Supporting Information for

Neuronal Calcium Recording with an Engineered TEV Protease

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General materials and methods

The fidelity of all DNA constructs was confirmed by DNA sequencing at the Stony Brook University DNA Sequencing facility. Mouse α-myc tag IgG1 (Clone Myc.A7) was purchased from Abcam (Cambridge, United Kingdom). E18 Sprague Dawley rat hippocampi, primary neuron culturing kit, NbActiv1 culture medium, and transfection medium were purchased from BrainBits, LLC (Springfield, IL). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Rabbit α-GFP IgG, TOP10 One-Shot and Mach1 competent cells, Lipofectamine 2000, and cell culture medias were purchased from Invitrogen (Carlsbad, CA). Secondary antibodies Alexa Fluor® 647-conjugated AffiniPure goat α-mouse IgG (H+L) and Alexa Fluor® 488 conjugated AffiniPure goat α-rabbit IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Millicell EZ 8-chamber slides were purchased from Millipore EMD (Darmstadt, Germany). Phusion DNA polymerase, Taq DNA polymerase, calf intestinal alkaline phosphatase (CIP), and restriction enzymes were purchased from New England BioLabs (Beverly, MA). Isopropyl β-D-1-thiogalactopyranoside (IPTG), Omega gel extraction kit, Omega E.Z.N.A. plasmid mini kit II, and Omega PCR cycle pure kit were purchased from Omega Bio-Tek, Inc. (Norcross, GA). Ionomycin was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Vectashield with DAPI was purchased from VWR (Radnor, PA). Natus neurology Genuine Grass® platinum subdermal needle electrodes were purchased from Fisher Scientific (Boston, MA).

Origin of DNA PCR templates and plasmids used in construction of SCANR constructs

SYS-V1046 (dsRed substrate plasmid) and SYS-V518 (GVER substrate plasmid) were purchased from Systasy Bioscience (Munich, Germany).[1] pcDNA3/Myc-DNMT3A was a gift from Arthur Riggs (Addgene plasmid #35521). pRK603 was a gift from David Waugh (Addgene plasmid #8831). G-CaMP3 was a gift from Loren Looger (Addgene plasmid #22692). pUC-dirTopo-P2A-aGFP was a gift from Hiroshi Udo (Addgene plasmid #61974). c-Flag pcDNA3 was a gift from Stephen Smale (Addgene plasmid #20011). pMAL-p5X plasmid (periplasmic directing maltose binding protein) was purchased from New England BioLabs (Beverly, MA).

Cloning of SCANR and control expression vectors

With the exception of the full-length TEV protease mammalian expression vector, all constructs were prepared using the Circular Polymerase Extension Cloning (CPEC) method, which uses overlapping regions in the DNA to assemble a linear vector component and all required inserts into a circular plasmid product.[2]

The oligonucleotides used for the CPEC reactions are shown in Tables S1–4 and were designed using the CPEC inventor's recommended guidelines for length and degree of overlap,[2] unless otherwise noted. The SCANR constructs for bacterial expression were built into the pMAL-p5x vector. The SCANR constructs for individual construct expression in mammalian systems were built into the pcDNA3/myc vector, and the SCANR constructs for multi-gene expression in mammalian systems were built into the pUC-dirTopo-P2A vector. The TEV fragments were constructed using S219D TEV protease (Addgene plasmid pRK603) as a template. The N-terminal fragment (nTEV) consisted of amino acids 1–118 and the C-terminal fragment consisted of amino acids 119–242 of the S219D TEV protease. The calmodulin and M13 components correspond to G-CaMP3 amino acids 38–58 (M13 peptide) and 304–450 (Calmodulin). The linkers between each fragment consisted of a short glycine alanine serine linker (TCCGCGGGT). The large linear vector components for CPEC were amplified by PCR using a thermocycler program with 30 cycles of 98 °C for 30 s, 54 °C for 30 s, and 72 °C for 2–6min. For PCR of the smaller CPEC reaction inserts, the thermocycler program was 30 cycles of 98 °C for 30 s, 54 °C for 30 s, and 72°C for 30 s. The PCR products for both the linear vectors and inserts were purified prior to use in CPEC reactions by agarose gel electrophoresis, product excision from the gel, and product isolation using the Omega gel extraction kit.

To combine the vector and inserts by CPEC, a two-step CPEC reaction was performed. First, each individual insert required for a particular construct was added to the CPEC reaction at a 1:1 molar ratio and the inserts were combined into a single unit by thermocycling for 25 cycles of 98 °C for 10 s, slow ramp annealing from 70 to 55 °C (1 °C/s), 55 °C for 30 s, and 72 °C for 30 s. After this first reaction was complete, the linear vector was added into the reaction at molar ratio of 1:2 (vector:insert) and the target plasmid was assembled by thermocycling for 25 cycles of 98 °C for 10 s, slow ramp anneal from 70 to 55 °C (1 °C/s), 55 °C for 30 s, and 72 °C for 2 min. In situations where the target DNA construct required the addition of only one insert, a single CPEC reaction using the molar ratios and thermocycler program for the second step described above was done. Finally, 4 μL of the CPEC reaction was transformed into Mach1 or TOP10 One-Shot chemically competent cells and individual clones were isolated and analyzed for proper insert incorporation by DNA sequencing.

The mammalian expression vector for full-length TEV protease Flag conjugate was constructed of aa 1–242 of S219D TEV protease (Addgene plasmid pRK603) using traditional restriction digest cloning using HindIII and BamHI restriction sites, which were included in its PCR primer sequences (Table S1). Essentially, full-length TEV was amplified using PCR with thermocycling at 30 cycles of 98 °C for 30 s, 54 °C for 30 s, and 72°C for 30 s, and the product was purified by excision from an agarose gel after electrophoresis and isolation using the Omega gel extraction kit. The c-terminal Flag vector (c-Flag pcDNA3, Addgene plasmid #20011) was digested with HindIII and BamHI in the presence of CIP and

purified by excision from an agarose gel followed by gel extraction using the Omega gel extraction kit before ligation at room temperature for 10 minutes with T4 DNA ligase. Finally, the plasmid was transformed into Mach1 chemically competent cells and individual clones were isolated and analyzed for proper insert incorporation by DNA sequencing.

Site directed mutagenesis of myc-Calmodulin-cTEV (Construct C1) and myc-M13-cTEV (Construct M3) was required due to the inadvertent addition of a base pair that produced a frame shift in the original PCR primers (Denoted with an asterisk in Table S1). The site-directed mutagenesis repair was performed using Pfx Turbo polymerase, 50 ng of the template DNA, and 100 ng of the forward and reverse mutagenesis primers. The reaction was completed with the following thermocycler program: 16 cycles of 95 °C for 30 s, 55 °C for 30 s, and 68°C for 6 min. The reaction was then digested with DpnI and the product was purified with the Omega PCR Cycle Pure kit prior to its transformation into TOP10 competent cells. Individual clones were isolated and analyzed for successful mutagenesis and construct integrity by DNA sequencing.

