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Supplemental Information

Stabilization of the Transcription Factor Foxp3 by the Deubiquitinase USP7 Increases Treg-Cell-Suppressive Capacity

Jorg van Loosdregt, Veerle Fleskens, Juan Fu, Arjan B. Brenkman, Cornelis P.J. Bekker, Cornelieke E.G.M. Pals, Jenny Meerding, Celia R. Berkers, Joseph Barbi, Andrea Gröne, Alice J.A.M. Sijts, Madelon M. Maurice, Eric Kalkhoven, Berent J. Prakken, Huib Ovaa, Fan Pan, Dietmar M.W. Zaiss, and Paul J. Coffer

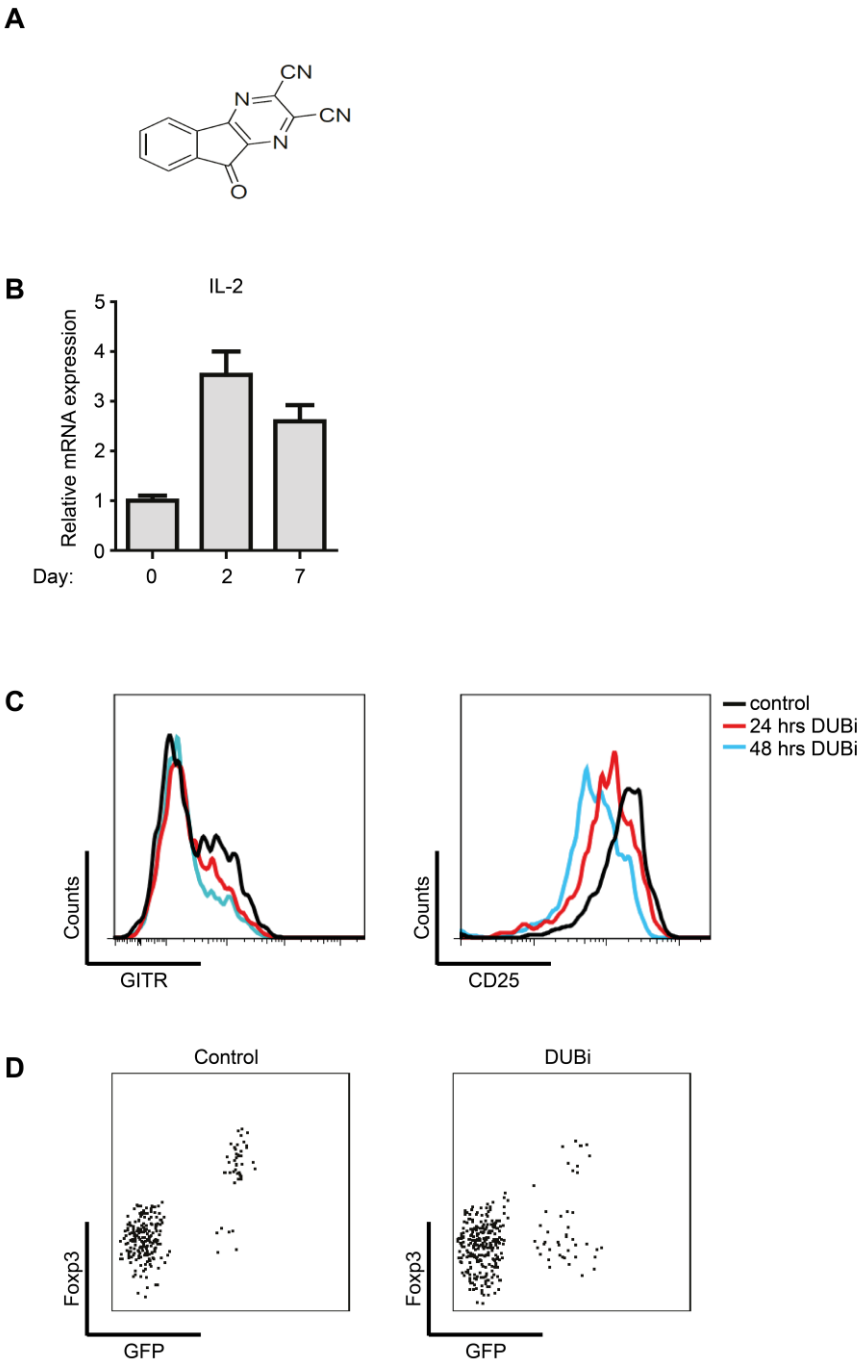

Figure S1. DUBs Regulate Treg-Cell-Mediated Suppression, Related to Figure 1

(A) Structure of DUB inhibitor (DUBi).

