Crystal structure of Mdm12 reveals the architecture and dynamic organization of the ERMES complex

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Abstract

The endoplasmic reticulum–mitochondria encounter structure (ERMES) is a protein complex that plays a tethering role in physically connecting ER and mitochondria membranes. The ERMES complex is composed of Mdm12, Mmm1, and Mdm34, which have a SMP domain in common, and Mdm10. Here, we report the crystal structure of *S. cerevisiae* Mdm12. The Mdm12 forms a dimeric SMP structure through domain swapping of the β 1-strand comprising residues 1–7. Biochemical experiments reveal a phospholipid-binding site located along a hydrophobic channel of the Mdm12 structure and that Mdm12 might have a binding preference for glycerophospholipids harboring a positively charged head group. Strikingly, both full-length Mdm12 and Mdm12 truncated to exclude the disordered region (residues 74–114) display the same organization in the asymmetric unit, although they crystallize as a tetramer and hexamer, respectively. Taken together, these studies provide a novel understanding of the overall organization of SMP domains in the ERMES complex, indicating that Mdm12 interacts with Mdm34 through head-to-head contact, and with Mmm1 through tail-to-tail contact of SMP domains.

Keywords crystal structure; ERMES complex; Mdm12; phospholipid binding; SMP domain

Subject Category Membrane & Intracellular Transport

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Introduction

Eukaryotic cells are composed of membrane-bound subcellular compartments that play distinct and essential roles for cell survival. The compartments not only work independently, but also they actively cooperate to achieve their ultimate roles. Apart from communication among subcompartments achieved through vesicular trafficking, direct contact sites of subcompartment membranes

have been discovered through electron microscopy (EM) [1–3]. Such membrane contact sites (MCSs) are involved in essential processes for cell survival, such as subcellular communications, ion homeostasis, metabolic pathways, and lipid biosynthesis [1–5].

Among several MCSs, ER–mitochondria direct contact sites have been extensively studied in terms of physical tethering of two membranes and their physiological relevancies, such as lipid trafficking and Ca^{2+} exchange [6–9]. The endoplasmic reticulum–mitochondria encounter structure (ERMES) components were first identified as molecular tethering factors in the formation of ER–mitochondrial junctions using synthetic biology screens in *S. cerevisiae* [10]. The ERMES complex consists of four proteins with different subcellular localizations. Mdm12 (mitochondrial distribution and morphology protein 12) is a soluble protein present in the cytosol, while Mmm1 (maintenance of mitochondrial morphology protein 1) and Mdm34/Mdm10 are integral membrane proteins that are anchored in the ER and mitochondrial outer membranes, respectively. Additionally, Gem1 (GTPase EF-hand protein of mitochondrial 1), a Ca^{2+} -binding Miro GTPase, associates with ERMES and regulates the number, size, and functions of these complexes in yeast [11,12]. In addition to its primary role in maintaining a close proximity (10–30 nm) between two membranes independently of fusion or fission, the ERMES complex also has been known to function in lipid trafficking to cooperatively synthesize phosphatidylcholine (PC) from phosphatidylserine (PS) in ER and mitochondria junctions [10,13–15]. However, there is a conflicting report that ERMES and Gem1 do not directly affect PS trafficking [16]. Recently, a couple of redundant pathways for lipid trafficking involved in the maintenance of mitochondrial lipid homeostasis have been reported. For example, the EMC (ER–membrane protein complex) located in the ER tethers a phosphatidylethanolamine (PE) to mitochondria by interacting with a TOM (translocase of the outer membrane) [17]. The vCLAMP (vacuole and mitochondria patch) is another alternative pathway for transferring lipids to the mitochondria [18,19]. Composite defects in these pathways result in severe disruption of mitochondrial lipid homeostasis. In addition to lipid trafficking, the Mdm12–Mmm1 complex plays an important role in β -barrel assembly of mitochondrial outer membrane proteins, and

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in the maintenance of mitochondrial morphology and mtDNA [6,20]. Furthermore, the ERMES complex has been repeatedly implicated in essential activities for cell survival such as mitophagy, inheritance, mtDNA inheritance, and mitochondrial dynamics [12,21–25].

Primary structure analyses of ERMES components reveal that Mdm12, Mmm1, and Mdm34 share a synaptotagmin-like mitochondrial-lipid-binding protein (SMP) domain, although their sequences are not closely related to each other [13]. In particular, full-length Mdm12 contains SMP domains across its entire sequence, while SMP domains in Mmm1 and Mdm34 account for half of the C-terminus and N-terminus, respectively. The remaining halves of the Mmm1 and Mdm34 protein sequences are predicted to be unstructured and not conserved among species, and the C-terminus of Mdm34 is known to be anchored into the outer mitochondrial membrane [26]. Structural studies demonstrated that the SMP domain adopts a dimer configuration rather than existing solely as a monomer [27–29]. The association of SMP domains might act as the driving force in the assembly of ERMES components and maintain intact membrane proximity. Biochemistry experiments combined with a negative-staining EM structure revealed that Mdm12–Mmm1 forms a hetero-tetramer through the direct association of SMP domains, generating an arch-shaped structure with dimensions of $\sim 210 \times 45 \times 35$ Å [30]. However, despite its importance in ER–mitochondria contact, no high-resolution structures of the ERMES complex are available. Therefore, the molecular details of how the SMP domains in the ERMES complex are organized to tether two organelles, and how ERMES recognizes certain lipids and facilitates their trafficking, remain unknown.

In this study, we determined the crystal structures of full-length Mdm12 and Δ Mdm12 ($\Delta 74$ –114) and elucidated the molecular details of the contact regions for self-association of SMP domains and of lipid coordination in Mdm12. Furthermore, we suggest that two interfaces between SMP domains, head-to-head and tail-to-tail, provide a mechanistic understanding of the assembly and organization of the ERMES tetrameric complex at a molecular level.

