Oxysterol-binding protein-related protein 5 (ORP5) promotes cell proliferation by activation of mTORC1 signaling

**Ximing Du**¹, *, **Armella Zadoorian**¹, **Ivan E. Lukmantara**¹, **Yanfei Qi**¹, **Andrew J. Brown**¹, and **Hongyuan Yang**¹, *

From the ¹School of Biotechnology and Biomolecular Sciences, the University of New South Wales, Sydney, NSW, 2052, Australia

*Running title: ORP5 regulates mTORC1*

*To whom correspondence should be addressed: Ximing Du, tel: 61-2-93858112, fax: 61-2-93851483, x.r.du@unsw.edu.au; Hongyuan Yang, tel: 61-2-93858133, fax: 61-2-93851483, h.rob.yang@unsw.edu.au.

**Keywords:** OSBP, ORP5, ORP8, mTOR, mTORC1, lipid, cell proliferation, mechanistic target of rapamycin, oxysterol-binding protein related protein 5, cancer

**ABSTRACT**

Oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) constitute a large family of proteins that mainly function in lipid transport and sensing. ORP5 is an endoplasmic reticulum (ER)-anchored protein implicated in lipid transfer at the contact sites between the ER and other membranes. Recent studies indicate that ORP5 is also involved in cancer cell invasion and tumor progression. However, the molecular mechanism underlying ORP5’s involvement in cancer is unclear. Here, we report that ORP5 promotes cell proliferation and motility of HeLa cells, an effect that depends on its functional OSBP-related domain (ORD). We also found that ORP5 depletion or substitutions of key residues located within ORP5-ORD and responsible for interactions with lipids interfered with cell proliferation, migration and invasion. ORP5 interacted with the protein mechanistic target of rapamycin (mTOR), and this interaction also required ORP5-ORD. Of note, whereas ORP5 overexpression induced mTOR complex 1 (mTORC1) activity, ORP5 down-regulation had the opposite effect. Finally, ORP5-depleted cells exhibited impaired mTOR localization to lysosomes, which may have accounted for the blunted mTORC1 activation. Together, our results suggest that ORP5 expression is positively correlated with mTORC1 signaling and that ORP5 stimulates cell proliferation, at least in part, by activating mTORC1.

**INTRODUCTION**

Alterations in cell signaling pathways that control normal cell growth are very common in cancer. In particular, deregulation of the mechanistic target of rapamycin (mTOR) signaling is known to play an important part in cancer development (1,2). mTOR is an evolutionarily conserved protein kinase that is present in two distinct complexes, each containing several other proteins, termed mTOR complex 1 (mTORC1) and mTORC2.
mTORC1 is a central regulator of cell proliferation. It senses nutrients, especially amino acids, as well as energy levels and stress, and regulates cell proliferation in response to these signals. In this regard, activated mTORC1 phosphorylates and activates its major downstream effector ribosomal protein S6 kinase (S6K). Once activated, S6K phosphorylates the ribosomal S6 protein to control fundamental cellular processes including protein synthesis, lipid metabolism, and cell proliferation (3,4).

Oxysterol-binding protein (OSBP)-related protein 5 (ORP5) belongs to a large family of lipid transfer proteins. In mammalian cells, the OSBP/ORPs protein family consists of twelve members with variant sequence identities, giving rise to six subfamily groups (5,6). OSBP/ORPs share a characteristic feature of a conserved carboxyl-terminal OSBP-related domain (ORD) or ligand-binding domain. The N-termini of these proteins often possess a FFAT motif (diphenylalanine in an acidic tract) and a pleckstrin homology (PH) domain. The FFAT motif and the PH domain are responsible for targeting OSBP/ORPs to endoplasmic reticulum (ER) membranes and non-ER organelle membranes, respectively (7-9). These two membrane-targeting determinants enable OSBP and some ORPs to function at ER-associated membrane contact sites, where they contribute to the intracellular exchange of lipids (10). For example, OSBP associates with the Golgi through its PH domain and the ER through FFAT/VAPs interaction (9). Tethering the ER and Golgi, OSBP has been shown to transport cholesterol from the ER to the Golgi by the ORD, where it exchanges cholesterol with PI(4)P (phosphatidylinositol-4-phosphate) and transfers it back to the ER (11).

Within the OSBP/ORPs family, ORP5 and ORP8, which lack the FFAT motif, are the only members that have a transmembrane domain anchoring them to the ER (12,13). ER-anchored ORP5 has been identified as a phosphatidylserine (PS) transporter through its ORD (14,15). In a counter-transport process occurring at the membrane contact sites, ORP5 and ORP8 were shown to transfer PS from the ER to the plasma membrane and PI(4)P and PI(4,5)P2 from the plasma membrane to the ER (15,16). Interestingly, ORP5 and ORP8 were also reported to transport PS from the ER to mitochondria and maintain proper mitochondrial function (17). Despite seemingly working together to transport lipids at the membrane contact sites, ORP5 and ORP8 appear to have different functions in the regulation of cellular homeostasis (18). Notably, ORP8 expression reportedly inhibits cell proliferation and tumor growth while enhancing apoptosis (19). In contrast, ORP5 expression has been linked to increased cancer cell invasion and metastasis. For example, a previous report showed that the invasion rate of both hamster and human pancreatic cancer cells is enhanced by ORP5 overexpression and reduced by ORP5 depletion (20). Importantly, analysis of clinical samples suggested that poor prognosis in human pancreatic cancer is associated with high expression levels of ORP5 (20). In a recent study, tissue microarray analysis revealed that ORP5 is highly expressed in lung tumor tissues, especially in the lung tissues of metastasis-positive cases (21).

Why overexpression of ORP5 is positively correlated with cancer cell invasion and tumor progression is unknown. Specifically, it is unclear whether this correlation involves alterations of cell signaling pathways. In the current study, we provide evidence that mTORC1 signaling is positively regulated by ORP5. Our data suggest that ORP5 facilitates cancer cell proliferation and motility at least in part through mTORC1 activation.

