

Expanded View Figures

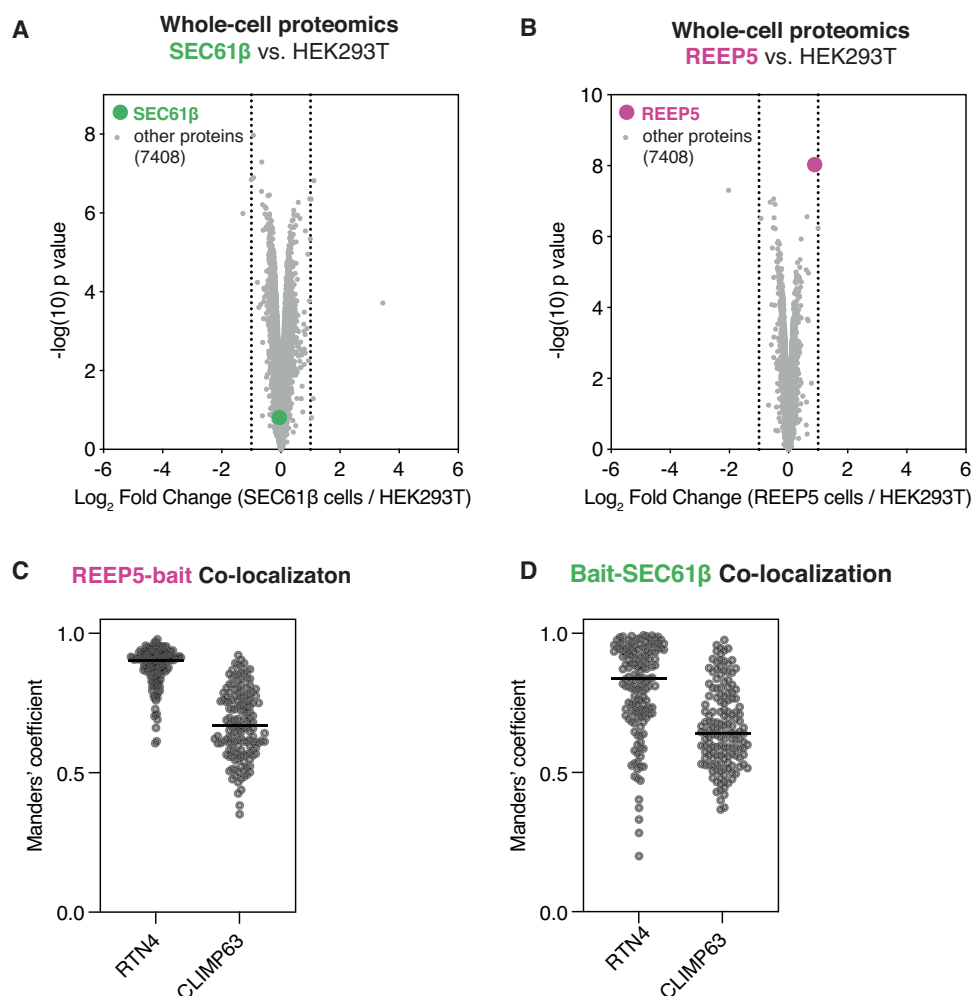


Figure EV1. Impact of bait protein expression on HEK293T proteomes and colocalization analysis of two MemPreps via the REEP5-bait and bait-SEC61β.

(A) Limma *t*-test analysis of a non-targeted, TMT-labeling proteomics experiment performed with $n=3$ biological replicates comparing cell lysates between a bait-SEC61β-expressing cell line and HEK293T cells. (B) Limma *t*-test analysis of a non-targeted, TMT-labeling proteomics experiment performed with $n=3$ biological replicates comparing cell lysates between a REEP5-bait-expressing cell line and HEK293T cells. (C, D) Quantification of the colocalization of the indicated bait proteins with the ER subdomain markers RTN4 (tubular ER) and CLIMP63 (ER sheets) (examples in Fig. 1E, F). Each data point represents the Manders' coefficient of one individual cell. Per condition, 50 cells were quantified in three independent experiments ($n=150$). Source data are available online for this figure.