Mutagenesis of myc- D133E Calmodulin-cTEV (Construct C1) to reduce the Ca^{2+} binding ability of calmodulin, was produced using the linearization PCR primers (Table S5). The linear vector was amplified by PCR using a thermocycler program with 30 cycles of 98 °C for 30 s, 54 °C for 30 s, and 72 °C for 2–6min. The band was purified by agarose gel electrophoresis, product excision from the gel, and product isolation using the Omega gel extraction kit. The linear mutated vector was circularized without nicks via thermocycling for 25 cycles of 98 °C for 10 s, slow ramp annealing from 70 to 55 °C (1 °C/s), 55 °C for 30 s, and 72 °C for 30 s. Finally, 4 μL of the CPEC reaction was transformed into Mach1 or TOP10 One-Shot chemically competent cells and individual clones were isolated and analyzed for proper mutation by DNA sequencing

Bacterial SCANR protein expression and solubility assays

Periplasmic-directing maltose binding protein (MBP) construct fusion proteins were transformed into Rosetta Blue competent cells and utilized for protein expression. Cultures in carbenicillin/chloramphenicol 2xYT rich media were grown at 37 °C with continuous 250 rpm agitation. When the cultures reached an O.D of 0.8., the *E. coli* were induced by addition of IPTG to a concentration of 0.4 mM and allowed to express overnight at 16 °C with continuous 250 rpm agitation. Cells were then pelleted by centrifugation at 4000 x g (10 min 4 °C) and lysed by sonication using a Fisher Scientific Sonic Dismembrator Model 100 with 6 W continuous 30-s on, 1-min off, in 0.03 M Tris-HCl, 0.25 M NaCl, at pH 7.5 unless otherwise stated. Following sonication, the cell debris was pelleted by centrifugation at 39,000 x g for 1 h at 4 °C. The soluble portion was isolated from the insoluble cell debris in the pellet by decanting the supernatant. SDS PAGE was used to analyze the solubility and expression of proteins (Figure S1). Protein solubility assays were performed with *E. coli* whole cell pellets being re-suspended in 1 mL of test lysis buffer (Figure S1) in 10% lysozyme, incubated overnight with agitation, and sonicated as described above before being pelleted by centrifugation at 17,000 x g for 10 minutes at room temperature.

HEK293 cell culture and transfection

HEK293 cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS and 50,000 U penicillin-streptomycin at 37 °C in air with 5% CO_2. Cells were passaged on alternating days when they reached approximately 85% confluency. Cells were transfected at 75–85% confluence in Millipore 8-chamber culture slides with Lipofectamine 2000 according to the manufacturer's instructions. Cells were cultured for 24 h before ionomycin assays or immunohistochemistry analyses were performed.

Ionomycin assay for increasing intracellular Ca^{2+} in HEK293 cells

Media was removed from the cells and the cells were washed twice with 400 μL phosphate buffered saline (PBS) pH 7.4. Cells were incubated in 5 μM ionomycin in Hanks balanced salt solution (HBSS) for 1, 2, 3 ,4, or 5 minutes at room temperature. The ionomycin solution was removed via aspiration and the cells were washed 2 times with 400 μL PBS to remove all traces of ionomycin. The cells were put into fresh DMEM solution and allowed to incubate for 24 h at 37 °C in air with 5% CO_2. After 24 h, the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes. Vectashield with 4',6-diamidino-2-phenylindole (DAPI) was added to the plate before addition of the coverslip and subsequent imaging by confocal microscopy.

Quantification of DsRed-positive nuclei in SCANR-expressing HEK293 cells ± ionomycin

At time points of 0, 12, and 24 h after exposure of SCANR- and caged DsRed substrate-expressing HEK293 cells to ionomycin or vehicle control, the number of DsRed-fluorescent nuclei per well of an 8-well chamber slide was manually counted by visualization of the live cells with a Zeiss Axio Vert A.1 inverted microscope equipped with a Zeiss EC Plan-Neofluar 20x NA0.50 objective.

Immunohistochemistry analysis of HEK293 cells

Transfected cells were washed with 400 μL PBS at 24 h post transfection and incubated in 4% paraformaldehyde in PBS for 15 minutes at room temperature. The cells were washed and then permeabilized for 3 min at room temperature by treatment with a solution of 1% bovine serum albumin (BSA) and 0.1% Triton-X 100 in PBS. After washing three times with 400 μL of PBS, the cells were blocked with 1% BSA in PBS for 10 minutes. For cells being probed with α-myc, cells were incubated 1:200 α-myc overnight at 4 °C in 1% BSA in PBS. The cells were washed 3 times with 400 μL PBS before incubation with a 1:100 dilution of goat α-mouse Alexa Flour® 647 in 1% BSA/PBS overnight at 4 °C. Subsequently, the cells were washed 3 times with 400 μL PBS before addition of vectashield with DAPI was added to the plate before addition of the coverslip and subsequent imaging with confocal microscopy.

Primary rat hippocampal neuron culture and transfection

One pair of E18 Sprague Dawley Rat hippocampus was cultured as previously described.[3,4] Neurons were incubated at 37 °C in air with 5% CO_2. Approximately half of the media was exchanged with fresh NbActiv1 every 3–4 days. Neurons were transfected with Lipofectamine 2000 after 5 days *in vitro* and allowed to express undisturbed for 24 h prior to field stimulation. Preliminary assessment of the neurons was performed with a Zeiss 2 Axio Vert A.1 inverted microscope with a 10x EC Plan-NeoFluar NA0.3 or 20x EC Plan-NeoFluar NA0.5 objective.

Primary rat hippocampal neuron field stimulation assay

Platinum electrodes were placed 1 cm apart to fit within an 8-cell microscope slide culture will. Neurons were stimulated for 1 ms every 33 ms for a duration of 30 s at 15 V using a Grass SD9 stimulator. Non-stimulated neurons were left undisturbed at 37 °C and 5% CO_2.

