

Supporting Information

Figure S1 – Schematic diagram for the generation of S49 (OS 4-15) cells. S49 (Neo) cells were treated with 500 mOsm mannitol for 4 hours followed by a period of recovery in normal media. Each round of mannitol treatment required 7 to 14 days to generate a viable population of cells. Successive rounds of mannitol treatment were carried out for 15 rounds to generate the S49 (OS 4-15) cells.

Figure S2 - Analysis of phos-ERK in the S49 (Neo) and S49 (OS 4-15) cells upon hyperosmotic stress. Changes in ERK phosphorylation was examined over time after an acute exposure to 250 mM mannitol. While an overall increase in phos-ERK was observed in the S49 (OS 4-15) cells, compared to the S49 (Neo) cell, no significant difference was apparent between time-matched samples. PMA was included the analysis as a positive control.

Figure S3- Analysis of phos-PDK1 and phos-mTOR in the S49 (Neo) and S49 (OS 4-15) cells upon hyperosmotic stress. Changes in PDK1 and mTOR phosphorylation were examined over time after an acute exposure to 250 mM mannitol. Active phos-PDK1 was observed at the early times after hyperosmotic stress, while active phos-mTOR was observed at a later time in S49 (OS 4-15) cells, suggesting both kinases may play a role in the sustained phosphorylation of AKT.

Figure S4 – Flufenamic acid does not inactive AKT in the presence or absence of osmotic stress. S49 (OS 4-15) cells were treated with 250 mOsm mannitol for various times in the presence and absence of 200 μ M flufenamic acid (FFA) and examined for phospho-AKT. A sustained increase in AKT activity was observed in the S49 (OS 4-15) cells regardless of the presence of FFA, suggesting that FFA does not direct effect AKT activity.

Figure S5 – Various non-steroidal anti-inflammatory drug family members and inhibitors of the Na^+/H^+ exchanger do not induce apoptosis in S49 (OS 4-15) cells. Since FFA, a nonsteroidal anti-inflammatory drug, is known to also induce the mitochondrial permeability transition (MPT) and promote mitochondrial calcium efflux (1,2), we examined the effect of other drugs in this family known not have an effect on cell volume regulation as to their ability to sensitize S49 (OS 4-15) cells to undergo cell death. Both mefenamic acid (MFA; 150 μ M final) and sodium salicylate (SS; 1 mM final) had no effect in inducing the apoptotic program. This suggests that FFA was not acting solely as a un-coupler and inducer of the mitochondrial membrane potential, but was working through an inhibition of the newly gained RVI response to activate the cell death program upon hyperosmotic stress. Additionally, 5-(N-methyl-N-isobutyl)-amiloride (MIA; 100 μ M final) and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 100 μ M final), known inhibitors of the Na^+/H^+ exchanger did not sensitize the S49 (OS 4-15) cells to undergo apoptosis. Additionally, we show that neither MIA nor EIPA prevent the RVI response on S49 (OS 4-15) cells. Data represents the mean (\pm SEM) of at least 3 independent experiments, *, $p < 0.01$ versus control.

Supporting References

1. Jordani, M.C., Santos, A.C., Prado, I.M.R., Uyemura, S.A., and Curti, C. (2000) *Mol. Cell. Biochem.* **210**, 153-158
2. Gardam, K.E., Geiger, J.E., Hickey, C.M., Hung, A.Y., and Magoski, N.S. (2008) *J. Neurophysiol.* **100**, 38-49