SUPPLEMENTAL INFORMATION

Supplemental Figure 1 (related to Figure 1): PP1 analog screen for a specific inhibitor of Csk^{AS}.

Supplemental Figure 2 (related to Figure 2): Phosphorylation of ζ-chain and associate with ZAP-70 upon Csk^{AS} inhibition.

Supplemental Figure 3 (related to Figure 3): Inhibition of Csk^{AS} in primary mouse T cells activates Lck and ERK.

Supplemental Figure 4 (related to Figure 4): Csk^{AS} activity regulates surface TCR expression.

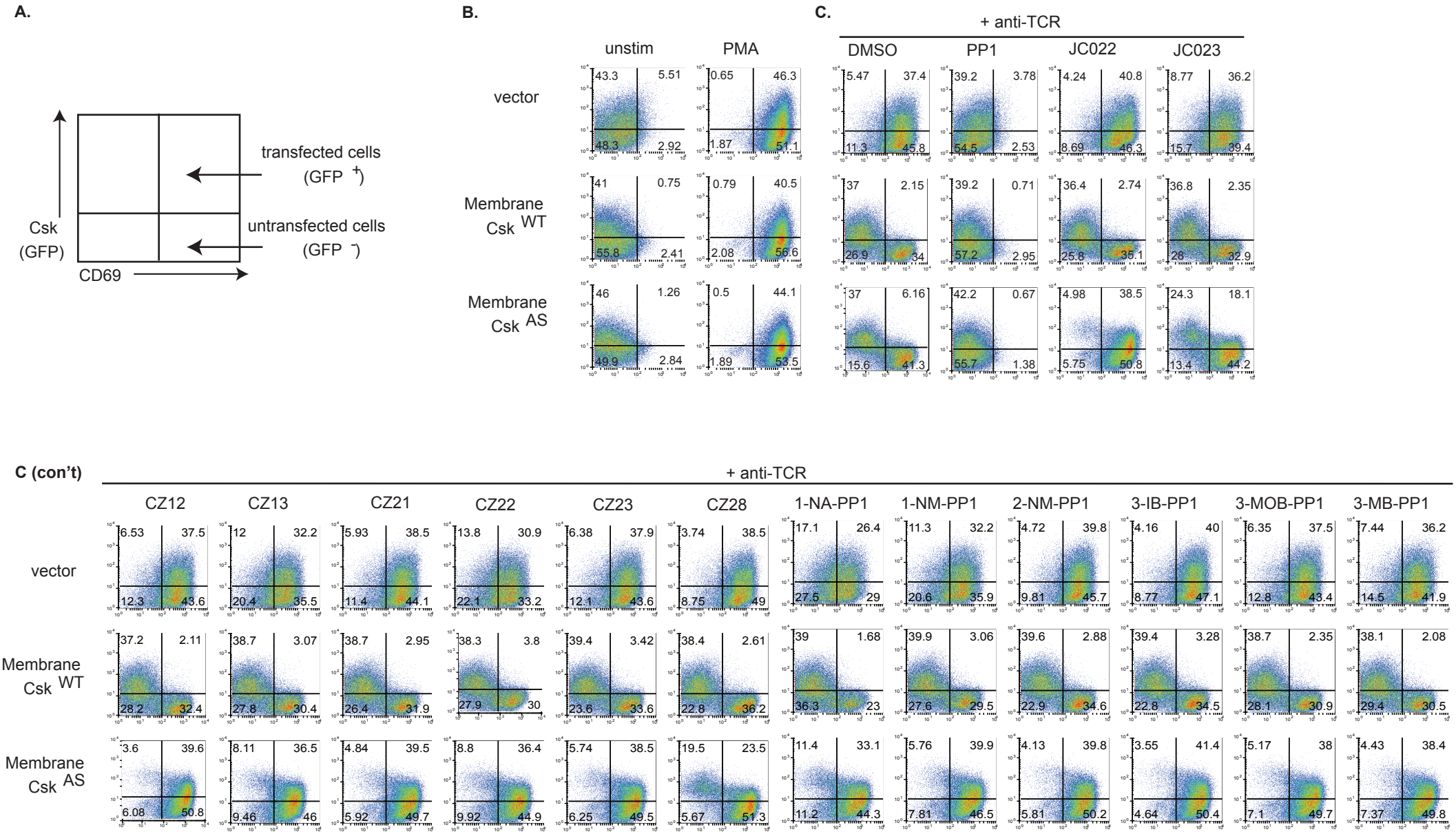
Supplemental Figure 5 (related to Figure 5): Activation induced by Csk^{AS} inhibition requires TCR surface expression, but is not dependent on the amount of TCR.

Supplemental Figure 6 (related to Figure 5): Supplemental Figure 6: TCR upregulation in response to Csk^{AS} expression is not required for activation upon Csk^{AS} inhibition.

