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Supplementary Information for

Anthrax toxin requires ZDHHC5-mediated palmitoylation of its surface processing host enzymes

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Supplementary Materials & Methods

SILAC cell surface protein pull-down coupled to mass spectrometry

Wildtype (WT) and ΔZDHHC5 cells were grown for 7 passages in medium supplemented with heavy, 84 mg/L $^{13}C_6$ $^{15}N_4$ L-Arginine (Cambridge Isotope Laboratories CNLM-539-H) and 146 mg/L $^{13}C_6$ L-Lysine (Thermo Fisher Scientific #89988), or light isotopes (Thermo Fisher Scientific #89989 for L-Arginine and #89987 for L-Lysine), both with an excess of light 115 mg/L L-Proline (Thermo Fisher Scientific #88211). Cells were lysed normally or their surface was labeled with biotin, as described in the Main Materials and Methods. After lysis, protein concentrations were measured, and cells were mixed in equal amounts between the light ΔZDHHC5 and heavy WT (forward reaction) or vice-versa (reverse reaction). Each sample was digested by Filter Aided Sample Preparation (FASP)(1) with minor modifications. Dithiothreitol (DTT) was replaced by Tris (2-carboxyethyl)phosphine (TCEP) as reducing agent and Iodoacetamide by Chloroacetamide as alkylating agent. A combined proteolytic digestion was performed using Endoproteinase Lys-C and Trypsin. Peptides were desalted on C18 Stage Tips (2) and dried down by vacuum centrifugation. Samples were then fractionated into six fractions by strong cation exchange (SCX) chromatography on Stage Tips (3) and dried down again by vacuum centrifugation. For liquid chromatography mass spectrometry (LC MS/MS) analysis, peptides were resuspended and separated by reversed-phase chromatography on a Dionex Ultimate 3000 RSLC nanoUPLC system in-line connected with an Orbitrap Q Exactive HF Mass-Spectrometer (Thermo Fisher Scientific). Database search was performed using MaxQuant 1.6.0.1 (4) against the UniProt human database (UniProt release 2017_05). Carbamidomethylation was set as fixed modification, whereas oxidation (M) and acetylation (protein N-terminus) were considered as variable modifications. SILAC quantifications was performed by MaxQuant using the standard settings with the re-quantification mode enabled. Significance B values were calculated using Perseus Software (5).

Toxin purification

Bacillus anthracis protective antigen (PA) and lethal factor (LF) were expressed in *E. coli* and purified as documented previously with minor modifications (6). Briefly, *E. coli* BL21(DE3) cells transformed with the PA-expressing plasmid were grown to O.D. 0.6 and induced with 1 mM IPTG overnight at 16 °C. The periplasm fraction was isolated using sucrose and $MgSO_4$ buffers. The periplasm was passed over an anion exchange column and PA was eluted using a gradient up to 1 M NaCl. Finally, PA was isolated from the peak fractions using size exclusion chromatography and aliquots were flash frozen in liquid nitrogen. *E. coli* BL21(DE3) cells transformed with an LF-His-tag-expressing plasmid were grown the same way as for PA above. The cells were lysed via sonication, lysate was applied to a nickel-NTA column, and eluted using a gradient to 0.5 M imidazole. Fractions containing LF were pooled and the His-tag was cleaved using thrombin. Finally, the protein was passed over an anion exchange column from which it was eluted by a gradient of up to 0.5 M NaCl. Both proteins were approximately 95% pure and recognized by their corresponding antibodies as full-length species. Aerolysin toxin was purified as previously described by our laboratory (7).

Quantitative real-time PCR and RNA-Seq

RNA was extracted from cells using the Qiagen RNAeasy kit with use of the QIAshredder. RNA concentration was measured and 500 ng of total RNA was used for cDNA synthesis using iScript (Bio-Rad 1708891). A 1:5 dilution of cDNA was used to perform quantitative real-time PCR using Applied Biosystems SYBR Green Master Mix (Thermo Fisher Scientific) on 7900 HT Fast QPCR System (Applied Biosystems) with SDS 2.4 Software. Primers used were (all 5' to 3'): Furin F: AGG GAT AGG AGC CTG ACT GTT and R: AGC ACC TGG GAT TCA TCC TG; and PC7 F: CGG TCT GTA GCC TTT GCT GA and R: GGC TAA CCA CGT CAC ACT CA. The data (always in triplicate) were normalized using three human housekeeping genes (TATA-binding protein, β-microglobulin and β-glucoronidase). For RNA-Seq, total RNA from WT or ΔZDHHC5 RPE-1 cells was isolated as mentioned above, from which mRNA libraries were prepared. Paired-end sequencing was done using the Illumina NextSeq 500 and data was processed using R (8).

