Supplementary Figure Legends

Figure S1 - Expression profile of CB *in vivo* and MST binding curves

A A four-times longer exposure of the Western blot shown in Fig 1B.

B Interaction of the SH3 domain with $CB2_{SH3-}$. Fluorescently labeled $CB2_{SH3-}$ or $CB2_{SH3-}$/E202A were kept at 50 nM while SH3, SH3/R70A, and SH3/W24 were titrated starting at 1500 µM in serial dilutions. Upon binding the fluorescent signal decreased. K_Ds were fitted (solid lines) as shown in the table. The dashed line indicates the fit of the SH3/R70A vs. $CB2_{SH3-}$ titration, which did not allow for a K_D calculation due to incomplete saturation.

Figure S2 - Additional features of the CB structures and similarity of the primary sequences of CB and Asef.

A Placement of the SH3 domain. The SH3 domain has been positioned incorrectly in the electron density map obtained after DEN refinement in CNS (Schröder *et al*, 2010) to illustrate the map quality. Correlation coefficients between the models of the SH3 domain and the corresponding map have been calculated with Phenix resulting in values of 0.74 for the correct orientation (top) vs. 0.16, 0.12 and 0.22 (from top to bottom) for the misplaced models.

B Alignment of the primary sequences of CB_{SH3+} and human Asef. SH3, DH and PH domains are highlighted in green, magenta, and blue, respectively.

C The helix connecting the DH and PH domains adopts different conformations in different CB structures. CB_{SH3+} is shown in magenta (DH domain) and blue (PH domain). The two $CB2_{SH3-}$ molecules from the Cdc42-$CB2_{SH3-}$ cocrystal structure are shown in light and dark gray, respectively, and the apo $CB1_{SH3-}$ structure is shown in cyan. Vectors describing the direction of parts of the helix connecting the DH and PH

domains are shown in corresponding colors, except for CB_{SH3+}, where the vectors are presented in red. The regions where the helix is kinked (Gln290/Ile291 or Ile301/Asp302) are highlighted in black. For simplicity the structures have been terminated at Gln308 so that the core of the PH domain is not visible.

D SDS gels of dissolved protein crystals. $CB1_{SH3+}$ (58.2 kDa, left panel, red arrow) is degraded during crystallization resulting in an approximate weight of 39 kDa (left panel, black arrow). In contrast, CB_{SH3+} is running at the same height on the SDS gel before and after crystallization, indicating no degradation (right panel, red and black arrow, respectively).

Figure S3 - Kratky plots and EOM-distribution for the E262A mutant of CB.

A The Kratky plots for CB_{SH3+} (blue curve) and CB_{SH3+}/E262A (red curve) show maxima at 0.64 s/nm. However, the maximum for CB_{SH3+}/E262A is less pronounced indicating a higher flexibility than in the WT.

B Comparison of the initial random pool (blue line) and the selected ensemble (red line) of the EOM D_{max} distribution for CB_{SH3+}/E262A indicates a conformational heterogeneity of the protein in solution. Three coexisting conformational clusters are suggested by the ensemble distribution, which might be classified as compact/intermediate/extended.

Figure S4 - Distributions of AFM-volumes.

AFM-volume distributions of CB_{SH3+} WT (top) and CB_{SH3+}/E262A (bottom) with peak values of 45.5±13.3 nm^3 and 54.5±10.4 nm^3, respectively, corresponding to a monomer in each case. Note that for CB_{SH3+}/E262A a secondary peak at a volume of 119.9±15.4 nm^3 indicating a dimer is present, however, the corresponding particles were omitted in the structural analysis since only the shapes of monomeric particles were analyzed.

Depicted are two representative experiments with n-values corresponding to the number of particles analyzed.

Figure S5. CB binds to phosphoinositides via its PH domain and to the NL2 cytoplasmic tail via its SH3 domain.

A GST-pulldown assay to test binding of GST-CB2$_{SH3+}$ and GST-CB2$_{SH3-}$ to gephyrin.

B Protein-lipid overlay assays using commercially available PIP-Strips. Upper left, template for the location of lipids on the membrane. LPA, lysophosphatidic acid; S1P, sphingosine-1-phosphate; LPC, lysophosphocholine; PI, phosphatidyl-inositol (PtdIns); PI(3)P, phosphatidyl-inositol-3-phosphate; PI(4)P, phosphatidyl-inositol-4-phosphate; PI(5)P, phosphatidyl-inositol-5-phosphate; PI(3,4)P$_2$, phosphatidyl-inositol-3,4-bisphosphate; PI(3,5)P$_2$, phosphatidyl-inositol-3,5-bisphosphate; PI(4,5)P$_2$, phosphatidyl-inositol-4,5-bisphosphate; PI(3,4,5)P$_3$, phosphatidyl-inositol-3,4,5-triphosphate; PA, phosphatidic acid; PE, phosphatidyl-ethanolamine; PS, phosphatidyl-serine; PC, phosphatidyl-choline. Upper right, purified GST-fusion proteins used in the assay. Bottom, bound proteins were detected using an anti-GST antibody. A GST tagged PH domain of PLCδ1, which binds specifically to PI(4,5)P$_2$ was used as a positive control. GST-CB2/ΔSH3 binds most strongly to PI(3)P. GST-CB2$_{SH3+}$ and GST-CB2$_{SH3+}$/ΔPH bind PI(3)P substantially weaker.

