The dual roles of RPE65 S-palmitoylation in membrane association and visual cycle function

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Supplemental Figure Legends

Figure *S1*. **Co-existence of palmitoylated and non-palmitoylated populations of RPE65**. Acyl-RAC analysis of RPE65 showed that a major population of RPE65 did not pull down with thiopropyl-sepharose beads (unbound fraction). The palmitoylation status of this unbound RPE65 was checked by twice sequentially re-incubating unbound fraction (unbound fraction 1 and 2) with fresh beads, followed by immunoblot analysis using anti-RPE65 antibody. This showed that there was no pulldown of this population of RPE65, confirming its lack of palmitoylation.

Figure *S2*. **Amino acid sequence alignment of RPE65 from different species.** Sequence alignment shows the position of non-conserved (in blue color) and conserved (in red color) cysteine residues among different species. Note that only in one of three paralogs in teleost fishes and in hagfish RPE65 is C146 not completely conserved. There are 12 cysteine residues in bovine RPE65. Dog RPE65 has 11 cysteine residues, with substitution of the non-conserved cysteine 396 (present in human and bovine RPE65s) to an arginine residue.

Figure *S3*. **Structural view of surface exposed cysteine residues.** Surface view of dog RPE65 structure was visualized and generated using PyMol software. Close inspection of the cysteine residues on the three-dimensional structure of RPE65 revealed five cysteines (C112, C169, C195, C278 and C448, as shown in red color; highlighted in circle) that have their thiol groups facing to the solvent. The iron (Fe) atom in the center of the RPE65 structure is represented by orange color.

Figure *S4*. **Identification of cysteine residues involved in RPE65 palmitoylation.** HEK293F cells were transfected with cysteine mutants of RPE65 to determine the residues that undergo palmitoylation. Cell lysate was prepared (as described in Materials and Methods section) and used for ABE and acyl-RAC analysis. (A) ABE result of cysteine 112, 146 and 195 mutants compared to wild type RPE65. (B) Acyl-RAC results for both alanine and serine substituted mutants of cysteine residues other than C112, C146 and C195 showed a protein band in the HAM-treated samples and so did not affect palmitoylation of RPE65. (C) Serine substituted C112, C146 and C195 residues showed reduced or no RPE65 band in the HAM-treated samples, and thus are involved in RPE65 palmitoylation. Samples were treated with 0.5 M hydroxylamine (HAM; indicated as "+") or 0.5 M NaCl (indicated as "-"), respectively. Results were calculated as mean ± S.D. from three independent experiments. *P<0.005, **P<0.05, unpaired student's t-test.

Figure *S5*. **Mass spectrometric analyses of control and hydroxylamine-treated samples for rhodopsin and CRALBP.** Comparison of relative abundance of NEM- and 4-VP modified peptides in the control and HAM-treated samples for rhodopsin (A) and CRALBP (B). N-ethyl maleimide (NEM) and 4-vinyl pyridine (4-VP) modification represents the non-palmitoylation and palmitoylation of cysteine residues, respectively. We identified the peptide containing 4-VP modifications of cysteines 322 and 323 from the bovine rhodopsin sample. In the case of CRALBP, only NEM-modified peptides were detected, indicating lack of palmitoylation. The square box represents the technical replicates of the sample) and the line represent the comparative behavior of the peptide ion of interest in the – HAM and + HAM samples.

Figure *S6*. **MS-coupled acyl-labeling of bovine microsome RPE using different MS instruments, Synapt G2-Si HDMS, AB Sciex 6600 w/SelexION**. Rhodopsin and CRALBP were used as positive and negative control. NEM- and and 4VP-modified cysteine indicates indicates non-palmitoylation and palmitoylation of cysteine residue, respectively. The ratio of modified peptide shown was relatively high in hydroxylamine-treated samples compared to untreated samples.

Figure *S7*. **Immunoblot analysis of proteins in HEK293F-based heterologous visual cycle system.** HEK293F cells transfected with pVitro2/RPE65+CRALBP and pVitro3/LRAT or $LRAT^{C161S}$ mutant+RDH5 plasmids were analysed by western blotting for RPE65, CRALBP, LRAT, and RDH5.

Figure *S8*. **Expression profiles of wild type and C112, C146 and C195 mutant RPE65 protein.** HEK293F cells were transfected with pVitro2/RPE65 wild type or cysteine mutants +CRALBP and ~10µg of total protein were analysed by western blotting for expression analysis. The same samples were then subjected to subcellular fractionation shown in Figure 6D.

Figure *S9*. **Presence of C112 mutant protein in lysosomal fraction.** HEK293F cells transfected with pVitro2/RPE65 wild type or C112 mutants +CRALBP were subjected to lysosomal extraction kit (Sigma) and then the lysosomal fractions were analysed by western blotting for RPE65 and cathepsin D (lysosomal marker protein).

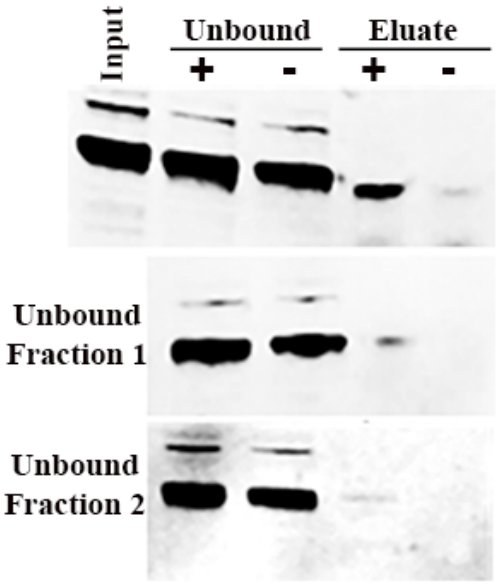
Figure *S10*. **Enlarged view of the catalytic core of RPE65 showing rotamer variability of cysteine 195.** Structural representation of RPE65 (PDB ID: 4RSC; chain A) in complex with the non-retinoid inhibitor emixustat and palmitate. Iron (Fe) atom in the center is represented in orange color. Residues C146 and C195 are denoted as stick figures on the cartoon to mark the orientation of the thiol group. The thiol group (marked with arrows) of C195 exists in two different conformations Asterisks (*) represent the location of the unresolved loop (residues G196-S201) in the ligand bound RPE65.

Figure *S11.* **Surface view of crystal structure of RPE65.** A, RPE65 structure showing three hydrophobic regions. The region consisting of aa109-125 is generated by the ITASSER server (shown in yellow). B, hydrophobic surface view of RPE65 as predicted by PyMOL server. Red color indicates the hydrophobic surface.

Figure *S12*. **Enlarged view of the catalytic core of RPE65 showing the approximate distance of 8 Å between the bound palmitate and C146 thiol atom.** The dashed yellow line represent the measured distance between the bound palmitate and C146-thiol atom in the palmitate-bound crystal structure of RPE65 (PDB ID: 4RSC).

Figure *S13*. **Original western blot panels with gel markers for Figures 1A, 1C, 2B, 5A, 5B, 5C, 6, S1, S2, S3, S6A, S6C, S8, and S9.** Complete scanned gels for western blots shown. Red dashed line identifies cropped region shown in respective figure.