Additional S-palmitoylation methods

For non-radioactive metabolic labeling followed by click chemistry, cells were fed 10 μM 17-octadecynoic acid (17-ODYA; Chayman Chemical 90270) in media supplemented with 5% dialyzed FBS for 3 h at 37 °C. Cells were washed and lysed normally, then lysates were immunoprecipitated with antibodies against the protein of interest. The immunoprecipitated samples still on the beads underwent a click chemistry reaction by incubation with PBS containing 250 μM Tetramethylrhodamine (TAMRA) azide (Thermo Fisher Scientific T10182), 1 mM TCEP, 200 μM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA; Sigma-Aldrich 678937), and 1 mM $CuSO_4$ (Sigma-Aldrich C7631) for 1 h at room temperature. Samples were eluted off the beads as above. Samples were divided for immunoblot (one fourth) and fluorescent signal capture (three fourths). To assay fluorescence from the clicked TAMRA, the gels were washed 6-8 times in water and fluorescent signal was developed using the Typhoon imager.

Supplementary References

1. Wiśniewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. *Nat Methods* 6(5):359–362.

2. Rappsilber J, Mann M, Ishihama Y (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 2(8):1896–1906.

3. Kulak NA, Pichler G, Paron I, Nagaraj N, Mann M (2014) Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. *Nat Methods* 11(3):319–324.

4. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26(12):1367–1372.

5. Tyanova S, et al. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* 13(9):731–740.

6. Kintzer AF, Sterling HJ, Tang II, Williams ER, Krantz BA (2010) Anthrax Toxin Receptor Drives Protective Antigen Oligomerization and Stabilizes the Heptameric and Octameric Oligomer by a Similar Mechanism. *PLoS ONE* 5(11):e13888.

7. Gonzalez MR, et al. Pore-forming toxins induce multiple cellular responses promoting survival. *Cell Microbiol* 13(7):1026–1043.

8. R Core Team (2013) *R: A language and environment for statistical computing* (R Foundation for Statistical Computing, Vienna, Austria). Available at: http://www.R-project.org/.

