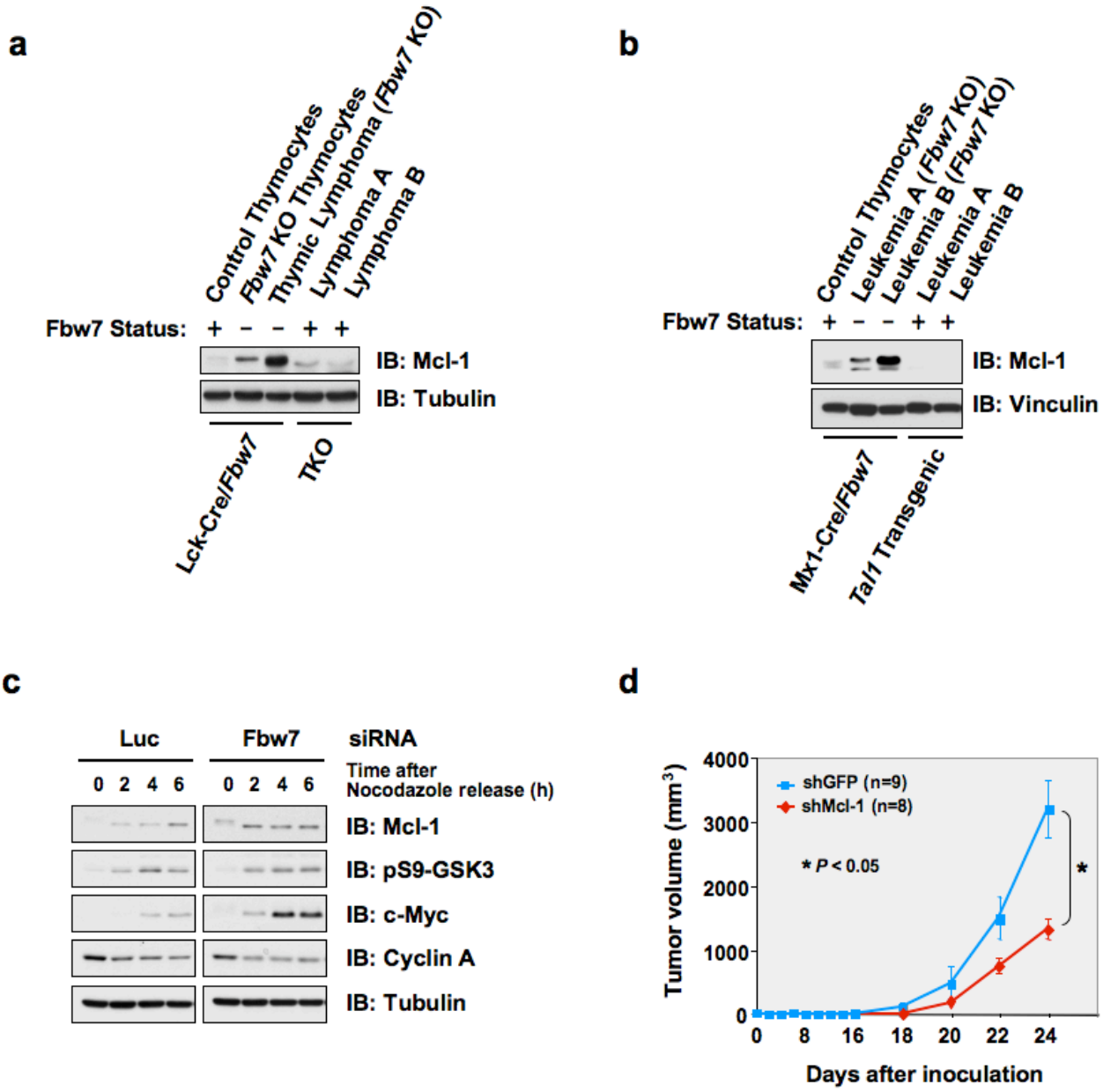


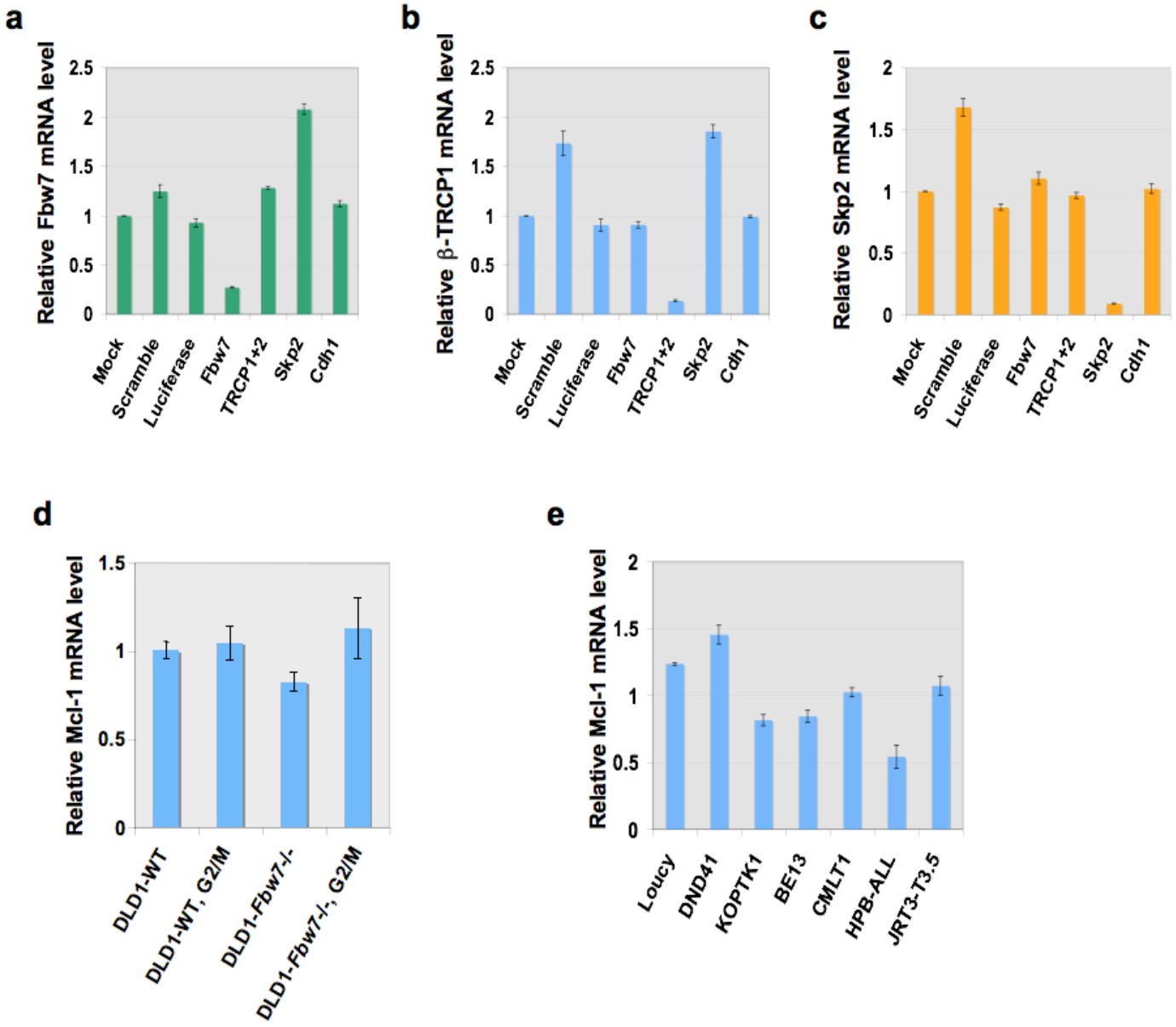
Supplementary Figure 1: Depletion of Fbw7 results in elevated Mcl-1 abundance.

a, Total thymocytes from 8-wk-old Lck-Cre/*Fbw7*$^{+/fl}$ (Control) or Lck-Cre/*Fbw7*$^{fl/fl}$ (Fbw7 KO) mice were subjected to immunoblot analysis with the indicated antibodies. Thymic lymphoma cells were from a 15-wk-old Lck-Cre/*Fbxw7*$^{fl/fl}$ (Fbw7 KO) and *Terc*$^{-/-}$*ATM*$^{-/-}$*p53*$^{-/-}$ (TKO) mice.

b, Total thymocytes from 12-wk-old Mx1-Cre/*Fbw7*$^{+/fl}$ (Control), leukemic *Fbw7* KO or *Tal1* transgenic mice were subjected to immunoblot analysis with the indicated antibodies.

c, Immunoblot analysis of HeLa cells transfected with the indicated siRNA oligos after synchronization with nocodazole and release.

d. *In vivo* effects of Mcl-1 depletion in Fbw7-deficient T-ALL cells. An *in vivo* xenograft model of Fbw7-deficient T-ALL was created by subcutaneous injection of 1.2x10^7 CMLT1 cells (CMLT1-shGFP or CMLT1-shMcl-1) in SCID mice. Tumor burden was determined by measuring the diameters of the tumor size. The tumor volume was calculated by using the formula, 1/2 x (tumor length) x (tumor width)^2. Data was represented as the mean of tumor volume (mm^3) $\pm$ SEM with statistical significance determined by Student's t-test.

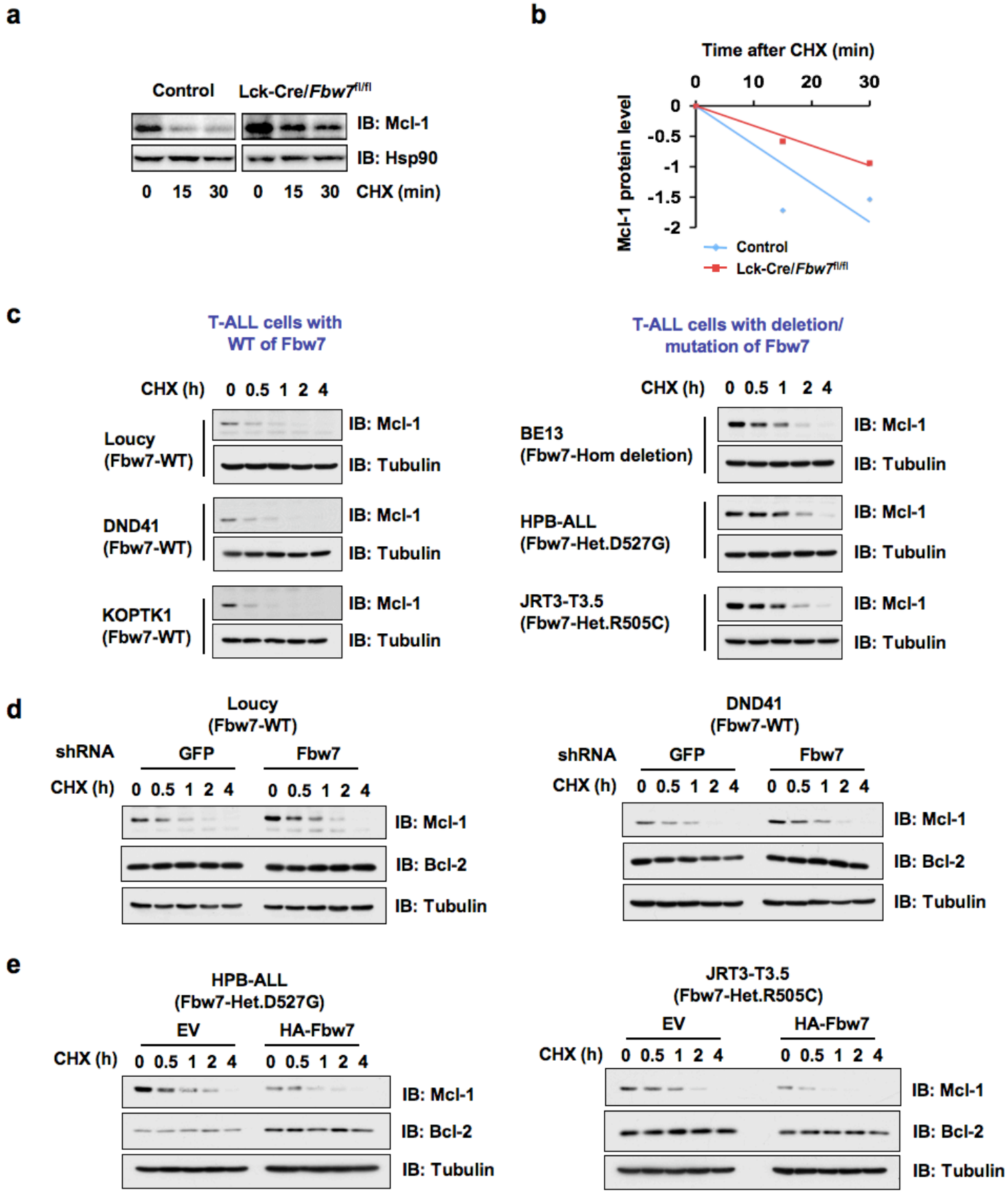


Supplementary Figure 2: Inactivation of Fbw7 does not affect Mcl-1 mRNA expression levels.

