Supplementary Information for:

Essential biphasic role for JAK3 catalytic activity in IL-2 receptor signaling

Geoffrey A. Smith[1,2], Kenji Uchida[1†], Arthur Weiss[2,3] and Jack Taunton[1*]

[1]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California, USA. [2]Rosalind Russell and Ephraim P. Engleman Arthritis Research Center, Division of Rheumatology, Department of Medicine, University of California, San Francisco, California, USA. [3]Howard Hughes Medical Institute, University of California, San Francisco, California, USA.

[†] Present Address: Chemical Research Laboratories, R&D Division, Kyowa Hakko Kirin Co., Ltd.

*e-mail: jack.taunton@ucsf.edu

Supplementary Results

Supplementary Tables

Supplementary Table 1 | In vitro selectivity profile among JAK-family (100 μM ATP)

Kinase	IC_{50} (nM)
JAK1	1,520
JAK2	8,680
JAK3	0.43
TYK2	>10,000

Supplementary Table 2 | In vitro selectivity profile among kinases with similar cysteine (1 mM ATP)

Kinase	IC_{50} (nM)
JAK3	7.43 nM
BTK	363 nM
ITK	4630 nM
EGFR	>10 μM

Supplementary Table 3 | Cellular effects of JAK3i and tofacitinib.

	JAK3i	Tofacitinib (pan-JAKi)
pSTAT5 (15 min)	47.2 ± 4.5 (n=5)	24.7 ± 5.7 (n=8)
CD25	3.1 ± 0.9 (n=7)	7.7 ± 4.9 (n=7)
proliferation	3.7 ± 1.6 (n=9)	12.5 ± 5.6 (n=13)
		IC_{50} in nM

Average IC_{50} values ± S.D. from all experiments assessing effects of JAK3i and tofacitinib on 15 min pSTAT5, CD25 upregulation and proliferation as described in **Fig**. **2.** n refers to the number of independent experiments, which were each 10-pt, 3-fold titrations from 10 μM to 0.51 nM or 1 μM to 0.05 nM (CD25 and proliferation), or 7-pt, 3 fold titrations from 1 μM to 1.37 nM (pSTAT5). Each proliferation experiment was conducted in technical triplicate.

Supplementary Table 4. | Cellular selectivity of JAK3i.

Cellular Assay	Kinases	IC_{50}
IFN-γ pSTAT1	JAK1/2	>10 μM (n=2)
IL-2 proliferation	JAK1/3	3.7 ± 1.6 nM (n=9)
α-IgM CD69 induction	BTK	>10 μM (n=2)
TCR IL-2 production	ITK/RLK	1.12 ± 0.5 μM (n=3)

Average IC_{50} values (± SD when n ≥ 3) of JAK3i in indicated cellular assays of potential off target kinases. n indicates number of independent experiments. See **Fig. 3c** for details.

Supplementary Table 5 | FACS Antibody Information

Target	Fluor	Clone	Source	Dilution
CD4	PerCP Cy5.5 PE-Cy7	GK1.5	BD	1:200
CD4	BUV395	GK1.5	BD	1:100
CD25	PerCP Cy5.5 APC Biotin	PC61.5	BD	1:200
CD45.1	PE PE-Cy7	A20	Biolegend	1:200
CD45.2	APC	104	eBioscience	1:200
CD90.1	PE	HIS51	eBioscience	1:200
CD90.2	eFluor780	53-2.1	eBioscience	1:200
STAT1 pY701	PE	4a	BD	1:3.75
STAT5 pY694	AlexaFluor647	47/STAT5(pY694)	BD	1:5
Rb pS807/pS811	PE	J112/906	BD	1:5
pS6 S240/244	Unconjugated PE	D68F8	Cell Signaling	1:200
Anti-rabbit	PE		Jackson Immuno	1:200

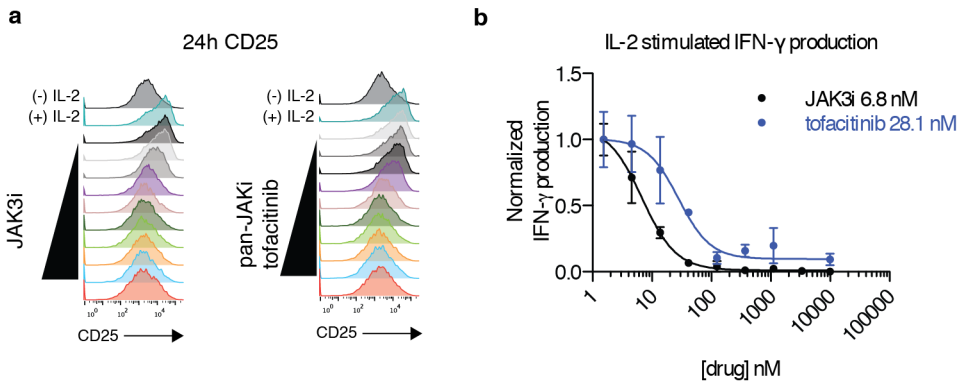
Supplementary Table 6 | qPCR Primer & Probe Information

