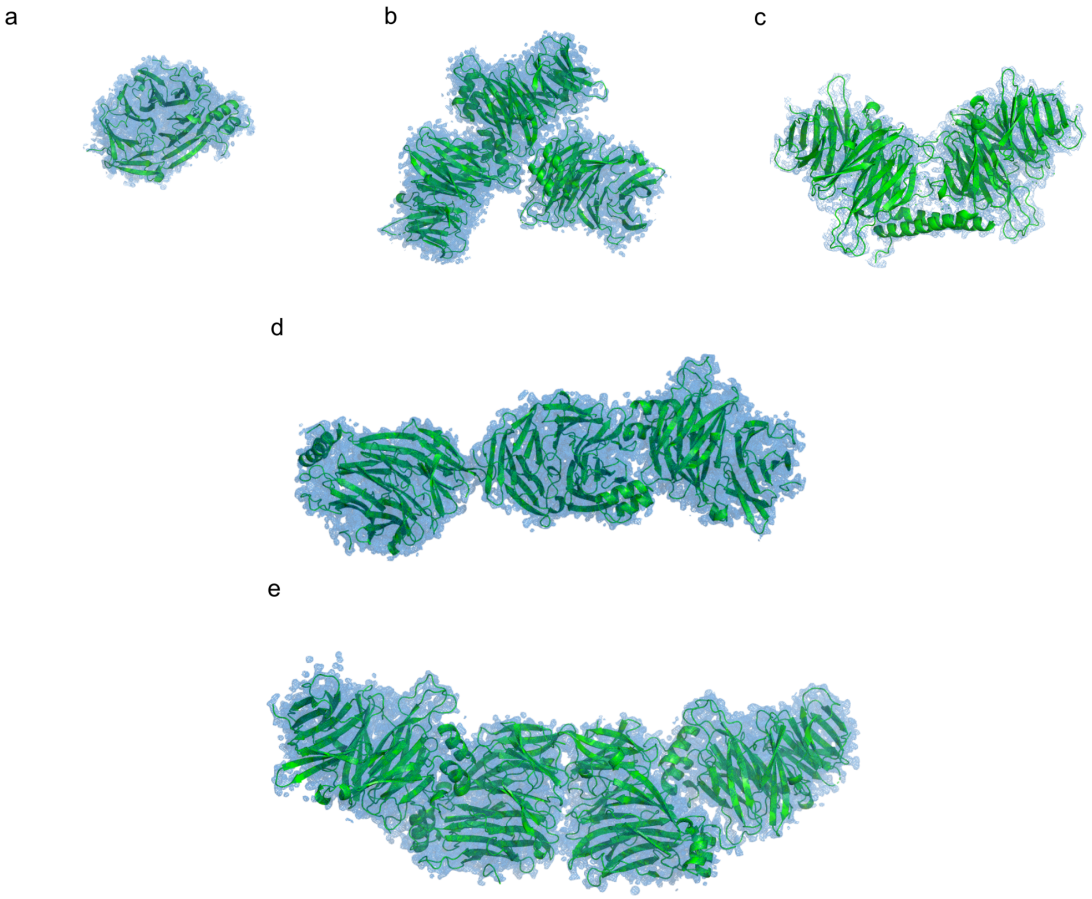
# Supplementary information for “Subtleties in Clathrin Heavy Chain Binding Boxes provide selectivity among Adaptor Proteins of Budding Yeast”

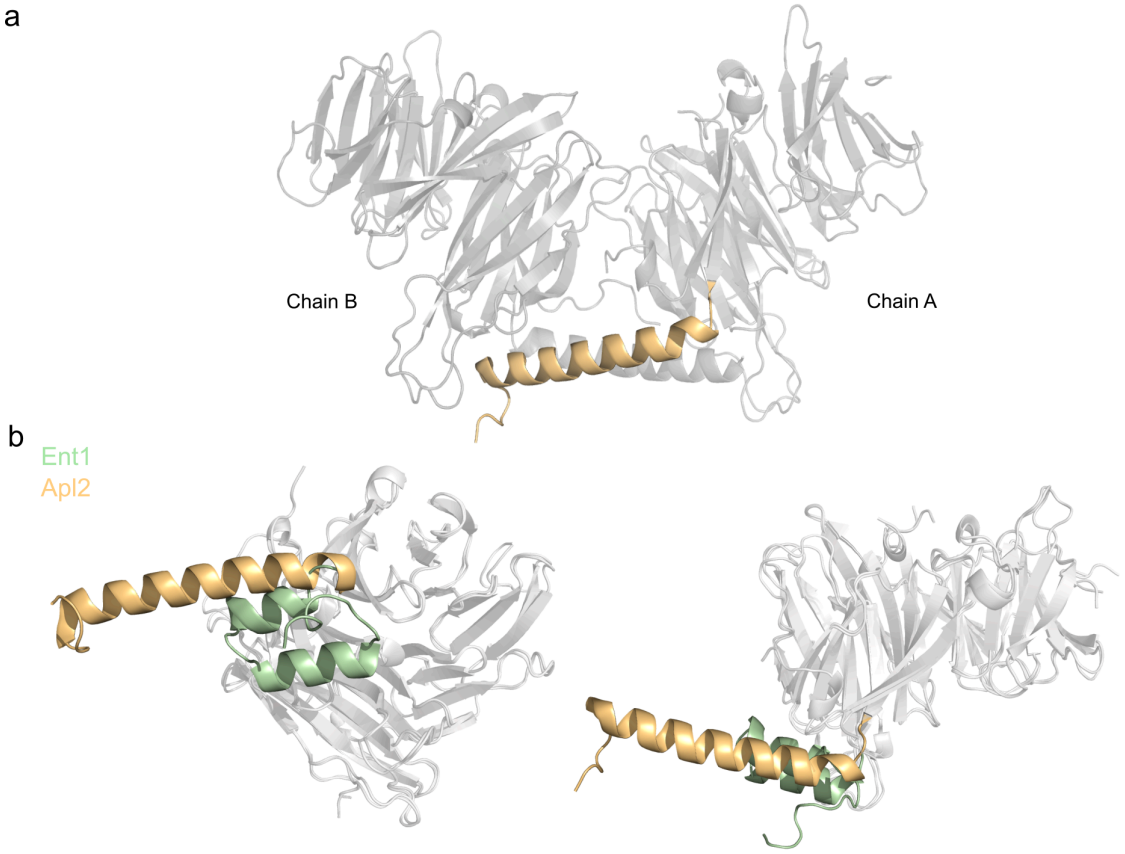
Lucas A. Defelipe[1,2], Katharina Veith[1,2], Osvaldo Burastero[1,2] ,Tatiana Kupriianova[1,2], Isabel Bento[1], Michal Skruzny[3,4§], Knut Kölbel[2,5,6], Charlotte Uetrecht[2,5,6,7], Roland Thuenauer[2,5,8] and Maria M. García-Alai[1,2*]

1. European Molecular Biology Laboratory - Hamburg Unit, Notkestraße 85, 22607 Hamburg, Germany.
2. Centre for Structural Systems Biology, Notkestraße 85, 22607 Hamburg, Germany.
3. Cell Biology and Biophysics Unit, European Molecular Biology Laboratory, Heidelberg, Germany.
4. Department of Systems and Synthetic Microbiology, Max Planck Institute for Terrestrial Microbiology, 35043 Marburg, Germany.
5. Leibniz Institute of Virology (LIV), Martinistraße 52, 20251 Hamburg, Germany.
6. Deutsches Elektronen Synchrotron - DESY, Notkestraße 85, 22607 Hamburg, Germany.
7. Institute of Chemistry and Metabolomics, University of Lübeck, Ratzeburger Allee 160, 23562 Lübeck, Germany.
8. Technology Platform Light Microscopy (TPLM), Universität Hamburg (UHH), Notkestraße 85, 22607 Hamburg, Germany