Supplementary Figures

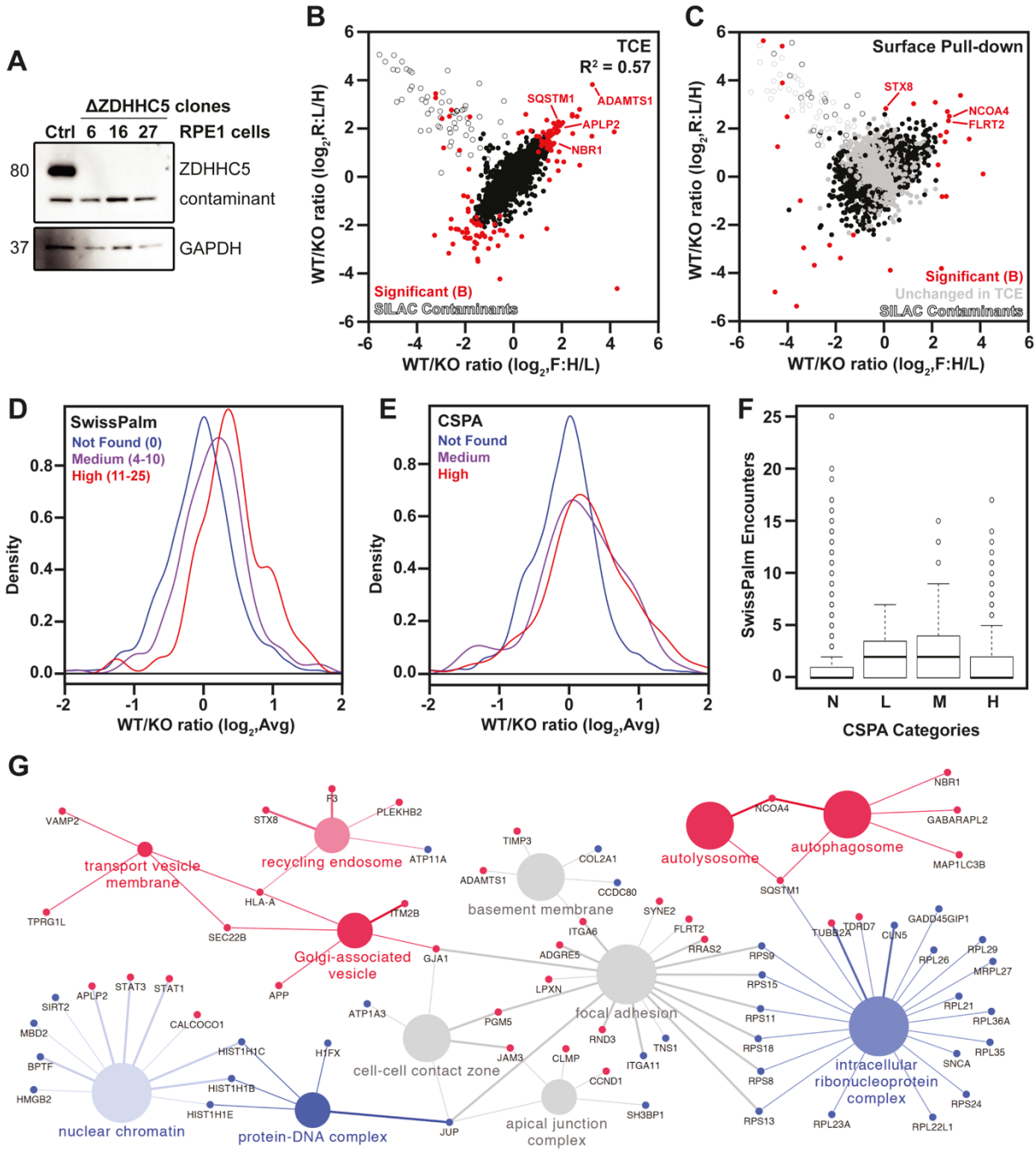

Fig. S1. ZDHHC5 controls presence of specific proteins at the cell surface.
A. RPE-1 cells knocked out for ZDHHC5 (3 different clones) or WT are verified by immunoblot against ZDHHC5 with GAPDH shown as a control. Clone 16 was used for experiments with ΔZDHHC5 cells. WT or ΔZDHHC5 RPE-1 cells were labeled with either heavy or light SILAC isotopes, then the total cell extract (TCE) samples (**B**) or the surface proteins (**C**), which were labeled with biotin and isolated, were subjected to mass spectrometry analysis. Both forward (WT cells labeled with heavy isotopes) and reverse reactions are shown with the known contaminants in open circles. The two directions of the TCE samples had an R^2 of 0.57. The proteins whose $\log_2$ average of the TCE forward and reverse reactions was between -0.5 and 0.5 (meaning unchanged) are shown in gray on the graph for the cell surface proteins. The proteins that are statistically different (B significant) between the two cell types are shown in red with a few key proteins labeled. **D.** An average of the forward and reverse SILAC

total cell extract (TCE) experiments was probed for palmitoylated proteins using SwissPalm (accessed in April 2018): *blue line* is those that are annotated 0 times in SwissPalm palmitomes or validation articles (n= 2612; p-value=0.82 in Welch 2-sample 2-sided t-test against the total of n=4481), the *purple line* is those that are annotated 5-10 times in SwissPalm (n=218, p-value = $1.6x10^{-5}$ in same test as above), and the *red line* are those that are annotated 11-25 times in SwissPalm (n=51, p-value = $1x10^{-7}$ in same test as above). **E.