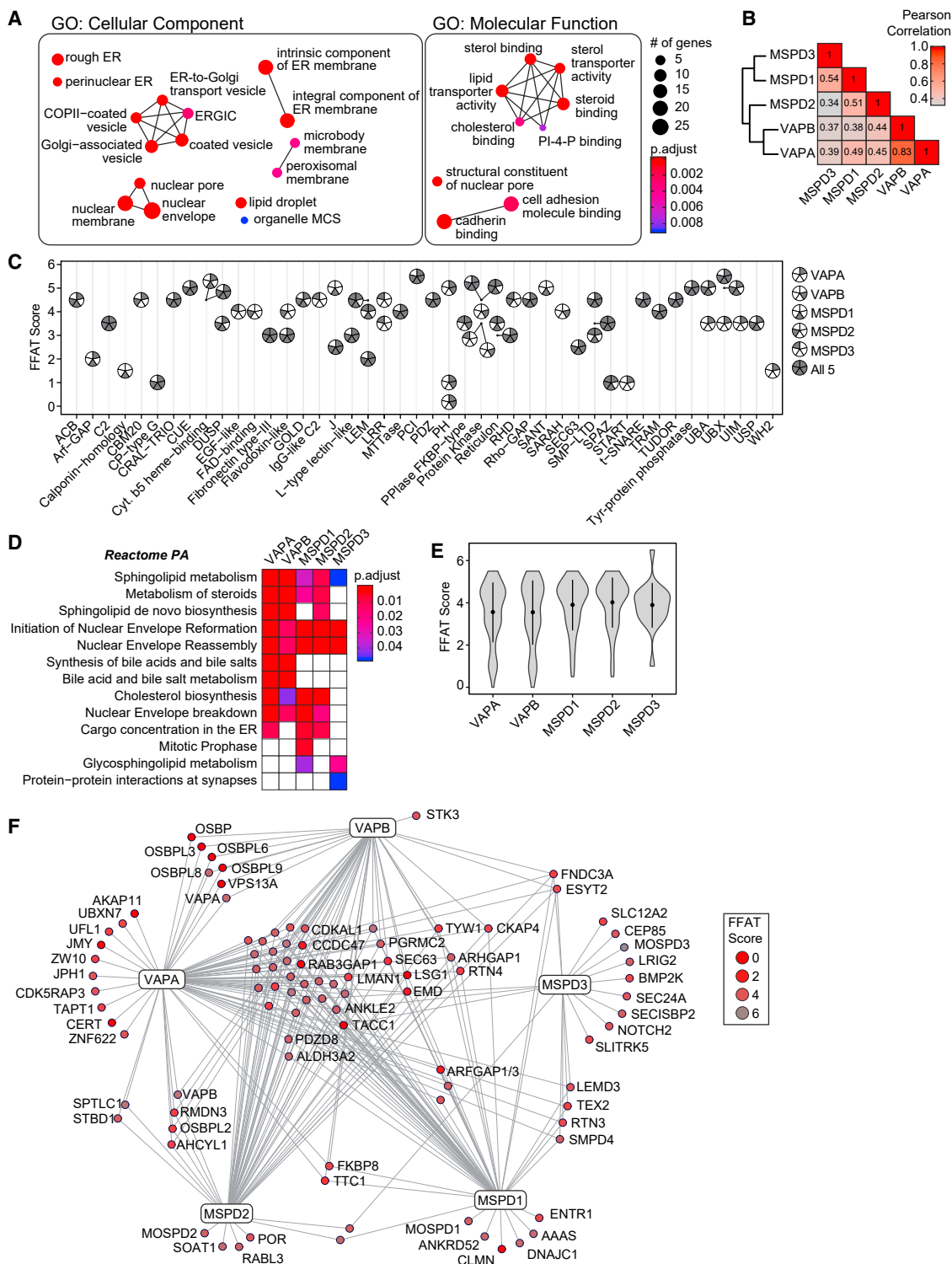


Figure 2. Extended Protein Interaction Map of VAP Proteins

(A) Gene Ontology (GO) analysis of proteins identified with BioID.

(B) Correlation and overlap of identified proteins interacting with individual VAP proteins.

(C) Domains annotated in BioID identified proteins. The y axis depicts FFAT scores of the proteins with the designated domains, and the shading of pie charts demonstrates the interacting VAP as shown at the right side of the figure.

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VAPB, and MSPD2. We named these motifs FFNT (two phenylalanines [FF] in a neutral tract) motifs. This segregation in motif selectivity is also reflected in these proteins' ability to self-associate into two ER-resident protein complexes. As we report that MSPD1 and MSPD3 can also form MCS between the ER and other organelles, we found that MSPD3 is critical for the timely reformation of NE in post-mitotic cells. We characterized many interactions of individual VAP and MSPD proteins that may act as a basis for further understanding the complex and dynamic interplay between the ER and other cellular compartments.

RESULTS

MSPD1 and MSPD3 Contain Functional MSP Domains

Numerous studies have shown that the ER-localized proteins VAPA, VAPB, and MSPD2 interact with FFAT motifs in other proteins on various organelles and macromolecular structures to ensure their recruitment to the ER (Di Mattia et al., 2018; Murphy and Levine, 2016; Slee and Levine, 2019). This interaction between FFAT motifs and VAPA/VAPB/MSPD2 is mediated by MSP domains (Figure 1B). In addition to VAPA, VAPB, and MSPD2, the human genome encodes two other MSP domain-containing proteins, MSPD1 and MSPD3, and all MSP domain-containing proteins are ubiquitously expressed in various tissues throughout development and found to be expressed in various cultured cell lines (Figures S1A and S1B) (Cardoso-Moreira et al., 2019). However, the ability of MSPD1 and MSPD3 to interact with FFAT motifs is yet to be determined.

To assess whether all of VAPA, VAPB, MSPD1, MSPD2, and MSPD3 (hereafter collectively referred to as VAP proteins) can interact with FFAT motifs, we tested their ability to interact with several FFAT motif-containing proteins by co-immunoprecipitation. We first used the MCS-localized cholesterol transfer protein OSBP, which contains the canonical FFAT motif, as prey (Figures 1B and 1C) (Murphy and Levine, 2016). Although VAPA and VAPB strongly interacted with OSBP, the interaction between the recently characterized FFAT-binding protein MSPD2 and OSBP was much weaker (Figure 1D). Similarly, the ceramide transfer protein CERT, another FFAT motif-containing protein, was immunoprecipitated only with VAPA and VAPB (Figures S1D and S1E). Interestingly, VAPA, VAPB, and MSPD2 showed comparable affinities toward late endosomal proteins ORP1L and STARD3 (Figures 1E and S1F). Although VAPA, VAPB, and MSPD2 are all reported to interact with FFAT motifs, we observed that they display varying affinities toward different FFAT motif-containing proteins, indicating a degree of selectivity among VAP proteins. Concurrently, no interaction between MSPD1/MSPD3 and these four proteins (OSBP, ORP1L, CERT, and STARD3) was detected.

In contrast, all five VAP proteins displayed some degree of affinity toward the mitochondrial protein RMDN3 (Mikitova and Levine, 2012) (Figure 1F). Mutations in the characterized (VAPA: K94D/M96D; VAPB: K87D/M89D; MSPD2: R404D/

L406D) and predicted (MSPD1: K94D/R96D; MSPD3: R111D/R113D) critical residues of their MSP domains reduced the interactions between VAPs and RMDN3 (Figures 1A and 1G). These findings indicated that the interactions with RMDN3 are facilitated by the FFAT binding of MSP domains and substantiate that all VAP proteins consist of functional MSP domains. Collectively, our findings invited us to speculate that VAP proteins are selective in their FFAT motif interactions.

BioID Identification of VAP Interactors and Extended Human VAPome

To further investigate the proposed selectivity of the five VAP proteins toward FFAT motifs, we set out to identify proteins interacting with each VAP. To achieve this, we performed BioID (Roux et al., 2012). We identified >600 proteins, of which ~100 were consistently found in three independent experiments (Figure S2A; Table S1). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses presented known biological processes associated with VAPA and VAPB, such as cholesterol transport and anterograde vesicular transport, as well as biological processes such as NE assembly (Figures 2A and S2B–S2D). Moreover, protein domains found on identified proteins highlight the multitude of VAP-associated biological processes (Figure 2C). Distribution of identified interaction partners shows a varying degree of correlation among VAP proteins (Figure 2B). As VAPA and VAPB interactomes presented the highest correlation observed, MSPD1 and MSPD3 appeared somehow distant from the other three VAP proteins. This variation in individual VAP interactomes (VAPomes) was also reflected in their pathway analysis (Figures 2D and S2E).

Next, we assigned FFAT scores to the identified proteins (Murphy and Levine, 2016; Slee and Levine, 2019). Here, the numeric value of FFAT score represents how distant a predicted FFAT motif is from the canonical FFAT motif of OSBP, which has a FFAT score of 0 (Figure 1C). It appears that MSPD1, MSPD2, and MSPD3 tend to interact with proteins of higher FFAT score or more distant from the canonical FFAT motif compared with VAPA and VAPB (Figure 2E). On the basis of the proteins identified in the BioID experiments and on their FFAT scoring, we expanded the current VAPome dataset, which provides information on the individual and collective VAPomes (Figures 2F and S2F; Table S1). Next, we used this dataset to validate additional VAP interaction partners.

