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Supplemental information

The IMiD target CRBN determines HSP90 activity toward transmembrane proteins essential in multiple myeloma

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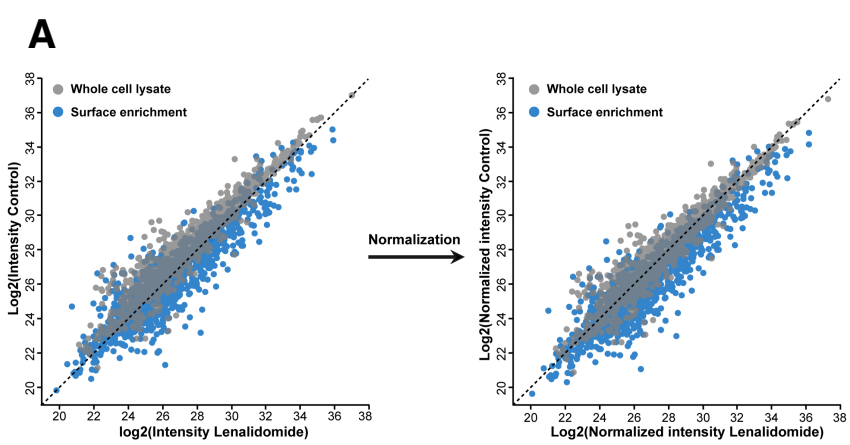
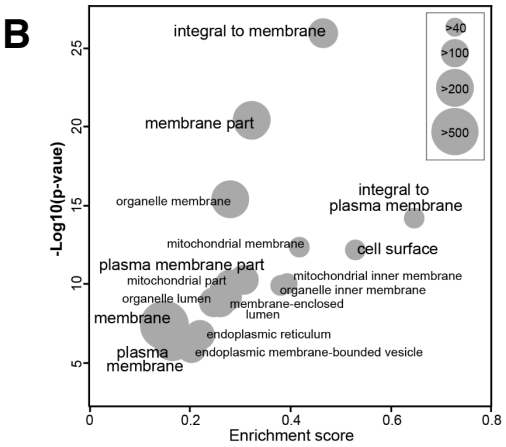
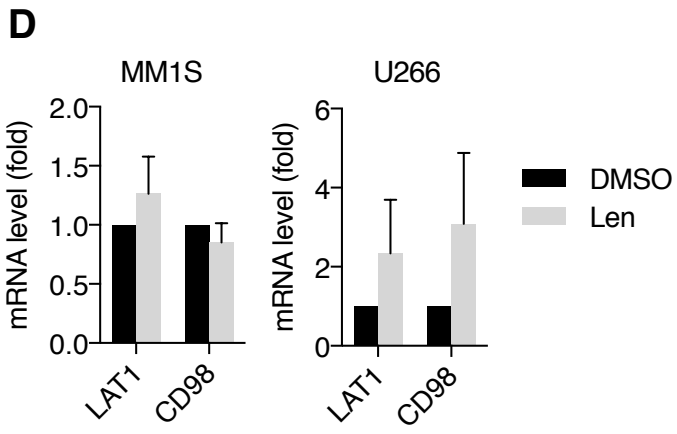
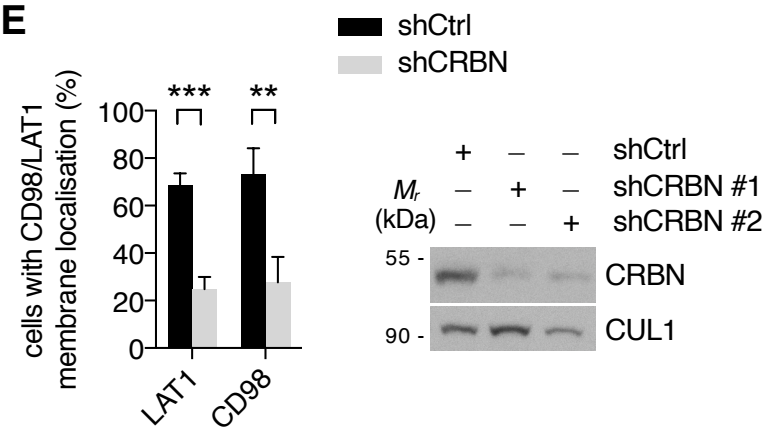


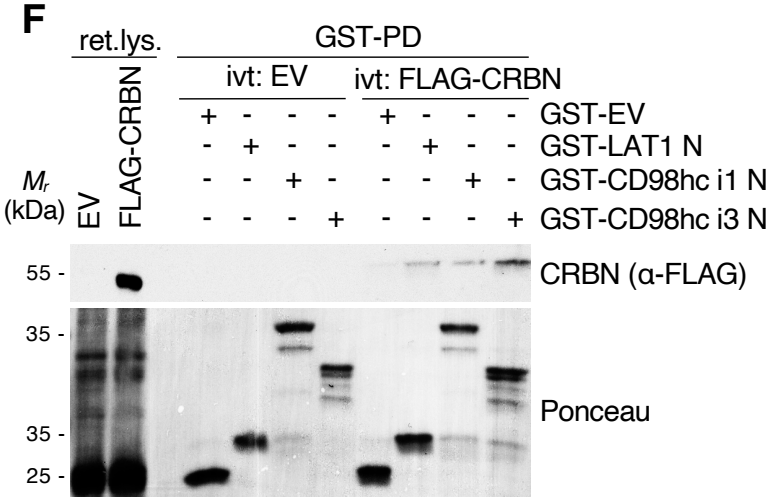


C

Interactors	M_r (kDa)	EV	CRBN-SF
DDB1	127	59	1756
CUL4A	102	0	291
CD147	29	0	24
MCT1	52	0	16
CD98hc	58	0	19
AHA1	38	0	9







