

## Supplementary Materials for

### Feedback Circuits Monitor and Adjust Basal Lck-Dependent Events in T Cell Receptor Signaling

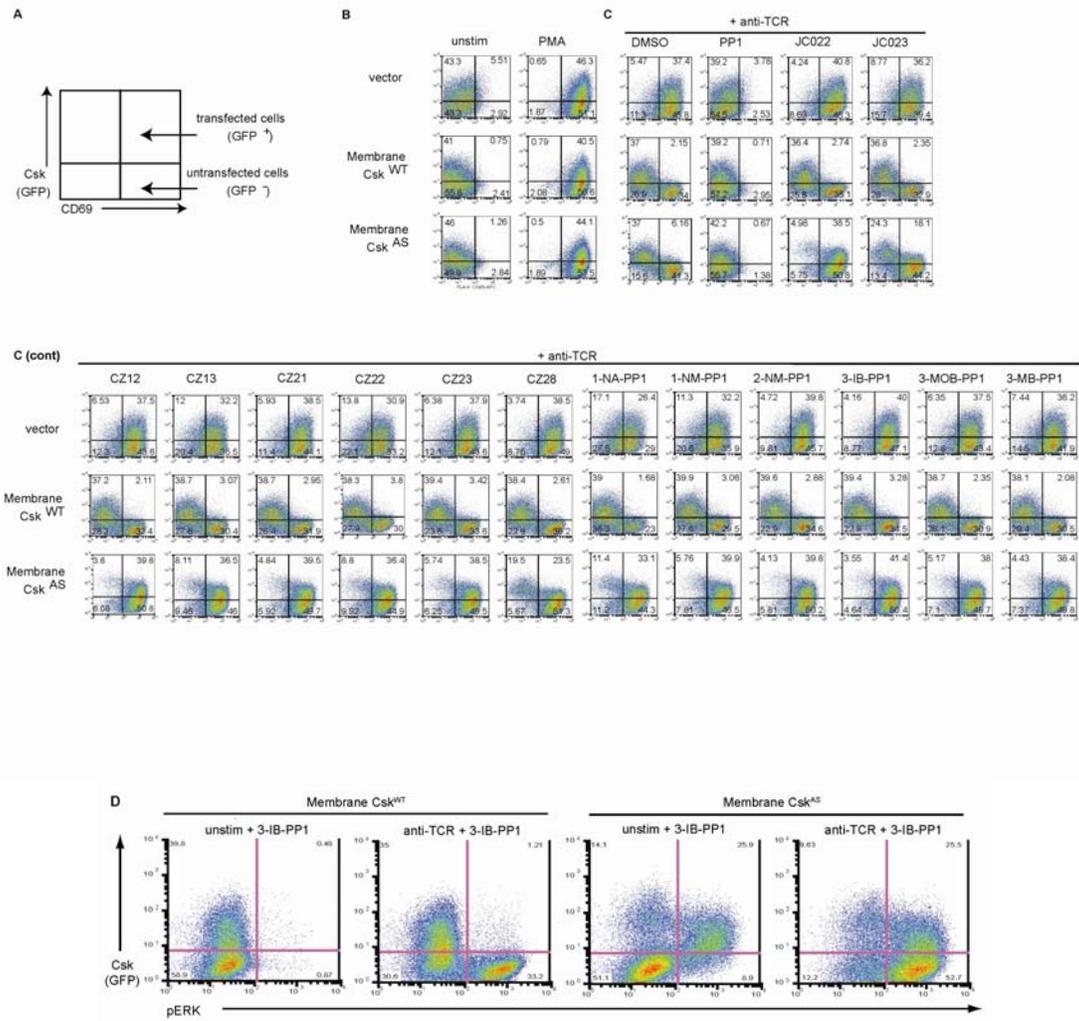
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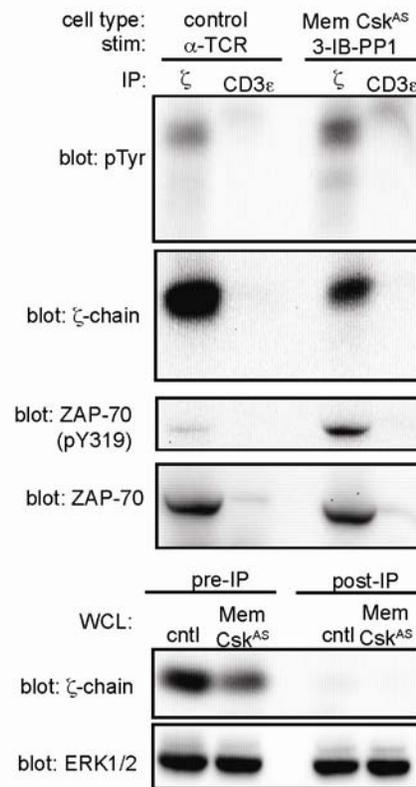
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#### The PDF file includes:

