

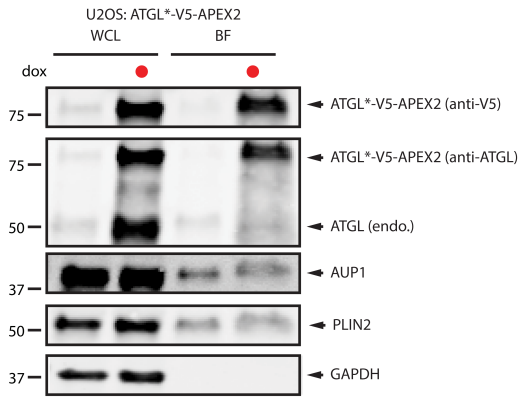
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Supplemental Information

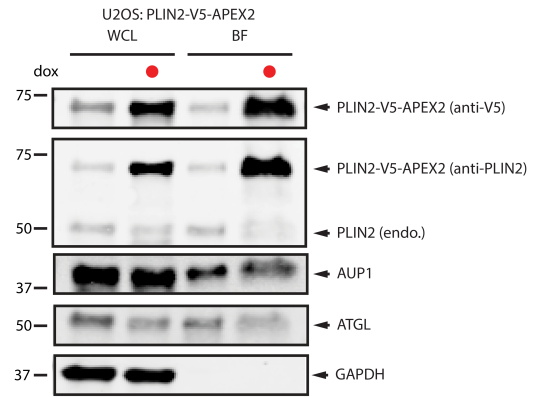
**A Proximity Labeling Strategy Provides Insights
into the Composition and Dynamics
of Lipid Droplet Proteomes**

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A



B



C

$$CS = (\text{Eq. 1}) \times (\text{Eq. 2})$$

$$\text{Eq. 1} = \sum_{LD=1}^{LD=k} R_{LD,P}$$

$$\text{Eq. 2} = \sum_{LD=1}^{LD=k} (X_{LD,P} - X_{C,P})$$

$$CS_N = \frac{CS}{CS_T}$$

$$X_{LD,P} \begin{cases} \text{SAF for protein P from LD-APEX2} \\ \text{fusion protein (ATGL* or PLIN2)} \end{cases}$$

$$X_{C,P} \begin{cases} \text{SAF for protein P from Cyto-APEX2} \\ \text{fusion protein} \\ 0; X_{LD,P} = 0 \end{cases}$$

$$R_{LD,P} \begin{cases} 0; X_{LD,P} = 0 \\ 1; X_{LD,P} > 0 \end{cases}$$

SAF = TSC / #aa x 10
NSAF = SAF / (average SAF, if SAF > 0)
k = total number of LD-targeted APEX2 runs
15% validated < CS_T < 85% validated

Figure S1 – Characterization of APEX2 U2OS cells and calculation of LD confidence score.

(A and B) U2OS cells stably expressing ATGL*-APEX2 or PLIN2-APEX2 were treated with 10 ng/mL dox for 48 hr. Whole cell lysates (WCL, normalized by total protein levels) or buoyant fractions (BF, normalized by AUP1 levels) were separated by SDS-PAGE and analyzed by blotting with the indicated antibodies. Endo., endogenous protein.

(C) The LD confidence score (CS) for a protein “P” is calculated by multiplying a replication value (Eq. 1), which is the sum of the number of times the protein was detected in LD-targeted APEX2 samples ($R_{LD,P}$), by an abundance value (Eq. 2), which is the sum of the spectral abundance factor (SAF) for the protein in the LD-targeted APEX2 samples ($X_{LD,P}$) minus the SAF for the protein in the corresponding control Cyto-APEX2 sample ($X_{C,P}$). SAF is calculated by dividing the total spectral counts (TSC) by the number of amino acids (aa) in the protein, multiplied by 10. The normalized SAF

(NSAF) is calculated by dividing the SAF by the average SAF for proteins in the sample (based on proteins with an SAF > 0).

