Supplementary Material for "PTEN-inhibition by zinc ions augments interleukin-2-mediated Akt phosphorylation"

List of the Supplementary Materials:

Supplementary Methods

In general, experiments were performed as described in the Materials and Methods section of the manuscript. Additional procedures used only for the supplementary experiments are described below.

Viability assay with propidium iodide (PI)

Cells were collected by centrifugation at 300 g for 5 min followed by resuspension in PBS containing 10 µg/ml PI (Sigma-Aldrich, Germany). After incubation for 5 min in the dark, viability was assessed using a FACSCalibur flow cytometer (Becton Dickinson, USA).

Viability assay with neutral red

Cells were seeded into a 96 micro-wellplate and cultured for 24 h. Subsequently, cells were incubated for 3 h in culture medium containing 55 µM neutral red, washed with PBS and disrupted in a mixture of water:EtOH:acetic acid (50:50:1) by gentle shaking for 20 min. Neutral red uptake was analyzed by measurement of absorbance at 540 nm with a reference wavelength of 612 nm in a Tecan Sunrise wellplate reader (Tecan, Germany).

Isolation and culture of primary human T-cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral venous blood from healthy donors by centrifugation over Ficoll-Hypaque (Biochrom, Germany) and cultured in RPMI 1640 containing 10 % heat inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. For enrichment of activated T-lymphocytes, PBMC were incubated for 2 days with 2.5 µg/mL phytohaemagglutinin (PHA). Monocytes and B cells were depleted by adherence to plastic and supernatants transferred for 4 days into fresh culture medium containing 50 U/mL IL-2, yielding T-cell populations that were >95% $CD3^+$ and >93% $CD25^+$.

Inactivation of IL-2 by KOH

IL-2 was inactivated by treatment with KOH (final concentration 0.33 M) for 15 min. KOH was neutralized by addition of equimolar HCl and the inactivated IL-2 used for incubations.

Analysis of CD data

The CD spectra depicted in figure 6D were analyzed with the DichroWeb on-line analysis platform for protein Circular Dichroism spectra (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [S1]. Two different algorithms were used to calculate the relative contributions of structural elements, based on protein reference data set No. 4 (for input data ranging from 190 to 240 nm) [S2]: CONTIN [S3] and SELCON3 [S4]. The resulting relative content of α-helix, β-sheet and turns/unstructured structural elements is shown in table S1 for both methods individually and as means of both datasets.

Supplemental References

S1 L. Whitmore, B. A. Wallace, *Nucleic Acids Res.*, 2004, **32**, W668-W673.

S2 N. Sreerama, R. W. Woody, *Anal. Biochem.*, 2000, **287**, 252-260.

S3 S. W. Provencher, J. Glockner, *Biochemistry*, 1981, **20**, 33-37.

S4 N. Sreerama, R. W. Woody, *Anal. Biochem.*, 1993, **209**, 32-44.

