Steed et al., http://www.jcb.org/cgi/content/full/jcb.201304115/DC1

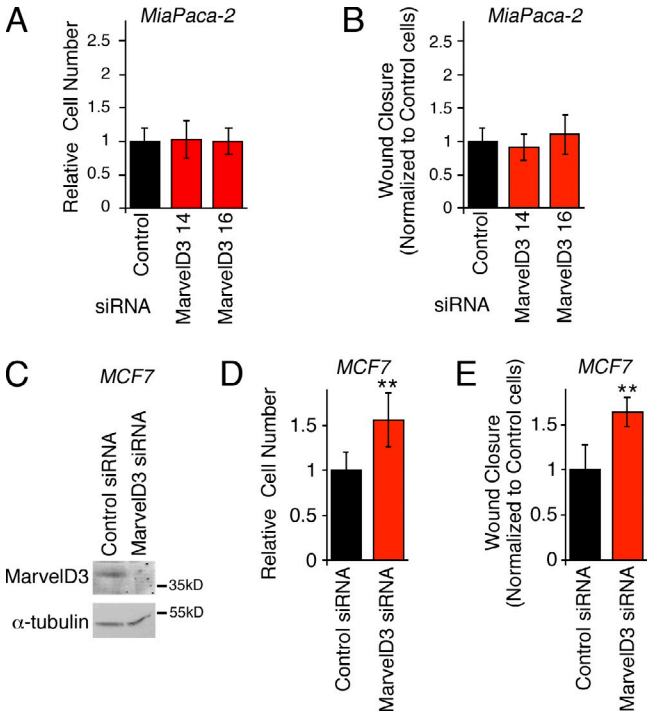

Figure S1. **MarvelD3 siRNAs on MiaPaca-2 and MCF7 cells.** (A–E) MiaPaca-2 (A and B) and MCF7 (C–E) cells were transfected with siRNAs. After 3 d, the cells were analyzed for expression, migration, and proliferation as described in Fig. 1 for Caco-2 cells. Note, MiaPaca-2 cells do not express MarvelD3, and hence, no immunoblot is shown (see Fig. 2). All quantifications show means ± 1 SD; $n = 6$ (A and D) and $n = 8$ (B and E). **, $P < 0.01$.

