Supplement Material

Methods

Reagents

Chemical reagents were purchased from Sigma-Aldrich and tissue culture reagents from Invitrogen, except where specified. LDL and acetylated LDL (AcLDL) were purchased from Biomedical Technologies, Inc (MA). Aggregated LDL (AggLDL) was made by vortexing (1 min) and sonicating LDL with a Branson sonifier (10 min, 70% duty cycle) on ice. Moderately oxidized LDL (OxLDL) was made by dialyzing LDL against 0.9% NaCl containing 20 μM $CuSO_4$ at 37ºC for 2 hours. Cyclodextrin-cholesterol complex (CD-chol) was prepared by sonicating cholesterol in medium containing cyclodextrin. Acyl-coenzyme A-cholesterol acyltransferase (ACAT) inhibitor 58035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide was from J. Heider, formerly of Sandoz (East Hanover, NJ). MAP kinase inhibitors (Calbiochem), Toll-like receptor (TLR) ligands (InvivogGen) were purchased. A molecular imaging agent for cysteinyl proteinase activity (ProSense 680) was from VisEn Medical, MA

Mice Littermates *Apoe*$^{-/-}$, *Npc*$^{+/+}$ were used as control for *Apoe*$^{-/-}$, *Npc*$^{-/-}$ mice. p38$^{flox/flox}$ mice were backcrossed four generations with C57BL6/J mice. p38$^{flox/flox}$ mice were crossed with LysMCre/C57BL6 mice to generate p38$^{flox/flox}$, LysMCre+/- mice. These mice were intercrossed. p38$^{flox/flox}$, LysMCre+/- mice were used as source for p38 deficient peritoneal macrophages and literemates p38$^{flox/flox}$, LysMCre-/- mice were used as controls. *Myd88*/*Trif*$^{-/-}$ mice were backcrossed for 10 generations with C57BL6/J mice.

Wild type C57BL6/J mice were used as controls for *Myd88*/*Trif*$^{-/-}$ mice. *Mitf*$^{mi/mi}$ mice were also on C57BL6/J background.

Cell culture-Mouse peritoneal macrophages were isolated by peritoneal lavage with PBS 3 days after intraperitoneal injection with 40 ug concanavalin A (ConA), or 1ml of 3.85% thioglycollate (Thio), or no injection (Resi). Bone marrow cells were flushed from femurs and tibias with PBS. These cells were cultured in DMEM/10% FBS. Bone marrow cells derived from *Myd88*/*Trif*$^{-/-}$ mice were cultured in 20% L-cell-conditioned DMEM/10% FBS medium for 7 days before treatment.

Spleen-derived macrophages-Spleen-derived precursors were harvested in DMEM, dissociated with a 23-1/2 gauge needle, passed through a 40-micron filter, and resuspended in DMEM/10% heat-inactivated low endotoxin FBS/ PenStrep /1X glutamate supplement/50 ng/ml recombinant colony-stimulating factor 1 (CSF-1). Cultured cells (1 spleen/plate) were incubated 3 days (7%CO_2, 37ºC,), flushed out of plates and seeded onto 10 cm tissue culture plates at 2-3 million cells/plate. The cells expressed macrophage markers within 2- 3 days.

Immunofluorescent staining of aortic cross-sections- Aortic sections were dewaxed, rehydrated, blocked with 10% goat serum in PBS, and incubated overnight at 4 °C with the first antibody in 1% BSA in PBS: affinity purified rabbit polyclonal anti-cathepsin K (1:50), mouse monoclonal antibody against SMC α-actin (1:100, Biomeda, Foster City, CA). Mouse IgG at the same dilutions were used as negative controls. Sections were then incubated with the appropriate secondary antibody: goat anti-rabbit Cy3-conjugated and goat anti-mouse Cy2-conjugated antibody (1:200, Rockland, Gilbertsville, PA) for cathepsin K and VSMC staining.

Microarray Gene Expression Profiling-RNA was extracted and pooled from thioglycollate-elicited peritoneal macrophages derived from BALB or BALB- *Npc*$^{-/-}$ mice (5 each group). Labeling, hybridization and data analysis were provide by Gene Expression Center at University of Wisconsin using Affymetrix Mouse Genome 430 2.0 Genechips.

