Supplement: **Selective Inhibition of Mitochondrial JNK Signaling Achieved Using Peptide Mimicry of the Sab Kinase Interacting Motif-1 (KIM1).**

Supplemental Materials:

I. **Supplemental Methods**
II. **Supplemental Figure Legends**
III. **Supplemental Figures**
IV. **Supplemental Table**
V. **References**

I. Supplemental Methods

Materials: c-jun siRNAs were purchased from Cell Signaling Technologies. The Amplex Red Assay Kit was purchased from Invitrogen, and the Kinase-Glo Assay Kit was obtained from Promega. FITC-TatSab$_{KIM1}$ (FTIC-GFESLSVPSPLDLSGPRVVAPPRRRQRRKKRG-NH_2) peptide was purchased from NeoPeptide.

Cell Lysis and Western Blotting: Following cellular experiments cells were washed twice in ice-cold phosphate buffered saline (PBS). To acquire cell lysates for Western blot analysis, cells were treated with radioimmunoprecipitation assay buffer (RIPA; 50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SDS)) supplemented with protease inhibitors (1mM PMSF and Protease Inhibitor Cocktail Set III (Calbiochem)) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set V (Calbiochem)). Cells were incubated in lysis buffer for 5 minutes while rocking gently at 4°C. Cell lysates were removed from cell culture plate or flask by scraping and transferred to a microcentrifuge tube.

After two minutes of incubation on ice, the cells were briefly sonicated, and then the protein was separated from the cell homogenate by centrifugation at 14,000xg for 15 minutes. Protein concentration of the supernatant was determined by the Pierce BCA Kit protocol. Proteins were resolved by SDS-PAGE, and transferred to nitrocellulose membranes for Western blot analysis. Briefly, membranes were incubated in blocking buffer (1xTBST with 5% non-fat milk, 20mM Tris-HCl, pH 6.7, 137mM NaCl, 0.1% Tween-20, and 5% non-fat milk) for one hour at room temperature or overnight at 4°C. Phospho-Western blot membranes were blocked using blocking buffer with 5% bovine serum albumin (BSA) instead of non-fat milk. Membranes next were incubated with primary antibodies (diluted 1:1000 in blocking buffer) according to manufacturer's suggestions. Primary antibodies specific for JNK (Cell Signaling Technology, #9252), Phospho-JNK (Thr183/Tyr185) (Cell Signaling Technology, #9251), c-jun (Cell Signaling #9165), Phospho-c-jun (Ser73) (Cell Signaling #3270), Sab (Novus Biologicals, H00009467-M01), Phospho-Bcl-2(Ser70) (Cell Signaling #2827), Bcl-2 (Cell Signaling #2875), α-Tubulin (Cell Signaling Technology, #2144), Calnexin (Cell Signaling #2679), Enolase-1 (Cell Signaling #3810), and Histone H3 (Cell Signaling #5192) were used during this study. The membranes were then washed in 1xTBST. Membranes were next incubated with secondary antibodies (diluted 1:2000 in blocking buffer) for one hour at room temperature with gentle rocking. Secondary antibodies conjugated to horseradish-peroxidase (HRP-conjugated antibodies) used in this study were Anti-rabbit IgG, HRP-linked (Cell Signaling Technology, #7074) and Anti-mouse IgG, HRP-linked (Cell Signaling Technology, #7076). Western blots were developed using chemiluminescence detection using the Pierce Dura-HRP chemiluminescent substrate.

Mitochondrial Isolation: Isolation was performed similar to Pallotti and Lenaz(*1*). Briefly, $2x10^8$ cells were grown on two $150mm^2$ tissue culture plates, and then washed twice with PBS at room temperature. The cells were scraped gently from the culture surface, and pelleted by room temperature centrifugation (1000xg, 15 minutes). The pellet was suspended in ice-cold homogenization buffer (150mM $MgCl_2$, 10mM KCl, 10mM Tris-HCl pH 6.7) at six times the pellet volume. The suspension incubated on ice for 2 minutes. The cells were homogenized with up to 10 up-and-down strokes of an ice-cold homogenizer, and cell disruption was confirmed by microscopy. Homogenization buffer containing 0.25M sucrose was added to the cell homogenate at one third the suspension volume. Gentle inversion was used to mix the homogenate and buffer thoroughly. Nuclear fractions were obtained by centrifuging homogenates at 1000xg for 5 minutes at 4^oC. The supernatant of the nuclear fraction was again centrifuged at 5000xg for 10 minutes at 4^oC. Following centrifugation, the pellet was suspended in ice-cold sucrose/Mg^{2+} buffer (150mM $MgCl_2$, 250mM sucrose, 10mM Tris-HCl, pH 6.7). Using an ice-cold Dounce homogenizer, the pellet was disrupted with three strokes on average. This suspension was centrifuged at 5,000xg for 10 minutes at 4^oC. The mitochondrial pellet was suspended in the buffers required by the experiments hereafter. Mitochondria were diluted to a concentration of 80mg/mL, then frozen on dry-ice/ethanol slurry and stored at -80^oC until use. Mitochondrial enrichment purity was assessed by Western blot analysis. The relative abundance of mitochondrial resident protein, cyclooxygenase IV (COX-IV), cytosolic protein, enolase, nuclear contamination with histone-H3 and microsomal constituent, calnexin were evaluated and compared. Mitochondrial fractions possessing greater than 75% purity were used for experiments. The functional capacity of the mitochondrial enrichments was determined by examining the individual enrichments for citrate synthase activity(*1, 2*). Citrate synthase activity,