Results

The oligomeric state of full-length Mdm12 and Mmm1

We prepared the Mdm12 protein from *S. cerevisiae* by expression in *E. coli* bacterial cells. Interestingly, the *S. cerevisiae* Mdm12 migrated differently on size-exclusion columns, depending on the presence or absence of N-terminus hexa-histidine (His₆) tag plus TEV cleavage site (ENLYFQS) for full-length Mdm12 proteins. Full-length Mdm12 without His₆ eluted from the column at a volume corresponding to approximately the mass of the Mdm12 dimer. On the other hand, His₆-Mdm12 eluted from the column at a mass corresponding to the Mdm12 monomer (Fig 1A and B). The TEV cleavage site existing between His₆ tag and Mdm12 was not vulnerable to proteases, suggesting that the N-terminus including the TEV cleavage site of Mdm12 was somehow masked by the protein itself. To further investigate the oligomeric state of Mdm12 and measure the molecular weights in solution, we conducted analytical ultracentrifugation with native Mdm12 and His₆-Mdm12 proteins. Consistent with gel-filtration chromatography, Mdm12 and His₆-Mdm12 were

measured as 58.3 kDa (dimer) and 34.5 kDa (monomer), respectively (Figs 1C and EV1). From this observation, we propose that the N-terminus of Mdm12 could be critically involved in self-association and that the extra amino acid sequences consisting of the His₆ tag and TEV cleavage sequence might disturb the dimerization of the protein.

Mmm1 from *S. cerevisiae* was eluted in the void volume fraction during gel-filtration column chromatography, indicating that by itself Mmm1 is aggregated in solution (Fig 1B). However, when we co-expressed Mmm1 with Mdm12 in BL21 (DE3) bacterial cells, the complex displayed a monodisperse profile on the gel-filtration column, with an estimated molecular weight of around 200 kDa, suggesting that the Mdm12–Mmm1 complex exists as a hetero-tetramer in solution. This result was confirmed by analytical ultracentrifugation (Fig 1C, M.W. 122.7 kDa) and is consistent with previous data [30].

Crystal structure determination for *S. cerevisiae* Mdm12

Full-length Mdm12 proteins from *S. cerevisiae* were crystallized under various conditions. The best crystals grew in a P2₁2₁2 space group and diffracted to 3.1 Å resolution at a synchrotron source. The initial electron density map was calculated to 3.5 Å resolution from Se-Met-derivatized crystals using a single-wavelength anomalous diffraction (SAD) experiment, and the structure was phase extended and refined to 3.1 Å resolution with native crystal with $R_{\text{work}}/R_{\text{free}}$ values of 21.2/26.5%. Statistics for data collection and refinement are presented in Table 1.

Overall structure of Mdm12

The molecular models of Mdm12 are presented in Fig 2A–D. As observed by size-exclusion chromatography, the full-length Mdm12 forms dimers in the crystals with the asymmetric unit containing two Mdm12 dimers (four Mdm12 monomers in total) related to twofold symmetry. The four Mdm12 molecules are almost identical with a RMSD of < 0.3 Å. The crystal structure reveals that the Mdm12 dimer adopts an elongated tubular structure with dimensions of $40 \text{ Å} \times 60 \text{ Å} \times 110 \text{ Å}$ (Fig 2A). The Mdm12 monomer consists of three structural elements: (i) β 1-dimerization center; (ii) β -barrel with incomplete and highly twisted β -strands and three α -helices, which are comparably organized as shown in most synaptotagmin (SMP) domain-containing proteins [27–29]; and (iii) proline rich region, which protrudes from the SMP domain from the middle of the last strand of the β -barrel (Figs 2B and EV2). The truncated cone-shaped structure of the Mdm12 monomer forms an extensive hydrophobic channel through the elongated cavity, which was reported to provide a binding channel for particular fatty acids (discussed below) in previous studies [27–29]. Two Mdm12 molecules are arranged in a twofold symmetry and associate with each other through domain swapping of the N-terminus β -strand (β 1) comprising residues 1–7 as detailed below. Overall, the Mdm12 dimer structure resembles that of members of the TULIP family such as E-SYT2 (extended synaptotagmin 2, RMSD: 5.71), CETP (cholesterol ester transfer protein, RMSD: 4.47), and BPI (bactericidal/permeability-increasing protein, RMSD: 4.26) despite the absence of any significant sequence similarity among them [27–29]. Notably, BPI and CETP exist as monomers containing two separate SMP

Figure 1. Mdm12 and Mmm1 organization.

A Schematic diagrams showing the domain structures of Mdm12 and Mmm1 used in this study.

B Size-exclusion chromatography (SEC) experiments of Mdm12, tMmm1, and the Mdm12–tMmm1 complex comparing the molecular size of these proteins in solution. The proteins indicated were injected into a Superdex 200 column (GE Healthcare) with a buffer containing 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 5 mM DTT. The standard molecular masses for the SEC experiments (top) are shown for relative molecular weight comparison (blue dextran, void; ferritin, 440 kDa; aldolase, 158 kDa; conalbumin, 75 kDa; ovalbumin, 44 kDa; and carbonic anhydrase, 29 kDa).

C Graph indicating the molecular weights of Mdm12, His₆–Mdm12, and the Mdm12–tMmm1 complex in solution as measured by analytical ultracentrifugation.

Table 1. Data collection and refinement statistics.

