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Supplemental information

The Ubiquitin Ligase Stub1 Negatively Modulates Regulatory T cell Suppressive Activity by Promoting Degradation of the Transcription Factor Foxp3

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1. Supplemental Figures and Tables

2. Supplemental Experimental Procedures

Figure S1.

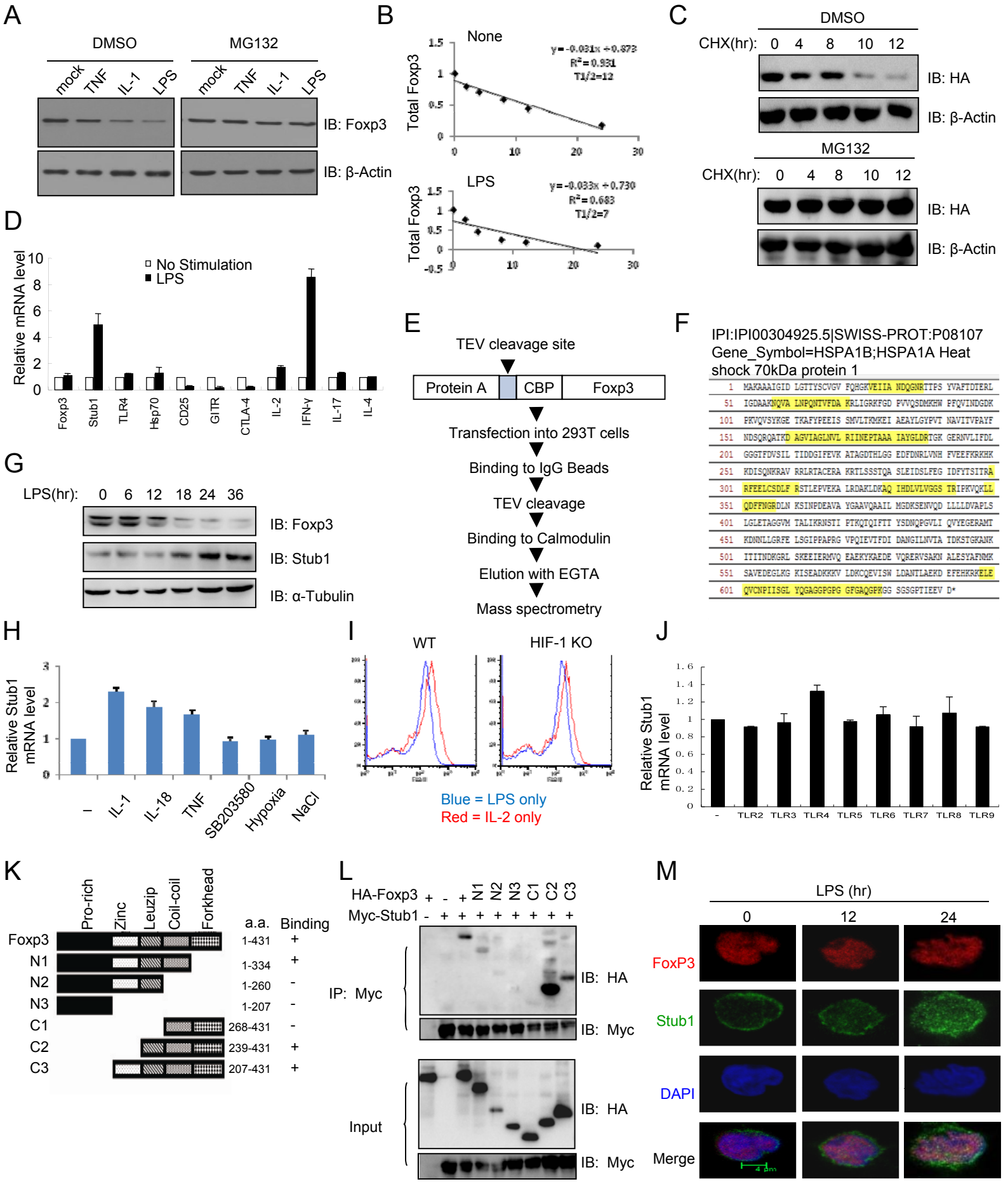

A
DMSO
MG132
mock
TNF
IL-1
LPS
IB: Foxp3
IB: β-Actin
B
None
y = -0.031x + 0.872
R² = 0.931
T1/2=12
LPS
y = -0.033x + 0.730
R² = 0.683
T1/2=7
Total Foxp3
C
DMSO
CHX(hr): 0 4 8 10 12
IB: HA
IB: β-Actin
MG132
D
No Stimulation
LPS
Relative mRNA level
Foxp3
Stub1
TLR4
Hsp70
CD25
GITR
CTLA-4
IL-2
IFN-γ
IL-17
IL-4
E
TEV cleavage site
Protein A
CBP
Foxp3
Transfection into 293T cells
Binding to IgG Beads
TEV cleavage
Binding to Calmodulin
Elution with EGTA
Mass spectrometry
F
IPI:IPI00304925.5|SWISS-PROT:P08107
Gene_Symbol=HSPA1B;HSPA1A Heat shock 70kDa protein 1
1 MAKAAAIGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
51 IGDAAKNQVA LNPQNTVFDA KRLIGRKFGD PVVQSDMKHW PFQVINDGDK
101 PKVQVSYKGE TKAFYPEEIS SMVLTKMKEI AEAYLGYPVT NAVITVPAYF
151 NDSQRQATKD AGVIAGLNVL RIINEPTAAA IAYGLDRTGK GERNVLIFDL
201 GGGTFDVSIL TIDDGIFEVK ATAGDTHLGG EDFDNRLVNH FVEEFKRKHK
251 KDISQNKRAV RRLRTACERA KRTLSSSTQA SLEIDSLFEG IDFYTSITRA
301 RFEELCSDLF RSTLEPVEKA LRDAKLDKAQ IHDLVLVGGS TRIPKVQKLL
351 QDFFNGRDLN KSINPDEAVA YGAAVQAAIL MGDKSENVQD LLLLDVAPLS
401 LGLETAGGVM TALIKRNSTI PTKQTQIFTT YSDNQPGVLI QVYEGERAMT
451 KDNNLLGRFE LSGIPPAPRG VPQIEVTFDI DANGILNVTA TDKSTGKANK
501 ITITNDKGRL SKEEIERMVQ EAEKYKAEDE VQRERVSAKN ALESYAFNMK
551 SAVEDEGLKG KISEADKKKV LDKCQEVISW LDANTLAEKD EFEHKRKELE
601 QVCNPIISGL YQGAGGPGPG GFGAQGPKGG SGSGPTIEEV D*
G
LPS(hr): 0 6 12 18 24 36
IB: Foxp3
IB: Stub1
IB: α-Tubulin
H
Relative Stub1 mRNA level
–
IL-1
IL-18
TNF
SB203580
Hypoxia
NaCl
I
WT
HIF-1 KO
Blue = LPS only
Red = IL-2 only
J
Relative Stub1 mRNA level
-
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
K
Pro-rich
Zinc
Leuzip
Coil-coil
Forkhead
a.a.
Binding
Foxp3 1-431 +
N1 1-334 +
N2 1-260 -
N3 1-207 -
C1 268-431 -
C2 239-431 +
C3 207-431 +
L
HA-Foxp3 + - + N1 N2 N3 C1 C2 C3
Myc-Stub1 - + + + + + + + +
IP: Myc
IB: HA
IB: Myc
Input
IB: HA
IB: Myc
M
LPS (hr)
0
12
24
FoxP3
Stub1
DAPI
Merge

