PIP4K2A regulates intracellular cholesterol transport through modulating PI(4,5)P$_2$ homeostasis

Ao Hu$^1$, Xue-Tong Zhao$^1$, Heng Tu$^1$, Ting Xiao$^1$, Ting Fu$^1$, Yan Wang$^1$, Yong Liu$^1$, Xiong-Jie Shi$^1$, Jie Luo$^{1,*}$ and Bao-Liang Song$^{1,*}$

$^1$Hubei Key Laboratory of Cell Homeostasis, College of Life Sciences, the Institute for Advanced Studies, Wuhan University, Wuhan 430072, China

*Correspondence: jieluo@whu.edu.cn or blsong@whu.edu.cn

Running title: PIP4K2A regulates lysosome-peroxisome membrane contacts
Abstract

The transport of LDL-derived cholesterol from lysosomes to peroxisomes is mediated by membrane contacts, which are facilitated by the lysosomal protein synaptotagmin VII and the peroxisomal lipid phosphatidylinositol 4, 5-bisphosphate (PI(4,5)P₂). Here, we used RNA interference to search for regulators of PI(4,5)P₂ and to study the effects of altered PI(4,5)P₂ homeostasis on cholesterol transport. We found that knockdown of phosphatidylinositol 5-phosphate 4-kinase type-2 alpha (PIP4K2A) reduced peroxisomal PI(4,5)P₂ levels, decreased lysosome-peroxisome membrane contacts, and increased accumulation of lysosomal cholesterol in human SV-589 fibroblasts. Forced expression of peroxisome-localized, kinase-active PIP4K2A in the knockdown cells reduced cholesterol accumulation, and in vitro addition of recombinant PIP4K2A restored membrane contacts. These results suggest that PIP4K2A plays a critical role in intracellular cholesterol transport by up-regulating PI(4,5)P₂ in the peroxisome membrane. Further research into PIP4K2A activity may inform future therapeutic interventions for managing lysosomal storage disorders.

Key words:
ABCD1, lysosomes, LPMC, Syt7, Synaptotagmin
Introduction

Cholesterol is the most abundant sterol in mammalian cells. It is a basic constituent of membrane structure and regulates membrane fluidity. Cholesterol is also implicated in various biological processes, such as the synthesis of steroid hormones and bile acids, the trafficking of endomembrane systems, and the covalent modification of hedgehog and smoothened proteins (1-3).

Mammalian cells can acquire cholesterol from lipoproteins through receptor-mediated endocytosis (4). The circulating low-density lipoprotein (LDL) particles bind to the LDL receptor on the cell surface and are internalized through receptor-mediated endocytosis. The free cholesterol is released in the late endosome/lysosomes (5). The luminal Niemann Pick type C2 (NPC2) protein binds to free cholesterol in lysosomes and delivers cholesterol to the multi-spanning membrane protein NPC1 by a hydrophobic handoff model (6, 7). NPC1-bound cholesterol is inserted into lysosome membrane and further delivered to other organelles. Deficiency in NPC1 or NPC2 leads to the neurodegenerative NPC disease. Our previous study shows that peroxisomes are involved in the egress of lysosomal cholesterol by forming direct and dynamic membrane contacts with lysosome (8). The tethering between the two organelles is formed between Synaptotagmin VII (SYT7) on lysosome and phosphatidylinositol-4, 5-bisphosphate (PI(4,5)P2) on peroxisome. Depletion of peroxisomal PI(4,5)P2 results in cholesterol accumulation in lysosomes (8). Lysosome-peroxisome association is also observed in mouse neurons (9). Peroxisomal dysfunction caused expanded lysosomes likely due to impairment of the organelle contacts (9).
PI(4,5)P₂ can either be synthesized from PI(4)P or PI(5)P by distinct phosphatidylinositol phosphate (PIP) kinases, or dephosphorylated from PI(3,4,5)P₃ by the phosphatase and tensin homolog (PTEN) (Figure 1A)(10). Mammalian PI4P-kinases and PI5P-kinases are encoded by three individual genes. All PIP kinases share a highly conserved central kinase core domain called PIPKc (10). However, the direct evidence linking these PIP kinases to cholesterol transport is still lacking.

