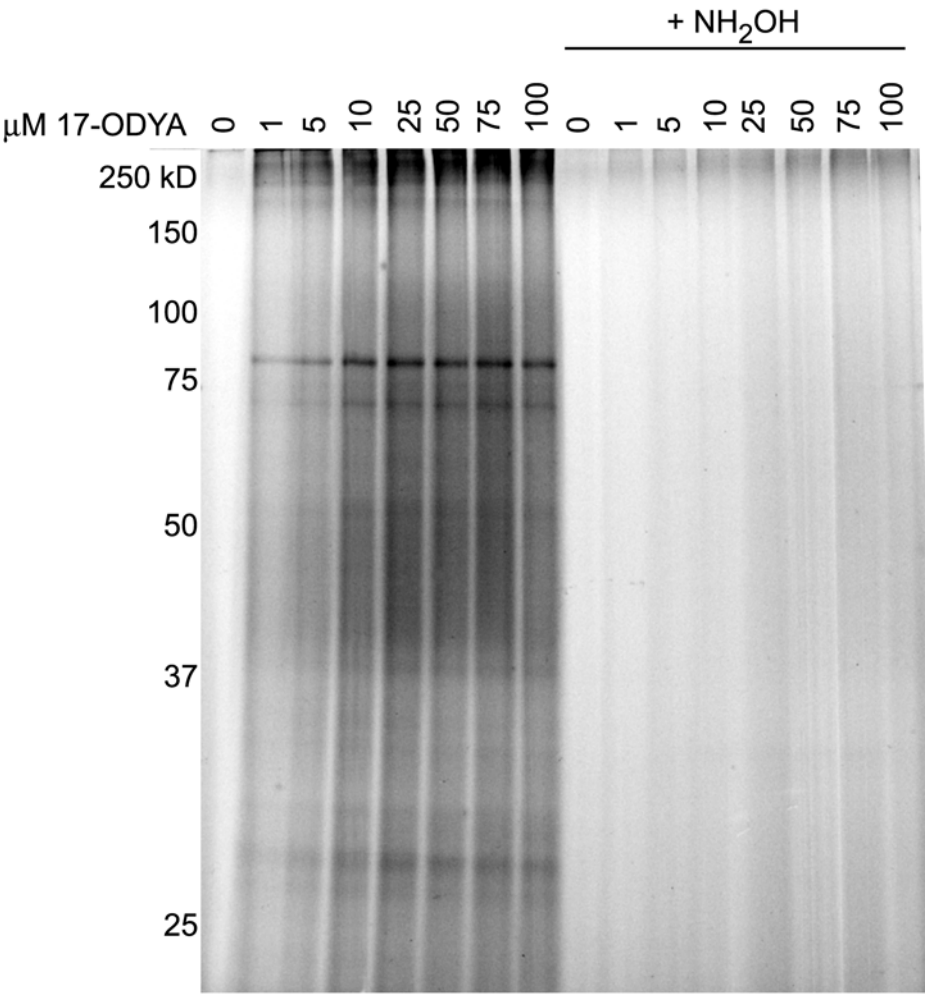
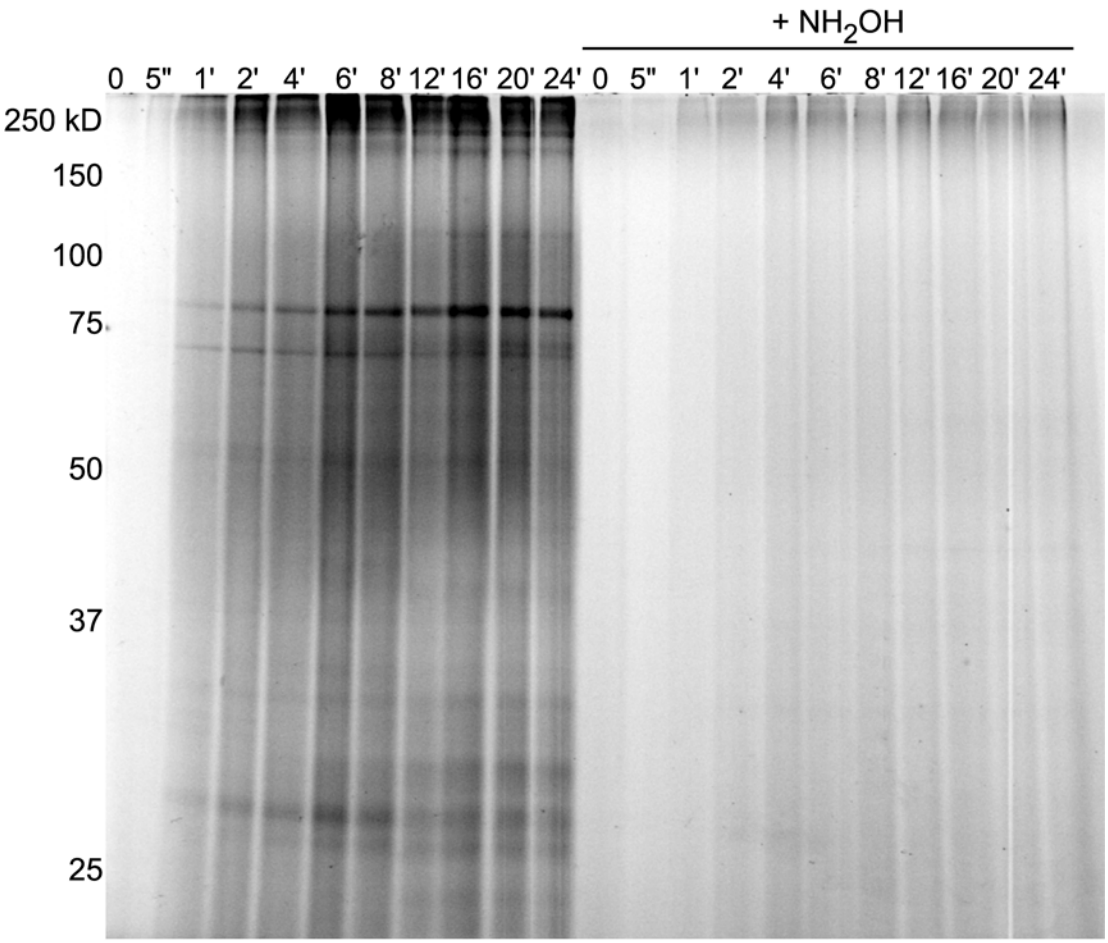
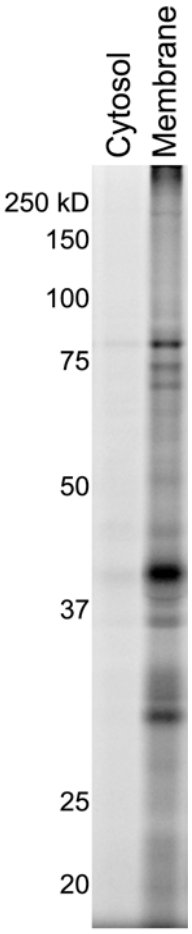
Supplementary Figure 1. Titration of 17-ODYA labeling in Jurkat T-cells. Cells were metabolically labeled for 10 hours in the presence of increasing concentrations of 17-ODYA. Next, 50 µg of membrane particulate fraction was reacted with rhodamine-azide, half was boiled in the presence of 2.5% neutral hydroxylamine (NH_2OH), and 20 µg of protein was loaded in each lane of an SDS-PAGE gel for separation and fluorescence analysis. Bands were generally smeared due to the exclusion of reducing agent from the gel loading buffer.



