Cell Reports, Volume *28*

Supplemental Information

CRISPR/Cas9 Screens Reveal Multiple Layers of B cell CD40 Regulation

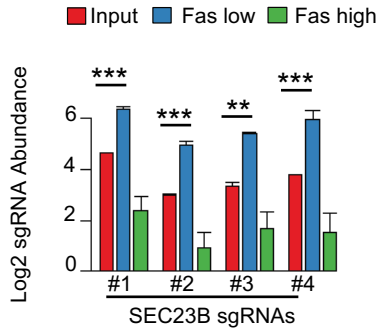
Chang Jiang, Stephen J. Trudeau, Taek-Chin Cheong, Rui Guo, Mingxiang Teng, Liang Wei Wang, Zhonghao Wang, Chiara Pighi, Carole Gautier-Courteille, Yijie Ma, Sizun Jiang, Chong Wang, Bo Zhao, Luc Paillard, John G. Doench, Roberto Chiarle, and Benjamin E. Gewurz

Figure S1

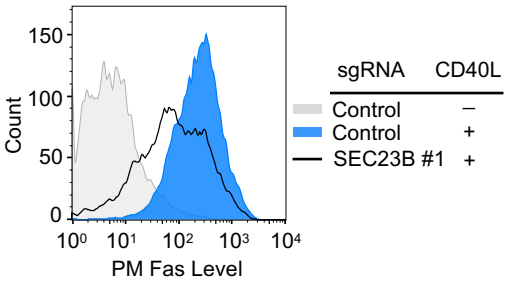
A

Fas^{low} Screen GSEA Enriched Term	FDR q-Value
KEGG_PROTEIN_EXPORT	0
BIOCARTA_CD40_PATHWAY	0
REACTOME_RNA_POL_III_TRANSCRIPTION	0.003
BIOCARTA_RELA_PATHWAY	0.021
REACTOME_TAK1_ACTIVATES_NFKB_BY_PHOSPHORYLATION_AND_ACTIVATION_OF_IKKS	0.024
REACTOME_RNA_POL_III_TRANSCRIPTION_INITIATION_FROM_TYPE_2_PROMOTER	0.028
BIOCARTA_NFKB_PATHWAY	0.030
BIOCARTA_TNFR2_PATHWAY	0.034

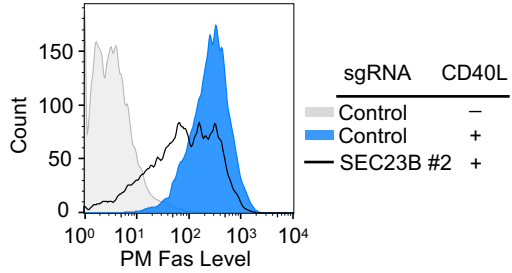
B



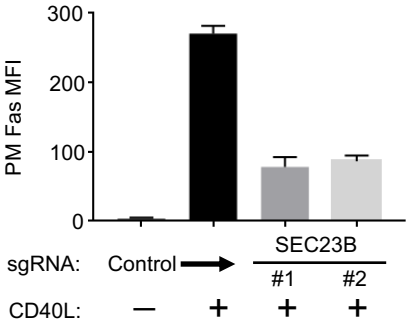
C



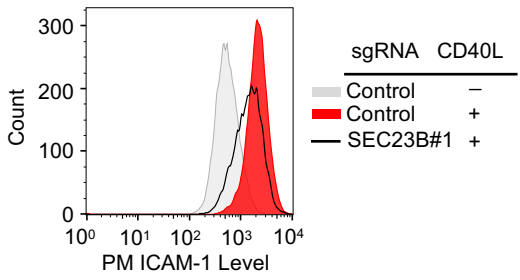
D



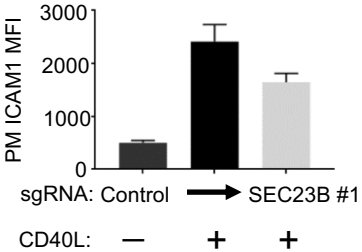
E



F



G



H

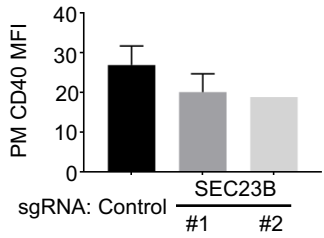


Figure S1. Related to **Figure 1**; SEC23B is a Dependency Factor for CD40 PM Expression.

(A) Top gene set enrichment analysis (GSEA) terms and multiple-hypothesis test adjusted false discovery rate (FDR) q-values for the 87 Faslow screen $q < 0.05$ hits. GSEA highlighted protein export and CD40 pathway as the most highly enriched terms.

(B) Log2-normlized CRISPR screen SEC23B sgRNA abundances of input, sorted Fas low and Fas high populations. Mean + standard deviation (SD) of two input libraries and four screen replicates are shown. ** $p < 0.01$, *** $p < 0.001$.

(C-D) FACS analysis of PM Fas abundances in Cas9+ Daudi B-cells expressing the indicated control or independent SEC23B targeting sgRNAs (SEC23B #1 or #2), stimulated with Mega-CD40L (50 ng/ml for 48 hours) as shown.

(E) FACS analysis of PM Fas MFI in Cas9+ Daudi B-cells expressing control or independent sgRNAs targeting SEC23B and stimulated by Mega-CD40L (50 ng/mL for 48 hours), as indicated.

(F) FACS analysis of PM ICAM-1 levels in Cas9+ Daudi B-cells cells expressing the indicated sgRNAs and stimulated by Mega-CD40L (50 ng/mL for 48 hours), as shown.

(G) PM ICAM1 MFI as in (F) from $n = 3$ independent experiments.

