Ceramides bind VDAC2 to trigger mitochondrial apoptosis

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Ceramides draw wide attention as tumor suppressor lipids that act directly on mitochondria to trigger apoptotic cell death. However, molecular details of the underlying mechanism are largely unknown. Using a photoactivatable ceramide probe, we here identify the voltage-dependent anion channels VDAC1 and VDAC2 as mitochondrial ceramide binding proteins. Coarse-grain molecular dynamics simulations reveal that both channels harbor a ceramide binding site on one side of the barrel wall. This site includes a membrane-buried glutamate that mediates direct contact with the ceramide head group. Substitution or chemical modification of this residue abolishes photolabeling of both channels with the ceramide probe. Unlike VDAC1 removal, loss of VDAC2 or replacing its membrane-facing glutamate with glutamine renders human colon cancer cells largely resistant to ceramide-induced apoptosis. Collectively, our data support a role of VDAC2 as direct effector of ceramide-mediated cell death, providing a molecular framework for how ceramides exert their anti-neoplastic activity.

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Sphingolipids are essential components of eukaryotic membrane structures that participate in a broad range of cellular processes by controlling vital physical membrane properties and as signaling molecules in response to physiological cues and stresses. Namely, ceramide mediates the pro-apoptotic activity at least in part by interacting directly and specifically with the voltage-dependent anion channel VDAC2, a mitochondrial platform for Bax/Bak translocation. Identification of VDAC2 as an effector of ceramide-mediated cell death provides new opportunities for exploiting the therapeutic potential of ceramides as tumor suppressor lipids.

**Results**

A chemical screen for ceramide-binding proteins yields VDAC2. To identify proteins involved in ceramide-mediated stress signaling and apoptosis, we used a bifunctional ceramide analog carrying a photoactive diazirine and clickable alkyne group in its N-linked acyl chain (pacCer, Fig. 1a). Total membrane fractions from human HeLa cells were incubated with pacCer-containing liposomes, subjected to UV crosslinking and click reaction with Alexa Fluor 647 azide (AF647-N3). In-gel fluorescence (IGF) analysis revealed a subset of membrane-bound proteins with affinity for the pacCer probe, which included a mitochondria-associated protein of ~33 kDa that was prominently photolabeled (Fig. 1b, Supplementary Fig. 1). As ceramide exerts its apoptogenic activity in mitochondria, we set out to identify the 33 kDa candidate ceramide-binding protein (CBP).

To this end, mitochondria were photolabeled with pacCer and then click reacted with a PEG-based reagent containing an azide, a biotin and a TAMRA fluorophore as functional groups (Fig. 1c). Next, pacCer-crosslinked proteins were isolated using NeutrAvidin agarose and visualized by IGF (Fig. 1d). The fluorescent 33 kDa protein band was cut from the gel, trypsin-digested, and identified by LC-MS/MS analysis as the voltage-dependent anion channel VDAC1 and VDAC2 (Supplementary Table 1). In line with the MS data, pretreatment of HeLa cells with VDAC1 and VDAC2-targeting siRNAs effectively depleted the fluorescent 33 kDa protein band from pacCer-labeled and Alexa click-reacted mitochondria (Fig. 1e, f). The photolabeled 33 kDa protein band also cross-reacted with both anti-VDAC1 and anti-VDAC2 antibodies (Fig. 1g, h). In contrast, VDAC3 and TOM40—an outer mitochondrial membrane (OMM) channel protein with a cellular copy number close to that of VDAC3—lacked affinity for pacCer, indicating that VDAC1 and VDAC2 are genuine mitochondrial CBPs.

MD simulations uncover a ceramide-binding site on VDACs. To search for a putative ceramide-binding site on VDAC1 and VDAC2, we performed coarse-grain molecular dynamics (CG-MD) simulations using the Martini model. Main simulations were performed with VDAC channels at an aggregate time of 1.23 ms (Supplementary Table 2)—only attainable using CG-MD. A well-resolved structure of mouse VDAC1 (PDB: 4C69) was constructed with ~630 lipids. To the assumption of identical secondary structure we mutated VDAC1 side chains to the mouse sequences of VDAC2 and VDAC3. The underlying mechanisms remain to be established. Interestingly, ceramides have been shown to form pores in model bilayers as well as in the outer membrane of isolated mitochondria that are large enough to mediate passage of cytochrome c across the outer membrane. However, the underlying mechanisms remain to be established. Interestingly, the remaining mechanisms are to be explained. Ceramide has been shown to form pores in model bilayers as well as in the outer membrane of isolated mitochondria that are large enough to mediate passage of cytochrome c. Formed of ceramide channels do not rely on any particular protein but is disrupted by anti-apoptotic Bcl-2 proteins.

