Supporting Information for

Leveraging new definitions of the LxVP SLiM to discover novel Calcineurin regulators and substrates

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		K_D (μM)
NFATc1	^{383}DDQYLAVPQHPYQWAKPK^{400}	5.9 ± 1.2
NHE1	^{678}QKINNYLTVPAHKLDSP^{694}	18 ± 1
Nup53	^{143}GQPRKTTLSPAQLDPFYTQ^{161}	35 ± 4
RANBP3	^{324}GQNMSERVLSPPKLNEVS^{341}	85 ± 17
NFATc2	^{366}ESILLVPPTWPKPLVP^{381}	265 ± 33
Nup160	^{39}GALERSFVELSGAERERPR^{57}	460 ± 179
SKP1.1	^{76}ENKEKRTDDIPVWDQEFLK^{94}	616 ± 208
MCM2	^{36}DALTSSPGRDLPPFEDESE^{54}	864 ± 147
GCFC2	^{86}EGSESRTLDVSTDEEDKIH^{104}	882 ± 116
IPO7	^{238}RDVPNETLQVEEDDRPE^{254}	1244 ± 442
	πφLxVP	

Table S1: Peptides used for SPR measurements and their associated affinities for $CN_{A: 1-370; B: 1-169}$

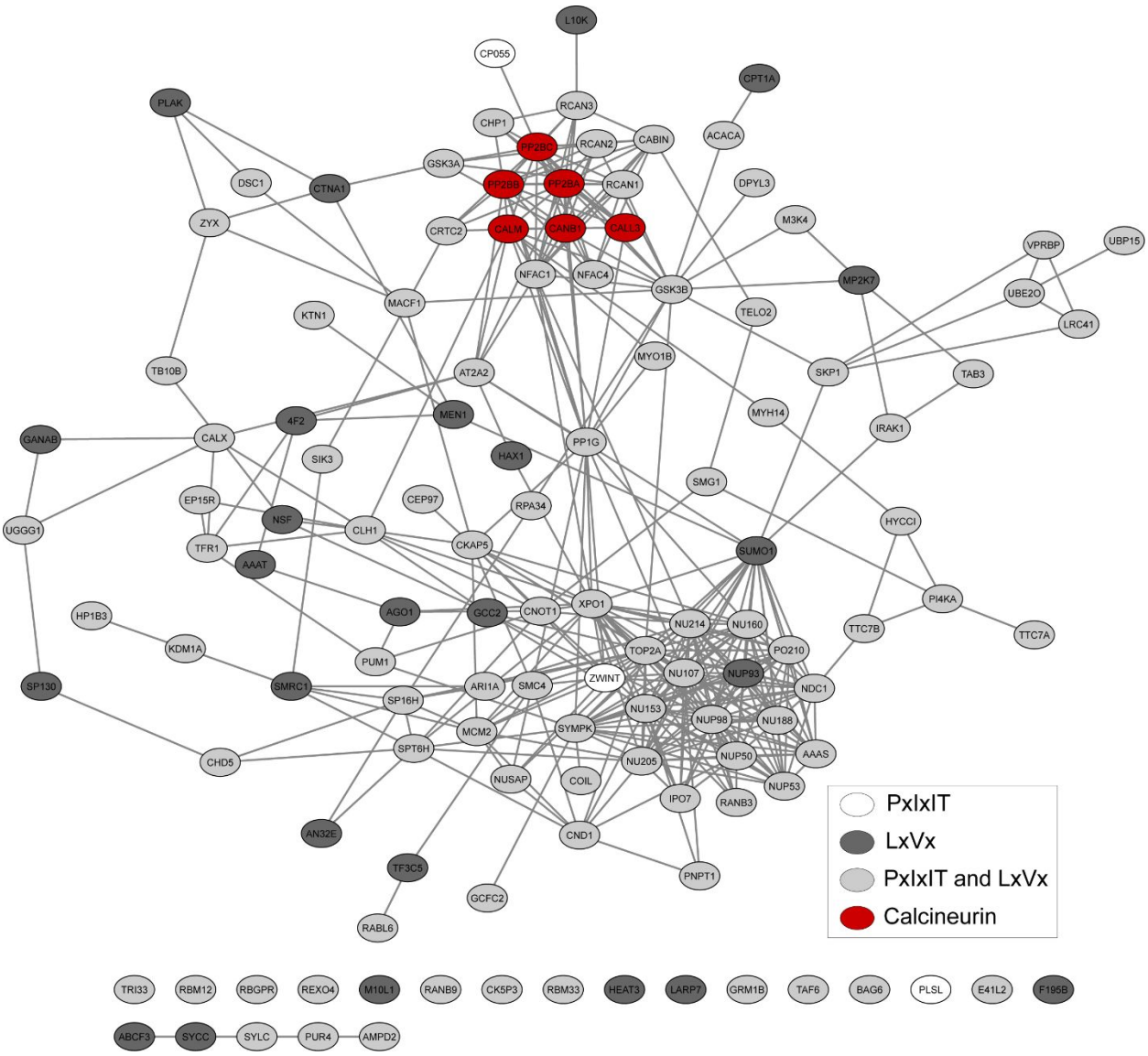


Figure S1: CN Interaction network based on the STRING database for PxIxIT and LxVx motif containing proteins. Nodes are colored based on the presence of a PxIxIT (white), LxVx (dark grey), PxIxIT and LxVx (light grey) SLiM motif. CN subunits and associated proteins are shown in red.

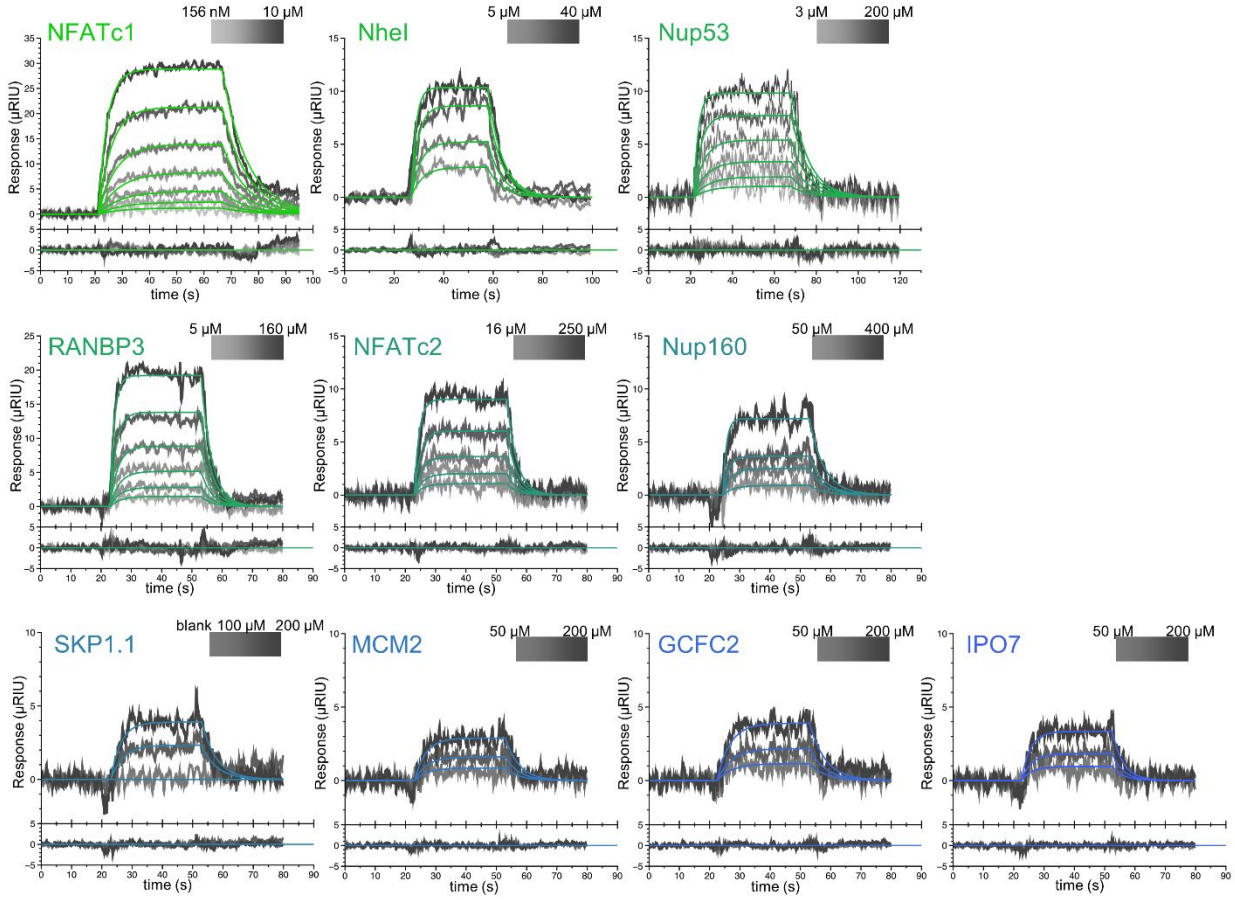
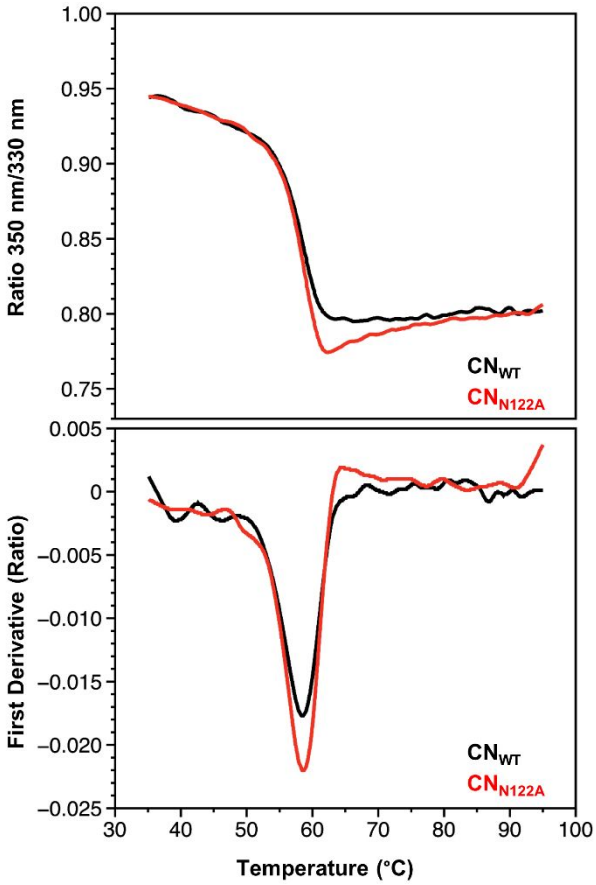