	Mdm12 native	Mdm12 Se-SAD	ΔMdm12 native
Dataset			
PDB accession #	5GYD		5GYK
X-ray source	Beamline 5C, PAL	Beamline 5C, PAL	Beamline 5C, PAL
Temperature (K)	100	100	100
Space group	P ₂ ₁ ₂ ₁ ₂	P ₂ ₁ ₂ ₁ ₂	P ₂ ₁ ₂ ₁ ₂
Cell parameters a, b, c (Å)	142.592, 219.073, 73.097	142.592, 219.017, 73.268	109.239, 148.243, 212.394
Data processing			
Wavelength (Å)	0.97933	0.97928	0.97957
Resolution (Å)	35.0–3.10 (3.15–3.10)	50.0–3.50 (3.55–3.50)	50.0–3.60 (3.66–3.60)
R _{merge} (%) ^a	11.0 (84.5)	14.3 (65.8)	14.2 (67.9)
CC1/2	0.995 (0.626)	0.994 (0.841)	0.995 (0.648)
I/σ	19.9 (2.22)	20.6 (3.98)	11.1 (2.21)
Completeness (%)	99.6 (100.0)	99.8 (100.0)	99.7 (100.0)
Redundancy	5.3 (5.3)	6.4 (6.6)	3.6 (3.7)
Measured reflections	221,431	190,622	146,540
Unique reflections	41,953	29,933	40,722
Refinement statistics			
Data range (Å)	35.0–3.10		50.0–3.60
Reflections	41909		40625
Non-hydrogen atoms	7409		10865
R.m.s. Δ bonds (Å) ^b	0.009		0.007
R.m.s. Δ angles (°) ^b	1.490		1.369
R-factor (%) ^c	21.2		22.5
R _{free} (%) ^{c,d}	26.5		26.7
Ramachandran plot, residues in			
Most favored regions (%)	91.9		88.3
Additional allowed regions (%)	7.6		10.2
Generously allowed regions (%)	0.5		1.5
Disallowed regions (%)	0		0

Highest resolution shell is shown in parenthesis.

^a $R_{\text{merge}} = 100 \times \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \langle I(h) \rangle$, where $I_i(h)$ is the i^{th} measurement and $\langle I(h) \rangle$ is the weighted mean of all measurements of $I(h)$ for Miller indices h .

^bRoot-mean-squared deviation (r.m.s. Δ) from target geometries.

^c $R\text{-factor} = 100 \times \sum |F_o - F_{\text{p(calc)}}| / \sum F_o$.

^d R_{free} was calculated with 5% of the data.

domains that show no significant sequence conservation between them.

No electron density was observed for residues 74–114 of Mdm12, suggesting that this region might be highly flexible. Furthermore, these residues are not conserved in several Mdm12 orthologs. We obtained another orthorhombic crystal from the construct excluding

the disordered region (Δ74–114, referred to as ΔMdm12 hereafter) in full-length Mdm12. The crystals of ΔMdm12 grew in a P₂₁₂₁₂ space group and diffracted to 3.6 Å resolution. The structure of ΔMdm12 was solved by molecular replacement using the full-length Mdm12 structure as the search model and refined to 3.6 Å resolution. ΔMdm12 also crystallized as a dimer, and the structures and twofold arrangement of Mdm12 and ΔMdm12 are almost identical with a RMSD of 0.5 Å.

The highly conserved β1-strand of Mdm12 forms the dimeric interface for self-association

The N-terminus of Mdm12 is highly conserved among Mdm12 orthologs (Fig 3A). In the Mdm12 dimer, residues 1–7 from one monomer fold into a β-strand that inserts itself between β1 and β2 from the second monomer, running antiparallel with β1 and parallel with β2 in the twofold center of the Mdm12 dimer. They are systematically associated with each other by forming a hydrogen bond network among main chains of the protein between β1 (residues 4–7) and β2 (residues 53–56) from counter molecules, and two β1 (residues 1–6) strands from two molecules (Fig 3A). The buried surface area caused by the dimerization of Mdm12 is around 1,400 Å². E-SYT2 makes a twofold dimerization interface between two separate SMP domains using a highly conserved helix (residues 167–180) located at the beginning of each SMP domain (Fig 3C). The dimeric interface of Mdm12 closely resembles the twofold-like interface of CETP and BPI involving two SMP domains, an interface consisting of the central β-sheets comprising six antiparallel β-strands (Fig 3C). However, it is a distinctive feature of Mdm12 that the dimer is formed through domain swapping of the central β-strand located between the two SMP domains.

To further investigate whether the role of the β1-strand in the dimerization of Mdm12 as observed in the crystal structure also applied to Mdm12 in solution, we generated a point mutant (I5P) aimed at disrupting the β1-strand structure. In the dimer, the main chain of I5 forms H-bonds with the main chain of M1 from the second Mdm12 molecule, and its side chain makes van der Waals interactions with the hydrophobic side chains of M1, F3, W7, and I54 in the second molecule. As expected, both gel-filtration and analytical ultracentrifugation experiments revealed that the I5P mutant could not form a homo-dimer (Fig 3B), supporting the critical involvement of the highly conserved β1-strand in Mdm12 homo-dimerization in solution.

The SMP domain of Mdm12 binds phospholipid

Initial electron density maps clearly displayed a lipid-like molecule inside the hydrophobic channel of the Mdm12 monomer (Fig 4A and B). We were unable to identify the bound phospholipid using only electron density maps because of (i) the mid-range resolution (~3.1 Å) of this structure and (ii) the disordered electron density corresponding to the head group of phospholipid. However, it was previously reported that the recombinant Mdm12 proteins expressed in bacteria bind PE (~80%) and PG (~15%) species [30]. Therefore, we inferred that the diacyl glycerophospholipid bound to Mdm12 might be a PE or PG. To identify the phospholipids present in the Mdm12 structure, we performed denaturing quantitative APCI-MS using purified Mdm12 expressed in *E. coli*. The major

Figure 2. Overall structure of Mdm12.

- A Ribbon diagram of the yeast Mdm12 dimer. The crystal structure of full-length Mdm12 was determined by SAD and refined with native data to 3.1 Å resolution. Lipids bound to Mdm12 are drawn with black stick models.
- B Schematic diagram indicating the secondary structure elements and their organization in Mdm12. Three structural elements of Mdm12 are highlighted in different colored boxes.
- C, D Surface representations of the Mdm12 dimer are shown in different orientations.

phospholipid bound to Mdm12 was observed to have an m/z of 704.5 (Fig EV3B), which identified the molecule as PE, consistent with a previous lipidomic analysis in which PE (33:1) with an m/z of 704.5 was the predominant phospholipid co-purified with Mdm12 expressed in bacteria [30]. We built a PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) into the diacyl-like ligand density (Fig 4A), and the lipid-bound Mdm12 structure was well refined with native diffraction data. Ligand positioning is almost identical among three Mdm12 molecules within the asymmetric unit, except in one Mdm12 molecule, where the hydrocarbon chain of lipid is displaced and the head region is disordered (Fig EV3A). This displacement might be the result of crystal packing because the hydrophobic cavity of this molecule was slightly shrunk through the formation of close contacts with the symmetry-related molecules in the crystal.