IDT Primetime Assays		
Target		**Sequence**
Fos	Probe	/56-FAM/CAGCCGACT/ZEN/CCTTCTCCAGCATG/3IABkFQ/
	Primer 1	GGCACTAGAGACGGACAGAT
	Primer 2	ACAGCCTTTCCTACTACCATTC
Lta	Probe	/56-FAM/AGCCCCAGG/ZEN/AGGAAGACAGGA/3IABkFQ/
	Primer 1	AGAAGCGGACACCAGAGA
	Primer 2	CACAGCAGGTTCTCCACAT
Myc	Probe	/56-FAM/CGGTGTCTC/ZEN/CTCATGCAGCACT/3IABkFQ/
	Primer 1	CTTCCTCATCTTCTTGCTCTTCT
	Primer 2	TTCTCTCCTTCCTCGGACTC
Ifng	Probe	/56-FAM/TCTTGGCTT/ZEN/TGCAGCTCTTCCTCA/3IABkFQ/
	Primer 1	TCCACATCTATGCCACTTGAG
	Primer 2	CTGAGACAATGAACGCTACACA
Ccne1	Probe	/56-FAM/AAGAACTGC/ZEN/TCTCATCCTCGCCTG/3IABkFQ/
	Primer 1	CTGTGGAGCTTATAGACTTCGC
	Primer 2	GACTTACCTGAGAGATGAGCAC
Life Technologies Taqman Assays		
Target	**Exons Targeted**	**Assay ID**
Socs1	1-2	Mm01342740_g1
B2m	1-2	Mm00437762_m1

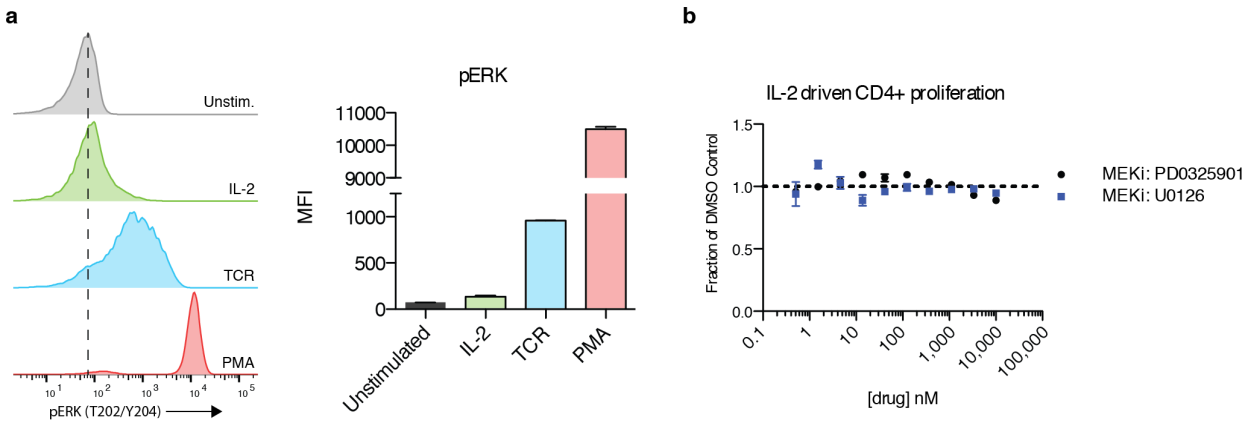
Supplementary Figures

Supplementary Figure 1 | JAK Family Sequence Alignment

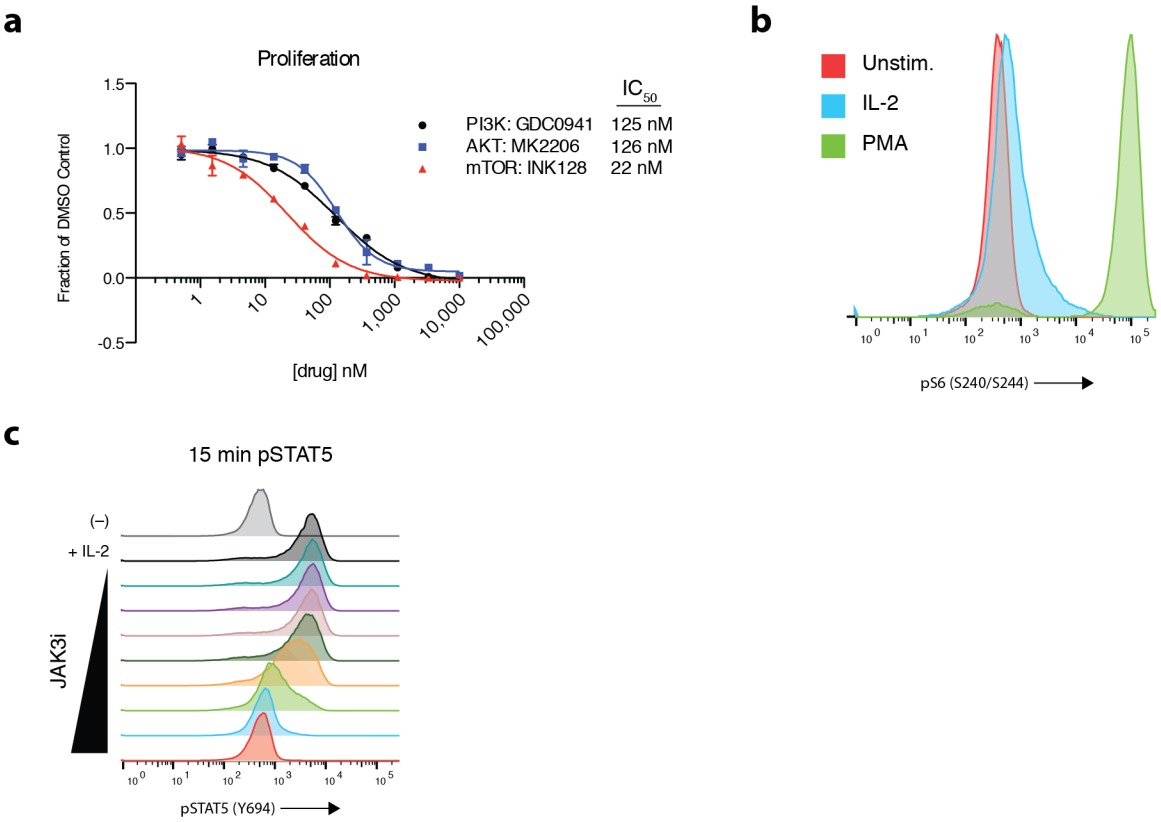
Alignment of JAK-family kinases showing the unique cysteine (C905) in JAK3.