Figure S1. LPS is a potent stimulus inducing the association between Foxp3 and Stub1, Foxp3 degradation and altering the characteristic gene expression of primary Tregs. Related to Figure 1.

(A) Mouse nTregs ($CD4^{+}CD25^{High}$) were stimulated with LPS or other cytokines as indicated in the absence or presence of 5μM MG132. Cells were harvested and subjected to Western blotting.

(B) LPS decreased Foxp3 half-life in Treg cells. Band densities indicating Foxp3 amount after CHX treatment as indicated in Figure 1D was quantified using Image J software.

(C) Foxp3 levels are stabilized by proteasome inhibition. Jurkat T cells expressing HA-Foxp3 were treated with 5μg/ml CHX with or without the addition of MG132. Cells were harvested at the indicated time-points and analyzed by Western blotting.

(D) LPS stimulation upregulates Stub1 and effector genes in Tregs while downregulating those involved in Treg function. qRT-PCR analysis was used to detect expression levels of Foxp3, Stub1 or the indicated genes.

(E) Schematic summary of tandem affinity purification of TAP-tagged Foxp3 Complex (See Methods Section).

(F) The proteins pulled down by TAP-Foxp3 were resolved by gel electrophoresis and subjected to Mass Spectrometry analysis. The identified peptides of Hsp70 are highlighted in the full length sequence.

(G) LPS downregulated Foxp3 and upregulated Stub1 in Treg cells. Human Treg cells were treated with 1μg/ml LPS. Cells were harvested at the indicated time-points. Cell lysates were analyzed by Western blotting.

(H) Human primary Tregs were treated with different cytokines or other stress stimulus as indicated. Cells were harvested and subjected to qRT-PCR analysis.

(I) HIF-1α is not required for LPS induced Foxp3 degradation in nTregs.

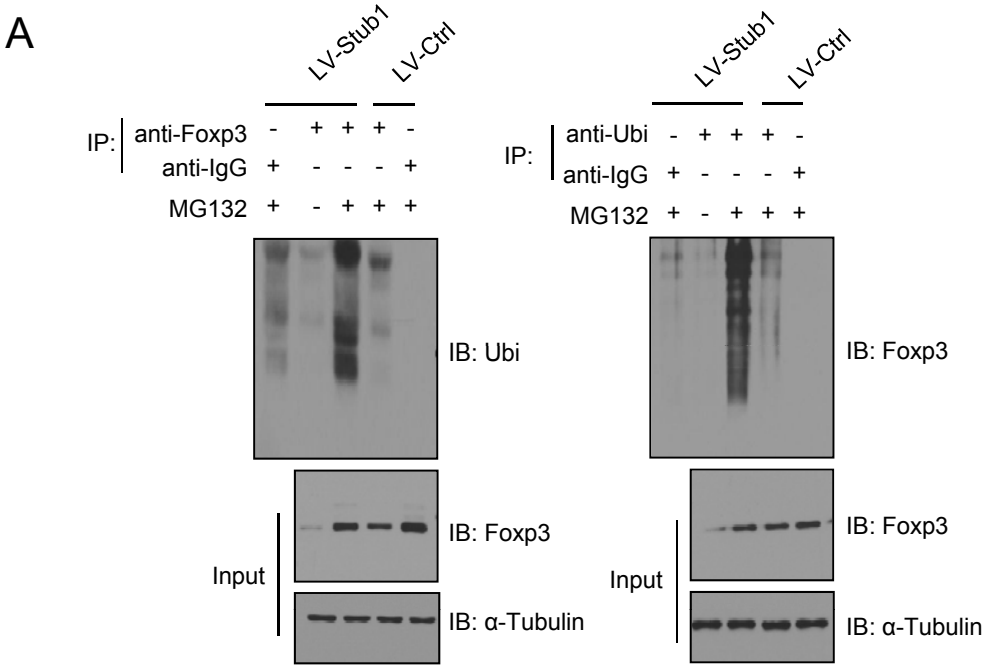
(J) Human primary Tregs were treated with different TLR agonists as indicated. Cells were harvested and subjected to qRT-PCR analysis.

(K) and **(L)** Different truncated Foxp3 constructs were generated as shown, and were cotransfected with or without Myc-Stub1 into 293T cells. Cell lysates were immunoprecipitated using anti-Myc antibody and Foxp3 levels were detected by Western blotting.

(M) Stub1 enters into the nucleus and colocalizes with Foxp3 under LPS stimulation. HA-Foxp3 Jurkat cells were stimulated with LPS and treated with 5μM MG132 in order to preserve Foxp3 protein for visualization. Cell samples were harvested at the indicated time-points and stained with PE conjugated anti-Foxp3 antibody (eBioscience, Cat 12-4777-42) (Red), and anti-Stub1

antibody (Bethyl, Cat IHC-00345), followed by 488-labeled anti-rabbit antibody (Green) and nuclei stained with DAPI (Blue). The fluorescence signal was detected on a laser confocal microscope. Shown are representative findings from at least three independent experiments.

