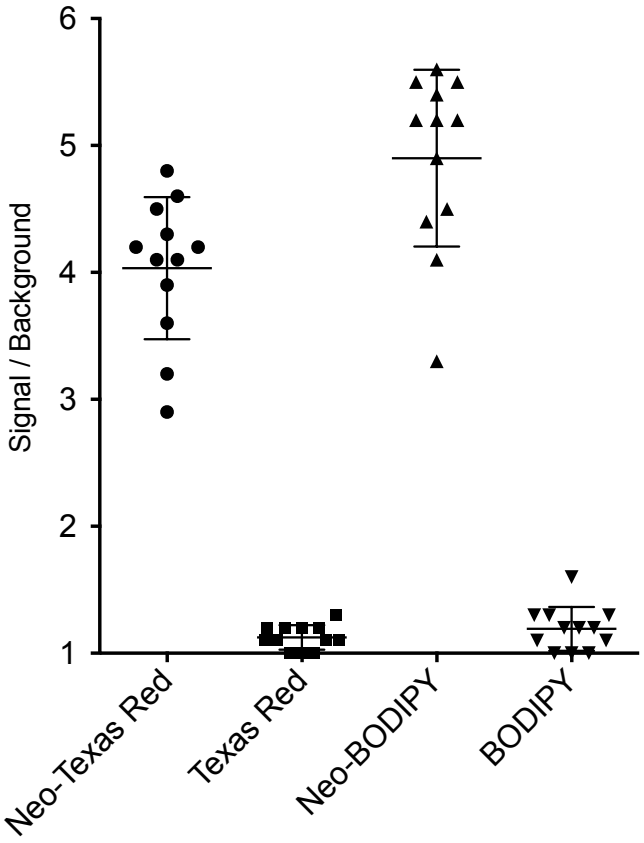
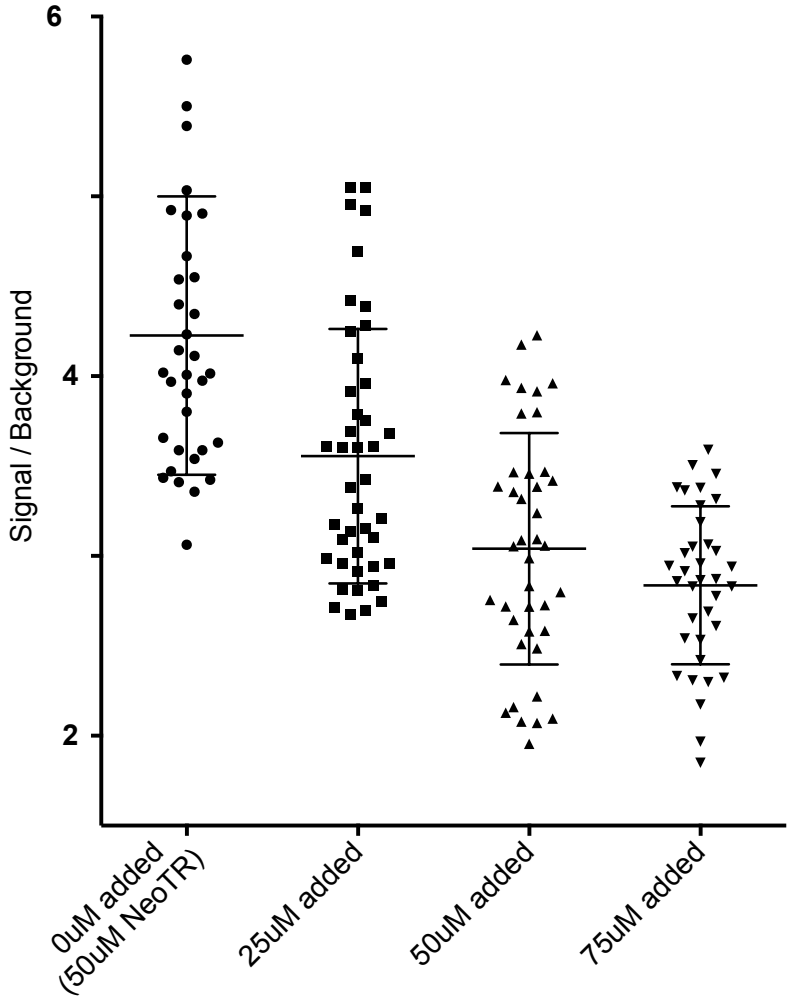
Supplemental Figure 1