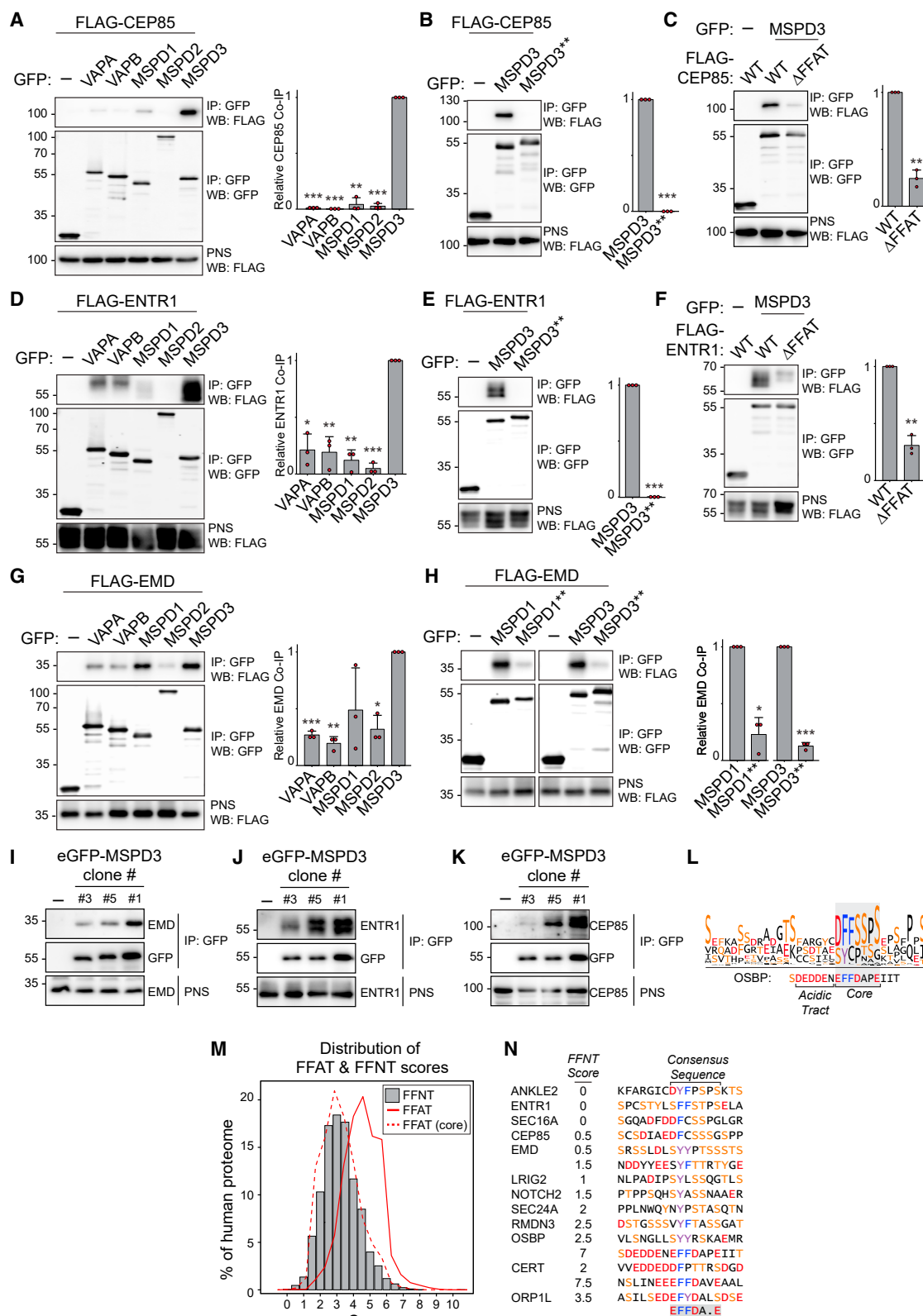
VAP Proteins Are Selective in Their Interactions with FFAT Motifs

We started validating interactions with two proteins found to interact with all five VAP proteins: fibronectin type-III domain-containing protein 3A (FNDC3A) and extended synaptotagmin-2 (ESYT2) (Figures 2F and S2F). Co-immunoprecipitations verified that both FNDC3A and ESYT2 interact with all VAP proteins (Figures S3A and S3B). However, the FFAT-binding-deficient mutants of VAPs did not weaken these interactions, implying

(D) Reactome pathway analysis comparison of individual VAP interactomes.

(E) Distribution of FFAT scores in individual VAP interactomes.

(F) Interaction map of human VAPome. Proteins confirmed in three independent BioID experiments are shown. The colors of nodes represent the FFAT score of the proteins, as indicated. For a full list, see Table S1 and Figure S2F.



(legend on next page)

that the interaction between VAP proteins and FNDC3A/ESYT2 is not mediated by a FFAT motif (Figures S3C and S3D, data not shown). As both FNDC3A and ESYT2 are predicted transmembrane proteins and found to localize in the ER (Figure S3E), we speculate that they interact with VAP proteins via their transmembrane regions.

Next, we studied interactions specific to MSPD1 and MSPD3. Centrosomal protein 85 (CEP85) interacted strongly with MSPD3 and showed some affinity toward MSPD1 (Figure 3A). Also, this interaction was abolished when the FFAT-binding-deficient MSPD3 mutant (MSPD3**) was used (Figure 3B), implying that CEP85 contains a FFAT motif to interact with MSPD3. As anticipated, the removal of the predicted FFAT motif by mutating two phenylalanine positions to alanines (F71A/C72A) reduced the interaction between MSPD3 and CEP85 drastically (Figures 1C and 3C), further establishing CEP85 as an MSPD3-favoring FFAT motif-containing protein.

Similar to CEP85, ankyrin repeat and LEM domain containing 2 (ANKLE2) and endosome-associated-trafficking regulator 1 (ENTR1) demonstrated strong affinities for MSPD3, and these interactions were weaker with MSPD3** (Figures 3D, 3E, S3F, and S3G). Furthermore, mutations in the predicted FFAT motifs of ANKLE2 (Y256A/F257A) and ENTR1 (F159A/F160A) diminished these interactions (Figures 1C, 3F, and S3H). A similar pattern of interaction was observed with nuclear lamina protein emerin (EMD), where MSPD1 and MSPD3 are preferred interaction partners over VAPA and VAPB (Figure 3G). These interactions were also reduced in the cases of the FFAT-binding-deficient mutants of MSPD1/MSPD3 and FFAT motif deletion mutant of EMD (Figures 3H and S3I).

As the plasmid overexpression of VAPs can result to a 3–4 log-fold increase above endogenous expression levels, we investigated if any of these interactions take place at endogenous levels (Figure S1C). For this purpose, we endogenously tagged MSPD3 with GFP in MeJuSo cells using CRISPR-Cas9 and validated three independent clones by immunoblotting (Figure S3J). GFP pull-downs in the endogenously tagged MSPD3 cells, endoGFP-MSPD3, further confirmed that interactions of MSPD3 with EMD, ENTR1, and CEP85 occurred at endogenous expression levels (Figures 3I–3K). Collectively, our data indicate a sub-specialization among VAP proteins, where group 1 VAP proteins (VAPA, VAPB, and MSPD2) interact with more canonical FFAT motifs and MSPD1/MSPD3, as group 2, interact with motifs that are related, and therefore could be predicted with the current search algorithm, yet different from classical FFAT motifs. We define these FFAT-related motifs interacting with MSPD1/MSPD3 as FFNT (two phenylalanines [FF] in a neutral tract) motifs.

Proteome-wide Search for FFNT Motifs

Next, we set to define a consensus sequence for FFNT motifs recognized by MSPD1/MSPD3. As the confirmed interactors of group 2 proteins are poorly conserved outside mammals, we aligned FFNT motif sequences derived from mammalian homologs of ANKLE2, ENTR1, and CEP85, the three best interactors of MSPD3. The sequence logo generated from the alignment revealed that the core residues are somehow better conserved than upstream elements, unlike the FFAT motif (Figure 3L). On the basis of this alignment and using an online tool specialized in short linear motifs, we created a position weight matrix (PWM) for FFNT motifs (Figure S3K) (Krystkowiak et al., 2018). Then, we adapted the currently available FFAT motif search PWM to this online-generated PWM with adjustments (Figure S3L). Using the PWM, we mapped the human proteome for FFNT motifs (Figure 3M; Table S2).

We found that frequency distributions of the core FFAT and FFNT scores in human proteome are highly similar (Figure 3M). As expected, the proteins that were the basis of the FFNT PWM have low FFNT scores: ANKLE2 (0), ENTR1 (0), and CEP85 (0.5) (Figure 3N). Even though it was not included in the alignment led to the PWM, the confirmed FFNT motif of EMD also presented a low score (1.5). Similarly, interacting with all VAP proteins but favoring group 1, RMDN3 has a higher score of 2.5 according to FFNT PWM. Finally, the canonical FFAT motifs of OSBP and CERT have FFNT scores of 7 and 7.5, respectively (Figure 3N). As our FFNT PWM and proteome list constitute the first step in understanding group 2 VAP proteins and FFNT motifs, it must be stressed that each predicted FFAT/FFNT motif must be validated experimentally.

VAP Proteins Are ER Localized and MSPD3 Overexpression Induces OSER Formation

Next, we investigated whether all VAP proteins localize in the ER to potentially recruit proteins containing FFAT or FFNT motifs to the ER. We found that all VAP proteins displayed ER-like reticular localization (Figure 4A). Transmembrane helix predictions suggested that VAPA, VAPB, and MSPD2 are single-pass transmembrane proteins and that MSPD1 and MSPD3 are also membrane anchored each with two transmembrane helices (Figure S4A). To test whether MSPD1 and MSPD3 are membrane-bound proteins, we performed detergent-free membrane fractionations and confirmed that all VAP proteins are associated with cellular membranes (Figure 4B). On the basis of the transmembrane helix predictions, as well as our microscopy and biochemical observations, we depict the membrane topology of VAP proteins as in Figure 4C.

Figure 3. MSPD1 and MSPD3 Prefer FFNT Motifs over FFAT Motifs

(A, D, and G) colP of GFP-tagged VAP proteins with FLAG-tagged CEP85 (A), ENTR1 (D), and EMD (G). Three independent experiments were quantified and normalized.
(B, E, and H) colP of GFP-tagged MSPD1, MSPD3, MSPD1**, and MSPD3** with FLAG-tagged CEP85 (B), ENTR1 (E), and EMD (H).
(C and F) colP of GFP-MSPD3 with FFAT motif deleted mutants of CEP85 (C) and ENTR1 (F). $n = 3$ for all experiments.
(I, J, and K) colP of endogenous MSPD3 with endogenous EMD, ENTR1, and CEP85 using endoGFP-MSPD3 cells (eGFP-MSPD3). Note that each colP was confirmed in multiple independent clones.
(L) Sequence logo from FFAT motif alignment of mammalian homologs of CEP85, ENTR1, and ANKLE2. Note that residues corresponding to the acidic tract of the canonical FFAT motif are not well conserved.
(M) Distribution of core FFAT and FFNT motifs in human proteome.
(N) FFNT scores and motifs of proteins examined in this study. All error bars indicate standard deviation.