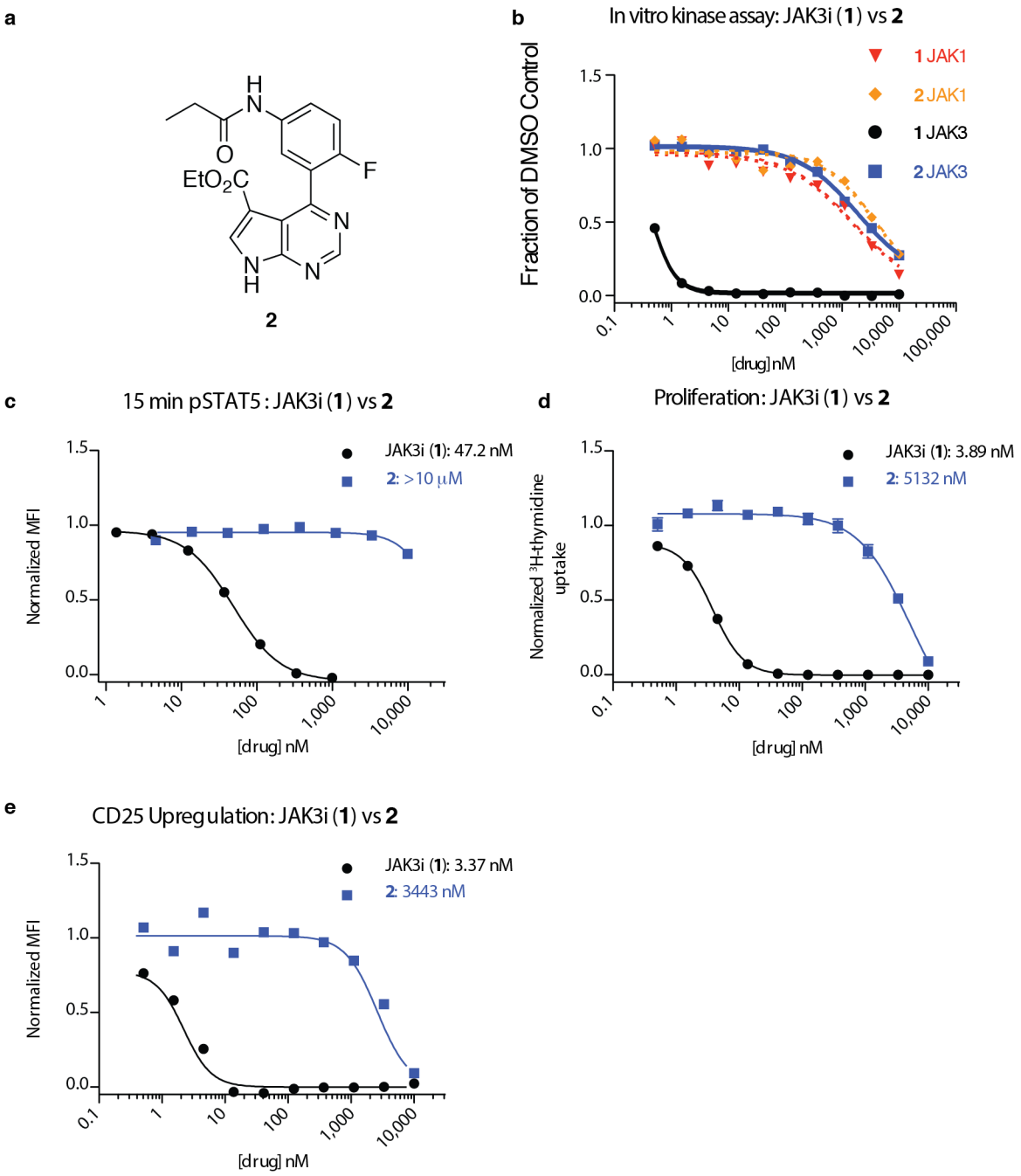
Supplementary Figure 2 | Role of JAK3 in downstream readouts.
(a) FACS Histograms of CD25 upregulation after 24 hours of IL-2 stimulation in the presence of JAK3i or tofacitinib, as graphed in **Fig. 2 b,d**. (b) JAK3i potently inhibits IL-2 stimulated IFN-γ production, measured by ELISA after 24 hours. Plotted as mean ± SEM of triplicates. Data are representative of 7 (a) and 2 (b) independent experiments.