(H) PM CD40 levels in Daudi Cas9+ B-cells expressing the indicated control or independent SEC23B targeting sgRNAs and stimulated by Mega-CD40L (50 ng/mL for 48 hours), as shown.

Mean and SD from 3 independent experiments are shown in (E), (G) and (H).

Figure S2

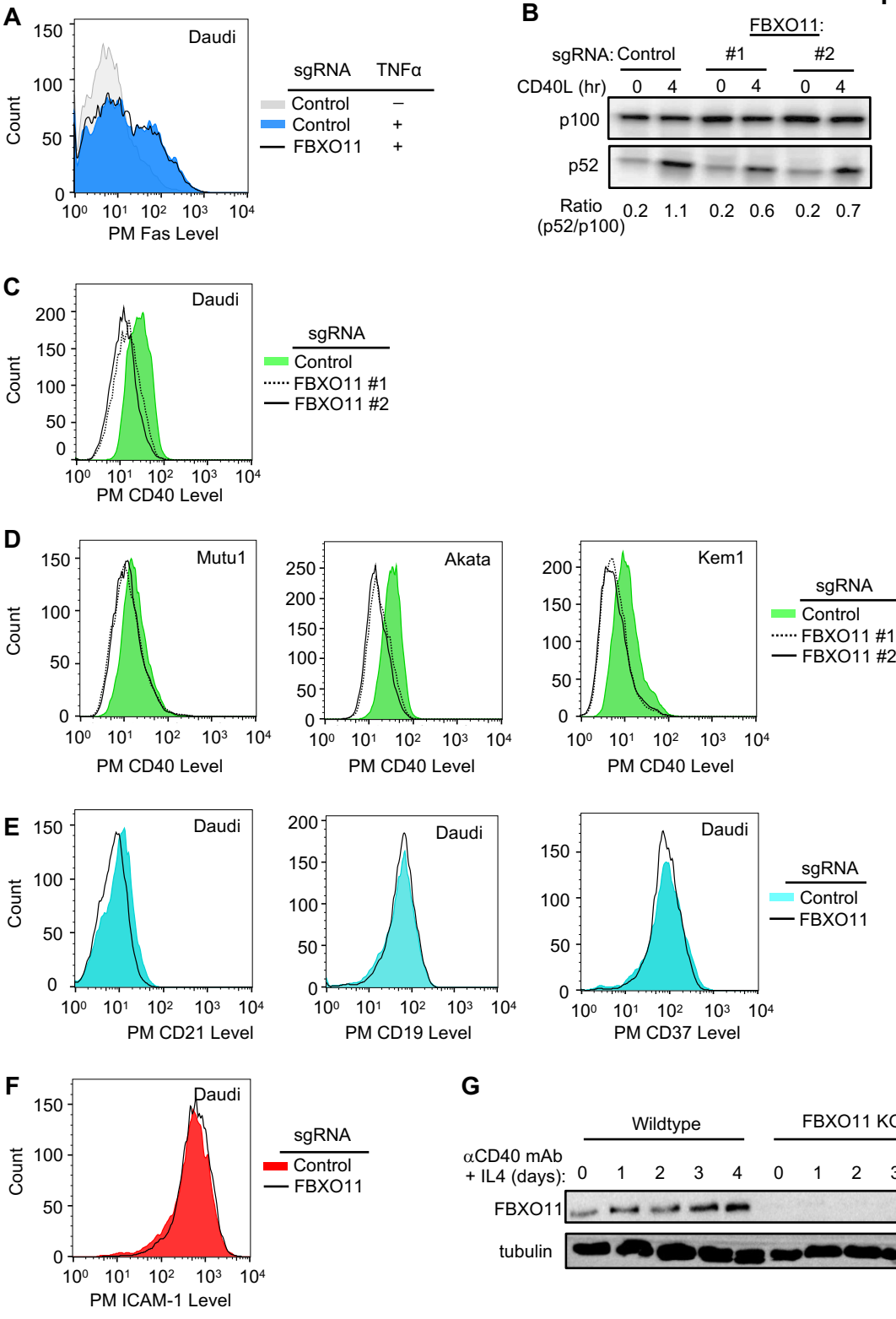

A
Daudi
Count
PM Fas Level
sgRNA TNFα
Control –
Control +
FBXO11 +
B
FBXO11:
sgRNA: Control #1 #2
CD40L (hr) 0 4 0 4 0 4
p100
p52
Ratio (p52/p100) 0.2 1.1 0.2 0.6 0.2 0.7
C
Daudi
PM CD40 Level
sgRNA
Control
FBXO11 #1
FBXO11 #2
D
Mutu1
Akata
Kem1
PM CD40 Level
E
Daudi
PM CD21 Level
PM CD19 Level
PM CD37 Level
sgRNA
Control
FBXO11
F
Daudi
PM ICAM-1 Level
G
Wildtype
FBXO11 KO
αCD40 mAb + IL4 (days): 0 1 2 3 4 0 1 2 3 4
FBXO11
tubulin

Figure S2. Related to **Figure 2**; FBXO11 is a Key Dependency Factor for CD40 Expression.

(A) FACS analysis of PM Fas levels in Cas9+ Daudi B-cells expressing the indicated control or FBXO11 targeting sgRNAs and stimulated with TNFα 10 ng/ml for 24 hours, as shown.

(B) Immunoblot analysis of non-canonical NF-κB pathway p100 and p52 abundances WCE from Cas9+ Daudi B-cells expressing the indicated control or independent FBXO11 targeting sgRNAs and stimulated by Mega-CD40L (50 ng/ml) for the indicated hours (hr). Ratios of p100:p52 abundances, indicative of non-canonical NF-kB pathway activity, were quantitated and are shown beneath each lane.