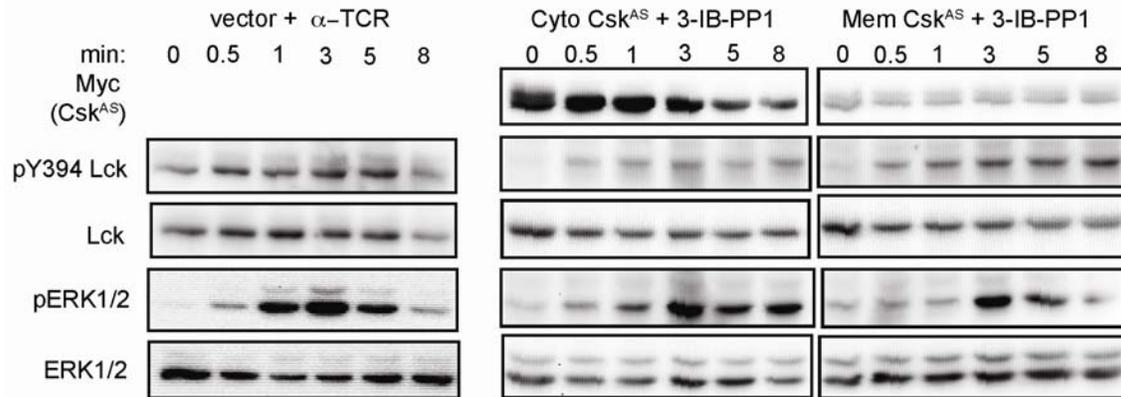
- Fig. S1. Screen of PP1 analogs for a specific inhibitor of Csk<sup>AS</sup>.
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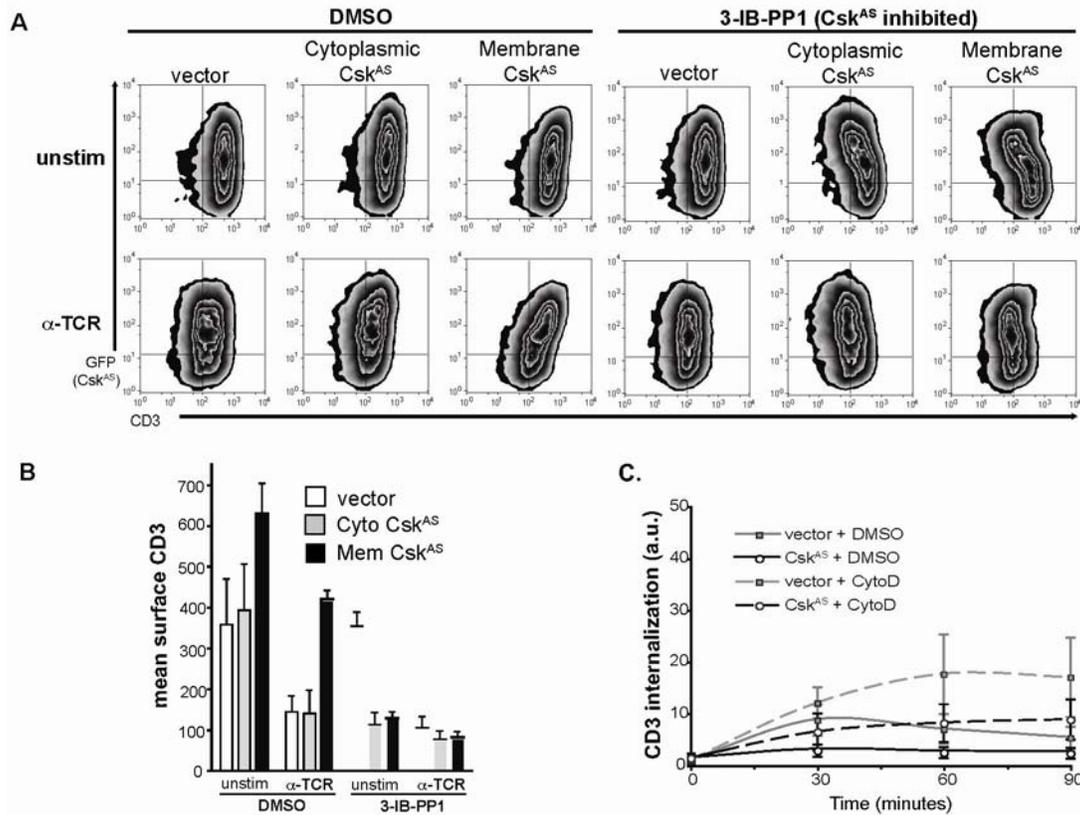
**Fig. S1.** Screen of PP1 analogs for a specific inhibitor of Csk<sup>AS</sup>. Jurkat cells were transiently cotransfected with plasmid encoding GFP together with control vector, plasmid encoding wild-type (WT) membrane-Csk, or plasmid encoding analog-sensitive (AS) membrane-Csk. Cells were stimulated through the TCR in the presence of PP1 and its analogs for 16 hours as denoted, and then analyzed by flow cytometry for surface CD69. **(A)** Flow cytometry schematic of transfected cells. **(B)** Unstimulated cells were all CD69<sup>lo</sup>, whereas PMA-treated cells were all CD69<sup>hi</sup>. **(C)** Cells were treated with antibody against the TCR and inhibitors (all at 10  $\mu$ M). Note the ability of 3-IB-PP1 to revert GFP<sup>+</sup> transfected cells to CD69<sup>+</sup> cells where cells contained membrane-Csk<sup>AS</sup> but not membrane-Csk<sup>WT</sup>. **(D)** Cells containing membrane-targeted WT or AS Csk were treated with 3-IB-PP1 and were either left unstimulated or were stimulated through the TCR for 5 min, after which they were analyzed by flow cytometry for ERK phosphorylation. 3-IB-PP1 impaired the inhibitory effect of Csk<sup>AS</sup>, but not Csk<sup>WT</sup>, on TCR stimulation; furthermore, 3-IB-PP1 had no stimulatory effect on cells transfected with plasmid encoding Csk<sup>WT</sup>.