Figure S2


A
LV-Stub1
LV-Ctrl
IP:
anti-Foxp3 - + + + -
anti-IgG + - - - +
MG132 + - + + +
IB: Ubi
Input
IB: Foxp3
IB: α-Tubulin
LV-Stub1
LV-Ctrl
IP:
anti-Ubi - + + + -
anti-IgG + - - - +
MG132 + - + + +
IB: Foxp3
Input
IB: Foxp3
IB: α-Tubulin

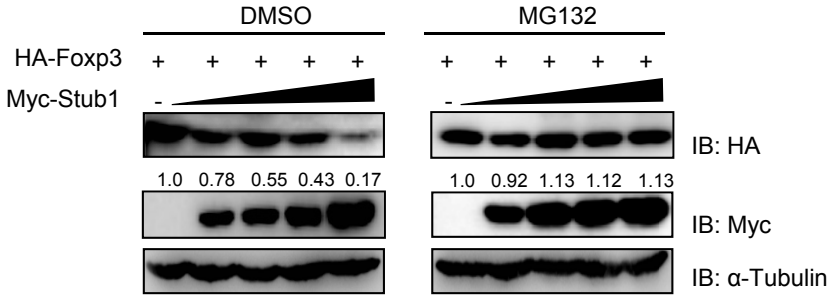

B
DMSO
MG132
HA-Foxp3 + + + + + + + + + +
Myc-Stub1 - -
IB: HA
1.0 0.78 0.55 0.43 0.17
1.0 0.92 1.13 1.12 1.13
IB: Myc
IB: α-Tubulin

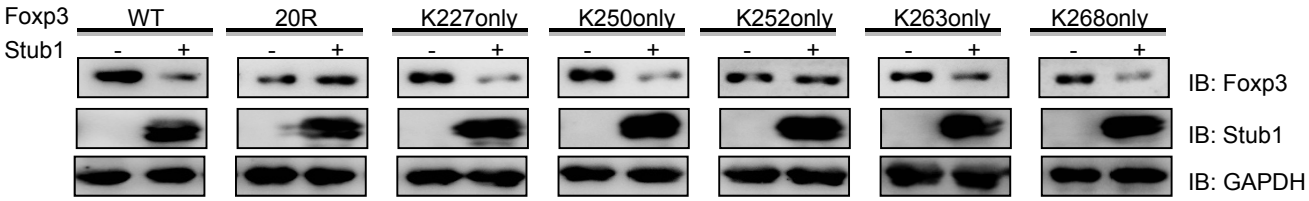
Figure S2. Stub1 ubiquitinates Foxp3 in Treg cells and destabilizes Foxp3 protein levels in a dose dependent manner. Related to Figure 2.

(A) Primary mouse T cells were transduced with either lentivirus-based control vector (LV-Ctrl) or LV-Stub1 expression plasmid carrying an IRES-GFP marker for monitoring transduction efficiency. GFP^+ cells were sorted and cultured under iTreg skewing conditions for an additional 3 days before being harvested and lysed, followed by immunoprecipitation with anti-Foxp3 or control IgG antibodies. The pulled down proteins along with an input control were resolved by SDS-PAGE and blotted with the indicated antibodies. Depicted are typical findings from three independent experiments.

(B) 293T cells were co-transfected with HA-Foxp3 and the indicated doses of Myc-Stub1 plasmid. 24-hr post-transfection cells were treated with MG132 or DMSO for 6 hr followed by harvesting and Western blotting analysis using the indicated antibodies. Relative band densities indicating protein amount is shown below each band.

Figure S3.

A


Foxp3
Stub1
WT
20R
K227only
K250only
K252only
K263only
K268only
- + - + - + - + - + - + - +
IB: Foxp3
IB: Stub1
IB: GAPDH

B

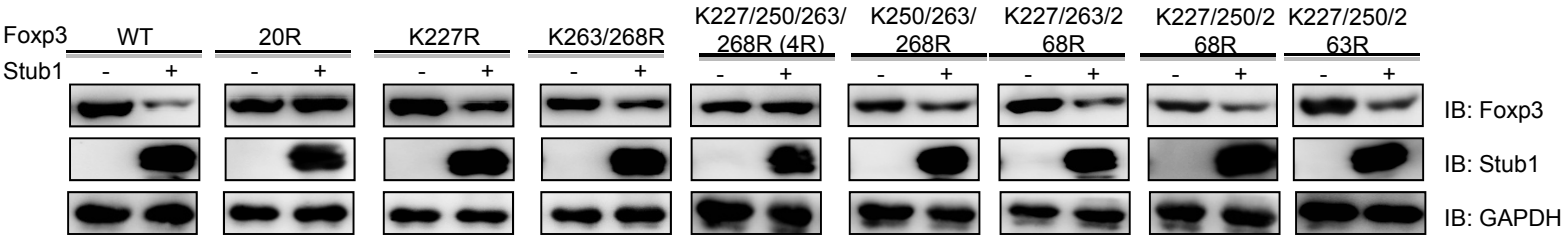

Foxp3
Stub1
WT
20R
K227R
K263/268R
K227/250/263/ 268R (4R)
K250/263/ 268R
K227/263/2 68R
K227/250/2 68R
K227/250/2 63R
- + - + - + - + - + - + - + - + - +
IB: Foxp3
IB: Stub1
IB: GAPDH

Figure S3. Determination of the lysine residues of Foxp3 responsible for Stub1-mediated degradation. Related to Figure 3. A panel of Foxp3 mutants in which an individual lysine residue, or multiple lysine residues were mutated (K→R) was screened for their resistance to degradation by Stub1. Cells were transfected with the indicated Foxp3 mutant constructs with or without a Stub1 overexpression plasmid. Foxp3 protein levels were assessed by Western blotting.

(A) The resistance of wild type (WT) Foxp3 to Stub1-mediated degradation was compared to that of a mutant having all 20 lysine residues substituted and mutants with specific individual residues left unaltered.

(B) Additionally, the stability of Foxp3 mutants having individual lysine resides or combinations of individual mutants were also examined.

Figure S4.

