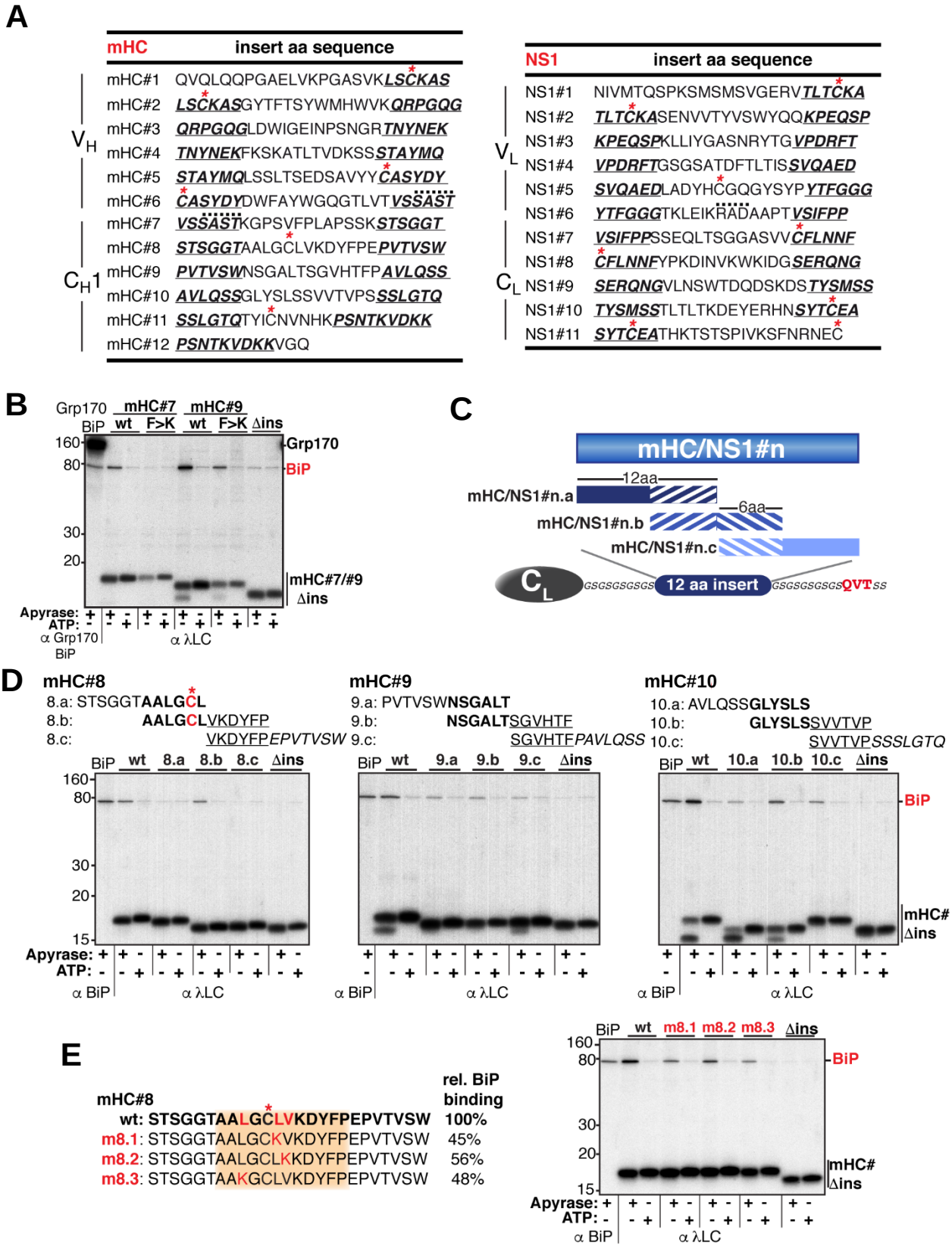
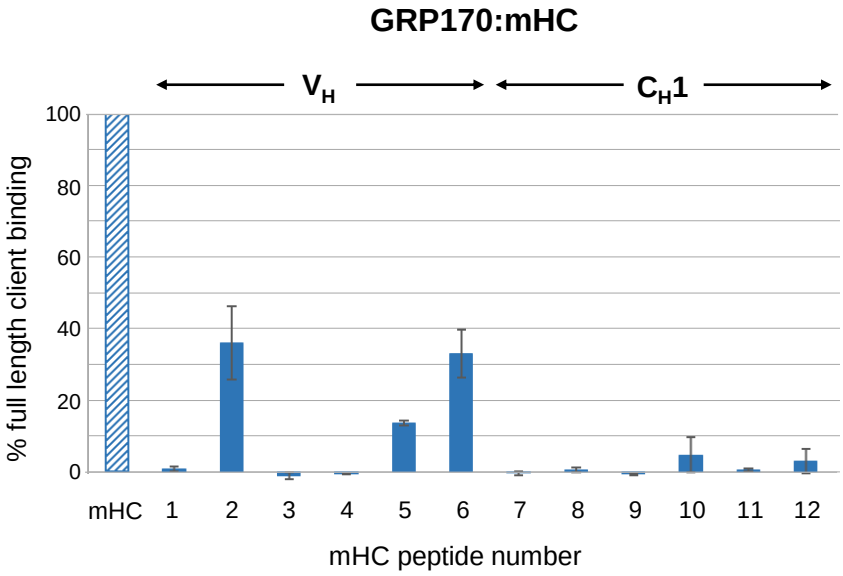