Supplementary Figure S1

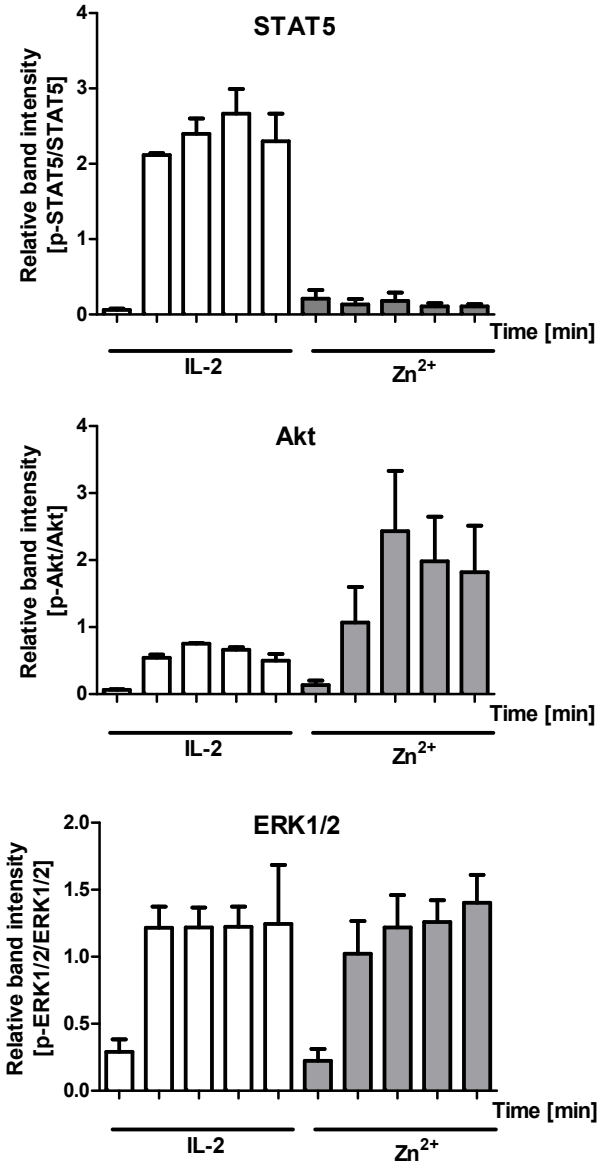

Fig. S1: Impact of IL-2 and Zn^{2+} on the phosphorylation of STAT5, Akt and ERK1/2

CTLL-2 cells were treated with IL-2 (30 U/ml) or Zn^{2+} (20 µM) and pyrithione (Pyr, 10 µM) for the indicated times and phosphorylated and total levels of STAT5, Akt, and ERK1/2 were detected by Western blot (representative blots are shown in figure 2A). Blots were analyzed using ImageJ software and data are shown as means of n=3 independent experiments +S.E.M.

Supplementary Figure S2

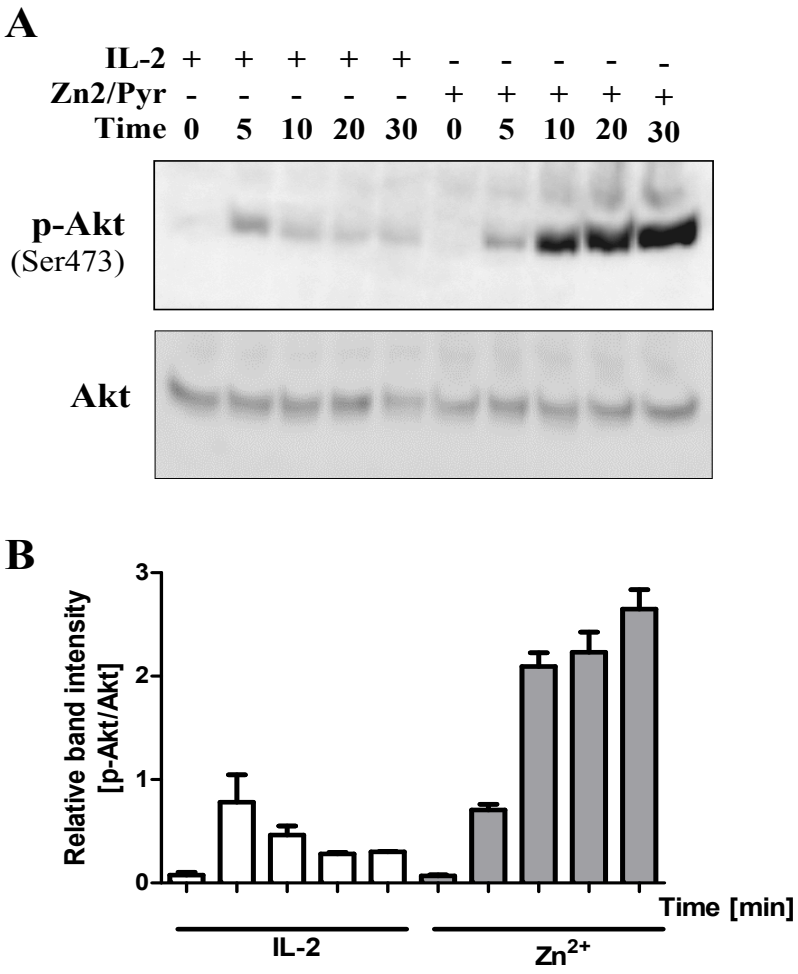

Fig. S2: Impact of IL-2 and Zn^{2+} on Akt phosphorylation in primary human T-cells

Primary human T-cells were treated with IL-2 (100 U/ml) or Zn^{2+} (20 µM) and pyrithione (Pyr, 10 µM) for the indicated times and phosphorylated and total levels of STAT5, Akt, and ERK1/2 were detected by Western blot. Data are shown (A) as representative images or (B) means + S.E.M. quantified with ImageJ software from n=3 different donors.