Immunohistochemistry analysis of SCANR-expressing rat hippocampal neurons

After stimulation, primary rat hippocampal neurons were incubated at 37 °C and 5% CO_2 for approximately 24 h to permit uncaged DsRed fluorophore maturation. Subsequently, neurons were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized by incubation with 0.1% Triton X-100 and 10% Normal Goat Serum (NGS) in PBS for 30 min at room temperature, and incubated overnight at 4 °C with primary antibody α-GFP (1:200) in PBS containing 0.1% Triton X-100 and 10% NGS. After primary antibody incubation, the neurons were washed twice with 400 μL of PBS at room temperature prior to incubation with the secondary antibody, goat α-rabbit IgG (H+L) conjugated to Alexa Flour® 488 at a dilution of 1:100 overnight at 4 °C in 0.1% Triton X-100 and 10% NGS in PBS. Subsequently, the neurons were washed twice with 400 μL of PBS before addition of Vectashield with DAPI, coverslip, and imaging by confocal microscopy.

Confocal imaging and image analysis

Confocal microscopy was performed using a Zeiss Axio Examiner.D1 modified with an Andor Differential Scanning Disk 2 confocal unit equipped with a 40x NA1.0 water immersion objective and Piezo objective holder for acquiring Z-stacks. Optimal z-stack step size was calculated using the Andor iQ3 software to provide Nyquist sampling. Excitation and emission filters used were: Blue channel excitation 390/40, emission 452/45; Green channel excitation 482/18, emission 525/45; Red channel excitation 556/20, emission 609/54; Far red channel excitation 640/14. Emission 676/29. Confocal images were converted to maximum intensity projections of the z-stack and level-normalized across all images using ImageJ. Image cropping and organization into figure illustrations was performed using Adobe Illustrator CC. Fixed cells were randomly sampled for each condition. A maximum intensity z-projection of the red channel confocal image for each cell was created containing the slices with the nucleus. An ROI was drawn around the nucleus of each cell and the mean intensity of the pixels in that region was collected.

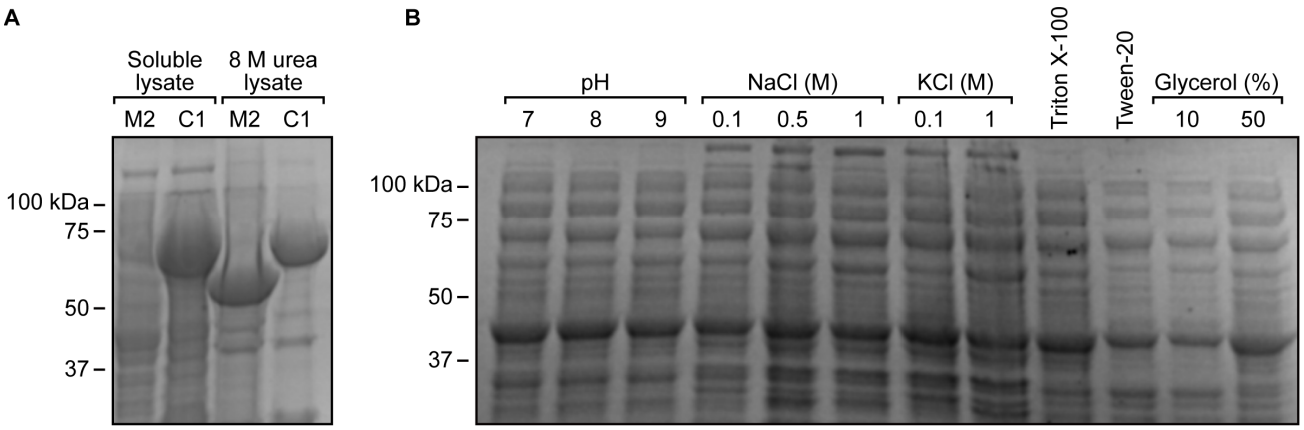

Figure S1: *E. coli* expression and solubility assays of protein constructs. (**A)** Expression and solubility products of the cTEV constructs – M2 and C1. Expected molecular weights are 60.45 kDa (M2) and 63.14 kDa (C1). (**B**) Solubility panel of construct M1 with varying lysis buffers. From left to right: (Lanes 1–3) 50 mM Tris, 1 mM EDTA, pH 7–9; (Lanes 4–8) 50 mM Tris, 1 mM EDTA, pH 7.5, 0.1–1 M NaCl or KCl; (Lanes 9–12) 20 mM Tris, 50 mM NaCl, pH 7.5, and 0.2% Triton X-100, or 0.2% Tween-20, or 10–50% glycerol. Expected molecular weight of M1 is 63.14 kDa.