a-c, Real-time RT-PCR analysis to examine the depletion efficiency of the siRNA oligos against Fbw7 (**a**), β-TRCP1 (**b**) and Skp2 (**c**) used in Figure **1b**. Data was shown as mean ± SD for three independent experiments.

d, Real-time RT-PCR analysis to examine the relative Mcl-1 mRNA expression levels in wild-type (WT) and *Fbw7*-/- DLD1 cells. Data was shown as mean ± SD for three independent experiments.

e, Real-time RT-PCR analysis to examine the relative Mcl-1 mRNA expression levels in various T-ALL cell lines. Data was shown as mean ± SD for three independent experiments.


a
Control
Lck-Cre/Fbw7fl/fl
IB: Mcl-1
IB: Hsp90
0 15 30 0 15 30 CHX (min)
b
Time after CHX (min)
0 10 20 30
Mcl-1 protein level
0
-0.5
-1
-1.5
-2
Control
Lck-Cre/Fbw7fl/fl
c
T-ALL cells with WT of Fbw7
T-ALL cells with deletion/ mutation of Fbw7
CHX (h) 0 0.5 1 2 4
Loucy (Fbw7-WT)
DND41 (Fbw7-WT)
KOPTK1 (Fbw7-WT)
BE13 (Fbw7-Hom deletion)
HPB-ALL (Fbw7-Het.D527G)
JRT3-T3.5 (Fbw7-Het.R505C)
IB: Mcl-1
IB: Tubulin
d
Loucy (Fbw7-WT)
DND41 (Fbw7-WT)
shRNA
GFP
Fbw7
CHX (h) 0 0.5 1 2 4 0 0.5 1 2 4
IB: Mcl-1
IB: Bcl-2
IB: Tubulin
e
HPB-ALL (Fbw7-Het.D527G)
JRT3-T3.5 (Fbw7-Het.R505C)
EV
HA-Fbw7
CHX (h) 0 0.5 1 2 4 0 0.5 1 2 4
IB: Mcl-1
IB: Bcl-2
IB: Tubulin

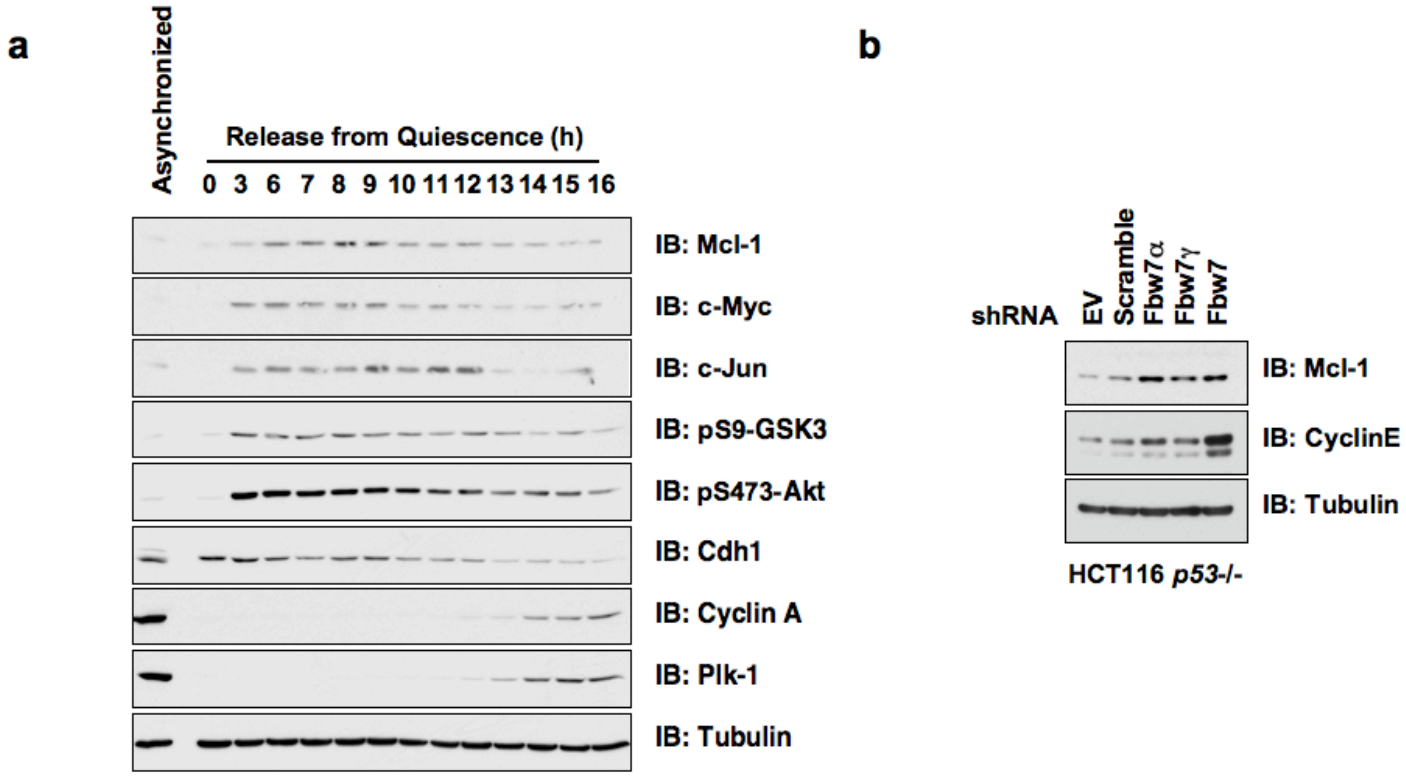
Supplementary Figure 3: Mcl-1 half-life is controlled by Fbw7.

a-b, The thymocytes from 8-wk-old Lck-Cre/*Fbw7*$^{+/fl}$ (Control) or Lck-Cre/*Fbw7*$^{fl/fl}$ (*Fbw7* KO) were treated with 100 μg/ml cycloheximide (CHX). At the indicated time points, whole cell lysates were prepared and immunoblots were probed with the indicated antibodies (**a**). Band intensity was measured, normalized by that of Hsp90, and expressed as a percentage of the corresponding normalized value for time zero (**b**).

c, The indicated T-ALL cell lines were treated with 20 μg/ml cycloheximide (CHX). At the indicated time points, whole cell lysates were prepared and immunoblots were probed with the indicated antibodies.

d, DND41 and Loucy cells, which contain wild-type Fbw7, were infected with the indicated lentiviral shRNA construct and selected with 1 μg/ml puromycin to eliminate the non-infected cells. Afterwards, the indicated cell lines were treated with 20 μg/ml cycloheximide (CHX). At the indicated time points, whole cell lysates were prepared and immunoblots were probed with the indicated antibodies.

e, HPB-ALL and JRT3-T3.5 cells with deficient Fbw7 were infected with the Fbw7-expressing retrovirus construct (or an empty vector as a negative control) and selected with 1 μg/ml puromycin to eliminate the non-infected cells. Afterwards, the indicated cell lines were treated with 20 μg/ml cycloheximide (CHX). At the indicated time points, whole cell lysates were prepared and immunoblots were probed with the indicated antibodies.

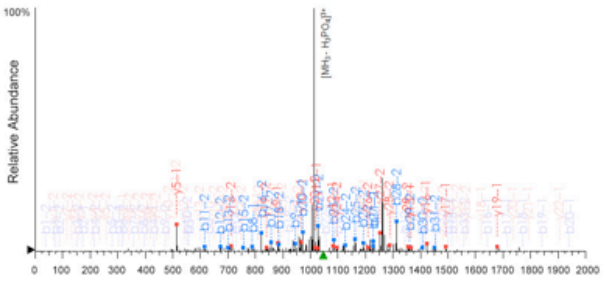


Supplementary Figure 4: Endogenous Mcl-1 levels inversely correlate with GSK3 activity during cell cycle progression, and Fbw7 depletion-induced Mcl-1 upregulation is p53 independent.

a. Immunoblot analysis of T98G cells induced to enter the G0 phase by serum starvation for 72 hours and then released for the indicated time periods.

b, Immunoblot analysis of HCT116 *p53*-/- cells transfected with the indicated shRNA constructs.

a



Predicted Fragmentation Pattern

Seq	#	b (+1)	y (+1)		b (+2)	y (+2)	
A	1	72.05	---	33	36.53	---	33
T	2	173.09	3063.44	32	87.05	1532.23	32
E	3	302.14	2962.40	31	151.57	1481.70	31
K	4	430.23	2833.35	30	215.62	1417.18	30
E	5	559.27	2705.26	29	280.14	**1353.13**	29
A	6	630.31	2576.22	28	315.66	**1288.61**	28
S	7	717.33	2505.18	27	359.17	**1253.09**	27
A	8	**788.37**	2418.16	26	394.69	**1209.58**	26
R	9	**944.47**	2347.12	25	472.74	1174.06	25
R	10	1100.57	2191.02	24	550.79	**1096.01**	24
E	11	**1229.61**	2034.92	23	**615.31**	**1017.96**	23
I	12	1342.70	1905.87	22	**671.85**	953.44	22
G	13	1399.72	1792.79	21	**700.36**	896.90	21
G	14	1456.74	1735.77	20	728.87	868.39	20
G	15	1513.76	**1678.75**	19	**757.39**	**839.88**	19
E	16	1642.81	1621.73	18	**821.91**	811.37	18
A	17	1713.84	**1492.68**	17	**857.43**	746.85	17
G	18	1770.86	**1421.65**	16	**885.94**	**711.33**	16
A	19	1841.90	**1364.62**	15	921.45	682.82	15
V	20	1940.97	1293.59	14	**970.99**	647.30	14
I	21	2054.05	1194.52	13	**1027.53**	597.76	13
G	22	2111.08	**1081.43**	12	1056.04	541.22	12
G	23	2168.10	**1024.41**	11	**1084.55**	512.71	11
S	24	2255.12	**967.39**	10	**1128.06**	484.20	10
A	25	2326.16	**880.37**	9	**1163.58**	440.69	9
G	26	2383.18	809.33	8	**1192.09**	405.17	8
A	27	2454.22	752.31	7	**1227.61**	376.66	7
S#	28	2621.21	681.27	6	**1311.11**	341.14	6
P	29	2718.26	**514.28**	5	**1359.63**	257.65	5
P	30	2815.31	417.23	4	**1408.16**	209.12	4
S	31	2902.33	320.18	3	**1451.67**	160.59	3
T	32	3003.38	233.15	2	1502.19	117.08	2
L	33	---	132.11	1	---	66.56	1