Supplementary Figure S3

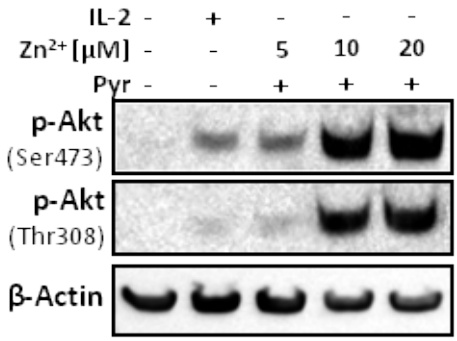

Fig. S3: Impact of Zn^{2+} on Akt phosphorylation on Ser473 and Thr308

CTLL-2 cells were stimulated for 30 minutes with IL-2 (30 U/ml) or the indicated concentrations of Zn^{2+} together with pyrithione (Pyr, 10 µM). Phosphorylated Akt (Ser473 and Thr308) and total β-Actin were assessed by Western blot. All blots are representative of n=3 independent experiments.

Supplementary Figure S4

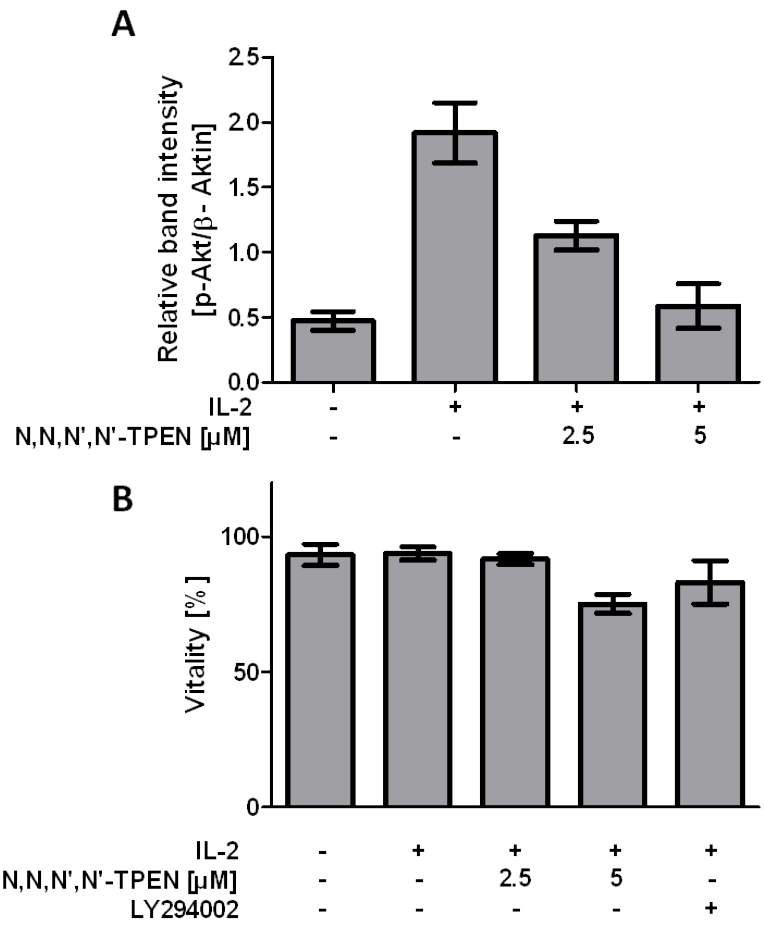

Fig. S4: Impact of N,N,N',N'-TPEN on Akt phosphorylation and CTLL-2 vitality

(A) Densitometric quantification of the Western Blots shown in figure 3A. (B) CTLL-2 cells were stimulated for 12 h with IL-2 (30 U/ml) alone, and in combination with the indicated concentrations of N,N,N',N'-TPEN or LY294002 (50 µM). Afterwards, cells were stained with 10 µg/ml PI for 5 min in the dark before vitality was assessed by flow cytometry. All data show means ± SEM and are representative of n=3 independent experiments.