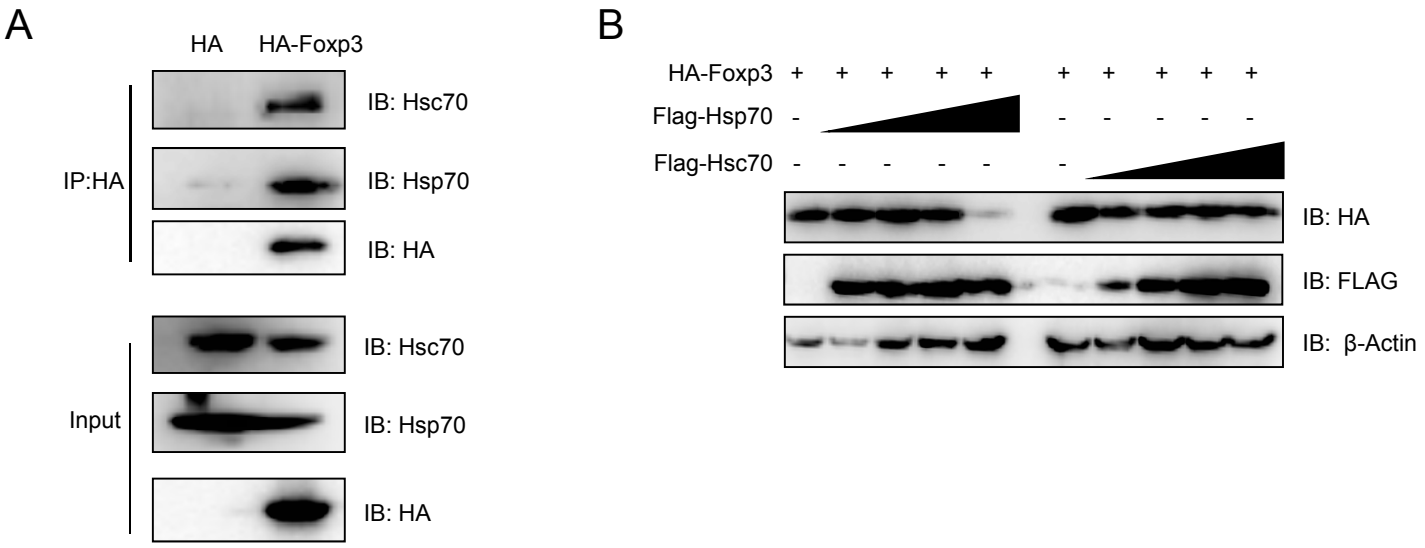

A
HA
HA-Foxp3
IP:HA
IB: Hsc70
IB: Hsp70
IB: HA
Input
IB: Hsc70
IB: Hsp70
IB: HA
B
HA-Foxp3 + + + + + + + + + +
Flag-Hsp70 - - - - - - -
Flag-Hsc70 - - - - - - -
IB: HA
IB: FLAG
IB: β-Actin

Figure S4. Hsp70 but not Hsc70 promotes Foxp3 degradation. Related to Figure 4.

(A) HEK293T cells were transfected with HA-Foxp3 or empty vector. Cells were lysed and immunoprecipitated using anti-HA antibody 48-hours post-transfection. Pulled-down endogenous Hsp70 and Hsc70 were detected by Western blotting.

(B) 293T cells were transfected with HA-Foxp3 along with increasing amounts of Flag-Hsp70 or Flag-Hsc70. Cell lysates were harvested and analysed by Western blotting.

Figure S5.