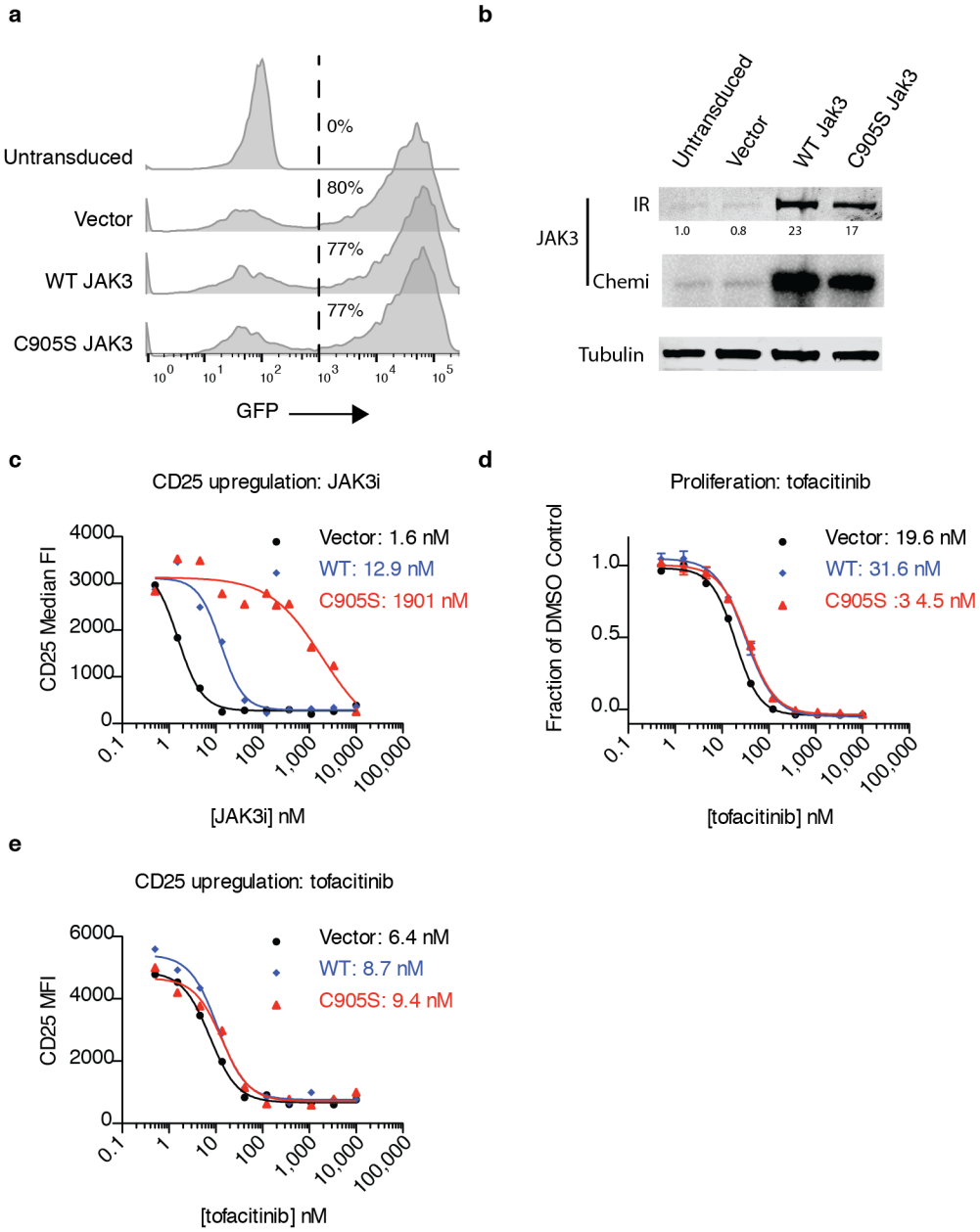
Supplementary Figure 3 | MEK/ERK Signaling is dispensable for IL-2-driven T-cell proliferation.
(a) CD4+ T-cell blasts were stimulated with IL-2 (50 U/mL) or PMA (50 ng/mL) for 15 minutes or αCD3 (10 μg/mL) crosslinking for 5 minutes and pERK was monitored by phosphoflow. Results quantified at right, MFI ± SEM of pooled results from three independent experiments. (b) Titration of MEK inhibitors PD0325901 and U0126 in ^{3}H-thymidine proliferation assay. Plotted as mean ± SEM of technical triplicates. Representative of 3 independent experiments.