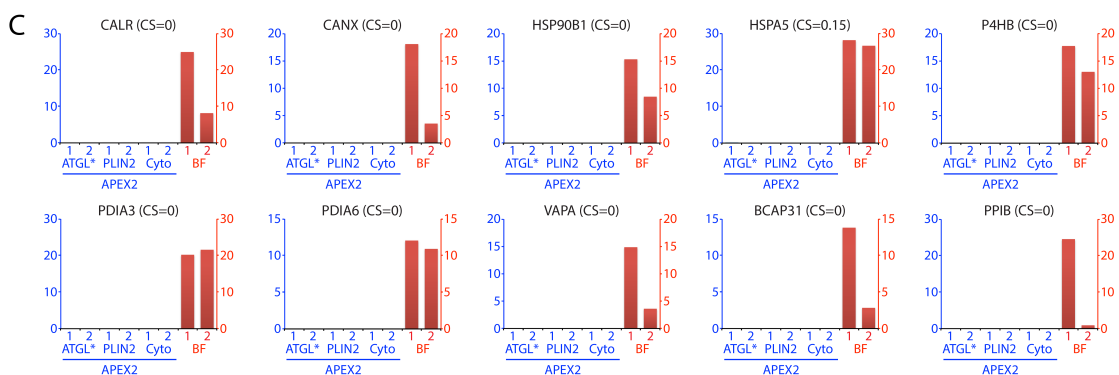
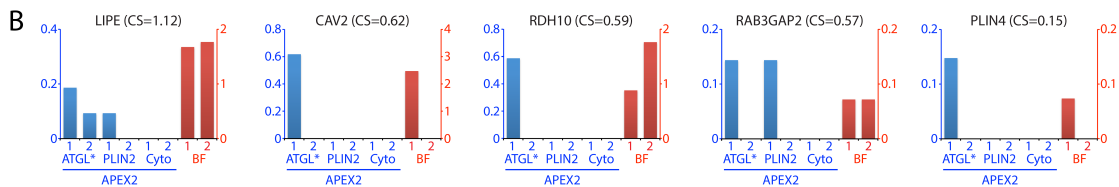
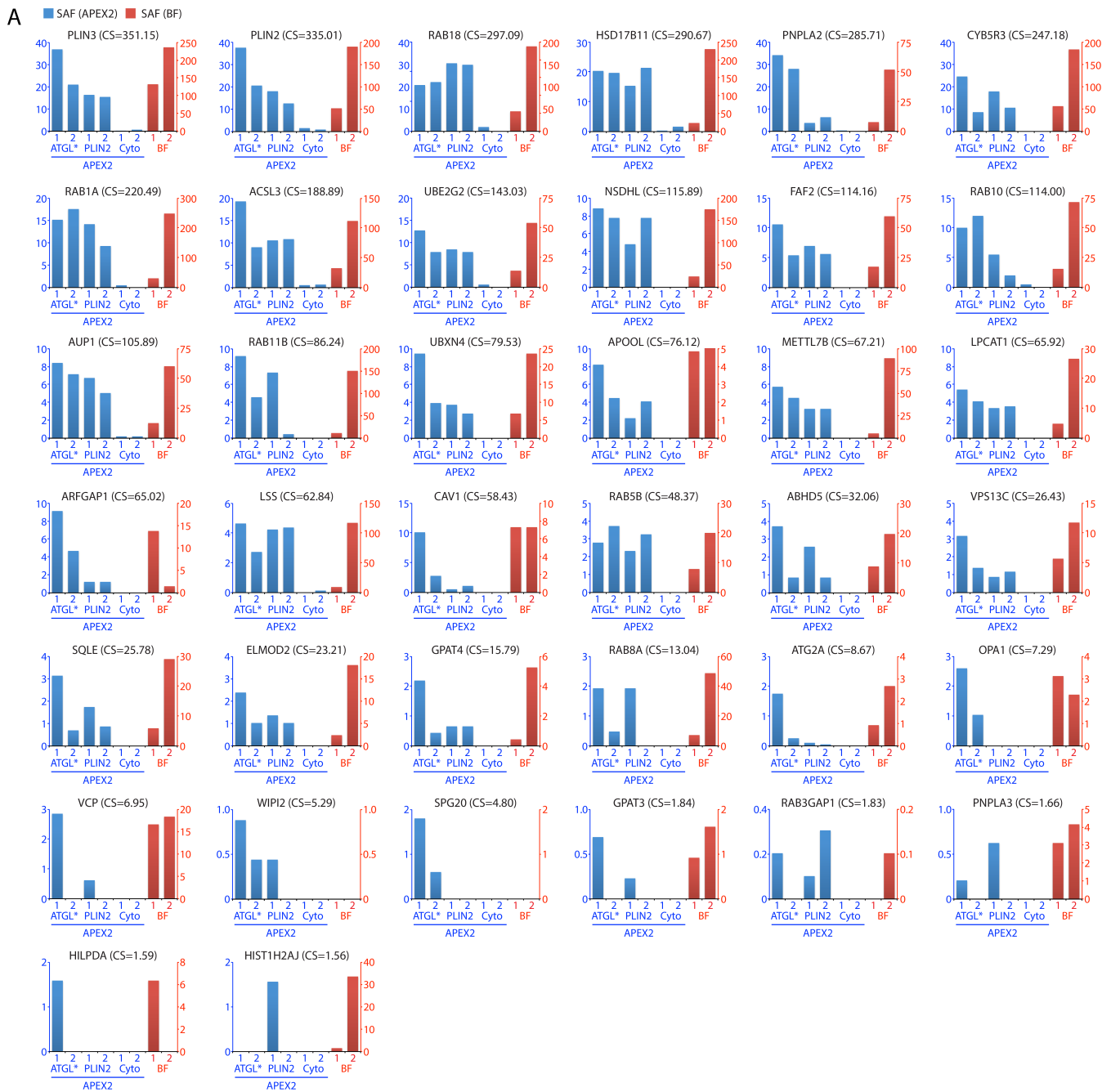


Figure S2 – Spectral profiles of validated lipid droplet proteins and select contaminants in U2OS cells.

