Supporting Information

SI Materials and Methods

Mice: Themis KO (1), Shp1$^{fl/fl}$ CD4-Cre^{+} (23) and *Themis*$^{-/-}$ Shp1$^{fl/fl}$ CD4-Cre^{+} (26) mice have been described previously. All animal experiments were approved and carried out according to the institutional guidelines set by the Animal Care and Use Committee of National University of Singapore. Mice were used at 6-8 weeks of age.

Immunoprecipitation and Western Blotting: Thymocytes were freshly isolated from thymi from dissected mice. 15-20 million cells were lysed using a maltoside based lysis buffer (20 mM Tris pH7.0, 150 mM NaCl, 1% n-dodecyl β-D-maltoside with protease inhibitor tablets (Thermo Scientific, USA, Cat. No.88666) at 4°C in shaker for 20-30 min. The cell lysate was spun at 13000 rpm in a tabletop centrifuge for 15 min at 4°C and the supernatant was transferred to a new tube. 5 µl of the protein G beads were then added to it for 10 min on ice for pre-clearing in order to reduce non-specificity. Meanwhile, antibody dilution was made by adding 2-3 µg of antibody (Ab) into 100 µl of lysis buffer, which was then added to the cleared lysate. This mixture was incubated for up to 4 hr. at 4°C. Then, 20-30 µl of Protein G beads (Invitrogen) were added to the lysate with the Ab. This complex was then incubated for 2 hr. at 4° C. For some of the experiments as mentioned in the results, immunoprecipitates were treated with Shp1/2 inhibitors (Merck, USA, Cat. No. 565851). Using a magnetic separator, the supernatant was removed and the beads were washed thrice with the lysis buffer. This was followed by boiling the samples for 5 min in SDS sample buffer (Nacalai Tesque Inc.) to elute the protein. The samples were then resolved on 4-12% pre cast SDS gel (Invitrogen) and blotted. The membrane was blocked for 1 hr. with the LI-COR blocking buffer, and incubated with the primary antibody overnight at 4°C with shaking. After rinsing the membrane with TBS 0.1% Tween buffer, the blot was incubated with the required secondary antibody diluted in LI-COR buffer for 1 hr. at room temperature and developed using LI-COR Odyssey imaging system. Band intensities were calculated using background subtraction method with the help of LI-COR Odyssey software.

Phosphatase assay: IP was done in the same way as described above but using 1% Nonidet P40 based lysis buffer with 5 mM DTT. However, at the end of IP, beads were washed in Tris - NaCl buffer, followed by two washes with 1X phosphatase buffer (2 × phosphatase buffer: 50 mM HEPES [pH 6.5], 0.2 mM EDTA, 10 mM DTT, 200 µg/ml BSA, 0.02 % Brij 35). Beads were finally resuspended in 150 µl of phosphatase buffer and 5 µl of phosphopeptide that is provided with the kit (Promega V2471) was added to each sample. The mixture was kept at 25°C for 10 min to allows the enzyme (Shp1 in this case) to interact with the phosphopeptide and produce free phosphate. Final product was added into each well of a 96 well plate (in triplicates) after removing the beads through magnetic separator. Following this, malachite green dye/ additive mix was added to the sample and the absorbance at 620 nm was read using the plate reader. Standards were made with the 1M-phosphate standard that was provided with the kit. The molar concentration of the free phosphate released in each sample was calculated using the phosphate standards with the help of Microsoft Excel.

Antibodies used: The following were used as primary antibodies: rabbit anti-Themis (ED Millipore 06-1328), mouse anti-Shp2 (BD Biosciences, 610622), rabbit anti-Shp1 (Santa Cruz, SH-PTP1 C-19), rabbit anti-Shp1 (Thermo Fisher Scientific, PA5- 27803), rabbit anti-phospho-Shp1 (Y564) (Cell Signaling, 8849S), rabbit anti-phospho-Erk (Cell Signaling, 9101S) and mouse anti-Total Erk (BD Biosciences, 610031). Odyssey Goat anti-Mouse 800 CW and Odyssey Goat anti-Rabbit 680 LT were used as the secondary antibodies. Goat anti-mouse IgG H&L Alexa fluor 488 (Abcam, ab150113) and Goat anti-rabbit IgG H&L Alexa fluor 568 (Abcam, ab175471) were used as secondary antibodies for imaging experiments.

Sequential Immunoprecipitation: 5-10 million cells were lysed using 300 µl of 1% maltoside buffer for 20 min on ice followed by centrifugation at 13000 rpm for 15 min at 4°C. 50 µl of the whole cell lysate was separated to be used as 1st Whole cell lysate (WCL) and remaining was used to precipitate Themis after adding 50 µl of beads-Ab complex. After the first IP, the lysate was magnetically separated from the Protein-Ab-beads complex. The beads complex was then washed and the proteins eluted as with standard IP. Whereas, 50 µl of the lysate that was separated after the 1st IP (now has

much less Themis remaining in it as most of it has already been precipitated) was used as 2nd WCL and remaining 250 µl was used to precipitate again by adding 50 µl of Themis Ab-beads complex. Process was repeated till 3rd or 4th IP to completely deplete Themis. All the samples were then subjected to SDS-PAGE followed by western blotting. Quantification was done using background subtraction method with the help of LI-COR Odyssey software. Shp1 was first normalized with Total Erk from WCL samples. Amount of free Shp1 was calculated by dividing normalized Shp1 from last WCL once Themis is completely depleted with the total amount of normalized Shp1 from all the WCL samples.

