Supplementary Information

- Supplementary Experimental Procedures
- Supplementary Tables: Captions
- Supplementary Figures

Supplementary Experimental Procedures

SILAC of primary macrophages – splitting procedure

To split whole cultures, consisting of non-adherent progenitor cells and adherent cells that developed under the influence of M-CSF, the following procedure was applied: Non-adherent cells were collected in 250 ml cell culture centrifuge beakers and placed on ice. Adherent cells were detached with accutase (PAA laboratories) diluted 1:4 in sterile PBS (4 ml per 10 cm plate, 37 °C, 15 min). After dilution of accutase with 4 ml PBS cells were collected with a cell scraper in a separate cell centrifuge beaker and plates were washed with 4 ml sterile PBS to collect as many cells as possible. Detached cells were spun down (Sorvall RC 26Plus, SLA-1500, 300g, 4°C, 10 min) and re-suspended in the medium with non-adherent cells plus fresh SILAC medium with cytokines and M-CSF for re-plating. Whole cultures were split 1:2 or higher, depending on cell density. On day 13 all cells were deprived of medium to remove cytokines before re-plating in medium with 10 % LCCM.

Reduction and alkylation of protein lysates

Mixed protein lysates were reduced at RT under shaking (1200 rpm) for 30 min by addition of 1 M dithiothreitol (DTT) to a final concentration of 1 mM DTT and then alkylated for 30 min in the dark by addition of 550 mM iodoacetamide (IAA-) solution to final 5.5 mM.

In-solution digest of proteins

After reduction and alkylation 20 ng endoproteinase Lys-C (Wako) per µg protein were added and the lysates were digested at RT under shaking (1200 rpm) for 4 h. The resulting peptide mixtures were diluted with water to achieve a final urea concentration below 2 M. For double-digestion, 20 ng sequencing grade modified trypsin (Promega) per µg of protein were added

and the mixtures were shaken at RT overnight. Trypsin activity was quenched by acidification using tri-fluoro acetic acid (TFA) to a final concentration of 1 %.

In-gel digest of in-soluble protein fraction

Proteins from the in-soluble chromatin pellet including transcription factors were extracted by DNA digest with benzonase (Merck) and re-solubilisation in 8 M urea followed by incubation with SDS-loading dye under rotation at 95 °C for 5 min. Proteins were reduced and alkylated as described above, resolved by SDS-PAGE on a gradient gel (4-15 % Tris-HCl Ready Gel Precast Gel, Bio-Rad) and stained with Coomassie. Each lane was loaded with 40 µg of total protein and cut into 5 slices containing equal protein amounts. Enzymatic digestion in-situ was performed essentially as described (Shevchenko et al., 2006). Briefly, gel slices were minced, de-stained (20 mM ammonium hydrogen carbonate / 50 % ethanol absolute, 1200 rpm, 3 x 20 min), de-hydrated (ethanol absolute, 1200 rpm, 2 x 10 min) and digested by saturating the gel with 12.5 ng/µL sequencing grade modified trypsin (Promega) in 20 mM ammonium bicarbonate (500 rpm, overnight). Peptide mixtures were acidified with tri-fluoro acetic acid (TFA, final concentration 3 %) and extracted from the gel in 3 rounds (30 % acetonitrile (ACN) / 3 % TFA, 80 % ACN / 0.05 % acetic acid, 100 % ACN; 30 min, 800 rpm, take supernatant).

Strong cation exchange (SCX) chromatography of digests

Trifluoracetic acid (TFA) was added to peptide mixtures to adjust their pH to 2. If conductivity of the samples was too high, samples were desalted on C18 cartridges (PhosphoScan Kit, Cell Signalling) according to the manufacturer's instructions. 10 mg per sample were loaded onto a 1 ml resource S column (Pharmacia) equilibrated with 0.1 % TFA, 30 % acetonitrile and 5 mM potassium dihydrogen phosphate using an ÄKTA Purifier chromatography system (Amersham Biosciences) with a fraction collector. The peptides were separated by a linear gradient of potassium chloride from 0 to 350 mM with a flow rate of 1 ml/min. 17 peptide

collections were collected including the flow through. Adjacent fractions were combined to a total of 11 fractions for enrichment of phosphopeptides.

Titansphere enrichment of phosphopeptides

Phosphopeptides from each fraction were enriched by using Titansphere-chromatography (TiO_2) columns as described (Olsen et al., Cell 2006) with minor modifications.

Acidified peptide fractions (pH below 2) were incubated with approximately 5 µL Titansphere-material (GL Sciences, Japan) in 0.5 g/L 2,5-dihydrobenzoic acid (DHB) / 80 % acetonitrile (ACN) / 0.1 % tri-fluoro acetic acid (TFA) under rotation for 30 min. Beads were washed once with 100 µL 40 % ACN / 0.1 % TFA and twice with 80 % ACN / 0.1 % TFA and transferred home-made C8-Stage Tips in 200 µL pipet-tips. The columns were washed once with more with 80 % ACN / 0.1 % TFA. Phosphopeptides were eluted from the TiO_2-C8-Stage Tips into a 96-well plate with 2x 20 L of 40 % acetonitrile in 15 % ammonia-water solution (pH~11) and dried to 2 µL in a speed-vac. The dried phosphopeptide mixtures were acidified with 5 % acetonitrile in 0.3 % tri-fluoro acetic acid (TFA) to an end volume of 8 µL.

Mass spectrometric analysis

Phosphopeptide mixtures were analysed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Olsen et al., 2006) with a few modifications. Briefly, all nanoLC-MS/MS-experiments were performed on an EASY-nLC™ system (Proxeon Biosystems, Odense, Denmark) connected to the LTQ-Orbitrap XL (Thermo Electron, Bremen, Germany) through a nanoelectrospray ion source. The phosphopeptides were auto-sampled directly onto the 15 cm long 75 µm-inner diameter (i.d.) analytical column packed with reversed-phase C18 Reprosil AQUA-Pur 3 µm particles at a flow rate of 500 nl/min. The flow rate was reduced to 250 nl/min after loading, and the phosphopeptides were separated with a linear gradient of acetonitrile from 5-40 % in 0.5 % acetic acid for 100 min. The effluent from the column was directly electrosprayed into the mass spectrometer.

The LTQ Orbitrap XL instrument under Xcalibur 2.0 was operated in the data dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300 – 2000) were acquired in the orbitrap with resolution R=60,000 at m/z 400 (after accumulation to a 'target value' of 1,000,000 in the linear ion trap). The ten most intense multiply-charged ions ($z \geq 2$) were sequentially isolated and fragmented in the linear ion trap by collisionally induced dissociation (CID) at a target value of 5,000 or a maximum ion time of 150 ms. All tandem mass spectra were acquired with the multi-stage activation (MSA) option enabled for neutral losses of m/z 32.66, 48.99 and 97.97. For all full scan measurements in the orbitrap detector a lock-mass ion from ambient air (m/z 445.120025) was used for internal calibration as described earlier (Olsen et al., 2005). Typical mass spectrometric conditions were: spray voltage, 2.2 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200 °C; normalized CID collision energy 40 % for MSA in LTQ. The ion selection threshold was set to 100 counts for MS/MS. An activation q = 0.25 and activation time of 30 ms for MSA acquisition were used.