Based on our crystal structure, the head group of phospholipid is exposed into the solvent and makes no direct contacts with neighboring residues of Mdm12, indicating that Mdm12 might have no clear selectivity for specific phospholipids. However, the fatty acyl chain of PE was tightly coordinated by the hydrophobic side chains of neighboring amino acids including I20, F45, L47, L177, F179, F251, L256, I262, and L264 (Fig 4C). We tested the ability of phospholipids to bind directly to the SMP domain of Mdm12 *in vitro*.

We used the fluorescently labeled PE (7-nitro-benz-2-oxa-1,3-diazol-4-yl-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, referred to as NBD-PE) and full-length Mdm12 purified from *E. coli* to measure their binding, as previously described [29,30]. Mdm12 proteins incubated with NBD-PE were run onto native PAGE to remove unbound NBD-PE, and NBD-PE-bound Mdm12 was quantified with fluorescence detection. Figure 4D and E shows that Mdm12 binds NBD-PE in a concentration-dependent manner. Unexpectedly, while around half of the full-length Mdm12 appeared as a dimer (46% of total Mdm12), the other half ran as a monomer (54%) in the native PAGE, as compared with Mdm12 (I5P) that migrated only as a monomer. The monomer and dimer distribution of Mdm12 observed in native PAGE was not correlated with NBD-PE incorporation (Figs 4D and EV4B). More surprisingly, monomeric Mdm12 had a higher affinity for NBD-PE than did dimeric Mdm12. Interestingly, the I5P mutant showed the highest affinity for NBD-PE, suggesting that the N-terminal β 1-strand of Mdm12 might be involved in regulating lipid trafficking, including access. Indeed, the structure shows the lipid-binding region, including the head group, to be very close to the dimerization interface. The dimerization of Mdm12 could thus sterically occlude lipid access, and the perturbation of the β 1-strand by mutation therefore increased the affinity for NBD-PE (Fig EV4).

Figure 3. Dimer interface of Mdm12.

A Ribbon diagram showing the twofold dimerization interface of Mdm12. Oxygen and nitrogen atoms are shown in red and blue, respectively. The orange dotted lines indicate intermolecular hydrogen bonds between two protomers of Mdm12. The sequence alignment of yeast Mdm12 orthologs is shown to highlight the sequence conservation in the N-terminus β 1-strand. Ten orthologs are aligned from residues 1–11. Absolute and highly conserved residues are indicated in red and orange, respectively.

B The molecular weight of the Mdm12 (I5P) mutant was measured by size-exclusion chromatography (below) and ultracentrifugation (top) as in Fig 1B and C.

C Ribbon diagram showing the structures of the SMP domain in E-SYT2, CETP, and BPI for the comparison of dimeric interfaces among SMP domains. Note that CETP and BPI are not dimers but monomers containing two tandem SMP domains.

Next, to validate the lipid coordination shown in our Mdm12 structure, we generated a construct harboring mutations in L256 and I262, both mutated to tryptophan residues. Our rationale was that the bulky side chain of tryptophan introduced by these mutations would occupy the hydrophobic cavity and generate a steric hindrance for lipid interaction. Compared with wild-type Mdm12 (I5P), the affinities of I262W and the L256W/I262W double mutants for NBD-PE were reduced by twofold and fourfold, respectively (Fig 4F). In this experiment, the Mdm12

(I5P) mutant was used as a reference to compare the effects of I262W and L256W/I262W because it migrated as a monomer during native PAGE.

Mdm12 has a clear preference for positively charged phospholipids

We further investigated the structure to determine whether Mdm12 might have a preference for certain phospholipids under native

Figure 4.

Figure 4. Mdm12 binds lipid through the SMP domain.

- A Simulated annealing omit map (Fo-Fc, contoured at 1.5 σ) showing the molecule bound to Mdm12 (left). The final model for the bound PE is shown as in stick representation. The electron density (2Fo-Fc) calculated in the final model is shown with the stick model of PE in the right (3.1 Å resolution, contoured at 0.8 σ).
- B Surface representation of the Mdm12 dimer. Hydrophobic amino acids lining the Mdm12 channel are indicated by a blue mesh. Lipids built in Mdm12 are in space-filling representation.
- C Ribbon diagram showing lipid coordination by Mdm12. Mdm12 residues and lipid fatty acids are colored in green and yellow, respectively.
- D Mdm12 binds NBD-PE. Wild-type and monomeric (ISP mutant) Mdm12 were incubated with NBD-PE and separated from free NBD-PE in native PAGE. Coomassie staining (left) and fluorescent (right) detection indicates that Mdm12 directly interacts with NBD-PE *in vitro*.
- E Quantitative data showing binding affinities for NBD-PE by Mdm12. The binding affinities of Mdm12 (monomer/dimer shown in native PAGE and ISP mutant) for NBD-PE was measured with a NBD-PE concentration-dependent manner. All experiments were carried out three times, and the means $\pm$ SD are given.
- F Mdm12 mutants (L256W, I262W, and L256W/I262W double mutants) were incubated with NBD-PE and subjected to native PAGE. Because wild-type Mdm12 separates as both monomer and dimer on native PAGE, the purely monomeric form (ISP) of Mdm12 was used as the wild type for clarity. The graph in the right indicates the quantities measured in the experiments. The bar shows the relative amounts of the band ratio (fluorescence/Coomassie). Values represent the means and SD from three independent experiments.