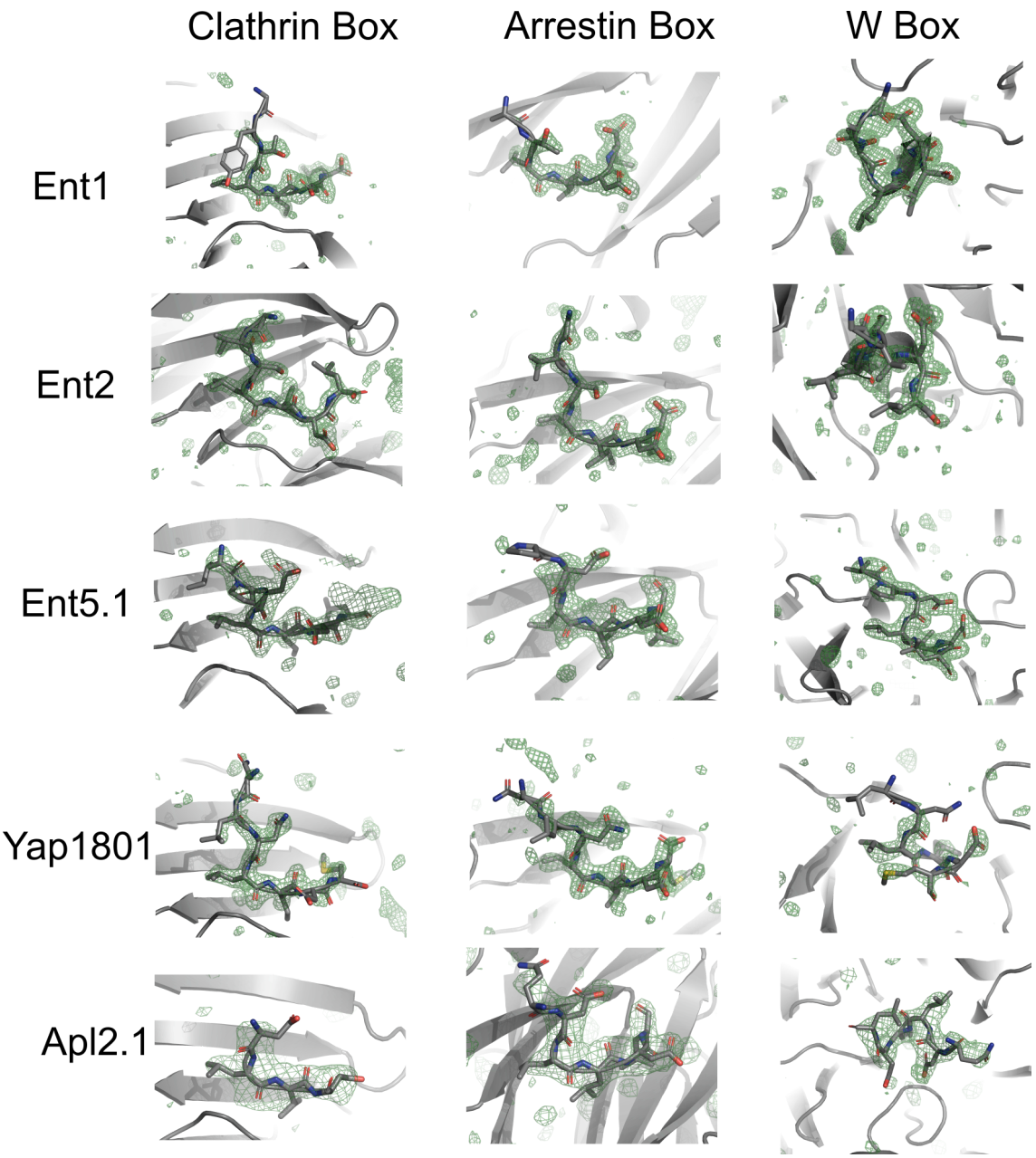
* To which correspondence should be addressed: maria.garcia@embl-hamburg.de



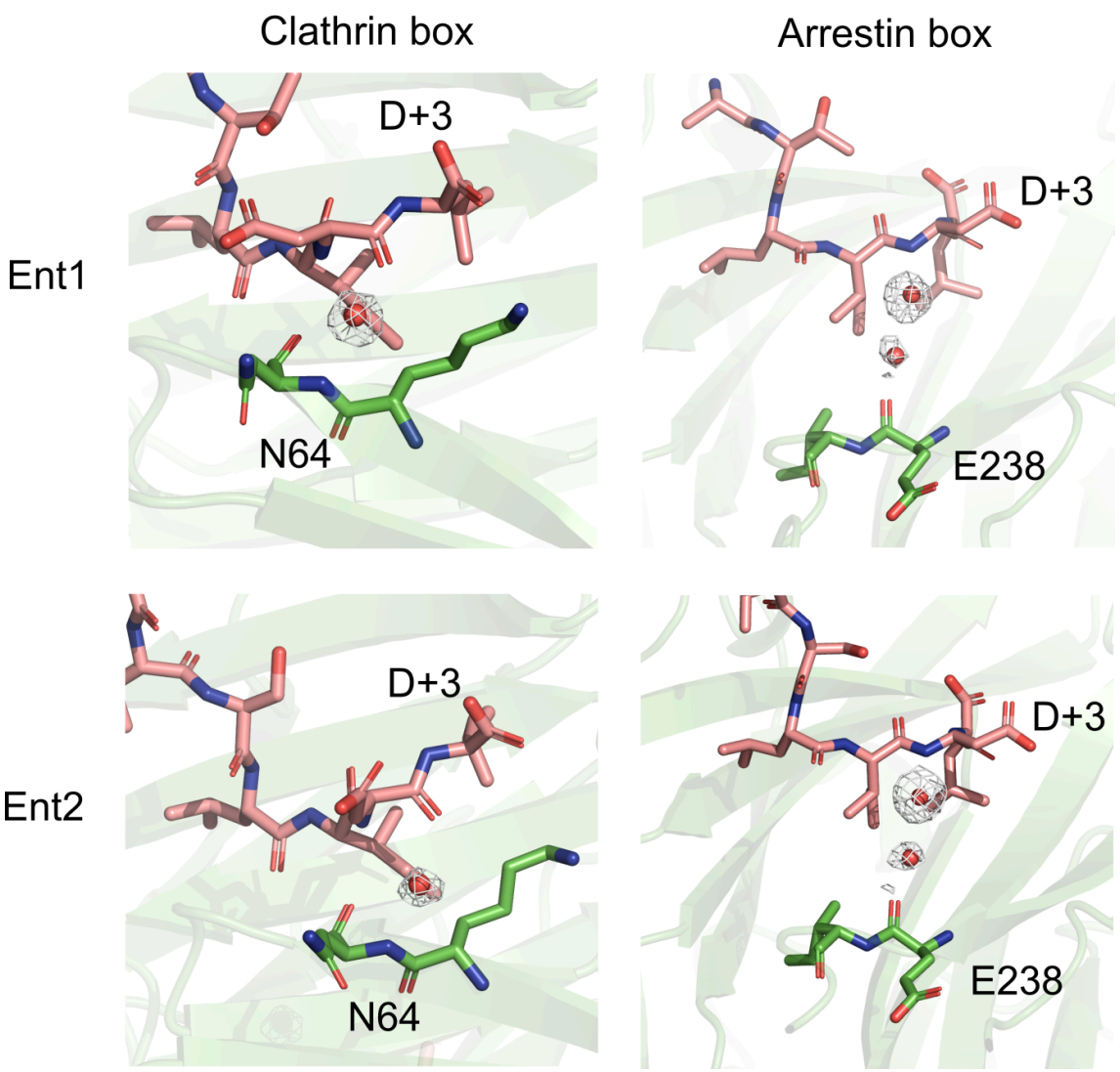

**Supplementary Figure 1. Density map ($2F_o$-$F_c$) of CHC in complex with different peptides.** a) Ent1, b) Ent 2, c) APL2, d) Ent5 and e) YAP1801. Density is shown as a blue mesh at a contour level of 1σ (standard deviation) above the mean electron density.



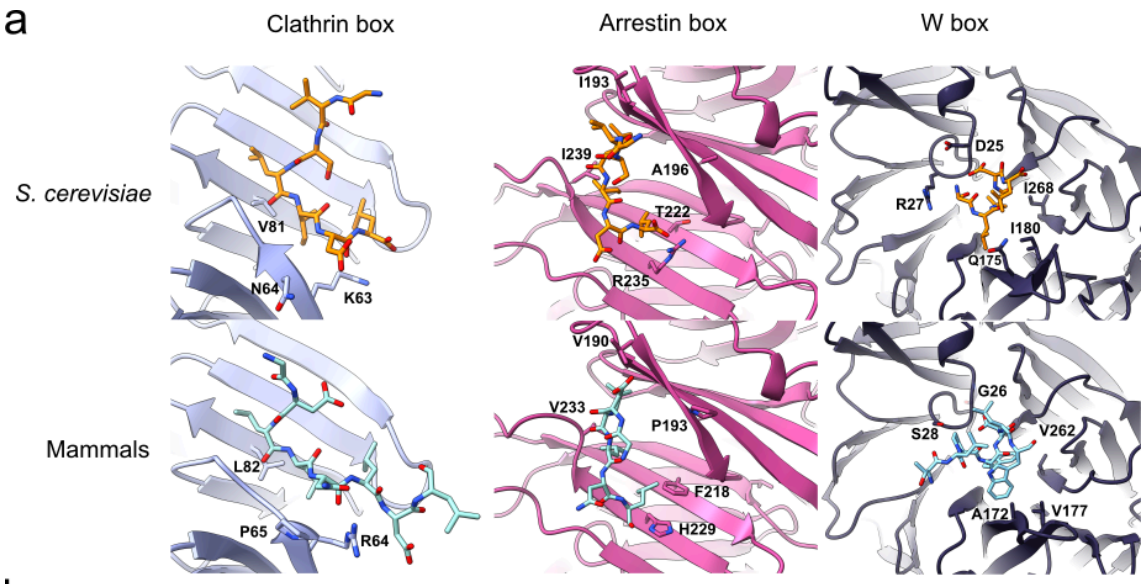

**Supplementary Figure 2.** a) Crystallographic dimer of NTD complexed with the Apl2.1 peptide showing an alternative conformation of the C-terminal helix. b) Comparison of the NTD C-terminal helix for the Ent1 (green) and Apl2 (orange) bound structures.




**Supplementary Figure 3. Omit map of each ScCHC-NTD/Peptide complex.** The electron density map ($F_o$-$F_c$) surrounding these peptides is shown as a green mesh at a contour level of 3σ (standard deviation) above the mean electron density.