Here, we performed an small-scale RNAi screening to search for regulators of peroxisomal PI(4,5)P₂. We found that disruption of PIP4K2A, a PI(5)P-kinase, caused robust cholesterol accumulation in lysosomes. Meanwhile, reduced lysosome-peroxisome membrane contacts (LPMC) were detected in PIP4K2A-knockdown cells as revealed by immunostaining, the proximity-dependent biotinylation assay, and the in vitro reconstitution assay. Interestingly, PIP4K2A deficiency also reduced peroxisomal PI(4,5)P₂ level and this impairment was successfully reverted by re-expression of the wild-type or peroxisome-anchoring form of PIP4K2A. Together, we conclude that PIP4K2A regulates LMPC and cholesterol transport through modulating the homeostasis of PI(4,5)P₂ on peroxisome.
Materials and Methods

Reagents

The anti-LAMP1 (H4A3) antibody was purchased from Developmental Studies Hybridoma Bank. The anti-PMP70 antibody, filipin and D-biotin were purchased from Sigma. ALLN was purchased from Calbiochem. Fluorophore-conjugated secondary antibodies were purchased from Invitrogen. Anti-PI(4,5)P₂ and PI(4,5)P₂ standard were purchased from Echelon Biosciences.

Cell culture

SV589 and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. Cells were grown at 37 °C with 5% CO₂.

Immunofluorescence

Cells grown on coverslips were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for 10 min, blocked in 3% BSA in PBS for 1 hour, and incubated overnight at 4 °C with 3% BSA in PBS containing primary antibodies in 1:1000 dilution (for anti-LAMP1 antibody) or 1μg/ml (for anti-PMP70 antibody). Secondary antibodies were applied in 1:1000 dilution (2 μg/ml) for 1 hour at room temperature. Slides were coverslipped with FluorSave mounting medium (Millipore) and dried at room temperature (11).

Filipin staining
Cells were washed and fixed as indicated before (12). Fixed cells were incubated with PBS containing 10% FBS, 50 µg/ml filipin and primary antibodies for 1 hour at room temperature. Cells were then incubated with secondary antibodies diluted in PBS containing 10% FBS and 50 µg/ml filipin for 1 hour at room temperature. Slides were coverslipped as previously described.

**Generation of CRISPR-Cas9 mediated PIP4K2A knockout cell line**

Guide RNA targeting to the first exon of human PIP4K2A gene (sequence TGGCGACCCCCGGCAACCTA) was designed using [http://crispr.mit.edu](http://crispr.mit.edu) and cloned to pX330-U6-Chimeric-bb-CBh-SpCas9 vector. The guide RNA-containing construct were co-transfected with a puromycin resistant expression plasmid. Cells were selected with 2 µg/ml puromycin for 4 days and seeded onto 96-well plates. Colonies from single cells were expanded after 10 days. Genomic region flanking the targeted regions were amplified by PCR and sequenced.

**Analysis of SREBP-2 cleavage**

Wild-type and PIP4K2A-knockout SV-589 cells were seeding in 60-mm petri dish in triplicate. In the second day, cells were switched to medium A (DMEM containing 5% LPDS, 1 µM lovastatin and 10 µM mevalonate) for 16 hours. Cells were then treated with LDL at indicated concentrations for 4 hours, followed by 1.5 hour incubation in the presence of 25 µg/ml ALLN as before (13). Cells were harvested in RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% SDS, 1.5% NP-40, 0.5% deoxycholate and 2mM MgCl₂) plus protease inhibitors. Cell lysates were subjected to standard western blot procedure.
Measurement of PM cholesterol

The measurement of PM cholesterol was performed as previously described (8) with minor modifications. In brief, wild-type and PIP4K2A-knockout SV-589 were seeding in 6-well plate in duplicate and were switched to medium A on the second day for 16 hours. Cells were treated in DMEM plus 3.5% (w/v) hydroxypropyl-β-cyclodextrin for 15 min and then cultured in LDL-containing medium A for 4 hours. Cells were incubated with or without 1U/ml cholesterol oxidase. Cholesterol was extracted with chloroform/methanol (2:1), dried under nitrogen and measured with Amplex Red Cholesterol Assay Kit (Invitrogen) under the manufacturer’s instructions.

Generation of shPIP4K2A-stably expressing cell line

Human PIP4K2A-targeting oligo (sequence GCACCTCGTAGCGCAGAAAGT) was inserted into pLKO.1 vector. The HEK293T cell was co-transfected with pLKO.1-shPIP4K2A, psPAX2 and pMD2.G in a mass ratio of 4:3:1. The medium was refreshed on the second day of transfection, and the supernatant was collected 48 hours post transfection. The lentiviral particle-containing supernatant was mixed in equal volume with fresh medium to infect HeLa cell. Cells stably expressing shRNA cassette were selected and maintained in culture medium containing 1 µg/ml puromycin.