Assigning peptide sequences using MASCOT and MaxQuant

Raw Orbitrap full-scan MS and ion trap MSA spectra were processed by MaxQuant as described (Olsen et al., 2006). In brief, all identified SILAC triplets were quantified, accurate precursor masses determined using the entire LC elution profiles and MS/MS spectra were merged into peak-list files (*.msm), and searched against the mouse IPI protein database version 3.37. Peptides and proteins were identified by Mascot (Matrix Science, London, UK) via automated database matching of all tandem mass spectra against an in-house curated concatenated target/decoy database; a forward and reversed version of the mouse International Protein Index (IPI) sequence database (version 3.37; 102,934 forward and reversed protein sequences from EBI (http://www.ebi.ac.uk/IPI/)) supplemented with common contaminants such as human keratins, bovine serum proteins and porcine trypsin. Tandem mass spectra were initially matched with a mass tolerance of 7 ppm on precursor masses and 0.5 Da for fragment ions, and strict trypsin specificity and allowing for up to 3 missed tryptic cleavage sites. Cysteine carbamidomethylation (Cys +57.021464 Da) was searched

as a fixed modification, whereas N-acetylation of protein (N-term +42.010565 Da), N-pyro-glutamine (Gln -17.026549), oxidized methionine (+15.994915 Da) and phosphorylation of serine, threonine and tyrosine (Ser/Thr/Tyr +79.966331 Da) were searched as variable modifications.

Analysis of assigned peptide sequences

The resulting Mascot result files (*.dat) were loaded into the MaxQuant software for further processing. In MaxQuant we fixed the estimated false discovery rate (FDR) of all peptide and protein identifications at 1 %, by automatically filtering on peptide length, mass error and Mascot score of all forward and reversed peptide identifications. Finally, to pinpoint the actual phosphorylated amino acid residue(s) within all identified phosphopeptide sequences in an unbiased manner, we calculated the localization probabilities of all putative serine, threonine and tyrosine phosphorylation sites using the PTM score algorithm as described (Olsen et al., 2006). Quantification of phosphorylation sites was done by MaxQuant software (Olsen et al., 2006). Data were normalised such that the median log-transformed ratio of all peptides identified were zero, to correct for unequal sample mixing. Phosphopeptide ratios referring to un-stimulated wild type were calculated for each genotype and time point.

Exclusion of contaminating proteins

Samples can be contaminated with FCS proteins during cell culture or with human keratins during sample preparation. Exclusion of contaminating proteins is especially important for the analysis of down-regulated phosphorylation sites, since contaminating proteins do not contain labelled amino acids and are therefore often characterized by very low SILAC-ratios in all conditions. Most contaminating proteins were identified via an in-house curated database of contaminating proteins (human keratins, bovine serum proteins and porcine trypsin). Furthermore, phosphorylation sites with SILAC-ratios ≤ 0.3 in all conditions were manually checked for keratins or secreted proteins using PubMed Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed). For proteins with unclear annotation,

homology of the identified peptide sequences with bovine and murine proteins was analysed using NCBI-BLAST2 (http://www.ebi.ac.uk/Tools/blastall/). If the BLAST scores for murine and bovine proteins were equal, the protein was regarded as contaminant. Phosphorylation sites on contaminating proteins were removed from the dataset.

Metabolic labelling, purification and analysis of nascent RNA

Metabolic labelling and purification of nascent RNA were performed essentially as described (Dolken et al., 2008), with minor modifications for use with primary macrophages. In brief, 4 x 10^7 bone marrow derived macrophages differentiated under the same conditions as for the phosphoproteome analysis (except SILAC) were stimulated on 15 cm dishes with 100 ng/ml LPS *Escherichia coli* (Sigma Aldrich) for 45 min or 4.5 h or were left untreated. For metabolic labelling, the medium was supplemented with 200 µM 4-thiouridine (4sU, Sigma Aldrich, Cat. No. T4509) during the last 35 min of stimulation. Cells were lysed by addition of 10 ml Trifast (PeqLab, Germany) and total RNA was extracted. Biotinylation of 4sU labeled RNA was performed in a total volume of 1 ml containing 120 µg RNA, 10 mM Tris (pH 7.4), 1 mM EDTA and 0.2 mg/ml Biotin-HPDP (Pierce) by rotation at RT for 90 min, followed by two rounds of phenol-chloroform extraction and precipitation. For separation of biotinylated nascent and pre-existing unlabelled RNA paramagnetic streptavidin-coated beads and MACS columns were used (Miltenyi, Germany). RNA and beads were mixed and incubated at RT for 15 min, transferred to the columns and washed extensively with washing buffer. The flow through and the first wash volume were collected for recovery of unlabelled, pre-existing RNA. Labelled RNA was eluted with two rounds of 100 µl DTT (100 mM) into buffer RLT (Qiagen, Germany) and cleaned up using RNeasy MinElute Spin Columns. Concentration and integrity of total and labelled RNA were determined by spectrophotometry (Nanodrop) and Experion automated electrophoresis system (Biorad, Munich, Germany).

Microarrays

To investigate the changes in nascent and total mRNA after LPS stimulation of macrophages, RNA samples from two independent experiments were processed and hybridised to Affymetrix Mouse Gene ST 1.0 GeneChips according to the manufacturer's protocols. In brief, 200 ng total RNA and 100 ng nascent RNA were reverse transcribed introducing by random priming a T7-binding site into the cDNA that allows *in vitro* transcription. The resulting cRNA was subjected to a second round of random primed cDNA synthesis in the presence of dUTP, that allow fragmentation of the cDNA with uracil DNA glycosylase and apurinic/apyrimidinic endonuclease 1. Biotinylation of the fragmented cDNA was accomplished by incubation with Terminal Deoxynucleotidyltransferase (TdT). 5 µg of biotinylated DNA were hybridized to Mouse Gene ST 1.0 GeneChips overnight, followed by washing and staining procedures, and scanning, following Affymetrix protocols. For generation of probe set expression values, CEL files containing probe level data were normalized using RMA (Affymetrix Expression Console). Microarray data have been deposited in Gene Expression Omnibus and will be publicly accessible after acceptance of the paper.