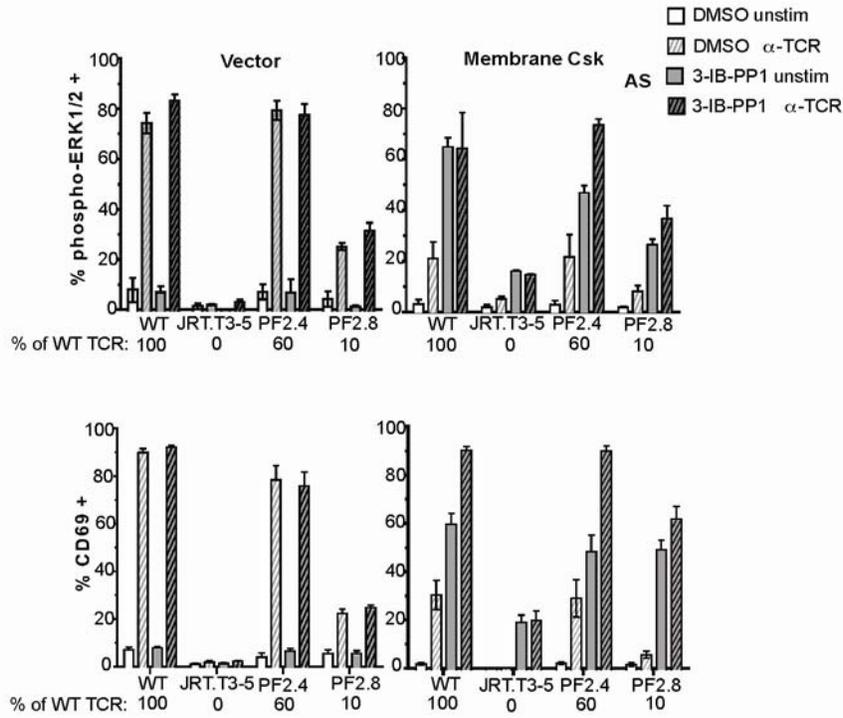
**Fig. S2.** Phosphorylation of  $\zeta$  chain and its association with ZAP-70 upon inhibition of Csk<sup>AS</sup>. Jurkat cells transfected with vector control plasmid or with plasmid encoding membrane-CskAS were starved of serum and then stimulated with antibody against the TCR or 3-IB-PP1 for 5 min. Whole-cell lysates (WCLs) were sequentially subjected to immunoprecipitation with antibodies against the  $\zeta$ -chain and CD3 $\epsilon$  (top); pre- and post-immunoprecipitation (IP) WCLs were 1/10 the volume of input (bottom). Data represent at least two experiments.