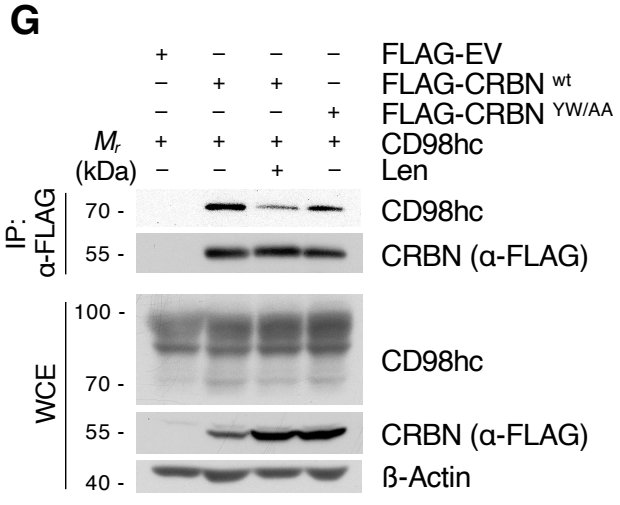


Fig. S1

Figure S1. Cell surface proteomics reveal a broad effect of IMiDs on transmembrane protein abundance and specify CD98hc/LAT1 as novel CRBN clients (related to Figure 1)
Cells treated with Lenalidomide ("light" SILAC label) were mixed with non-treated control cells ("heavy" SILAC label) and subjected to an enrichment of cell surface proteins. An aliquot of the initial cell mix used as input for the enrichment and the enriched fraction were analyzed using mass spectrometry. (A) Protein intensities in treated cells are plotted against those in control cells. Protein ratios in the surface enrichment (blue dots) were corrected for unequal cell mixing by applying the same normalization factor that shifted the median of intensity ratios in the initial cell mix (grey dots) to the bisectrix. (B) The enrichment of surface proteins was checked in a 1D enrichment analysis (at 1% Benjamini-Hochberg FDR) using gene ontology cellular component (GOCC) annotations and comparing protein intensity ratios of the surface fraction to those of the initial cell mix. Membrane associated categories are clearly overrepresented in the enriched surface fraction. (C) Interactors of SF-CRBN identified by mass-spectrometry. Numbers of individual peptides are shown. (D) Real-time qPCR analysis of MM1S and U266 cell lines treated with 10 μM Len. or DMSO. The amount of mRNA in the control sample was set as 1. Data are mean ± s.d.. (E) *left panel*: Quantification of the percentage of cells in Figure 1D that showed membrane localization of CD98hc and LAT1 (n = 3 independent experiments). Data are mean ± s.d. ** $P < 0.01$, *** $P < 0.001$ by Student's t-test. *right panel*: Representative immunoblot of cells shown in Figure 1D. (F) GST pull-down analysis, as determined by immunoblotting, of the indicated GST-tagged intracellular fragments of CD98hc (isoform 1: aa 1-184 and isoform 3: aa 1-122) and LAT1 (aa 1-49), from reticulocyte lysates with *in-vitro* translated FLAG-CRBN or FLAG-EV control. (G) IP analysis of FLAG-$CRBN^{wt}$ or $CRBN^{YW/AA}$ transfected into $CRBN^{-/-}$ HEK293T co-expressing CD98hc. Cells were pretreated with lenalidomide (50μM) or DMSO for 24 hours prior to harvest. Immunocomplexes and respective whole cell extracts (WCE) were probed with antibodies to the indicated proteins