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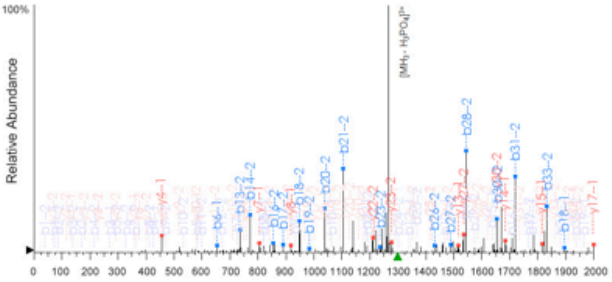
Ascore = 16.58

b



Predicted Fragmentation Pattern

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P	2	169.10	3820.69	34	85.05	1910.85	34
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R	4	426.25	3622.59	32	213.63	1811.80	32
R	5	582.35	3466.49	31	291.68	1733.75	31
A	6	**653.38**	3310.39	30	327.20	**1655.70**	30
A	7	724.42	3239.35	29	362.71	1620.18	29
P	8	821.47	3168.32	28	411.24	1584.66	28
L	9	934.56	3071.26	27	467.78	**1536.14**	27
E	10	1063.60	2958.18	26	532.30	1479.59	26
E	11	1192.64	2829.14	25	596.83	1415.07	25
M*	12	1339.68	2700.09	24	670.34	1350.55	24
E	13	1468.72	2553.06	23	**734.86**	**1277.03**	23
A	14	1539.76	2424.02	22	**770.38**	**1212.51**	22
P	15	1636.81	2352.98	21	818.91	1176.99	21
A	16	1707.85	2255.93	20	**854.43**	1128.47	20
A	17	1778.89	2184.89	19	**889.95**	1092.95	19
D	18	**1893.91**	2113.85	18	**947.46**	1057.43	18
A	19	1964.95	**1998.83**	17	**982.98**	999.92	17
I	20	2078.03	1927.79	16	**1039.52**	964.40	16
M	21	2209.07	**1814.70**	15	**1105.04**	907.86	15
S#	22	2376.06	**1683.66**	14	1188.54	842.34	14
P	23	2473.12	**1516.67**	13	**1237.06**	758.84	13
E	24	2602.16	1419.62	12	1301.58	710.31	12
E	25	2731.20	1290.58	11	1366.10	645.79	11
E	26	2860.24	1161.54	10	**1430.63**	581.27	10
L	27	2973.33	1032.49	9	**1487.17**	516.75	9
D	28	3088.36	**919.41**	8	**1544.68**	460.21	8
G	29	3145.38	**804.38**	7	1573.19	402.70	7
Y	30	3308.44	747.36	6	**1654.72**	374.18	6
E	31	3437.48	584.30	5	**1719.25**	292.65	5
P	32	3534.54	**455.26**	4	1767.77	228.13	4
E	33	3663.58	358.20	3	**1832.29**	179.61	3
P	34	3760.63	229.16	2	1880.82	115.08	2
L	35	---	132.11	1	---	66.56	1

APTRRAPLEEMEAPAADAIMS(p)PEEELDGYEPEPL

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XCorr = 2.864
Ascore = 19.00

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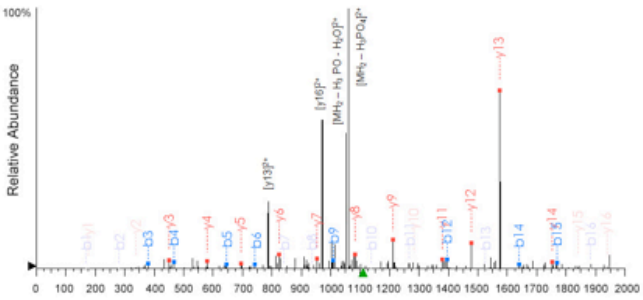

Predicted Fragmentation Pattern

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S	4	**465.16**	1841.74	15
T#	5	**646.17**	**1754.72**	14
P	6	**743.22**	**1573.71**	13
P	7	840.28	**1476.65**	12
P	8	937.33	**1379.60**	11
A	9	**1008.37**	1282.55	10
E	10	1137.41	**1211.51**	9
E	11	1266.45	**1082.47**	8
E	12	**1395.49**	**953.43**	7
E	13	1524.54	**824.38**	6
D	14	**1639.56**	**695.34**	5
E	15	**1768.61**	**580.31**	4
L	16	1881.69	**451.27**	3
Y	17	2044.75	338.19	2
R	18	---	175.12	1

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XCorr = 3.964
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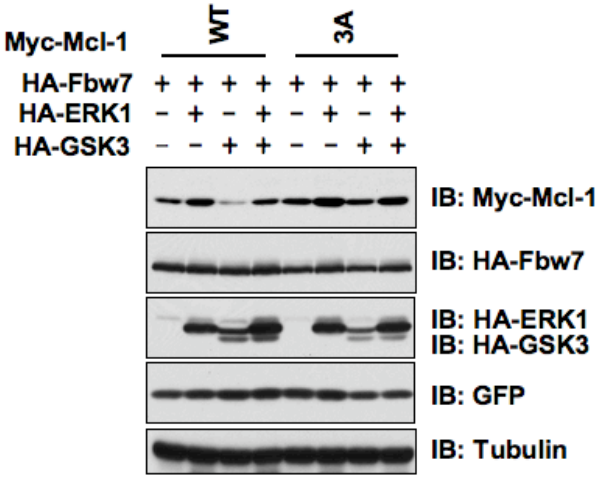
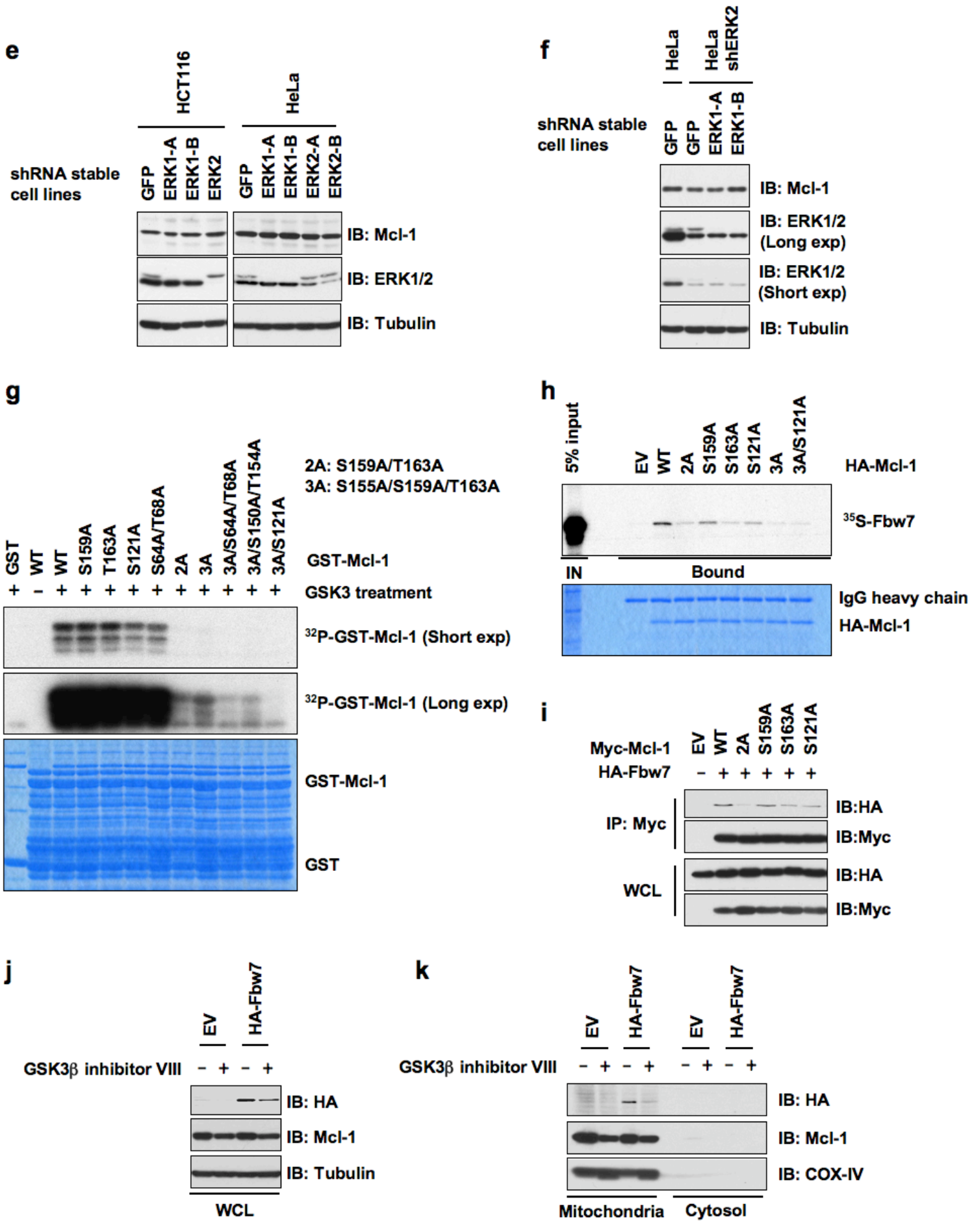
d

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HCT116
HeLa
shRNA stable cell lines
GFP
ERK1-A
ERK1-B
ERK2
GFP
ERK1-A
ERK1-B
ERK2-A
ERK2-B
IB: Mcl-1
IB: ERK1/2
IB: Tubulin
f
HeLa
HeLa shERK2
shRNA stable cell lines
GFP
GFP
ERK1-A
ERK1-B
IB: Mcl-1
IB: ERK1/2 (Long exp)
IB: ERK1/2 (Short exp)
IB: Tubulin
g
2A: S159A/T163A
3A: S155A/S159A/T163A
+ GST
– WT
+ WT
+ S159A
+ T163A
+ S121A
+ S64A/T68A
+ 2A
+ 3A
+ 3A/S64A/T68A
+ 3A/S150A/T154A
+ 3A/S121A
GST-Mcl-1
GSK3 treatment
32P-GST-Mcl-1 (Short exp)
32P-GST-Mcl-1 (Long exp)
GST-Mcl-1
GST
h
5% input
EV
WT
2A
S159A
S163A
S121A
3A
3A/S121A
HA-Mcl-1
35S-Fbw7
IN
Bound
IgG heavy chain
HA-Mcl-1
i
Myc-Mcl-1
HA-Fbw7
EV
WT
2A
S159A
S163A
S121A
– + + + + +
IP: Myc
IB:HA
IB:Myc
WCL
IB:HA
IB:Myc
j
EV
HA-Fbw7
GSK3β inhibitor VIII – + – +
IB: HA
IB: Mcl-1
IB: Tubulin
WCL
k
EV
HA-Fbw7
EV
HA-Fbw7
GSK3β inhibitor VIII – + – + – + – +
IB: HA
IB: Mcl-1
IB: COX-IV
Mitochondria
Cytosol

Supplementary Figure 5: Phosphorylation of Mcl-1 by GSK3 triggers Mcl-1/Fbw7 interaction.

a-c, Detection of *in vivo* Mcl-1 phosphorylation status by mass spectrum analysis. HA-Mcl-1 was transfected into 293T cells, then immunoprecipitated with anti-HA in the presence of phosphatase inhibitors. The immunoprecipitate was resolved by SDS-PAGE and phosphorylation was detected by mass spectrum analysis. The Ser64 site (**a)**, Ser121 site (**b)**, Ser159 and Thr163 sites (**c)** were detected to be phosphorylated *in vivo*.

d, Immunoblot analysis of 293T cells transfected with the indicated Myc-Mcl-1 and HA-Fbw7 plasmids in the presence or absence of HA-GSK3 and/or HA-ERK1. A plasmid encoding GFP was used as a negative control for transfection efficiency.

