Sorting Nexin 27 (SNX27) regulates the trafficking and activity of the glutamine transporter ASCT2

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Running title: SNX27 regulates ASCT2 localization and glutamine homeostasis

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ABSTRACT

The Alanine, Serine, Cysteine-prefering Transporter 2 (ASCT2; SLC1A5) is responsible for the uptake of glutamine into cells, a major source of cellular energy and a key regulator of mammalian Target of Rapamycin (mTOR) activation. Furthermore, ASCT2 expression has reported in several human cancers making it a potential target for both diagnostic and therapeutic purposes. Here we identify ASCT2 as a membrane trafficked cargo molecule, sorted through a direct interaction with the PDZ domain of Sorting Nexin 27 (SNX27). Using both membrane fractionation and subcellular localisation approaches we demonstrate that the majority of ASCT2 resides at the plasma membrane. This is significantly reduced within CrispR-mediated SNX27 Knock-Out (KO) cell lines, as it is missorted into the lysosomal degradation pathway. The reduction of ASCT2 levels in SNX27 KO cells leads to a decreased glutamine uptake, which in turn inhibits cellular proliferation. SNX27 KO cells also present impaired activation of mTOR complex 1 (mTORC1) pathway and enhanced autophagy. Taken together, our data reveals a role for SNX27 in glutamine uptake and amino acid-stimulated mTORC1 activation via the modulation of ASCT2 intracellular trafficking.

Glutamine is a major energy source utilized by mammalian cells to maintain cellular homeostasis and growth (1). Several subsets of solute carrier (SLC) protein families have been identified as glutamine transporters, regulating uptake of glutamine from the extracellular environment into cells, including SLC1A5, also known as the Alanine, Serine, Cysteine-prefering Transporter 2 (ASCT2; SLC1A5) (2). The transport of glutamine into the cell by ASCT2 primes SLC7A5/LAT1, an antiporter for glutamine, to exchange it for essential amino acids (EAAs) leading to EAA-stimulated mTORC1 activation (3). The pathological importance of ASCT2 is demonstrated by its upregulated expression in multiple cancer types, such as prostate, lung and breast cancer (4,5). There have been significant efforts to pharmacologically target ASCT2 for the inhibition of cancer cell growth. For example, monoclonal antibodies against ASCT2 or chemical ASCT2 inhibitors, such as benzylserine and gamma-L-Glutamyl-p-nitroanilide (GPNA), have been shown to inhibit cell growth of various cancers (5-7). While the impact of cell proliferation through ASCT2 activity is clear, the molecular details of how ASCT2 activity is regulated are poorly understood.

The localization of ASCT2 on the plasma membrane is critical for glutamine uptake (8-10). Specifically, N-glycosylation is required for the trafficking of newly synthesized ASCT2 to the plasma membrane as a non-glycosylated ASCT2 mutant presents considerably less cell surface expression than wild type ASCT2 (10). Furthermore, the rate of glutamine uptake in cells expressing this mutant is only half of the wild type; despite the fact that they have the similar intrinsic transport activity (10).

Various signaling proteins, such as protein kinases and ubiquitin ligases, are suggested to play a role in the subcellular localization and protein expression of the SLC protein families (11). In the case of ASCT2, EGF stimulation increases ASCT2 localization at the cell surface, which is regulated by multiple kinases, including EGFR, MAPK, Rho and PI3K (12). The serum and glucocorticoid inducible kinase 1 (SGK1), SGK3 and AKT/PKB are also implicated in the modulation of ASCT2 levels on the cell surface, as the overexpression of the constitutively active forms of these kinases can enhance the abundance of...
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ASCT2 at the cell surface (13). In contrast, the association of the ubiquitin ligase RNF5 with ASCT2 leads to the ubiquitination and degradation of ASCT2 (4). Many SLC proteins undergo constant cycles of endocytosis and return back to the cell surface via the recycling endosomes; and the disruption of the intracellular trafficking machineries affects the sorting of the transporters to the desired subcellular compartments, thereby impacting on the recycling efficiency of the transporters back to the cell surface.

Despite the physiological and clinical importance of ASCT2 little is known about the details of its cellular trafficking. SNX27, a member of sorting nexin (SNX) family, is known to regulate the trafficking of endosomal cargo proteins from early endosomes to the cell surface (14-16). SNX27’s FERM domain interacts with NPXY motif containing cargoes, whereas its PDZ domain can interact with PDZ-binding motifs (PDZbm) at the C-terminus of cargo proteins (15,17). Proteomic studies revealed that the cell surface levels of numerous SLC family proteins including ASCT2 were modulated by SNX27 (15), while structural bioinformatics found that SLC family proteins were one of the most abundant classes of PDZbm-containing SNX27 cargos (18). In this study, we identify a direct interaction of SNX27 via its PDZ domain with ASCT2 that controls its subcellular distribution. SNX27 knock-out (KO) cell lines miss-sort ASCT2 to the lysosome for degradation, leading to decreased levels of the transporter and a subsequent decrease in glutamine uptake. Furthermore, SNX27 KO cells demonstrate decreased cell proliferation and delayed cell cycle progression, while the levels of autophagy are increased due to a reduced level of mTORC1 signaling.