Supplementary Figure 2. Timecourse of 17-ODYA labeling in Jurkat T-cells. Cells were labeled with 25 µM of 17-ODYA and harvested at the described time-points. Membrane particulate fractions were collected and 50 µg was reacted with rhodamine-azide, then half was boiled in the presence of 2.5% hydroxylamine (NH_2OH), and 20 µg of protein was loaded in each lane of a SDS-PAGE gel for separation and fluorescence analysis. Evidence of labeling was first observed at 1 hour and reached equilibrium by 6 hours.

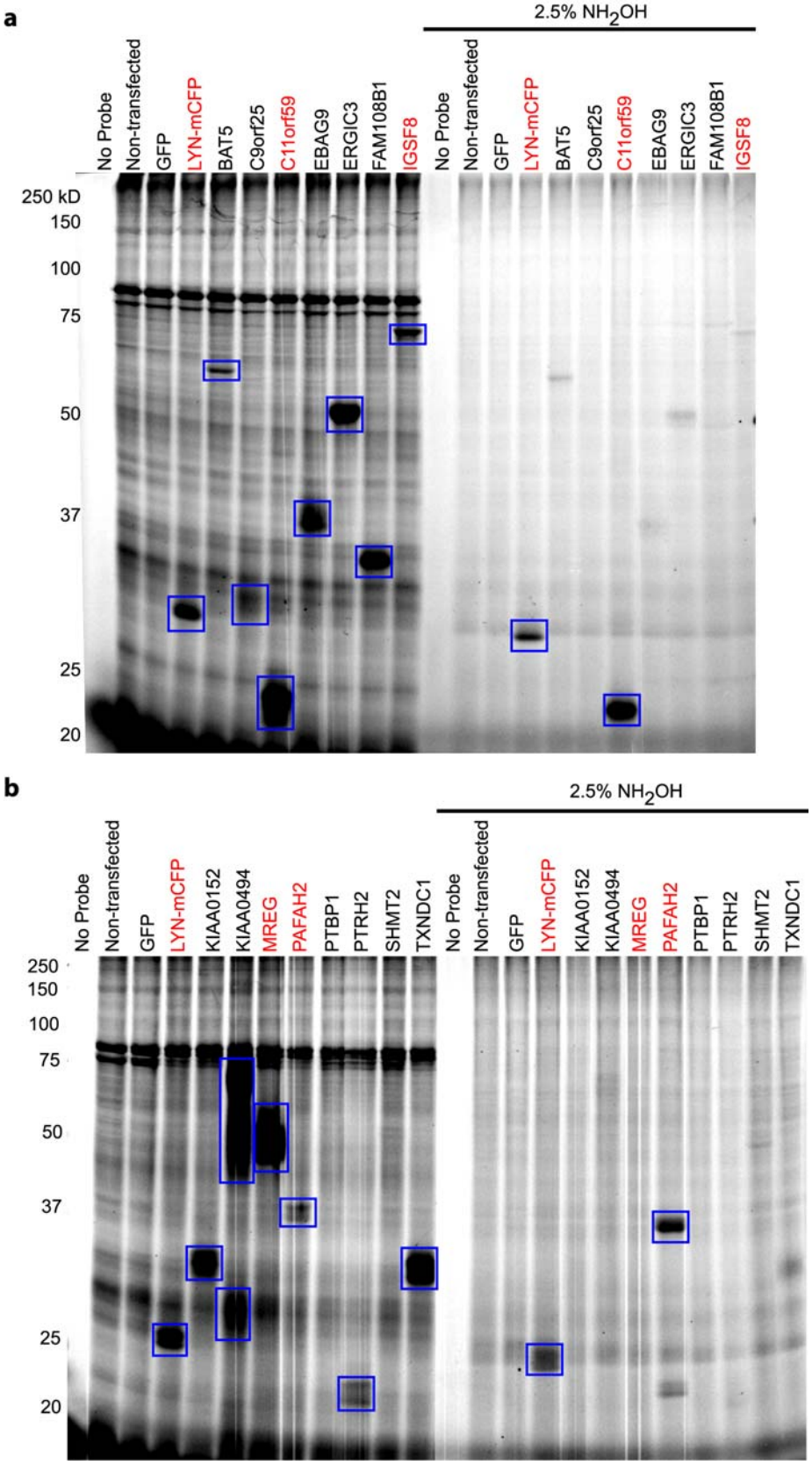


Supplementary Figure 3. 17-ODYA labeled proteins are exclusively found in the membrane fraction. Cells were labeled for 6 hrs, lysed, and centrifuged at 100,000 x g for 45 minutes. The supernatant (soluble fraction of the cell) and pellet (membrane fraction of the cell) were reacted with rhodamine-azide, and 20 µg of protein for each fraction was separated by SDS-PAGE and analyzed by in-gel fluorescence.