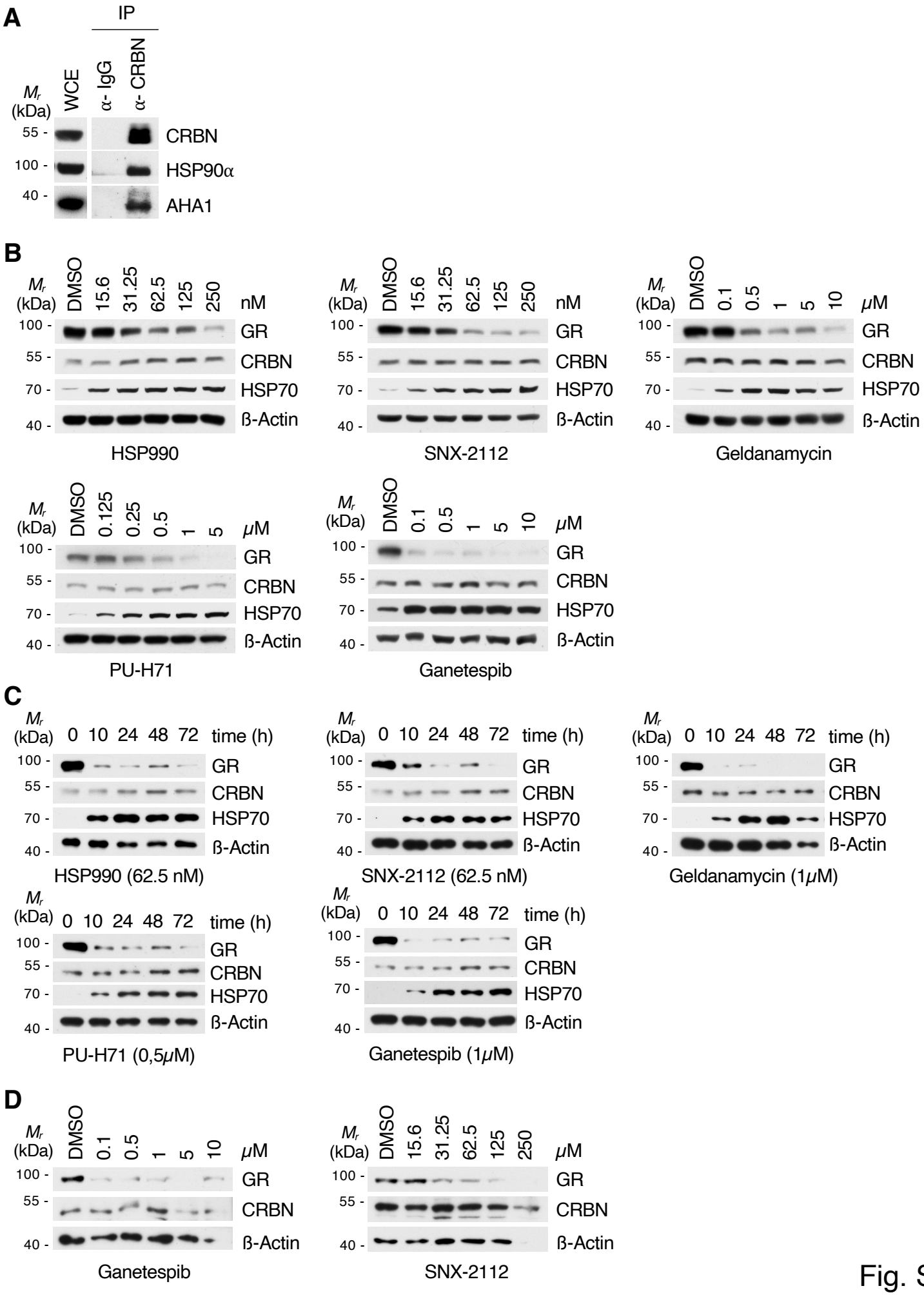

A
IP
WCE
α- IgG
α- CRBN
Mr (kDa)
55 -
100 -
40 -
CRBN
HSP90α
AHA1
B
Mr (kDa)
DMSO
15.6
31.25
62.5
125
250
nM
100 -
55 -
70 -
40 -
GR
CRBN
HSP70
ß-Actin
HSP990
SNX-2112
0.1
0.5
1
5
10
μM
Geldanamycin
0.125
0.25
PU-H71
Ganetespib
C
0
10
24
48
72
time (h)
HSP990 (62.5 nM)
SNX-2112 (62.5 nM)
Geldanamycin (1μM)
PU-H71 (0,5μM)
Ganetespib (1μM)
D
Ganetespib
SNX-2112

Fig. S2

Figure S2. Endogenous CRBN interacts with HSP90α in MM cells, while its levels are not influenced by HSP90 inhibition (related to Figure 2)

(A) Endogenous IP of CRBN from MM1S cells analyzed with the indicated antibodies (B) Immunoblot analysis of MM1S cells, which were treated with the indicated concentrations of HSP990, SNX-2112, Geldanamycin, PU-H71, Ganetespib or DMSO for 48hrs prior to harvest. (C) Immunoblot analysis of MM1S cells, which were treated with the indicated concentrations of HSP990, SNX-2112, Geldanamycin, PU-H71 or Ganetespib for the indicated times before harvest. (D) Immunoblot analysis of the insoluble fraction of MM1S cells, which were treated with the indicated concentrations of Ganetespib, SNX-2112 or DMSO for 48hrs prior to harvest.