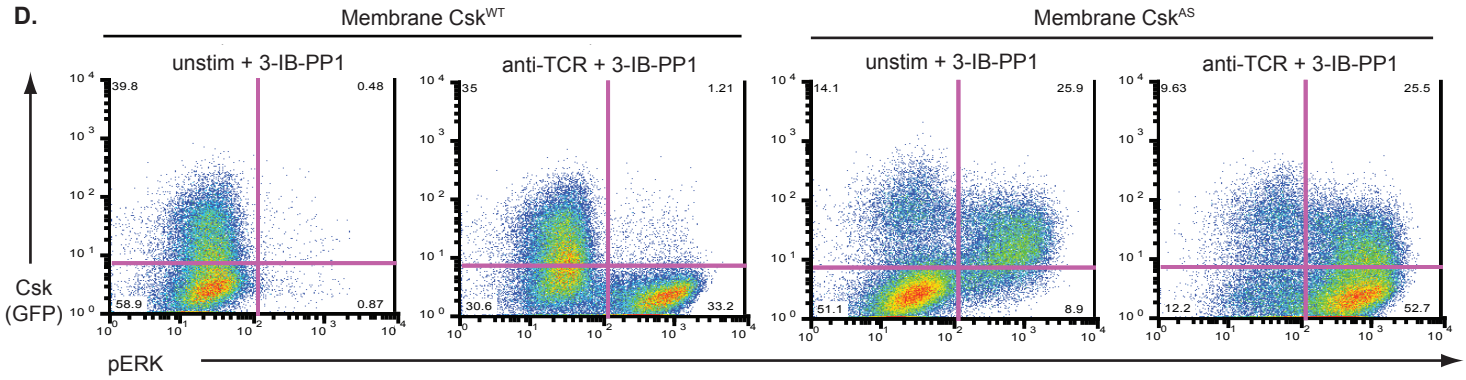
Supplemental Figure 7 (related to Figure 6): Identification of Dok-1 as p65 by mass spectrometry of Csk^{AS} immunoprecipitates.

Supplemental Experimental Procedures: procedures specific to supplemental data that are not used in the main text nor figures; comprehensive and in-depth description of cloning of the *Csk^{AS}* allele, characteristics of 3-IB-PP1 compound, and peptide separation, mass spectrometry and protein identification.

Supplemental References: references cited within supplemental experimental procedures.

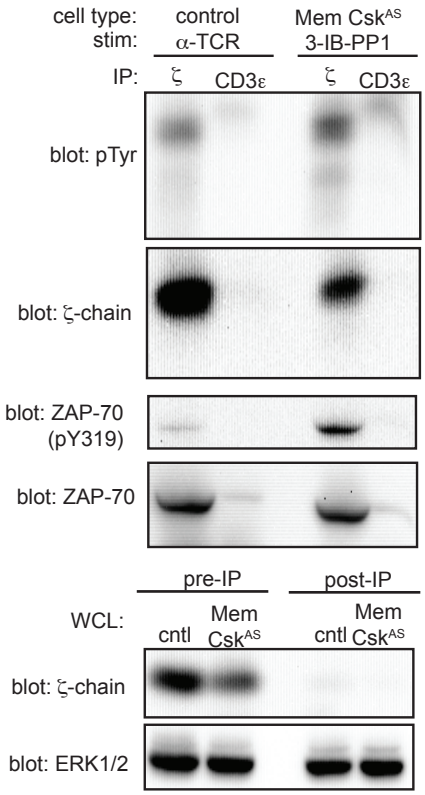


Supplemental Figure 1: PP1 analog screen for a specific inhibitor of Csk AS



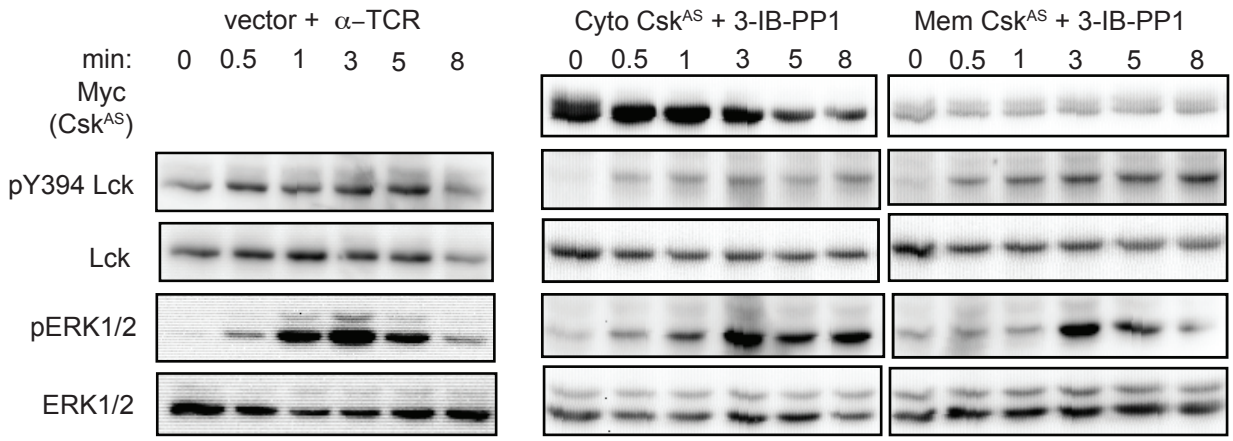
Supplemental Figure 1 (continued): PP1 analog screen for a specific inhibitor of Csk^{AS}