Figure *S1*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Fraction 2

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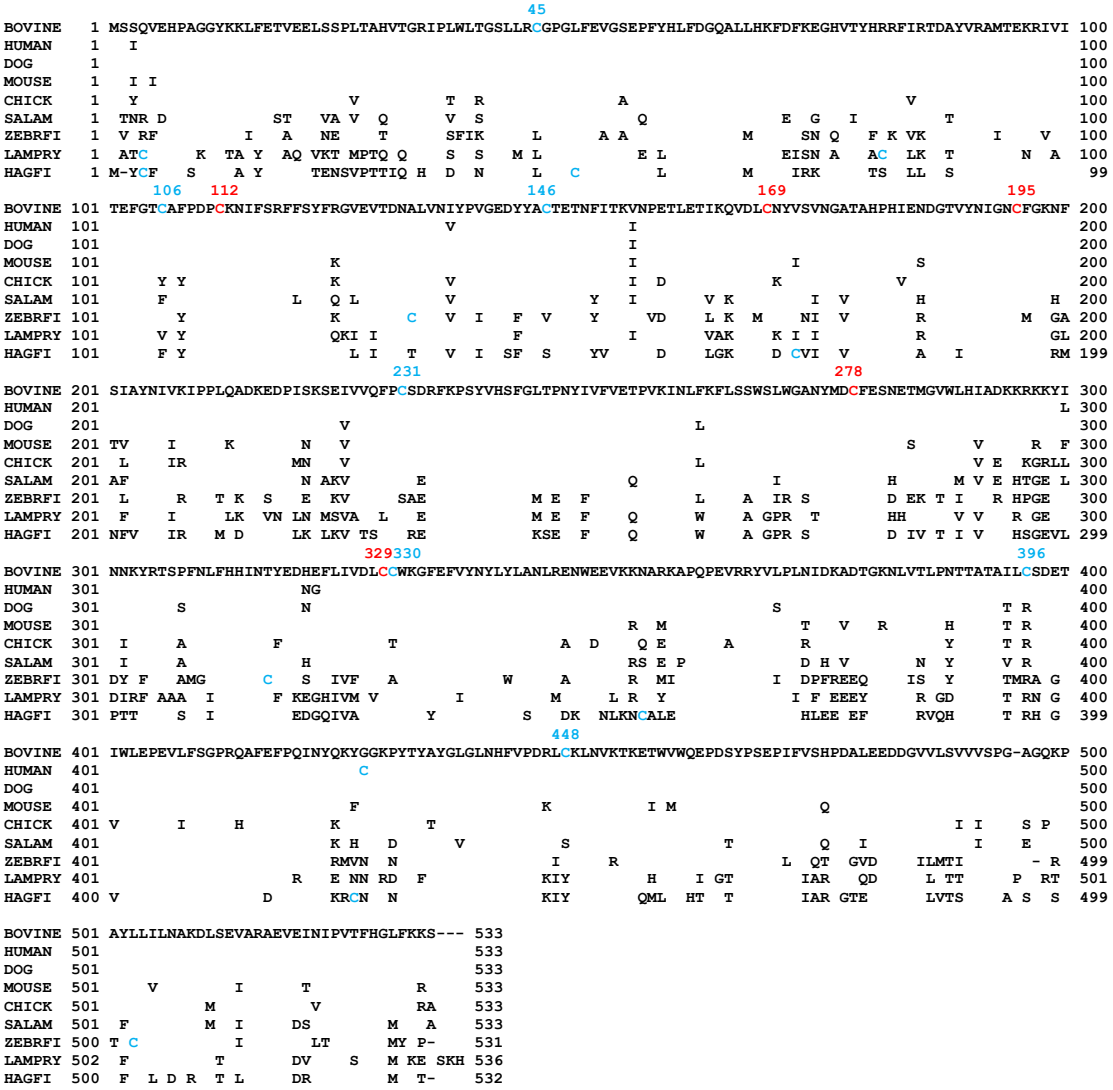
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HUMAN 1 I 100
DOG 1 100
MOUSE 1 I I 100
CHICK 1 Y V T R A V 100
SALAM 1 TNR D ST VA V Q V S Q E G I T 100
ZEBRFI 1 V RF I A NE T SFIK L A A M SN Q F K VK I V 100
LAMPRY 1 ATC K TA Y AQ VKT MPTQ Q S S M L E L EISN A AC LK T N A 100
HAGFI 1 M-YCF S A Y TENSVPTTIQ H D N L C L M IRK TS LL S 99
106 112 146 169 195
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HUMAN 101 V I 200
DOG 101 I 200
MOUSE 101 K I I S 200
CHICK 101 Y Y K V I D K V 200
SALAM 101 F L Q L V Y I V K I V H H 200
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HAGFI 101 F Y L I T V I SF S YV D LGK D CVI V A I RM 199
231 278
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LAMPRY 201 F I LK VN LN MSVA L E M E F Q W A GPR T HH V V R GE 300
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329330 396
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DOG 301 S N S T R 400
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CHICK 301 I A F T A D Q E A R Y T R 400
SALAM 301 I A H RS E P D H V N Y V R 400
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LAMPRY 301 DIRF AAA I F KEGHIVM V I M L R Y I F EEEY R GD T RN G 400
HAGFI 301 PTT S I EDGQIVA Y S DK NLKNCALE HLEE EF RVQH T RH G 399
448
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HUMAN 401 C 500
DOG 401 500
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CHICK 401 V I H K T I I S P 500
SALAM 401 K H D V S T Q I I E 500
ZEBRFI 401 RMVN N I R L QT GVD ILMTI - R 499
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HAGFI 400 V D KRCN N KIY QML HT T IAR GTE LVTS A S S 499
BOVINE 501 AYLLILNAKDLSEVARAEVEINIPVTFHGLFKKS--- 533
HUMAN 501 533
DOG 501 533
MOUSE 501 V I T R 533
CHICK 501 M V RA 533
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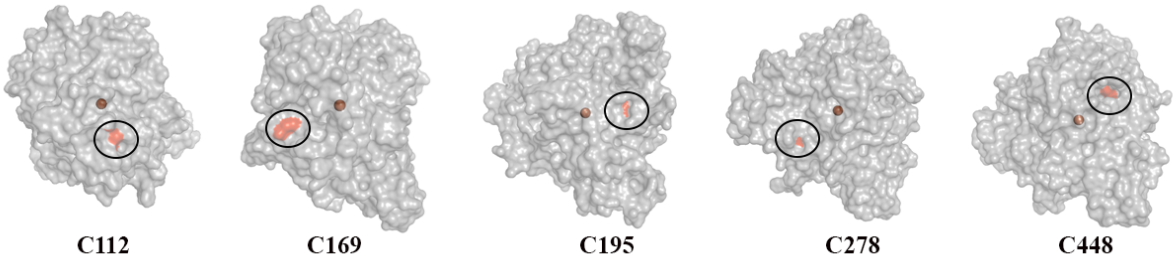
Figure *S3*



