Supplementary Information for

Zn^{2+} influx activates ERK and Akt signaling pathways

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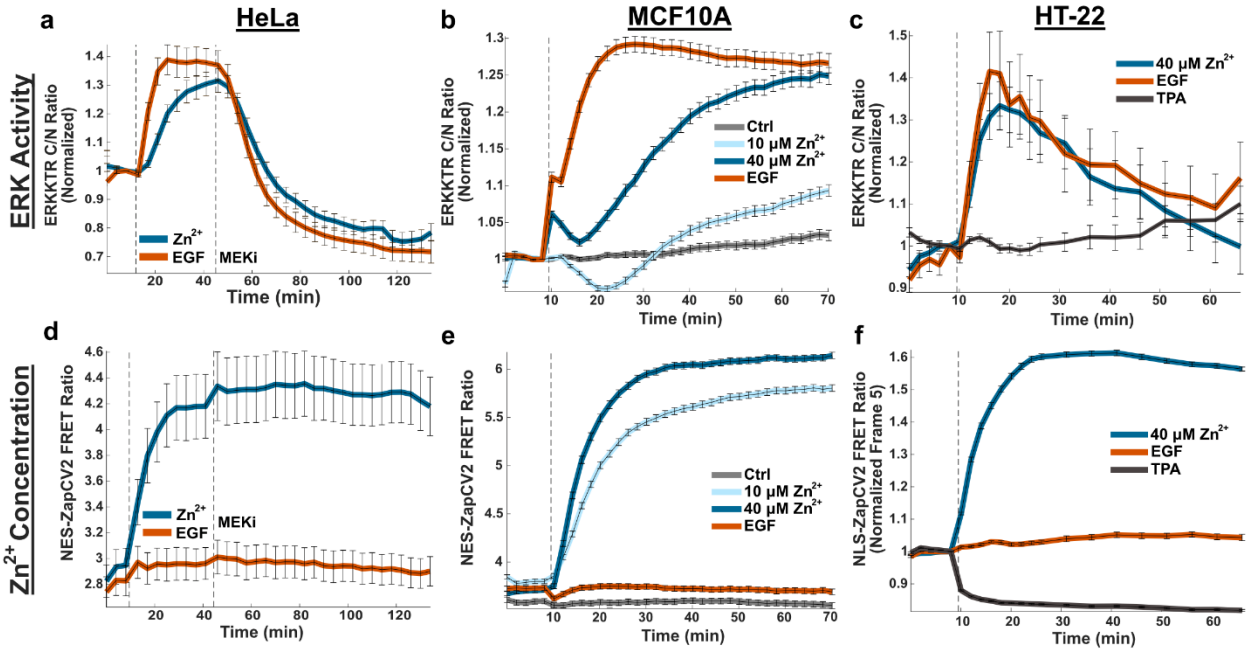
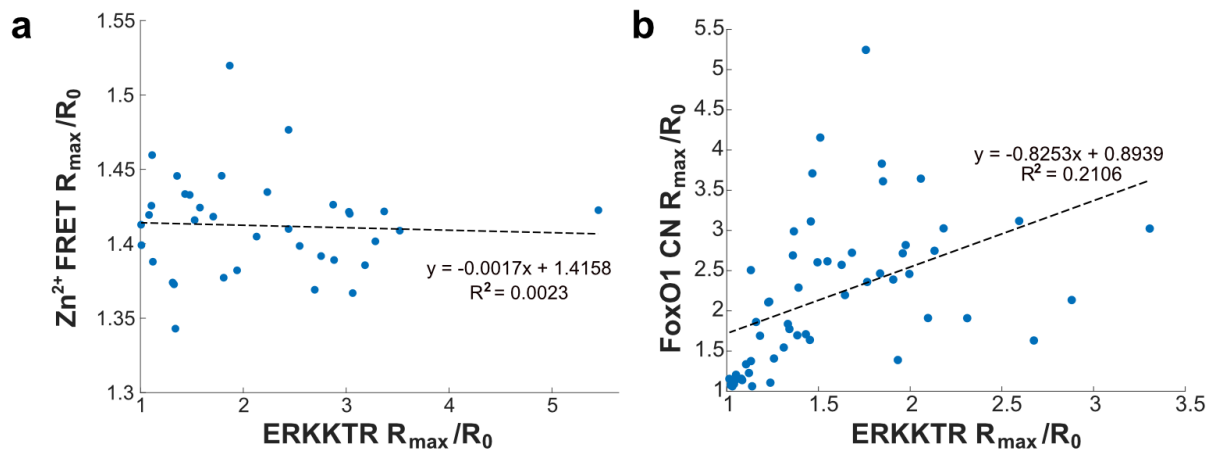
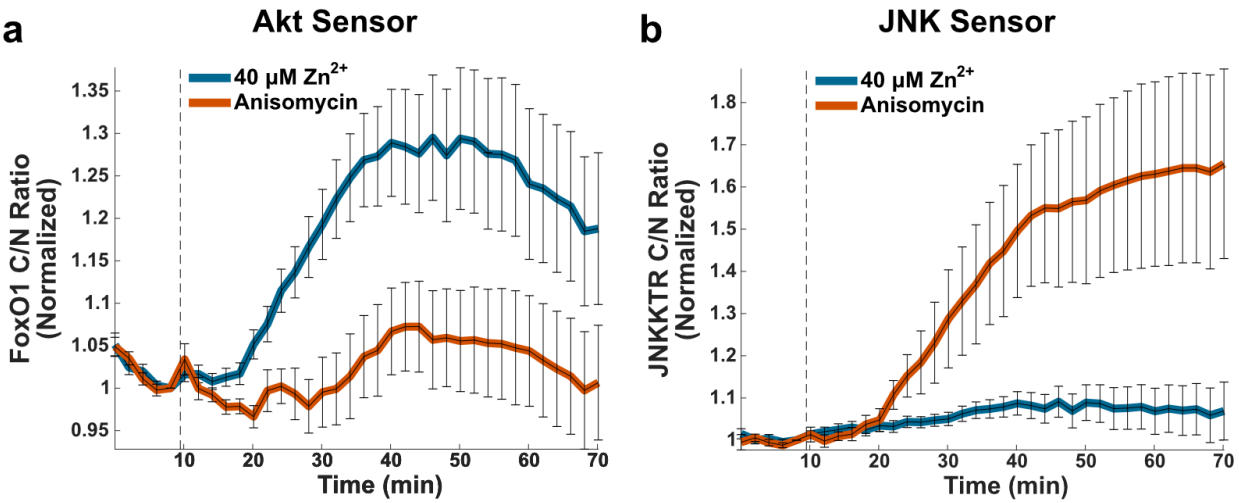