Jurkat T cells were transiently co-transfected with GFP and vector or wild-type (WT) or analog-sensitive (AS) membrane-Csk. Cells were TCR stimulated in the presence of PP1 and its analogs for 16 hours as denoted, then stained for surface CD69. (A) FACS schematic of transfected cells. (B) Unstimulated cells are all CD69lo, whereas phorbol myristate acetate (PMA)-treated cells are all CD69hi. (C) Cells were treated with anti-TCR and inhibitors (all a t 10 μM). Note ability of 3-IB-PP1 to revert GFP+ transfected cells to CD69+ in the membrane-CskAS but not membrane-CskWT cells. (D) Cells expressing membrane-targeted wild-type (WT) or analog-sensitive (AS) were treated with 3-IB-PP1 and either left unstimulated or TCR-stimulated for 5 minutes, followed by analysis of ERK phosphorylation. 3-IB-PP1 is able to impair the inhibitory effect of CskAS, but not CskWT, on TCR stimulation; furthermore, 3-IB-PP1 has no stimulatory effect on cells transfected with CskWT.



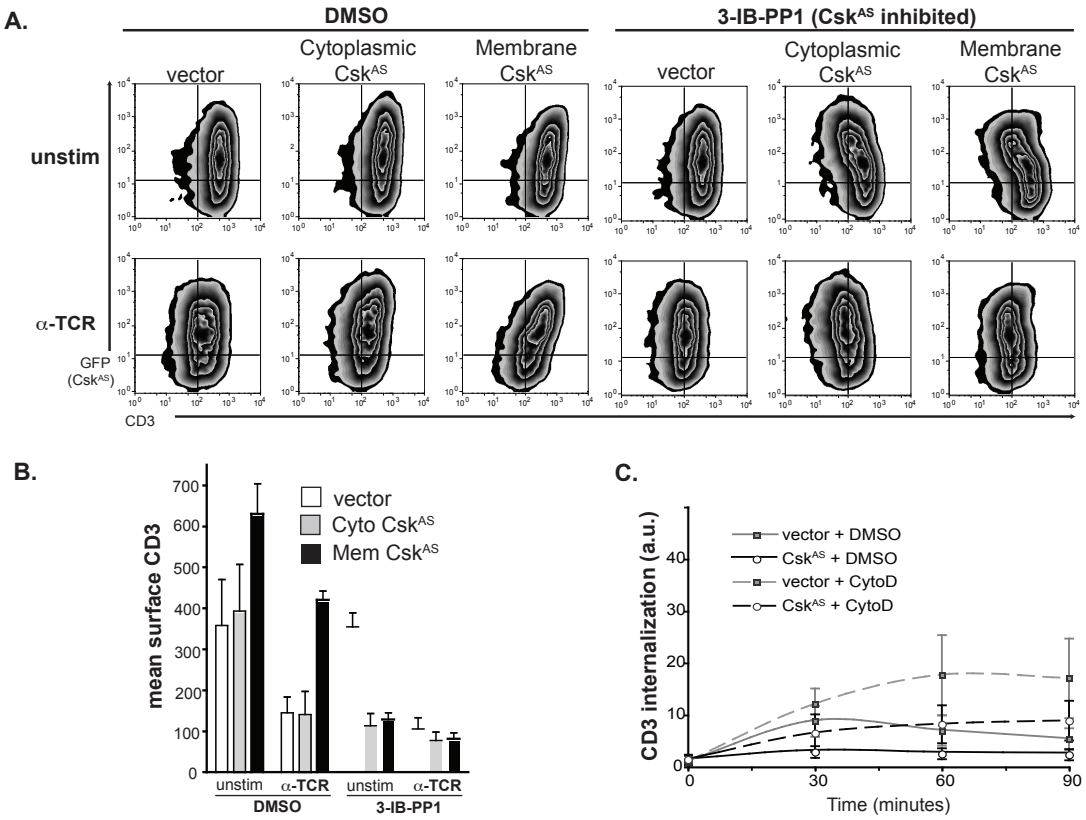
Supplemental Figure 2: Phosphorylation of ζ-chain and association with ZAP-70 upon Csk^{AS} inhibition

Jurkat T cells expressing vector or membrane-CskAS were serum-restricted, then stimulated with anti-TCR antibodies or 3-IB-PP1 for 5 minutes. Lysates were sequentially immunoprecipitated for ζ-chain and CD3ε (top); pre- and post-IP WCL are 1/10 volume of input (bottom). Data are indicative of at least 2 independent experiments.



