Oxysterol-binding Protein (OSBP)-related Protein 4 (ORP4) Is Essential for Cell Proliferation and Survival*

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Oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) comprise a large gene family with sterol/lipid transport and regulatory activities. ORP4 (OSBP2) is a closely related paralogue of OSBP, but its function is unknown. Here we show that ORP4 binds similar sterol and lipid ligands as OSBP and other ORPs but is uniquely required for the proliferation and survival of cultured cells. Recombinant ORP4L and a variant without a pleckstrin homology (PH) domain (ORP4S) bind 25-hydroxycholesterol and extract and transfer cholesterol between liposomes. Two conserved histidine residues in the OSBP homology domain ORP4 are essential for binding phosphatidylinositol 4-phosphate but not sterols. The PH domain of ORP4L also binds phosphatidylinositol 4-phosphate in the Golgi apparatus. However, in the context of ORP4L, the PH domain is required for normal organization of the vimentin network. Unlike OSBP, RNAi silencing of all ORP4 variants (including a partial PH domain truncation termed ORP4M) in HEK293 and HeLa cells resulted in growth arrest but not cell death. ORP4 silencing in non-transformed intestinal epithelial cells (IEC-18) caused apoptosis characterized by caspase 3 and poly(ADP-ribose) polymerase processing, DNA cleavage, and JNK phosphorylation. IEC-18 transformed with oncogenic H-Ras have increased expression of ORP4L and ORP4S proteins and are resistant to the growth-inhibitory effects of ORP4 silencing. Results suggest that ORP4 promotes the survival of rapidly proliferating cells.

Oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) comprise a 12-member mammalian gene family characterized by a conserved OSBP homology domain (OHD) that binds sterols and lipids, as well as the pleckstrin homology (PH) domain and two phenylalanines in an acidic tract (FFAT) motif that mediate interaction with organelle membranes. OSBP and ORPs have been broadly implicated in sterol and lipid transport, cell signaling, and membrane trafficking (reviewed in Ref. 1), but the primary function of the protein family remains controversial. Most mammalian OSBP/ORPs and yeast OSBP homologue (Osh) proteins bind sterol ligands with high affinity and could be involved in the transport of cholesterol, oxysterols and/or ergosterol between organelles (2–4). However, yeast Osh4 and OSBP also transport phosphatidylinositol 4-phosphate (PI-4P) between liposomes competitively with ergosterol (5, 6), OSBP binds PI-4P and cholesterol competitively (7), Osh3 binds PI-4P but not sterols (8), and phosphatidylycerine (PS) is bound and transported by Osh6 and Osh7 (9). Osh proteins and ORPs also mediate sterol-dependent recruitment of protein complexes that regulate endosome positioning (10, 11), polarized exocytosis at bud sites (12), and PI-4P metabolism by Sac1p at plasma membrane-endoplasmic reticulum contact sites (13). These data suggest an expanded role for the OSBP family in lipid and sterol transport and signaling. Despite these variations in ligand specificity and function, complementation studies in yeast indicate that the OSBP family has a fundamental activity that is confined to the OHD (14).

The mammalian OSBP family can be divided into phylogenetically related subgroups whose members localize to similar cellular compartments and interact with each other and common protein partners (15, 16). As an example, OSBP and ORP4 (OSBP2) paralogues share sequence similarity, physically interact, bind vesicle-associated membrane protein-associated protein (VAP), and have similar affinity for sterol ligands (17, 18). However, there is no evidence that the proteins functionally overlap. Unlike OSBP, alternate transcription start sites in the ORP4 gene result in expression of ORP4L, a full-length version

Background: The function of oxysterol-binding protein-related protein (ORP4) is unknown.
Results: Silencing ORP4L or all ORP4 isoforms triggers growth arrest and apoptosis, which is suppressed by H-Ras and involves the C-terminal lipid binding domain.
Conclusion: ORP4 is a sterol and PI-4P-binding protein required for survival and proliferation of immortalized and transformed cells.
Significance: This is the first study to identify a mammalian ORP with growth regulatory activity.
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that includes an N-terminal PH domain, and ORP4S, a truncated variant missing the PH domain (18). OSBP distributes between the endoplasmic reticulum and Golgi apparatus, where it mediates sterol-dependent ceramide transport to the Golgi apparatus (19, 20). ORP4L does not localize to the Golgi apparatus in response to these stimuli; nor does it affect sphingolipid regulation (18).4 Instead, the ORP4 OHD binds vimentin in vitro and, when overexpressed in cells, interacts with and collapses or “bundles” the vimentin network (17, 18). The functional relevance of the ORP4-vimentin interaction is unknown, but other ORPs and VAP also bind and affect the activity of the actin and microtubule cytoskeleton (reviewed in Ref. 1).

OSBP and ORP4 were recently implicated in the anticancer activity of a set of natural products (21). Cephalostatin, OSW-1, ritterazine B, and schweinfurthin A, collectively termed ORPphilins, competitively inhibited 25-hydroxycholesterol (25OH) binding to OSBP and ORP4L with affinities that correlated with their cytotoxic activity. In addition, silencing or overexpression of OSBP affected the sensitivity of HCT-116 cells to the cytotoxic effects of ORPphilins. Exogenous 25OH competed with ORPphilin binding to OSBP and/or ORP4L, reducing sensitivity to these drugs. However, transient or stable silencing of OSBP by RNAi has no apparent effect on the proliferation or survival of cultured cells (19, 20, 22), indicating that ORP4L and/or other ORPs are targets for these growth-regulatory compounds, or ORPphilins stimulate an antiproliferative activity of OSBP and ORPs. To understand the involvement of ORP4, its three variants were individually or collectively silenced by RNAi to reveal an essential prosurvival and proliferative activity of ORP4L. ORP4L silencing was used to examine the effects of ORPphilins on OSBP or ORP4L constructs (18, 23). PC, PS, phosphatidylethanolamine (PE), lactosyl-PE, and phosophatidylinositol polyphosphates (PIPs) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, free sterols, and phosphatidylcholine were purchased from PerkinElmer Life Sciences.