Proximity-dependent biotinylation assay

The BirA* fragment carrying a R118G mutation was described before (14). The plasmid encoding NPC1-BirA*-EGFP fusion protein was constructed with standard method using the pEGFP-N1 plasmid as a backbone. HEK293T cells stably expressing NPC1-BirA*-EGFP
fusion protein were transfected with indicated siRNAs using RNAiMAX (Invitrogen). 48 hours after transfection, cells were incubated in 10% FBS/DMEM either with or without 50 µM biotin for 24 hr. Total proteins were extracted in RIPA buffer containing protease inhibitors. Whole cell lysate was dialyzed against RIPA to remove residual free biotin. Biotinylated proteins were precipitated with NeutrAvidin agarose beads (Invitrogen). Pellet fractions were isolated by boiling beads in laemmli buffer and separated by SDS-PAGE.

**Lipid dot blot**

Peroxisomes and lysosomes from SV589 cells transfected with indicated siRNAs were purified with peroxisome or lysosome isolation kit (Sigma Aldrich) according to the manufacturer’s instructions, respectively. Acidic lipids from organelles were extracted with methanol-12N HCl (10:1) followed by adding chloroform at a volumetric ratio of 2:1. The organic phase was separated by centrifugation, collected, and dried under nitrogen. Pellets were resuspended for lipid blot analysis. A Hybond-C nitrocellulose membrane was spotted with extracted lipids, dried, blocked with 3% bovine serum albumin (BSA) and blotted with antibody against PI(4,5)P₂.

**In vitro reconstitution of LPMC**

*In vitro* reconstitution assay was performed as previously described (8). In brief, lysosomes and peroxisomes were isolated from either scramble shRNA or *PIP4K2A*-targeting shRNA stably expressing PEX3-EGFP-His₆ HeLa cells. Peroxisomes were pulled down with Ni-NTA, washed with the reconstitution buffer (250 mM sucrose, 1 mM DTT, 1 mM MgCl₂, 50 mM KCl, and 20 mM HEPES pH 7.2) containing 2 mM EGTA, and incubated with lysosomes in
the presence or the absence of 1 mg/ml cytosol or 50 μg/ml purified Flag-PIP4K2A at 37 °C for 30 min. Ni-NTA beads were spun down, boiled with sample buffer and subjected to western blotting.

### Purification of recombinant PIP4K2A

HEK293T cells were seeded in 15-cm petri dish, and transfected with 12 μg Flag-PIP4K2A plasmid per dish. 48 hours after transfection, cells were scraped, washed with ice-cold PBS, and resuspended in IP buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) containing protease inhibitors. The lysate was clarified by centrifugation at 13,200 g at 4 °C for 10 min. Supernatant were incubated with anti-Flag M2 resin (Sigma) on a rotator at 4 °C for 4 hr. Then the M2 beads were spun down and washed extensively with IP buffer. Bound proteins were competitively eluted with 0.1 mg/ml 3×Flag peptide, and eluate was collected and dialyzed against PBS. Protein concentration was determined by BCA assay (Pierce), and protein purity was assessed by SDS-PAGE followed by Coomassie brilliant blue.
Results

**PIP4K2A is required for intracellular cholesterol transport**

The homeostasis of PI(4,5)P2 is controlled by the kinases and phosphatases shown in Figure 1A. To identify the key enzyme(s) regulating peroxisomal PI(4,5)P2 and then affecting cholesterol transport, we individually knocked down each gene using small interfering RNA (siRNA) in SV589 cells. The RNAi efficiency was shown in Figure 1B. Among the PI(4,5)P2-metabolizing genes, only the cells expressing siRNA against *PIP4K2A* exhibited significant perinuclear cholesterol accumulation, resembling the phenotype induced by *NPC1* or *ABCD1* deficiency (Figure 1C, 1D) (8). These cholesterol-rich puncta co-localized with late endosome/lysosome marker LAMP1 (Figure 1E). PIP4K2A catalyzes the conversion of PI(5)P to PI(4,5)P2. According to previous research, PIP4K2A is distributed mainly in the cytosol and partially in the nucleus (15). It has the highest catalytic activity among the three isoforms. PIP4K2A is responsible for the clearance of PI(5)P upon oxidative stress (16) and its mutations are associated with leukemia (17-19) and schizophrenia (20-22).

To further confirm that lysosomal cholesterol accumulation was resulted from the loss of PIP4K2A, we generated *PIP4K2A*-knockout SV589 cell using the CRISPR/Cas9 technique. Similarly, these *PIP4K2A*-knockout cells showed drastic cholesterol accumulation (Figure 1F). To analyze the effects on downstream cholesterol transport, we monitored the inhibition of sterol regulatory element-binding protein 2 (SREBP-2) cleavage. The *PIP4K2A*-knockout cell exhibited delayed response of inhibition of SREBP-2 cleavage towards LDL, indicating lowered level of the ER cholesterol (Figure 1G). In addition, the cholesterol level on the plasma membrane was decreased in *PIP4K2A*-knockout cell upon LDL treatment (Figure...
PIP4K2A deficiency reduces LPMCs