In this study, we present evidence for an alternative mechanistic view, namely that ceramides mediate their pro-apoptotic activity at least in part by interacting directly and specifically with the voltage-dependent anion channel VDAC2, a mitochondrial platform for Bax/Bak translocation. Identification of VDAC2 as an effector of ceramide-mediated cell death provides new opportunities for exploiting the therapeutic potential of ceramides as tumor suppressor lipids.
In its deprotonated state, this residue seemed to promote direct contact with the ceramide head group (Fig. 2c; Supplementary Information Videos 1 and 2). In VDAC3, which does not bind ceramide (Figs. 1h and 2a), the bilayer-facing Glu residue is replaced by a glutamine (Gln73; Fig. 2b). Substitution of Gln for Glu73 in VDAC1 or Glu84 in VDAC2 strongly reduced the ceramide occupancy and residence time at the binding sites (Fig. 2d, e). Protonation of the bilayer-facing Glu also greatly diminished ceramide-binding (Fig. 3a, b) while substitution of a deprotonated aspartate for the Glu residue retained ceramide binding (Supplementary Fig. 2). This indicates that a negative charge on the membrane-buried Glu residue is critical for ceramide binding.

In line with a previous computational study, we also found a number of binding sites for cholesterol. These displayed no overlap with the ceramide-binding site (Fig. 2d). To exclude the possibility that competition with ceramide prevented cholesterol to occupy the ceramide-binding site, additional simulations were performed.
performed in the absence ceramide. Also in those simulations no cholesterol binding near the membrane-facing Glu was observed (Supplementary Fig. 3). We observed zero-specific binding events between phosphatidylcholine (PC) and VDACs in the OMM mimics. Yet when VDAC1 was simulated in a bilayer of 100% dimyristoyl-phosphatidylcholine (DMPC) following the setup of Supplementary Fig. 4c). In sum, CG-MD simulations revealed that VDAC1 and VDAC2 were produced recombinantly in E. coli and then reconstituted in egg PC liposomes (Supplementary Fig. 5). Density gradient fractionation analysis revealed that reconstitution efficiencies of wild type and mutant channels were practically indistinguishable. The reconstituted channels were then subjected to photolabeling with pacCer and bifunctional analogs of diacylglycerol (pacDAG), PC (pacPC), phosphatidylethanolamine (pacPE), and cholesterol (pacChol; Supplementary Fig. 6). VDAC1 and VDAC2 could be efficiently and reproducibly photolabeled with pacCer, pacPC, and pacChol, but not with pacDAG or pacPE (Fig. 4). In agreement with the simulations, replacing the membrane-facing Glu with Gln virtually abolished labeling of both channels with pacCer and pacPC, whereas labeling with pacChol was not or only slightly affected (Figs. 4 and 5a). Moreover, reducing the pH from 7 to 5 caused a significant reduction in E73-dependent photolabeling of VDAC1 with pacCer (Fig. 3c, d). This suggests that ceramide binding is critically dependent on the protonation state of the membrane-exposed Glu, as predicted by the simulations.

The pronounced labeling of the wild-type channels with pacPC was somewhat unexpected as simulations revealed that specific

**Ceramide binding relies on a membrane-facing glutamate.** We next sought to verify the relevance of the membrane-facing Glu residue in VDACs for ceramide binding. To this end, human VDAC1 and VDAC2 and the mutant channels VDAC1E73Q and VDAC2E84Q were produced recombinantly in E. coli and then reconstituted in egg PC liposomes (Supplementary Fig. 5). Density gradient fractionation analysis revealed that reconstitution efficiencies of wild type and mutant channels were practically indistinguishable. The reconstituted channels were then subjected to photolabeling with pacCer and bifunctional analogs of diacylglycerol (pacDAG), PC (pacPC), phosphatidylethanolamine (pacPE), and cholesterol (pacChol; Supplementary Fig. 6). VDAC1 and VDAC2 could be efficiently and reproducibly photolabeled with pacCer, pacPC, and pacChol, but not with pacDAG or pacPE (Fig. 4). In agreement with the simulations, replacing the membrane-facing Glu with Gln virtually abolished labeling of both channels with pacCer and pacPC, whereas labeling with pacChol was not or only slightly affected (Figs. 4 and 5a). Moreover, reducing the pH from 7 to 5 caused a significant reduction in E73-dependent photolabeling of VDAC1 with pacCer (Fig. 3c, d). This suggests that ceramide binding is critically dependent on the protonation state of the membrane-exposed Glu, as predicted by the simulations.
46. In contrast to the present work, the latter study was the bilayer-facing Glu to a major cholesterol binding pocket on binding sites on VDACs, a recent photolabeling study mapped were click-reacted with AF647-N3, subjected to SDS-PAGE, and analyzed by IGF and CB staining. 47. Summing all points’ y-values yields the fraction of total simulation time when ceramide was bound. 48. Pretreatment of pacCer was progressively reduced by C16-ceramide when added in 3- to 27-fold excess (Fig.5b), arguing against the idea that pacCer labeling of VDACs is primarily driven by af 49. Data are means ± s.d.; n = 4; **p < 0.01 by two-tailed paired t-test. Source data encounters of PC molecules with the membrane-facing Glu are relatively rare. This discrepancy could be due to the irreversible nature of photoaffinity labeling in combination with the major difference in timescales between CG-MD simulations (μs range) and photoaffinity labeling (second range). While our data revealed no obvious overlap between the ceramide and cholesterol binding sites on VDACs, a recent photolabeling study mapped the bilayer-facing Glu to a major cholesterol binding pocket on VDAC1. In contrast to the present work, the latter study was performed on VDAC1-containing birecules with cholesterol probes that carry the photoactive diazirine in the aliphatic tail or at C7. Thus, additional work will be necessary to resolve the discrepancy between the simulations and photolabeling studies of cholesterol binding to VDACs.