**EXPERIMENTAL PROCEDURES**

*Materials—*25-[26,27-3H]Hydroxycholesterol and [1,2-3H]-cholesterol were purchased from PerkinElmer Life Sciences. [14C]Dipalmitoyl-phosphatidylcholine (PC) was purchased from American Radiolabeled Chemicals (St. Louis, MO). OSBP and ORP4 polyclonal antibodies were described previously (18). OSBP affinity purification used the immunizing peptide (18, 23). PC, PS, phosphatidylethanolamine (PE), lactosyl-PE, and phosphatidylinositol phospholipids (PIPs) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol and 25OH were purchased from Seraloidics, Inc. (Newport, RI). Secondary antibodies (IRDye 800CW-conjugated goat anti-rabbit and IRDye 680LT-conjugated goat anti-mouse) for immunoblotting were purchased from LI-COR Biosciences (Lincoln, NE). Lentiviral vectors (pLKO.1) encoding shRNA, pCMV-D8.2, and pCMV-VSVG using polyethyleneimine. After 48 h, lentivirus-containing medium was collected, supplemented with 5 μg/ml Polybrene, and applied to HeLa or HEK293 cells. Cells were subsequently selected in medium A with 2 μg/ml puromycin for 24–48 h. Because ORP4 silencing in IEC-18 caused a loss of cell viability and growth arrest by 24–30 h, puromycin selection could not be used, and cells were not subcultured after viral transduction. The efficiency of ORP4 silencing was determined by RT-PCR or immunoblotting.

**Immunoblotting—**Cell lysates were resolved by SDS-polyacrylamide and transferred to nitrocellulose membranes, which were incubated with primary and secondary fluorophore-conjugated antibodies in TBS containing 0.1% Tween 20 and 20% Odyssey blocking buffer (v/v) (LI-COR Biosciences). Proteins were visualized and quantified using an Odyssey infrared imager (LI-COR Biosciences) and application software (version 3.0).

**Expression and Purification of Recombinant ORP4—**cDNAs for ORP4L and ORP4S were amplified by polymerase chain reaction (PCR), cloned into pENTR/D-TOPO, and verified by sequencing. pENTR-ORP4S and -ORP4L were recombined into Baculodirect linear DNA containing a C-terminal His6 tag, transfected into SF21 monolayer cultures, and selected with ganciclovir (100 μM). SF21 cells were transduced with high titer viral stocks (>107 plaque-forming units/ml) at a 0.1–0.2 multiplicity of infection for 72 h, and ORP4 was purified from cell supernatants by metal affinity chromatography as described previously for OSBP (1).

**Sterol-binding Assays—**[3H]25OH and [3H]cholesterol binding by ORP4 was assayed using a Talon resin pull-down method (7). Recombinant OSBP, ORP4L, or ORP4S (8 pmol) was incubated in 75 μl of binding buffer (10 mM HEPES (pH 7.4), 150 mM KCl, 2% (w/v) polyvinyl alcohol) containing radioactive sterols (with or without a 40-fold excess of unlabeled sterol). After 2 h at 20 °C, a 25-μl slurry of Talon resin (1:1, v/v) was added and mixed for 10 min. Talon resin and bound protein were washed three times with 300 μl of 10 mM HEPES (pH 7.4) and 150 mM KCl and sedimented by brief centrifugation. Bound proteins were eluted from the resin with 150 mM imidazole (pH 7.4), the resin was sedimented by centrifugation, and radioactivity bound to protein in the supernatant was measured by liquid scintillation counting.

**Cholesterol and PI-4P Extraction and Transfer Assays—**The cholesterol extraction activity of ORP4L and ORP4S was measured utilizing liposomes prepared by rehydrating lipids in liposome buffer (25 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.4) for

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1 h at 20 °C to a final concentration of 0.5 mm, followed by extrusion through a 400-nm diameter filter (Avestin, Inc., Ottawa, ON). Liposomes were precleared by centrifugation at 13,000 × g for 5 min in a microcentrifuge and stored at 4 °C. [14C]PC was included in donor liposomes as a tracer to measure their presence in supernatant and pellet fractions following extraction and transfer. For extraction assays, donor liposomes contained [14C]PC/PE/PS/PI/lactosyl-PE/[3H]cholesterol (49:20:10:10:1, mol/mol). Liposomes (10 nmol) were incubated with 0–400 pmol of recombinant OSBP, ORP4L, or ORP4S and 3 μg of fatty acid-free BSA in 100 μl of liposome buffer for 25 min at 25 °C and subsequently placed on ice. Donor liposomes were precipitated by the addition of Ricinus communis agglutinin (10 μg) on ice for 15 min and centrifugation at 13,000 × g for 5 min. Radioactivity in the supernatant was measured (2). Liposomes were incubated with 100 pmol of recombinant OSBP, ORP4L, or ORP4S and 3 μg of fatty acid-free BSA in 80 μl of liposome buffer at 25 °C. After 20 min, 20 μl of acceptor liposomes (10 nmol) containing 10 mol% PI or PI-4P was added at 25 °C for an additional 10 min before transfer to ice and precipitation with R. communis agglutinin by centrifugation at 13,000 × g for 5 min. Radioactivity in the supernatant and pellet was measured by liquid scintillation counting. Parallel extraction assays were performed for an additional 20 min to determine the percentage extraction over the full 30 min of the assay. Extraction and transfer values were expressed as a percentage of total [3H]cholesterol extracted from liposomes was calculated as the fraction of input in the supernatant after subtracting background (assay without protein) and donor liposome contamination ([14C]PC).

We used a transfer assay (1) that was modified to include pre-extraction of [3H]cholesterol from donor liposomes prior to acceptor liposome addition. Donor and acceptor liposomes were composed of [14C]PC/PE/PS/[3H]cholesterol (69:20:10:1, mol/mol) and PC/PE/PS/PI-4P/lactosyl-PE (50:20:10:10, mol/mol), respectively. Donor liposomes (10 nmol) were incubated with 100 pmol of recombinant OSBP, ORP4L, or ORP4S and 3 μg of fatty acid-free BSA in 80 μl of liposome buffer at 25 °C. After 20 min, 20 μl of acceptor liposomes (10 nmol) containing 10 mol% PI or PI-4P was added at 25 °C for an additional 10 min before transfer to ice and precipitation with R. communis agglutinin by centrifugation at 13,000 × g for 5 min. Radioactivity in the supernatant and pellet was measured by liquid scintillation counting. Parallel extraction assays were performed for an additional 20 min to determine the percentage extraction over the full 30 min of the assay. Extraction and transfer values were expressed as a percentage of total [3H]cholesterol input and were corrected for background (assays without protein addition) and donor liposome contamination ([14C]PC).

[32P]PI-4P was isolated from HeLa cells that were radiolabeled with [32P]PO4 (0.5 mCi/dish) for 16 h. [32P]PI-4P (40 dpm/pmol) was incorporated into PC/PE/PS/lactosyl-PE/PI-4P (59.5:20:10:10:0.5, mol/mol), and extraction by recombinant ORP4 and GST fusion proteins was assayed as described previously for OSBP (7).