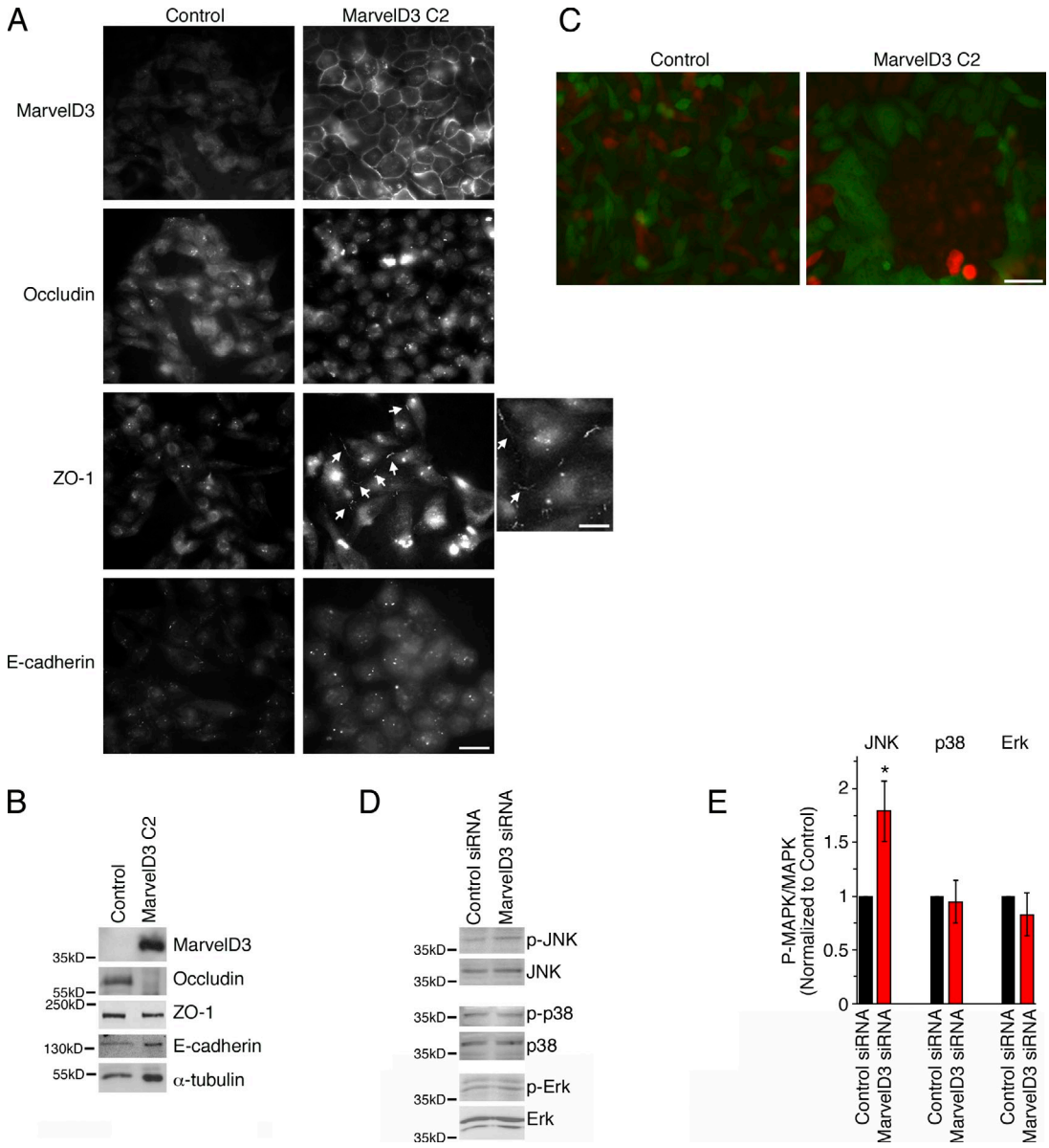

Figure S2. **Ectopic MarvelD3 expression in MiaPaca-2 cells.** (A and B) Control and MarvelD3-expressing MiaPaca-2 cells were analyzed by immunofluorescence or immunoblotting using the antibodies indicated. Note, only ZO-1 was recruited to MarvelD3-induced cell–cell contacts and only infrequently (arrows mark areas of ZO-1 at the plasma membrane; shown is also a twofold magnified section of the main image). (C) GFP- and RFP-expressing pools of control or MarvelD3-expressing cells were co-cultured, and distribution of the cells was then analyzed by fluorescence microscopy. Note, control MiaPaca-2 cells mix freely, whereas MarvelD3-expressing cells form concrete islands of either green or red cells, indicating that mobility is reduced. (D and E) MAPK activation in siRNA-transfected MCF7 cells was monitored by immunoblotting as described in Fig. 3 for Caco-2 and MiaPaca-2 cells. E shows means ± 1 SD; $n = 3$. *, $P < 0.05$. Bars: (A, left) 20 µm; (A, right) 10 µm; (C) 50 µm.