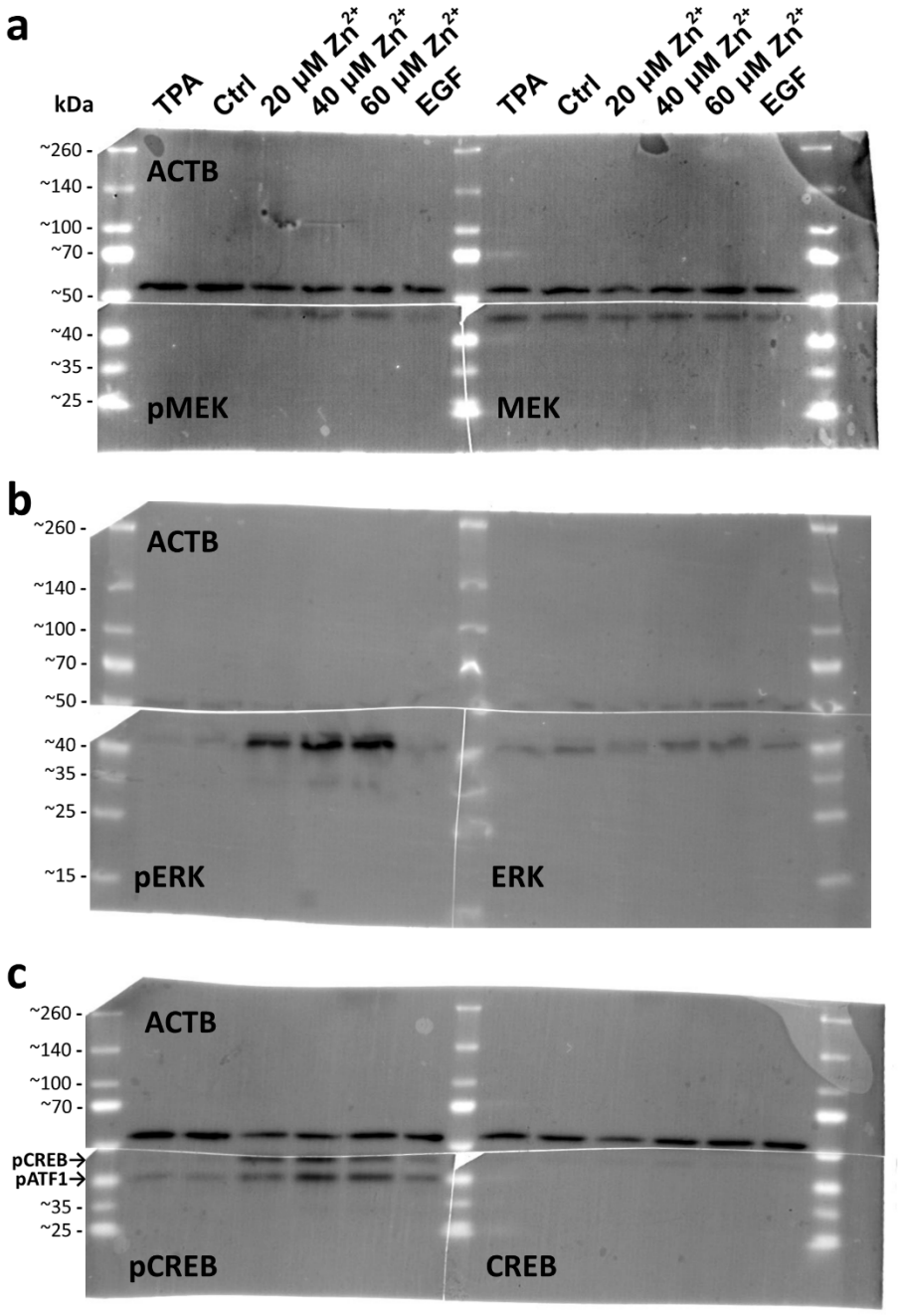
Supplemental Fig S1: Extracellular Zn^{2+} leads to rapid cytosolic Zn^{2+} influx and ERK activation in multiple cell types. A) Activation of ERK by Zn^{2+} or EGF in HeLa cells showing the normalized mean and SD of at least 38 cells (from Fig 6c). B) Activation of ERK by Zn^{2+} or EGF in MCF10A mammary epithelial cells showing the normalized mean and SD of at least 200 cells. C) Activation of ERK by Zn^{2+} or EGF compared to Zn^{2+}-deficient control in HT-22 mouse hippocampal neuronal cell line showing the normalized mean of at least 19 cells. D-E) Cytosolic Zn^{2+} as shown by NES-ZapCV2 in the same cells as above upon stimulation by extracellular Zn^{2+} and EGF. Results show that treatment with EGF and changing the media (Ctrl in middle panel) do not lead to changes in cytosolic Zn^{2+}.