Figure S2: SPR traces for peptides in Table S1. Concentration series for each peptide are 2-fold dilutions of maximum concentration highlighted.



CN variant	T_m (°C)	n
CN_{WT}	59.5 ± 0.6	4
CN_{N122A}	58.5 ± 0.1	5

Figure S3: Data for measurement of unfolding stability measurements (TM) for CN_{WT} and CNN_{122A}. Table of statistics for unfolding TM for CN_{WT} and CNN_{122A}.

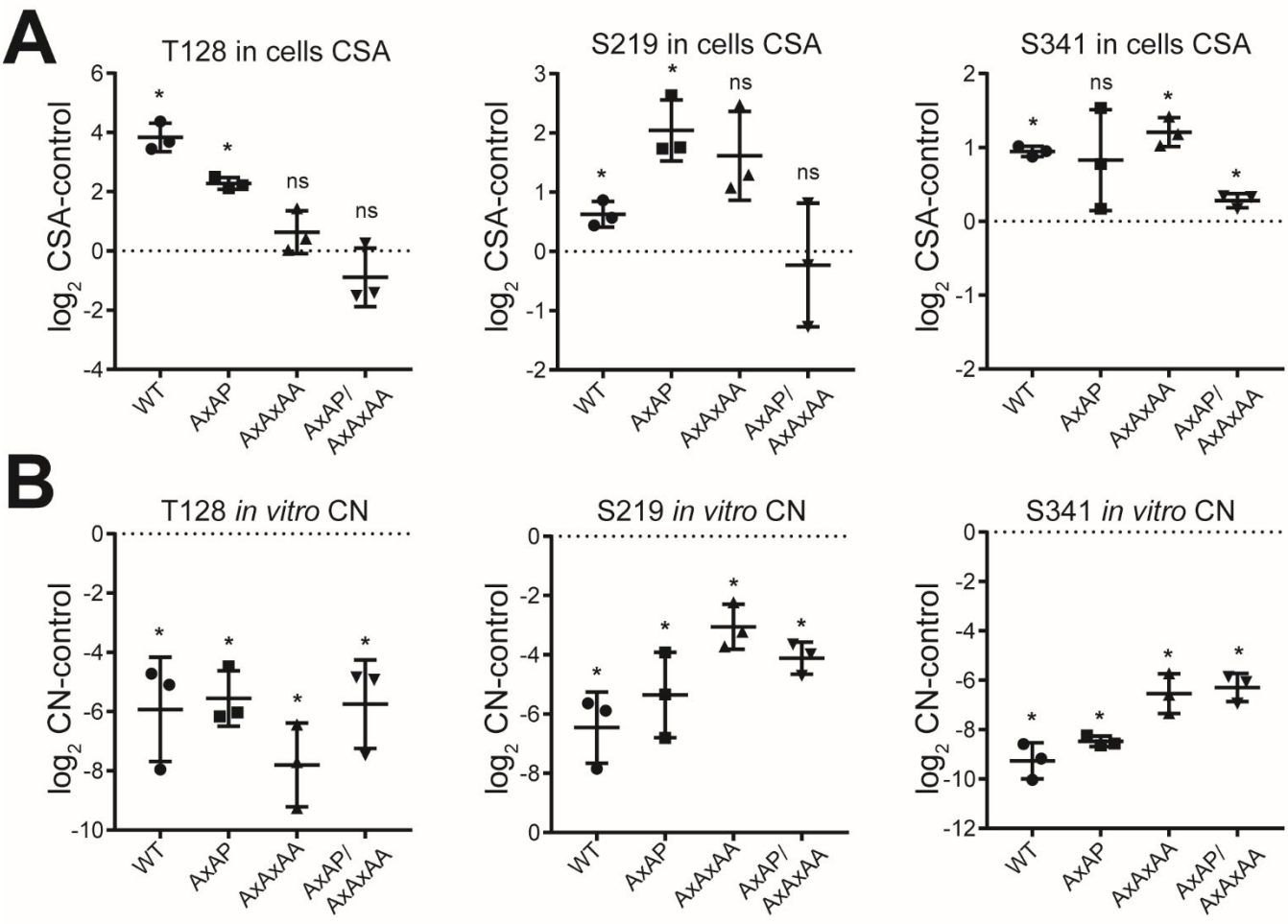


Figure S4: Plot of phosphorylation site abundance of RANBP3 WT, LxVP (AxAP), PxIxIT (AxAxAA), and double (AxAP/AxAxAA) mutants in CSA treated HEK293T cells **A**) and in CN *in vitro* phosphatase assays **B**) (*p<0.05).

Supplementary Methods

Antibodies. Pan-Calcineurin (1:1000, Catalog No. 2614S), pRANBP3 Ser58 (1:1000, Catalog No. 9380S), RANBP3 (1:1000, Catalog No. 93706S) and SMAD2 (1:1000, Catalog No. 5339S) antibodies were purchased from Cell Signaling; anti-Flag M2 (1:2000, Catalog No. F3165) and actin-HRP (1:10000, Catalog No. A3854) antibodies were purchased from Millipore-Sigma.

Peptide pulldowns. CNBr-activated sepharose was rehydrated in 1 mM ice-cold HCl for 15 minutes before washing with coupling buffer (0.1 M $NaHCO_3$ + 0.5 M NaCl pH 8.0). 200 µg peptide was added to 100 µL of resin suspended in coupling buffer and incubated for 4 hours, rotating at 4°C. The peptide-resin was blocked with 0.1 M Tris/HCl pH 8.0 overnight, rotating at 4°C. For the pulldown, HEK293T cells were lysed in lysis buffer + 1 mM $CaCl_2$, sonicated, cleared by