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Supplementary Information for:

Autophagy is required for proper cysteine homeostasis in pancreatic cancer through regulation of SLC7A11

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Supplementary information

Chemicals. N-Acetyl-L-cysteine (Sigma Aldrich, # A9165, 10mM), L-Cysteine ethyl ester hydrochloride (Sigma Aldrich-C121908, 0.4mM), DMEM (high glucose, no glutamine, no methionine, no cystine, TFS:#21013024), LysoTracker™ Blue DND-22 (TFS:#L7525), L-Methionine (Sigma Aldrich:#64319, 0.2mM), GlutaMAX™ Supplement (TFS:#35050061), L-Cystine dihydrochloride (Sigma Aldrich, #C6727, 0.25mM), Hoechst 33342 (TFS:H3570), MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma:5655), Chloroquine (Sigma:#C6628, 25µM unless otherwise mentioned), Transferrin from human serum, Alexa Fluor™-488 Conjugate (TFS:#T13342), BioTracker™ Cystine-FITC Live Cell Dye (Sigma:#SCT047), Trolox (Cayman Chemicals, #10011659, 10µM), MnTBAP chloride (Santa Cruz Biotechnology, #sc-221954, 10 µM), Ebselen (Cayman:#70530, 10µM), EUK 134 (Cayman:#10006329, 10µM), Catalase (Sigma:#C1345, 300U/ml), SBI-0206965 (Sigma:#SML1540-5MG, 10µM), Vps34-IN1(Cayman Chemicals:#17392, 10µM).

Plasmids and RNAi. siRNA pools comprised of 3 target-specific 19-25nt siRNAs to knock down gene expression for human ATG5 (sc-41445), LC3 (sc-43390), ATG7 (sc-41447), mTOR (sc-35410), Raptor (sc-44069), Rictor (sc-61478), TBC1D5 (sc-78234), VPS35 (sc-63218) were purchased from Santa Cruz Biotechnology, Inc. Control siRNA (sc-37007) consisted of a scrambled sequence.

shATG7#1-(Human): 5’-GCCTGCTGAGGAGCTCTCCAT-3’-(TRCN0000007584),

shATG7#2-(Human): 5’-CCCAGCTATTGGAACACTGTA-3’-(TRCN0000007587),

shAtg7#1-(Mouse): 5’-TTCTGTCACGGTTCGATAATG-3’-(TRCN0000305991),

shAtg7#2-(Mouse): 5'- GCCAACATCCCTGGATACAAG-3'-(TRCN0000375444), shATG5#1: 5'-CCTGAACAGAATCATCCTTAA-3'-(TRCN0000330394), shATG5#2: 5'-CCTGAACAGAATCATCCTTAA-3'-(TRCN0000151963), shLC3:5'-CCTGCTGTGTGGTTCATCTTT-3'-(TRCN0000181068), shSLC7A11: 5'-TATTCTATGAGTCGCACAATT-3'-(TRCN0000296035), shGFP: 5'-TGCCCGACAACCACTACCTGA-3'-(TRCN0000072186). *SLC7A11* cDNA (Genscript, #OHu13066) was tagged with GFP and cloned into pBabe vector. Site directed mutagenesis was performed to generate SLC7A11-S26E, SLC7A11-S26A using following primers SLC7A11-S26E-Forward: 5'-GAGGCTGCCTgaaCTGGGCAACA-3', SLC7A11-S26E-Reverse: 5'- CCGTTAACATTTCCCTGC, SLC7A11-S26A-Forward: 5'-GAGGCTGCCTgcaCTGGGCAACA-3', SLC7A11-S26A-Reverse: 5'-CCGTTAACATTTCCCTGCAGGTAAC-3' respectively using KLD enzyme mix (NEB, #M0554S) following the manufacturer's instructions.

S26A/S26E/Wild type SLC7A11 plasmids were used to generate stably expressing cells with SLC7A11 expression in shSLC7A11 (Blasticidin) containing cells.

Quantitative proteomics in details. TMT isobaric reagents were from Thermo Scientific (Rockford, IL, USA). Water and organic solvents were from J.T. Baker (Center Valley, PA, USA). Cells were homogenized by passing through a needle and using a lysis buffer (8M Urea, 200mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.5, 1× Roche protease inhibitors, 1× Roche PhosphoStop phosphatase inhibitors). Cellular homogenates were collected by centrifuging at 20,000×g for 5min at 4°C followed by disulfide bond reduction with 5mM dithiotreitol (37°C, 25 min) and alkylation with 10mM iodoacetamide (room temperature, 30 min in the dark). Chloroform–methanol precipitation