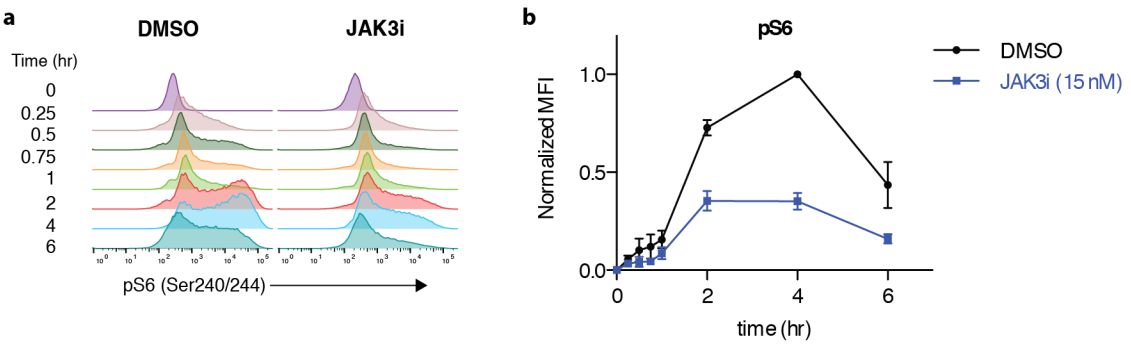
Supplementary Figure 4 | PI3K, AKT, and mTOR are required for IL-2-driven T-cell proliferation.
(a) Titration of PI3K (GDC0941), AKT (MK2206) or mTOR (INK128) inhibitors in the ^{3}H-thymidine proliferation assay, plotted as mean ± SEM of three replicates. Representative of 4 (GDC0941), 3 (MK2206), or 2 (INK128) independent experiments. (b) Phosphorylation of ribosomal protein S6 Ser240/244 after stimulation with IL-2 (50 U/mL) or PMA (50 ng/mL) for 15 minutes. (c) pSTAT5 histograms from cells costained with pS6 (see Fig. 2e). (b) and (c) are representative data from 2 independent experiments.



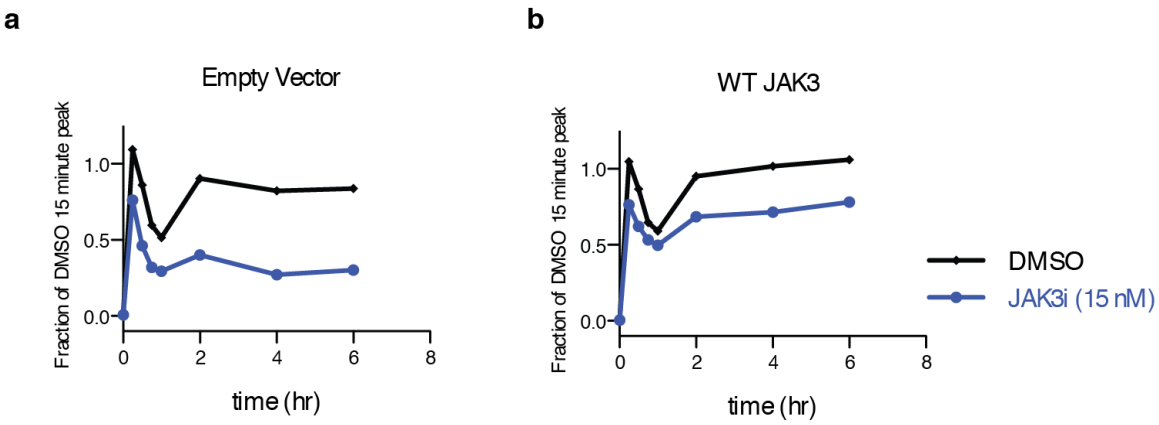
Supplementary Figure 5 | Propionamide 2 is much less potent than JAK3i.
(a) Structure of propionamide **2.** (b) In vitro kinase assays with JAK1 and JAK3**.** (c-e) IL-2-stimulated STAT5 phosphorylation (15 min, c), proliferation (d), and CD25 upregulation (e) (EC_{50} values shown for **1** and **2**). Proliferation data (d) are plotted as mean ± SEM of triplicates. Panels are representative of one (b) or two (c-e) independent experiments.