As aliphatic diazirines display photochemical preference for nucleophilic amino acids, the deprotonated side chain of the membrane-buried Glu in VDACs may provide a site of insertion for the diazirine in pacCer, which may diffuse a short distance to reach such a nucleophile. Therefore, we next labeled VDACs with pacCer in the presence of excess C16-ceramide. Labeling by pacCer was progressively reduced by C16-ceramide when added in 3- to 27-fold excess (Fig. 5b), arguing against the idea that pacCer labeling of VDACs is primarily driven by affinity of the aliphatic diazirine for the negatively charged Glu. Excess C16-ceramide did not affect labeling of VDACs with pacChol. In line with the simulations, these data indicate that ceramides bind VDACs at the membrane-facing Glu residue. To verify that this concept not only holds for recombinant channel proteins in synthetic bilayers but also for their native counterparts in mitochondrial membranes, we made use of the carboxyl-modifying reagent dicyclohexylcarbodiimide (DCCD). This hydrophobic compound irreversibly reacts with a number of integral membrane proteins through covalent modification of membrane-embedded Asp or Glu residues and was previously shown to modify Glu73 in VDAC1. Pretreatment of mitochondria with DCCD selectively abolished photolabeling of the 33 kDa protein band with pacCer (Supplementary Fig. 7), hence providing complementary proof that the membrane-facing Glu residue in VDAC1 and VDAC2 is part of an authentic ceramide-binding site.

**Loss of VDAC2 disrupts ceramide-induced apoptosis.** VDAC channels are active participants in the cytosolic release of apoptogenic proteins from mitochondria. VDAC2 serves as a platform for the mitochondrial recruitment of pro-apoptotic Bcl-2 proteins Bak and Bax33–35, which can commit cells to death by permeabilizing the OMM for cytochrome c. In response to various apoptotic stimuli, VDAC1 forms oligomers and participates in the assembly of a cytochrome c-conducting pore. Identification of a ceramide-binding site on VDAC1 and VDAC2 raised the question whether ceramides exert their apoptotic activity by interacting with these proteins. To address this, we employed an engineered ceramide transfer protein equipped with an OMM anchor, mitoCERT (Fig. 6a). We previously demonstrated that human HCT116 colon cancer cells expressing mitoCERT undergo Bax-dependent apoptosis by mistargeting newly synthesized ER ceramides to mitochondria (Fig. 6b). This led us to determine the impact of VDAC removal on mitoCERT-induced apoptosis in HCT116 cells. Loss of VDAC1, VDAC2, or both was verified by immunoblotting and IGF analysis of mitochondrial photolabeled with pacCer (Supplementary Fig. 8). Expression of mitoCERT in wild-type HCT116 cells triggered apoptosis, as indicated by cleavage of caspase substrate PARP1 (Fig. 6c). No PARP1 cleavage was observed in cells expressing a mitoCERT variant that lacked the ceramide transfer or START domain,mitoCERTΔSTART. Loss of VDAC1 had no effect on the ability of mitoCERT to induce PARP1 cleavage. In contrast, VDAC2 removal rendered cells partially resistant to mitoCERT-induced PARP1 cleavage, especially in the absence of VDAC1 (Fig. 6c).
Glutamate 84 in VDAC2 is critical for ceramide-induced apoptosis.

We next analyzed individual wild-type and ceramide-binding defective VDAC channels for their ability to support mitoCERT-induced apoptosis. To this end, HCT116 VDAC1/2 double KO cells were stably transduced with haemagglutinin (HA)-tagged VDAC1, VDAC1E73Q, VDAC2, or VDAC2E84Q (Fig. 7a). Mitochondrial localization of the tagged channels was confirmed by immunofluorescence microscopy (Supplementary Fig. 9). Heterologous expression of VDAC2, but not VDAC1, restored mitoCERT-induced apoptosis in the double KO cells, as evidenced by PARP1 cleavage and proteolytic activation of caspase-3, the principal executioner caspase in apoptotic cells (Fig. 7a–c). This confirmed that VDAC2, unlike VDAC1, is a key player in ceramide-mediated cell death. Strikingly, replacing Glu84 with Gln in VDAC2 greatly reduced its ability to support mitoCERT-induced apoptosis in the double KO cells (Fig. 7a–c), indicating that cell death triggered by a rise in mitochondrial ceramides critically relies on a uniquely positioned charged Glu residue that mediates direct contact with the ceramide head group in the bilayer interior, potentially driven by electrostatic attraction. We speculate that an amide-containing backbone combined with a small polar head group renders ceramide the preferred lipid binding partner of VDAC isoforms containing the membrane-buried Glu residue. Although it is energetically unfavorable for charged residues to face the bilayer’s hydrophobic core, recent work revealed that the pKa of Glu73 in VDAC1 is closely tuned to the physiological pH of the cytosol (pKa ~7.4)51. This implies that under stress-free conditions, the membrane-buried Glu is in its deprotonated fully charged state at least a significant amount of time. Interestingly, ceramide binding to late endosomal protein LAPTM4B depends on a membrane-embedded aspartate52. We anticipate that also other proteins with charged acidic residues to their membrane spans may have affinity for ceramide and potentially participate in ceramide-operated signaling pathways. While ceramide-induced apoptosis requires Bax8,24,25, a recent study revealed that VDAC2 specifies Bax recruitment to the finding that the Bak-stabilizing activity of VDAC2 requires isoform-specific sequence motifs located outside of the region involved in ceramide binding33.