(B) Human Treg cells were transiently treated with 10 µM DUBi for one hour. Two or seven days after treatment IL-2 and USP7 mRNA expression was analyzed by qRT-PCR. All values were normalized for housekeeping gene GAPDH.

(C) Human Treg cells were cultured in the presence of 2 µM DUBi for 24 or 48 hours and GITR or CD25 expression of $CD4^{+}Foxp3^{+}$ Treg cells was analyzed utilizing flow cytometry.

(D) Representable FACS plots of Figure 1E.

Data are represented as mean + SEM.

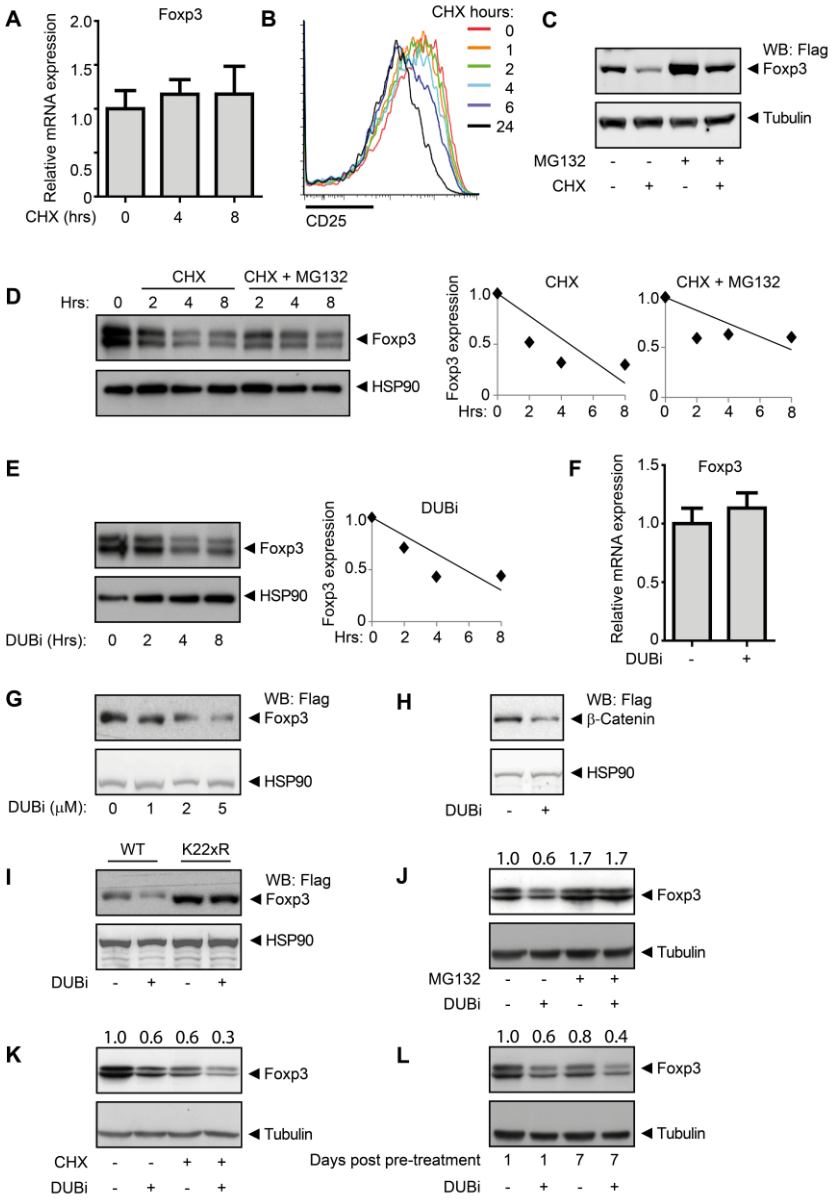

Figure S2. DUBs Regulate Foxp3 Protein Stability, Related to Figure 2

(A) Human Treg cells were transiently treated with 150 μg/ml Cycloheximide (CHX) for 4 or 8 hours. Foxp3 mRNA expression was analyzed by qRT-PCR. All values were normalized for housekeeping gene GAPDH.