Supplementary Figure S5

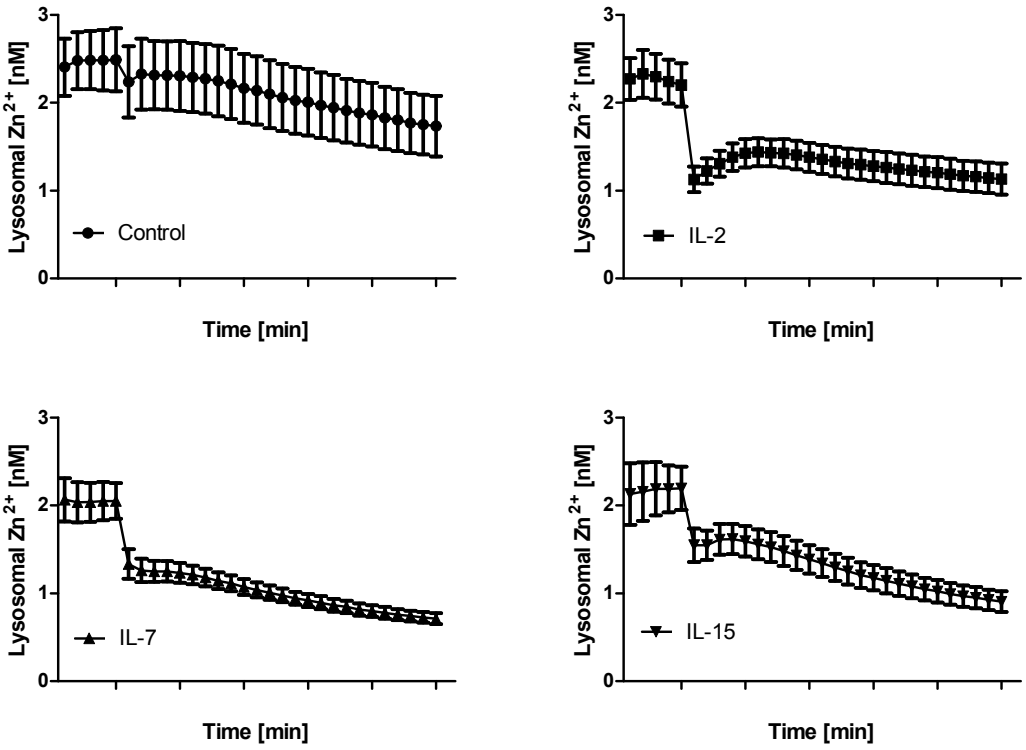

Fig. S5: Release of lysosomal Zn^{2+} in response to IL-2, -7, and -15

Primary human T-cells were loaded with FluoZin-3 to measure the lysosomal free Zn^{2+} concentration. After 10 minutes measuring the baseline fluorescence, IL-2, -7, or -15 (100 U/ml) were added. Data are shown as means from n=4 different donors ± SEM.

Supplementary Figure S6

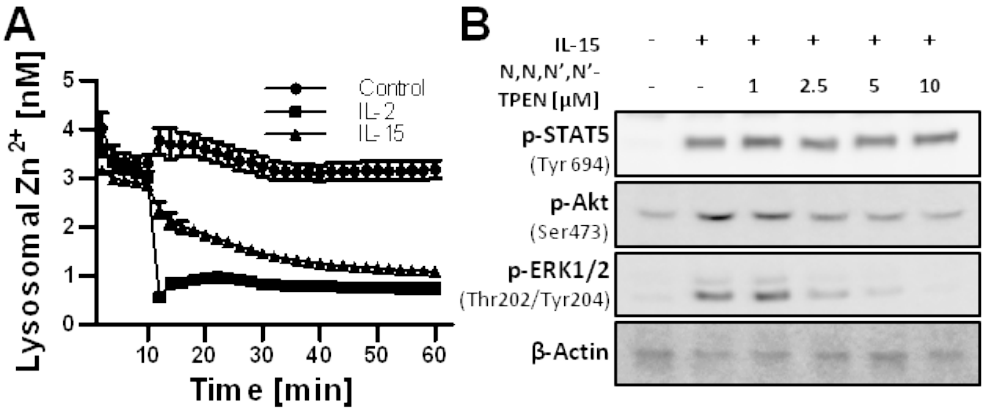

Fig. S6: Role of free Zn^{2+} in IL-15-dependent signal transduction.

(A) CTLL-2 cells were loaded with FluoZin-3. After 10 minutes measuring the baseline fluorescence, IL-2 or -15 (100 U/ml) were added. Data are shown as means from triplicates ± SEM. (B) CTLL-2 cells were pre-treated with N,N,N',N'-TPEN (30 min), followed by stimulation with IL-15 (100 U/ml, 30 min). Western blot was performed to analyze STAT5, Akt, and ERK1/2 phosphorylation and total β-Actin expression. (A,B) All data are representative of n=3 independent experiments.