**Supplementary Figure 4.** Visualization of structural waters within NTD and Ent1 or Ent2 peptides. The electron density map ($2F_o$-$F_c$) surrounding these water molecules is depicted as a light blue mesh at a contour level of 1σ (standard deviation) above the mean electron density.




b

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ScChc NTD    1 -MSDLPIEFTELVDLMSLGISPQFLDFRSTTFESDHFVTVRETKDGTNSVAIVDLAKGNEVT  61
HsChc NTD    1 MAQILPIRFQEHLQLQNLGINPANIGFSTLTMESDKFICIREKVGEQAQVVIIDMNDPSNPI  62

ScChc NTD   62 RKNMGGDSAIMHPSQMVISVRANGTIVQIFNLETKSKLKSFTLDEPVIFWRWLSETTLGFVT 123
HsChc NTD   63 RRPISADSAIMNPASKVIALKA-GKTLQIFNIEMKSKMKAHTMTDDVTFWKWISLNTVALVT 123

ScChc NTD  124 ARSILTSNVFDGNVNAKPQLLTLRHANLNNTQIINFVANKNLDWFAVVGILQENGRIAGRIQ 185
HsChc NTD  124 DNAVYHWS---MEGESQPVKMFDRHSSLAGCQIINYRTDAKQKWLLLTGISAQQNRVVGAMQ 182

ScChc NTD  186 LFSKQRNISQAIDGHVAIFTNILLEGNGSTPVQVFVTGNRNATTGAGELRIIEIDHDASLPS 247
HsChc NTD  183 LYSVDRKVSQPIEGHAASFAQFKMEGNAEESTLFCF-AV--RGQAGGKLHIIEVGTPPTGNQ 241

ScChc NTD  248 QYQKETTDIFFPPDATNDFPIAVQVSEKYGIIYLLTKYGFIHLYELETGTNLFVNRITAESV 309
HsChc NTD  242 PFPKKAVDVFFPPEAQNDFPVAMQISEKHDVVFLITKYGYIHLYDLETGTCIYMNRISGETI 303

ScChc NTD  310 FTAAPYNHENGIACINKKGQVLAVEISTSQIVPYILNKLSNVALALIVATRGGLPGADDL  369
HsChc NTD  304 FVTAPHEATAGIIGVNRKGQVLSVCVEEENIIPYITNVLQNPDLALRMAVRNNLAGAEEL  363
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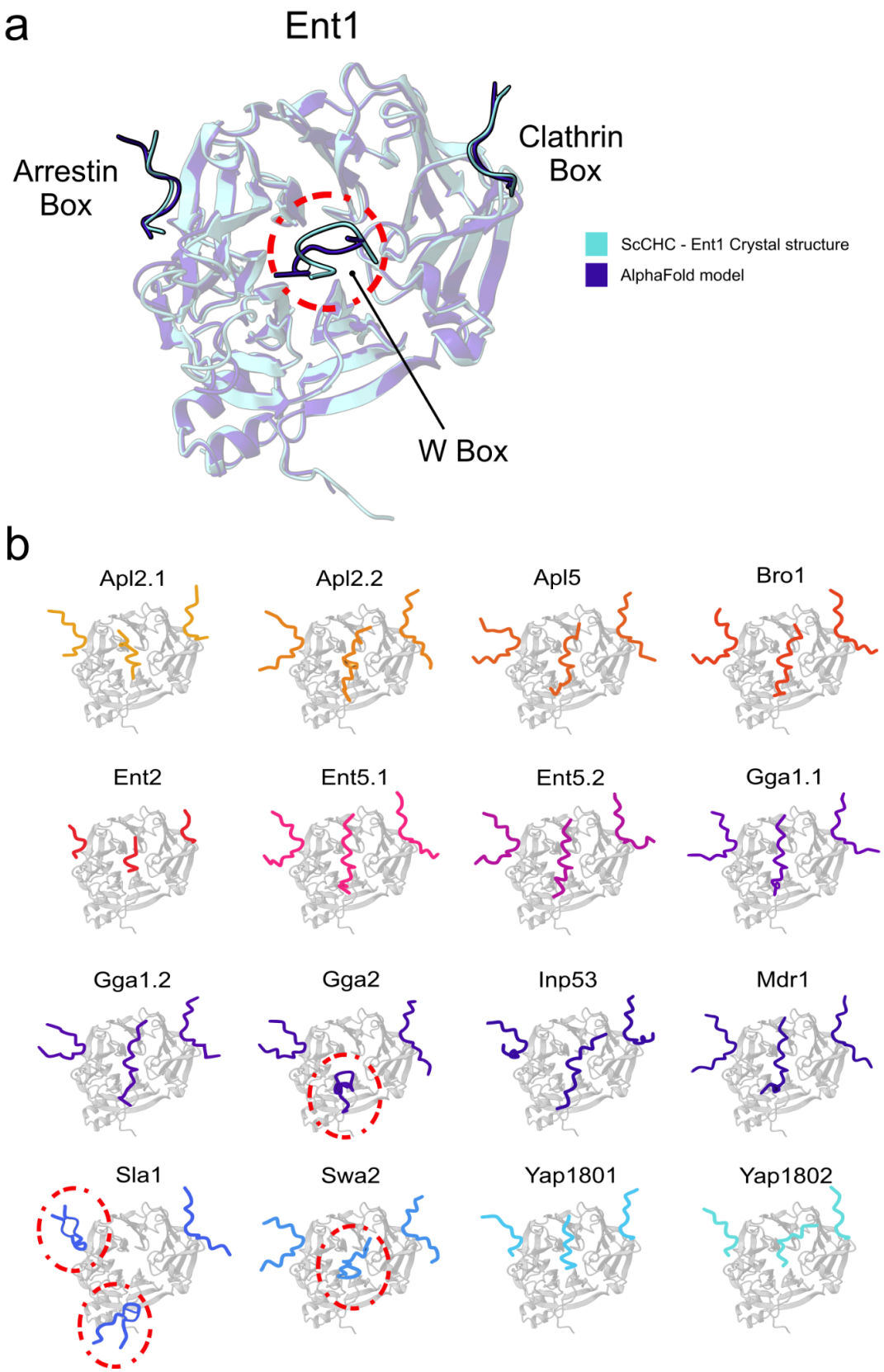
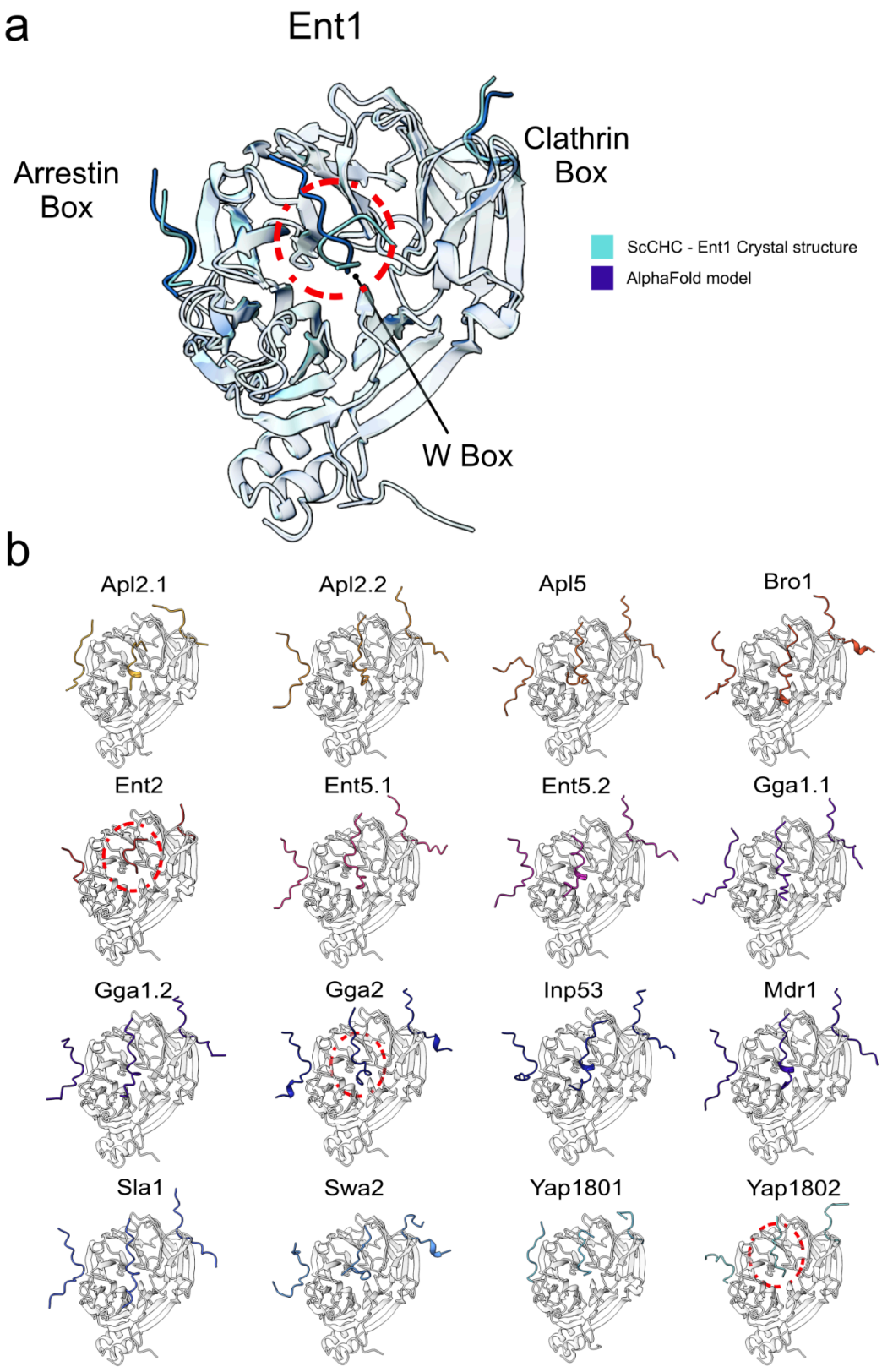
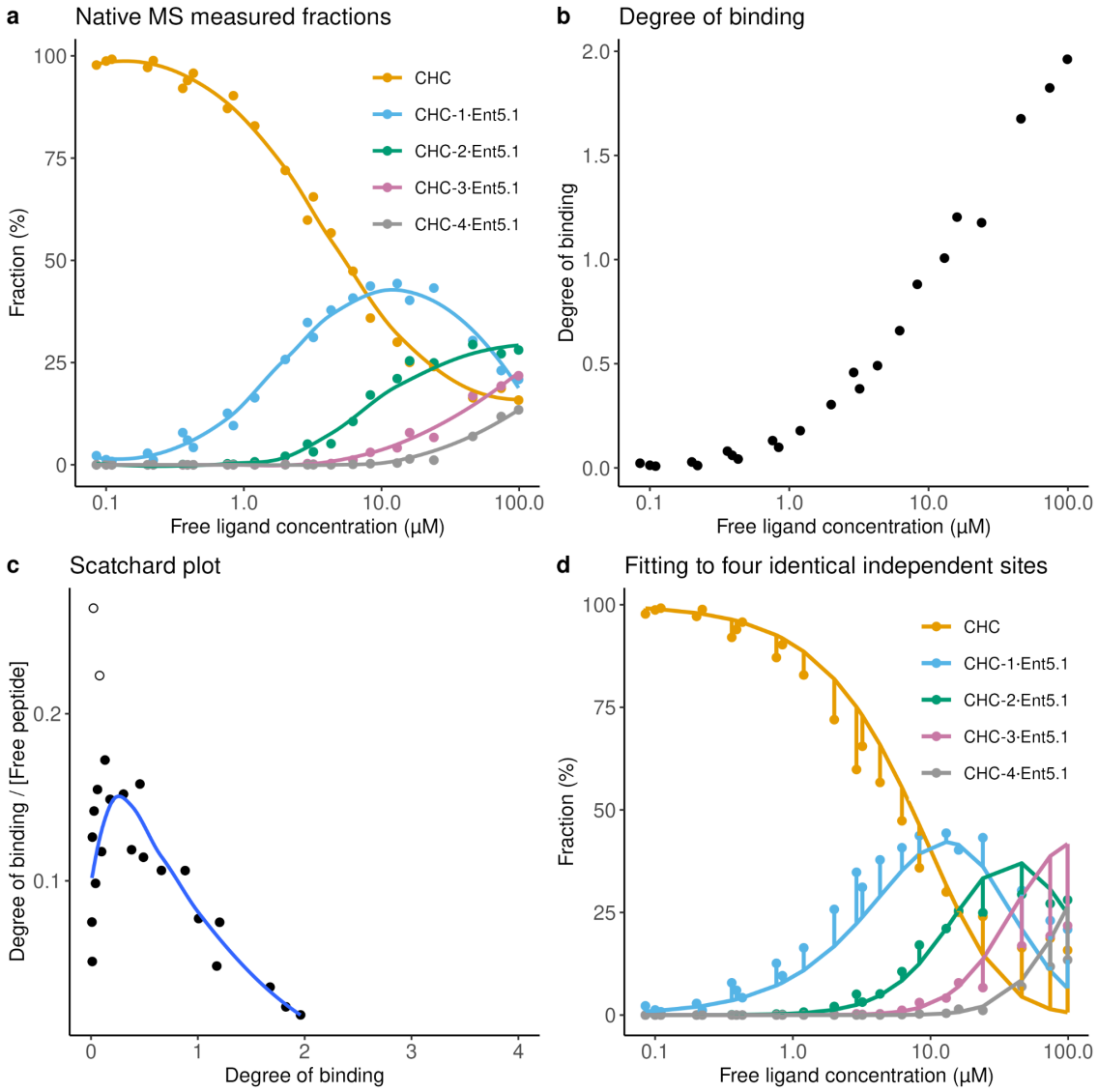
**Supplementary Figure 5. Structural comparison of *S. cerevisiae* and mammalian Chc boxes.** a) Crystal structures of *S.cerevisiae* and mammalian (*Bos taurus* and human) NTD: Clathrin box (violet), Arrestin box (pink) and W-box (gray). *S. cerevisiae*: Cartoon representation of the three boxes bound to the Ent2 peptide. Mammalian: *Bos taurus* Clathrin and Arrestin boxes bound to human AP-2 Beta peptide (PDB 5M5R) and human W-box bound to human Amphiphisin peptide (PDB 1UTC). b) Sequence alignment between *S. cerevisiae* and *Bos taurus* NTD.



