

Appendix A. Supplemental Data and Experimental Methods

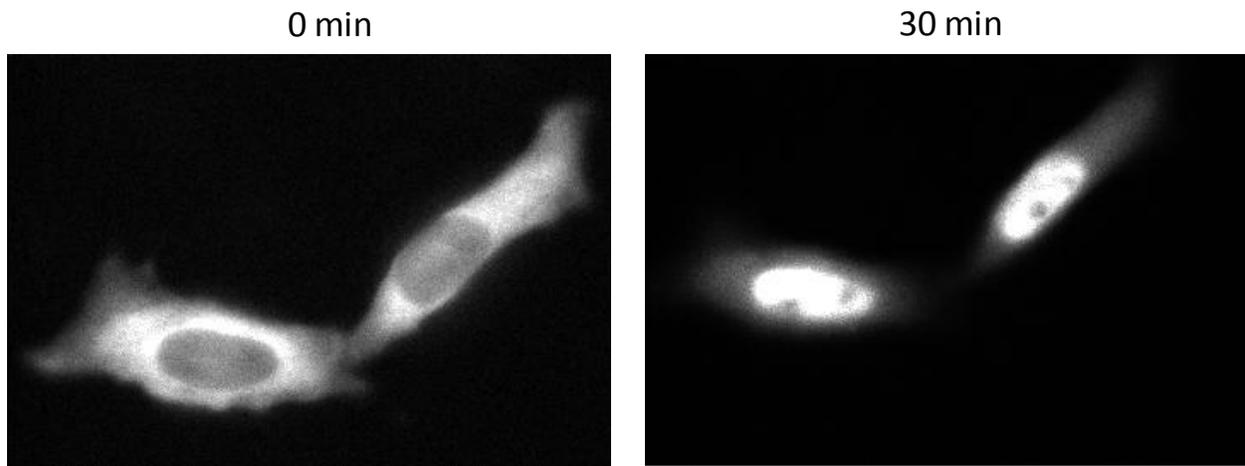


Figure S1: ECFP-NFAT1(1-415) undergoes nuclear import following ionomycin treatment. The sub-cellular distribution of ECFP-NFAT(1-415) was measured before (first panel) and 30 minutes after (second panel) ionomycin treatment (1 μ M).