A
mHC
insert aa sequence
VH
CH1
mHC#1 QVQLQQPGAELVKPGASVKLSCKAS
mHC#2 LSCKASGYTFTSYWMHWVKQRPGQG
mHC#3 QRPGQGLDWIGEINPSNGRTNYNEK
mHC#4 TNYNEKFKSKATLTVDKSSSTAYMQ
mHC#5 STAYMQLSSLTSEDSAVYYCASYDY
mHC#6 CASYDYDWFAYWGQGTLVTVSSAST
mHC#7 VSSASTKGPSVFPLAPSSKSTSGGT
mHC#8 STSGGTAALGCLVKDYFPEPVTVSW
mHC#9 PVTVSWNSGALTSGVHTFPAVLQSS
mHC#10 AVLQSSGLYSLSSVVTVPSSSLGTQ
mHC#11 SSLGTQTYICNVNHKPSNTKVDKK
mHC#12 PSNTKVDKKVGQ
NS1
insert aa sequence
VL
CL
NS1#1 NIVMTQSPKSMSMSVGERVTLTCKA
NS1#2 TLTCKASENVVTYVSWYQQKPEQSP
NS1#3 KPEQSPKLLIYGASNRYTGVPDRFT
NS1#4 VPDRFTGSGSATDFTLTISSVQAED
NS1#5 SVQAEDLADYHCGQGYSYPYTFGGG
NS1#6 YTFGGGTKLEIKRADAAPTVSIFPP
NS1#7 VSIFPPSSEQLTSGGASVVCFLNNF
NS1#8 CFLNNFYPKDINVKWKIDGSERQNG
NS1#9 SERQNGVLNSWTDQDSKDSTYSMSS
NS1#10 TYSMSSTLTLTKDEYERHNSYTCEA
NS1#11 SYTCEATHKTSTSPIVKSFNRNEC
B
Grp170
mHC#7
mHC#9
BiP
wt
F>K
wt
F>K
Δins
160
80
30
20
Grp170
BiP
mHC#7/#9
Δins
Apyrase:
ATP:
α Grp170 BiP
α λLC
C
mHC/NS1#n
12aa
mHC/NS1#n.a
6aa
mHC/NS1#n.b
mHC/NS1#n.c
CL
GSGSGSGSGS
12 aa insert
GSGSGSGSGSQVTSS
D
mHC#8
8.a: STSGGTAALGCL
8.b: AALGCLVKDYFP
8.c: VKDYFPEPVTVSW
mHC#9
9.a: PVTVSWNSGALT
9.b: NSGALTSGVHTF
9.c: SGVHTFPAVLQSS
mHC#10
10.a: AVLQSSGLYSLS
10.b: GLYSLSSVVTVP
10.c: SVVTVPSSSLGTQ
BiP
wt
8.a
8.b
8.c
9.a
9.b
9.c
10.a
10.b
10.c
Δins
160
80
30
20
15
BiP
mHC#
Δins
Apyrase:
ATP:
α BiP
α λLC
E
mHC#8
rel. BiP binding
wt: STSGGTAALGCLVKDYFPEPVTVSW 100%
m8.1: STSGGTAALGCKVKDYFPEPVTVSW 45%
m8.2: STSGGTAALGCLKKDYFPEPVTVSW 56%
m8.3: STSGGTAAKGCLVKDYFPEPVTVSW 48%
BiP
wt
m8.1
m8.2
m8.3
Δins
160
80
30
20
15
BiP
mHC#
Δins
Apyrase:
ATP:
α BiP
α λLC

Figure S1

Figure S1, related to Figure 1. Sequences of the initial peptide library and mutational analysis of BiP binding sequences. (A) Sequences of the 25 amino acid peptides derived from the mini HC and NS1 LC proteins. Overlapping regions are underlined and in bold italics. Cysteines are highlighted with a red asterisk and linker residues connecting the V_H and C_H1 and the V_L and C_L domains are indicated with a dotted line. (B) The indicated BiP-binding peptides in the C_H1 domain were mutated as indicated and examined as in Figure 1C. (C) Schematic showing the procedure for subdividing the 25 amino acid inserts into three overlapping 12 amino acid sequences. (D) The specified 25-amino acid BiP binding peptides were subdivided into 12 amino acid peptides as shown above the gel images. Binding of BiP to these peptides in the context of the reporter constructs was analyzed as in Figure 1C. (E) The indicate substitutions were made in the mHC#8 peptide and analyzed as above for BiP binding. Relative binding was quantified with the wild-type sequence set to 1 (N≥3).