Alkaline phosphatase treatment: 15-20 million cells were lysed in 250 µl of maltoside lysis buffer with protease inhibitors. 2 µg of recombinant His-tagged Themis (see below) was then added in some of the thymocytes samples as mentioned in the results section of manuscript and incubated for 30 min on ice. Immunoprecipitation was done using protein G beads and the protein-Ab-beads complex was then resuspended in alkaline phosphatase buffer along with protease inhibitors, EDTA free and with (for treated samples) or without (for untreated samples) 1µl (≅10 units) of Alkaline phosphatase enzyme and incubated for 1 hr. at 37°C. This was followed by washing and elution of the immunoprecipitated samples, which were then used for phosphatase assay or SDS-PAGE.

Recombinant Themis: Themis-His was expressed in BL21-pET28a vector, and grown in LB media with Kanamycin (Kan) overnight at 220 rpm. 50 ml of this BL21 pET28a-Themis-His culture was then added to 1 L LB (Kan) for expansion by shaking at 37°C at 220 rpm till 0.6 OD (600 nM) was reached. 500 µl of 1 M IPTG was then added into the media to induce expression of Themis, and incubated overnight with shaking at 220 rpm. Next day, this culture was centrifuged at 4000 rpm for 30 min. Cell pellet was either used immediately or stored at -80°C. For protein purification, pellet was resuspended in 20 ml of resuspension buffer (10 mM Imidazole, pH 7.8, 20 mM Tris, 10% Glycerol, 300 mM NaCl, 10% sucrose, EDTA free anti-proteinase, 3U Dnase I, 0.1 mg/ml lysozyme, 2 mM $MgCl_2$). The resuspended bacteria was incubated on ice for 30 min and sonicated 5 times at intervals of 10 sec. These lysed cells were then centrifuged at 16,000 rpm, 4°C for 30