(A-C) Graphs indicating the SAF in APEX2 (blue) and BF (red) samples for validated proteins identified as high confidence LD proteins (A), validated proteins that were detected, but were below the threshold value and were not designated as high confidence LD proteins (B), and select common contaminant proteins (C). CS, confidence score.

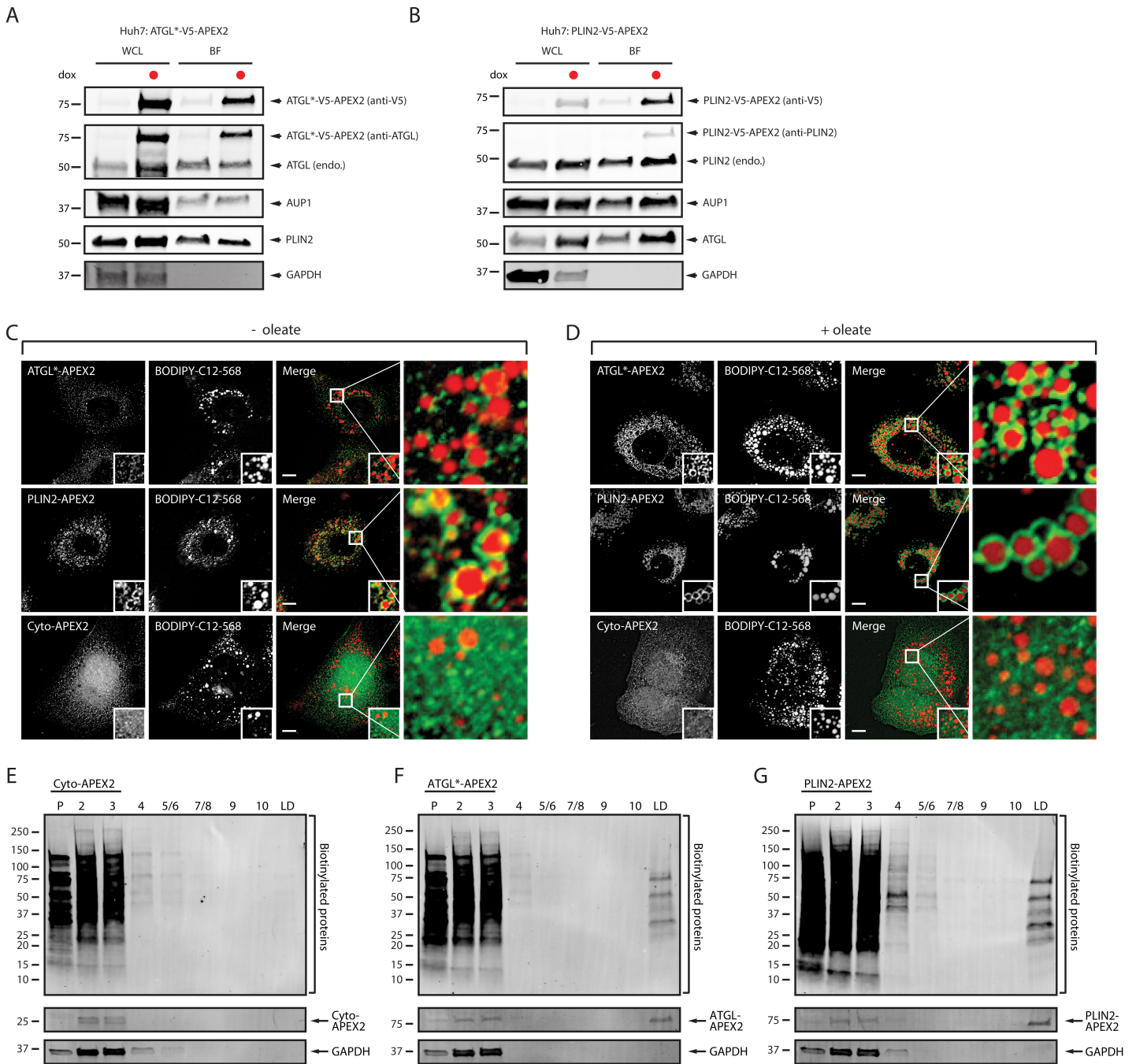


Figure S3 – Lipid droplet-targeted APEX2 biotinylates lipid droplet proteins in Huh7 cells

(A and B) Huh7 cells stably expressing ATGL*-APEX2 or PLIN2-APEX2 were treated with 10 ng/mL dox for 48 hr. Whole cell lysates (WCL, normalized by total protein levels) or buoyant fractions (BF, normalized by AUP1 levels) were separated by SDS-PAGE and analyzed by blotting with the indicated antibodies. Endo., endogenous protein.

