# Supporting Online Material for

## Phosphorylation by p38 MAPK as an Alternative Pathway for GSK3β Inactivation

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## MATERIAL AND METHODS

**Mice.** The MKK6(Glu) transgenic mice have been previously described (*1, 2*). Wildtype and Rag1$^{-/-}$ mice were purchased from Jackson Laboratory. Procedures that involved mice were approved by institutional guidelines for animal care.

**Plasmids.** pGEX-GST-GSK3β and pGEX-GST-GSK3β T$^{43}$A both with the K$^{84}$A kinase inactivating mutation were gifts from Dr. Mien-Chie Hung. Site directed mutagensis using the Transformer kit (Clontech, Mountain View, CA) was used to generate kinase inactive pGEX-GST-GSK3β-T$^{390}$A and pGEX-GST-GSK3β-T$^{390}$A /T$^{43}$A double mutants for use as substrates. The same site directed mutagensis technique was used to reverse the K$^{84}$A mutation to generate kinase active pGEX-GST-GSK3β wildtype and mutant constructs for their use in GSK3β *in vitro* kinase assays. Wildtype human GSK3β was subcloned into the expression vector pEGZ-HA, a gift from Ingolf Berberich (University of Wurzburg, Wurzburg, Germany). Expression plasmids for wildtype p38 MAPK and constitutively active MKK6 (*3*) were also used. Mouse GSK-3β with a C-terminal 3XFLAG tag was subcloned into the mammalian expression vector pEF5/FRT/V5/D-TOPO (Invitrogen). The point mutation at S$^{389}$ (Ala) was introduced using the QuikchangeII XL mutagenesis kit (Stratagene).

**Cell Cultures.** Thymocytes were isolated from wildtype, Rag$^{-/-}$ or MKK6(Glu) transgenic mice. 293T cells were transiently transfected with the indicated expression constructs using calcium phosphate. When specified, 293T cells were treated with SB203580 (5 μM) and Wortmanin (1 μM) (Calbiochem, San Diego, CA). Wildtype, GSK3α$^{-/-}$ and GSK3β$^{-/-}$ ES cells (*4*), as well as WT and MKK3$^{-/-}$

MKK6$^{-/-}$ MEF (*5*) have been previously described, and were also treated with SB203580 as described above for the indicated periods of time. For *in vivo* inhibition of p38 MAPK, wildtype mice were intraperitoneally (i.p.) injected with SB203580 or vehicle alone, and after 18 hours brain and thmocytes were harvested to prepare whole cell lysates.

**Reverse transcription-polymerase chain reaction.** Total RNA was extracted using RNAeasy mini kit (Qiagen) and transcribed into cDNA using oligo(dT) primers and reverse transcriptase (Invitrogen) that was used to examine β-catenin expression by semiquantitative RT-PCR. The primers for β-catenin were (5'ACAGCACCTTCAGCACTCT3' and 5'AAGTTCTTGGCTATTACGACA) and the primers for Actin were (5'GTGGGGGCGCCCCAGGCACCA3' and 5'CTTCCTTAATGTCACGCACGATTTC3')**.**

**Western blots and Immunoprecipitations.** Whole cell extracts were prepared in Triton lysis buffer (TLB) as previously described (*6, 7*). Nuclear extracts were made from cells as previously described (*8*). Immunoprecipitations and Western blotting was performed as described in (*2*). Anti-Akt, anti-p38 MAPK, anti-Lef, anti-c-Myc and anti-Histone (Santa Cruz Biotechnology), anti-GSK3β, anti-β-catenin, anti-phospho-p38 MAPK and anti-phospho-S$^{9}$ (Cell Signaling, Beverly, MA) and Anti-Flag (Stratagene, La Jolla, CA) were used for Western blot analysis. The phospho-S$^{389}$ GSK3β antibody was made by Proteintech, Chicago, IL using N-C-ARIQAAA(phos-S)PPANATA for immunization. Anti-Akt, anti-p38 MAPK and Anti-GSK3β were used for immunoprecipitations. Anti-rabbit-HRP,

anti-mouse-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) and anti-goat-HRP (Santa Cruz) were used as secondary antibodies.