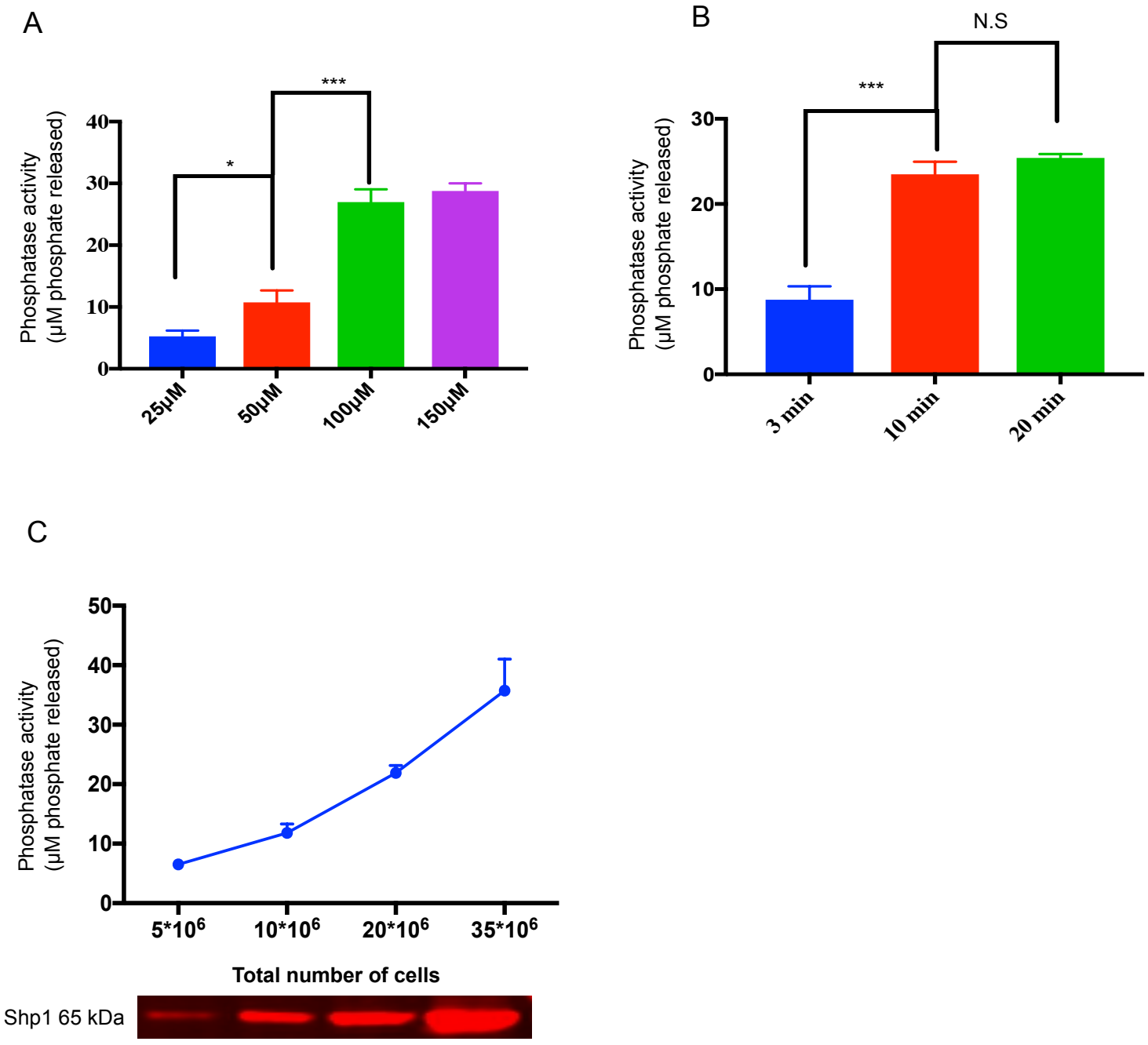
min, and the supernatant was transferred into a new falcon tube and pH was adjusted to 7.8. After that, 5 ml of Ni-NTA beads was used to purify Themis from bacterial lysate. Using a 10 ml filter syringe, 5 ml of Ni-NTA beads were added and the beads storing solution was drained out via gravitation. The beads were washed twice with 10 ml of resuspension buffer (10 mM Imidazole, pH 7.8, 20 mM Tris, 10% Glycerol, 300 mM NaCl). The previously collected lysate was allowed to pass through the beads twice. The beads were washed 5 times with 10 ml of washing buffer (20 mM Imidazole, pH 7.8, 20 mM Tris, 10% Glycerol, 300 mM NaCl). Themis was eluted out from the beads with 15 ml of elution buffer (250 mM Imidazole, pH 7.8, 20 mM Tris, 10% Glycerol, 300 mM NaCl). Eluted Themis was concentrated to 6 ml with vivaspin (10 kDa) and further purified with FPLC using size-exclusion column (S200-120 ml) with running buffer (pH7.8, 20 mM Tris, 300 mM NaCl, 10% glycerol); Themis was collected at 66 ml - 75 ml during the FPLC run and concentrated to 500 µl with vivaspin (10 kDa). Concentration of the purified Themis was determined with standard bradford assay. Purity of the protein was checked with 12% SDS-PAGE stained with coomassie blue and western blotting with anti-Themis antibody.

Total Internal Reflection Microscopy: 0.1-0.2*10^6 of thymocytes at a concentration of 1 million cells per ml from OT-I *β2m*$^{-/-}$*Themis*$^{+/+}$ and OT-I *β2m*$^{-/-}$*Themis*$^{-/-}$ mice were stimulated with plate coated anti-CD3 (1 µg/ml) antibody on 8 well microscopy chamber for 45 min. Following stimulation, 200 µl of freshly prepared 8% PFA was added and incubated for 12 min at room temperature. Cells were then washed with PBS and 200 µl of 0.3% triton X was added to the cells for 3 min, following which, cells were again washed extensively few more times. Blocking was done by adding 10% normal goat serum in PBS for 1 hr. at room temperature. 200 µl of anti-p-Shp1 antibody at a concentration of 1:250 in 1% normal goat serum was then added to the cells and incubated at room temperature for 1 hr. Cells were washed with PBS and secondary antibody of Goat anti-rabbit Alexa fluor 568 was added at a concentration of 1:1000 for 1 hr. at room temperature. Cells were again washed and blocked for 1 hr. at room temperature with 10% normal goat serum. Anti-Shp1 Ab was then added at 1:300 at room temperature for 1 hr. followed by washing of cells with PBS. Secondary antibody of Goat anti-