(C and D) Huh7 cells stably expressing cytosolic or LD-targeted APEX2 were treated for 24 hr with 1 μ M BODIPY-C12-568 or 200 μ M oleate and 1 μ M BODIPY-C12-568 (red). Cells were imaged by fluorescence microscopy using antibodies against the V5 epitope tag (green). Magnified insets show cellular regions with LDs. Scale bars represent 10 μ m.

(E-G) Lysates from Huh7 cells stably expressing LD-targeted or cytosolic APEX2 were fractionated by sucrose gradient centrifugation. Proteins in individual fractions were separated by SDS-PAGE and analyzed by blotting with fluorescent streptavidin-568 and antibody against the V5 epitope tag.

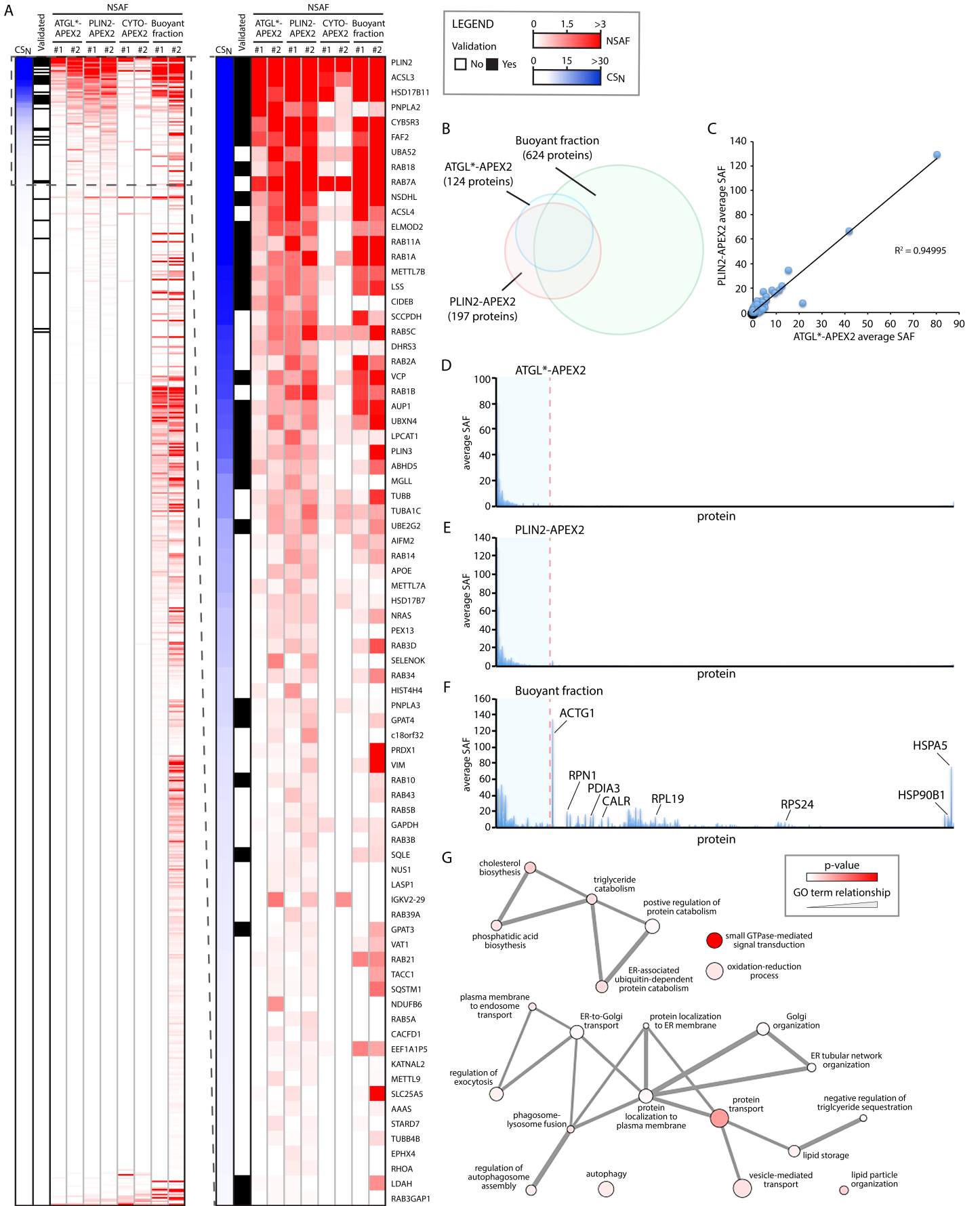


Figure S4 – Proteomic analysis of biotinylated lipid droplet proteins in Huh7 cells

(A) Proteins identified in total buoyant fractions and in streptavidin affinity purifications from the indicated Huh7 APEX2 cell lines were ranked by descending LD confidence score (CS_N). Data from two independent experimental replicates for each sample are shown. The intensity of the blue color represents the CS_N value and the intensity of the red color represents the normalized spectral abundance factor (NSAF) value. The heat map scale is linear. The black color indicates if a protein was previously validated as an LD protein by microscopy. The boxed inset shows the high confidence LD proteins ($CS_N > 1$).