Definition of genes expressed in macrophages

Genes expressed in macrophages were defined from our previously published genome-wide analysis of LPS tolerance in macrophages (Mages, 2007) (dataset is available in Gene Expression Omnibus database, identifier GSE8621). All genes represented by at least one probe set with present-calls on at least 2 of 12 microarray samples and a normalised expression value of at least 80 were considered as expressed in macrophages.

Pharmacological inhibitors

The following table lists all chemicals that were used for inhibition of kinases and pathways in LPS-stimulated macrophages at the indicated concentrations. The results depicted in Fig. 4B were obtained using the higher concentration of inhibitors.

Kinase/Pathway	***Inhibitor***	***Supplier Cat. No.***	***Solvent***	***Stock concentration***	***Final Concentration***
MEK	PD98059	Sigma	DMSO	10 mM	10 µM 1 µM
Akt	Akt Inhibitor VIII	Calbiochem 124018	DMSO	10 mM	10 µM 1 µM
mTOR	Rapamycin	Calbiochem 553210	DMSO	100 µM	100 nM 10 nM
PI3K	LY294002	Calbiochem 440202	DMSO	50 mM	20 µM 2 µM
ATM	ATM Kinase Inhibitor	Calbiochem 118500	DMSO	5 mM	10 µM 1 µM
F-Actin	Cytochalasin D	Calbiochem	DMSO	4 mM	10 µM 1 µM
Camk2	KN93	Calbiochem 422708	DMSO	10 mM	10 µM 1 µM
Rho	tatC3	Tebu-bio 027CT04-A	50% Glycerol	200 µg/ml	2 µg/ml 0.5 µg/ml
PKD	Gö6976	Calbiochem 365250	DMSO	6.6 mM	2.3 nM

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was reverse transcribed using random primers. Real-time PCR was carried out on an ABI 7900 system in 384-well plates employing the Roche Universal Probe Library primer and probe combinations. Expression of the housekeeping gene Hprt was used for normalization and untreated samples were used as calibrators, applying the ΔΔCT method for calculation of fold changes.

siRNA transfection of primary macrophages

For efficient knockdown of gene expression in primary macrophages, a recently published protocol was employed (Wiese JIM 2009). In brief, macrophages were differentiated from

C57BL/6 bone marrow cells in M-CSF containing medium in hydrophobic Teflon bags for seven days. 2 x 10^6 macrophages were electroporated in 100 µl OptiMEM with 0.4 nmol Dharmacon On-Target plus SMART pool siRNA. After electroporation, cells were distributed onto four wells of a 24-well plate, incubated for 30 minutes in DMEM alone, followed by addition of FCS and antibiotics. 24 and 48 h after electroporation, macrophages were used for stimulation with LPS.

nCounter expression analysis

5 x 10^5 macrophages were lysed in 450 µl RLT buffer (RNeasy kit, Qiagen). Aliquots of 100 µl were set aside for nCounter expression analysis .

Supplementary Tables: Captions

Tab. S1. Reproducibly identified macrophage phosphorylation sites.

See Excel spreadsheet.

Tab. S2. Phosphorylation sites on known TLR signalling molecules.

See Excel spreadsheet.

Tab. S3. LPS-regulated phosphoproteins associated with enriched Gene Ontology terms.

See Excel spreadsheet.

Tab. S4. Phosphorylation sites on transcription factors with binding site enrichment in LPS-regulated promoters.

See Excel spreadsheet.

Amino acid sequences of murine TFs and available orthologs from other species were derived from Uniprot (http://www.uniprot.org), aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2) and analysed for evolutionary conservation of amino acid residues that showed LPS-regulated phosphorylation in the phosphoproteome study.

Fig. S1

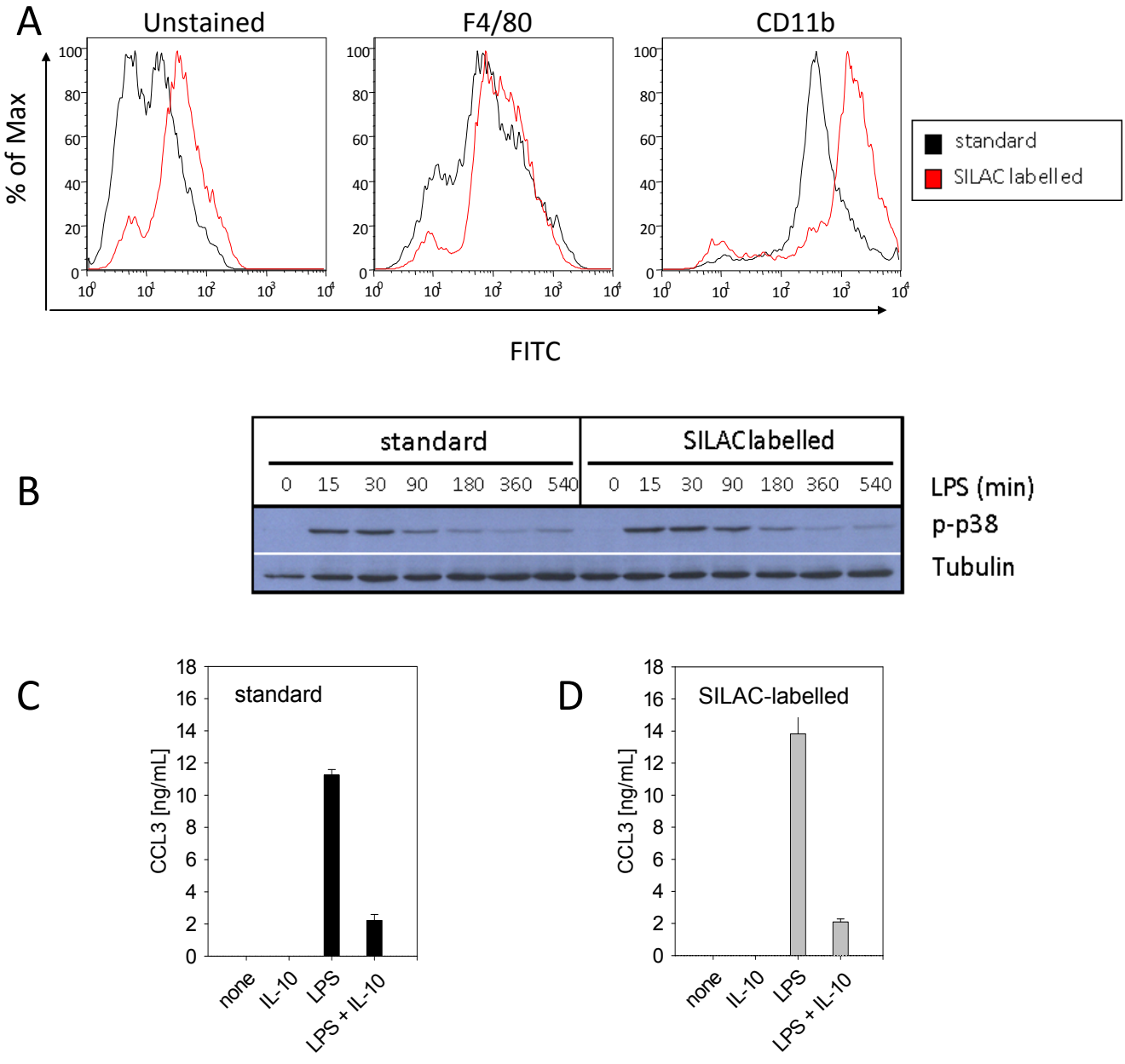


Fig. S1. Quality control of macrophages obtained by the SILAC-adapted protocol.