Chromatin immunoprecipitation assay-spleen derived macrophages were plated at density of 2X10^6 cells per 10 cm dish and treated with or without 5 mM CD-cholesterol (2.5:1, M:M) for 18 hours. Cells were cross linked with 1% final concentration of formaldehyde at 37ºC for 10 min. Soluble chromatin was prepared following sonication with a Branson 250 digital sonifier (Branson Ultrasonics, Danbury, CT) to an average DNA length of 200-1000 bp. ~5 x 10^5 cell equivalent (one-sixth) of the sheared soluble chromatin was pre-cleared with tRNA-blocked Protein G-agarose, and 10% of the pre-cleared chromatin was set aside as input control. Immunoprecipitation was carried out with 5 µg of antibodies as indicated in the figures overnight at 4 °C. Immune complexes were pulled down using Protein G-agarose, washed, and eluted twice with 250 µl of elution buffer (0.1 M $NaHCO_3$, 1% SDS), and cross-linking was reversed in 200 mM NaCl at 65 °C overnight with 20 µg of RNase A (Sigma). DNA was purified following proteinase K treatment (Invitrogen) with the Qiagen PCR purification kit using the manufacturer's instructions. Samples were analyzed by real-time PCR either by SYBR Green super mix (Bio-Rad). The threshold for the promoter being studied was adjusted by that of input values and represented as relative abundance. All qPCR reactions were analyzed by melt curve analysis and agarose gels to confirm the presence of a single specific band.

Media Transfer- Bone marrow cells of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice were flushed from femurs and tibias with PBS. These cells were grown in DMEM/10%FBS for 24 hours as donors and media were collected as conditioned media. Another group of bone marrow cells were collected 24 hours after donors. These were recipient cells. The recipient cells were plated and grew in DMEM/10%FBS or conditioned media from *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ donors. mRNA from recipient cells were collected for *Ctsk* expression four days after plating.

Statistics

Results from at least three independent experiments, performed in triplicate, were used to calculate mean±S.D unless specified in the legend. Statistical significance was determined by 2-tailed Student's *t* test.

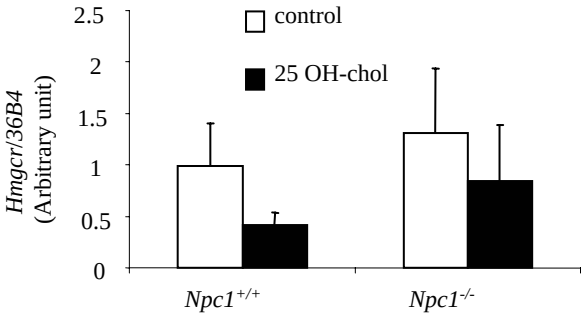
Online Table I. Fold changes of genes induced in *Npc1*$^{-/-}$ macrophages as determined by Taqman real-time PCR.

Gene	Thio	ConA	Resi.	BM day 0	BM day 4
Ctsk	2.5	14	12	3.0	2.8
Mmp14	2.4	5	3.4	N.D.	N.D.
Mmp8	3.5	4.4	1.7	8.9	N.S.
Mmp9	N.S.	N.S.	↓	N.D.	N.D.
S100a8	5.8	5.5	2.6	N.D.	N.D.
S100a9	13	15	1.7	4.2	↓
Ym1	13	18	52	45	N.S.

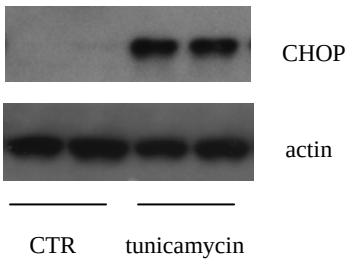
The expression levels of genes are normalized to ribosomal protein 36B4. The ratio of expression levels between *Npc1*$^{-/-}$ and wild type are shown. N>3, P<0.05. Thio, thioglycollate-elicited peritoneal macrophages; ConA, conconavalin A-elicited macrophages; Resi., resident peritoneal macrophages; BM, bone marrow derived cells. N.S., no significant change. N.D. not determined. ↓, decreased.