***In vitro* kinase assays.** *In vitro* kinase assays for p38 MAPK and ERK were performed as described in (*9*) with immunoprecipitated p38 MAPK, purified recombinant active p38 MAPK (Cell Signaling) and ERK (Cell Signaling) Kinase using 2 μg inactive wildtype GST-GSK3β, GST-GSK3β $T^{43}A$ mutant, GST-GSK3β $T^{390}A$ mutant and GST-GSK3β $T^{390}A$ /$T^{43}A$ double mutant (2 μg), GST-GSK3α (2 μg) (Cell Signaling) (boiled to inactivate) and GST-ATF2 fusion protein (2 μg) (Cell Signaling) as substrates in the presence or absence of SB203580 (2.5 μM) (Calbiochem, San Diego, CA). The reactions were terminated after 30 min at 30°C by addition of SDS-PAGE sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. Protein was visualized by staining with PonceauS and incorporated $^{32}P$ was visualized by autoradiography. In specific experiments, whole cell extracts were first depleted of Akt prior to p38 MAPK immunoprecipitation by pre-incubated with anti-Akt antibody prebound to protein A-Sepharose followed by one incubation with protein A-sepharose alone. Active GST-GSKβ and GST-GSK3β-$T^{43}A$ and GST-GSK3β-$T^{390}A$ mutants were expressed and purified as described in (*10*). The specific activity of the wildtype and the mutants was determine using the GSM substrate peptide (Upstate, Lake Placid, NY) and were as follows: GST-GSK3β (91 pmol/μg x min) and GST-GSK3β-$T^{43}A$ (53 pmol/μg x min) and GST-GSK3β-$T^{390}A$ (65 pmol/μg x min). *In vitro* GSK3β kinase assays were performed by incubating purified active wildtype GST-GSK3β or GST-GSK3β-$T^{43}A$ and GST-GSK3β-$T^{390}A$ mutants (400ng) at

30°C for 10 minutes in kinase buffer supplemented with 1mM ATP containing [$\gamma$-$^{32}$P] ATP and GSM substrate peptide (62.5 $\mu$M) (Upstate, Lake Placid, NY). Reactions were terminated by spotting onto P81 filters. Filters were washed extensively and counted in a scintillation counter. To examine the effect of p38 MAPK and Akt on GSK3$\beta$ activity, purified active GSK3$\beta$ or mutants were preincubated for 15 minutes at 30°C with 500 ng recombinant active p38 MAPK or active Akt (Cell Signaling) prior to performing the GSK3$\beta$ activity assay as described above.

For peptide inhibition assays, Phospho-S$^{9}$ (GRPRTTS($PO_3H_2$)FAE) (*11*), Phospho-T$^{390}$ (RIQAAAST($PO_3H_2$)PTN) and non-phospho-T$^{390}$ (RIQAAASTPTN) peptides were synthesized and purified by UVM Protein Core Facility. *In vitro* kinase assays were performed with purified GST-GSK3$\beta$ (400 ng) in Kinase buffer supplement with 1mM ATP containing [$\gamma$-$^{32}$P] ATP and the indicated concentrations of GSM and inhibitory peptide.

**Mass spectrometric analysis by LC-MS/MS.** Kinase-inactive GSK3$\beta$ was preincubated with or without active recombinant p38 MAPK for 30 min in the presence of ATP. The reaction mix was treated with an ammonium bicarbonate buffer containing DTT (10 mM) to reduce cysteines, incubated with iodoacetamide and digested with trypsin (40ng/µl). Digestion was stopped with acetic acid (10%), centrifuged and the supernatant was used for analysis by electrospray ionization (ESI) liquid chromatography-mass spectrometry (LC-MS). A fused-silica microcapillary LC column (12 cm x 75 µm id) packed with C18 reversed phase resin (Magic C18AQ, 5-µm particle size, 20-nm pore size,