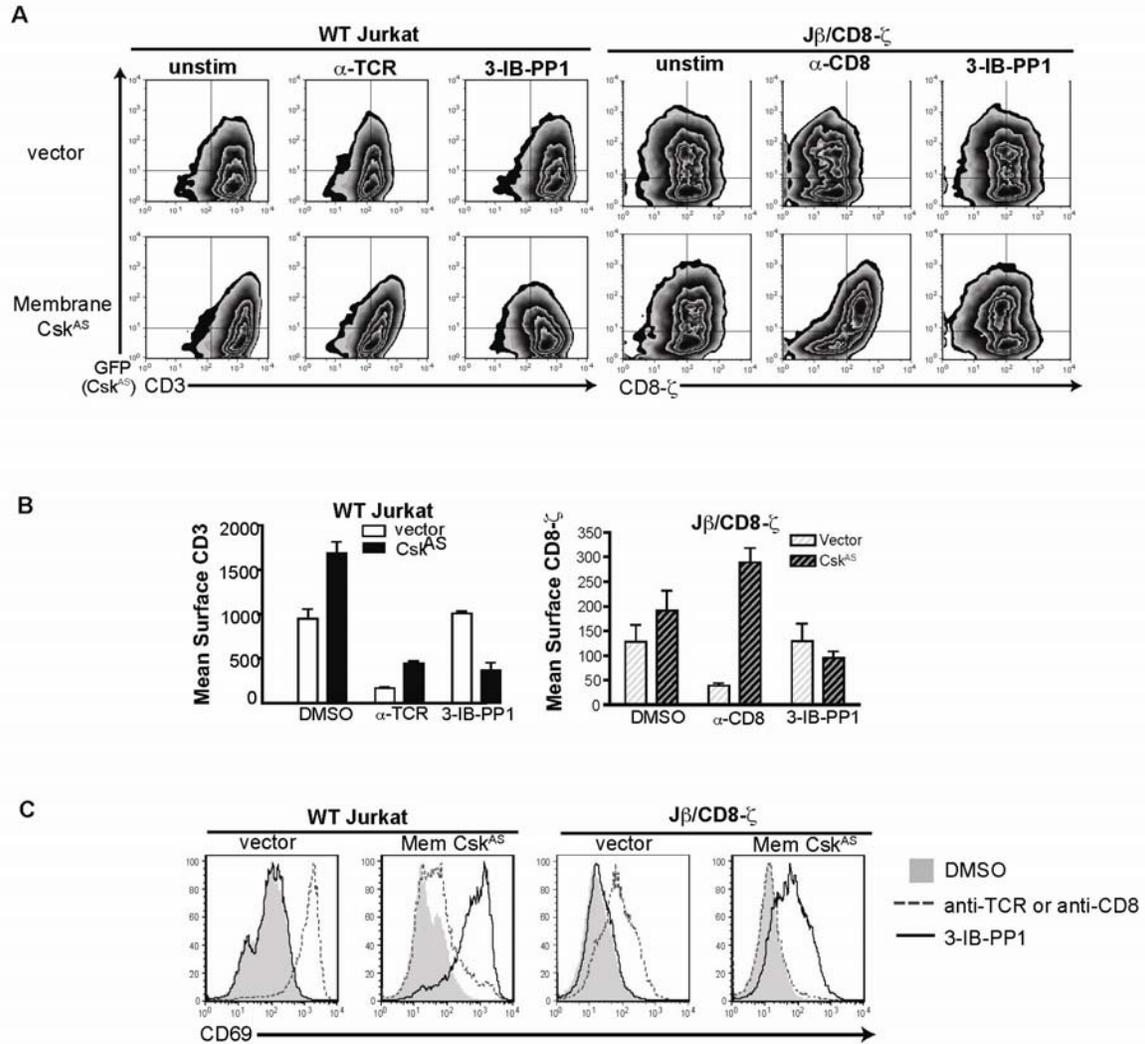
**Fig. S3.** Inhibition of Csk<sup>AS</sup> in primary mouse T cells activates Lck and ERK. Mouse CD4<sup>+</sup> T cells were transduced with control retrovirus or with retrovirus expressing the indicated form of Csk<sup>AS</sup>. Cells were rested in the absence of exogenous IL-2 for 16 hours and were starved of serum in the final 30 min. Cells were harvested directly or after stimulation with antibody against TCR or treatment with 3-IB-PP1 for the indicated times. Cell lysates were analyzed by Western blotting for the phosphorylation of Lck and ERK proteins. Data are representative of at least three experiments.