Online Figure I

A

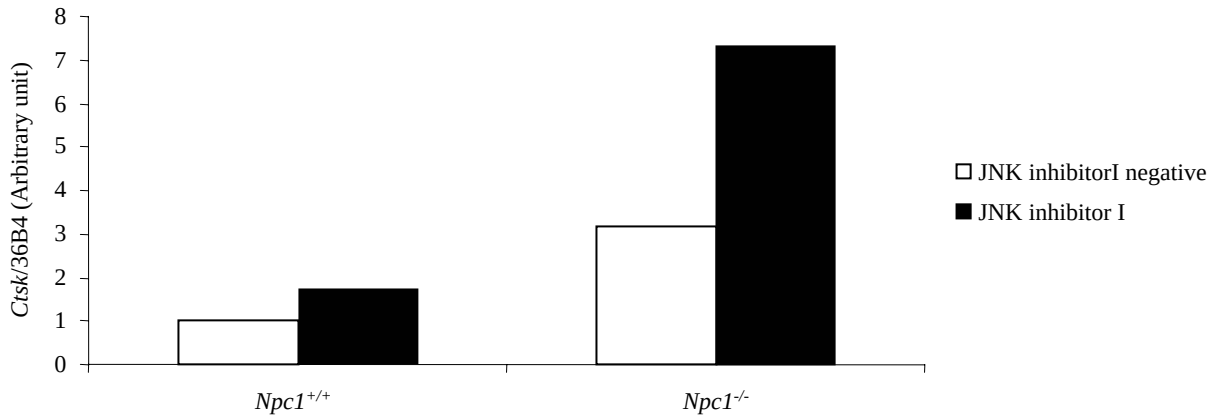


B



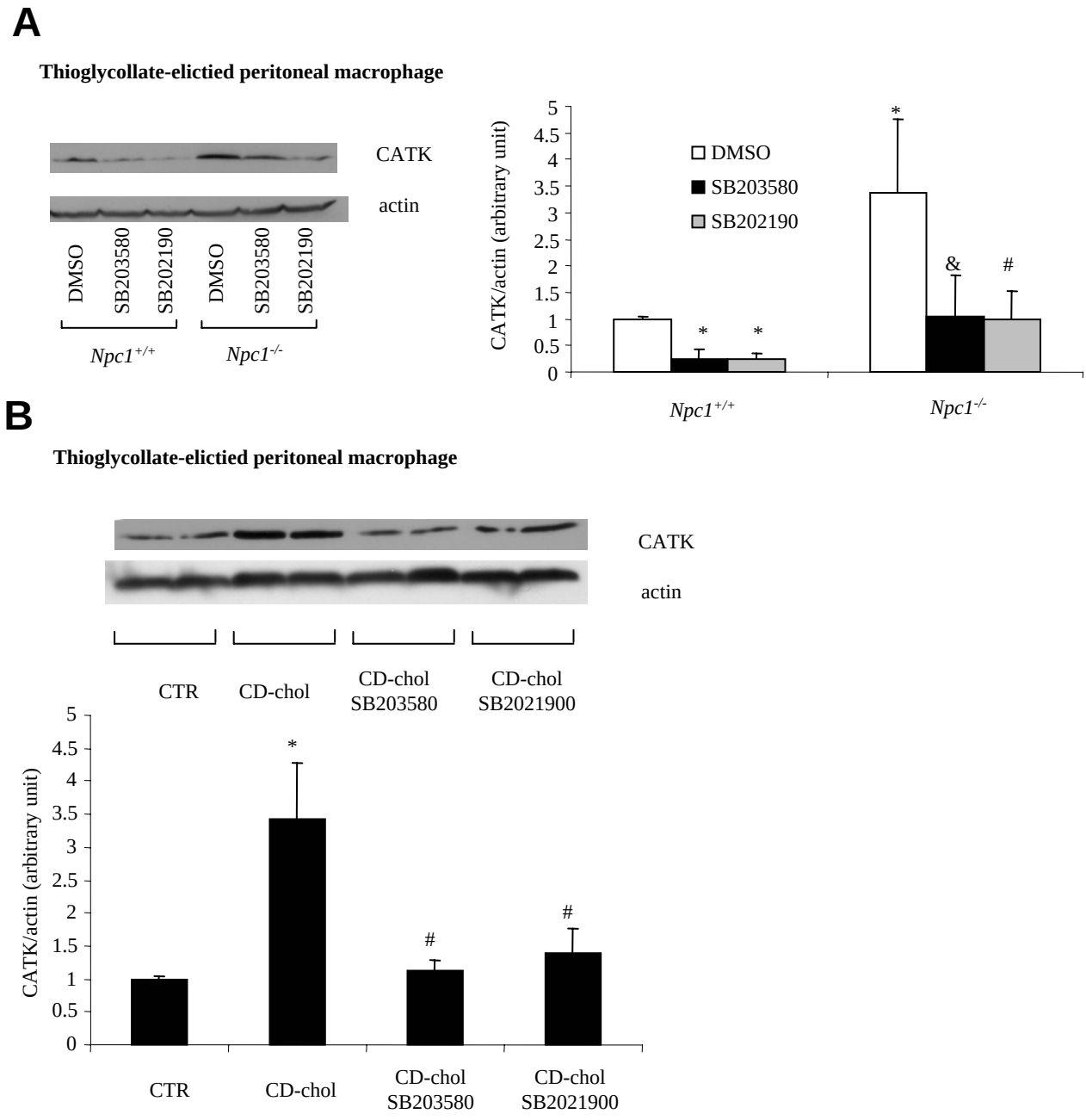
Online Fig I. Positive controls for 25-hydroxylcholesterol and tunicamycin treatment. A. ConA-elicted peritoneal macrophages from *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ mice were treated with 10 uM 25-OH-chol for 24 hours. The levels of HMG-CoA reductase mRNA were measured by real-time PCR and normalized to 36B4. **B.** ConA-elicted peritoneal macrophages from wt mice were treated with 2ug/ml tunicamycin for 18 hours. CHOP and actin protein levels were measured by western blot.