** An average of the two SILAC TCE experiments was probed for cell surface protein using the Cell Surface Protein Atlas (CSPA): *blue line* is those that don't appear in CSPA (n=4014; p-value = 0.24 in Welch 2-sample 2-sided t-test against the total n=4481), the *purple line* is those that appear in CSPA as medium confidence (n=82, p-value = 0.0094 in same test as above), and the *red line* are those that appear in CSPA as high confidence (n=264, p = $2.3x10^{-6}$ in same test as above). **F.** CSPA categories (N: None, L: Low, M: Medium, H: High) have no correlation with the numbers of times that protein is found in SwissPalm. This confirms that the two observations of cell surface and palmitoylated proteins in the TCEs of ΔZDHHC5 vs. WT RPE-1 cells are independent. **G.** Cytoscape ClueGO Cellular Component GO term analysis on those proteins that are B-significant in either the TCE or cell surface pull-down proteomes, shows that proteins that are lower expressed in ΔZDHHC5 cells as compared to control cells (red) are more associated with autophagy and cellular trafficking, while in the other direction (blue), proteins are more associated with ribosomes and chromatin. GO terms that were not specific for proteins from one direction or another, but were still enriched (gray) include focal adhesion and cell-cell contact zone. Size of the circles corresponds to the number of B-significant proteins that belong to those annotations. Settings for this cellular component GO Term analysis were as follows: 2 clusters (B-significant genes higher in ΔZDHHC5 cells as compared to control cells, and vice versa, from both TCE and pull-down proteomes); 2 genes minimum per GO term with at least 2% of genes represented; at least 60% counted as specific; and, GO Tree Interval 1-8 with GO Term Fusion.