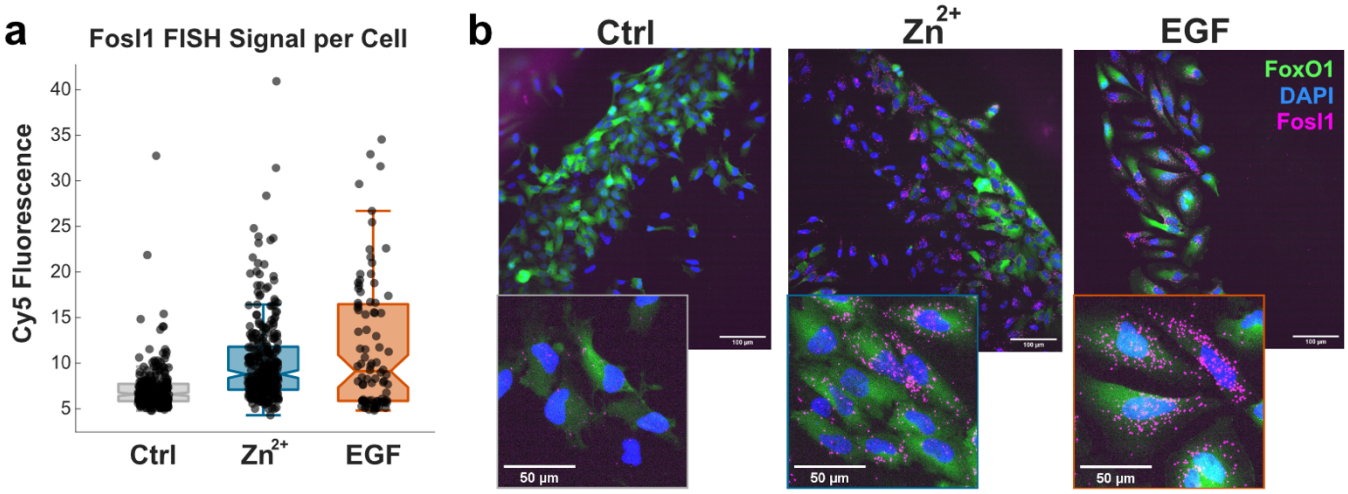
Supplemental Fig S2: Comparison of cell variability in response to Zn^{2+} stimulation. A) Variability in Zn^{2+} signal as a function of variability in ERK signal. The FRET ratio R_{max}/R_0 for the ZapCV2 sensor (representative of Zn^{2+} levels) for each HeLa cell is plotted as a function of the ERKKTR R_{max}/R_0 signal, where R_0 represents the initial ratio for each sensor. There is no correlation between the variability in the two signals. B) Variability in Akt signal from the FoxO1-Clover translocation sensor as a function of variability in ERK signal upon 40 µM Zn^{2+} addition. The Akt sensor R_{max}/R_0 for each HeLa cell is plotted as a function of the ERKKTR R_{max}/R_0 signal, where R_0 represents the initial ratio for each sensor.



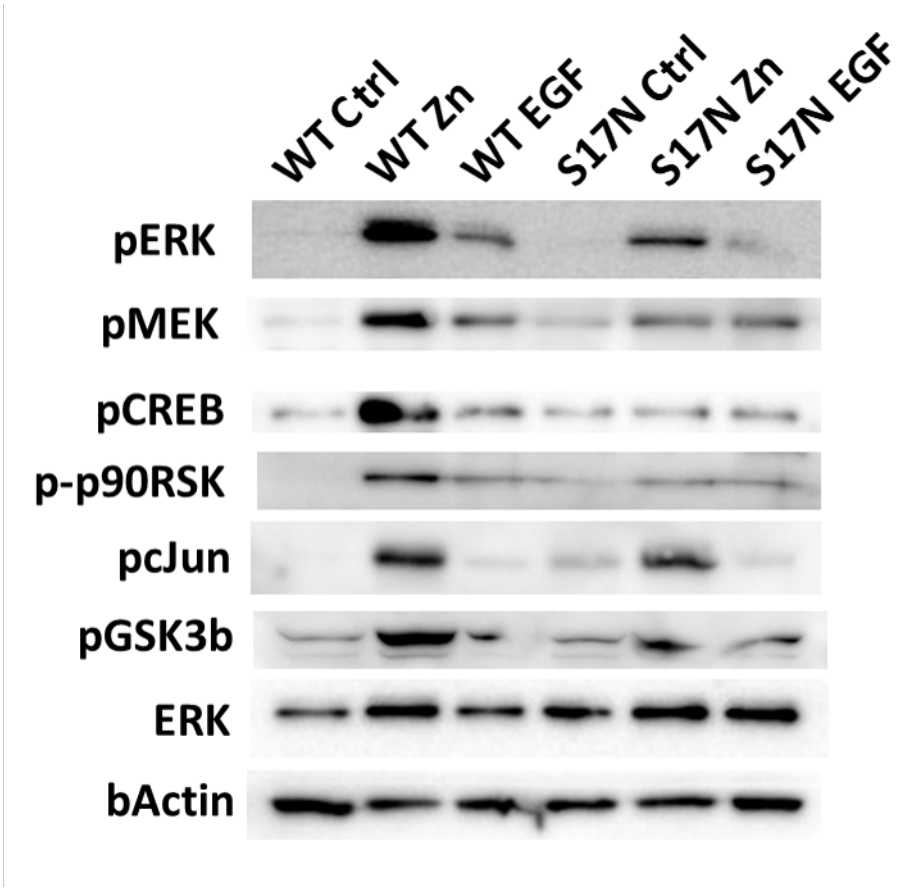
Supplemental Fig S3: Zn^{2+} does not activate JNK. Akt (a) or JNK (b) translocation sensors upon stimulation with either 40 μM Zn^{2+} or 100 ng/mL Anisomycin at the time indicated by dashed line (~ 10 minutes). The normalized mean and SD from at least 10 cells are plotted against time. Traces are representative of three separate experiments.