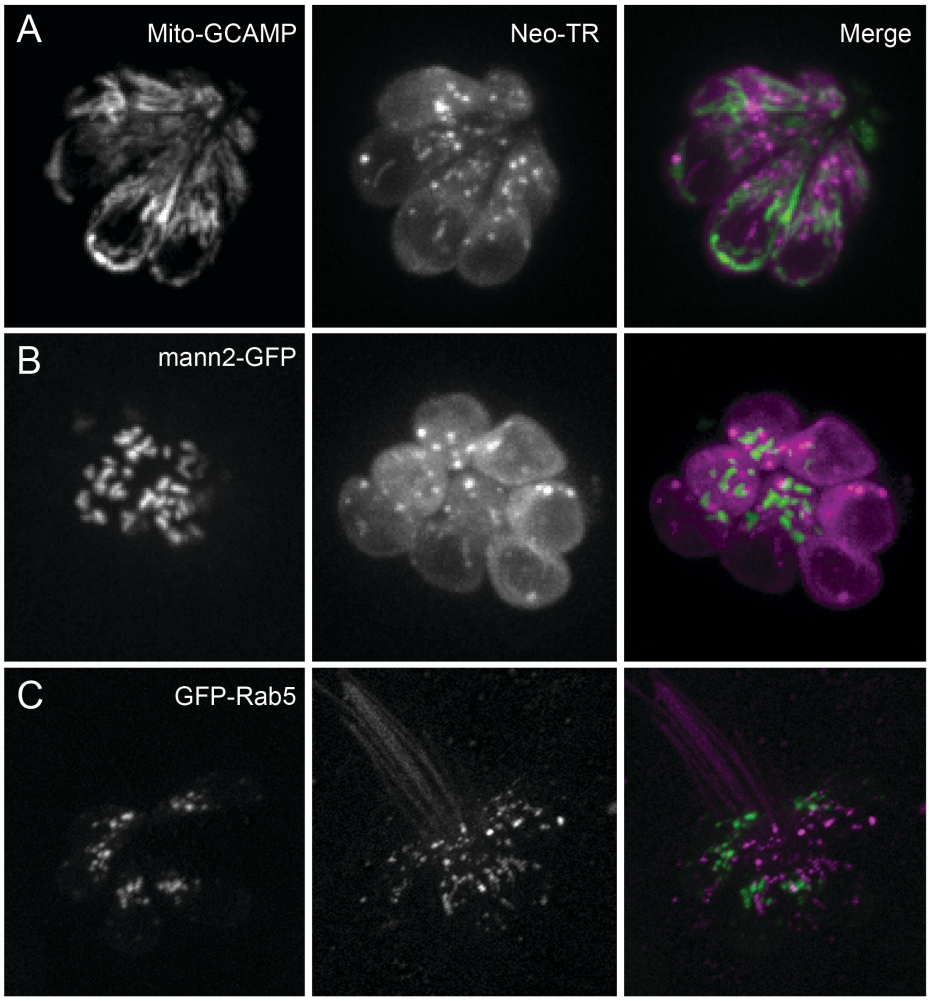
Supplemental Figure 1: The fluorescent labels Texas Red and BODIPY do not appreciably enter hair cells unless they are conjugated to aminoglycosides. 5 dpf zebrafish were treated with 50μM of the indicated compound for 5 minutes, washed 3 times, and imaged. Fluorescence present in neuromasts was quantified as described in the text for Neo-Texas Red and Texas Red. For Neo-BODIPY and BODIPY, DIC images were used to draw ROIs around the neuromasts. Values are expressed as fluorescent signals relative to the signal present outside the neuromasts. Each graphed symbol represents 1 neuromasts (3 fish sampled). Error bars: +/- 1 SD.