Michrom Bioresourses, Auburn, CA) was used with nanospray ESI. The nanospray-ESI was fitted onto a linear quadrupole ion trap (LTQ) mass spectrometer (Thermo Electron, San Jose, CA) that was operated in a collisional-induced dissociation mode to obtain both MS and MS/MS spectra. Samples of tryptic peptides were loaded onto the microcapillary column and separated by applying a gradient from 5-80% acetonitrile in 0.5% acetic acid at a flow rate of 250 nL/min for 55 min. Mass spectrometry data were acquired in data-dependent acquisition mode, in which a full MS scan was followed by 10 MS/MS spectra of the 10 most abundant ions. Spectra were searched against the human International Protein Index (IPI) database using SEQUEST (Bioworks software package, version 3.3, Thermo Electron, San Jose, CA). GSK3β (Swiss-Prot entry P49841) was identified. The search parameters permitted ± 1.0 Da peptide MS tolerance, and ± 1.0 Da MS/MS tolerance. Phosphorylation (a +80 mass increase) was sought on serine (S), threonine (T), and tyrosine (Y) residues, together with allowance of oxidation of methionine and carboxymethylation of cysteines. Up to two missed tryptic cleavages of peptides were considered. The cutoff for SEQUEST assignment was a cross-correlation score (Xcorr) greater than 1.9, 2.5, and 3.8 for peptide charge states of 1,2, and 3, respectively; and a delta-correlation score ($\Delta$Cn) >0.10. Manual identifications were also performed to define the exact location of phosphorylation sites based upon the b- and y-ions of the corresponding identifying MS/MS spectra.

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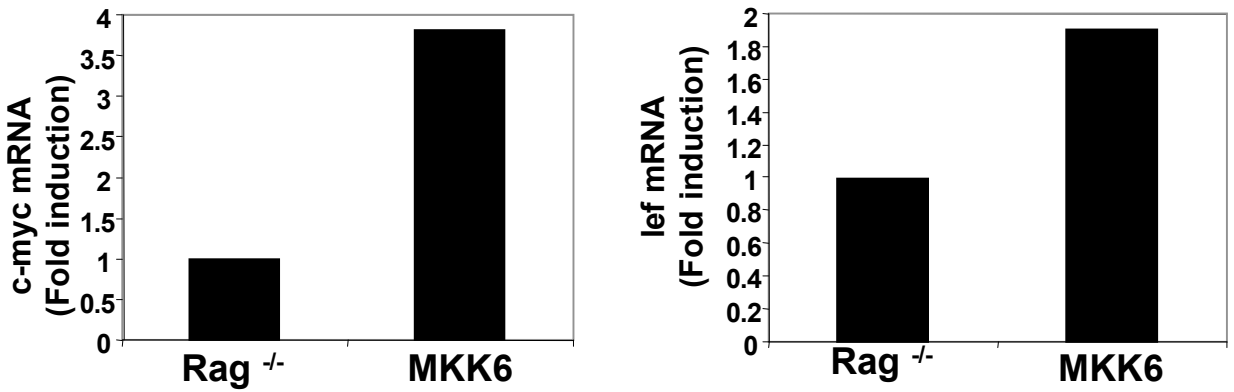



**Figure S1.** MKK6 transgenic thymocytes have increased expression of c-myc and lef mRNA. Gene expression profile was examined by Affymetrix gene chip analysis using mRNA isolated from MKK6 and $Rag^{-/-}$ thymocytes. Fold increase of MKK6 c-myc and lef mRNA relative to $Rag^{-/-}$ mRNA are shown.