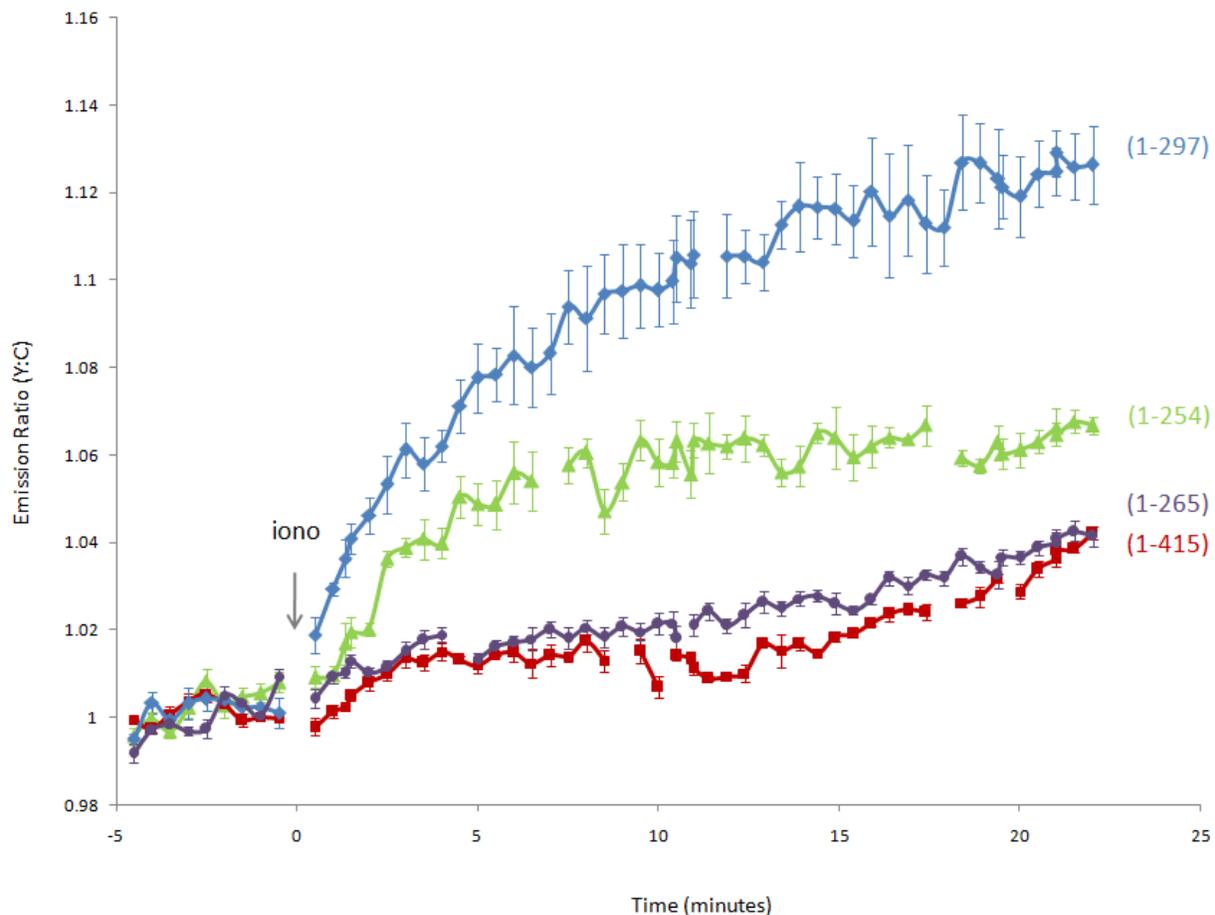


Figure S2: Representative time courses of HeLa cells expressing various test constructs stimulated by ionomycin (1 μ M). The emission ratio of each candidate construct was plotted as a function of time. The standard error for each time point is shown. Constructs were composed of either NFAT1(1-415) (red squares, $n = 4$), NFAT1(1-297) (blue diamonds, $n = 2$), NFAT1(1-265) (purple cross-hairs, $n = 2$), or NFAT1(1-254) (green triangles, $n = 2$) flanked by ECFP and cpV(L194) at the N- and C-terminus, respectively.

EXPERIMENTAL METHODS

Gene construction. The entire regulatory domain of NFAT1 (amino acid residues 1–415) and several C-terminally truncated versions of this region (encoding residues 1-254, -265 and -297) were generated by PCR using HA-NFAT1 (1-415) as the template.¹⁵ SphI and SacI restriction sites were engineered into the primary nucleotide sequence for cloning. Enhanced cyan fluorescent protein (ECFP)¹⁶ and a circularly-permuted form of Venus (cpV(L194))¹⁷⁻¹⁸ were fused to the 5'- and 3'-ends of each gene construct, respectively (Figure 2B). Each fusion construct was first generated in pRSET B (Invitrogen) and subcloned into pcDNA3 (Invitrogen) behind a Kozak sequence to enhance mammalian expression.

Cell culture and imaging. HeLa cells were plated onto sterilized glass coverslips in 35-mm dishes and grown to ~ 40% confluency in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C under 5 % CO₂. Cells were transfected with calcium phosphate and grown for 20–25 h prior to imaging. Before imaging, transfected cells were washed twice with Hanks' balanced salt solution (HBSS) and maintained in HBSS in the dark at 20–25 °C. At the indicated times, ionomycin (iono; Sigma) and CaCl₂ (Sigma) were added to the imaging media to final concentrations of 1 μM and 5 mM, respectively. Inhibition studies were conducted as described above except 10 μM of cyclosporin A (CsA; Sigma) was added to the imaging buffer 10 minutes prior to ionomycin treatment. Cells were imaged on a Zeiss Axiovert 200M microscope with a 40×/1.3NA oil-immersion objective lens and cooled CCD camera as previously described.²⁸ Briefly, dual emission ratio imaging used a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters (475DF40 for cyan and 535DF25 for yellow). The ratios of yellow-to-cyan were then calculated at different time points and normalized by dividing all ratios by the average emission ratio before stimulation. For dual imaging experiments, CaNAR1-transfected cells were incubated with 1 μM of Fura-2/AM ester for 30 minutes at 37 °C. Fura-2-loaded cells were then washed twice with HBSS imaging solution and incubated for an additional 10 minutes at RT prior to imaging. Imaging experiments were conducted as described above except 340- and 380-nm excitation filters and a 520-nm emission filter were used for Fura-2 dual-wavelength excitation ratio imaging.

Western blotting. HeLa cells were grown to ~ 50 % confluency and transfected with CaNAR1 as described above. Transfected cells were grown for 15-16 h in DMEM/ 10 % FBS at 37 °C under 5 % CO₂. Prior to drug addition, the culture media was replaced with DMEM supplemented with 2.5 mM of CaCl₂.¹³ To stimulate the cells, ionomycin was added to the media to a final concentration of 1 μM and the cells were incubated at 37 °C with 5 % CO₂ until lysis. As controls, transfected cells were either pre-incubated with 10 μM of CsA for 10 minutes before ionomycin addition or left untreated. At various time-points after Ca²⁺ stimulation, the cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS; Gibco) and lysed in 200 μL of 2X Laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 4 % SDS, 20 % glycerol, 200 mM β-mercaptoethanol, 0.004 % bromophenol blue). To promote complete lysis, samples were transferred to microcentrifuge tubes, incubated at 90 °C for 10 minutes and sonicated for 50 seconds using a 550 Sonic Dismembrator (Fisher Scientific). Lysates were then electrophoresed on a 7.0 % SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad).

Non-specific sites were blocked with TBS-T (20 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.05 % Tween-20) supplemented with 5 % non-fat dry milk. To detect CaNAR1, the blot was probed with anti-GFP polyclonal antibody (Invitrogen) diluted 1:4000 in blocking buffer followed by an anti-rabbit IgG horseradish peroxidase conjugate (Pierce) diluted 1:20000 in blocking buffer. The blot was developed using SuperSignal chemiluminescence reagents (Pierce) as described by the manufacturer.