centrifugation, and 25 µL peptide-resin was added. The peptide-resin was incubated overnight, rotating at 4 °C, collected by centrifugation, lysate removed, 1 ml for fresh lysis buffer was added (wash), and collected again by centrifugation. The wash step was performed for a total of 3 times. The resin was eluted in 100 µL 2x SDS/Laemlli solution, 1mM DTT for 15 minutes at 95 °C.

In Vitro Phosphatase assays. GCFC2, SKP1, and IPO7 were cloned into the p3xFLAG-CMV10 construct. All p3xFLAG-CMV10 constructs (GCFC2, SKP1, IPO7, MCM2, RANBP3, XPO1, and NUP53) were transiently expressed in HEK293FT cells and affinity enriched. For *in vitro* dephosphorylation, purified proteins were incubated in Buffer A (1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1:100 TCEP in TBS) with or without purified CN

for 45 min at 28°C. Samples were denatured with 2% SDS in 100 mM Tris, pH 8.7, reduced with 5 mM DTT, and alkylated with 6 mM iodoacetamide for 1 hour in the dark. The reaction was quenched with DTT and the samples separated by SDS-PAGE, stained with Coomassie, and excised. Gel slices were destained overnight at 37 °C, dehydrated, and trypsin digested overnight at 37 °C. Peptides were extracted, desalted, and analyzed by LC-MS/MS. Peptide abundance was normalized to protein abundance. Missing values in CN treated samples were imputed in Perseus[47] from a normal distribution. Statistical analysis of protein quantification was carried out in Excel by two-tailed Student's T-Test.