of proteins was performed before protease digestion in HEPES buffer (200mM, pH 8.5). Each sample comprising 100µg protein was digested at 1:100 protease-to-protein ratio, with LysC (37°C, 3h) followed by digestion with trypsin (37°C, overnight). About 50µg of peptides from each sample was labelled with 100µg TMT which were dissolved in ACN to achieve a final concentration of 30% v/v. TMT labelled samples was acidified, vacuum centrifuged to dryness and subjected to C18 SPE (Sep-Pak, Waters). Samples were subjected to basic pH reverse phase HPLC and data acquisition was done with Orbitrap Fusion mass spectrometer (Thermo) coupled with a Proxeon EASY-nLC 1000 LC pump (Thermo). Peptide separation was done using Magic C4 resin (5mm, 100Å, Michrom Bioresources) followed by Accucore C18 resin (2.6mm, 100Å, Thermo) for 3h using a gradient of 6–30% ACN in 0.125% formic acid with a flow rate of 300nl /min.

Antibodies. Proteins were separated on 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels by SDS PAGE electrophoresis followed by transferring on nitrocellulose membranes (GE Healthcare Amersham™ Protran™ NC Rolls, Fisher, #10600000). Subsequently the membrane was blocked in 5% bovine serum albumin for 2 h and incubated overnight at 4 °C with the following antibodies as mentioned in the text under indicated experimental condition. Immunoreactive bands were detected using peroxidase-conjugated antibodies and enhanced chemiluminescence detection reagents (Clarity Western ECL Substrate, BioRad, #1705061) and were analysed using the ChemiDoc™ Imaging System (BioRad, #12003153). The following antibodies were used in this paper. ATG5 (Novus Biologicals, #NB110-53818), ATG7 (Sigma, #A2856), β -actin (Sigma, #A5441), LC3B (Novus Biologicals, #NB100-220), xCT/SLC7A11 (Cell signaling technology, #12691S; Novus Biologicals, #NB300-318), P62/SQSTM1 (Abnova, #H00008878-M01 and Cell signaling

technology, #23214), Cathepsin C (Novus, #NBP2-55936, SLC7A1 (LS-BIO, #LS-C749764-100), mTOR (Cell signaling technology, #2983S, 2972S), TBC1D5 (Santa Cruz Biotechnology, #sc-376296), Phospho-Akt Substrate (RXXS*/T*) (110B7E) (Rabbit mAb Magnetic Bead Conjugate, Cell signaling technology, #8050), VPS35 (abcam, #ab10099), Rictor (Cell Signaling Technology, #2114S), COX7A2L (Proteintech, #11416), Anti-Sodium Potassium ATPase antibody [EP1845Y] (abcam, # ab76020) Calreticulin (abcam, # ab2907), HA tag (BioLegend, #901513), LC3 (Nanotools, # 0231-100/LC3-5F10).