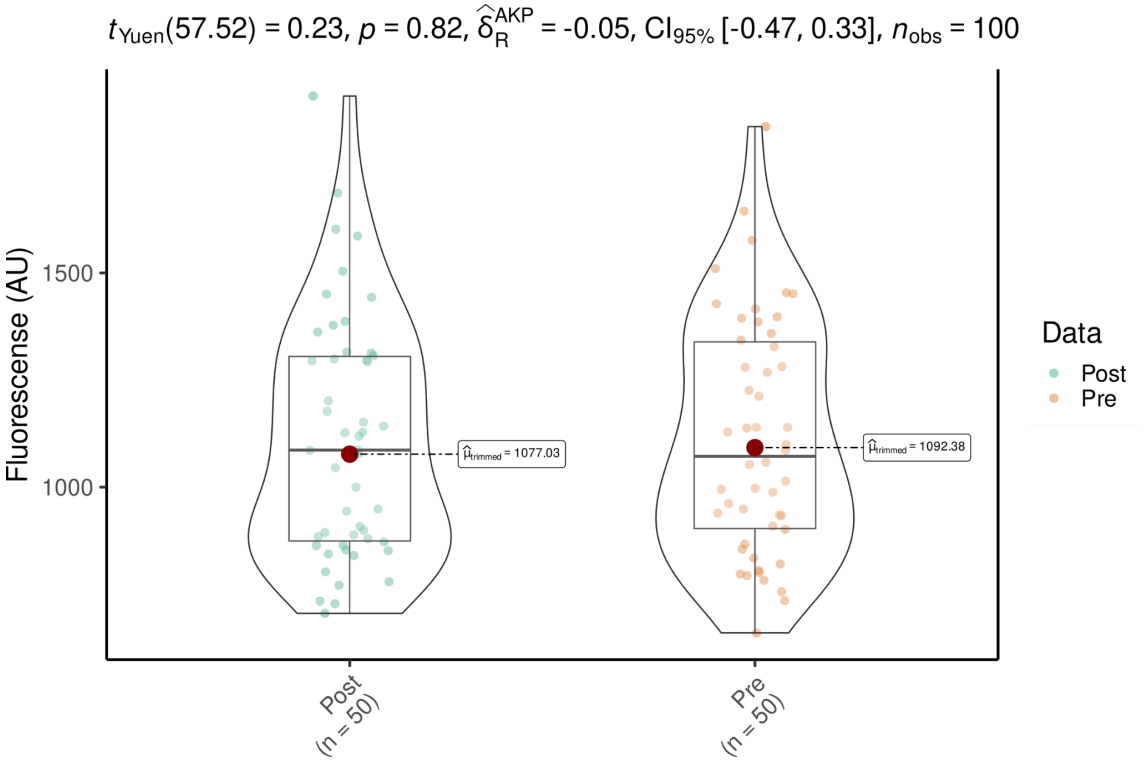

**Supplementary Figure 6. AlphaFold2 predictions for the NTD CBM complexes.** a) Superposition of the crystal structure of NTD bound to Ent1 peptide (cyan) and the AF2 best model (blue). b) AF2 best predictions for NTD in complex with various CBM-containing peptides. Dashed red circles highlight wrong predictions in all five models.