Supplemental Figure 2



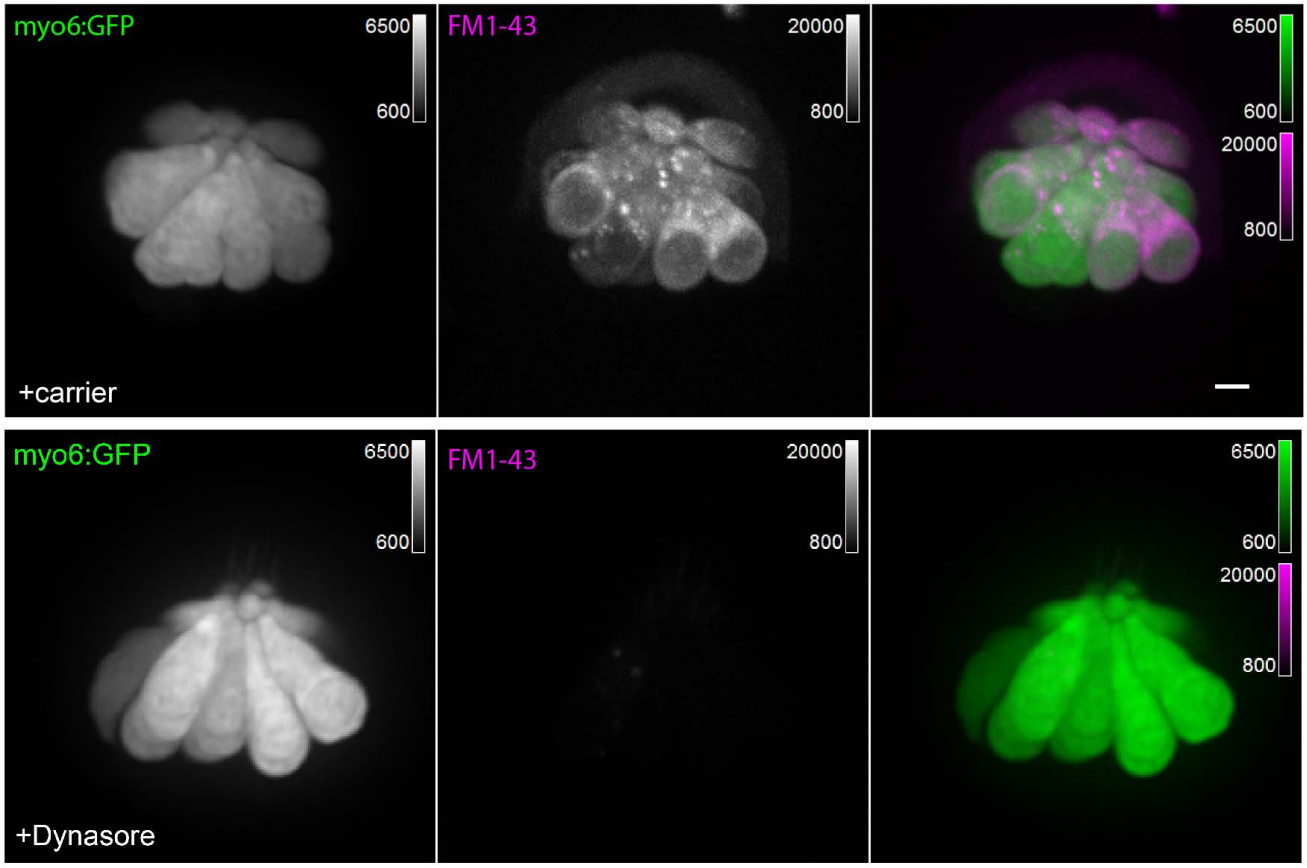
Supplemental Figure 2: Unlabeled neomycin competes with labeled neomycin in the HC loading assay. 5 dpf zebrafish were treated with a mixture of 50μM Neo-TR and unlabeled neomycin at indicated concentrations. Fish were exposed for 5 minutes, washed 3 times, and imaged. Neo-TR fluorescence present in neuromasts was quantified as described above. Values are expressed as Neo-TR signal relative to the signal present outside the HCs. Each graphed symbol represents 1 neuromasts (at least 5 fish sampled). Error bars: +/- 1 SD.