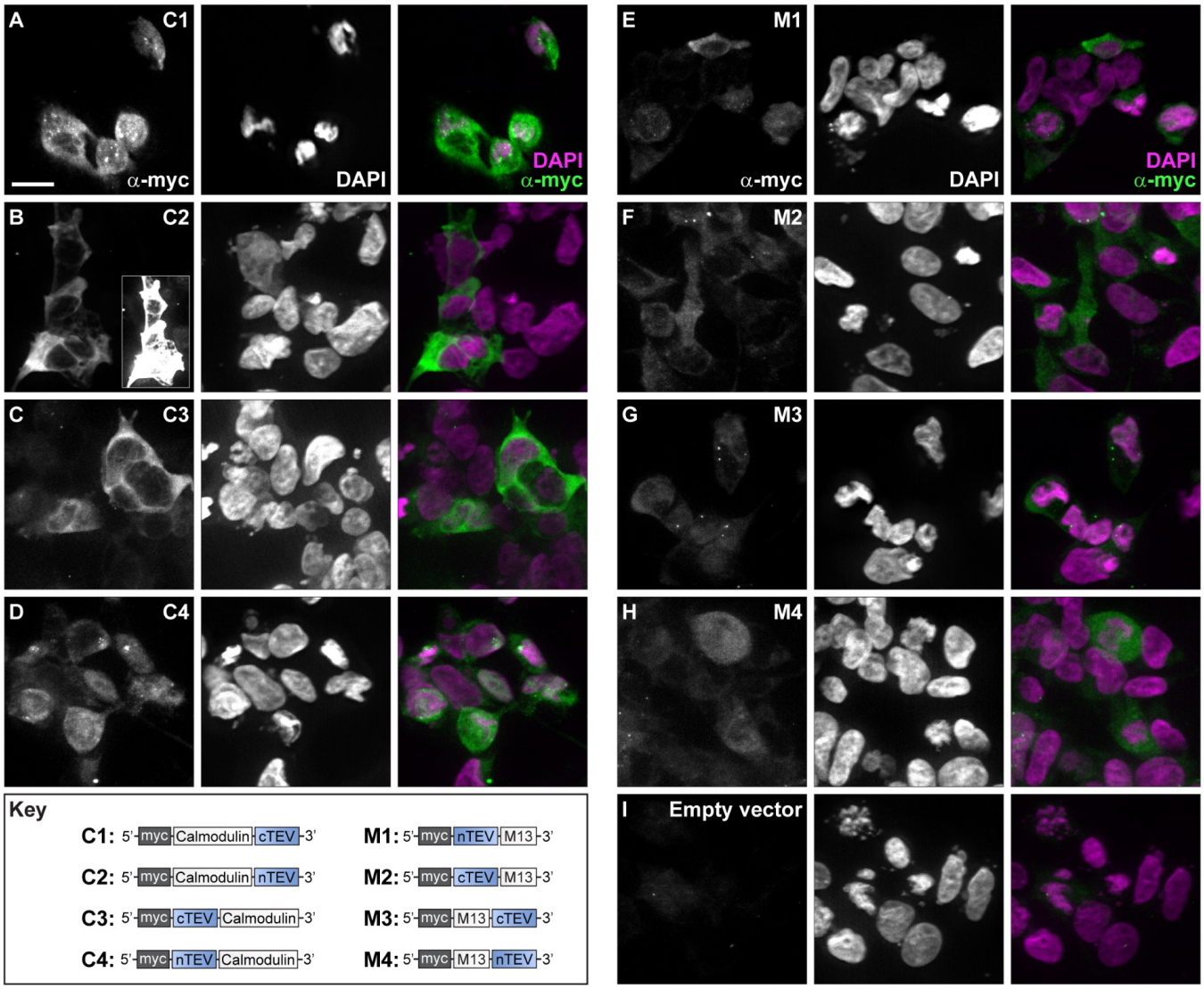

Figure S2. Candidate SCANR construct HEK293 expression analysis. (**A**–**I**) Constructs C1–4 (**A–D**), M1–4 (**E–H**), or empty vector control (**I**) were transfected into HEK293 cells and, at 24 h post transfection, the cells were fixed, probed with α-myc to visualize construct expression, and imaged by confocal microscopy. Inset in (**B**) shows the fluorescence intensity of thehigh expressing construct C2 at settings comperable to the other α-myc panels. All images are maximum intensity projections of a confocal z-stack. Scale = 20 μm.

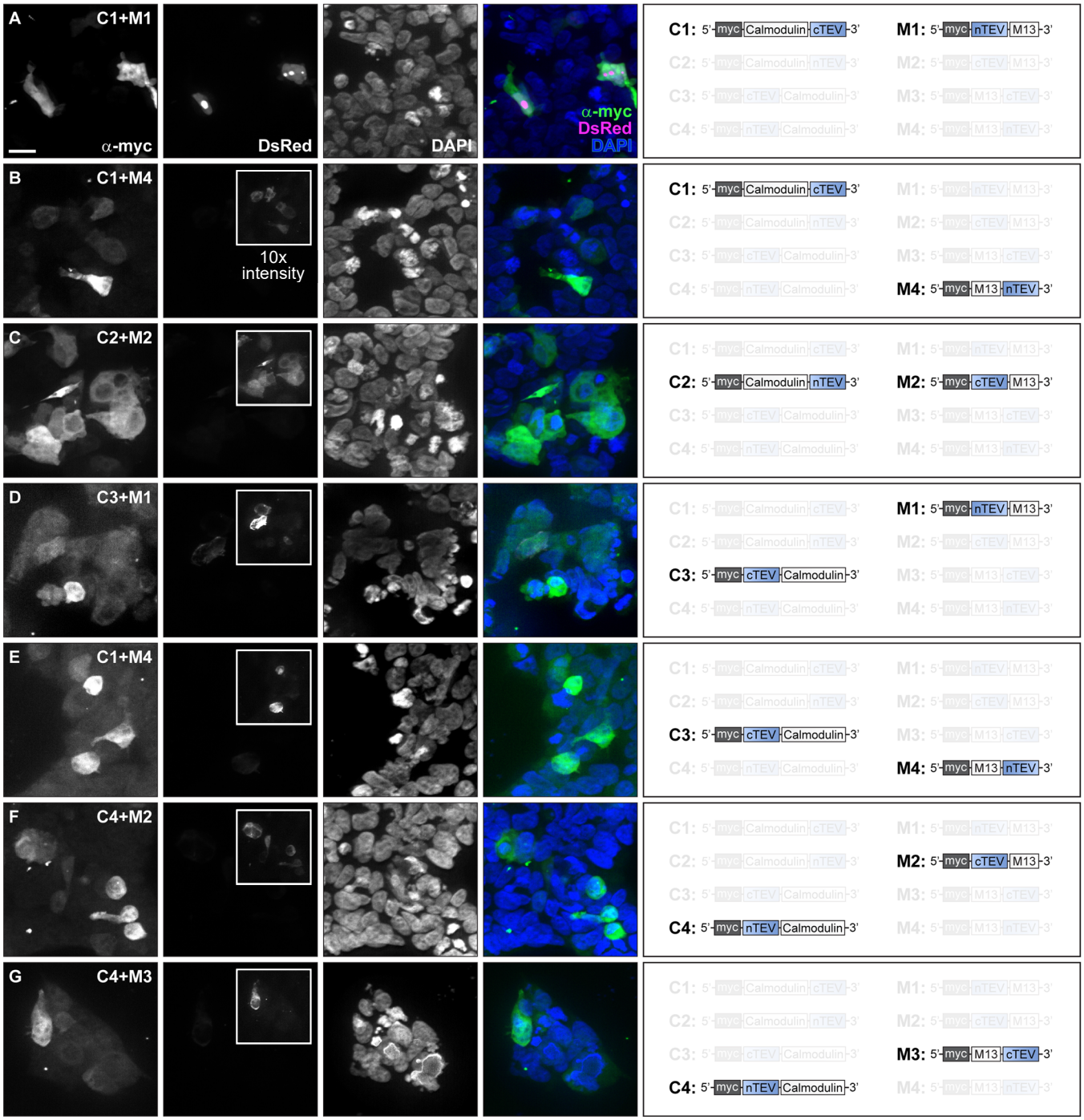

Figure S3. Candidate SCANR chimera analyses in HEK293 cells. (**A**–**G**) Binary sense combinations of chimeras C1–4 and M1–4 (*i.e.*, combinations that contain one each of calmodulin, M13, cTEV, and nTEV) as well as the caged DsRed TEV substrate were transfected into HEK293 cells. At 24 h post-transfection, cells were exposed to ionomycin to produce a transient high intracellular Ca^{2+} concentration and then allowed to recover for 24 h to permit TEV-liberated DsRed substrate to oligomerize, translocate to the nucleus, and form a mature fluorophore. Insets in panels B–G show DsRed fluorescence at 10x intensity to visualize uncleaved DsRed substrate. All images are maximum intensity projections of a confocal z-stack. Scale bar = 20 μm.