RESULTS
SNX27 interacts with ASCT2 cytoplasmic PDZbm
To confirm the proposed interaction between ASCT2 and SNX27 (15), HeLa cells were transiently transfected with plasmids encoding GFP-tagged SNX27 (SNX27-GFP) or GFP-tagged truncated SNX27 lacking the PDZ domain (SNX27ΔPDZ-GFP). Cells were then harvested for immunoprecipitation with GFPTrap agarose beads. Overexpression of SNX27-GFP or SNX27ΔPDZ-GFP had no detectable effect on endogenous ASCT2 protein expression when whole cell lysates were immunoblotted with ASCT2 antibodies (Fig.1A). Immunoprecipitation with GFPTrap beads, demonstrated that SNX27-GFP co-precipitated with endogenous ASCT2 (Fig.1A). In contrast, SNX27ΔPDZ-GFP failed to co-precipitate with endogenous ASCT2 (Fig.1A), indicating that the PDZ domain is required for the interaction between SNX27 and ASCT2. The C-terminal sequence of ASCT2 (ASEKESVM) is a putative SNX27 interacting PDZbm (18), Isothermal titration calorimetry (ITC) experiments using the isolated SNX27 PDZ domain and a synthetic ASCT2 peptide indicated that this peptide binds to SNX27 with an affinity typical of other PDZbms (K_D = 13.6 ± 0.5 µM) (Fig.1B), confirming that the SNX27 and ASCT2 interaction directly depends on the binding of the PDZ domain to the PDZbm. Bioinformatics analysis predicts that the specific phosphorylation sites upstream of PDZbm sequence are required for maintaining its interactions with SNX27 (18). Consistent with this model, ITC experiments demonstrated that substituting serine (S) residue at -2 position by glutamate (E) mimicking serine phosphorylation within ASCT2 PDZbm abolished its interaction to SNX27 PDZ domain, whereas the substitution at -6 position slightly increased the interaction (K_D = 6.9 ± 0.6 µM) (Fig.1B). This data indicates that phosphorylation of the -2 serine within the PDZbm could regulate the interaction between SNX27 and ASCT2. Indirect immunofluorescence of endogenous or HA-tagged ASCT2 (HA-ASCT2) predominantly localizes it on the cell surface (Fig.1C). When HeLa cells were co-transfected with HA-ASCT2 and SNX27-GFP constructs, fluorescence imaging demonstrated that HA-ASCT2 co-localized with SNX27-GFP on the intracellular structures (Fig.1C). Whilst the truncation mutant SNX27ΔPDZ-GFP was unable to bind to ASCT2 (Fig.1A), it is still recruited to the same intracellular structures containing ASCT2 (Fig.1C) indicating that its intracellular membrane recruitment is independent of its interaction with ASCT2.

The efficient retrieval of ASCT2 from endosomes to the plasma membrane requires SNX27
Having established the direct interaction between SNX27 and ASCT2, we next sought to determine if SNX27 controls the intracellular trafficking of ASCT2. To address this question a HeLa SNX27 KO cell line (18) were examined. We initially examined any changes of ASCT2 along with SNAT1 (SLC38A1) and SNAT2 (SLC38A2), the two other amino acid transporters implicated in cellular glutamine uptake (19), at the transcriptional level. Quantitative real time PCR analysis by TaqMAN assay demonstrated that the KO of SNX27 did not affect the gene expressions levels of these transporters (Fig.2A). Comparison of the uptake of glutamine into the cells as measured by 3H-labelled glutamine incorporation demonstrated that SNX27 KO cells showed reduced glutamine uptake compared to HeLa control cells.
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(Fig.2B). To determine if the molecular interaction of SNX27 with ASCT2 influences ASCT2 protein expression, thereby glutamine uptake, western immunoblotting was performed. The total level of ASCT2 protein in SNX27 KO cells was significantly decreased compared to HeLa control cells (Fig.2C). Differential ultracentrifugation and sucrose cushion techniques were then used to separate cell lysates into the purified plasma membrane (PM), intracellular membrane, and cytosol fractions as reported previously (20). Consistent with the observations made with immunofluorescence microscopy, ASCT2 was mainly detected in the PM fraction of HeLa control cells, with lesser amounts in the intracellular membrane fraction and none in cytosolic fraction (Fig.2C). Furthermore, the levels of ASCT2 within the PM fractions from SNX27 KO cell were significantly decreased, compared to HeLa control (Fig.2C). Within these same samples two other membrane proteins, Na+/K+-ATPase and Transferrin Receptor (TfR), whose trafficking is not dependent on SNX27, were examined and the total levels and distribution across the fractions remained similar to control (Fig.2C). Similar to the fractionation data, immunofluorescence staining of ASCT2 within SNX27 KO cells displayed a decreased cell surface localization with a pronounced increase in intracellular punctate staining for ASCT2, which co-localized with LAMP1 – a lysosome marker (Fig.3A). To examine the potential mechanisms for the reduced ASCT2 protein levels within SNX27 KO cells, the cells were incubated with 50 µM chloroquine, a lysosomotropic agent to increase lysosomal pH and inhibit lysosomal hydrolase activities (21), leading to the increased accumulation of ASCT2 within intracellularly lysosomal compartments in both HeLa control and SNX27 KO cells (Fig.3A). In addition, when cells were treated with 200 nM Bafilomycin A1, a specific vacuolar type H+-ATPase inhibitor (22) in the presence of cycloheximide to inhibit newly protein synthesis, increased ASCT2 protein levels were observed in SNX27 KO cells treated with Bafilomycin A1 relative to untreated SNX27 KO cells. The ASCT2 levels in Bafilomycin A1-treated SNX27 KO cells were significantly increased however these levels were still lower than Bafilomycin A1-treated HeLa control cells (Fig.3B). Taken together, the data suggests that the depletion of SNX27 leads to reduced ASCT2 localization at the plasma membrane partly due to an inability recycle the transporter from the early endosome thus exposing it to the degradative pathways within the late endosome/lysosome system.