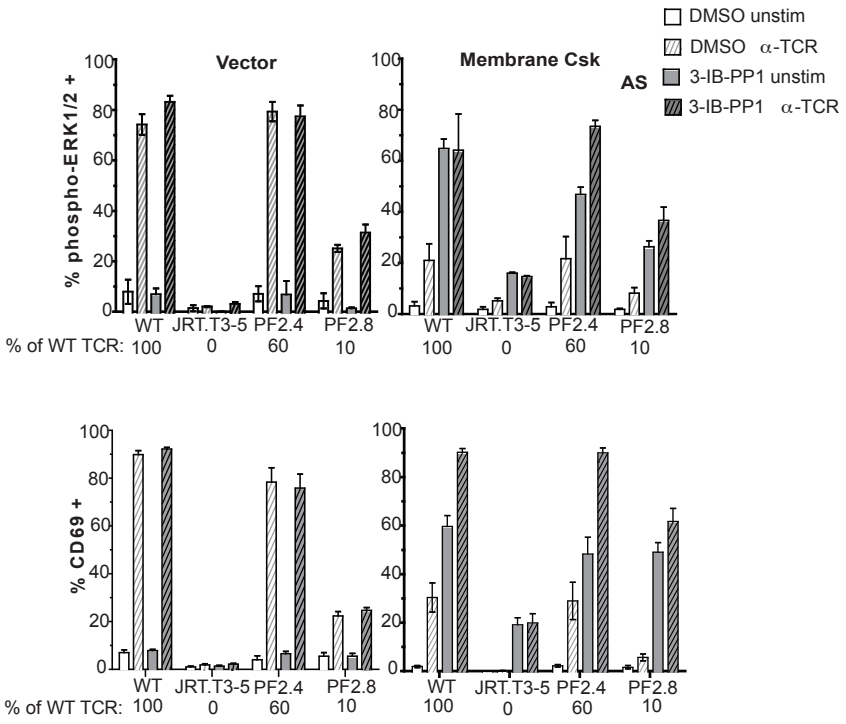
Supplemental Figure 3: Inhibition of Csk^{AS} in primary cells activates Lck and ERK

Mouse CD4+ T cells were retrovirally infected with viral supernatants expressing vector or CskAS as noted. Cells were rested out of exogenous IL-2 for 16 hours and serum-restricted in the final 30 minutes. Cells were harvested directly or following anti-TCR cross-linking or 3-IB-PP1 treatment for the times denoted. Lysates were immunoblotted for phosphorylation of Lck and ERK proteins. Data are representative of at least 3 experiments.



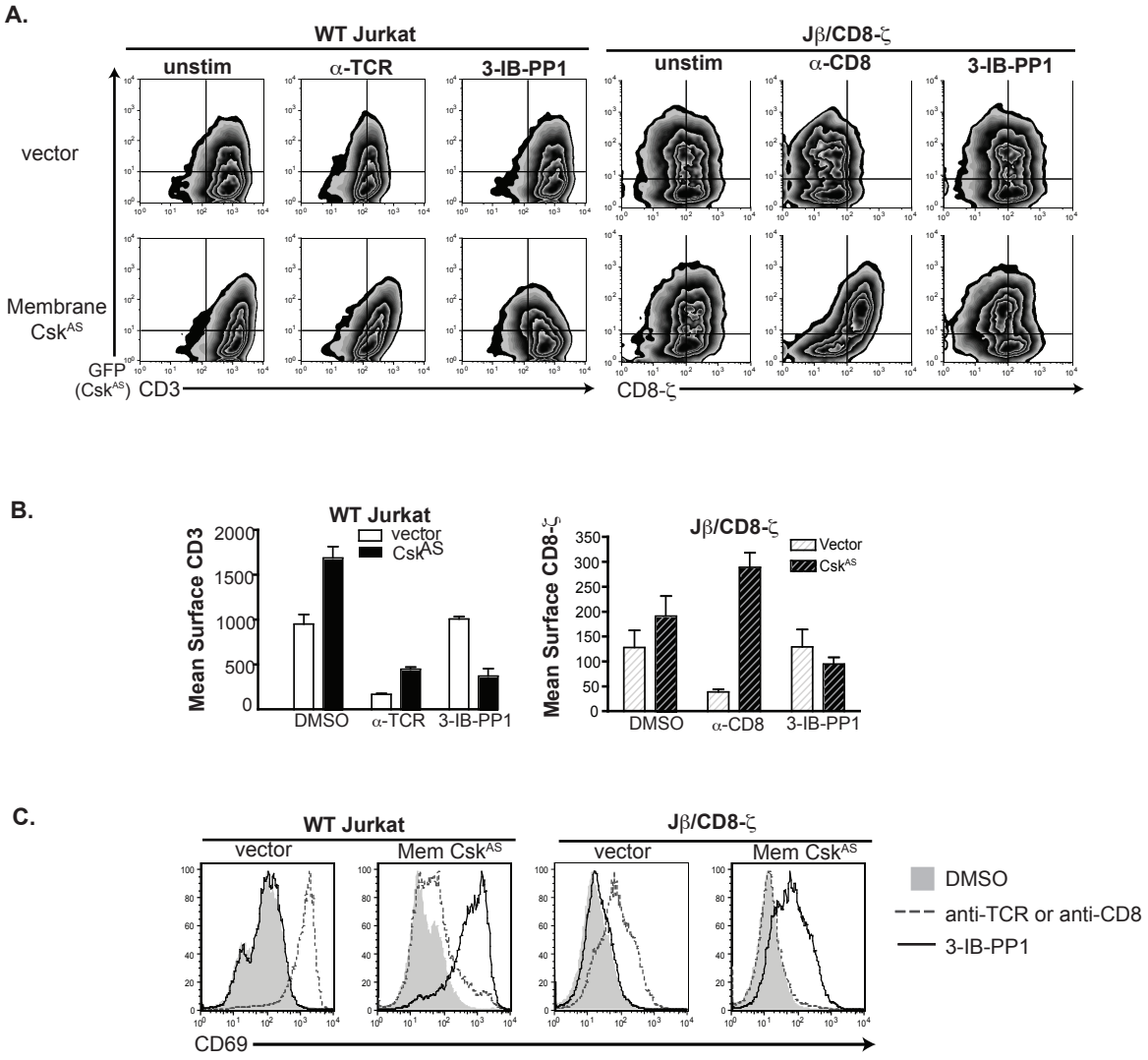
Supplemental Figure 4: Csk ^{AS} activity regulates surface TCR expression