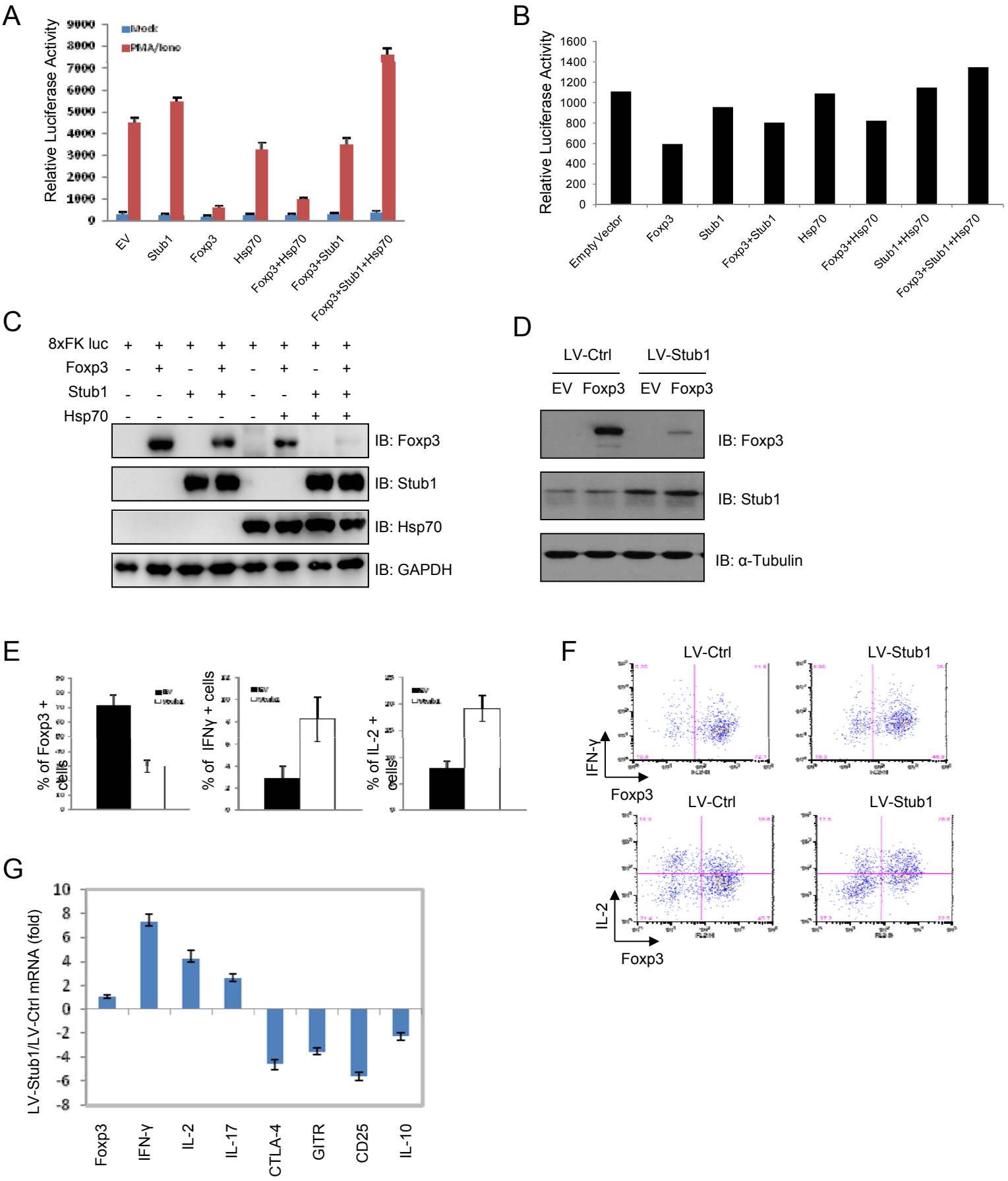

A
Relative Luciferase Activity
Mock
PMA/Iono
EV
Stub1
Foxp3
Hsp70
Foxp3+Hsp70
Foxp3+Stub1
Foxp3+Stub1+Hsp70
B
Relative Luciferase Activity
Empty Vector
Foxp3
Stub1
Foxp3+Stub1
Hsp70
Foxp3+Hsp70
Stub1+Hsp70
Foxp3+Stub1+Hsp70
C
8xFK luc
Foxp3
Stub1
Hsp70
IB: Foxp3
IB: Stub1
IB: Hsp70
IB: GAPDH
D
LV-Ctrl
LV-Stub1
EV
Foxp3
IB: Foxp3
IB: Stub1
IB: α-Tubulin
E
% of Foxp3 + cells
% of IFNγ + cells
% of IL-2 + cells
F
LV-Ctrl
LV-Stub1
IFN-γ
Foxp3
IL-2
G
LV-Stub1/LV-Ctrl mRNA (fold)
Foxp3
IFN-γ
IL-2
IL-17
CTLA-4
GITR
CD25
IL-10

Figure S5. Effects of Stub1 overexpression or knockdown on Foxp3-mediated gene suppression *in vitro*. Related to Figure 5.

(A) Jurkat T cells were co-transfected with the indicated expression constructs along with an IL-2 promoter-driven firefly luciferase reporter. 24-hours post-transfection, cells were either treated with PMA and ionomycin or left untreated for 8 hours prior to the analysis of luciferase activity, which was normalized to renilla luciferase activity. Data is presented as the mean plus s.d. of triplicate transfection experiments.

(B) The 8xFK luciferase reporter gene was co-transfected with Myc-Stub1, Flag-Hsp70 and/or HA-Foxp3 into 293T cells. Luciferase activity was detected by standard methods with β-gal activity serving as a control. The figure is representative of at least 3 independent experiments showing the mean of 3 replicates +/- SEM.

(C) The 8xFK luciferase reporter gene was co-transfected with Myc-Stub1, Flag-Hsp70 and/or HA-Foxp3 into 293T cells. Luciferase activity was determined (as in B) and the level of Foxp3 protein in each sample was determined by Western blotting.

(D) Primary mouse T cells were transduced with either lentivirus-based control vector (LV-ctrl) or LV-Stub1 expression plasmid carrying an IRES-GFP marker for monitoring transduction efficiency. GFP^+ cells were sorted, and then transduced with either a biscistronic retroviral empty vector (carrying an IRES-dsRed2 marker) or the vector expressing full-length Foxp3. GFP^+ $dsRed2^+$ cells were sorted from each group, and cultured in the presence of anti-CD3 plus anti-CD28 antibodies overnight. IL-2 production was determined by ELISA (Figure 5A) and the levels of Foxp3 protein were determined by Western blotting.

(E) $CD4^+CD25^+$ nTreg cells were isolated by FACS and transduced with either Stub1 overexpression vector or a control empty vector. Transductants were then activated by anti-CD3/anti-CD28 antibodies and levels of Foxp3, IFN-γ and IL-2 were assessed by intracellular staining and flow cytometry analysis after 48 hours of activation. Shown are the mean results of at least three independent experiments +/- SEM.

(F) nTregs were transduced as in (A) and re-stimulated for ICS staining of proinflammatory cytokines after a brief re-culture period. Depicted is the intracellular staining for Foxp3 and either IL-2 or IFN-γ in normal cells or those receiving Stub1 overexpression vector.

(G) Forced expression of Stub1 by nTreg cells alters expression of Foxp3 target genes. Shown are the changes in mRNA levels of the indicated genes in nTregs transduced with lentiviral Stub1 over-expression vector relative to that seen in nTregs carrying an empty vector control. Depicted are the mean +/-SEM results of at least three independent experiments.

Figure S6.