(C) FACS analysis of PM CD40 abundances in Cas9+ Daudi B-cells expressing the indicated control or independent FBXO11 targeting sgRNAs.

(D) FACS analysis of PM CD40 levels in Cas9+ Mutu I, Akata or Kem I Cas9+ B cells expressing control or independent FBXO11 sgRNAs.

(E) FACS analysis of PM CD21, CD19 and CD37 levels in Cas9+ Daudi B cells expressing either control or FBXO11 targeting sgRNAs, as indicated.

(F) FACS analysis of PM ICAM1 levels in Cas9+ Daudi B cells expressing either control or FBXO11 targeting sgRNAs, as indicated.

(G) Immunoblot analysis of FBXO11 or control tubulin levels in WCE from primary spleen B-cells obtained from wildtype (WT) or FBXO11 knockout (KO) mice, purified by negative selection and stimulated by anti-CD40 agonist antibody (1 μg/mL) and IL4 (20 ng/ml) for the indicated number of days.

All immunoblots and FACS results were representative of at least n = 3 independent experiments.

Figure S3

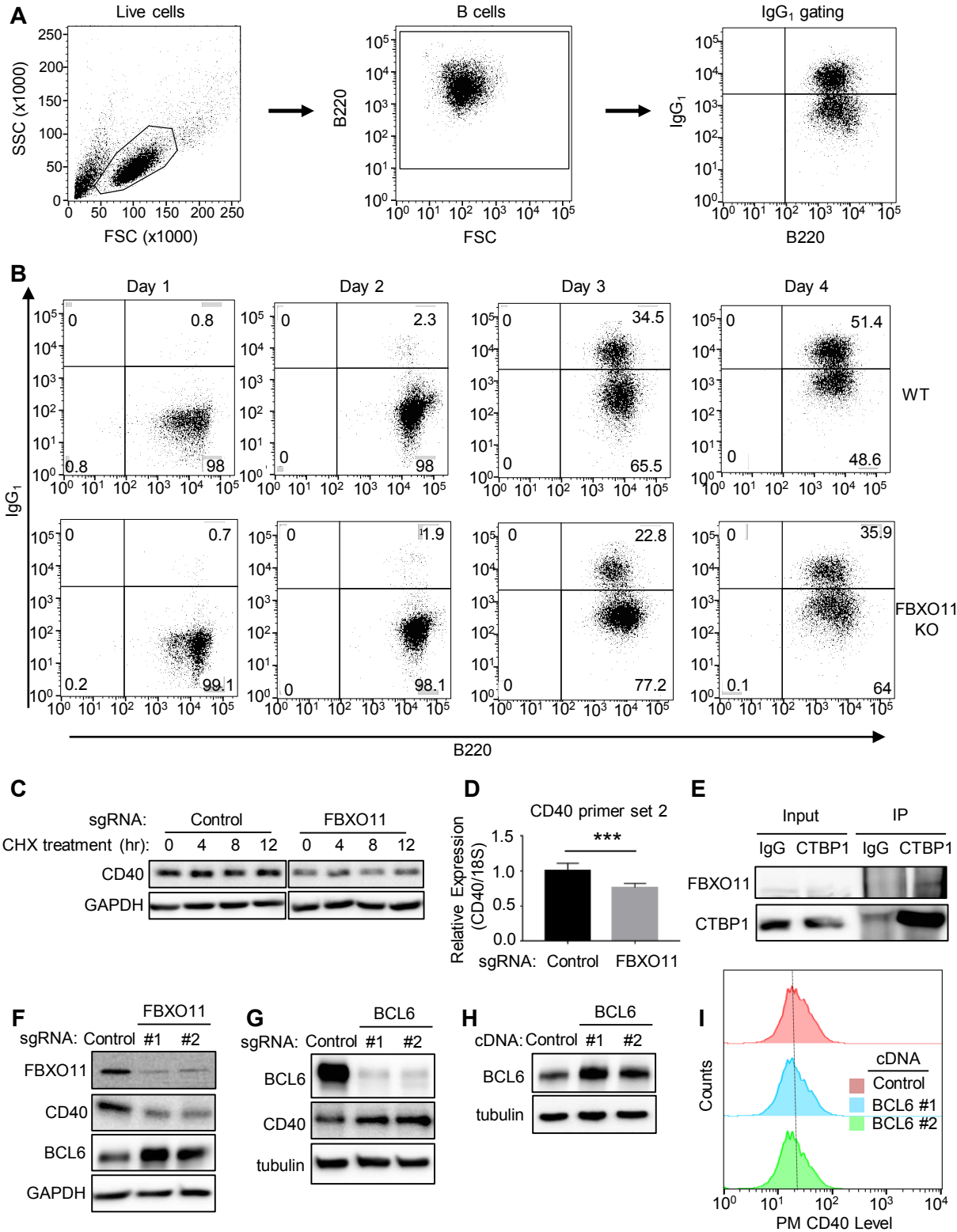

A
Live cells
B cells
IgG1 gating
SSC (x1000)
FSC (x1000)
B220
FSC
IgG1
B220
B
Day 1
Day 2
Day 3
Day 4
WT
FBXO11 KO
IgG1
B220
C
sgRNA: Control FBXO11
CHX treatment (hr): 0 4 8 12 0 4 8 12
CD40
GAPDH
D
CD40 primer set 2
***
Relative Expression (CD40/18S)
sgRNA: Control FBXO11
E
Input IP
IgG CTBP1 IgG CTBP1
FBXO11
CTBP1
F
FBXO11
sgRNA: Control #1 #2
FBXO11
CD40
BCL6
GAPDH
G
BCL6
sgRNA: Control #1 #2
BCL6
CD40
tubulin
H
BCL6
cDNA: Control #1 #2
BCL6
tubulin
I
cDNA
Control
BCL6 #1
BCL6 #2
Counts
PM CD40 Level

Figure S3. Related to **Figures 2-3**; FBXO11 is Important for CD40 Expression Through Targeting of The Transcription Repressors BCL6 and CTBP1.