(A, B) Membrane-Csk^{AS} protects cells from TCR degradation, even in a basal state. Jurkat T cells were transiently co-transfected with vector or Csk^{AS} and a GFP transfection marker. Cells were treated with 3-IB-PP1 or DMSO and were TCR-stimulated for 18 hours prior to surface staining for CD3ε. FACS plots in (A) show CD3 expression in transfected (upper quadrants) and untransfected (bottom quadrants) in a representative experiment; (B) mean MFI of CD3 in GFP+ transfected cells in three independent experiments. (C) Membrane-Csk^{AS} cells do not basally internalize CD3. Vector or membrane-Csk^{AS} cells were co-transfected with GFP plasmid. Cells were serum restricted in the presence of vehicle or cytochalasin D for 30 minutes. Cells were then allowed to internalize receptor at 37°C for times indicated. Cells were washed and amount of internalized CD3 was measured as described in supplemental materials and methods. Data are the means of three independent experiments.



Supplemental Figure 5: Activation induced by Csk^{AS} inhibition requires TCR expression, but is not dependent on amount of TCR.

WT Jurkats, TCRβ-deficient JRT.T3-5, or stable lines reconstituted to express 10% (JRT.T3-5 PF2.8) and 60% (JRT.T3-5 PF2.4) of WT TCR were transiently transfected to express vector or membrane-Csk^{AS} and a GFP co-transfection plasmid. Cells were serum-restricted, then pretreated with DMSO or 10 μM 3IB-PP1 (A) for 15 minutes prior to TCR stimulation for 5 minutes and stained for pERK, or (B) for 18 hours and stained for surface CD69 expression. Plots show percentage of total live cells GFP+ transfected cells that are positive for phospho-ERK or CD69 upregulation. Data are average of three independent experiments.