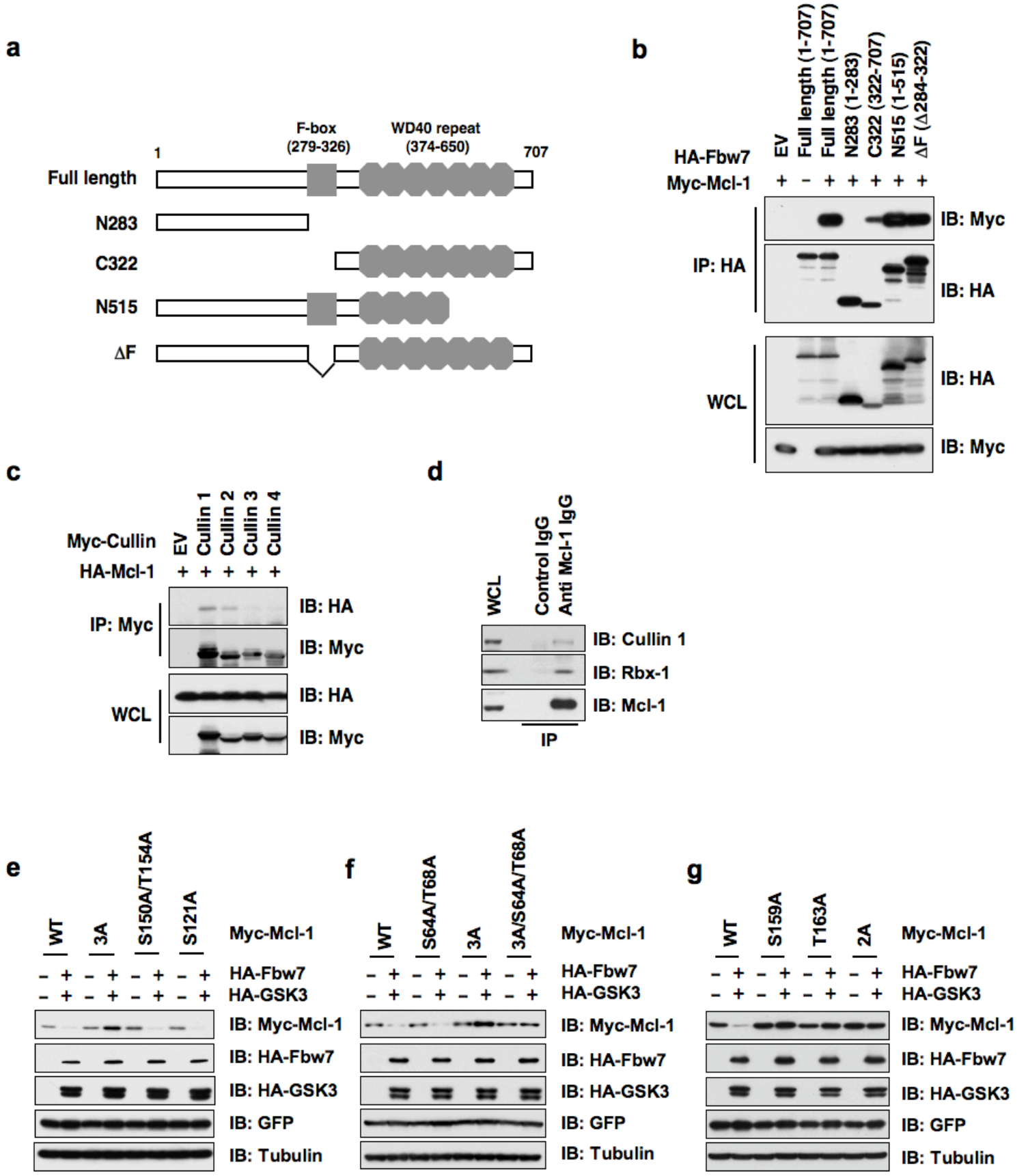
e-f, HeLa or HCT116 cells were infected with the indicated lentiviral shRNA constructs (with shGFP as a negative control) and selected with 1 μg/ml puromycin to eliminate the non-infected cells. Whole cell lystates were collected for immunoblot analysis.

g, GSK3 phosphorylates Mcl-1 *in vitro* at multiple sites. Purified GSK3 protein (from New England Biolabs) was incubated with 5 μg of the indicated GST-Mcl-1 proteins in the presence of γ-^{32}P-ATP. The kinase reaction products were resolved by SDS-PAGE and phosphorylation was detected by autoradiography.

h, Phosphorylation of Mcl-1 at multiple sites *in vivo* triggers its interaction with Fbw7 *in vitro.* Autoradiograms showing recovery of ^{35}S-labeled Fbw7 protein bound to the indicated HA-Mcl-1 proteins immunoprecipitated from 293T cells. IN, input (5% as indicated).

i, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with HA-Fbw7 together with the indicated Myc-Mcl-1 constructs. Thirty hours post-transfection, cells were pretreated with 10 μM MG132 for 10 hours to block the proteasome pathway before harvesting.

j-k, HeLa cells were transfected with the pcDNA3-HA-Fbw7 construct (with empty vector as a negative control) and selected with 800 μg/ml G418 to generate a cell line stably expressing HA-Fbw7. Cells were pretreated with 20 μM MG132 for 8 hours to block the proteasome pathway before harvesting. Where indicated, 25 μM of the GSK3β inhibitor VIII (with DMSO as a negative control) was added for 8 hours before harvesting for immunoblot analysis (**j**). Under the same experimental conditions, another set of cells were collected and mitochondrial and cytosolic fractions were separated by ultracentrifuge before immunoblot analysis with the indicated antibodies (**k**).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707
Full length
N283
C322
N515
ΔF
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Myc-Mcl-1
EV
Full length (1-707)
Full length (1-707)
N283 (1-283)
C322 (322-707)
N515 (1-515)
ΔF (Δ284-322)
IP: HA
IB: Myc
IB: HA
WCL
IB: HA
IB: Myc
c
Myc-Cullin
HA-Mcl-1
EV
Cullin 1
Cullin 2
Cullin 3
Cullin 4
IP: Myc
IB: HA
IB: Myc
WCL
IB: HA
IB: Myc
d
WCL
Control IgG
Anti Mcl-1 IgG
IB: Cullin 1
IB: Rbx-1
IB: Mcl-1
IP
e
WT
3A
S150A/T154A
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HA-Fbw7
HA-GSK3
IB: Myc-Mcl-1
IB: HA-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin
f
WT
S64A/T68A
3A
3A/S64A/T68A
Myc-Mcl-1
HA-Fbw7
HA-GSK3
IB: Myc-Mcl-1
IB: HA-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin
g
WT
S159A
T163A
2A
Myc-Mcl-1
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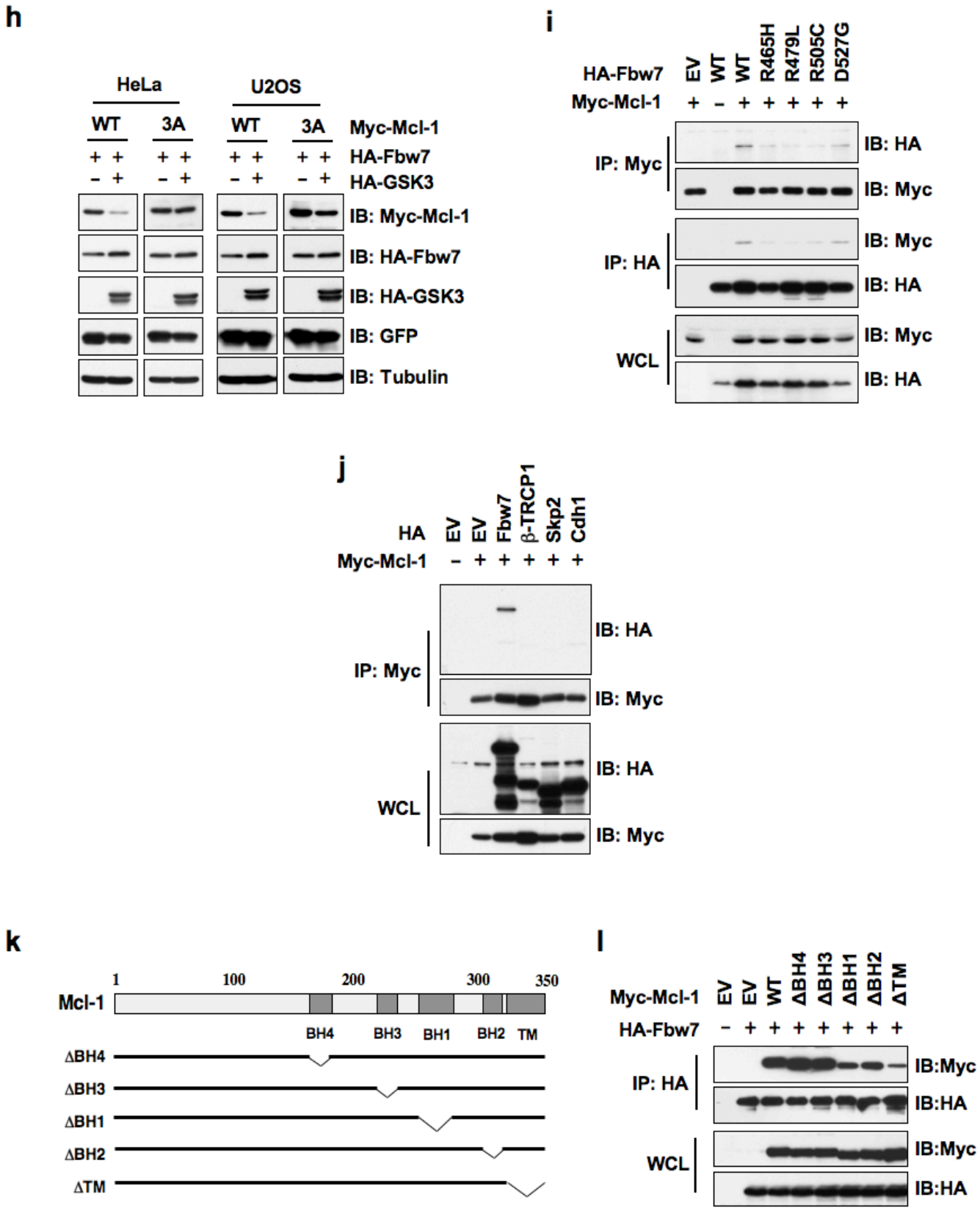
IB: HA-GSK3
IB: GFP
IB: Tubulin


h
HeLa
U2OS
WT
3A
WT
3A
Myc-Mcl-1
HA-Fbw7
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IB: HA-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin
i
HA-Fbw7
EV
WT
WT
R465H
R479L
R505C
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WCL
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IB: HA
j
HA
EV
EV
Fbw7
β-TRCP1
Skp2
Cdh1
Myc-Mcl-1
IP: Myc
WCL
IB: HA
IB: Myc
IB: HA
IB: Myc
k
1
100
200
300
350
Mcl-1
BH4
BH3
BH1
BH2
TM
ΔBH4
ΔBH3
ΔBH1
ΔBH2
ΔTM
l
Myc-Mcl-1
EV
EV
WT
ΔBH4
ΔBH3
ΔBH1
ΔBH2
ΔTM
HA-Fbw7
IP: HA
WCL
IB:Myc
IB:HA
IB:Myc
IB:HA

Supplementary Figure 6: Mcl-1 interacts specifically with Cullin 1 and Fbw7 *in vivo*.

a, Illustration of the various Fbw7 deletion constructs used in **b**.

b, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with Myc-Mcl-1 and various HA-tagged Fbw7 constructs. Twenty hours post-transfection, cells were treated with 10 μM MG132 overnight before harvesting.

c, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with HA-Mcl-1 and various Myc-tagged Cullin constructs. Twenty hours post-transfection, cells were treated with 10 μM MG132 overnight before harvesting.

d, Immunoblot (IB) analysis of HEK-293 whole cell lysates (WCL) and anti-Mcl-1 immunoprecipitates (IP). Mouse IgG was used as a negative control for the immunoprecipitation procedure. Cells were treated with 10 μM MG132 overnight before harvesting.

e-g, Immunoblot analysis of 293T cells transfected with the indicated Myc-Mcl-1 and HA-Fbw7 plasmids in the presence or absence of HA-GSK3. A plasmid encoding GFP was used as a negative control for transfection efficiency.

