Supplemental Information

JNK interaction with Sab mediates ER stress induced inhibition of mitochondrial respiration and cell death

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SUPPLEMENTAL METHODS

Hela cell culture, shRNA mediated gene silencing, and measurement of oxygen consumption—Hela cells were cultured with DMEM supplemented with 10% FBS, L-glutamine and penicillin/streptomycin in 5% CO_2 humidified 37C incubator. To knockdown Sab, Hela cells were incubated with purified Ad shSab at MOI 10 in culture medium for 7-10 days. shSab sequence used are 5'CACCGGATGACAAGCGGCAGTTTGACGAATCAAACTGCCGCTTGTCATCC -3' and 5'- AAAAGGATGACAAGCGGCAGTTTGATTCGTCAAACTGCCGCTTGTCATCC-3'. XBP1(S) (spliced form) and XBP1 mRNA from Hela cells was determined by primers ACAGCGCTTGGGGAT GGATGC & CCATGGGGAGATGTTCTGGG. To measure oxygen consumption by Seahorse analyzer, Hela cells were seeded for 24 hours and oxygen consumption was measured in unbuffered modified DMEM with L-glutamine 2mM.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. JNK- Sab dependent inhibition of mitochondrial respiration and hepatocellular death by Brefeldin A induced ER stress

(A) PMH were treated with brefeldin A (BFA) 10 - 20μg/ml with or without JNK inhibitor (SP600125) pretreatment. Cell death was determined by SYTOX-green staining at 24hours after BFA treatment. Dose dependent cell death induced by BFA was prevented by JNKi. #*P*<0.05 versus DMSO; **P*<0.05 versus without JNKi. **(B)** Early after BFA treatment, mitochondrial oxidative-phosphorylation and reserved capacity were inhibited. **P*<0.05 versus DMSO. **(C)** BFA activation of JNK at 1-2 hr after treatment was inhibited by SP600125. **P*<0.05 versus without JNKi. **(D)** Knockdown of Sab by shRNA prevented the sustained activation of JNK after BFA treatment. **P*<0.05 shlacZ versus shSab. **(E)** Knockdown of Sab prevented BFA induced cell death. **P*<0.05 shlacZ versus shSab. **(F)** Knockdown of Sab did not affect BFA induced ER stress as shown by XBP1 splicing (XBP1(s)). **(G)** Knockdown of Sab prevented inhibition of mitochondrial respiration by ER stress induced by BFA 10μg/ml. The data are presented as means ± S.D of five wells of each sample for each experiment, **P*<0.05 versus DMSO, N = 3 experiments.

Figure S2. Tunicamycin induced ER stress and inhibition of mitochondrial respiration in Hela cells was JNK-Sab mediated

(A) Hela cell death induced by tunicamycin (10-30μg/ml) at 24 hours was determined by SYTOX-green staining. #*P*<0.05 versus DMSO and **(B)** cell death was prevented by JNKi. **P*<0.05 versus without JNKi. **(C)** JNKi prevented tunicamycin induced JNK sustained activation. **P*<0.05 versus

without JNKi. **(D)** Knockdown of Sab by shRNA prevented the sustained activation of JNK after tunicamycin treatment. *P<0.05 shlacZ versus shSab. **(E)** Knockdown of Sab prevented tunicamycin induced Hela cell death. **(F)** Knockdown of Sab in Hela cells did not affect tunicamycin induced ER stress as shown by XBP1 splicing (XBP1(s)).

Figure S3. Brefeldin A induced ER stress and inhibition of mitochondrial respiration in Hela cells was JNK-Sab mediated

(A) Hela cell death induced by BFA (10-30μg/ml) at 24 hours was determined by SYTOX-green staining. #P<0.05 versus DMSO and **(B)** cell death was prevented by JNKi. *P<0.05 versus without JNKi. **(C)** JNKi prevent BFA induced JNK sustained activation. *P<0.05 versus without JNKi. **(D)** Knockdown of Sab by shRNA prevented the sustained activation of JNK after BFA treatment. *P<0.05 shlacZ versus shSab. **(E)** Knockdown of Sab prevented BFA- induced Hela cell death. *P<0.05 shlacZ versus shSab. **(F)** Knockdown of Sab in Hela cells did not affect BFA induced ER stress as shown by XBP1 splicing (XBP1(s)).

Figure S4. Tunicamycin or brefeldin A induced ER stress inhibited mitochondrial respiration in Hela cells which depends on presence of mitochondrial protein Sab

Hela cells cultured with adenoviral shlacZ or shSab (MOI 10) for 7-10days were plated overnight. Oxygen consumption was measured before and after tunicamycin or BFA 10μg/ml injection into unbuffered modified DMEM with L-glutamine 2mM. Tunicamycin or BFA inhibited mitochondrial oxidative phosphorylation and reserved capacity of Hela cells. Knockdown of Sab prevented these changes. The data are presented as means ± S.D of five wells of each sample for each experiment, *P<0.05 shlacZ versus shSab, N = 3 experiments.

