Online Supplemental Methods

MK2 non-enzymatically promotes nuclear translocation of caspase-3 and resultant apoptosis

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The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols.

Male C57BL/6J (wild type, WT) mice aged 10-12 weeks (Jackson Laboratory, Bar Harbor, ME) and *MK2*$^{-/-}$ mice, C57BL/6J background (6) were exposed to intravenous (IV) PBS or lipopolysaccharide (LPS, 0127:B8, product # L3129, Sigma) via retro-orbital injection (8) for up to 6hrs. After exposure to the experimental conditions, lungs were flushed free of blood, removed and homogenized in cell lysis buffer. Lung lysates were frozen by immersion into liquid nitrogen and subsequently stored for later analyses.

Cell Lines: Non-small cell lung carcinoma (NSCLC) cell lines- H23 and A549 cell lines and small cell lung carcinoma (SCLC) cell line- H446 were purchased from ATCC (Manassas, VA). H23 and H446 cells were cultured in RPMI 1640 media (ThermoFisher A1049101) supplemented with 10% (v/v) FBS (Hyclone). A549 cells were cultured in FK12 medium (ThermoFisher 21127022) supplemented with 10% (v/v) FBS (Hyclone). Cells were maintained in full growth media in 75-cm^2 flasks. Cells were grown at 37 C with 5% CO_2.

Adenoviral vectors: adenoviral vectors encoding wild-type MK2 (Ad-WT MK2), constitutively active MK2 (Ad-Active-MK2; T222E, T334E)(3), dominant negative MK2 (Ad-Dom Neg-MK2; K93R)(4), a mutated nuclear export sequence MK2 (Ad-Mut-NES; L360A) (2), a mutated nuclear localization sequence MK2 (Ad-Mut-NLS-MK2; K372A, K373A, K388A, K389A) (1, 5), a wild-type MK2 fused to a biotin protein ligase on the c-

terminus (Ad-WT-MK2-BioID-C) or the n-terminus (Ad-WT-MK2-BioID-N) were directly purchased from Vector Builder (Chicago, IL). The sequences of the plasmids encoding these vectors were verified by Sanger sequencing (The Genetics Resources Core Facility, Johns Hopkins University).

Infections: Cells were seeded in 6-well plates at a cell density of 5x10^5 cells per well. Cells were grown at 37 C with 5% CO_2 for approximately 6 hours to allow adherence. After adherence, media was replaced with media plus viral vector. Cells were incubated with viral vector to have a final plaque-forming unit number of up to 100. Cells were left to incubate with the viral media at 37 C with 5% CO_2 for approximately 24 hours. Viral media was then replaced with the appropriate subsequent media, depending on experimental conditions.

Immunoblot analyses: Cell cultures were lysed using cell lysis buffer (CST 9803s, Cell Signaling, Boston, MA) supplemented with protease inhibitors cocktail (Sigma P8340), PMSF 1mM, Thermo Fischer 36978), NaF (1mM, Sigma, 201154) and NaOV (1mM, Sigma, S6508). Protein lysates were denatured using Laemmli Sample buffer (BioRad 1610747), 2-Mercaptoethanol (Millipore Sigma M6250), and 100 C heat (5-minute exposure). Proteins were separated by SDS-PAGE (Thermo Fisher XP00122BOX), and transferred to PVDF membranes (BioRad 1620177). Membranes were blocked in 5% non-fat dry milk (BioRad 1706404) in TBS (Quality Biological 50983267) with 0.5% Tween-20 (Thermo Fisher BP337-500). Membranes were incubated with primary

antibodies at 1:1000 dilution overnight in 2.5% non-fat dry milk. PVDF membranes were then incubated with horse radish peroxidase-linked secondary antibodies, anti-mouse (CST 7076) or anti-rabbit (CST 7074), at 1:5000 dilution for 1 hour in 1% non-fat dry milk. Immunoblots for Streptavidin conjugated to horse radish peroxidase utilized bovine serum albumin instead of nonfat dry milk. Protein bands were visualized using chemiluminescent detection methods. Band intensities were quantified using ImageJ software.

Phospho-specific anti-total antibodies directed at HSP27(p-HSP27- CST-2401; t-HSP27- CST-2402) and anti-total antibodies directed at MK2 (CST-3042), caspase 3 (CST-9662), β-tubulin (CST-5346), GAPDH (CST-3683), PARP1 (CST-9542) (Cell Signaling, Boston, MA) were used. Streptavidin conjugated to horse radish peroxidase was also used (CST-3999).