Altered cell cycle progression upon SNX27 Knock-Out

Glutamine is one of the major metabolites required by proliferating cells for protein synthesis, as well as being an important nitrogen and carbon source for nucleotide synthesis (1). To investigate the effect of SNX27 KO on cellular homeostasis, cell proliferation rates were firstly measured in the presence of the complete DMEM medium containing glutamine. Cell proliferation rates as measured by MTT assay showed that the growth rate in SNX27 KO cell is significantly reduced when compared to parental HeLa control cells (Fig.4A). During proliferation, cells progress through the different stages of the cell cycle prior to cell division, and glutamine levels plays a critical role in the cell cycle progression (23). Propidium iodide (PI) staining was used to monitor the proportion of cells in G1 and G2 phase. 20.8% ± 3.5% of parental HeLa control cells are in G1 and 55.1% ± 4.3% are in G2 phase, while SNX27 KO showed a shift with 61.5% ± 2.2% in G1 and 15.9% ± 1.9% in G2 (Fig.4B). This progression through the different stages of the cell cycle is tightly controlled by cyclin-dependent kinases (CDKs) and cyclin proteins. Among them, cyclin D1 is one of the most essential regulators to mediate G1 phase progression (24). Overexpression of cyclin D1 is frequently associated with human cancer, whereas cyclin D1 degradation is sufficient to cause cell cycle arrest (25). By western immunoblotting we found that the expression level of cyclin D1 in SNX27 KO cells was significantly reduced compared with control cells (Fig.4C). Therefore, our data shows that a consequence of SNX27 depletion is a decrease in cell proliferation and inhibition of cell cycle progression.

Altered autophagy and mTOR activation upon SNX27 knock-out

In addition to cell proliferation, glutamine levels also modulate other signaling pathways to maintain cellular homeostasis. Notably, internalised glutamine can be exchanged by the Large Neutral Amino Acid Transporter (LAT1; SLC3A2) for the uptake of essential amino acids (EAA) leading to mTORC1 activation, which in turn inhibits autophagy (3). LC3, a ubiquitin-like modifier consisting of A, B, B2 and C members, is generally present as form I (LC3-I) at steady state, but is converted to form II (LC3-II) by the conjugation a phosphatidylethanolamine (PE) group during autophagy (26). The induction of LC3-II is critical for the selection of cargos for autophagic degradation, and is also important for fusion between endosomes/lysosomes with autophagosomes. Consistent with previous studies (27), the amount of LC3B-I in HeLa cells was undetectable, however in SNX27 KO cells LC3B-II dramatically increased compared to HeLa controls (Fig.5A).

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Autophagosome maturation requires the fusion between autophagosomes and lysosomes to complete the autophagic cycle. Cells are always undergoing basal levels of autophagy, as marked by the co-localization between LC3B and the lysosome marker LAMP1 (Fig.5B). Strikingly, the co-localization between LC3B and LAMP1 is significantly increased in SNX27 KO cells suggestive of elevated autophagy (Fig.5B). The induction of autophagy is suppressed by active signaling from the mTORC1 pathway. In mammalian cells, EAA stimulated mTOR activation is characterized by the recruitment of mTORC1 from the cytosol to the lysosomal compartment in a GTPase-dependent manner (28). In HeLa control cells, mTOR is mostly localized to the cytosol and little colocalization with the lysosome is seen in the absence of EAA addition, whereas stimulation with EAAs significantly increases the colocalization of mTOR with the lysosome compartment as measured by Pearson’s Correlation coefficient (Fig.5C). In contrast to HeLa control cells, EAA-stimulated mTOR recruitment to the lysosome is significantly decreased in SNX27 depleted cells (Fig.5C). Consequently, EAA-stimulated activation of mTOR signaling in the SNX27 depleted cells is also decreased, as determined by the phosphorylation levels on mTOR, as well as its downstreams, p70S6K and 4E-BP1 (Fig.5D). In addition to EAA, glutamine can also activate mTOR pathway via glutaminolysis pathway to increase intracellular α-ketoglutarate level (29,30). Similar as EAA stimulation, immunoblotting showed that glutamine stimulated phosphorylation levels on mTOR, p70S6K and 4E-BP were decreased in SNX27 KO cells, compared with HeLa control (Fig.5D). One of the consequences of mTOR activation is an increase in cell size and cell mass through promotion of protein synthesis and cell growth in a p70S6K and 4E-BP1-dependent manner. Consistent with this notion, FACS analysis showed that the relative cell sizes of SNX27 KO cells are significantly smaller than HeLa control cells (Fig.5E).