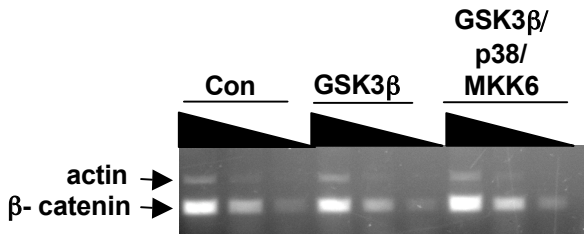



**Figure S2.** p38 MAPK does not affect β-catenin mRNA levels. The mRNA levels of β-catenin and actin in non-transfected control (Con) 293T cells and cells transiently transfected with expression constructs for GSK3β alone or in combination with p38 MAPK and MKK6 were examined by semi-quantitative RT-PCR. Three serial 1/2 dilutions of cDNA were used to examine β-catenin and actin expression

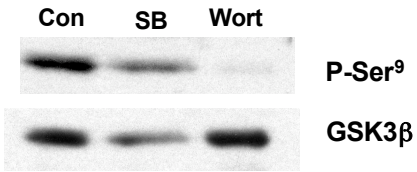



**Figure S3.** p38 MAPK does not regulate AKT-mediated Ser$^9$ phosphorylation of GSK3β. 293T cells were treated with vehicle control (Con), SB203580 (SB) or Wortmanin (Wort) for 40 minutes. The levels of phospho-Ser$^9$ and GSK3β were determined by Western Blot analysis.

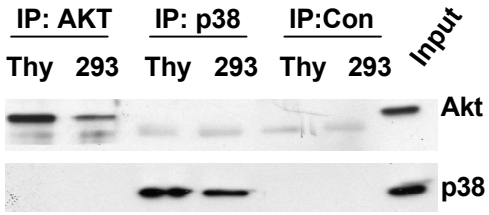



**Figure S4.** Akt and p38 MAPK were immunoprecipitated (IP) from whole cell extracts from MKK6 thymocytes (Thy) or MKK6-transfected 293T cells (293). Immunoprecipitation of p21 was included as a negative control (Con). Total cell lysate (Input) from MKK6 thymocytes and the immunoprecipitates were examined for Akt and p38 MAPK by Western blot analysis.