Supplementary Figure S7

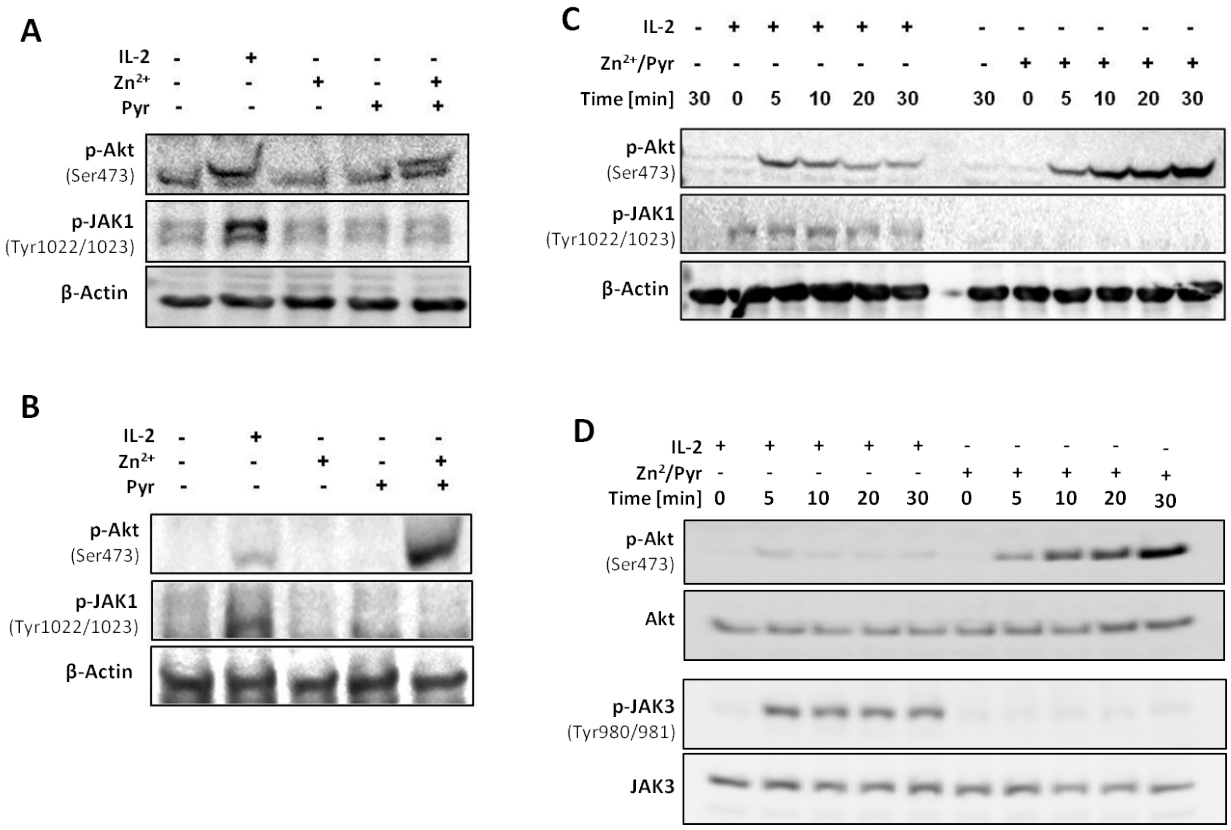

Fig. S7: Effect of Zn^{2+}/pyrithione on p-Akt and p-JAK levels

(A) CTLL-2 cells were treated with IL-2 (30 U/ml), Zn^{2+} (20 µM), and pyrithione (Pyr, 10 µM) for 30 minutes. Phosphorylated Akt (Ser473) and JAK (Tyr1022/1023) as well as total β-Actin were analyzed by Western blot. (B,C,D) Primary human T-cells were cultured without IL-2 for 16 h. Cells were then incubated as indicated with IL-2 (50 U/ml), Zn^{2+} (20 µM) and pyrithione (Pyr, 10 µM), either for 20 minutes (B), or for different times between 0 and 30 min (C,D). Western blot analysis was used to detect phosphorylated Akt (Ser473), phosphorylated JAK1 (Tyr1022/1023), phosphorylated JAK3 (Tyr980/981), and total Akt, JAK3, or β-Actin. Data are representative of independent experiments with cells from n=3 different donors.