Figure S5. Measurement of anaerobic glycolysis in tunicamycin 20µg/ml treated cells.

Cellular anaerobic glycolysis capacity was determined by EACR (extra cellular acidification rate) in Seahorse XF24 analyzer simultaneously while measuring OCR. The EACR was quantified after CCCP injection. EACR was decreased in tunicamycin 20µg/ml treatment at 1 hour (not shown) and 2 hour. JNKi did not prevent tunicamycin inhibition of EACR and thus anaerobic glycolysis, **P*<0.05, N = 3 experiments.

Figure S6. Effect of Sab knockdown on tunicamycin induced cytochrome c release

Mice were tail vein injected with shlacZ or shSab adenovirus and 10 days later PMH were isolated and cultured cells were treated with tunicamycin 20µg/ml and fixed with 3% NBF and immunofluorescence staining of cytochrome c was performed. Mitochondrial localization of cytochrome c was observed in DMSO treated cells. After tunicamycin treatment, cytochrome c was released into cytosol in shlacZ hepatocytes, but cytochrome c release was prevented in Sab knocked down hepatocytes. Representative images were at 4 hours after tunicamycin treatment.

Figure S7. Effect of tunicamycin on isolated mitochondrial respiration

OCR of isolated mitochondria was determined as before (Rogers et al., 2011). Mitochondria was isolated and resuspended in respiration buffer with pyruvate/malate substrate. 10µg of mitochondria / well in 50µl buffer in 24 wells XF24 cell culture microplates was spun at 2000xg for 20 minutes at 4°C. After centrifugation, 450µl of respiration buffer was added to each well. Tunicamycin 10-20µg/ml (final) was added to medium. ADP (4 mM final) from port A, oligomycin (2.5 mg/ml final) from port B, CCCP (4 mM final) from port C, Antimycin

A (4 mM final) from port D were injected. State III respiration after ADP injection and state IV respiration after oligomycin injection were same as DMSO vs tunicamycin treated mitochondria.

Figure S8. Effect of CaMKII inhibitor KN93, Ca^{2+} chelator BAPTA-AM, mitochondrial Ca^{2+} uniporter inhibitor Ru360 on tunicamycin 20µg/ml induced JNK activation

(A) KN93 did not prevent Tm20 induced sustained JNK activation or p-PERK. CHOP expression was decreased somewhat at later time (8hr). **(B)** BAPTA-AM did not block JNK, PERK activation, or CHOP expression. **(C)** Neither KN93, BAPTA-AM, Ru360 nor IP3R inhibitor (Xestospongin D) preincubation prevented tunicamycin 20µg/ml induced inhibition of mitochondrial respiration at 1hr incubation. Thus, inhibition of mitochondrial respiration by tunicamycin was not mediated by Ca^{2+}. Release of Ca^{2+} from ER store by IP3 treatment alone for 1hr significantly increased mitochondrial oxidative-phosphorylation compared to DMSO control, #$p<0.05$ versus DMSO. Mitochondrial reserve oxidative capacity was decreased without significant change in cellular OCR and proton leak. Thus physiological ER Ca^{2+} release activates and expands the respiring pool of mitochondria. On the other hand, IP3R inhibitor alone did not change OCR significantly compared to DMSO control. Neither IP3 (D-myo-Inositol-1,4,5-triphosphate) nor IP3R inhibitor prevented tunicamycin 20µg/ml inhibition of cellular and mitochondrial respiration; *$p<0.05$ versus DMSO. IP3 was permeabilized to PMH as described before (Streb et al., 1983).

To confirm these findings, PMH were preloaded with intracellular BAPTA-AM and treated with tunicamycin 20µg/ml for 1hr and mitochondrial oxidative-phosphorylation was measured. Irrespective to intracellular Ca^{2+} level, mitochondrial oxidative phosphorylation was

inhibited by severe ER stress induced by tunicamycin 20μg/ml. Similar to this finding, mitochondria Ca^{2+} uptake blocker (Ru360) and cytoplasmic Ca^{2+} sensitive kinase CaMKII blocker (KN93) did not prevent tunicamycin 20μg/ml inhibition of mitochondrial oxidative-phosphorylation. Thus tunicamycin inhibits mitochondrial respiration through a signal other than changes of intracellular Ca^{2+}. Of note, P-CaMKII (Thr286) activation in PMH by tunicamycin 20μg/ml treatment was seen only at 4 and 8 hours but not at 1 hour incubation (data not shown) (Timmins et al., 2011). Thus, involvement of P-CaMKII in tunicamycin 20μg/ml induced inhibition of mitochondrial respiration was unlikely, especially in initial phase of P-JNK activation.

SUPPLEMENTAL REFERENCE

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