Because peroxisomal PI(4,5)P2 is required for LPMC, we next sought to investigate whether PIP4K2A deficiency decreases lysosome-peroxisome association. Indeed, a more than 30% reduction in LPMCs was observed in PIP4K2A-knockdown cells (Figure 2A, 2B). We further developed a proximity-dependent biotinylation assay to analyze the interorganellar contacts. BirA* is a biotin ligase that covalently attaches biotin to the nearby lysine residues when cells are supplemented with extra biotins (14). We thus generated a HEK293T cell line stably expressing NPC1 fused with BirA* and EGFP, allowing the spatial distance between lysosome and peroxisome to be determined by monitoring the biotinylation of peroxisomal proteins (Figure 3A). The NPC1-BirA*-EGFP fusion protein was successfully targeted to lysosome, as evidenced by the colocalization of EGFP with LAMP1 (Figure 3B). We first validated the peroxisome biotinylation assay in SYT7-knockdown cells where the close apposition between lysosome and peroxisome is known to be impaired (8). PMP70 is a peroxisome membrane-integrated protein, which belongs to the superfamily of ATP-binding cassette (ABC) transporters. PMP70 (also named ABCD3) is one of major components of peroxisomal membranes, and is responsible for the transport of long chain fatty acyl-CoA across peroxisomal membrane (23). We then used biotinylated PMP70 as the readout of the proximity between lysosome and peroxisome. We observed reduced levels of the biotinylated PMP70 upon SYT7 deficiency, suggestive of an increased spatial distance between lysosome and peroxisome. The biotinylation of gp78, an ER membrane-anchored protein, remained unchanged, suggesting that the contacts between lysosome and ER were not affected (Figure
3C). With this technique, we then analyzed the effect of PIP4K2A on LPMC. The results showed that silencing \textit{PIP4K2A} reduced the biotinylated PMP70 level to about one-third of control cells without altering the biotinylation of the NPC1-BirA*-GFP fusion protein itself (Figure 3D, 3E). These data suggest that PIP4K2A regulates the membrane contacts between lysosome and peroxisome \textit{in vivo}.

**Peroxisomal PIP4K2A is required for LPMCs \textit{in vitro}\**

To directly show that PIP4K2A in peroxisome is required for LPMC, we purified peroxisomes and lysosomes from control or \textit{PIP4K2A}-knockdown cells and performed the \textit{in vitro} reconstitution assay as shown before (8). In the presence of cytosol and ATP/GTP, lysosomes and peroxisomes from control shRNA-expressing cells formed tight associations with each other (Figure 4B, lane 1). However, the peroxisomes from \textit{PIP4K2A}-knockdown cells failed to co-precipitate with lysosomes (Figure 4B, lane 2). On the contrary, the lysosomes from \textit{PIP4K2A}-knockdown cells were still associated with peroxisomes (Figure 4B, lane 3). Furthermore, we purified recombinant Flag-tagged PIP4K2A from HEK293T cells (Figure 4C) and applied it to the \textit{in vitro} reconstitution system. Addition of the recombinant protein successfully rescued LPMC when the peroxisomes from \textit{PIP4K2A}-knockdown cells were incubated with the lysosomes from control cells (Figure 4D, compare lane 5 with lane 4). Together, these results demonstrated that PIP4K2A in peroxisome, rather than lysosome, is required for LPMC formation.

**PIP4K2A controls peroxisomal PI(4,5)P2 homeostasis**

To test whether silencing PIP4K2A alters the peroxisomal PI(4,5)P\textsubscript{2} level, we extracted the
lipids from the isolated peroxisomes and lysosomes, and performed the dot blot assay using the anti-PI(4,5)P$_2$ antibody. The peroxisomal PI(4,5)P$_2$ level of $PIP4K2A$-knockdown cells was approximately one fourth that of control cells (Figure 5A). The lysosomal PI(4,5)P$_2$ level remained unchanged (Figure 5A).

We next transfected $PIP4K2A$-deficient cell with constructs encoding various forms of $PIP4K2A$ followed by filipin staining (Figure 5B). Re-expressing of the wild-type $PIP4K2A$-GFP, as well as the peroxisome-anchoring, kinase active PEX3-$PIP4K2A$-GFP, completely reversed cholesterol accumulation in $PIP4K2A$-knockout cells. However, delivery of the construct encoding a kinase-dead mutation failed to rescue the phenotype. Interestingly, a schizophrenia-associated, kinase-active mutation N251S (24) did not reversed the cholesterol accumulation. Notably, cells expressing $PIP4K2A$ fused to the carboxyl terminus of NPC1 still exhibited robust lysosomal cholesterol accumulation, proving that $PIP4K2A$ does not modulate cholesterol transport by altering lysosomal PI(4,5)P$_2$ level. Collectively, these data strongly support that $PIP4K2A$ is required for LPMC and intracellular cholesterol transport through modulating peroxisomal PI(4,5)P$_2$ level.
Discussion