RESULTS
Overexpression of ORP5 promotes cell proliferation and migration

Elevated expression of ORP5 has been associated with pancreatic and lung cancers (20,21). One of the aims of this study is to verify whether increased expression of ORP5 can drive cell proliferation. For this purpose, we chose HeLa cells because the level of endogenous ORP5 in HeLa cells is relatively low. This system not only allowed us to test wild type ORP5, but also mutant forms of ORP5 in cell proliferation. To study the effect of ORP5 overexpression, we used a retroviral vector system (Clontech pQCXIN) and generated a stable line of HeLa cells overexpressing ORP5. Using a goat polyclonal ORP5 antibody, we detected a robust band of ~110 kDa corresponding to ORP5 in the stable cell line (HeLa/ORP5) (Figure 1A, lane 2). It has been shown that overexpression of ORP5 induces pancreatic cancer invasion and lung tumor formation (20,21). We predicted that overexpression of ORP5 in HeLa cells may facilitate cell proliferation and motility. To test this hypothesis, we carried out cell proliferation and migration assays in HeLa/ORP5 and Mock cells. Indeed, compared to the control, HeLa/ORP5 cells had a significantly higher proliferation rate (increased by ~50%) (Figure 1B). Cell migration rate was also significantly enhanced in HeLa/ORP5 cells by ~60% compared with the Mock control (Figure 1C). If ORP5 overexpression facilitates cell proliferation and migration, depletion of ORP5 should blunt this effect. We used a set of three specific siRNAs against ORP5 to efficiently reduce ORP5 expression in HeLa/ORP5 cells (Figure 1A, lane 6-8). As expected, depletion of ORP5 inhibited both cell proliferation and migration to an extent comparable to that of the mock control cells (Figure 1D and 1E).

A functional ORP5-ORD is critical for facilitating cell growth and motility

ORP5 is a lipid transfer protein and its ORD domain is responsible for extracting, binding and transporting lipid cargos between the ER and other organelle membranes (12,16,17). To investigate whether a functional ORD of ORP5 is required for facilitating cell proliferation and migration, we mutated some critical residues within ORP5-ORD conserved for binding PS (L389D) (14), PI(4)P (K446A, H478A/H479A, K670A) or possibly cholesterol (H538A/K540A) (16,22) (Figure 2A). Transient transfection in HeLa cells with GFP-fused wildtype ORP5 and these mutant cDNAs displayed comparably high transfection efficiency under fluorescent microscope (70-80%, Figure S1). The transiently transfected HeLa cells were used for testing cell proliferation and migration. Compared with empty vector control, wild-type ORP5 transfection significantly enhanced cell proliferation and migration (Figure 2B, C and D), which is consistent with the observations in HeLa/ORP5 vs HeLa control cells (Figure 1B and C). A significant reduction of cell proliferation and migration rates was seen in the cells transfected with lipid binding mutants compared with wild-type ORP5 (Figure 2B, C and D). Importantly, the PS binding mutation (L389D) and the PI(4)P binding mutations (H478A/H479A) also resulted in a significant reduction of cell invasiveness based on the assay using the Matrigel invasion chamber (Figure 2E), highlighting the critical importance of a functional ORP5-ORD in facilitating cell motility. Together, these data suggest that lipid binding/transfer function of ORP5 is essential for its role in promoting cell growth.

ORP5 interacts with mTOR

We hypothesized that the role of ORP5 in promoting cell proliferation may involve other ORP5-interacting proteins. To this end, we performed liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis to identify potential ORP5 interacting proteins.
The goat polyclonal ORP5 antibody was incubated with the cell lysates prepared from HeLa/ORP5 and immunoprecipitation performed with normal goat IgG as a control. After immunoprecipitation, the efficiency of ORP5 pull down was verified by Coomassie blue staining and anti-ORP5 Western blot analysis (Figure S2A, B). LC-MS/MS analysis of ORP5 immunoprecipitates identified a list of proteins (Figure S2C), among which we selected mTOR (MASCOT ID score: 32.5 – 49.6; peptide hits = 26) for further investigation since this serine/threonine-protein kinase is a master regulator of cell proliferation and survival (1).

We performed immunoprecipitation experiments to confirm ORP5-mTOR interaction. Firstly, we analyzed samples pulled-down by the control or ORP5 antibody from the HeLa/ORP5 cell lysates. Western blot analysis using antibodies to ORP5, mTOR, Calnexin (an ER integral-membrane protein), Akt (an upstream effector of mTOR) and p70-S6K (a downstream of mTOR signaling) showed that only mTOR was detected along with ORP5 (Figure 3A, lane 4). Secondly, we transiently transfected HeLa cells with cDNAs for mCherry empty vector (EV), mCherry-fused ORP5 or ORP8, and used anti-RFP antibody to carry out immunoprecipitation. Anti-mTOR Western blot analysis demonstrated that mCherry-ORP5, and not mCherry-ORP8, interacted with mTOR (Figure 3B, lane 5). We next used the in situ proximity ligation assay (PLA) to further verify ORP5-mTOR interaction. PLA allows direct visualization and quantitation of protein-protein interaction. When HeLa/ORP5 cells were incubated with ORP5 primary antibody alone, only negligible PLA signal was observed and hardly detectable. However, when the cells were treated with both ORP5 and mTOR primary antibodies, there was a robust, significantly amplified PLA signal that could be clearly detected in every single cell (Figure 3C, D). The PLA signal observed in these experiments was specific, as the signal was significantly weakened in cells depleted with ORP5 by siRNAs (Figure 3E, F).

Next, we wanted to examine which domain of ORP5 is required for its interaction with mTOR. We constructed GFP-fused ORP5 deletion mutants that lack the N-terminal PH domain (GFP-ORP5ΔPH), ORD (GFP-ORP5ΔORD), as well as both the PH domain and ORD (GFP-ORP5ΔPHΔORD) (Figure 4A). GFP empty vector (EV), GFP-fused wild-type ORP5, or the deletion mutants were co-transfected into HEK-293 cells together with Myc-mTOR cDNA. Anti-GFP immunoprecipitation analysis revealed that the PH domain of ORP5 is dispensable for its interaction with mTOR as GFP-ORP5ΔPH similarly associated with myc-mTOR when compared to wild-type GFP-ORP5 (Figure 4B, lane 8). In stark contrast, deletion of ORD severely diminished the physical association between ORP5 and mTOR (Figure 4B, lane 9, lane 10), suggesting that ORP5-ORD is required for ORP5-mTOR interaction. Consistent with this observation, the reverse anti-Myc immunoprecipitation also demonstrated that mTOR strongly associates with wild-type GFP-ORP5 but not GFP-ORP5ΔORD (Figure 4C, lane 5 vs lane 6). The dependency on ORP5-ORD appears to be separable from its lipid transfer activity, as the point mutations within ORP5-ORD (L389D and H478A/H479A) abolishing PS or PI(4)P binding did not affect ORP5-mTOR interaction (Figure 4D, 4E). This may be due to the observation that mutations abolishing lipid transfer capability do not alter the overall conformation of ORP5-ORD (14,22). Taken together, these results demonstrate that ORP5 interacts with mTOR and that ORP5-ORD is required for this interaction.