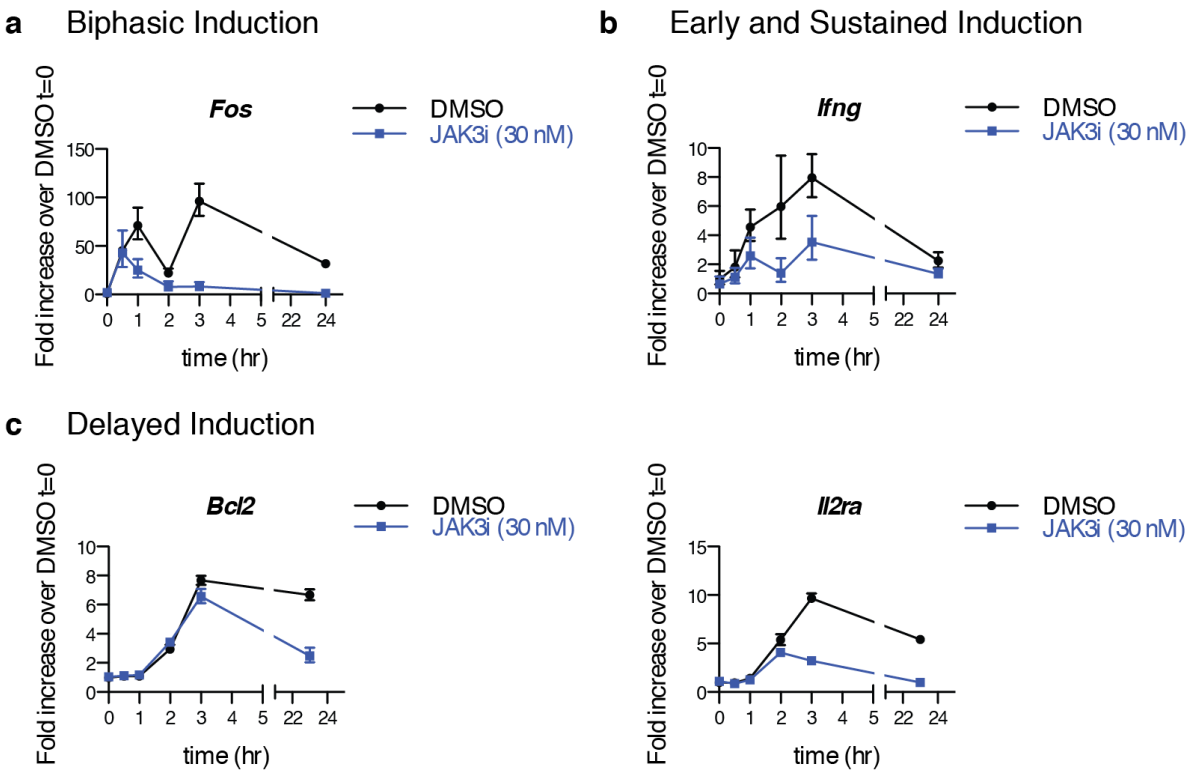
Supplementary Figure 6 | C905S JAK3 Mutant Rescue.
(a) Transduction efficiency of primary CD4+ T cells used without sorting in proliferation assays (Fig. 3a). (b) Overexpression of JAK3 in unsorted cells detected by Western blotting. Quantification from Licor data (IR), normalized to tubulin and relative to untransduced cells, is indicated below. For comparison, overexposed blots with chemiluminescence detection (Chemi) are shown. Full gel in **Supplementary Fig. 12**. (c) CD25 upregulation after 24 hours of IL-2 stimulation in GFP+ cells transduced with WT JAK3, C905S JAK3 or empty vector. (d-e) Tofacitinib inhibition of IL-2-driven proliferation (c) and CD25 upregulation (d) is unaffected by C905S JAK3. Proliferation results (d) are plotted as the mean ± SEM of three replicates. All data are representative of two independent experiments.