(A) Surface marker expression of macrophages differentiated with the normal or SILAC-adapted protocols, as analysed by flow cytometry. Note that SILAC-macrophages have increased autofluorescence. (B) Westernblot for phosphorylation of p38 MAPK after stimulation with 100 ng/mL LPS for the indicated times. (C) Cytokine expression in response to overnight stimulation with 100 ng/mL LPS and/or 5 ng/mL IL-10 was measured by ELISA (mean ± SD from two technical replicates). Data are representative for two independent experiments.

Fig. S2

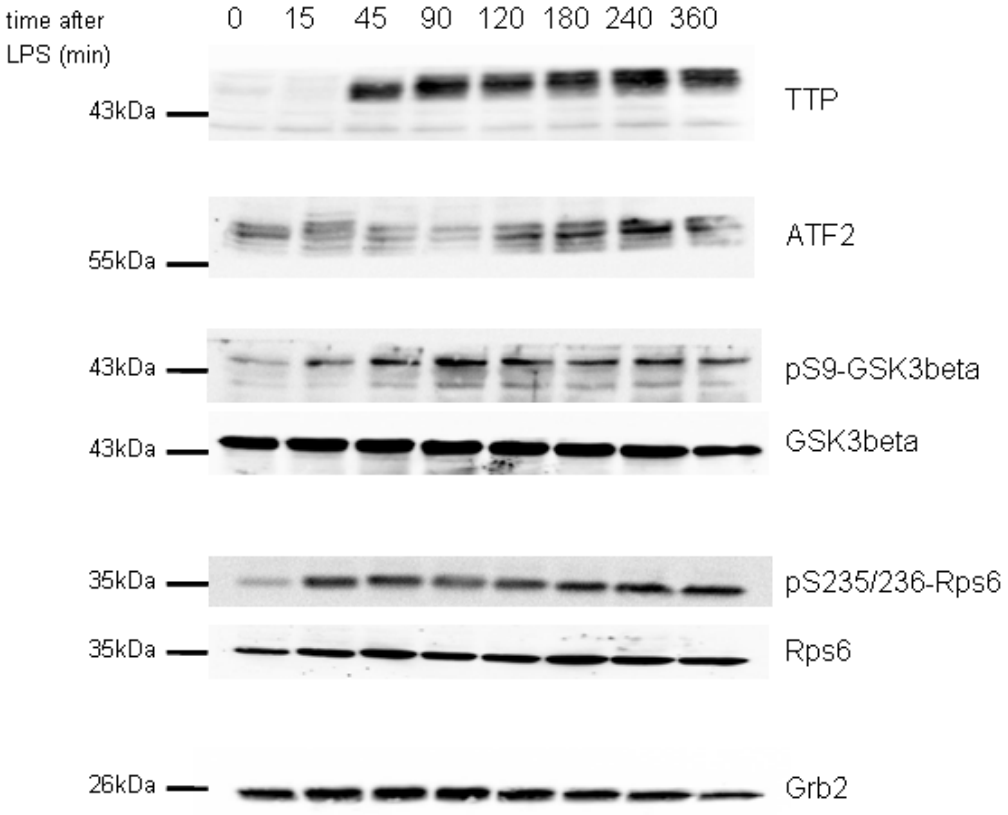


Fig. S2.Validation of phosphorylation of selected proteins by Western blot. Bone marrow derived macrophages were stimulated with LPS for the indicated times. Phosphorylation of Gsk3b and Rps6 was analysed using antibodies for specific phosphosites as indicated. Antibodies to TTP (=Zfp36) and ATF2 detect changes in molecular weight indicative of the extensive phosphorylation on multiple sites after LPS that was detected by mass spectrometry. Grb2 was included to control for loading differences.

Fig. S3

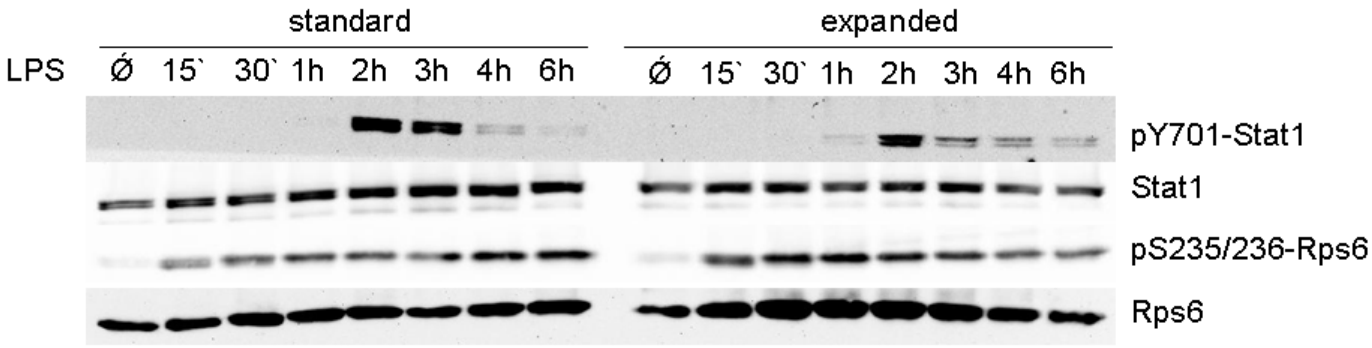


Fig. S3. Comparison of Stat1- and ribosomal protein S6 phosphorylation in SILAC- and standard protocol macrophages.

Bone marrow derived macrophages grown for 7 days in M-CSF (standard protocol) or expanded by the SILAC protocol for a total of 17 days were stimulated with LPS for the indicated times. Protein lysates were analysed by Westernblot for the levels of phosphorylated Y701-Stat1 and ribosomal protein S6 phosphorylation. Total Stat1 and Rps6 were detected as controls.