C GST-pulldown assay to test binding of GST-SH3 and GST-SH3/W24A and exogenously expressed NL2 cytoplasmic domain. Both GST-fusion constructs interact with NL2.

Figure S6. Total gephyrin cluster intensity and mean intensity of VIAAT clusters in CB$^{-/-}$ and NL2$^{-/-}$ neurons transfected with CB variants.

A Total gephyrin cluster intensity (mean intensity of gephyrin clusters x average size of gephyrin clusters x density of gephyrin clusters) induced by overexpression of CB

variants in $CB^{-/-}$ neurons, arbitrary units (a.u.). $CB2_{SH3+}$: 0.88 ± 0.08; $CB2_{SH3+}$/W52A: 0.47 ± 0.05, P<0.001; $CB2_{SH3+}$/W24A-E262A: 1.43 ± 0.12, P<0.001; CB2/ΔSH3: 2.34 ± 0.17, P<0.001; GFP (no CB): 0.04 ± 0.01, P<0.001.

B Total gephyrin cluster intensity (mean intensity of gephyrin clusters x average size of gephyrin clusters x density of gephyrin clusters) induced by overexpression of CB variants in $NL2^{-/-}$ neurons, arbitrary units (a.u.). $CB2_{SH3+}$: 0.46 ± 0.09; $CB2_{SH3+}$/W52A: 0.39 ± 0.08, n.s.; $CB2_{SH3+}$/W24A-E262A: 1.21 ± 0.18, P<0.001; CB2/ΔSH3: 1.85 ± 0.18, P<0.001; GFP (no CB): 0.49 ± 0.24, n.s.

C Average size of perisomatic VIAAT clusters in $CB^{-/-}$ neurons overexpressing CB variants, in μm^2. $CB2_{SH3+}$: 0.202 ± 0.006; $CB2_{SH3+}$/W52A: 0.212 ± 0.009, n.s.; $CB2_{SH3+}$/W24A-E262A: 0.219 ± 0.009, n.s.; CB2/ΔSH3: 0.270 ± 0.009, P<0.001; GFP (no CB): 0.233 ± 0.010, P<0.01.

D Average size of perisomatic VIAAT clusters in $NL2^{-/-}$ neurons overexpressing CB variants, in μm^2. $CB2_{SH3+}$: 0.230 ± 0.013; $CB2_{SH3+}$/W52A: 0.244 ± 0.013, n.s.; $CB2_{SH3+}$/W24A-E262A: 0.243 ± 0.012, n.s.; CB2/ΔSH3: 0.243 ± 0.012, n.s.; GFP (no CB): 0.264 ± 0.014, n.s.

E Mean intensity of VIAAT clusters in $CB^{-/-}$ neurons overexpressing CB variants, arbitrary units (a.u.). $CB2_{SH3+}$: 62.5 ± 0.7; $CB2_{SH3+}$/W52A: 61.7 ± 1.2, n.s.; $CB2_{SH3+}$/W24A-E262A: 63.8 ± 1.0, n.s.; CB2/ΔSH3: 67.0 ± 1.2, P<0.001; GFP (no CB): 68.7 ± 1.2, P<0.001.

F Mean intensity of VIAAT clusters in $NL2^{-/-}$ neurons overexpressing CB variants, arbitrary units (a.u.). $CB2_{SH3+}$: 67.3 ± 1.3; $CB2_{SH3+}$/W52A: 69.3 ± 1.9, n.s.; $CB2_{SH3+}$/W24A-E262A: 68.4 ± 1.4, n.s.; CB2/ΔSH3: 70.1 ± 1.7, n.s.; GFP (no CB): 75.6 ± 1.3, P<0.001.

Table S1. Data collection and refinement statistics.