Online Figure II



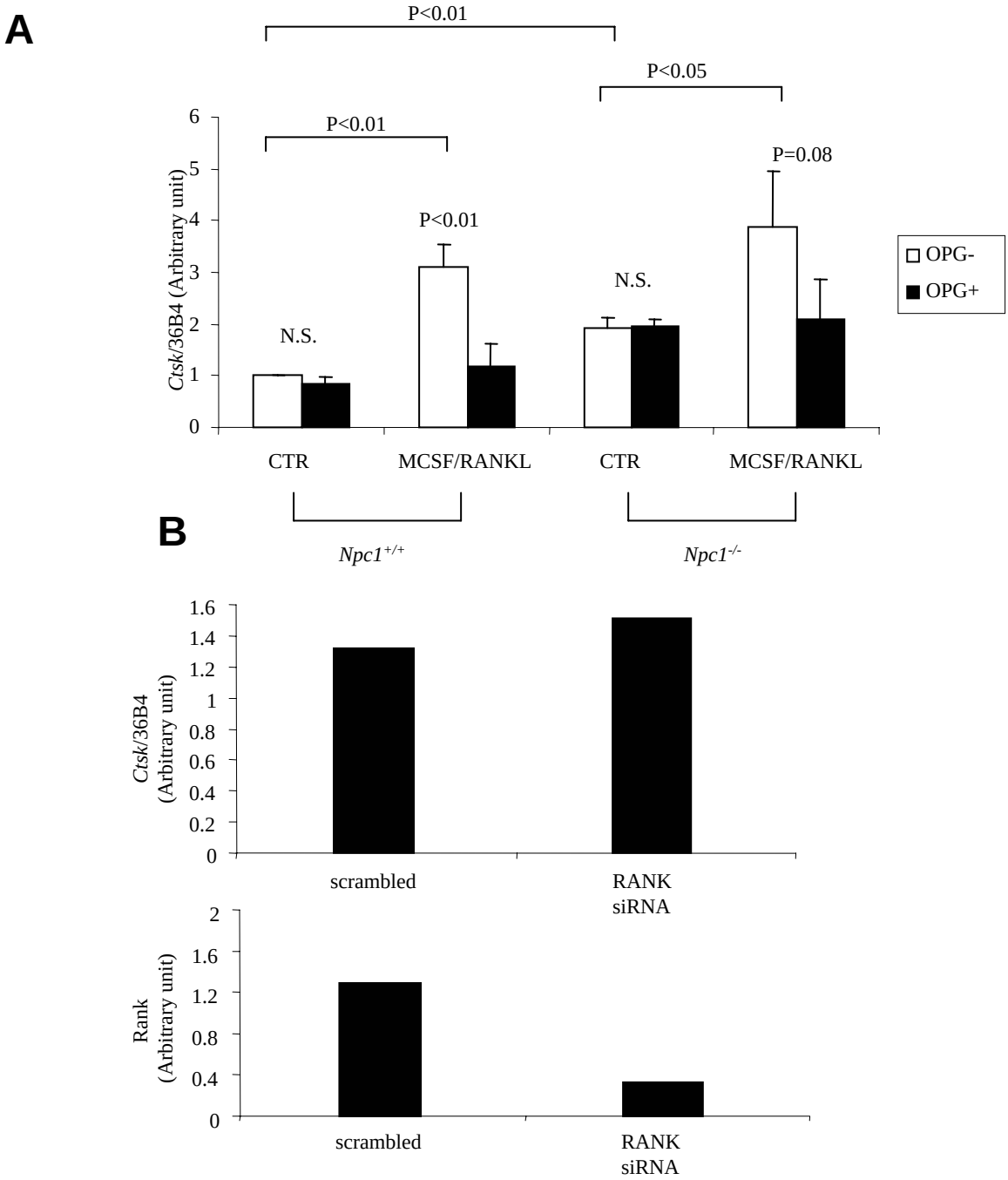
Online Fig II. A peptide inhibitor of JNK also increased *Ctsk* mRNA. ConA-elicited peritoneal macrophages from *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ mice were treated with JNK inhibitor I (Calbiochem) or negative control for 24 hours. Cells were then harvested for RNA analysis.