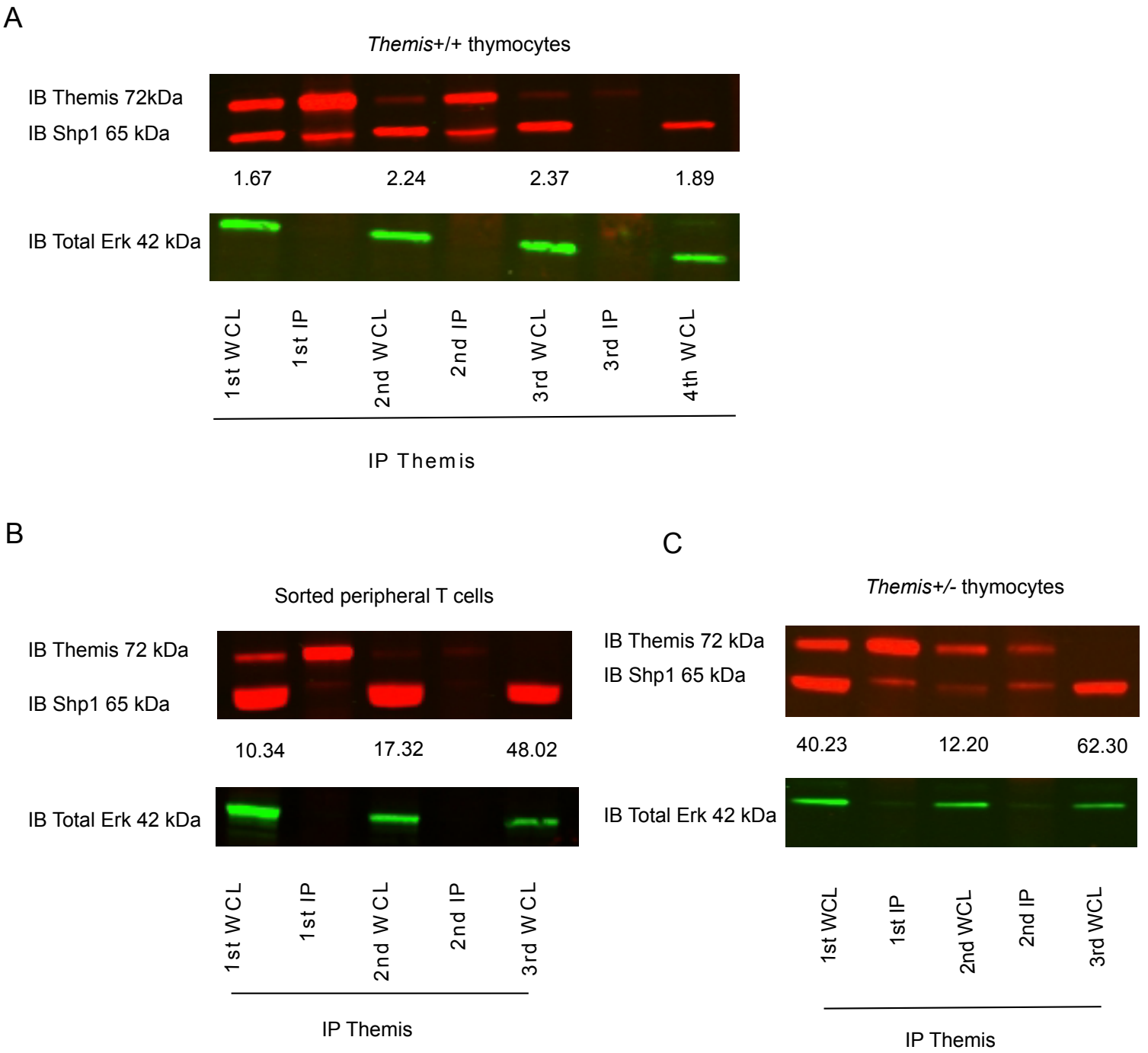
mouse Alexa fluor 488 was added at a concentration of 1:1000 for 1 hr. at room temperature. Cells were then washed twice with PBS and finally 200 µl of PBS was added to each chamber with the cells. Microscopy was then done using TIRF microscope with the help of Olympus IX83 inverted microscope. 488 and 561 nm lasers were aligned to strike on the coverslip at such an angle that it yields a field depth (*d*) <100 nm for internal reflection. The images were recorded using 100 ms exposures and were obtained in a Hamamatsu ORCA Flash 4.0 camera. Data was analyzed using Olympus cellSens software.

Flow cytometry and cell sorting: Single cell suspensions were stained with fluorochrome labeled monoclonal antibodies for 30 min on ice. Samples were run and data were acquired on Fortessa X-20 (BD Biosciences) and analyzed using FlowJo Software version 9.0. For the samples that needed to be sorted, they were stained in the same way and later sorted using Sy2000.