Source data are available online for this figure.

conditions. Interestingly, the surface for Mdm12 in which the phospholipid head group is placed represents a negative charge according to analysis of the surface electrical potential [31] (Fig 5A). The negative electric potential comes from mainly the C-term dipole end of helix 3, the main chain carbonyl oxygen of the loop comprising residues 250–255, and negatively charged side chains from E65, E73, E255, and D265. An investigation using the ConSurf [32] server revealed that these residues of Mdm12 are highly conserved among different species. We propose that Mdm12 might have a higher affinity for phospholipids with positive charges, such as PC or PE, than for negatively charged lipids. This hypothesis is supported by previous biochemical studies, which show that Mdm12 has a higher affinity for PC and PE than for PA and PS [30]. To measure quantitatively and kinetically the natural lipid-binding ability of Mdm12, we carried out lipid replacement experiments as previously described [29,30]. NBD-PE-preloaded Mdm12 (GST-tagged at the C-terminus) was incubated with a series of phospholipids in a dose-dependent manner, and the amount of NBD-PE replaced by non-labeled phospholipids was estimated by measuring the decrease in fluorescence. Figure 5B shows that PC and PG have the highest affinity for Mdm12 among the phospholipids tested, consistent with a previous report in which PC was confirmed as a *bona fide* ligand of Mdm12 purified from yeast *in vitro* [30]. Interestingly, acidic phospholipids such as PA and PS were unable to replace NBD-PE, even when present at a high concentration (~0.25 mM). To investigate the involvement of negatively charged amino acids in phospholipid selection, we engineered the E255R mutant of Mdm12 and measured its binding affinity for PC. Interestingly, the binding affinity of the E255R mutant for PC was reduced by ~1.4-fold compared with the wild-type protein, which supports our suggestion that the negatively charged surface of Mdm12 underpins its preference for positively charged phospholipids (Fig 5B).

In E-SYT2, no apparent weighted surface charge for the hydrophilic head group of lipid is apparent (Fig EV4C), which is consistent with data demonstrating that E-SYT2 has no preference for specific phospholipids. However, while E-SYT2 might recruit other proteins for their lipid selectivity [29], no additional proteins have yet been suggested to provide lipid selectivity in the ERMES complex. Taken together, we propose that Mdm12 might have a preference for binding of positively charged phospholipids through its negatively charged surface.

Putative architecture of Mdm12–Mmm1 complex

In the crystal asymmetric unit, there are four Mdm12 molecules (Fig 6A). In addition to the N-terminus dimerization interface (referred to as “head”), two Mdm12 dimers make another twofold rotation arrangement through the highly conserved C-terminal helices (referred to as “tail”), resulting in an extended arch-shaped structure with a 200 Å long dimension. Here, two Mdm12 dimers self-associate through a tail-to-tail junction, burying a surface accessible area of around 765.8 Å² (Fig 6C). We initially considered that the tail-to-tail junction of the SMP domain shown in the Mdm12 structure might represent a crystal contact, not a biological one, based on previous biochemical experiments demonstrating that Mdm12 forms a homo-dimer in solution. The structure of Δ Mdm12 lacking residues 74–114 has the same arrangements of the molecules in the asymmetric unit, although Δ Mdm12 crystals are differently packed from wild-type Mdm12 (Fig 6B). Crystals of Δ Mdm12 contain six Δ Mdm12 molecules in the asymmetric unit, and three Δ Mdm12 dimers are organized through a tail-to-tail junction in a similar fashion to wild-type Mdm12. Previous biochemical data show that Mdm12 interacts with Mmm1 in a 1:1 ratio stoichiometry, and four molecules are depicted in an elongated organization as a series of Mdm12–(Mmm1)₂–Mdm12 [30]. We propose a new model in which Mmm1 forms a homo-dimer through a head-to-head interaction of each SMP domain in the center, and a hetero-dimer with Mdm12 through a tail-to-tail interaction of their respective SMP domains. Consistent with the hypothesis, the size-exclusion chromatography experiment of Mmm1–Mdm12 (Δ 1–10) revealed that the N-terminus-truncated Mdm12 retained its ability to interact with Mmm1, suggesting that the N-terminus of Mdm12 is not involved in the interaction with Mmm1 (Fig 6D). Based on these results, we suggest that the tail-to-tail contact of two SMP domains from the Mdm12 tetramer and Δ Mdm12 hexamer, as shown in the two crystal structures, might provide a novel structural binding interface from two SMP domains between Mmm1 and Mdm12.

Discussion

In this study, we have elucidated the crystal structure of Mdm12 and the organization of SMP domains from self-associated molecules in crystal asymmetric unit. To ensure the correct organization

Figure 5. Glycerophospholipid selectivity of Mdm12.

A Structural views suggesting that Mdm12 might have a preference for certain phospholipids. The surface representation of Mdm12 positioned around the binding site for the head group of PE is shown with charge distribution (left) and the sequence conservation (right) in the same orientation. Surface electrostatics and sequence conservation were calculated using an APBS program [31] with the nonlinear Poisson–Boltzmann equation and contoured at ± 3 kT/e, and ConSurf website (consurf.tau.ac.il) [32] with 34 different yeast orthologs, respectively. The ribbon diagram shown in the middle indicates the overall orientation of Mdm12.

B Lipid displacement experiments to identify the natural ligands of Mdm12 *in vitro* (see Materials and Methods section for details). NBD-PE-preloaded Mdm12–GST was mixed with a series of glycerophospholipids at different concentrations, and displacement of NBD-PE by non-labeled ligand was estimated from the decrease in fluorescence. Means $\pm$ SD are shown ($n = 3$ independently performed experiments).

Source data are available online for this figure.

of ERMES components and eventually the facilitation of direct contact between the two organelles, the interactions among the SMP domains of the different ERMES components are of critical importance. A potential model for the formation of the Mdm12–Mmm1–

Mdm34 ternary complex is represented in Fig 7A and C. Similar to most SMP domains, Mmm1 forms a homo-dimer through the head region of its SMP domain, while the tail region of Mmm1 forms a hetero-dimer with Mdm12 through the highly conserved tail region

Figure 6. The Mdm12/ΔMdm12 molecules in the asymmetric unit provide an insight into the organization of the Mdm12–Mmm1 binary complex.

A Overall structure (left) and electron density (right) of Mdm12 in the asymmetric unit. Four molecules (two Mdm12 dimers) are organized with twofold rotation symmetry. The twofold axes are indicated with a black dotted line.

B Overall structure (left) and electron density (right) of ΔMdm12 in the asymmetric unit. Six ΔMdm12 molecules (three Mdm12 dimers) are arranged with twofold rotation symmetry as shown above.