Fig.S1

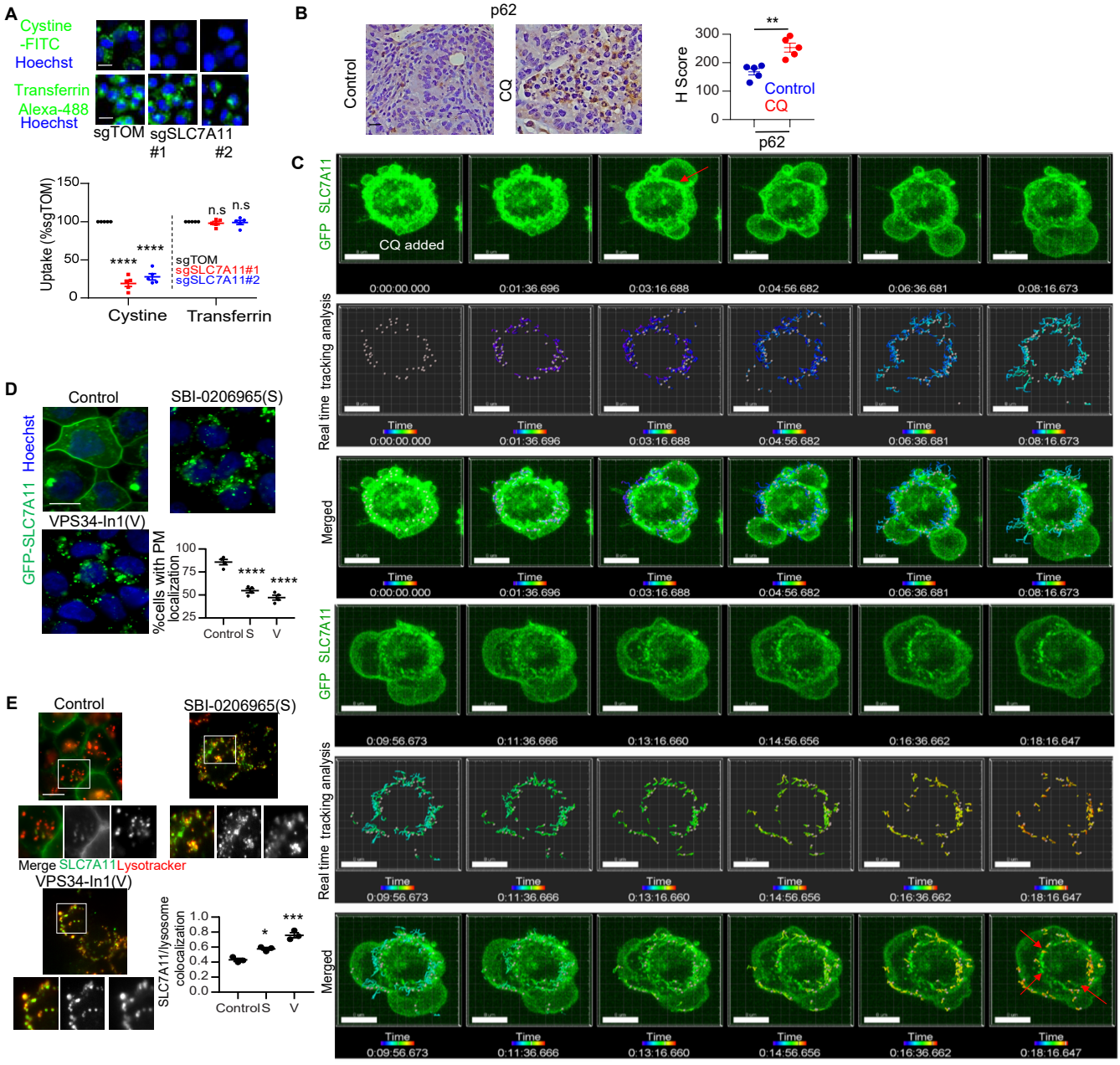

Fig.S1: A, Validation of fluorescent cystine uptake assay was performed using sgSLC7A11-8988T cells. Fluorescent transferrin was used as an unrelated control of uptake (n=151 cells, 5 experiments). B, Tumor (n=5) sections from mice treated with CQ were analyzed for p62 by IHC followed by their quantification. *P*-values: two-tailed unpaired t-test. C, Montage of confocal laser-scanning microscopy time lapse video images was performed immediately after CQ treatment in GFP-SLC7A11 stably expressing 8988T cells. Red arrow illustrates SLC7A11 internalization. Color coding represents real time tracking of the GFP-SLC7A11 with time (refer Supplementary-Movie S1, S2). D, After SBI-0206965, Vps34-IN1 (36h) treatment, plasma membrane (PM) localization of SLC7A11 (n=52cells, 4 experiments) or lysosomal colocalization (n=52cells, 3 experiments, E) was performed. Data: mean±s.e.m, *P* values:one-way ANOVA with post-hoc-Tukey. Scale-bar:15μm. ****:*P*<0.0001, ***:*P*<0.001, **:*P*<0.01, *:*P*<0.05, n.s: *P*>0.05.