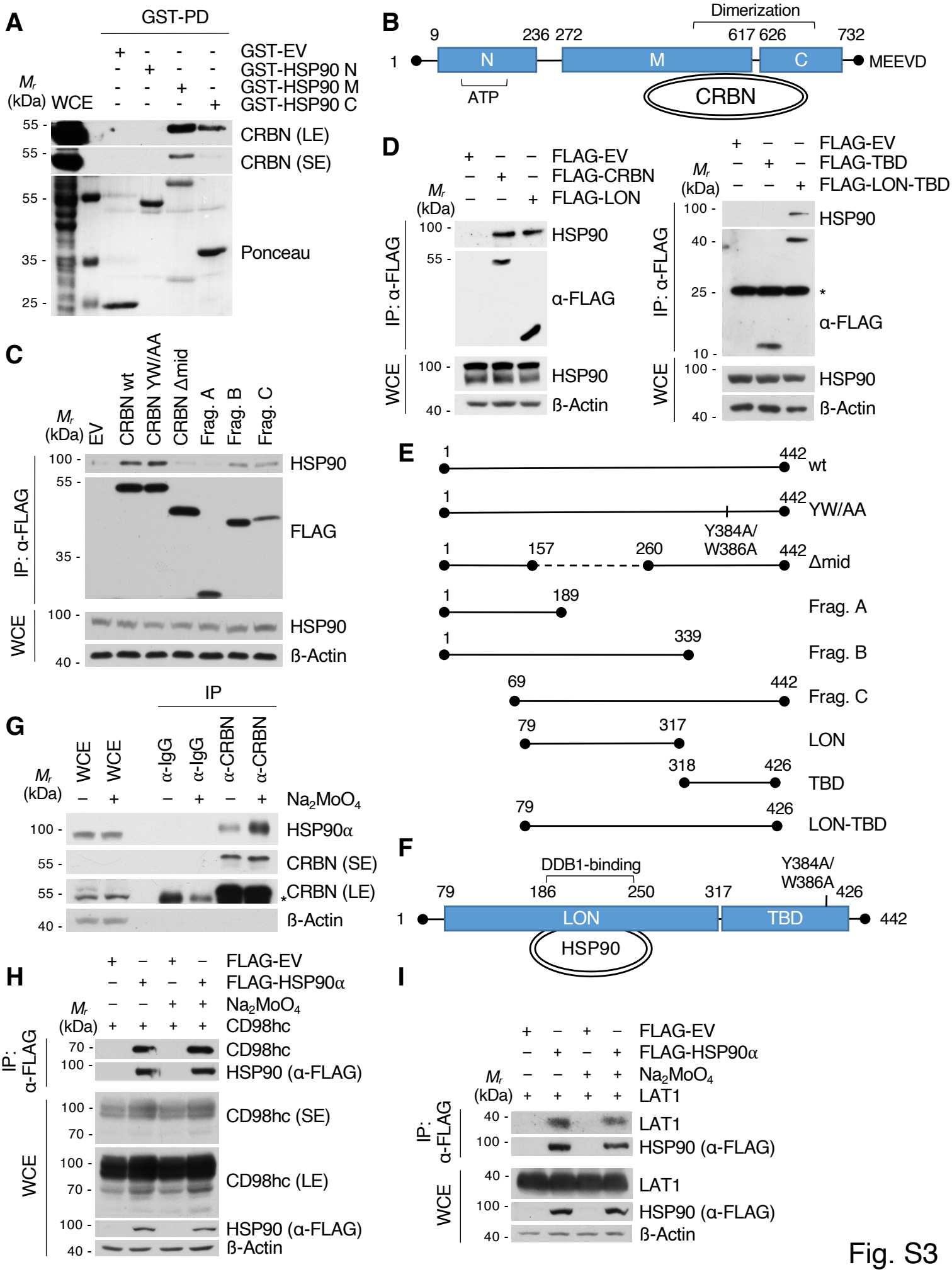

A
GST-PD
GST-EV
GST-HSP90 N
GST-HSP90 M
GST-HSP90 C
Mr (kDa)
WCE
CRBN (LE)
CRBN (SE)
Ponceau
B
Dimerization
N
M
C
MEEVD
ATP
CRBN
C
CRBN wt
CRBN YW/AA
CRBN Δmid
Frag. A
Frag. B
Frag. C
IP: α-FLAG
WCE
HSP90
FLAG
ß-Actin
D
FLAG-EV
FLAG-CRBN
FLAG-LON
FLAG-TBD
FLAG-LON-TBD
α-FLAG
E
wt
YW/AA
Y384A/
W386A
Δmid
Frag. A
Frag. B
Frag. C
LON
TBD
LON-TBD
F
DDB1-binding
LON
TBD
HSP90
G
IP
α-IgG
α-CRBN
Na2MoO4
HSP90α
CRBN (SE)
CRBN (LE)
H
FLAG-EV
FLAG-HSP90α
CD98hc
HSP90 (α-FLAG)
CD98hc (SE)
CD98hc (LE)
I
LAT1

Fig. S3

Figure S3. Mapping of the HSP90-CRBN interaction (related to Figure 2)
(A) GST pull-down analysis, as determined by immunoblotting, of the indicated GST-tagged HSP90 domains (N: aa 9-236, M: aa 272-617, C: aa 626-732) from WCEs of MM1S cells. (B) Schematic representation of the CRBN-HSP90 binding site. (C) IP analysis of the indicated FLAG-CRBN mutants and fragments transfected into $CRBN^{-/-}$ HEK293T cells. (D) *left panel:* IP analysis of full length FLAG-CRBN and FLAG-LON domain transfected into $CRBN^{-/-}$ HEK293T. *right panel*: IP analysis of FLAG-TBD (thalidomide binding domain) and FLAG-LON-TBD transfected into $CRBN^{-/-}$ HEK293T. (E) Schematic representation of various FLAG-CRBN constructs used in (C) and (D). (F) Schematic representation of HSP90-CRBN binding. (G) Endogenous IP analysis of CRBN from WCEs of U266 cells. Lysis and IP was performed in absence or presence of 20mM sodium molybdate. The asterisk denotes an unspecific band. (H) IP analysis of FLAG-HSP90α transfected into HEK293T cells co-expressing CD98hc. Lysis and IP was performed in absence or presence of 20mM sodium molybdate. (I) IP analysis of FLAG-HSP90α transfected into HEK293T cells co-expressing LAT1. Lysis and IP was performed in absence or presence of 20mM sodium molybdate.