C Ribbon diagram showing a twofold interface (tail-to-tail) in two crystal structures (see text for details).

D Size-exclusion chromatography revealing that the N-terminus-truncated version of Mdm12 (residues 11–271) retains the ability to interact with Mmm1. The experiment was performed as in Fig 1B. Eluted fractions indicated by shading were subjected to SDS–PAGE followed by Coomassie Blue staining.

Source data are available online for this figure.

of its SMP domain. Since both the biochemical data and EM structure showed that Mdm12–Mmm1 forms a hetero-tetramer rather than a hetero-hexamer, accommodating the organization of the

Mdm12–Mmm1 complex in our model would first require the dissociation of the Mdm12 homo-dimer. The results of native PAGE described above showed that Mdm12 alone exists in a dynamic

Figure 7. Putative architecture of the Mdm12-Mmm1-Mdm34 ternary complex.

- A** Schematic representation of the SMP domains of Mdm12 and E-SYT2 showing different head structures depending on the presence of helix or strand. Based on our structural and biochemical data, we modeled Mdm12-Mmm1-Mdm34 ternary complex according to the organization of their respective SMP domains using head or tail regions. Two major contact regions among SMP domains are highlighted: H-H (head-to-head) and T-T (tail-to-tail) contacts.
- B** Sequence alignment between Mdm12 and Mdm34 along the N-terminal region (residues 1–7) critical for self-association or hetero-interaction. Each of the Mdm12 and Mdm34 sequences is displayed as a WebLogo [38] representation to highlight sequence conservation. The sequences for Mdm12 and Mdm34 were analyzed using 34 and 60 orthologs, respectively.
- C** Schematic diagram showing the putative organization of the Mdm12 (yellow)–Mmm1 (red)–Mdm34 (blue)–Mdm10 (green) tetramer. Mmm1 forms a homo-dimer with a head-to-head contact in the center, capped on each end by a Mdm12 monomer through a tail-to-tail contact. Mdm12 associates with Mdm34 through a head-to-head contact. The hexameric SMP model was derived from the structure of six Δ Mdm12 molecules within the asymmetric unit.

Source data are available online for this figure.

monomer/dimer equilibrium, and the crystal structure revealed that Mdm12 self-associates through its N-terminus. Given that the interaction of Mdm12 with Mmm1 occurs through tail-to-tail contact of their respective SMP domains, the exposed head region of the Mdm12 monomer would then be free to associate with the SMP domain of Mdm34, which is one of the core components in the ERMES complex. Interestingly, the N-terminal sequences of Mdm34 (residues 1–7, sequence “MSFRFNE”) are highly conserved among other species and are precisely aligned with those in Mdm12 (MSFDINW) (Fig 7B), suggesting that (i) the N-terminus (residues 1–7) of Mdm34 might fold into a β -strand, and (ii) the Mdm34 might form a complex with Mdm12 using this β -strand through head-to-head contact as seen in the Mdm12 dimer. To test whether the β 1-strand of Mdm12 is involved in the interaction with the

Mdm34 SMP domain, the full-length or the N-terminus-truncated Mdm12 was incubated with the SMP domain of Mdm34 (residues 1–188) fused with MBP and analyzed using a MBP pull-down assay. Full-length Mdm12 interacted with the Mdm34 SMP domain, while the truncated Mdm12 did not (Fig EV5A). We also demonstrated a direct interaction between Mdm12 and the N-terminal fragment of Mdm34 comprising residues 1–22 tagged with GST by co-expressing the two proteins and a GST pull-down assay (Fig EV5A). The I5P homologous mutant of Mdm34 (residues 1–22, F5P) lost its ability to interact with Mdm12. The data support our proposed model that the interaction between Mdm12 and Mdm34 would be very similar to that seen in the Mdm12 dimer interface, namely the domain-swapped structure of β 1-strands from two SMP domains.

A previous structural study identified a unique contact site comprising an α -helix in the SMP domain of E-SYT2 that was required for its homo-oligomerization [29]. From our crystal structures and biochemical experiments, we propose two novel interfaces (head-to-head and tail-to-tail) for contact among SMP domains in ERMES components (Fig 7A and C). Mdm12 in particular, a soluble component, acts as a bridge to physically connect two membrane-anchored components, Mmm1 in the ER and Mdm34 in mitochondria, by providing both head- and tail-interacting surfaces through its SMP domain. The observed self-association of Mdm12 was unexpected; however, it is necessary to further test how the self-association of Mdm12 could be related to biological functions such as lipid trafficking. We observed that the self-oligomerization of Mdm12 is dynamic between monomers and dimers from the native PAGE of full-length Mdm12, and the Mdm12 monomer has even higher affinity for NBD-PE than the Mdm12 dimer (Fig 4D and E). In the same vein, it is necessary to examine whether the self-association of Mdm12 might have a negative effect on the lipid trafficking or the organization of the ERMES tetramer complex by inhibiting the interaction with Mdm34.

The structure of the Mdm12 dimer interface and the pairwise sequence alignment between Mdm12 and Mdm34 in N-terminus residues 1–7 reveals that the Mdm12–Mdm34 interaction would be mediated by the crossover of their N-terminus β -strand as shown in the Mdm12 dimer interface. The highly conserved N-terminus sequences of Mdm34 would have the ability to form homo- and hetero-complexes with Mdm12. The dimeric conformation of Mdm34 has been already verified by size-exclusion chromatography with Mdm34 SMP (residues 1–188), and the GST- or GFP-fused SMP domain of Mdm34 [30]. The interactions occurring through the flexible β -strand among the Mdm12 dimer, Mdm34 dimer, and Mdm12–Mdm34 complex appear to be relatively weak, a characteristic that could be associated with the dynamics for the assembly and disassembly of membrane contact mediated by the ERMES complex. The dynamics of the SMP domain also might be important in lipid trafficking, as shown with the Mdm12 monomer that has a higher affinity for lipids than the Mdm12 dimer. Likewise, the association between Mdm12 and Mdm34 might be implicated in lipid trafficking. We observed that the glycerophospholipid-binding site is located very close to the dimerization interface of Mdm12, a distinctive feature for lipid binding by the SMP domain shared by other TULIP family proteins including E-SYT2 (Fig EV4A). The proximity of the lipid interaction and dimerization sites in Mdm12 could enable a direct and more efficient transfer of lipids from Mdm12 to Mdm34. Our structure thus provides indirect evidence for lipid translocation from Mdm12 to Mdm34 or in the reverse direction. We also observed that self-association of Mdm12 inhibits lipid access and incorporation, maybe because new lipids cannot be introduced into the ERMES complex when it is intact, assembled, and transferring lipids. Gem1 has been characterized as a regulator of ERMES activity [11]. The weak interactions and dynamics of ERMES components might contribute to the regulation of membrane contact and lipid trafficking by this small GTPase. Further studies will be required to test this hypothesis.