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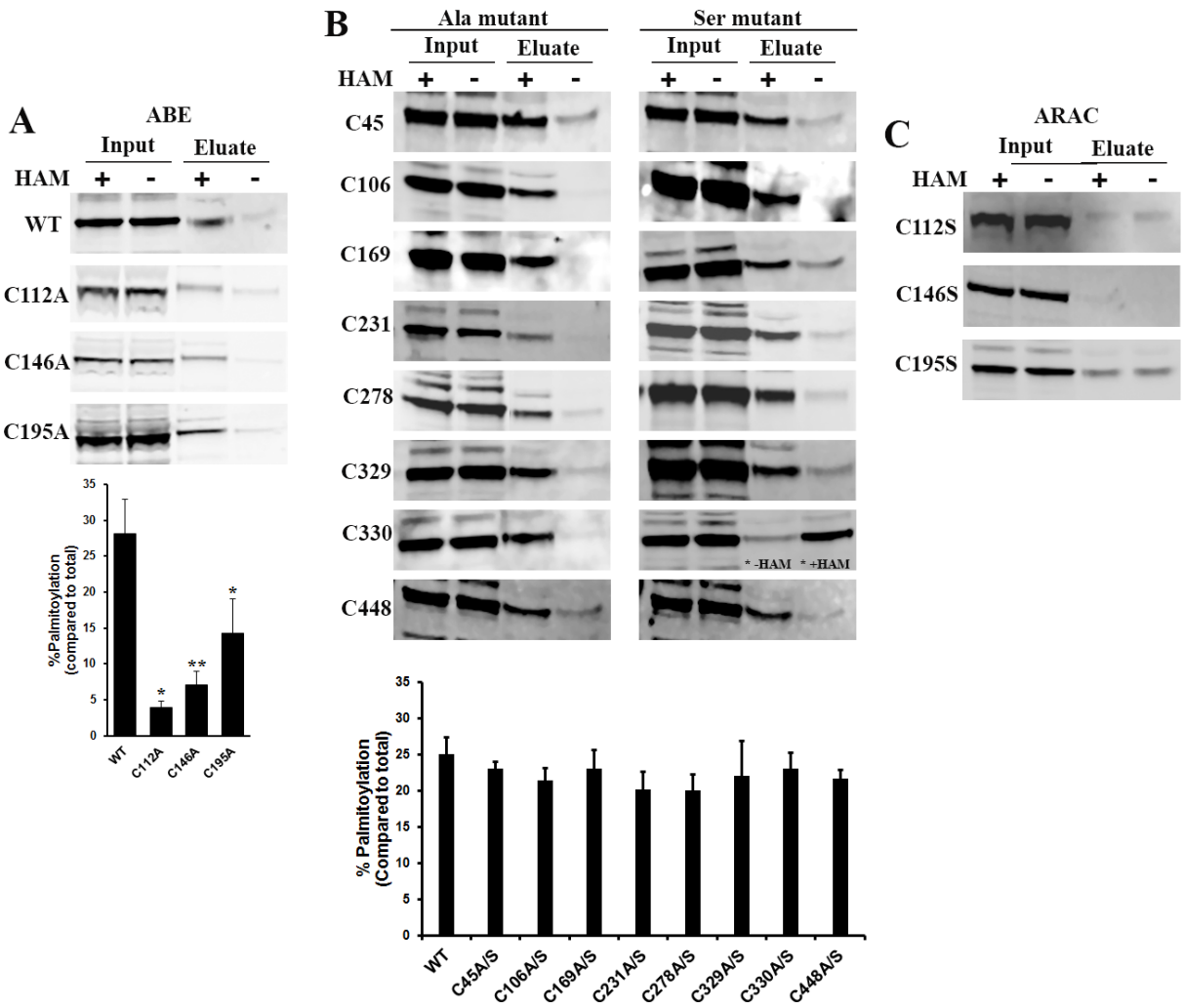
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ABE
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C112A
C146A
C195A
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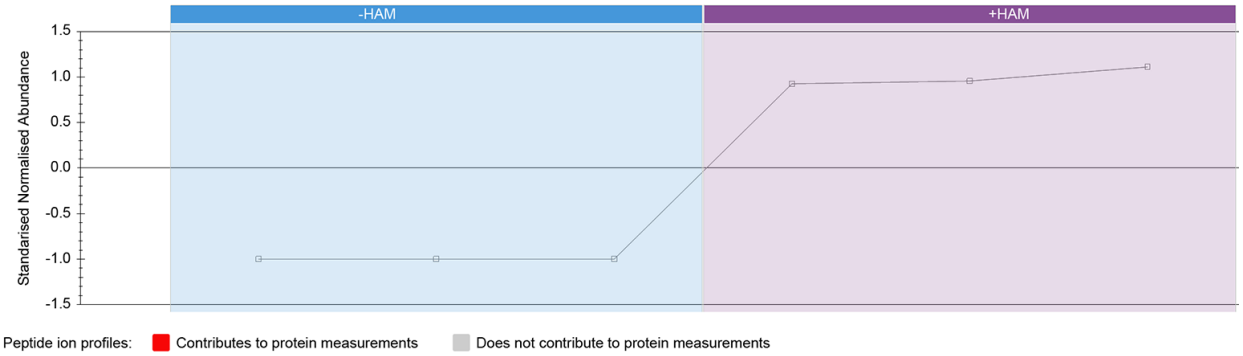
C146A
C195A
B
Ala mutant
Ser mutant
Input
Eluate
HAM + - + -
C45
C106
C169
C231
C278
C329
C330
C448
* -HAM * +HAM
% Palmitoylation (Compared to total)
WT
C45A/S
C106A/S
C169A/S
C231A/S
C278A/S
C329A/S
C330A/S
C448A/S
C
ARAC
Input
Eluate
HAM + - + -
C112S
C146S
C195S

Figure *S5*

A Rhodopsin: QFRN$^{316}C_{NEM}$MVTTL$^{322}C_{4\text{-}VP}$$^{323}C_{4\text{-}VP}$GKNPLGDDEASTTVSK