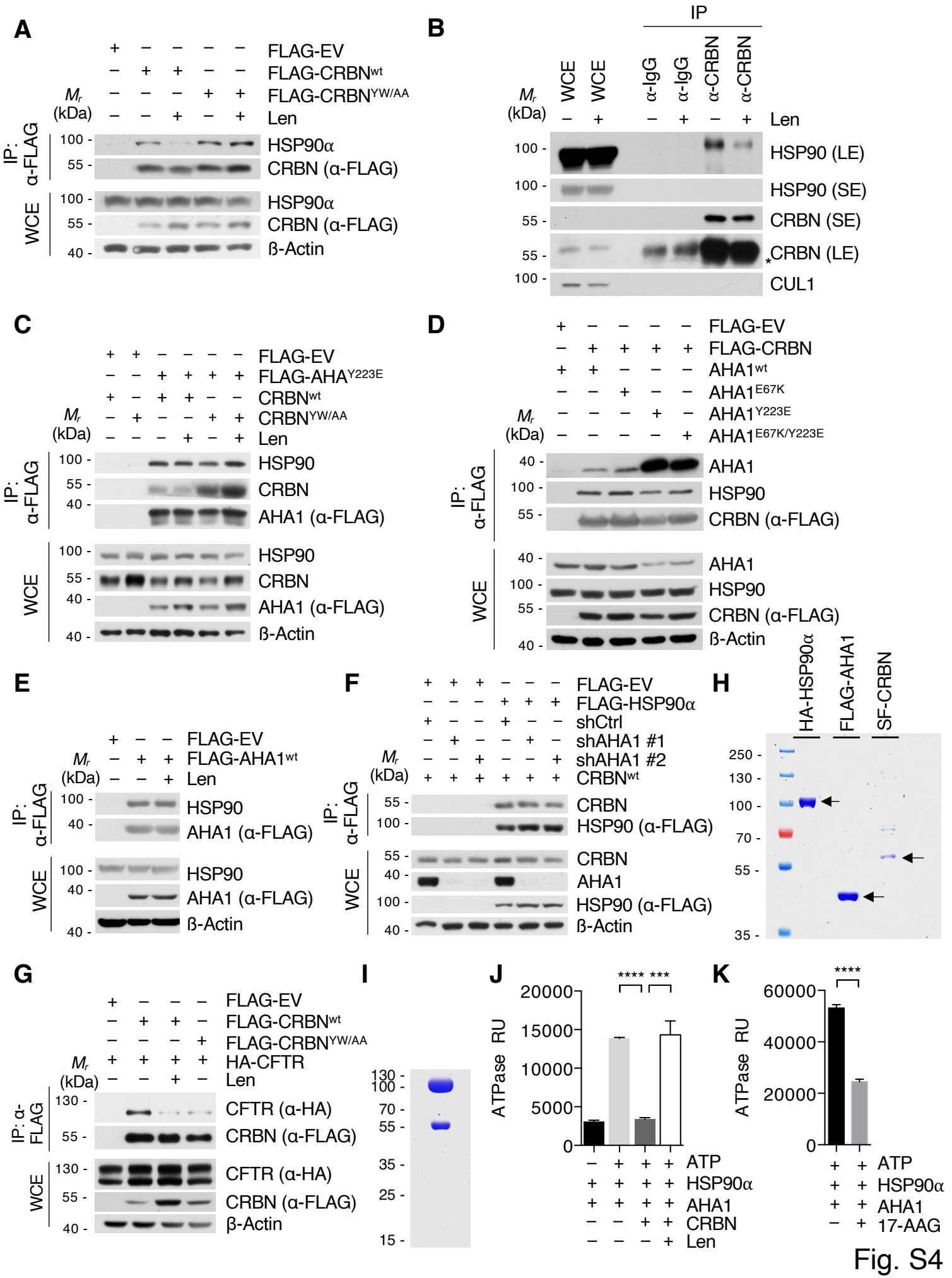

A
FLAG-EV
FLAG-CRBNwt
FLAG-CRBNYW/AA
Mr (kDa)
Len
IP: α-FLAG
HSP90α
CRBN (α-FLAG)
WCE
HSP90α
CRBN (α-FLAG)
ß-Actin
B
IP
WCE
α-IgG
α-CRBN
Len
HSP90 (LE)
HSP90 (SE)
CRBN (SE)
CRBN (LE)
CUL1
C
FLAG-EV
FLAG-AHAY223E
CRBNwt
CRBNYW/AA
Len
HSP90
CRBN
AHA1 (α-FLAG)
ß-Actin
D
FLAG-EV
FLAG-CRBN
AHA1wt
AHA1E67K
AHA1Y223E
AHA1E67K/Y223E
AHA1
HSP90
CRBN (α-FLAG)
ß-Actin
E
FLAG-EV
FLAG-AHA1wt
Len
HSP90
AHA1 (α-FLAG)
ß-Actin
F
FLAG-EV
FLAG-HSP90α
shCtrl
shAHA1 #1
shAHA1 #2
CRBNwt
CRBN
HSP90 (α-FLAG)
AHA1
ß-Actin
H
HA-HSP90α
FLAG-AHA1
SF-CRBN
G
FLAG-EV
FLAG-CRBNwt
FLAG-CRBNYW/AA
HA-CFTR
Len
CFTR (α-HA)
CRBN (α-FLAG)
ß-Actin
I
J
ATPase RU
****
***
ATP
HSP90α
AHA1
CRBN
Len
K
ATPase RU
****
ATP
HSP90α
AHA1
17-AAG

Fig. S4

Figure S4. CRBN modulates HSP90-AHA1 activity towards client proteins (panels A and B related to Figure 2, panels C-K related to Figure 3)

(A) IP analysis of FLAG-CRBN^{wt} or CRBN$^{YW/AA}$ from the lentivirally infected and sorted human MM cell line U266. Cells were pretreated with lenalidomide (10μM) or DMSO for 72 hours prior to harvest. (B) Endogenous IP analysis of CRBN from WCEs of MM1S cells in low salt buffer. Cells were pretreated with lenalidomide (10μM) or DMSO for 72 hours prior to harvest. The asterisk denotes an unspecific band. (C) IP analysis of FLAG-AHA^{Y223E} transfected into CRBN$^{-/-}$ HEK293T reconstituted with CRBN^{wt} or CRBN$^{YW/AA}$ in low salt buffer. Cells were pretreated with lenalidomide (50μM) or DMSO for 24 hours prior to harvest. (D) IP analysis of FLAG-CRBN transfected into HEK293T cells co-expressing AHA1^{wt} or various AHA1 mutants in low salt buffer. (E) IP analysis of FLAG-AHA1^{wt} transfected into HEK293T cells. Cells were pretreated with lenalidomide (50μM) or DMSO for 24 hours prior to harvest. (F) IP analysis of FLAG-HSP90α transfected into HEK293T cells, in which AHA1 was lentivirally silenced using the indicated shRNAs. (G) IP analysis of FLAG-CRBN^{wt} or CRBN$^{YW/AA}$ transfected into CRBN$^{-/-}$ HEK293T co-expressing HA-CFTR. Cells were pretreated with lenalidomide (50μM) or DMSO for 24 hours prior to harvest. (H) Coomassie stain of HA-HSP90, FLAG-AHA1 and STREP-FLAG-CRBN purified from 293T cells and eluted with respective peptides. Proteins are depicted with an arrow. (I) Coomassie stain of CRBN/DDB1 complex purified from insect cells. The upper band corresponds to DDB1, the lower band to CRBN (55kDa). (J) In vitro ATPase activity of HSP90 with addition of AHA1 purified from HEK293T cells. The CRBN-DDB1 complex purified from insect cells and lenalidomide (1μM) were added as indicated. (K) In vitro ATPase activity of HSP90 with addition of AHA1 purified from HEK293T cells. The HSP90 inhibitor 17-AAG was added as indicated. Data are expressed as mean ± s.d. *** $P < 0.001$, **** $P < 0.0001$ by Students t-test.