ORP5 is involved in mTORC1 signaling
In mammalian cells, mTOR forms two functionally distinct complexes, namely mTORC1 and mTORC2. mTORC1 contributes to cell growth and proliferation by directly phosphorylating S6 kinase (S6K), which activates ribosomal S6 protein, ultimately promoting protein synthesis (3). The role of ORP5 in facilitating cell proliferation and the association between ORP5 and mTOR prompted us to ask whether ORP5 plays any role in mTORC1 signaling. We treated HeLa/Mock and HeLa/ORP5 cells with control or ORP5 siRNAs and analyzed the phosphorylation of S6K and S6. When treated with control siRNA, a significant increase of S6K and S6 phosphorylation was seen in HeLa/ORP5 cells (Figure 5A, lane 3 vs lane 1, 5B, 5C), indicating that overexpression of ORP5 in HeLa cells induces mTORC1 activation. ORP5 depletion resulted in downregulation of the phosphorylation of S6K and S6, and this effect was more drastic in HeLa/ORP5 cells (Figure 5A, lane 4, B, C). Treatment of HeLa/ORP5 cells with two different ORP5 siRNAs showed similar results in terms of S6 phosphorylation (Figure 5D, E). The downregulation of S6 phosphorylation by ORP5 knockdown appears to be mTORC1-dependent, since in our system a complete inhibition of S6K activation by rapamycin treatment also shut down S6 phosphorylation (Figure S3). Nascent protein synthesis assay in HeLa/ORP5 cells treated with control or ORP5 siRNAs indicated that ORP5 knockdown significantly decreased protein synthesis (Figure 5F, G), a phenotype that reflected the downregulation of S6K and S6 phosphorylation downstream of mTORC1. These data indicate that, at least when overexpressed, ORP5 is positively associated with mTORC1 activation.

Knockdown of endogenous ORP5 impairs S6K activation and cell proliferation

Next, we investigated whether endogenous ORP5 regulates mTORC1 signaling. The expression of ORP5 has been linked to the induction of pancreatic cancer cell invasion (20). This prompted us to examine the effect of ORP5 depletion on mTORC1 signaling and cell proliferation in PANC-1 cells, a pancreas ductal adenocarcinoma cell line. The two specific ORP5 siRNAs efficiently reduced endogenous ORP5 protein levels in PANC-1 cells (Figure 5H). Anti-phosphorylated S6K immunoblotting analysis clearly revealed that S6K phosphorylation was downregulated upon the depletion of ORP5 (Figure 5H). Accordingly, ORP5 depletion severely impaired normal cell growth and cell number expansion (Figure 5I). This observation was also in accordance with the results from cell proliferation assay, which indicated a significant impairment of cell proliferation when ORP5 was depleted in PANC-1 cells (Figure 5J). We observed similar results in another pancreatic cancer cell line, Capan-1 cells. In Capan-1 cells, there was a clear decrease of mTORC1 activation and significant reduction of cell growth when ORP5 was depleted (Figure S4). Importantly, the growth defect in ORP5-depleted Capan-1 cells was rescued when TSC1, an inhibitory factor of mTORC1 activation (23), was silenced (Figure S4), highlighting the link between ORP5 and mTORC1.

ORP5 depletion impairs the localization of mTOR to lysosomes

Targeting of mTORC1 to the lysosomal surface is essential for its activation (24). We hypothesized that depletion of ORP5 may interfere with the translocation of mTORC1 to lysosomes, thereby impairing mTORC1 signaling. To test this hypothesis, we examined the co-localization of endogenous mTOR with LAMP-1, a lysosomal membrane marker, by immunofluorescence analysis in HeLa/ORP5 cells. In control siRNA treated cells and in agreement with the previous study (23), the majority of endogenous mTOR localized to LAMP-1-positive lysosomal
ORP5 regulates mTORC1 structures (Figure 6A, top panel). Quantification of mTOR/LAMP-1 co-localization revealed a significant decrease of the association of mTOR with lysosomes (Figure 6A, 6B) in ORP5 siRNAs-treated cells. Similar results were obtained from the immunofluorescence experiments performed in PANC-1 cells. Knocking down of endogenous ORP5 in PANC-1 cells also significantly decreased mTOR/LAMP-1 co-localization (Figure 6C, 6D), without affecting overall mTOR protein levels in the cells examined (Figure 6E).

**DISCUSSION**

OSBP/ORPs are unified by their characteristic ORDs that render them capable of sensing, binding, and transporting lipids between intracellular membranes. Previous studies have implicated some members of the OSBP/ORPs family in the regulation of cell signaling pathways, such as OSBP in ERK1/2 activation (25) and ORP4L in Ca\(^{2+}\) signaling (26). Here we uncover another connection between OSBP/ORPs and cellular signaling events. We show that overexpression of ORP5 promotes cell proliferation and motility, and that ORP5 expression is positively correlated with mTORC1 signaling. In this context, we present evidence that ORP5 interacts with mTOR and contributes to the targeting of mTORC1 to lysosomal surface for activation.

As a lipid transfer protein, ORP5 has been shown to play a role in intracellular lipid transport at membrane contact sites associated with the ER (12,15-17). Membrane lipid homeostasis regulated by ORP5 and other lipid transfer proteins has an impact on mTORC1, which itself is a key regulator of lipid metabolism, as well as protein synthesis and cell proliferation (4). Notably, ORP5 appears to be a phosphatidylinositol transporter (14) and has been shown to deliver phosphatidylinositol from the ER to the plasma membrane and mitochondria (15-17). Phosphatidylinositol is critical for the organized distribution of cholesterol in the cytosolic leaflet of the plasma membrane and possibly other organelles (27). Hence, phosphatidylinositol transport mediated by ORP5 may be coupled with cholesterol movements between intracellular membranes. Since proper intracellular cholesterol trafficking is essential for mTOR signaling (28), it is possible that phosphatidylinositol transport by ORP5 is favored for mTORC1 activation in certain cancer cells, contributing to increased cell proliferation. It is worth noting that membrane phosphatidylinositol binds to specific residues in the PH and regulatory domain of Akt, an upstream effector of mTORC1 (29). The phosphatidylinositol-Akt interaction is required for Akt activation and downstream signaling (29). This may represent another possible explanation of why ORP5 expression is positively correlated with mTORC1 signaling. In agreement with this possibility, we found that in HepG2 cells, ORP5 depletion impaired insulin-induced Akt activation and mTORC1 signaling (Du and Yang, unpublished data). Therefore, the ability of ORP5 to transport phosphatidylinositol seems to be critical for the regulation of mTORC1. In line with this, mutation of a key residue in ORP5-ORD (L389D) responsible for phosphatidylinositol binding and transfer failed to promote cell proliferation, migration, and invasion. Similar effects obtained from ORP5-ORD mutants incapable of transporting phosphoinositides or other lipids could be secondary to the impairment of phosphatidylinositol transport. However, we could not rule out the possibility that these mutants have a direct impact on the homeostasis of membrane phosphoinositides, resulting in the downregulation of the Akt/mTORC1 pathway (30). It should also be noted that while ORP5 overexpression in HeLa cells was needed for assessing the gain-of-function effects of ORP5 and its mutants, it
remains possible that the use of ORP5 overexpression in HeLa cells yields non-physiological results.