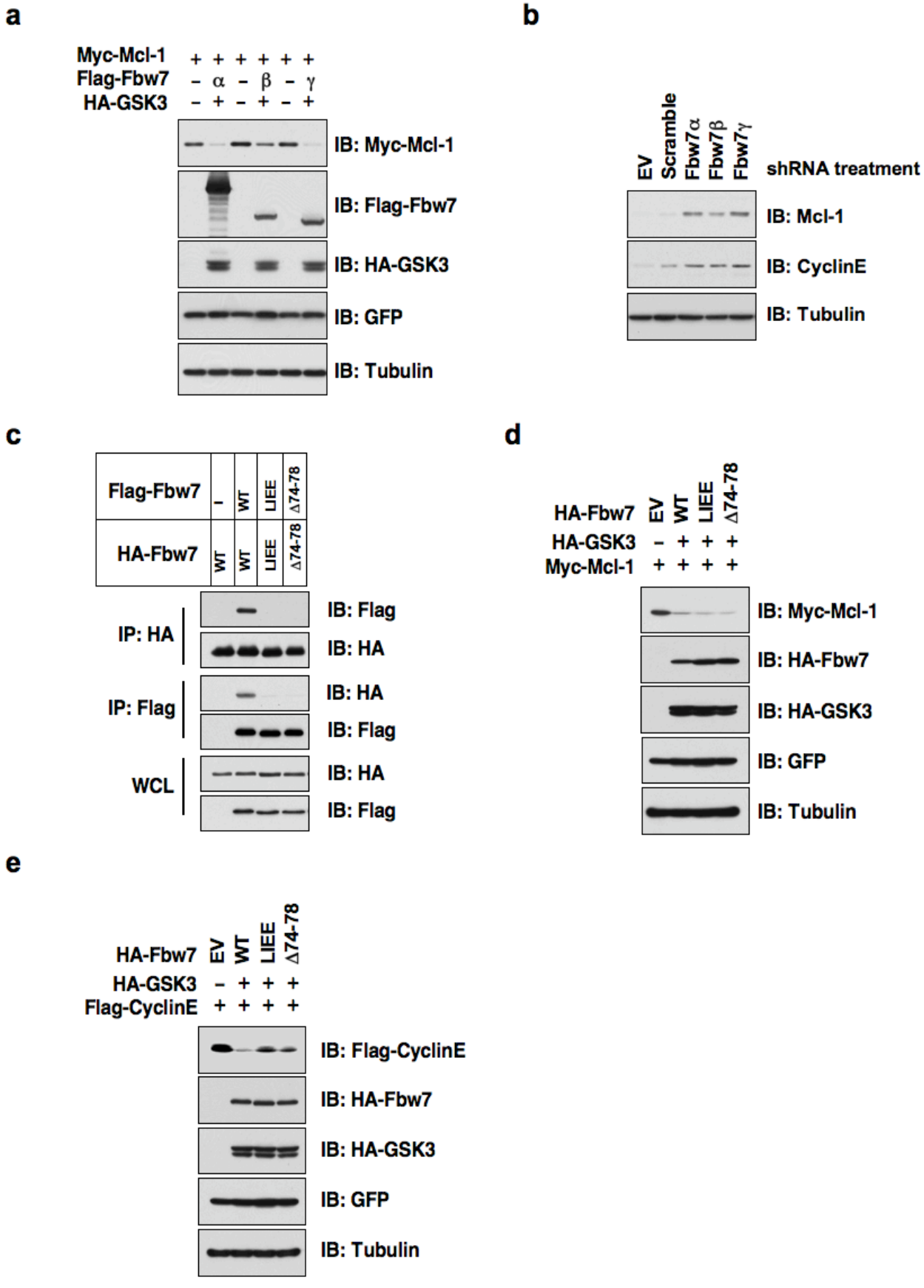
h, Immunoblot analysis of HeLa or U2OS cells transfected with the indicated Myc-Mcl-1 and HA-Fbw7 plasmids in the presence or absence of HA-GSK3. A plasmid encoding GFP was used as a negative control for transfection efficiency.

i, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with HA-tagged Fbw7 and the indicated Myc-Mcl-1 constructs. Twenty hours post-transfection, cells were treated with 330 nM Nocodazole for 18 hours to arrest cells in the M phase and 25 μM MG132 for 8 hours before harvesting.

j, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with Myc-Mcl-1 and the indicated HA-tagged F-box protein constructs (or HA-Cdh1 as a negative control). Twenty hours post-transfection, cells were treated with 330 nM Nocodazole for 18 hours to arrest cells in the M phase and 25 μM MG132 for 8 hours before harvesting.

k, Illustration of the various Mcl-1 deletion constructs used in **l**.

l, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with HA-Fbw7 and various Myc-Mcl-1 constructs. Twenty hours post-transfection, cells were treated with 10 μM MG132 overnight before harvesting. IP analyses were performed to demonstrate the role of the individual BH domains and the transmembrane domain in mediating Mcl-1/Fbw7 interaction. Deletion of the BH3 or BH4 domains was found to have no effect on Mcl-1/Fbw7 interaction, and deletion of the BH1 or BH2 domains only moderately decreased Mcl-1/Fbw7 interaction. On the other hand, deletion of the transmembrane domain has a more dramatic effect on Mcl-1/Fbw7 interaction.


a
Myc-Mcl-1 + + + + + +
Flag-Fbw7 – α – β – γ
HA-GSK3 – + – + – +
IB: Myc-Mcl-1
IB: Flag-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin
b
EV
Scramble
Fbw7α
Fbw7β
Fbw7γ
shRNA treatment
IB: Mcl-1
IB: CyclinE
IB: Tubulin
c
Flag-Fbw7 – WT LIEE Δ74-78
HA-Fbw7 WT WT LIEE Δ74-78
IP: HA
IB: Flag
IB: HA
IP: Flag
IB: HA
IB: Flag
WCL
IB: HA
IB: Flag
d
HA-Fbw7 EV WT LIEE Δ74-78
HA-GSK3 – + + +
Myc-Mcl-1 + + + +
IB: Myc-Mcl-1
IB: HA-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin
e
HA-Fbw7 EV WT LIEE Δ74-78
HA-GSK3 – + + +
Flag-CyclinE + + + +
IB: Flag-CyclinE
IB: HA-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin

Supplementary Figure 7: Fbw7 isoform specificity and dimerization requirement for its ability to promote Mcl-1 destruction.

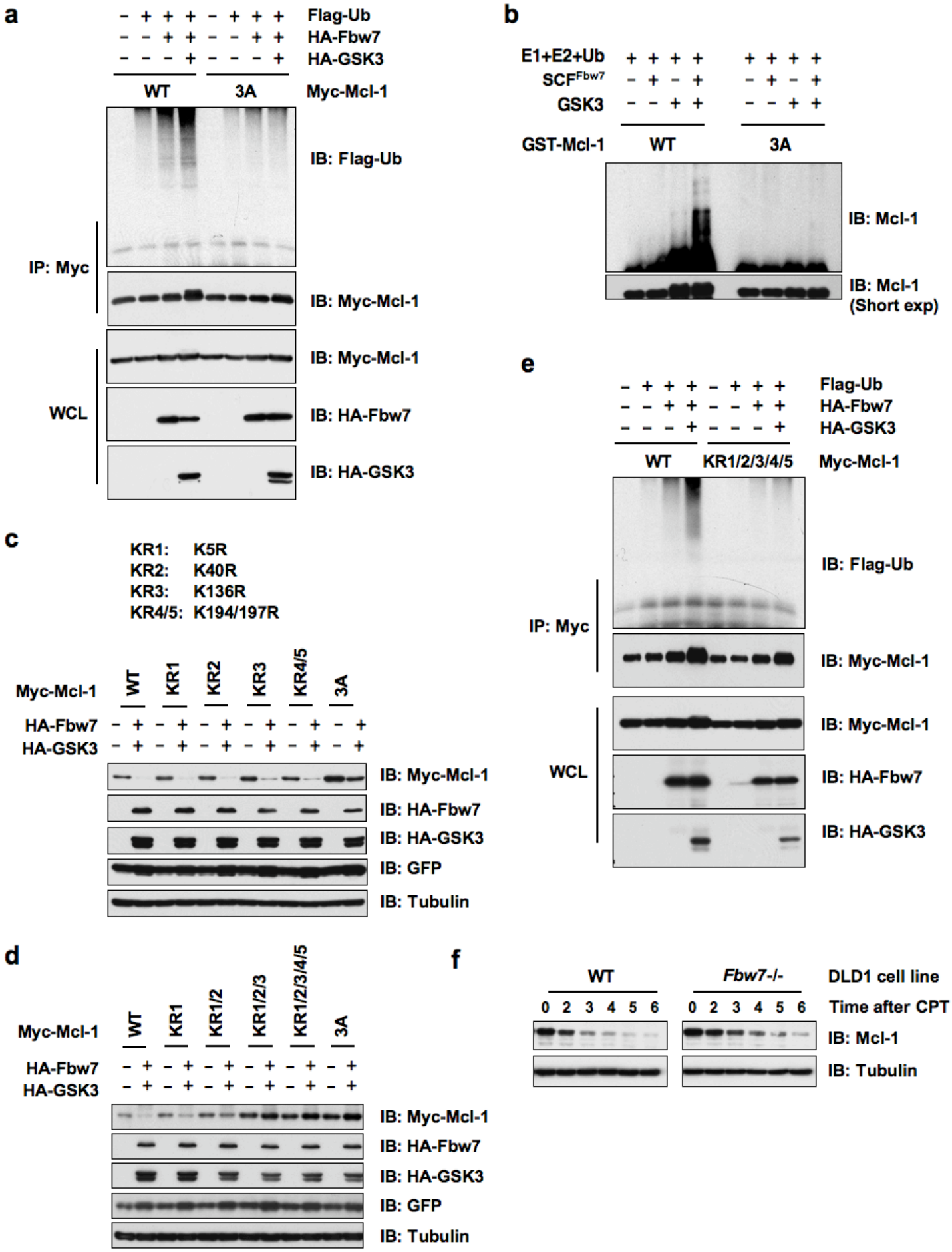
a, Immunoblot analysis of 293T cells transfected with the indicated Myc-Mcl-1 and Flag-Fbw7 plasmids in the presence or absence of HA-GSK3. A plasmid encoding GFP was used as a negative control for transfection efficiency.

b, HEK-293 cells were transfected with the indicated shRNA constructs. Whole cell lysates were collected for immunoblot analysis.

c, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with the indicated HA-tagged and Flag-tagged Fbw7 constructs. Twenty hours post-transfection, cells were treated with 10 μM MG132 overnight before harvesting. LIEE, Fbw7$\alpha^{L256E/I257E}$

d, Immunoblot analysis of 293T cells transfected with the indicated Myc-Mcl-1 and HA-Fbw7 plasmids in the presence or absence of HA-GSK3. A plasmid encoding GFP was used as a negative control for transfection efficiency.

e, Immunoblot analysis of 293T cells transfected with the indicated Flag-Cyclin E and HA-Fbw7 plasmids in the presence or absence of HA-GSK3. A plasmid encoding GFP was used as a negative control for transfection efficiency.


a
Flag-Ub
HA-Fbw7
HA-GSK3
WT
3A
Myc-Mcl-1
IB: Flag-Ub
IP: Myc
IB: Myc-Mcl-1
IB: Myc-Mcl-1
WCL
IB: HA-Fbw7
IB: HA-GSK3
b
E1+E2+Ub
SCF^Fbw7
GSK3
GST-Mcl-1
WT
3A
IB: Mcl-1
IB: Mcl-1 (Short exp)
c
KR1: K5R
KR2: K40R
KR3: K136R
KR4/5: K194/197R
Myc-Mcl-1
WT
KR1
KR2
KR3
KR4/5
3A
HA-Fbw7
HA-GSK3
IB: Myc-Mcl-1
IB: HA-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin
d
Myc-Mcl-1
WT
KR1
KR1/2
KR1/2/3
KR1/2/3/4/5
3A
HA-Fbw7
HA-GSK3
IB: Myc-Mcl-1
IB: HA-Fbw7
IB: HA-GSK3
IB: GFP
IB: Tubulin
e
Flag-Ub
HA-Fbw7
HA-GSK3
WT
KR1/2/3/4/5
Myc-Mcl-1
IB: Flag-Ub
IP: Myc
IB: Myc-Mcl-1
IB: Myc-Mcl-1
WCL
IB: HA-Fbw7
IB: HA-GSK3
f
WT
Fbw7-/-
DLD1 cell line
0 2 3 4 5 6
0 2 3 4 5 6
Time after CPT (h)
IB: Mcl-1
IB: Tubulin

Supplementary Figure 8: Fbw7 promotes Mcl-1 ubiquitination.

a, Immunoblot (IB) analysis of whole cell lysates (WCL) and anti-Myc immunoprecipitates of 293T cells transfected with the indicated plasmids. Twenty hours post-transfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting.