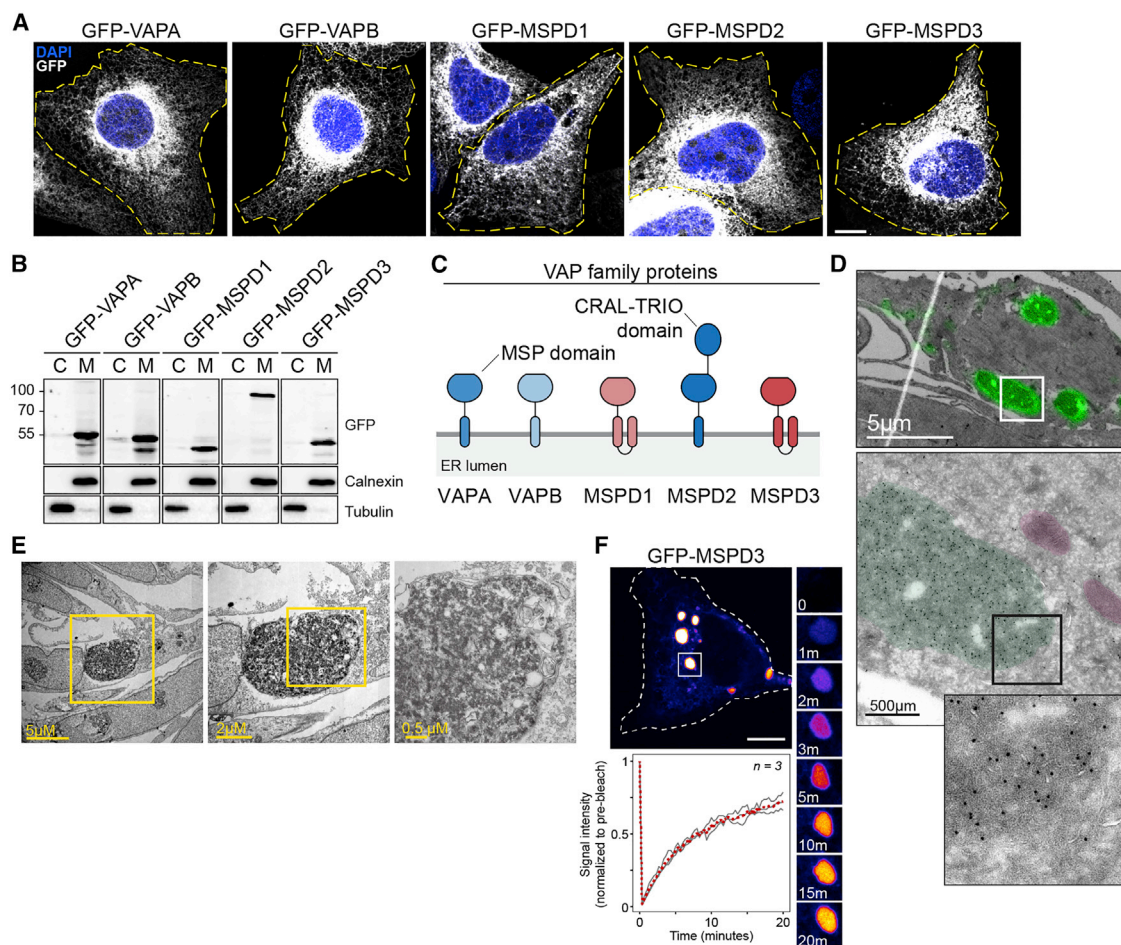


Figure 4. All VAP Proteins Are Localized in the ER and GFP-MSPD3 Overexpression Causes OSER Formation

(A) Confocal images of U2OS cells expressing GFP-tagged human VAPA, VAPB, MSPD1, MSPD2, and MSPD3. Cell boundaries are marked with dashed lines. Scale bar is 10 μ m.

(B) Membrane fractionations from HEK293T cells expressing GFP-tagged VAP proteins. Cytosol (C) and total membrane (M) fractions were validated using tubulin and calnexin antibodies, respectively.

(C) Predicted membrane topology of VAP proteins.

(D) Immuno-CLEM images of a U2OS cell demonstrate that ultra-structures GFP-MSPD3 is localized are composed of ER membrane subdomains of highly clustered GFP-MSPD3. ER and mitochondria are highlighted in green and magenta, respectively.

(E) Transmission EM of GFP-MSPD3-transfected U2OS cells consist structures resembling OSER.

(F) FRAP of GFP-MSPD3 ultra-structures reveal that these structures are connected to the rest of the ER network.

We often observed GFP-MSPD3 concentrating in cytoplasmic ultra-structures (Figure S4B). Over-exposure of these images displayed that MSPD3 additionally localizes at the ER in these cells. Immuno-CLEM (correlative light-electron microscopy) images revealed that MSPD3-positive structures contain GFP-MSPD3 clustered in a dense bundle (Figure 4D). Transmission electron microscopy (EM) images further revealed that these ultra-structures are made of stacked membrane arrays, resembling organized smooth ER (OSER) (Figures 4E and S4C) (Snapp et al., 2003). Meanwhile, no such structure was observed in control cells, implying that these structures are induced by GFP-MSPD3 overexpression (Figure S4D). The GFP-MSPD3-induced OSER presented similar characteristics as previously described OSER (Snapp et al., 2003). GFP-MSPD3-OSER is attached to

the remaining ER membrane, as its fluorescent signal recovered from photobleaching (Figure 4F). Moreover, these structures were positive for multiple ER markers: calnexin and endogenous and overexpressed VAPA/VAPB (Figure S4E).

As the overexpression of a GFP-tagged resident ER protein can induce OSER formation, we examined whether it is GFP or MSPD3 overexpression causing OSER formation (Snapp et al., 2003). For this, we used a tandem FLAG tag, as it is less prone to dimerization because of its net charge of -6 . The overexpression of both N- and C-terminally FLAG-tagged MSPD3 also localized in cytoplasmic ultra-structures, suggesting that MSPD3 overexpression is sufficient to form OSER structures (Figures S4F and S4G). Furthermore, neither GFP- nor FLAG-tagged MSPD3** was able to induce OSER structures (Figures

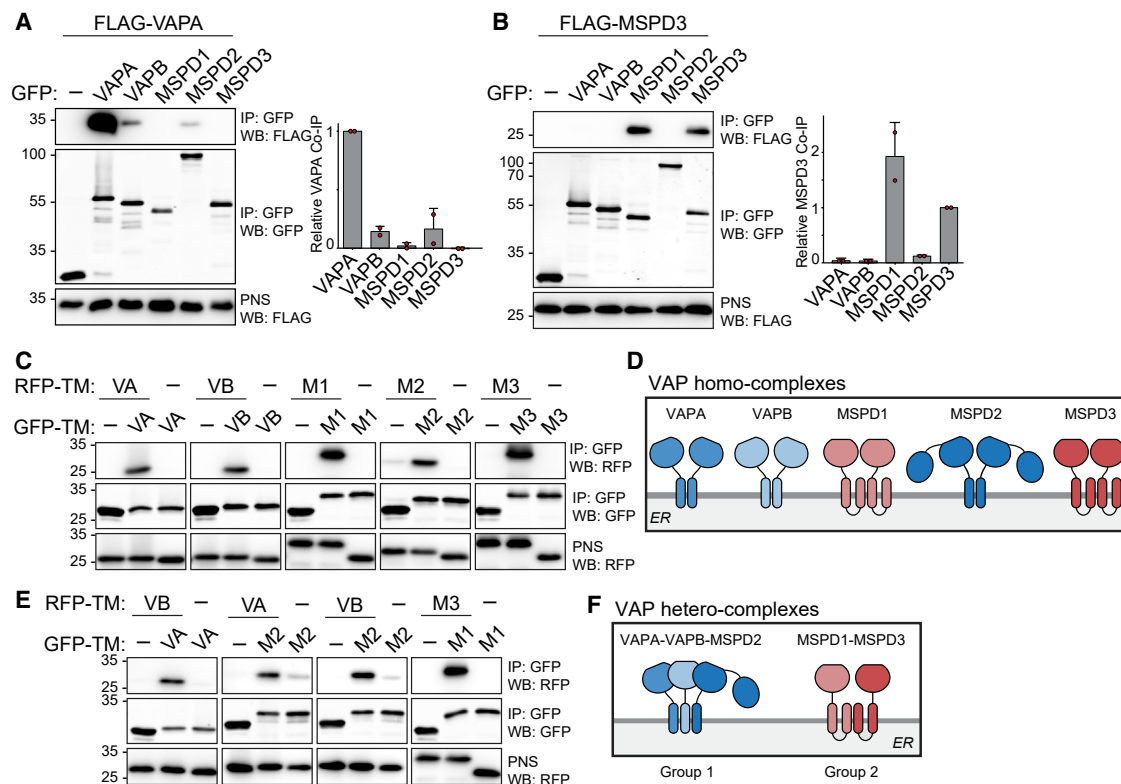


Figure 5. VAP Proteins Self-Associate into Two Distinct Complexes via Their Transmembrane Regions

(A and B) coIP of GFP-tagged VAP proteins with VAPA (A) and MSPD3 (B) (n = 2).
(C and E) coIP of GFP-tagged VAP transmembrane regions with RFP-tagged VAP transmembrane regions.
(D) Model of VAP homo-dimerization.
(F) Model of VAP hetero-dimers. All error bars indicate standard deviations.

S4B and S4G), implying that MSPD3 requires the motif-binding interface of its MSP domain for OSER induction. To show that the lack of OSER in MSPD3** is not due to a difference in expression levels between wild-type and mutant MSPD3, we analyzed OSER formation in cells expressing comparable levels of MSPD3 and MSPD3**. To achieve this, we pooled and fluorescence-activated cell sorting (FACS)-sorted U2OS cells either co-transfected with GFP-MSPD3 and RFP or transfected only with GFP-MSPD3** (Figure S3H). Different pools of sorted cells expressing comparable levels of GFP revealed that OSER formation was present only in RFP-positive GFP-MSPD3-transfected cells (Figure S3I). Meanwhile, RFP-negative cells expressing GFP-MSPD3** did not display the characteristic GFP-MSPD3-OSER ultra-structures. This further suggests that MSPD3 requires its motif-binding interface for OSER induction.

VAP Proteins Self-Associate into Groups

In addition to interacting with FFAT motifs, VAPA and VAPB form homo- and hetero-dimers (Kim et al., 2010; Nishimura et al., 1999). We wondered whether MSPD1/MSPD2/MSPD3 also forms homo- and hetero-dimers. As reported previously, VAPA and VAPB demonstrated a strong degree of self-association as well as interactions with each other, but not with MSPD1/MSPD3 (Kim et al., 2010; Murphy and Levine, 2016; Weir et al., 2001)

(Figures 5A and S5B). Also, MSPD2 forms homo-dimers and interacts only with VAPA and VAPB (Figure S5C). Interestingly, both MSPD1 and MSPD3 self-associated and interacted with the other, but not with VAPA/VAPB/MSPD2 (Figures 5B and S5D).

Although VAPA/VAPB dimerization can occur independently of MSP domains, both the coiled coil and transmembrane regions contribute to their dimerization. MSPD1, MSPD2, and MSPD3 do not contain a predicted coiled coil region or a G-X-X-X-G motif, which facilitates the self-assembly of VAPA and VAPB transmembrane helices (Figures S4A and S5A) (Kim et al., 2010). Instead, the second predicted transmembrane helices of MSPD1/MSPD3 both contain A-X-X-X-G weak dimerization motif (Schneider and Engelman, 2004). To test if the transmembrane helices of VAP proteins can drive homo-dimerization, we performed co-immunoprecipitations using transmembrane regions only. We found that transmembrane region of each VAP is sufficient to form homo-dimers (Figures 5C and 5D).

Remarkably, as all VAP proteins form homo-dimers, the interactions among VAP proteins are subjected to the same grouping as in their FFAT/FFNT motif interactions (i.e., VAPA-VAPB-MSPD2 and MSPD1-MSPD3 complexes are segregated from each other). It is conceivable that the hetero-assembly of VAP proteins is due to their MSP-mediated common interaction partners. Alternatively, the grouping of VAP proteins could be also

preserved outside MSP domains. Co-immunoprecipitations with transmembrane regions revealed that indeed the grouping of full-length VAPs is also inherent in their transmembrane regions (Figure 5E). Overall, our data show that the VAP proteins form two separate ER-localized protein complexes to interact with FFAT and FFNT motifs (Figure 5F).