B CRALBP: DHGPVFGP$^{38}C_{NEM}$SQLPR and $^{138}C_{NEM}$TVEAGYPGVLSTR

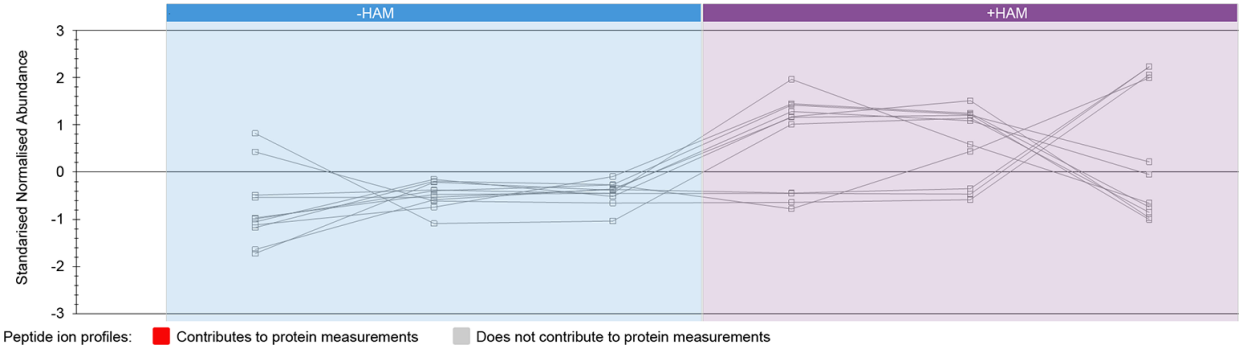


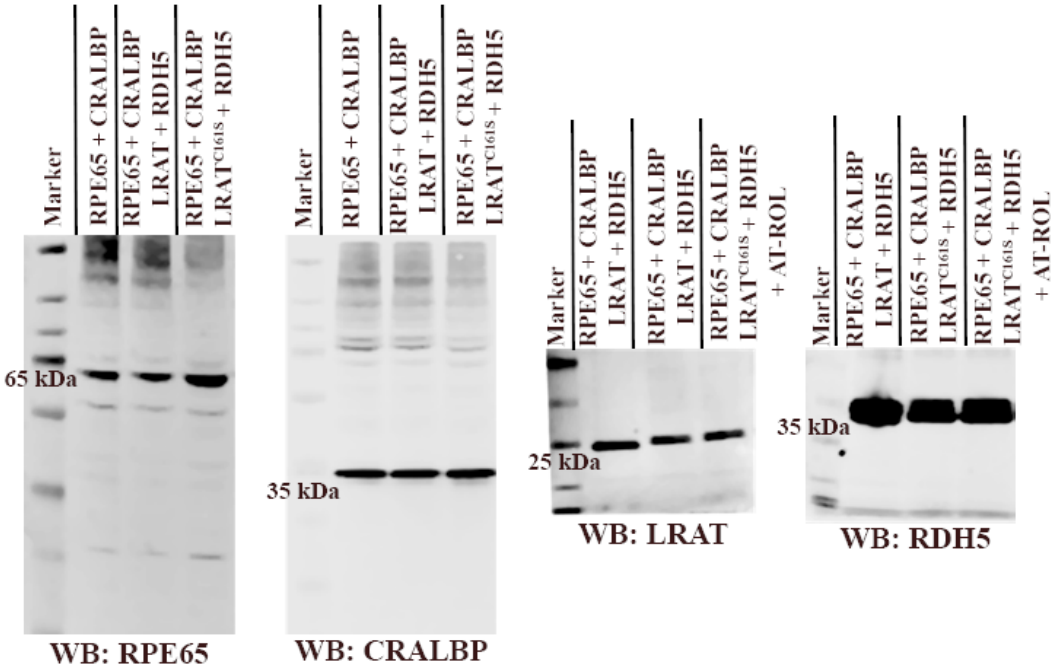
Figure *S6*

Rhodopsin (palmitoylated protein)		
Peptides	**Synapt G2-Si HDMS^E**	**AB Sciex6600 w/SelexION**
K.QFRNCMVTTL$_{322}$C$_{323}$CG KNPLGDDEASTTVSK.T	$_{322}$C: 4VP $_{323}$C: 4VP	$_{322}$C: 4VP $_{323}$C: 4VP

CRALBP (non-palmitoylated protein)		
Peptides	**Synapt G2-Si HDMS^E**	**AB Sciex6600 w/SelexION**
K.DHGPVFGP$_{38}$CSQLPR.H	$_{38}$C: NEM	$_{38}$C: NEM
R.$_{138}$CTVEAGYPGVLSTR.D	$_{138}$C: NEM	$_{138}$C: NEM

RPE65		
Peptides	**Synapt G2-Si HDMS^E**	**AB Sciex6600 w/SelexION**
R.IVITEFGT$_{106}$CAFPDP$_{112}$CK.N	$_{106}$C: NEM $_{112}$C: NEM; 4VP	$_{106}$C: NEM $_{112}$C: 4VP
R.GVEVTDNALVNIYPVGEDY YA$_{146}$CTETNFITK.V	$_{146}$C: 4VP	$_{146}$C: NEM
K.QVDL$_{169}$CNYVSVNGATAHP HIENDGTVYNIGN$_{195}$CFG.K	$_{169}$C: NEM $_{195}$C: NEM	$_{169}$C: NEM $_{195}$C: NEM

Figure *S7*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RPE65 + CRALBP
RPE65 + CRALBP LRAT + RDH5
RPE65 + CRALBP LRAT^{C161S} + RDH5
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WB: RPE65
35 kDa
WB: CRALBP
25 kDa
WB: LRAT
+ AT-ROL
35 kDa
WB: RDH5

Figure *S8*

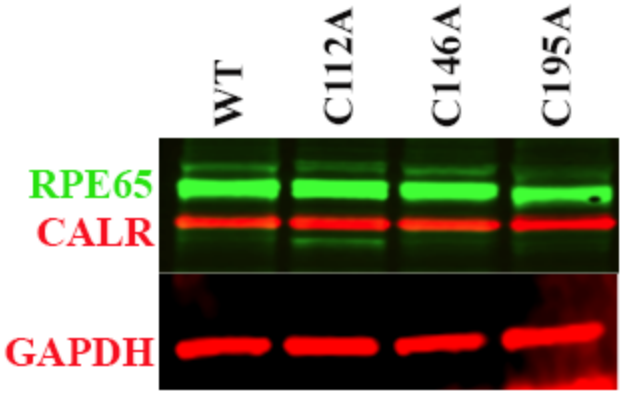
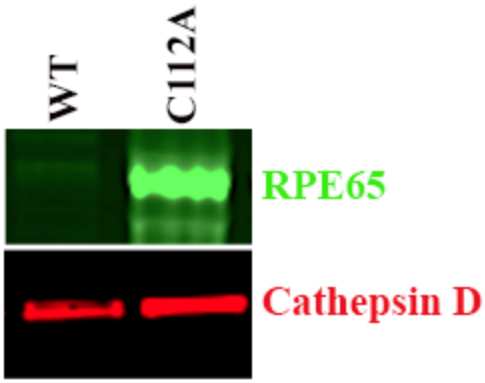


Figure S9



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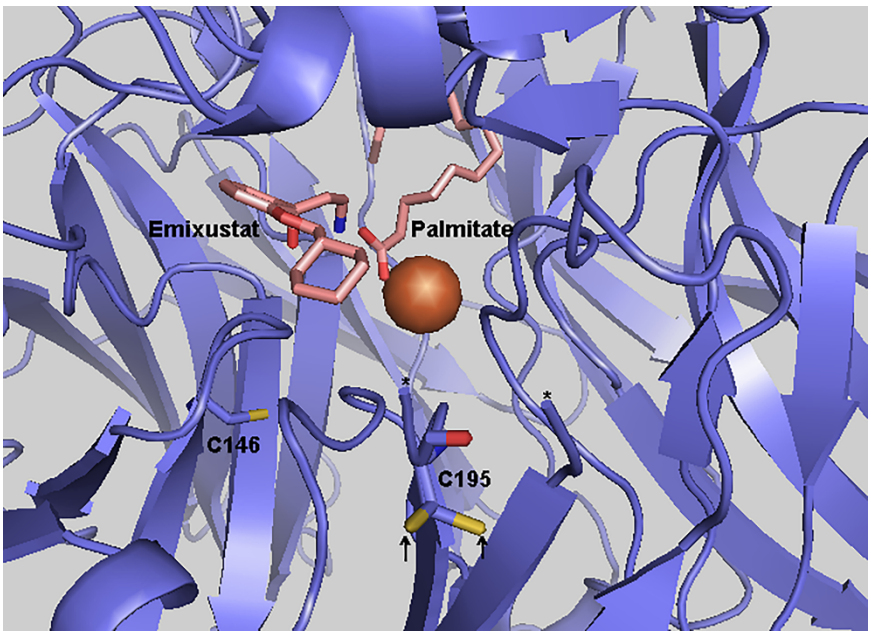