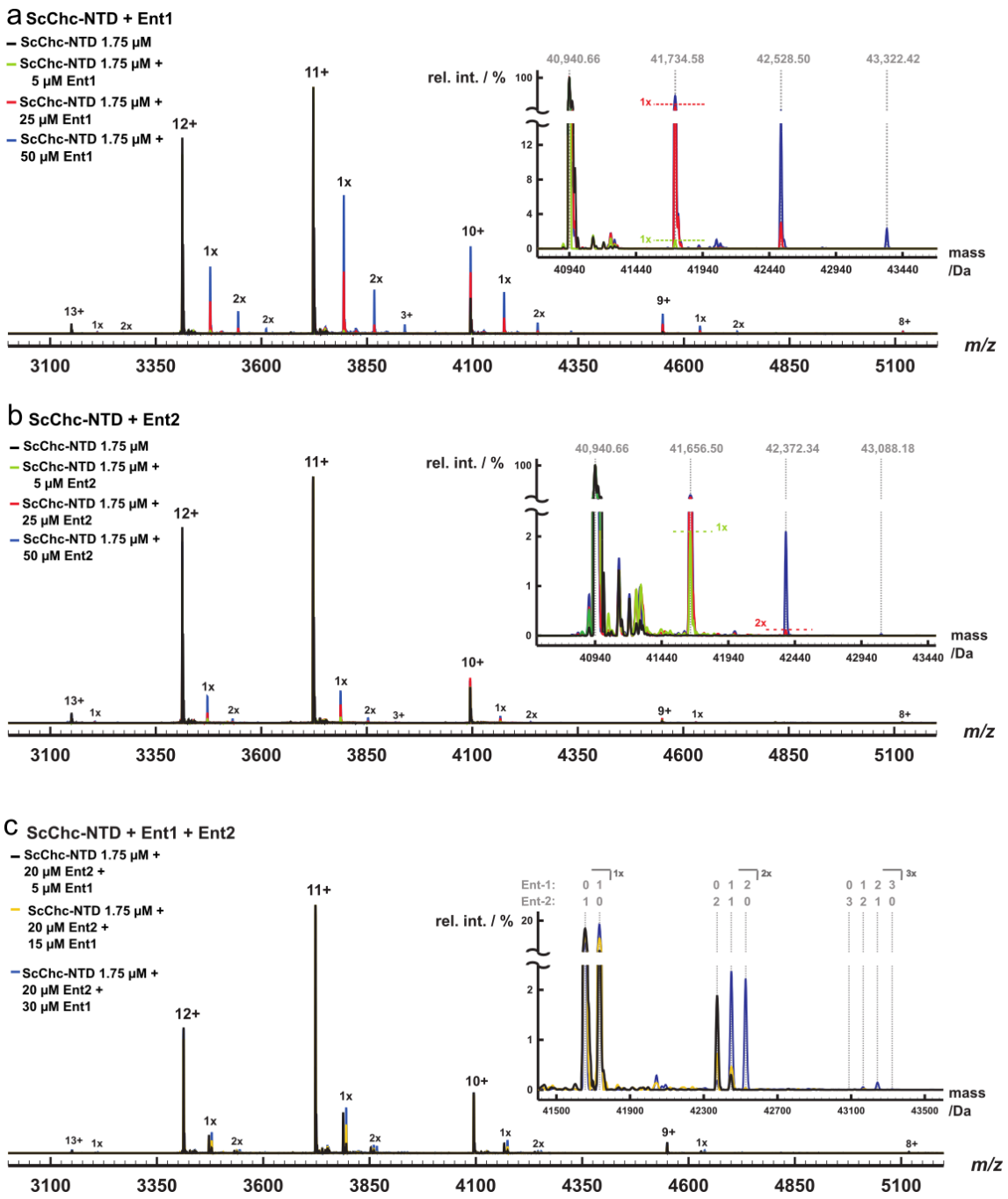

**Supplementary Figure 7. AlphaFold3 predictions for the NTD CBM complexes.** a) Superposition of the crystal structure of NTD bound to Ent1 peptide (cyan) and the AF3 best model (blue). b) AF3 best predictions for NTD in complex with various CBM-containing peptides. Dashed red circles highlight wrong predictions in all five models.



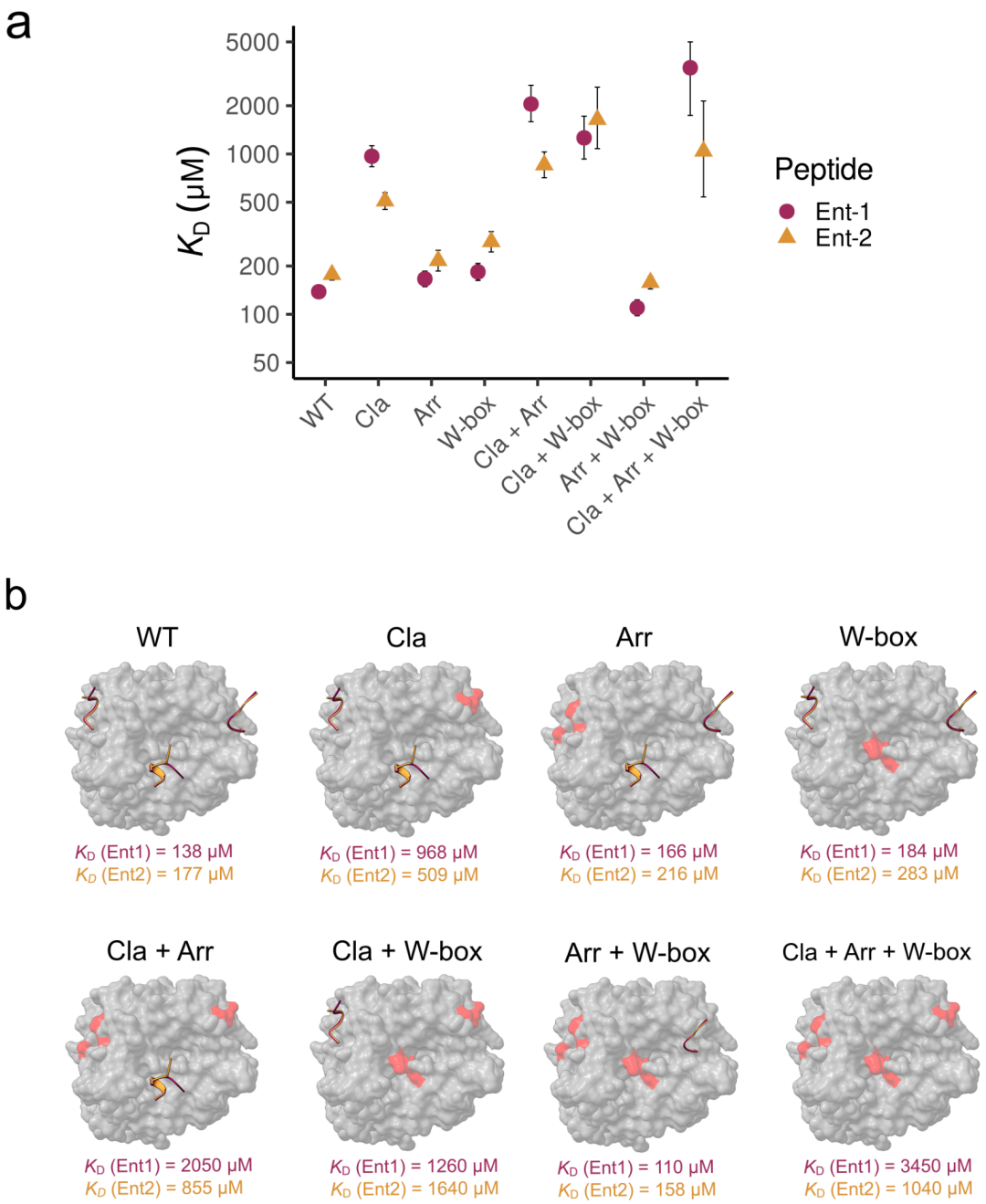

**Supplementary Figure 8. Native mass spectrometry (native MS) analysis of the interaction between Clathrin heavy chain (CHC) and the Ent5.1 peptide**. See Section Supplementary Methods. a) Points: measured fraction versus free ligand concentration (μM). Lines: Smoothed lines based on the locally estimated scatterplot smoothing method (LOESS). b) Degree of binding versus free peptide (ligand) concentration (μM). c) Scatchard plot (free peptide concentration in μM). Blue line: LOESS-based smoothing without the two empty dots. d) Points: Measured fraction versus free ligand concentration (μM). Lines: Estimation based on a microscopic $K_D$ of 39.1 μM assuming four independent sites with the same binding affinity. Vertical lines: Difference between the data and the predicted fractions. The microscopic $K_D$ was obtained by performing a global non-linear square fitting of the five different measured fractions.



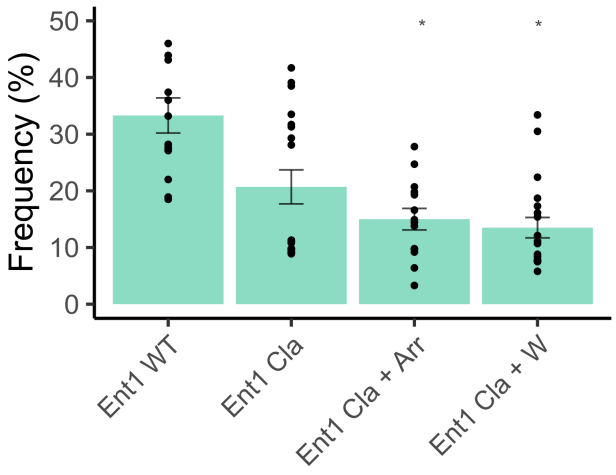

**Supplementary Figure 9. FRET Specificity.** Fluorescence intensity of Ent1-mNeonGreen-tagged cells excited with the 488 nm laser was measured before (Pre) and after photobleaching (Post) in the mScarlet channel (561 nm and 640 nm lasers). Data were analyzed using Yuen's trimmed means test[1], and no significant differences were observed, demonstrating that the photobleaching process does not affect donor fluorescence.




**Supplementary Figure 10. Native mass spectrometry spectra.** a) Native MS of free ScCHC-NTD (black) and in presence of Ent1 at 5 µM (green), 25 µM (red) and 50 µM (blue). Right, inset of the 11+ ions showing single, double and triple bound ScCHC-NTD with Ent1. b) Native MS of free ScCHC-NTD (black) and in presence of Ent2 at 5 µM (green), 25 µM (red) and 50 µM (blue). Right, inset of the 11+ ions showing single, double and triple bound ScCHC-NTD with Ent2. c) Native MS of ScCHC-NTD in presence of both Ent1 and Ent2. Right, inset of the 11+ ions showing single, double and triple bound ScCHC-NTD with Ent1 and Ent2.




**Supplementary Figure 11. Binding Affinities of Ent1 and Ent2 for WT Chc-NTD and Mutants.** a) Determination of apparent dissociation constants ($K_D^{App}$) by nano Differential Scanning Fluorimetry (nDSF) based on $T_m$ shifts for Ent1 and Ent2 peptides with WT NTD and mutants at Clathrin (K63E, I87D, Q89A and K98E), Arrestin (Q195A, I197T and K251E), and W-box (F26A and Q155A) binding sites. Data is shown as mean asymmetric IC95 as implemented in FoldAffinity (spc.embl-hamburg.de). b) Schematic representation of these $K_D^{App}$ values mapped onto the NTD structure (surface in gray). The presence of a peptide (Ent1 in red and Ent2 in yellow) indicates assumed peptide binding. Mutated residues are shown in red.




**Supplementary Figure 12. Abp presence in Ent1-mNeonGreen endocytic sites of different CHC mutants.** * indicates a p-value of $8.1e^{-5}$ , denoting statistical significance in a two-tailed Mann–Whitney U-test corrected for multiple comparisons (WT n=15, Cla n=18, Cla + Arr n=14, Cla + W n=19).