MSPD1 and MSPD3 Can Recruit Proteins to the ER Membrane and Form Contact Sites with Mitochondria

The ability of VAPA and VAPB to interact with FFAT motifs facilitates their function as molecular hubs to recruit proteins and organelles to the ER. Although the ER recruitment function of group 1 proteins is well established, we questioned whether MSPD1/MSPD3 can also form MCS. So far, we have shown that all VAP proteins were able to interact with the outer mitochondrial membrane protein RMDN3 (Figure 1F). The interaction of RMDN3 with VAPB and MSPD2 is sufficient to form MCS between the ER and mitochondria, which exhibits clustered ER and mitochondria (Di Mattia et al., 2018; Stoica et al., 2014; De Vos et al., 2012) (Figure S6A). Similar to VAPB and MSPD2, GFP-MSPD1 was able to recruit RMDN3 to the ER and form MCS between two organelles (Figures 6A and 6B). Although MSPD3 demonstrated the same ability to form MCS, this was less efficient than other VAP proteins (Figures 6B and S6A). This is most likely related to the affinity of MSPD3 toward RMDN3 being insufficient to engage the entire mitochondrial network to the ER surface, as deduced from the biochemical analyses (Figure 1F).

Although each VAP can form MCS between the ER and mitochondria, it is plausible to think that each VAP also plays a unique role at these contact sites that may be essential for mitochondrial function or morphology. We observed that the small interfering RNA (siRNA)-mediated depletion of group 1 and group 2 proteins resulted in a redistribution of mitochondria in MeJuSo cells. Characteristically, mitochondria in these cells are dispersed throughout the cytoplasm, often reaching to the peripheral regions of the cell (Figure 6C). We found that the depletion of group 1 proteins collapsed the mitochondrial distribution, resulting in a more perinuclear localization of these organelles (Figure 6C). The same was observed following the depletion of group 2 proteins, although not as pronounced. To quantify these observations, we sectioned the cytoplasm to five concentric circles emanating from the nucleus to the plasma membrane and determined the fraction of mitochondria localized in each “ring” using an in-house ImageJ script (Figure 6D). The quantifications further demonstrated that the depletion of either group 1 or group 2 results in a collapse of the mitochondrial network (Figure 6E). Moreover, a 5-fold reduction of all VAP proteins demonstrated slightly yet significantly stronger phenotype than the depletion of either VAP groups (see rings 1 and 3). It is possible that the inefficiency of 5-fold knockdown is due to incomplete depletions by siRNAs (Figure S6C). Also, the depletions of VAP groups did not cause any obvious aberrations in the ER morphology (Figure S6B). Overall, these results showed that both VAP groups contribute to the peripheral distribution of mitochondria and implied that this event is facilitated via the contact sites formed between the ER and mitochondria.

As MSPD3 overexpression induces OSER formation, we wondered whether MSPD3 can recruit its interacting partners to these domains. We found the globular FLAG-ENTR1 localizing around OSER structures formed by GFP-MSPD3 (Figures 6F and S6D). Similarly, FLAG-EMD was localized at MSPD3-induced OSER (Figures 6G and S6F). It is possible that these proteins are recruited to OSER structures independent of MSPD3. To examine this notion, we overexpressed GFP-SEC61B, an ER-resident protein that can also induce OSER formation (Snapp et al., 2003). Although GFP-SEC61B occasionally induced OSER formation, these structures were devoid of FLAG-ENTR1, suggesting that its OSER localization is specific to MSPD3 (Figure S6E). In contrast, FLAG-EMD was found localized at SEC61B-OSER structures (Figure S6G). This suggests that FLAG-EMD co-localization with GFP-MSPD3 at OSER structures is likely due to OSER formation and not due to its FFNT motif. Nevertheless, although EMD may localize to OSER structures independent of its interaction with MSPD3, our biochemical data confirm that the interaction between these proteins facilitated via MSP domain and a FFNT motif (Figures 3G, 3H, and S3I).

MSPD3-EMD Interaction Takes Place Predominantly in the ER

EMD is an inner nuclear membrane (INM) protein also localizing in the ER, and GFP-MSPD3 localizes exclusively in the ER when overexpressed (Berk et al., 2013) (Figure 4A). This raises the question of where these two proteins interact. Cells expressing endogenously GFP-tagged MSPD3 (endoGFP-MSPD3) demonstrated ER localization for MSPD3 at endogenous levels without inducing OSER formation (Figure S7A). Furthermore, these images were devoid of a clear NE localization, unlike endogenous stainings of EMD, implying that MSPD3 is not enriched in the NE membranes (Figure 7A). To further investigate the possibility of MSPD3 and EMD co-localizing in the INM, we compared this interaction with the one between EMD and Lamin A/C, which exclusively takes place in the INM (Berk et al., 2013). Immunofluorescence images of endogenous EMD and Lamin A/C displayed co-localization at the NE membrane (Figure 7A). Moreover, line scans of these images revealed two overlapping peaks, most likely representing the INM. On the other hand, co-staining of EMD with endoGFP-MSPD3 did not exhibit any obvious co-localization around the NE (Figure 7A). Furthermore, the peak of endoGFP-MSPD3 in the line scan did not overlap with the peak of EMD but was adjacent on the cytoplasmic side. Overall, these data imply that MSPD3 is not enriched in the INM, where majority of cellular EMD pools are placed.

To determine the subcellular localization of MSPD3-EMD interaction, we used proximity ligation assay (PLA). PLA signal of the GFP~EMD antibody pair in endoGFP-MSPD3 cells was concentrated in the cytoplasm, but not the nucleus (Figure 7B). As controls, GFP-negative wild-type cells and siMSPD3- or siEMD-treated cells were also tested with PLA, and they displayed no or reduced PLA signal for GFP~EMD (Figures 7B, 7C, S7B, S7C, and S3J). Meanwhile, PLA signal for the EMD~Lamin A/C antibody pair was almost exclusively detected around the nucleus, representing the nature of their interaction

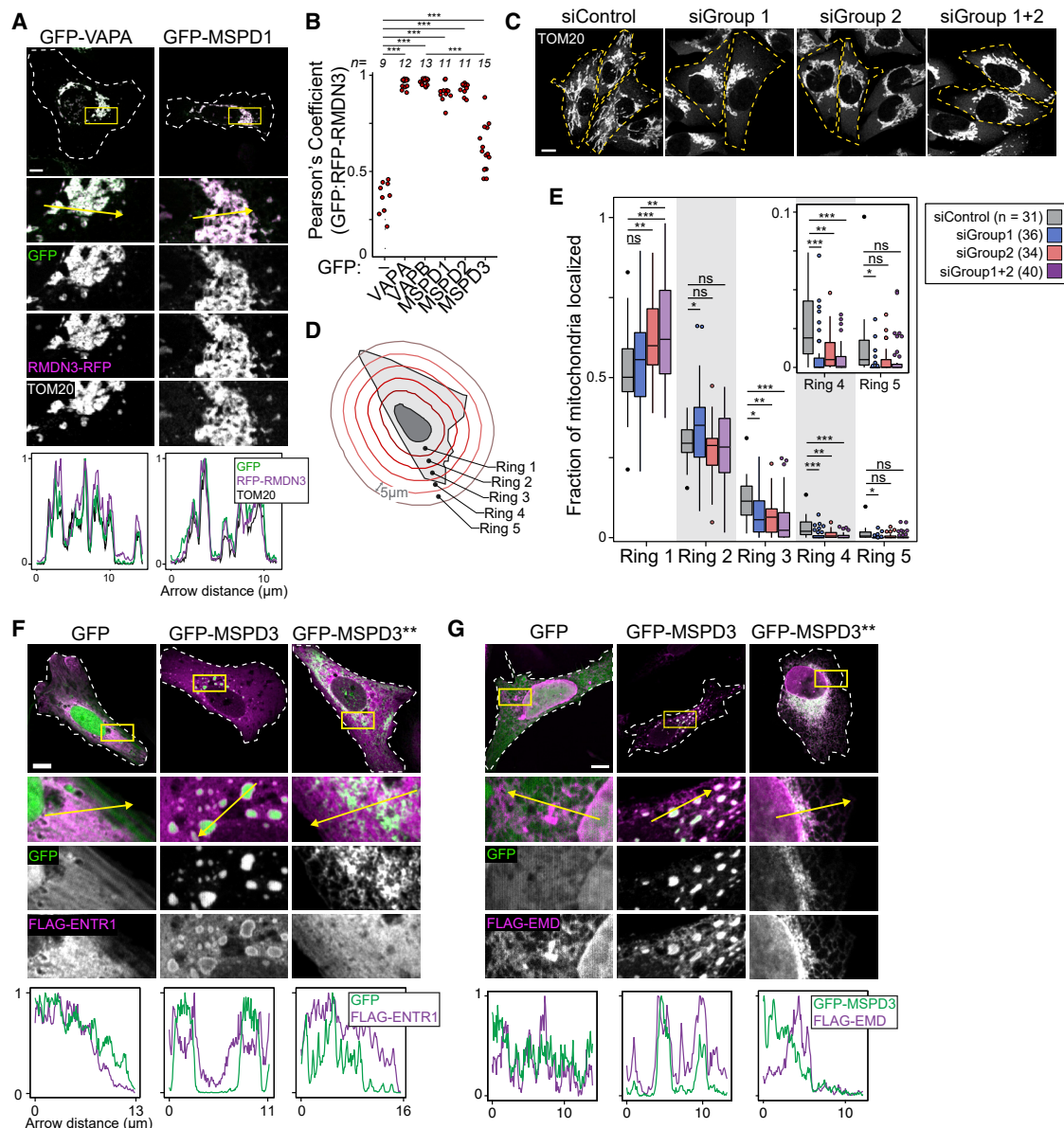


Figure 6. MSPD1 and MSPD3 Recruit Proteins to the ER Membrane

(A) Confocal images of U2OS cells expressing RMDN3-RFP with GFP-tagged VAPA and MSPD1. Full panel is depicted in Figure S6A.

(B) Quantification of VAP protein co-localizations with RMDN3-RFP from three independent experiments.

(C) Immunofluorescence images of mitochondria after siRNA depletion of group 1 or group 2 VAP proteins in MeJuSo cells.

(D) Work model of ShAMi analysis: the cell analyzed is divided into five concentric circles around nucleus and the fraction of signal corresponding to each “ring” is calculated.

(E) Peripheral distribution quantification of mitochondria after depletion of VAP groups using ShAMi analysis. Note that depletion of either VAP group increases the fraction of mitochondria in ring 1 and decreases it in rings 3–5. Moreover, depletion of both groups advances this phenotype in rings 1 and 3. Data are from two independent experiments.

(F) U2OS cells co-expressing FLAG-ENTR1 with GFP, GFP-MSPD3, or GFP-MSPD3**. Full panel is depicted in Figure S6D.