Liposome and Lipid Binding Assays—Liposomes (10 nmol) composed of PC/PE/PI (85:10:5, mol/mol) were mixed with 100 pmol of recombinant ORP in 100 μl of liposome buffer for 25 min at 25 °C and transferred to ice. Liposomes were sedimented by centrifugation at 100,000 × g for 30 min, and the ORP4 distribution between pellet and supernatant was analyzed by SDS-PAGE and staining with Coomassie Blue.

Phosphatidylinositol (PI) and its phosphorylated species (300 pmol), PC, and a solvent control were spotted onto a Hybond-C nitrocellulose membrane for 1 h and incubated overnight in blocking buffer (20 mm Tris–HCl, 150 mm NaCl, 3% (w/v) fatty acid-free BSA, and 0.1% (v/v) Tween 20, pH 7.4) at 4 °C. Membranes were incubated with 50 nm ORP4S or 100 nm of either ORP4L or ORP4L for 1 h in blocking buffer. Filters were probed with antibodies against OSBP or ORP4, followed by a fluorophore-conjugated secondary antibody and detection using an Odyssey infrared imager (LI-COR Biosciences).

Apoptosis Assays—Nucleosome release from detergent-permeabilized IEC-18 and IEC-RAS cells was assayed using an sandwich ELISA kit (Cell Death ELISA Plus, Roche Applied Science). DNA fragmentation was visualized by 2% agarose gel electrophoresis and ethidium bromide staining of genomic DNA extracted from IEC-18 and IEC-RAS cells (24). Caspase 3, poly(ADP-ribose) polymerase (PARP), and Jun N-terminal kinase (JNK)/phospho-JNK were monitored by immunoblotting of whole cell lysates.

ORP4 Expression Vectors—Human ORP4L and ORP4S cDNAs were amplified by PCR, subcloned into pcDNA-V5/His-TOPO, and verified by sequencing. shRNA-resistant ORP4 cDNAs with four silent nucleotide changes in the shORP4-targeting site were made by site-directed mutagenesis of pcDNAV5/His constructs.

Genomic and expressed sequence tag databases indicated a potential ORP4 splice variant encoding a 743-amino acid protein with a PH domain truncation (accession number AK131374). The presence of this new variant (termed ORP4M) in HEK293 and HeLa cells was confirmed by PCR amplification and sequencing, and a protein of the predicted mass (85 kDa) was observed in HeLa, HEK293, and IEC-18 by immunoblotting (see Figs. 5 and 7). An ORP4M expression vector was constructed by PCR amplification of HeLa cell cDNA using primers for exon 1′ (CCCTATTTGTGAGAGACCCG) and exon 3 (CCGCTCTACAGACCTTCTC) (see Fig. 5A). The product was cloned into pcR2.1-TOPO, digested with HindIII and AfeI, and ligated into pcDNA-ORP4L-V5/His that was digested with the same enzymes. The construct was verified by sequencing.

Reverse Transcription-PCR—Total RNA harvested from HEK293 cells using TRIZol reagent was used to synthesize a first strand cDNA using the Thermoscript RT-PCR system (Invitrogen). PCR primer sets were designed to amplify specific ORP4 isoforms or total ORP4 transcripts in HEK293 cells: ORP4L, CGTTAAAGCCCTGCTCTTCTGC and GTGTTTACATA- CGCGGAAGCCCTTCTTG; ORP4M, GAAGGCGCTTTGGCATG- AACCAGTAG and GTGTTACATACGCAGAGCCAGC; ORP4S, TGGTGTCTCTTGGCAATTAC and TTGG- ATGTGATGCGGAAGAGGC; ORP4M transcripts, GTG- AGTGAGACGCCAGGAGCTG (exon 12) and CGGCGC- CGACAGGCCGTCTGCTATT (exon 13). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified using a commercial primer set (Invitrogen). PCR conditions were optimized (15–30 cycles), and the products were separated by 2% (w/v) agarose gel electrophoresis and visualized with ethidium bromide.

Immunomicroscopy and Electron Microscopy—Cells cultured on glass coverslips were fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.05% Triton X-100 at 4 °C for 10 min. Slides were incubated with primary and secondary AlexaFluor-conjugated secondary antibodies in PBS containing 1% (w/v) BSA and mounted in Mowiol-4–88. Images were captured using a Zeiss Axiovert 200M inverted microscope.
equipped with a × 100 oil immersion objective (numerical aperture 1.4) and AxioCam HRm CCD camera.

Recombinant vimentin was purified and stored in tetramer buffer (5 mM Tris-HCl, pH 7.4, 1 mM DTT) containing 8M urea, 1mM EDTA, 0.1 mM EGTA, and 10 mM methyl ammonium chloride (25). Prior to use, vimentin was dialyzed at 4 °C for 30-min intervals in tetramer buffer containing 6, 4, and 2M urea and then overnight in tetramer buffer. For polymerization experiments, 20 pmol of vimentin tetramers and recombinant ORP4L, ORP4S, or buffer (no addition) were incubated on ice in 30 μl of tetramer buffer. Filament assembly was initiated by adding an equal volume of 25 mM Tris-HCl (pH 7.4) and 100 mM NaCl at 37 °C. After 1 h, samples were fixed in 0.1% (w/v) glutaraldehyde, adsorbed onto carbon-coated copper grids and stained with 2% (w/v) uranyl acetate. Images were captured using a JEOL JEM 1230 transmission electron microscope operating at 80 kV.

RESULTS

Sterol and Lipid Binding Properties of ORP4—To determine whether ORP4 variants had differential interaction and/or affinity for lipid ligands, we undertook a comparison of the sterol and PI-4P binding activities of recombinant ORP4L, ORP4S, and OSBP. The His-tagged proteins were expressed by baculovirus transduction of Sf21 cells, purified by metal affinity chromatography, and resolved by SDS-8% PAGE (Fig. 1A). ORP4L displayed high affinity, saturable binding of 25OH ($K_d = 17 \text{ nM}$) and cholesterol ($K_d = 68 \text{ nM}$) (Fig. 1B and C). ORP4S had similar affinity for 25OH ($K_d = 23 \text{ nM}$) and cholesterol ($K_d = 60 \text{ nM}$) but had reduced maximal binding for 25OH compared with ORP4L. $K_d$ values for cholesterol and 25OH are similar to those for His-tagged OSBP assayed under similar conditions (7).