(A) Flow cytometry gating strategy for analysis of primary mouse B-cell IgG1 class switch recombination. Mouse splenic B-cells were stained for the B cell marker B220 and for IgG1. Live mouse B-cells were identified by forward scatter (FSC) and side scatter (SSC) analysis. B220 and IgG1 double-positive B cells were then identified using the gates shown.

(B) Representative FACS analyses of WT and FBXO11 KO mouse B-cells stained with anti-B220 and IgG1 antibodies at the indicated day post stimulation by anti-CD40 agonist antibody (1 μg/ml) and IL4 (20 ng/ml). Shown are data from one representative mouse out of three analyzed. IgG1 switched B cells were measured each day up to Day 4 post-stimulation.

(C) Immunoblot analysis of WCE from Cas9+ Daudi B-cells expressing the indicated control or FBXO11 targeting sgRNAs and subjected to cycloheximide (CHX) chase (10 μg/ml) for the indicated hours (hr).

(D) RT-qPCR analysis of 18S rRNA normalized CD40 mRNA steady-state levels in Cas9+ Daudi B-cells expressing control vs. FBXO11 targeting sgRNAs, using a second primer set (distinct from the one used in Figure 2). Mean + SD are shown from n = 3 replicates.

(E) Co-immunoprecipitation analysis of endogenous CTBP1 and FBXO11 in Daudi B-cell lysates. 1% input or material immunopurified by control IgG versus anti-CTBP1 antibody was subject to immunoblot analysis for FBOX11 and CTBP1.

(F) Immunoblot analysis of WCE from Cas9+ Daudi B-cells expressing the indicated control or independent FBXO11-targeting sgRNAs for FBXO11, CD40, BCL6 and control GAPDH abundances.

(G) Immunoblot analysis of WCE from Cas9+ Daudi B-cells expressing the indicated control or independent BCL6-targeting sgRNAs for CD40, BCL6 and control tubulin abundances.

(H) Immunoblot analysis of WCE from Daudi cells with stable lentivirus-driven expression of control BCL6 cDNA, as indicated. BCL6 #1 and #2 are technical replicates.

(I) FACS analysis of PM CD40 abundances in Daudi B-cells stably expressing the indicated cDNAs. BCL6 #1 and #2 are technical replicates.

All immunoblots and FACS results were representative of at least n = 3 independent experiments.

