SUPPLEMENTARY MATERIAL EITELHUBER ET AL.:

SUPPLEMENTARY MATERIAL AND METHODS

Yeast two hybrid screen
The GUK domain (aa 932-1147) and the C-terminus of Carma1 (aa 600-1147) were fused in-frame to the GAL4 DNA-binding domain of the pAS2-1 vector (Clontech). With these plasmids screenings of a B-cell cDNA library in pAct were performed according to the manufacturer´s protocols (Clontech). The yeast strain AH109 was used for screening. No auto-activation of the Carma1 constructs was observed. Selection was performed on Trp⁻ Leu⁻ His⁻ medium and positive clones were further verified by a filter assay for β-galactosidase activity.

Calcium Assay
siRNA transfected Jurkat T cells were transferred to RPMI medium without phenolred. 10⁶ cells per ml were stained with Indo1-AM (Molecular Probes; final concentration 5 μM) for 25 min at 37°C. After washing twice in RPMI without phenolred cells were diluted to 3,3x10⁵ per ml. Cells were stained with propidium iodide (PI) to distinguish dead cells. The ratiometric intracellular calcium concentration indicator Indo-1 was excited at 355nm, PI at 488nm. Analysis was done by flow cytometry on a FACSstarplus (Becton Dickinson) as described previously (Grundler et al, 2000). Emission fluorescence was recorded with bandpath filters for Indo-1 at 395nm and 530nm and PI at wavelength longer than 600nm. Besides the three emission wavelength, forward- and sidescatter were recorded together with time. Stimulation with CD3/CD28 leads to calcium release and the ratio of the fluorescence emissions I (395nm) / I (530nm) is used as a measure for intracellular calcium concentration.
and plotted versus time. Stimulation with ionomycin, a selective calcium ionophore, functioned as a positive control.

**NF-AT EMSA**

Nuclear extracts of P/I stimulated cells were performed by lysis in buffer containing 10 mM HEPES, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 0.2% NP-40 and protease and phosphatase inhibitors. After centrifugation the supernatant contains cytoplasmic fraction and the pellet is lysed in 20 mM HEPES, 10 % Glycerol, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol and protease and phosphatase inhibitors and contains the nuclear fraction. NF-AT EMSA was essentially performed as described using an oligonucleotide (GATCAGAAAGGAGGAAAAACTGTGTTCATACAGAAGGCGTT) comprising the NF-AT1 DNA binding sequence from the IL-2 enhancer (Boise et al, 1993). DNA binding buffer contained 1000ng poly(dI-dC), 50 mM KCl, 10 mM Tris (pH 7.5), 10 mM HEPES, 1.25 mM dithiothreitol, 1.1 mM EDTA and 15 % Glycerol. NF-AT1 antibody was a kind gift of Anjana Rao.

**Gel filtration chromatography**

Gel filtration analysis using Superose 6 column (GE Healthcare) was performed as previously described (Oeckinghaus et al, 2007). Peak elution of standard marker proteins is depicted. Fractions were pooled as depicted and subjected to anti-Bcl10 IP (see Figure 3C).

**PKCθ kinase assay**

Cells were lysed in co-IP buffer and PKCθ-IP was performed. Precipitates were washed twice with co-IP buffer without inhibitors and washed twice with kinase assay buffer containing 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM β-glycerophosphate, 50 μM sodium vanadate, 1
mM dithiothreitol. Precipitate was incubated in kinase assay buffer containing 20 μM ATP, 1μg substrate MBP, 3μCi γ 32P ATP for 25 minutes at 37°C.

SUPPLEMENTARY REFERENCES


SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: PPP2R1A interacts with the C-terminal Carma1 fragments in yeast. Yeast strain AH109 was transfected with plasmids PPP2R1A 159-589 expressing pAct (contains Leucin gene) and Carma1 fragments 600-1147 or 932-1147 expressing pAS2-1 (contains Tryptophan gene). Selection was performed on Trp’ Leu’ His’ medium. Positive interaction activates the Histidin reporter and the lacZ reporter, which is visualized by fast growing colonies positive in a β-galactosidase assay (blue). Controls were performed using PPP2R1A fragment in pAct and empty vector pAS2-1 as well as Carma1 fragments in pAS2-1 and empty vector pAct. Note that due to leakiness we observed still growth colonies on the control plate even in the absence of exogenous Histidin. However, growth was reduced and the colonies are negative in β-galactosidase assay.