Online Figure III



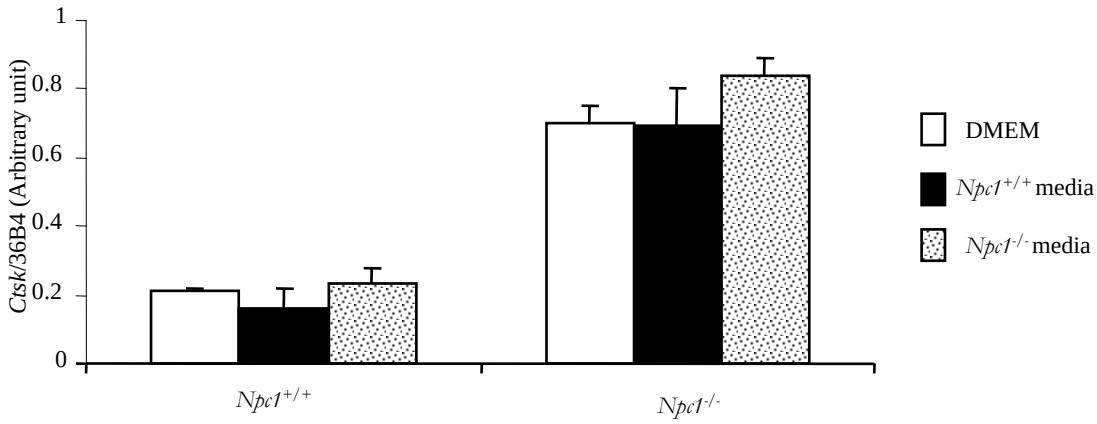
Online Fig III. *Npc1*$^{-/-}$- or CD-cholesterol (CD-chol)- induced cathepsin K protein (CATK) levels were reduced by p38 inhibitors. A. Thioglycollate-elicited peritoneal macrophages from *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ mice were treated with 10 uM p38 inhibitors SB203580 or SB202190 for 48 hours. *, P<0.05 compared with *Npc1*$^{+/+}$/DMSO; #, P<0.05 compared with *Npc1*$^{-/-}$/DMSO; &, P=0.06 compared with *Npc1*$^{-/-}$/DMSO; n= 3. **B.** Thioglycollate- elicited peritoneal macrophages from *Npc1*$^{+/+}$ mice were loaded with 5 mM CD-chol (2.5:1, M:M) at the absence or presence of p38 inhibitors for 72 hours. *, P<0.05 compared with CTR; #, P<0.05 compared with CD-chol. CATK levels were measured by western blots in duplicates and normalized to β-actin. Data from three independent experiments were used to calculate mean±S.D..

Online Figure IV



Online Fig IV. RANKL-RANK pathway was not involved in *Npc1* deletion induced Ctsk induction. A. Osteoprotegerin treatment does not reverse the induction of *Ctsk* in *Npc1*$^{-/-}$ bone marrow cells. Bone marrow derived cells were flushed out from femur bone cavity with PBS or PBS containing 50ng/ml Osteoprotegerin. The cells were then plated in DMEM/10%FBS. The cells were maintained for 4 days with indicated treatments. The doses for treatments are, MCSF, 10ng/ml, Rank ligand, 25 ng/ml, Osteoproteterin, 50 ng/ml. **B.** Knockdown of RANK did not result in decreased Ctsk expression in *Npc1*$^{-/-}$ macrophages. ConA elicited *Npc1*$^{-/-}$ macrophages were transfected with scrambled or RANK siRNA. 72 hours after transfection, cells were harvested for RNA analysis.

Online Figure V



Online Fig V. The level of *Ctsk* was determined by cell genotype not factors in the media. Fresh bone marrow derived cells from *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ mice were grown in normal cell media (DMEM/10%FBS) or conditioned media from *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ cells for 4 days. Then cells were harvested for RNA analysis.