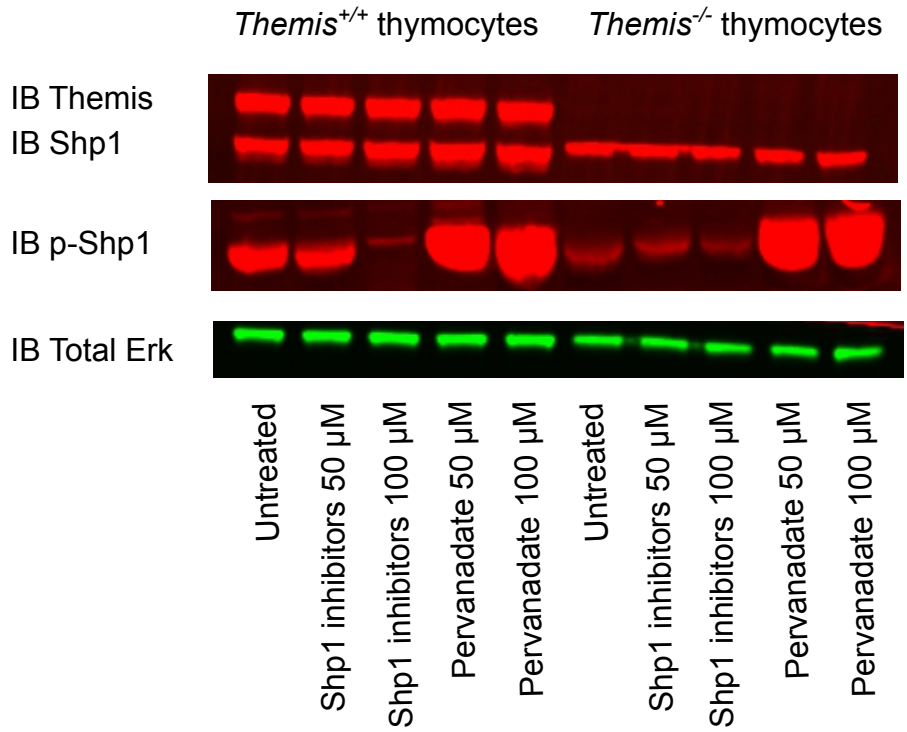
Statistical testing: Statistical differences were calculated using the mean difference hypothesis of Student's two-tailed t-test assuming different variances and confidence level of 95%. Graphs were made using Graph Pad Prism 6.0.



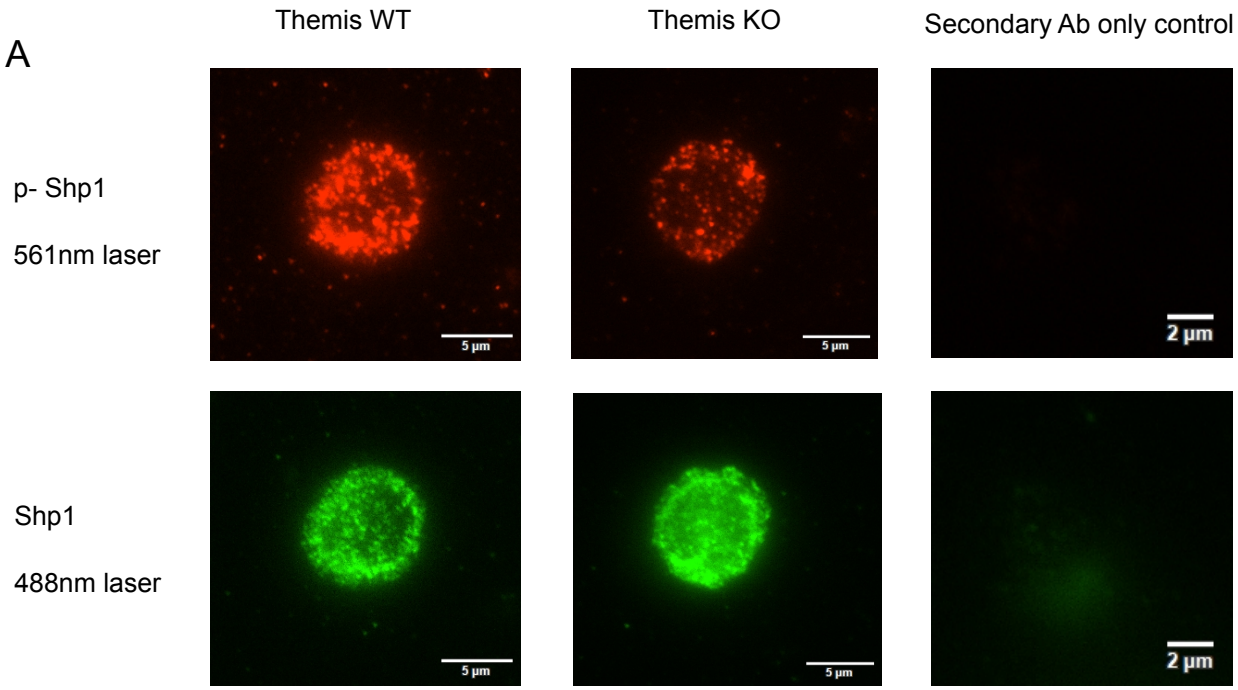
Supplementary Figure 1. Optimisation of different parameters for tyrosine phosphatase assay using immunoprecipitated Shp1. Amount of free phosphate released A) with different concentrations of phosphopeptide substrate. B) after different time of incubation for reaction to occur between Shp1 and the substrate. C) Shp1 phosphatase activity precipitated from different numbers of thymocytes.