which catalyzes the conversion of oxaloacetate and acetyl-coenzyme A to citrate and reduces coenzyme-A, can be detected using a specrophotmetric assay. Briefly, the sulfhydryl group on conenzyme-A is reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB, and the reaction mixture turns yellow; subsequently, this change can be measured by monitoring absorbance at a wavelength of 412nm. Our reaction was based on the method from Kirby et al. (*2*). 35µg of mitochondria enrichment and was added to citrate synthase assay mixture (100mM Tris-HCl, pH 8.0, 0.25mM oxaloacetate, 50µM acetyl-coenzyme A, 0.1mM DTNB, and 0.1% Triton X-100.

Outer Mitochondrial Membrane and Mitoplast Preparation: This method was altered slightly to the one proposed by Schnaitman *et al(3).* Briefly, 100µg of mitochondrial enrichment was diluted in 0.2mL of Milli-Q water and incubated on ice for 20 minutes. Following the incubation, samples were centrifuged at 10,000xg for 15 minutes at 4°C. The supernatant was supplemented with RIPA, protease inhibitors, and phosphatase inhibitors, as this was considered the outer mitochondrial membrane fraction. The pellet, or mitoplast fraction was resuspended in the assay buffers indicated below or in RIPA (with protease and phosphatase inhibitors) for protein analysis. For experiments exclusively using mitoplasts, a digitonin-based isolation was employed for mitoplast preparation. Mitochondria enrichments were diluted to 20µg/mL in Clark electrode buffer (described above). Digitonin was added at a ratio of 0.1mg digitonin per 1mg protein. The samples were incubated on ice for 15 minutes. The samples then were diluted in three volumes of 250mM sucrose in $T_{10}E_{20}$ buffer (10mM Tris-HCl, 1mM EDTA, pH 7.6) (*1*). Mitoplasts were recovered by centrifugation at 15,000xg for 15 minutes at 4°C. The pellet was washed in 250mM sucrose $T_{10}E_{20}$ buffer, and the mitoplasts were recovered again. Mitoplasts were reconstituted in assay buffers as indicated.

Cloning, Expression and Pruification of Recombinant Sab: A clone containing the cDNA of full-length human Sab was obtained from the Cell-Based Screening core facility of Scripps Florida. The sequence was amplified by PCR using oligonucliotide primers of the following sequences: Forward: 5'-CACCATGGACCAGGGGCTGGAG-3' and Reverse: 5'-TCAGCCAATCTGCACCATTTTT-3'. The amplified sequence was subcloned into a pENTR/SD/D-TOPO entry vector for Gateway cloning (Invitrogen). The resulting entry clone was subcloned into pDEST17 destination vector (Invitrogen), which encodes an N-terminal 6X His fusion tag. The sequence of the construct was confirmed externally (Genewiz). The pDEST17-Sab construct was transformed into the BL21(DE3)Star (Invitrogen) strain of *E. coli.* A 10 mL overnight culture was grown in Luria Broth containing 100 μg/mL ampicillin and this culture was used to innoculate 1L of Overnight Express Instant TB media (Novagen). The culture was grown at 37° C with shaking until OD_{600} reached 0.5, at which point the termperature was lowered to 18° and left overnight. The cells were spun down and stored at -80 °C until use.

The frozen cells were thawed and resuspended in lysis buffer containing 20 mM Tris-Cl, pH 7.4, 500 mM NaCl, 30 mM imidazole, 10% glycerol, 1 mM DTT, 1X Halt protease inhibitor (Thermo Scientific), and 0.02 mg/mL DNAse1 (Sigma) and lysozyme (Sigma). Lysis was aided by sonication in 30 sec. bursts at 30W, with 30 sec. intervals on ice in between bursts. The lysate was centrifuged at 20,000 rpm in a Beckman JA-20 rotor at 4° C. for 30 min. and the supernatant containing His-Sab retained.

