

**Table S1.  $K_d$  values of  $\text{Ca}^{2+}$  binding to CBD12 mutants.**

Protein	Maximal Binding Capacity (mol/mol)	Microscopic $K_d$ Values ( $\mu\text{M}$ )
WT	$6.1 \pm 0.04$	$0.03 \pm 0.003$ , $0.04 \pm 0.001$ , $0.1 \pm 0.42$ , $2.0 \pm 0.6$ , $37.3 \pm 13.4$ , $107.9 \pm 7.5$
H501A	$5.5 \pm 0.08$	$0.03 \pm 0.02$ , $0.07 \pm 0.01$ , $0.9 \pm 0.5$ , $2.8 \pm 1.1$ , $14.2 \pm 7.9$ , $136.7 \pm 16.7$
A502P	$6.0 \pm 0.2$	$0.04 \pm 0.02$ , $0.08 \pm 0.02$ , $1.4 \pm 0.7$ , $6.3 \pm 1.8$ , $48.3 \pm 24.9$ , $64.0 \pm 24.7$
G503A	$3.1 \pm 0.3$	$0.11 \pm 0.01$ , $0.12 \pm 0.02$ , $4.6 \pm 1.9$ , $31.2 \pm 1.5$
G503P	$3.7 \pm 0.1$	$0.13 \pm 0.03$ , $0.62 \pm 0.08$ , $1.9 \pm 0.7$ , $3.9 \pm 0.9$
I504A	$5.8 \pm 0.2$	$0.03 \pm 0.01$ , $0.12 \pm 0.01$ , $0.31 \pm 0.08$ , $2.7 \pm 1.0$ , $13.0 \pm 7.4$ , $50.9 \pm 11.7$
I504P	$4.0 \pm 0.1$	$0.24 \pm 0.07$ , $0.58 \pm 0.14$ , $1.0 \pm 0.1$ , $1.6 \pm 0.2$
F505A	$4.9 \pm 0.2$	$0.12 \pm 0.02$ , $0.37 \pm 0.15$ , $1.9 \pm 0.3$ , $72.6 \pm 13.7$ , $99.7 \pm 11.8$
F505P	$3.7 \pm 0.1$	$0.14 \pm 0.04$ , $0.42 \pm 0.14$ , $0.63 \pm 0.14$ , $3.18 \pm 0.95$
T506A	$6.0 \pm 0.1$	$0.01 \pm 0.003$ , $0.06 \pm 0.02$ , $1.4 \pm 0.5$ , $3.2 \pm 0.5$ , $9.8 \pm 2.5$ , $22.3 \pm 2.6$
T506P	$3.0 \pm 0.1$	$0.16 \pm 0.06$ , $0.39 \pm 0.17$ , $5.2 \pm 2.0$

Equilibrium  $^{45}\text{Ca}^{2+}$  binding was measured as described in "Materials and methods". The  $\text{Ca}^{2+}$ -titration curves were fit to Adair equation for the appropriate number of sites. "Best fit" was obtained according to the  $\chi^2$  weighting criteria. Values are presented as mean  $\pm$  SEM ( $n = 3$  for all the preparations).

**Table S2.  $\text{Ca}^{2+}$  off-rates and amplitudes values of CBD12 mutants.**

Protein	Number of exponents	Amplitudes (%)			Rate constants ( $\text{s}^{-1}$ )		
		$A_f$	$A_s$		$k_f$	$k_s$	
WT	2	$43.7 \pm 0.5$	$56.3 \pm 0.5$		$5.3 \pm 0.8$	$0.6 \pm 0.02$	
H501A	2	$47.1 \pm 3.7$	$52.9 \pm 3.7$		$4.1 \pm 1.0$	$0.9 \pm 0.1$	
A502P	2	$52.2 \pm 0.4$	$47.8 \pm 0.4$		$6.4 \pm 0.4$	$1.1 \pm 0.03$	
		$A_r$	$A_f$	$A_s$	$k_r$	$k_f$	$k_s$
I504A	3	$28.2 \pm 1.7$	$44.6 \pm 3.9$	$27.2 \pm 3.6$	$122.9 \pm 12.5$	$1.8 \pm 0.3$	$0.6 \pm 0.1$
F505A	3	$27.6 \pm 3.9$	$43.3 \pm 3.2$	$29.1 \pm 0.8$	$96.6 \pm 14.5$	$14.9 \pm 3.8$	$1.0 \pm 0.1$
T506A	3	$17.5 \pm 0.6$	$41.1 \pm 0.9$	$41.4 \pm 0.8$	$116.7 \pm 8.3$	$2.0 \pm 0.1$	$0.6 \pm 0.03$

Traces were fit to double or triple exponential equations, as indicated, and are presented as mean  $\pm$  SEM (n = 6).

### Supplementary Figure Legend

**Figure S1.  $^{45}\text{Ca}^{2+}$  titration curves of isolated CBD1 and CBD2.** Curves were fitted with the following parameters: For CBD1, capacity = 4 ions/protein and the  $K_d$ s are 0.1, 0.2, 2, 196  $\mu\text{M}$ . For CBD2, capacity = 2 ions/protein and the  $K_d$ s are 8.5 and 47  $\mu\text{M}$ .

Figure S1