The extraction of [3H]cholesterol from liposomes by ORP4L and ORP4S was assayed by measurement of radioactivity in the supernatant following liposome precipitation (Fig. 1D). ORP4S had similar cholesterol extraction activity as OSBP, which was 50% greater than ORP4L. The transfer of [3H]cholesterol from donor to acceptor liposomes by ORP4L, ORP4S, and OSBP was assayed by an initial 20-min extraction from donor liposomes, followed by the addition of acceptor liposomes (with and without 5 mol% PI-4P) for 10 min (Fig. 1E). The extraction component of the assay was also continued for 30 min to determine whether additional cholesterol was extracted during the remaining 10 min of the assay in the absence of acceptor liposomes (extraction, 30 min). Consistent with the results in Fig. 1D, 100 pmol of OSBP, ORP4L, and ORP4S extracted ~5% of donor liposome cholesterol at 20 min, with no further extraction at 30 min. OSBP transferred over 10% of donor liposome cholesterol to acceptor liposomes independent of PI-4P, indicating that an additional 5% of donor liposome cholesterol was extracted and transferred in the presence of acceptor liposomes. ORP4L transferred <5% of donor cholesterol representing the pre-extracted pool, with no additional extraction/transfer occurring upon the addition of acceptor liposomes. In contrast, ORP4S transferred >15% of donor cholesterol, indicating sustained extraction and transfer in the presence of
acceptor liposomes. This suggests that [3H]cholesterol extraction from donor liposomes was complete by 20 min, but the presence of acceptor liposomes stimulated further rounds of extraction and subsequent transfer. In contrast to previous results (2), cholesterol transfer was not stimulated by PI-4P in this two-stage assay.

We tested whether ORP4 bound PI-4P by measuring its extraction from donor liposomes containing the [32P]-labeled lipid (Fig. 2). Similar to OSBP (7), ORP4L extracted [32P]PI-4P from liposomes in a dose-dependent manner to a maximum of ~20% (Fig. 2A). ORP4S had reduced activity, and maximum extraction was not reached at 200 pmol. To determine the requirements for PI-4P binding, we utilized previously characterized mutants of the ORP4 OHD fused to glutathione S-transferase (GST-ORP4) (17). GST-ORP4 bound 25OH and cholesterol (17) and also extracted [32P]PI-4P from liposomes (Fig. 2B). The mutation of two histidine residues that are predicted to hydrogen-bond with the 4-phosphate of PI-4P (GST-ORP4 H588A/H589A) did not affect sterol-binding activity (17) but reduced PI-4P extraction activity by 60%. The sterol-binding activity of GST-ORP4 ΔS501–505 is reduced by >95% (17), but PI-4P extraction was similar to that of wild-type GST-ORP4.

Characterization of ORP4 PH Domain Specificity—The PH domains of OSBP and ORP4L share a high degree of similarity, including residues predicted to be involved in PI-4P binding (26). However, ORP4L does not localize to organelles enriched in PI-4P, such as the Golgi apparatus, in the presence or absence of oxysterols. A comparison of PI-P binding specificity using a lipid overlay assay showed that ORP4L and OSBP primarily bind PI-4P (Fig. 3A). ORP4S, which does not have a PH domain, displayed nonspecific binding to PIPs but did not bind phosphetidic acid or PS. OSBP bound to liposomes containing PI-4P in preference to other monophosphorylated derivatives but also bound di- and triphosphorylated species, perhaps reflecting nonspecific electrostatic interactions (Fig. 3B). ORP4L binding of liposomal PIPs was relatively nonspecific compared with OSBP, and ORP4S binding to liposomes was not enhanced by the inclusion of PIPs. To test whether the ORP4L PH domain associates with cellular membranes in a PI-4P-dependent manner, a PH domain-GFP fusion was transiently expressed in HeLa cells. GFP-PH-ORP was present on the Golgi apparatus based on co-localization with giantin (Fig. 3C). This interaction appears to be mediated by PIPs because mutating arginine residues at positions 163 and 164 (GFP-PH-ORP4-RR/EE), which are essential for PI-4P binding by OSBP (27), prevented Golgi localization (Fig. 3C). GFP-PH-ORP4 was also associated with small non-Golgi structures that were also evident in cells expressing the R163E/R164E mutant.

In contrast to the isolated ORP4 PH domain, full-length ORP4L is not associated with the Golgi apparatus but has a diffuse cytoplasmic localization as well as partial overlap with the vimentin filament network (Fig. 4, A and D) (17). ORP4S containing only the OHD and FFAT domains strongly localized with vimentin in unusual perinuclear bundles and aggregates. The association with vimentin can also be visualized in vitro by electron microscopy (Fig. 4B). When ORP4L was incubated with purified polymerized vimentin and subjected to negative staining, an electron-dense coat was evident on the surface of the filaments that was absent in controls. ORP4L did not cause appreciable aggregation of vimentin filaments compared with control. In contrast, ORP4S did not coat filaments but instead caused multiple aggregated foci (indicated by arrowheads in Fig. 4B). An explanation for the vimentin aggregation phenotype became apparent when we identified a new ORP4 variant with a truncation of the PH domain that replaces four β-strands at the N terminus with a novel sequence from an alternate exon (Fig. 5A). When transiently overexpressed in CHO cells, this variant (hereafter referred to as ORP4M) had the expected mass on SDS-PAGE (Fig. 4C) and co-localized with aggregated vimentin similar to ORP4S (Fig. 4A). The truncated PH domain of ORP4M is predicted to be non-functional with respect to PI-4P binding, suggesting that the PH domain suppresses the vimentin aggregation phenotype. If this prediction is correct, ORP4L R163E/R164E should also aggregate vimentin because GFP-PH-ORP4-RR/EE is defective in binding PIPs in the Golgi apparatus (Fig. 3C). ORP4L R163E/R164E expressed in CHO cells caused extensive aggregation of the vimentin network but only partially co-localized with vimentin aggregates (Fig. 4D). Thus, a functional PH domain maintains ORP4L in a conformation that prevents vimentin aggregation.

