Supplemental Materials

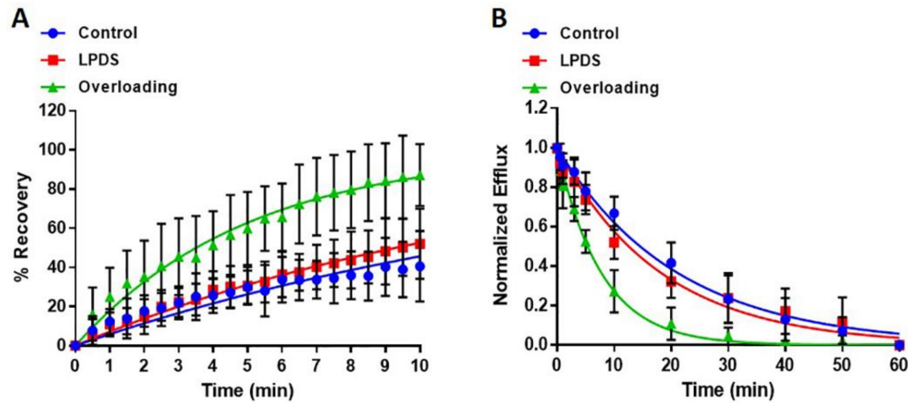
Molecular Biology of the Cell

Iaea et al.

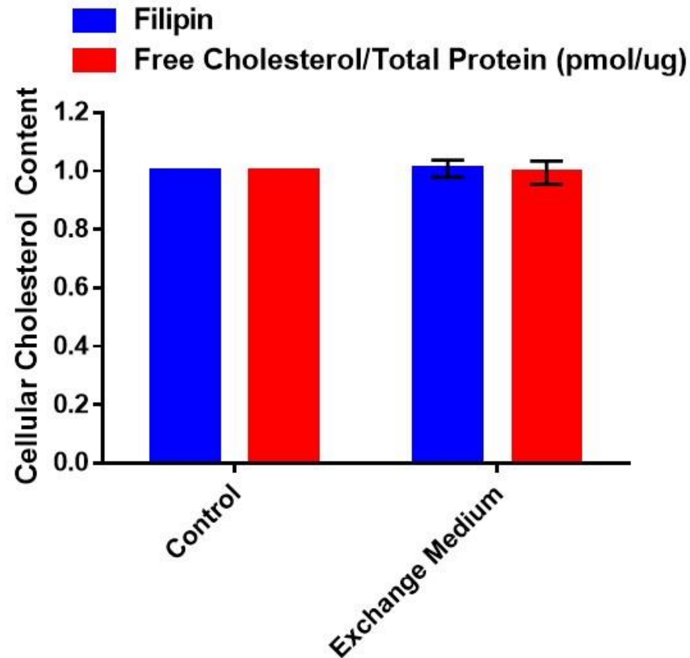
Supplementary Table 1- Complementary oligonucleotides for cloning sgRNAs into PX461

Set #1		
alpha (A)-1	GAAGGTCATCTATAACACCT	Oligo A
	AGGTGTTATAGATGACCTTC	Oligo B
beta (B)-1.1	GAATAGACCATATACGCCCA	Oligo A
	TGGGCGTATATGGTCTATTC	Oligo B
beta (B)-1.2	GTAATAGACCATATACGCCC	Oligo A
	GGGCGTATATGGTCTATTAC	Oligo B
Set #2		
alpha (A)-2	GGTCTATTATACTATAGACA	Oligo A
	TGTCTATAGTATAATAGACC	Oligo B
beta (B)-2.1	GCGCCCAGGGCCTTGTCGTT	Oligo A
	AACGACAAGGCCCTGGGCGC	Oligo B
beta (B)-2.2	GAGGGCCTTGTCGTTTGGAT	Oligo A
	ATCCAAACGACAAGGCCCTC	Oligo B

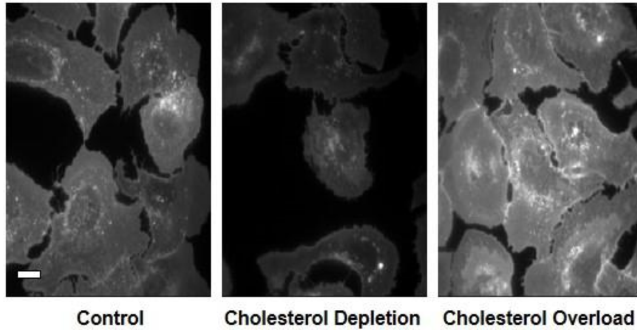
PCR primers for Surveyor assay	
STARD4_F	CACATGCTCTGGCTCACTCA
STARD4_R1	CACCTCAGCCTCCCAGAATG
STARD4_R2	ACAGGTTTTTGCCACGTTGG
STARD4_R3	CTCAGCCTCCCAACTAGCTG

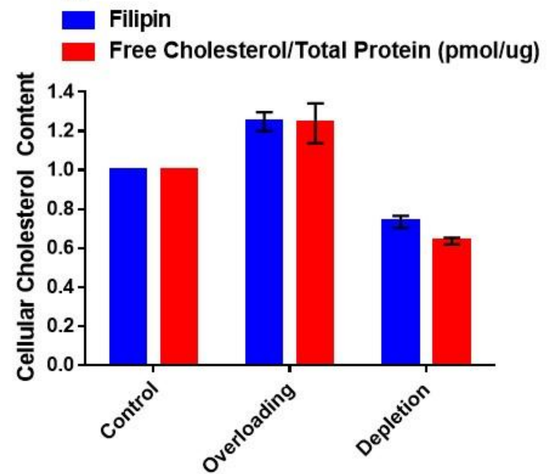


Supplementary Figure 1- Bidirectional sterol transport. For both FRAP and efflux experiments, U2OS-SRA cells were labeled with DHE for 1 minute and equilibrated for 120 minutes. In the last 20 minutes, cells were incubated with 20 μg/ml Tf-Alexa546 37°C in Medium 2/glucose to identify the ERC. Cells were maintained at 37°C. (A) FRAP measurements for control, LPDS and cholesterol overloaded cells. (B) DHE efflux curves for control, LPDS and cholesterol overloaded cells. Each data point is derived from an average of at least eight experiments (± SD). Data are fit to single exponential curves.

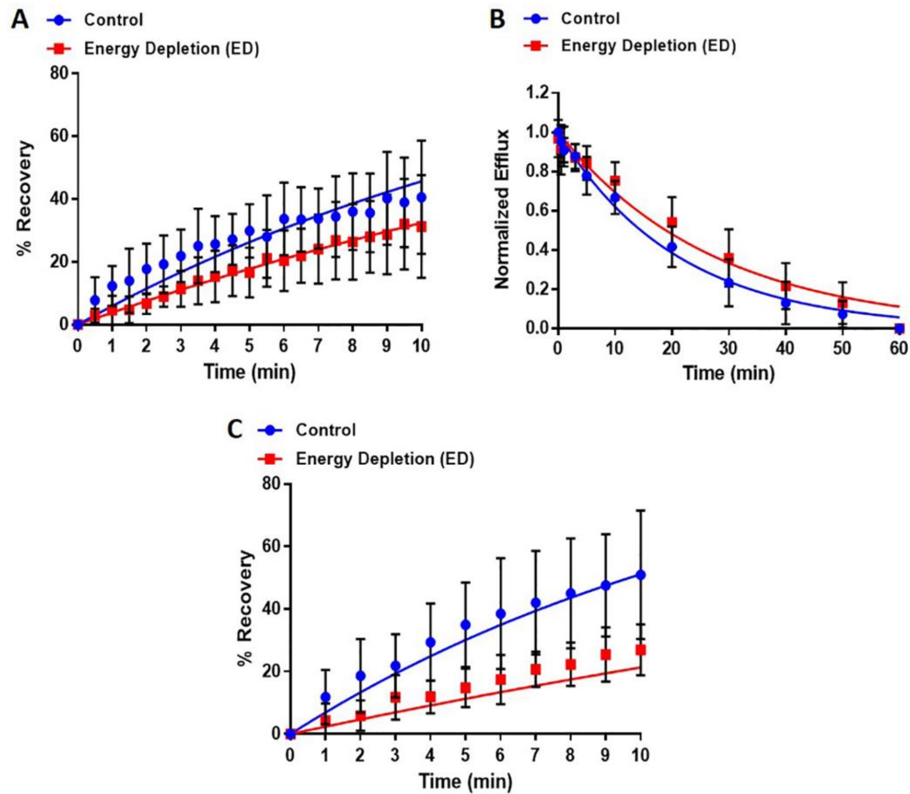


Supplementary Figure 2- Cellular cholesterol content is not altered by efflux medium. Blue bars, quantification (± SE) of the average cellular filipin fluorescence power of U2OS-SRA cells following 60 min incubation in control or efflux medium. Filipin fluorescence was normalized to the control experiment. Intensity measurements were performed on background-subtracted images from an average of 50 fields of cells in each condition. Red bars, GC/MS measurement of free cholesterol levels of U2OS-SRA cells following 60 min incubation in control or efflux medium. Cellular lipids were extracted and analyzed by GC/MS. Data represent averages (± SE) of three independent experiments normalized to control value.