**DISCUSSION**

Despite the pathophysiological significance of ASCT2 in the etiology of cancer, the molecular mechanisms that regulate ASCT2 function in cells are still unclear. Understanding how ASCT2 protein levels are regulated at the plasma membrane is critical as only cell surface ASCT2 can transport glutamine from the extracellular environment into cells. ASCT2 contains a class I PDZbm sequence at its C-terminus that also closely matches the longer consensus sequence for high affinity SNX27 interaction (18). A previous study found that PDZK1 interacts with ASCT2 via its PDZ domain, although the functional significance of this interaction underlying is unclear (31). We describe here that SNX27 serves as an important binding partner for ASCT2. We demonstrate that the interaction between ASCT2 and SNX27 is mediated via the PDZ domain of SNX27 and the PDZbm of ASCT2. SNX27, one of many PX-domain containing proteins, serves as an endosomal scaffold to regulate the trafficking of transmembrane cargo proteins from the endosome to the cell surface, such as GPCRs and SLC1A1/GLUT1 (15). Consistent with a role for SNX27 in the cargo sorting, we observed that SNX27 knock-out in HeLa cells leads to the decreases in ASCT2 protein expression as well as ASCT2 localization on the cell surface, resulting in the suppression of glutamine uptake. Interestingly, ASCT2 contains multiple putative phosphorylation, ubiquitination and glycosylation sites. Several studies have indicated the treatment of the cells with EGF can increase the levels of ASCT2 at the cell surface by PI3K-AKT kinase or MAPK-dependent manners (12,13). Although the mechanisms are unclear, it is possible that the phosphorylation on ASCT2 triggered by kinase activations could modulate its interaction with SNX27 to facilitate retention in endosomes and/or recycling back to the plasma membrane. The ASCT2’s PDZbm contains multiple potential phosphorylation sites and it is established that phosphorylation of residues within the PDZbms of various cargo proteins can either enhance or block their interaction with SNX27 depending on the sites (18). Indeed, the findings from ITC experiments showed that mimicking phosphorylation at serine-2 position within ASCT2 PDZbm abolished its interaction with the SNX27 PDZ domain. This data indicates a role for phosphorylation events within the ASCT2’s PDZbm for the regulating its interaction with SNX27 in vivo.

Cells in which SNX27 has been genetically knocked-out displayed a decreased glutamine uptake and altered glutamine-dependent cellular processes, suggesting that SNX27 is required to maintain normal progression through the cell cycle and cellular proliferation kinetics. Glutamine uptake via ASCT2 is required for the exchange of amino acid uptake, which directly regulates mTORC1 activation (3). Consistent with this, we found SNX27 depletion also leads to the decreased activation of mTORC1 pathways, consequently leading to the smaller cell sizes in SNX27 depleted cells. The activation of autophagy is generally antagonized by mTORC1 activation, and SNX27 depleted cells had increased autophagy due to suppressed mTORC1 activation. Overall, our data from SNX27 knock-out cells...
supports various studies that ASCT2 inhibition using pharmacological inhibitors, such as L-gamma-glutamyl-p-nitroanilide (GPNA) or BenSer, or knockdown approaches which also decreased glutamine uptake also impacted on the cell proliferation rates in various cancer cell models (5,6). Disruption of ASCT2 protein trafficking represents another mechanism to regulate ASCT2 levels at the plasma membrane, which in turn will impact on glutamine metabolism within cells.

**EXPERIMENTAL PROCEDURES**

Chemicals, plasmids and antibodies - All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Ann Arbor, MI, USA). Mouse monoclonal antibodies against Na⁺/K⁺-ATPase (Catalog: AB7671) and human SNX27 (Catalog: 77799) were purchased from Abcam (Melbourne, VIC, Australia). Mouse monoclonal antibody against β-tubulin (Catalog: T9026) was purchased from Sigma-Aldrich. Mouse monoclonal antibody to LAMP1 (Catalog: 555798) was from BD (Franklin Lakes, NJ, USA); mouse monoclonal antibody against transferrin receptor (Catalog: 136800) and rabbit polyclonal antibody to GFP were from Thermo Fisher Scientific (Mulgrave, VIC, Australia). Rabbit polyclonal antibody against cyclin D (Catalog: 06-137) was from Merck Millipore (Bayswater, VIC, Australia). Rabbit monoclonal antibodies against HA-tag (C29F4) (Catalog: 3724), ASCT2 (D7C12) (Catalog: 8057), LC3B (D11) (Catalog: 3868), mTOR (7C10) (Catalog: 2983), phosphor-Ser2448 TOR (D9G2) (Catalog: 5536), phosphor-Thr389 p70S6K (108D2) (Catalog: 9234) and phosphor-Thr37/46 4E-BP1 (236B4) (Catalog: 2855) were purchased from Cell Signaling Technology (CST) (Danvers, MA, USA). HRP conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Life Technologies. IRdye 800 and IRdye 680 conjugated fluorescence secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE, USA). The plasmids for EGFP tagged SNX27 or SNX27ΔPDZ (SNX27-GFP, SNX27ΔPDZ-GFP) were described previously (32), and HA-ASCT2 plasmid is the gift from Professor Shao-Cong Sun (MD Anderson Cancer Center, The University of Texas) (33). Peptide for ASCT2 (ASEKESVM) was synthesized from GenScript (Piscataway, NJ).