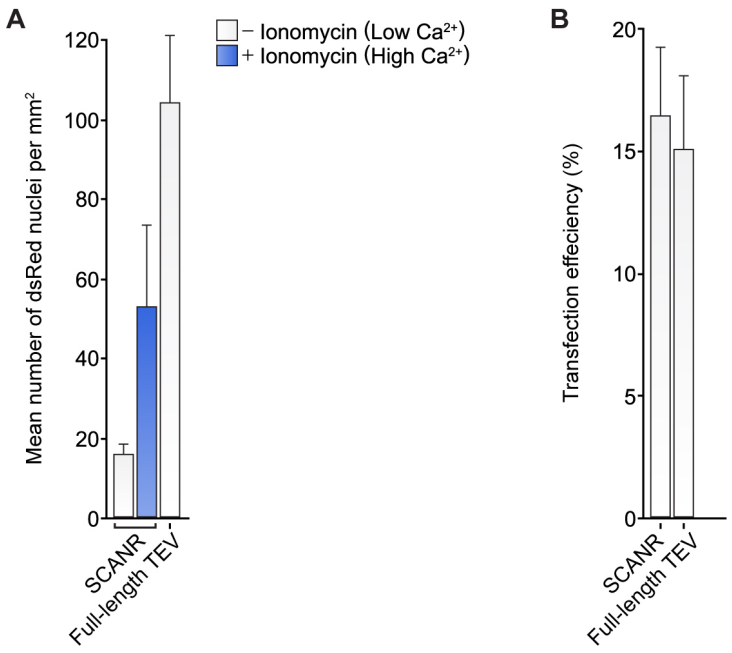

Figure S4. Comparison to of SCANR +/- ionomycin exposure to full length TEV protease. Hek293 cells were transfected with SCANR components and caged DsRed substrate or full-length TEV protease and DsRed substrate and exposed to ionomycin or vehicle control solutions. After 24 h (**A**) the number of DsRed positive nuclei were counted and (**B**) transfection effeciency was determined by quantifying transfected cells (α-myc positive) vs. the total number of cells (DAPI positive). Error bars represent the standard deviation.

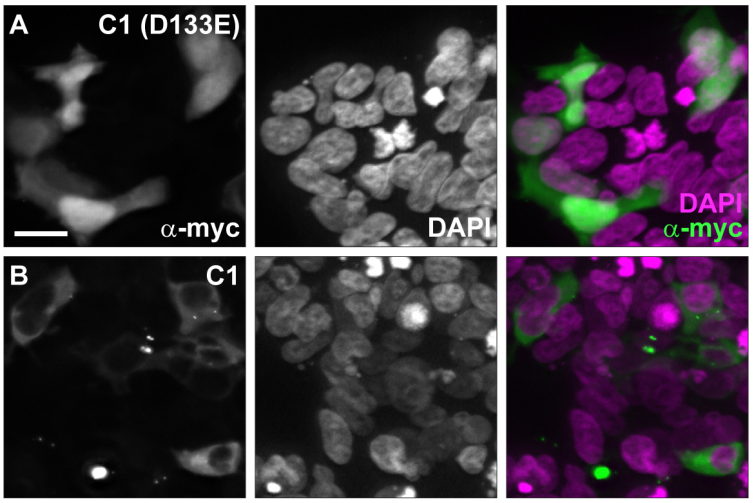

Figure S5: **Evaluation of C1 D133E expression levels.** HEK293 cells were transfected with C1 D133E or C1, both containing a myc tag, and expression was determined by probing the cells with α-myc. Scale = 20 μm.

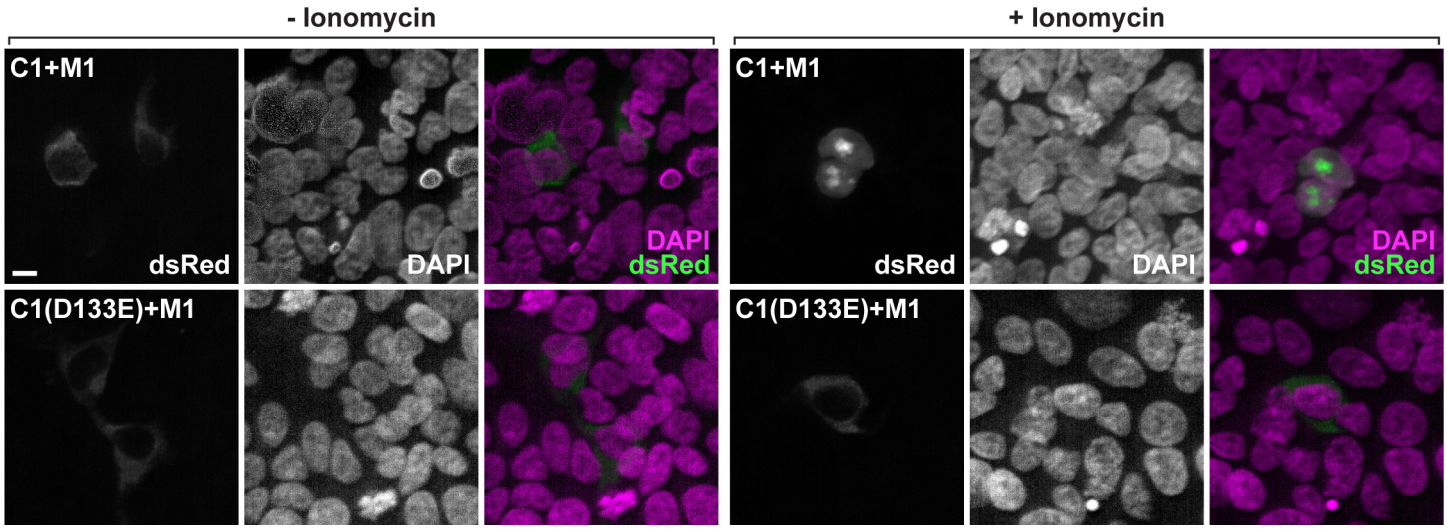

Figure S6: **C1 D133E vs. C1 responses to ionomycin-induced Ca^{2+} concentration increases.** HEK293 cells were transfected with the indicated constructs (C1+M1 or C1(D133E)+M1) and the caged DsRED TEV substrate. 24 h post-transfection the cells were fixed and imaged by confocal microscopy. Images are maximum intensity projections of a confocal z-stack. Scale = 10 μm.