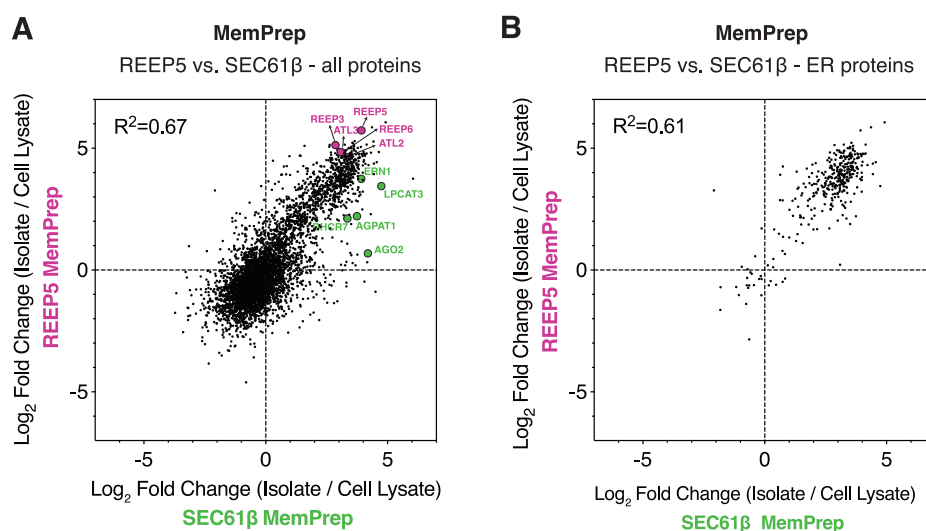


Figure EV2. Correlation analysis of two MemPreps via the REEP5-bait and bait-SEC61β.

(A) The relative fold change enrichment of proteins in the REEP5 and SEC61β MemPreps over the respective whole cell lysates were compared after non-targeted, TMT-labeling proteomics, consistently detecting 4816 proteins. Plotting the log₂ fold change in the isolate over the respective cell lysate from both MemPreps on the two axes allows for a correlation analysis of the data. The correlation coefficient was determined by a linear fit forced through the origin. (B) Correlation analysis as in (A) using a subset of the ER-annotated proteins in the MemPrep isolates. Each data point in (A, B) is from $n = 3$ biological replicates and from the same experiment as shown in Fig. 3. Source data are available online for this figure.

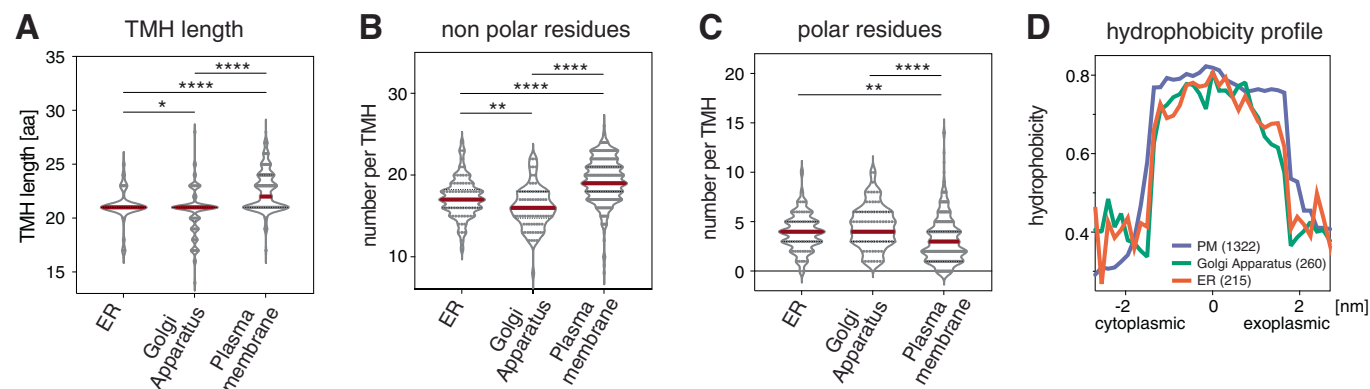


Figure EV3. Systematic analysis of the transmembrane helix length and amino acid composition of single-pass ER proteins.