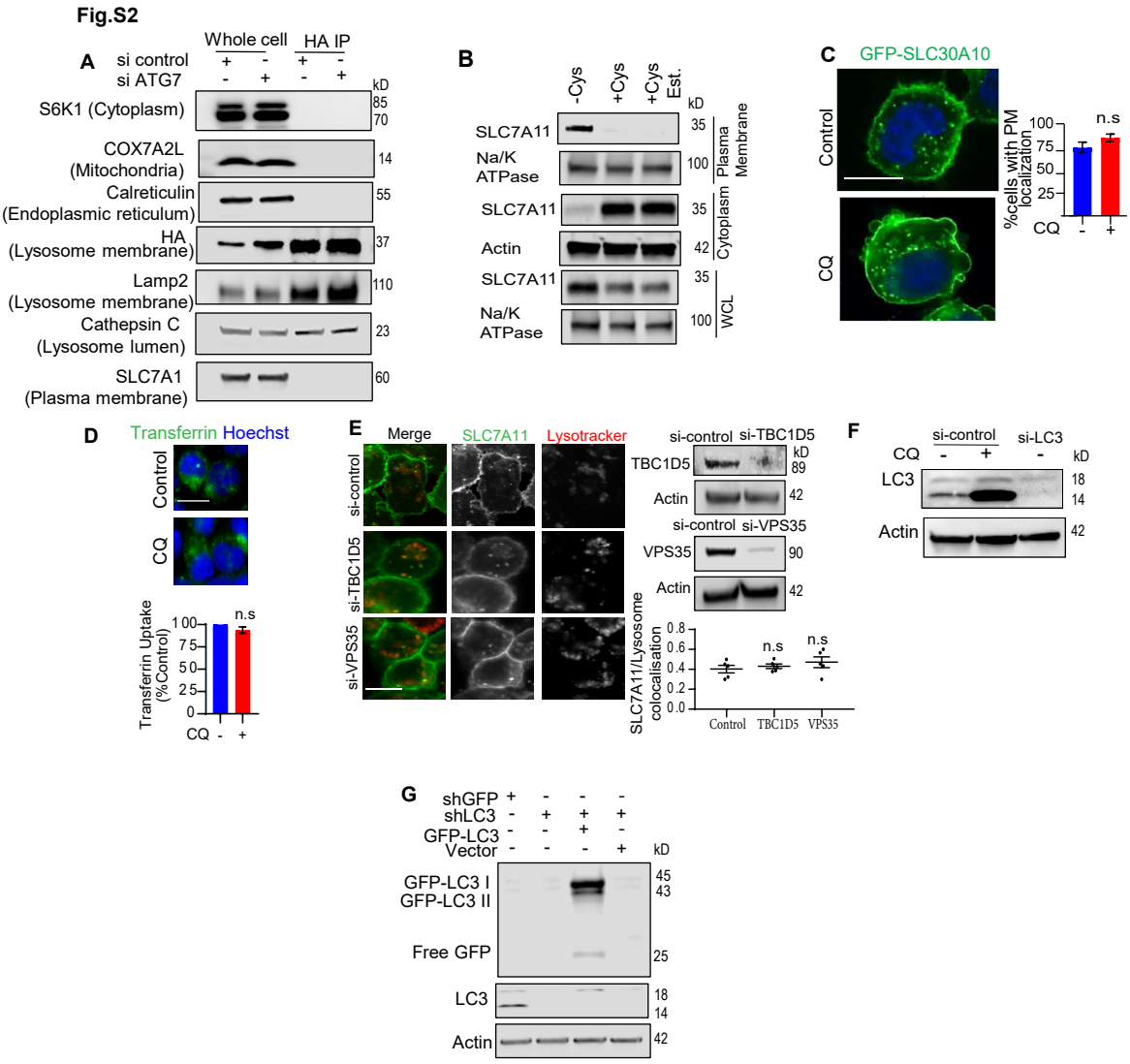

Fig.S2: A, Validation of the quality of the immunopurified lysosomes was assessed by immunoblotting using different organelle markers. B, Plasma membrane and cytoplasm was purified from 8988T cells which were cystine starved overnight and refed with cys/ cys ester (*) for 30min and probed for SLC7A11. C, CQ treated GFP-SLC30A10-8988T cells were analyzed as a control to assess the effect of autophagy inhibition on the internalization of an unrelated solute carrier (SLC) (n=125cells, 5 experiments). D, Transferrin uptake was investigated after CQ (24h) treatment in 8988T (n=210cells, 5experiments). E, Colocalization analysis of SLC7A11 and lysotracker was performed after knockdown of essential retromer components TBC1D5 or VPS35 (n=75cells, 5 experiments); right panel shows the western blot demonstrating knockdown of retromer components in GFP-SLC7A11-8988T cells. F, Autophagy inhibition in 8988T cells by CQ treatment and LC3 knockdown, followed by western blot analysis of LC3 levels. G, Western blots were performed using indicated proteins with lysates from 8988T cells where endogenous LC3 was suppressed and rescued with a non-degradable GFP-LC3. Data: mean±s.e.m, *P* values: two tailed unpaired t-test (C, D) while in (E) it is one-way ANOVA with post-hoc-Tukey; n.s: *P*>0.5. Scale-bar:15μm.

Movie S1. GFP-SLC7A11 trafficking in autophagy inhibited cells. Live time lapse imaging was performed in 8988T cells stably expressing GFP-SLC7A11 immediately after (t=1s) addition of CQ using the Zeiss LSM 880 Airyscan Fast Live Cell using Zen software at 20sec interval, 7slice z stack, 60cycles. Images were acquired for 20 min. Scale bar is present on left hand side and time lapse duration is indicated in right hand side.

Movie S2. GFP-SLC7A11 trafficking in autophagy comepetent cells. Live time lapse imaging was performed in 8988T cells stably expressing GFP-SLC7A11 using the Zeiss LSM 880 Airyscan Fast Live Cell using Zen software at 20sec interval, 7slice z stack, 60cycles. Images were acquired for 20 min. Scale bar is present on left hand side and time lapse duration is indicated in right hand side.