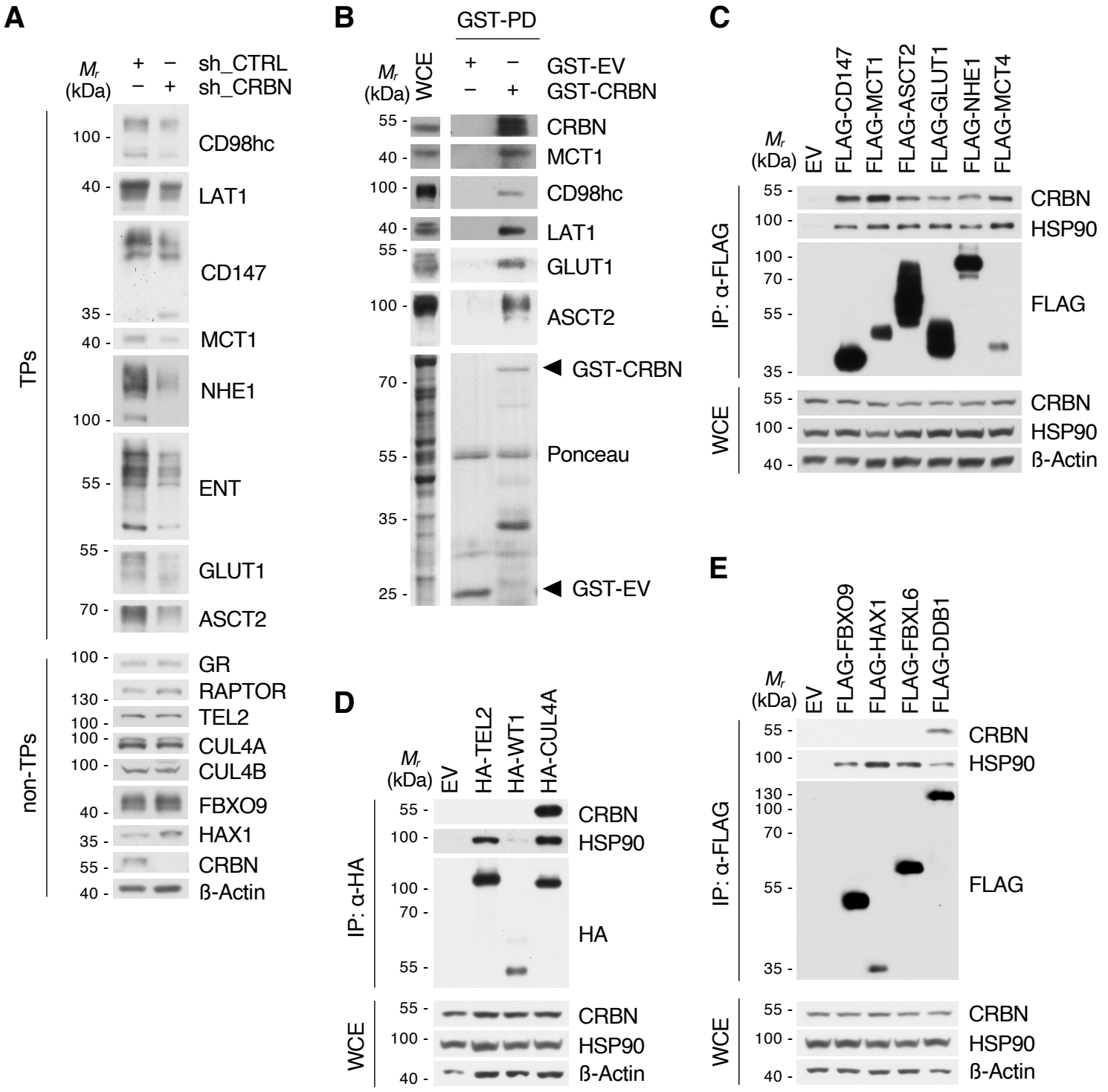


Figure S5. CRBN is a transmembrane protein-specific co-chaperone of the HSP90-AHA1 axis (related to Figure 4)

(A) Immunoblot analysis of MM1S cells, in which CRBN was lentivirally silenced with shRNAs as indicated. (B) GST pull-down analysis, as determined by immunoblotting, of GST-CRBN in WCEs of MM1S cells. (C) IP analysis of further FLAG-tagged TM clients identified in the cell surface screen (Fig. 1A) transfected into HEK293T cells. (D) IP analysis of HA-tagged non-TM HSP90 clients transfected into HEK293T cells. (E) IP analysis of FLAG-tagged non-TM HSP90 clients/interactors transfected into HEK293T cells.