In this study, we discovered that the PI(5)P kinase PIP4K2A is involved in the regulation of intracellular cholesterol transport. There are several lines of evidence supporting that PIP4K2A is required for cholesterol transport from lysosome to peroxisome. First, disruption of PIP4K2A results in cholesterol accumulation in lysosomes (Figure 1). Second, knockdown of *PIP4K2A* reduces LPMC as evidenced by fluorescent microscopy (Figure 2), proximity-dependent biotinylation assay (Figure 3) and *in vitro* reconstitution assay (Figure 4). Third, knockdown of *PIP4K2A* greatly reduces peroxisomal, but not lysosomal PI(4,5)P2 levels. Re-expression of peroxisome-anchored, but not lysosome-anchored PIP4K2A ameliorates cholesterol accumulation in *PIP4K2A*-knockdown cells (Figure 5). Fourth, the peroxisomes, but not lysosomes, purified from *PIP4K2A*-knockdown cell displayed reduced LPMC *in vitro* (Figure 4). These data collectively suggest that PIP4K2A is involved in intracellular cholesterol transport through regulating PI(4,5)P2 homeostasis on peroxisomes.

PIP4K2A has been identified as one of the three isozymes responsible for the conversion of PI(5)P to PI(4,5)P2. In fact, the catalytic activity of PIP4K2A is far more active than the 2B and 2C isoforms (15, 25). The PIP4K2B and PIP4K2C modulate PI(5)P by recruiting high-activity isoform PIP4K2A (15). Interestingly, we did not observe noticeable cholesterol accumulation upon knockdown of *PIP4K2B* or *PIP4K2C*, suggesting that PIP4K2A-mediated peroxisome PI(4,5)P2 generation is independent of the other two isoforms. The genetic polymorphisms of *PIP4K2A* are associated with chronic lymphoblastic leukemia (CLL) and schizophrenia. These genetic variants can alter the binding of transcriptional factors and...
reduced the protein level of PIP4K2A (17). We here find that lowered PIP4K2A decreases LPMC and impairs cholesterol transport. In addition, it has been reported that the CLL patients exhibit lower serum cholesterol (26). The N251S variant of PIP4K2A was found to associate with schizophrenia without affecting the enzyme activity. We showed that PIP4K2A(N251S) failed to reverse the cholesterol accumulation phenotype in PIP4K2A-knockdown cells (Figure 5B). Together, it is possible that cholesterol might play a role in the development and progression of CLL and schizophrenia.

NPC disease is one of lysosome storage disorders (LSD) with massive cholesterol accumulation in lysosomes. It is also known that peroxisomal disorders are accompanied with cholesterol accumulation in lysosomes (8, 9), demonstrating that peroxisome plays an important role in cholesterol transport from lysosome. Although the regulation of PIP4K2A is not well characterized, there are some clues suggesting PIP4K2A may be involved in LSD in previous studies. For example, the reactive oxygen species (ROS) are increased in the cells of LSDs (27, 28). ROS can impair PIP4K2A expression through modulating nuclear factor erythroid 2-related factor 2 (NRF2) and its downstream genes (16), and therefore exacerbates the lysosomal storage phenotypes.

In summary, our work demonstrates that PIP4K2A is involved in cholesterol transport from lysosome to peroxisome through modulating the PI(4,5)P₂ homeostasis on peroxisome. Enhancing PIP4K2A activity might serve as a potential therapeutic approach for treating LSDs such as NPC disease.
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References

1. Lippincott-Schwartz, J., and R. D. Phair. 2010. Lipids and cholesterol as regulators of traffic in the endomembrane system. *Annual review of biophysics* 39: 559-578.

2. Porter, J. A., K. E. Young, and P. A. Beachy. 1996. Cholesterol modification of hedgehog signaling proteins in animal development. *Science* 274: 255-259.

3. Xiao, X., J. J. Tang, C. Peng, Y. Wang, L. Fu, Z. P. Qiu, Y. Xiong, L. F. Yang, H. W. Cui, X. L. He, L. Yin, W. Qi, C. C. Wong, Y. Zhao, B. L. Li, W. W. Qiu, and B. L. Song. 2017. Cholesterol Modification of Smoothened Is Required for Hedgehog Signaling. *Molecular cell* 66: 154-162 e110.

4. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34-47.