Isothermal Titration Calorimetry (ITC) - ITC experiments were performed as described previously (18) using synthetic ASCT2 peptides - ASEKESVM or ASEKEEVVM, AEEKESVM, where serine (S) residue was substituted with glutamate (E) residue at -2 and -6 position, respectively (GenScript, USA), and purified SNX27 PDZ domain protein. The peptide at 1 mM was titrated into the SNX27 PDZ domain at 50 μM in 50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM DTT.

Cell culture and transfection - HeLa control and HeLa SNX27 knock-out cell line (SNX27 KO) (18) were cultured in high glucose Dulbecco’s Modified Eagles Medium (DMEM) (Thermo Fisher Scientific), supplemented with 10% Fetal Bovine Serum (FBS), 5 mg/ml penicillin and streptomycin, 2 mM L-glutamine, and maintained in 5% CO₂ at 37°C. Transient transfection was performed by using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer instruction.

Glutamine starvation and amino acid stimulation - For glutamine starvation, HeLa cells were starved in RPMI medium 1640 (Thermo Fisher Scientific, Catalog: 21870092) (without L-glutamine) supplement with 10% FBS for overnight.

For amino acid stimulation, glutamine starved HeLa cells were subjected to amino acid withdrawal in modified DMEM medium without glutamine and amino acid (USBiological, Catalog: D9800-13) for 2 hrs before treated with 2 x MEM essential amino acid (EAA) solution supplemented with 2 mM of L-glutamine.

For glutamine stimulation, HeLa cells were subjected to amino acid withdrawal in modified DMEM medium without glutamine and amino acid for 2 hrs before treated with amino acid-free DMEM medium supplemented with 20 mM of L-glutamine (30).

Quantitative RT-PCR – RT-PCR assay was performed using TaqMAN assay as described previously (27,34). In brief, total RNA was extracted from HeLa control and SNX27 KO cells using QiAshredder and RNeasy Mini Kit from Qiagen (Chadstone, VIC, Australia), and cDNA was synthesized using a SuperScript III First Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative RT-PCR assays for the gene expression of SNAT1 (SLC38A1), SNAT2 (SLC38A2) and ASCT2 using inventoried TaqMAN gene expression assays (Thermo Fisher Scientific, assay ID: Hs01056542_m1: ASCT2; Hs01562175-m1: SNAT1; Hs01089954_m1: SNAT2). Relative gene expression was normalized to expression of housekeeping gene GAPDH (assay ID: Hs02758991_g1) using a comparative method (2−ΔΔCt).
Subcellular fractionation, co-immunoprecipitation and SDS-PAGE/immunoblotting - Subcellular fractionation was performed as described previously (20). In brief, cells were collected and homogenized in the buffer containing 20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA and protease inhibitor cocktails. Lysates were centrifuged at 500xg for 5 min, followed by 17,200xg for 20 min to generate crude plasma membrane (PM) fraction. Resulting supernatant was further centrifuged at 175,000xg for 75 min to separate high-speed pellet (HSP) fraction and cytosol fraction. Crude PM fraction was gently loaded onto sucrose cushion solution containing 20 mM HEPES, pH 7.4, 1 mM EDTA and 1.12 M sucrose, spin at 100,000 g for 1 hrs to generate purified plasma membrane fraction.

Co-immunoprecipitation was performed as described previously (18). In brief, cells were washed with cold PBS and lysed in lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitor cocktails. After centrifugie for 10 min at 17,000g, equal amounts of cleared cell lysates were immunoprecipitated with GFPnanotrap beads (AIBN, The University of Queensland) for 1 hr at 4 degree. Immunoprecipitated proteins were thoroughly washed with 3 times of lysis buffer, and eluted with 2 x SDS-loading buffer with DTT.

For SDS-PAGE/immunoblotting, protein samples were subjected to bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) to determine protein concentration. Equivalent amounts of protein per sample were resolved on SDS-PAGE according to procedures performed in previous studies (20). Proteins were transferred onto Immobilon-PDVF membranes (Merck Millipore), incubated with primary antibodies overnight, before processing to detect signals either by HRP conjugated secondary antibodies using Super Signal ECL detection kit (Thermo Fisher Scientific) or by IRdye fluorescence secondary antibodies using Odyssey Infrared Imaging System (Li-COR). The calculated relative molecular weight for each protein is shown. Membrane analysis and quantification was conducted using Image J (NIH) or Odyssey Imaging software. Protein band intensities for the samples from cell lysates, purified PM fractions of SNX27 KO cells were used to compare with the samples for whole cell lysates or PM fractions from Hela controls.