His-Sab was purified on an AKTA purifier (GE Healthcare) using a three-column protocol: The supernatant was loaded at 2 mL/min onto a 5 mL HisTrap Ni^{++}Sepharose column (GE Healthcare) and washed with lysis buffer (minus protease inhibitor, DNAse1, and lysozyme)

until UV absorbance at 280 nm returned to baseline. The bound protein was eluted by switching to 100% of elution buffer of the same composition but containing 250 mM imidazole. The eluted fractions were combined and diluted 10X with Buffer A (20 mM Tris-Cl pH 7.4, 5% glycerol, 1 mM DTT) and loaded onto a Mono Q 4.6/100 PE column (GE Healthcare) at 1 mL/min. The column was washed until absorbance returned to baseline and the protein eluted using a linear gradient of 0-100% Buffer B (20 mM Tris-Cl pH 7.4, 5% glycerol, 1 mM DTT, 1M NaCl) over a period of 30 min. The fractions containing His-Sab were determined using SDS-PAGE, combined, and concentrated to 1 mL. The sample was then run over a Tricorn 10/300 column (GE Healthcare) packed with Superdex 200 (GE Healthcare) resin at 0.5 mL/min in Buffer C (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 5% glycerol, 1 mM DTT). The fractions containing His-Sab were concentrated to approx. 3 mg/mL in a Amicon Ultra-4 concentrator (Millipore), flash-frozen in liquid nitrogen, and stored at -80° C until use. SDS-PAGE analysis indicated that the protein was ≥95% pure.

The cDNA for ATF2 (2-115) was cloned into a bacterial expression vector (pET104.1DEST) using the Gateway® technology (Invitrogen, Carlsbad, CA) as a fusion protein with an N-terminal terminal Bioease-Flag-tag for *in vivo* biotinylation. *Escherichia coli* strain BL21(DE3) was transformed with the plasmid for Bioease-Flag-ATF2 [(2-115) B-F-ATF2] expression and a plasmid (pBirAcm) for BirA (biotin protein-ligase A) co-expression. Transformed cells were grown in Luria broth (LB) containing ampicilin and chloramphenicol and protein expression was induced during log phase with 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of 50μM d-biotin for 3 h at 30 °C. Cells were collected by centrifugation and lysed on ice with B-Per reagent (Pierce, Rockford, IL) containing lysozyme, benzonase (Novagen, Gibbstown, NJ) and protease inhibitor cocktail (Sigma, St. Louis, MO). Cell lysate was clarified

by spinning at 20,000 x g for 60 min at 4°C. Obtained supernatant was filtered and loaded onto an anti-Flag M2-antibody column (Sigma, St. Louis, MO) equilibrated with 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% NaN_3. After exhaustively washing column with equilibration buffer, B-F-ATF2 was eluted with 0.1 M glycine HCl pH 3.5 into tubes containing neutralization buffer 1 M Tris pH 8. Fractions with the highest protein concentration were pooled together and concentrated in Amicon Ultra centrifugation devices with 5000 MWCO (molecular weight cut-off). Buffer was exchanged to 15 mM HEPES pH 7.3, 100 mM KCl, 6.25% glycerol, 1 mM dithiothreitol (DTT) using the same centrifugation devices. Purified B-F-ATF2 was stored at -80°C.

Luciferase Assay: The assay below was similarly described by Brasier and Fortin(*4*). The cells were scraped from the culture apparatus, and then lysed in 25 mM glycylglycine, pH 7.8, 1% (v/v) Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA by sonication. Protein was quantitated by BCA assay. The luciferase activity was determined by adding 2mM ATP to 10µg of protein from cell lysates in 15 mM potassium phosphate (pH 7.8), 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA. Data were reported as relative luciferase units normalized to sample protein used for assay.

Nuclear Subcellular Fractionation: Cells (~2.5×10^6) were harvested in ice-cold PBS by gently scraping from the tissue culture surface. Cells were transferred to a microcentrifuge tube and pelleted by centrifugation (1,000xg for 10 minutes at 4°C). Following supernatant aspiration, cells were resuspended in nuclear isolation lysis buffer 1 (10mM HEPES, pH 7.9, 10mM KCl, and 0.1mM EDTA) and incubated on ice for 30 minutes. NP-40 was added to a final concentration of 0.2%, and the mixture was incubated at room temperature for 5 minutes. Samples were then vortexed for 20 seconds, and immediately centrifuged for 1.5 minutes

(4,000xg at 4°C). The pellet was washed once in nuclear isolation lysis buffer 1 followed by centrifugation at 2,000xg for 1.5 minutes at 4°C. The pellet was then resuspended in nuclear isolation lysis buffer 2 (20mM HEPES, pH 7.9, 400mM NaCl, and 1mM EDTA). The suspended pellet was shaken for 60 minutes at 4°C, and the sample was centrifuged at 14,000xg for 20 minutes at 4°C. The final supernatant was retained as the nuclear fraction. The supernatant from the 4,000xg centrifugation step was transferred to a new tube. Save the supernatant as the cytosol/small organelle fraction. Nuclear/cytosolic contamination was evaluated by Western blot analysis for nuclear resident protein Histone H3 and cytosolic marker GAPDH. Only nuclear preparations with less than 5% cytosolic contamination were used for Western blot analysis of JNK translocation to the nucleus.