(A) The transmembrane domains of single-pass proteins from the ER (82), Golgi apparatus (90), and the plasma membrane (273) were systematically analyzed for their predicted TMH length. (B, C) The number of non-polar (B) and polar (C) amino acids in each TMH of our dataset are plotted and sorted by the annotated subcellular localization. The following amino acids (single letter code) were considered non-polar: L, I, V, A, M, F, W, G, P; the following as polar: K, R, H, S, N, Q, T, C, Y, E, D. (D) Average hydrophobicity profiles of all human single-pass TMDs from the ER (orange), the Golgi apparatus (green) and the PM (purple) along the membrane axis (cytoplasmic to exoplasmic) taken from (Lorent et al, 2025). Statistical test: Unpaired *t*-tests were performed with Welch's correction. *P* values in (A): 1.196E-10 (ER vs. plasma membrane), 0.0455 (ER vs. Golgi apparatus), 4.1805E-12 (Golgi apparatus vs. plasma membrane). *P* values in (B): 2.791E-10 (ER versus plasma membrane), 0.0024 (ER vs. Golgi apparatus); 7.059E-20 (Golgi apparatus vs. plasma membrane). *P* values in (C): 3.481E-3 (ER vs. plasma membrane), 5.466E-2 (ER vs Golgi apparatus), 1.7768E-6 (Golgi apparatus vs. plasma membrane). Source data are available online for this figure.