The self-association of Mmm1 could be mediated by a helix, as in the case of the E-SYT2 SMP domain (Figs 3C and EV5C). The sequences of Mmm1 encoding the first helix in the SMP domain (residues 198–210) are well aligned with those of E-SYT2 and are predicted to fold into an α -helix, suggesting that Mmm1 might form

a homo-dimer through this helical interface (Fig EV5B–D). Given that the SMP domains in Mmm1 and E-SYT2 are located in the middle of the protein primary sequences, and that the association of Mdm12–Mmm1 is stronger than that of Mdm12–Mdm34, control of the assembly of the ERMES complex would likely occur through regulation of the Mdm12–Mdm34 complex, which involves the N-terminus β -strands of the two proteins. Future work will be required to address this biological hypothesis. A high-resolution structure of the ERMES tetramer complex including Mdm10 would be required to elucidate in molecular detail how the tetramer cooperatively and efficiently facilitates direct contact with the membrane and lipid exchange. In the absence of such a structure, our study provides a first understanding of the molecular mechanisms involved in the recognition of lipids by Mdm12 as well as of the dynamics and organization of the ERMES complex in its entirety.

Materials and Methods

Cloning and protein production

The DNA fragments encoding full-length Mdm12, truncated Mmm1 (residues 160–426), and Mdm34 (residues 1–188) were amplified by PCR using *S. cerevisiae* genomic DNA as a template, and cloned into pET28b-SMT3 vector with BamHI/SalI restriction sites, pCDF-Duet vector with NdeI/XhoI sites, and pMBP-Parallel1 fusion vector with EcoRI/SalI sites, respectively. For production of Mdm12 proteins, the plasmids were transformed into *E. coli* strain BL21 (DE3) cells, and proteins were expressed by induction with 0.4 mM IPTG at 18°C for 18 h after cell density reached an A_{600} of 0.5–0.6. The harvested cells were lysed in 25 mM sodium phosphate (pH 7.8), 400 mM sodium chloride, 4 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. Mdm12 was purified by Ni^{2+} -immobilized affinity chromatography (IMAC) followed by ULP1 cleavage of the SMT3 tag overnight during dialysis against 25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM β -mercaptoethanol at 4°C. The non-cleaved Mdm12 were removed by another round of Ni^{2+} -IMAC, and the Mdm12 collected from the flow-through was concentrated and applied onto a Superdex 200 column (GE Healthcare) equilibrated with buffer A containing 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM dithiothreitol. For selenomethionine-derivatized protein, the Mdm12 plasmid was transformed and expressed in B834 (DE3) grown in M9 minimal media plus selenomethionine. Prior to crystallization experiments, the proteins were concentrated by ultrafiltration to 10 mg/ml and flash frozen in liquid nitrogen for storage. For the Mdm12–tMmm1 complex, two plasmids containing pCDF-Duet with Mdm12 (no-tag) and pET28b-SMT3-tMmm1 were transformed into BL21 (DE3) and expressed and purified as above. All mutants including point mutants and deletion mutant (5′-E₇₃-GGSGG (extra sequences)-S₁₁₅-3′, for Δ Mdm12) were generated by PCR-based methods, and the mutations were confirmed by sequencing.

Crystallization and SAD structure determination

Native and Se-Met-derivatized crystals of Mdm12 of maximum diffraction quality were grown in different crystallization conditions. Native crystals were grown in a well solution containing

2.9 M sodium formate, 100 mM ADA (pH 7.5), 5 mM DTT at 4°C by hanging-drop vapor diffusion. Crystals were cryoprotected by soaking them in a well solution plus 30% glycerol. Diffraction data at 3.1 Å resolution were collected at the Pohang synchrotron at 100 K and processed with HKL2000 [33]. Se-Met-derivatized crystals were grown in a crystallization buffer consisting of 12% polyethylene glycol (PEG) 3350, 100 mM bis tris propane (BTP) pH 6.5, and 200 mM magnesium sulfate at room temperature. Crystals were cryoprotected by soaking them into a well solution plus 30% ethylene glycol, and 3.5 Å resolution Se-SAD data were collected at the same synchrotron beamline and processed as above. Phase determination with the SAD dataset was carried out using Phenix, and excellent electron density was produced with a figure-of-merit of 0.4 [34]. The final model was refined to R/R_{free} values of 0.212/0.265 with native data via successive rounds of model building and refinement using Coot and Phenix [34,35]. The final model includes four molecules of Mdm12 without any disallowed geometry. The following residues were not modeled owing to disordered electron density: residues 74–113 and 268–271 in the first copy, residues 73–113 and 267–271 in the second copy, residues 74–112 and 268–271 in the third copy, and residues 74–117 and 266–271 in the fourth copy.

Δ Mdm12 was crystallized using the hanging-drop method by mixing 1 μ l of 15 mg/ml Δ Mdm12 proteins with 1 μ l of crystallization buffer comprising 1.2 M ammonium sulfate, 100 mM Tris-HCl (pH 8.5), 100 mM lithium sulfate at room temperature. A complete dataset was collected to a resolution of 3.6 Å at the same beamline and processed with HKL2000 as above. Using the Mdm12 structure as a search model, a molecular replacement solution was determined using Phaser [36]. Refinement and model building were performed with Phenix and Coot, respectively. The final model contains six Mdm12 (Δ 74–114) molecules in the asymmetric unit without any disallowed geometry. The following residues were not modeled owing to the presence of disordered electron density: residues 268–271 in the first copy, residues 73 and 265–271 in the second copy, residues 73 and 268–271 in the third copy, residues 268–271 in the fourth copy, residues 267–271 in the fifth copy, and residues 266–271 in the sixth copy. The coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5GYD (native Mdm12) and 5GYK (Δ Mdm12).