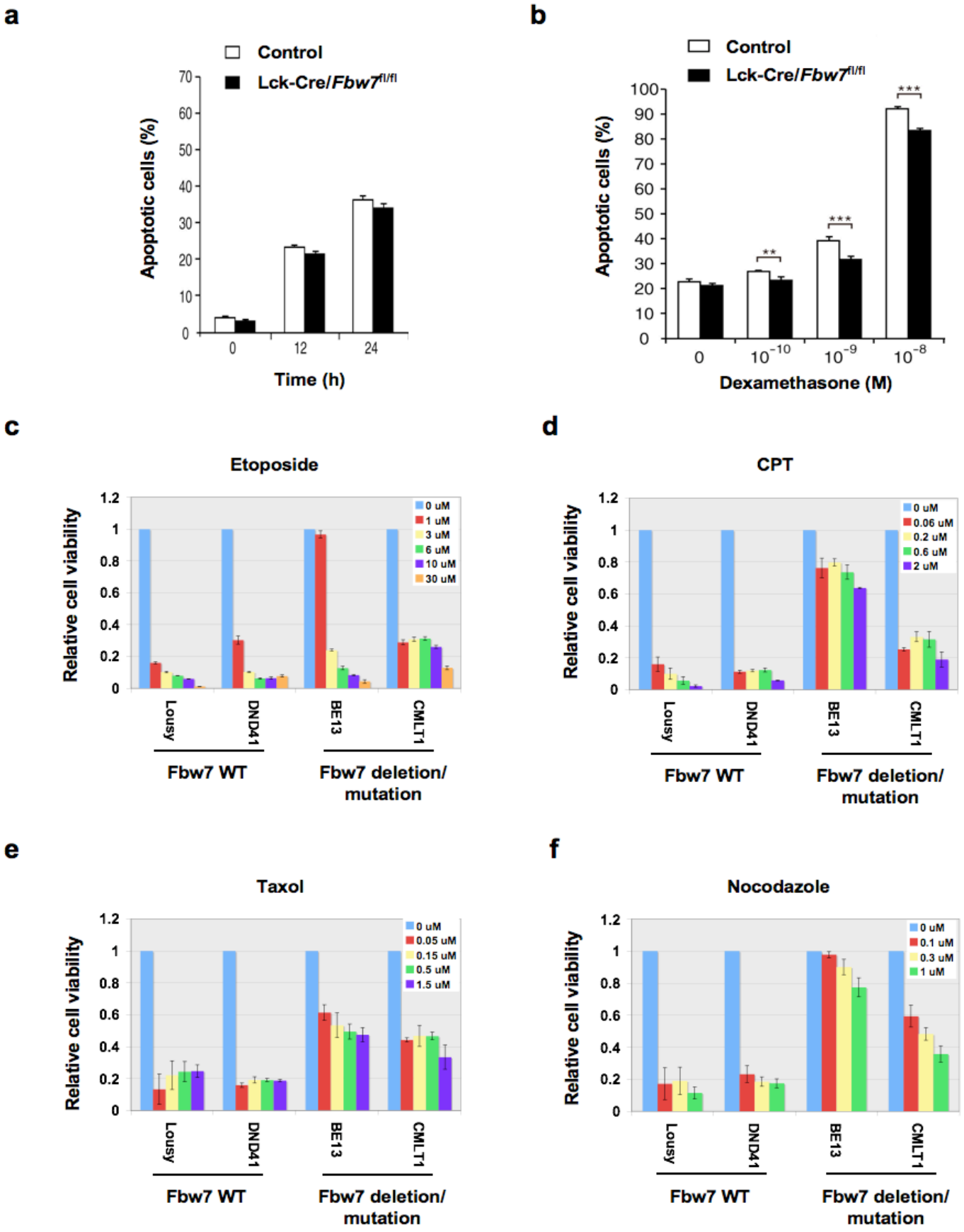
b, The SCF^{Fbw7} complex promotes Mcl-1 ubiquitination *in vitro*. Affinity-purified SCF^{Fbw7} complexes were incubated with purified recombinant GST-Mcl-1 proteins, purified E1, E2 and ubiquitin as indicated at 30°C for 45 minutes. The ubiquitination reaction products were resolved by SDS-PAGE and probed with the anti-Mcl-1 antibody.

c, Inactivation of the individual putative ubiquitination sites in Mcl-1 does not impair Fbw7-mediated Mcl-1 destruction. Immunoblot analysis of 293T cells transfected with the indicated Myc-Mcl-1 and HA-Fbw7 plasmids in the presence or absence of HA-GSK3. A plasmid encoding GFP was used as a negative control for transfection efficiency.

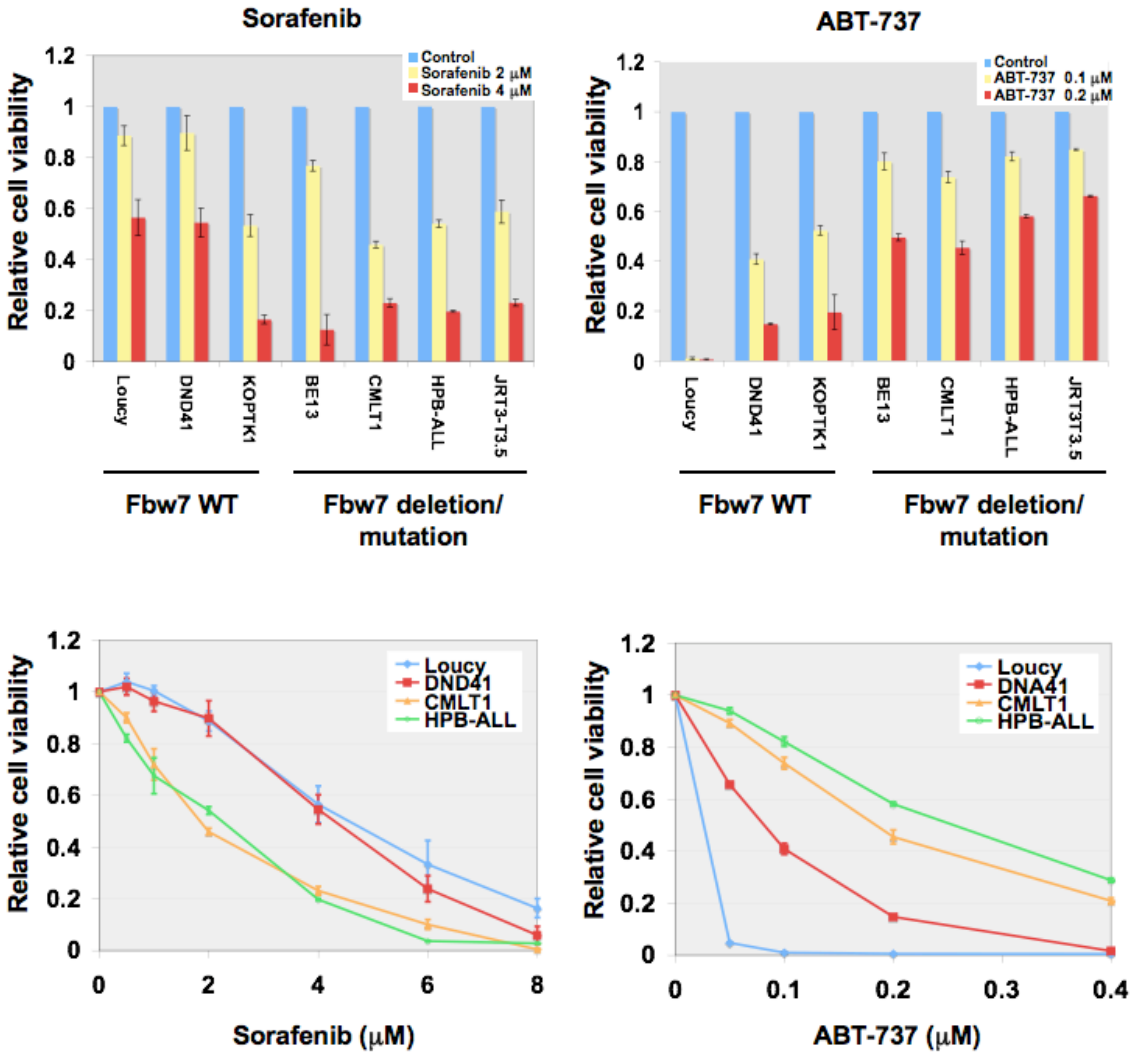
d, Combinational inactivation of the putative ubiquitination sites in Mcl-1 leads to a progressive resistance to Fbw7-mediated Mcl-1 destruction. Immunoblot analysis of 293T cells transfected with the indicated Myc-Mcl-1 and HA-Fbw7 plasmids in the presence or absence of HA-GSK3. A plasmid encoding GFP was used as a negative control for transfection efficiency.

e, Inactivation of the five putative ubiquitination sites impairs the Fbw7-mediated ubiquitination of Mcl-1 *in vivo*. Immunoblot (IB) analysis of whole cell lysates (WCL) and anti-Myc immunoprecipitates of 293T cells transfected with the indicated plasmids. Twenty hours post-transfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting.

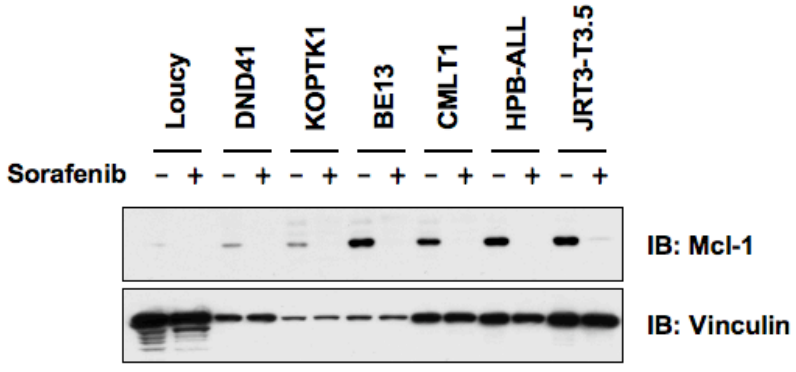
f, Immunoblot analysis of wild-type (WT) or *Fbw7*-/- DLD1 cells treated with 10 μM camptothecin (CPT) for the indicated durations of time.

