

## SUPPLEMENTAL MATERIALS:

### Methods

**Erythrocyte membrane immunoblots:** Following erythrocyte stress assays, samples were spun at 1500xg for 10 minutes at 4°C and the supernatant discarded. From the pellet, packed erythrocytes were washed two times with chilled ghosting<sup>1</sup> lysis buffer<sup>1</sup> containing protease and phosphatase inhibitors (5 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 15.4 mmol/L NaN<sub>3</sub>, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 mmol/L sodium pyrophosphate, 1 mmol/L sodium fluoride; pH 8.0). Wash steps included 30 minutes rocking at 4°C, followed by a spin (3000 xg, 15 minutes, 4°C). Following the second wash, chilled radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Sigma Aldrich; cat. no R0278; plus 1 mmol/L PMSF, 2 mmol/L sodium pyrophosphate, 1 mmol/L sodium fluoride) was added to the ghost pellet and vortexed. Protein level was quantified with the Pierce BCA Protein Assay Kit (ThermoFisher; cat. no 23225).

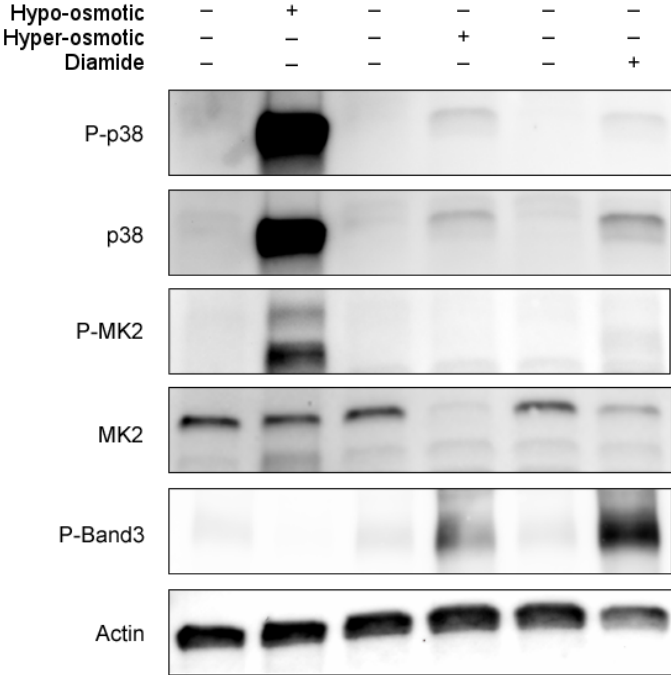
**Immunoblotting:** Protein samples were loaded together with 4x Laemmli Sample Buffer (Bio-Rad; cat. no 1610747) into Any kD Mini-PROTEAN TGX precast gels (Bio-Rad; cat. no 4569034). Proteins were transferred onto 0.2 µm PVDF membranes (Bio-Rad; cat. no 1704156) using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc.). Membranes were then blocked with either SuperBlock (TBS) Blocking Buffer (ThermoFisher; cat. no 37537) or Blocker BLOTTO in TBS (ThermoFisher; cat. no 37530) for 1 hour at room temperature and then washed 3 x 10 minutes in tris-buffered saline with 0.05% Tween-20 (TBST; ThermoFisher; cat. no 28360). The membranes were rocked with primary antibodies diluted as indicated at 4°C overnight. The following primary antibodies were used: anti-Band3 (Cell Signaling Technologies (CST); cat. no 23276; 1:1000), anti-phosphorylated (p)-Band3 (1:1,000; Abcam; cat. no ab77236), anti-beta actin (1:1000; CST; cat. no 3700), anti-MK2 (1:1000; CST; cat. no 3042), anti-p-MK2 (1:1000; CST; cat. no 3007), anti-MKK3 (1:1000; CST; cat. no 8535), anti-p-MKK3/p-MKK6 (1:1000; CST; cat. no 12280), anti-p38 (1:1000; CST; cat. no 8690), anti-p-p38 (1:1000; CST; cat. no 4511), anti-RSK2 (1:1000; CST; cat. no 5528), anti-p-RSK2 (1:1000; CST; cat. no 3556), anti-p-Src (1:1000; CST; cat. no 6943). Membranes were then washed 3 x 10 minutes in TBST before rocking in secondary antibodies at room temperature for 1 hour. The following secondary antibodies were used: anti-mouse IgG-Dylight 680 (CST; cat. no 5470; 1:15,000) and anti-rabbit-horseradish peroxidase (HRP) (CST; cat. no 7074; 1:10,000). Membranes went through final wash steps of 3

x 10 minutes in TBST followed by 2 x 5 minutes in PBS. For membranes that were blotted with an HRP-conjugated secondary antibody, protein bands were activated using Clarity Max Western ECL Substrate (Bio-Rad; cat. no 1705062). Imaging was performed with a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.). Stripping of membranes was performed with Restore Western Blot Stripping Buffer (ThermoFisher; cat. no 21059) for 20 minutes, followed by 3 x 10 min washes in TBST. Such blots were re-blocked in SuperBlock or Blocker BLOTTO as described above before incubation with the next primary antibody. Band densities were quantified using Image Lab Software (Bio-Rad Laboratories, Inc.). Band densities were normalized to the corresponding actin band. Statistical analysis was performed using GraphPad Prism 9.3.1 (GraphPad Software).