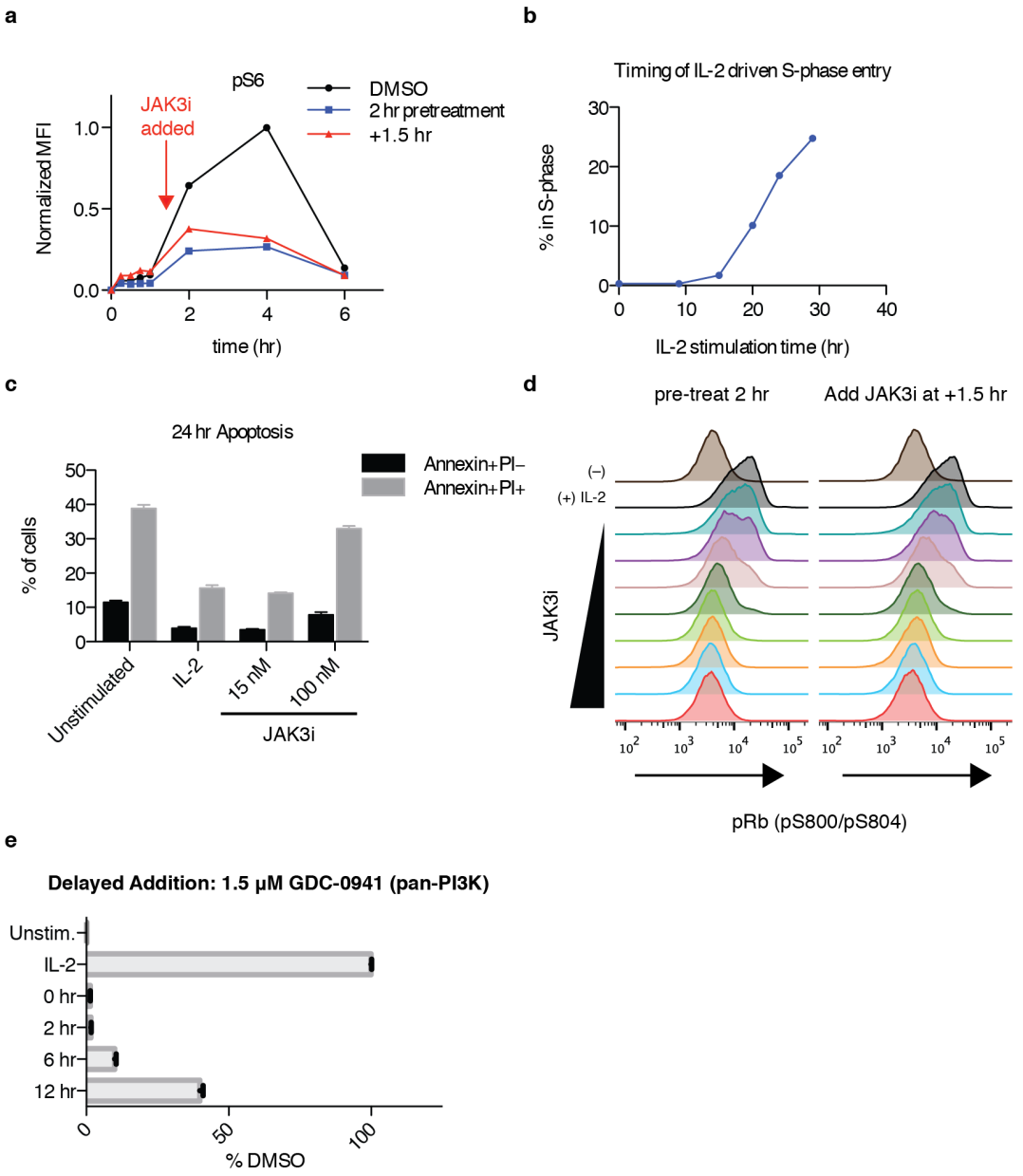
Supplementary Figure 7 | Time course of S6 phosphorylation.
(a) pS6 (Ser240/244) was followed by phosphoflow in the presence of DMSO or JAK3i (15 nM) over 6 hours in IL-2-stimulated CD4+ T cells. Representative of three independent experiments. (b) cumulative quantification of all three experiments, plotted as mean ± SEM.



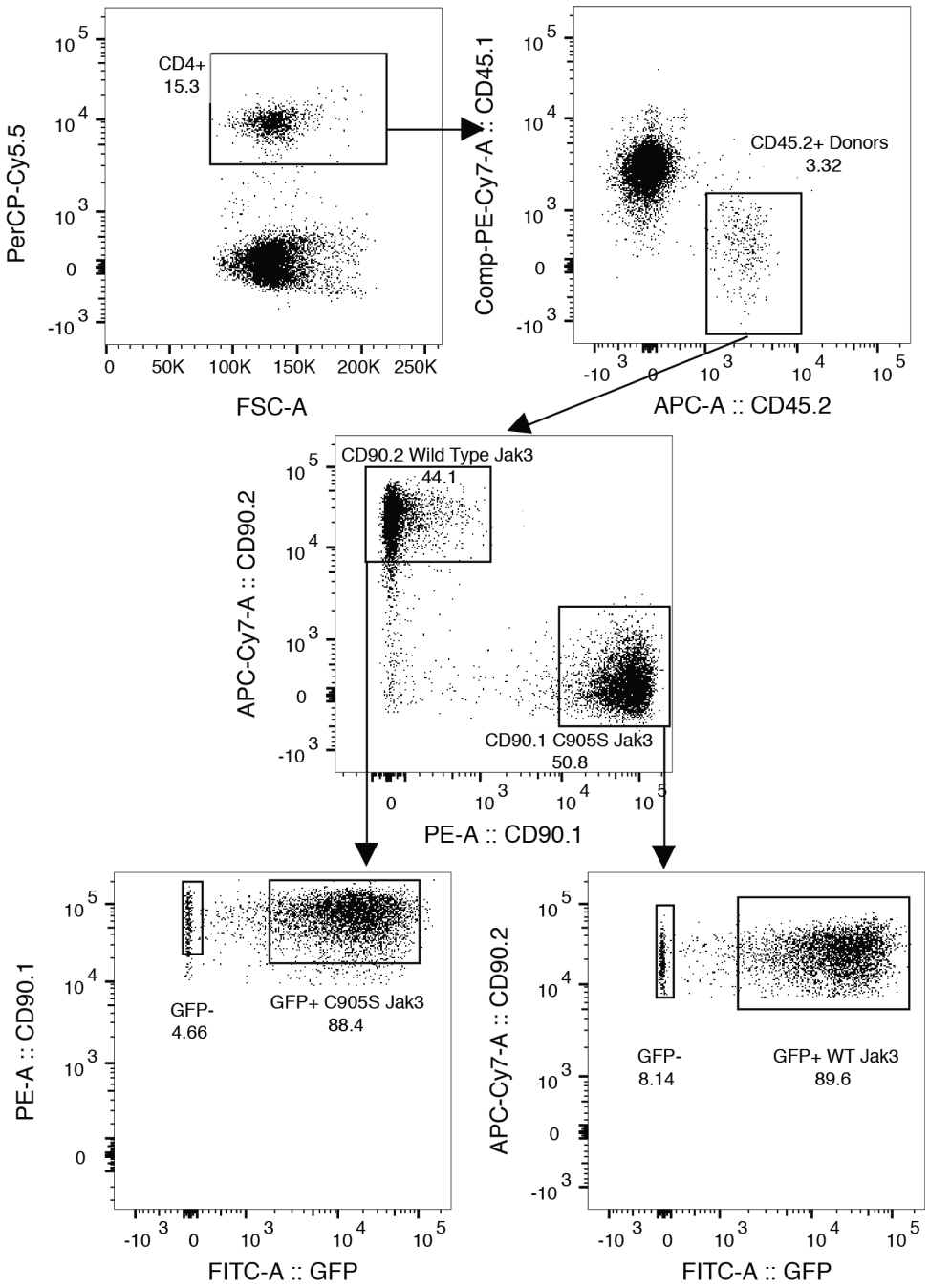
Supplementary Figure 8 | Signaling time course in overexpression controls.
STAT5 phosphorylation was monitored over 6 hours in cells transduced with an empty vector (a) or WT JAK3 (b) and treated with or without 15 nM JAK3i. Data are representative of two independent experiments.