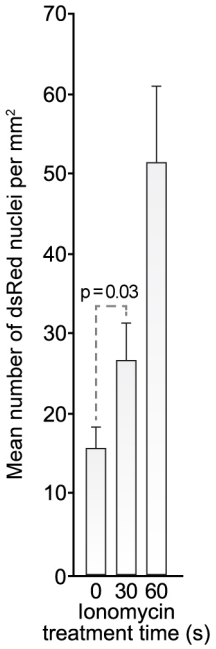

Figure S7: **Evaluation of SCANR activation after 0, 30, and 60 s ionomycin treatment times.** HEK293 cells were transfected with C1 + M1 and the caged DsRED TEV substrate. 24 h post-transfection dsRed positive nuclei were quantified. Error bars represent standard deviation for a sample size of 3.

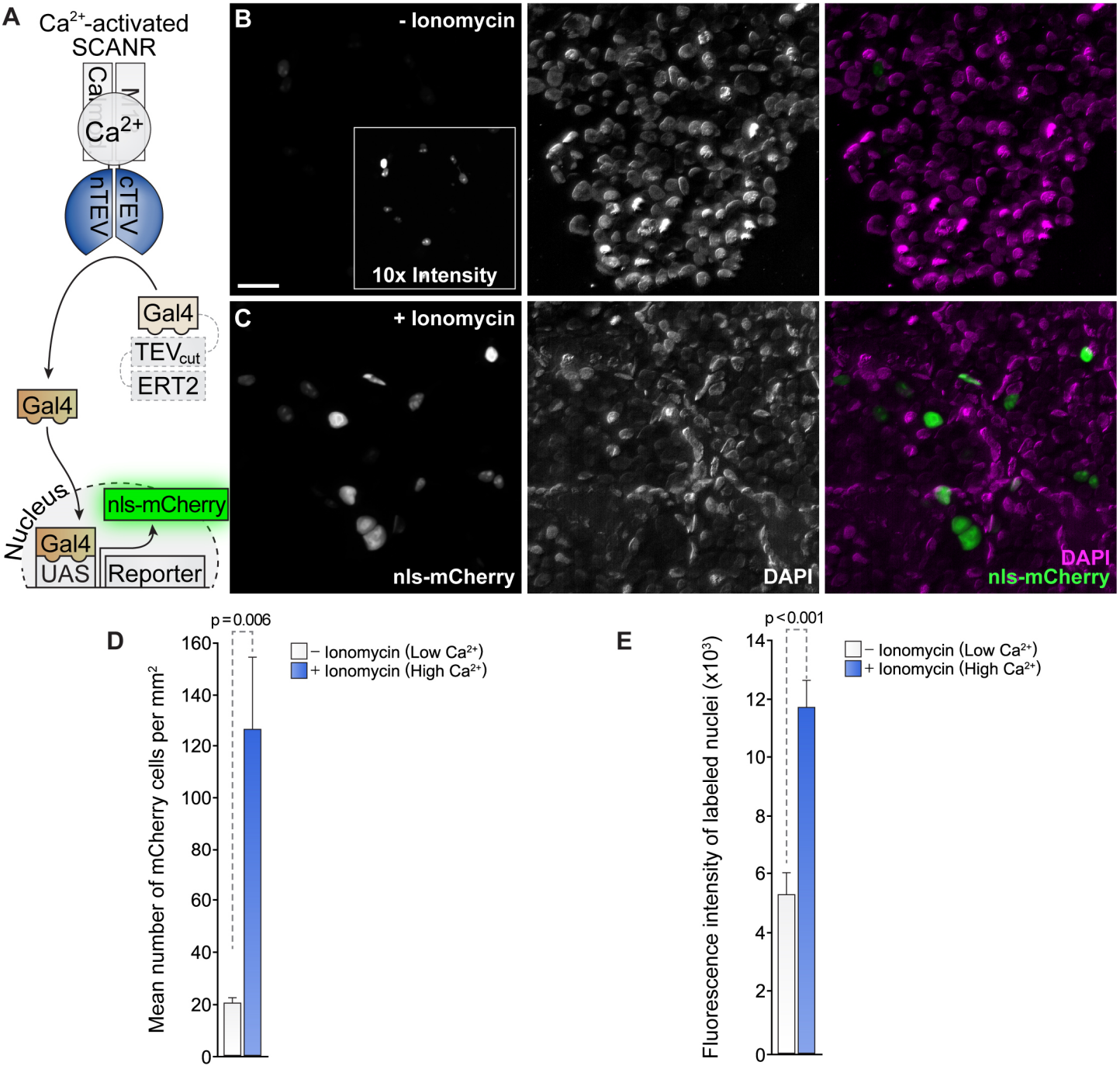

Figure S8: **SCANR use with a Gal4-driven mCherry-nls.** (**A**) Schematic for Gal4 substrate and reporter function with SCANR. (**B**–**C**) Maximum intensity projections of HEK293 cells transfected with the SCANR constructs, Gal4 TEV substrate and UAS -mCherry-nls and exposed to control or high Ca^{2+} conditions by exposure to vehicle (B) or ionomycin (C), respectively. (**D**–**E**) Quantification of the number (D) and fluorescence intensity (E) of mCherry labeled cells from the experiment described in B and C. Images are maximum intensity projections of a confocal z-stack. Error bars represent standard deviation for a sample size of 3 (D) or standard error of the mean for a sample size of 105 (+ ionomycin) or 17 (- ionomycin). Scale = 50 μm.

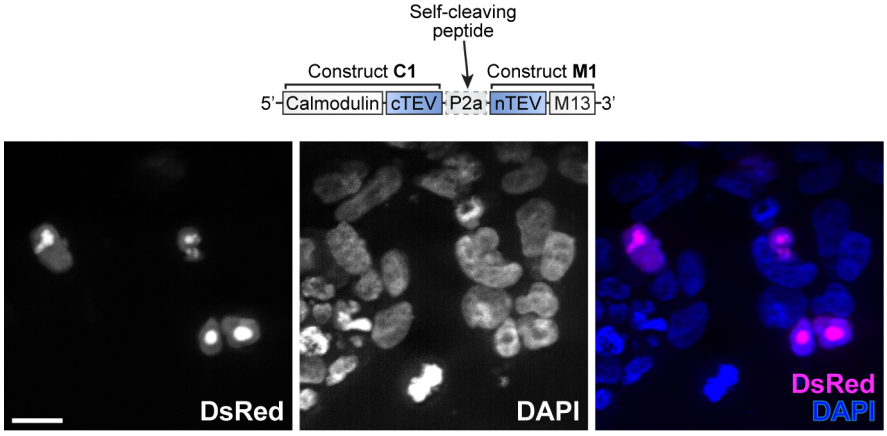

Figure S9: **Direct linkage of SCANR constructs with a P2A self-cleaving peptide results in active TEV protease at resting intracellular Ca^{2+} concentrations.** HEK293 cells were transfected with the indicated P2A SCANR construct and the caged DsRED TEV substrate. 24 h post-transfection the cells were fixed and imaged by confocal microscopy. Images are maximum intensity projections of a confocal z-stack. Scale = 20 µm.