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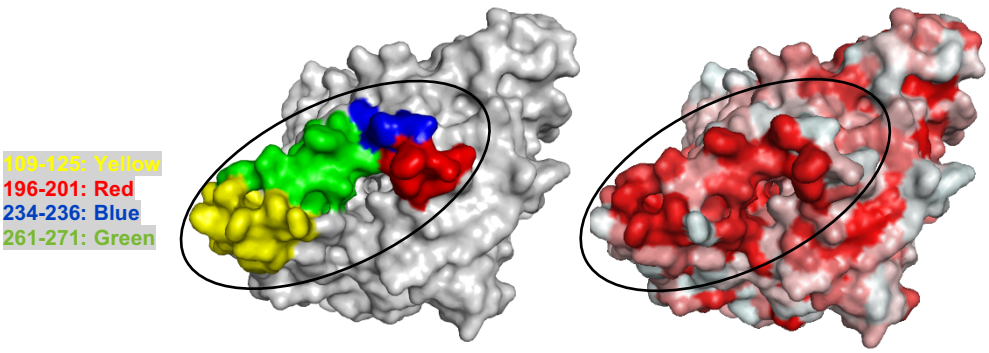

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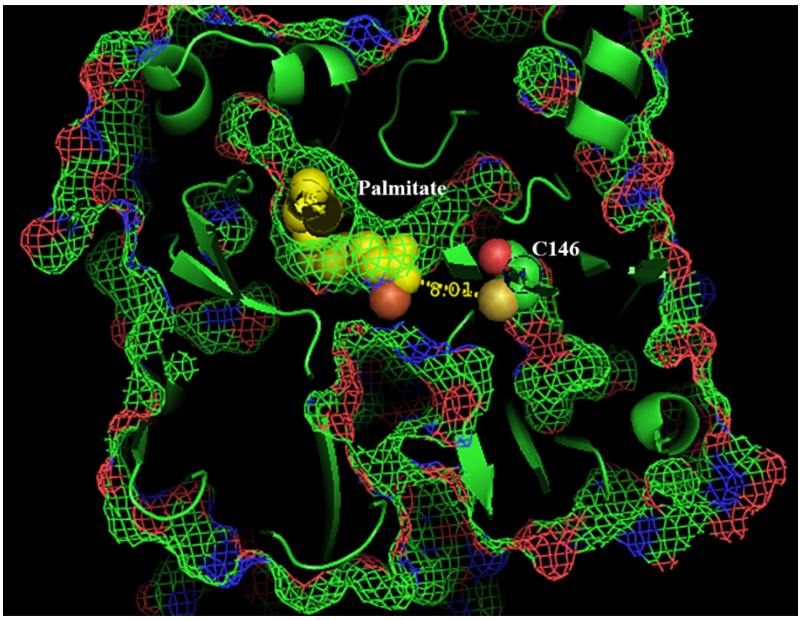

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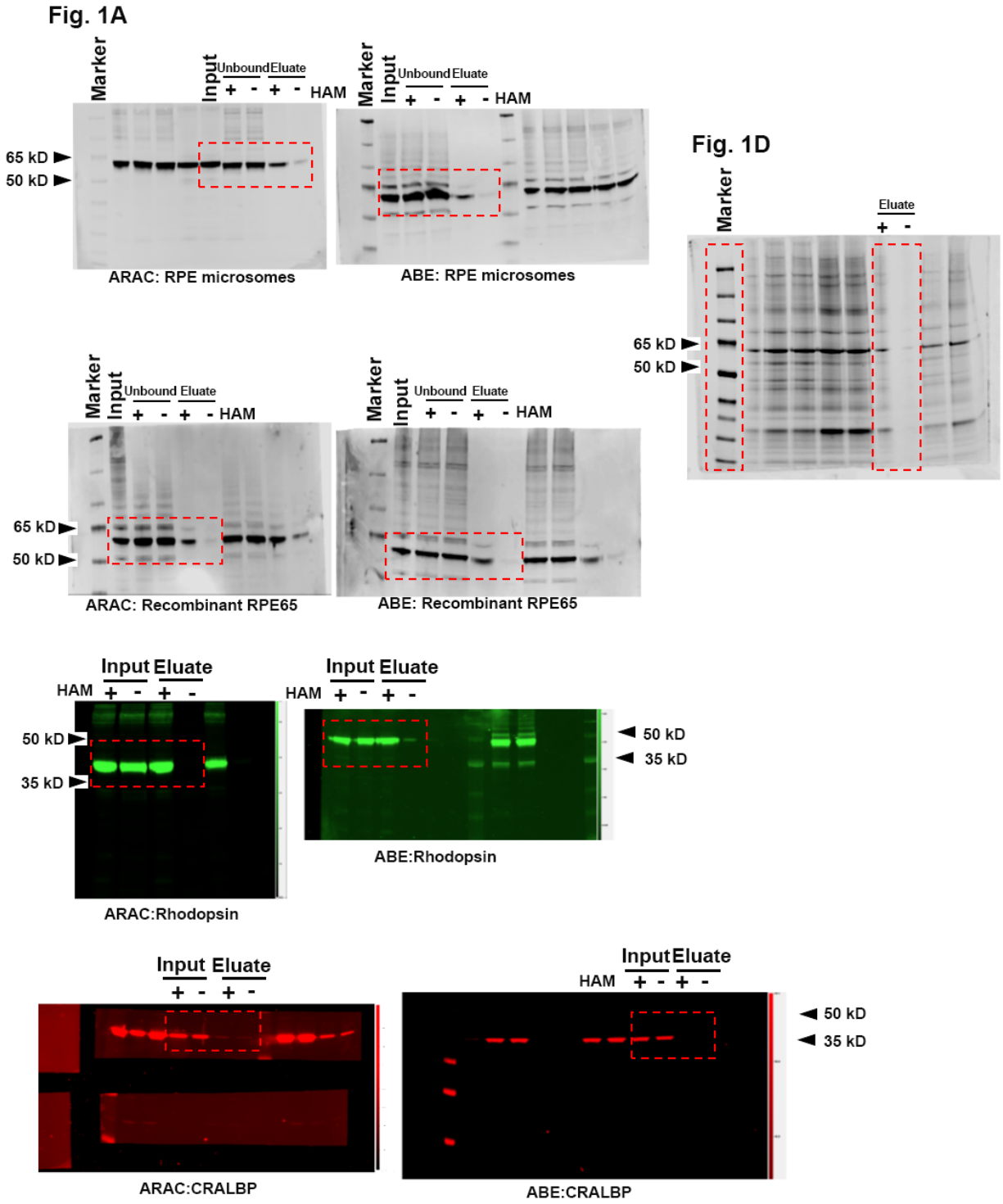
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50 kD
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ABE: RPE microsomes
Fig. 1D
Eluate
ARAC: Recombinant RPE65
ABE: Recombinant RPE65
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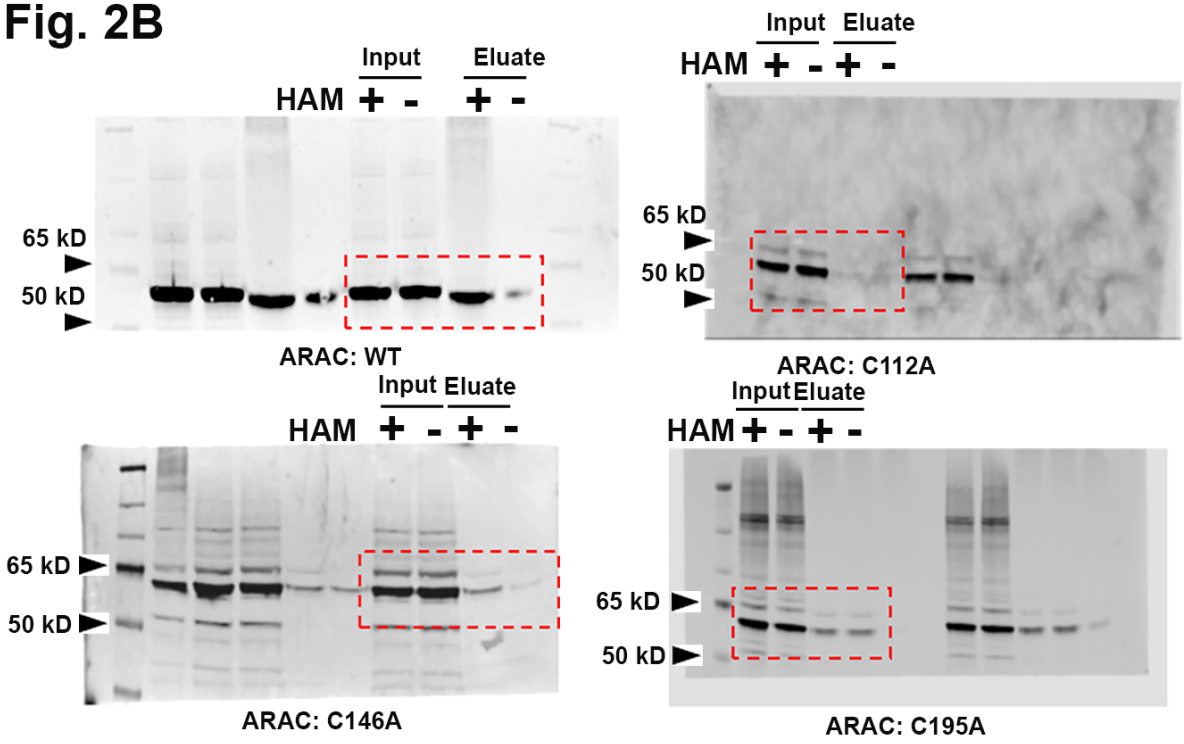
ARAC:Rhodopsin
ABE:Rhodopsin
ARAC:CRALBP
ABE:CRALBP