Surface Plasmon Resonance Spectroscopy. Measurements were conducted using a four channel SPR instrument fitted with an autosampler and degassing pump (Reichert Technologies). Gold sensorships modified with NiNTA-functionalized matrices (Xantec) were used to bind His_6-tagged CN (200 nM) to the surface in a buffer of 20 mM Tris pH 7.5, 50-100 mM NaCl, 0.5 mM TCEP, 1 mM $CaCl_2$, and 0.05% Tween 20. CN was loaded onto Channels 1 and 2 to achieve between 1500-3500 μRIU of binding on the surface (channels 3 and 4 were used as references). CN-doped surfaces were allowed to equilibrate under 50 μL/min flow conditions for 30 minutes until the baseline had stabilized. Serial injections of increasing concentrations of peptides were applied to the chip between 30-45 s followed by a 60 s dissociation step. Technical replicates were achieved by stripping the sensorchip with 350 mM EDTA pH 8.0, reconditioning the surface with 40 mM $NiSO_4$, and loading fresh CN on the surface. Kinetic and equilibrium fits of the data were determined by curve fitting using TraceDrawer (Ridgeview Instruments AB). Statistics were calculated using Prism8 (GraphPad Inc)

Mutagenesis. The QuickChange (Agilent) site-directed mutagenesis method was used with Calcineurin (CNA: 1-370, CNB: 1-169) as a template to produce the CNB:N122A mutant and RANBP3 WT as a template to produce the RANBP3-AxAP, RANBP3-AxAP, RANBP3-AxAxAA, and RANBP3-AxAP/AxAxxAA mutants

Expression and purification of Calcineurin. Calcineurin (CNA: 1-370, CNB:1-169) WT and CNB:N122A were expressed in BL21 (DE3) RIL *E. coli* as previously described[16,19]. To purify Calcineurin for SPR measurements, pellets were resuspended in lysis buffer (50 mM Tris pH 8.5, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100) and lysed via high pressure homogenization (Avestin Emulsiflex C3). Cell lysates were clarified by centrifugation at 45,000 x *g* for 60 minutes at 4 °C. The clarified supernatant was passed over a 7 mL Ni-NTA gravity column and eluted using lysis buffer supplemented with 250 mM imidazole. Elution fractions containing Calcineurin were further purified and buffer exchanged by size exclusion chromatography (SEC; Superdex 200 26/60; GE Healthcare) into 20 mM Tris pH 7.5, 500 mM NaCl, 1 mM $CaCl_2$, 0.5 mM TCEP at 4 °C. Peak fractions containing Calcineurin were pooled and dialyzed overnight into 50 mM Sodium Acetate pH 5.0, 1 mM $CaCl_2$, 0.5 mM TCEP at 4 °C. Aliquots of Calcineurin were flash-frozen in liquid nitrogen and stored at -80 °C.

Protein Stability Measurements. Protein stability measurements report on the melting temperature (T_m) of CN (in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $CaCl_2$ and 0.5 mM TCEP) under investigation and were performed on a Tycho NT.6 (Nanotemper) using

standard capillaries (10 µl) using a 30°C/min ramp (from 35°C to 95°C) and evaluated using the Tycho NT.6 software version 1.1.5.668.

Immunofluorescence. HeLa cells were plated at 10,000 cells per No. 1.5 glass coverslip and allowed to adhere overnight before treatment or not with 500 nM CSA for 45 minutes. Cells were fixed in 3% formaldehyde in PBS for 7 minutes, permeabilized with PBS + 0.1% Triton X-100 (PBST), and blocked in 3% milk in PBST. Cells were probed for anti-SMAD2 antibody at 1:1000 concentration in milk for half an hour, followed by Alexa Fluor 488 for half an hour. DNA was stained with bis-benzamide for 5 minutes. Cover slips were mounted using ProLong Diamond and imaged on a Zeiss LSM 800 with Airyscan. Cytoplasmic verses nuclear SMAD2 signal was analyzed in FIJI[45]. Cells and nuclei were outlined by hand, and SMAD2 signal intensity was measured for the whole cell area and the nucleus. Nuclear SMAD2 signal intensity was subtracted from whole cell intensity to yield cytoplasmic SMAD2 signal intensity. The percentage of nuclear and cytoplasmic SMAD2 signal intensity was calculated for each cell and compared between control and CSA treated cells.