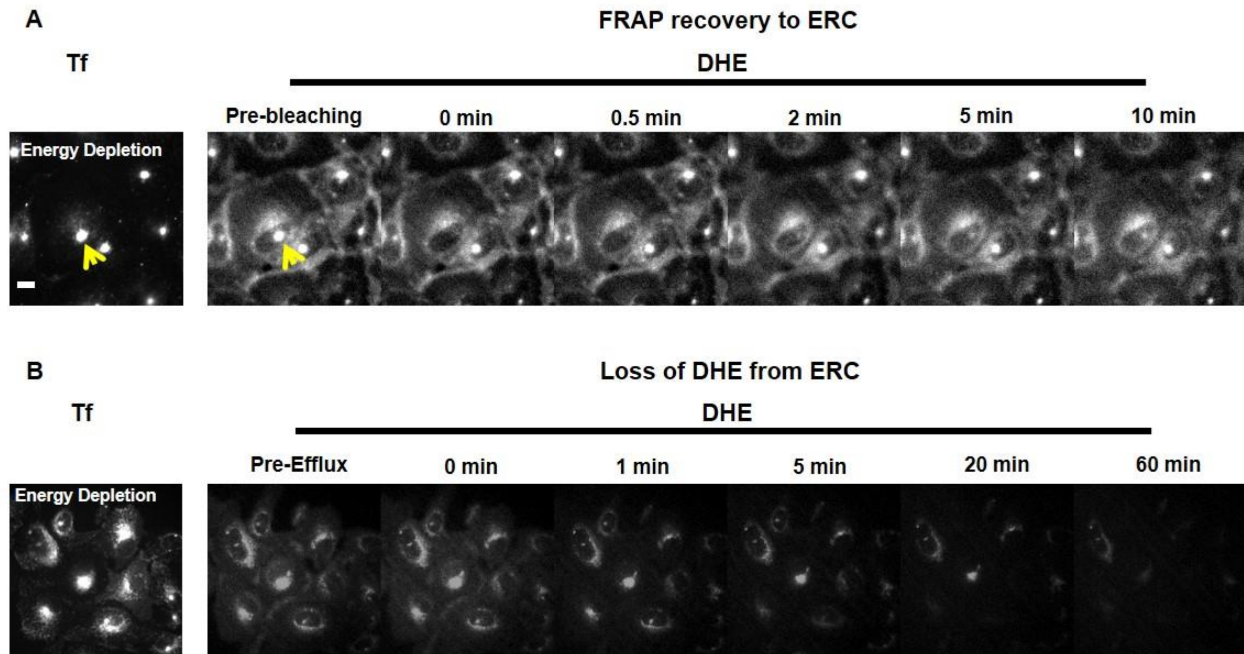




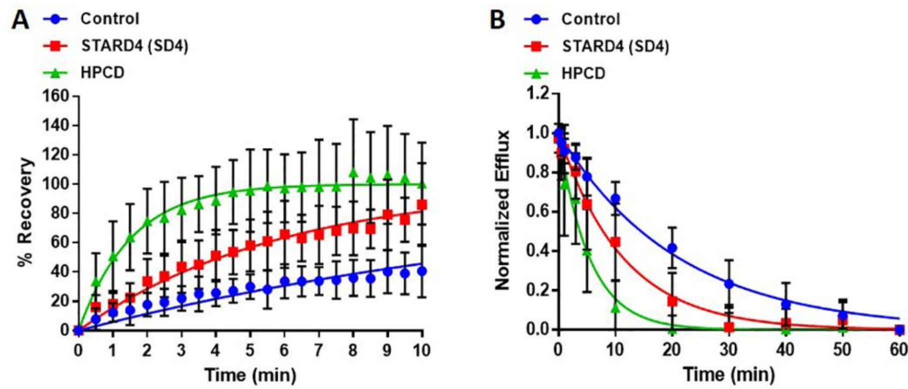
Supplementary Figure 3- Modulation of cellular cholesterol levels. (A) Epifluorescence microscopy images of cells that were fixed and stained with filipin. Scale bar: 10 μm. (B, blue) Quantification (± SE) of the average cellular filipin fluorescence power following cholesterol depletion or overloading. Filipin fluorescence was normalized to the control experiment. Intensity measurements were performed on background-subtracted images from an average of 20 fields of cells in each condition. (B, red) GC/MS measurement of free cholesterol levels in cells following cholesterol depletion or overloading. Cellular lipids were extracted and analyzed by GC/MS. Data represent averages (± SE) of three independent experiments normalized to control value.



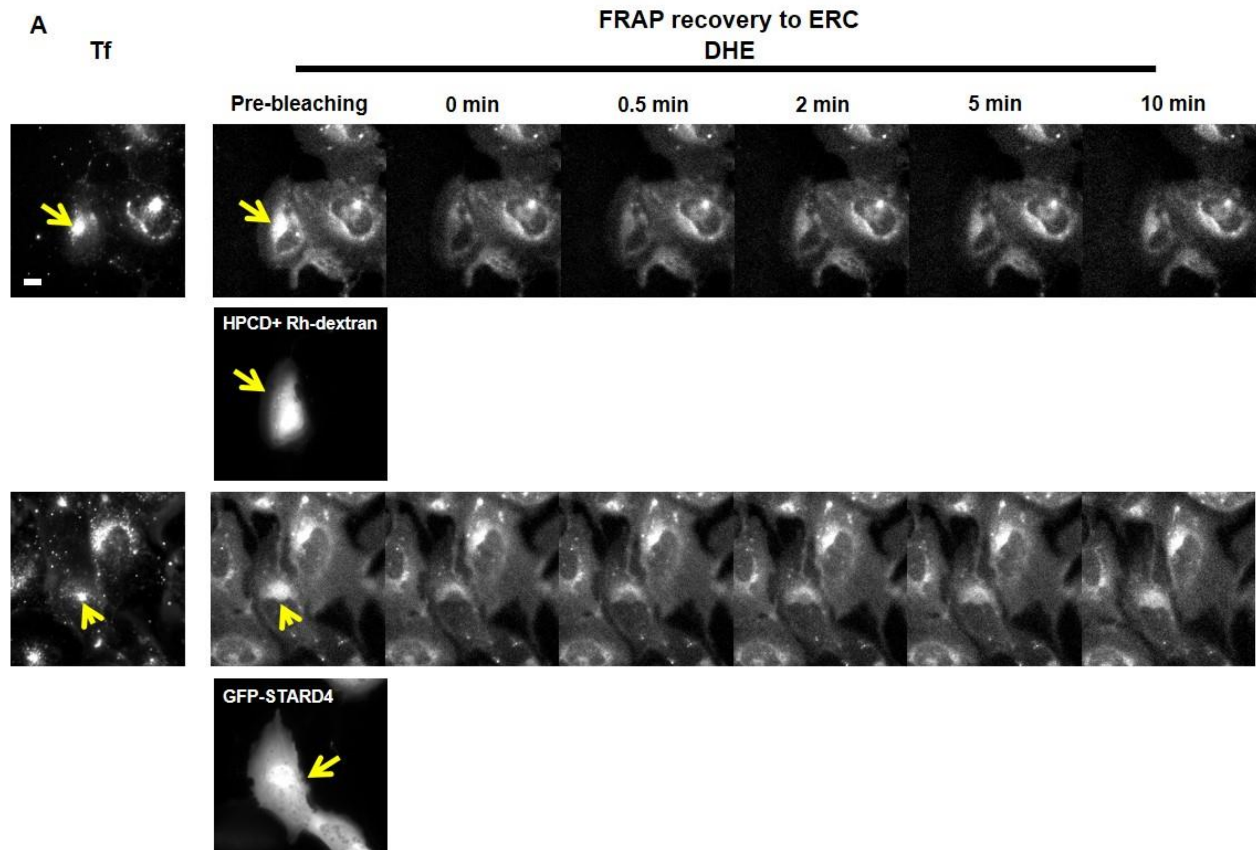
Supplementary Figure 4- Role of vesicular sterol transport. U2OS-SRA cells were treated with energy depletion (ED) medium to deplete cellular ATP levels. (A) FRAP measurements for control and ED. (B) DHE efflux curves for control and ED. (C) Extended FRAP measurement for control and ED U2OS-SRA cells. Each data point is derived from an average of at least eight experiments (± SD). Data are fit to single exponential curves.