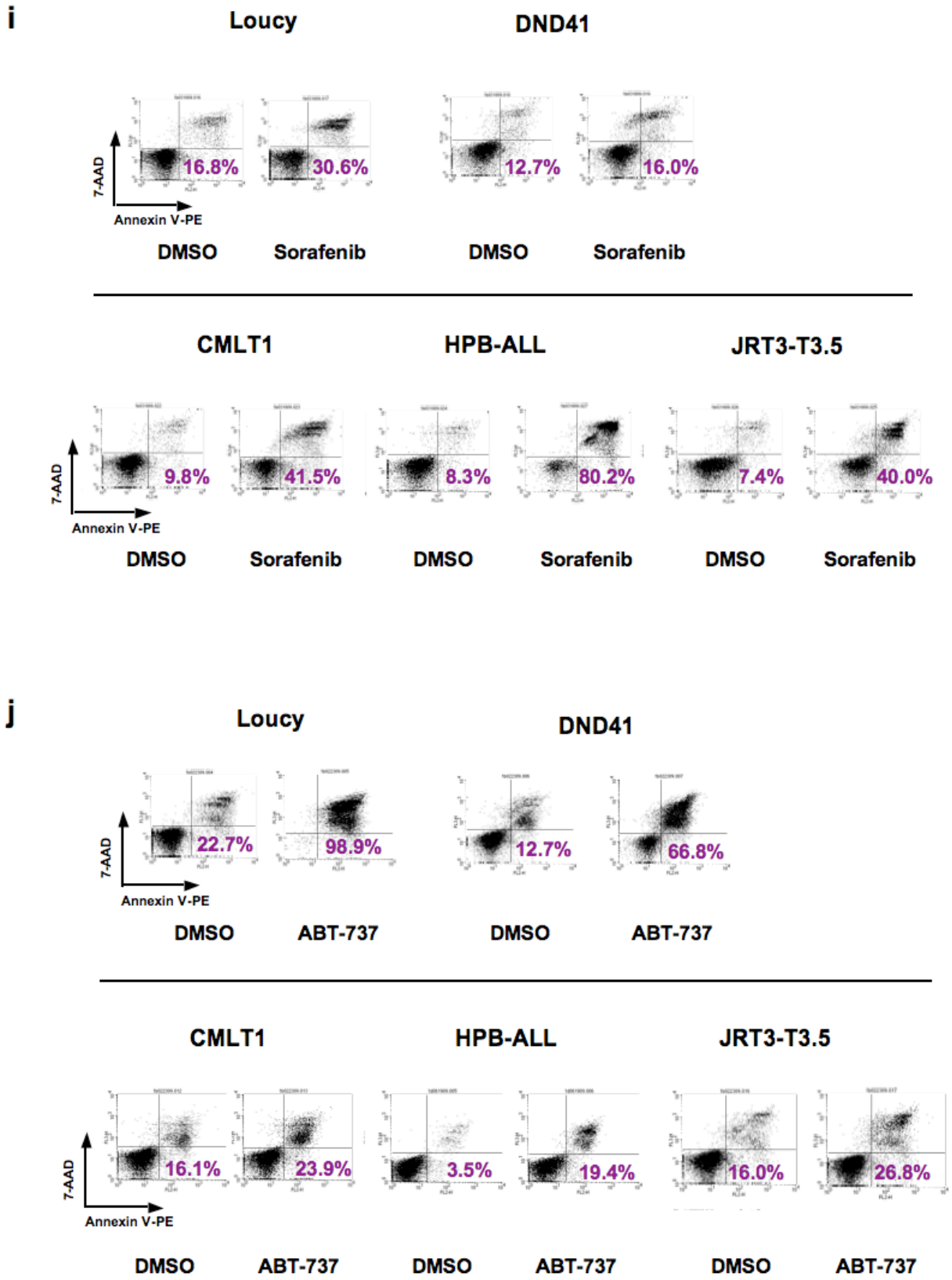

a
Control
Lck-Cre/Fbw7fl/fl
Apoptotic cells (%)
Time (h)
b
Control
Lck-Cre/Fbw7fl/fl
Apoptotic cells (%)
Dexamethasone (M)
c
Etoposide
Relative cell viability
0 uM
1 uM
3 uM
6 uM
10 uM
30 uM
Lousy
DND41
BE13
CMLT1
Fbw7 WT
Fbw7 deletion/ mutation
d
CPT
Relative cell viability
0 uM
0.06 uM
0.2 uM
0.6 uM
2 uM
Fbw7 WT
Fbw7 deletion/ mutation
e
Taxol
Relative cell viability
0 uM
0.05 uM
0.15 uM
0.5 uM
1.5 uM
Fbw7 WT
Fbw7 deletion/ mutation
f
Nocodazole
Relative cell viability
0 uM
0.1 uM
0.3 uM
1 uM
Fbw7 WT
Fbw7 deletion/ mutation

g


Sorafenib
ABT-737
Relative cell viability
Control
Sorafenib 2 μM
Sorafenib 4 μM
ABT-737 0.1 μM
ABT-737 0.2 μM
Loucy
DND41
KOPTK1
BE13
CMLT1
HPB-ALL
JRT3-T3.5
Fbw7 WT
Fbw7 deletion/ mutation
DNA41
Sorafenib (μM)
ABT-737 (μM)

h


Loucy
DND41
KOPTK1
BE13
CMLT1
HPB-ALL
JRT3-T3.5
Sorafenib
IB: Mcl-1
IB: Vinculin


i
Loucy
DND41
Fbw7 WT cell lines
7-AAD
Annexin V-PE
16.8%
30.6%
12.7%
16.0%
DMSO
Sorafenib
DMSO
Sorafenib
CMLT1
HPB-ALL
JRT3-T3.5
Fbw7 mutated cell lines
9.8%
41.5%
8.3%
80.2%
7.4%
40.0%
DMSO
Sorafenib
DMSO
Sorafenib
DMSO
Sorafenib
j
Loucy
DND41
Fbw7 WT cell lines
22.7%
98.9%
12.7%
66.8%
DMSO
ABT-737
DMSO
ABT-737
CMLT1
HPB-ALL
JRT3-T3.5
Fbw7 mutated cell lines
16.1%
23.9%
3.5%
19.4%
16.0%
26.8%
DMSO
ABT-737
DMSO
ABT-737
DMSO
ABT-737

Supplementary Figure 9: Fbw7-deficient T-ALL cell lines are more sensitive to sorafenib, but have increased resistance to ABT-737 treatment.

a, Thymocytes isolated from 8-wk-old Lck-Cre/*Fbw7*$^{+/fl}$ (Control) or Lck-Cre/*Fbw7*$^{fl/fl}$ (*Fbw7* KO) were cultured at 37°C for the indicated times and then stained with annexin V for determination of the proportion of apoptotic cells by flow cytometry. Data are represented as mean ± SD from three independent experiments.

b, Thymocytes of 8-wk-old Lck-Cre/*Fbw7*$^{+/fl}$ (Control) or Lck-Cre/*Fbw7*$^{fl/fl}$ (*Fbw7* KO) were cultured for 12 h at 37°C with the indicated concentrations of dexamethasone and then analyzed as in (**a**). **, $P < 0.01$ using the Student *t* test. ***, $P < 0.005$ using the Student *t* test.

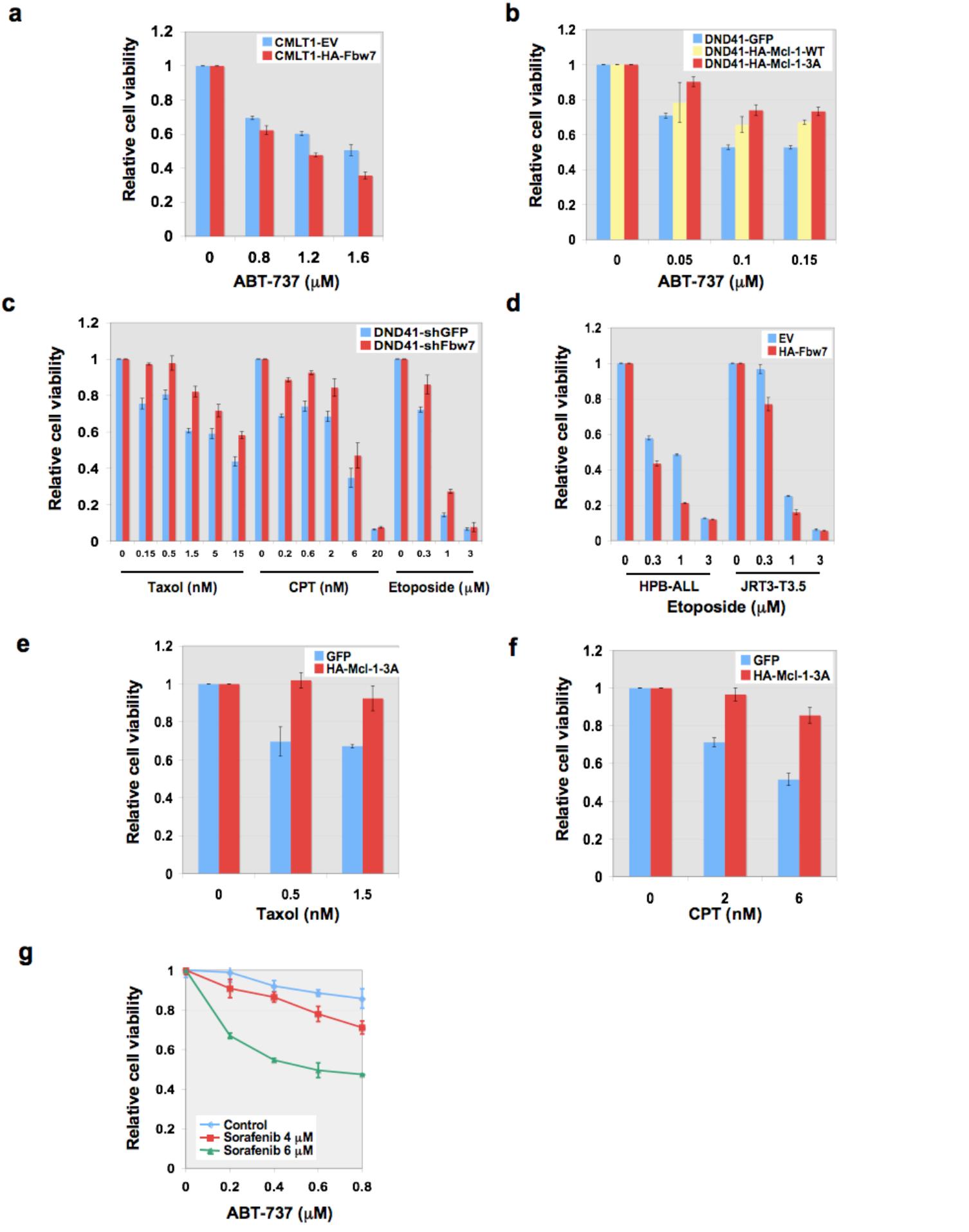
c-f, Cell viability assays showing that compared with T-ALL cell lines with wild-type Fbw7, Fbw7-deficient T-ALL cell lines were more resistant to multiple apoptotic stimuli including etoposide (**c**), camptothecin (CPT) (**d**), Taxol (**e**) and Nocodazole (**f**). Data was shown as mean ± SD for three independent experiments.