GRP170:mHC
V_H
C_H1
% full length client binding
100
80
60
40
20
0
mHC
1
2
3
4
5
6
7
8
9
10
11
12
mHC peptide number

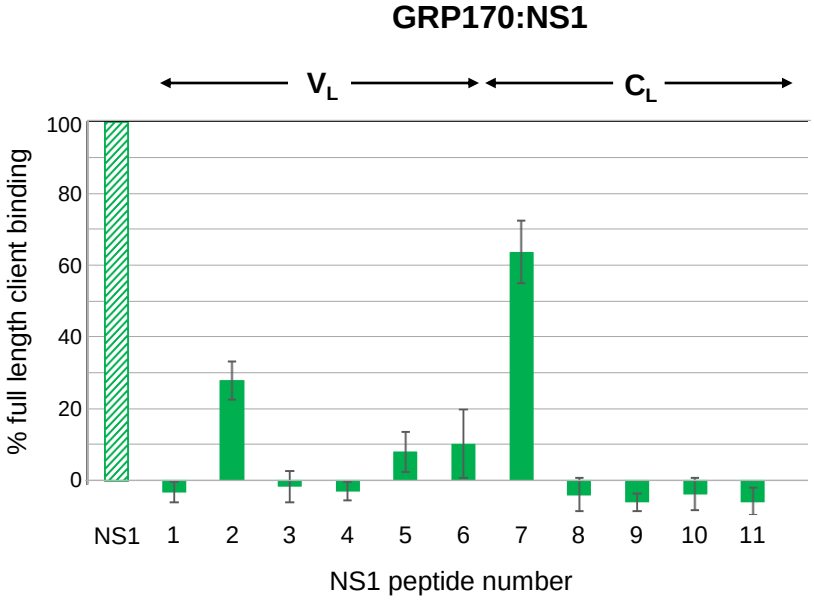

GRP170:NS1
V_L
C_L
% full length client binding
100
80
60
40
20
0
NS1
1
2
3
4
5
6
7
8
9
10
11
NS1 peptide number

Figure S2

Figure S2, related to Figure 1. Quantification of Grp170 binding to the mHC and NS1 peptide libraries. (A) COS-1 cells were co-transfected with Grp170, BiP, and either the full-length mini HC or each of the peptide constructs. Cells were metabolically labeled for 2.5 hr, lysed in NP-40 buffer, and clients were immunoprecipitation with monoclonal anti-HA in the case of the mini HC or anti-λ antiserum for the peptide library. After SDS-PAGE, the gel was scanned using a Typhoon FLA 9500 phosphorimager. The signal for Grp170 was divided by the signal of the client. In each case, the quotient of the Grp170 signal divided by that of Δins was subtracted. The value for mHC was set to 100% and that for each of the peptide constructs was expressed as a percent of the full-length client. Results obtained are from three experiments and shown as standard error of the mean (SEM). (B) COS-1 cells were co-transfected with Grp170, BiP, and either full-length NS1 or each of the peptide constructs. Detection of Grp170 binding was performed and quantified as in (A), except NS1 was isolated with anti-κ antiserum.