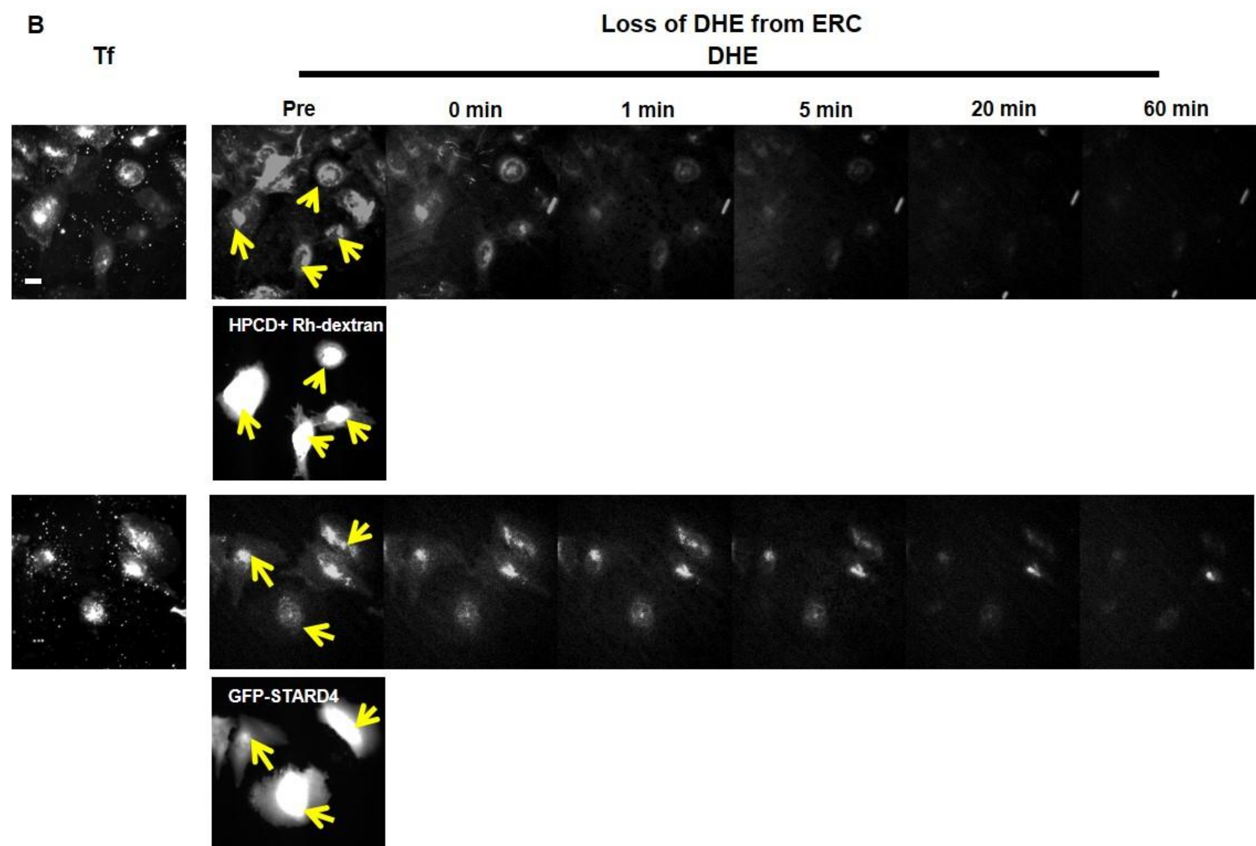


Supplementary Figure 5- Role of vesicular sterol transport. U2OS-SRA cells were treated with energy depletion (ED) medium to deplete cellular ATP levels. (A) For FRAP recovery to the ERC, an image was taken before photobleaching. DHE in the ERC was photobleached (yellow arrow), and images were taken every 30 seconds. (B) To record the loss of DHE from the ERC, an image was taken before exchanging with efflux medium. Images were taken as described in materials and methods. Cells were maintained at 37°C. Scale bar: 10 µm.

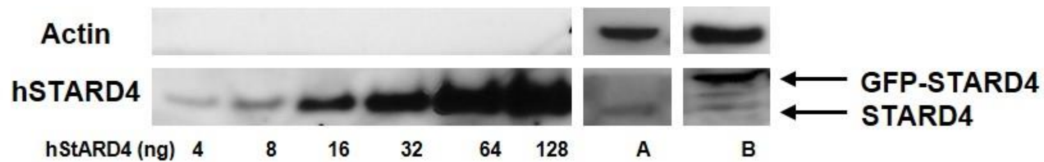


Supplementary Figure 6- Microinjected HPCD and STARD4 overexpression enhances bidirectional sterol transport. HPCD and rhodamine-dextran were microinjected immediately before labeling cells with DHE. U2OS-SRA cells were transfected with GFP-STARD4 for 18 h before the experiment. (A) FRAP measurements for single cells microinjected with HPCD or transfected with GFP-STARD4. (B) DHE efflux curves for cells microinjected with HPCD or transfected with GFP-STARD4. Each data point is derived from an average of at least ten experiments (± SD). Data are fit to single exponential curves.