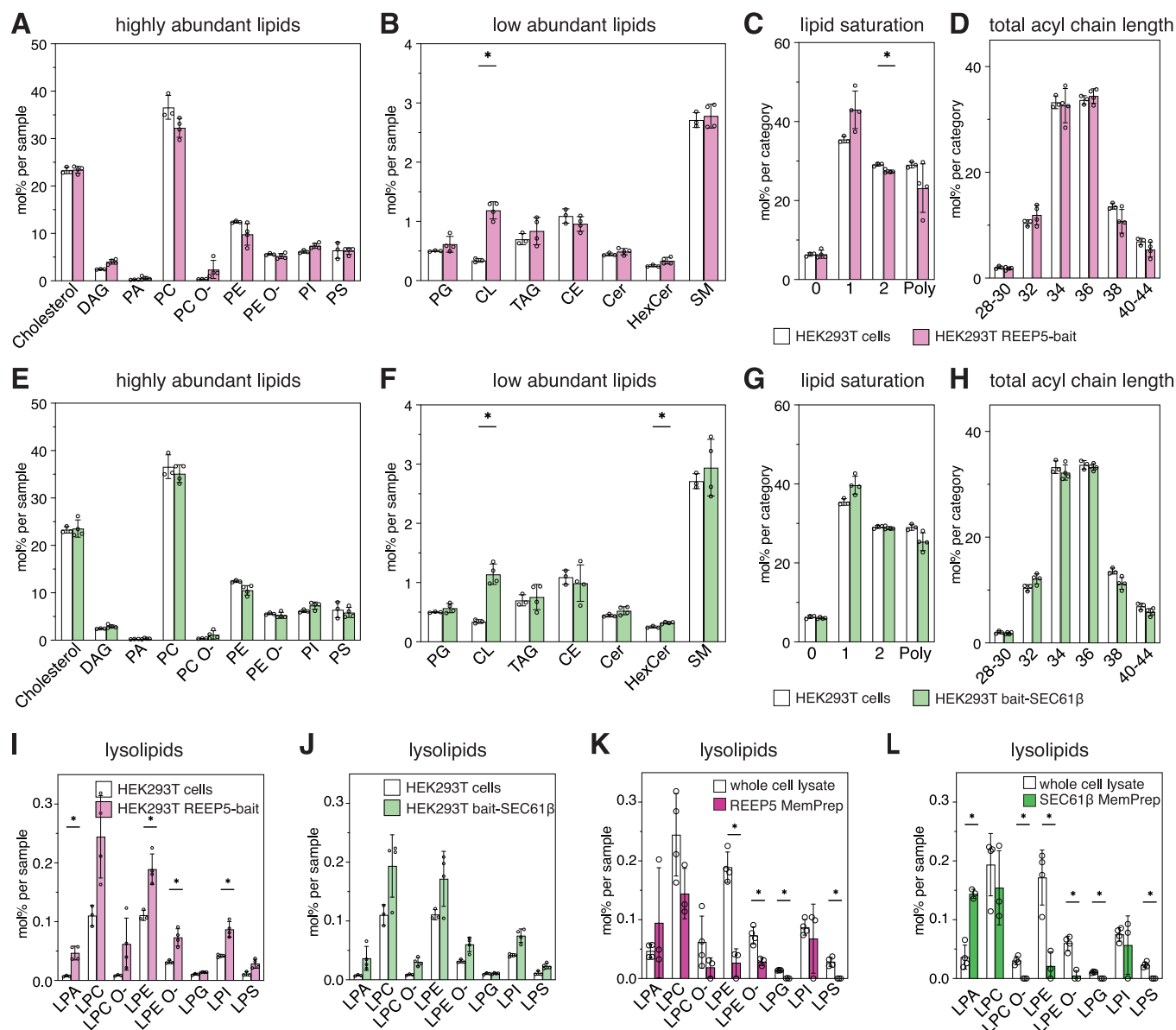


Figure EV4. Expression of bait constructs has only a minor impact on the cellular lipidome.

(A–H) Quantitative lipidomics reveals the lipid class composition given as mol% of all identified lipids in the extract from a whole cell lysate from HEK293T WT cells, and cells expressing either the REEP5-bait or bait-SEC61β constructs. Except for cholesterol, each bar corresponds to a lipid class composed of several lipid species. (A, E) highly abundant lipids—DAG diacylglycerol, PA phosphatidic acid, PC phosphatidylcholine, PC O- phosphatidylcholine (-ether), PE phosphatidylethanolamine, PE O- Phosphatidylethanolamine (-ether), PI phosphatidylinositol, PS phosphatidylserine. The shotgun analysis does not distinguish plasmalogen and plasmalogen lipids. (B, F) low-abundant lipids—PG phosphatidylglycerol, CL cardiolipin, TAG triacylglycerol, CE cholesteryl ester, Cer ceramide, HexCer hexosylceramide, SM sphingomyelin. (C, G) Lipid unsaturation of whole cell lysates from HEK293T WT cells, and cells expressing REEP5-bait or bait-SEC61β given as the total number of double bonds in membrane glycerolipids except for CL (i.e., DAG, PA, PC, PC O-, PE, PE O-, PI, PS, PG) as mol% in this group. All lipids with more than two unsaturations in the lipid acyl chains are categorized as polyunsaturated lipids (Poly). (D, H) The relative abundance of membrane glycerolipids with different total acyl chain lengths (calculated as the sum of both fatty acyl chains) in whole cell lysates and the corresponding MemPrep isolate are plotted in mol% of this category. (K, L) Quantitative lipidomics reveals differences in lysolipid abundance in HEK293T whole cell lysates and the corresponding MemPrep isolates. The lysolipid composition is given as mol% of all identified lipids. Data information: Data from $n = 4$ biological replicates in (A–L) are represented as individual data points and as the mean $\pm$ SD. The data in (K, L) are from the same experiments as shown in Fig. 6 for comparison. Statistical test: Multiple unpaired t -tests with Welch's correction were performed by the Holm–Šidák method with a p value threshold of 0.05. Adjusted p value in (B) 0.0061 (CL). Adjusted p value in (C) 0.016 (2). Adjusted p value in (F) 0.0135 (CL), 0.0195 (HexCer). Adjusted p value in (I): 0.0377 (LPA), 0.0377 (LPE), 0.0431 (LPE O-), 0.0377 (LPI). Adjusted p values in (K): 0.0025 (LPE), 0.0230 (LPE O-), 0.000003 (LPG), 0.0092 (LPS). Adjusted p value in (L) 0.0027 (LPA), 0.0035 (LPC O-), 0.0121 (LPE), 0.0047 (LPE O-), 0.0001 (LPG), 0.0026 (LPS). Source data are available online for this figure.