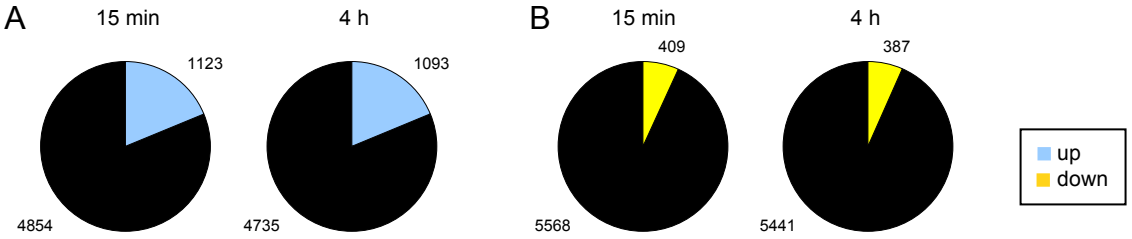
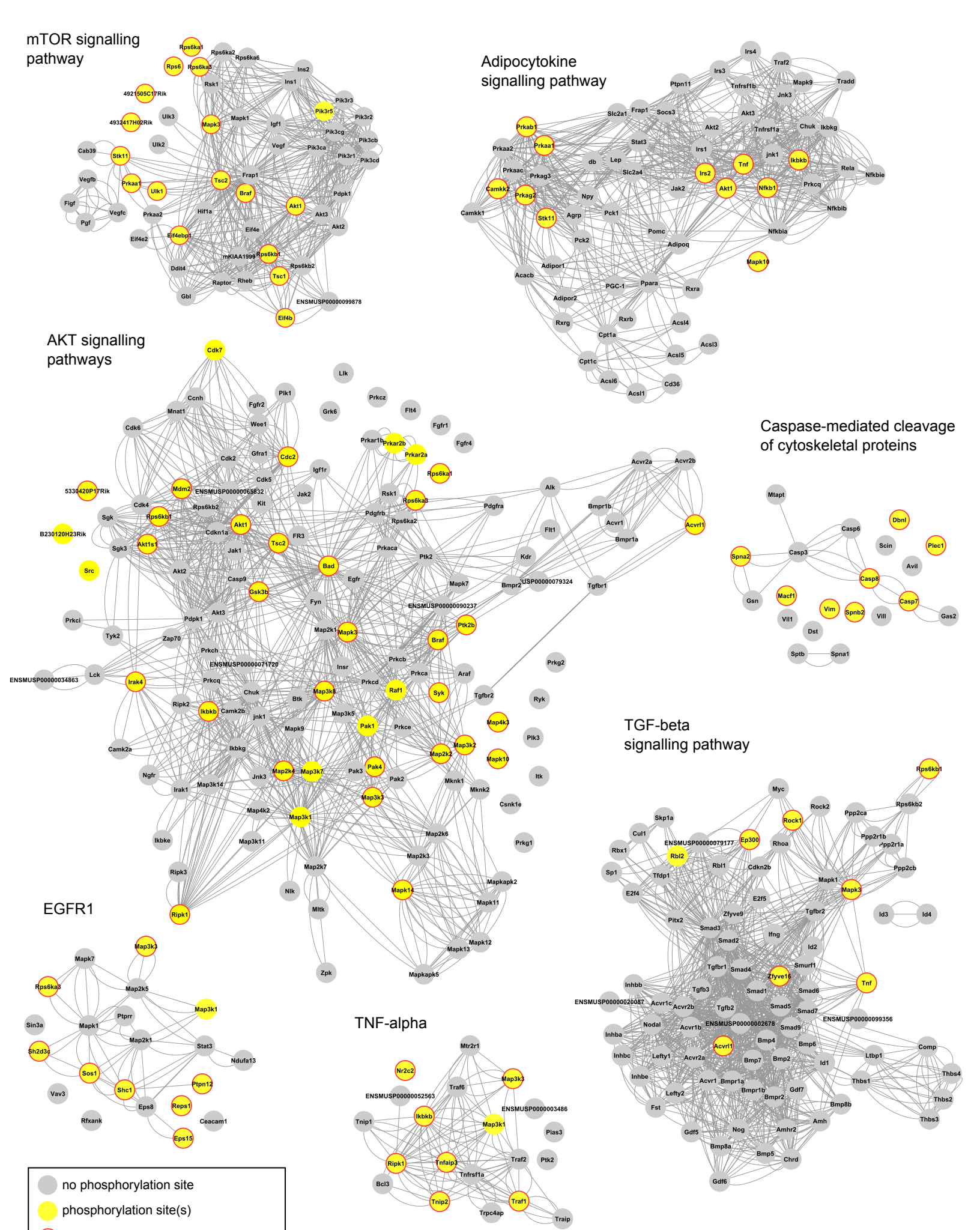


Fig. S4

Fig. S4. Regulation of phosphorylation by LPS.

(A) Up- and (B) down-regulation of phosphorylation in wild type cells 15 min and 4 h after LPS stimulation (fold-change ≥ 1.5 in both experiments).


mTOR signalling pathway
Adipocytokine signalling pathway
AKT signalling pathways
Caspase-mediated cleavage of cytoskeletal proteins
TGF-beta signalling pathway
EGFR1
TNF-alpha
no phosphorylation site
phosphorylation site(s)
LPS-regulated phosphorylation site(s)