Mitochondrial Superoxide Detection: MitoSOX Red staining was employed similar to the techniques described in previous studies(*5-7*). Cells were stained with 10μM MitoSOX-Red for microscopy and 100nM dye was used for fluorescence quantitation in 96-well plates. Cells were incubated with dye for 10 minutes prior to washing and visualization. Rotenone treatment (100μM for 4 hours) was used as a positive control for mitochondrial superoxide generation in HeLa cells. To confirm mitochondrial localization of the dye, cells were counterstained with 5μM Mito-Tracker Green (Invitrogen) for 10 minutes. Normalization of fluorescent data was done using counterstaining with Hoescht 33342 (1μM) for 5 minutes. Fluorescent wavelength pairs for the individual dyes were 510nm/580nm for MitoSOX-Red, 490nm/516nm for Mito-Tracker Green, and 350nm/461nm Hoescht 33342 on a Spectromax M5e plate reader (Molecular Devices). The data is presented as normalized mitochondrial superoxide, as the superoxide produced was corrected for cell number.

Mitochondrial Membrane Potential: JC-1 staining was used to determine mitochondrial membrane potential as described by Ryan *et al(8)*. Mitochondrial membrane potential was determined using JC-1 staining in a 96-well plate format. Cells were grown to ~80% confluency and stained with 2μM JC-1 for 10 minutes following anisomycin treatment. Fluorescence was monitored for green fluorescence (depolarization) at 488nm and red fluorescence (polarized) at 590nm on a Spectromax M5e plate reader (Molecular Devices). Actively growing cells were used as a negative control, while cells treated with 2mM FCCP and 1mM valinomycin served as the positive control. Normalization to cell number was achieved by Hoechst 33342 staining described earlier. Data are presented as percent cells viable based on presumed 95-100% depolarization observed in FCCP/valinomycin-treated cells.

Cell Viability: Cell viability was monitored using trypan blue exclusion using the BioRad TC10 Automated Cell Counter. Briefly, cells were trypsinized and then pelleted by centrifugation. Cell pellets were gently resuspended in Accumax cell counting solution. Cells (10μL) were combined with 10μL 0.4% Trypan Blue dye (BioRad). The cells and dye were mixed gently. Cells were loaded onto a TC10 slide and counted. Data is displayed as percent cells viable. The data was validated using SYTOX Green (Invitrogen) nucleic acid staining in a 96-well plate format (Data not shown).

Cell Staining: HeLa cells were incubated with 1μM DAPI for 5 minutes at 37°C. HeLa cells were stained with 5μM MitoTracker Red for 15 minutes at 37°C. Cells were washed and covered in cell imaging buffer (described above) and visualized by fluorescence microscopy.

Amplex Red Detection of Hydrogen Peroxide: For the detection of mitochondrial produced ROS in the presence of respiratory substrates (2mM glutamine/malate/ADP) and active JNK1α1, we

employed an amplex red detection method using the Amplex Red Hydrogen Peroxide Detection kit protocol from Invitrogen. Briefly, 20μg of isolated mitochondria were incubated with 30μg of JNK1α1 for 15 minutes at 30°C. The mitochondria/JNK mixture was then incubated with assay buffer and Amplex Red for 30 minutes at 30°C. Hydrogen peroxide production by mitochondria was measured by fluorescence at 571nm (excitation) and 585nm (emission).

II. Supplemental Figure Legends:

Supplemental Figure 1: JNK translocated to the outer mitochondrial membrane during stress. (A) Proteinase K treatment of mitochondria isolated from HeLa cells stressed with 25μM anisomycin for 30 minutes demonstrated that JNK is on the mitochondria. Digestion of the proteins on the outer mitochondrial membrane with Proteinase K indicated that JNK was present on the outer mitochondrial membrane, as it is not recovered with the Proteinase K-treated mitochondria. (B) Mitochondrial JNK was found in the mitochondrial outer membrane enrichment of HeLa treated with 25μM anisomycin for 30 minutes. Mitoplasts isolated from mitochondrial enrichments do not possess mitochondrial JNK; however, hyponically removed outer mitochondrial membrane fractions demonstrate JNK's presence by western blotting.

Supplemental Figure 2: FITC-Tat-Sab$_{KIM1}$ peptide demonstrated cellular permeability. (A) HeLa cells were treated with 3μM FITC-Tat-Sab$_{KIM1}$ for 1 hour, and then cells were stained with MitoTracker Red (Invitrogen) and DAPI. Cells demonstrated that FITC-Tat-Sab$_{KIM1}$ was throughout the cell, and did not demonstrate any distinct subcellular localization. (B) Cellular stability of the FITC-Tat-Sab$_{KIM1}$ peptide was monitored by peptide fluorescence for 24 hours. At 24 hours, greater than 90% of the fluorescent signal was still detectable in HeLa cells.