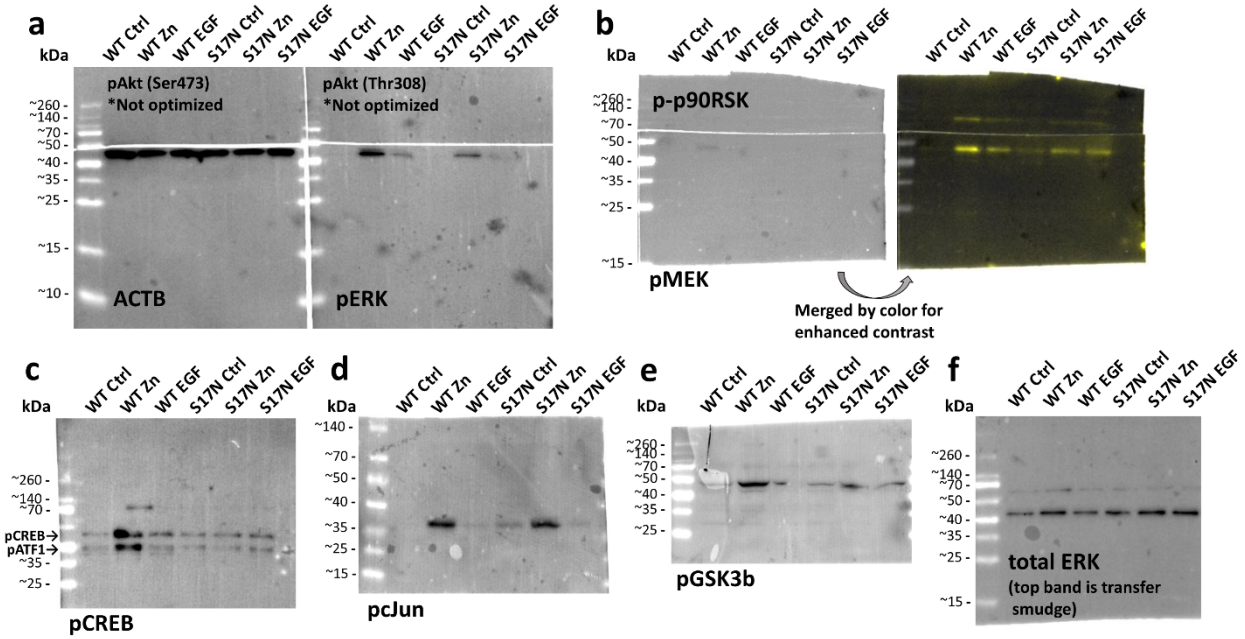
Supplemental Figure S4: Western blots for total and phosphorylated protein of (a) MEK, (b) ERK, and (c) CREB from Figure 3b. Full blot, with β-actin (ACTB) loading control. Protein was loaded in volumes controlled by BCA assay, and membrane was cut (indicated by white space) to incubate different regions of the membrane with different antibodies. Luminescence images were overlayed with digital images to highlight separation between antibodies and show protein ladder using average intensity z projection. Phospho-ATF1 band is labeled as indicated by the manufacturer.



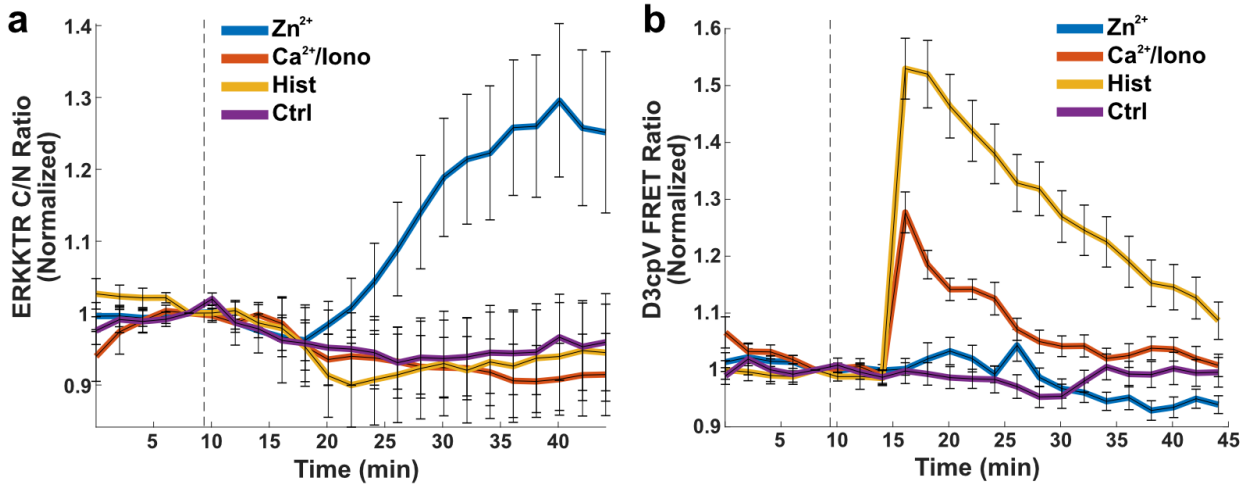
Supplemental Fig S5: RNA FISH against Fosl1 (also called FRA1) reveals that EGF and Zn2+ each increase expression of Fosl1 compared to control. (a) Quantification of Cy5 fluorescence in a 4-pixel ring around the nuclear mask. Each dot represents an individual cell. (b) Representative images showing FoxO1 signal (green), DAPI DNA stain (blue) and Fosl1 mRNA (magenta). Scale bar = 100 µm. Inset shows zoomed image for detail. Statistical analysis was carried out by a one-way ANOVA which showed that the difference in Zn^{2+} and EGF compared to Ctrl was significant with a $p < 0.0001$. Scale bar = 50 µM.