A Growth-regulatory Function for ORP4 Revealed by shRNA Silencing—To identify a function for ORP4, we silenced its expression in HeLa and HEK293 cells. As mentioned, the ORP4 gene encodes ORP4L as well as two truncated variants from alternate transcription start sites (Fig. 5A). After employing numerous RNAi methodologies, we identified a lentiviral shRNA that targeted exon 3 and silenced the expression of all three variants (shORP4) as well as an shRNA that targeted only ORP4L expression by targeting exon 1 (shORP4L). Compared with a non-targeting control (shNT), shORP4 transduction of HEK293 cells reduced the mRNA for ORP4L, ORP4M, and ORP4S and the total ORP4 transcript pool (total ORP4) (Fig. 5B). shORP4L effectively reduced ORP4L mRNA compared with shNT (Fig. 5C). Immunoblotting with an ORP4 polyclonal antibody detected expression of all three variants in lysates from HEK293 cells transduced with shNT (Fig. 5D). Transduction with shORP4 resulted in silencing of all three ORP4 variants by 70–90% (Fig. 5, D and E).
A striking phenotype of ORP4 silencing in HEK293 and HeLa cells was growth arrest. After culturing HEK293 cells transduced with shNT or shORP4 at the indicated densities for 3 days, it was apparent that ORP4 silencing strongly inhibited cell growth (Fig. 6A). In related experiments, HEK293 and HeLa cells were transduced with shORP4 or shORP4L for up to 3 days, and cell density was quantified (Fig. 6, B and C). Again silencing with shORP4 significantly inhibited HEK293 and HeLa cell proliferation compared with the non-targeting control. Reduction of ORP4L expression significantly inhibited HEK293 cell proliferation but only partially suppressed HeLa cell growth. To determine whether the expression of ORP4 isoforms could rescue growth inhibition, shRNA-resistant ORP4 isoforms were transiently expressed in shNT- and shORP4-transduced HeLa cells, and viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 6, D and E). Relative to shNT controls, proliferation of HeLa cells transduced with shORP4 could be partially rescued by transient expression of ORP4L, ORP4S, or ORP4M. Inhibition of HEK293 and HeLa cell proliferation by ORP4 silencing was not associated with caspase 3 activation or DNA cleavage (based on nucleosome release assays), but DNA replication measured by [3H]thymidine incorporation was inhibited by 50–60% (results not shown). Finally, shNT- and shORP4-silenced HeLa cells were immunostained for vimentin and actin to assess changes in cell morphology (Fig. 6E). It was apparent that the actin and vimentin networks were more disorganized in ORP4 knockdown cells compared with controls. shORP4-transduced cells were also misshaped, with many cells having actin- and vimentin-positive plasma membrane extensions. However, morphology of the vimentin network in shORP4-silenced cells was unlike that observed in cells overexpressing ORP4S or ORP4M.

Apoptosis of Intestinal Epithelial Cells Triggered by ORP4 Deficiency Is Blocked by H-Ras Transformation.—To understand the growth arrest phenotype, we compared the effect of ORP4 silencing in non-malignant rat IEC-18 with three IEC-18 clones that had been transformed with human oncogenic H-Ras (IEC-RAS) (28). Unlike IEC-18, IEC-RAS3, -RAS4, and -RAS7 grow independent of the extracellular matrix in culture and are highly tumorigenic when injected into mice. The expression of ORP4 variants in IEC-18- and H-Ras-transformed clones was compared (Fig. 7). IEC-18 cells primarily express the ORP4L and ORP4M variants. ORP4L was significantly overexpressed (7-fold) in IEC-RAS3 and to a lesser extent in IEC-RAS4 and IEC-RAS7 cells, whereas the ORP4M variant was not affected. We previously showed that ORP4S has two variants of ~50 and 60 kDa (18), both of which are increased in all three IEC-RAS clones compared with IEC-18. Total OSBP expression was not affected by H-Ras transformation, but the distribution of phosphorylated species varied between the three clones (7). ORP9L expression was increased only in IEC-RAS3.

Next, we assessed the effect of ORP4 silencing on the growth and viability of IEC-18 and IEC-RAS clones. IEC-18 and IEC-RAS were transduced with lentiviral shRNAs for the indicated times, and expression of ORP4 was assessed by immunoblotting (Fig. 8, A and B). Knockdown with shORP4 for 36 h
resulted in loss of all ORP4 variants, but the degree of silencing was difficult to assess because of variable actin expression due to diminished cell viability. shORP4L caused selective knock-down of only the ORP4L variant (Fig. 8A). Although ORP4 silencing in IEC-RAS3 was not as effective as IEC-18, there was reduced expression of all variants by shORP4 at 48 and 72 h (Fig. 8B). shORP4L silenced the expression of ORP4L in IEC-RAS3 but also reduced the expression of ORP4M. Compared with shNT-transduced cells, there was a substantial 5- and 50-fold increase in the release of soluble nucleosomes in IEC-18 after transduction with shORP4 for 36 and 48 h (Fig. 8C). IEC-18 transduced with shORP4L also had a 10-fold increase in nucleosome release at 48 h. In contrast, there was no significant nucleosome release in IEC-RAS3 transduced with shORP4 or shORP4L. DNA cleavage in IEC-18 was also examined by agarose gel electrophoresis (Fig. 8D). DNA laddering was evident in ORP4-depleted IEC-18, but it was primarily high molecular weight compared with IEC-18 that were treated with camptothecin for 6 or 24 h.

Consistent with nucleosome release and DNA cleavage results shown in Fig. 8, IEC-18 transduced with shORP4 displayed caspase 3 and PARP processing at 36 and 48 h that was similar in magnitude to that of cells treated with camptothecin (Fig. 9A). Also, IEC-18 transduced with shORP4L had significant caspase 3 and PARP processing at 48 h but not at 36 h. IEC-RAS3 were relatively resistant to ORP4 and ORP4L silencing and camptothecin treatment (Fig. 9B).

Activation of JNK is frequently associated with apoptosis induced by genotoxic agents (29, 30). To determine whether loss of ORP4 expression also triggers an apoptotic pathway leading to JNK activation, we quantified the level of Thr-183/Tyr-185-phosphorylated JNK in IEC-18 and IEC-RAS3 cells
Silencing the expression of all ORP4 variants, but not ORP4L alone, in IEC-18 caused a significant 2–3-fold induction of JNK phosphorylation at 30 and 48 h relative to shNT controls. IEC-RAS3 had low levels of phospho-JNK that were not increased by shORP4 and shOPR4L transduction. Silencing with shORP4 or shORP4L in IEC-18 had no effect on extracellular signal-regulated kinase (ERK) phosphorylation (Fig. 9, E and F).

**DISCUSSION**

OSBP and ORPs have a conserved, high affinity lipid and sterol binding domain that, together with the PH and FFAT domains, implicate these proteins in sterol/lipid transfer or signaling between organelle membranes. ORP4 is a closely related paralogue of OSBP that interacts with VAP but is not involved in Golgi activity (17). Here we show that the ORP4 OHD has sterol and PI-4P binding and *in vitro* cholesterol transfer activity, and the PH domain targets PI-4P in the Golgi apparatus. Unlike OSBP, however, ORP4 variants interact with vimentin in a PH domain-dependent manner and have a unique and essential role in proliferation and survival of immortalized and transformed cells.