Supplemental Figure 3: Mitochondrial priming was required for mitochondrial JNK induction of ROS generation and MMP dissipation. Mitochondrial were isolated from JNK-null MEFs following 25μM anisomycin treatment for 0, 5,10, 15,20, 30, 45, and 60 minutes. Mitochondria were then incubated with 50ng active JNK1α1 for 60 minutes at 30°C, and (A) mitochondrial reactive oxygen species generation was monitored by Amplex Red assay for hydrogen peroxide (Invitrogen) and (B) mitochondrial membrane potential was measured with JC-1 fluorescence.

Supplemental Figure 4: Tat-Sab$_{KIM1}$ prevented JNK kinase activity on the mitochondria. Mitochondria isolated from JNK-null MEFs treated with DMSO or 25μM anisomycin for 30 minutes. Mitochondria were pre-incubated with 10μM Tat-Scramble, 10μM Tat-Sab$_{KIM1}$, or 1μM Tat-TI-JIP for 10 minutes, and then treated with 50ng of active JNK1α1 for 60 minutes at 30°C. JNK kinase activity was determined using a Kinase-Glo Assay (Promega).

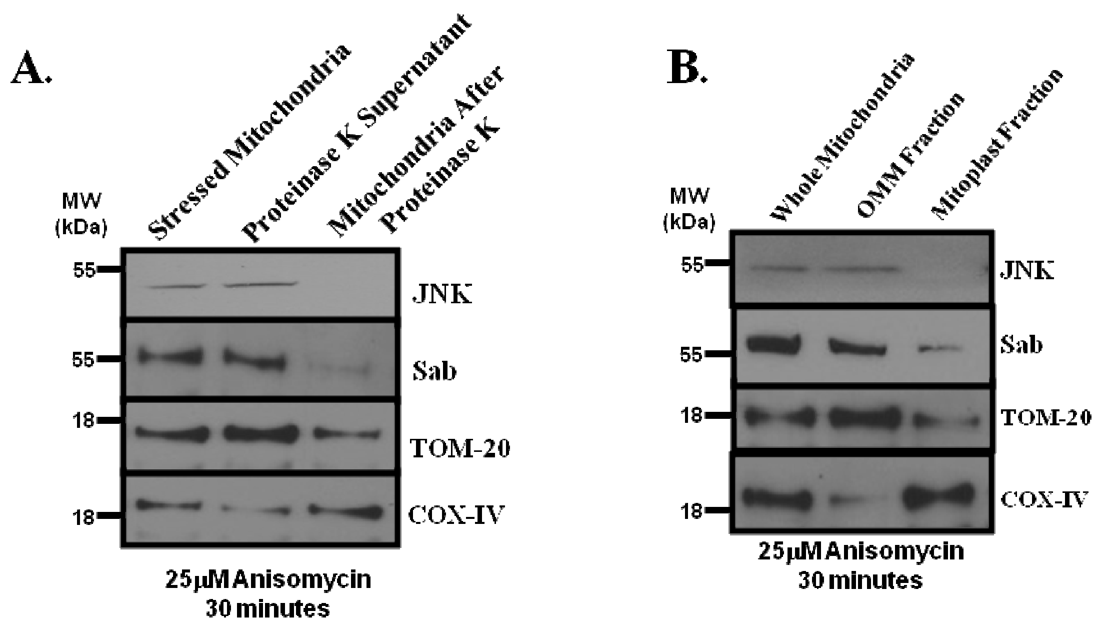
Supplemental Figure 5: Tat-Sab$_{KIM1}$ does not inhibit JNK1α1 phosphorylation of ATF2. Active JNK1α1 was incubated with increasing amounts of TI-JIP (dashed line) and Tat-Sab$_{KIM1}$ (solid line). The substrate ATF2 (1μM) was then added to the kinase assay. JNK activity was determined using the Kinase-Glo Assay (Promega).

Supplemental Figure 6: c-jun knockdown did not impact JNK-induced mitochondrial dysfunction during anisomycin stress in HeLa cells. (A) Silencing c-jun was done by transfecting HeLa cells with 50nM c-jun-specific siRNAs. Knockdown was greater than 80% as indicated by western blotting, and control siRNAs had no impact on c-jun expression as compared to mock transfected cells. (B) HeLa cells incubated with 25μM anisomycin for 40 minutes had elevated levels of superoxide generation as indicated by MitoSOX Red fluorescence. Moreover, c-jun knockdown had no impact on superoxide generation. (C) Mitochondrial membrane potential was

also measured by JC-1 fluorescence at 60 minutes following 25μM anisomycin treatment. Similar to superoxide generation, silencing c-jun had a small impact on the dissipation of mitochondrial membrane potential. (D) Silencing c-jun increased cell viability (by ~15%) at 4 hours following 25μM anisomycin stress compared to mock and control siRNA transfected cells.