(B) Human Treg cells were transiently treated with CHX for 0, 1, 2, 4, 6 or 24 hours. CD25 expression was analyzed by FACS.

(C) Flag-Foxp3 transfected HEK293 cells were treated with CHX and MG132. Foxp3 expression was determined by Western blot utilizing anti-Flag antibodies.

(D) nTreg cells were treated with CHX +/- MG132 for 0, 2, 4, or 8 hours and Foxp3 expression was determined by Western blot.

(E) nTreg cells were incubated with DUBi (5 µM) for 0, 2, 4, or 8 hours and Foxp3 expression was determined by Western blot.

(F) Treg cells were treated with 10 µM DUBi for 16 hours. qRT-PCR was utilized to determine Foxp3 mRNA expression.

(G) Flag-Foxp3 transfected HEK293 cells were treated with increasing concentrations of DUBi for 18 hours and the amount of Foxp3 was determined by Western blot.

(H) Flag-β-catenin transfected HEK293 cells were incubated with 5 µM DUBi for 18 hours and expression was analyzed by Western blot utilizing specific antibodies.

(I) HEK293 cells were transfected with epitope tagged wild-type (WT) Foxp3 or Foxp3 K22xR and cultured in the presence or absence of 5 µM DUBi for 18 hours.

(J and K) Human Treg cells were treated with 150 µg/ml CHX or 2 µM MG132 for 16 hours in the presence or absence of 5 µM DUBi. Foxp3 protein expression was determined by Western blotting using antibodies directed against Foxp3 and tubulin.

(L) Treg cells were transiently treated with 10 µM DUBi (for one hour), or control samples were left untreated. One or seven days after treatment cell lysates were prepared, Western blotted, and analyzed for Foxp3 expression utilizing specific antibodies.

Data are represented as mean + SEM.