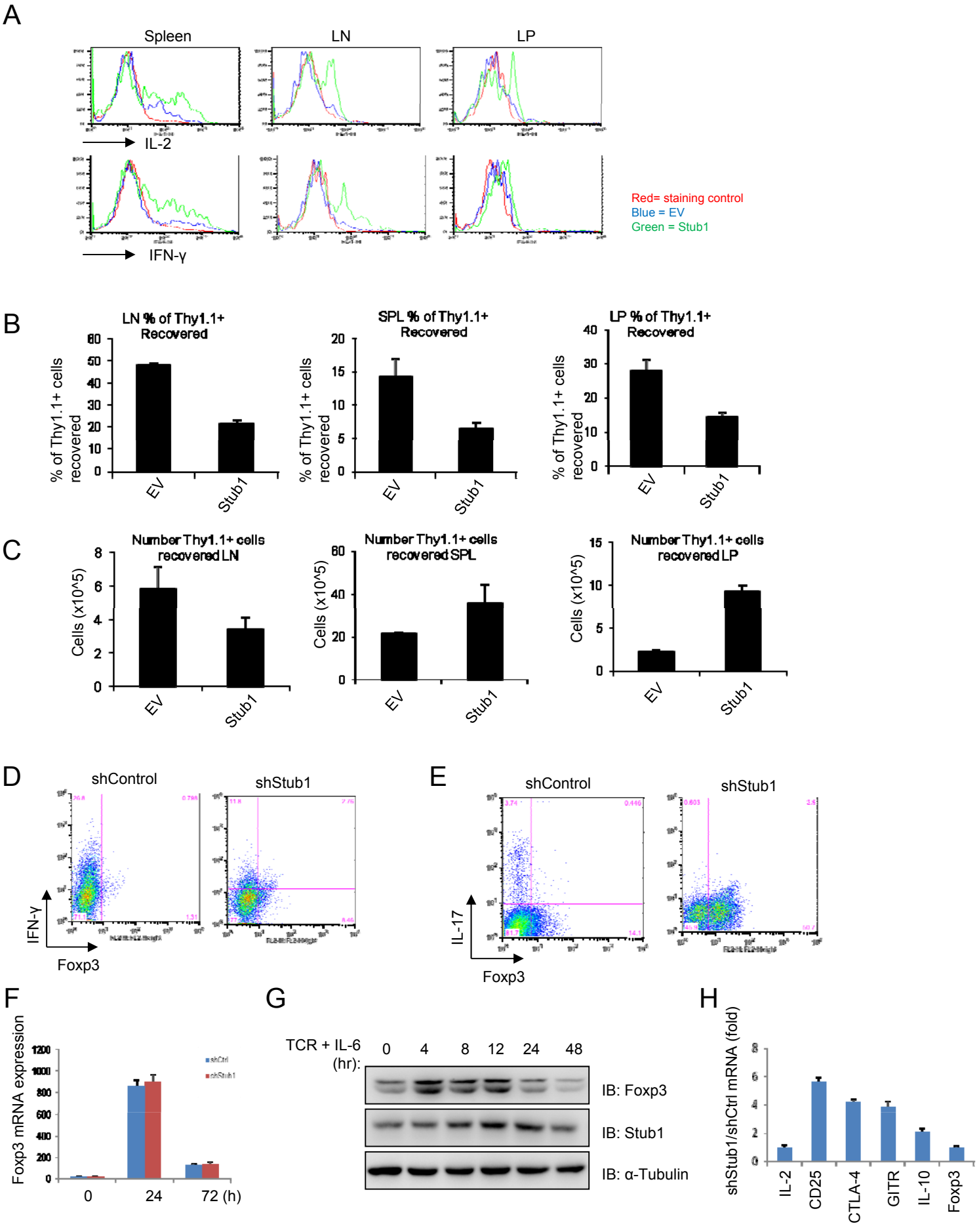

A
Spleen
LN
LP
IL-2
IFN-γ
Red= staining control
Blue = EV
Green = Stub1
B
LN % of Thy1.1+ Recovered
SPL % of Thy1.1+ Recovered
LP % of Thy1.1+ Recovered
% of Thy1.1+ cells recovered
EV
Stub1
C
Number Thy1.1+ cells recovered LN
Number Thy1.1+ cells recovered SPL
Number Thy1.1+ cells recovered LP
Cells (x10^5)
D
shControl
shStub1
IFN-γ
Foxp3
E
shControl
shStub1
IL-17
Foxp3
F
Foxp3 mRNA expression
shCtrl
shStub1
0
24
72 (h)
G
TCR + IL-6 (hr):
0
4
8
12
24
48
IB: Foxp3
IB: Stub1
IB: α-Tubulin
H
shStub1/shCtrl mRNA (fold)
IL-2
CD25
CTLA-4
GITR
IL-10
Foxp3

Figure S6. The effect of Stub1 overexpression and silencing on Treg cytokine expression and survival *in vivo* and *in vitro*. Related to Figure 6.

(A) Proinflammatory cytokine expression by Tregs during adoptive transfer induced colitis. IL-2 and IFN-γ levels in adoptively transferred Treg cells carrying either control vector (blue line) or Stub1-expression vector (green line) was determined after isolating the cells from the indicated tissues and staining for surface markers and intracellular cytokines. Staining controls are shown in red. Depicted are representative histograms gated on $CD4^+Thy1.1^+$ events (original Treg cells).

(B, C) The relative percentages of $Thy1.1^+$ cells among the cells of the indicated tissues as well as the absolute number of $Thy1.1^+$ cells were determined. Panel (A) represents the results of at least three independent experiments. Panels (B) and (C) are the mean of three replicate samples from at least two independent experiments.

(D and E) shRNA knockdown of Stub1 in naïve T cells prevents Th1 and Th17 differentiation while increasing Foxp3 expression. Naïve T cells were FACS purified and transduced with LV-sh-Stub1 (right panel) or LV-sh-control (left panel) before anti-CD3/CD28 activation and the addition of the Th1-polarizing cytokine, IL-12 (D) or Th17-polarizing cytokines TGF-β and IL-6 **(E)**. After two days of culture, cells were restimulated with PMA/ionomycin and intracellular IFN-γ, IL-17 and Foxp3 were stained. Panels were gated on GFP^+ events (transductants). Shown are the representation findings from at least two independent experiments.

(F) Stub1 knockdown does not impact Foxp3 transcription in T cells under Th17-skewing conditions. RNA was extracted from the cells treated in (E) and qRT-PCR analysis was carried out to measure Foxp3 transcript levels in sh-control and sh-Stub1 transduced T cells.

(G) Stub1 was upregulated under TCR and IL-6 stimulation. Expanded human Treg cells were rested for 2 days and then stimulated with anti-CD3/CD28 antibodies plus 10ng/ml IL-6. Cells were harvested at the indicated time-points. Cell lysates were analyzed by Western blotting.

(H) sh-RNA-mediated knockdown of Stub1 in nTregs enhanced expression of Treg-associated genes. nTreg cells were transduced with lentiviral vectors (carrying GFP marker as an internal control) in order to express either sh-Stub1 or control constructs. The transduced nTregs were stimulated with anti-CD3/CD28 antibodies in the presence of IL-2 (100U/ml) for 3 days. GFP^+ cells were sorted out, and subjected to the qRT-PCR analysis to assess levels of the indicated gene transcripts. Shown are the mean results from at least three independent experiments +/- SEM.

Supplemental Experimental Procedures

Mice

All animal experiments were performed in the specific-pathogen-free facilities of the Johns Hopkins Animal Resource Center in accordance with national, state and institutional guidelines. Animal protocols were approved by the Johns Hopkins Animal Care and Use Committee. Wild type C57BL/6, Foxp3-IRES-GFP mice (originally developed in T.Chatila's laboratory) as well as Thy1.1^{+}, Thy1.2^{+}, and *Rag2*$^{-/-}$ mice on a BALB/c genetic background were purchased from Jackson and bred in our facility. Foxp3-GFP mice and Foxp3-DTR-IRES-GFP (Fontenot et al., 2005) were kindly provided by A. Rudensky. *Myd88*$^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Labs.

Isolation of Human Treg cells

Human PBMCs were isolated from the buffy coat of healthy donors (Shanghai Blood Center). Human CD4^{+}CD25^{hi}CD127^{lo} Treg cells were purified using a FACS ARIA II cell sorter (BD). The purity of the sorted cells was 95-99%. The purified human Treg cells were expanded with anti-CD3/CD28 beads (Invitrogen) and 500U/ml IL-2 (R&D). The expanded human Treg cells were rested with 100U/ml IL-2 for 2 days and then stimulated with the indicated cytokines.