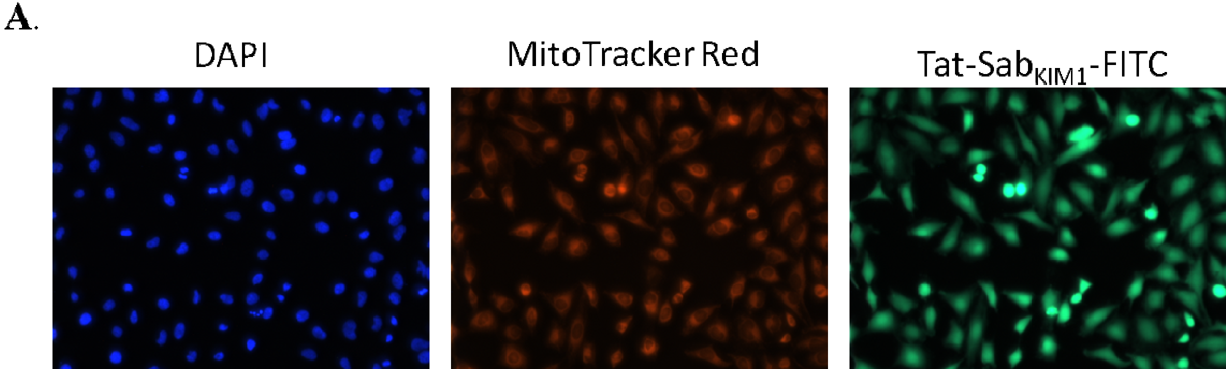
III. Supplemental Figures

Supplemental Figure 1



Supplemental Figure 2

A.



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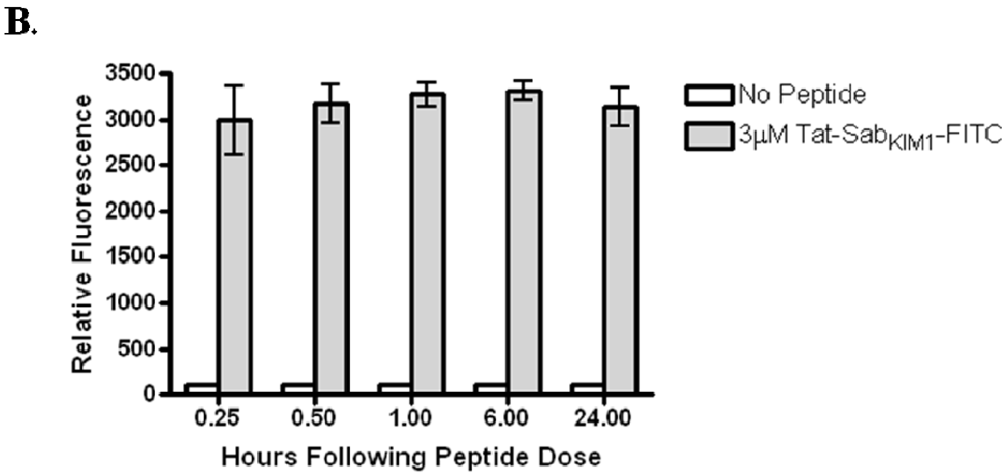
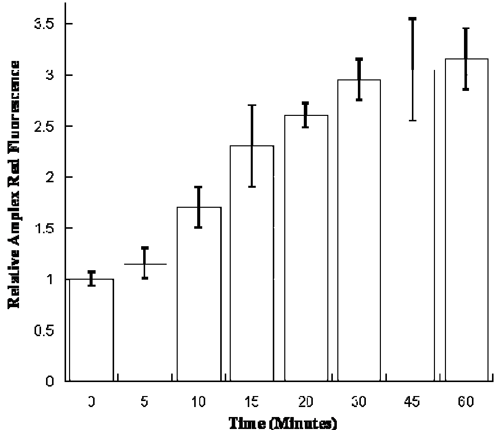


Supplemental Figure 3

A.