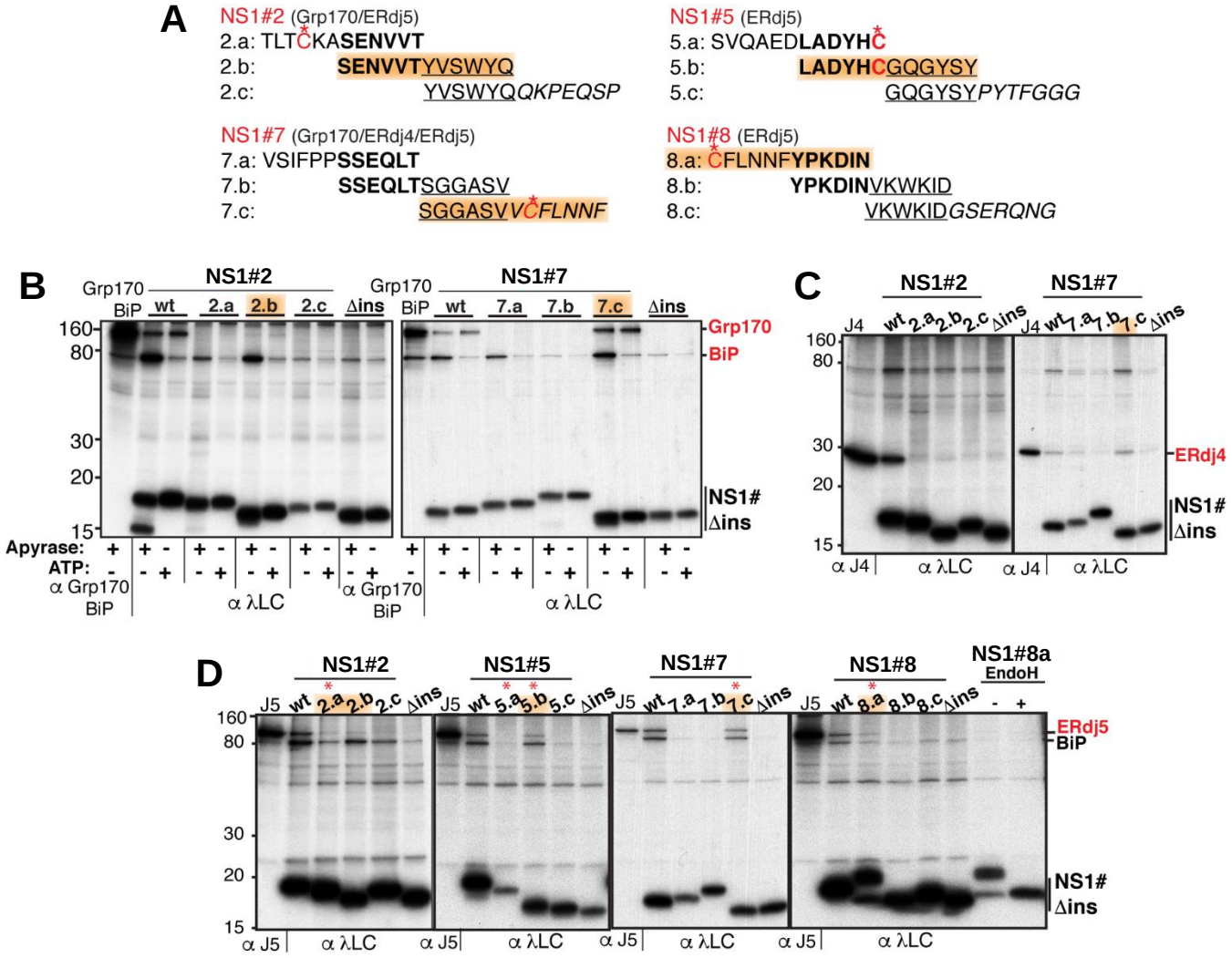

A
NS1#2 (Grp170/ERdj5)
2.a: TLTCKASENVVT
2.b: SENVVTYVSWYQ
2.c: YVSWYQQKPEQSP
NS1#5 (ERdj5)
5.a: SVQAEDLADYHC
5.b: LADYHCGQGYSY
5.c: GQGYSYPYTFGGG
NS1#7 (Grp170/ERdj4/ERdj5)
7.a: VSIFPPSSEQLT
7.b: SSEQLTSGGASV
7.c: SGGASVVCFLNNF
NS1#8 (ERdj5)
8.a: CFLNNFYPKDIN
8.b: YPKDINVKWKID
8.c: VKWKIDGSERQNG
B
Grp170
BiP
NS1#2
wt
2.a
2.b
2.c
Δins
NS1#7
7.a
7.b
7.c
160
80
30
20
15
Grp170
BiP
NS1#
Δins
Apyrase:
ATP:
α Grp170
BiP
α λLC
C
NS1#2
NS1#7
J4
ERdj4
α J4
D
NS1#2
NS1#5
NS1#7
NS1#8
NS1#8a
EndoH
J5
ERdj5
BiP
α J5

Figure S3

Figure S3, related to Figure 3. Binding of (co-)chaperones to NS1 subdivided peptides. (A) The subdivided sequences of each of the NS1 peptides that bound the (co-)chaperones are shown, and the subdivided peptide for which interactions could be detected are highlighted in orange and the (co-)chaperones they bound are indicated in parentheses. The binding of GRP170 (B), ERdj4 (C), and ERdj5 (D) to subdivided peptides was performed as described in Figure 2. In the case of NS1#8, the insertion of the 8a peptide, which ended with an asparagine, into the GS linker created a glycosylation site (NGS) that was glycosylated as revealed by EndoH treatment.

