SUPPLEMENTARY DATA

Supplementary Research design and methods

Western Immunobloting

Total cell extracts were prepared using RIPA buffer. Briefly, ST-2 cells were washed twice with ice cold PBS and then the cells were lysed using ice cold modified RIPA buffer (50mM Tris-HCl pH=7.4, 1% NP-40, 0.25% Na deoxycholate, 150 mM Nacl, 1mM EDTA, 1mM PMSF, 1μg/ml of aprotinin, leupeptin and pepstatin, 1mM Na3VO4, 1mM NaF). Protein electrophor esis was performed in a 12% acrylamide gel and immunoblotting was performed as previously described(1). The primary antibodies used were anti-Nrf2 C-20 (Santa Cruz, Santa Cruz, CA), anti-α-tubulin T8203 (Sigma, St Louis, MO) and the secondary HRP conjugated antibodies (mouse #7076 and rabbit #7074S) were from Cell Signaling Technology (Beverly, MA).

Fluorescence staining of ST-2 cells

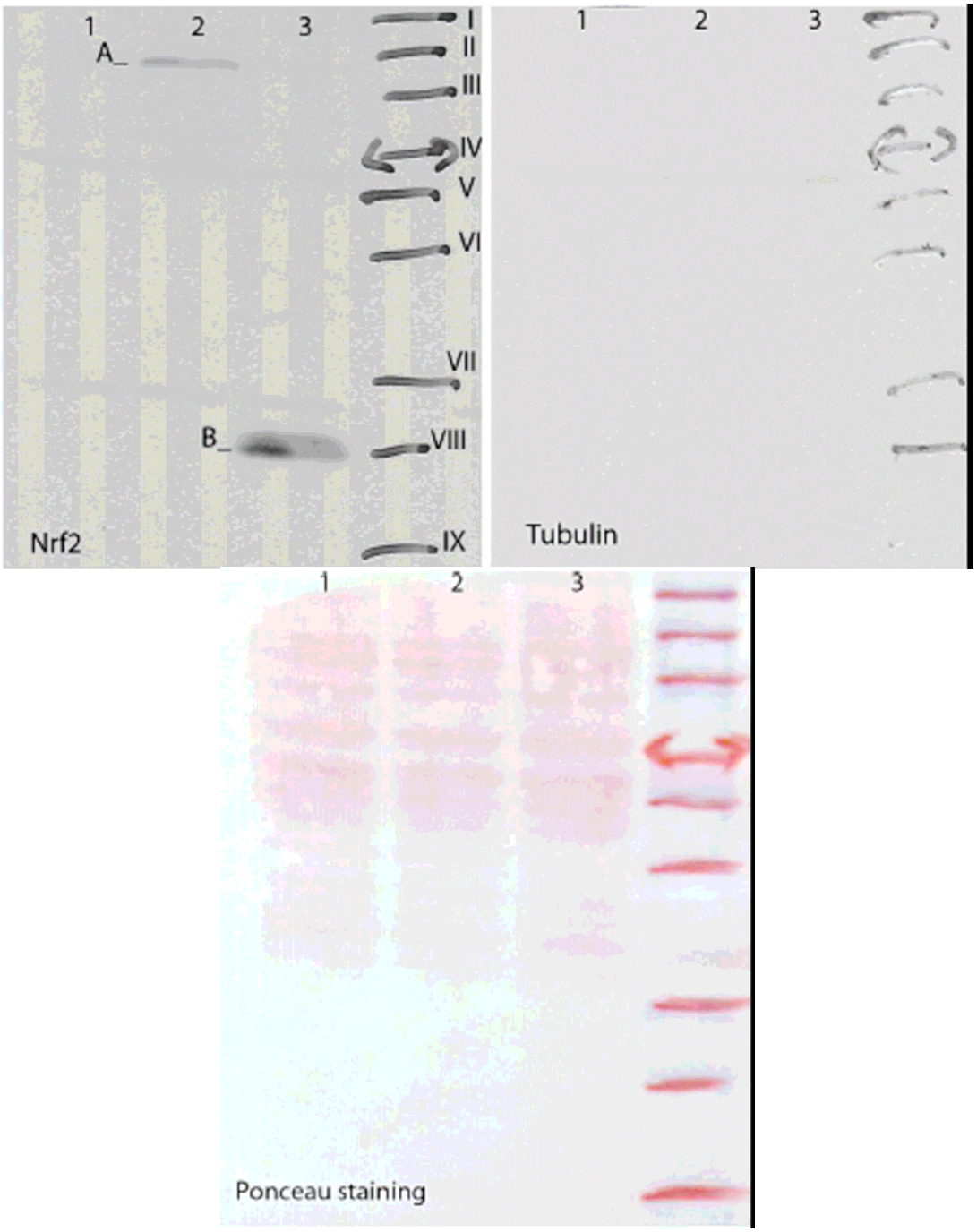
Cells were grown and transfected on sterile glass coverslips. Before the staining procedure, they were washed briefly with PBS 1x and then they were fixed with cold 4% paraformaldehyde (in PBS pH=7.4) for 10 minutes. After three 5-minute washing steps with PBS 1x (the first washing step with PBS 1x 1% glycine), they were incubated for 10 minutes with 1% Triton-x 100 in PBS 1x. Then, after three 5-minute washing steps with PBS 1x, cells were mounted in Vectashield Mounting Medium with DAPI (Vector labs, Burlingame, CA). Cells were observed and photographed using the Nikon Eclipse TE2000-U microscope of the Advanced Light Microscopy facility of the Medical School, University of Patras.