Supplementary Figure 9 | Transcriptional time course.
qPCR analysis of genes induced by IL-2 stimulation, grouped by pattern (mean of 3 replicates with 95% confidence interval): (a) biphasic, (b) early and sustained, and (c) delayed induction. Data are representative of three independent experiments.

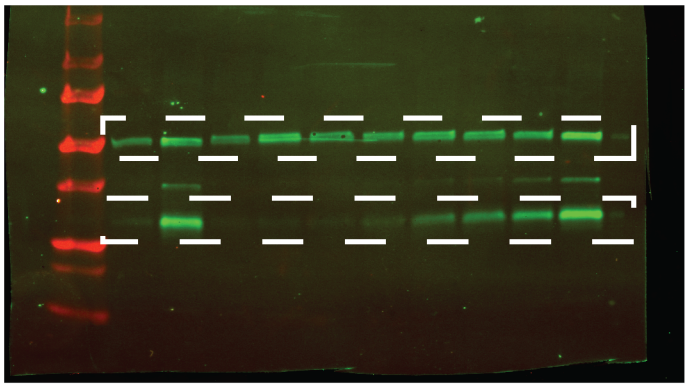


Supplementary Figure 10 | Delayed addition studies. (a) pS6 (Ser240/244) levels were monitored over time in T cells treated with JAK3i (15 nM) either 2 hours before or 90 min after IL-2 stimulation, as in Fig. 5a. (b) Kinetics of IL-2 stimulated S-phase entry was assessed by 1-hour pulse of EdU at the indicated times. (c) T cells were treated with 15 nM or 100 nM JAK3i, stimulated with IL-2, and incubated for 24 hours before staining with Annexin and PI to mark cells undergoing apoptosis (Annexin+PI–) and dead cells (Annexin+PI+). Plotted as mean ± SEM of triplicates. (d) FACS histograms of pRB (pS800/p804) after 24 hours of IL-2 stimulation, with an 8-point JAK3i titration series added 2 hours before or 90 minutes after stimulation. MFI plotted in **Fig. 5f**. (e) PI3K inhibitor GDC-0941 was added at the indicated times after IL-2 stimulation (1.5 μM, EC_{90} for blocking proliferation, **Supplementary Fig. 3a**). After 24 hrs, cells were labeled with EdU for 1 hr, and the percentage of EdU+ cells was measured by FACS and normalized to DMSO (± SEM, n = 3). Data from all panels are representative of two independent experiments.

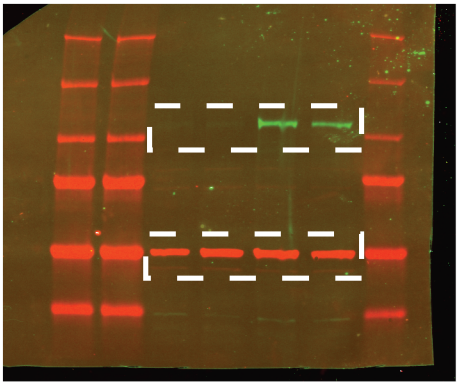


Supplementary Figure 11 | In vivo rescue experiment gating scheme. Splenocytes were gated on FSC/SSC lymphocyte profile and single cells (not shown), then on CD4+ cells. CD45.1−CD45.2+ identified adoptively transferred donor cells. CD90.1 and CD90.2 staining distinguished cells transduced with C905S JAK3 and WT JAK3, respectively. Finally, cells were gated on GFP+ to confirm viral transduction.

Figure 5d - tubulin & Cyclin D3



Supplementary Figure 6b - JAK3 (green) and tubulin (red)



Supplementary Figure 12 | Full gels