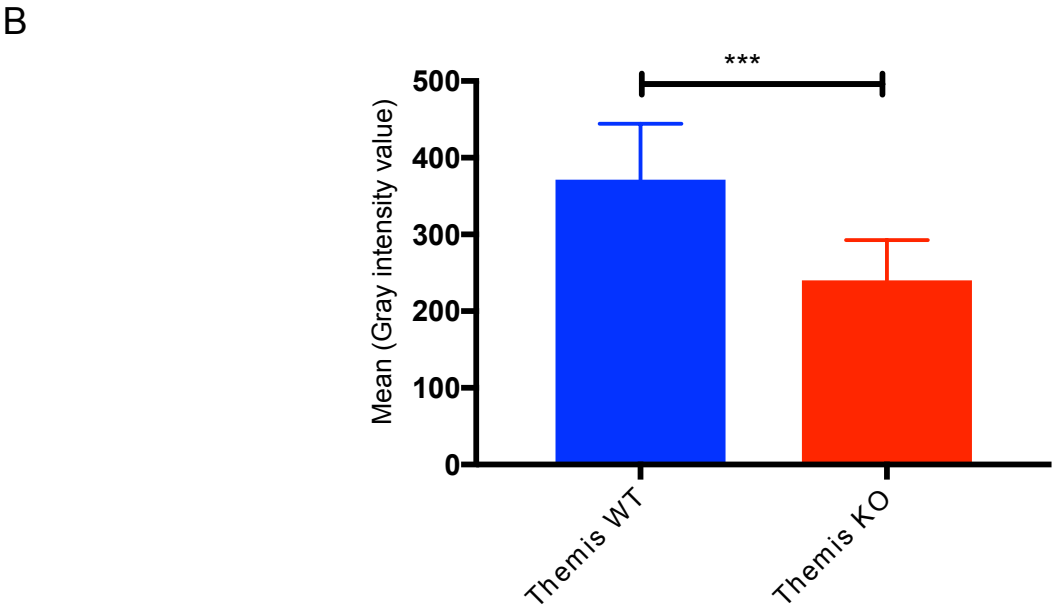


Supplementary Figure 2. More Shp1 is associated with Themis in thymus as compared to periphery. Western blot showing amount of free Shp1 once Themis is sequentially depleted from A) $5*10^6$ thymocytes from *Themis*$^{+/+}$ mice. B) $8*10^6$ sorted peripheral T cells. C) $8*10^6$ thymocytes from *Themis*$^{+/-}$ mice. Shp1 values from WCL were normalised to that of total Erk.

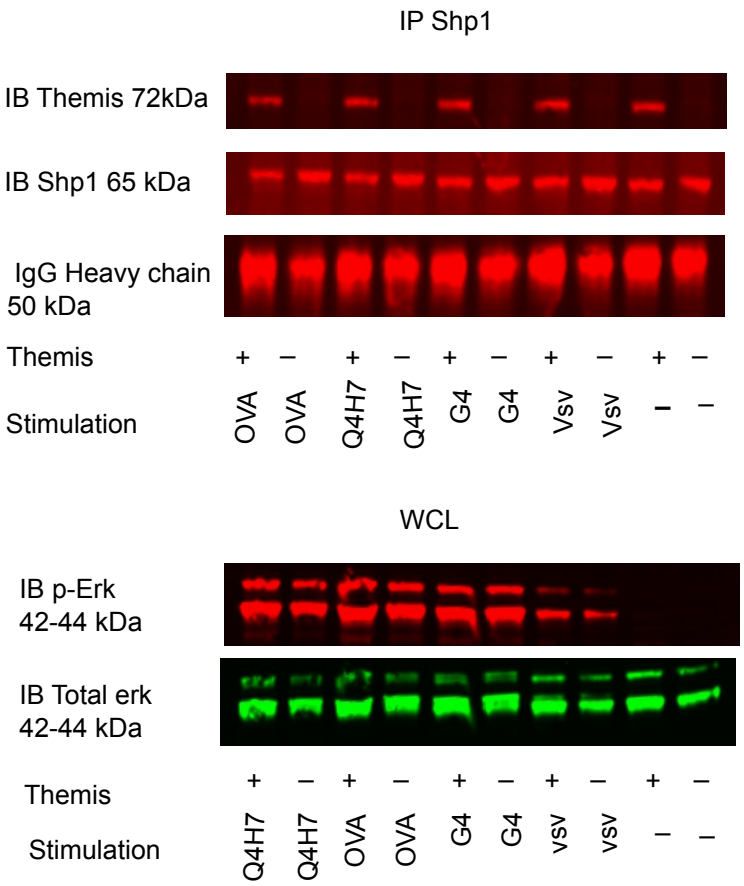


Supplementary Figure 3. p-Shp1 corresponds to its phosphatase activity in thymocytes. Western blot showing reduced p-Shp1 on specific inhibition of Shp1 with Shp inhibitors and increased p-Shp1 on inhibition with pervanadate. Data is representative of three independent experiments.