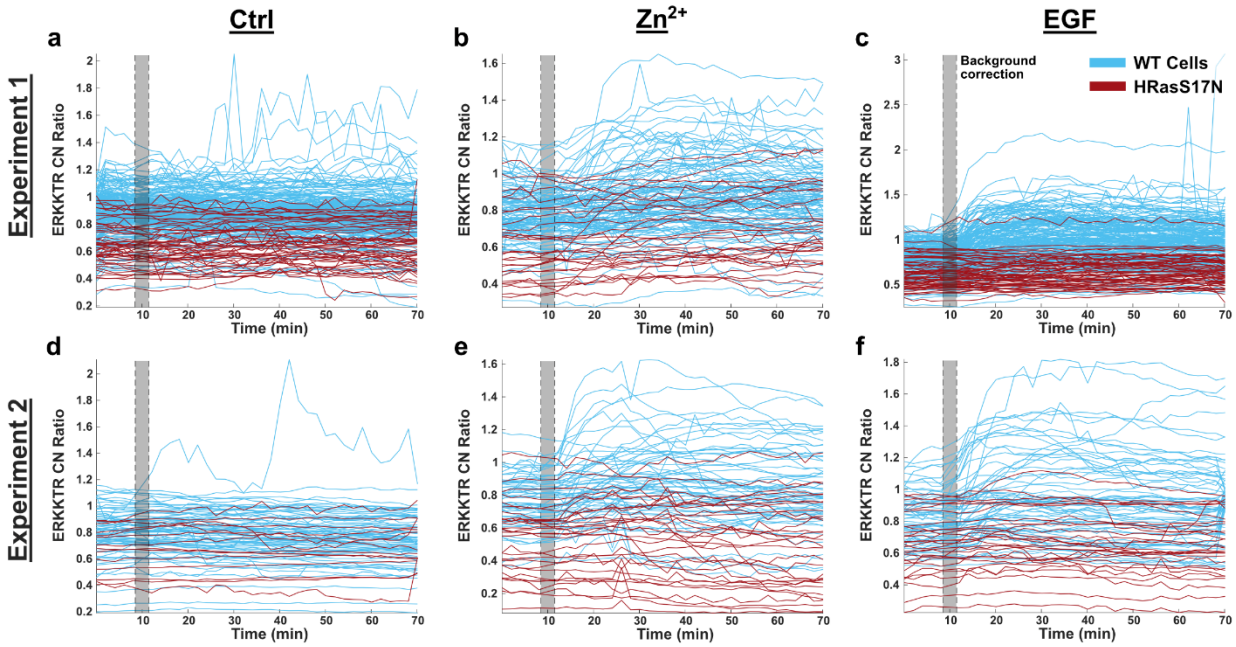
Supplemental Fig S6: Western blot results for key proteins in the MAPK or Akt signaling pathways, where "p" indicates detection of the phosphorylated state of the protein. MEK is upstream of ERK. CREB, p90RSK, c-Jun are downstream of ERK. GSK3β is downstream of Akt. Beta-Actin and total ERK are used as loading controls. HeLa cells were treated with Ctl (media change), 40 μM Zn^{2+}, or 20 nM EGF. The dominant negative EGFP-HRas-S17N was transiently transfected 48 hours prior to treatment. Because the transfection was variable, a complete block of Zn^{2+}- or EGF-induced activation was not observed in the S17N samples, although a decrease in the amount of phosphorylated protein was consistently observed. Each image represents the protein band of interest from different membranes that were incubated with the antibodies indicated. Full gel membranes are shown in Supplementary Figure S7. Details regarding the antibodies used are provided in the Resources Table.