Measurement of cellular glutamine uptake - Glutamine starved HeLa cells in 24-well plate format were incubated with glutamine-free medium supplemented with 0.5 µCi/ml 2-deoxy-H\textsuperscript{3} -glutamine (Perkin Elmer Biosciences, Waltham, MA, USA) for 15 min. Cells were harvested in 1% Triton x-100 and radioactivity was counted on a MicroBeta liquid scintillation counter (Perkin Elmer Biosciences, Waltham, MA, USA). The counts per min (cpm) were normalized to total protein concentrations calculated by BCA assay.

Cell proliferation, cell cycle and cell size measurement - Cell proliferation was measured by using MTT assay. HeLa cells were seeded at 7500 cells in 100 µl of complete DMEM medium containing L-glutamine and 10% FBS per well in a 96-well plate format. Cells were growing for 48 hrs before incubation with 20 µl of 5 mg/ml of MTT in PBS for 3.5 hrs at 37 degree incubator. Following, medium was removed, and cells were dissolved in 150 µl of MTT solvent containing 4 mM HCl and 0.1% NP-40 in isopropanol. After 15 min incubation, optical absorbance was measured under 590 nm wavelength using a microplate reader (BioTek Instruments, Winooski, VT, USA).

Cell cycle analysis was performed by flow cytometry using propidium iodide (PI) staining. Briefly, 10\textsuperscript{6} HeLa cells in PBS were fixed in 70% ethanol for at least 2 hrs on ice. After fixation step, cells were pelleted and washed with PBS before suspending cells with staining solution containing 20 µg/ml of PI, 0.1% of Triton X-100 and 0.2 mg/ml of DNase-free RNase A in PBS for 15 min at 37 degree. Cell suspension are then subjected to flow cytometry analysis using BD FACS\textregistered Aria Cell Sorter at 488 nm excitation. Relative cell sizes are determined by using Forwards Scatter (FSC) parameter on BD FACS\textregistered Aria Cell Sorter.

Immunofluorescence microscopy and Image analysis - Cells grown on coverslips were routinely fixed and permeabilised in ice-cold methanol, unless otherwise cited. After blocking with 2% BSA in PBS for 30 min, cells were labelled with anti-ASCT2 (1:400), anti-LC3B (1:200), anti-LAMP1 (1:100), anti-mTOR (1:400) primary antibodies for 1 hr at room temperature followed by the incubation with Alexa Fluor 488, 546 and 647 conjugated secondary antibodies (Thermo Fisher Scientific). For HA-ASCT2 staining, cells were fixed in 4% PFA in PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and subjected to the incubation with HA antibody (1: 400) for 1 hr. Coverslips were mounted
on glass microscope slides using fluorescence mounting medium and analysed using a Zeiss LSM 710 META confocal laser scanning microscope using x 63 oil objective.

Co-localization analysis was performed as described previously (35). Multi-channel images were threshold in each channel; the co-localization was quantified using Image J plugin and was represented as co-localization Pearson’s correlation coefficient (r).

Statistical analysis - Statistical analysis and graphs generation were performed using Prism6 (Graphpad). Error bars on the graphs were represented as the standard deviation (± SD). All p values were calculated using a two-tailed student t-test. p < 0.05 was considered as significant.

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Author contributions: Z.Y., B.M.C and R.D.T conceived the project and designed the experiments. Z.Y., J.F, M.C.K, T.C and M.C executed the experiments, and all authors contributed to data analyses and interpretations of the results. Z.Y. and R.D.T wrote the manuscript, and all authors added critical reviews and modifications.

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FOOTNOTES
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FIGURE LEGENDS

**FIGURE 1.** SNX27 interacts directly with ASCT2. (A) Ectopically expressed GFP-SNX27 fusion proteins were immunoprecipitated by GFP-TRAP and the binding of ASCT2 was determined by western immunoblotting as indicated and the calculated molecular weight for each protein is indicated. (B) The ASCT2 PDZbm peptide binds directly to the SNX27 PDZ domain *in vitro*. Top, raw ITC data; bottom, integrated normalized data and calculated *Kd* values. Peptides with phosphomimetic mutation at the -2 position are unable to bind SNX27, while phosphomimetic mutation at the -6 position enhances binding affinity and enthalpy. Binding parameters with SDs from three experiments are provided in the table. (C) Fixed and permeabilised HeLa cells were co-stained with endogenous ASCT2 and SNX27 antibodies. Alternatively, HA-ASCT2 was co-transfected with GFP-SNX27 constructs, as indicated, in HeLa cells. Indirect immunofluorescence was performed on fixed transfected cells to detect the HA epitope. Images were captured on a Zeiss LSM710 confocal microscopy using a 60 x objective (Scale Bar, 5 μm).