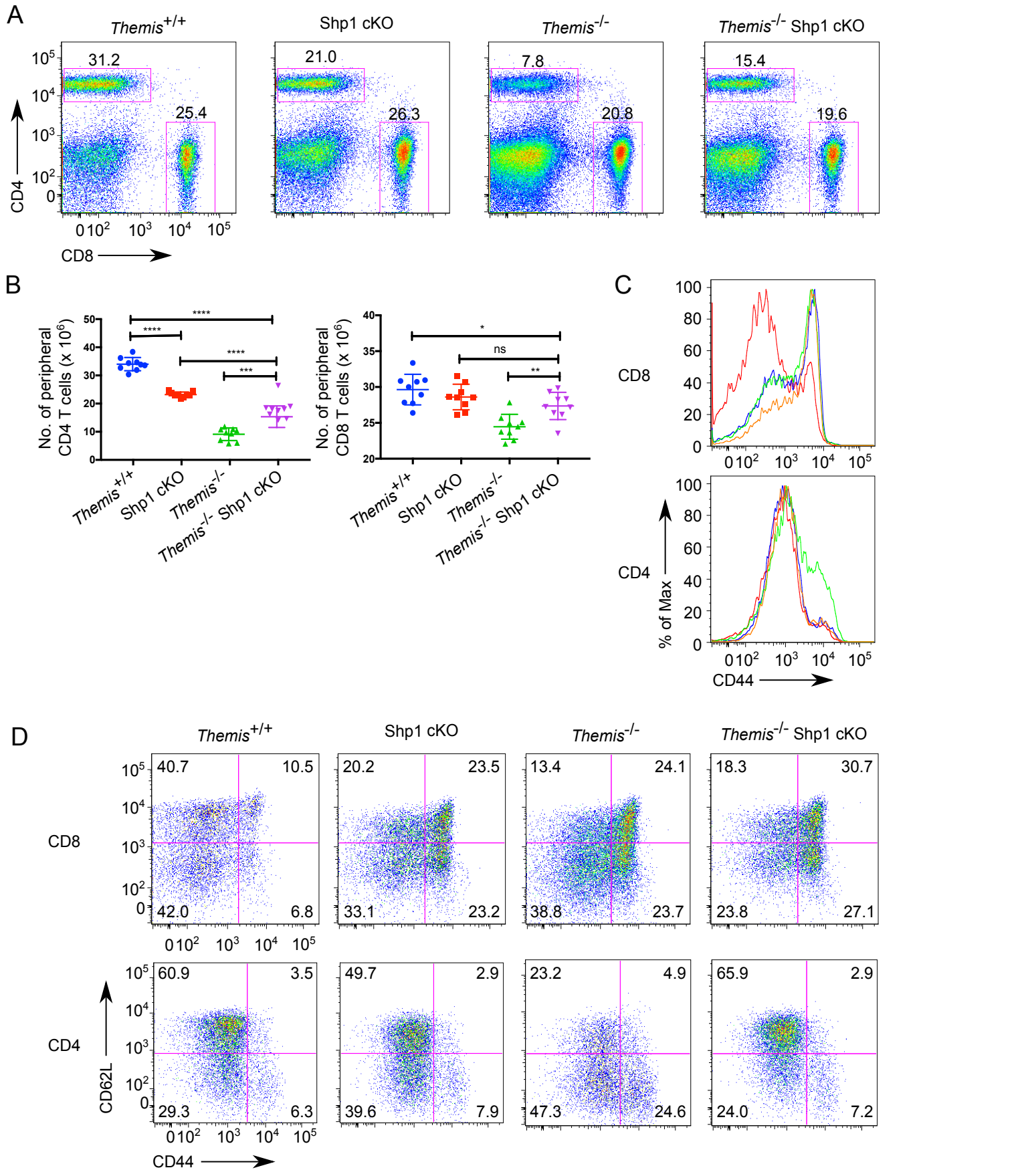




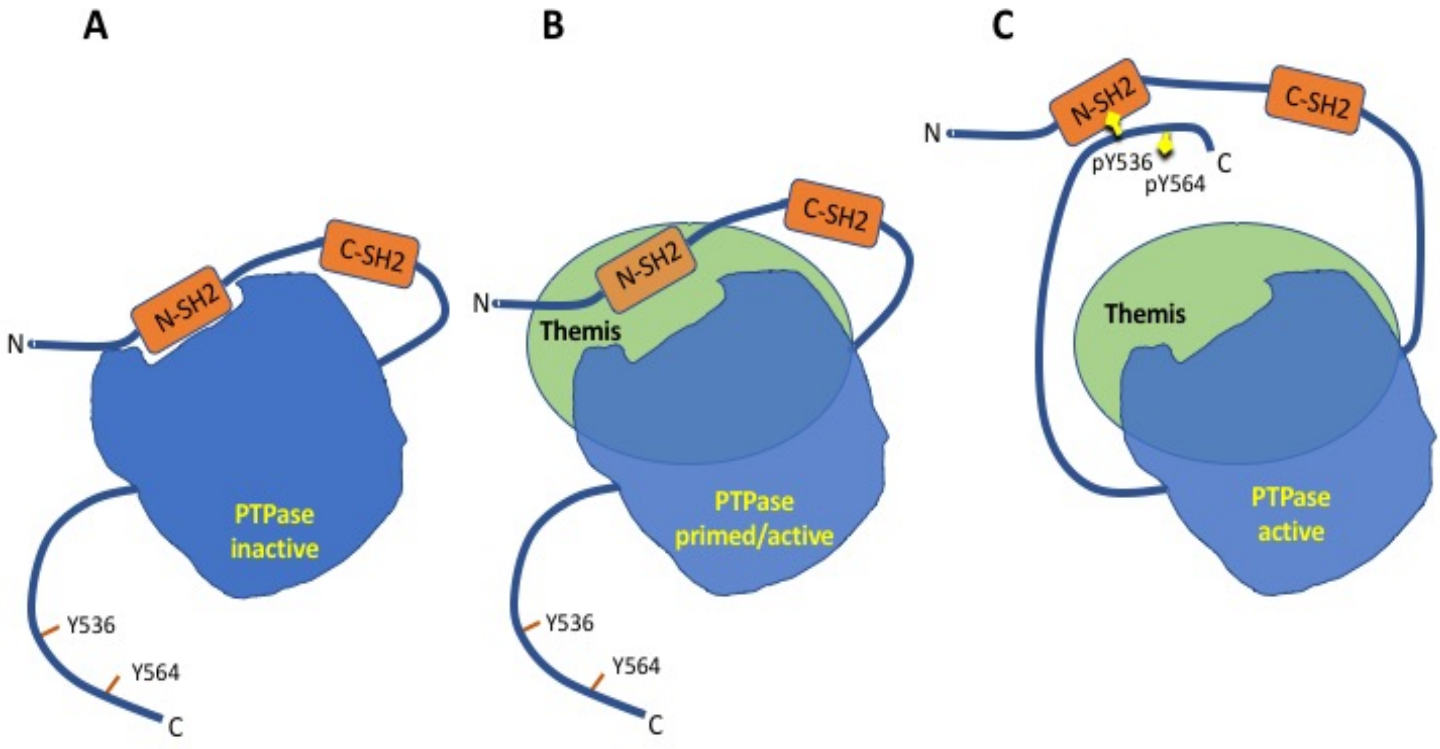
Supplementary figure 4. p-Shp1 is mainly localized at the membrane and is reduced in Themis KO. A) Representative of TIRF images of thymocytes stained with antibodies for p-Shp1 (in red) and Shp1 (in green) from OT-I *β2m*$^{-/-}$*Themis*$^{+/+}$ and OT-I *β2m*$^{-/-}$*Themis*$^{-/-}$ mice . Cells stained with secondary antibody only were used as a negative control. B) Graph showing quantification of mean intensity values of p-Shp1 from OT-I *β2m*$^{-/-}$ *Themis*$^{+/+}$and OT-I *β2m*$^{-/-}$ *Themis*$^{-/-}$ thymocytes, after subtracting the background values from negative control samples. Values were calculated using Olympus cellSens software.



Supplementary Figure 5. Amount of Themis associated with Shp1 is similar in all stimulation conditions. Western blot showing Themis associated with Shp1 in OT-I *β2m*$^{-/-}$ *Themis*$^{+/+}$ and OT-I *β2m*$^{-/-}$ *Themis* $^{-/-}$ thymocytes stimulated with different tetramers. p-Erk was used as a positive control for stimulations. Total Erk was used as a loading control.



Supplementary Figure 6. Partial rescue of peripheral phenotype of *Themis*$^{-/-}$ Shp1 cKO mice as compred to *Themis*$^{-/-}$ mice. A) Representative of flow cytometry showing percentages of CD4 and CD8 T