Fig. 2B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Input Eluate
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65 kD
50 kD
ARAC: C146A
Input Eluate
HAM + - + -
65 kD
50 kD
ARAC: C195A

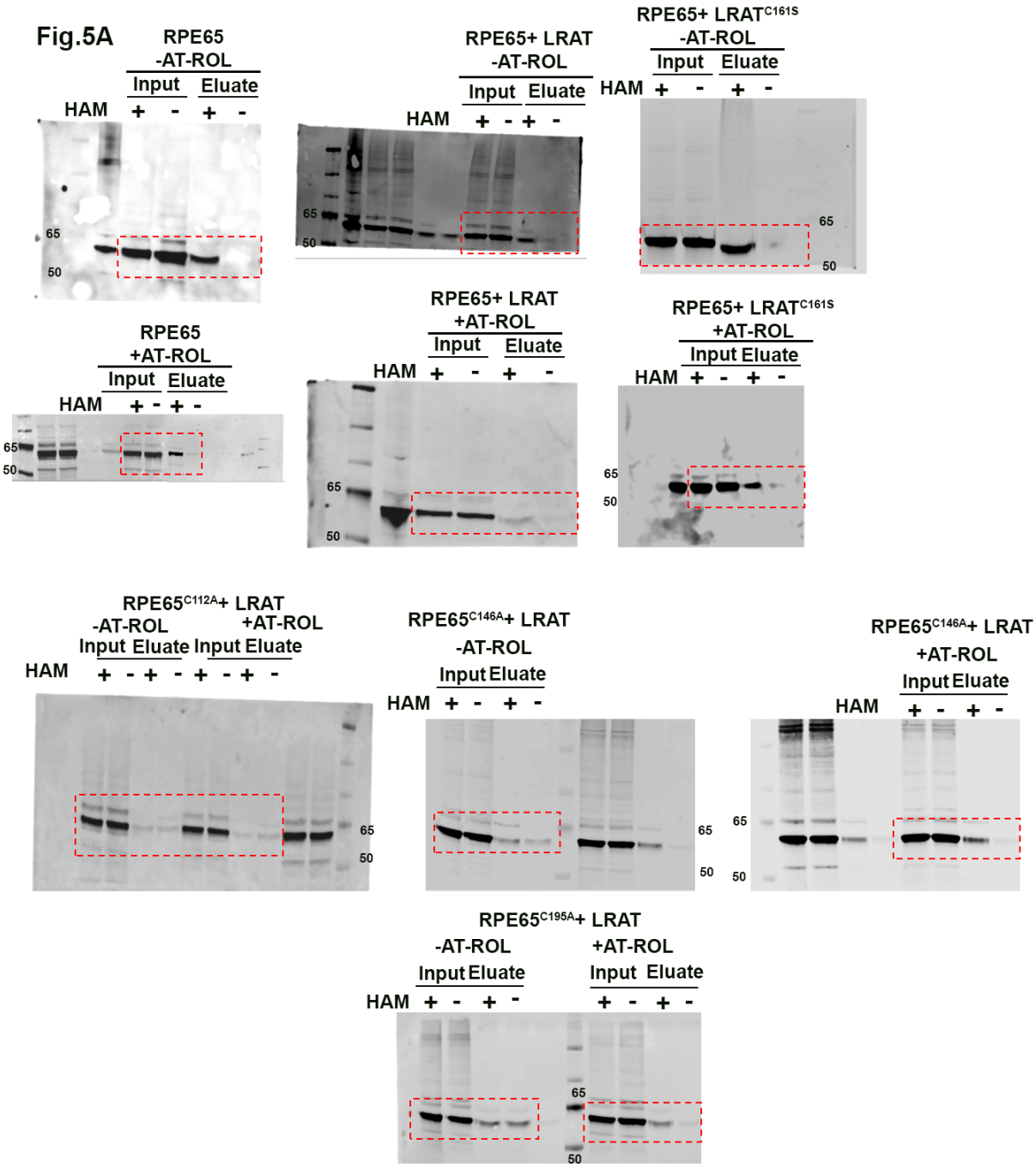
Fig.5A
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RPE65+ LRAT
-AT-ROL
Input Eluate
HAM + - + -
65
50
RPE65+ LRATC161S
-AT-ROL
Input Eluate
HAM + - + -
65
50
RPE65
+AT-ROL
Input Eluate
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RPE65+ LRATC161S
+AT-ROL
Input Eluate
HAM + - + -
65
50
RPE65C112A+ LRAT
-AT-ROL +AT-ROL
Input Eluate Input Eluate
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50
RPE65C146A+ LRAT
-AT-ROL
Input Eluate
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Input Eluate
HAM + - + -
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HAM + - + - + - + -
65
50