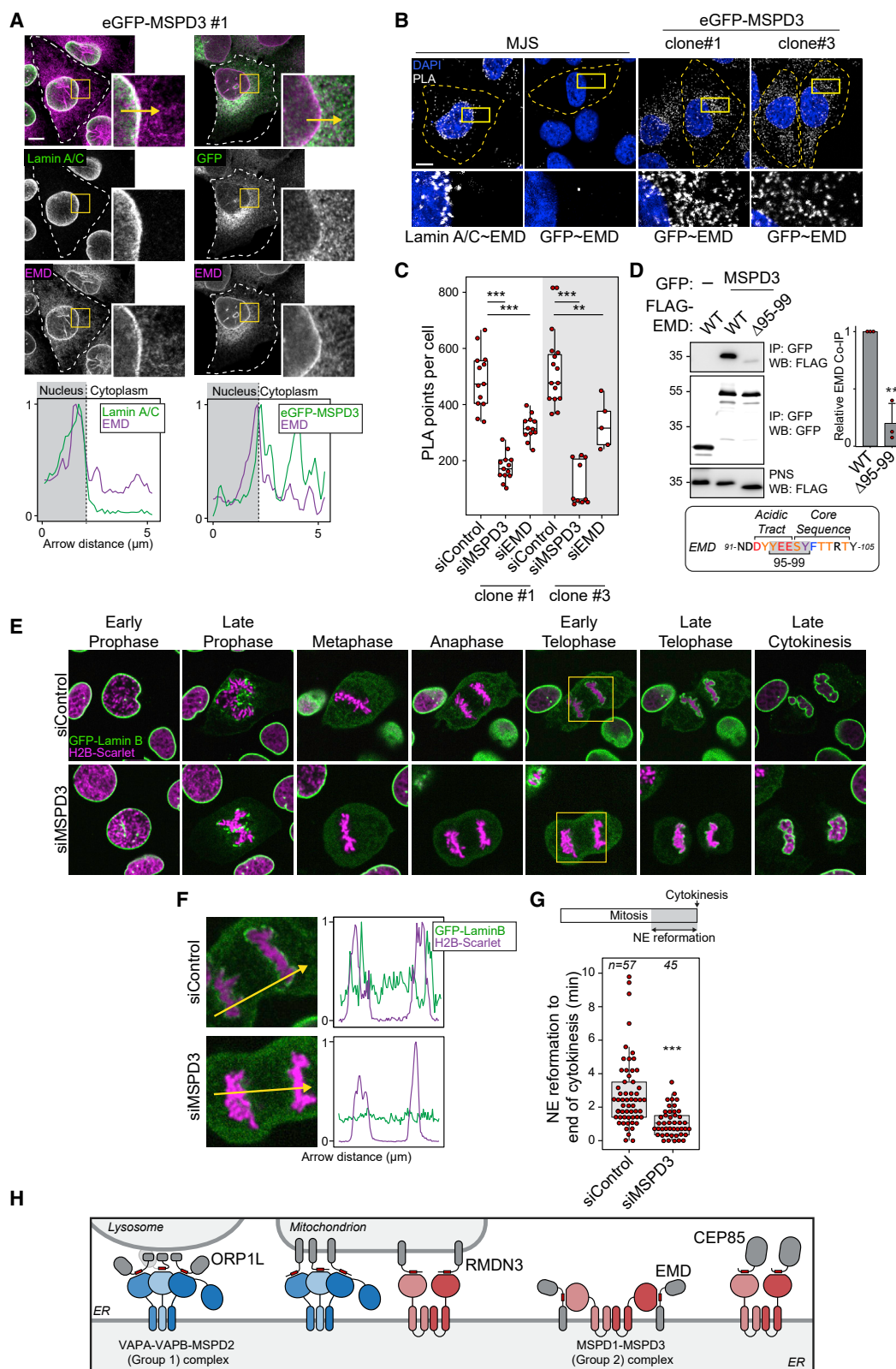
(G) Confocal images of U2OS cells co-expressing FLAG-EMD with GFP, GFP-MSPD3, or GFP-MSPD3**. Full panel is depicted in Figure S6F.

All scale bars are 10 μ m.

(Figure 7B). Collectively, our findings highlight that MSPD3-EMD interaction displays a different subcellular localization than that of EMD-Lamin A/C and suggest that MSPD3 and EMD mainly interact with each other in the ER.

MSPD3 Depletion Delays NE Reformation after Mitosis

Numerous mutations affecting EMD protein are affiliated with type 1 Emery-Dreifuss muscular dystrophy (EDMD) (Berk et al., 2013). Patients with EDMD display joint deformities, progressive



(legend on next page)

muscle weakness, and heart abnormalities (Storey et al., 2020). One of the reported mutations causing EDMD, deletion of residues from 95 to 99, removes five residues involving the FFNT motif (Ellis et al., 1998; Yates et al., 1999). Therefore, we wondered whether this mutation also interferes with MSPD3-EMD interaction. We found that the deletion of residues 95–99 diminished the interaction between EMD and MSPD3 significantly (Figure 7D). Interestingly, the same deletion also blocks the ability of EMD to interact with Lamin A, actin, and HDAC3 (Berk et al., 2013). This suggested that MSPD3 might play a role in NE dynamics.

Two confirmed high-affinity interaction partners of MSPD3, EMD and ANKLE2, belong to LEM family of INM proteins. The observations that MSPD3 interacts with two LEM proteins involved in post-mitotic NE assembly led us to the notion that MSPD3 takes part in NE dynamics during mitosis (Asencio et al., 2012; Barton et al., 2015; Haraguchi et al., 2001). To monitor NE dynamics in mitosis, we endogenously tagged Lamin B1 and the histone H2B with GFP and mScarlet using CRISPR, respectively (Figure S7D) (Qiao et al., 2020). Using live-cell imaging, we observed that the post-mitotic NE reformation is delayed in MSPD3-depleted cells (Figure 7E; Video S1). This event often takes place at the early stages of telophase and could be readily visualized by the concentration of Lamin B signal around the newly separated sister chromatids (Figure 7E). In MSPD3-depleted cells, this event was significantly delayed and occurred around late telophase or even late stages of cytokinesis (Figures 7F and S7E). We quantified this delay by measuring the time between the initial positioning of GFP-Lamin B around chromatids and the completion of cytokinesis. We found that MSPD3 depletion consistently delayed NE reformation (i.e., reducing the time between the initiation of NE reformation and the completion of cytokinesis) (Figure 7G). In contrast, the NE breakdown in prophase or the integrity of the NE membrane in interphase was not affected (Figures 7E and S7F). Collectively, our results imply that MSPD3 is involved in the timing of post-mitotic NE reassembly.

DISCUSSION

MCS are the emerging focus of interorganellar communications, as they participate in lipid trafficking, organelle positioning, organelle fission, and many other biological processes (Cabukusta and Neeffjes, 2018; Holthuis and Menon, 2014; Neeffjes et al., 2017). The ER reaches to the farthest corners of the cell and can thus make extensive contacts with other organelles. In

this context, VAPA and VAPB play an important role as they facilitate a multitude of MCS. VAPA and VAPB interact with proteins containing FFAT motifs for their ER recruitment to create MCS. In addition to VAPA and VAPB, MSPD2 is recently described to interact with a subset of FFAT motif-containing proteins known to interact with VAPA/VAPB (Di Mattia et al., 2018). As the human genome encodes two other MSP domain-containing proteins, MOSPD1 and MOSPD3, we investigated the ability of these proteins to interact with FFAT motifs. As a result, we identified MSPD1 and MSPD3 as two scaffolds interacting with proteins containing FFNT motifs.

To better understand the nature of the five different VAP family proteins and the diversity of their interactomes, we identified their unique and shared interaction partners. It is suggested that domain-motif interactions have lower affinity than domain-domain interactions, and co-purification methods can be too harsh to identify interactions between proteins and their low-affinity interactors (Kim et al., 2014). Therefore, we used the BioID method, which allows the identification of transient and low-affinity interactions. With this approach, we expanded the current human VAPome dataset. In this dataset, we found known interactors of VAPA and VAPB, identified proteins with low and high FFAT scores possibly interacting with VAPA/VAPB/MSPD2 and characterized interaction partners for MSPD1 and MSPD3.

We found that MSPD1/MSPD3 does not interact with canonical FFAT motifs, but motifs related to FFAT and still mostly predictable with the available FFAT motif search algorithm (Slee and Levine, 2019). We classify these short linear motifs preferred by MSPD1/MSPD3 as FFNT motifs. The motif preferences of VAP proteins allowed us to propose a grouping present within VAP family of proteins. This grouping is also reflected to their ability to form ER-resident VAP hetero-complexes. Accordingly, VAPA-VAPB-MSPD2 complexes interact with FFAT motifs, whereas MSPD1-MSPD3 complexes interact with FFNT motifs.

On the basis of the motifs facilitated interactions with MSPD1/MSPD3, we developed a PWM to score FFNT motifs. Characteristically, the FFAT motifs contains an N-terminally adjacent acidic tract. One exception to this is STARD3, which does not contain an adjacent acidic tract but contains two acidic patches further up- and downstream of the core (Alpy et al., 2013). Interestingly, in our FFNT motif alignments, we noticed that the residues corresponding to the acidic tract were poorly conserved. Moreover, no region further up- or downstream of the core was significantly conserved. In this PWM, the similarities to the canonical FFAT motifs are penalized by higher scores, as the FFAT motifs of OSBP and CERT scored 7 and 7.5, respectively.

Figure 7. MSPD3 Interacts with EMD in the ER and Is Critical for the Timing of NE Reformation after Mitosis

- (A) Immunofluorescence image of endoGFP-MSPD3 (eGFP-MSPD3) cells using EMD, Lamin A/C, and GFP antibodies. Note that the peaks of endogenous EMD and GFP-MSPD3 do not overlap. Scale bar is 10 μ m.
- (B) Proximity ligation assay of MSPD3-EMD and EMD-Lamin A/C interactions in MeJuSo (MJS) and endoGFP-MSPD3 MeJuSo (eGFP-MSPD3) cells. Scale bar is 10 μ m.
- (C) Quantification of PLA points in endoGFP-MSPD3 cells following the depletion of MSPD3 or EMD. See also Figure S7C.
- (D) coIP of GFP-MSPD3 with the EDMD-causing EMD Δ 95-99 mutant. Error bars indicate standard deviations.
- (E) Still images from live-cell imaging of U2OS/GFP-LaminB+H2B-mScarlet cells treated with control or MSPD3 siRNA. Also see Video S1.
- (F) Zoomed-in images of early telophase cells from (E). Note that MSPD3 depletion delays recruitment of Lamin B to newly separated sister chromatids.
- (G) Quantification of NE reformation delay in MSPD3-depleted cells. Time between the initial positioning of GFP-Lamin B around the chromatids and the completion of cytokinesis is quantified. Data are from three independent experiments on two independent clones of U2OS/GFP-LaminB+H2B-mScarlet cells.
- (H) Model of VAP-mediated ER recruitment of proteins from different compartments.

Furthermore, the FFAT motifs of EMD and RMDN3, which interact with all VAPs albeit with different for each VAP group, present comparable FFAT and FFNT scores. Overall, the presence of proteins interacting with both groups of VAPs hints at an elasticity between two groups of VAP proteins.

We also noted a sub-segregation among group 1 VAP proteins. In our study, MSPD2 poorly interacted with OSBP, CERT, and RMDN3 and better with STARD3 and ORP1L. Consistent with this, the MSPD2 interactome diverged somewhat from the VAPA/VAPB interactome. Similarly, MSPD3 in general displayed better affinity toward FFNT motifs than MSPD1. This sub-division of group 1 and group 2 proteins further underscores the complexity of FFAT motif interactions among all VAP proteins and implies a sub-specialization of the different VAPs.