5. Chang, T. Y., C. C. Chang, N. Ohgami, and Y. Yamauchi. 2006. Cholesterol sensing, trafficking, and esterification. *Annual review of cell and developmental biology* 22: 129-157.

6. Infante, R. E., M. L. Wang, A. Radhakrishnan, H. J. Kwon, M. S. Brown, and J. L. Goldstein. 2008. NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. *Proceedings of the National Academy of Sciences of the United States of America* 105: 15287-15292.

7. Kwon, H. J., L. Abi-Mosleh, M. L. Wang, J. Deisenhofer, J. L. Goldstein, M. S. Brown, and R. E. Infante. 2009. Structure of N-terminal domain of NPC1 reveals distinct subdomains for binding and transfer of cholesterol. *Cell* 137: 1213-1224.

8. Chu, B. B., Y. C. Liao, W. Qi, C. Xie, X. Du, J. Wang, H. Yang, H. H. Miao, B. L. Li, and B. L. Song. 2015. Cholesterol transport through lysosome-peroxisome membrane contacts. *Cell* 161: 291-306.

9. Kleinecke, S., S. Richert, L. de Hoz, B. Brugger, T. Kungl, E. Asadollahi, S. Quintes, J. Blanz, R. McGonigal, K. Naseri, M. W. Sereda, T. Sachsenheimer, C. Luchtenborg, W. Mobius, H. Willison, M. Baes, K. A. Nave, and C. M. Kassmann. 2017. Peroxisomal dysfunctions cause lysosomal storage and axonal Kv1 channel redistribution in peripheral neuropathy. *eLife* 6.

10. Sasaki, T., S. Takasuga, J. Sasaki, S. Kofuji, S. Eguchi, M. Yamazaki, and A. Suzuki. 2009. Mammalian phosphoinositide kinases and phosphatases. *Progress in lipid research* 48: 307-343.

11. Ge, L., J. Wang, W. Qi, H. H. Miao, J. Cao, Y. X. Qu, B. L. Li, and B. L. Song. 2008. The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1. *Cell metabolism* 7: 508-519.

12. Wei, J., Y. Y. Zhang, J. Luo, J. Q. Wang, Y. X. Zhou, H. H. Miao, X. J. Shi, Y. X. Qu, J. Xu, B. L. Li, and B. L. Song. 2017. The GARP Complex Is Involved in Intracellular Cholesterol Transport via Targeting NPC2 to Lysosomes. *Cell reports* 19: 2823-2835.

13. Tang, J. J., J. G. Li, W. Qi, W. W. Qiu, P. S. Li, B. L. Li, and B. L. Song. 2011. Inhibition of SREBP by a small molecule, betulin, improves hyperlipidemia and insulin resistance and reduces atherosclerotic plaques. *Cell metabolism* 13: 44-56.

14. Roux, K. J., D. I. Kim, M. Raida, and B. Burke. 2012. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *The Journal of cell biology* 196: 801-810.

15. Bulsma, Y., W. J. Keune, and N. Divecha. 2010. PIP4Kbeta interacts with and modulates nuclear localization of the high-activity PtdIns5P-4-kinase isoform PIP4Kalpha. *The Biochemical journal* 430: 223-235.
16. Keune, W. J., D. R. Jones, Y. Bulitsma, L. Sommer, X. Z. Zhou, K. P. Lu, and N. Divecha. 2012. Regulation of phosphatidylinositol-5-phosphate signaling by Pin1 determines sensitivity to oxidative stress. *Science signaling* 5: ra86.

17. Liao, F., D. Yin, Y. Zhang, Q. Hou, Z. Zheng, L. Yang, Y. Shu, H. Xu, and Y. Li. 2016. Association Between PIP4K2A Polymorphisms and Acute Lymphoblastic Leukemia Susceptibility. *Medicine* 95: e3542.

18. Migliorini, G., B. Fiege, F. J. Hosking, Y. Ma, R. Kumar, A. L. Sherborne, M. I. da Silva Filho, J. Vijayakrishnan, R. Koehler, H. Thomsen, J. A. Irving, J. M. Allan, T. Lightfoot, E. Roman, S. E. Kinsey, E. Sheridan, P. Thompson, P. Hoffmann, M. M. Nothen, T. W. Muhleisen, L. Eisele, M. Zimmermann, C. R. Bartram, M. Schrappe, M. Greaves, M. Stanulla, K. Hemminki, and R. S. Houlston. 2013. Variation at 10p12.2 and 10p14 influences risk of childhood B-cell acute lymphoblastic leukemia and phenotype. *Blood* 122: 3298-3307.