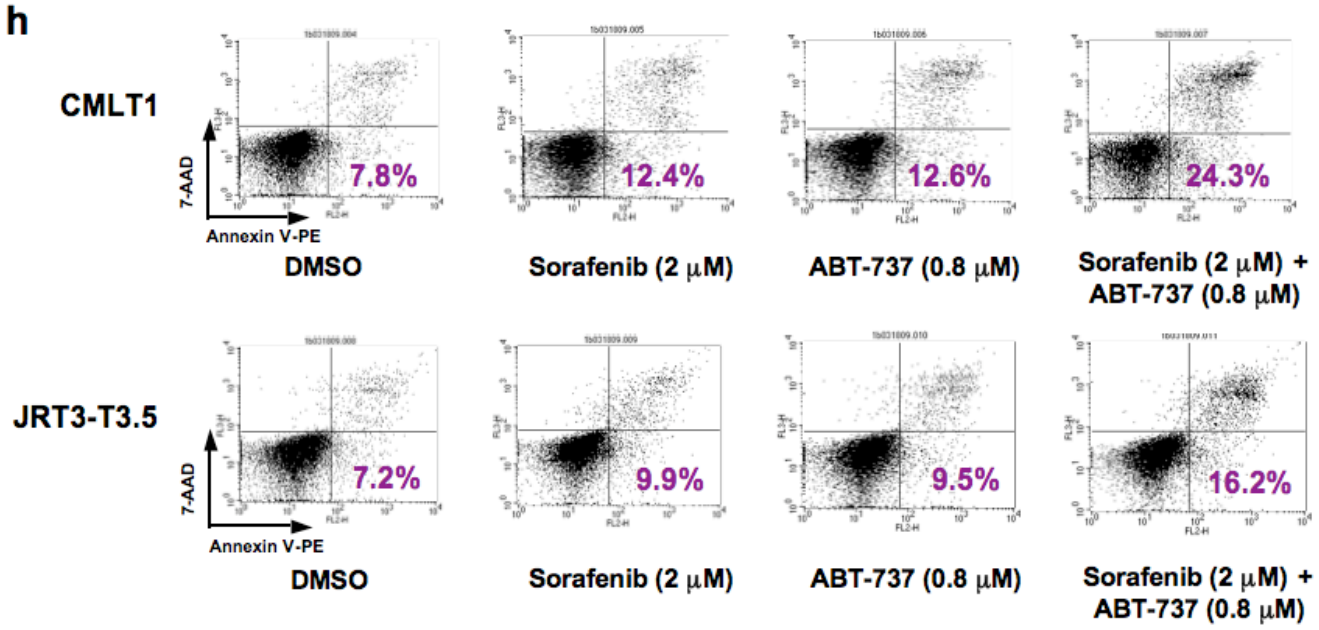
g, Cell viability assays showing that compared with T-ALL cell lines with wild-type Fbw7, Fbw7-deficient T-ALL cell lines were more sensitive to sorafenib, but resistant to ABT-737 treatment. T-ALL cells were cultured in 0.5% FBS-containing medium with the indicated concentrations of sorafenib or ABT-737 for 48 hours before performing the cell viability assays. Data was shown as mean ± SD for three independent experiments.

h, Immunoblot analysis of the indicated human T-ALL cell lines with or without sorafenib treatment in 0.5% FBS-containing medium.

i, 7-AAD/Annexin V double staining FACS analysis to detect the percentage of sorafenib-induced apoptosis of the indicated Fbw7-deficient T-ALL cell lines. Various T-ALL cells were cultured in 0.5% FBS-containing medium with or without sorafenib (2 μM) treatment for 48 hours before the FACS analysis. Numbers indicate the percentage of apoptotic cells.

j, 7-AAD/Annexin V double staining FACS analysis to detect the percentage of ABT-737-induced apoptosis of the indicated Fbw7-deficient T-ALL cell lines. Various T-ALL cells were cultured in 10% FBS-containing medium with or without ABT-737 (0.8 μM) treatment for 48 hours before the FACS analysis. Numbers indicate the percentage of apoptotic cells.


a
Relative cell viability
CMLT1-EV
CMLT1-HA-Fbw7
ABT-737 (μM)
b
DND41-GFP
DND41-HA-Mcl-1-WT
DND41-HA-Mcl-1-3A
ABT-737 (μM)
c
DND41-shGFP
DND41-shFbw7
Taxol (nM)
CPT (nM)
Etoposide (μM)
d
EV
HA-Fbw7
HPB-ALL
JRT3-T3.5
Etoposide (μM)
e
GFP
HA-Mcl-1-3A
Taxol (nM)
f
GFP
HA-Mcl-1-3A
CPT (nM)
g
Control
Sorafenib 4 μM
Sorafenib 6 μM
ABT-737 (μM)



Supplementary Figure 10: Manipulating Fbw7 activity changes ABT-737 sensitivity.

a, Cell viability assays showing that re-introduction of wild-type Fbw7 into the Fbw7-deficient T-ALL (CMLT1) cell line partially restored its sensitivity to ABT-737 treatment. The CMLT1 cells were cultured in 10% FBS-containing medium with the indicated concentrations of ABT-737 treatment for 48 hours before performing the cell viability assays. Data was shown as mean ± SD for three independent experiments.

b, Cell viability assays showing that re-introduction of wild-type or Mcl-1-3A into DND41 cells results in an increase in resistance to ABT-737 treatment. Indicated DND41 cells were cultured in 10% FBS-containing medium with the indicated concentrations of ABT-737 treatment for 48 hours before performing the cell viability assays. Data was shown as mean ± SD for three independent experiments.

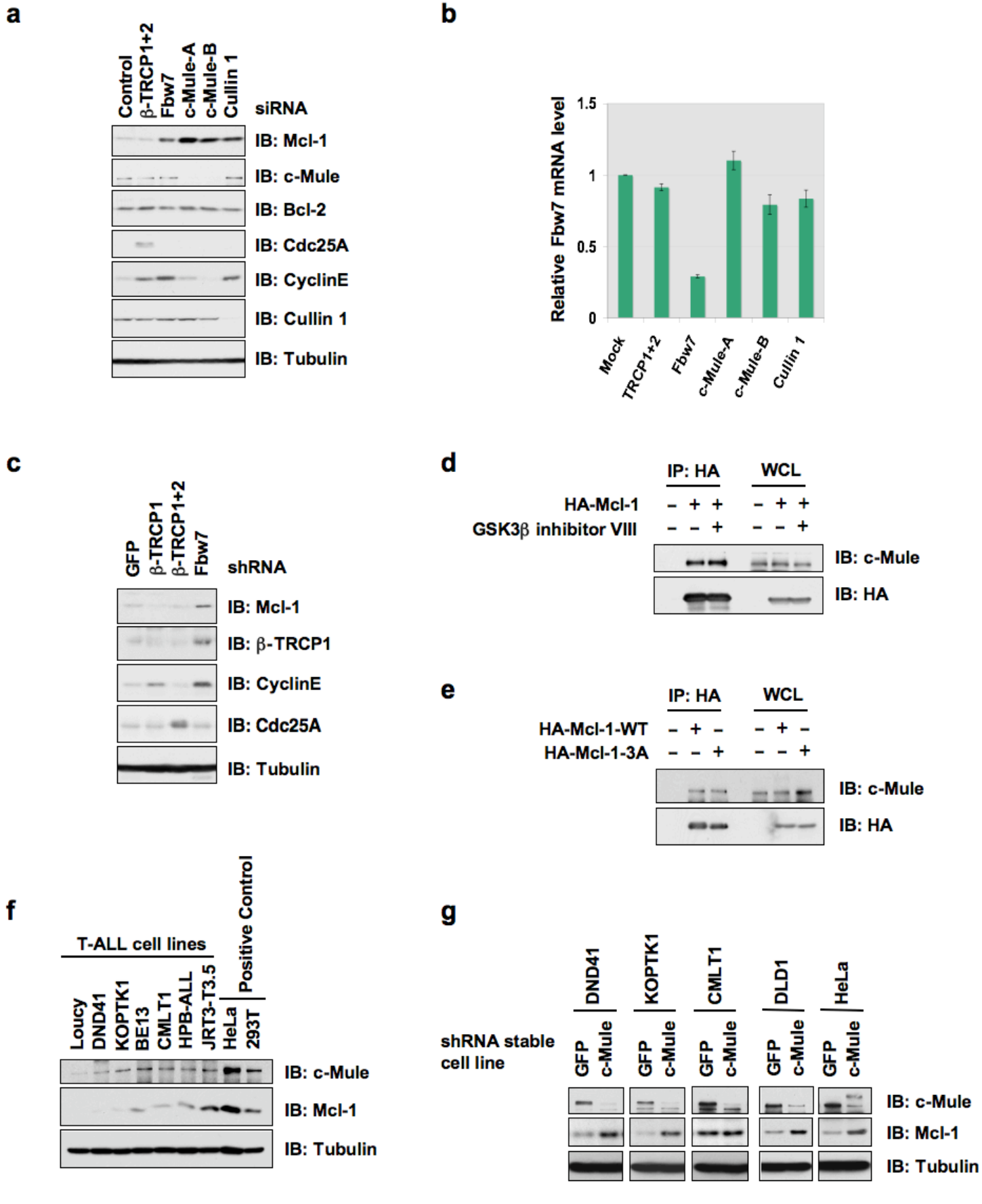
c, Cell viability assays showing that depletion of Fbw7 in DND41 cells resulted in elevated resistance to multiple apoptotic stimuli. Data was shown as mean ± SD for three independent experiments.

d, Cell viability assays showing that re-introduction of wild-type Fbw7 into the Fbw7-deficient T-ALL cell lines (HPB-ALL and JRT3-T3.5) partially restored their sensitivity to etoposide-induced apoptosis. Data was shown as mean ± SD for three independent experiments.

e-f, Cell viability assays showing that re-introduction of Mcl-1-3A into DND41 cells results in an increase in resistance to Taxol (**e**) and CPT (**f**) treatments. Data was shown as mean ± SD for three independent experiments.

g, Cell viability assays to demonstrate that sorafenib treatment restored ABT-737 sensitivity in Fbw7-deficient HPB-ALL cells. HPB-ALL cells were cultured in 10% FBS-containing medium with the indicated concentrations of sorafenib and ABT-737 for 48 hours before performing the cell viability assays. In order to score the effects of increasing concentrations of ABT-737 on cell viability, each reading was scaled relative to the respective sorafenib treatment with 0 μM ABT-737 set as 100%. Data was shown as mean ± SD for three independent experiments.

h, 7-AAD/Annexin V double staining FACS analysis to demonstrate that sorafenib treatment restored ABT-737 sensitivity of Fbw7-deficient CMLT1 and JRT3-T3.5 cells. CMLT1 and JRT3-T3.5 cells were cultured in 10% FBS-containing medium with the indicated concentrations of sorafenib and/or ABT-737 for 48 hours before the FACS analysis. Numbers indicate the percentage of apoptotic cells.


a
Control
β-TRCP1+2
Fbw7
c-Mule-A
c-Mule-B
Cullin 1
siRNA
IB: Mcl-1
IB: c-Mule
IB: Bcl-2
IB: Cdc25A
IB: CyclinE
IB: Cullin 1
IB: Tubulin
b
Relative Fbw7 mRNA level
1.5
1
0.5
0
Mock
TRCP1+2
Fbw7
c-Mule-A
c-Mule-B
Cullin 1
c
GFP
β-TRCP1
β-TRCP1+2
Fbw7
shRNA
IB: Mcl-1
IB: β-TRCP1
IB: CyclinE
IB: Cdc25A
IB: Tubulin
d
IP: HA
WCL
HA-Mcl-1
GSK3β inhibitor VIII
IB: c-Mule
IB: HA
e
IP: HA
WCL
HA-Mcl-1-WT
HA-Mcl-1-3A
IB: c-Mule
IB: HA
f
T-ALL cell lines
Positive Control
Loucy
DND41
KOPTK1
BE13
CMLT1
HPB-ALL
JRT3-T3.5
HeLa
293T
IB: c-Mule
IB: Mcl-1
IB: Tubulin
g
shRNA stable cell line
DND41
KOPTK1
CMLT1
DLD1
HeLa
GFP
c-Mule
IB: c-Mule
IB: Mcl-1
IB: Tubulin

Supplementary Figure 11: c-Mule is not the physiological E3 ubiquitin ligase for Mcl-1 in T-ALL cell lines.

a, Immunoblot analysis of HeLa cells transfected with the indicated siRNA oligonucleotides.

b, Real-time RT-PCR analysis to examine the Fbw7 mRNA levels after treatments with the various siRNA oligos in (**a**). Data was shown as mean ± SD for three independent experiments.

c, Immunoblot analysis of HeLa cells transfected with the indicated shRNA constructs.

d, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with HA-Mcl-1. Thirty hours post-transfection, cells were pretreated with 20 μM MG132 for 8 hours to block the proteasome pathway before harvesting. Where indicated, 25 μM GSK3β inhibitor VIII (with DMSO as a negative control) was added for 8 hours before harvesting.

e, Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with the indicated HA-Mcl-1 constructs. Thirty hours post-transfection, cells were pretreated with 10 μM MG132 for 10 hours to block the proteasome pathway before harvesting.

f, Immunoblot analysis of the indicated human T-ALL cell lines cultured in 10 % FBS-containing medium. HeLa and 293T cell lines were included as positive controls for detection of the endogenous c-Mule expression.

g, Various cell lines were infected with the lentiviral sh-c-Mule construct (with shGFP as a negative control) and selected with 1 μg/ml puromycin to eliminate the non-infected cells. Whole cell lystates were collected for immunoblot analysis with the indicated antibodies.