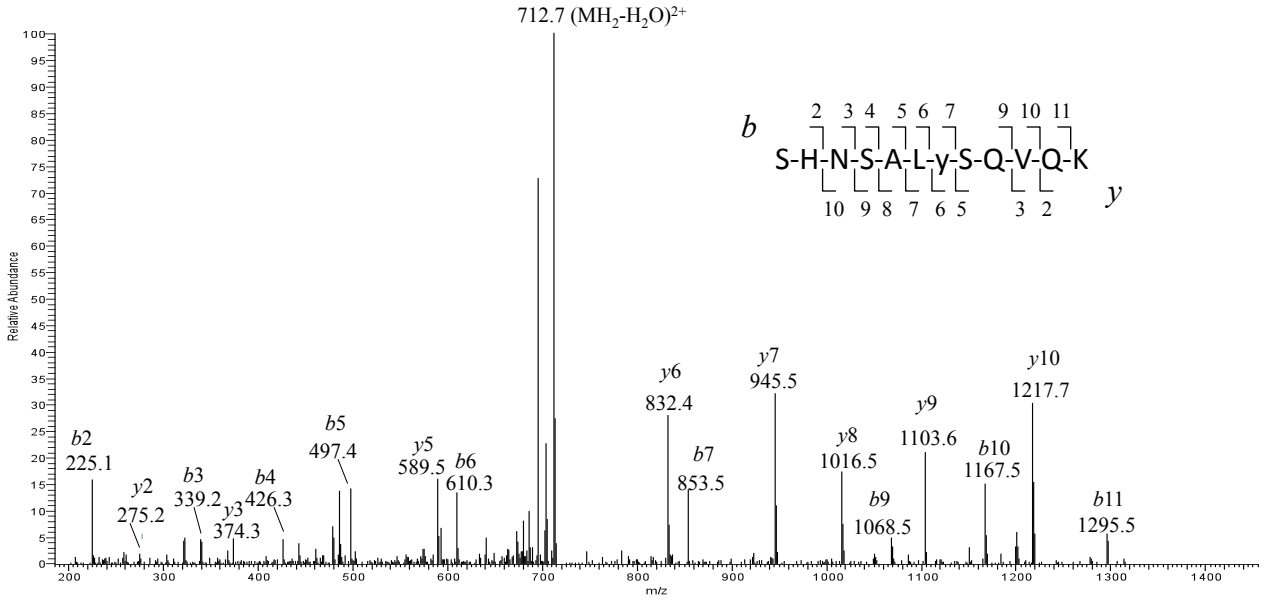
Supplemental Figure 6: TCR upregulation in response to Csk^{AS} expression is not required for activation upon Csk^{AS} inhibition

WT and TCRβ-deficient JRT.T3-5 stably expressing a chimeric CD8-ζ molecule containing the intracellular ITAM motifs of ζ-chain were transiently transfected with vector or membrane-Csk^{AS}. Cells were treated with either vehicle and left unstimulated, stimulated with anti-TCR (WT Jurkats) or anti-CD8 (Jβ/CD8-ζ), or treated with 10 μM 3-IB-PP1 for 18 hours. Cells were then surface stained for CD3ε, CD8-ζ chain, and CD69. FACS plots (A) show one representative experiment and (B) bar graphs show average of mean receptor expression in GFP+ transfected cells in three independent experiments. (C) Histograms show CD69 induction in GFP+ transfected cells as noted. Grey filled, unstimulated; dark grey dashed line, receptor stimulation (anti-TCR or anti-CD8); solid black line, 3-IB-PP1 treatment. Data are representative of two independent experiments.

A.

Accession #	Entry Name	Protein Name	Protein Score [1]	# Peptides (p <0.05)	Ion Score of the Best Peptide [2]	Expectation Value of the Best Peptide [3]	% Protein Sequence Coverage	Protein Mass (Da)
P10809	CH60_HUMAN	60 kDa heat shock protein, mitochondrial	612	10	93	4.2E-07	18.5	61187
P386486	GRP75_HUMAN	Stress-70 protein, mitochondrial	456	7	88	1.1E-06	13.1	73920
P41240	CSK_HUMAN	Tyrosine-protein kinase CSK	452	7	83	1.4E-06	17.3	51242
P31146	COR1A_HUMAN	Coronin-1A	273	5	73	3.7E-05	9.8	51678
P40227	TCPZ_HUMAN	T-complex protein 1 subunit zeta	149	3	70	1.0E-04	6	58444
P17987	TCPA_HUMAN	T-complex protein 1 subunit alpha	138	2	83	2.9E-06	4.5	60819
P48643	TCPE_HUMAN	T-complex protein 1 subunit epsilon	83	1*	85	1.5E-06	1.8	60089
P61978	HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprotein K	71	1*	71	3.7E-05	2.6	51230
P61626	LYSC_HUMAN	Lysozyme C	65	1*	68	1.2E-04	8.1	16982
Q5D862	FILA2_HUMAN	Filaggrin-2	58	1*	58	6.5E-04	0.5	249296
P62988	UBIQ_HUMAN	Ubiquitin	58	1*	60	8.6E-04	21.1	8560
Q99704	**DOK1_HUMAN**	**Docking protein 1 (p62^{Dok1})**	**57**	**1***	**63**	**3.9E-04**	**2.5**	**52815**
P52294	IMA1_HUMAN	Importin subunit alpha-1	46	1*	49	9.0E-03	2	60952

B.



C.

b				*y*
---	1	S	12	---
225.10	2	H	11	1354.62
339.14	3	N	10	1217.56
426.17	4	S	9	1103.51
497.21	5	A	8	1016.48
610.29	6	L	7	945.44
853.32	7	**y**	6	**832.36**
940.36	8	S	5	**589.33**
1068.41	9	Q	4	502.30
1167.48	10	V	3	374.24
1295.54	11	Q	2	275.17
---	12	K	1	147.11

Supplemental Figure 7: Identification of Dok-1 as p65 by mass spectrometry of Csk^{AS} immunoprecipitates.