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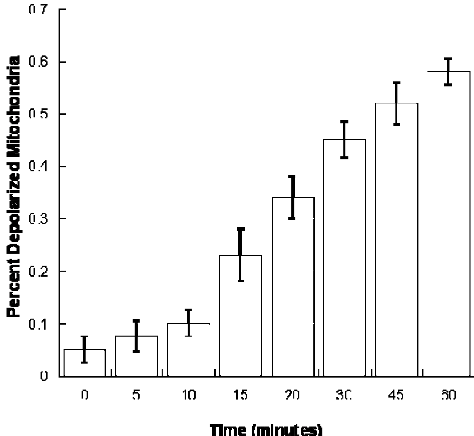
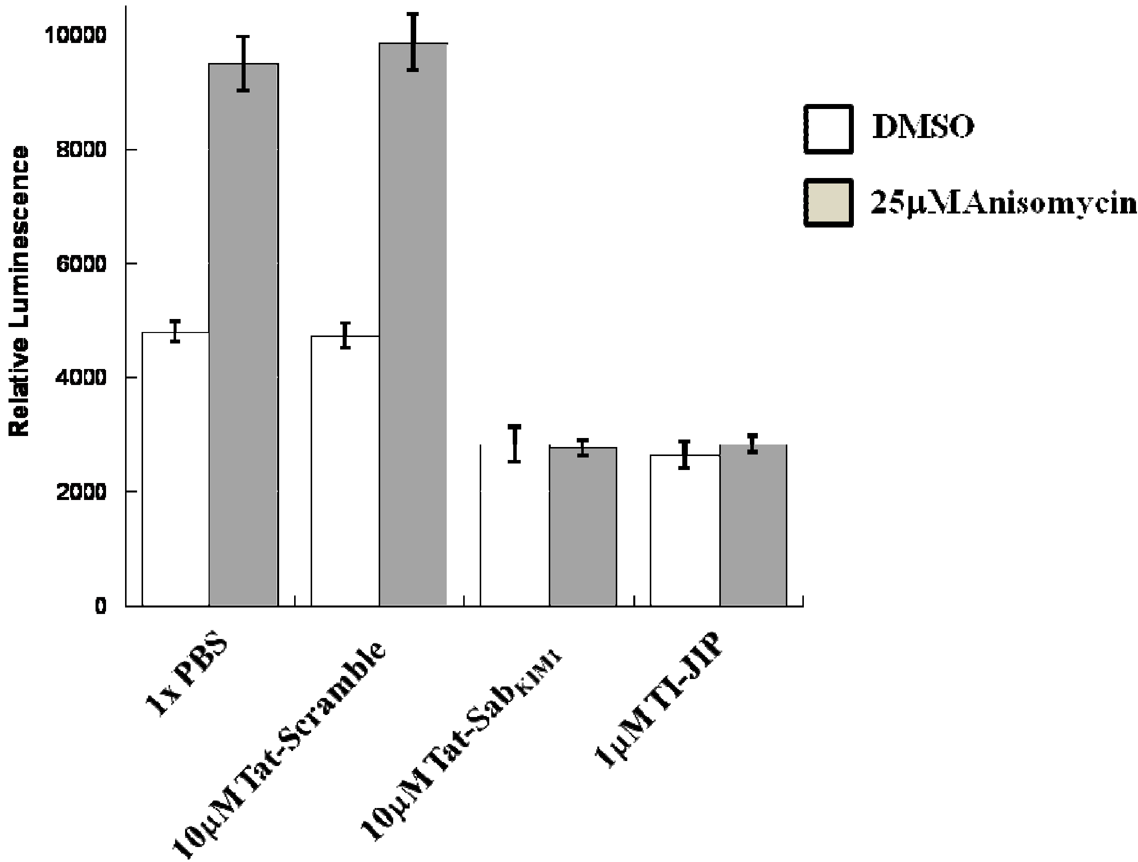
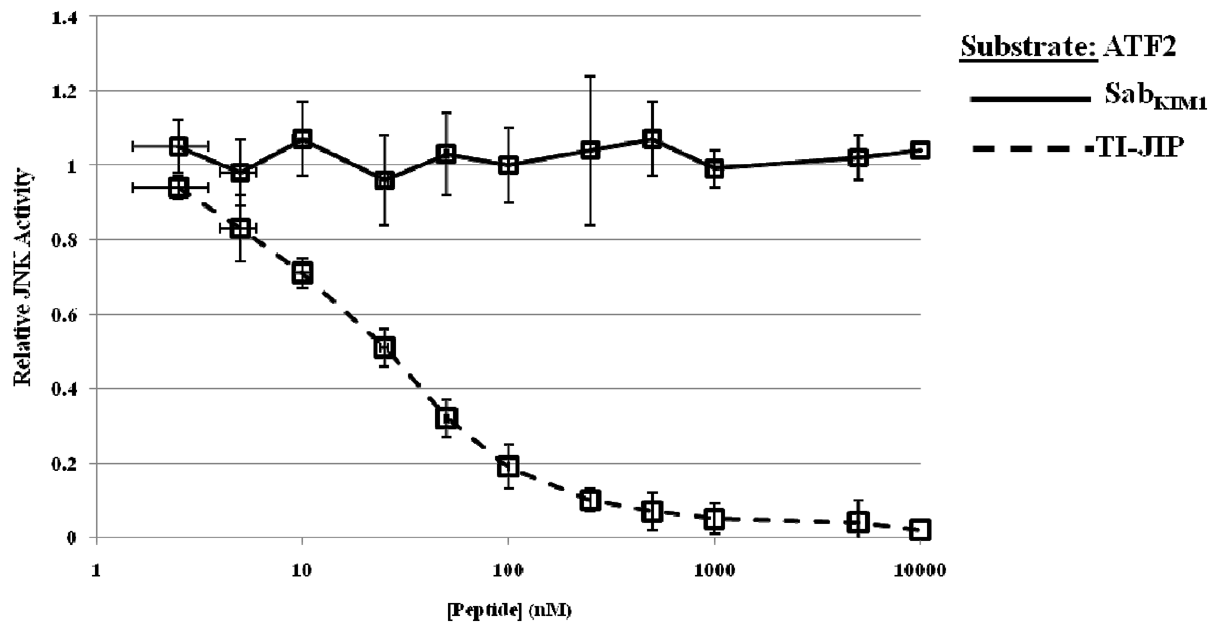