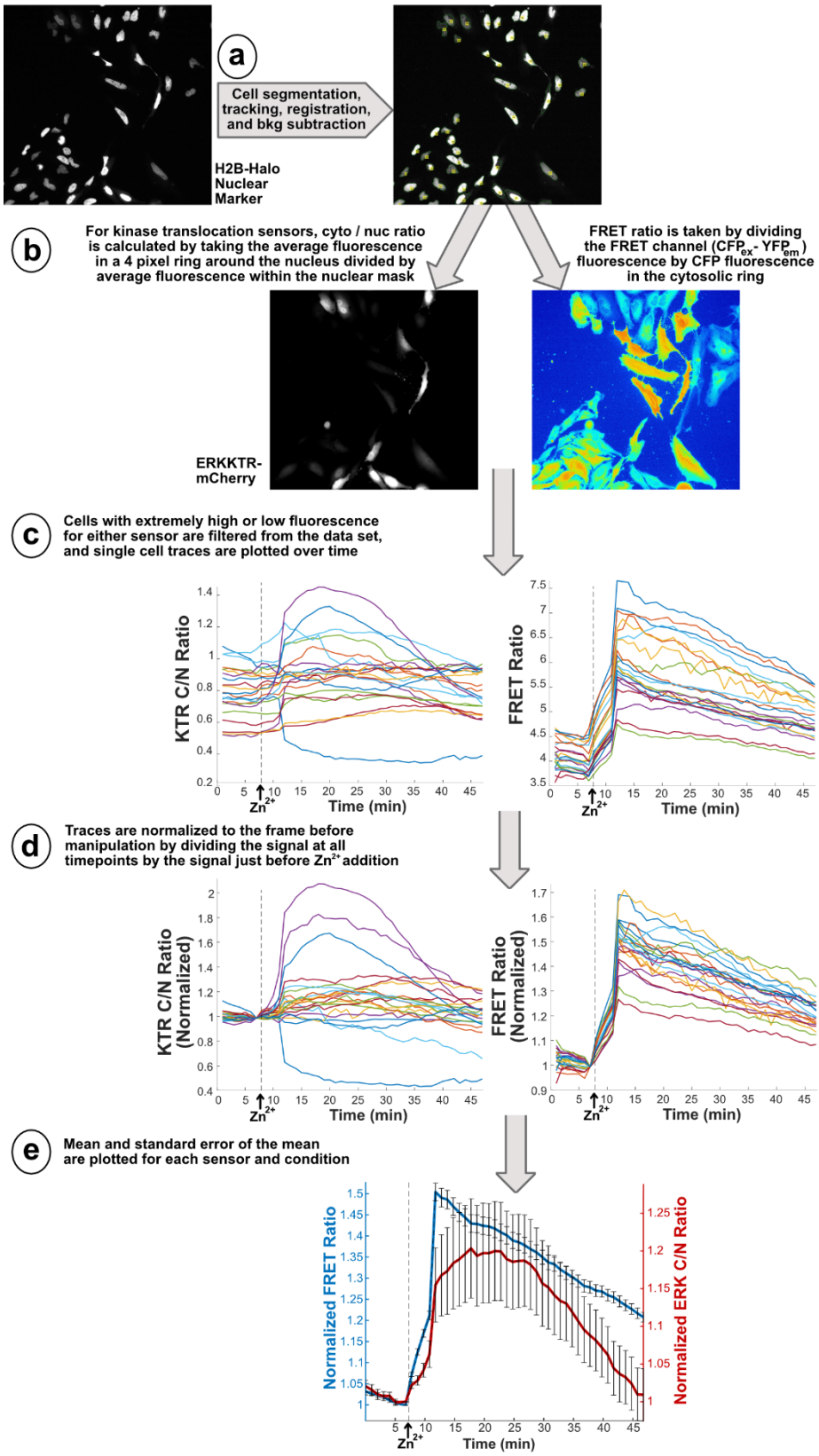
Supplemental Figure S7: Luminescence full-gel images from Supp Fig S6 western blots were overlayed with digital images to show full gel with protein ladder and any cuts used to test multiple antibodies on the same gel. Results are shown using protein from a single biological replicate. (a) ACTB loading control and phospho-ERK blots, along with two phospho-Akt antibodies that were not optimized at the time of the experiment. (b) phospho-p90RSK (S380) and phospho-MEK antibodies were both low-contrast compared to the digital image, so a color overlay where luminescence is shown in yellow is used to highlight the antibody bands. Antibodies for downstream targets of ERK and Akt (c) phospho-CREB and phospho-ATF1, annotated as indicated by the manufacturer, (d) phospho-cJun, and (e) phospho-GSK3β. (f) Total ERK control was bumped slightly during transfer.



Supplemental Fig S8: Zn^{2+} and calcium are independent within our system of Zn^{2+} activation. Cells with an ERK-KTR translocation sensor (a), and D3cpV FRET calcium sensor (b) were imaged with addition of 40 µM Zn^{2+}, 10 mM calcium + 5 µM ionomycin, or 100 µM histamine just before 10 minutes. Zn^{2+}, but not treatments that elevate cytosolic Ca^{2+} (Ca^{2+}/ionomycin and Histamine), increase ERK activity. Conversely, Ca^{2+}/ionomycin and Histamine increase cytosolic Ca^{2+} while Zn^{2+} does not. The normalized mean and SD from at least 23 cells are plotted against time.



Supplemental Fig S9: Single cell traces of ERK activation in cells with the RasS17N mutation. A-C) Single cell traces from Fig 6e, showing very low ERK response to both Zn^{2+} and EGF in cells containing the dominant negative RasS17N mutation. D-F) Single cell traces from a similar experiment conducted on a separate day.


a
Cell segmentation, tracking, registration, and bkg subtraction
H2B-Halo Nuclear Marker
b
For kinase translocation sensors, cyto / nuc ratio is calculated by taking the average fluorescence in a 4 pixel ring around the nucleus divided by average fluorescence within the nuclear mask
FRET ratio is taken by dividing the FRET channel (CFP$_{ex}$- YFP$_{em}$) fluorescence by CFP fluorescence in the cytosolic ring
ERKKTR-mCherry
c
Cells with extremely high or low fluorescence for either sensor are filtered from the data set, and single cell traces are plotted over time
KTR C/N Ratio
FRET Ratio
Zn2+
Time (min)
d
Traces are normalized to the frame before manipulation by dividing the signal at all timepoints by the signal just before Zn2+ addition
KTR C/N Ratio (Normalized)
FRET Ratio (Normalized)
e
Mean and standard error of the mean are plotted for each sensor and condition
Normalized FRET Ratio
Normalized ERK C/N Ratio