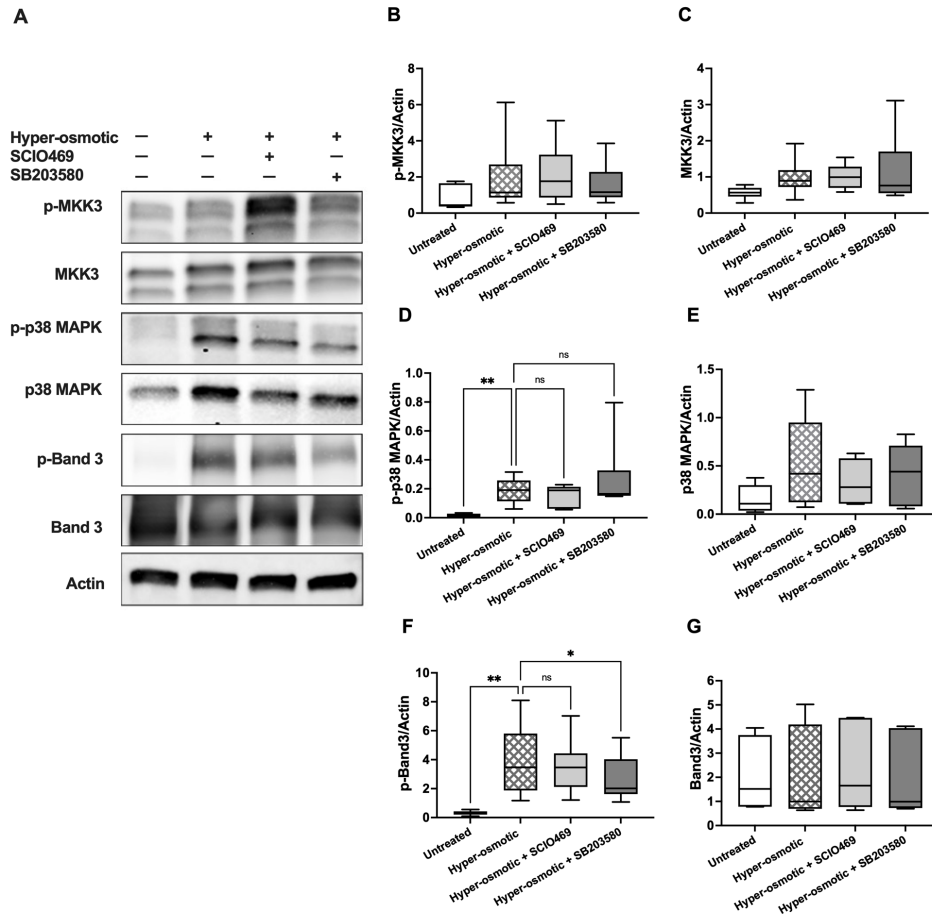
#### **REFERENCES:**

1. Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys* 1963;**100**: 119-30.

**SUPPLEMENTAL FIGURES:**



**Supplemental Figure S1.** Differential MAPK and Band 3 protein expression in response to hypo-osmotic stress (pink test assay), hyper-osmotic stress (500 mmol/L sucrose), or diamide-induced oxidative stress (2 mmol/L). Erythrocytes were collected from the same individual and samples were loaded on the same gel (Western blotting) to reflect differences in phosphorylated and total p38 MAPK, MAPKAPK2 (MK2), Band 3 expression under each stress test.



**Supplemental Figure S2.** Western blotting analyses of human erythrocyte membranes following incubations in hypertonic buffer (500 mmol/L sucrose; 1h) in the presence or absence of p38 MAPK inhibitors SCIO469 (50  $\mu\text{mol/L}$ ) or SB203580 (50  $\mu\text{mol/L}$ ). **A.** Representative immunoblots of phosphorylated (p-) and total MKK3, p38 MAPK, Band 3, and actin. Quantified protein expressions (normalized to actin) are shown for **B.** phospho-MKK3. **C.** total MKK3. **D.** phospho-p38 MAPK. **E.** total p38 MAPK. **F.** phospho-Band 3. **G.** total Band 3. N=6-9. Data are presented as box and whiskers (median and min to max). \* $p < 0.05$ , \*\* $p < 0.01$  by repeated measures ANOVA with mixed-effects analysis as described in Methods.