Figure S4

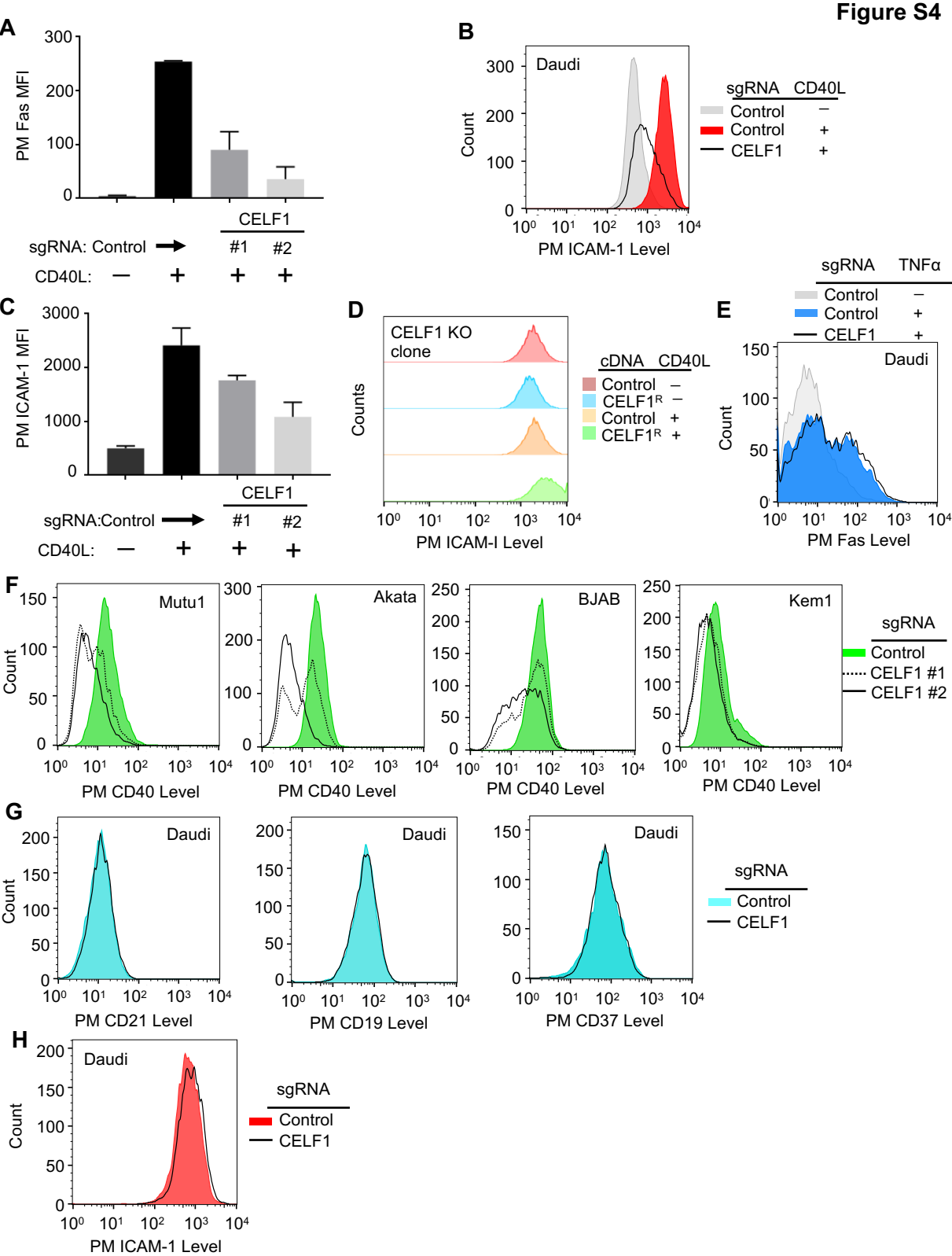

A
PM Fas MFI
300
200
100
0
CELF1
sgRNA: Control
#1
#2
CD40L:
—
+
+
+
B
Daudi
Count
PM ICAM-1 Level
sgRNA
CD40L
Control
—
Control
+
CELF1
+
C
PM ICAM-1 MFI
3000
2000
1000
0
CELF1
sgRNA:Control
#1
#2
CD40L:
—
+
+
+
D
CELF1 KO clone
Counts
PM ICAM-I Level
cDNA
CD40L
Control
—
CELF1R
—
Control
+
CELF1R
+
E
sgRNA
TNFα
Control
—
Control
+
CELF1
+
Daudi
Count
PM Fas Level
F
Mutu1
Akata
BJAB
Kem1
Count
PM CD40 Level
sgRNA
Control
CELF1 #1
CELF1 #2
G
Daudi
Count
PM CD21 Level
PM CD19 Level
PM CD37 Level
sgRNA
Control
CELF1
H
Daudi
Count
PM ICAM-1 Level
sgRNA
Control
CELF1

Figure S4. Related to **Figure 4**; CELF1 is a Key Dependency Factor for CD40 Expression.

(A) FACS analysis of mean +SD PM Fas levels from n = 3 replicates in Cas9+ Daudi B-cells expressing the indicated control or independent CELF1 targeting sgRNAs and stimulated by Mega-CD40L (50 ng/ml for 48 hours) as indicated.

(B) FACS analysis of PM ICAM-1 levels in Cas9+ Daudi B-cells expressing the indicated sgRNAs and stimulated by Mega-CD40L (50 ng/ml for 48 hours) as indicated.

(C) FACS analysis of mean PM ICAM-1 + SD levels from n = 3 replicates in Cas9+ Daudi B-cells with the indicated sgRNAs and stimulated Mega-CD40L (50 ng/ml for 48 hours) as indicated.

(D) FACS analysis of PM ICAM-1 levels in a single cell CELF1 KO Daudi cell clone stably expressing the indicated control or CELF1 cDNA rescue construct (CELF1R).

(E) FACS analysis of PM Fas levels in Cas9+ Daudi B-cells expressing the indicated control or CELF1-targeting sgRNAs and stimulated with TNFα 10 ng/ml for 24 hours, as shown.

(F) FACS analysis of PM CD40 levels in Cas9+ Mutu I, Akata, BJAB or Kem I B cells expressing control or independent CELF1 sgRNAs.

(G) FACS analysis of PM CD21, CD19, and CD37 levels in Cas9+ Daudi B-cells with control or CELF1 sgRNA expression.

(H) FACS analysis of PM ICAM1 levels in Cas9+ Daudi B-cells with control or CELF1 sgRNA expression.

All FACS plots are representative of at least n = 3 independent experiments.