Discussion

The current study identified a role of VDAC2 as a direct and specific effector of ceramide-mediated cell death. This function critically relies on a uniquely positioned charged Glu residue that mediates direct contact with the ceramide head group in the bilayer interior, potentially driven by electrostatic attraction. We speculate that an amide-containing backbone combined with a small polar head group renders ceramide the preferred lipid binding partner of VDAC isoforms containing the membrane-buried Glu residue. Although it is energetically unfavorable for charged residues to face the bilayer’s hydrophobic core, recent work revealed that the pKa of Glu73 in VDAC1 is closely tuned to the physiological pH of the cytosol (pKa ~7.4)51. This implies that under stress-free conditions, the membrane-buried Glu is in its deprotonated fully charged state at least a significant amount of time. Interestingly, ceramide binding to the late endosomal protein LAPTM4B depends on a membrane-embedded aspartate52. We anticipate that also other proteins with charged acidic residues in their membrane spans may have affinity for ceramide and potentially participate in ceramide-operated signaling pathways. While ceramide-induced apoptosis requires Bax8,24,25, a recent study revealed that VDAC2 specifies Bax recruitment to the finding that the Bak-stabilizing activity of VDAC2 requires isoform-specific sequence motifs located outside of the region involved in ceramide binding33.
mitochondria and concomitantly ensures Bax inhibition by mediating its retrotranslocation into the cytosol\textsuperscript{34}. By establishing a dynamic equilibrium between mitochondrial and cytosolic Bax pools, this VDAC2-dependent shuttling is ideally suited for regulation by pro- and anti-apoptotic cues. Our present findings suggest that ceramide binding to VDAC2 may commit cells to death by blocking Bax retrotranslocation. This concept is distinct from previous models postulating that ceramides accumulating in the OMM: (i) self-assemble into cytochrome c-conducting channels\textsuperscript{27,28}; (ii) form lipid macrodomains into which Bax inserts and functionalizes as a pore\textsuperscript{30,31}; (iii) affect mitochondrial shape to facilitate Bax recruitment and apoptosis\textsuperscript{53}; (iv) require metabolic conversion to gain apoptogenic activity\textsuperscript{32}. How ceramide binding tips the balance in VDAC2-mediated shuttling of Bax to trigger mitochondrial apoptosis remains to be established. Our simulations and photoaffinity experiments

![Graphs and images showing competitive inhibition of pacCer photolabeling of VDACs by C\textsubscript{16}-ceramide.](image-url)
indicate that ceramide binding to VDACs is pH sensitive and controlled by the protonation state of the membrane-buried Glu. Interestingly, acidification has been shown to promote association of two VDAC1 monomers into a dimer\(^{34}\). Assembly of a high-affinity dimer relies on protonation of the membrane-facing Glu residue and a serine (Ser43) at the dimer interface\(^{31}\). VDAC oligomerization has been implicated as a component of the mitochondrial pathway of apoptosis\(^{39,50}\) and may be part of the mechanism by which VDAC2 stabilizes the mitochondrial pool of Bax in response to apoptotic stimulation\(^{34}\). In view of our present findings, a prospect that merits further investigation is whether binding of ceramide to the charged Glu residue lowers the threshold for VDAC oligomerization at neutral pH. Ceramide binding to VDACs may also influence interactions with other proteins, as the membrane-facing Glu residue is critical for association of VDAC1 with hexokinase I\(^{54,55}\). VDAC-bound hexokinases are thought to play a pivotal role in promoting cell growth and survival in rapidly growing, hyperglycemic tumors\(^{36,57}\). Consequently, our current study establishes a molecular framework to unravel how ceramides execute their tumor suppressor functions.