(A) Table of proteins identified by mass spectrometry of Csk^{AS} immunoprecipitates. [1]Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. [2]Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 40 indicate identity or extensive homology ($p<0.05$). [3]Expectation value denotes the number of times that peptide score as good or better than the one observed can be expected by chance. *MS/MS spectrum was manually inspected. (B) MS/MS spectrum of tryptic peptide ^{443}SHNSALYSQVQ^{454}K derived from Dok1_Human protein. Spectrum was generated by collision-induced dissociation using a linear ion trap mass spectrometer (LTQ, Thermo Scientific). The observed *b* and *y* fragment ion series are annotated on the spectrum and shown on a peptide sequence drawing (top right). Fragment ion pairs *b*6:*b*7 and *y*5:*y*6 confirm the presence of phosphorylation at ^{449}Tyr. (C) Table of theoretical m/z values of *b* and *y* fragment ions. The observed fragment ions are shown in red. Fragment ion pairs that are diagnostic for phosphotyrosine are shown in bold.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of the *Csk^{AS}* allele: The *Csk^{AS}* allele was generated by cloning mouse *Csk* from C57BL/6 splenocyte cDNA (SuperScript II, Invitrogen) and subcloned into pCR2.1-TOPO vector (Invitrogen). The 11-amino terminal residues of Lck (MGCVCSSNPED) and Myc tag were added onto the membrane-*Csk^{AS}* allele via flexible linker regions. Stratagene's QuikChange kit was used to generate the threonine-to-glycine (T266G) mutation of the gatekeeper residue. Correctly targeted Csk^{T266G} mutations were sequence verified and ligated into pEF6/*myc*-His A(Invitrogen) via BamHI and EcoRV digest. Final construct: 5'-BamHI-(Lck$_{11}$-SAGGSAGG)-Csk^{AS}-SAGGSAGG-Myc-EcoRV-3', where SAGGSAGG is a flexible linker region. Jurkat T cells were transfected with either cytoplasmic- or membrane-*Csk^{AS}* alleles and cellular localization was confirmed via fluorescence microscopy and western blot analysis of membrane and cytoplasmic cellular fractions. Whereas only a small portion of cytoplasmic-Csk^{AS} was recruited to the membrane, likely representing interactions with endogenous membrane adaptor proteins, membrane-Csk^{AS} was found almost exclusively at the plasma cell membrane and enriched in lipid rafts (data not shown).

Primers for generation of *Csk^{AS}* allele:

Cyto-Csk Forward: 5'- gga tcc atc atg tcg gca ata cag gcc gcc t -3'

Lck$_{11}$-Csk Forward (external): 5'- gga tcc atc atg ggc tgt gtc tgc agc tca aac cct gaa gat agt gct ggt ggt agt gct ggt ggt tc -3'

Lck$_{11}$-Csk Forward (internal): 5'- agt gct ggt ggt agt gct ggt ggt tcg gca ata cag gcc gcc tgg cca t -3'

Csk-Myc Reverse (external) : 5'- ga tat cta cag atc ctc ttc tga gat gag ttt ttg ttc acc acc agc act acc acc agc act cag gtg -3'

Csk-Myc Reverse (internal) : 5'- acc acc agc act acc acc agc act cag gtg cag ctc gtg ggt ttt gat gt -3'

Mutagenesis Forward: 5'- ggc tct aca tcg tcg gag agt aca tgg cca agg g -3'

Mutagenesis Reverse: 5'- ccc ttg gcc atg tac tct ccg acg atg tag agc c -3'

Description of 3-IB-PP1 (3-(3-Iodobenzyl)-1-*tert*-butyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine): White powder; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.79 (s, 9H), 4.24 (s, 2H), 4.90 (s, 2H), 7.04 (t, 1H), 7.13 (d, 1H), 7.58 (s, 1H), 7.60 (d, 1H), 8.26 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 29.4, 34.9, 60.3, 95.4, 100.8, 127.8, 131.0, 136.6, 137.5, 140.0, 140.8, 154.8, 154.9, 157.7; HRMS (EI) molecular ion calculated for $C_{16}H_{18}IN_5$ 407.0685, found 407.0705. 10mM stock solutions were prepared in DMSO, and individual use aliquots were stored at -80°C.

In-gel digestion, peptide separation, mass spectrometry and protein identification:
Immunoprecipitates from 10^9 membrane-Csk^{AS} transfected cells were eluted from Protein G beads with 0.1M glycine, pH 2.5 and concentrated by centrifugal filtration to 60 µl (Vivaspin 500, Sartorius). The concentrates were separated by SDS-PAGE and protein bands were visualized with SYPRO Ruby stain (Invitrogen) according to manufacturer's protocol. The 65kD band of interest was excised and an in-gel trypsin digest was performed along with the appropriate negative control band (1). Modified porcine trypsin (Promega) was used at a final concentration of 12.5 ng/μl. Mixtures of proteolytically generated peptides were analyzed by nanoLC MS/MS utilizing a 2DLC nanoHPLC System (Eksigent) interfaced with an LTQ XL mass spectrometer (ThermoFisher Scientific) equipped with an Advance ion source