**Fig. S4.** Csk<sup>AS</sup> activity regulates the amount of the TCR at the cell surface. (**A** and **B**) Membrane-Csk<sup>AS</sup> protects cells from TCR degradation, even in a basal state. Jurkat cells were transiently cotransfected plasmid encoding GFP and with vector control or plasmid encoding the indicated form of Csk<sup>AS</sup>. Cells were treated with 3-IB-PP1 or DMSO and were stimulated through the TCR for 18 hours before flow cytometric analysis for the surface expression of CD3 $\epsilon$ . (**A**) Flow cytometry plots show the surface expression of CD3 in transfected (upper quadrants) and untransfected (bottom quadrants) cells in a representative experiment. (**B**) MFIs of CD3 in GFP<sup>+</sup> (transfected) cells from three independent experiments. (**C**) Cells containing membrane-Csk<sup>AS</sup> cells do not exhibit basal internalization of CD3. Cells were with plasmid encoding GFP and with vector control or with plasmid encoding membrane-Csk<sup>AS</sup>. Cells were starved of serum in the presence of vehicle or cytochalasin D for 30 min, after which receptor internalization was allowed to occur at 37°C for the indicated times. Cells were then washed and the amount of internalized CD3 was measured as described in the Materials and Methods. Data are the means of three experiments.



**Fig. S5.** Cellular activation induced by Csk<sup>AS</sup> inhibition requires TCR at the cell surface, but is not dependent on the amount of TCR. WT Jurkat cells, TCRβ-deficient JRT.T3-5 cells, and stable cell lines reconstituted to have 10% (JRT.T3-5 PF2.8) or 60% (JRT.T3-5 PF2.4) of the amount of TCR on the surface of WT Jurkat cells, were transiently transfected with plasmid encoding GFP together with vector control or plasmid encoding membrane-Csk<sup>AS</sup>. (Top) Cells were starved of serum and then pretreated with DMSO or 3-IB-PP1 (10 μM) for 15 min before undergoing TCR stimulation for 5 min and analysis for the presence of pERK. (Bottom) Cells were starved of serum and then pretreated with DMSO or 3-IB-PP1 (10 μM) for 18 hours before being analyzed for surface CD69 expression. Plots show the percentages of total live GFP<sup>+</sup> (transfected) cells that contained pERK or surface CD69. Data are the averages of three experiments.