(B) Venn diagram illustrating the degree of overlap between proteomes identified in the Huh7 LD-targeted APEX2 cell lines and in the buoyant fraction.

(C) Comparison of average spectral abundance factors (SAF) for proteins identified in the affinity purifications from ATGL*-V5-APEX2 and PLIN2-V5-APEX2 Huh7 cells. Each symbol corresponds to an LD protein identified in both cell lines. The R^2 coefficient for the linear regression line is indicated.

(D-F) The average SAF for proteins identified in the affinity purifications from ATGL*-V5-APEX2 (D) or PLIN2-V5-APEX2 (E) Huh7 cells or in the total buoyant fractions isolated from parental cells (F).

(G) Selected enriched GO-Term categories for high confidence LD proteins in Huh7 cells.

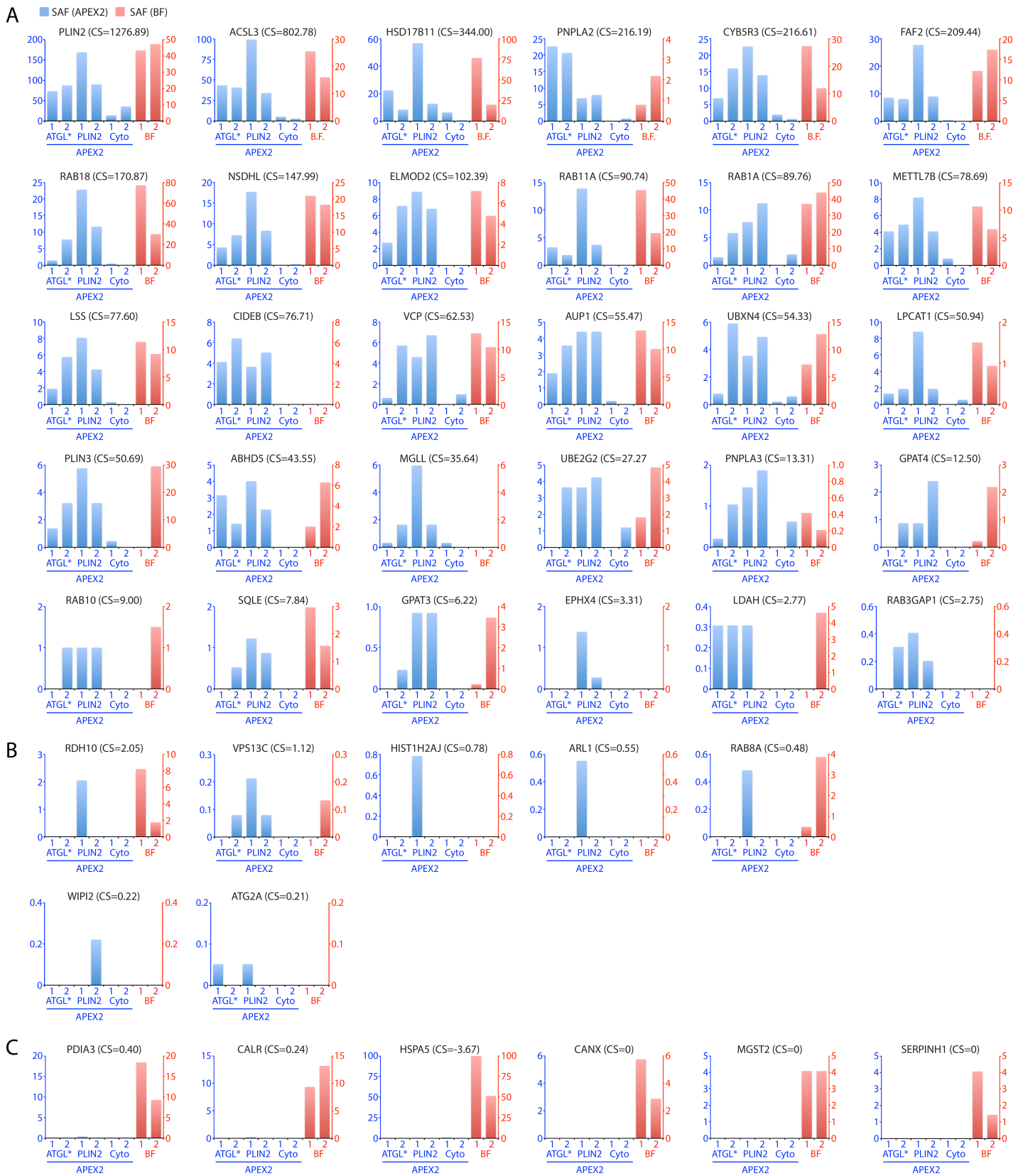
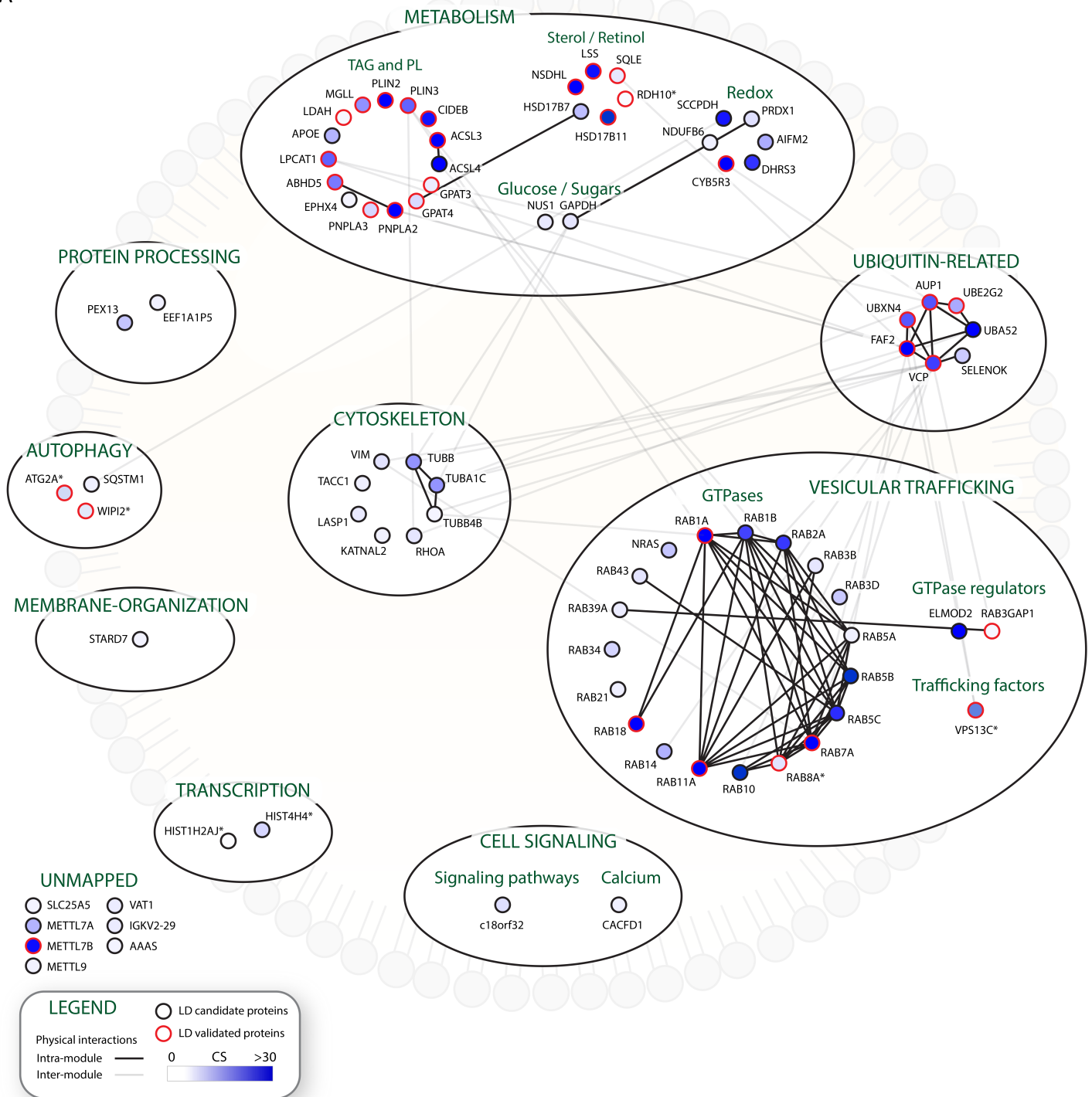


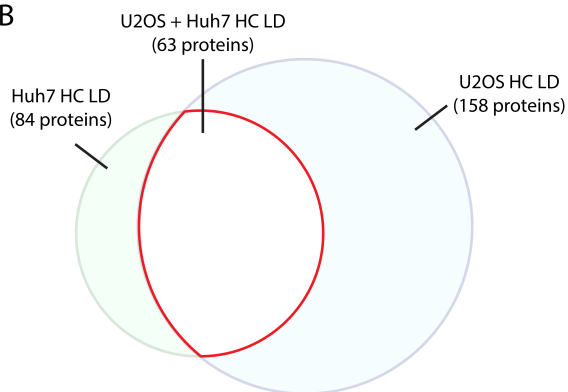
Figure S5 – Spectral profiles of validated lipid droplet proteins and select contaminants in Huh7 cells

(A-C) Graphs indicating the SAF in APEX2 (blue) and BF (red) samples for (A) validated proteins identified as high confidence LD proteins, (B) validated proteins that were detected, but were below the threshold value and were not designated as high confidence LD proteins, and (C) select common contaminant proteins. CS, confidence score.