An important finding of the current study is that proteomics analysis identified mTOR as one of possible ORP5 interacting proteins. The results from the co-immunoprecipitation and proximity ligation assays confirmed that ORP5 is closely associated with mTOR, suggesting that ORP5 might be able to directly regulate mTORC1. Interestingly, the association between ORP5 and mTOR depends on ORP5-ORD, and this dependency does not seem to rely on the lipid transfer activity of ORP5. Future studies are needed to pinpoint critical regions or residues responsible for ORP5-mTOR interaction. It is also possible that deletion of ORD influences the localization of ORP5 and hence the interaction between ORP5 and mTOR. For example, loss of ORD has been shown to dissociate ORP5 from the mitochondrial surface (17). Given the observation that mTORC1 can be found on mitochondria and regulates mitochondrial functions (31), the dissociation of ORP5 from mitochondria due to ORD deletion could justify the loss of the interaction between ORP5 and mTOR. For instance, ORP5 may recruit mTORC1 to lysosomes through the interaction with mTOR. This would explain why mTORC1 translocation to the lysosomal surface is impaired when ORP5 is deficient. However, it is also possible that the regulation of mTORC1 activation by ORP5 could be independent of the interaction between ORP5 and mTOR. As mentioned earlier, lipid transfer or targeting activity by ORP5 may be the key to regulate mTORC1 activation. For instance, ORP5 may regulate the homeostasis of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2), which is concentrated in endolysosomal membrane (32). Of note, PI(3,5)P2 has been shown to bind to the mTORC1 component raptor and could contribute to lysosomal targeting of mTORC1 (33). Future studies involving co-localization between PI(3,5)P2 and mTORC1, as well as in vitro lipid transfer assay and lipidomics analysis, are required to test this hypothesis.

In summary, we show that the lipid transfer function of ORP5 is critical for ORP5 to promote cell proliferation. Importantly, we identify a novel link between ORP5 and mTORC1 signaling which provides another explanation for the involvement of ORP5 in cancer cell proliferation. Our work suggests that
ORP5 may be a therapeutic target, alongside mTORC1 inhibition, to treat certain cancers.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

HeLa, PANC-1 and Capan-1 cells were obtained from ATCC. Monolayers of cells were maintained in DMEM supplemented with 10% FBS (for Capan-1 cells 20% FBS was used), 100 units/ml penicillin, and 100 µg/ml streptomycin sulphate in 5% CO2 at 37°C. DNA transfection was performed using Lipofectamine™ LTX and Plus Reagent (Life Technologies) according to manufacturer’s instruction. siRNA transfection was carried out in cells grown in full serum medium according to standard methods using Lipofectamine™ RNAiMAX transfection reagent (Life Technologies).

RNAi and cDNA constructs

siRNA against ORP5 was obtained from Sigma-Aldrich (Product # SASI_Hs02_00365256, SASI_Hs01_00039221, SASI_Hs02_00365257). ORP5 cDNA was cloned between NotI and PacI sites of the pQCXIN retroviral vector (Clontech) using standard subcloning procedure. GFP-ORP5 and pmCherry-ORP8 were descried previously11. GFP-ORP5 point or deletion mutations (L389D, K446A, H478A/H479A, H538A/K540A, K670A, ΔPH, ΔORD, ΔPHΔORD) were generated by site-directed mutagenesis using the following oligos: L389D, F5’-ATGGACCTGTCCCGCGTGGTGGACCCAC TGTTGTACTGGGAGGCGG-3’; R5’-CGGCTCCAGTACGAACGTGGGGTCCACC ACGCGGGACAGGTCCAT-3’; K446A, F5’-GGCCCAAAGGTGAGACGCTGGGCTCCA TCACACAGCCCTGCC-3’, R5’-CTCACCTTTGGGCGCTTACCGGTGCTCA GCCATGATGACCCAGC-3’; H538A/K540A, F5’-CCCTTACCATGCCTACGCGCCTGTCAG CAGGAATCCTGTATGGCAGAT, R5’-ATCGTGCATACAGGATCTCTGGGACGG CGCGTACGCCATGTGAAGG; ΔPH, F5’-GATTCCCTTGGGTATCCTCTCCACCG CAGCCCTGGGAGAGCAG-3’, R5’-AAGCCCAAAGGAATCAAGAGCCCATTAC ACCCCATCTGGGGGAG-3’, ΔORD, F5’-GAGGACACCAGCCCTGGGACCCCTGAG AAGCATGCCCGCATGG-3’, R5’-GGGGCTGTGCTCCTCTACGACACGCGCG GACAGGTCCATGCTGG-3’. myc-mTOR was a gift from David Sabatini (Addgene plasmid # 1861).

Generation of HeLa/ORP5 stable cell line

Retrovirus particles were produced in the packaging cell line 293T Phoenix-AMPHO expressing pQCXIN-ORP5. HeLa cells were transduced with the retrovirus encoding ORP5 or mock control for 48 h. Transduced cells were selected with growth medium containing 500 µg/ml G418 (Sigma Aldich) for 10 d. During the selection, cells were passaged every 2 d until a stable line of cells expressing ORP5 was established.