**FIGURE 2.** SNX27 is essential for maintaining ASCT2 levels at the plasma membrane. (A) Relative mRNA expression of SNAT1, SNAT2 and ASCT2 in HeLa control and SNX27 KO cells were determined by
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TaqMAN gene expression assays, and normalized to the expression of a house-keeping gene – GAPDH. Graph represents the difference of gene expressions within HeLa control and SNX27 KO cells (means ± SD). (B) Glutamine starved HeLa and SNX27 KO HeLa cells were incubated with glutamine-free DMEM medium supplemented with 0.5 μCi/ml 2-deoxy-H\(^{3}\)-glutamine for 15 min. Cells were harvested and incorporated radioactivity was quantified on a MicroBeta liquid scintillation counter. The counts per min (cpm) were normalized to total protein concentrations calculated by BCA assay, and the value presented represents the fold-difference in glutamine uptake (means ± SD) from three experiments. Two tailed student’s t test indicated the difference between HeLa and SNX27 KO HeLa cells, *p < 0.05. (C) HeLa and SNX27 KO HeLa cells were subjected to subcellular fractionation to generate purified plasma membrane (PM), intracellular membrane as well as cytosolic fractionations. Equal amounts of protein from whole cell lysates (WCL, 30 µg) and each fraction (10 µg) were used for western blot to determine the protein expressions of ASCT2, Na\(^{+}/K^{+}\)-ATPase, TfR, SNX27 and tubulin as indicated. Representative blots from at least three independent experiments are shown, and the calculated molecular weight for each protein is indicated. The fold differences for ASCT2, TfR and Na\(^{+}/K^{+}\)-ATPase between HeLa cells and SNX27 KO HeLa cells are presented (means ± SD). Two tailed student’s t test indicated the difference between HeLa and SNX27 KO HeLa cells, *p < 0.05, HeLa vs SNX27 KO.

FIGURE 3. The knock-out of SNX27 miss-sorts ASCT2 for the lysosomal degradation. (A) HeLa control and SNX27 KO cells were untreated or treated with 50 μM of Chloroquine for 16 hrs before fixation and immunolabeling with ASCT2 and LAMP1 antibodies. (B) HeLa control and SNX27 KO cells were untreated or treated with 200 nM Bafilomycin A1 for 10 hrs in the presence of 100 µg/ml of cycloheximide. Equal amounts of HeLa control and SNX27 KO HeLa cells were subjected to western immunoblotting, and stained with the antibodies against ASCT2, SNX27 and tubulin. Representative blots from three independent experiments are shown, and the calculated molecular weight for each protein is indicated. The fold differences for ASCT2 between HeLa cells and SNX27 KO HeLa cells are presented (means ± SD). Two tailed student’s t test indicated the difference between HeLa parental and SNX27 KO cells, **p < 0.01, HeLa vs SNX27 KO, no Bafilomycin A1; *p < 0.05, HeLa vs SNX27 KO, Bafilomycin A1 treated; *p < 0.05, untreated SNX27 KO vs Bafilomycin A1 treated SNX27 KO.

SNX27 is required for normal cell proliferation. (A) HeLa and SNX27 KO HeLa cells were growing in complete DMEM medium for 48 hrs before subjected to MTT assay. Optical absorbance was measured under 590 nm wavelength using a microplate reader, and the value representing the fold difference in cellular proliferation rate as measured by MTT assay is presented (means ± SD). Two tailed student’s t test indicated the difference between HeLa and SNX27 KO HeLa cells, ****p < 0.0001. (B) HeLa and SNX27 KO HeLa cells were grown in complete DMEM for 48 hrs before fixed and stained with propidium iodide (PI) solution. Cell suspensions are then subjected to flow cytometry analysis using BD FACSAria Cell Sorter with 488 nm excitation. The peaks for P3 and P4 within the graph represent G1 and G2 phase of cell cycle, respectively. Graph summaries the different stages of cell cycle between HeLa cells and SNX27 KO HeLa cells from three-independent experiments (means ± SD). Two tailed student’s t test indicated the difference between HeLa parental and SNX27 KO cells, ***p < 0.001, HeLa parental vs SNX27 KO, G1 phase; **p < 0.01, HeLa parental vs SNX27 KO, G2 phase. (C) Equal amounts of HeLa and SNX27 KO HeLa cells were subjected to western blot and stained with the antibodies against cyclin D and SNX27. Representative blots from at least three independent experiments are shown, and the calculated molecular weight for each protein is indicated. The fold differences for cyclin D between HeLa cells and SNX27 KO HeLa cells is presented (means ± SD). Two tailed student’s t test indicated the difference between HeLa parental and SNX27 KO cells, ****p < 0.0001, HeLa parental vs SNX27 KO.