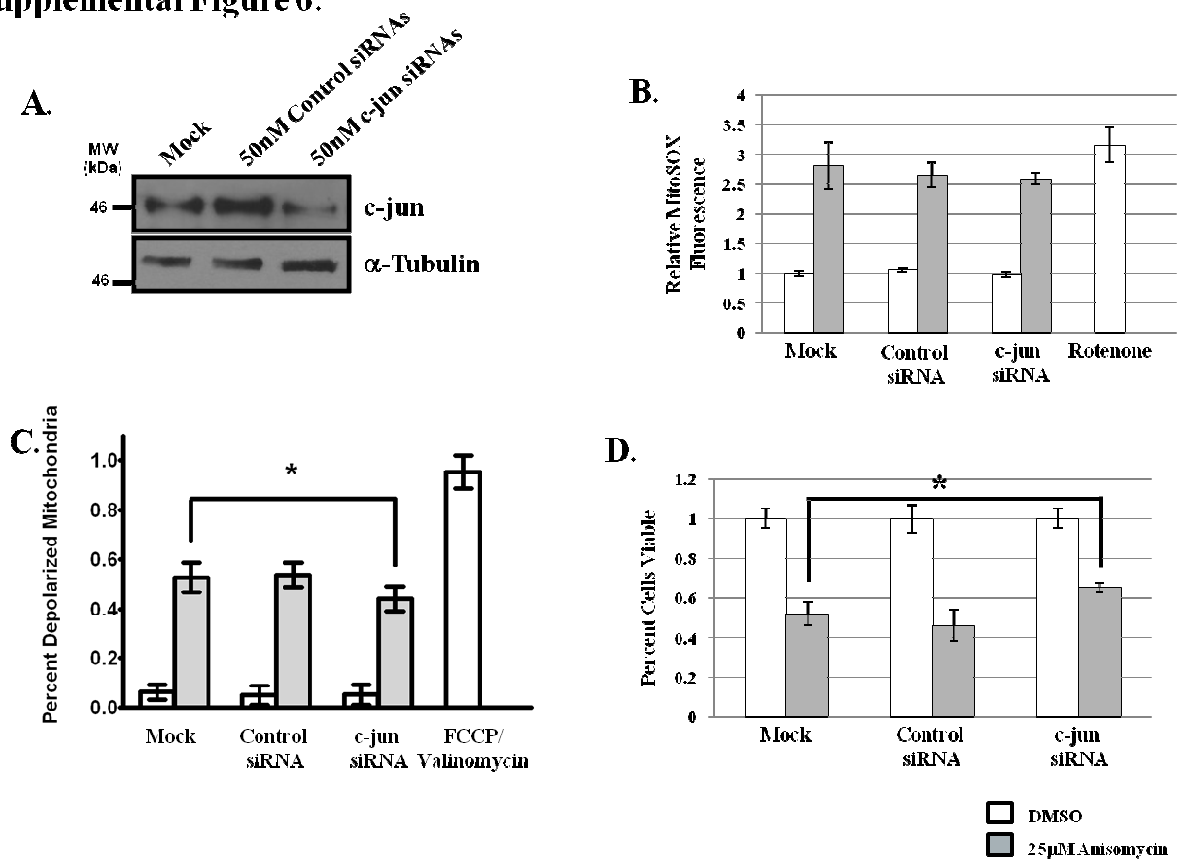
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



IV. Supplemental Table

Supplemental Table S1: Peptide Inhibition Data for JNK-interacting Peptides for JNK1 α1.

Peptide	IC_{50} -vs- Sab	IC_{50} -vs- c-jun	IC_{50} -vs- ATF2
TI-JIP	22±10nM	32±8nM	43±14nM
Sab_{KIM1}	270±85nM	~10% at 10μM	n.i.*
Scramble	n.i.*	n.i.*	n.i.*

* The peptide demonstrated less than 10% inhibition at a concentration of 10μM. IC_{50}s were determined using a Kinase-Glo assay and data were fit to a 4-parameter nonlinear regression model..

V. References

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