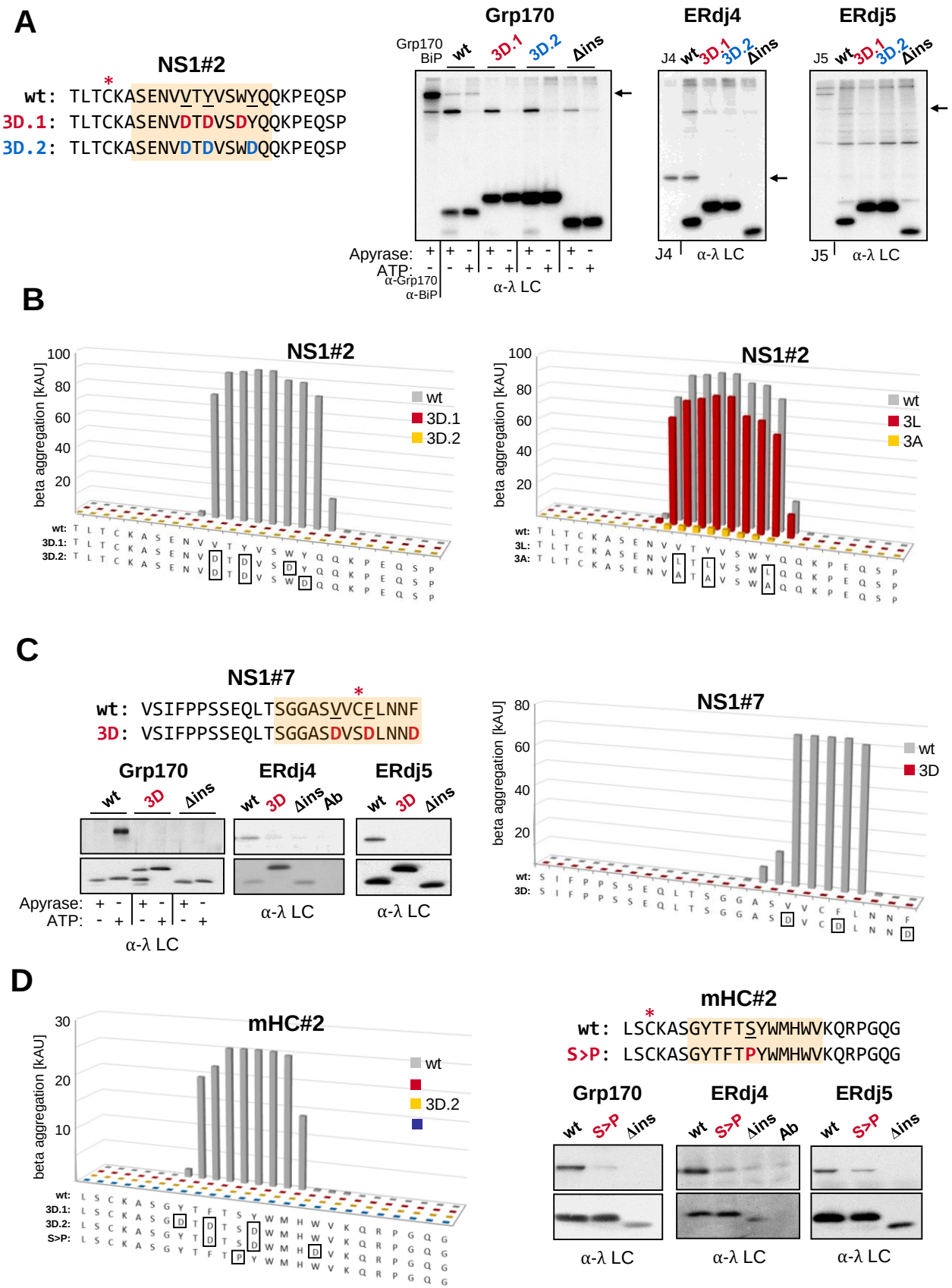
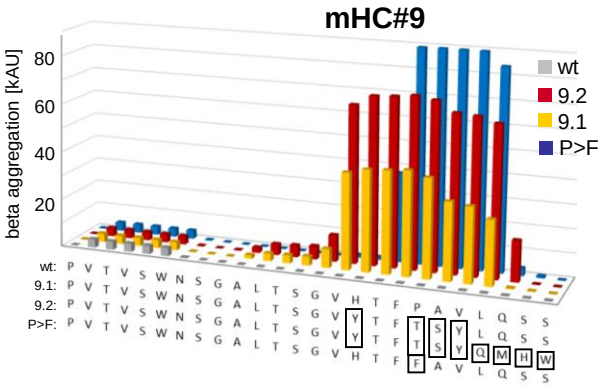

A
NS1#2
wt: TLTCKASENVVTYVSWYQQKPEQSP
3D.1: TLTCKASENVDTDVSDYQQKPEQSP
3D.2: TLTCKASENVDTDVSWDQQKPEQSP
Grp170
ERdj4
ERdj5
Grp170
BiP
wt
3D.1
3D.2
Δins
J4
J5
Apyrase:
ATP:
α-Grp170
α-BiP
α-λ LC
B
NS1#2
beta aggregation [kAU]
wt
3D.1
3D.2
3L
3A
C
NS1#7
wt: VSIFPPSSEQLTSGGASVVCFLNNF
3D: VSIFPPSSEQLTSGGASDVSDLNND
Grp170
ERdj4
ERdj5
wt
3D
Δins
Ab
Apyrase:
ATP:
α-λ LC
D
mHC#2
beta aggregation [kAU]
wt
3D.2
S>P
wt: LSCKASGYTFTSYWMHWVKQRPGQG
S>P: LSCKASGYTFTPYWMHWVKQRPGQG
Grp170
ERdj4
ERdj5
Ab
α-λ LC

Figure S4

Figure S4, related to Figures 3 and 4. Disruption of (co-)chaperone binding sites on peptide constructs by limited point mutations. (A) The residues that were mutated in the NS1#2 peptides are highlighted with color and core binding sequence for the wild-type peptide is boxed in orange. COS-1 cells were co-transfected with wild-type NS1#2 or the two mutant constructs together with either a combination of BiP and Grp170, ERdj4, or ERdj5 and metabolically labeled. Peptide constructs were immunoprecipitated with anti-λ LC and analyzed by reducing SDS-PAGE. Binding of the various (co-)chaperones was performed respectively as described in Figures 1 and 2. (B) The NS1#2 wild-type and mutants sequences were subjected TANGO algorithm. Respective plots are color coded and mutated residues are boxed in the sequences below the plots. (C-D) Cells expressing the indicated (co-)chaperones and either NS1#7 (C) or mHC#2 (D) constructs were subjected to TANGO analyses and ip-coupled western blotting as described in Figure 3F. In the case of Grp170 and ERdj5 immunoprecipitated material was separated by reducing SDS-PAGE, whereas non-reducing SDS-PAGE was used to detect ERdj4 interaction. In the latter case, the anti-λ antiserum used to isolate the peptides is included in a separate lane as a control, because the LC from this antiserum co-migrates with the ERdj4 protein. The sequences of each of the NS1#7 and mHC#2 peptides are shown and mutated residues are in color. The cysteine residue in each is indicated with an asterisk, and the 12-aa core binding fragments is identified for both with an orange box.