Supplemental Figure S10: Diagram of ZapCV2 FRET and ERKKTR image processing pipeline. The Nikon ND2 file is imported into MATLAB. (a) Cells are segmented by nuclear marker in either Cy5 (H2B-Halo + JF646, this image) or BFP (Hoechst nuclear dye) channels, using the watershed method to generate nuclear masks. Frames are registered automatically anytime the experiment is paused to account for small shifts when media is manipulated. Nuclei are tracked between frames with adjustable link distance set at 100 pixels and all channels are subjected to local background subtraction. (b) To differentiate between cytosol and nucleus, the nuclear mask was dilated 4 pixels and this region was termed "cytosol". The cytosol ring size was chosen to maintain separation between small or densely packed cells while enabling robust signal measurement. For translocation sensors, average intensity in the nucleus and cytosol of each cell is measured. For FRET sensors, average intensity in the cytosol for the acceptor channel (CFP ex, YFP em) is divided by average intensity in the donor channel (CFP) to get a FRET ratio. YFP fluorescence is also tracked over time to monitor photobleaching. (c) Using fluorescence cutoffs, cells that have very low or high sensor fluorescence were omitted from analysis. Cells that lose tracking or die during the experiment are also omitted. Ratio data for each cell is then plotted over time. (d) The ratio at each timepoint is then divided by the ratio at the frame immediately before Zn^{2+} addition to normalize the data. (e) Mean and standard error of the mean are taken for each sensor and plotted over time.

Supplemental Table S1: Zn^{2+} Curve Fitting: $y = a * e^{(-b*x)} + c$

Sensor	[Zn^{2+}] added	a	b	c	Sum of squares due to error	Adjusted R-square	Root mean squared error
ZapCV2	10 µM	-2.589	0.091	6.807	0.0117	0.9983	0.0300
	20 µM	-2.897	0.119	6.769	0.0030	0.9997	0.0153
	40 µM	-3.384	0.213	7.430	0.0706	0.9940	0.0737
ZapCV5	10 µM	-0.475	0.125	3.604	0.0028	0.9892	0.0146
	20 µM	-0.737	0.094	3.836	0.0004	0.9994	0.0058
	40 µM	-0.757	0.122	4.077	0.0033	0.9949	0.0159

Supplemental Table S2: Kinase Array Raw Data

Kinase	Phospho-site	Fluorescence signal				Log_2 fold change	Negative log_2 p-value
		Ctrl-1	Ctrl-2	Zinc-1	Zinc-2		
ERK1/2	T202/Y204, T185/Y187	5.37	8.25	42.30	46.51	2.70	7.47
CREB	S133	4.69	4.53	14.97	14.58	1.68	8.09
Akt1/2/3	S473	4.19	3.48	14.58	9.63	1.66	3.65
WNK1	T60	5.20	4.68	16.44	12.15	1.53	3.98
HSP27	S78/S82	4.11	3.34	11.05	7.95	1.35	3.98
GSK-3a/b	S21/S9	22.16	23.74	49.17	44.13	1.02	4.50
PRAS40	T246	5.57	4.81	11.96	7.73	0.92	3.14
STAT3	Y705	1.40	1.08	1.75	1.54	0.41	4.52
JNK1/2/3	T183/Y185, T221/Y223	10.21	8.36	12.37	11.03	0.33	4.93
p38a	T180/Y182	3.85	3.41	4.92	3.81	0.27	2.89
MSK1/2	S376/S360	10.81	10.47	11.88	12.76	0.21	3.17
p70 S6 Kinase	T389	2.68	1.52	2.73	1.93	0.15	2.23
c-Jun	S63	3.73	2.27	3.46	2.76	0.05	1.29
STAT5a/b	Y694/Y699	4.84	4.55	4.19	4.90	-0.05	1.29
Yes	Y426	6.48	4.84	5.83	5.10	-0.05	1.44
Chk-2	T68	5.77	4.93	5.36	4.56	-0.11	5.72
EGF R	Y1086	4.12	3.23	3.65	3.11	-0.12	2.60
STAT5b	Y699	4.38	3.30	3.21	3.70	-0.15	1.50
PDGF Rb	Y751	3.49	2.99	2.81	3.00	-0.16	1.97
Hck	Y411	5.06	4.45	4.22	4.30	-0.16	2.38
FAK	Y397	7.64	5.64	5.63	6.08	-0.18	1.65
HSP60	--	23.27	18.35	17.30	18.75	-0.21	1.88
Src	Y419	6.47	4.50	4.70	4.75	-0.22	1.76
Fyn	Y420	2.59	1.25	1.49	1.77	-0.23	1.35
STAT6	Y641	5.85	4.72	4.07	4.66	-0.28	2.06
STAT5a	Y694	3.37	2.03	2.13	2.32	-0.28	1.63
p27	T198	1.64	0.60	1.00	0.77	-0.34	1.58
AMPKa1	T183	4.59	3.40	3.35	2.96	-0.34	2.82
mTOR	S2448	7.09	5.91	5.00	5.19	-0.35	2.80
AMPKa2	T172	8.97	6.98	5.87	6.23	-0.40	2.52
STAT2	Y689	9.80	7.54	6.76	6.37	-0.40	2.91
b-Catenin	--	3.73	2.66	2.20	2.49	-0.45	2.22
RSK1/2/3	S380/S386/S377	3.03	1.80	1.86	1.64	-0.46	2.27
p70 S6 Kinase	T421/S424	3.64	2.30	2.37	1.88	-0.48	2.75
PYK2	Y402	2.73	2.10	1.92	1.52	-0.49	4.23
Lyn	Y397	2.92	1.86	1.71	1.57	-0.54	2.51
Fgr	Y412	1.78	0.79	0.78	0.94	-0.57	1.74
STAT3	S727	2.84	1.60	1.52	1.21	-0.70	2.67
Akt1/2/3	T308	3.94	2.50	2.02	1.83	-0.74	2.81