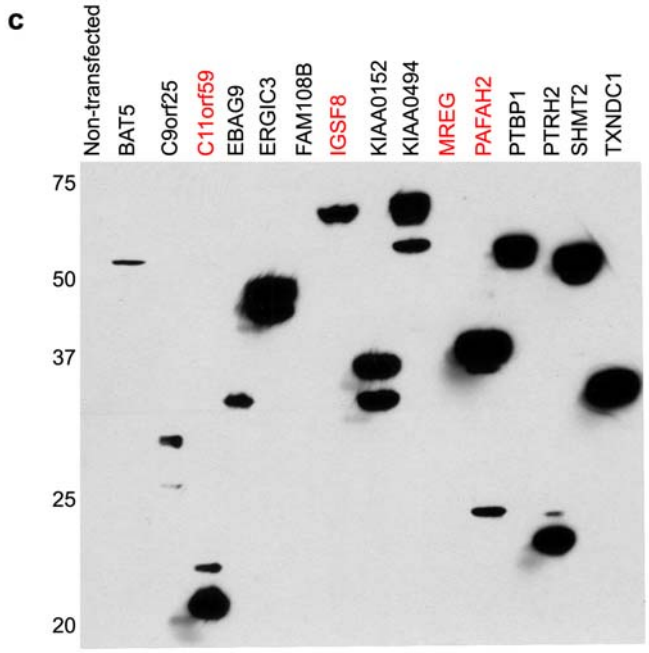


Supplementary Figure 4. Validation of 15 representative predicted palmitoylated proteins by recombinant expression in HEK-293T cells. Each experiment contains four controls: unlabeled (No Probe), labeled (Non-transfected), mock transfected (GFP), and the positive control LYN-mCFP, which contains an N-terminal dual-acylation motif from Lyn fused to the N-terminus of mCFP (MGCIKSKRKDNLNDDE-mCFP). Proteins with a consensus myristoylation site (*N*-Met-Gly) are colored in red. Unique fluorescent bands are highlighted with blue boxes. (**a**) Validation of 7 putative acylated target proteins. Estimated molecular weights (including the epitope fusion which adds 3 kD) are shown in parentheses below: LYN-mCFP (29 kD), BAT5 (66 kD), C9orf25 (22 kD), C11orf59 (21 kD), EBAG9 (32 kD), ERGIC3 (47 kD), FAM108B1 (35 kD), IGSF8 (69 kD). C9orf25 and EBAG9 both migrate higher than their expected molecular weights, suggesting additional post-translational modifications. Both LYN-mCFP and C11orf59 show a significant reduction, but not elimination, of fluorescence upon hydroxylamine treatment suggesting both myristoylation and palmitoylation. (**b**) Validation of 8 additional putative acylation targets. Calculated molecular weights based on the identified Ensemble entries are shown in parentheses: KIAA0152 (35 kD), KIAA0494 (58 kD), MREG (28 kD), PAFAH2 (43 kD), PTBP1 (63 kD), PTRH2 (22 kD), SHMT2 (59 kD), TXNDC1 (35 kD). The N-myristoylated protein PAFAH2 demonstrates hydroxylamine resistant fluorescence. MREG migrates approximately at the same molecular weight as a dimer, despite boiling in reducing SDS-loading buffer. CHST11, HMOX2, and MYADM1 similarly show positive 17-ODYA labeling (data not shown). For **a** and **b**, probe-labeled protein bands at the correct predicted molecular weights are highlighted by blue boxes. (**c**) Anti-FLAG western blot of transfected HEK293T membrane particulate lysates. KIAA0152 shows a higher molecular weight species that was not detected by 17-ODYA labeling. MREG and FAM108B1 are not detected by FLAG western blot, suggesting proteolysis of the C-terminal epitope tag.

Supplementary Figure 4.



Supplementary Figure 4.



Supplementary Figure 5. N-terminal cysteine domain-dependent subcellular distribution of FAM108A1 and FAM108C1 in HeLa cells. Scale bar = 30 µm. (**a**) FAM108A1-GFP(green) is exclusively localized to the plasma membrane. DNA is stained with DAPI (blue). A *z*-projection is displayed above showing membrane localization. (**b**) Deletion of the N-terminal cysteine-rich domain of FAM108A1 (FAM108A1Δ1-19) eliminates the plasma membrane localization. (**c**) FAM108C1 is diffusely localized to the plasma membrane and internal membranes. (**d**) Deletion of the N-terminal cysteine-rich domain (FAM108C1Δ1-19) prevents membrane association and allows partial nuclear localization of the enzyme.