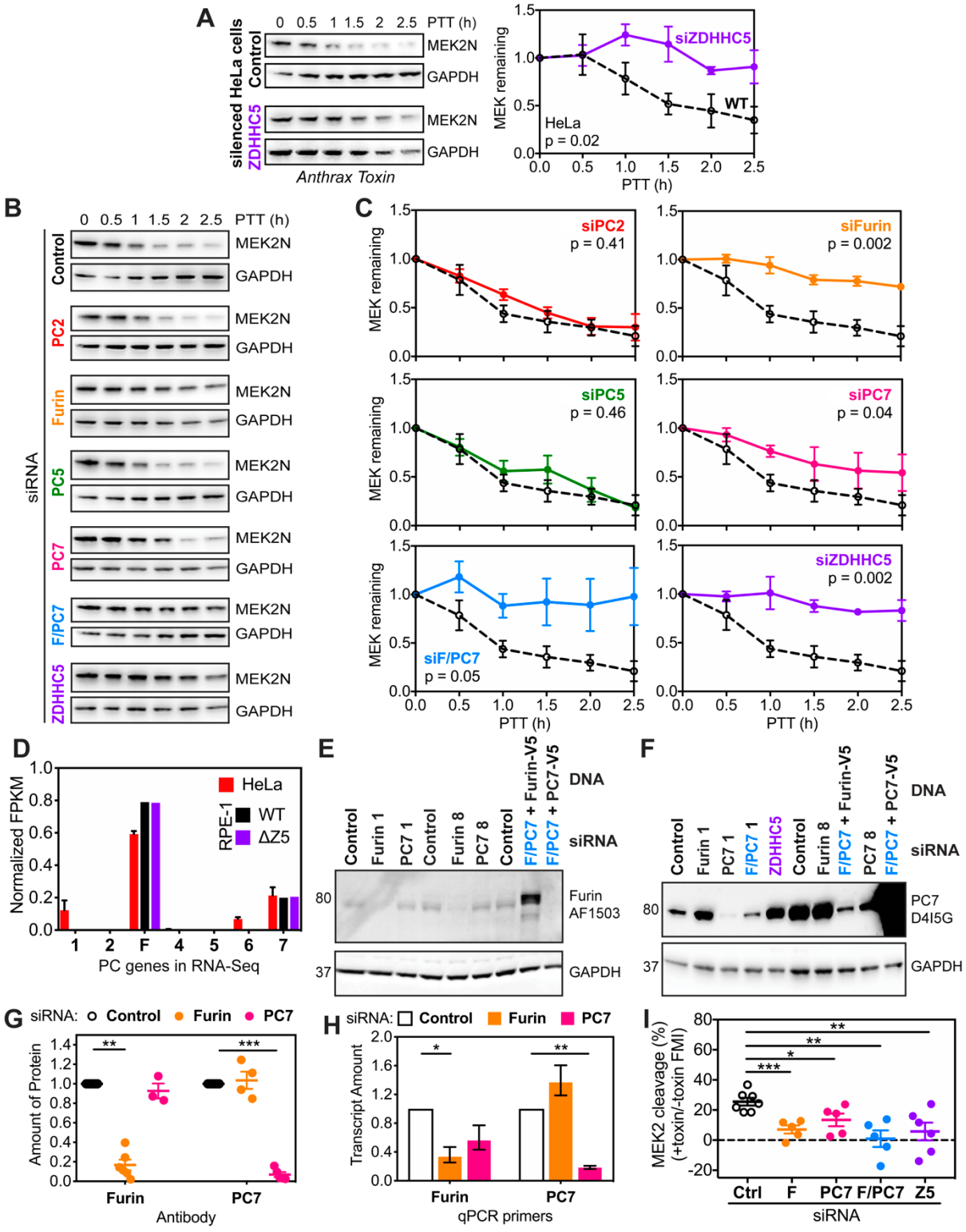

Fig. S2. ZDHHC5 silencing is similar to Furin and PC7 knockdown.
A. Toxin entry assays were carried out as in in *Fig. 1A-B* but in HeLa cells. *Statistics*: unpaired 2-tailed t-test on areas under curves [AUC] with n=3. **B**. Toxin entry assays were carried out as in *Fig. 1A-B*. Representative blots are shown for cells depleted of Control, PC2, Furin, PC5, or PC7, Furin/PC7 (both silenced together; labeled as F/PC7) or ZDHHC5. **C.** Graphs shows quantifications from experiments as in *B* with control silencing repeated in each graph and the graph of ZDHHC5 repeated from *Fig. 1B*. *Statistics*: unpaired 2-tailed t-test on AUCs with n = 4-7. **C.** Using available

HeLa RNA-seq data (Nagaraj et al. 2011, Higareda-Almaraz et al. 2013, and Ly et al. 2014) and our RPE-1 RNA-Seq data, the expression of PCs in these cell types were examined by checking their fragments per kilobase of exon per million mapped reads (FPKM). The values were normalized within each data set for ease of comparisons between datasets. Anti-Furin (**D**) and anti-PC7 (**E**) antibodies were tested along with silencing of two different Furin and PC7 siRNAs (1 is in coding region, 8 is outside of coding region, for both genes). Transfection was also verified using the Furin and PC7 V5-tagged constructs. GAPDH was used as a loading control. **F.** Immunoblots as in *D* and *E* were quantified. *Statistics*: ratio paired 2-tailed t-test on the original data. **G**. Representative results from real-time quantitative PCR (RT-qPCR) on the silenced cells are shown, with transcript amount normalized to housekeeping genes. *Statistics*: unpaired 2-tailed t-test on the original data. **H.** Toxin entry experiments were repeated using the flow cytometry read out shown in *Fig. 1C* with ZDHHC5 values repeated from there. *Statistics*: unpaired 2-tailed t-test. *P < 0.05, **P < 0.01, and ***P < 0.001.