(Michrom Bioresources). An LC Phenomenex Onyx monolithic column (100 μm i.d., 15 cm length) was used for both desalting and reversed phase peptide separation. A 20 minute linear gradient from 2% B to 40% B was run at 750 nL/min flow rate, utilizing solvent A: 2% acetonitrile/0.1% formic acid and solvent B: 90% acetonitrile/ 0.1% formic acid. External calibration of the LTQ XL mass spectrometer was performed in MS/MS mode using fragment ions of Angiotensin I as references. Peptide fragment ion spectra were obtained by precursor ion selection, which employed an automated routine that consisted of a series of one survey MS scan (m/z 400-1700), followed by six MS/MS scans (m/z 60-1500)where helium served as the trap gas and collision energy was set to 35. Protein identification was accomplished by using the MASCOT 2.2 (Matrix Science) search engine. Mammalia taxonomy was searched within the Uniprot database (11/27/2009 513877 sequences; 180750753 residues; taxonomy, homo sapiens; 20401 sequences) utilizing the following parameters: precursor ion mass tolerance: 0.8 Da; fragment mass tolerance: 0.8 Da; tryptic digestion with 3 missed cleavages, fixed modifications: S-carboxyamidomethyl, variable modifications: Deamidation (Asn and Gln); Met-sulfoxide; and Pyro-Glu (from N-terminal Gln). In-gel digestion of candidate proteins was performed according to the established protocol.

Cell lines, primary mouse T cell culture, retroviral transduction and stimulations: TCRβ-deficient Jurkat T cell line stably reconstituted to express 10% and 60% of wild type levels of surface TCR has been previously described (2, 3). TCRβ-deficient cells reconstituted with a chimeric CD8-ζ receptor containing the extracellular and transmembrane portions of the CD8 coreceptor linked to the cytoplasmic domain of the ζ-chain have been reported (4).

Mouse $CD4^+$ T cells were purified using CD4 T cell Isolation Kit (Miltenyi Biotech) according to protocol. Purified T cells were stimulated on anti-CD3 + anti-CD28 coated plates in the presence of IL-2 (100 U/ml) for 24-32 hours prior to retroviral transduction. Csk^{AS} was cloned into pMIGR plasmid and utilized to infect Phoenix packaging cells to generate supernatants for transduction, as previously described by G. Nolan. For transductions, media was gently aspirated from cells and replaced with 1ml of viral supernatent + 8 μg/ml polybrene (Sigma). Cells were spun at 2500 rpm, 30°C for 2 hours. Retroviral supernatents were left on cells during overnight culture. Cultures were supplemented with IL-2 for a period of 12-24 hours, during which time ~80-90% of cells were GFP^+. Cells were rested from IL-2 for 12 hours and serum restricted for 30 minutes prior to stimulation. Cells were stimulated with anti-CD3 (2C11, 10 μg/ml) and goat anti-armenian hamster (Jackson Immunolabs, 50 μg/ml) as indicated.

Lysates were prepared by directly lysing cells in an equivalent volume of SDS-PAGE sample buffer. For immunoprecipitations, cells were stimulated as described and immediately washed in ice-cold PBS. Cells were resuspended in NP-40 lysis buffer with protease and phosphatase inhibitors and immunoprecipitations were carried out according to Abcam's protocol. DTT was added to a final 1% concentration in all lysates, and samples were boiled prior to running on SDS-PAGE. Proteins were visualized using SuperSignal ECL reagent (Pierce Biotechnology) and a Kodak Imaging Station.

TCR internalization assay: To quantify TCR internalization, Jurkat cells were serum-restricted for 30 min at 37°C, then stained on ice in serum-free RPMI for 30 min. Cells were washed in FACS buffer, and warmed up to 37°C for stimulation or 3-IB-PP1 treatment for times indicated. TCR internalization was stopped by incubating cells in ice-cold FACS buffer. Samples were divided into 2 fractions for total CD3ε and internalized CD3ε staining. Cells stained for total CD3ε were washed in FACS buffer and fixed immediately. Cells analyzed for internalized CD3ε were washed in FACS buffer and had surface bound anti-CD3ε-PE acid stripped by resuspending in RPMI + 3% FBS, pH2.0 for 30 sec at RT. Cells were washed immediately in ice-cold FACS buffer and fixed prior to FACS analysis of MFI of CD3ε. To calculate the amount internalized, the following equation was used: $(S_{tx}-S_{t0}) / (T_{t0}-S_{t0})*100$, where S_{tx} is MFI of cells stripped following stimulation; St_0 is MFI of cells stripped without treatment, and Tt_0 is total

MFI of cells without stripping. Controls to verify cell viability and other surface markers were used to verify quality of data.

SUPPLEMENTAL REFERENCES

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4. B. A. Irving, A. Weiss, The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell* **64**, 891-901 (1991).