Antibodies

Antibodies used were rabbit polyclonal to mTOR [Cell Signaling Technology (CST), #2983, clone 7C10], S6K (CST, #9202), p-T389 S6K (CST, #9205), S6 (CST, #2217, clone 5G10), p-S240/244 S6 (CST, #5364, clone D68F8), β-Actin (CST, #4970, clone 13E5), calnexine (CST, #2433), GAPDH (CST, #2118, clone 14C10), Akt (CST, #9272), RFP (Abcam, ab167453), and ORP5 (Sigma-Aldrich, HPA03835); goat polyclonal to ORP5 (Abcam, ab59016); mouse monoclonal to LAMP1 (Santa Cruz Biotechnology, sc-20011, clone H4A3), RFP (Santa Cruz Biotechnology, sc-390909,
ORP5 regulates mTORC1

clone E-8), actin (Abcam, ab8226), and GFP (Santa Cruz Biotechnology, sc-9996, clone B-2). For immunoblotting, we obtained horseradish peroxidise-conjugated secondary antibodies from Jackson ImmunoResearch. For immunofluorescence, we obtained Alexa-Fluor conjugated secondary antibodies from Molecular Probes/Life Technologies.

Immunoblot Analysis

Samples were mixed with 2× laemmli buffer, boiled for 5 min at 95°C or incubated for 10 min at 70°C, and then subjected to 7.5% or 10% SDS-PAGE. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose filters (GE Healthcare). Incubations with primary antibodies were performed at 4°C overnight. Secondary antibodies were peroxidase-conjugated AffiniPure donkey anti-rabbit or donkey anti-mouse IgG (H+L; Jackson ImmunoResearch Laboratories) used at a 1:5000 dilution. The bound antibodies were detected by ECL Western blotting detection reagent (GE Healthcare or Merck Millipore) and visualised with Molecular Imager® ChemiDocTM XRS+ (Bio-Rad Laboratories).

Immunofluorescence and Confocal Microscopy

Cells grown on coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature. Immunofluorescence was carried out as previously described (34), and the primary antibodies used were goat anti-ORP5 (Abcam Cat.# Ab59016, 1:50), rabbit anti-mTOR (CST #2983, 1:400), and mouse anti-LAMP-1 (Santa Cruz Biotechnology, Cat.# sc-18821, 1:50). Cells were mounted in ProLong® Gold antifade reagent (LifeTechnology). Confocal images were acquired on an Olympus FV1200 laser-scanning microscope. The manufacturer’s software and Fiji software (35) were used for data acquisition and analysis. For co-localization study, Fiji Coloc 2 plugin was used.

Immunoprecipitation Assay

Transfected cells grown in 100-mm dishes were harvested, washed with cold PBS, resuspended in 1 ml of cell lysis buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1% Triton X-100) containing Protease Inhibitor Cocktail (100X, Sigma-Aldrich) and Phosphatase Inhibitor Cocktail (100X, CST). Cell lysates were passed through a 22-gauge needle twenty times, incubated on ice for 30 min, and clarified by centrifugation at 18,000 × g for 15 min at 4°C. Immunoprecipitation of the lysates with a polyclonal antibody against NPC1 was performed using the Dynabeads® Protein G (Life Technologies) according to manufacturer’s instructions. The immunoprecipitated pellets were resuspended in 60 µl 2× laemmli buffer (Sigma-Aldrich) and then incubated for 10 min at 70°C. The resultant samples were subjected to SDS/PAGE and immunoblotting.

MTS Assay

Cells were seeded in 96-well plates at 2.5×10³ cells/well and transfected with siRNA (20 nM) for 72 h. For non-transfected or plasmid cDNA transfected cells (24 h), cells were seeded at 5×10³ cells/well in 96-well plates. Six wells were seeded for each transfection. Cell proliferation was measured with a MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). For each reading, 20 µl of MTS solution was added, plates were incubated for 1-2 h, and absorbance read on a Spectra MAX340 Microplate Reader at 490 nm.

Crystal Violet staining

Cells were fixed with 4% paraformaldehyde for 5 min at room temperature. The fixed cells were stained with 0.05% (w/v) crystal violet diluted in distilled water for 30 min at room temperature. Cells
ORP5 regulates mTORC1

were washed twice with water and drained to dry. The dishes were then scanned and cell numbers were counted using Fiji software (35).

**Cell Migration Assay**

Cells were seeded in 24-well plates containing Culture-Insert 24 (ibidi® cells in focus, Planegg, Germany). For overexpression or knockdown experiments, cells were transfected with plasmid cDNAs for 24 h or siRNAs for 48 h, respectively, prior to seeding. After Culture-Insert was removed, images were taken at 0 and 24 h using an Olympus CKX41 microscope (Olympus, Japan) fitted with an Infinity 1-2CB camera (Lumenera Corporation, Ontario, Canada) connected to a computer. Percentage of the cell migration was quantitated using the Fiji Software (35). Data from 24 h were normalised to those from 0 h.

**Proximity ligation assay**

HeLa/ORP5 cells were seeded in 6-well plate containing coverslips at 4×10^5 cells/well and grown for 48 h. For knockdown experiment, cells were seeded at 2×10^5 cells/well and treated with siRNAs for 72 h. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature. The slides were then blocked with 3% BSA in PBS for 1 h and incubated with appropriate combinations of goat anti-ORP5 (Abcam Cat.#Ab59016, 1:50) and rabbit anti-mTOR (CST #2983, 1:400) antibodies diluted in blocking solution for 1 h at room temperature. After washing, the coverslips were incubated with Duolink PLA Rabbit MINUS and PLA Mouse PLUS proximity probes (Olink Bioscience, Uppsala, Sweden) and proximity ligation was performed using the Duolink detection reagent kit (Olink Bioscience) according to the manufacturer’s protocol. Fluorescence images were acquired using an Olympus Fluoview FV1200 confocal microscope with a 60x/1.35 UPlanSApo objective. Images were prepared and analyzed with Fiji software (35). All data were analyzed using GraphPad PRISM software.

**Cell invasion assay**

Invasion assay was performed using the Corning® BioCoat™ Matrigel® Invasion Chambers (Corning Life Sciences). The chambers of the 24-well cell culture inserts were rehydrated with serum-free medium at 37°C, 5% CO₂ atmosphere for 2 h, then transfer to a 24-well plate containing normal growth medium. To the upper chamber, 0.5 ml of cell suspension (2.5×10^4 cells) were added. The invasion chambers were incubated for 24 h at 37°C in the cell culture incubator. Non-invasive cells on the upper insert membranes were removed using cotton tipped swabs by gentle scrubbing. Invasive cells on the lower insert membranes were fixed with 100% methanol and stained with 1% Toluidine Blue (Sigma-Aldrich). The membranes from the insert housing were removed by cutting and mounted on microscope slides. The invading cells were counted under the microscope at 100X magnification. Data is expressed relative to cell numbers migrating through control membranes.