A



B



C

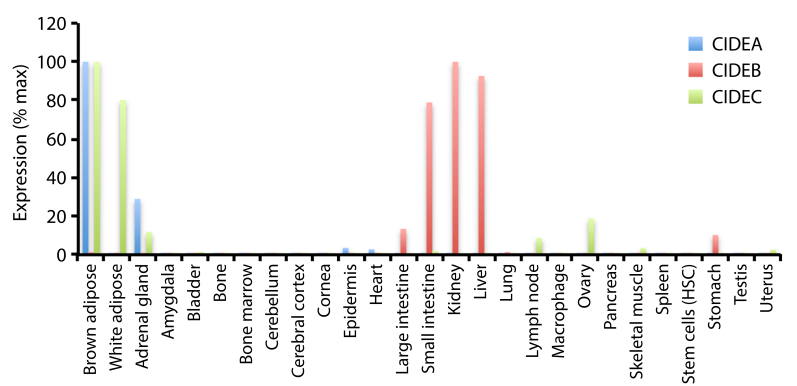


Figure S6 – Illustration of the high confidence lipid droplet proteome in Huh7 cells

(A) High confidence LD proteins identified in Huh7 cells ($CS_N > 1$) are grouped into functional modules based on GO analysis and UNIPROT functional annotations. Solid lines represent physical interactions within functional modules and transparent lines represent interactions between proteins in distinct modules, as annotated in BIOGRID. The intensity of the blue color in a node indicates the confidence score. Nodes outlined in red represent proteins that have been previously validated to localize to LDs by microscopy.

(B) Venn diagram illustrating the degree of overlap between high confidence LD proteins identified in U2OS and Huh7 cells.

(C) Expression of CIDEA, CIDEB, and CIDEA transcripts in mouse tissues. Expression data was downloaded from BioGPS and normalized to the maximum expression level.