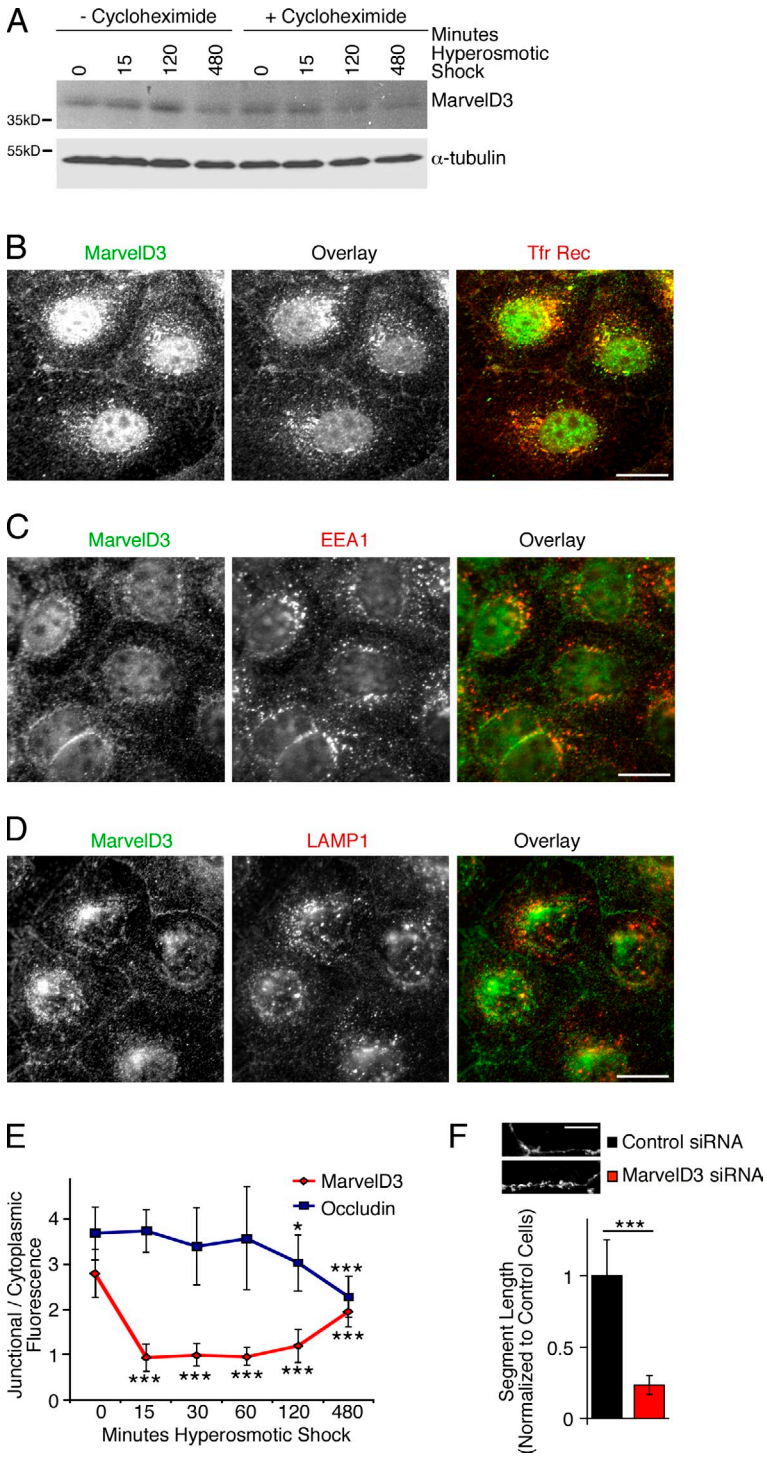

Figure S3. **Internalization of MarvelD3 in response to osmotic stress.** (A) Caco-2 cells were osmotically stressed in the absence or presence of cycloheximide. Expression levels of MarvelD3 were then analyzed by immunoblotting. (B–D) Caco-2 cells were osmotically stressed for 1 h (B and C) or 2 h (D) before fixation. The samples were then stained for MarvelD3 and the indicated endocytic markers. Tfr Rec, transferrin receptor. (E) Quantification of junction-associated MarvelD3 and Occludin during osmotic stress. Note, MarvelD3 is removed from the junctions within the first 15 min, whereas Occludin is internalized only at later time points. The values indicated represent means ± 1 SD; $n = 10$. (F) Distribution of junctional Occludin in control and MarvelD3-depleted cells was quantified by measuring the mean length of continuous labeling obtained with Occludin antibodies along cell–cell junctions. The values indicated represent means ± 1 SD; $n = 6$. *, $P < 0.05$; ***, $P < 0.001$. Bars: (B–D) 10 µm; (F) 3 µm.