Supplementary Figure 2: The very C-terminus of Carma1 and PPP2R1A associate upon overexpression in HEK293 cells. The C-terminal part of the GUK domain of Carma1 is
essential for PPP2R1A binding. Flag-PPP2R1A (aa 159-589) and HA tagged constructs of the Carma1 GUK domain were co-transfected in HEK293 cells as indicated. Deletion of the C-terminal part of the GUK domain (constructs aa 932-1048 and 932-1098) diminishes interaction after IP with HA-beads.

**Supplementary Figure 3**: Enhanced IL-2 production in PP2A knock-down Jurkat T cells after CD3/CD28 stimulation. (A) Jurkat T cells were transfected with siRNA against GFP control or PPP2R1A stimulated by anti-CD3/CD28 co-ligation. Secreted IL-2 amounts were measured by ELISA. Downregulation of PPP2R1A enhances IL-2 production. (B) Jurkat T cells were transfected with siRNAs against GFP or PPP2R1A and stimulated by anti-CD3/CD28 co-ligation for 3h. RNA was isolated and IL-2 transcript levels were investigated by quantitative RT PCR. IL-2 mRNA level in siPPP2R1A transfected cells is increased. Transcript levels were normalized using RNA polIII mRNA.

**Supplementary Figure 4**: PPP2R1A-1 knock-down enhances NF-κB activation. (A) Jurkat T cells were transfected with siRNA against GFP and PPP2R1A-1 and stimulated with CD3/CD28 as indicated. NF-κB DNA binding was determined by EMSA. (B) Supershift analysis of NF-κB-DNA complexes. Extracts of cells stimulated for 50 min by anti-CD3/CD28 antibodies. Migration of strong p50/p65 and weak p65/p65 complexes and supershifts is indicated.

**Supplementary Figure 5**: Ca\(^{2+}\) release and NF-AT activation is not affected by PP2A knock-down. (A) Jurkat T cells were transfected with siRNA against GFP, PPP2R1A-1 or PPP2R1A-2 and stimulated with CD3/CD28. Ca\(^{2+}\) release of intracellular stores was measured after stimulation with CD3/CD28. No significant alterations in relative intracellular Ca\(^{2+}\) concentrations were observed. (B) NF-AT DNA binding from nuclear extracts of siRNA
transfected cells were analyzed by EMSA. Downregulation of PP2A subunits did not significantly alter NF-AT activation. NF-AT-DNA complex was verified by anti-NF-AT supershift analysis. Incubation of anti-NF-AT1 antibody led to an inhibition and weak supershift of the depicted NF-AT-DNA complex.

**Supplementary Figure 6:** Elution profiles of Carma1, Bcl10, Malt1 and PPP2R1A after Superose6 gel filtration chromatography. Extracts of Jurkat T cells unstimulated or P/I stimulated (20 min) were fractionated by Superose6 gel filtration chromatography. Elution profiles of Carma1, Malt1, Bcl10 and PPP2R1A/B were analyzed by Western blotting. Molecular-weight standards depict the peak elution of marker proteins. Elution of the CBM complex after P/I stimulation peaked in fraction 11-14 (pool I), which is evident from the appearance of modified Malt1 that corresponds to ubiquitinated Malt1 within the CBM complex (compare (Oeckinghaus et al, 2007). Carma1 peak elution was detected in fractions 20-23 (pool II) and Bcl10-Malt1 predominately eluted in fractions 24-27.

**Supplementary Figure 7:** PKCθ activation is unaffected in PPP2R1A knock-down cells. PKCθ was immunoprecipitated from Jurkat T cells after downregulation of PPP2R1A and anti-CD3/CD28 ligation. PKCθ activity was measured by performing a kinase reaction using myelin basic protein (MBP). PKCθ activity is not significantly altered after PPP2R1A knock-down.
Supplementary Figure 1
Supplementary Figure 4
Supplementary Figure 5
Supplementary Figure 7