The concept that OSBP family members are solely sterol-binding proteins has been questioned by the recent identification of yeast Osh OHDs that bind PI-4P (Osh4 and Osh3) and PS (Osh6 and Osh7) (8, 9). The PI-4P headgroup interacts with histidine residues in the conserved OSBP signature sequence at the entrance to the OHD binding pocket of Osh3 and Osh4, indicating that this is a common activity in all Osh proteins. Our results show that ORP4 binds both phospholipid and sterol ligands; ORP4L and ORP4S bound cholesterol and 25OH with high affinity and extracted cholesterol and PI-4P from liposomes. ORP4L extracted a maximum of 20% of PI-4P from liposomes, but ORP4S had reduced activity that did not reach saturation, perhaps reflecting the absence of a PH domain that could enhance interaction with PI-4P-containing liposomes. PI-4P extraction by a GST-ORP4 OHD was inhibited by mutation of the histidine pair predicted to interact with the 4-phosphate but was not affected by a mutation that suppressed sterol binding. Although PI-4P and sterols seem to compete for OHD binding through interaction with mutually exclusive residues, we were unable to show that PI-4P affected ORP4L-, ORP4S-, or OSBP-mediated transfer of cholesterol to acceptor liposomes. This result could reflect differences in affinity for PI-4P...
and cholesterol or the design of the transfer assay, which involved preloading with cholesterol prior to acceptor liposome addition.

ORP4L also displayed PH domain-dependent interaction with PI-4P and other PIPs. Immobilization of PIPs on membranes was used to detect headgroup interaction with the PH domain or other surface residues. In this context, OSBP and ORP4L primarily bound to immobilized PI-4P, but ORP4S bound nonspecifically to PIPs. OSBP and ORP4L bound to liposomes containing PI-4P and other PIPs, indicating a nonspecific, charge-related interaction. However, the results of liposome binding assays should be interpreted with caution because the OHD and PH domain are potentially competing for PI-4P binding. The functionality of the ORP4 PH domain was confirmed by demonstrating that localization of a GFP fusion to the Golgi apparatus was ablated by mutation of residues predicted to interact with the PI-4P headgroup. Unlike OSBP, the PH domain does not mediate Golgi localization of ORP4L but regulates interaction with the vimentin network. Removal (ORP4S), truncation (ORP4M), or mutation (ORP4L-RR/EE) of PI-4P binding residues caused aggregation of the vimentin network. However, ORP4L-RR/EE was only partially localized with vimentin aggregates, indicating that other regions regulate interaction. This interpretation is consistent with previous results showing that mutation of an ORP4L leucine repeat motif caused aggregation and localization with vimentin (17). It is feasible that loss of protein and lipid interactions via the leucine repeat and/or PH domain of ORP4L increases its availability to bind and aggregate vimentin.

Structural and functional dissection of the Osh3 and Osh4 OHD points to PI-4P, and not sterol binding, as a core activity of the OSH family (5, 8). Similarly, OSBP and ORP4 paralogues

FIGURE 6. Silencing of ORP4 causes growth arrest of HEK293 and HeLa cells. A, HEK293 cells that were transduced with lentiviral shNT or shORP4 were seeded at the indicated densities on a 24-well plate, cultured for 3 days, and stained with crystal violet. B and C, HEK293 and HeLa cells seeded on 35-mm dishes were transduced with lentivirus and selected with puromycin for 48 h. Medium was replaced and cells were cultured for up to 3 days. Cells were stained with crystal violet and quantified by densitometry. Results are the mean and S.E. of 3–4 experiments. *, p < 0.05 compared with cells transduced with shNT. D and E, HeLa cells were transiently transfected with empty vector (V, pcDNA-V5/His) or shRNA-resistant ORP4L, ORP4M, ORP4S, or ORP4L and ORP4 cDNAs for 24 h and subsequently transduced with lentiviral shNT or shORP4. After 48 h, cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and expressed relative to shNT-transduced cells. Results are the mean and S.E. of 3–4 experiments. *, p < 0.05 compared with vector controls. The immunoblot (D) shows expression of shRNA-resistant ORP4 isoforms in total lysates of HeLa cells transduced with shNT (nt) or shORP4 (4). F, HeLa cells transduced with shNT or shORP4 for 48 h were immunostained for actin and vimentin using AlexaFluor-488 and -594 secondary antibodies, respectively. Images were acquired as described under “Experimental Procedures” using a ×63 objective.
also possess PI-4P binding activity (7), and residues predicted to interact with PI-4P are conserved in all ORP OHDs (8). The assumption of a core redundant activity, be it PI-4P or sterol binding, for all mammalian ORPs implies that silencing one or more members of this family would not affect essential cellular activities. This assumption has not been formally tested in mammalian cells due to the number of ORP genes and their complex cell-specific expression patterns. For this reason, it was surprising that silencing ORP4 was sufficient to inhibit proliferation of HeLa and HEK293 cells and kill non-malignant IEC. The expression of any one ORP4 variant appeared to be sufficient to support proliferation of HeLa or HEK239 cells. This is based on the observation that 1) shORP4L silencing partially inhibited HeLa cell proliferation and caused delayed apoptosis in IEC relative to shORP4, and 2) expression of shORP4-resistant ORP4L, ORP4M, or ORP4S cDNAs partially (30–40%) restored proliferation of HeLa cells in which all variants were silenced. ORP4 variants share a FFAT motif and

**FIGURE 7. ORP4 expression in normal and Ras-transformed IEC.** Immunoblots of total cell lysates from IEC-18, IEC-RAS3, IEC-RAS4, and IEC-RAS7 cells were probed with antibodies against ORP4, OSBP, ORP9, and actin. Protein expression in IEC-RAS relative to normal IEC-18 was quantified using LI-COR imaging software and normalization to the actin load control. Results are the mean and S.E. (error bars) of four experiments.

**FIGURE 8. DNA processing and nucleosome release in ORP4-depleted IEC-18 is prevented by H-Ras transformation.** A and B, total ORP4 and ORP4L expression was silenced in IEC-18 and IEC-RAS3 cells for 36 and 48 h, respectively, using lentiviral shORP4 and shORP4L. Total cell lysates were immunoblotted using an ORP4-specific polyclonal antibody. C, IEC-18 and IEC-RAS3 cells were transduced with shNT, shORP4, or shORP4L for the indicated times, and DNA fragmentation was assayed based on release of soluble nucleosomes. Results are expressed relative to shNT controls at each time point and are the mean and S.E. (error bars) of 3–5 experiments. D, DNA fragmentation in IEC-18 transduced with shNT, shORP4L, or shORP4 for 36 h or treated with camptothecin (CMT) was visualized by 2% agarose gel electrophoresis and ethidium bromide staining.
OHD, suggesting that prosurvival activity requires sterol, PI-4P, and/or VAP binding activity. However, lack of full restoration of growth by transfection of ORP4-depleted HeLa cells with shRNA-resistant ORP4 cDNAs implies that expression must be within a narrow range to maintain stoichiometric interactions of ORP4 with lipid, sterol, and protein ligands, or the relative proportion of ORP4 variants is a critical factor. Because all ORP4 variants partially supported HeLa cell proliferation to a similar extent but had different effects on the intermediate filament network, we assume that vimentin has a minimal role in the ORP4 prosurvival pathway.