	CB_{SH3+}	**$CB1_{SH3-}$**
Crystal parameters		
Space group	$I2_13$	$C222_1$
Cell dimension a (Å)	217.4	82.93
Cell dimension b (Å)	217.4	165.43
Cell dimension c (Å)	217.4	129.12
Data collection		
Wavelength (Å)	0.8726	0.8726
Resolution (Å)	108.7 - 5.5	64.6 – 3.5
R_{merge}*	0.181 (1.903)	0.080 (0.714)
R_{pim}	0.049 (0.489)	0.042 (0.372)
$\langle I/\sigma I \rangle$	10.8 (1.8)	9.5 (1.8)
Completeness (%)	100.0 (100.0)	99.9 (100.0)
Redundancy	11.0 (11.0)	4.5 (4.6)
$CC_{1/2}$	1.0 (0.46)	1.0 (0.80)
Refinement		
Resolution (Å)	108.7 - 5.5	50.9 - 3.5
No. of reflections	6,812	11,474
R_{work} / R_{free}	0.2367/0.2820	0.2207/0.2487
Average B factor ($Å^2$)	177.1	120.9
Number of nonhydrogen atoms	3717	2859
Stereochemistry		
Rmsd bond lengths (Å)	0.004	0.005
Rmsd bond angles (°)	0.682	0.923
Ramachandran outliers (%)	1.8	1.5
Ramachandran favored (%)	90.8	93.4

*Values in parentheses represent the highest-resolution shell.

$R_{sym}{}^{a} = \sum_{hkl} \sum_{i} | I_i - \langle I \rangle | / \sum_{hkl} \sum_{i} I_i$ where I_i is the ith measurement and $\langle I \rangle$ is the weighted mean of all measurements of I.

$R_{pim}{}^{b} = \sum_{hkl} 1/(N-1)^{1/2} \sum_{i} | Ii(hkl) - \overline{I}(hkl) | / \sum_{hkl} \sum_{i} I(hkl)$, where N is the redundancy of the data and $\overline{I}(hkl)$ the average intensity.

$\langle I/\sigma I \rangle^{c}$ indicates the average of the intensity divided by its standard deviation.

$R^{d} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl}|F_o|$ where F_o and F_c are the observed and calculated structure factor amplitudes.

R_{free}^{e} same as R for 5% of the data randomly omitted from the refinement. The number of reflections includes the R_{free} subset.

Table S2. Comparison of CB structures.

	CB_{SH3+}	CB_{SH3-}	$CB2_{SH3-}$ chain A	$CB2_{SH3-}$ chain C	domain
CB_{SH3+}	x	1.43Å	1.45 Å	1.52 Å	DH
CB_{SH3+}	x	1.30 Å (χ=59.1°)	1.22 Å (χ=62.7°)	1.31 Å (χ=92.3°)	PH
CB_{SH3-}	x	x	0.34 Å	0.48 Å	DH
CB_{SH3-}	x	x	0.44 Å (χ=54.7°)	0.68 Å (χ=70.0°)	PH
$CB2_{SH3-}$ chain A	x	x	x	0.44 Å	DH
$CB2_{SH3-}$ chain A	x	x	x	0.55 Å (χ=37.8°)	PH

RMSDs of the individual DH and PH domains and angles describing PH domain rotations. While almost no intra-domain conformational changes are present as reflected by low RMSD values, entire domains undergo significant rotations.

Table S3. SAXS data collection and analysis.

Data collection parameters		
Instrument (detector)	X33, EMBL, DORIS PILATUS 1M pixel (67 x 420 mm^2)	
Beam geometry	2 x 0.06 mm^2	
Wavelength (Å)	1.5	
s range ($Å^{-1}$)	0.006-0.6	
Exposure time (sec)	8 x 15	
	CB_{SH3+} WT	CB_{SH3+} E262A
Concentration range (mg ml^{-1})	0.8-6.6	0.27-1.0
Temperature (K)	283	283
Structural parameters		
I(0) (relative) [from P(r)]	51.8±20	75.3±20
Rg (Å) [from P(r)]	29.0±2	36.8±2
I(0) (relative) [from Guinier]	54.8±20	77.3±20
R_g (Å) [from Guinier]	31.0±2	36.7±2
D_{max} (Å) [from P(r)]	95±10	125±10
Porod volume estimate ($Å^3$)	79,000±1,000	92,000±1,000
Dammif excluded volume ($Å^3$)	114,000±1,000	134,000±1,000
Molecular mass determination		
Molecular mass Mr (kDa) [from Porod invariant]	48±5	55±5
Molecular mass Mr (kDa) [from excluded volume]	57±5	67±5
Calculated monomeric Mr (kDa) [from sequence]*	~53	~53
Software employed		
Primary data reduction	Automated PIPELINE (RADAVER)	
Data processing	PRIMUS	
Ab initio analysis	DAMMIF	
Validation and averaging	DAMAVER	
Rigid body modeling	BUNCH	
Flexibility	EOM	
Computation of model intensities	CRYSOL	
3D graphics representations	VMD	

Table S4. Comparison of overall structural parameters resulting from the use of either BUNCH or EOM for CB_{SH3+}/E262A.

	R_G (Å)	D_{max} (Å)	χ	N models
Experimental data	36.7±2.0	125±10	-	-
BUNCH	33.6	116.1	0.788	1
EOM	36.8	124.6	0.765	6