FIGURE 5. The knock-out of SNX27 increases autophagy induction and decreases mTOR activation in HeLa cells. (A) Equal amounts of protein from HeLa and SNX27 KO HeLa cells were subjected to western blot, labelled with the antibodies against LC3B, SNX27 and tubulin. Representative images are from at least three independent experiments, and the calculated molecular weight for each protein is indicated. The fold difference for LC3B level between HeLa and SNX27 KO HeLa cells is presented (means ± SD). Two tailed student’s t test indicated the difference between HeLa and SNX27 KO HeLa cells, ***p < 0.001, HeLa vs SNX27 KO HeLa cells. (B) HeLa and SNX27 KO HeLa cells cultured in complete DMEM medium were fixed,
and co-labeled with LC3B and LAMP1 antibodies. Analysis of co-localization between LC3B and LAMP1 of 20 cells each is presented by Pearson’s correlation coefficient. The value shows the difference between HeLa cells and SNX27 KO HeLa cells (means ± SD). Two tailed student’s t test indicated the difference between HeLa and SNX27 KO HeLa cells, **p < 0.01, HeLa vs SNX27 KO HeLa. (C) Amino acid (AA) - starved HeLa and SNX27 KO HeLa cells were untreated or treated with 2 x AA for 30 min in the presence of 2 mM L-glutamine. Cells were fixed and co-labeled with mTOR and LAMP1 antibodies. Analysis of co-localization between mTOR and LAMP1 of 20 cells each condition is presented by Pearson’s correlation coefficient. The value shows the differences between HeLa cells and SNX27 KO HeLa cells is presented (means ± SD). Two tailed student’s t test indicated the difference between HeLa and SNX27 KO HeLa cells, ****p < 0.0001, HeLa (no AA vs AA), *p < 0.05 SNX27 KO (no AA vs AA) and **p < 0.01, HeLa vs SNX27 KO HeLa (AA treated). (D) AA-starved parental and SNX27 KO HeLa cells were treated with 2 x AA for 30 and 60 min in the presence of 2 mM L-glutamine, or treated with L-glutamine alone for 60 and 120 min. After cell harvest, equal amounts of protein were used for western blot, and labeled with the antibodies against phosphor-Ser2448 mTOR, phosphor-Thr389 P70S6K, phosphor-Thr37/46 4E-BP1, SNX27 and tubulin antibodies. Representative blots from three independent experiments are shown, and the calculated molecular weight for each protein is indicated. (E) HeLa and SNX27 KO HeLa examined by flow cytometry analysis as indicated in Fig. 4B. The relative cell sizes are determined by using the Forwards Scatter (FSC) parameter. The graph represents the relative cell sizes (means ± SD). Two tailed student’s t test indicated the difference between HeLa and SNX27 KO HeLa cells, *p < 0.05, HeLa parental vs SNX27 KO.
Figure 1

A

WCL

IP: GFP

UT GFP SNX27-GFP SNX27APDZ-GFP

UT GFP SNX27-GFP SNX27APDZ-GFP

87kDa

GFP

27kDa

ASCT2

75kDa

Tubulin

55kDa

87kDa

GFP

27kDa

ASCT2

75kDa

B

Peptide | K_D (µM) | AH (kcal/mol) | AG (kcal/mol) | -TAS (kcal/mol) | N
---|---|---|---|---|---
Wild type ASEKESVM | 13.6 ± 0.5 | -25.4 ± 0.3 | -6.6 ± 0.04 | 18.9 ± 4.0 | 1.1
Phosphomimetic (6) ASEKESVM | 6.9 ± 0.6 | -33.5 ± 0.7 | -7.2 ± 0.2 | 26.3 ± 1.1 | 0.9
Phosphomimetic (2) ASEKESVM | No binding (NB) | NB | NB | NB | NB

C

ASCT2

SNX27

HA-ASCT2

SNX27-GFP

HA-ASCT2

SNX27APDZ-GFP

Figure 1
Figure 2

A. Relative mRNA Expression

B. Glutamine uptake

C. Western Blot Analysis

- WCL (Whole Cell Lysate)
- PM (Plasma Membrane)

- ASCT2
- Na/K-ATPase
- SNX27
- TIR
- Tubulin

- Total ASCT2 level
- ASCT2 level in the PM
- Total TIR level
- TIR level in the PM
- Total Na/K-ATPase level
- Na/K-ATPase level in the PM
Figure 3
Figure 4
Figure 5

(A) Western blot analysis showing the expression levels of LC3B-II, SNX27, and Tubulin in Control and SNX27 KO cells. LC3B protein expression levels are quantified and presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Immunofluorescence images showing the localization of LC3B and LAMP1 in Control and SNX27 KO cells.

(C) Immunofluorescence images of HeLa cells showing the localization of mTOR and LAMP1 in Control and SNX27 KO cells with or without AA treatment.

(D) Western blot analysis showing the expression levels of various proteins in Control and SNX27 KO cells treated with AA and GLN. 289 kDa, 85 kDa, 70 kDa, 55 kDa, and 60 kDa proteins are shown.

(E) Box plots showing the FSC-A intensity and cell size in Control and SNX27 KO cells. *p < 0.05.
Sorting Nexin 27 (SNX27) regulates the trafficking and activity of the glutamine transporter ASCT2
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