**Protein synthesis assay**

HeLa/ORP5 cells were seeded in 6-well plate containing coverslips at 2×10^5 cells/well treated control siRNA or ORP5 siRNAs for 72 h. The detection of nascent protein synthesis was carried out using the Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assays Kit (Life Technologies, Cat.#C10428) according to manufacturer’s instructions. Fluorescence images were acquired using an Olympus FluoView FV1200 confocal microscope equipped with DAPI filter and FITC filter for Alexa Fluor® 488. Nascent protein synthesis was assessed by determining the signal intensity in the fluorescent channel in the ring around the nucleus as defined by
NuclearMask™ Blue Stain. Images were prepared and analyzed with Fiji software (35). All data were analyzed using GraphPad PRISM software.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis

To identify potential ORP5 interacting proteins, HeLa/ORP5 cell lysates were immunoprecipitated with control goat IgG or goat anti-ORP5 polyclonal antibody. The immunoprecipitates were subject to gel electrophoresis and the gel was stained with Coomassie blue. Stained protein bands were excised and in-gel trypsin digestion was performed prior to LC-MS/MS analysis, which was carried out as previously described (36). All MS/MS spectra data from human proteins were searched using Mascot search engine and the results from control and ORP5 immunoprecipitates were combined and filtered using Scaffold software (Version: Scaffold_4.0.7). Positive hits from ORP5 immunoprecipitates were identified with the following criteria: peptide thresholds ≥ 90.0%; protein thresholds ≥ 99.0% and ≥ 2 peptides.

Statistical Analyses

Statistical analysis between groups was performed using GraphPad PRISM software (Version 6.03) with Student’s unpaired t tests or One-way ANOVA. Data are expressed as mean ± SD unless otherwise stated. Significant differences are indicated in the figures.
Acknowledgements: Mass spectrometric analysis was performed at the Bioanalytical Mass Spectrometry Facility, University of New South Wales and supported in part by infrastructure funding from the New South Wales Government. HY was supported by grants 1041301 and 1078117 from the National Health and Medical Research Council (NHMRC), Australia, and by grant DP130100457 from the Australian Research Council. HY is a Senior Research Fellow of the NHMRC. We thank Dr. Nigel Turner from the School of Medical Sciences, UNSW Sydney, Australia for providing PANC-1 cells, and Dr. Phoebe Phillips from the Lowy Cancer Research Centre, UNSW Sydney, Australia for providing Capan-1 cells.

Conflict of interest: The authors declare that they have no conflict of interests.

Author contributions: XD and HY conceived the project and designed the experiments. XD performed the experiments and analyses with advice from HY and AJB. AZ contributed to plasmid construction. IEL and YQ provided reagents and experimental assistance. XD wrote the manuscript with input from HY, AJB, AZ and IEL.
ORP5 regulates mTORC1

REFERENCES
1. Laplante, M., and Sabatini, D. M. (2012) mTOR signaling in growth control and disease. Cell 149, 274-293
2. Populo, H., Lopes, J. M., and Soares, P. (2012) The mTOR signalling pathway in human cancer. Int J Mol Sci 13, 1886-1918
3. Magnuson, B., Ekim, B., and Fingar, D. C. (2012) Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. Biochem J 441, 1-21
4. Ricoult, S. J., and Manning, B. D. (2013) The multifaceted role of mTORC1 in the control of lipid metabolism. EMBO Rep 14, 242-251
5. 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
6. Du, X., Turner, N., and Yang, H. (2017) The role of oxysterol-binding protein and its related proteins in cancer. Seminars in cell & developmental biology
7. Levine, T. P., and Munro, S. (1998) The pleckstrin homology domain of oxysterol-binding protein recognises a determinant specific to Golgi membranes. Curr Biol 8, 729-739
8. Loewen, C. J., Roy, A., and Levine, T. P. (2003) A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. EMBO J 22, 2025-2035
9. 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
10. Kentala, H., Weber-Boyvat, M., and Olkkonen, V. M. (2016) OSBP-Related Protein Family: Mediators of lipid transport and signaling at membrane contact sites. Int Rev Cell Mol Biol 321, 299-340
11. Mesmin, B., Bigay, J., Moser von Filsseck, J., Lacas-Gervais, S., Drin, G., and Antonny, B. (2013) 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
12. Du, X., Kumar, J., Ferguson, C., Schulz, T. A., Ong, Y. S., Hong, W., Prinz, W. A., Parton, R. G., Brown, A. J., and Yang, H. (2011) A role for oxysterol-binding protein-related protein 5 in endosomal cholesterol trafficking. J Cell Biol 192, 121-135
13. Yan, D., Mayranpaa, M. I., Wong, J., Perttila, J., Lehto, M., Jauhiainen, M., Kovanen, P. T., Ehnholm, C., Brown, A. J., and Olkkonen, V. M. (2008) OSBP-related protein 8 (ORP8) suppresses ABCA1 expression and cholesterol efflux from macrophages. J Biol Chem 283, 332-340
14. Maeda, K., Anand, K., Chiapparino, A., Kumar, A., Poletto, M., Kaksonen, M., and Gavin, A. C. (2013) Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins. Nature 501, 257-261
15. Ghai, R., Du, X., Wang, H., Dong, J., Ferguson, C., Brown, A. J., Parton, R. G., Wu, J. W., and Yang, H. (2017) ORP5 and ORP8 bind phosphatidylinositol-4, 5-biphosphate (PtdIns(4,5)P 2) and regulate its level at the plasma membrane. Nat Commun 8, 757
16. Chung, J., Torta, F., Masai, K., Lucast, L., Czapla, H., Tanner, L. B., Narayanaswamy, P., Wenk, M. R., Nakatsu, F., and De Camilli, P. (2015) INTRACELLULAR TRANSPORT. PI4P/phosphatidylserine countertransport at ORP5- and ORP8-mediated ER-plasma membrane contacts. Science 349, 428-432
17. Galmes, R., Houcine, A., van Vliet, A. R., Agostinis, P., Jackson, C. L., and Giordano, F. (2016) ORP5/ORP8 localize to endoplasmic reticulum-mitochondria contacts and are involved in mitochondrial function. *EMBO Rep* 17, 800-810

18. Li, J., Zheng, X., Lou, N., Zhong, W., and Yan, D. (2016) Oxysterol binding protein-related protein 8 mediates the cytotoxicity of 25-hydroxycholesterol. *J Lipid Res* 57, 1845-1853

19. Guo, X., Zhang, L., Zhang, Y., Zhang, D., Qin, L., Dong, S., and Li, G. (2016) Oxysterol binding protein-related protein 8 inhibits gastric cancer growth through induction of ER stress, inhibition of Wnt signaling and activation of apoptosis. *Oncol Res*