Methods

Reagents. 1,2-Dioleolyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanol-amine (DOPE), and \(\pm\)-a-phosphatidylcholine from chicken egg (egg PC), 1-palmitoyl-2-\(\pm\)-[\(\pm\)-nitro-2,13-decanedioate-4,6-yl]-a-amino[\(\pm\)-decanoyl]-\(\pm\)-glycerol-3-phosphocholine (NBD-PC) and CerSM were from Avanti Polar Lipids. Alexa Fluor 647-N\(_2\) (AF647-N\(_2\)) and Biotin-N\(_3\) were from Thermo Fischer Scientific, and TAMRA-Biotin-N\(_3\) from Click Chemistry Tools. The photoactive and clickable \(\pm\)-sterol probe (pacFA) was from Sigma-Aldrich. A 15 carbon-long fatty acid containing a photoactivatable diazirene and clickable alkyne group, pacFA, was synthesized in three steps from commercially available educts\(^{46}\). Next, pacFA was coupled to \(\beta\)-erythritol-sphingosine (Enzo Biochem) using a combination of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and hydroxybenzotriazole (HOBt) as condensing reagents, yielding the photactivatable and clickable C15-ceramide analog, pacCer (85% overall yield). pacPC was synthesized starting from 1-oleoyl-2-hydroxy-sn-glycerol-3-phosphocholine (Avanti Polar Lipids) and pacFA under the action of \(\beta\)-n,N-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) with satisfactory yield (39%). pacDAC was synthesized in three steps starting from 1-oleoyl-sn-glycerol (Santa Cruz Biotechnology). First, the primary HO-group was protected with the triphenylmethyl protecting group (trityl-chloride/pyridine; 92% overall yield). The 1-acetyl-3-trityloxy-glycerol obtained was coupled with pacFA using EDCI/DMAP activation (58% overall yield). The final deprotection step was achieved using trifluoroacetic acid (TFAA) to generate pacDAC (28% overall yield). pacPE was synthesized in three steps starting from 1-oleoyl-2-hydroxy-sn-glycerol-3-phosphoethanolamine (Avanti Polar Lipids). First, the amino-group was protected with the tert-butoxycarbonyl protecting group (di-tert-butyldicarbonate/triethylamine; 98% overall yield). The N-protected lyso-PE obtained was coupled with pacFA using EDCI/DMAP activation (58% overall yield). The final deprotection step was achieved using trifluoroacetic acid to generate pacPE (35%, overall yield).

Antibodies. Antibodies used were mouse monoclonal anti-\(\beta\)-actin (Sigma-Aldrich, A1978; IB 1:50,000), rabbit polyclonal anti-\(\beta\)-FLAG (Cell Signaling, 2338; IB 1:1,000), mouse monoclonal anti-mitochondrial surface protein p60 (Millipore, MAB1273; IB 1:1,000), rabbit polyclonal anti-VDAC2 (Abcam, Ab37985; IB 1:1,000), rabbit polyclonal anti-VDAC3 (Abcam, Ab80452; IB 1:1,000), mouse monoclonal anti-TOM20 (Millipore, MAB1273; IB 1:1,000), rabbit polyclonal anti-TOM40 (Abcam, Ab185543; IB 1:1,000), rabbit polyclonal anti-\(\beta\)-actin (Sigma-Aldrich, A1978; IB 1:1,000), rabbit polyclonal anti-VIN (Intravac, 715506; IB 1:1,000; IF 1:200), rat monoclonal anti-HA (Roche, 12158167001; IB 1:1,000), rabbit polyclonal anti-caspase-3 (Cell Signaling, 86611; IB 1:1,000), mouse monoclonal anti-PARP-1 (Santa Cruz, sc8007; IB 1:1,000) and rabbit polyclonal anti-calnexin (Santa Cruz, sc11397; IB 1:1,000). Goat anti-mouse (31430; IB 1:500), goat anti-rabbit (31460; IB 1:500) and donkey anti-goat IgG conjugated to horseradish peroxidase (pa1-28664; IB 1:5,000) were from Jackson ImmunoResearch Laboratories.