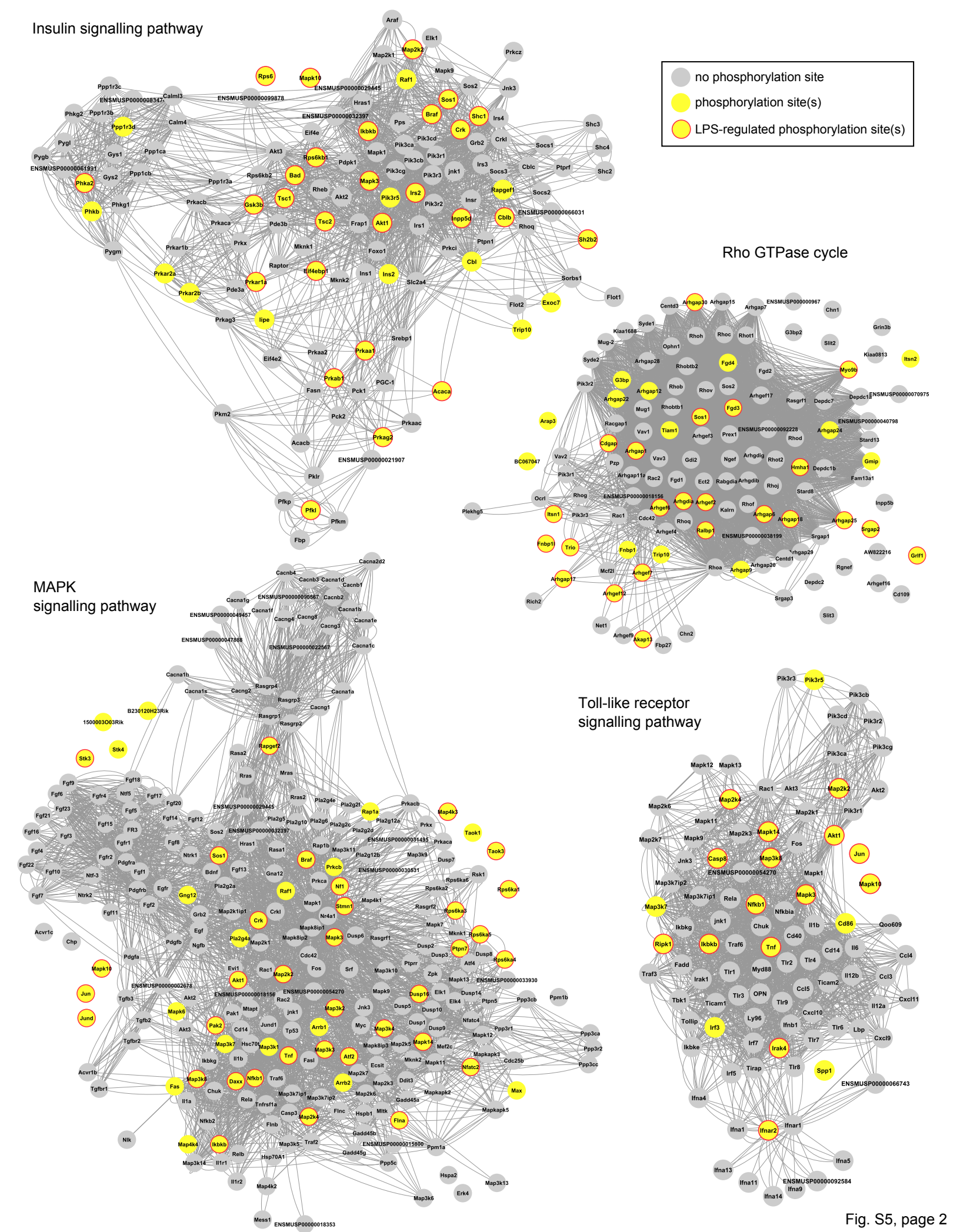
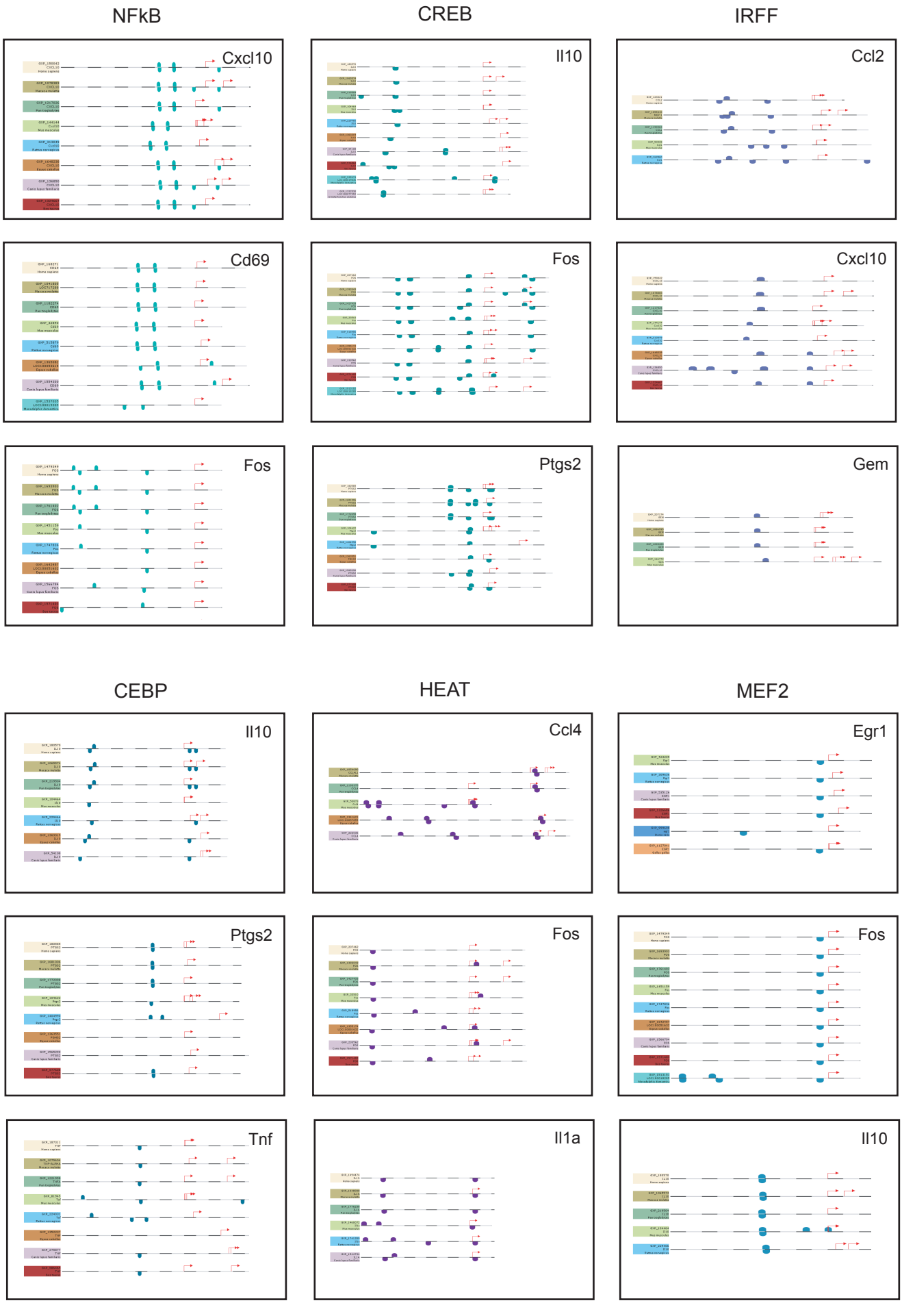

Insulin signalling pathway
no phosphorylation site
phosphorylation site(s)
LPS-regulated phosphorylation site(s)
Rho GTPase cycle
MAPK
signalling pathway
Toll-like receptor
signalling pathway

Fig. S5. Protein networks of over-represented signalling pathways.

Protein interaction networks for enriched InnateDB pathways enriched among LPS-regulated phosphoproteins were analysed with the STRING 8 database (http://string.embl.de) and visualised with Cytoscape (www.cytoscape.org). Depicted are over-represented pathways which did not show more than 75 % overlap with MAPK, AKT and mTOR signalling.