cells in the lymph nodes of *Themis*$^{+/+}$, Shp1 ckO, *Themis*$^{-/-}$ and *Themis*$^{-/-}$ Shp1 cKO mice. B) Graphs showing enumeration of CD4 and CD8 T cells in lymph nodes of *Themis*$^{+/+}$, Shp1 cKO, *Themis*$^{-/-}$ and *Themis*$^{-/-}$ Shp1 cKO mice. C) Expression of CD44 in CD8 (upper panel) and CD4 (lower panel) T cells in lymph nodes of *Themis*$^{+/+}$ (red), Shp1 cKO (blue), *Themis*$^{-/-}$ (green) and *Themis*$^{-/-}$ Shp1 cKO (orange) mice. D) Expression of CD62L vs CD44 in CD8 (upper panel) and CD4 (lower panel) T cells of *Themis*$^{+/+}$, Shp1 cKO, *Themis*$^{-/-}$, *Themis*$^{-/-}$ Shp1 cKO mice. Representative of 5 independent experiments. 3-4 mice of same age and sex were analysed from each genotype. Unpaired t-test, *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001.



Supplementary Figure 7. Proposed model of regulation of Shp1 activity by Themis. A) The inactive form of Shp1 in the absence of Themis, with the amino terminal SH2 domain bound to PTPase domain. In this condition, Shp1 PTPase is inactive. We report in this paper that when Themis is bound to Shp1, it has the effect of B) increasing Shp1 activity and C) increasing phosphorylation. We propose that Themis might act to reduce the inhibition by N-SH2 domain or in opening the PTPase site leading to more activity of Shp1.