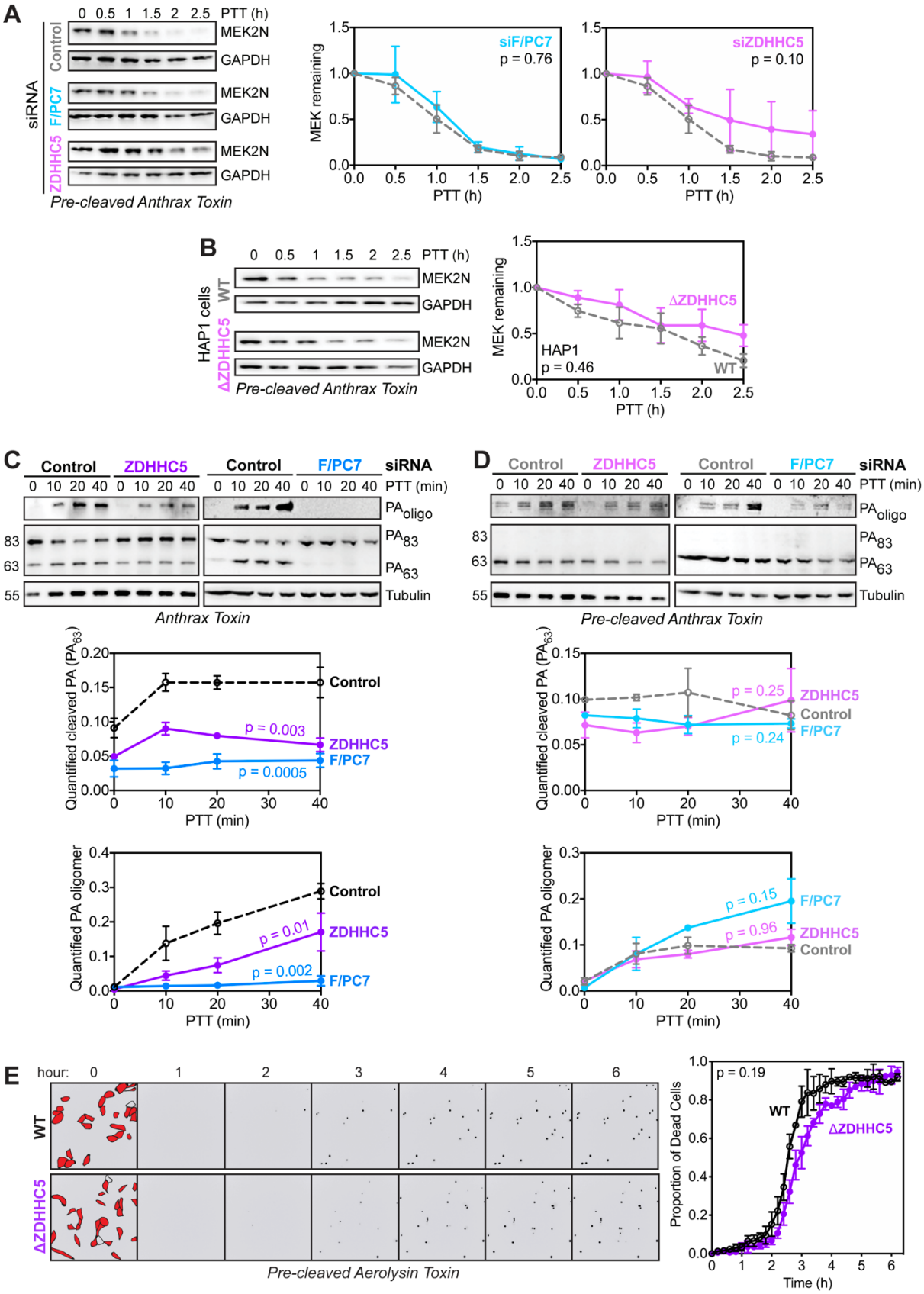

Fig. S3. ZDHHC5 is involved in cleavage of anthrax toxin.

A. Pre-cleaved PA (500 ng/mL) was combined with LF (50 ng/mL) and applied to Control-, Furin/PC7-(F/PC7), or ZDHHC5-silenced cells as in *Fig. 1A-B* and *SI Appendix, Fig. S2A-B*. *Statistics*: unpaired 2-tailed t-test on AUCs with n = 3. **B.** Experiments were repeated just as in *A* but for WT and

ΔZDHHC5 HAP1 cells. *Statistics*: unpaired 2-tailed t-test on AUCs with n = 3. **C.** As in *Fig. 1D*, anthrax toxin was applied to Control-, Furin/PC7- (F/PC7), or ZDHHC5-silenced cells and allowed to internalize for 0 to 40 min. The difference was already shown for cells lacking ZDHHC5 as compared to Control in *Fig. 1D*. *Statistics*: unpaired 2-tailed t-test on AUCs with n = 4. **D.** Experiments were repeated just as in *C*, but with pre-cleaved anthrax toxin generated the same way as in *A*. *Statistics*: unpaired 2-tailed t-test on AUCs with n = 3. **E.** Representative fields in live cell imaging (at 20X) of WT or ΔZDHHC5 cells treated with pre-cleaved WT aerolysin toxin. Cell outlines in the fields are shown at time 0 with cells in red as the ones that die at the end of the time course (more than 6 hours). Each time-point shows the channel in which a NucGreen Dead dye marks the nuclei of dead cells. *Statistics:* representative graph; unpaired 2-tailed t-test on AUCs with n = 3.