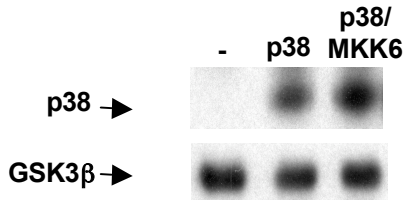
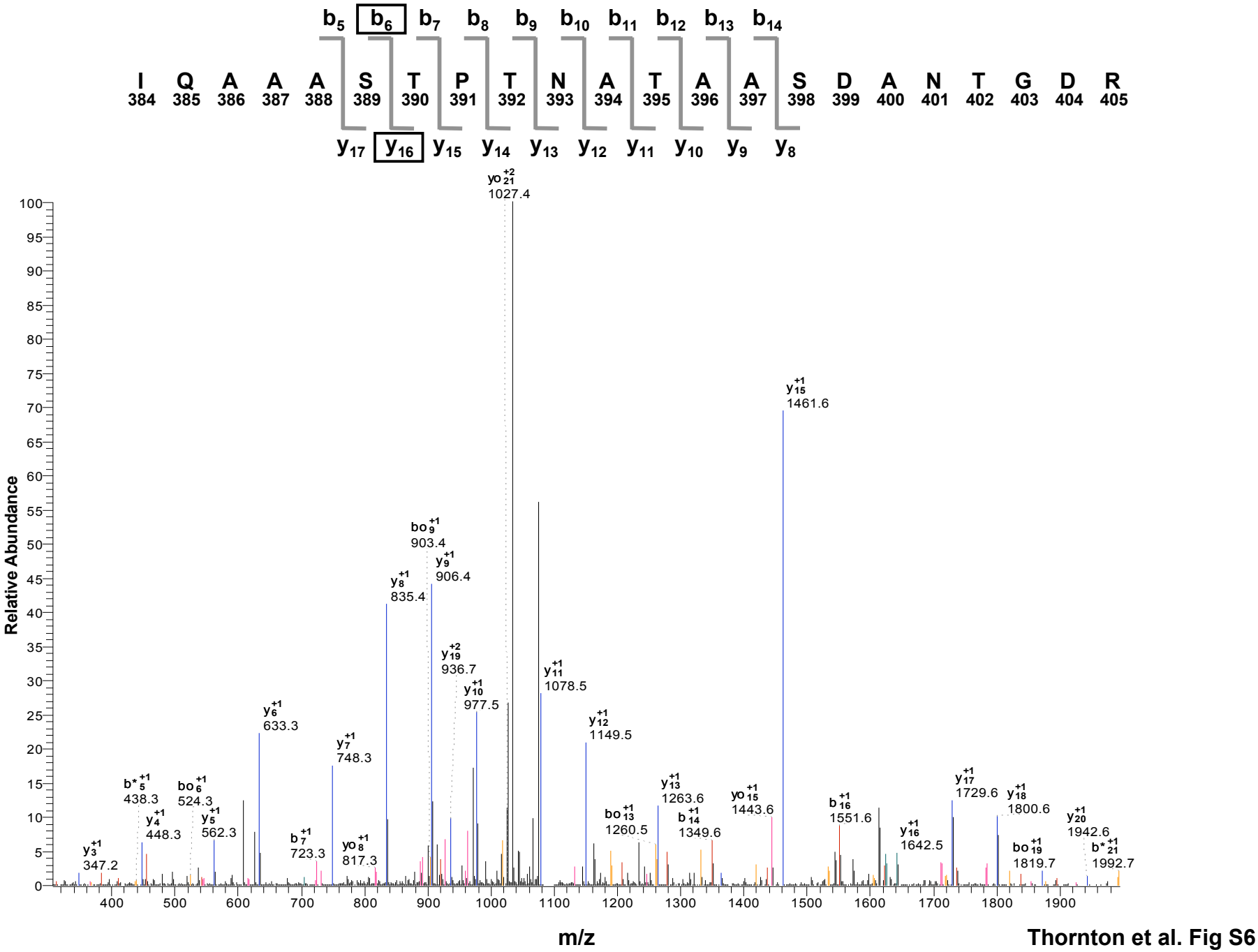
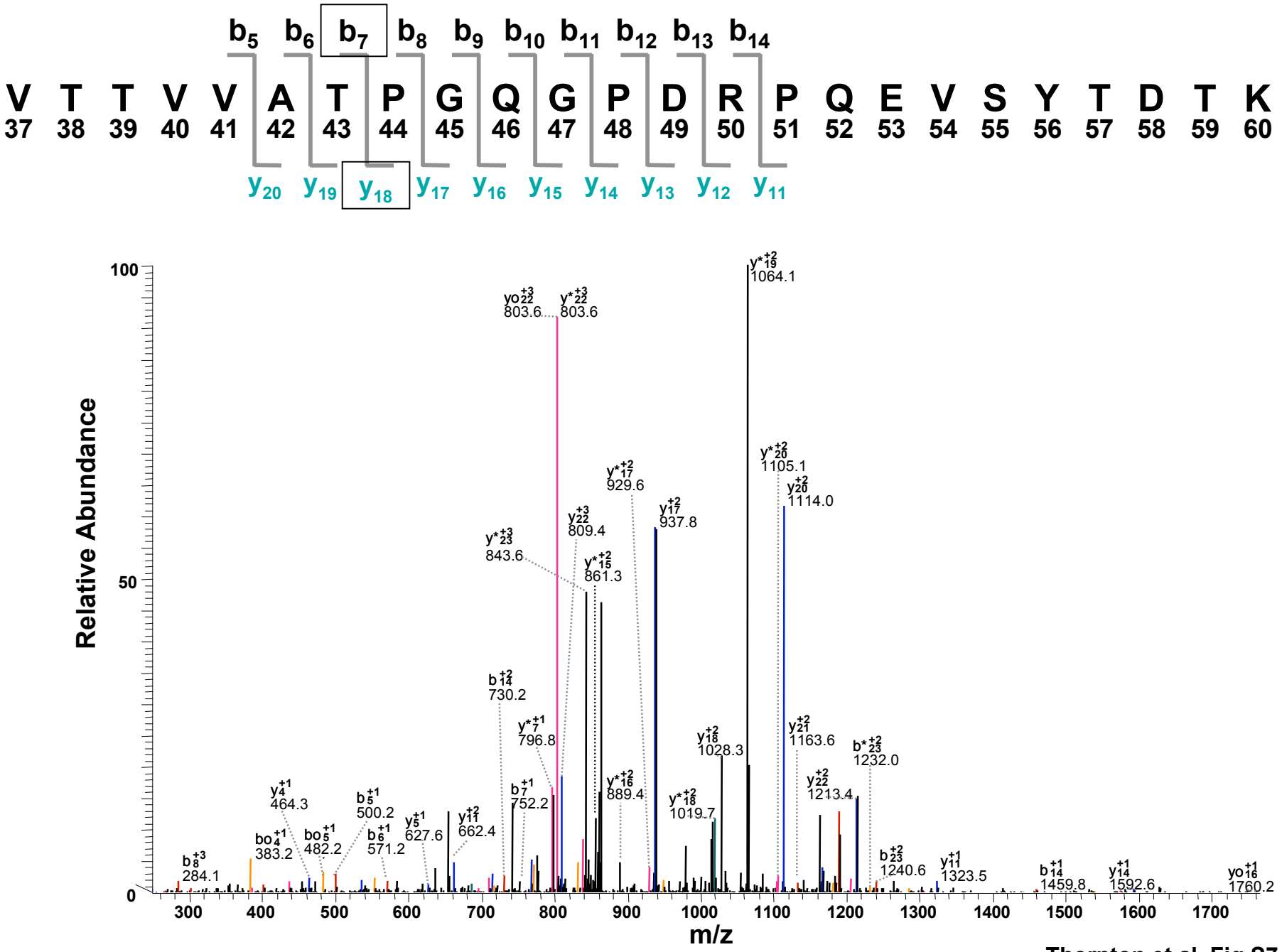