19. Walsh, K. M., A. J. de Smith, A. P. Chokkalingam, C. Metayer, G. V. Dahl, L. I. Hsu, L. F. Barcellos, J. L. Wiemels, and P. A. Buffler. 2013. Novel childhood ALL susceptibility locus BMI1-PIP4K2A is specifically associated with the hyperdiploid subtype. *Blood* 121: 4808-4809.

20. Thiselton, D. L., B. S. Maher, B. T. Webb, T. B. Bigdeli, F. A. O'Neill, D. Walsh, K. S. Kendler, and B. P. Riley. 2010. Association analysis of the PIP4K2A gene on chromosome 10p12 and schizophrenia in the Irish study of high density schizophrenia families (ISHDSF) and the Irish case-control study of schizophrenia (ICCSS). *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 153B: 323-331.

21. Schwab, S. G., M. Knapp, P. Sklar, G. N. Eckstein, C. Sewekow, M. Borrmann-Hassenbach, M. Albus, T. Becker, J. F. Hallmayer, B. Lerer, W. Maier, and D. B. Wildenauer. 2006. Evidence for association of DNA sequence variants in the phosphatidylinositol-4-phosphate 5-kinase IIalpha gene (PIP5K2A) with schizophrenia. *Molecular psychiatry* 11: 837-846.

22. Stopkova, P., T. Saito, C. S. Fann, D. F. Papolos, J. Vevera, I. Pacit, I. Zukov, R. Stryjer, R. D. Strous, and H. M. Lachman. 2003. Polymorphism screening of PIP5K2A: a candidate gene for chromosome 10p-linked psychiatric disorders. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 123B: 50-58.

23. Imanaka, T., K. Aihara, T. Takano, A. Yamashita, R. Sato, Y. Suzuki, S. Yokota, and T. Osumi. 1999. Characterization of the 70-kDa peroxisomal membrane protein, an ATP binding cassette transporter. *The Journal of biological chemistry* 274: 11968-11976.

24. Clarke, J. H., and R. F. Irvine. 2013. Enzyme activity of the PIP4K2A gene product polymorphism that is implicated in schizophrenia. *Psychopharmacology* 230: 329-331.

25. Wang, M., N. J. Bond, A. J. Letcher, J. P. Richardson, K. S. Lilley, R. F. Irvine, and J. H. Clarke. 2010. Genomic tagging reveals a random association of endogenous PtdIns5P 4-kinases Ialpha and Iibeta and a partial nuclear localization of the Ialpha isoform. *The Biochemical journal* 430: 215-221.

26. Yavasoglu, I., G. Sargin, F. Yilmaz, S. Altindag, G. Akgun, A. Tombak, B. Toka, S. Dal, H. Ozbas, G. Cetin, A. Donmez, Z. A. Yegin, O. Bilgir, N. Tiftik, S. Ertop, O. Ayyildiz, M. Sonmez, G. Pektas, G. Kadiyoluy, M. Tombuloglu, and Z. Bolaman. 2017. Cholesterol Levels in Patients with Chronic Lymphocytic Leukemia. *Journal of the National Medical Association* 109: 23-27.

27. Ivy, G. O., S. Kanai, M. Ohta, G. Smith, Y. Sato, M. Kobayashi, and K. Kitani. 1989. Lipofuscin-like substances accumulate rapidly in brain, retina and internal organs with cysteine protease inhibition. *Advances in experimental medicine and biology* 266: 31-45; discussion 45-37.

28. Terman, A., and U. T. Brunk. 1998. Ceroid/lipofuscin formation in cultured human fibroblasts: the
role of oxidative stress and lysosomal proteolysis. *Mechanisms of ageing and development* 104: 277-291.
Table 1. RNA oligo sequences used in Fig. 1B

| Target gene | #1 sequence       | #2 sequence                      |
|-------------|-------------------|----------------------------------|
| hNPC1       | GCACCAGGTCTTGACTTA|                                  |
| hABCD1      | GCAGTTTCTCATGAAGTAT|                               |
| hPIP5K1A    | GGTCTTGGAAGCTAGACAG| GGAAGCTAGACAGGATTCT             |
| hPIP5K1B    | CCATTAGATTCCGATATT| CCAGGCTATTACATGAATT             |
| hPIP5K1C    | GCTGCTGCCAAGTTCTAT| GCCACCTTCTTTGAGAA               |
| hPIP4K2A    | GCAGAAAGTGAAAGCTGT| GCACTTCGTAGCGCAGAAA             |
| hPIP4K2B    | CCTGTTTCTGCTAGCTAA| GCAAGATCAAGGGTGAGAA             |
| hPIP4K2C    | CCGATCCATTCAAGTCT| GCATTGATGACCAAGATTA             |
| hPTEN       | CCGGCAGCATCAAATGTT| GCAATCAAATGTTTCAGCTT           |
Figure 1: Cholesterol accumulated in lysosomes in PIP4K2A-deficient cells.