**Supplementary Table 1. Proteins from *Saccharomyces cerevisiae* exhibiting the Clathrin Binding Motifs (CBMs).** This table enumerates proteins identified in *Saccharomyces cerevisiae* that contain the designated motif, with uppercase letters delineating the motif itself and lowercase letters representing the sequences flanking the motif within the protein sequence.

| UniProt ID | Protein Name | Gene Name | Sequence | Seq Start | Seq Stop | Type of motif |
|---|---|---|---|---|---|---|
| P53309 | Clathrin coat assembly protein AP180B | YAP1802 | enpNLIDI | 564 | 568 | Canonical |
| P38856 | Clathrin coat assembly protein AP180A | YAP1801 | nnlNLIDM | 633 | 637 | Canonical |
| Q08562 | ATP-dependent helicase ULS1 | ULS1 | eviSLLDLpni | 411 | 415 | Canonical |
| Q01476 | Ubiquitin carboxyl-terminal hydrolase 2 | UBP2 | kdtSLIDLeme | 876 | 880 | Canonical |
| Q06677 | Auxilin-like clathrin uncoating factor SWA2 | SWA2 | iedSLLDFseg | 333 | 337 | Canonical |
| Q08204 | Structural maintenance of chromosomes protein 5 | SMC5 | mtSLIDLgry | 3 | 7 | Canonical |
| P32790 | Actin cytoskeleton-regulatory complex protein SLA1 | SLA1 | smqDLLDLqpl | 802 | 806 | Canonical |

| | | | | | | |
|---|---|---|---|---|---|---|
| P38814 | Protein SBE22 | SBE22 | snaSLLDFnem | 406 | 410 | Canonical |
| P43565 | Serine/threonine-protein kinase RIM15 | RIM15 | lsfSLLDIsrs | 1066 | 1070 | Canonical |
| P53258 | GTPase-activating protein GYP2 | MDR1 | eevNLIDLsdd | 759 | 763 | Canonical |
| Q08001 | Membrane-anchored lipid-binding protein LAM6 | LAM6 | ahaTLLDIpak | 674 | 678 | Canonical |
| Q04746 | Nuclear fusion protein KAR5 | KAR5 | ktgSLIDF | 500 | 504 | Canonical |
| Q12271 | Polyphosphatidylinositol phosphatase INP53 | INP53 | sesSLLDIdpi | 914 | 918 | Canonical |
| P39996 | Glutathione transferase 3 | GTT3 | tnfNLLDFstd | 140 | 144 | Canonical |
| P38817 | ADP-ribosylation factor-binding protein GGA2 | GGA2 | neiNLIDFndl | 351 | 355 | Canonical |
| Q06336 | ADP-ribosylation factor-binding protein GGA1 | GGA1 | kelNLIDFddd | 336 | 340 | Canonical |
| Q06336 | ADP-ribosylation factor-binding protein GGA1 | GGA1 | sgiDLLDFdsq | 400 | 404 | Canonical |
| Q99369 | Family of serine hydrolases 3 | FSH3 | ledDLLDMids | 255 | 259 | Canonical |
| Q03769 | Epsin-5 | ENT5 | sipDLIDLdds | 292 | 296 | Canonical |
| Q03769 | Epsin-5 | ENT5 | kidDLLDWdgp | 354 | 358 | Canonical |
| Q05785 | Epsin-2 | ENT2 | qgvSLIDL | 609 | 613 | Canonical |
| Q12518 | Epsin-1 | ENT1 | rgyTLIDL | 450 | 454 | Canonical |
| P48582 | Vacuolar-sorting protein BRO1 | BRO1 | pqpSLLDIddt | 537 | 541 | Canonical |
| P53958 | Protein BOP3 | BOP3 | kskSLLDIifg | 26 | 30 | Canonical |
| P53858 | Protein BNI4 | BNI4 | tspNLIDIdgs | 161 | 165 | Canonical |
| Q08951 | AP-3 complex subunit delta | APL5 | nskDLLDLnee | 745 | 749 | Canonical |

| | | | | | | |
|---|---|---|---|---|---|---|
| P36000 | AP-1 complex subunit beta-1 | APL2 | vsqDLLDLf | 721 | 725 | Canonical |
| P36000 | AP-1 complex subunit beta-1 | APL2 | nddVLLDFder | 637 | 641 | Canonical |
| P38817 | ADP-ribosylation factor-binding protein GGA2 | GGA2 | vndLLGDLtdl | 385 | 389 | Arrestin |
| P34758 | Protein SCD5 | SCD5 | ssdILGNLqsl | 850 | 854 | Arrestin |

**Supplementary Table 2**. Percentage of identity in the N-terminal domain region of the clathrin heavy chain (isoform 1) among selected mammalian sequences compared to the human sequence. The percentages were retrieved by running Blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (accessed on 07/03/2024) with the following non-default parameters: 'Database' - 'refseq_protein', 'Organism' - 'Mammalia' and 'Matrix' - 'BLOSUM45'. The query sequence was the first 369 residues of the Clathrin heavy chain (UniprotKb ID Q00610). The results were filtered to have a coverage greater than 99 %.

| **Description** | **Scientific Name** | **Per. ident** |
|---|---|---|
| PREDICTED: clathrin heavy chain 1 isoform X1 [Bison bison bison] | Bison bison bison | 100 |
| clathrin heavy chain 1 isoform X2 [Rattus norvegicus] | Rattus norvegicus | 100 |
| clathrin heavy chain 1 isoform X3 [Sus scrofa] | Sus scrofa | 100 |
| clathrin heavy chain 1 isoform X3 [Rattus norvegicus] | Rattus norvegicus | 100 |
| clathrin heavy chain 1 isoform X3 [Mirounga angustirostris] | Mirounga angustirostris | 100 |
| clathrin heavy chain 1 isoform X4 [Odocoileus virginianus texanus] | Odocoileus virginianus texanus | 100 |
| clathrin heavy chain 1 isoform X3 [Pongo abelii] | Pongo abelii | 100 |

| | | |
|---|---|---|
| PREDICTED: clathrin heavy chain 1 isoform X3 [Capra hircus] | Capra hircus | 100 |
| clathrin heavy chain 1 isoform X4 [Pongo abelii] | Pongo abelii | 100 |
| clathrin heavy chain 1 isoform X3 [Bubalus kerabau] | Bubalus kerabau | 100 |
| clathrin heavy chain 1 isoform X3 [Castor canadensis] | Castor canadensis | 100 |
| clathrin heavy chain 1 isoform X3 [Microcebus murinus] | Microcebus murinus | 100 |
| clathrin heavy chain 1 isoform X1 [Leptonychotes weddellii] | Leptonychotes weddellii | 100 |
| clathrin heavy chain 1 isoform X3 [Odocoileus virginianus texanus] | Odocoileus virginianus texanus | 100 |
| clathrin heavy chain 1 isoform X2 [Delphinapterus leucas] | Delphinapterus leucas | 100 |
| clathrin heavy chain 1 isoform X2 [Myotis lucifugus] | Myotis lucifugus | 100 |
| clathrin heavy chain 1 isoform X3 [Lagenorhynchus obliquidens] | Lagenorhynchus obliquidens | 100 |
| clathrin heavy chain 1 isoform X3 [Pteronotus parnellii mesoamericanus] | Pteronotus parnellii mesoamericanus | 100 |
| clathrin heavy chain 1 isoform X1 [Cynocephalus volans] | Cynocephalus volans | 100 |
| clathrin heavy chain 1 isoform X1 [Erinaceus europaeus] | Erinaceus europaeus | 100 |
| clathrin heavy chain 1 isoform X2 [Carlito syrichta] | Carlito syrichta | 100 |
| clathrin heavy chain 1 [Bos taurus] | Bos taurus | 100 |
| PREDICTED: clathrin heavy chain 1 [Bos indicus] | Bos indicus | 100 |
| clathrin heavy chain 1 isoform 1 [Mus musculus] | Mus musculus | 100 |

**Supplementary Table 3.** Crystallographic data collection and refinement. Values for the highest resolution shell are shown in parentheses.