Fig. S5

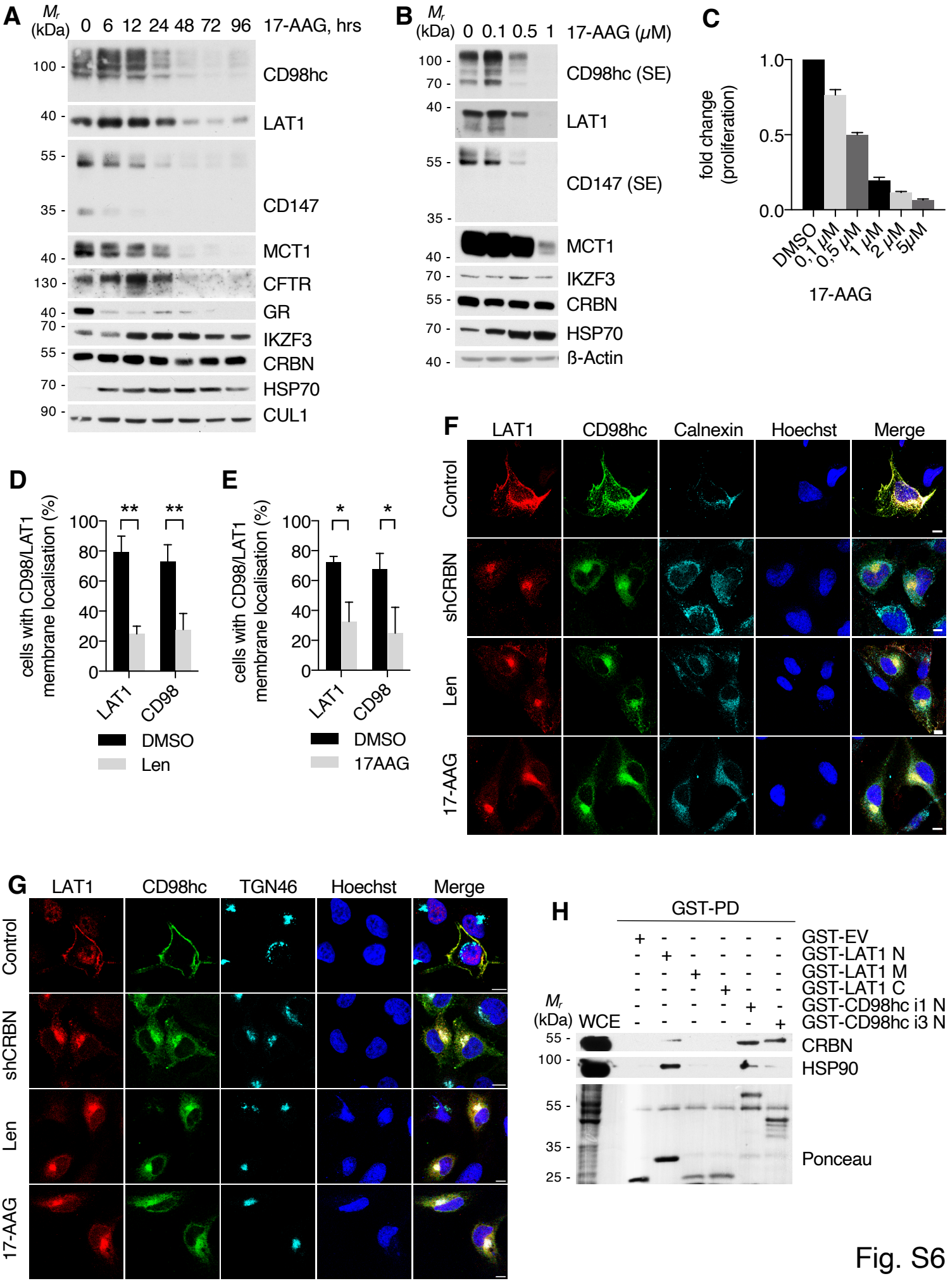

A
Mr (kDa)
0 6 12 24 48 72 96
17-AAG, hrs
100 -
CD98hc
40 -
LAT1
55 -
35 -
CD147
40 -
MCT1
130 -
CFTR
40 -
GR
70 -
IKZF3
55 -
CRBN
70 -
HSP70
90 -
CUL1
B
Mr (kDa)
0 0.1 0.5 1
17-AAG (μM)
100 -
70 -
CD98hc (SE)
40 -
LAT1
55 -
35 -
CD147 (SE)
40 -
MCT1
70 -
IKZF3
55 -
CRBN
70 -
HSP70
40 -
ß-Actin
C
fold change (proliferation)
1.0
0.5
0.0
DMSO
0,1 μM
0,5 μM
1 μM
2 μM
5μM
17-AAG
D
cells with CD98/LAT1 membrane localisation (%)
100
80
60
40
20
0
**
**
LAT1
CD98
DMSO
Len
E
cells with CD98/LAT1 membrane localisation (%)
100
80
60
40
20
0
*
*
LAT1
CD98
DMSO
17AAG
F
LAT1
CD98hc
Calnexin
Hoechst
Merge
Control
shCRBN
Len
17-AAG
G
LAT1
CD98hc
TGN46
Hoechst
Merge
Control
shCRBN
Len
17-AAG
H
GST-PD
GST-EV
GST-LAT1 N
GST-LAT1 M
GST-LAT1 C
GST-CD98hc i1 N
GST-CD98hc i3 N
Mr (kDa)
WCE
55 -
CRBN
100 -
HSP90
55 -
35 -
25 -
Ponceau

Fig. S6

Figure S6. CD98hc and LAT1 quality control requires an intact CRBN-HSP90-AHA1 axis (related to Figure 4)

(A) Immunoblot analysis of MM1S cells, which were treated with 1μM 17-AAG for the indicated times before harvest. (B) Immunoblot analysis of MM1S cells, which were treated with the indicated concentrations of 17-AAG or DMSO for 48hrs prior to harvest. (C) Cell proliferation analysis of MM1S cells, which were treated with the indicated concentrations of 17-AAG for 48hrs. (D) Quantification of the percentage of cells in Figure 4B that showed membrane localization of CD98hc and LAT1 (n = 3 independent experiments) upon treatment with 50μM lenalidomide for 16hrs. Data are mean ± s.d., ** $P < 0.01$ by Student's t-test. (E) Quantification of the percentage of cells in Figure 4B that showed membrane localization of CD98hc and LAT1 (n = 3 independent experiments) upon treatment with 50nM 17-AAG for 16hrs. Data are mean ± s.d., * $P < 0.05$ by Student's t-test. (F) and (G) Representative immunofluorescence images of HeLa cells that were transfected with constructs encoding both HA-CD98hc and FLAG-LAT1. The cells were stained with antibodies to HA (green), FLAG (red), and Calnexin (F) or TGN46 (G) (both cyan). DNA was stained with Hoechst (blue). Cells were treated with 50μM lenalidomide, 50nM 17-AAG and DMSO for 16 hours as indicated. Scale bars, 10μm. (H) GST pull-down analysis, as determined by immunoblotting, of the indicated GST-tagged intracellular fragments of CD98hc (isoform 1: aa 1-184 and isoform 3: aa 1-122) and LAT1 (N: aa 1-49, M: aa 340-395, C: aa 479-507) from MM1S WCEs.