Loss of ORP4 in IEC-18 results in rapid activation of a cell death pathway with the hallmarks of apoptosis, such as DNA fragmentation, nucleosome release, JNK phosphorylation, and caspase 3 and nuclear PARP proteolysis. These apoptotic responses were absent in shORP4-transduced IEC-RAS3, possibly due to constitutive activation of proliferative pathways and suppression of apoptotic pathways characteristic of oncogenic H-Ras-transformed IEC. Alternatively, resistance could stem from the 2–7-fold increased expression of ORP4L and ORP4S in IEC-RAS clones, suggesting that ORP4 expression is required for transformation and not suppressed by RNAi to a

FIGURE 9. ORP4 silencing induces caspase processing and JNK activation. A and B, IEC-18 and IEC-RAS3 cells were transduced with shNT (lanes 1 and 4), shORP4L (lanes 2 and 5), or shORP4 (lanes 3 and 6) for the indicated times or treated with camptothecin for 18 h. Total lysates were prepared and immunoblotted for caspase 3 and PARP. The positions of full-length and proteolyzed caspase 3 and PARP are indicated by arrows. The quantification of caspase 3 and PARP proteolysis (expressed as a ratio of the cleaved fragment/full-length protein) using LI-COR software is shown in the accompanying bar graphs. C and D, total lysates of IEC-18 and IEC-RAS3 transduced with lentiviral shNT, shORP4, or shORP4L were immunoblotted for JNK or phospho-JNK (p-JNK). Phospho-JNK/ JNK was quantified using LI-COR software and expressed relative to shNT controls. Results are the mean and S.E. (error bars) of 3–4 experiments. *, p < 0.05 compared with shNT controls. E and F, IEC-18 were transduced as described above, and the expression of ERK and phospho-ERK (p-ERK) was quantified by immunoblotting. Results are the mean and S.E. of 3–5 experiments.
threshold that triggers cell death. The results of ORP4 silencing in HEK293 and HeLa cells (growth arrest without indices of cell death) also indicate that cancer cells have acquired the ability to partially suppress the requirement for ORP4.

ORP4 is highly expressed in testis and, to a lesser extent, in brain and heart but is virtually absent from other human and mouse tissues (18, 31). A recent report that ORP4 knock-out mice are normal except for male infertility indicates that ORP4 has an essential function in tissues where it is highly expressed. In this case, lack of ORP4 resulted in apoptosis of postmeiotic spermatids and defects in germ cell differentiation (31). ORP4 could have an essential sterol and/or PI-4P transfer or sensing activity that has been re-established in immortalized and transformed cells to provide a growth advantage. In this regard, a series of natural products, collectively termed ORPphilins and including the bis-steroidal cephalostatin, exert their antiproliferative effects by inhibiting OSBP or ORP4L expression and activity (21). The possibility that the antineoplastic activity of these compounds is mediated by inhibiting ORP4L is supported by our RNAi findings. The ORPphilins induce both classical and atypical apoptosis pathways in leukemic cells (32, 33). For instance, cephalostatin promotes DNA fragmentation, caspase 3 activation, JNK phosphorylation, and PARP cleavage (but not cytochrome c), endoplasmic reticulum stress and caspase 9 activation by an apotosome-independent mechanism (33, 35). Some of these events are evident in ORP4-depleted cells, suggesting that cephalostatin and ORPphilins could inactivate an ORP4-dependent cell survival pathway in cancer cells (36). Although ORP4 is unique in the mammalian OSBP family for its essential role in a cell survival pathway, further research is required to more precisely define its cell-specific functions that support proliferation.