Fig. 6 VDAC2 removal disrupts ceramide-induced apoptosis. a Schematic outline of ceramide transfer protein CERT, mitoCERT, and mitoCERT\(^{\Delta}\)START. MitoCERT was created by swapping the Golgi-targeting pleckstrin homology domain of CERT against the OMM anchor of AKAP1. Removal of the ceramide transfer or START domain yielded mitoCERT\(^{\Delta}\)START. All three proteins bind the ER-resident protein VAP-A via their FFAT motif (F). Ceramide, P(\(\pm\))P phosphatidylinositol-4-phosphate, TGN trans-Golgi network. b Ceramides (Cer) are synthesized through N-acylation of long chain bases (LCB) by ceramide synthases (CerS) on the cytosolic surface of the ER and require CERT-mediated transfer to the Golgi for metabolic conversion into sphingomyelin (SM) by a Golgi-resident SM synthase (SMS). Expression of mitoCERT causes a diversion of this biosynthetic ceramide flow to mitochondria, triggering Bax-dependent apoptosis\(^{11}\). c Wild type (WT), VDAC1-KO (ΔVDAC1), VDAC2-KO (ΔVDAC2), and VDAC1/2 double KO (ΔVDAC1/2) human colon cancer HCT116 cells were transfected with empty vector (EV), Flag-tagged mitoCERT, or Flag-tagged mitoCERT\(^{\Delta}\)START. At 24 h post transfection, cells were processed for immunoblotting with antibodies against PARP1, the Flap-epitope, VDAC1, VDAC2, and \(\beta\)-actin. The percentage of PARP1 cleavage was quantified. Data are means ± s.d.; n = 3; *p < 0.05 and **p < 0.01 by two-tailed paired t-test. Source data.
**DNA constructs.** For expression of human VDACs in E. coli, the corresponding cDNAs were PCR amplified using Phusion high-fidelity DNA polymerase (Thermo Fischer Scientific) and inserted via Ndel and Xhol (VDAC1) or Xhol and Xhol sites (VDAC2) into bacterial expression vector pCold I (Takara Bio, USA). For retroviral transduction studies, DNA fragments encoding human VDACs with a C-terminal HA tag (YPYDVPDYA) were created by PCR and inserted via NotI and Xhol sites into lentiviral expression vector pLNCX2 (Takara Bio, USA). Single amino acid substitutions were introduced using the QuikChange II site-directed mutagenesis method (Agilent, USA). Primers used for cloning and site-directed mutagenesis are listed in Supplementary Table 3. Mammalian expression constructs encoding FLAG-tagged mitoCERT and mitoCERTΔSTART were described previously25. All expression constructs were verified by DNA sequencing.

**Cell culture and transfection and RNAi.** Human cervical carcinoma HeLa cells (ATCC CCL-2) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/l glucose, 2 mM l-glutamine and 10% FBS. Human colon carcinoma HCT116 cells (ATCC CCL-247) were cultured in McCoy’s medium supplemented with 10% FBS. Human embryonic kidney HEK293T cells (ATCC CRL-3216) were cultured in DMEM supplemented with 10% FBS. Cells were transfected with DNA constructs using FuGENE HD (Promega) according to the manufacturer’s instructions. The transfection efficiency was monitored by Immunoblotting analysis and immunofluorescence microscopy. All experiments were performed in triplicate. RNAi treatment of HeLa cells with siRNA (Qiagen) was performed using Oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions. The heparin-binding region was quenched with 1% polyethylene glycol (PEG) 6000, and 1 µg/ml polybrene was added to the transfection mixture. After 48 h, the retrovirus-containing medium was harvested, filtered through a 0.45 µm filter, mixed 1:1 (v/v) with McCoy’s growth medium, supplemented with 8 µg/ml puromycin, and used to transduce HCT116/ΔVDAC1/2 cells. After 3–5 days, positively transduced cells were selected and analyzed for expression of HA-tagged VDACs by immunoblot analysis and immunofluorescence microscopy.

**Retroviral transduction.** HCT116 ΔVDAC1/2 double KO cells stably expressing HA-tagged VDAC1, VDAC2, or VDAC2E84Q were created by retroviral transduction. To this end, HEK293T cells were co-transfected with pLNX2-mitoCERTΔSTART and packaging vectors (Clontech) using Lipo-SSectamine reagent (Invitrogen) according to the manufacturer’s instructions. The retrovirus-containing medium was harvested, filtered through a 0.45 µm filter, mixed 1:1 (v/v) with McCoy’s growth medium, supplemented with 8 µg/ml puromycin, and used to transduce HCT116/ΔVDAC1/2 double KO cells. Hygromycin (300 µg/ml) was added 6 h post-infection and selective medium was exchanged daily. After 2 weeks under selective pressure with 2 µg/ml puromycin, Individual drug-resistant clones were picked and analyzed for VDAC1 and VDAC2 expression by immunoblot analysis. A ΔVDAC1/2 double KO cell line was generated from ΔVDAC1 cells as described above following ejection of the puromycin selectable marker using Cre vector (Santa Cruz, sc-418923) according to the manufacturer’s instructions.