20. Koga, Y., Ishikawa, S., Nakamura, T., Masuda, T., Nagai, Y., Takamori, H., Hirota, M., Kanemitsu, K., Baba, Y., and Baba, H. (2008) Oxysterol binding protein-related protein-5 is related to invasion and poor prognosis in pancreatic cancer. *Cancer Sci* 99, 2387-2394

21. Nagano, K., Imai, S., Zhao, X., Yamashita, T., Yoshioka, Y., Abe, Y., Mukai, Y., Kamada, H., Nakagawa, S., Tsutsumi, Y., and Tsunoda, S. (2015) Identification and evaluation of metastasis-related proteins, oxysterol binding protein-like 5 and calumenin, in lung tumors. *Int J Oncol* 47, 195-203

22. de Saint-Jean, M., Delfosse, V., Douguet, D., Chicanne, G., Payrastre, B., Bourguet, W., Antonny, B., and Drin, G. (2011) Osh4p exchanges sterols for phosphatidylinositol 4-phosphate between lipid bilayers. *J Cell Biol* 195, 965-978

23. Menon, S., Dibble, C. C., Talbott, G., Hoxhaj, G., Valvezan, A. J., Takahashi, H., Cantley, L. C., and Manning, B. D. (2014) Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. *Cell* 156, 771-785

24. Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., and Sabatini, D. M. (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* 141, 290-303

25. Wang, P. Y., Weng, J., and Anderson, R. G. (2005) OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. *Science* 307, 1472-1476

26. Li, J. W., Xiao, Y. L., Lai, C. F., Lou, N., Ma, H. L., Zhu, B. Y., Zhong, W. B., and Yan, D. G. (2016) Oxysterol-binding protein-related protein 4L promotes cell proliferation by sustaining intracellular Ca2+ homeostasis in cervical carcinoma cell lines. *Oncotarget* 7, 65849-65861

27. Maekawa, M., and Fairn, G. D. (2015) Complementary probes reveal that phosphatidylserine is required for the proper transbilayer distribution of cholesterol. *J Cell Sci* 128, 1422-1433

28. Xu, J., Dang, Y., Ren, Y. R., and Liu, J. O. (2010) Cholesterol trafficking is required for mTOR activation in endothelial cells. *Proc Natl Acad Sci U S A* 107, 4764-4769

29. Huang, B. X., Akbar, M., Kevala, K., and Kim, H. Y. (2011) Phosphatidylserine is a critical modulator for Akt activation. *J Cell Biol* 192, 979-992

30. Dibble, C. C., and Cantley, L. C. (2015) Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol* 25, 545-555

31. Schieke, S. M., Phillips, D., McCoy, J. P., Jr., Aponte, A. M., Shen, R. F., Balaban, R. S., and Finkel, T. (2006) The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J Biol Chem* 281, 27643-27652

32. Hong, N. H., Qi, A., and Weaver, A. M. (2015) PI(3,5)P2 controls endosomal branched actin dynamics by regulating cortactin-actin interactions. *J Cell Biol* 210, 753-769

33. Bridges, D., Ma, J. T., Park, S., Inoki, K., Weisman, L. S., and Saltiel, A. R. (2012) Phosphatidylinositol 3,5-bisphosphate plays a role in the activation and subcellular localization of mechanistic target of rapamycin 1. *Mol Biol Cell* 23, 2955-2962
34. Du, X., Kazim, A. S., Brown, A. J., and Yang, H. (2012) An essential role of Hrs/Vps27 in endosomal cholesterol trafficking. *Cell Rep* 1, 29-35

35. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676-682

36. Fei, W., Zhong, L., Ta, M. T., Shui, G., Wenk, M. R., and Yang, H. (2011) The size and phospholipid composition of lipid droplets can influence their proteome. *Biochem Biophys Res Commun* 415, 455-462
FIGURE LEGENDS:

Figure 1. ORP5 facilitates cell proliferation and migration.
(A) Immunoblotting of ORP5 in HeLa/Mock and HeLa/ORP5 cells and in HeLa/ORP5 cells treated with control siRNA or three different ORP5 siRNAs.
(B) Cell proliferation assay in HeLa/Mock and HeLa/ORP5 cells (mean + SD; **** p < 0.0001; n = 12).
(C) Cell migration assay in HeLa/Mock and HeLa/ORP5 cells. Relative cell migration was analyzed using ImageJ (mean + SD; **** p < 0.0001; n = 6).
(D) Cell proliferation assay in HeLa/ORP5 cells treated with control siRNA or three different ORP5 siRNAs (mean + SD; **** p < 0.0001; n = 12).
(E) Cell migration assay in HeLa/ORP5 cells treated with control siRNA or three different ORP5 siRNAs. Relative cell migration was analyzed using ImageJ (mean + SD; **** p < 0.0001; n = 6).
All data are representative from three to four independent experiments with similar results.

Figure 2. Functional ORP5-ORD is critical for facilitating cell proliferation and motility.
(A) Diagram of the functional mutations within the ORD of GFP-ORP5.
(B) Cell proliferation assay in HeLa cells transfected with pGFP-ORP5 (WT) or pGFP-ORP5 L389D (mean + SD; **** p < 0.0001; n = 12).
(C) Cell proliferation assay in HeLa cells transfected with pGFP-ORP5 (WT) or indicated pGFP-ORP5 mutants (mean + SD; **** p < 0.0001; n = 12).
(D) Cell migration assay in HeLa cells transfected with pGFP-ORP5 (WT) or indicated pGFP-ORP5 mutants. Relative cell migration was analyzed using ImageJ (mean + SD; **** p < 0.0001; n = 6).
(E) Cell invasion assay in HeLa cells transfected with pGFP-ORP5 WT or the two mutants, pGFP-ORP5 L389D and pGFP-ORP5 H478A/H479A. Relative invasive cells per field were counted and analyzed using ImageJ (mean + SD; * p < 0.05, ** p < 0.01; n = 7).
All data are representative from three to five independent experiments with similar results.

Figure 3. ORP5 interacts with mTOR.
(A) Immunoprecipitation of cell lysates extracted from HeLa/ORP5 cells using control IgG or ORP5 antibody. Immunoblotting analyses of indicated proteins are shown.
ORP5 regulates mTORC1

(B) RFP Immunoprecipitation of cell lysates extracted from HeLa cells transfected with mCherry-empty vector (EV) or mCherry-tagged ORP5 or ORP8.

(C) Proximity ligation assay (PLA) using goat ORP5 antibody together with or without rabbit mTOR antibody in HeLa/ORP5 cells.