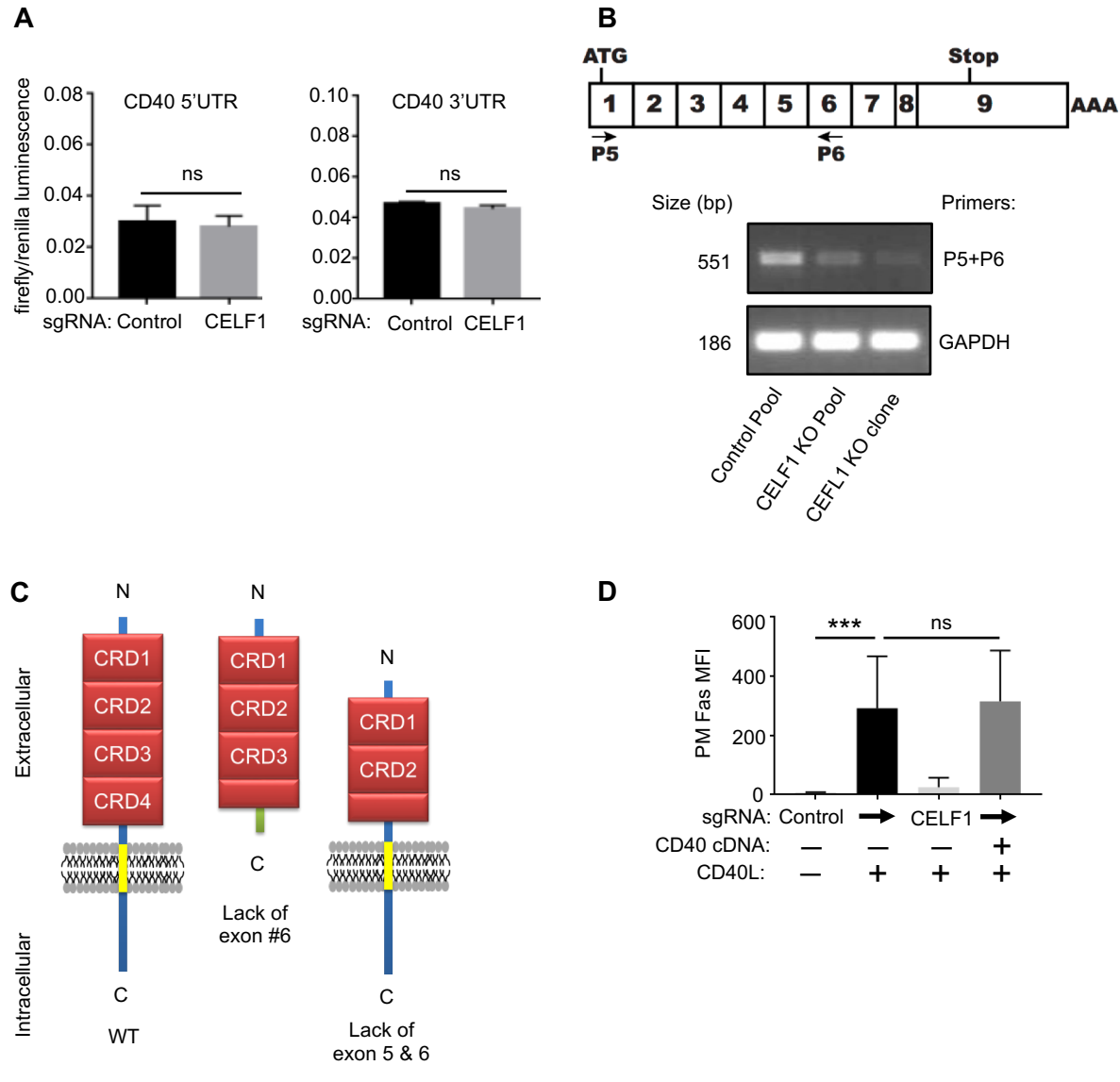

A
firefly/renilla luminescence
CD40 5'UTR
ns
0.08
0.06
0.04
0.02
0.00
sgRNA: Control CELF1
CD40 3'UTR
ns
0.10
0.08
0.06
0.04
0.02
0.00
sgRNA: Control CELF1
B
ATG
Stop
1 2 3 4 5 6 7 8 9
AAA
P5
P6
Size (bp)
Primers:
551
P5+P6
186
GAPDH
Control Pool
CELF1 KO Pool
CEFL1 KO clone
C
N
CRD1
CRD2
CRD3
CRD4
C
WT
Extracellular
Intracellular
N
CRD1
CRD2
CRD3
C
Lack of exon #6
N
CRD1
CRD2
C
Lack of exon 5 & 6
D
PM Fas MFI
600
400
200
0
***
ns
sgRNA: Control CELF1
CD40 cDNA: — — — +
CD40L: — + + +

Figure S5. Related to **Figure 5**; CELF1 is important for CD40 splicing rather than mRNA stability.

(A) Renilla control normalized firefly luciferase luminescence values, using extracts from control or CELF1 KO Daudi cells that stably express firefly luciferase constructs with either the CD40 5'UTR or 3' UTR.

(B) Using the P5/P6 primer pair indicated in the schematic above, CD40 mRNA abundance was analyzed by RT-PCR from control cells, a pool of cells expressing a CELF1 sgRNA, or a single cell CELF1 KO clone.

(C) Schematic diagram of CD40 in control cells (left) and CD40 isoforms whose mRNAs lack exon 6 or exons 5/6 in CELF1 KO cells (middle and right).

(D) Mean + SD PM Fas levels from cells expressing the indicated sgRNAs and control or CD40 cDNA, stimulated by CD40L as indicated, from n = 3 experiments. *** $p < 0.001$

(A) and (B) are representative of at least n = 3 experiments.