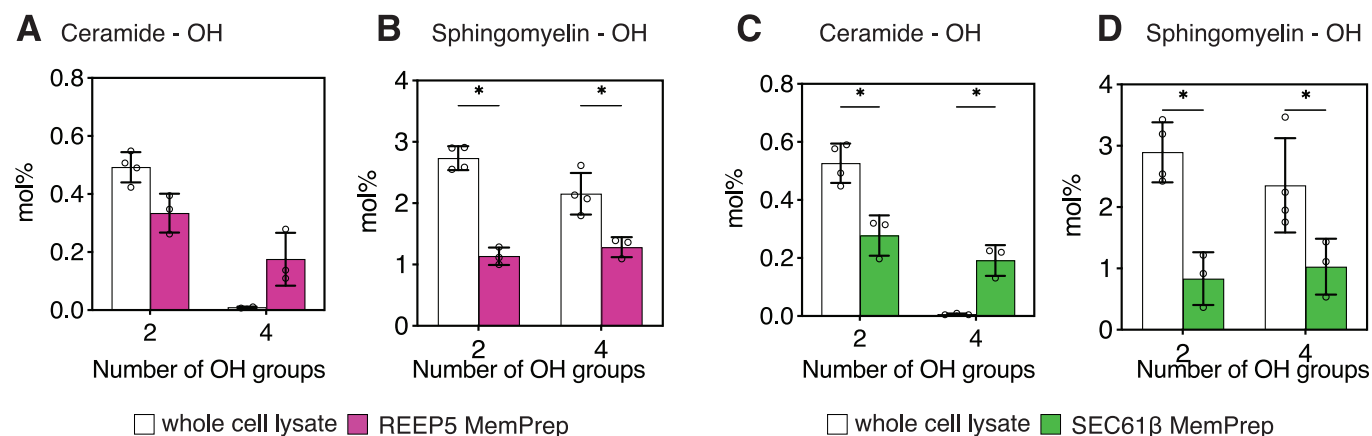


Figure EV5. Ceramide hydroxylation, but not sphingomyelin hydroxylation, distinguishes the ER from the whole cell lipidome.

(A–D) Quantitative lipidomics of differentially hydroxylated sphingolipids in the whole cell lysate and MemPrep isolates. (A) The level of differentially hydroxylated Ceramide species is given in mol% of all identified lipids in the sample, comparing a REEP5 MemPrep isolate with the corresponding whole cell lysate. (B) The level of differentially hydroxylated SM species is given in mol% of all identified lipids in the sample, comparing a REEP5 MemPrep isolate with the corresponding whole cell lysate. (C) The level of differentially hydroxylated Ceramide species from SEC61β MemPreps and the corresponding cells are given in mol% and plotted as in (A). (D) The level of differentially hydroxylated SM species for SEC61β MemPreps and the corresponding cells are given in mol% and plotted as in (B). Data information: Data from $n = 3$ biological replicates in (A–D) are represented as individual data points and as the mean $\pm$ SD. Statistical test: Multiple unpaired t -tests with Welch's correction were performed by the Holm–Šidák method with a p value threshold of 0.05. Adjusted p -values in (B): 0.0001 (2), 0.0082 (4). Adjusted p values in (C): 0.0142 (2) and 0.0260 (4). Adjusted p values in (D): 0.0046 (2), 0.0369 (4). Source data are available online for this figure.