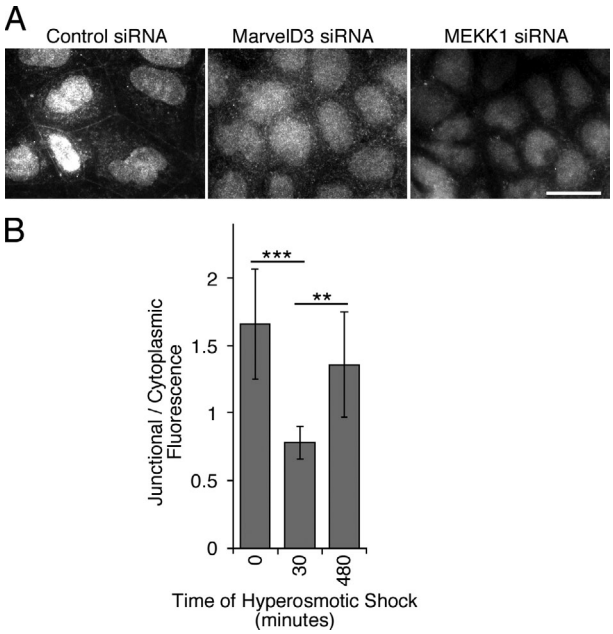

Figure S4. **Localization of MEKK1 in paraformaldehyde-fixed cells.** (A) Caco-2 cells transfected with the indicated siRNAs were fixed with paraformaldehyde and permeabilized with Triton X-100 before staining with mouse anti-MEKK1 antibodies. Note, chemical fixation reduces the cytoplasmic background staining in comparison to methanol (Fig. 5 H). In contrast, there is more nuclear staining, which, based on MEKK1 siRNA–transfected cells, is also nonspecific. However, chemical fixation facilitates the visualization of the cytoplasmic staining in osmotically stressed cells. (B) Quantification of junction-associated MEKK1 by fluorescent density analysis. Shown are means ± 1 SD; $n = 8$. **, $P < 0.01$; ***, $P < 0.001$. Bar, 10 µm.