| NTD Complex | Ent1 | Ent2 | Ent5 | YAP1801 | APL2 |
|---|---|---|---|---|---|
| PDB ID | 9EYT | 9EXG | 9EX5 | 9EXF | 9EXT |
| **Data collection** | | | | | |
| Beamline | PETRAIII/P13 | PETRAIII/P14 | PETRAIII/P14 | PETRAIII/P14 | PETRAIII/P14 |
| Space Group | P 43 21 2 | P 21 21 21 | C 2 2 21 | P 1 21 1 | P 41 21 2 |
| Cell Dimensions | | | | | |
| a, b, c (Å) | 56.31, 56.31, 254.60 | 46.50, 94.29, 269.03 | 77.00, 133.45, 285.53 | 51.08, 89.74, 188.12 | 141.78, 141.78, 168.597 |
| α, β, γ (°) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.39, 90.0 | 90.0, 90.0, 90.0 |
| Resolution (Å) | 63.73 – 1.74 (1.77-1.74) | 67.35 – 1.74 (1.77-1.74) | 71.38 – 2.01 (2.05-2.01) | 65.01 – 1.95 (1.98-1.95) | 100.3 - 2.75 (2.85-2.75) |
| Rpim | 0.019 (0.545) | 0.037 (0.457) | 0.049 (0.517) | 0.063 (0.751) | 0.022 (0.785) |
| <I/σI> | 27.2 (1.78) | 16.7 (1.80) | 15.1 (1.5) | 11.0 (1.79) | 30.0 (1.6) |
| CC 1/2 | 1 (0.843) | 0.999 (0.788) | 0.999 (0.533) | 0.998 (0.553) | 1 (0.589) |
| Completeness (%) | 100 (99.9) | 99.8 (99.8) | 95.9 (34.9) | 94.4 (86.3) | 99.99 (100) |
| Redundancy | 26 (27.5) | 13.1 (12.9) | 12.9(3.5) | 7.7 (7.1) | 13.1 (2.8) |
| **Refinement** | | | | | |
| No. of reflections (work/free) | 43582/2114 | 121660/6253 | 93850/4607 | 116855/5916 | 45265/2259 |
| Rwork/Rfree | 0.185/0.230 | 0.191/0.225 | 0.182/0.214 | 0.183/0.214 | 0.228/0.258 |
| Ramachandran favoured regions (%) | 96.6 | 98.1 | 98.3 | 98.7 | 96.51 |
| Ramachandran allowed regions (%) | 3.4 | 1.9 | 1.7 | 1.2 | 3.49 |
| Ramachandran outliers (%) | 0 | 0 | 0 | 0.07 | 0.0 |
| Rotamer outliers (%) | 0.91 | 1.31 | 1.11 | 2.22 | 1.07 |
| Clashscore | 1.17 | 2.00 | 1.01 | 2.91 | 8.62 |
| No. of atoms | | | | | |
| Protein | 2844 | 8511 | 8452 | 11485 | 5698 |
| Petide | 151 | 428 | 449 | 281 | 211 |
| Ligands | 0 | 5 | 0 | 1 | 0 |
| Water | 324 | 929 | 604 | 614 | 6 |
| B-factors (Average) | | | | | |
| Protein | 39.59 | 32.98 | 42.52 | 36.85 | 97.81 |
| Peptide | 48.48 | 37.71 | 51.28 | 50.99 | 114.20 |
| Ligands | - | 35.40 | - | 65.80 | - |
| Water | 43.43 | 35.53 | 45.74 | 36.51 | 92.60 |
| RMS deviations | | | | | |
| Bond lengths (Å) | 0.01 | 0.011 | 0.01 | 0.018 | 0.006 |
| Bond angles (°) | 1.7 | 1.57 | 1.45 | 1.83 | 0.93 |

**Supplementary Table 4.** Primers used for QuikChange mutagenesis in both pETM-30-GST-ScCHC-NTD and pRS315-5'UTR CHC-mScarlet plasmids.

| Name | Box | Mutation | Type | Sequence |
|---|---|---|---|---|
| Q89A_Fw | Clathrin | Q89A | Fw | GTTAGAGCAAATGGTACTATCGTGgccATATTTAATTTGGAAACTAAGAGCAAG |
| Q89A_Rev | Clathrin | Q89A | Rev | CTTGCTCTTAGTTTCCAAATTAAATATGGCCACGATAGTACCATTTGCTCTAAC |
| Q195A_Fw | Arrestin | Q195A | FW | CAATTATTCTCAAAACAACGTAACATCTCCgccGCTATCGACGGTCATGTTGCTATC |
| Q195A_Rev | Arrestin | Q195A | Rev | GATAGCAACATGACCGTCGATAGCGGCGGAGATGTTACGTTGTTTTGAGAATAATTG |
| Q155A_Fw | W-Box | Q155A | FW | CCTTGAGACACGCTAACTTGAACAATACCgccATTATCAATTTTGTGGCTAACAAAAACC |
| Q155A_Rev | W-Box | Q155A | Rev | GGTTTTTGTTAGCCACAAAATTGATAATGGCGGTATTGTTCAAGTTAGCGTGTCTCAAGG |
| F8A_Fw | Royle | F8A | FW | GAGTGACCTACCCATTGAAgccACCGAATTGGTCGATCTGATGTCCTTAGG |
| F8A_Rev | Royle | F8A | Rev | CCTAAGGACATCAGATCGACCAATTCGGTGGCTTCAATGGGTAGGTCACTC |
| K63E_Fw | Clathrin | K63E | Fw | GGCAATGAAGTGACAAGGGAGAATATGGGCGGTGATTCTGCTATCATG |
| K63E_Rev | Clathrin | K63E | Rev | CATGATAGCAGAATCACCGCCCATATTCTCCCTTGTCACTTCATTGCC |
| K98E_Fw | Clathrin | K98E | Fw | CAGATATTTAATTTGGAAACTAAGAGCGAGTTAAAGTCTTTTACTTTAGATG |
| K98E_Rev | Clathrin | K98E | Rev | CATCTAAAGTAAAAGACTTTAACTCGCTCTTAGTTTCCAAATTAAATATCTG |
| R191E_Fw | Arrestin | R191E | Fw | GAATTCAATTATTCTCAAAACAAGAGAACATCTCCCAAGCTATCGACGGTC |
| R191E_Rev | Arrestin | R191E | Rev | GACCGTCGATAGCTTGGGAGATGTTCTCTTGTTTTGAGAATAATTGAATTC |
| K251E_Fw | Arrestin | K251E | Fw | GCTTCATTGCCTTCTCAATATCAAGAGGAAACTACCGATATTTTCTTTCC |
| K251E_Rev | Arrestin | K251E | Rev | GGAAAGAAAATATCGGTAGTTTCCTCTTGATATTGAGAAGGCAATGAAGC |
| R27E_Fw | W-box | R27E | Fw | GGAATTTCCCCTCAATTCCTTGACTTCGAATCAACTACTTTCGAGAGTGAC |
| R27E_Rev | W-box | R27E | Rev | GTCACTCTCGAAAGTAGTTGATTCGAAGTCAAGGAATTGAGGGGAAATTCC |
| K326E_Fw | W-box | K326E | Fw | GAACGGTATTGCATGCATCAATGAAAAAGGTCAAGTTTTAGCAGTAGAG |
| K326E_Rev | W-box | K326E | Rev | CTCTACTGCTAAAACTTGACCTTTTTCATTGATGCATGCAATACCGTTC |
| R191E+Q195A_Fw | Arrestin | R191E+Q195A | Fw | CAATTATTCTCAAAACAAGAGAACATCTCCGCCGCTATCGACGGTC |
| R191E+Q195A_Rev | Arrestin | R191E+Q195A | Rev | GACCGTCGATAGCGGCGGAGATGTTCTCTTGTTTTGAGAATAATTG |
| F26A_Fw | Wbox | F26A | Fw | GGAATTTCCCCTCAATTCCTTGACGCCAGATCAACTACTTTCGAG |
| F26A_Rev | WBox | F26A | Rev | CTCGAAAGTAGTTGATCTGGCGTCAAGGAATTGAGGGGAAATTCC |
| I197T_Fw | Arrestin | I197T | Fw | CTCAAAACAACGTAACATCTCCgccGCTaccGACGGTCATGTTG |
| I197T_Rev | Arrestin | I197T | Rev | CAACATGACCGTCGGTAGCGGCGGAGATGTTACGTTGTTTTGAG |
| I193A_Fw | Arrestin | I193A | Fw | CTTAGTTTCCAAATTAAATATGGCCACGTCAGTACCATTTGCTCT |
| I193A_Rev | Arrestin | I193A | Rev | AGAGCAAATGGTACTGACGTGGCCATATTTAATTTGGAAACTAAG |
| I193A_Fw | Arrestin | I193A | Fw | CTCAAAACAACGTAACgccTCCgccGCTATCGACGGTCATGTTG |
| I193A_Rev | Arrestin | I193A | Rev | CAACATGACCGTCGATAGCGGCGGAGGCGTTACGTTGTTTTGAG |
| F91Q_Fw | Clathrin | F91Q | Fw | AGAGCAAATGGTACTATCGTGgccATAcaaAATTTGGAAACTAAG |
| F91Q_Rev | Clathrin | F91Q | Rev | CTTAGTTTCCAAATTTTGTATGGCCACGATAGTACCATTTGCTCT |
| I87D_Fw | Clathrin | I87D | Fw | AGAGCAAATGGTACTgacGTGgccATATTTAATTTGGAAACTAAG |
| I87D_Rev | Clathrin | I87D | Rev | CTTAGTTTCCAAATTAAATATGGCCACGTCAGTACCATTTGCTCT |
| I87D+Q89A+K98E | Clathrin | I87D+Q89A+K98E | Fw | CAAATGGTACTgacGTGgccATATTTAATTTGGAAACTAAGAGCgagTTAAAG |
| I87D+Q89A+K98E | Clathrin | I87D+Q89A+K98E | Rev | CTTTAACTCGCTCTTAGTTTCCAAATTAAATATGGCCACGTCAGTACCATTTG |

**Supplementary Table 5.** Primers used for endogenous tagging of ABP1 with mTurquoise2 and to knock out CHC.

| Name | Mutation | Type | Sequence |
|---|---|---|---|
| ABP1_S3 | ABP1_mTurquoise2 | Fw | GAATTTGTCGACGATGACTGGTGGCTAGGGGAACTAGAGAAAGACGGCTCAAAAGGTCTCTTCCCCAGCAATTATGTGTCTTTGGGCAACcgtacgctgcaggtcgac |
| ABP1_S2 | ABP1_mTurquoise3 | Rev | CGTGAAAGTATCTATACAAAAGCTTTAACGTCTCTGTAAGTATTTTTTTACGTAAGAATAATATAATAGCATGACGCTGACGTGTGATTatcgatgaattcgagctcg |
| CHC_S1 | CHC_KO | Fw | GAGAAAGAAGATATTCAACTATAAAAATAAATTATCACGAAAGTTCTAACCAATGcgtacgctgcaggtcgac |
| CHC_S2 | CHC_KO | Rev | AAAAAAAAAAATACACGATGGGGTACAGCAAACGAATTATTTTATCCACGTCttaatcgatgaattcgagctcg |