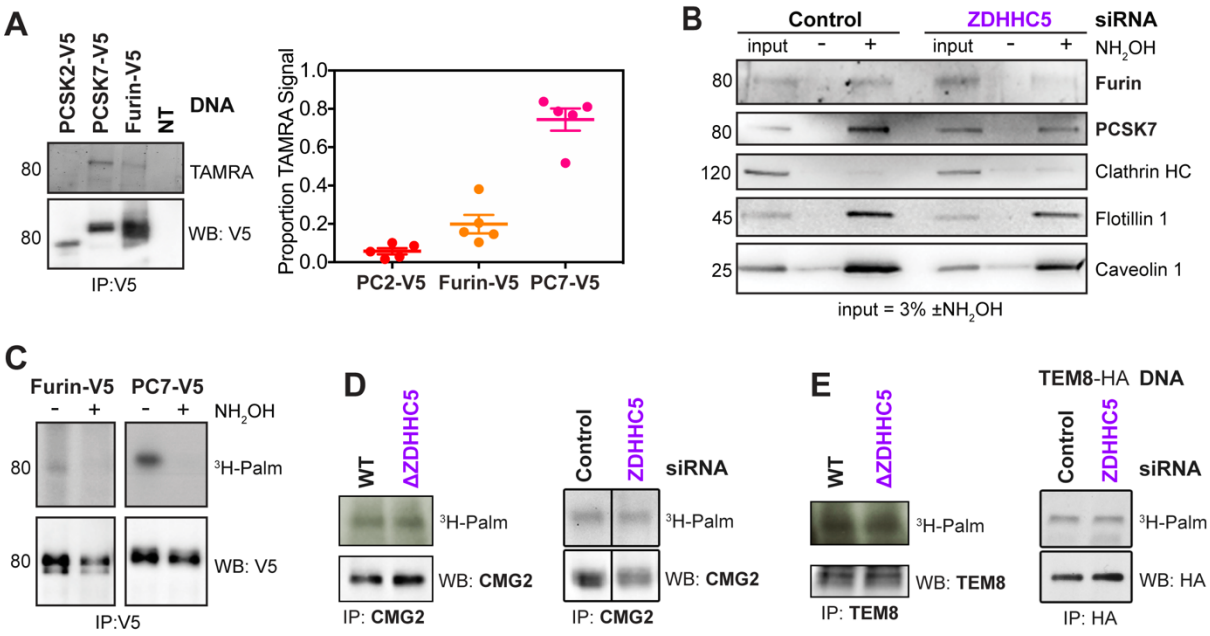

Fig. S4. Furin and PC7 palmitoylation controls; ZDHHC5 does not palmitoylate anthrax toxin receptors.

A. RPE-1 cells were transfected with PC2-, Furin-, or PC7- V5-tagged plasmids and then labeled for 4 h with 17-ODYA. Afterwards, cells were lysed, IP-ed with V5 beads, and a TAMRA fluorophore was attached to the 17-ODYA via a click reaction. Samples were run on SDS-PAGE and amount of fluorophore was visualized, and a Western blot was performed with V5 antibody. PC2 is consistent with background signal. **B.** Representative blot of Acyl-RAC performed on RPE-1 cells silenced with Control or ZDHHC5 siRNA as in *Fig. 2D*. **C.** Representative images of RPE-1 cells transfected with Furin- and PC7-V5 and labeled for 2 h with ^{3}H-palmitic acid, after which cells were lysed and immunoprecipitated with antibody beads against V5. Radioactive palmitate was cleaved from the proteins using hydroxylamine (NH_2OH). **D**. RPE-1 cells either knocked out or silenced for ZDHHC5, or not, were labeled for 2 h with ^{3}H-palmitic acid, after which cells were lysed and immunoprecipitated with an antibody against endogenous CMG2. **E.** WT or ΔZDHHC5 RPE-1 cells were labeled for 2 h with ^{3}H-palmitic acid, after which cells were lysed and immunoprecipitated with an antibody against endogenous TEM8. Complementarily, HeLa cells silenced or not for ZDHHC5 were transfected with TEM8-HA, and labeled for 2 h with ^{3}H-palmitic acid.