A. Phosphatidylinositol 4,5-bisphosphate related metabolic pathways.

B. Knockdown efficiencies of the siRNA duplexes. Scramble siRNAs were used as controls.
C. Filipin staining of intracellular cholesterol. Human SV589 fibroblasts were transfected with indicated siRNAs for 48 hours. Then the cells grown on coverslips were fixed, stained with filipin (pseudo-colored in red). Representative confocal pictures were selected and shown. Bar = 20 μm.

D. Quantification of filipin signal intensity in (B) by Image J. More than 30 cells were randomly selected and counted for each group, statistical analysis, one-way ANOVA. Data are expressed as mean ± SD.

E. Filipin staining of cholesterol in CRISPR-Cas9-mediated PIP4K2A-knockout SV589 cells. Genomic regions flanking guide RNA targeting sequences were amplified by PCR and validated by Sanger sequencing. Two individual subclones were selected and stained with filipin (blue).

F. Co-staining of cholesterol and LAMP1. Cholesterol and lysosomes were detected with filipin and antibody against endogenous LAMP1, respectively.

G. Analysis of SREBP-2 cleavage. Wild-type or PIP4K2A-KO cell were treated as described in Methods and Materials. Cell lysates were probed with monoclonal antibody 1D2 against human SREBP-2, and anti-β-actin.

H. Measurement of PM cholesterol. Cholesterol was measured with Amplex Red Cholesterol Assay Kit. Statistical analysis, one-way ANOVA. Data are expressed as mean ± SD.
Figure 2: Knockdown of PIP4K2A reduced lysosome-peroxisome membrane contacts in the cells.

A. Immunostaining of endogenous PMP70 (green) and LAMP1 (red). Insets show higher magnification of the area in white box. Bar=10μm.

B. Quantification of colocalization of LAMP1 and PMP70 signal. Confocal images were analyzed by Image J, with the JACoP plugin. Mander’s coefficients were defined by the fraction of LAMP1 signal overlapping PMP70 signal. 46 cells were counted for each group, statistical analysis, one-way ANOVA.
Figure 3: Silencing *PIP4K2A* reduced lysosome-peroxisome membrane contacts measured by proximity-dependent biotinylation assay.

A. Schematic illustration of proximity biotinylation assay.

B. Co-localization of NPC1-BirA-GFP (green) and lysosome marker LAMP1 (red).

C. Validation of proximity-dependent biotinylation assay. HEK-293T cells stably expressing NPC1-BirA-GFP fusion protein were transfected with indicated siRNAs and incubated with biotin-containing culture medium. Then the cells were lysed, and biotinylated proteins were pulled down with avidin beads and subjected to western blotting.

D. Proximity-dependent biotinylation assay in control and *PIP4K2A*-silencing cells. Experiments were performed as described in C.
E. Quantification of gray density of PMP70 in D. Triplicate pellet fraction were normalized with input and quantified by Image J.
Figure 4: Peroxisomal PIP4K2A was required for lysosome-peroxisome membrane contacts in vitro.

A. Schematic illustration of in vitro reconstitution assay. Peroxisomes or lysosomes from HeLa cells stably expressing either scramble or PIP4K2A-targeting shRNA were purified as previously described (8). Peroxisomes were pulldown with Ni-NTA, and further incubated with purified lysosomes. Ni-NTA resin was spun down, washed, and the
associated lysosomes were detected by western blot.

B. *In vitro* reconstitution of lysosome-peroxisome membrane contacts. Lysosomes and peroxisomes were purified from indicated cells and subjected to *in vitro* reconstitution illustrated in A.

C. Purified recombinant Flag-PIP4K2A from HEK-293T cell resolved on SDS-PAGE and stained with Coomassie Brilliant Blue.

D. *In vitro* lysosome-peroxisome membrane contacts rescued by the recombinant Flag-PIP4K2A protein. Reconstitution assay was performed with or without 1mg/ml cytosol, or 50 µg/ml Flag-PIP4K2A protein. Experiments were performed as described in A.
Figure 5: PIP4K2A affected peroxisomal PI(4,5)P₂ level.

A. PI(4,5)P₂ dot blot. Lipids were extracted from indicated fractions of cells. Extracts containing equal amount of protein from either lysosomes or peroxisomes were spotted with 2-fold dilution, on a Hybond membrane. PI(4,5)P₂ standards were spotted as indicated. Blots were detected with anti-PI(4,5)P₂ antibody.

B. Rescue experiments using various PIP4K2A constructs. PIP4K2A knockout cell were transfected with the indicated plasmids and stained with filipin. Cell boarders were marked with white dot-lines. Representative pictures from each experiment were shown.