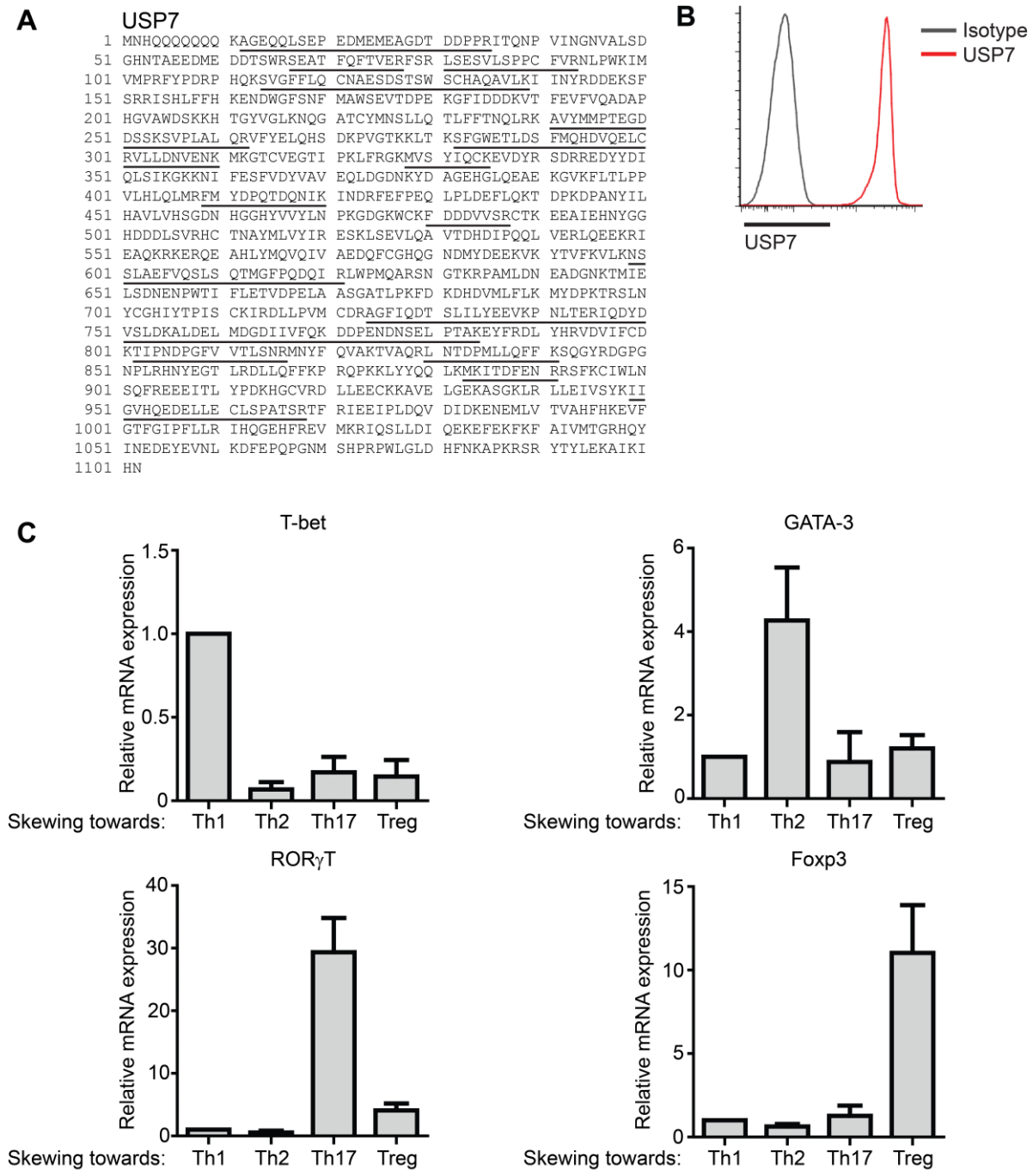

Figure S3. USP7 mRNA Is Specifically Upregulated in T Cells Differentiated to Treg Cells, Related to Figures 3 and 4

(A) Mass spectrometric analysis of Figure 4A, underlined amino acids represent the peptides that were identified.

(B) USP7 expression in $CD4^{+}CD25^{high}Foxp3^{+}$ Treg cells analyzed by flow cytometry.

(C) Naïve $CD4^+$ T cells were differentiated to Th1, Th2, Th17 and Treg cells. mRNA expression of T-bet, GATA-3, RORγt, and Foxp3, the hallmark transcription factors of these Th cell subsets, was analyzed by qRT-PCR. mRNA expression was normalized for housekeeping gene GAPDH.

Data are represented as mean + SEM.