NFkB
Cxcl10
CREB
Il10
IRFF
Ccl2
Cd69
Fos
Cxcl10
Fos
Ptgs2
Gem
CEBP
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HEAT
Ccl4
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Egr1
Ptgs2
Fos
Fos
Tnf
Il1a
Il10

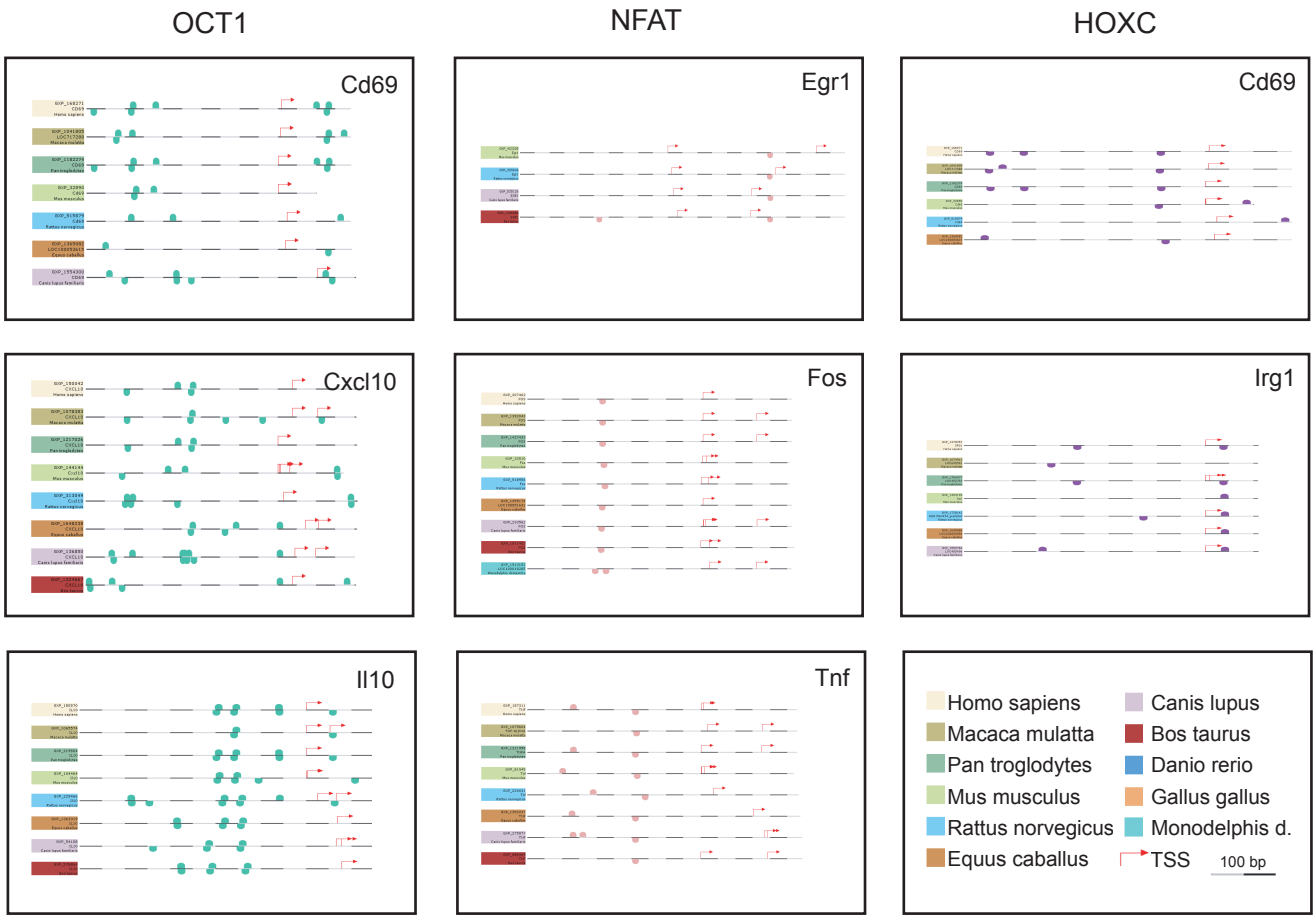

Fig. S6. Evolutionary conservation of TF binding sites in promoters of LPS-induced genes.

Promoter sequences of the 20 genes that were most strongly and rapidly induced in nascent RNA (45 min after LPS stimulation) and orthologous vertebrate promoters were analysed for evolutionary conservation of TF binding sites with Genomatix MatInspector. Each TF family with observed phosphorylation (15 min) and over-representation of binding sites in LPS-regulated promoters (45 min) had several target genes with evolutionary conserved binding sites, indicating potential functional relevance. Depicted are selected examples.

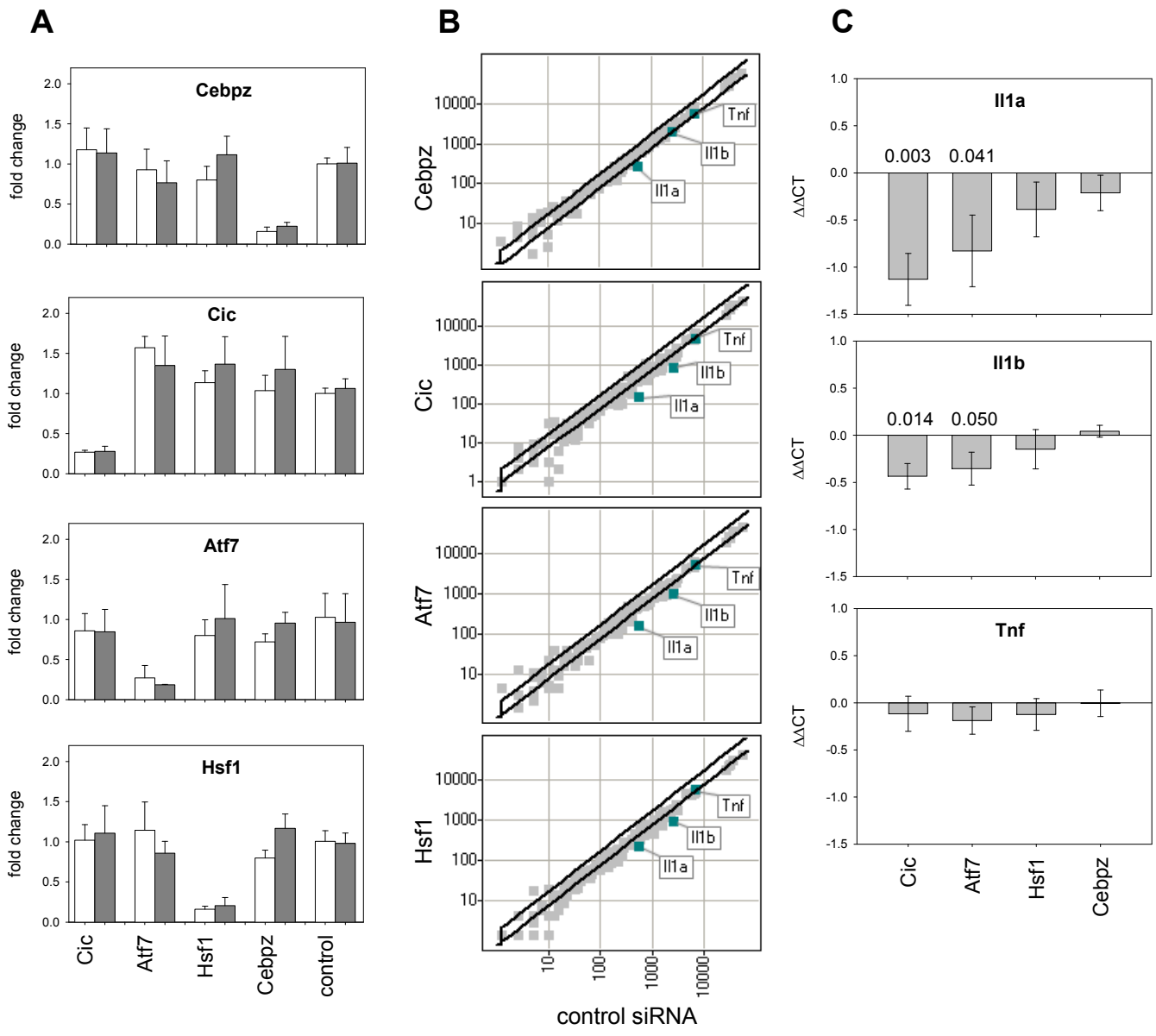
A
Cebpz
Cic
Atf7
Hsf1
fold change
Cic
Atf7
Hsf1
Cebpz
control
B
Cebpz
Cic
Atf7
Hsf1
Tnf
Il1b
Il1a
control siRNA
C
Il1a
Il1b
Tnf
0.003
0.041
0.014
0.050
ΔΔCT
Cic
Atf7
Hsf1
Cebpz