T Cell Differentiation

CD4^{+}CD62L^{hi}CD25^{-} T cells were purified using a FACS Aria high speed sorter. These cells were stimulated in 24-well plates containing immobilized anti-CD3ε and soluble ant-CD28 antibodies (1 and 4 μg per well, respectively; Biolegend). Th17 skewing conditions consisted of IMDM culture media supplemented with 5% FBS, 20ng/ml IL-6, 2.5ng/ml TGFβ (Peprotech), and 10ug/ml cytokine neutralizing antibodies directed against IFN-γ, IL-4, and IL-12 (all from Biolegend). iTreg-inducing conditions consisted of recombinant TGF-β (5ng/ml) and IL-2 (100 U/ml). For Th1 skewing, stimulation took place in the presence of anti-IL-4 antibody and 20ng/ml IL-12. At the indicated time periods, cells were removed from culture, restimulated with PMA/ionomycin in the presence of Golgi-stop for 5hrs and ICS analysis was performed as indicated to assay production of intracellular IFN-γ, IL-17 or expression of Foxp3.

RNA, Complementary DNA and Quantitative real-time PCR

Total RNA was isolated from whole cells using the Qiagen miniRNA extraction kit following the manufacturer's instructions. RNA was quantified and complementary DNA was reverse-transcribed using the cDNA archival kit (Applied Biosystems) following the manufacturer's

instructions. The cDNA samples were used at 20ng/well in a 96 well plate and run in triplicate. PCR reactions for detecting murine genes were set up in 25-μl volumes using TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI Prism 7500 Sequence Detection System. Quantification of relative mRNA expression was determined by the comparative CT (critical threshold) method where the amount of target mRNA, normalized to endogenous β-actin or 18s rRNA expression, is determined by the formula 2-ΔCT. The primers of murine genes were purchased from ABI. PCR reactions for detecting human genes were carried out using SYBR green mix (TAKARA) on ABI Prism 7900 Sequence Detection System. Quantification of relative mRNA expression was determined by the formula 2-ΔCT normalized to GAPDH expression. The primers of human genes were listed as follows: Foxp3-forward: 5'-tcccagagttcctccacaac-3' and Foxp3-reverse: 5'-attgagtgtccgctgcttct-3'; IL-2-forward: 5'-gcaactcctgtcttgcattg and IL-2-reverse: 5'-cagttctgtggccttcttgg-3'; GITR-forward: 5'-agtgggactgcatgtgtgtc-3' and GITR-reverse: 5'-gcagtctgtccaaggtttgc-3'; CTLA-4 forward: 5'-tggggaatgagttgaccttc-3' and CTLA-4 reverse: 5'-gcacggttctggatcaatta-3'; Stub1-forward: 5'-aggccaagcacgacaagtacat-3' and Stub1-reverse: 5'-ctgatcttgccacacaggtagt-3'; IL-17-forward: 5'-actacaaccgatccacctcac-3' and IL-17-reverse: 5'-actttgcctcccagatcacag-3'.

Cells, virus, and transfection

Human HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, penicillin/streptomycin and L-glutamine. The cells were transfected with the indicated plasmids using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions and used 48h post-transfection in the indicated assays. Jurkat T cells were grown in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, sodium pyruvate, non-essential amino acids and L-glutamine. FUGW-HA-Foxp3 was cotransfected with del 8.9 and VSV-G into 293T cells. The supernatant containing virus was harvested and used to transduce Jurkat cells to obtain the HA-Foxp3 Jurkat stable cell line. For Stub1 overexpression or knockdown experiments, primary Treg cells ($CD25^{High}/CD4^{+}$) were FACS purified and transduced with the lentiviral vector carrying either an expression (pLV-Stub1) or shRNA construct (pLV-shStub1) or an empty vector control encoding either GFP alone or a scrambled shRNA control (where appropriate). Transduction was accomplished by culturing Treg in the presence of retrovirus-containing supernatant and polybrene (5ug/ml) after centrifuging the virus/cell suspension at 2500 rpm for 1 hour at room temperature. GFP signal was used as an indicator of successful transfection or transduction.

Site-directed mutagenesis

The mutants were made using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's standard procedure, and confirmed by DNA sequencing. Primers were designed on PrimerX software.

Protein expression and purification

The Rosetta/PlysS *E. coli* strain was used for protein expression. 6His-E1, 6His-UbcH5b, 6His-Stub1, 6His-Hsp70 and 6His-Flag-Ubiqutin were eluted from the Ni-NTA slurry (QIAgen) and the elution fractions were purified by fast protein liquid chromatography (GE). MBP-Foxp3 and MBP were eluted from amylose resin (NEB) and the eluted proteins were dialyzed against buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM 2-ME, and 10% glycerol. All proteins were quantified using the Bradford assay (Beyotime).

***In vitro* ubiquitination**

0.1μg 6His-E1, 0.5μg 6His-UbcH5b, 2μg 6His-Stub1, 2μg 6His-Hsp70, 10μg 6His-Flag-Ubi, and 2μg MBP-Foxp3 or MBP were mixed and incubated at 30^{o}C for 2h in a total volume of 100μl containing 50mM Tris/HCl, pH 7.4, 2mM $MgCl_2$, 1mM DTT, 4mM ATP. After terminating the reaction by the addition of SDS containing loading buffer, the degree of ubiquitination was detected by Western blotting with an anti-MBP antibody.

Luciferase-based Transcription Activity and Repression Assays

The 8×FK or IL-2 luciferase reporter plasmid was co-transfected with a β-gal or renilla luciferase encoding plasmid into 293T or Jurkat T cells. The cells were lysed and analyzed using a luciferase assay normalized to β-gal or renilla luciferase activity according to the manufacturer's protocol (Beyotime and Promega). Results presented are the mean of three separate experiments, and the error bars indicate standard deviations from the mean.

Immunoblot analysis and immunoprecipitation

Cells were lysed in RIPA buffer containing 50 mM Tris/HCl, pH 7.4, 1% Nonidet P-40, 0.5% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, with 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor (Sigma), followed by immunoprecipitation with the indicated antibodies, separation by SDS/PAGE, and analysis by Western blotting. Where applicable, band density indicating protein amount was quantified using Image J software.