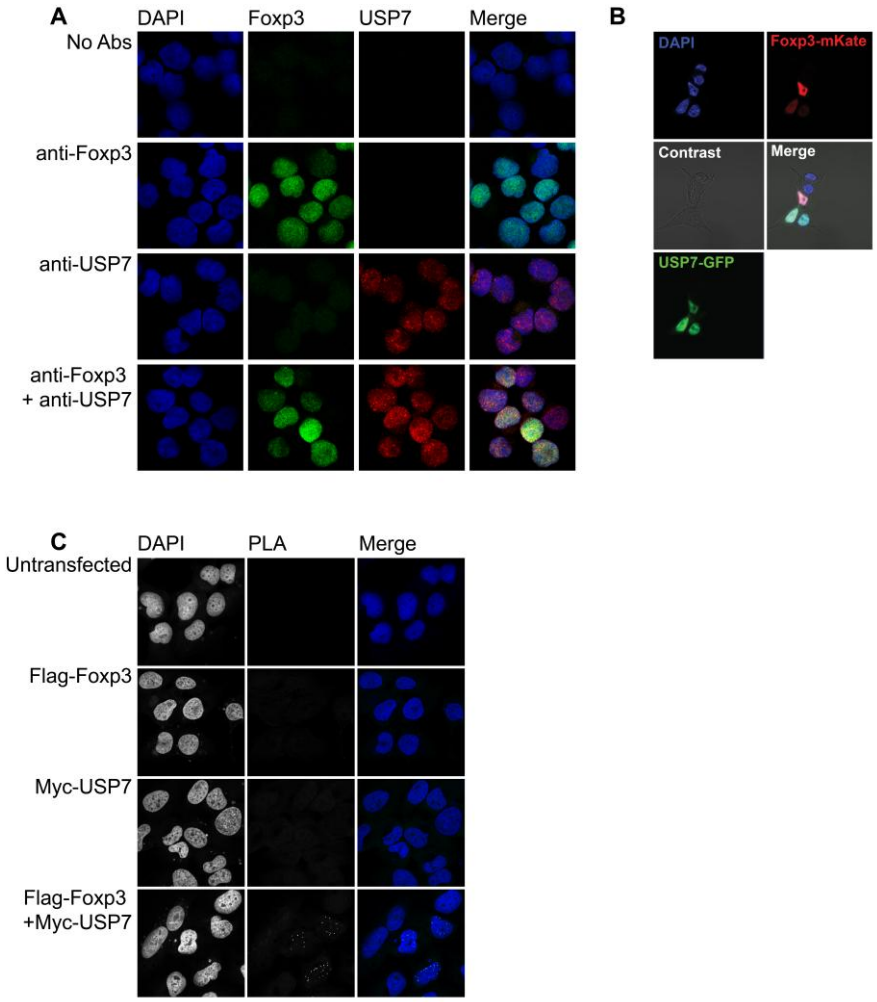

Figure S4. USP7 and Foxp3 Interact in the Nucleus of Treg Cells, Related to Figure 4

(A) Representative confocal microscopy images of human Treg cells. Endogenous USP7 (red) and Foxp3 (green) was visualized in human Treg cells utilizing specific antibodies, DAPI was used to visualize the nuclei (blue).

(B) Representative examples of HEK293 cells that were co-transfected with mKate-Foxp3 (red) and USP7-GFP (green). Nuclei were visualized using DAPI (blue).

(C) Foxp3-USP7 association of ectopically expressed Flag-Foxp3 and MYC-USP7 was visualized in HEK293 cells using an *in situ* proximity ligation assay (PLA) as described in the Experimental Procedures section. Punctate staining (green) indicates a Foxp3-USP7 interaction as detected by the assay, DAPI was used to visualize the nuclei (blue).

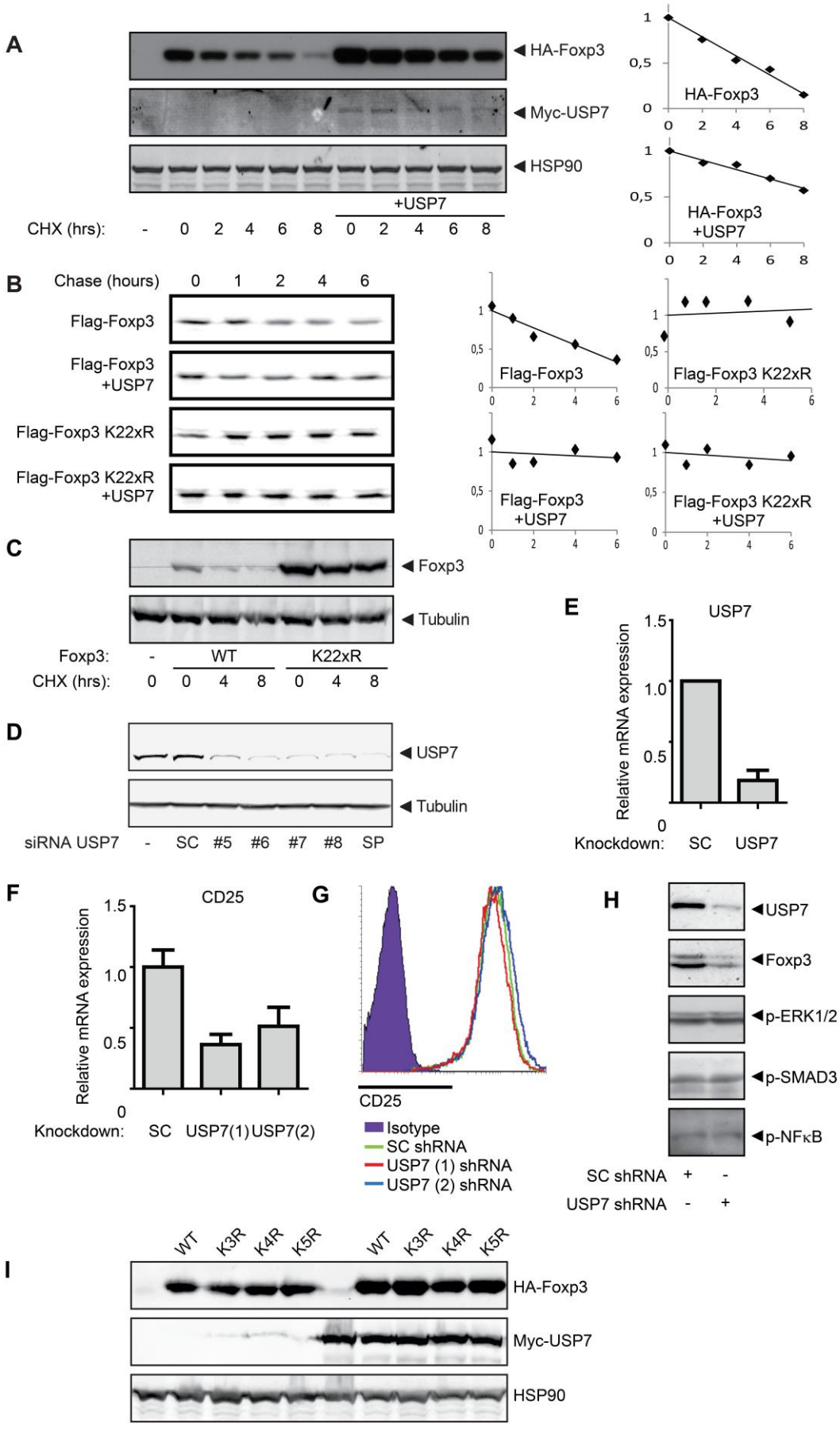
A
HA-Foxp3
Myc-USP7
HSP90
+USP7
CHX (hrs): - 0 2 4 6 8 0 2 4 6 8
HA-Foxp3
HA-Foxp3 +USP7
B
Chase (hours) 0 1 2 4 6
Flag-Foxp3
Flag-Foxp3 +USP7
Flag-Foxp3 K22xR
Flag-Foxp3 K22xR +USP7
Flag-Foxp3
Flag-Foxp3 K22xR
Flag-Foxp3 +USP7
Flag-Foxp3 K22xR +USP7
C
Foxp3
Tubulin
Foxp3: - WT K22xR
CHX (hrs): 0 0 4 8 0 4 8
D
USP7
Tubulin
siRNA USP7 - SC #5 #6 #7 #8 SP
E
USP7
Relative mRNA expression
Knockdown: SC USP7
F
CD25
Relative mRNA expression
Knockdown: SC USP7(1) USP7(2)
G
CD25
Isotype
SC shRNA
USP7 (1) shRNA
USP7 (2) shRNA
H
USP7
Foxp3
p-ERK1/2
p-SMAD3
p-NFκB
SC shRNA + -
USP7 shRNA - +
I
WT K3R K4R K5R WT K3R K4R K5R
HA-Foxp3
Myc-USP7
HSP90