**Generation of VDAC KO cell lines.** To knock out VDAC1 and VDAC2 in HCT116 cells, we obtained a mix of three different CRISPR/Cas9 plasmids per gene and the corresponding HDR plasmids from Santa Cruz (sc-418200, sc-416966). The VDAC1-specific gRNA sequences were: A′sense, 5′-TTGAGGAATTTCTAGC-3′; B′sense, 5′-CGAATTCATGAGGAGGC-3′; C′sense, 5′-CTTACA CATTAGTTGAAGC-3′. The VDAC2-specific gRNA sequences were: A′sense, 5′-AGAAATTCGAATTGAGAGGC-3′; B′sense, 5′-GCGCTAAGAAGCCGACAG CAT-3′; C′sense, 5′-TATGTAATGACTCTCA-3′. VDAC1/2 cells were transfected with both plasmid mixes and grown for 48 h without selection. Next, cells were grown for 2 weeks under selective pressure with 2 µg/ml puromycin. Individual drug-resistant clones were picked and analyzed for VDAC1 and VDAC2 expression by immunoblot analysis. A ΔVDAC1/2 double KO cell line was generated from ΔVDAC1 cells as described above following ejection of the puromycin selectable marker using Cre vector (Santa Cruz, sc-418923) according to the manufacturer’s instructions.
Reconstitution of reconstituted VDACs. VDAC-encoding pCold I constructs were transformed in E. coli BL21 (DE3) Omp95 cells (a kind gift from Dr. Lars-Oliver Hoyer and Prof. Kersten Marquardt, Goethe University in Frankfurt). Cultures were incubated at 37 °C to an early exponential phase in LB medium containing 100 µg/ml ampicillin and cooled for 30 min at 4 °C prior to addition of 1 mM IPTG. Growth was continued for 24 h at 15 °C. Cells were harvested by centrifugation and lysed in TEN buffer (50 mM Tris/HCl pH 8.0, 100 mM NaCl) supplemented with 2.5% Triton X-100 and 40 µM β-mercaptoethanol. The cell suspension was divided 1:10 by dropwise addition into 25 mM Na2PO4, pH 7.0, 100 mM NaCl, 6 M guanidine hydrochloride, 1 mM EDTA and 10 mM DTT while stirring, and stirred overnight at 4 °C. Next, the suspension was diluted 1:10 by dropwise addition to 25 mM Na2PO4, pH 7.0, 100 mM NaCl, 1 mM EDTA, and 2.2% lauryldimethylamine oxide (LDAO), and stirred overnight at 4 °C. Finally, the suspension was diluted 1:10 by dropwise addition to 25 mM Na2PO4, pH 7.0, 100 mM NaCl, 1 mM EDTA, 0.1% LDAO, and 1 mM DTT, stirred overnight at 4 °C, and loaded on a Fractogel EMD-SE Hicap cation-exchange column (Merck Millipore). VDAC proteins were eluted with a linear NaCl concentration gradient in 25 mM Na2PO4, pH 7.0, 100 mM NaCl, 1 mM EDTA, 0.1% LDAO, and 10 mM DTT, on a AKTAprime plus protein purification system (GE Healthcare Life Sciences). Peak fractions were pooled, concentrated on an Amicon Ultra-4 unit (MWCO 10 kDa; Merck Millipore), and loaded on a Superose 12 10/300 GL size exclusion column (GE Healthcare Life Sciences). Elution was in 10 mM Tris/HCl pH 7.0, 100 mM NaCl, and 0.05% LDAO. Purified VDAC proteins in peak fractions were divided into three aliquots. The first was loaded into a column that was equilibrated with buffer R (100 mM KCl, 10 mM MOPS/Tris pH 7.0) by vortexing and sonication. LDAO was added in 10-fold molar excess over lipids. The concentration of LDAO in the sample was adjusted to that in the lipid sample. Both samples were incubated separately at RT for 20 min, mixed at a 1:1 (v/v) ratio, and incubated again for 30 min. SM2 Biobeads (Bio-rad Laboratories) pretreated according to the manufacturer’s instructions were added to the lipid–protein mixture in 30-fold excess over detergent (w/w). After incubation overnight at 4 °C on a rotating wheel, the beads were removed by centrifugation and the proteoliposome-containing supernatant was aliquoted, snap-frozen in liquid N2, and stored at −80 °C. To check reconstitution efficiency, an aliquot of the proteoliposomes was subjected to density flotation analysis. To this end, proteoliposomes were mixed 1:1 (v/v) with 80% Accucent (Accurate Chemical & Scientific Corporation, USA) in buffer R, transferred to the bottom of a 5 ml ultracentrifuge tube, and overlayed with 30%, 20%, 10%, and 0% Accucent prepared in reconstitution buffer. After centrifugation for 1 h at 100,000 × g at 4 °C, 10 × 0.5 ml fractions were collected from top to bottom. Fractionation profiles of NBD-PC and VDACs were determined by TLC analysis and SDS-PAGE followed by Coomassie blue staining, respectively.

Photofluorophore labeling of reconstituted VDACs. Liposomes used for photofluorophore labeling of reconstituted VDACs were prepared from a defined lipid mixture (DOPC/DOPE/pacLipid, 80/20/1 mol%) in CHCl3/methanol (9/1, v/v). In brief, 10 µmol of total lipid was dried in a Rotavap and the resulting lipid film was resuspended in 10 mM Tris/HCl pH 7.0, 100 mM NaCl, 1 mM EDTA and 10 mM DTT while shaking. The beads were washed five times in 0.6 ml PBS/0.5% SDS and then incubated in Sample buffer for 5 min at 95 °C to elute bound proteins. Proteins in total were immunoprecipitated, washed with PBS/methanol precipitation and processed for IGF analysis as above. The pacCer-labeled 33-kDa protein band in the eluate fraction was excised from the gel and digested by trypsin (Promega, V5111) for 6 h at 37 °C. Protein fragments were collected, concentrated, and analyzed by LC-MS/MS in an amaToPend ETD Ion Trap mass spectrometer (Bruker Daltonics, Bremen). tryptic peptides were separated on a C18 column using a two-buffer system with water/acetonitrile/formic acid (99:1/1, v/v/v) and water/acetonitrile/formic acid (20/80, v/v/v). Raw MS data were converted to peak lists using Data Analyzer 4.1 (Bruker, USA) and noise filtered using an in-house developed script. The spectra were searched against the IPI human (IPID085543) and Swissprot (v56.2) databases. Peptide identifications were accepted with a score greater than 20 and a p-value smaller than 0.01, and peptides were identified with at least two unique peptides.