Nuclear and cytosolic fractionation: Cells were trypsinized and re-suspended in PBS. Nuclear and cytosolic fractions of the resulting cell suspensions were generated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher 78833, Rockford, IL). The purity of cytosolic fractions was assessed by lack of PARP1 immuno-reactivity and purity of the nuclear fraction was assessed by lack of GAPDH immuno-reactivity (Cell Signaling, Boston, MA) using standard immuno-blotting techniques.

Brightfield microscopy: Six hours after sub-confluent plating, H23 cells were infected with Ad-eGFP or Ad-WT MK2 and imaged over time. A mark was placed under side each dish to allow for sequential imaging of the same region. All images were anonymized and number of cells per image were counted by blinded investigators (AS, GS, MK). Cell counts obtained by each investigator were subsequently averaged together.

In vitro kinase assay: Reactions were performed in 50 mM sodium β-glycerophosphate containing 10 mM Mg-acetate, 0.1 mM ATP, 2 uCi γ-^{32}P-ATP (New England Nuclear, PerkinElmer, Waltham, MA) and 170 ng (6 U) of recombinant active MK2 (Millipore, Dundee, UK). Substrates were recombinant active caspase-3 (250 ng, Enzo, Farmingdale, NY) or recombinant HSP27 (14 ng, Enzo, Farmingdale, NY). Reactions were incubated at 30^{o}C for 30 or 60 min, and resolved on 4-12% NUPAGE gels (Life Technologies, Carlsbad, CA). After staining with Coomassie Blue, gels were dried and phosphorylated proteins were detected on a Molecular Imager FX phosphorimager (BioRad, Hercules, CA) using Quantity One software.

Bio-ID assay: H23 cells were infected with Ad-WT MK2, Ad-WT-MK2-BioID-C or Ad-WT-MK2-BioID-N for 24 hours after which the media was changed to include biotin (50 uM) (ThermoFisher, 29129) for an additional 18 hours, after which cell lysates were prepared. Cell lysates were incubated with 50 uL of Streptavidin Sepharose High Performance Beads (GE Healthcare, 17511301) overnight at 4˚C with gentle shaking.

Beads were washed four times with buffer to elute off non-specific proteins bound to the sepharose beads. The buffer was aspirated and beads were re-suspended in Laemmli buffer for Western blotting.

Co-Immunoprecipitation: H23 cells were infected with Ad-eGFP, Ad-WT MK2, Ad-Mut-NLS-MK2 or Ad-Mut-NES-MK2 and 48 hrs afterwards cell lysates were prepared. Appropriate cell lysates (50 µg) were treated with anti-MK2 (CST-3042, 1:100 dilution) or isotype control antibody (SC-2027X, 1:100) for overnight incubation at 4˚C with gentle shaking. Protein A sepharose beads (GE Healthcare 17-5280-01; 10 µl) were added on the next day and incubated with shaking for 3 hrs at 4˚C. Beads were washed two times with Krebs+ buffer to elute off non-specific proteins bound to the protein A sepharose beads. The buffer was aspirated and beads were re-suspended in 20 µl 2x Laemmli sample buffer. The samples were separated by 4-12% SDS-PAGE and immunoblotted.

Flow cytometry: Following experimental exposures, cell cultures were trysinized and single cell suspension was generated. 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, ThermoFisher D21490) was used to stain DNA of cells as a way to quantify condensed and fragmented nuclei, a hall mark of apoptosis(7). Data acquisition was performed on a custom FACSAria II instrument running FACSDiva acquisition software (BD Biosciences, San Jose, CA). A singly-stained aliquot of H23 cells for the DAPI fluorochrome and unstained cells were used to compensate for background auto-

fluorescence. 5 x 10^4 events were obtained per sample and analyzed using FCS Express 6 (De Novo Software, Pasadena, CA).

Statistical analysis: Data are shown as means (± SD). Since data is obtained using cell lines, biological replicates are not feasible. Data from separate individual cultures (each individual culture represents N of 1) are plotted for each condition. Sample size is identified in Figure Legends. A combination of parametric and nonparametric tests was used. The specific statistical test and post-hoc testing performed is identified within each Figure Legend. A *P* value of less than 0.05 was considered significant. Data were analyzed using GraphPad Prism 8 (La Jolla, CA).

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