**Supplementary Table 6.** Distribution of Bound Peptides in the Competition Experiment Between Ent1 and Ent2 with Ent1 at a Constant Concentration

| [Ent-1] / μM | [Ent-2] / μM | f 0x / % | f 1x / % | | f 2x / % | | | f 3x / % | | | | f 4x / % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ent1/Ent2 peptides bound | | | 1/0 | 0/1 | 2/0 | “1/1” | 0/2 | 3/0 | "2/1" | "1/2" | 0/3 | 4/0 | “3/1" | “2/2” | “1/3” | 0/4 |
| 20 | 0 | 85.76 ±0.9 | 12.78 ±0.7 | - | 1.36 ±0.2 | - | - | 0.10 ±0.03 | - | - | - | - | - | - | - | - |
| 20 | 5 | 84.31 ±1.7 | 11.93 ±1.1 | 1.82 ±0.2 | 1.21 ±0.3 | 0.54 ±0.1 | 0.03 ±0.01 | 0.10 ±0.04 | 0.04 ±0.02 | 0.003 ±0.003 | 0.006 ±0.006 | - | - | - | - | - |
| 20 | 10 | 79.40 ±1.0 | 13.00 ±0.4 | 3.70 ±0.3 | 1.72 ±0.1 | 1.21 ±0.1 | 0.34 ±0.1 | 0.21 ±0.03 | 0.27 ±0.04 | 0.10 ±0.04 | 0.04 ±0.01 | - | - | - | - | - |
| 20 | 15 | 75.99 ±0.7 | 13.50 ±0.4 | 5.62 ±0.4 | 1.74 ±0.1 | 1.80 ±0.1 | 0.67 ±0.1 | 0.16 ±0.1 | 0.26 ±0.04 | 0.21 ±0.05 | 0.05 ±0.01 | - | - | - | - | - |
| 20 | 20 | 73.89 ±1.1 | 12.31 ±0.7 | 7.48 ±0.3 | 1.60 ±0.1 | 2.35 ±0.2 | 1.14 ±0.2 | 0.18 ±0.03 | 0.38 ±0.1 | 0.47 ±0.03 | 0.17 ±0.03 | - | 0.01 ±0.01 | 0.01 ±0.01 | - | - |
| 20 | 25 | 71.68 ±1.0 | 12.26 ±0.2 | 9.23 ±0.5 | 1.47 ±0.04 | 2.64 ±0.1 | 1.42 ±0.04 | 0.16 ±0.1 | 0.43 ±0.1 | 0.47 ±0.1 | 0.23 ±0.1 | - | - | - | - | - |
| 20 | 30 | 67.10 ±1.8 | 12.01 ±0.6 | 11.22 ±0.6 | 1.60 ±0.2 | 3.56 ±0.3 | 1.99 ±0.2 | 0.23 ±0.02 | 0.65 ±0.1 | 0.78 ±0.1 | 0.38 ±0.1 | 0.01 ±0.01 | 0.10 ±0.04 | 0.16 ±0.1 | 0.15 ±0.1 | 0.04 ±0.02 |
| 20 | 50 | 63.05 ±2.6 | 9.40 ±0.5 | 15.21 ±1.1 | 1.12 ±0.1 | 4.17 ±0.3 | 3.82 ±0.4 | 0.08 ±0.01 | 0.615 ±0.1 | 1.45 ±0.1 | 0.79 ±0.1 | - | 0.06 ±0.03 | 0.18 ±0.03 | 0.22 ±0.02 | 0.14 ±0.03 |

**Supplementary Table 7.** Distribution of Bound Peptides in the Competition Experiment Between Ent1 and Ent2 with Ent2 at a Constant Concentration.

| [Ent-1] / μM | [Ent-2] / μM | f 0x / % | f 1x / % | | f 2x / % | | | f 3x / % | | | | f 4x / % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ent1/Ent2 peptides bound | | | 1/0 | 0/1 | 2/0 | “1/1” | 0/2 | 3/0 | "2/1" | "1/2" | 0/3 | 4/0 | “3/1" | “2/2” | “1/3” | 0/4 |
| 0 | 20 | 87.33 ±0.9 | - | 11.24 ±0.8 | - | - | 1.33 ±0.1 | - | - | - | 0.09 ±0.01 | - | - | - | - | - |
| 5 | 20 | 81.97 ±0.9 | 3.25 ±0.1 | 11.91 ±0.5 | 0.09 ±0.01 | 0.80 ±0.1 | 1.61 ±0.2 | - | 0.05 ±0.01 | 0.13 ±0.03 | 0.18 ±0.04 | - | - | - | - | - |
| 10 | 20 | 79.71 ±1.0 | 6.79 ±0.4 | 9.89 ±1.0 | 0.48 ±0.1 | 1.49 ±0.1 | 1.19 ±0.2 | 0.01 ±0.01 | 0.13 ±0.03 | 0.19 ±0.03 | 0.13 ±0.01 | - | - | - | - | - |
| 15 | 20 | 75.70 ±1.1 | 10.22 ±0.5 | 9.57 ±0.7 | 0.87 ±0.1 | 1.97 ±0.2 | 1.10 ±0.1 | 0.05 ±0.01 | 0.19 ±0.04 | 0.23 ±0.03 | 0.10 ±0.01 | - | - | - | - | - |
| 20 | 20 | 71.80 ±1.2 | 12.94 ±0.5 | 8.67 ±0.5 | 1.66 ±0.1 | 2.70 ±0.1 | 1.14 ±0.1 | 0.17 ±0.03 | 0.39 ±0.05 | 0.38 ±0.06 | 0.15 ± ±0.02 | - | - | - | - | - |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 20 | 71.53 ±2.5 | 13.97 ±0.9 | 7.14 ±0.7 | 2.19 ±0.2 | 2.70 ±0.3 | 0.91 ±0.2 | 0.30 ±0.1 | 0.58 ±0.1 | 0.44 ±0.1 | 0.12 ±0.04 | 0.02 ±0.01 | 0.04 ±0.02 | 0.04 0.03 | 0.02 ±0.02 | 0.01 ±0.01 |
| 30 | 20 | 68.80 ±1.9 | 16.25 ±1.2 | 6.97 ±0.3 | 2.72 ±0.3 | 2.98 ±0.3 | 0.89 ±0.1 | 0.32 ±0.1 | 0.55 ±0.1 | 0.36 ±0.03 | 0.09 ±0.01 | 0.01 ±0.01 | 0.03 ±0.03 | 0.02 ±0.02 | 0.01 ±0.01 | - |
| 50 | 20 | 66.39 ±6.8 | 19.31 ±3.2 | 5.33 ±0.8 | 3.97 ±1.2 | 2.74 ±0.9 | 0.68 ±0.1 | 0.525 ±0.3 | 0.65 ±0.3 | 0.27 ±0.1 | 0.05 ±0.02 | 0.03 ±0.03 | 0.03 ±0.03 | 0.04 ±0.04 | - | - |

**Supplementary Methods**

**Analysis of Native mass spectrometry (native MS) data - Interaction between Clathrin heavy chain (CHC) and the Ent5 peptide.**

The processed output of the native MS interaction data consists of the relative fractions of CHC bound to different numbers of peptides. In this case, we detected CHC bound to up to four peptides. From the bound fractions, the total protein concentration, and the total ligand (peptide) concentration, we can calculate the free ligand (peptide) concentration as:

$$[L] = [L_t] - ([CHC \cdot L_1] + 2[CHC \cdot L_2] + 3[CHC \cdot L_3] + 4[CHC \cdot L_4])$$

where $[L_t]$ is the total ligand concentration and $[CHC \cdot L_i]$ is the concentration of CHC bound to *i* ligands.

To average the measured fractions related to similar free ligand concentrations, we used log-spaced intervals with base 1.1 starting at 0.011 μM. Examples of two intervals are [0.011 - 0.0121) μM, centered at 0.0115 μM, and [64.23 - 70.72) μM, centered at 67.4 μM.

The degree of binding (average occupancy) was calculated as:

$$Degree\ of\ binding = \frac{([CHC \cdot L_1] + 2[CHC \cdot L_2] + 3[CHC \cdot L_3] + 4[CHC \cdot L_4])}{[P_t]}$$

where [Pt] is the total protein concentration (1.75 μM). The Scatchard plot graphs the degree of binding divided by the free ligand concentration against the degree of binding.

We fitted the five different measured fractions simultaneously to obtain the expected fractions from a four non-interacting binding sites model. The expected concentration of CHC (free protein) is given by

$$[CHC] = [P_t] / (1 + \gamma)$$

where

$$\gamma = \frac{[L]}{k_1} + \frac{[L]^2}{k_1 k_2} + \frac{[L]^3}{k_1 k_2 k_3} + \frac{[L]^4}{k_1 k_2 k_3 k_4}$$

where $k_i$ is the macroscopic dissociation constant of the ith binding event. $k_i$ depends on the microscopic dissociation constant ($k$) as follows:

$$k_i = k \frac{\Omega_{4,i-1}}{\Omega_{4,i}}$$

where $\Omega_{4,i}$ is the number of microstates for $i$ bound ligands and four binding sites. The microscopic dissociation constant ($k$) is the only fitted parameter.

Finally, the concentration of [CHC · $L_i$] is calculated as:

$$[CHC \cdot L_i] = [CHC] ([L] / k)^i \prod_{j=1}^{i} (4 - j + 1) / j$$

**References**


1. Yuen, K. K. The two-sample trimmed t for unequal population variances.

   *Biometrika* **61**, 165–170 (1974).