Supplementary Figure S8

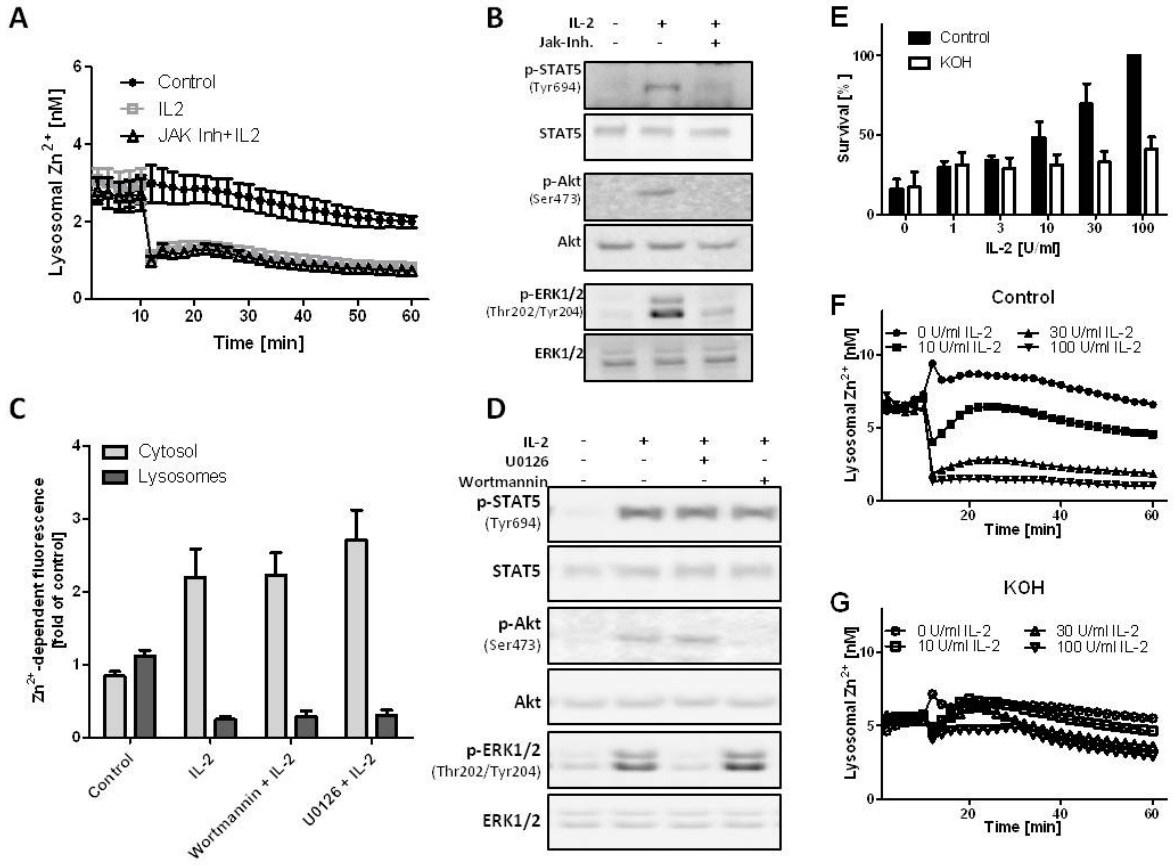

Fig. S8: Effect of inhibitors and IL-2 inactivation on lysosomal Zn^{2+} release and cellular viability

(A,B) After pre-treatment with Jak-inhibitor (300 nM, 30 min), CTLL-2 cells were used to (A) analyze phosphorylated and total levels of STAT5, Akt, and ERK1/2 by Western blot after incubation with IL-2 (100 U/ml, 30 min), or (B) measure free Zn^{2+} with FluoZin-3. After 10 minutes measuring the baseline fluorescence, IL-2 (100 U/ml) was added. (C,D) CTLL-2 cells were pre-treated with Wortmannin (200 nM) or U0126 (10 µM) for 30 minutes as indicated. Subsequently, cells were stimulated with IL-2 (30 U/ml). (C) Phosphorylated and total levels of STAT5, Akt, and ERK1/2 were analyzed by Western blot, and (D) free Zn^{2+} by measuring the fluorescence of cells loaded with Zinquin (cytosolic zinc) or FluoZin-3 (lysosomal zinc). All data are shown as representative blots or means of at least n=3 independent experiments. (E) CTLL-2 cells were cultured for 24 h with functional IL-2 or KOH-inactivated IL-2. Survival was assessed by measuring neutral red uptake. Data were calculated as percent of viability at 100 U/ml functional IL-2 (F,G) CTLL-2 cells were loaded with FluoZin-3. After 10 minutes measuring the baseline fluorescence, the indicated concentrations of functional IL-2 (F) or KOH-inactivated IL-2 (G) were added. Data are shown as means + SEM (C,E) or one representative (A,F,G) of n=3 independent experiments. All Western blots in this figure are representative of at least n=3 independent experiments.