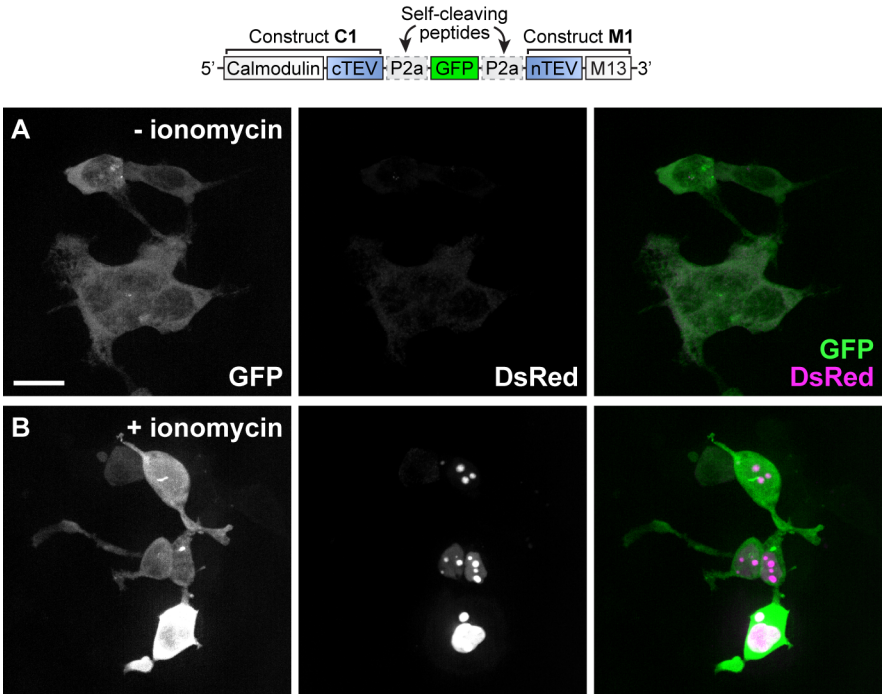

Figure S10: Multi-gene SCANR construct with GFP for expression control. HEK293 cells were transfected with the indicated P2A SCANR GFP construct and the caged DsRED TEV substrate. 24 h post-transfection the cells were exposed to control (- ionomycin) or ionomycin (+ ionomycin) to produce a transient high intracellular Ca^{2+} concentration. 24 h later the cells were fixed and imaged by confocal microscopy. Images are maximum intensity projections of a confocal z-stack. Scale = 20 μm.

Table S1: **Primers used for candidate SCANR cloning.** All primers written in the 5' to 3' direction. Asterisk denotes constructs that required repair by site directed mutagenesis with the indicated primers.

Target Construct		Amplicon		DNA Oligonucleotide Sequence
C1	5'-myc-Calmodulin-cTEV-3'	Calmodulin	*For*	CTCAGAGGAGGACCTGGAATTCATGGACCAACTGACTGAAGAGC
			Rev	CCATGCTAGACATACCCGCGGACTTCGCTGTCATCATTTGTAC
		cTEV*	*For*	TACAAATGATGACAGCGAAGTCCGCGGGTATGTCTAGCATGGTGTC
			Rev	CGAGCGGCCGCGGTACCTCACCATGAAAACTTTATGGCC
C2	5'-myc-Calmodulin-nTEV-3'	Calmodulin	*For*	CTCAGAGGAGGACCTGGAATTCATGGACCAACTGACTGAAGAGC
			Rev	CTTTCTCCCATACCCGCGGACTTCGCTGTCATCATTTGTAC
		nTEV	*For*	GATGACAGCGAAGTCCGCGGGTATGGGAGAAAGTTTGTTTAAG
			Rev	CTCGAGCGGCCGCGGTACCTCAAGTTTGGAAGTTGGTTGTC
C3	5'-myc-cTEV-Calmodulin-3'	cTEV	*For*	CTCAGAGGAGGACCTGGAATTCATGTCTAGCATGGTGTCAGAC
			Rev	CAGTTGGTCCATACCCGCGGACATGAAAACTTTATGGCCCCCC
		Calmodulin	*For*	CATAAAGTTTTCATGTCCGCGGGTATGGACCAACTGACTGAAGAGC
			Rev	CGAGCGGCCGCGGTACCTCACTTCGCTGTCATCATTTGTACAAAC
C4	5'-myc-nTEV-Calmodulin-3'	nTEV	*For*	CAGAGGAGGACCTGGAATTCATGGGAGAAAGTTTGTTTAAGGGGC
			Rev	GTTGGTCCATACCCGCGGAAGTTTGGAAGTTGGTTGTCAC
		Calmodulin	*For*	CTTCCAAACTTCCGCGGGTATGGACCAACTGACTGAAGAGCAG
			Rev	CGAGCGGCCGCGGTACCTCACTTCGCTGTCATCATTTGTACAAAC
M1	5'-myc-nTEV-M13-3'	nTEV	*For*	CAGAGGAGGACCTGGAATTCATGGGAGAAAGTTTGTTTAAGGGGC
			Rev	CGTGATGAACCCGCGGAAGTTTGGAAGTTGGTTGTCACAAG
		M13	*For*	CTTCCAAACTTCCGCGGGTTCATCACGTCGTAAGTGGAATAAG
			Rev	CGAGCGGCCGCGGTACCTCACGAGCTTGACAGCCGACCTATAGC
M2	5'-myc-cTEV-M13-3'	cTEV	*For*	CTCAGAGGAGGACCTGGAATTCATGTCTAGCATGGTGTCAGAC
			Rev	CTTACGACGTGATGAACCCGCGGACATGAAAACTTTATGGCCCC
		M13	*For*	AAAGTTTTCATGTCCGCGGGTTCATCACGTCGTAAGTGGAATAAG
			Rev	CGAGCGGCCGCGGTACCTCACGAGCTTGACAGCCGACCTATAGC
M3	5'-myc-M13-cTEV-3'	M13	*For*	CAGAGGAGGACCTGGAATTCTCATCACGTCGTAAGTGGAATAAG
			Rev	CCATGCTAGACATACCCGCGGACGAGCTTGACAGCCGACCTATAG
		cTEV*	*For*	GTCAAGCTCGTCCGCGGGTATGTCTAGCATGGTGTCAGACACTAG
			Rev	CGAGCGGCCGCGGTACCTCACCATGAAAACTTTATGGCC
M4	5'-myc-M13-nTEV-3'	M13	*For*	CAGAGGAGGACCTGGAATTCTCATCACGTCGTAAGTGGAATAAG
			Rev	CTCCCATACCCGCGGACGAGCTTGACAGCCGACCTATAGC
		nTEV	*For*	TAGGTCGGCTGTCAAGCTCGTCCGCGGGTATGGGAGAAAGTTTG
			Rev	CTCGAGCGGCCGCGGTACCTCAAGTTTGGAAGTTGGTTGTC
Linearized vector destination for C1–4 & M1–4 ligation independent cloning			*For*	GAATTCCAGGTCCTCCTCTGAGATCAGCTTCTGCTCCATGGTGGC
			Rev	TGAGGTACCGCGGCCGCTCGAGCATGCATCTAGAGGGCCCTATTC
Full-length TEV-flag		TEV S219D	*For*	TCATCAT**AAGCTT**ATGGGAGAAAGTTTGTTTAAG
			Rev	CTTC**GGATCC**GTTCACCATGAAAACTTTATG
C1 & M3 site-directed mutagenesis of cTEV component			*For*	GCCATAAAGTTTTCATGTGAGGTACCGCGGCCGCTCGAG
			Rev	CTCGAGCGGCCGCGGTACCTCACATGAAAACTTTATGGC