Supplemental Figure 3



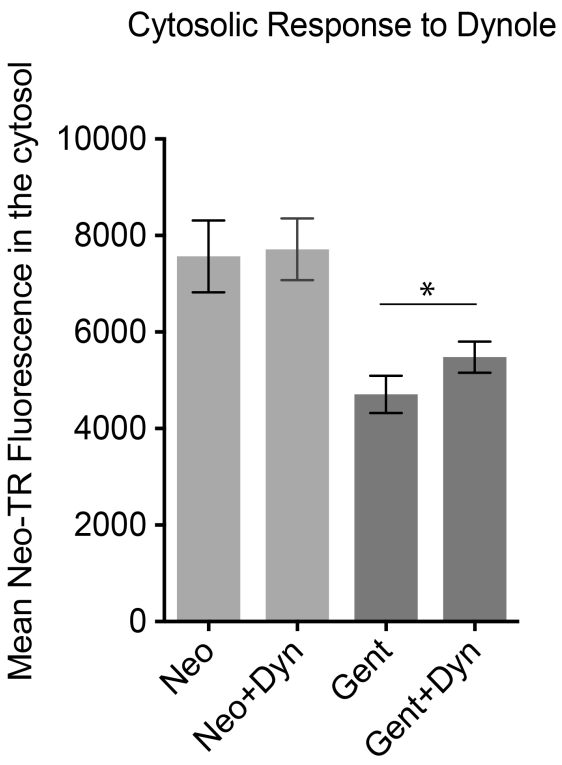
Supplemental Figure 3: Accumulations of labeled neomycin are not detectable in many intracellular compartments. Transgenic 5 dpf zebrafish expressing fluorescent proteins targeted to the mitochondria (A), Golgi (B), and the Rab5+ subset of early endosomes (C) were imaged 5 minutes after a pulse exposure to 50μM Neo-TR. None of these intracellular compartments showed apparent accumulations of Neo-TR. (These experiments do not rule out the presence of low concentrations that can not be distinguished from the cytosolic pool.)