It remains unclear what determines the differences in FFAT motif selectivity by MSP domains. As crystal and nuclear magnetic resonance (NMR) structures of VAPA in complex with ORP1L- and OSBP-FFAT motifs visualized the fundamentals of FFAT motif binding, they provide a possible explanation (Furuita et al., 2010; Kaiser et al., 2005). The acidic elements of the canonical FFAT motif electrostatically interact with the positive surface created collectively by K43, K45, R55, K85, K87, K118, and R120 residues of VAPA/VAPB. Of these seven positions, only two are positively charged in MSPD1 and MSPD3, which partially clarifies the lack of an acidic tract in FFNT consensus sequence. Furthermore, residues that create the hydrophobic grooves of VAPA accommodating the position 2 phenylalanine (K45, T47, K87, M89) and the position 5 alanine (V44, T46, V54, and N57) are also not well conserved in MSPD1/MSPD3. Collectively, the lack of a positively charged surface in conjunction with a poorly conserved hydrophobic groove might explain why MSPD1 and MSPD3 prefer less canonical motifs.

In addition to interacting with FFAT and FFNT motifs, all VAP proteins could recruit proteins to the ER membrane and form MCS between the ER and mitochondria. We found that each VAP group is necessary for the peripheral distribution of mitochondria in MeJuSo cells, possibly by contributing to the contact sites between the ER and mitochondria. We also observed that MSPD3 is critical for the correct timing for post-mitotic NE reformation and the silencing of MSPD3 delays the process of Lamin B condensation around freshly separated chromatids. Among the proteins interacting with MSPD3, the LEM family proteins EMD and ANKLE2 take part in post-mitotic NE reassembly (Asencio et al., 2012; Barton et al., 2015; Haraguchi et al., 2001). Both proteins are involved in the phosphorylation cycle of the chromatin-interacting INM protein BAF. In interphase, soluble BAF protein bridges chromatin to EMD and nuclear lamina proteins (Wagner and Krohne, 2007). Phosphorylation of BAF in early mitosis breaks the interaction among chromatin, BAF, and EMD, facilitating the NE breakdown (Nichols et al., 2006). At mitotic exit, BAF dephosphorylation, coordinated by ANKLE2, allows the interaction of BAF with chromatin and EMD, followed by nuclear lamina proteins. Consequently, both ANKLE2 and EMD are critical for NE reassembly (Asencio et al., 2012; Haraguchi et al., 2001). On the basis of our observations, it is tempting to speculate that MSPD3 is involved in recruiting ANKLE2 or EMD to the site of BAF to facilitate NE reformation.

Although VAPA, VAPB, and FFAT motifs are conserved from yeast to human, MSPD1, MSPD3, and FFNT motifs in interaction partners are found only in higher eukaryotes (i.e., they are not present in yeast). It remains puzzling why the human genome encodes five VAP proteins. Whereas VAPA and VAPB are virtually identical in their FFAT motif selectivity, MSPD1/MSPD2/MSPD3, on the other hand, display some variation in their selectivity toward FFAT and FFNT motifs. Furthermore, *de facto* contribution of each VAP to different MCS still remains to be addressed, as most studies involving VAPs are performed under overexpression conditions. In general, further work is required to understand the biological role of individual VAP proteins.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.108475>.

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AUTHOR CONTRIBUTIONS

B.C. designed the study and performed the majority of experiments with critical input from J.N. R.H.M.W., M.S., and I.F. provided technical support. I.B. performed live-cell experiments. J.J.L.L.A. created endogenously tagged cell lines. D.M.v.E. carried out EM studies with the support of A.W.M.d.J.

G.M.C.J. and P.A.v.V. performed proteomics mass spectrometry analysis. B.C. wrote the manuscript with input from J.N.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TOM20	Proteintech	Cat#11802-1-AP; RRID: AB_2207530
TOM20	Abcam	Cat#ab56783; RRID: AB_945896
Calnexin (C5C9)	Cell Signaling	Cat#2679; RRID: AB_2228381
Lamin A/C (H-110)	Santa Cruz	Cat#sc-376248; RRID: AB_10991536
Lamin A/C (4C11)	Cell Signaling	Cat#4777; RRID: AB_10545756
Emerin (D3B9G)	Cell Signaling	Cat#30853; RRID: AB_10545756
Emerin (H-12)	Santa Cruz	Cat#sc-25284; RRID: AB_2100051
GFP	ChromoTek	Cat#PABG1; RRID: AB_2749857
FLAG (M2)	Sigma-Aldrich	Cat#F3165; RRID: AB_259529
Anti-Rabbit IgG, Alexa Fluor 647	Thermo Fisher	Cat#A31573; RRID: AB_2536183
Anti-Mouse IgG, Alexa Fluor 647	Thermo Fisher	Cat#A31571; RRID: AB_162542
Anti-Mouse IgG, Alexa Fluor 405	Abcam	Cat#ab175660
GFP	Rocha et al., 2009	N/A
V5	Invitrogen	Cat#46-0705; RRID: AB_2556564
Tubulin	Sigma-Aldrich	Cat#T9026; RRID: AB_477593
Actin	Sigma-Aldrich	Cat# A5441; RRID: AB_476744
FLAG-HRP	Sigma-Aldrich	Cat#A8592; RRID: AB_439702
RFP (6G6)	ChromoTek	Cat#6G6-100; RRID: AB_2631395
ENTR1	Proteintech	Cat#15969-1-AP; RRID:AB_2183415
CEP85	Proteintech	Cat#26314-1-AP
Lamin B1	Proteintech	Cat#12987-1-AP; RRID: AB_2136290
HA (3F10)-HRP	Roche	Cat#12013819001; RRID: AB_390917
Anti-Rabbit, IRDye 800 CW	LI-COR	Cat#926-32211; RRID: AB_621843
Anti-Rabbit, IgG, HRP	Thermo Fisher	Cat#G-21234; RRID: AB_1500696
Anti-Mouse IgG, HRP	Thermo Fisher	Cat#G-21040; RRID: AB_2536527
Anti-GFP, biotin	Rockland	Cat#600-106-215; RRID: AB_218204
Anti-biotin	Rockland	Cat#100-4198; RRID: AB_219938
Chemicals, Peptides, and Recombinant Proteins		
X-treme GENE HP DNA Transfection Reagent	Roche	Cat#6366244001
Digitonin	Cayman Chemical	Cat#14952
cOmplete Protease Inhibitor Cocktail	Roche	Cat#11697498001
GFP-Trap agarose beads	Chromotek	Cat#gta-10
SuperSignal West Dura Extended Duration Substrate	ThermoFisher	Cat#34076
D-Biotin	Sigma-Aldrich	Cat#B4501
Pierce High Capacity NeutrAvidin Agarose	ThermoFisher	Cat#29204
DharmaFECT-1	Dharmacon	Cat#T-2001-03
Critical Commercial Assays		
Duolink <i>In Situ</i> Red Starter Kit Mouse/Rabbit	Sigma-Aldrich	Cat#DUO92101-1KT
SilverQuest Silver Staining Kit	ThermoFisher	Cat#LC6070
Isolate II RNA Mini Kit	Bioline	Cat#BIO-52073
cDNA Synthesis Kit	Roche	Cat#05081963001

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
SensiFAST SYBR® No-ROX Kit	Bioline	Cat#BIO-98020
Deposited Data		
Extended human VAPome	This study	N/A
List of FFNT motifs in human proteome	This study	N/A
Experimental Models: Cell Lines		
U2OS	ATCC	HTB-96
HEK293T	ATCC	CRL-3216
HeLa	ATCC	CCL-2
MelJuSo	Jongsma et al., 2020	N/A
U2OS / H2B-mScarlet	Qiao et al., 2020	N/A
U-118	This study	N/A
SK-MEL-28	This study	N/A
U-87	This study	N/A
A549	This study	N/A
THP-1	This study	N/A
SK-BR-3	This study	N/A
PC-3	This study	N/A
HCT-116	This study	N/A
DU-145	This study	N/A
BxPC-3	This study	N/A
BT-474	This study	N/A
MelJuSo/ endoGFP-MSPD3	This study	N/A
U2OS/H2B-Scarlet + GFP-LaminB1	This study	N/A
Oligonucleotides		
siVAPA	Dharmacon	MQ-021382-01-0002
siVAPB	Dharmacon	MQ-017795-00-0002
siMOSPD1	Dharmacon	MQ-020537-00-0002
siMOSPD2	Dharmacon	MQ-017039-01-0002
siMOSPD3	Dharmacon	MQ-016132-02-0002
siEMD	Dharmacon	M-011025-01-0005
siCTRL	Dharmacon	D-001206-13-20
VAPA-RT-PCR-F	Integrated DNA Tech.	N/A
5'-AGATCCTGGTCTCGATCCG-3'		
VAPA-RT-PCR-R	Integrated DNA Tech.	N/A
5'-TTTTCTATCCGATGGATTTCGCA-3'		
VAPB-RT-PCR-F	Integrated DNA Tech.	N/A
5'-AGATGGACTGCGGATGAGGAA-3'		
VAPB-RT-PCR-R	Integrated DNA Tech.	N/A
5'-CAGTTGGGGCTAATGCTGAAA-3'		
MSPD1-RT-PCR-F	Integrated DNA Tech.	N/A
5'-TGTGATTCGTCATCGAGATGTTTC-3'		
MSPD1-RT-PCR-R	Integrated DNA Tech.	N/A
5'-CTTTGCTCGGAACTTGGAGA-3'		
MSPD2-RT-PCR-F	Integrated DNA Tech.	N/A
5'-ATGCTCGATGAGAGTTTTTCAGTG-3'		
MSPD2-RT-PCR-R	Integrated DNA Tech.	N/A
5'-CCAACCAGAATGCTATGAGCTTC-3'		