Supplementary Figure S9

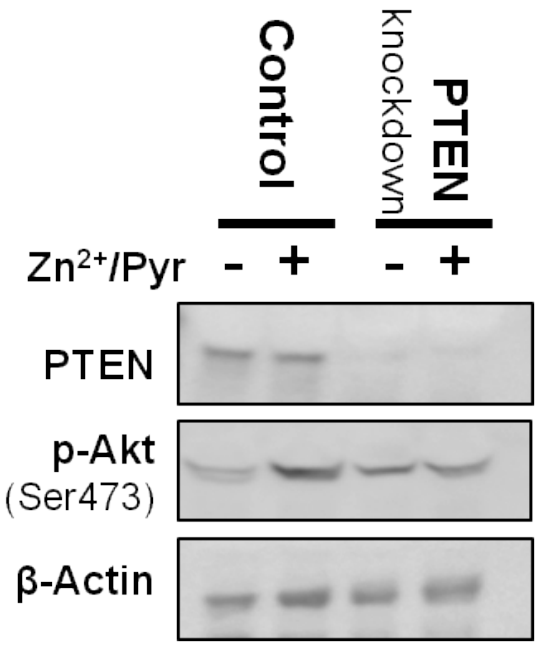

Fig. S9: siRNA knockdown of PTEN

72 h post transfection with siRNA, HeLa cells were serum-starved for 4 hours followed by stimulation with Zn^{2+} (20 µM) in the presence of pyrithione (Pyr, 10 µM) for 20 minutes. Western blot analysis was performed with antibodies against total PTEN, phosphorylated Akt (Ser473) and total β-Actin. Representative blots for n=5 independent experiments are shown.

Supplementary Figure S10

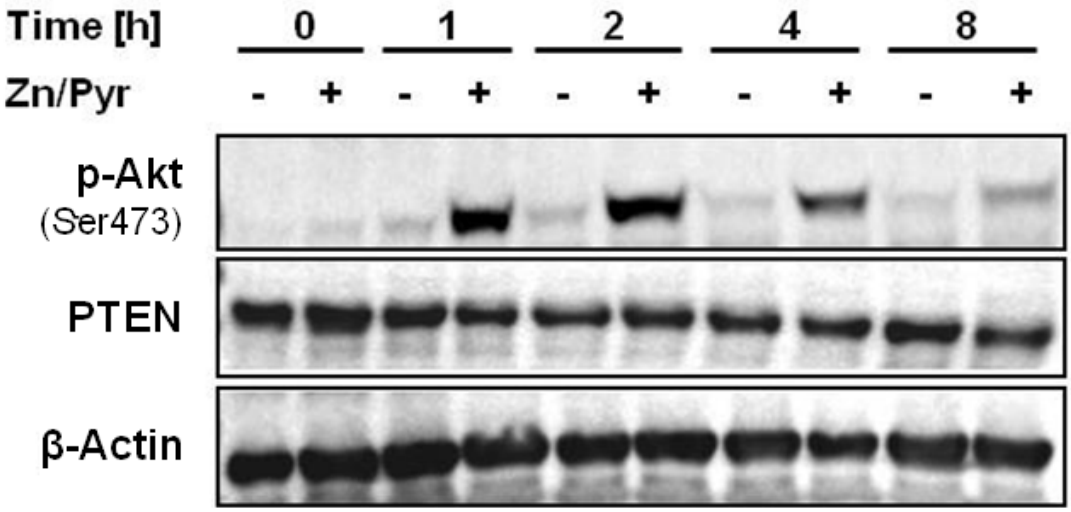

Fig. S10: Impact of Zn^{2+}/pyrithione on PTEN degradation

Primary human T-cells were cultured for 16 h without IL-2, followed by stimulation with Zn^{2+} (20 µM) and pyrithione (Pyr, 10 µM) for the times indicated. Protein levels of phosphorylated Akt (Ser473), total PTEN and β-Actin were assessed by Western blot. Blots are representative of independent experiments with cells from n=3 different donors.