Transfection of ARE-ST2 cells

ARE-ST2 cells are ST-2 cells that stably express a plasmid (pGL2-basic-luc) that bears the Antioxidant Response Element (ARE) sequence upstream of the luciferase gene. Generation of these cells has been described previously (2) . The ARE sequence is found in the promoter of Nrf2 target genes such as NQO1 (NADPH quinine oxidoreductase, HO-1 (Heme Oxygenase 1) etc. Thus, any increase in the transcriptional activity of Nrf2 is translated into increased luciferase activity. In this study, we transfected ARE-ST2 cells with Nrf2, DN-Nrf2 or empty vector under the conditions the transfection experiments with ST-2 cells were performed (in figure 6 of the main text).

SUPPLEMENTARY DATA

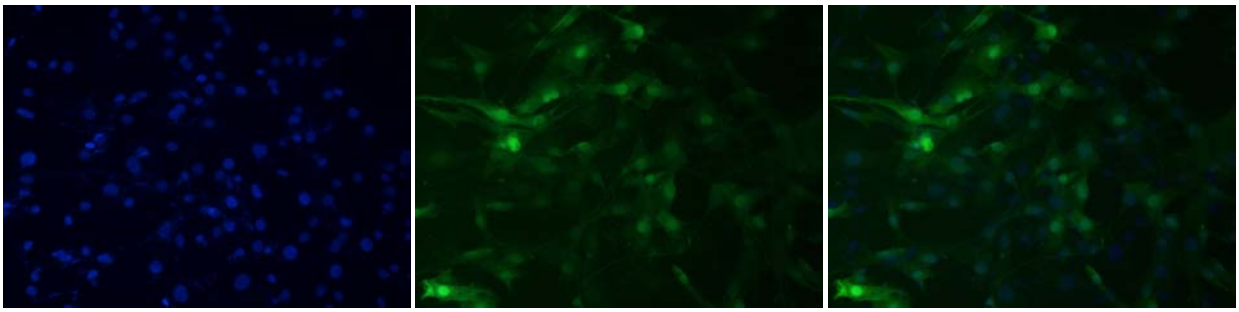
Supplementary Figure 1. Nrf2 and DN-Nrf2 protein expression after transfection of their corresponding plasmids in ST-2 cells.
Total protein extracts from ST-2 cells transfected with Nrf2 (lane 2), DN-Nrf2 (lane 3) or empty plasmid (lane 1). Nrf2 expression was assayed by Western Immunoblotting. Tubulin was employed as a loading control. Ponceau staining of the nitrocellulose membrane was employed as a loading control as well. Due to low exposure time (1 min) endogenous Nrf2 is not detected in lane 1. Band A: Nrf2 band, Band B: DN-Nrf2 band. Bands of the protein ladder, I: 181.8 kDa, II: 115.5 kDa, III: 82.2 kDa, IV: 64.2 kDa, V: 48.5 kDa, VI: 37.1 kDa, VII: 25.9 kDa, VIII: 19.4 kDa, IX: 14.8 kDa.