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
MSPD3-RTPCR-F	Integrated DNA Tech.	N/A
5'-CCCCGGATCTAGTATTTCAGGG-3'		
MSPD3-RTPCR-R	Integrated DNA Tech.	N/A
5'-CAAACACCGTGTATTTGGCAG-3'		
GAPDH-RTPCR-F	Integrated DNA Tech.	N/A
5'-TGTTGCCATCAATGACCCCTT-3'		
GAPDH-RTPCR-R	Integrated DNA Tech.	N/A
5'-CTCCACGACGTACTCAGCG-3'		
Recombinant DNA		
GFP-BirA-C1	Sapmaz et al., 2019	N/A
HA-VAPA	Wijdeven et al., 2016	N/A
HA-ORP1L	Wijdeven et al., 2016	N/A
STARD3-RFP	Alpy et al., 2013	N/A
mCherry-VAPB	Zewe et al., 2018	Addgene plasmid # 108126
GFP-EMD	Zuleger et al., 2011	Addgene plasmid # 61993
Myc-ESYT2	Giordano et al., 2013	Addgene plasmid # 66834
GFP-SEC61B	Ma and Mayr, 2018	Addgene plasmid # 121159
pX330-U6-Chimeric BB-CBh-hSpCas9	Cong et al., 2013	Addgene plasmid # 42230
MOSPD1 cDNA	Source Bioscience	IMAGE: 3450155; GenBank: BC005700.1
MOSPD2 cDNA	Source Bioscience	IMAGE: 4821861; Genbank: BC030641.1
MOSPD3 cDNA	Source Bioscience	IMAGE:3689026; Genbank: BC005042.1
OSBP cDNA	Source Bioscience	IMAGE: 4560111; GenBank: BC011581.1
FNDC3A cDNA	Source Bioscience	IMAGE: 9052821; GenBank: BC144301.1
CEP85 cDNA	Source Bioscience	IMAGE: 5585742; GenBank: BC064528.1
RMDN3 cDNA	Source Bioscience	IMAGE:6527521; GenBank: BC063844.1
ANKLE2 cDNA	ORFeome collaboration library	GenBank: NM_015114.1
CERT cDNA	ORFeome collaboration library	GenBank: NM_005713.3
ENTR1 cDNA	ORFeome collaboration library	GenBank: NM_001039707.1
FLAG-C1	This study	N/A
RFP-C1	This study	N/A
GFP-MOSPD1	This study	N/A
GFP-MOSPD2	This study	N/A
GFP-MOSPD3	This study	N/A
GFP-VAPA	This study	N/A
GFP-VAPB	This study	N/A
BirA-VAPA	This study	N/A
BirA-VAPB	This study	N/A
BirA-MOSPD1	This study	N/A
BirA-MOSPD2	This study	N/A
BirA-MOSPD3	This study	N/A
FLAG-VAPA	This study	N/A
FLAG-VAPB	This study	N/A
FLAG-MOSPD1	This study	N/A
FLAG-MOSPD2	This study	N/A
FLAG-MOSPD3	This study	N/A
MOSPD3-FLAG	This study	N/A
MOSPD3 (R111D/R113D)-FLAG	This study	N/A
GFP-VAPA (221-249)	This study	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
GFP-VAPB (216-243)	This study	N/A
GFP-MOSPD1 (150-213)	This study	N/A
GFP-MOSPD2 (479-518)	This study	N/A
GFP-MOSPD3 (173-235)	This study	N/A
RFP-VAPA (221-249)	This study	N/A
RFP-VAPB (216-243)	This study	N/A
RFP-MOSPD1 (150-213)	This study	N/A
RFP-MOSPD2 (479-518)	This study	N/A
RFP-MOSPD3 (173-235)	This study	N/A
GFP-VAPA (K94D/M96D)	This study	N/A
GFP-VAPB (K87D/M89D)	This study	N/A
GFP-MOSPD1 (K94D/R96D)	This study	N/A
GFP-MOSPD2 (R404D/L406D)	This study	N/A
GFP-MOSPD3 (R111D/R113D)	This study	N/A
FLAG-OSBP	This study	N/A
FLAG-ESYT2	This study	N/A
FLAG-FNDC3A	This study	N/A
FLAG-CEP85	This study	N/A
FLAG-EMD	This study	N/A
RMDN3-RFP	This study	N/A
FLAG-ANKLE2	This study	N/A
FLAG-CERT	This study	N/A
FLAG-ENTR1	This study	N/A
FLAG-ANKLE2 (Y256A/F257A)	This study	N/A
FLAG-CEP85 (F71A/C72A)	This study	N/A
FLAG-ENTR1 (F159A/F160A)	This study	N/A
FLAG-EMD (Y99A/F100A)	This study	N/A
FLAG-EMD (Δ 95-99)	This study	N/A
LaminB1/pX330	This study	N/A
MSPD3/pX330	This study	N/A
LaminB1/endoGFP	This study	N/A
MSPD3/endoGFP	This study	N/A
Software and Algorithms		
FIJI	Schindelin et al., 2012	https://imagej.net/Fiji
Muscle Sequence Alignment Tool	Madeira et al., 2019	https://www.ebi.ac.uk/Tools/msa/muscle/
R Software (version 3.5.2 "Eggshell Igloo")	N/A	https://www.r-project.org/
UNIPROT	N/A	https://www.uniprot.org
TMHMM	Krogh et al., 2001	http://www.cbs.dtu.dk/services/TMHMM/
SAINT	Choi et al., 2011	http://saint-apms.sourceforge.net/Main.html
clusterProfiler	Yu et al., 2012	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
ReactomePA	Yu and He, 2016	http://bioconductor.org/packages/release/bioc/html/ReactomePA.html
FFAT Score Calculation PWM	Slee and Levine, 2019	N/A
ShAMi: Shell Associated Mitochondria analysis	This study	N/A
JalView	Waterhouse et al., 2009	N/A
PSSM	Krystkowiak et al., 2018	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
FFNT search	This study	N/A
Other		
Expression map of VAP proteins	Cardoso-Moreira et al., 2019	N/A

RESOURCE AVAILABILITY

Lead contact

Further information or requests should be directed to Jacques Neefjes (j.j.c.neefjes@lumc.nl)

Materials availability

All materials are available without restrictions and requests for unique resources should be directed to the lead contact.

Data and code availability

The published article includes datasets generated in this study, including those in [Tables S1](#) and [S2](#). R code for searching FFNT motifs is available at <https://github.com/birolcabukusta/vapome/>

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and transfections

U2OS, HEK293T, U-87, U-118, SK-MEL-28, HeLa, A549 in DMEM, THP-1, SK-BR-3, PC-3, HCT-116, DU-145, BxPC-3 in RPMI, Mel-JuSo in IMDM, and BT-474 in DMEM/F12, all supplemented with 8% fetal calf serum.

For plasmid transfections, HEK cells were transfected with 3 μ g polyethylenimine (PEI) per μ g plasmid DNA. Briefly, separate DMEM solutions containing PEI and plasmid DNA were mixed, incubated 30 min at RT and added to cells. MJS and U2OS cells were transfected using X-treme GENE HP DNA Transfection Reagent.

For reverse siRNA transfections, 25 pmol siRNA oligos were mixed with 1 μ L DharmaFECT-1 in DMEM and incubated at RT for 30 min while constant shaking. Next, cells were added to transfection mixtures to bring final siRNA concentration to 50 nM and samples were processed 72 h after siRNA transfections.

Generation of endogenously tagged cell lines

Endogenous tagging of LaminB1 and MSPD3 were performed as described previously ([Qiao et al., 2020](#)). Briefly, U2OS/H2B-mScarlet cells were co-transfected with LaminB1/pX330 and LaminB1/endoGFP plasmids and MJS cells were co-transfected with MSPD3/pX330 and MSPD3/endoGFP plasmids. Transfected cells were single cell FACS sorted and validated by microscopy and western blotting.

LaminB1/pX330 plasmid was generated by cloning LaminB1 5' end-targeting guide sequence (CACGGGGGTGCGAGTCGCCA) into pX330-U6-Chimeric_BB-CBH-hSpCas9 plasmid. LaminB1/endoGFP plasmid was created by modifying GFP-C1 vector, where LaminB1 left homology arm (CACCTCGTCTTGATTTTCCCGCGTGCCTGTGTGAGTGGGTGTGTGTTTCTTACAAA GGGTATTTGCGATCGATCGATTGATTCGTAGTTCCTCCCGCGCGCTTTGCCCTTTGTGCTGTAATCGAGCTCCCGCCATCCC AGGTGCTTCTCCGTTCTCTAAACGCCAGCGTCTGGACGTGAGCGCAGGTGCGCGGTTTGTGCTTCGGTCCCGCTTCGCCCC CTGCGCTCCCTCTATCACGGTCCCGCTCGCGGCTCGCCGCCCGCTGTCTCCGCCGCCGCCATGG) was inserted using NdeI/NheI sites (replacing the CMV promoter) and the right homology arm (GCGACTGCGACCCCGTGCCGCCCGCGGATGGG CAGCCGCGCTGGCGGCCCCACCACGCCGCTGAGCCCCACGCGCTGTGCGGCTCCAGGAGAAGGAGGAGCTGCGCGAGCT CAATGACCGGCTGGCGGTGTACATCGACAAGGTGCGCAGCCTGGAGACGGAGAACAGCGCGCTGCAGCTGCAGGTGACGGAG CGCGAGGAGGTGCGCGGCCGTGAGCTCACCGGCCTCAAGGCGCTCTACGAGACCGAGCTGGCCGACGCGCGACGCGCGCTC GACGACACGGCCCCG) using BglII/Sall sites.

MSPD3/pX330 was generated by cloning MSPD3 5' end-targeting guide sequence (CAGAGCCTGCATGCGCCGTG) into pX330-U6-Chimeric_BB-CBH-hSpCas9 plasmid. MSPD3/endoGFP plasmid was created by modifying GFP-C1 vector, where left homology arm (GAAGCCGGGTTGGGAGCTGCGAGGGGCCGGTTTAGCGGGGTGGGCGTGCCGTGAGGTGGGGCTCGCGGGTCC GAAGGAACCTGGGGCAGAGCTGGAAGAGGAAAGGTCTTGGGGGCCCTGGGCTGGGTCTGCGGGAGCCCCATCTAGCGGGGT TCCAAGGCGGCGGCGGCATCAGTCGAGGCCCTGCTCCGGGAGCTCATCTTGTCCCTTTGCCCCCTACGCCCCCAGCTCTG ACTCCAGGCCCTGTCCCCTGAGCTCTGCCCTCGGATGTCCGACCGCCAGAGCCTGCATG) was inserted using NdeI/NheI restriction sites (replacing the CMV promoter) and right homology arm sequence (CGCCGTGGGGCGCCCCAGGACCG GAGCTGGTGGGTCCGGGGCCCCCTGGGCGGGGGTCCCGGGCGCCCCCTCTCCCTTGG- GACCCGTTGTCCCGTCTGCTTTTCCCCCGATCTAGTATTCAGGGCGGACCGAGCGGAGCCCGACAGCTGCTGACC CTCTATAACCCACAGGAAGTGCCTTCGCTTCGAGGTAAAGGGATCGGCGCCTCGTGGGGGCTGGTGGCCGACACTGGGG

GGTTGTGTTGGCTGGAGGAGTCAGATGGGTAGGGTTGCACGTCCAGCTTTGGTGGACTGGG) using EcoRI/BamHI restriction sites.

METHOD DETAILS

RNA isolation, cDNA synthesis, and qPCR

RNA isolation, cDNA synthesis and quantitative RT-PCR were performed according to the manufacturer's instructions. Obtained signal was normalized to GAPDH, and expression was calculated using the Pfaffl formula.

Immunofluorescence microscopy

Cells seeded on coverslips were washed with PBS and fixed with 3.7% (w/v) formaldehyde in PBS for 15 min at RT. Coverslips were washed twice with PBS and incubated with Permeabilization Buffer (PBS containing 0.1% (v/v) Triton X-100 and 0.2% (w/v) BSA) for 30 min at RT. Next, coverslips were incubated in primary and then secondary antibody solutions in permeabilization buffer, each 1 h at RT. Coverslips were washed and mounted. Samples were imaged using a Leica TCS SP8 microscope (Leica Microsystems, Wetzlar, Germany) equipped with 405/488/561/647 nm laser lines. Images were analyzed using ImageJ Software.