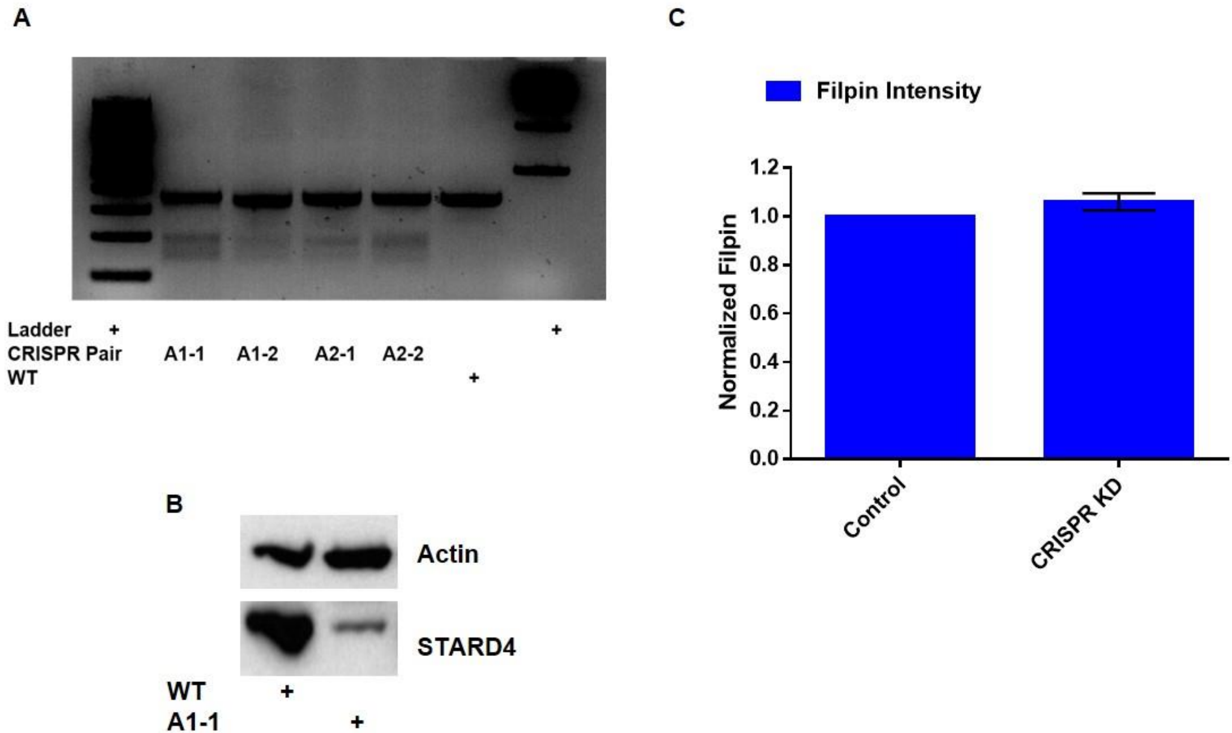

A
Tf
FRAP recovery to ERC
DHE
Pre-bleaching
0 min
0.5 min
2 min
5 min
10 min
HPCD+ Rh-dextran
GFP-STARD4


B
Tf
Loss of DHE from ERC
DHE
Pre
0 min
1 min
5 min
20 min
60 min
HPCD+ Rh-dextran
GFP-STARD4

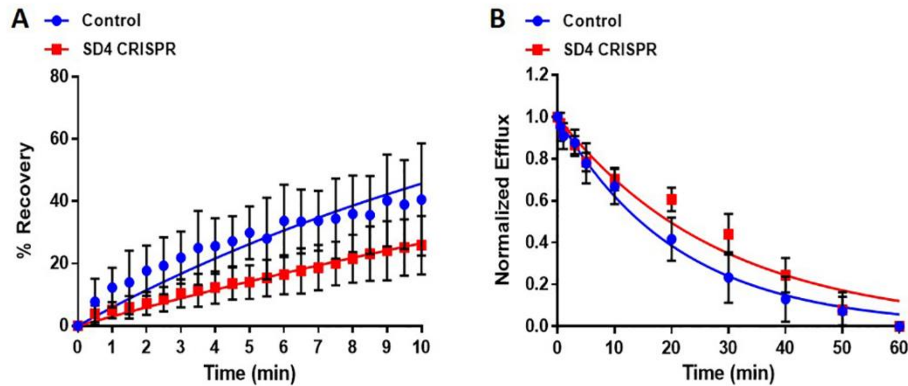
Supplementary Figure 7- Microinjected HPCD and STARD4 overexpression enhances bidirectional sterol transport. HPCD and rhodamine-dextran were microinjected immediately before labeling cells with DHE. U2OS-SRA cells were transfected with GFP-STARD4 for 18 h before the experiment. (A) An image was taken before photobleaching. DHE in the ERC was photobleached (yellow arrow), and images were taken every 30 seconds. Cells were maintained at 37°C. Scale bar: 10 μm. (B) To record the loss of DHE from the ERC, an image was taken before exchanging with efflux medium. Images were taken as described in materials and methods. Cells were maintained at 37°C. Scale bar: 10 μm.



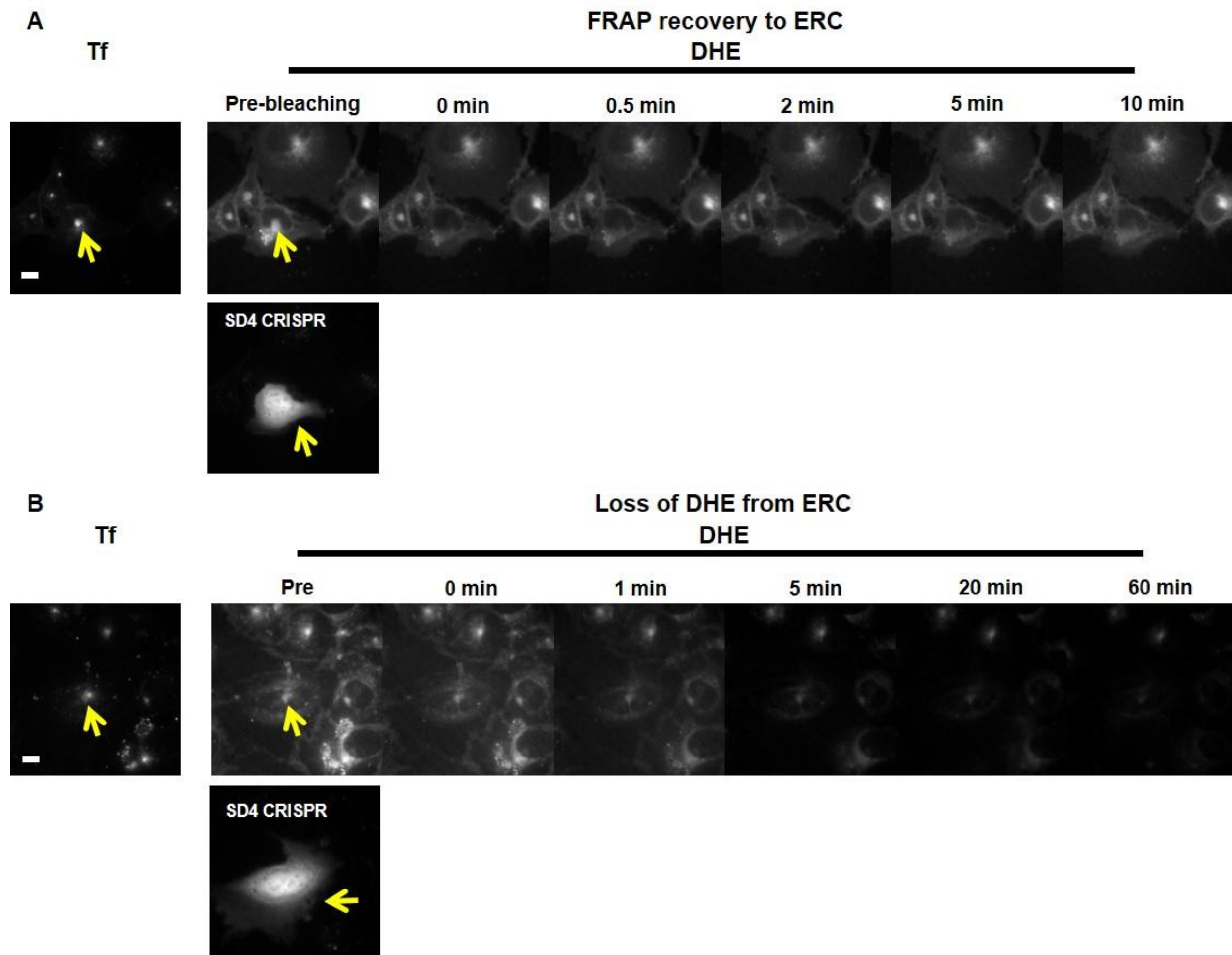
Supplementary Figure 8- Quantification of STARD4 molecules per U2OS-SRA cell. To estimate the number of STARD4 molecules per cell, U2OS-SRA cells were counted and lysed. Proteins from an aliquot of the cell lysate were separated by 16% SDS-PAGE and immunoblotted with anti-STARD4. Known amounts of purified hSTARD4 were loaded in parallel as standard references. 15% of the volume of a lysate of 2.0×10^6 cells was loaded. The band corresponding to STARD4 was quantified and compared to the protein standards. We found that one U2OS cell has ~250,000 molecules. The same measurement was performed on lane B with cells expressing GFP-STARD4. In this case 10% of the cells used for quantification were transfected with GFP-STARD4. The band corresponding to GFP-STARD4 was quantified, from which the number of GFP-STARD4 molecules per cell was calculated. We found that GFP-STARD4 is ~5 fold more abundant than endogenous STARD4 in transfected cells, with up to ~1,200,000 molecule/cell



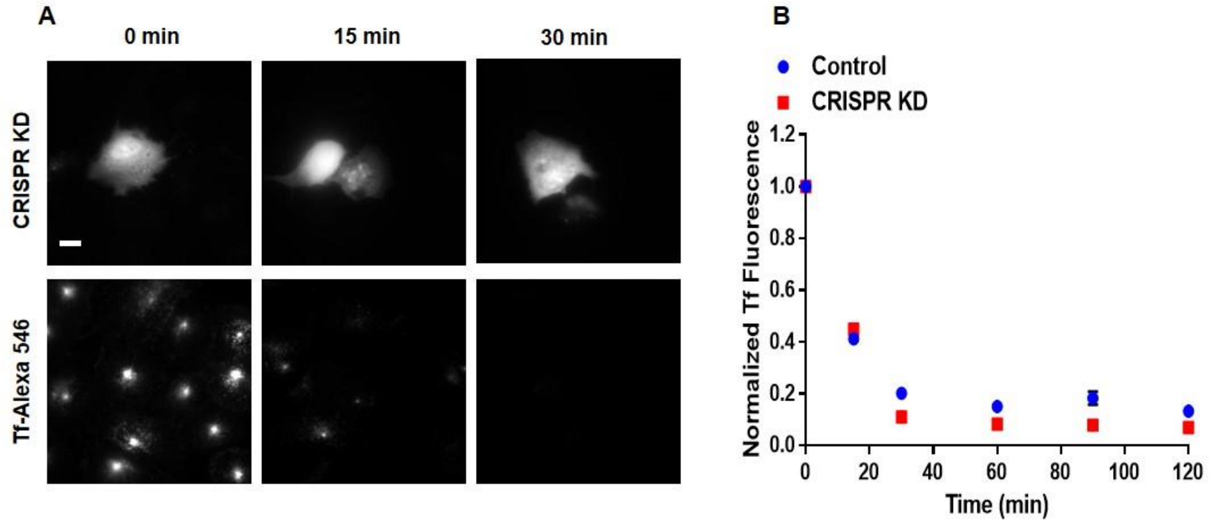
Supplementary Figure 9- Genomic disruption of STARD4 for 18 hours does not alter cholesterol levels. Surveyor assay of the *STARD4* loci following 18 hour expression of GFP-Cas9 (CRISPR-STARD4) constructs. U2OS-SRA cells expressing paired SD4 CRISPR constructs were sorted and collected using GFP fluorescence. (B) Western blot of whole cell lysates from U2OS-SRA control cells and cells expressing the A1-1 GFP-Cas9 CRISPR-STARD4) construct. Cells were transfected and sorted after 18 hours using GFP fluorescence. Cells expressing GFP-Cas9 show reduction of STARD4, compared to control cells. Pan-Actin is used as a loading control. (C) Quantification of the average cellular filipin fluorescence power of cells expressing CRISPR-STARD4 construct. Filipin fluorescence was normalized to the non-transfected cells. Intensity measurements were performed on background-subtracted images from an average of 20 fields of cells in each condition. Data represent averages (± SE) of three independent experiments normalized to control value.



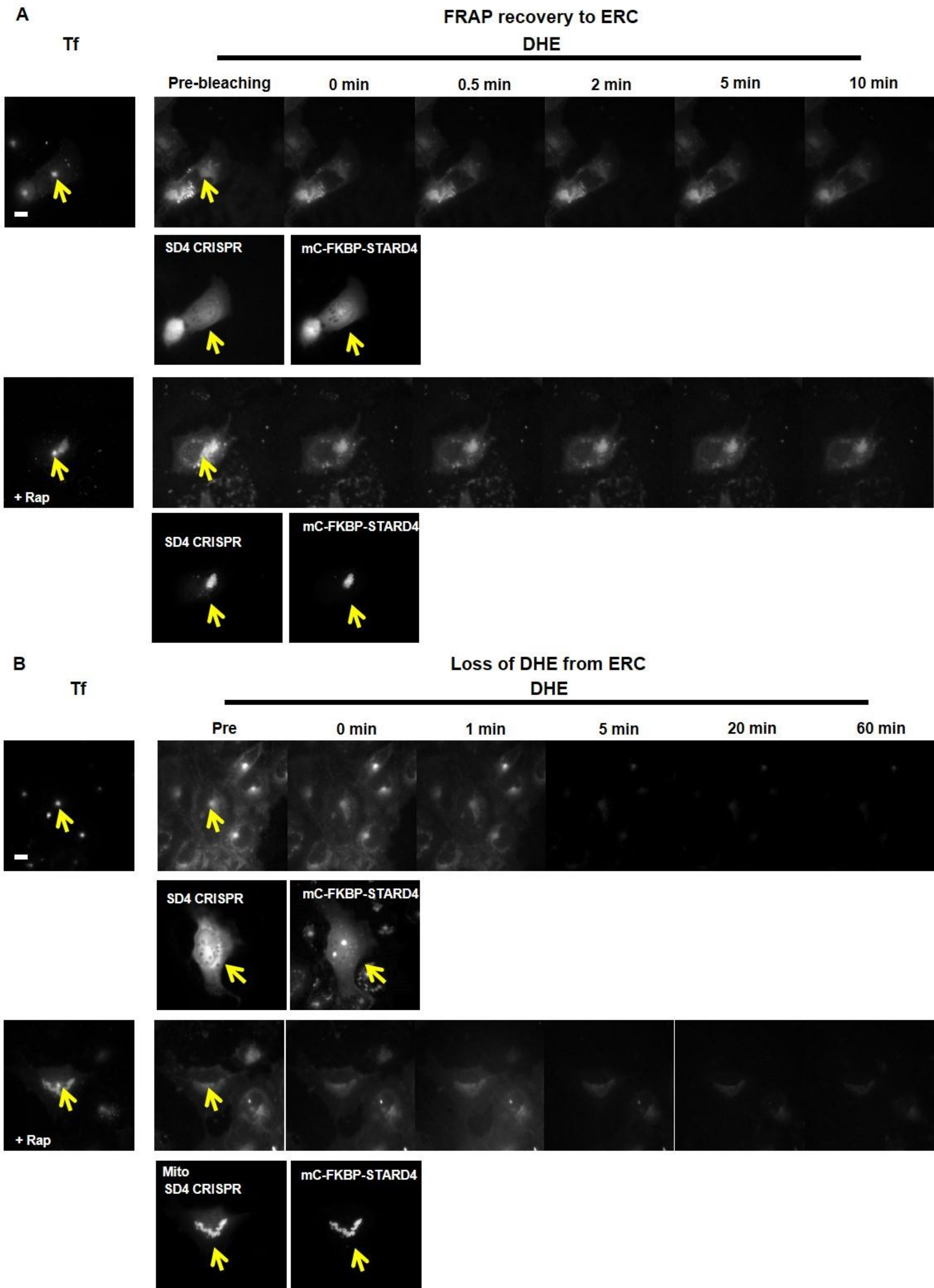
Supplementary Figure 10- Depletion of STARD4 slows sterol transport between ERC and plasma membrane. U2OS-SRA cells were transfected with GFP-Cas9 (STARD4 CRISPR) constructs for 18 h before the experiment. (A) FRAP measurements for single cells expressing STARD4 CRISPR constructs. (B) DHE efflux curves for cells expressing STARD4 CRISPR constructs. Each data point is derived from an average of at least eleven experiments (± SD). Data are fit to single exponential curves.



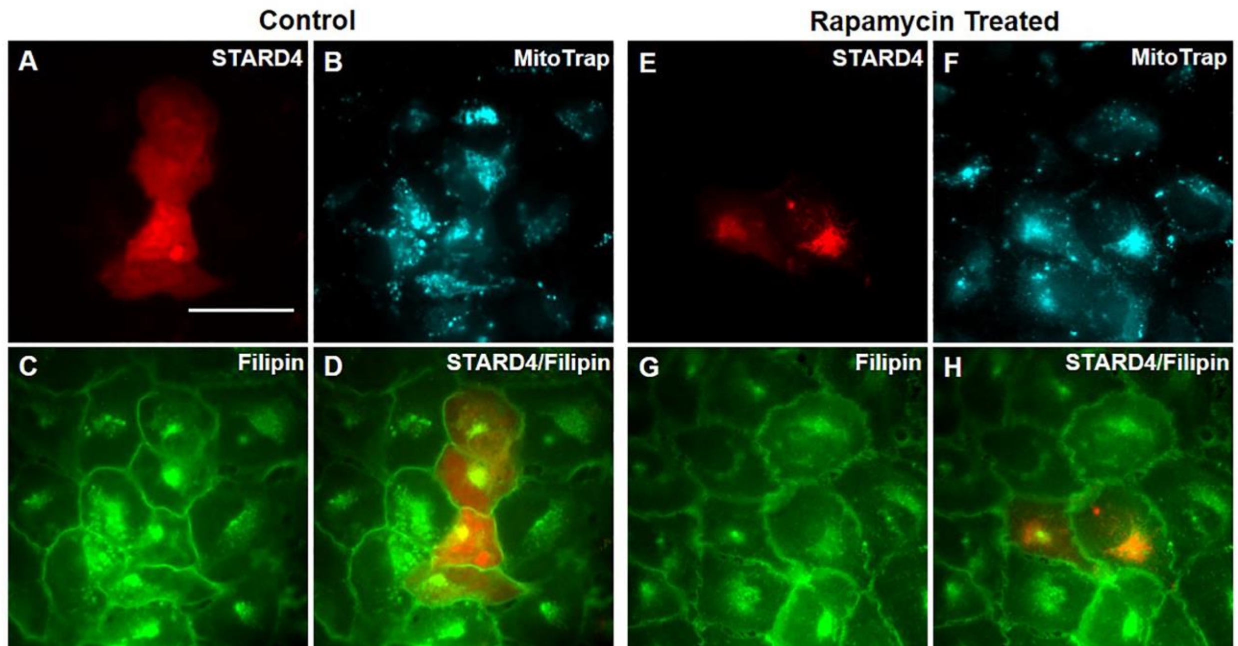
Supplementary Figure 11- U2OS-SRA cells transfected with GFP-Cas9 (STARD4 CRISPR) constructs for 18 hours. (A) For FRAP recovery to the ERC, an image was taken before photobleaching. DHE in the ERC was photobleached (yellow arrow), and images were taken every 30 seconds. (B) To record the loss of DHE from the ERC, an image was taken before exchanging with efflux medium. Images were taken as described in materials and methods. Cells were maintained at 37°C. Scale bar: 10 μm.



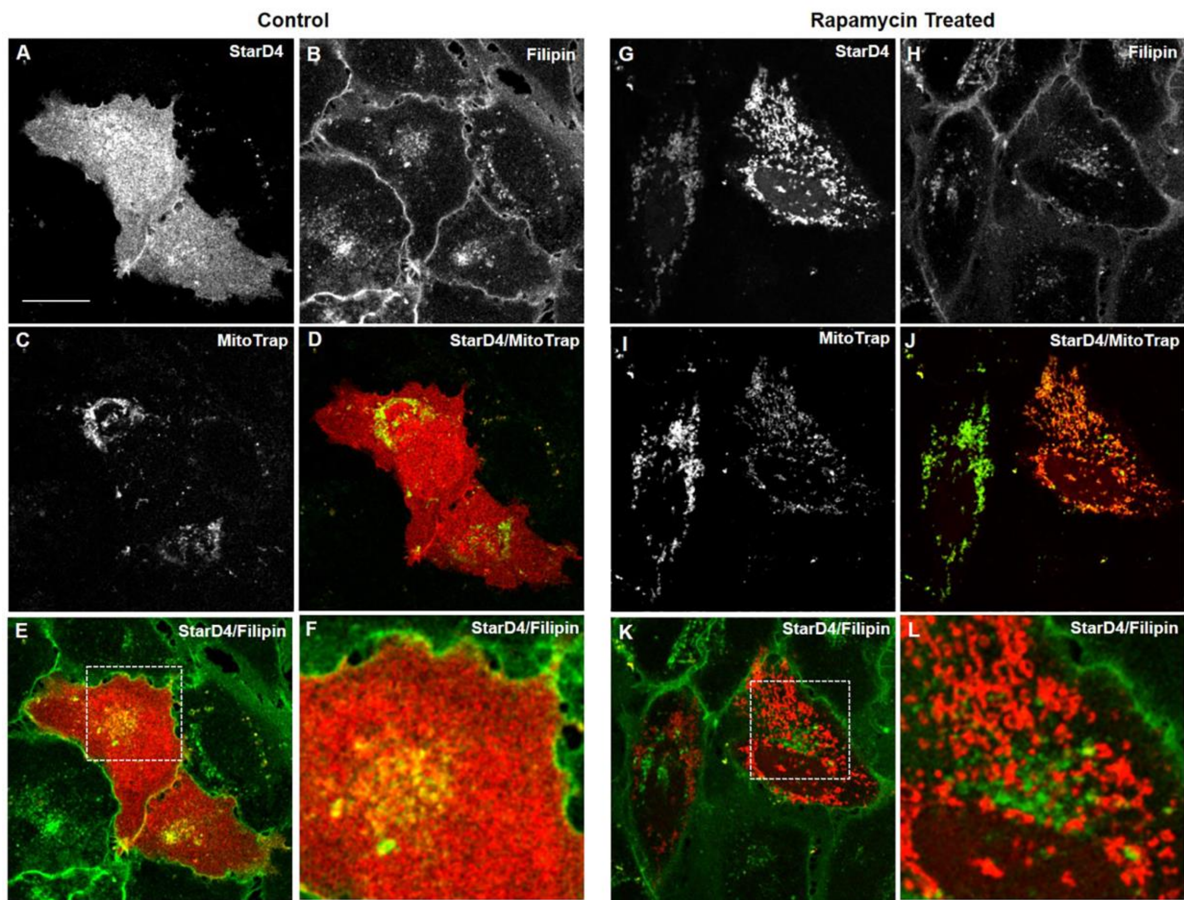
Supplementary Figure 12- Genomic disruption of STARD4 does not alter vesicular trafficking. U2OS-SRA cells transiently transfected with GFP-Cas9 (CRISPR-STARD4) constructs were incubated with Tf-Alexa546 for 20 minutes and subjected to a chase in the presence of a 100-fold molar excess of unlabelled transferrin and deferoxamine for 120 minutes. (A) Epifluorescence microscopy images of U2OS-SRA cells expressing CRISPR-STARD4 constructs that were labeled with Tf-Alexa546. Scale bar: 10 µm. (B) Total integrated intensity per field was obtained for Tf-Alexa546, after background correction. The integrated intensity values were then averaged. Plots were constructed for the normalized Tf-Alexa546 fluorescence intensity as a function of time. Data represent averages (± SE) of three independent experiments normalized to control value.


A
FRAP recovery to ERC
Tf
DHE
Pre-bleaching
0 min
0.5 min
2 min
5 min
10 min
SD4 CRISPR
mC-FKBP-STARD4
+ Rap
SD4 CRISPR
mC-FKBP-STARD4
B
Loss of DHE from ERC
Tf
DHE
Pre
0 min
1 min
5 min
20 min
60 min
SD4 CRISPR
mC-FKBP-STARD4
+ Rap
Mito
SD4 CRISPR
mC-FKBP-STARD4

Supplementary Figure 13- Expression of wild type, cytosolic localized STARD4 restores sterol transport between ERC and plasma membrane. U2OS-SRA cells were transfected with mCherry-FKBP-STARD4 with or without MitoTrap 48 hours prior to the experiment. STARD4 CRISPR constructs were transfected for 18 h before the experiment. To redistribute STARD4 from the cytosol to the mitochondria, cells were treated with 1 μM rapamycin for 10 minutes prior to imaging. Following rapamycin treatment, cells were washed and fresh medium 2 was added. (A) For FRAP recovery to the ERC, an image was taken before photobleaching. DHE in the ERC was photobleached (yellow arrow), and images were taken every 30 seconds. (B) To record the loss of DHE from the ERC, an image was taken before exchanging with efflux medium. Images were taken as described in materials and methods. In the efflux experiment, there is signal cross-over from Alexa633-Tf into mCherry-FKBP-STARD4 fluorescence signal. Cells were maintained at 37°C. Scale bar: 10 μm.

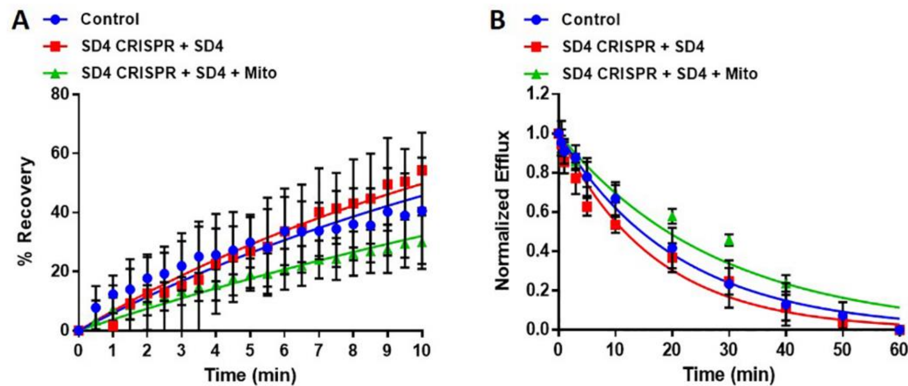


Supplementary Figure 14- Recruitment of STARD4 to the mitochondria results in cholesterol redistribution. U2OS-SRA cells were transfected with mCherry-FKBP-STARD4 with MitoTrap 18 hours prior to the experiment. To redistribute STARD4 from the cytosol to the mitochondria, cells were treated with 1 μM rapamycin for 10 minutes prior to imaging. Control cells were incubated in medium 2 without rapamycin. Following rapamycin treatment, cells were washed and fixed. Cells were subsequently stained with filipin prior to imaging. Control cells, STARD4 (A), MitoTrap (B) and filipin (C). (D) Overlay of STARD4 (red) and filipin (green) in control cells. Rapamycin treated cells, STARD4 (E), MitoTrap (F) and filipin (G). (H) Overlay of STARD4 (red) and filipin (green) in rapamycin treated cells.

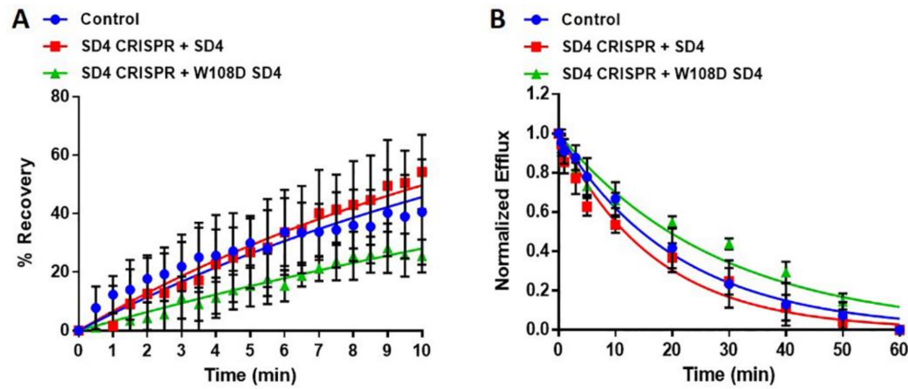


Supplementary Figure 15- STARD4 recruited to the mitochondria does not overlap with filipin. U2OS-SRA cells were transfected with mCherry-FKBP-STARD4 with MitoTrap 18 hours prior to the experiment. To redistribute STARD4 from the cytosol to the mitochondria, cells were treated with 1 μM rapamycin for 10 minutes prior to imaging. Control cells were incubated in medium 2 without rapamycin. Following rapamycin treatment, cells were washed and fixed. Cells were subsequently stained with filipin prior to imaging by confocal microscopy. STARD4 (A), filipin (B) and MitoTrap (C) in non-treated control cells. (D) Overlay of STARD4 (red) and MitoTrap (green) in control cells. (E) Overlay of STARD4 (red) and filipin (green) in control cells. (F) Zoom in of area in panel E. STARD4 (G), filipin (H) and MitoTrap (I) in rapamycin treated cells. (J) Overlay of STARD4 (red) and MitoTrap (green) in rapamycin treated

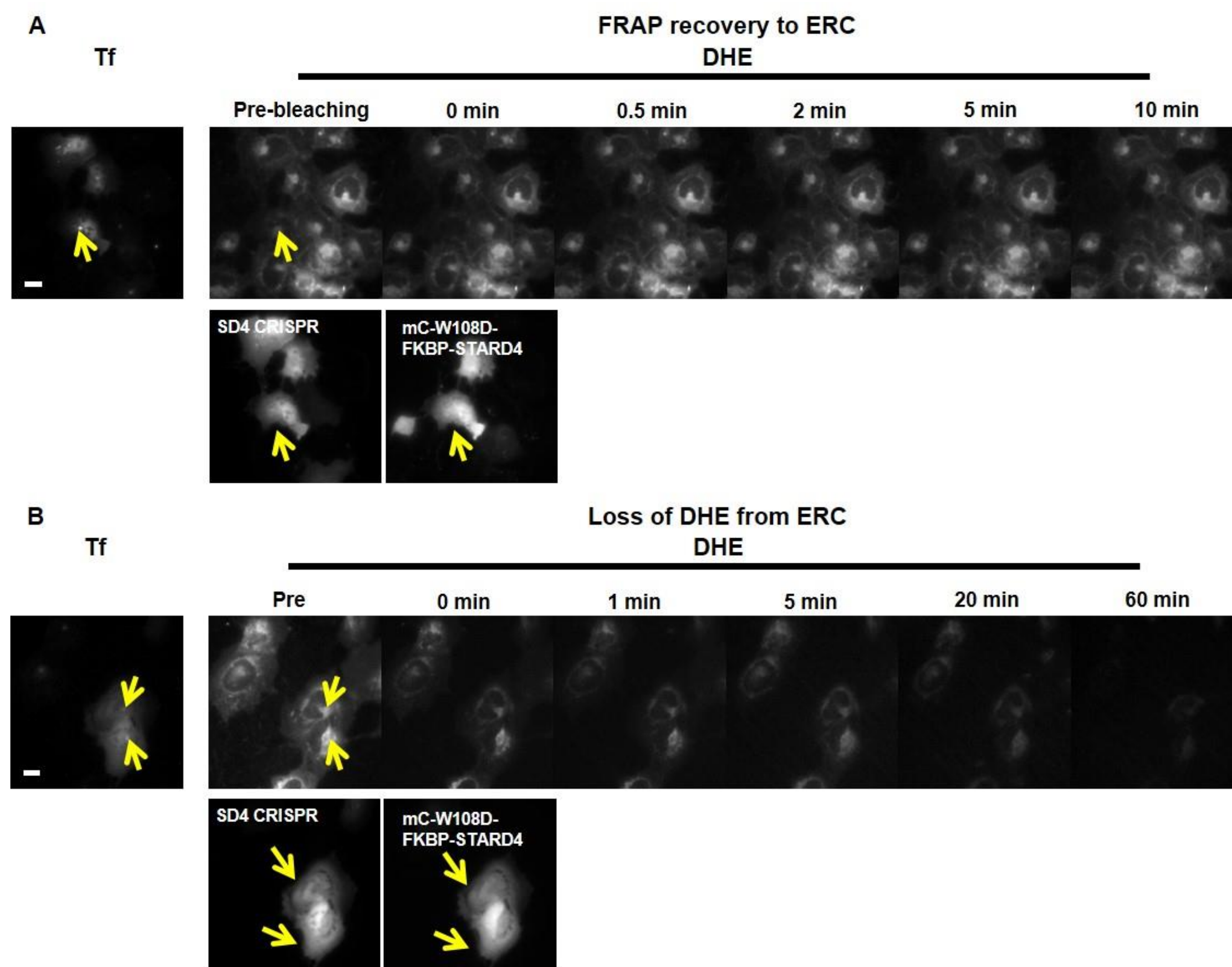
cells. (K) Overlay of STARD4 (red) and filipin (green) in control cells. (L) Zoom in of area in panel K.



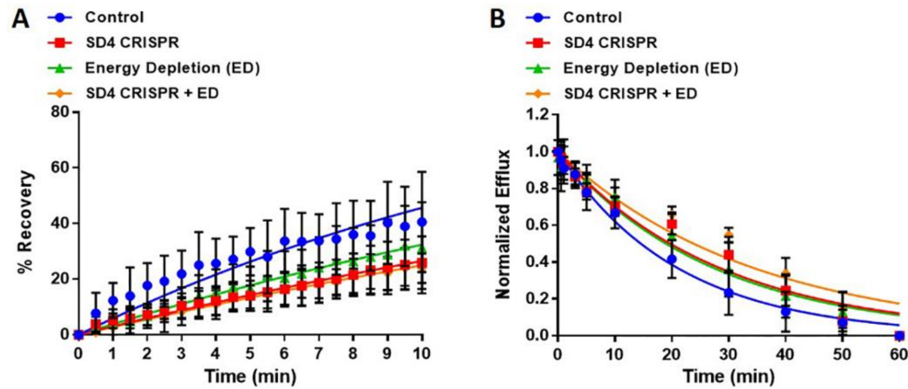
Supplementary Figure 16- Expression of wild type, cytosolic localized STARD4 restores sterol transport between ERC and plasma membrane. U2OS-SRA cells were transfected with mCherry-FKBP-STARD4 with or without MitoTrap 48 hours prior to the experiment. STARD4 CRISPR constructs were transfected for 18 h before the experiment. To redistribute STARD4 from the cytosol to the mitochondria, cells expressing MitoTrap were treated with 1 μM rapamycin for 10 minutes prior to imaging. Following rapamycin treatment, cells were washed and fresh medium 2 was added. (A) FRAP measurements for single cells expressing mCherry-FKBP-STARD4 and MitoTrap in cells expressing STARD4 CRISPR constructs. (B) DHE efflux curves for cells expressing mCherry-FKBP-STARD4 and MitoTrap in cells expressing STARD4 CRISPR constructs. Each data point is derived from an average of at least ten experiments (± SD). Data are fit to single exponential curves.



Supplementary Figure 17- Expression of Omega-1 loop mutant (W108D) of STARD4 does not restore sterol transport. (A) U2OS-SRA cells were transfected with either wild-type or W108D mCherry-FKBP-STARD4 48 hours prior to the experiment. STARD4 CRISPR constructs were transfected for 18 h before the experiment. FRAP measurements for cells expressing wild-type or W108D mCherry-FKBP-STARD4 in cells expressing STARD4 CRISPR constructs. (B) DHE efflux curves for cells expressing wild-type or W108D mCherry-FKBP-STARD4 in cells expressing STARD4 CRISPR constructs. Each data point is derived from an average of at least ten experiments (± SD). Data are fit to single exponential curves.

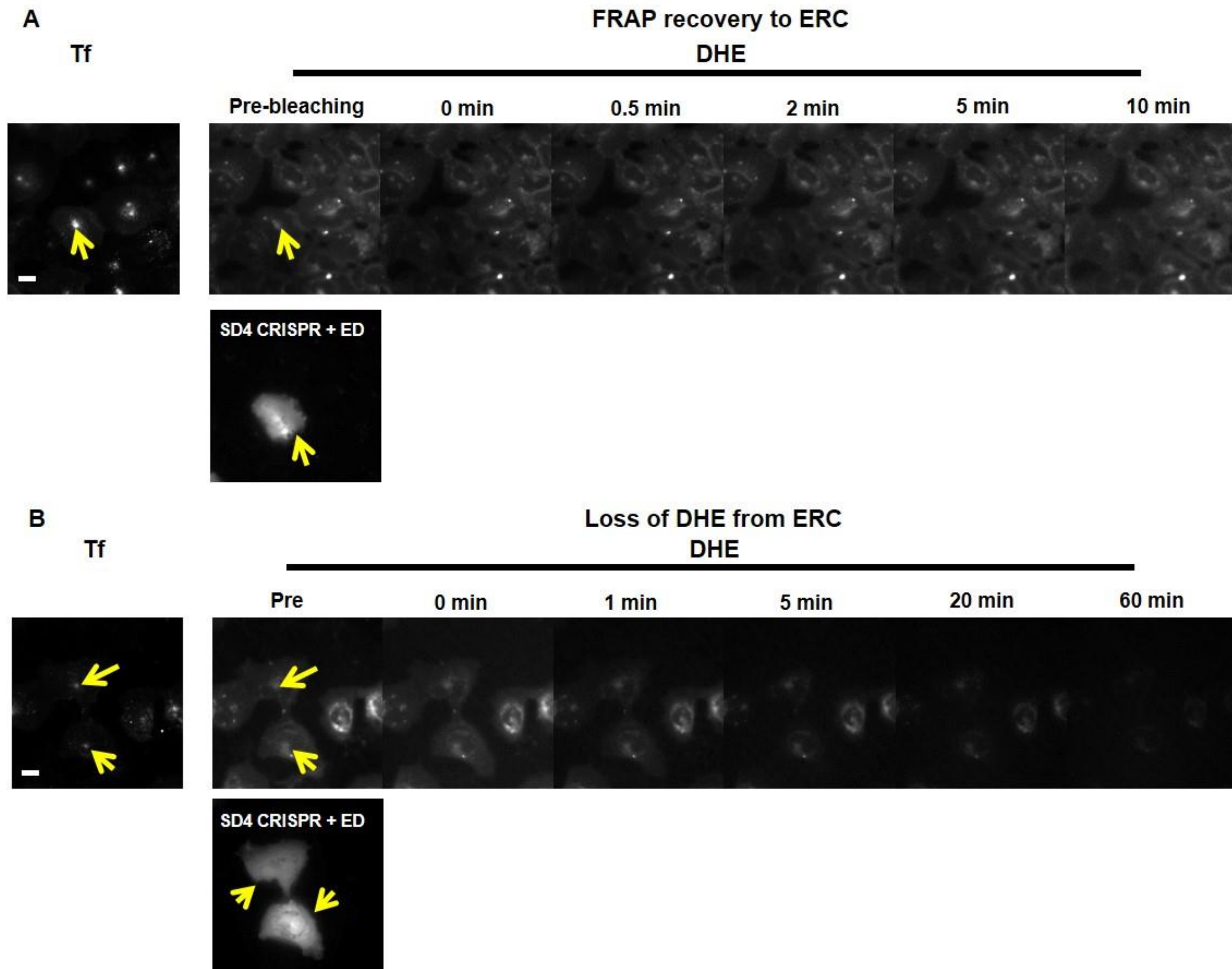


Supplementary Figure 18- Expression of Omega-1 loop mutant (W108D) of STARD4 does not restore sterol transport. U2OS-SRA cells were transfected with either wild-type or W108D mCherry-FKBP-STARD4 48 hours prior to the experiment. STARD4 CRISPR constructs were transfected for 18 h before the experiment. (A) For FRAP recovery to the ERC, an image was taken before photobleaching. DHE in the ERC was photobleached (yellow arrow), and images were taken every 30 seconds. (B) To record the loss of DHE from the ERC, an image was taken before exchanging with efflux medium. Images were taken as described in materials and methods. Cells were maintained at 37°C. Scale bar: 10 µm.



Supplementary Figure 19- STARD4 is a major non-vesicular sterol transport protein.

U2OS-SRA cells were transfected with STARD4 CRISPR constructs and/or treated with energy depletion (ED) medium. (A) FRAP measurements for cells expressing STARD4 CRISPR constructs with or without ED. (B) DHE efflux measurements for cells expressing STARD4 CRISPR constructs with or without ED. Each data point is derived from an average of at least nine experiments (± SD). Data are fit to single exponential curves.



Supplementary Figure 20- STARD4 is a major non-vesicular sterol transport protein. U2OS-SRA cells were transfected with STARD4 CRISPR constructs and treated with energy depletion (ED) medium. (A) For FRAP recovery to the ERC, an image was taken before photobleaching. DHE in the ERC was photobleached (yellow arrow), and images were taken every 30 seconds. (B) To record the loss of DHE from the ERC, an image was taken before exchanging with efflux medium. Images were taken as described in materials and methods. Cells were maintained at 37°C. Scale bar: 10 μm.