Table S2: **Primers used for cloning SCANR construct bacterial expression vectors.** All primers written in the 5' to 3' direction.

Target Construct	Amplicon	DNA Oligonucleotide Sequence	
pMBP-C1	Calmodulin	*For*	GAAGGATTTCACATATGTCCATGATGGACCAACTGACTGAAGAGC
		Rev	CCATGCTAGACATACCCGCGGACTTCGCTGTCATCATTTGTAC
	cTEV	*For*	TACAAATGATGACAGCGAAGTCCGCGGGTATGTCTAGCATGGTGTC
		Rev	GTGGTGGTGGTGGTGCTCGAGCATGAAAACTTTATGGCCCC
pMBP-M1	nTEV	*For*	GGATTTCACATATGTCCATGATGGGAGAAAGTTTGTTTAAGGGGC
		Rev	CGTGATGAACCCGCGGAAGTTTGGAAGTTGGTTGTCACAAG
	M13	*For*	CTTCCAAACTTCCGCGGGTTCATCACGTCGTAAGTGGAATAAG
		Rev	GTGGTGGTGGTGGTGCTCGAGCGAGCTTGACAGCCGACCTATAGC
pMBP-M2	M13	*For*	GAAGGATTTCACATATGTCCTCATCACGTCGTAAGTGGAATAAG
		Rev	CCATGCTAGACATACCCGCGGACGAGCTTGACAGCCGACCTATAG
	cTEV	*For*	GTCAAGCTCGTCCGCGGGTATGTCTAGCATGGTGTCAGACACTAG
		Rev	GTGGTGGTGGTGGTGCTCGAGCATGAAAACTTTATGGCCCC
pMBP-C1; M1; M3	Linear pMBP	*For*	CATGGACATATGTGAAATCCTTCCCTCGATCCCGAGGTTGTTG
		Rev	CTCGAGCACCACCACCACCACCACTGAGATGGATCCGAATTC

Table S3: **Primers used for cloning UAS-EGFP, UAS-mCherry-nls for use with the caged Gal4 substrate.** All primers written in the 5' to 3' direction.

Target Construct	Amplicon	DNA Oligonucleotide Sequence	
5xUAS-EGFP	EGFP	*For*	GGTACTGTTGGTAAAGCCACCATGGTGAGCAAGGGCGAGG
		Rev	CCCCGACTCTAGAGTCGCGGCCTTACTTGTACAGCTCGTCCATGC
	Linear 5x UAS	*For*	GGTGGCTTTACCAACAGTACCGGATTGCCAAGC
		Rev	GGCCGCGACTCTAGAGTCGGGGCGGCCGGCCGCTTCGAG
5xUAS-mCherry-nls	*mCherry*	*For*	GGTACTGTTGGTAAAGCCACCATGGTGAGCAAGGGCGAGG
		Rev	GACAGGCGGCCCTTGTACAGCTCGTCCATGCCGCCGGTG
	nls	*For*	GACGAGCTGTACAAGGGCCGCCTGTCTCGAGCTGATC
		Rev	AGAGTCGCGGCCTTATCTAGATCCGGTGGATCCTACCTTTCTCTTC
	Linear 5x UAS	*For*	CACCGGATCTAGATAAGGCCGCGACTCTAGAGTCGGGGCGG
		Rev	CTTGCTCACCATGGTGGCTTTACCAACAGTACCGGATTGCC

Table S4: **Primers used for cloning P2A SCANR constructs.** All primers written in the 5' to 3' direction.

Target Construct	Amplicon	DNA Oligonucleotide Sequence	
C1-P2A-EGFP-P2A-M1	C1	*For*	GATAACAATTTCCGGAGGAGGCCCTTCACCATGGACCAACTGACTG
		Rev	GAAGTTAGTAGCATTGGATCCCATGAAAACTTTATGGCCCCCCC
C1-P2A	Linear P2A for C1	*For*	GAAGGGCCTCCTCCGGAAATTGTTATCCGCTC
		Rev	GGATCCAATGCTACTAACTTCAGCCTGCTCAAGC
C1-P2A-EGFP	EGFP	*For*	GGCTGAAGTTAGTAGCATTGGATCCCTTGTACAGCTCGTCCATG
		Rev	GAGGAGAACCCTGGACCTGTCGACATGGTGAGCAAGGGCGAGG
C1-P2A-EGFP-P2A	Linear C1 P2A for EGFP	*For*	CATATGTATATCTCCTTCTTATAC
		Rev	GAGTCTGGTAAAGAAACCGCTGCTGCG
C1-P2A-EGFP-P2A-M1	Myc-M1	*For*	GCATGGACGAGCTGTACAAGGGATCCAATGCTACTAACTTCAG
		Rev	CAGTTATCTAGATTACTCGAGCGAGCTTGACAGCCGACCTATAG
C1-P2A-EGFP-P2A-M1	linear C1-P2A-EGFP-P2A for M1	*For*	CTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGC
		Rev	CTCGAGTAATCTAGATAACTGATCATAATCAGCCATACCACATTTG

Table S5: **Primers used for cloning D133E C1.** All primers written in the 5' to 3' direction.

Target Construct	DNA Oligonucleotide Sequence	
Mutagenesis of D133E calmodulin of C1	*For*	GGGAAGCAGACATCGATGGGGAGGGTCAGGTAAACTACGAAGAG
	Rev	CTCTTCGTAGTTTACCTGACCCTCCCCATCGATGTCTGCTTCCC

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