Supplemental Figure 4



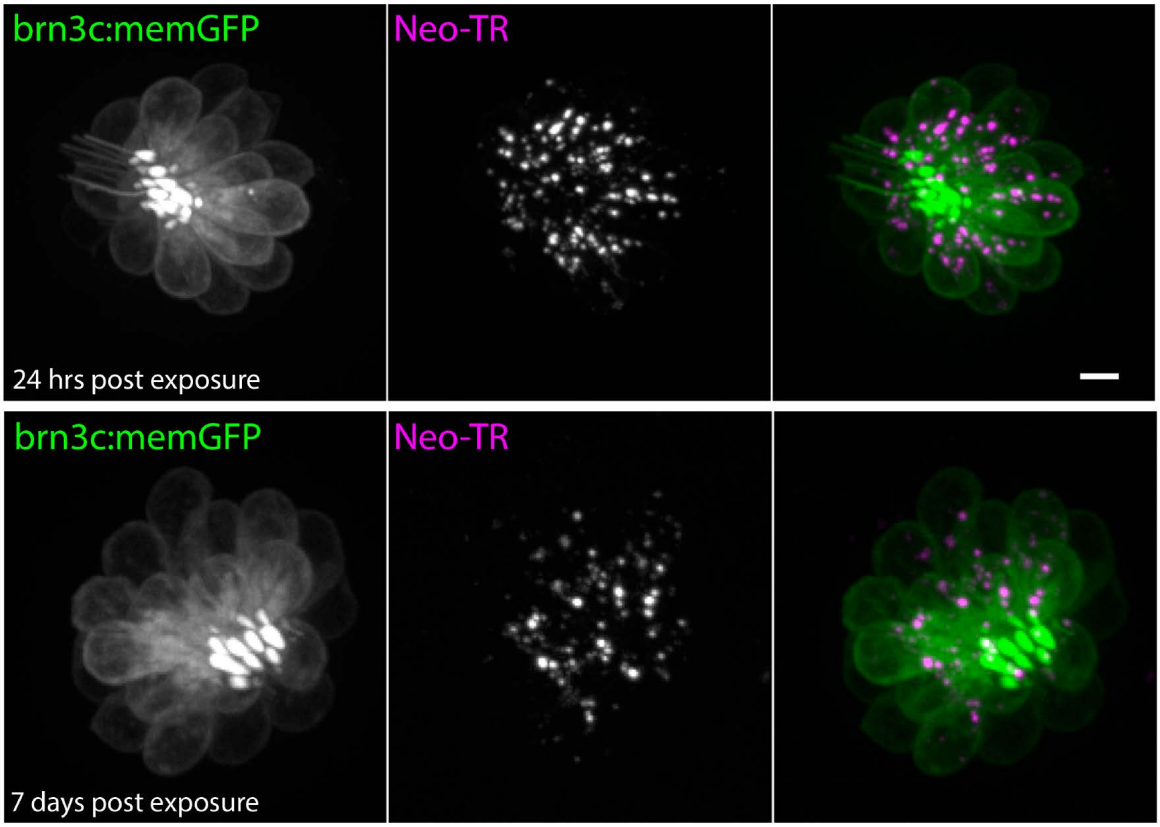
Supplemental Figure 4: FM1-43 is not taken up by hair cells treated with the dynamin inhibitor Dynasore. Transgenic 5 dpf zebrafish larvae expressing GFP in hair cells (myo6:GFP) readily take up FM1-43 during a 5 minute 500nM pulse exposure. Treatment with Dynasore abolishes this uptake, indicating that in addition to inhibiting endocytic processes in the LL hair cells, Dynasore blocks MET activity. This is in contrast to the effect of the dynamin inhibitor Dynole 34-2 which blocks the appearance of puncta (both FM1-43 and Neo-TR) but not the diffuse signal throughout the cytosol (see **Fig6A and 6B).** Scale bar: 5um

Supplemental Figure 5



Supplemental Figure 5: Inhibiting endocytosis increases cytosolic gentamicin but not neomycin. Hair cells were pretreated with Dynole 34-2 to inhibit dynamin-mediated endocytosis, and then exposed to either Neo-TR or Gent-TR for five minutes. Bright puncta were excluded, and the mean cytosolic signal was quantified. For Neomycin-TR, Dynole34-2 treatment did not significantly alter loading of Neo-TR into the cytosol. In contrast, gentamicin did show a significant increase in mean cytosolic signal when the dynamin-mediated endocytic entry path was inhibited, suggesting competition between these pathways for gentamicin. *P Value from Mann-Whitney test: 0.03. Error bars = 1 SD.

Supplemental Figure 6



Supplemental Figure 6: Neo-TR persists in lysosomes for more than a week in exposed lateral line hair cells. Hair cells were exposed to 20uM NeoTR for five minutes, washed out, and maintained in an incubator for either 1 day or a week following the pulse exposure. In both cases, Neo-TR is easily detected in puncta. The exposed hair cells do not show obvious signs of stress at either time point. Membranes are intact and no blebbing is apparent at the apical region or kinocilia. (Scale bar: 5um)