REFERENCES
1. Ngo, M. H., Colbourne, T. R., and Ridgway, N. D. (2010) Functional implications of sterol transport by the oxysterol-binding protein gene family. Biochem. J. 429, 13–24
2. Ngo, M., and Ridgway, N. D. (2009) Oxysterol binding protein-related Protein 9 (ORP9) is a cholesterol transfer protein that regulates Golgi structure and function. Mol. Biol. Cell 20, 1388–1399
3. Raychaudhuri, S., Im, Y. J., Hurley, J. H., and Prinz, W. A. (2006) Nonvesicular sterol movement from plasma membrane to ER requires oxysterol-binding protein-related proteins and phosphoinositides. J. Cell Biol. 173, 107–119
4. Schulz, T. A., Choi, M. G., Raychaudhuri, S., Mears, J. A., Ghirlando, R., Shinshaw, J. E., and Prinz, W. A. (2009) Lipid-regulated sterol transfer between closely apposed membranes by oxysterol-binding protein homologues. J. Cell Biol. 187, 889–903
5. de Saint-Jean, M., Dellosse, V., Douget, D., Chichane, G., Payrauste, B., Bourguet, W., Antonny, B., and Grin, G. (2011) Osh4p exchanges sterols for phosphatidylinositol 4-phosphate between lipid bilayers. J. Cell Biol. 195, 965–978
6. Mesmin, B., Bigay, J., Moser von Fileck, J., Lacas-Gervais, S., Drin, G., and Antonny, B. (2010) A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI(4)P exchange by the ER-Golgi tether OSBP. Cell 155, 830–843
7. Goto, A., Liu, X., Robinson, C. A., and Ridgway, N. D. (2012) Multisite phosphorylation of oxysterol-binding protein regulates sterol binding and activation of sphingomyelin synthase. Mol. Biol. Cell 23, 3624–3635
8. Goto, A., Liu, X., Robinson, C. A., and Ridgway, N. D. (2012) Multisite phosphorylation of oxysterol-binding protein regulates sterol binding and activation of sphingomyelin synthase. Mol. Biol. Cell 23, 3624–3635
9. Maeda, K., Anand, K., Chiapparino, A., Kumar, A., Poletto, M., Kaksonen, M., and Gavin, A. C. (2013) Interactive map uncovers phosphatidylycerine transport by oxysterol-binding proteins. Nature 501, 257–261
10. Johansson, M., Lehto, M., Tanhuopää, K., Cover, T. L., and Olkkonen, V. M. (2005) The oxysterol-binding protein homologue ORP1L interacts with Rab7 and alters functional properties of late endocytic compartments. Mol. Biol. Cell 16, 5480–5492
11. Johansson, M., Rocha, N., Zwart, W., Jorens, I., Janssen, L., Kuijl, C., Olkkonen, V. M., and Neefjes, J. (2007) Activation of endosomal dynein motors by stepwise assembly of Rab7–RILP–p150Glued, ORP1L, and the receptor β1β1 spectrin. J. Cell Biol. 176, 459–471
12. Alfaro, G., Johansen, J., Dighe, S. A., Duamel, G., Kozminski, K. G., and Beh, C. T. (2011) The sterol-binding protein Kes1/Osh4p is a regulator of polarized exocytosis. Traffic 12, 1521–1536
13. Stefan, C. J., Manford, A. G., Baird, D., Yamada-Hannf, J., Yao, M., and Emr, S. D. (2011) Osh proteins regulate phosphatidinositide metabolism at ER-plasma membrane contact sites. Cell 144, 389–401
14. Beh, C. T., Cool, L., Phillips, J., and Rine, J. (2001) Overlapping functions of the yeast oxysterol-binding protein homologues. Genetics 157, 1117–1140
15. Ridgway, N. D. (2010) Oxysterol-binding proteins. Subcell. Biochem. 51, 159–182
16. Vihervaara, T., Janssen, M., Uronen, R. L., Ohkaki, Y., Ikonen, E., and Olkkonen, V. M. (2011) Cytoplasmic oxysterol-binding proteins: sterol sensors or transporters? Chem. Phys. Lipids 164, 443–450
17. Wyles, J. P., Perry, R. J., and Ridgway, N. D. (2007) Characterization of the sterol-binding domain of oxysterol-binding protein (OSBP)-related protein 4 reveals a novel role in vimentin organization. Exp. Cell Res. 313, 1426–1437
18. Wang, C., JeBailey, L., and Ridgway, N. D. (2002) Oxysterol-binding-protein (OSBP)-related protein 4 binds 25-hydroxysterols and interacts with vimentin intermediate filaments. Biochem. J. 361, 461–472
19. Perry, R. J., and Ridgway, N. D. (2006) Oxysterol-binding protein and vescicle-associated membrane protein-associated protein are required for sterol-dependent activation of the ceramide transport protein. Mol. Biol. Cell 17, 2604–2616
20. Banerji, S., Ngo, M., Lane, C. F., Robinson, C. A., Minogue, S., and Ridgway, N. D. (2010) Oxysterol binding protein (OSBP)-dependent activation of sphingomyelin synthesis in the Golgi apparatus requires PtdIns 4-kinase IIa. Mol. Biol. Cell 21, 4141–4150
21. Burgett, A. W., Poulsen, T. B., Wangkanont, K., Anderson, D. R., Kikuchi, C., Shimada, K., Okubo, S., Fortner, K. C., Mimaki, Y., Kuroda, M., Murphy, J. P., Schwabl, D. J., Petrella, E. C., Cornella-Taracio, I., Schirle, M., Tallarico, J. A., and Shair, M. D. (2011) Natural products reveal cancer cell dependence on oxysterol-binding proteins. Nat. Chem. Biol. 7, 639–647
22. Nishimura, T., Inoue, T., Shibata, N., Sekine, A., Takabe, W., Noguchi, N., and Ara, H. (2005) Inhibition of cholesterol biosynthesis by 25-hydroxysterol is independent of OSBP. Genes Cells 10, 793–801
23. Wyles, J. P., McMaster, C. R., and Ridgway, N. D. (2002) Vesicle-associated membrane protein-associated protein-A (VAP-A) interacts with the oxysterol-binding protein to modify export from the endoplasmic reticulum. J. Biol. Chem. 277, 29908–29918
24. Duke, R. C., Chervenak, R., and Cohen, J. H. (1983) Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytosis. Proc. Natl. Acad. Sci. U.S.A. 80, 6361–6365
25. Herrmann, H., Hofmann, I., and Franke, W. W. (1992) Identification of a nonapeptide motif in the vimentin head domain involved in intermediate filament assembly. J. Mol. Biol. 223, 637–650
26. Perry, R. J. and Ridgway, N. D. (2005) Molecular mechanisms and regulation of ceramide transport. Biochim. Biophys. Acta 1734, 220–234
27. Levine, T. P., and Munro, S. (2002) Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components. Curr. Biol. 12, 695–704
28. Rak, J., Mitsuhashi, Y., Erdos, V., Huang, S. N., Filmus, J., and Kerbel, R. S. (1995) Massive programmed cell death in intestinal epithelial cells induced by three-dimensional growth conditions: suppression by mutant c-H-ras oncogene expression. J. Cell Biol. 131, 1587–1598
29. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995)
Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326–1331

30. Helbig, L., Damrot, J., Hülsenbeck, J., Köberle, B., Brozovic, A., Osmak, M., Fiket, Z., Kaina, B., and Fritz, G. (2011) Late activation of stress-activated protein kinases/c-Jun N-terminal kinases triggered by cisplatin-induced DNA damage in repair-defective cells. J. Biol. Chem. 286, 12991–13001

31. Udagawa, O., Ito, C., Ogonuki, N., Sato, H., Lee, S., Tripvanuntakul, P., Ichi, I., Uchida, Y., Nishimura, T., Murakami, M., Ogura, A., Inoue, T., Toshimori, K., and Arai, H. (2014) Oligo-astheno-teratozoospermia in mice lacking ORP4, a sterol-binding protein in the OSBP-related protein family. Genes Cells 19, 13–27

32. Rudy, A., López-Antón, N., Dirsch, V. M., and Vollmar, A. M. (2008) The cephalostatin way of apoptosis. J. Nat. Prod. 71, 482–486

33. Dirsch, V. M., Müller, I. M., Eichhorst, S. T., Pettit, G. R., Kamano, Y., Inoue, M., Xu, J. P., Ichihara, Y., Wanner, G., and Vollmar, A. M. (2003) Cephalostatin 1 selectively triggers the release of Smac/DIABLO and subsequent apoptosis that is characterized by an increased density of the mitochondrial matrix. Cancer Res. 63, 8869–8876

34. Müller, I. M., Dirsch, V. M., Rudy, A., López-Antón, N., Pettit, G. R., and Vollmar, A. M. (2005) Cephalostatin 1 inactivates Bcl-2 by hyperphosphorylation independent of M-phase arrest and DNA damage. Mol. Pharmacol. 67, 1684–1689

35. López-Antón, N., Rudy, A., Barth, N., Schmitz, L. M., Pettit, G. R., Schulze-Osthoff, K., Dirsch, V. M., and Vollmar, A. M. (2006) The marine product cephalostatin 1 activates an endoplasmic reticulum stress-specific and apoptosome-independent apoptotic signaling pathway. J. Biol. Chem. 281, 33078–33086

36. García-Prieto, C., Riaz Ahmed, K. B., Chen, Z., Zhou, Y., Hammoudi, N., Kang, Y., Lou, C., Mei, Y., Jin, Z., and Huang, P. (2013) Effective killing of leukemia cells by the natural product OSW-1 through disruption of cellular calcium homeostasis. J. Biol. Chem. 288, 3240–3250