mHC#9
beta aggregation [kAU]
80
60
40
20
wt
9.2
9.1
P>F
wt: P V T V S W N S G A L T S G V H T F P A V L Q S S
9.1: P V T V S W N S G A L T S G V Y T F S Y L Q S S
9.2: P V T V S W N S G A L T S G V Y T F S Y Q M H W
P>F: P V T V S W N S G A L T S G V H T F F A V L Q S S

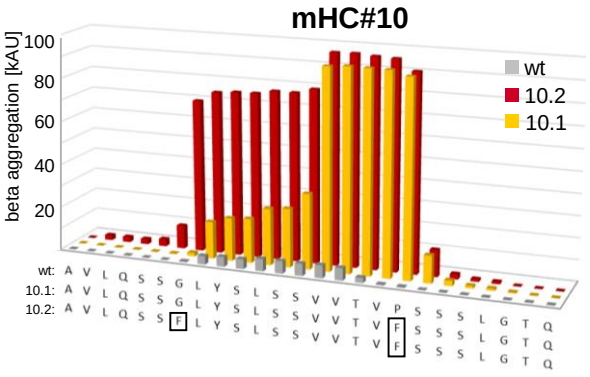

mHC#10
beta aggregation [kAU]
100
80
60
40
20
wt
10.2
10.1
wt: A V L Q S S G L Y S L S S V V T V P S S S L G T Q
10.1: A V L Q S S G L Y S L S S V V T V F S S S L G T Q
10.2: A V L Q S S F L Y S L S S V V T V F S S S L G T Q

Figure S5

Figure S5, related to Figure 5. Mutations that increase the computed aggregation potential of peptides lead to (co-)chaperone binding. The TANGO algorithm was used to calculate the potential for β-aggregate formation for the mHC#9 and mHC#10 wild-type constructs and their various mutants. The calculations for each wild-type and mutant peptide is color-coded with sequences indicated below and mutated residues boxed for each.