Figure S6

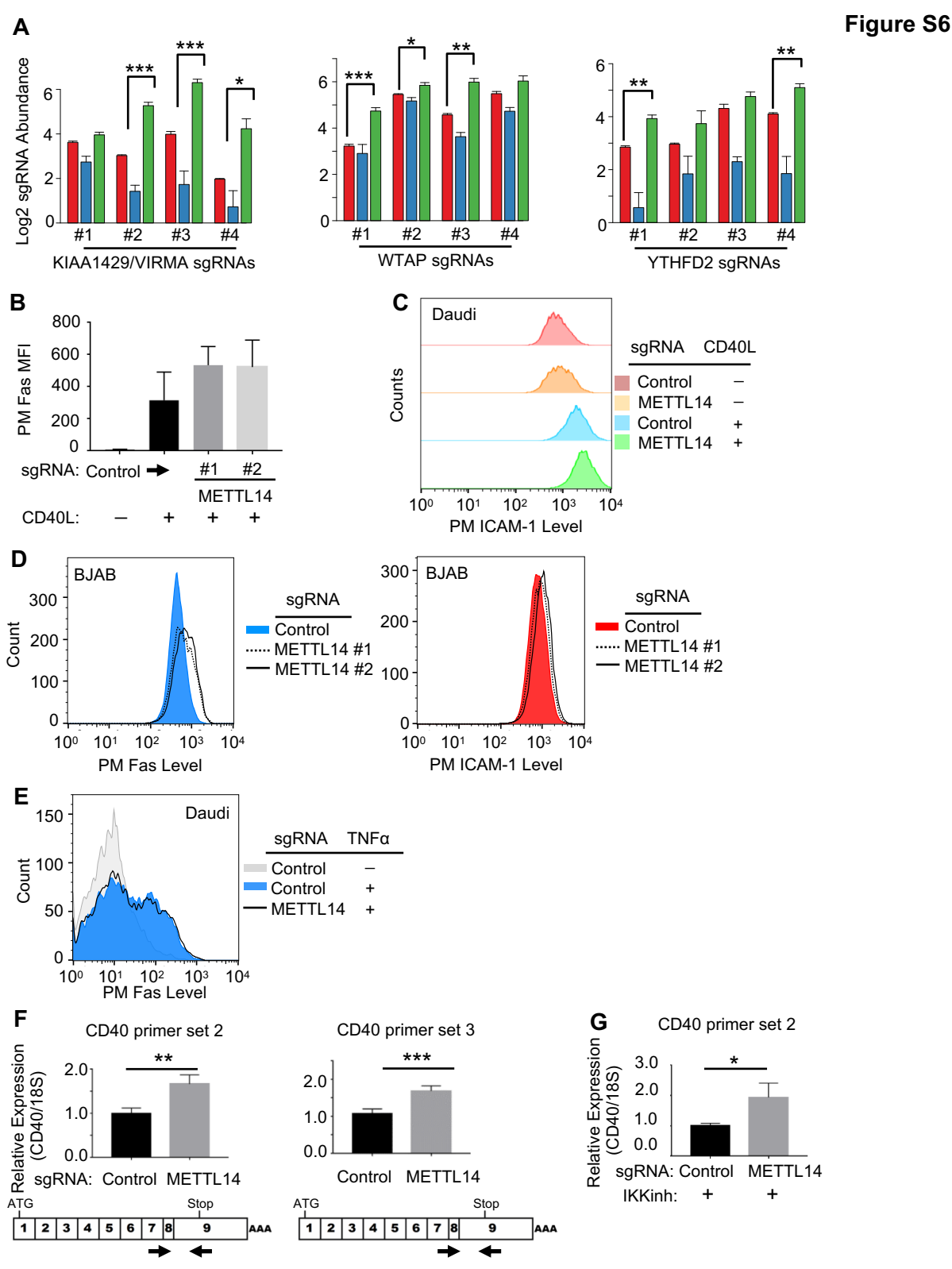

A
Log2 sgRNA Abundance
KIAA1429/VIRMA sgRNAs
WTAP sgRNAs
YTHFD2 sgRNAs
#1
#2
#3
#4
B
PM Fas MFI
sgRNA: Control
METTL14
CD40L:
C
Daudi
Counts
PM ICAM-1 Level
sgRNA
CD40L
Control
METTL14
D
BJAB
Count
PM Fas Level
PM ICAM-1 Level
sgRNA
Control
METTL14 #1
METTL14 #2
E
Daudi
Count
PM Fas Level
sgRNA
TNFα
F
CD40 primer set 2
CD40 primer set 3
Relative Expression (CD40/18S)
sgRNA: Control METTL14
ATG
Stop
AAA
G
CD40 primer set 2
Relative Expression (CD40/18S)
sgRNA: Control METTL14
IKKinh:

Figure S6. Related to Figure 6; The m6A writer WTAP complex negatively regulates CD40 mRNA abundance.

(A) Log2-normlized CRISPR screen KIAA1429/VIRMA, WTAP and YTHFD2 sgRNA abundances of input, sorted Fas low and Fas high populations. Mean + standard deviation (SD) of two input libraries and four screen replicates are shown.

(B) FACS analysis of PM Fas levels in Cas9+ Daudi B cells expressing the indicated sgRNAs and stimulated with Mega-CD40L (50 ng/ml for 48 hours), as shown.

(C) FACS analysis of PM ICAM-1 levels in Cas9+ Daudi B cells expressing control or independent METTL14 sgRNAs and stimulated with Mega-CD40L (50 ng/ml for 48 hours), as shown.

(D) FACS analysis of PM Fas and ICAM-1 levels in Cas9+ BJAB B cells expressing control or METTL14-targeting sgRNAs.

(E) FACS analysis of PM Fas levels in Cas9+ Daudi B-cells expressing control or METTL14-targeting sgRNAs and stimulated with TNFα (10 ng/ml) for 24 hours, as indicated.

(F) RT-PCR analysis of 18S rRNA-normalized CD40 mRNA levels in Cas9+ Daudi cells expressing control vs. METTL14 sgRNAs, using two independent primer pairs distinct from that used in Figure 6E. Schematic diagrams of CD40 exonic locations of primer pairs used is shown beneath each panel.

(G) RT-PCR analysis of 18S rRNA normalized CD40 mRNA levels in Cas9+ Daudi cells expressing control or METTL14-targeting sgRNAs and that were treated with 5 μM IKK inhibitor (IKK inh) for 24 hours, using primer pair set #2.

Mean and SD from n = 3 experiments are shown in (B), (D), (G) and (H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C), (E) and (F) are representative of at least n = 3 experiments.