Fig. 6A

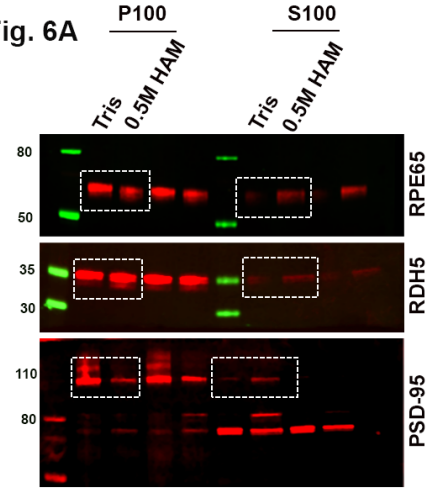
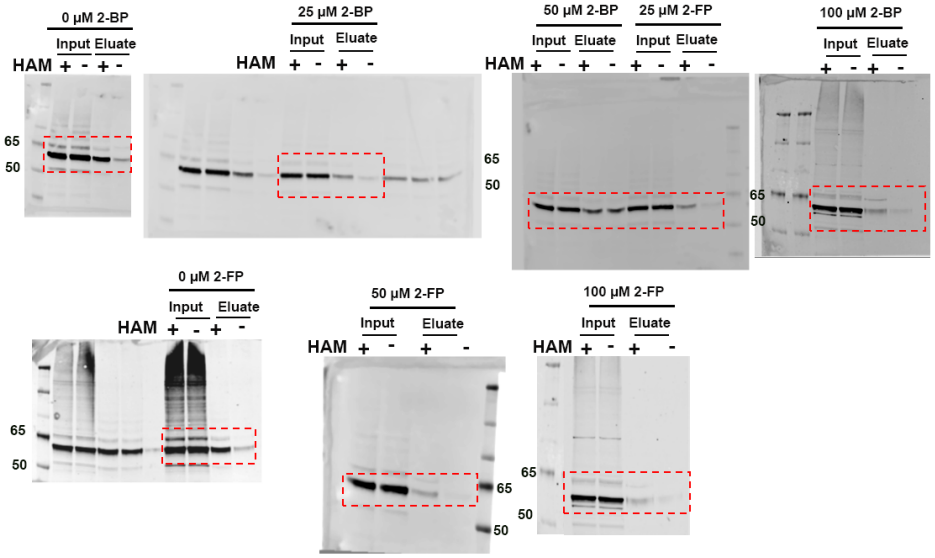


Fig. 6B



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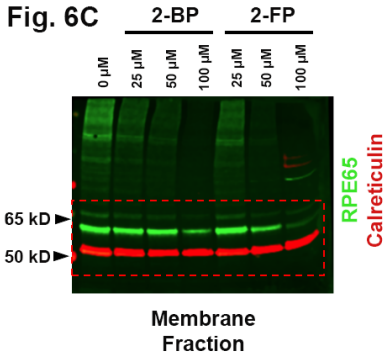

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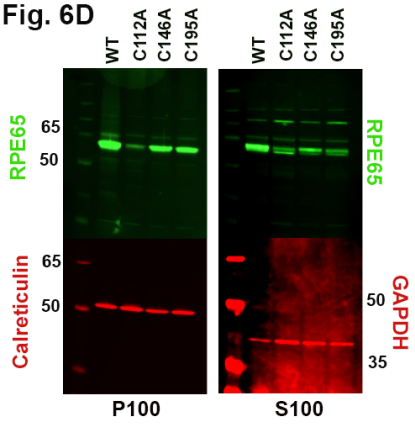
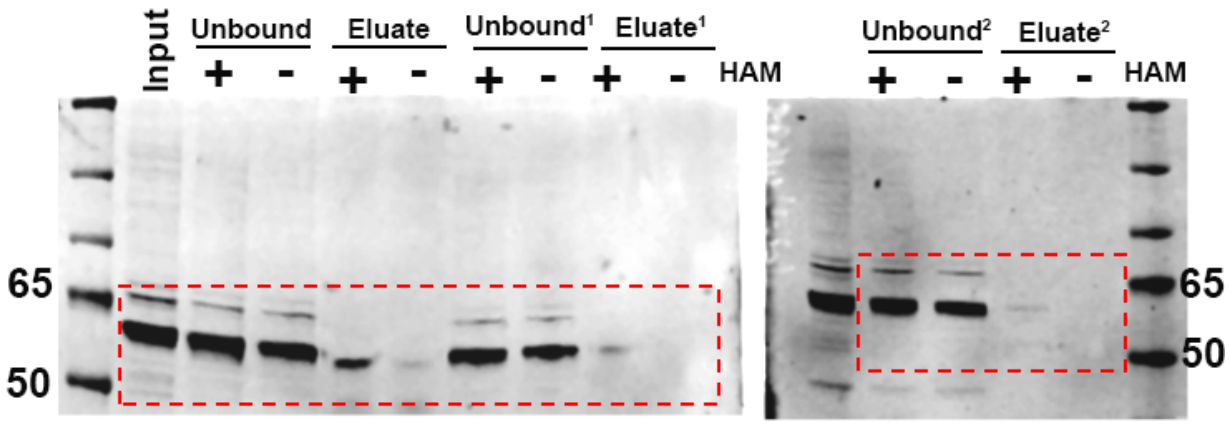


Fig. S1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Eluate
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Unbound²
Eluate²
+ - + - HAM
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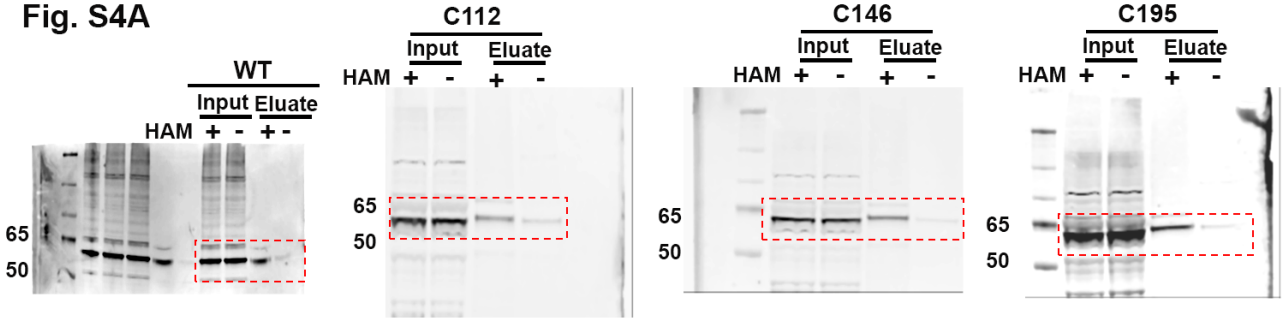
50
65
50