Figure S5. USP7 Regulates Foxp3 Protein Stability, Related to Figure 5

(A) HEK293 cells were transfected with HA-Foxp3 and Myc-USP7 and treated with 150 μg/ml CHX for 0, 2, 4, or 8 hours. Foxp3 protein was determined by Western blot.

(B) HEK293 cells were transfected with Foxp3, or Foxp3 K22xR in the presence or absence of USP7. Radioactive pulse-chase experiments were performed to visualize Foxp3 stability.

(C) Jurkat T cells were transfected with epitope tagged Foxp3 or Foxp3 K22xR and treated with 150 μg/ml CHX for 0, 4, or 8 hours. Foxp3 protein expression was assessed by Western blotting utilizing anti-Flag antibodies.

(D) HEK293 cells were transfected with different USP7 siRNAs. Endogenous USP7 expression was analyzed by Western blotting utilizing anti-USP7 antibodies, tubulin was used as control. Scrambled (SC), Smart Pool (SP).

(E) USP7 was knocked down in Treg cells using shRNA lentivirus containing a puromycin resistance cassette. USP7 mRNA expression was analyzed on puromycin selected cells and normalized for the housekeeping gene GAPDH.

(F) USP7 was knocked down in Treg cells and CD25 expression was analyzed by flow cytometry.

(G) USP7 was knocked down in activated Foxp3 negative CD4 Tcells, and expression was analyzed by flow cytometry.

(H) USP7 was knocked down in Treg cells and cells were lysed. Multiple downstream targets of the TGF-β and TCR signaling pathway were analyzed by Western blot.

(I) HEK293 cells were transfected with Foxp3 K3R (31,263,267), K4R (249,251,263,267) or K5R (249,251,263,267,393) and USP7. Foxp3 expression was determined by Western blot.

Data are represented as mean + SEM.