Size-exclusion chromatography

To measure the relative molecular weights and oligomerization in solution, Mdm12 with or without His-tag, tMmm1, Mdm12–tMmm1 complex were prepared in buffer A at 4°C. Proteins were applied to a Superdex 200 16/60 column (GE Healthcare).

Lipid-binding assays and lipid displacement experiments

For the lipid-binding assay, 1 μ l of 10 mg/ml wild-type and mutant (I5P, I5P/L256W, I5P/I262W, and I5P/L256W/I262W) Mdm12 proteins was mixed with 1 μ l of 1 mg/ml 16:0 NBD-PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[7-nitro-2-1,3-benzoxadiazol-4-yl], purchased from Avanti Polar Lipids) in a total reaction volume of 20 μ l of buffer A for 2 h on ice. After reaction, the products were diluted with the sample buffer consisting of 25 mM Tris-HCl (pH 6.8), 20% glycerol, and 0.01% Bromophenol

blue, and subjected to 12% native PAGE. The reaction products were detected with fluorescence (ImageQuant LAS 4000, GE Healthcare) followed by Coomassie Blue staining. Signal intensities were quantified with ImageJ software, and statistical analysis of the results was performed using Excel 2015.

For lipid displacement experiments, the C-terminal GST-tagged Mdm12 (Mdm12-GST) was incubated with a twofold molar excess of NBD-PE for 2 h on ice with 0.3 mM N,N-dimethyldodecylamine N-oxide (LDAO, Sigma-Aldrich). To remove excess unbound NBD-PE, Mdm12-GST was mixed with glutathione sepharose 4B (GE Healthcare) beads and washed three times with buffer A supplemented with 0.3 mM LDAO. NBD-PE bound to Mdm12-GST was eluted with buffer A containing 10 mM reduced glutathione and concentrated to a final concentration of 0.5 mg/ml. Mdm12-GST (20 μ l) preloaded with NBD-PE was mixed with 1 μ l of phospholipids dissolved in methanol. Reactions were incubated for 2 h on ice and analyzed by native PAGE as described above. All phospholipids were purchased from Avanti Polar Lipids: PA (1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPA), PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPC), PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPE), PG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol); DOPG), and PS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DOPS).

Pull-down experiments

For the pull-down experiment shown in Fig EV5A, 200 μ g of MBP-tagged Mdm34 (residues 1–188) purified from *E. coli* was mixed with 5 μ l of beads of amylose resin (NEB) in a total reaction volume of 500 μ l. The beads were washed three times with buffer A. Purified wild-type or mutant Mdm12 (300 μ g) was added to the beads and incubated for 2 h at 4°C. The beads were washed again three times with buffer A, and the proteins were analyzed by 12% SDS-PAGE followed by Coomassie Blue staining.

The Mdm34 fragment (residues 1–22, wt or mutants)-GST fusion proteins were co-expressed with full-length Mdm12. Proteins were incubated with 5 μ l of a 50% (v/v) slurry of glutathione sepharose 4B beads (GE Healthcare) for 60 min at 4°C. Beads were washed twice with buffer A. Proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE.

Analytical ultracentrifugation

The molecular masses of Mdm12, His₆-Mdm12, Mdm12 (I5P), and the Mdm12–tMmm1 complex were analyzed by equilibrium methods using a Beckman An-60 Ti rotor in a Beckman Optima XL-A analytical ultracentrifuge at 15°C. Proteins at a concentration of 10–20 μ M were prepared in buffer B comprising 25 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 5 mM β -mercaptoethanol. The buffer density, viscosity, and sample partial specific volumes were calculated using SEDNTERP (<http://sednterp.unh.edu>) [37]. Data were evaluated using a nonlinear least-squares curve-fitting algorithm (XL-A data analysis software). The measurements were fit to a single species model using the Origin 6.03 software (Beckman Coulter, Inc.). Sedimentation velocity experiments were performed at 15°C and 20,124 g using two-channel 12-mm path length aluminum centerpieces loaded with 400 μ l of sample and 420 μ l of buffer B. Separate experiments were conducted with various concentrations of Mdm12 (0.5, 1, and 2 mg/ml). Scans were collected in 10-min

intervals using continuous scan mode with a radial spacing of 0.003 cm. Data were analyzed using the continuous $c(s)$ distribution in the SEDFIT program for fitting the frictional ratio, meniscus, and time-invariant noise.

APCI-MS

Purified Mdm12 was desalted using a HiTrap desalting column (GE healthcare) equilibrated with ultrapure grade water. Desalted Mdm12 (50 μ l) was mixed with 950 μ l of acetonitrile and incubated with vigorous vortexing for 1 h at room temperature. All analyses were performed with a Bruker HCT ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an atmospheric pressure chemical ionization (APCI) source. For direct infusion, samples were infused with a syringe pump (KD Scientific) at a flow rate of 240 μ l/h. The APCI source was operated in positive mode with a drying gas (N_2) flow of 5 l/min, nebulizer pressure of 30 psi, drying gas temperature of 250°C, vaporizer temperature of 400°C, capillary voltage of 4.5 kV, and corona current of 4,000 nA. The scanning mass to charge range was 430–1,000 m/z , with a scanning speed of 26,000 m/z per s. To control the instrument, a Compass 1.3 for HCT/esquire (EsquireControl Version 6.2) was employed, and an ESI Compass 1.3 for HCT/esquire (DataAnalysis Version 4.0) was used for data evaluation (both obtained from Bruker Daltonics).

Expanded View for this article is available online.

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Author contributions

CL, HJ, and JP designed the experiments and analyzed the data. HJ and JP performed the experiments. CL, HJ, and JP prepared the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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