Fig. S4A



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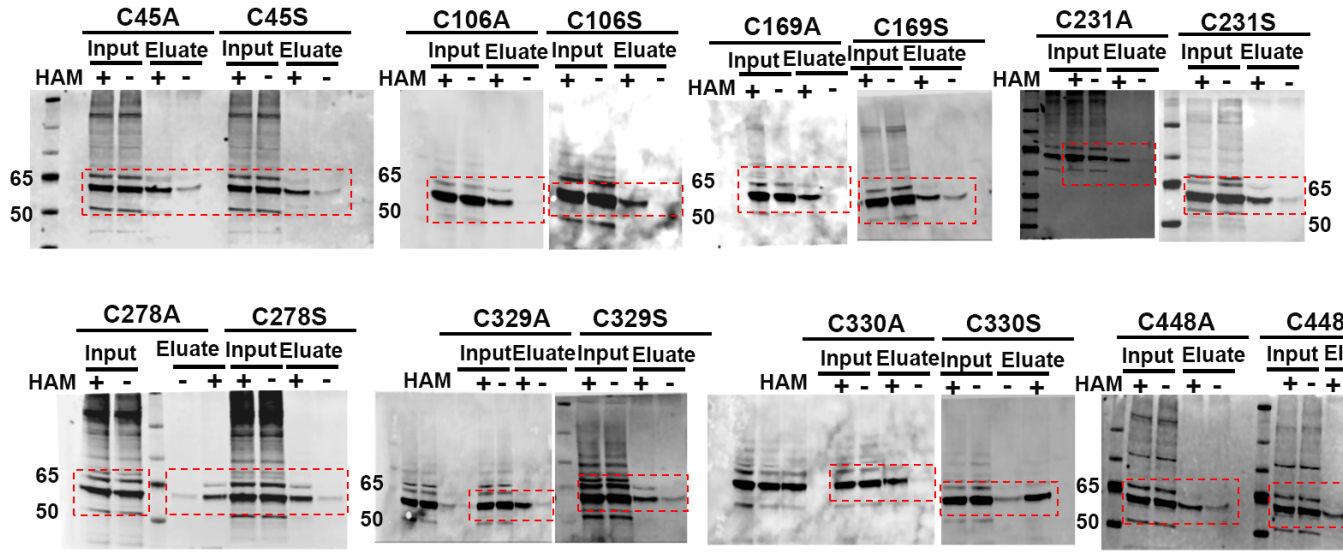

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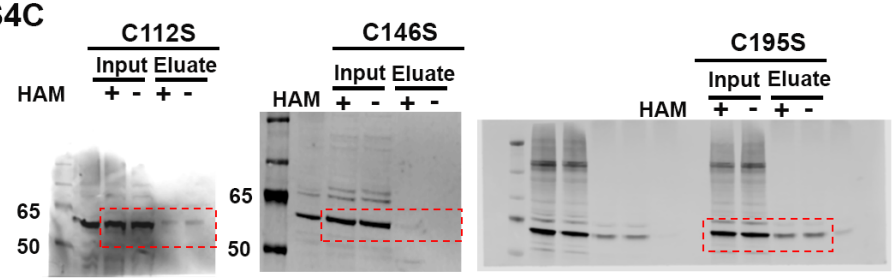

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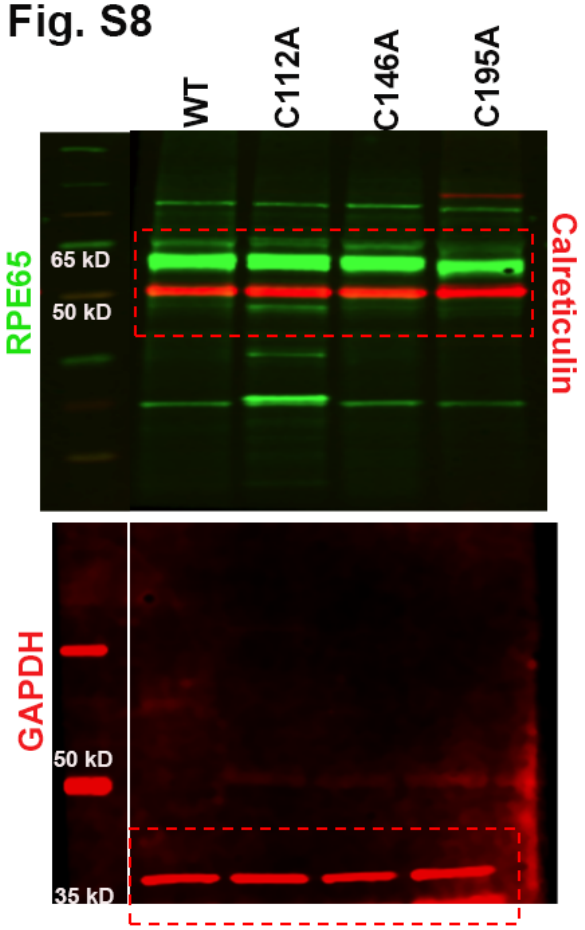
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C146A
C195A
RPE65
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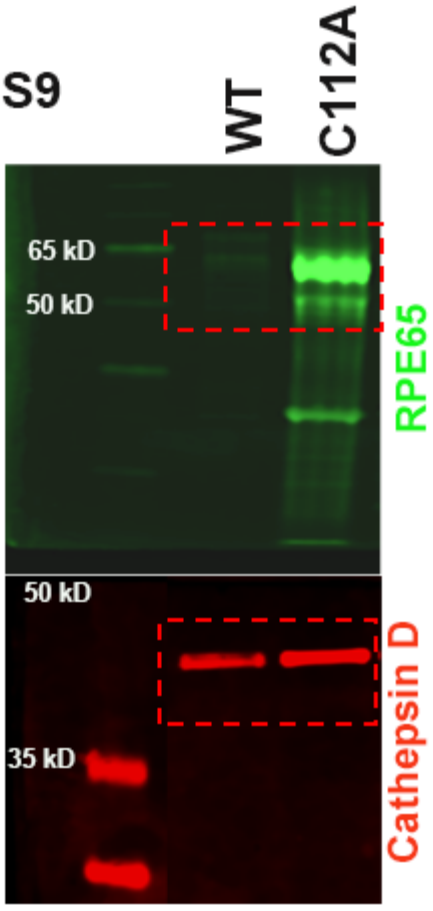
50 kD
Calreticulin
GAPDH
50 kD
35 kD

Fig.S9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C112A
65 kD
50 kD
RPE65
50 kD
35 kD
Cathepsin D