Figure S7

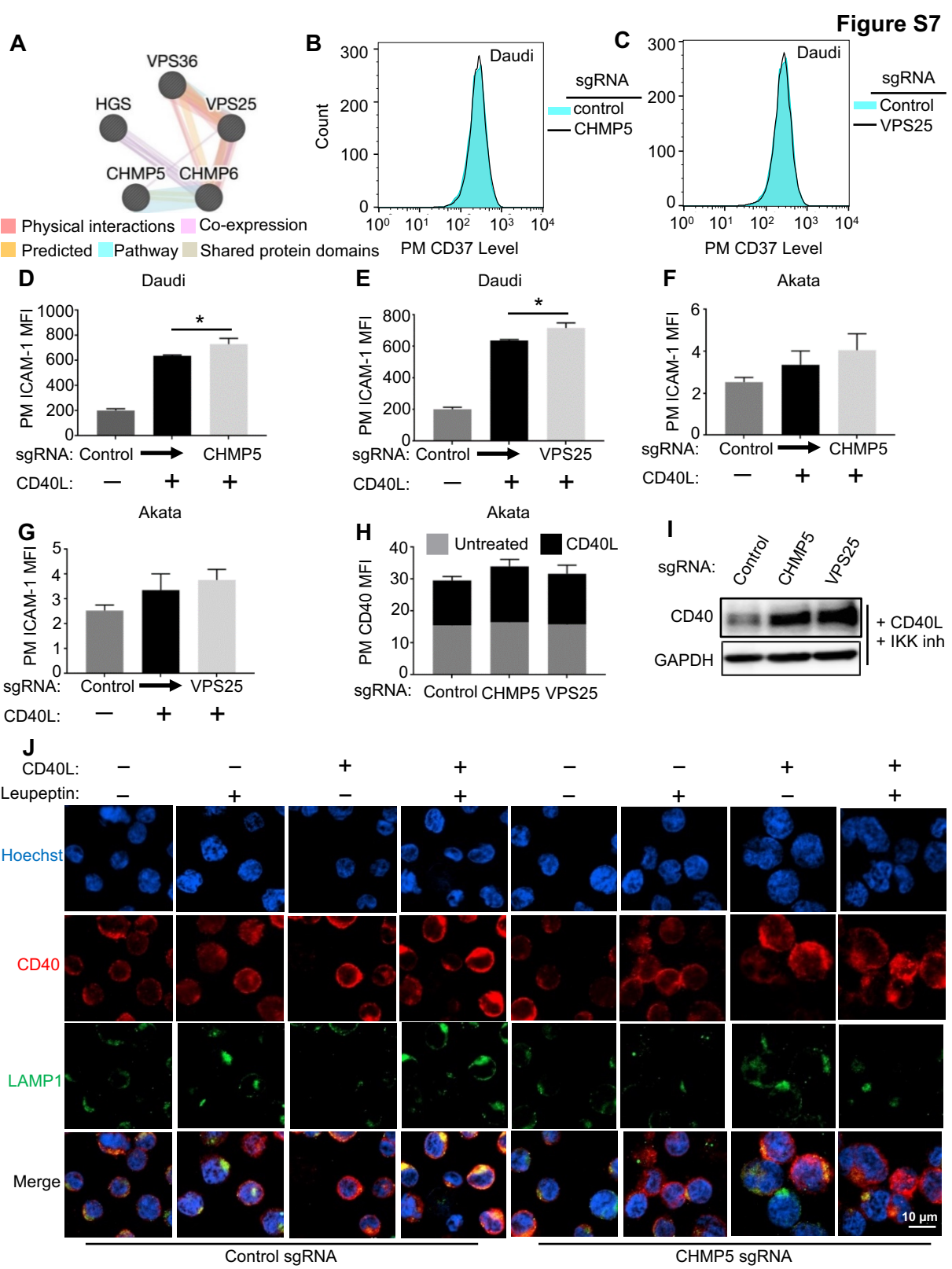

A
VPS36
HGS
VPS25
CHMP5
CHMP6
Physical interactions
Co-expression
Predicted
Pathway
Shared protein domains
B
Daudi
Count
sgRNA
control
CHMP5
PM CD37 Level
C
Daudi
sgRNA
Control
VPS25
PM CD37 Level
D
Daudi
PM ICAM-1 MFI
sgRNA: Control CHMP5
CD40L:
E
Daudi
PM ICAM-1 MFI
sgRNA: Control VPS25
CD40L:
F
Akata
PM ICAM-1 MFI
sgRNA: Control CHMP5
CD40L:
G
Akata
PM ICAM-1 MFI
sgRNA: Control VPS25
CD40L:
H
Akata
Untreated
CD40L
PM CD40 MFI
sgRNA: Control CHMP5 VPS25
I
sgRNA: Control CHMP5 VPS25
CD40
GAPDH
+ CD40L
+ IKK inh
J
CD40L:
Leupeptin:
Hoechst
CD40
LAMP1
Merge
10 μm
Control sgRNA
CHMP5 sgRNA

Figure S7. Related to **Figure 6**; ESCRT downregulates activated CD40 receptor abundance.

(A) Protein-protein interaction map of ESCRT components identified in the CD40 negative regulator screen.

(B,C) FACS analysis of PM CD37 MFI in Cas9+ Daudi B cells expressing control, CHMP5 or VPS25 sgRNAs, as indicated.

(D,E) FACS analysis of ICAM-1 PM levels in Cas9+ Daudi B cells expressing control, CHMP5 or VPS25 targeting sgRNAs and stimulated with Mega-CD40L (50 ng/mL) for 48 hours, as indicated.

(F,G) PM ICAM-1 MFI in Cas9+ Akata B cells expressing either control sgRNA or sgRNAs targeting CHMP5 (f) or VPS25 (g) sgRNAs and stimulated with Mega-CD40L (50 ng/ml), as indicated.

(H) FACS analysis of PM CD40 levels in Cas9+ Akata B cells with control sgRNA or sgRNAs targeting CHMP5 or VPS25 and stimulated with Mega-CD40L (50 ng/mL for 48 hours), as shown.

(I) Immunoblot analysis of WCE from Cas9+ Daudi cells expressing the indicated control, CHMP5 or VPS25 targeting sgRNA and stimulated with Mega-CD40L (50 ng/mL) for 24h in the presence of Calbiochem IKK inhibitor VIII at 5 μM.

(J) Confocal immunofluorescence microscopy analysis of Cas9+ Daudi B-cells expressing control sgRNA (left) or CHMP5 targeting sgRNA (right) and treated with Mega-CD40L (50 ng/mL) for 12 hours and/or 10 μM leupeptin for 13 hours (leupeptin was given 1 hour prior to CD40L treatment), as indicated. Scale bar indicates 10 μM and was the same for all panels.

Mean and SD from n = 3 experiments are shown in (D-H). (B), (C), (I) and (J) are representative of n = 3 independent experiments.