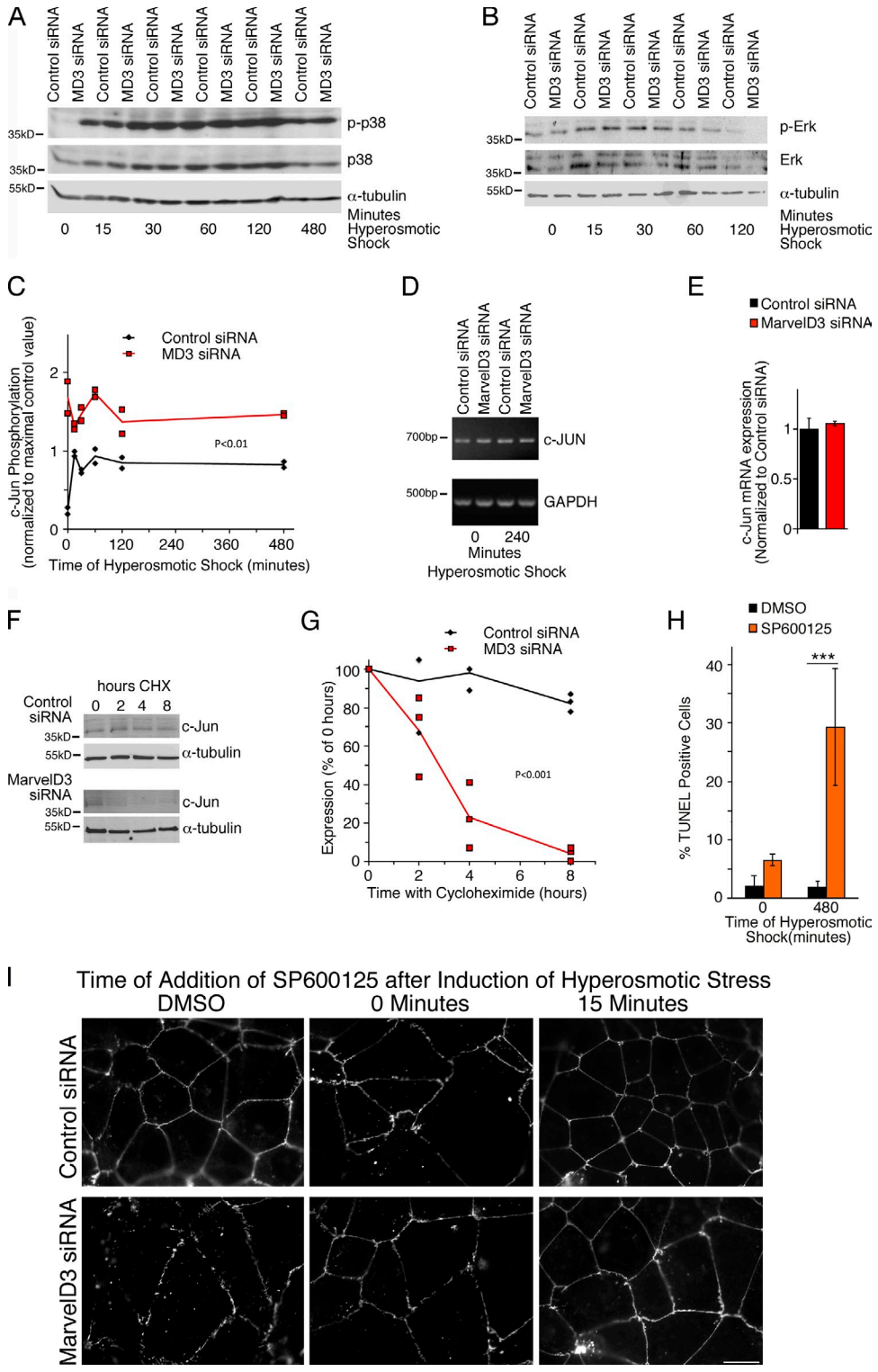

Figure S5. **Analysis of MAPK signaling during osmotic stress.** (A–C) Caco-2 cells were transfected with control and MarvelD3-targeting siRNAs before applying an osmotic stress as described in Fig. 8. Total cell extracts were then immunoblotted to analyze the activation profiles of p38 and Erk (A and B) or c-Jun phosphorylation (C; given is a quantification of immunoblots as shown in Fig. 8 H; indicated are individual determinations, and the lines were drawn through the corresponding means for each time point). p-Erk, phospho-Erk. (D and E) Total RNA was isolated from siRNA-transfected Caco-2 cells, and mRNA expression of c-Jun was determined by RT-PCR (D) or quantitative PCR (E; shown are means ± 1 SD; $n = 3$.). (F and G) Control and MarvelD3 siRNA–transfected Caco-2 cells were incubated for cycloheximide (CHX) for different periods of time before analysis of c-Jun expression by immunoblotting. The quantification shows individual determinations, and the lines were drawn through the corresponding means for each time point. (H) Cells incubated with DMSO or SP600125 were osmotically stressed for 8 h before fixation and analysis of apoptosis using the TUNEL assay. Shown are means ± 1 SD; $n = 5$; ***, $P < 0.001$. (I) Cells transfected with the indicated siRNAs were hyperosmotically shocked for 8 h. The JNK inhibitor SP600125 was added either together with the hypertonic medium or 15 min after shock induction. The cells were then fixed, and junctional integrity was monitored by staining for ZO-1. Bar, 10 μm.