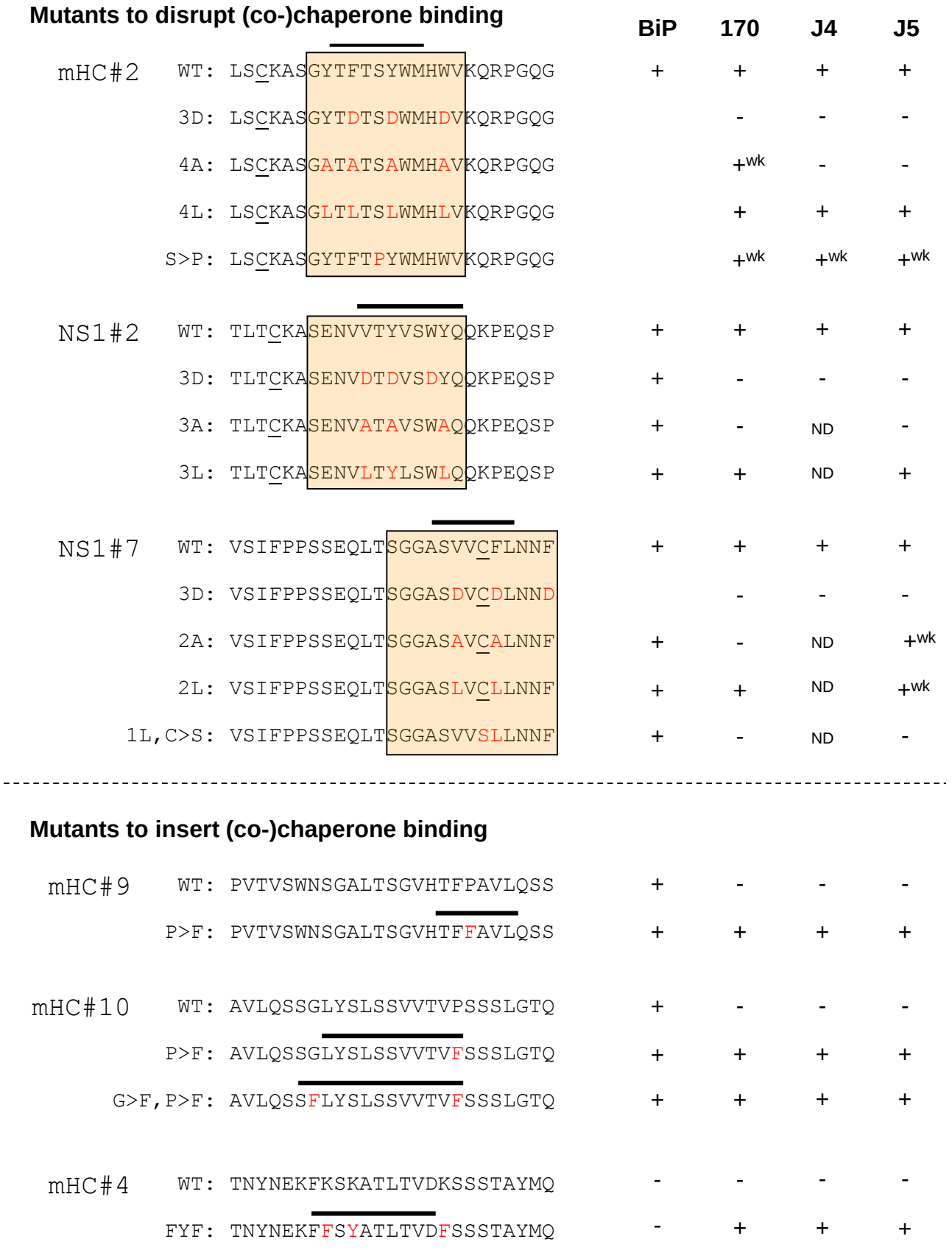

Mutants to disrupt (co-)chaperone binding
BiP
170
J4
J5
mHC#2
WT: LSCKASGYTFTSYWMHWVKQRPGQG + + + +
3D: LSCKASGYTDTSDWMHDVKQRPGQG - - -
4A: LSCKASGATATSAWMHAVKQRPGQG +wk - -
4L: LSCKASGLTLTSLWMHLVKQRPGQG + + +
S>P: LSCKASGYTFTPYWMHWVKQRPGQG +wk +wk +wk
NS1#2
WT: TLTCKASENVVTYVSWYQQKPEQSP + + + +
3D: TLTCKASENVDTDVSDYQQKPEQSP + - - -
3A: TLTCKASENVATAVSWAQQKPEQSP + - ND -
3L: TLTCKASENVLTYLSWLQQKPEQSP + + ND +
NS1#7
WT: VSIFPPSSEQLTSGGASVVCFLNNF + + + +
3D: VSIFPPSSEQLTSGGASDVCDLNND - - -
2A: VSIFPPSSEQLTSGGASAVCALNNF + - ND +wk
2L: VSIFPPSSEQLTSGGASLVCLLNNF + + ND +wk
1L,C>S: VSIFPPSSEQLTSGGASVVSLLNNF + - ND -
Mutants to insert (co-)chaperone binding
mHC#9
WT: PVTVSWNSGALTSGVHTFPAVLQSS + - - -
P>F: PVTVSWNSGALTSGVHTFFAVLQSS + + + +
mHC#10
WT: AVLQSSGLYSLSSVVTVPSSSLGTQ + - - -
P>F: AVLQSSGLYSLSSVVTVFSSSLGTQ + + + +
G>F,P>F: AVLQSSFLYSLSSVVTVFSSSLGTQ + + + +
mHC#4
WT: TNYNEKFKSKATLTVDKSSSTAYMQ - - - -
FYF: TNYNEKFFSYATLTVDFSSSTAYMQ - + + +

Figure S6

Figure S6, related to Figures 4, S4, 5, and S5. Compilation of (co-)chaperone binding data for the peptides and the various mutations of these. The core binding sequences in mHC#2, NS1#2 and NS1#7 are boxed and the predicated aggregation-prone regions are indicated with a black line above the sequence. Cysteines are underlined and mutated residues are in red. Binding is scored as positive or negative, although in a few cases a very weak signal was observed that was slightly above the negative control; these are shown as ($+^{wk}$). For several of the mutants it was not possible to determine ERdj4 binding due to background bands from the precipitating antibody; these are indicated as not detectable (ND). (Co-) chaperone binding sequences were introduced into mHC#9, #10, and #4. The altered residues are in red, and the sequence predicted to be aggregation prone by TANGO is indicated with a bold line.

Supplemental Experimental Procedures

Expression vectors

The pSVL expression vectors encoding the truncated γHC with a double HA-tag (mHC-HA) (Lee et al., 1999), NS1 LC (Skowronek et al., 1998), full length γ HC (Gaut and Hendershot, 1993), and λC_L (Hellman et al., 1999) have been described, as have pMT-hamster BiP (Gaut and Hendershot, 1993) pSG5-human ERdj3 (Shen and Hendershot, 2005), pcDNA3.1-Grp170 (Behnke and Hendershot, 2014). pCMV-ERdj5 vector was obtained from Dr. Kazuhiro Nagata (Kyoto Sangyo University, Japan). For this study, two additional HA-tags were added to the C-terminus of HA-tagged mouse ERdj4 in the pSG5 mammalian expression vector (Shen et al., 2002) resulting in ERdj4-3HA, allowing us to distinguish between NS1 LC and ERdj4, which have similar molecular weights. In experiments to examine the interaction of ERdj4 with the complete mini-HC, the HA-tag in mHC-HA was substituted with a FLAG-tag (mHC-FL).