Fig. S7

Fig. S7. Knockdown of phosphorylated transcription factors by siRNA transfection of primary macrophages.

Bone marrow cells were differentiated to macrophages by culture in Teflon-bags in the presence of M-CSF for 7 days. Following harvest, 2x106 macrophages were transfected with the indicated siRNAs by electroporation using an established protocol (Wiese et al. 2009. J Immunol Methods *doi:10.1016/j.jim.2009.12.002*). 24-48 hours after transfection, cells were stimulated with LPS for 4.5 h, followed by preparation of total RNA.

Efficiency of target gene knockdown was determined by qRT-PCR using the Roche Universal Probe Library system. Sequences of primers and probes are available upon request. Expression of the un-stimulated, non-silencing siRNA control was set to 100%. Mean and SD from quadruplicate determinations from a representative experiment out of five performed.

(B) nCounter analysis of expression of 128 regulator genes (Amit et al 2009. Unbiased reconstruction of a mammalian transcriptional network mediating pathogen responses. Science *326*, 257-263) in macrophages transfected with indicated siRNA and stimulated 48 h later with LPS for 4.5 h. Shown are signal intenisites from one experiment. Black lines indicate 1.5-fold up- and down-regulation, respectively. Il1a, Il1b and Tnf are highlighted in green.

(C) Relative change in the level of expression of Il1a (top), Il1b (middle) and Tnf (bottom) in LPS-stimulated macrophages by siRNA knockdown of the indicated genes. DDCT values were calculated by calibrating to the LPS-treated, non-silencing siRNA control. A DDCT value of 1 or -1 equals a up- or down-regulation by a factor of 2. Mean and SEM of 6 independent knockdown experiments. T-test p-values for comparison of DCT values between control and specific siRNAs are indicated.

Fig. S8

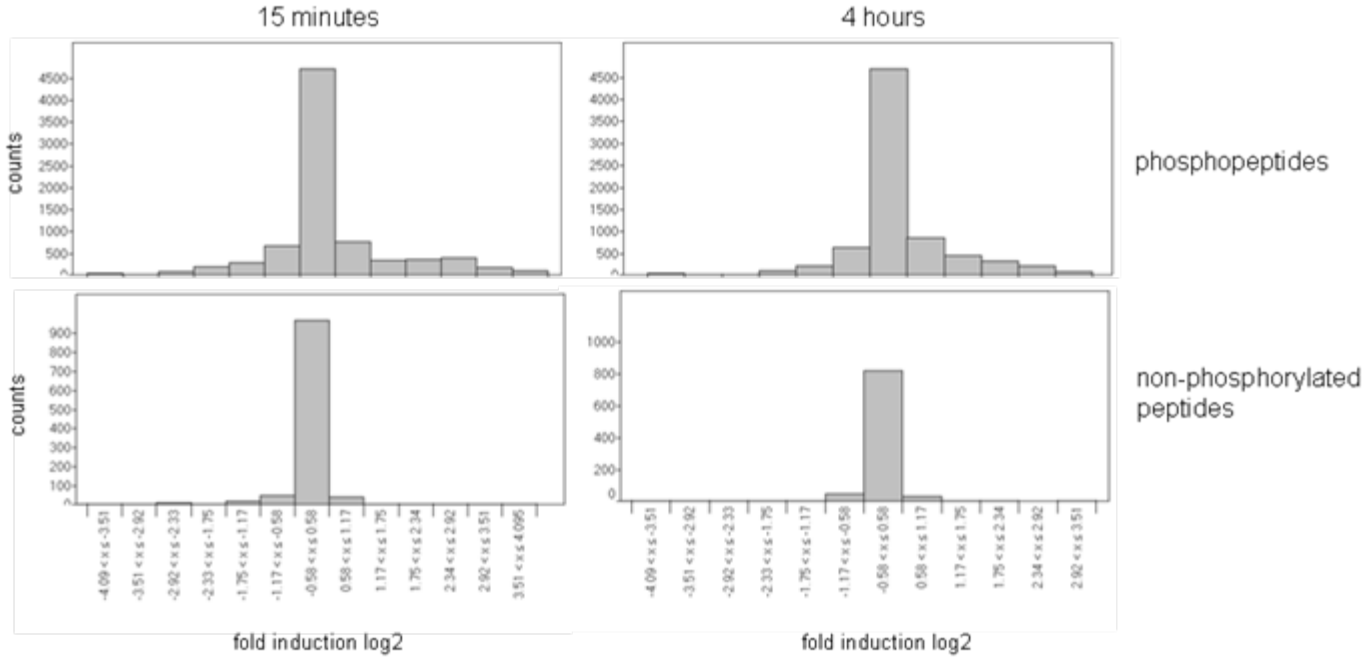

Fig. S8. Distribution of ratios for phosphopeptides and non-phosphorylated peptides.
Ratios were log2-transformed and binned in steps of 0.585=log21.5 to group the data consistent with the criteria used to identify regulated peptides. Histograms are shown depicting the data from experiment 1.