SUPPLEMENTARY DATA

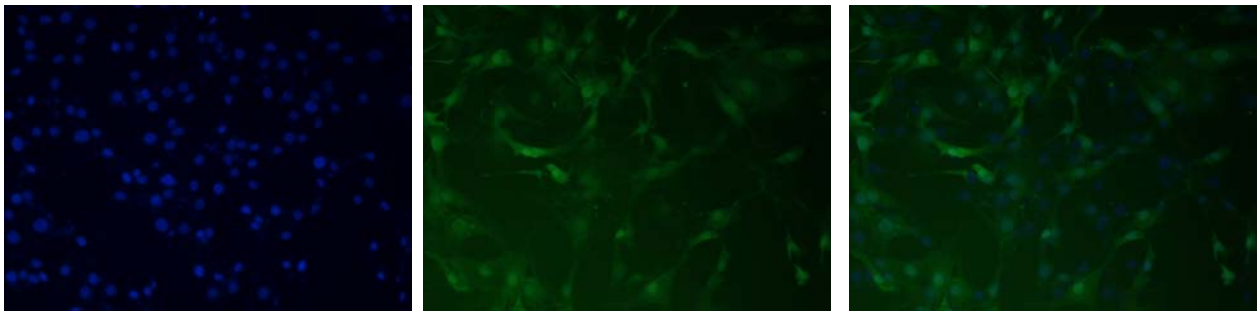
Supplementary Figure 2. Transfection efficiency of ST-2 cells.
To check the efficiency of the delivery of plasmids in our experimental settings, ST-2 cells were transfected with pcDNA3-EGFP plasmid along with the plasmids used in the transfection experiments described in figure 6 of the main text. Then, the cells were stained with Dapi and were observed and photographed under UV microscope. The percentage of cells transfected is about the same (70%) in each experiment, consistent with the expected transfection efficiency of lipofectamine.

Empty vector



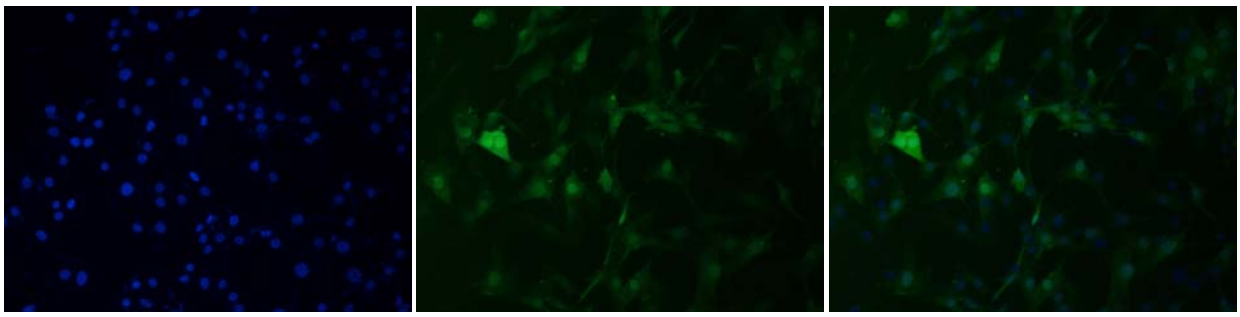
Dapi GFP Merge (Dapi+GFP)

Nrf2 plasmid



Dapi GFP Merge (Dapi+GFP)

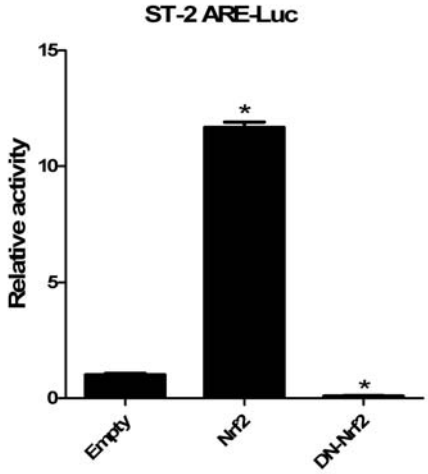
DN-Nrf2 plasmid



Dapi GFP Merge (Dapi+GFP)

SUPPLEMENTARY DATA

Supplementary Figure 3. Transcriptional activity of Nrf2 in ARE-ST2 cells after transfection with Nrf2, DN-Nrf2 or empty plasmid. Data show means±SEM. *p<0.001 compared to empty vector. The results are indicative of three different experiments performed on three different days. The statistical analysis was performed using ANOVA with Tukey's post-test.
Transfection of Nrf2 increases Nrf2 transcriptional activity more than 10-fold while transfection of DN-Nrf2 almost eliminates Nrf2 transcriptional activity.



Supplementary References

1. Ziros PG, Gil AP, Georgakopoulos T, Habeos I, Kletsas D, Basdra EK, Papavassiliou AG: The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. *J Biol Chem* 277:23934-23941, 2002
2. Habeos IG, Ziros PG, Chartoumpekis D, Psyrogiannis A, Kyriazopoulou V, Papavassiliou AG: Simvastatin activates Keap1/Nrf2 signaling in rat liver. *J Mol Med* 86:1279-1285, 2008