Cell Culture and transfection

COS-1cells were cultured as previously described (Behnke and Hendershot, 2014). Cells were plated 24 h prior to transfection, which was performed using GeneCellin (BioCellChallenge, France) according to the manufacturer's protocol. When NVT versions of the peptide library were expressed alone, 4 μg of plasmid DNA were used to transfect one p60 dish. For the interaction studies 1 μg of Grp170 and 1 μg of BiP were co-transfected with 3 μg of each peptide construct. The ERdj proteins were examined separately, in which case 2 μg of ERdj3, ERdj4 or ERdj5 were co-transfected with 3 μg each peptide construct in a p60 dish unless otherwise indicated. Transfectants were analyzed 24 hrs later using metabolic labeling or immunoprecipitation-coupled western blotting as described.

Antibodies

The polyclonal anti-rodent BiP (Hendershot et al., 1995), anti-ERdj3 (Shen and Hendershot, 2005), and anti-Grp170 antisera (Behnke and Hendershot, 2014) and monoclonal anti-ERdj4 antibody (Lai et al., 2012) have been described. The monoclonal anti-HA antibody (12CA5) producing cell line was kindly provided by Dr. Al Reynolds (Vanderbilt University, USA). All other antibodies were obtained commercially including the anti-FLAG antibody (F7425, Sigma-Aldrich, USA), goat anti-mouse κ LC (1050-01) and goat anti-mouse λ LC (1060-01) (both SouthernBiotech, USA); HRP-conjugated goat anti-rabbit (sc-2054) and HRP-conjugated donkey anti-goat (sc-2020) (both Santa Cruz Biotechnology, USA); monoclonal anti-ERdj5 (H00054431-M01, Abnova, Taiwan).

Metabolic Labeling and Immunoprecipitation

For metabolic labeling experiments, cells were labeled with Express [^{35}S] Labeling Mix (PerkinElmer) in DMEM labeling media without Met/Cys (Gibco) for 2.5 hrs unless otherwise indicated. In the case of pulse-chase experiments, cells were first starved in DMEM labeling media for 30 minute prior to labeling for 30 minutes and chasing as shown on the figure. Cells were lysed either in RIPA buffer supplemented with 0.1 mM PMSF and 1x Roche complete protease inhibitor tablets w/o EDTA (Roche Applied

Science, Indianapolis, IN, USA) as described previously (Behnke and Hendershot, 2014) or in NP-40 lysis buffer with the following modifications. NP-40 lysates were additionally precleared with CaptivA™ PriMAB Protein A agarose slurry for 60 min at 4°C before adding the immunoprecipitating antibody and incubating at 4°C overnight. Subsequently, Protein A agarose was added for 1hr, and isolated immune complexes were analyzed by SDS-PAGE. In cases where isolated proteins were to be examined under non-reducing conditions, cells were washed first 2X with PBS supplemented with 20 mM N-ethylmaleimide (NEM), which was also included in the lysing buffer. In this case, the 2x Laemmli buffer was supplemented with 40 mM NEM instead of ß-mercaptoethanol.
NP-40 lysis buffer was supplemented with 10 U/ml apyrase (Sigma) to maintain BiP binding, whereas 2 mM Mg-ATP combined with 25 mM KCl was added to release BiP. The detection of ERdj3 binding required stabilization with the cell-permeable cross-linker 3,3-dithio-bis(propionic acid N-hydroxysuccinimideester) (DSP), which was conducted as described (Shen and Hendershot, 2005). Samples to be queried for ERdj5 interaction were washed and lysed in the presence of 20 mM N-ethylmaleimide (NEM) to prevent post-lysis disulfide bond reshuffling. Glycosylation of the NVT version of the library was determined by treatment of immunoprecipitated proteins with Endoglycosidase H (EndoH) (NEB) according to the manufacturer's instructions.

Quantitation of (co-)chaperone binding to peptide constructs
A Hyphoon FLA 9500 phosphorimager (GE Healthcare, Pittsburgh, PA) was used to detect signals, which were analyzed with the ImageQuant TL software (GE Healthcare, Pittsburgh, PA). To quantify the interaction of Grp170 with the 25 amino acid peptide libraries, BiP with the subdivided mHC#8 peptide, and the three (co-)chaperones with the aspartate substituted mHC#2 peptide, the signals of the indicated (co-)chaperone were divided by the signal of the respective peptides, and the quotient obtained for (co-)chaperone binding to Δins was subtracted from this. The value for the wild-type was set to 1 or 100% as shown in the figures, and the value for the peptide constructs was represented as a fraction or percent of wild-type. For the analysis of pulse chase experiments, the value of the constructs at t=0 was set to 1 and all other time points were calculated relative to t=0. Results are shown as standard error of the mean (SEM) and at least 3 experiments were performed for each analysis.

Western Blotting
Unlabeled cell lysates from COS-1 cell transfectants were immunoprecipitated with specific immune reagents as shown on the figures. Isolated proteins were separated on SDS-polyacrylamide gels and transferred to a PVDF membrane (Biorad, USA). After blocking, specific proteins were detected by incubation with the indicated antiserum followed by the appropriate species-specific secondary antiserum conjugated to HRP. Signals were detected by ECL (Thermo Fisher Scientific, USA). In the case of ERdj4 binding, the samples were electrophoresed under non-reducing conditions to minimize detection of the light chain (~25 kDa) from the precipitating antibody by the secondary antibody used to develop the western. The precipitating antibody alone was added to one lane to control for residual signal.

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