Distribution of mitochondrial network was analyzed using a custom-made ImageJ script, ShAMi: Shell Associated Mitochondria. Briefly, cell and nucleus boundaries are defined by the user. Next, the script draws five concentric circles 5 μ m apart and registers areas in between circles as ring regions and calculates the fraction of mitochondria affiliated mitochondria circle after thresholding. ShAMi ImageJ script is available upon request.

Live-cell imaging and FRAP

For mitosis analysis, siRNA-transfected U2OS/H2B-Scarlet+GFP-LaminB1 cells were imaged using Andor Dragonfly 200 confocal spinning disk on a Leica DMI8 microscope equipped with a 63x/1.40 HCX PL APO oil objective and a Zyla sCMOS camera using 525/50 and 620/60 bandpass filters.

For FRAP experiments, a Leica SP8 White Light Laser microscope equipped with a 63x/1.40 HCX PL APO oil objective was used. Both microscopes were equipped with a humidified climate control system for 37°C supplemented with 5% CO₂.

Membrane isolations

HEK293T cells were transfected with GFP-tagged VAPA, VAPB, MOSPD1, MOSPD2, and MOSPD3. Total membranes were isolated as in Vance (1991). Briefly, cells were washed twice with 0.25M sucrose and scraped in Isolation Medium (0.25M D-Mannitol, 0.5mM EGTA, 5mM HEPES at pH 7.4). Cells were passed 10-times through a 18G needle. Nuclei were separated by centrifugation at 600xg at 4°C for 10 min. Post-nuclear supernatant was subjected to 100,000xg at 4°C for 1 h to separate cytosol and membranes. Cytosol and resuspended membranes were subjected to SDS-PAGE and western blotting.

Co-immunoprecipitation

HEK cells were seeded on 6-cm culture dishes and transfected using PEI. The day after transfections, cells were harvested in ice-cold lysis buffer (0.5% (v/v) NP-40, 50mM Tris-HCl 8.0, 150mM NaCl, 5mM MgCl₂ and cOmplete Protease Inhibitor Cocktail). Lysates were incubated on ice for 15 min and centrifuged at 15,000xg for 10 min. Supernatants were incubated with GFP-Trap agarose beads for 90 min at 4°C. Next, samples were washed three times with lysis buffer and eluted in SDS sample buffer (10% glycerol, 1% SDS, 63.5mM Tris-HCl pH 6.8 and 0.001% (w/v) Bromophenol Blue).

Western blotting

Samples prepared in sample buffer were incubated at 95°C for 10 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked overnight with 5% (w/v) milk in PBST (PBS containing 0.05% (v/v) Tween 20), incubated with primary antibody solution in PBST, washed thrice with PBST, incubated with secondary antibody solution in PBST, washed thrice with PBST and twice with PBS before imaging. For HRP detection, membranes were incubated with the substrate and imaged using Amersham Imager 600 (GE Healthcare). Fluorescent secondary antibodies were detected using Odyssey CLx Imaging System (LI-COR). Western blots were quantified using ImageJ software and normalized first to bait then to highest co-immunoprecipitated sample.

Bio-ID and label-free mass spectrometry quantification

HEK293T cells were seeded on 10-cm culture dishes and transfected using PEI. The day after transfections, culture medium was replaced with fresh medium containing 100 μ M D-Biotin and further cultured for 4 h. Next, cells were washed twice with PBS and lysed in ice-cold lysis buffer. Lysates were incubated on ice for 15 min and centrifuged at 15,000xg for 10 min. Supernatants were added 0.5% (w/v) SDS to final concentration and incubated overnight with NeutrAvidin agarose beads at 4°C. Next, beads were washed three times with lysis buffer containing 0.5% (w/v) SDS and twice with lysis buffer containing 1% (w/v) SDS. Beads were dried and continued with mass spectrometry.

For MS analysis, triplicate protein samples from pull down experiments were incubated at 90°C with LDS sample buffer (Life Technologies). Proteins were run for 3 cm on a 4%–12% PAGE (NuPAGE Bis-Tris Precast Gel, Life Technologies) and stained with silver (SilverQuest Silver Stain, Life Technologies). The lane was cut into four parts, and subjected to reduction with dithiothreitol, alkylation with iodoacetamide and in-gel trypsin digestion using Proteineer DP digestion robot (Bruker). Tryptic peptides were extracted from the gel slices with 50/50/0.1 v/v/v water/acetonitrile/formic acid, lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitrile/formic acid and subsequently analyzed by on-line C18 nanoHPLC MS/MS with a system consisting of an Easy nLC 1200 gradient HPLC system (Thermo, Bremen, Germany), and an orbitrap Fusion LUMOS mass spectrometer (Thermo). Samples were injected onto a homemade precolumn (100 μ m $\times$ 15 mm; Reprosil-Pur C18-AQ 3 μ m, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (30 cm $\times$ 50 μ m; Reprosil-Pur C18-AQ 3 μ m). The gradient was run from 2% to 36% solvent B (20/80/0.1 water/acetonitrile/formic acid v/v) in 30 min. The nano-HPLC column was drawn to a tip of $\sim$ 5 μ m and acted as the electrospray needle of the MS source. The LUMOS mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 s, with a HCD collision energy at 32 V and recording of the MS2 spectrum in the orbitrap. In the master scan (MS1) the resolution was 120,000, the scan range 400–1500, at an AGC target of 400,000 @maximum fill time of 50 ms. Dynamic exclusion was set to $n = 1$ with exclusion duration of 10 s. Charge states 2–5 were included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2 Da. First mass was set to 110 Da. The MS2 scan resolution was 30,000 with an AGC target of 50,000 @maximum fill time of 60 ms.

In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.2 (Thermo Electron), and then submitted to the Uniprot *Homo sapiens* canonical database (67911 entries), using Mascot v. 2.2.07 (www.matrixscience.com) for protein identification. Mascot searches were with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Up to two missed cleavages were allowed. The following modifications were set for the searches: oxidation (on M) and acetylation (on protein N terminus) were set as a variable modification; carbamidomethyl (on C) was set as a fixed modification. Label-free protein abundance calculation and statistical analysis was performed using Proteome Discoverer software v2.2. A maximum FDR of 1% at both the peptide and the protein level was allowed. Raw data files from each lane were grouped. Normalization was on total peptide amount. The four bands from each lane and technical replicates were processed as merged, generating one list of identified proteins for each biological sample. The MS raw data have been uploaded to PRIDE with the submission nr PXD01547.

Protein sequence alignment

Alignment of MSP domains was performed with Muscle Sequence Alignment Tool using following sequences: human VAPA (UNIPROT: Q9P0L0), mouse VAPA (UNIPROT: Q9WV55), human VAPB (UNIPROT: Q95292), mouse VAPB (UNIPROT: Q9QY76), human MSPD1 (UNIPROT: Q9UJG1), mouse MSPD1 (UNIPROT: Q8VEL0), human MSPD2 (UNIPROT: Q8NHP6), mouse MSPD2 (UNIPROT: Q9CWP6), human MSPD3 (UNIPROT: O75425) and mouse MSPD3 (UNIPROT: Q8BGG6).

Immunogold CLEM

For immunogold CLEM, U2OS cells transfected as indicated were prepared for cryosectioning, as described ([van Elsland et al., 2016](#); [Peters et al., 2006](#)). Briefly, cells were fixed for 2 h in freshly prepared 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer. Fixed cells were scraped, embedded in 12% gelatine (type A, bloom 300, Sigma) and cut with a razor blade into 0.5 mm³ cubes. The sample blocks were infiltrated in phosphate buffer containing 2.3M sucrose. Sucrose-infiltrated sample blocks were mounted on aluminum pins and plunged in liquid nitrogen. The vitrified samples were stored under liquid nitrogen. Ultrathin cell sections of 75nm were obtained essentially as described elsewhere ([van Elsland et al., 2016](#)). Briefly, the frozen sample was mounted in a cryo-ultramicrotome (Leica). The sample was trimmed to yield a squared block with a front face of about 400 $\times$ 300 μ m (Diatome trimming tool). Using a diamond knife (Diatome) and antistatic devise (Leica) a ribbon of 75nm thick sections was produced that was retrieved from the cryo-chamber with the lift-up hinge method ([Bos et al., 2011](#)). A droplet of 2.3M sucrose was used for section retrieval. Obtained sections were transferred to a specimen grid previously coated with formvar and carbon.

The CLEM approach used was adapted from [Vidicini et al. \(2010\)](#). Grids containing thawed cryo-sections of fixed cells were incubated on the surface of 2% gelatine at 37°C. Subsequently sections were rinsed to remove the gelatine and sucrose, were blocked with 1% BSA in PBS, and then labeled with the indicated antibodies and 10 nm protein A-coated gold particles (CMC, Utrecht University). Next grids were labeled with DAPI (2 μ g/ml in PBS), washed with 50% glycerol and placed on a glass slide (pre-cleaned with 100% ethanol). Grids were then covered with a small drop of 50% glycerol, after which a coverslip was mounted over the grid. Coverslips were fixed using Scotch Pressure-Sensitive Tape. Samples were imaged with a Leica TCS SP8 confocal microscope (63x oil lens, N.A. = 1.4). After confocal microscopy grids were removed from the glass slide, rinsed in distilled water and embedded in 1.8% methylcellulose and 0.6% uranyl acetate. Electron microscopy imaging was performed with a Tecnai 20 transmission electron microscope (FEI) operated at 120 kV acceleration voltage. Correlation of confocal images and electron micrographs was performed in Adobe Photoshop CC 2018. In Adobe Photoshop CC 2018, the confocal image was copied as a layer into the electron micrograph and made 50% transparent. Transformation of the confocal image was necessary to match it to the larger scale of the electron micrograph. This was performed via isotropic scaling and rotation using interpolation settings; bicubic smoother. Alignment at low magnification was carried out with the aid of nuclear DAPI staining in combination with the shape of the fluorescently labeled cells. At high magnification, alignment was performed using the fiducial beads ([Kuipers et al., 2015](#)).

For conventional EM analysis cells transfected as indicated were fixed for 30 min with 1.5% (vol/vol) GA in 0.1 M cacodylate buffer (pH 7.4). After fixation, the samples were washed with 0.1 M cacodylate buffer. Next, the samples were treated with 1% (wt/vol) OsO₄.

at 4°C for 1 h, washed with 0.1 M cacodylate buffer and Milli-Q water, and stained at room temperature for 1 h with 1% (wt/vol) uranyl acetate in Milli-Q. Following a new washing step with Milli-Q water, the samples were dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, 100%), embedded in epoxy resin (LX-112, Ladd Research), and polymerized at 60°C. Sections were collected on mesh-100 copper EM grids covered with a carbon-coated Pioloform layer, and poststained with 7% (wt/vol) uranyl acetate and Reynold's lead citrate. Electron microscopy imaging was performed with a Tecnai 20 transmission electron microscope (FEI) operated at 120 kV acceleration voltage.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

All statistics are performed using Student's t test using R Software. p values are indicated by asterisks: * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.001$. All error bars indicate standard deviation.