**Figure S5.** p38 MAPK associates with GSK3β. GSK3β was immunoprecipated from whole cell lysates from 293T cells transiently transfected with expression constructs for GSK3β alone (-), GSK3β and p38 MAPK (p38), or GSK3β, p38 MAPK and MKK6 (p38/MKK6). The levels of p38 and GSK3β in the immunoprecipitates were determined by Western blot analysis.




Thornton et al. Fig S6

**Figure S6.** MS/MS spectra identifying a TP motif-containing phosphopeptide IQAAASTPTNATAASDANTGDR at the C-terminus of GSK3β as a target for p38 MAPK. The b and y ion layout is shown for the middle of the peptide. Phosphorylation of serine-389 ($S^{389}$) will produce a b6 ion that is 80 Da heavier, while phosphorylation of threonine-390 ($T^{390}$) will produce a y16 ion that is 80 Da heavier. These are the only ions that will differentiate the difference in phosphorylation of $S^{389}$ and $T^{390}$. All other b and y ions will be the same for both species. The b7, b8, y14, and y15 ions will differentiate between phosphorylation of $T^{390}$ and $T^{392}$ (or higher). Similar b and y ions can be used to differentiate phosphorylations of $T^{395}$, etc. The ions actually identified as b and y ions are indicated in the figure. There was no b6 ion present, but the y16 ion at m/z = 1642.5 and the presence of the y15 ion at m/z = 1461.6 defines that the phosphorylation site is at $T^{390}$ and not on any another serine or threonine.




Thornton et al. Fig S7

**Figure S7.** MS/MS spectra identifying a P-Thr$^{43}$Pro phosphopeptide VTTVVATPGQGPDRPQEVSYTDTK as a target for p38 MAPK as described in S6.