Identification of pacCer-photo labeled proteins. After photolabeling, all liposomes were suspended in mitochondrial or cell lysate and separated by either centrifugation or immunoprecipitation. Proteins were recrystallized and subjected to high-resolution 2D gel electrophoresis according to the manufacturer’s instructions (InvitroGen). The mixture was transferred onto a PVDF membrane using a semi-dry blotter. Western blotting was performed using the following antibodies: 1) an anti-VDAC1/D2/D3 antibody (BD Transduction Laboratories) and 2) an anti-total VDAC antibody (Pro-Sci). VDAC proteins were visualized using Amido Black58, while shaking. The beads were washed five times in 0.6 ml PBS/0.5% SDS and then incubated in Sample buffer for 5 min at 95 °C to elute bound proteins. Proteins in total were immunoprecipitated, washed with PBS/methanol precipitation and processed for IGF analysis as above. The pacCer-labeled 33-kDa protein band in the eluate fraction was excised from the gel and digested by trypsin (Promega, V5111) for 6 h at 37 °C. Protein fragments were collected, concentrated, and analyzed by LC-MS/MS in an amaToPend ETD Ion Trap mass spectrometer (Bruker Daltonics, Bremen). tryptic peptides were separated on a C18 column using a two-buffer system with water/acetonitrile/formic acid (99:1/1, v/v/v) and water/acetonitrile/formic acid (20/80, v/v/v). Raw MS data were converted to peak lists using Data Analyzer 4.1 (Bruker, USA) and noise filtered using an in-house developed script. The spectra were searched against the IPI human (IPID085543) and Swissprot (v56.2) databases. Peptide identifications were accepted with a score greater than 20 and a p-value smaller than 0.01, and peptides were identified with at least two unique peptides.
systems was 1.23 ms (Supplementary Table 2). Trajectories were saved and analyzed every 600 ps.

Simulation analysis. Two main types of analysis were performed: contact analysis and space occupation analysis. Contact analyses were performed either as residue-discriminated analyses or as residence-time distributions. Residue-discriminated contacts were calculated by finding all frames of ceramide (particles AM1 or AM2) or cholesterol (particle ROH) head groups within 7 Å of any relevant residue particle. To allow comparison between simulations of different lengths, the number of in-contact frames was normalized by the total number of frames. Contact residence-time distributions were plotted by logarithmically histogramming the duration of all individual continuous binding events of each single ligand to the VDAC-binding site. To correctly convey the impact of long-term binding events, the duration of all individual continuous binding events of each single ligand to the VDAC-binding site. To correctly convey the impactful events of long-term binding events, the duration of all individual continuous binding events of each single ligand to the VDAC-binding site.

Received: 26 July 2018 Accepted: 22 March 2019

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Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Projects SFB944-P14 and HO3539/1-1) to J.C.M.H., a Marie Curie Intra-European Fellowship to J.G.M.M. (Project 298278), and a German Egyptian Research Long-term Scholarship to D.G.H. (Project 5722240). M.N.M was supported by Project LISBOA-01-0145-FEDER-007660 (Microbiologia Molecular, Estrutural e Celular) funded by FEDER funds through COMPETE20–Programa Operacional Competitividade e Internacionalização (POCI) and by national funds through FCT—Fundação para a Ciência e a Tecnologia. M.N.M. and G.R. acknowledge the National Laboratory for Scientific Computing (LNCC/MCTI, Brazil) for providing HPC resources of the SDumont supercomputer (http://sdumont.lncb.br). Additional support was provided by the NIH (Project 1R21AI124225-01A1) to F.G.T.

Author contributions

J.C.M.H. conceptualized the study and wrote the manuscript, with critical input from S.D., S.B., J.G.M.M., and M.N.M. J.G.M.M. designed and performed the photolabeling, fractionation, purification, and identification of ceramide-binding proteins. M.N.M. designed, performed, and analyzed the computer simulation studies, with critical input from G.R. and S.J.M. S.K. synthesized photoactive and clickable lipid analogs. S.B., D.G.H., H.J., and M.S. designed and performed the reconstitution and photo-labeling of recombinant mitochondrial channels. S.D. and P.N. created and characterized mutant cell lines, with critical input from F.G.T. S.D. designed and performed transfection studies and photolabeling of purified mitochondria, with critical input from D.G.H.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09654-4.

Competing interests: The authors declare no competing interests.

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Journal peer review information: Nature Communications thanks Alex Evers, Oliver Beckstein, and the other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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