Lck	Y394	1.80	0.95	0.79	0.80	-0.79	2.30
eNOS	S1177	2.75	1.62	1.30	1.22	-0.79	2.59
p53	S392	4.72	3.37	2.45	2.19	-0.80	3.36
p53	S46	3.25	1.84	1.50	1.36	-0.83	2.60
p53	S15	2.40	1.14	1.08	0.87	-0.86	2.43
PLC-g1	Y783	2.73	1.35	1.20	0.95	-0.92	2.56

*Fold change was taken by averaging the replicates for each condition and dividing the Zn^{2+} added condition by control

** p-value was the result of a one-tailed t test. Green boxes indicate $p > 0.05$.

*** To visualize differences in signal, the fluorescence signal for each replicate was shaded from white (0) to deep orange (50).

Supplemental Table S3: Making Buffered Zn^{2+}

Chelator	Competing Cation	Buffered Zinc Concentrations									
NTA	none	Total Zn^{2+} (mM)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
		Accessible Zn^{2+} (M)	**6.27E-10**	**1.41E-09**	2.42E-09	3.76E-09	**5.64E-09**	8.47E-09	**1.32E-08**	2.26E-08	5.08E-08
EGTA	Sr^{2+}	Total Zn^{2+} (mM)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
		Accessible Zn^{2+} (M)	7.71E-09	1.89E-08	3.51E-08	**5.89E-08**	9.46E-08	**1.51E-07**	2.5E-07	**4.53E-07**	**1.07E-06**
EGTA	Ca^{2+}	Total Zn^{2+} (mM)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
		Accessible Zn^{2+} (M)	2.22E-06	**5.38E-06**	9.82E-06	**1.61E-05**	2.49E-05	**3.74E-05**	5.55E-05	***8.16E-05***	**1.19E-04**

* Bolded concentrations were used in buffered zinc MKP3 inhibition experiment

** Italicized concentration was used in Zn^{2+} sensor calibration experiments

Log $K_{(ZnChelator)}$		Log $K_{(SrChelator)}$		Log $K_{(CaChelator)}$	
EGTA	12.7	EGTA	8.5	EGTA	10.97
NTA	10.67				

	NTA	EGTA
pKa_1	9.71	9.47
pKa_2		8.85

Captions for Supplemental Movies

Supplementary Movie S1&2:

Companion to Figure 2. Simultaneous imaging of Zn^{2+} and ERK activity demonstrates that in individual HeLa cells Zn^{2+} increase precedes the increase in ERK activity. 1) Individual cells expressing ERKKTR-mCherry, imaged over 70 minutes. 2) Individual cells expressing NES-ZapCV2 pseudo-colored by FRET Ratio where warmer colors indicate higher FRET, imaged over 70 minutes.

Supplementary Movie S3&4:

Companion to Figure 4. Zn^{2+} activates both ERK and Akt in a concentration-dependent manner. 3) Individual cells expressing ERKKTR-mCherry, imaged over 70 minutes. 4) Individual cells expressing FoxO1-Clover Akt sensor, imaged over 70 minutes.

Supplemental Methods

Adenovirus Generation

Adenovirus plasmid cloning and viral amplification were conducted as previously described[1,2] with the generous help of Dr. Stephen Langers in the Lab of Professor Leslie Leinwand (University of Colorado Boulder). Briefly, primers in the Resources Table were used to amplify ERKKTR-mCherry with overhangs for InFusion cloning into the pShuttle plasmid, which was then cut with Pme1 restriction enzyme and transformed into BJ5183 (RecA+, pAdEasy-1) bacteria for homologous recombination into pAdEasy-1 plasmid. Successful clones were transformed into DH5α *E.coli*, DNA was extracted using QIAGEN midi kit and digested with Pac1 to linearize. Linear DNA was extracted using phenol-chloroform and ethanol precipitation and transfected into HEK293pr cells. Virus was amplified six times in HEK293pr cells and harvested by cesium chloride gradient. Virus was added at MOI = 10 to MCF10A cells for plasmid expression.

RNA FISH

Cells were imaged as above with the FoxO1-Clover sensor and Hoechst nuclear dye. Briefly, cells were incubated in HHBSS with Hoechst for 15 minutes and then further incubated for 15 min in HHBSS at 37°C and 0% CO_2. FoxO1-Clover Akt sensor was measured for 10 minutes and 40 µM $ZnCl_2$ or 20 nM EGF was added for 30 minutes. Cells were fixed following this time-course by incubation in 4% paraformaldehyde for 15 min and processed according to the manufacturer's instructions (ThermoFisher QVC0001). The Fosl1 ViewRNA Type 6 probe (ThermoFisher) mRNA probe was hybridized at 40 °C for 3 hr, followed by standard amplification and fluorescent labelling steps also at 40 °C. FISH fluorescence was detected on the Nikon Ti-E with 20X 0.8 NA Plan Apo objective lens. Mean Cy5 fluorescence was detected in the 4-pixel ring around the nuclear mask.

Supplemental References

1. He, T.-C. *et al.* A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci.* **95**, 2509–2514 (1998).
2. Sommese, R. F. *et al.* Molecular consequences of the R453C hypertrophic cardiomyopathy mutation on human -cardiac myosin motor function. *Proc. Natl. Acad. Sci.* **110**, 12607–12612 (2013).