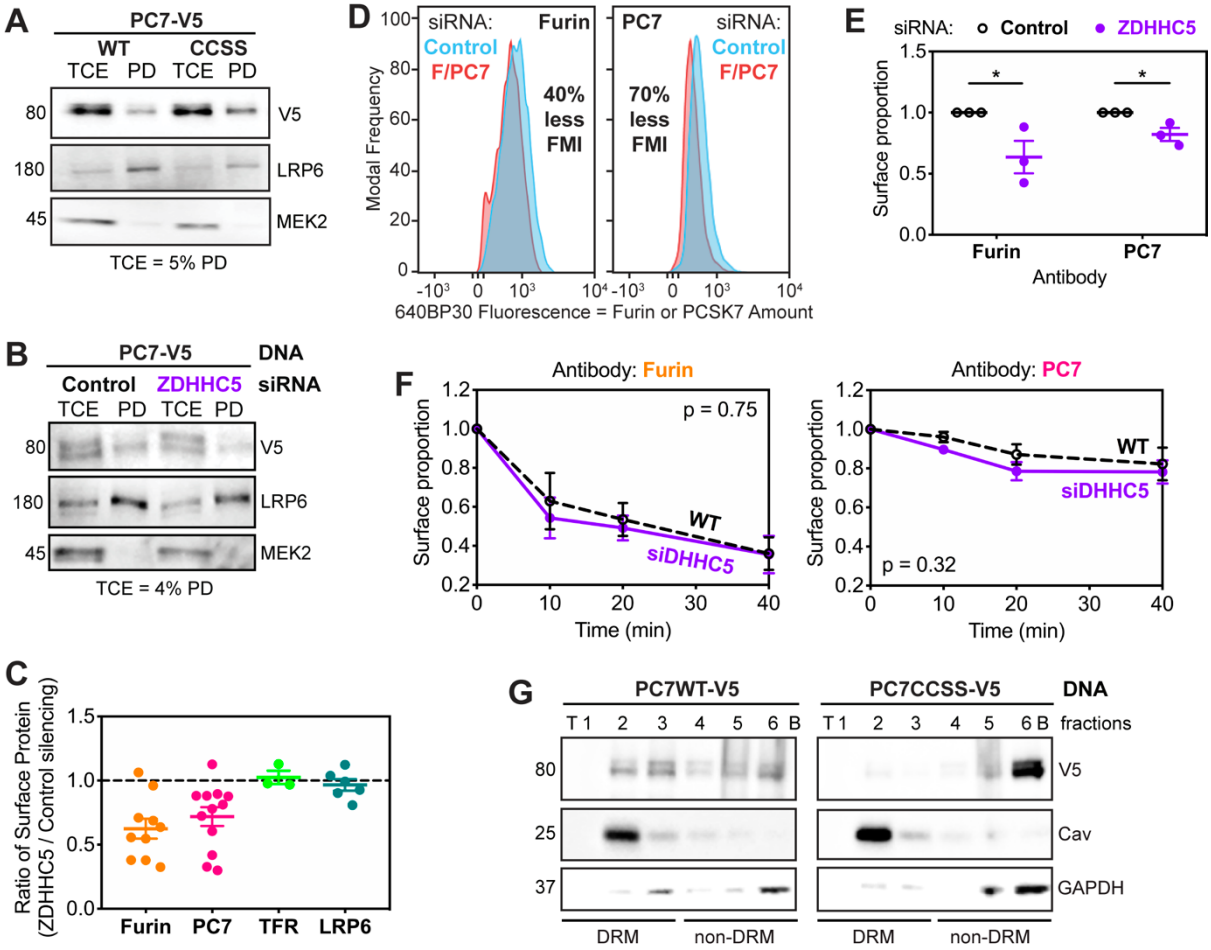

Fig. S5. Cell surface expression controls and supporting experiments.
A. Representative gel for PC7 for the experiment quantified in *Fig. 4A* is shown. **B.** Representative gel for PC7 for the experiment quantified in *Fig. 4B* is shown. **C.** Ratios of surface protein in ZDHHC5-silenced cells as compared to Control-silenced cells are shown for transfected Furin and PC7 and endogenous Transferrin Receptor (TFR) and LDL-Receptor related Protein 6 (LRP6). **D.** To verify that the anti-Furin and anti-PC7 antibodies work for flow cytometry, they were tested using Control- and Furin/PC7-silenced cells. A decrease in Fluorescence Median Intensity (FMI) of 40% for Furin (left) and 70% for and PC7 (right) was found using their respective antibodies. **E.** RPE-1 cells were silenced with Control or ZDHHC5 and permeabilized (total) or not (surface) with saponin and then stained with primary antibodies against Furin and PC7, checked in *D*. Using flow cytometry, the ratio of FMI of the fluorescent secondary antibody for the surface over total was calculated for both Furin and PC7. *Statistics:* ratio paired 2-tailed t-test (*P < 0.05). **F.** RPE-1 cells silenced or not for ZDHHC5 were first labeled with primary antibodies and then allowed to internalize at 37 °C for 0 to 40 min, after which all cells were put on ice and labeled with secondary antibody. Only the fraction of Furin (left) or PC7 (*right*) remaining at the surface was quantified by flow cytometry. *Statistics:* unpaired 2-tailed t-test on AUCs; n = 3. **G.** Representative gel for PC7 for the experiment quantified in *Fig. 4G* is shown.