Immunofluorescence

Cells were treated with LPS and a proteasome inhibitor to prevent Foxp3 loss as indicated. They were then fixed in 4% formaldehyde, permeabilized in 0.5% Triton-X 100, blocked, and incubated with antibody to Stub1 and Foxp3. Cell nuclei were stained with DAPI dye. And slides were imaged on a laser confocal microscope (LEICA SP5).

Proliferation assay

Treg cells modified by transduction were isolated from Thy1.2$^+$ mice. Responder cells ($CD4^+CD25^-CD62L^{high}$) were isolated from Thy1.1$^+$ mice, then labeled with 1 μM CFSE (Invitrogen) and these cells were then mixed at the indicated ratios and cultured for 80 hrs in the presence of anti-CD3 antibody (1ug/ml). Cells were harvested and proliferation for Thy1.1$^+$ responder cells was assessed by flow cytometric detection of CFSE dilution.

LPS treatment

We obtained LPS from Sigma (extracted from *E.coli* O111B4) and purified by ion exchange, which contains less than 1% protein and RNA. Isolated mouse Treg cells or resting Human Treg cells were stimulated with anti-CD3/CD28 beads (Invitrogen) for one day and treated with 1μg/ml LPS for one day. For *in vivo* treatment, C57BL/6 mice were injected i.p. with 100 ul 1X PBS (controls) or an equal volume of an LPS solution (0.5μg/kg) for four weeks. Mice were humanely sacrificed and the frequency of $Foxp3^+/CD4^+$ cells in the spleen was determined by intracellular staining and flow cytometry.

Colitis induction and histological assessment

Naïve $CD4^+CD25^-CD62L^{high}$ T cells were isolated from BALB/c mice and injected via the tail vein (i.v.) into BALB/c $RAG2^{-/-}$ immunodeficient recipients ($1x10^6$/mouse). BALB/c wild-type $CD4^+CD25^+$ Treg or Stub1 transduced Treg cells or those receiving an empty vector ($2x10^5$) were co-injected i.v. as indicated. In other experiments, si-Stub1 or a si-Control construct was transfected into Tregs prior to adoptive co-transfer into *Rag2* deficient recipients. A lower number of Tregs were used in these experiments in order to better demonstrate enhancement of Treg suppressive function. In all experiments, mice were monitored weekly for wasting disease and mice losing more than 20% of its starting body weight or showing severe signs of disease were humanely sacrificed. Colons were removed from mice 8 weeks after T cell reconstitution and fixed in 10% formalin. Five-micrometer paraffin-embedded sections were cut and stained with haematoxylin and eosin (H&E). The pathology of colon tissue was scored in a

blinded fashion, on a scale of 0-5 where a grade of 0 was given when there were no changes observed. Changes associated with other grades were as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submusoca and associated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands. Transferred naïve and Treg cell populations were characterized by surface marker and intracellular staining for cytokines and Foxp3. Leukocytes recovered from recipient lymph node, spleen and lamina propria were restimulated as previously described before surface staining for CD4 and Thy1.1 and Thy1.2. Intracellular Foxp3, IL-2 and IFN-γ were also stained after fixation and permeabilization. Analysis of ICS data was carried out using CellQuest and FlowJo software.

Plasmids, antibodies and reagents

pIPHA2-Foxp3, its truncations, and pIPMyc2-Stub1 were constructed as described previously (Li et al., 2007). HA-Ubi and Flag-Ubi was a gift from Huiwu Zhao. Plasmids FUGW, del8.9 and VSV-G for lentivirus production were gifts from Ke Lan. shRNA construct vector pLKO.1 was from Addgene. shRNA sequence for Stub1 was 5'-TGCCGCCACTATCTGTGTAAT-3'; and for Hsp70: 5'-GGCCAACAAGATCACCATCTT-3'. E1, UbcH5b and Hsp70 were amplified from human PBMC cDNA. The primers used for gene amplification were as follows: E1-forward: 5'-atgtccagctcgccgctgtcc-3', E1-reverse: 5'-tcagcggatggtgtatcggac-3'; UbcH5b-forward: 5'-atggctctgaagagaatccacaag-3', UbcH5b-reverse: 5'-ttacatcgcatacttctgagtccattc-3'; Hsp70-forward: 5'-atggccaaagccgcggcgatc-3', Hsp70-reverse: 5'-taatctacctcctcaatggtggggcctg-3'. The vector pIPHA2, pIPMyc2, pIPFlag2 were used for mammalian expression and the vector pET28a and pET21-MBP for bacterial expression. The antibodies we used were as follows: anti-Flag (M2, Sigma), anti-Myc (9E10, Santa Cruz), anti-HA (F-7, Santa Cruz), anti-α-Tubulin (DM1A, Sigma), anti-GAPDH (1C4, Sungene Biotech) anti-Foxp3 (hFOXY, eBioscience) and anti-Stub1 (H-231, Santa Cruz). MG132 was purchased from Merck and protein AG-beads were obtained from Santa Cruz.

Tandem affinity purification and MS sequencing

HEK293T cells were transduced with a construct carrying Foxp3 fused to a tag (TAP) including Protein A-, a TEV cleavage site and calmodulin binding protein. The transfected cells were lysed with Lysis buffer (10 mM Tris-HCl, pH8.0, 150 mM NaCl, 2 mM EDTA, 0.1%NP-40,2 mM DTT,10% glycerol) containing 10 mM NaF, 1 mM $NaVO_3$ and 1 mM PMSF on ice for 30 min. After removal of cell debris by centrifugation, crude cell lysates were incubated with Rabbit IgG agarose (Sigma) for 2-4 hours. The bound proteins were washed three times with Lysis buffer. TEV cleavage buffer (10 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.2%NP-40, 0.5 mM EDTA and 1 mM DTT) and 20 µl homemade TEV protease were added to Rabbit IgG agarose, and incubated overnight at 4C with gentle agitation. After centrifugation, supernatant was saved, and incubated with calmodulin affinity resin (Stratagene) in CaM binding buffer (10 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.1%NP-40,1 mM Mg Acetate, 1 mM Imidazol, 2 mM $CaCl_2$ and 1 mM 2-mercaptoethanol), 1/250 volume 1 M $CaCl_2$ for 2 hours at 4C. Bound proteins were washed three times with CaM binding buffer and then eluted with CaM elution buffer (50 mM Ammonium bicarbonate, 25 mM EGTA). The elution was TCA precipitated, resolved by SDS-PAGE and visualized by silver staining. Visible protein bands were excised and subjected to trypsin digestion followed by mass spectrometry analysis.