Supplementary Figure S11

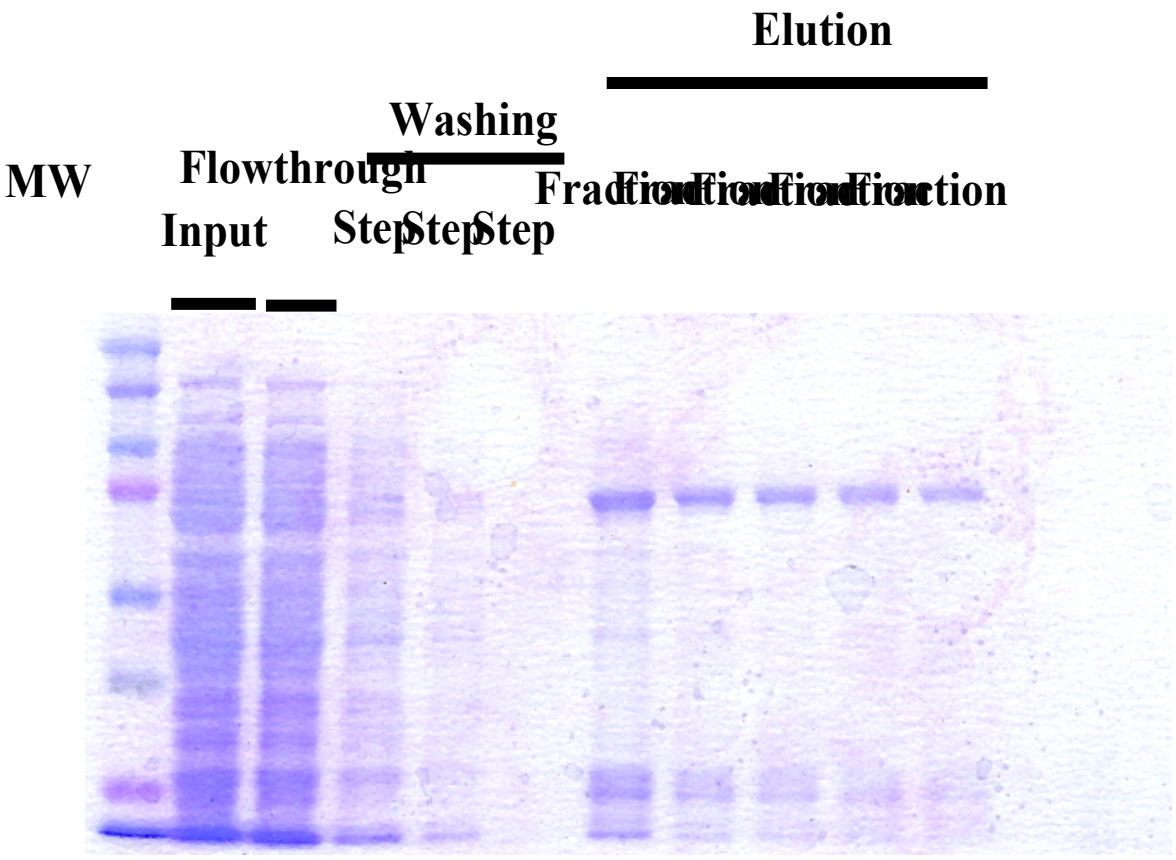

Fig. S11: Purification of recombinant PTEN

Coomassie blue stained SDS-PAGE, showing purity of PTEN preparations after each step of the purification process.

Table S1: Calculated relative contribution of structural elements to the CD spectra shown in fig. 6D

		w.t.			Cys124Ser			Cys71Ser		
		α-helix	β-strand	turns/ unordered	α-helix	β-strand	turns/ unordered	α-helix	β-strand	turns/ unordered
Control	SELCON3	0.356	0.114	0.571	0.376	0.188	0.475	0.333	0.200	0.502
	CONTIN	0.465	0.398	0.137	0.313	0.034	0.653	0.207	0.048	0.746
	Average	**0.411**	**0.256**	**0.354**	**0.345**	**0.111**	**0.564**	**0.270**	**0.124**	**0.624**
Zn^{2+}	SELCON3	0.764	0.013	0.255	0.311	0.216	0.503	0.273	0.208	0.562
	CONTIN	0.652	0.111	0.237	0.428	0.048	0.524	0.224	0.024	0.752
	Average	**0.708**	**0.062**	**0.246**	**0.370**	**0.132**	**0.514**	**0.249**	**0.116**	**0.657**
H_2O_2	SELCON3	0.744	0.015	0.283	0.330	0.204	0.510	0.288	0.206	0.549
	CONTIN	0.758	0.068	0.173	0.356	0.045	0.600	0.311	0.037	0.652
	Average	**0.751**	**0.042**	**0.228**	**0.343**	**0.125**	**0.555**	**0.300**	**0.122**	**0.601**

Table S2: Properties of cells used in this study

Name	Cell type	Origin	PTEN expression	SHIP1 expression
CTLL-2	T lymphocyte	mu	+	+
HUT-78	Cutaneous T lymphocyte	hu	+	+
Jurkat	T lymphocyte	hu	-	-
Molt-4	T lymphoblast	hu	-	+
HeLa	Epithelial adenocarcinoma	hu	+	n. d.
Primary T- cells	T-cells	hu	+	+
SHIP1 $^{+/+}$	Thymocytes	mu	+	+
SHIP1 $^{-/-}$	Thymocytes	mu	+	-

hu, human; mu, murine; n.d., not determined