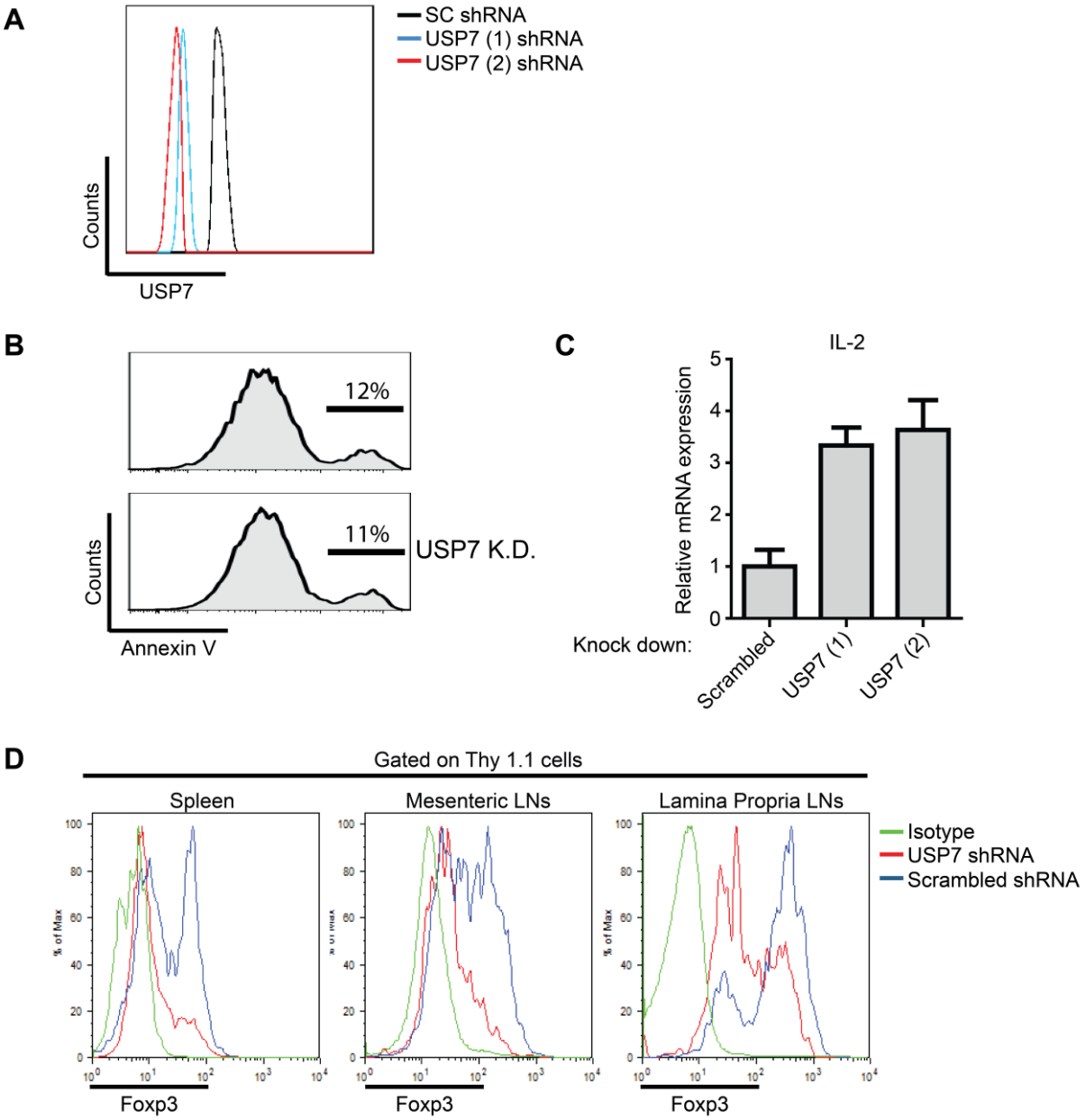

Figure S6. Modulation of Foxp3 Degradation by USP7 Regulates Treg-Cell-Mediated Suppression, Related to Figure 6

(A) Analysis of USP7 knock down efficiency for the experiment shown in Figure 6B.

(B) USP7 was knocked down in Treg cells using shRNA lentivirus containing a puromycin resistance cassette. Puromycin selected cells were washed, cultured for four days and apoptosis was analyzed by annexin V staining utilizing flow cytometry.

(C) USP7 was knocked down in Treg cells using shRNA lentivirus containing a puromycin resistance cassette. IL-2 mRNA of puromycin selected cells was analyzed by quantitative PCR.

(D) Representative FACS plots of the experiment depicted in Figure 6G.

Data are represented as mean + SEM.