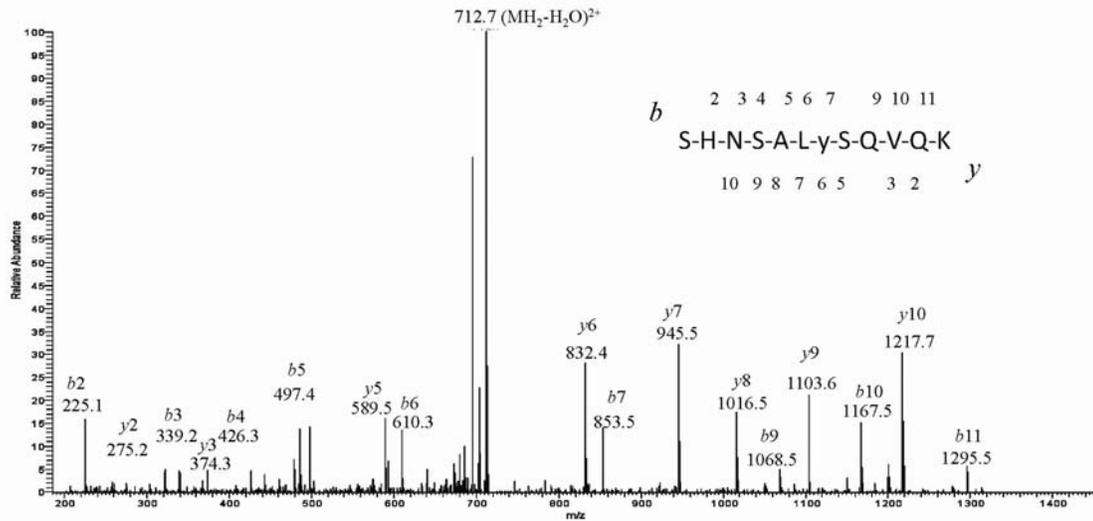


**Fig. S6.** Increase in TCR abundance in response to Csk<sup>AS</sup> is not required for cellular activation upon inhibition of Csk<sup>AS</sup>. WT Jurkat cells and TCR $\beta$ -deficient JRT.T3-5 cells stably expressing a chimeric CD8- $\zeta$  molecule containing the intracellular ITAM motifs of the  $\zeta$ -chain were transiently cotransfected with vector control or with plasmid encoding membrane-Csk<sup>AS</sup>. Cells were treated with vehicle and left unstimulated or were treated with antibody against the TCR (WT Jurkat cells) or with antibody against CD8 (J $\beta$ /CD8- $\zeta$  cells), or were treated with 3-IB-PP1 (10  $\mu$ M) for 18 hours. Cells were then analyzed by flow cytometry for the surface expression of CD3 $\epsilon$ , the CD8- $\zeta$  chain, and CD69. **(A)** Flow cytometry plots from one representative experiment. **(B)** Bar graphs show the average mean surface receptor expression in GFP<sup>+</sup> (transfected) cells from three experiments. **(C)** Histograms show the increase in CD69 abundance in GFP<sup>+</sup> (transfected) cells expressing the indicated receptors. Gray, filled histograms indicate unstimulated cells; the dark gray dashed line indicates cells whose receptors (either TCR or CD8) had been stimulated; whereas the solid black line indicates cells that were treated with 3-IB-PP1. Data are representative of two independent experiments.

A

Accession #	Entry Name	Protein Name	Protein Score <sup>1</sup>	# Peptides (p < 0.05)	Ion Score of the Best Peptide <sup>2</sup>	Expectation Value of the Best Peptide <sup>3</sup>	% 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
<b>Q99704</b>	<b>DOK1_HUMAN</b>	<b>Docking protein 1 (p62<sup>Dok1</sup>)</b>	<b>57</b>	<b>1*</b>	<b>63</b>	<b>3.9E-04</b>	<b>2.5</b>	<b>52815</b>
P52294	IMA1_HUMAN	Importin subunit alpha-1	46	1*	49	9.0E-03	2	60952

B



C

<i>b</i>				<i>y</i>
---	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

**Fig. S7.** Identification of Dok-1 as p65 by mass spectrometric analysis of Csk<sup>AS</sup> immunoprecipitates. (A) Table of proteins identified by mass spectrometric analysis of Csk<sup>AS</sup> immunoprecipitates. <sup>1</sup>Protein scores are derived from ions scores as a non-

probabilistic basis for ranking protein hits. <sup>2</sup>Ions score is  $-10 \cdot \log(P)$ , where  $P$  is the probability that the observed match is a random event. Individual ions scores  $> 40$  indicate identity or extensive homology ( $P < 0.05$ ). <sup>3</sup>Expectation value denotes the number of times that the peptide score was as good as or better than the one observed could be expected by chance. \*MS/MS spectrum was manually inspected. (B) MS/MS spectrum of the tryptic peptide <sup>443</sup>SHNSALYSQVQ<sup>454</sup>K derived from Dok1\_Human protein. The spectrum was generated by collision-induced dissociation with 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 b6:b7 and y5:y6 confirm the presence of phosphorylation at Tyr<sup>449</sup>. (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.