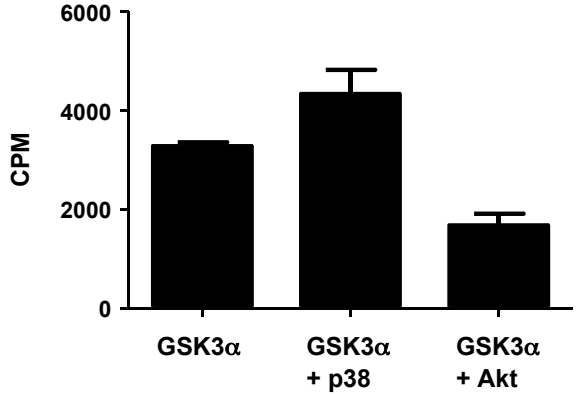



**Figure S8.** p38 MAPK does not inhibit GSK3α activity *in vitro*. Recombinant kinase-active GSK3α was pre-incubated in the reaction buffer alone (GSKα), with activated p38 MAPK or activated Akt (15 min). I*n vitro* GSK3α kinase assays were then performed using GSM as the substrate. Error bars represent SD (n=3).

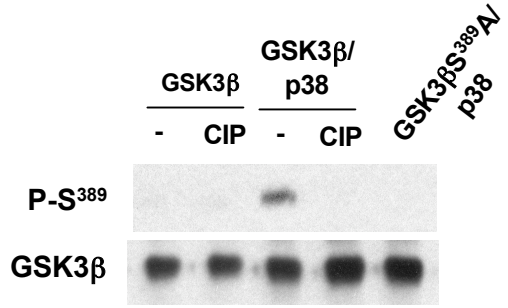



**Figure S9.** The phospho-S$^{389}$ Ab specifically recognizes GSK3β phosphorylated at S$^{389}$ by p38 MAPK. Recombinant mouse wildtype GSK3β was incubated with or without recombinant active p38 MAPK for 15 min, followed by an incubation with or without (-) Calf Intestinal Phosphatase (CIP). Recombinant GSK3β-S$^{398}$A mutant was also incubated with active p38 MAPK as described for wildtype GSK3β, but not treated with CIP. The presence of phospho-S$^{389}$ was determined by Western blot analysis using the anti-phospho-S$^{389}$ Ab. Blots were re-probed for total GSK3β.

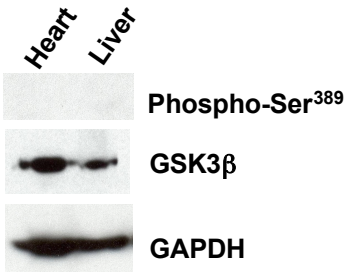



**Figure S10.** Phospho-Ser$^{389}$ GSK3β and total GSK3β in whole cell lysates from mouse heart and liver were examined by Western blot analysis. GAPDH was examined as a loading control.

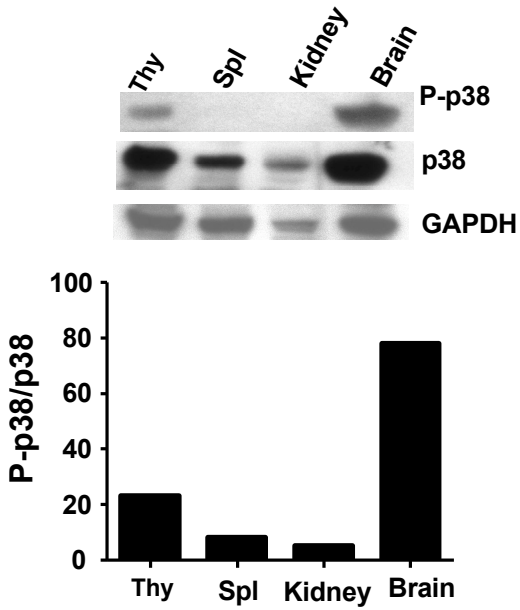



**Figure S11.** Activated p38 MAPK in different tissues. Phospho-p38 (P-p38) and total p38 levels in whole cell extracts for thymocytes (Thy), splenocytes (Spl), kidney and brain were determined by Western blot analysis. The level of GAPDH was examined as a loading control. Ratio of phospho-p38 to total p38 is provided in the lower panel.