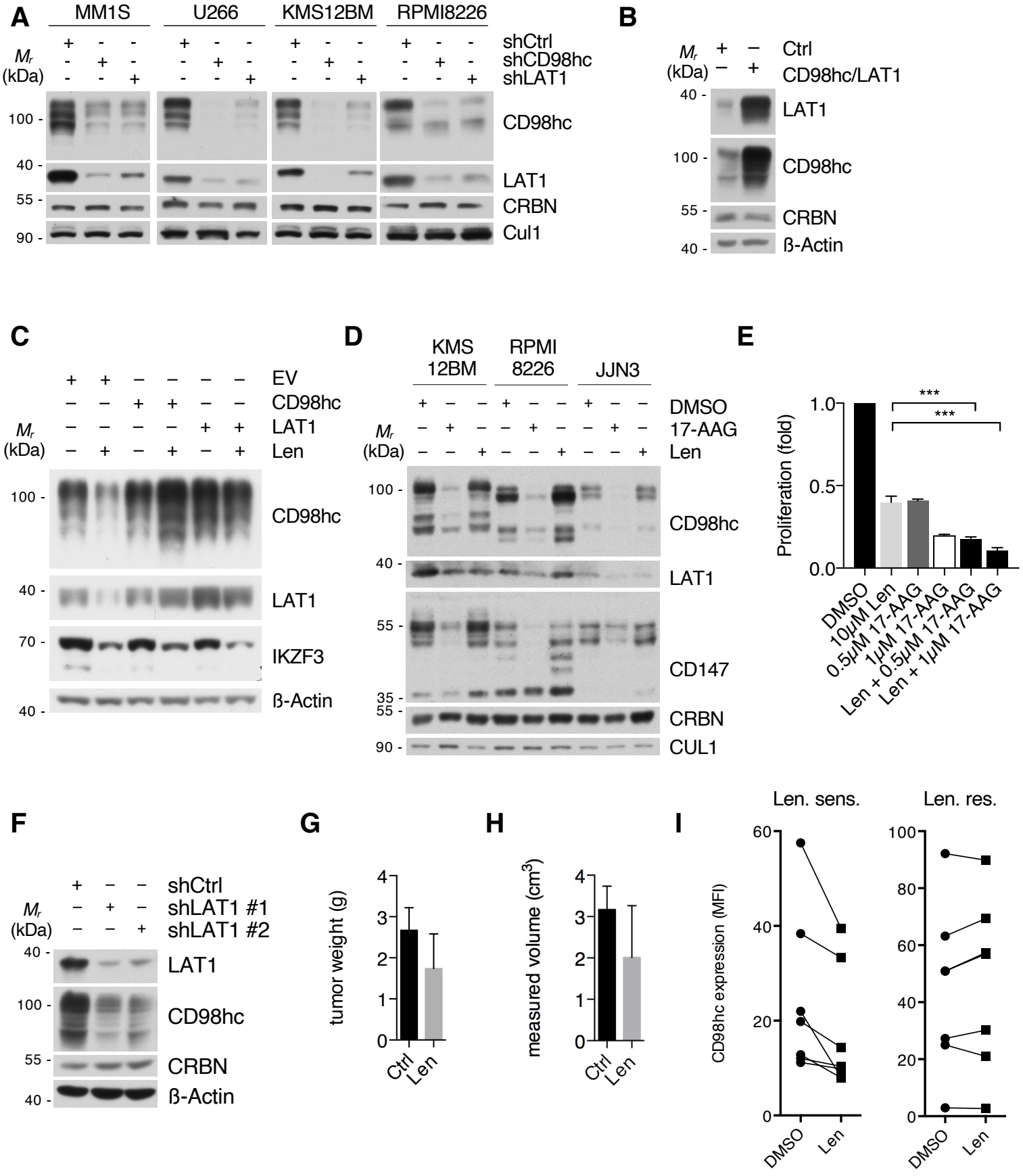

A
MM1S
U266
KMS12BM
RPMI8226
shCtrl
shCD98hc
shLAT1
Mr (kDa)
100 -
40 -
55 -
90 -
CD98hc
LAT1
CRBN
Cul1
B
Ctrl
CD98hc/LAT1
40 -
100 -
55 -
40 -
LAT1
CD98hc
CRBN
ß-Actin
C
EV
CD98hc
LAT1
Len
100 -
40 -
70 -
40 -
CD98hc
LAT1
IKZF3
ß-Actin
D
KMS 12BM
RPMI 8226
JJN3
DMSO
17-AAG
Len
100 -
40 -
55 -
35 -
55 -
90 -
CD98hc
LAT1
CD147
CRBN
CUL1
E
Proliferation (fold)
1.0
0.5
0.0
***
***
DMSO
10μM Len
0.5μM 17-AAG
1μM 17-AAG
Len + 0.5μM 17-AAG
Len + 1μM 17-AAG
F
shCtrl
shLAT1 #1
shLAT1 #2
40 -
100 -
55 -
40 -
LAT1
CD98hc
CRBN
ß-Actin
G
tumor weight (g)
4
3
2
1
0
Ctrl
Len
H
measured volume (cm3)
4
3
2
1
0
Ctrl
Len
I
Len. sens.
Len. res.
CD98hc expression (MFI)
60
40
20
0
100
80
60
40
20
0
DMSO
Len
DMSO
Len

Fig. S7

Figure S7. CD98hc/LAT1-destabilization blocks MM cell growth in vitro and in vivo and perturbs cellular amino acid metabolism (panels A-F related to Figure 5, panels G-I related to Figure 6)

(A) Immunoblot analysis of cells shown in Figure 5A, in which CD98hc or LAT1 were lentivirally silenced with the indicated shRNAs. (B) Immunoblot analysis of cells shown in Figure 5B, which were lentivirally infected with empty vector constructs (EV) or constructs to induce both CD98hc and LAT1 expression. (C) Immunoblot analysis of cells shown in Figure 5F, which were lentivirally infected with empty vector constructs (EV) or constructs to induce CD98hc or LAT1 expression. Cells were treated with DMSO or 10μM lenalidomide for 72hrs. (D) Immunoblot analysis of cells shown in Figure 5H, which were treated with 1μM 17-AAG or 10μM lenalidomide for 72hrs. (E) Cell proliferation analysis of MM1S cells, which were treated with the indicated concentrations of lenalidomide and/or 17-AAG for 96hrs. (F) Immunoblot analysis of MM1S cells, in which LAT1 was lentivirally silenced with the indicated shRNAs. Radioactive amino acid uptake analysis in these cells is presented in Figure 5J. (G-H) Metric quantification of tumors obtained after necropsy (n=5 tumors per condition) (I) Flow cytometry analysis of endogenous CD98hc cell surface expression of primary $CD38^{+}$ cells that were obtained from patients with lenalidomide-sensitive (Len. sens.) or lenalidomide-resistant (Len. res.) disease and which were cultured *in vitro* with DMSO or 10μM lenalidomide for 48 hours.