(D) Average PLA intensity in (C) was quantitated using ImageJ (mean +SD; ** p < 0.01; n > 50 cells).

(E) Proximity ligation assay in HeLa/ORP5 cells treated with control siRNA or ORP5 siRNA.

(F) Average PLA intensity in (E) was quantitated using ImageJ (mean +SD; *** p < 0.001; n > 30 cells).

Figure 4. ORP5-ORD is required for ORP5-mTOR interaction.

(A) Diagram of GFP-ORP5 and its deletion mutants lacking the domains of PH, or ORD, or both PH and ORD.

(B) GFP immunoprecipitation of cell lysates extracted from HEK-293 cells transfected with cDNAs for myc-mTOR together with empty vector (EV) or GFP-ORP5 variants as shown in (A).

(C) c-Myc immunoprecipitation of cell lysates extracted from HEK-293 cells transfected cDNAs for myc-mTOR together with EV control or GFP-ORP5 or GFP-ORP5ΔORD.

(D) Diagram of the functional mutations (L389D, H478A/H479A) within the ORD of GFP-ORP5.

(E) GFP immunoprecipitation of cell lysates extracted from HEK-293 cells transfected with cDNAs for myc-mTOR together with EV or GFP-ORP5 variants (WT, ΔORD, H478A/H479A, and L389D).

Figure 5. ORP5 depletion downregulates mTORC1 signaling.

(A) HeLa/Mock and HeLa/ORP5 cells were treated with control or ORP5 siRNAs. Cells were starved in serum free medium overnight prior to harvest for immunoblotting analysis.

(B) Densitometry of activated p70 S6 kinase (p-S6K) in (A) (mean + SD; * p < 0.05; n = 4).

(C) Densitometry of activated S6 ribosomal protein (p-S6) in (A) (mean + SD; * p < 0.05, ** p < 0.01; n = 4).

(D) HeLa/ORP5 cells were treated with control and two different ORP5 siRNAs. Cells were starved in serum free medium overnight prior to harvest for immunoblotting analysis.

(E). Densitometry of p-S6 in (D) (mean + SD; * p < 0.05, ** p < 0.01; n = 3).
(F) Protein synthesis assay in HeLa/ORP5 cells treated with control or ORP5 siRNAs. Newly synthesized proteins in the cytosol were detected by the Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit.

(G) The Click-iT HPG signals in (F) were quantified using ImageJ and plotted relative to the values of control siRNA treated cells (mean + SD; **** p < 0.0001; n = 16 to 24 cells).

(H) PANC-1 cells were treated with control or ORP5 siRNAs for 72 h followed by harvest for immunoblotting analysis.

(I) PANC-1 cells were treated with control or ORP5 siRNAs for 72 h. Cells were imaged under a wide-field microscope.

(J) Cell proliferation assay in PANC-1 cells treated with control siRNA or three different ORP5 siRNAs (mean + SD; **** p < 0.0001; n = 12).

Figure 6. ORP5 knockdown impairs the localization of mTOR to lysosomes.

(A) HeLa/ORP5 cells were treated with control siRNAs or siORP5 for 72 h followed by immunofluorescence of LAMP-1 and mTOR.

(B) Co-localization of mTOR and LAMP-1 in (A) was analyzed using ImageJ. Pearson’s R value is shown (mean + SD; * p < 0.05, ** p < 0.01; n = 15 to 20 cells).

(C) PANC-1 cells were treated with control siRNAs or siORP5 for 72 h followed by immunofluorescence of LAMP-1 and mTOR.

(D) Co-localization of mTOR and LAMP-1 in (C) was analyzed using ImageJ. Pearson’s R value is shown (mean + SD; ** p < 0.01; *** p < 0.001; n = 10 cells).

(E) PANC-1 cells were treated with control or ORP5 siRNAs for 72 h followed by harvest for immunoblotting analysis.
A

| HeLa/Stable Mock ORP5 | HeLa/ORP5 siRNA Ctrl ORP5-1 ORP5-2 ORP5-3 |
|-----------------------|---------------------------------------------|
| Stable ORP5           | 1                                           |
| Mock ORP5             | 2                                           |

B

![Graph showing relative cell proliferation](image)

C

![Graph showing relative cell migration](image)

D

![Graph showing relative cell proliferation](image)

E

![Graph showing relative cell migration](image)
Du et al. Figure 2

A

GFP-ORP5

GFP

PH

ORD

TM

H538A/H540A

L389D

K446A

K670A

H478A/H479A

B

Cell proliferation

Control

WT

L389D

C

Cell proliferation

Control

WT

H538A/K540A

K446A

K670A

H478A/H479A

D

Cell proliferation

Control

WT

H538A/K540A

K446A

K670A

H478A/H479A

E

Relative Invasive Cells

Control

WT

L389D

H478A/H479A

H538A/H540A

K446A

K670A

H478A/H479A
Du et al. Figure 3

**A**

| HeLa/ORP5 | Input | IP |
|-----------|-------|----|
| Ab        | Ctrl  | ORP5 | Ctrl | ORP5 |
| 1         | 2     | 3    | 4    |

- ORP5
- mTOR
- Calnexin
- p70-S6K
- Akt

**B**

| Input | mCherry- EV ORP5 | mCherry- EV ORP5 |
|-------|------------------|------------------|
| IP: RFP | 1 | 2 | 3 | 4 | 5 | 6 |

- IB: RFP
- mTOR

**C**

| Proximity Ligation Assay |
|--------------------------|
| Primary Ab | ORP5 | ORP5 |
| mTOR        | **mTOR** |

**D**

**Average PLA Intensity**

| mTOR | ORP5 |
|------|------|
| -150 | 40   |

**E**

| Proximity Ligation Assay |
|--------------------------|
| Primary Ab | ORP5 + mTOR |
| siRNA | Ctrl | ORP5 |

**F**

**Average PLA Intensity**

| siCtrl | siORP5 |
|--------|--------|
| 30     | **10** |

*Abbreviations: Ab = antibody, ORP5 = Oryzalin Resistant Protein 5, mTOR = mammalian target of rapamycin, IB = immunoblot, PLA = proximity ligation assay.*
Du et al. Figure 6
Oxysterol-binding protein-related protein 5 (ORP5) promotes cell proliferation by activation of mTORC1 signaling
Ximing Du, Armella Zadoorian, Ivan E. Lukmantara, Yanfei Qi, Andrew J. Brown and Hongyuan Yang

*J. Biol. Chem.* published online January 22, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.001558

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts