Supplementary Experimental Procedures

Late endosome/lysosome fusion assay

The fusion between late endosomes and lysosomes was evaluated in fibroblasts using a method described by Jahrus *et al.* (REF) with modifications to allow quantitative assessments based on fluorescence resonance energy transfer (FRET) of late endosome/lysosome contents upon fusion. Jahrus et al. originally observed the fusion interactions in live cells using incubations of sucrose to form swollen lysosome compartments full of sucrose, termed sucrosomes. Subsequently, a large latex bead conjugated with invertase was pulsed into late endosomes. The disappearance of the sucrosomes, as observed with light microscopy, was evaluated over time as the two compartments interacted. Upon interacting, the invertase enzyme is able to cleave sucrose into fructose and glucose, which are then able to escape the lysosome. To quantitatively evaluate this phenomenon, we employed a similar strategy. First, non-degradable dextran was pulsed into lysosomes. This dextran was conjugated with biotin and fluorescently labeled with Alexa Fluor 647 (FRET acceptor). To label late endosomes, a large latex bead of the same diameter used previously by Jahrus was pulsed into late endosomes. This bead was conjugated with streptavidin and fluorescently labeled with Alexa Fluor 555 (FRET donor). Interactions between the two labeled compartments was evaluated using FRET signals originating from the biotinylated dextran binding to the streptavidin conjugated latex bead, which brings the FRET donor in close proximity to the FRET acceptor.

To construct the FRET donor and acceptor, standard labeling strategies were employed. For the lysosome tracer, Biotinylated-dextran-amine (10,000 M.W., BDA-10000) was fluorescently labeled with the amine-reactive succinimidyl ester derivative of AlexaFluor 647 per Invitrogen's instructions. First, the AlexaFluor 647 was reconstituted in dry DMSO to a concentration of 10 mg/ml. BDA-10000 was reconstituted to 50 mg/ml in 0.1 M sodium bicarbonate buffer (pH = 9.0, Buffer A) of which a 100 µl aliquot was added to 75 µl of Buffer A and 25 µl of 10 mg/ml AlexaFluor 647 stock solution. The reaction was carried out at room temperature for 2 hs and was quenched with the addition of 90 mM Tris. The resulting solution was desalted five times using protein desalting columns from Thermo Scientific (Rockford, IL) to remove unreacted Alexa Fluor 647. Aliquots of the labeled biotin dextran were stored at -20^{o}C until use. For the late endosome tracer, carboxylate-modified monodisperse polystyrene beads (2.5% solids, 1.08 x 10^{11} beads/ml, 0.792 ± 0.037 µm) were used. First, the beads were activated using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDAC). To accomplish this, 4 ml of the polystyrene beads were washed 3 times with 0.1 M MES buffer (pH = 6.0, Buffer B) by centrifuging the beads at 5,000 x g for 20 mins and resuspending with Buffer B. Immediately after the final wash, the beads were resuspended to a 2% solids mixture in Buffer B and 2 ml of freshly prepared 50 mg/ml EDAC was added drop-wise to the suspension. The EDAC was allowed to activate the carboxylate groups for 15 mins at room temperature under gentle mixing followed by three washes with 0.1 M PBS (pH = 7.4, Buffer

C). The activated beads were then resuspended in 5 ml of Buffer C for subsequent streptavidin conjugation. Lyophilized streptavidin, purchased from Invitrogen, was reconstituted in Buffer C at a concentration of 1 mg/ml of which 5 ml of the stock solution was added to the activated beads solution. The conjugation was allowed to take place at room temperature for 3 hs under gentle mixing. Unconjugated streptavidin was removed by extensive washing with PBS. Degree of conjugation was determined using the method of Bradford on fractions of streptavidin pre-conjugation, post-conjugation, and from subsequent washes. It was determined that approximately 47,000 molecules of streptavidin were conjugated per polystyrene bead. The beads were centrifuged and stored at 4^{o}C in a solution of Buffer C containing 0.1% glycine to mask any unreacted sites at a 2% solids mixture. Fluorescence labeling of the streptavidin conjugated beads was carried out using the amine-reactive succinimidyl ester derivative of AlexaFluor-555 per Invitrogen's instructions. First, 1 ml of streptavidin conjugated beads were washed 2 times with Buffer A to remove the glycine storage buffer and resuspended back to 1 ml. The AlexaFluor-647 was reconstituted to 10 mg/ml using dry DMSO, and an aliquot of 10 µl was added to the freshly washed beads. Labeling was performed room temperature for 2 hs under gentle mixing and subsequently quenched with 90 mM Tris. Unreacted AlexaFluor 555 was removed with extensive washing with PBS. Subsequent dialysis was performed against PBS using a dialysis cassette with a 3.5 kDa cutoff. Beads were centrifuged once more, resuspended in PBS, and stored at 4^{o}C until needed.

To evaluate the fusion of late endosomes and lysosomes, cells were seeded in 8-well culture chambers at 75,000 cells per well. Cells were incubated for 2 hs with 2.5 mg/ml AlexaFluor 647 biotinylated dextran in complete medium, washed once with PBS, and given chase in complete phenol red-free DMEM medium for 20 hs to ensure localization into the lysosome. After 20 hs post-chase, cells were exposed to 1.28 x 10^7 beads/ml of AlexaFluor 555 streptavidin-conjugated latex beads for 2 hs and chased for 1 h to label the late endosomal compartment. All treatments, as well as FRET measurements, were started immediately after the localization of the latex bead to the late endosome. Monolayers were analyzed using a Photon Technologies International (Birmingham, NJ) Ratiomaster microscope-mounted spectrofluorimeter with PMT detection. For each treatment, three measurements were made, corresponding to the donor excitation/emission (AF555), donor excitation/acceptor emission (FRET), and acceptor excitation/emission (AF647). Equation 1 was used to correct for both spectral cross-talk of the FRET tracers and the propensity of cell populations to endocytose differential amounts of the tracers. Correction factors α and β were determined to be 0.011 and 0.799, respectively for our experimental setup (1,2).

Eqn 1. $$\text{FRET ratio} = \frac{\text{FRET} - \alpha \times \text{AF555} - \beta \times \text{AF647}}{\sqrt{\text{AF555} \times \text{AF647}}}$$

Supplemental References

1. Hillebrand, M., Verrier, S. E., Ohlenbusch, A., Schafer, A., Soling, H. D., Wouters, F. S., and Gartner, J. (2007) *The Journal of biological chemistry* **282**(37), 26997-27005
2. Xia, Z., and Liu, Y. (2001) *Biophysical journal* **81**(4), 2395-2402

Supplemental Figure Legends

Fig. S1. Normal fibroblasts ($NPC1^{+/+}$) treated with 70 μM NR form vacuoles that do not colocalize with the Golgi specific protein GM130 (green) nor with the early endosome specific protein EEA1 (red).

Fig. S2. Full ^{3}[H]-dextran release profiles for both $NPC1^{+/+}$ and $NPC1^{-/-}$ cell lines treated or untreated with indicated molecules. Release into the medium was monitored over a period of 24 hrs after localizing the dextran into the lysosomes for 17 hrs and providing treatments for 3 hrs. Data points represent an average ± SD for the percent dextran released as a function of time. For treatments other than control and NR, only the 24 hr time point was evaluated. Increases in dextran release were observed for the lysosomotropic amines neutral red (70 μM, NR, n=12), morpholine (10mM, MOR, n=8) and imidazole (10 mM, IMDZ, n=7) in $NPC1^{+/+}$ fibroblasts relative to the untreated control. These differences were found to be statistically significant ($p < 0.001$). Statistical differences were not observed in $NPC1^{-/-}$ cells treated with the same compounds. Rhodamine-123 (R123, 70 μM, n=7), an amine-containing compound known not to accumulate in the lysosome, showed no deviation from the control $NPC1^{+/+}$ cells. U18666A (10 μM, n=7) inhibited NPC1 function in dextran secretion in normal cells and had no

influence on NPC cells. The formation of sucrosomes with sucrose (0.1% w/v, n=4) had no statistically significant influence of dextran secretion. Statistical analysis was performed using an unpaired t-test.

Fig. S3. Full profiles for late endosome/lysosome fusion assay. Cells were observed over a period of 2 hrs after the final chase of the late endosome FRET tracer as described in the Supplementary Experimental Procedures. Treatments of amines and nocodazole were started at the initial time point, which was normalized to zero for comparison purposes. Increased FRET signals, indicative of increased late endosome/lysosome fusion, were observed for the lysosomotropic amines morpholine (5mM, MOR, n=3) and imidazole (10mM, IMDZ, n=3). These differences were found to be statistically significant ($p < 0.01$) at the 2 hr time point. Statistically significant decreases in fusion events were observed for U18666A (10 μM, n=3) and nocodazole (NOC, 50 μM, n=3) at 2 hrs. Differences were not observed in $NPC1^{-/-}$ cells for any treatment.

Figure S1.

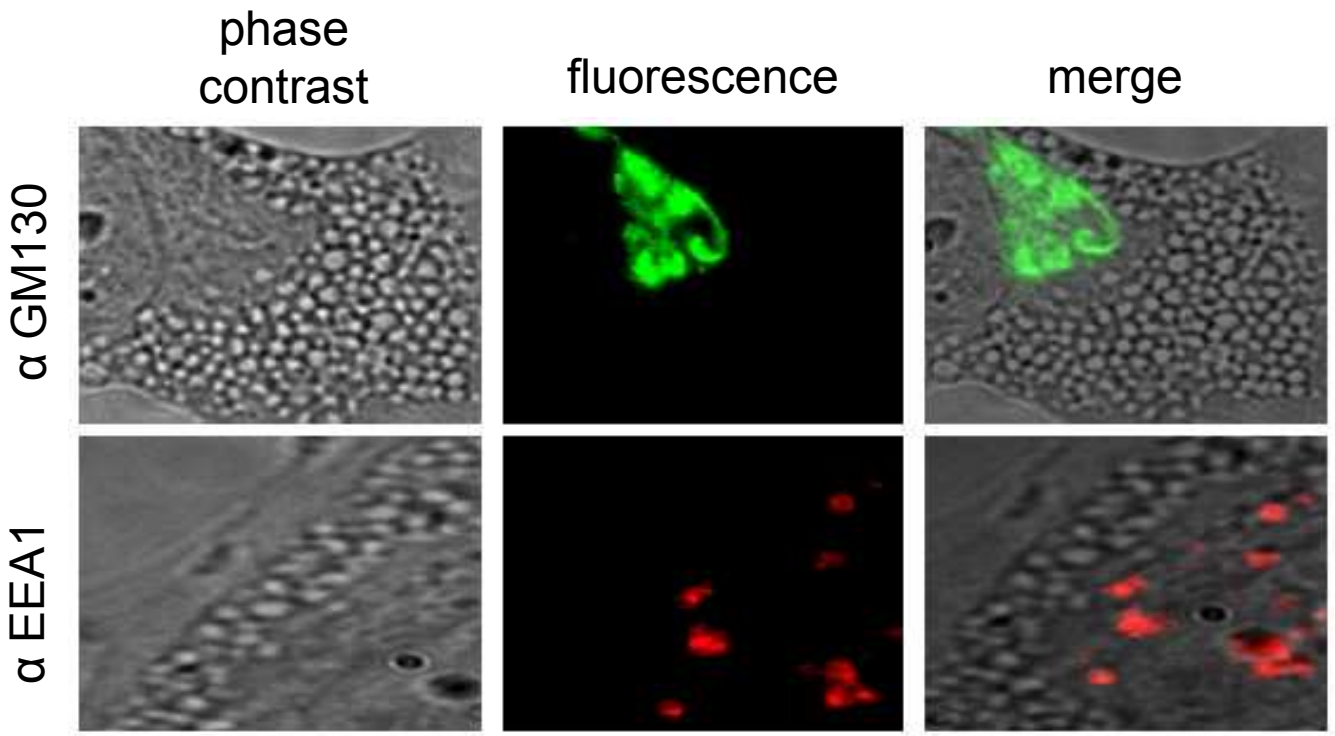


Figure S2.

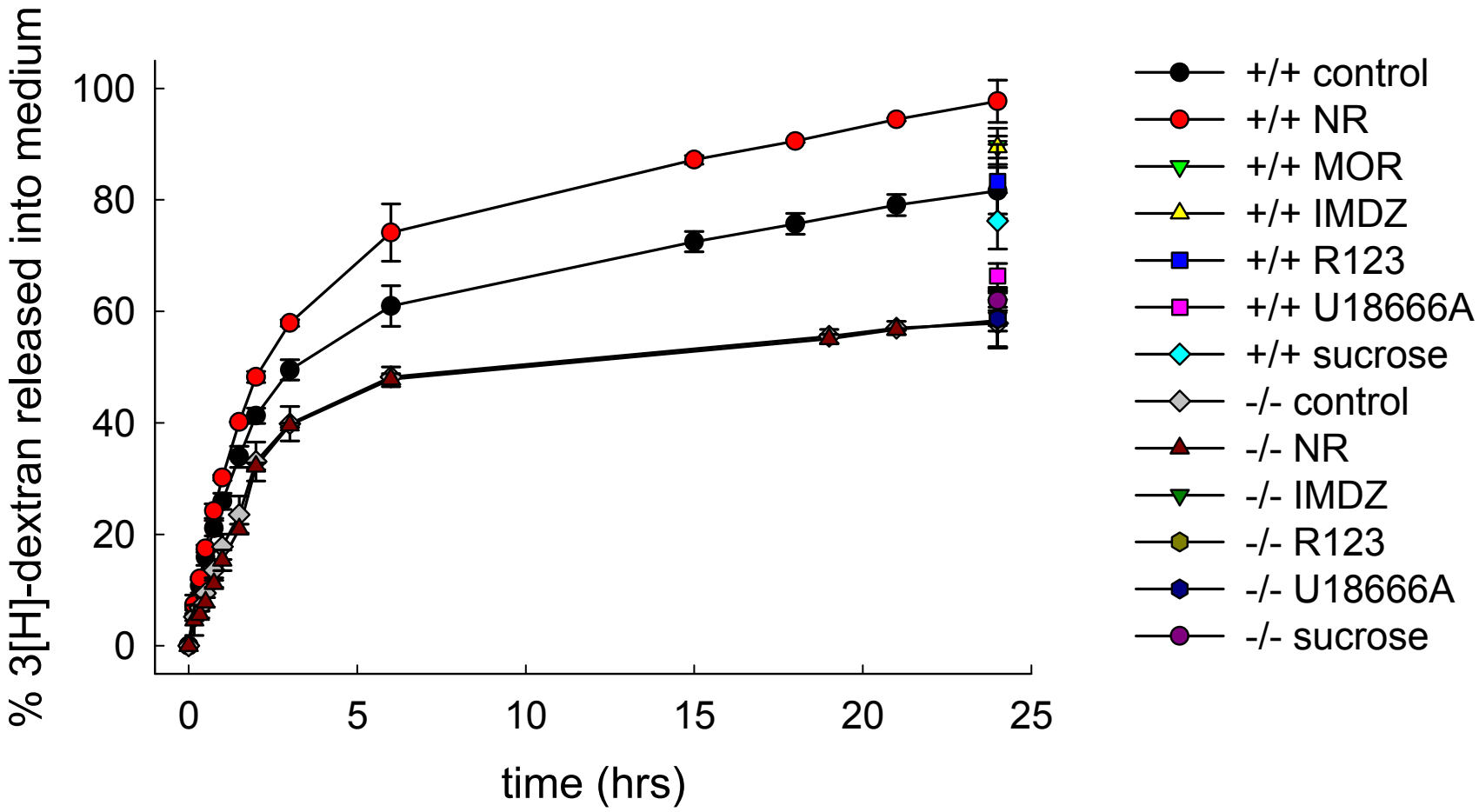
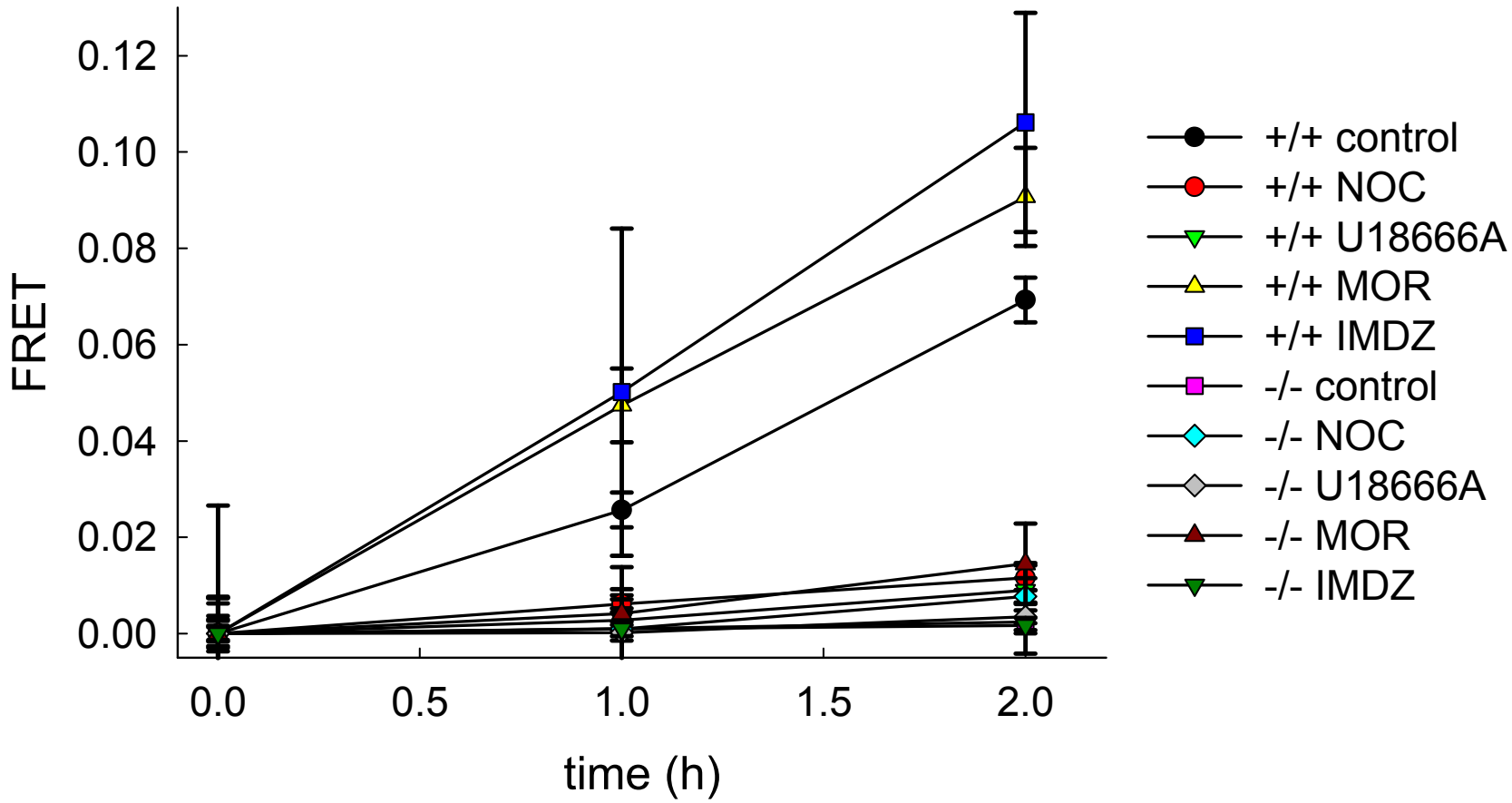

% 3[H]-dextran released into medium
100
80
60
40
20
0
0
5
10
15
20
25
time (hrs)
+/+ control
+/+ NR
+/+ MOR
+/+ IMDZ
+/+ R123
+/+ U18666A
+/+ sucrose
-/- control
-/- NR
-/- IMDZ
-/- R123
-/- U18666A
-/- sucrose

Figure S3.


0.12
0.10
0.08
0.06
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FRET
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1.5
2.0
time (h)
+/+ control
+/+ NOC
+/+ U18666A
+/+ MOR
+/+ IMDZ
-/- control
-/- NOC
-/- U18666A
-/- MOR
-/- IMDZ