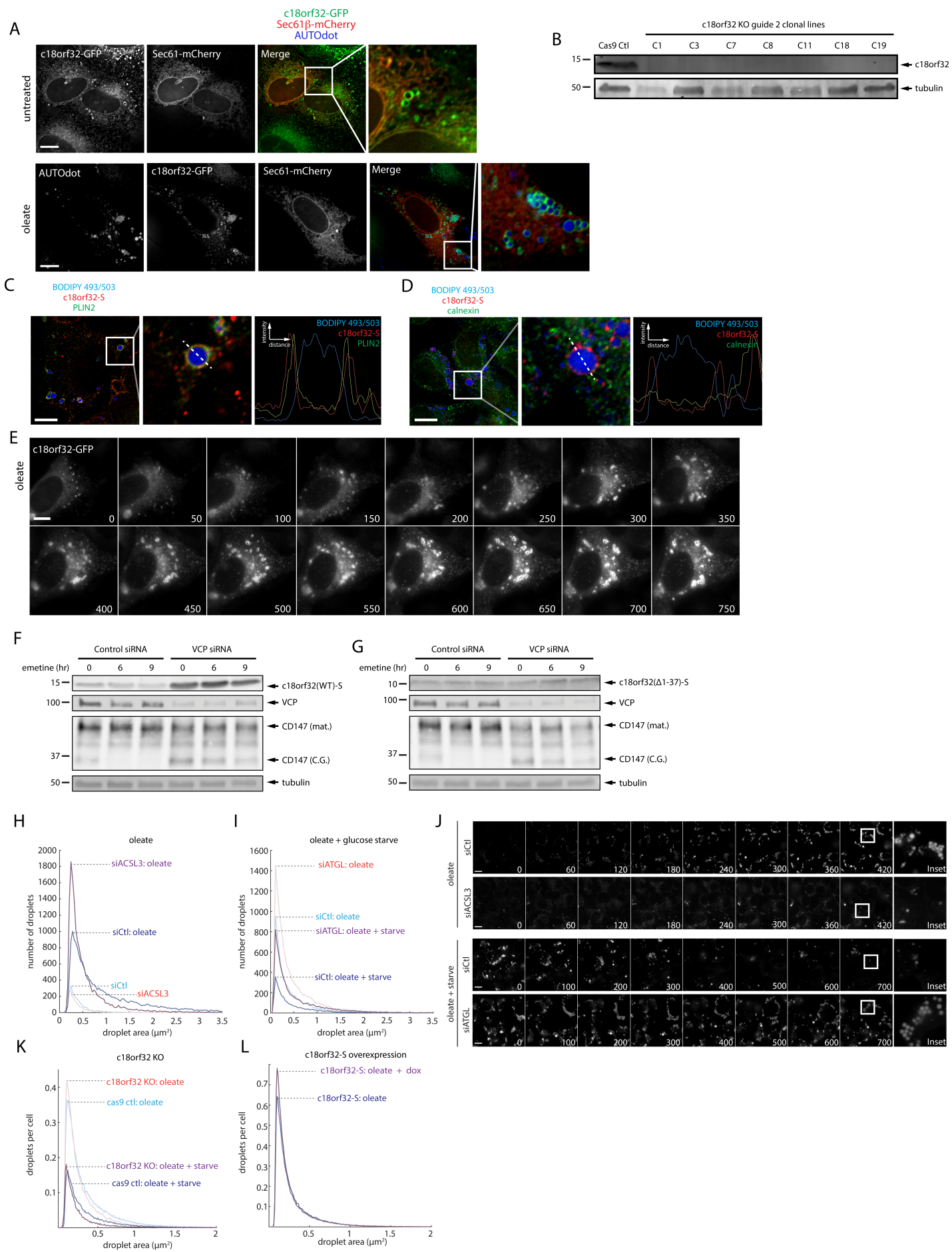


Figure S7 – c18orf32 is an ER-LD protein that is degraded by a VCP-dependent pathway

(A) U2OS cells stably expressing c18orf32-GFP (green) were transiently transfected with Sec61 β -mCherry (red) and incubated in the presence or absence of 200 μ M oleate for 24 hr. AUTOdot (blue) was added to oleate-treated cells to stain LDs. Magnified insets show cellular regions with LDs. Scale bars represent 10 μ m.

(B) Proteins in lysates from U2OS control and c18orf32 clonal null cell lines were separated by SDS-PAGE and analyzed by blotting with antibodies against c18orf32 and tubulin.

(C and D) U2OS cells stably expressing c18orf32-S were incubated with 200 μ M oleate for 24 hr, and fixed cells were imaged by fluorescence microscopy using the neutral lipid dye BODIPY 493/503 and antibodies against S-tag and PLIN2 (C) or calnexin (D). Magnified insets show regions with LDs and graphs show line scan intensity profiles (% max, white dashed line). Scale bars represent 10 μ m.

(E) U2OS cells stably expressing c18orf32-GFP were imaged by time-lapse fluorescence microscopy following the addition of 200 μ M oleate for the indicated times (min). Scale bar represents 10 μ m. Time is indicated in minutes.

(F and G) U2OS cells stably expressing c18orf32(WT)-S (F) or c18orf32(Δ 1-37)-S (G) were transfected with control or VCP-targeting siRNAs. After 48 hr, proteins from cell lysates were separated by SDS-PAGE, and analyzed by blotting with antibodies against S-tag, VCP, CD147, and tubulin. C.G., core-glycosylated; mat., mature.

(H) U2OS cells were transfected with control or ACSL3-targeting siRNA for 24 hr and treated with 200 μ M oleate for 24 hr. LDs stained with BODIPY 493/503 in live cells were imaged by fluorescence microscopy and LD size distributions were quantified.

(I) U2OS cells were transfected with control or ATGL-targeting siRNA for 24 hr and treated with 200 μ M oleate for 24 hr. A separate cell population transfected with siRNA was incubated in glucose-free media for 16 hr after the oleate treatment. LDs stained with BODIPY 493/503 in live cells were imaged by fluorescence microscopy, and LD size distributions were quantified.

(J) U2OS cells were transfected with control, ACSL3-targeting siRNA (*upper montage*) or ATGL-targeting siRNA (*lower montage*) for 48 hr. To image LD biogenesis, LDs were stained with BODIPY 493/503 and imaged by time-lapse

fluorescence microscopy after addition of 200 μ M oleate. To image LD turnover, cells were treated with 200 μ M oleate for 24 hr and then imaged in glucose-free media lacking oleate. The scale bars indicate 10 μ m.

(K) Clonal c18orf32-null U2OS cells or cas9-expressing control cells were treated with 200 μ M oleate for 24 hr or starved in glucose-free media for 16 hr after oleate treatment. LDs stained with BODIPY 493/503 and nuclei stained with Hoechst were imaged in live cells by fluorescence microscopy. LD distributions were quantified and normalized by the number of nuclei ($n > 10,000$). The result is representative of at least two independent experiments.

(L) U2OS cells stably expressing dox-inducible c18orf32-S were treated with dox for 48 hr and 200 μ M oleate for 24 hr. LDs stained with BODIPY 493/503 and nuclei stained with Hoechst were imaged in live cells by fluorescence microscopy. LD distributions were quantified and normalized by the number of nuclei ($n > 10,000$). The result is representative of at least two independent experiments.