Table S1. Data Collection and Refinement Statistics, Related to Figure 1

Data Collection	
Space group	P 2 2_1 2_1
a, b, c (Å)	73.3, 102.6, 183.0
Wavelength (Å)	0.87260
Resolution range (Å)	51.31 - 3.50 (3.83 - 3.50)
Total Reflections	153,163
Unique Reflections	18,047
Completeness (%)	99.8 (99.8)
Multiplicity	8.5 (8.7)
R_{merge} (%)	20.2 (129.5)
$R_{p.i.m.}$ (%)	7.5 (47.8)
$CC_{1/2}$ (%)	99.8 (66.5)
I / σ (I)	11.2 (2.9)
Wilson B factor (Å^2)	69.4
Refinement	
Resolution range (Å)	51.3 - 3.50
R_{work} (%)	20.47
R_{free} (%)	25.80
Number of total reflections	18,003
Number of R_{free} reflections	920
Number of protein atoms	6,871
B factors (Å^2)	97.70
Rmsd Bond length (Å)	0.011
Rmsd Bond angles (°)	1.592
Ramachandran Plot	
Favoured (%)	93.71
Allowed (%)	6.06
Outliers (%)	0.23

The highest resolution shell is in parentheses. R_{merge} is the merging R factor. $R_{p.i.m.}$ is the precision-indicating merging R factor (Weiss and Hilgenfeld, 1997) and $CC_{1/2}$ the half dataset correlation coefficient (Karplus and Diederichs, 2012).

Table S2. Structural Comparison of the α-Actinin-2 Spectrin-like Repeats SR1-SR4, Related to Figures 2 and S1

SR ID		**SR1**	**SR1**	**SR2**	**SR2**	**SR3**	**SR3**	**SR4**	**SR4**
	PDB	1HCI	4D1E	1HCI	4D1E	1HCI	4D1E	1HCI	4D1E
SR1	1HCI		1.890	2.663	2.838	2.586	2.455	2.772	4.054
SR1	4D1E	0.716		2.917	3.129	2.623	2.571	3.329	2.949
SR2	1HCI	0.427	0.355		**0.790**	1.710	2.677	1.615	1.719
SR2	4D1E	0.387	0.344	**0.871**		1.722	2.035	2.624	1.691
SR3	1HCI	0.537	0.496	0.589	0.543		**0.999**	1.720	1.848
SR3	4D1E	0.561	0.513	0.410	0.475	**0.886**		2.060	2.113
SR4	1HCI	0.407	0.379	0.555	0.372	0.615	0.555		**0.868**
SR4	4D1E	0.294	0.354	0.539	0.488	0.615	0.567	**0.859**	

Left column, upper row: spectrin repeat identifier; second left column, second upper row: PDB entry (1HCI the rod domain, 4D1E the full length structure). Values in bold denote the highly similar structures. Rmsd (upper right values, in Å) and Q-score (lower left values). Q-score represents the quality function of Cα-alignment, taking into account both the alignment length and rmsd.

Table S3. Rotamer Library Simulation, Related to Figure 3

Cysteine residue	No. of rotamers	α-actinin-2 domain	Cysteine pair	Distance
			Intrachain	
161	1[2]	ABD	<u>483 – 487</u>	<u>7 ± 3</u>
187	1[2]	ABD	<u>48 – 251</u>	<u>15 ± 3</u>
48	5	ABD	251 – 270	30 ± 4
862	7	EF3-4	48 – 270	34 ± 4
251	18	ABD	270 – 339	36 ± 6
339	21	SR1	781 – 862	37 ± 2
483	34	SR2	161 – 187[2]	13 ± 1
781	36	EF1-2	187 – 251[2]	14 ± 3
270	61	NECK	161 – 251[2]	17 ± 1
487	70	SR2	161 – 270[2]	26 ± 3
			48 – 161[2]	27 ± 1
			48 – 187[2]	27 ± 1
			187 – 270[2]	35 ± 3
			Interchain	
			<u>270 – 862</u>	<u>12 ± 2</u>
			251 – 862	27 ± 2
			339 – 781	28 ± 4
			48 – 862	32 ± 4
			339 – 862	36 ± 4
			270 – 781	38 ± 3
			48 – 781	39 ± 4

Number of rotamers for each spin labelled cysteine residue and main distances up to 45 Å[1] (± rmsd, in Å) between spin labels of all cysteine residues based on the crystal structure of α-actinin-2, calculated by the software MMM (Polyhach et al., 2011). Most probable cysteine pairs contributing to the distance distribution < 20 Å (**Figures 3A-B, S3**) are underlined.

[1] The DEER dipolar evolution time (d2) of 2.8 μs used for the Q-band experiments does not allow detection of distances above 45 Å.

[2] Sites 161, 187 and 48 can be labelled with few rotamers of the library, meaning they are probably too tight to be labelled experimentally. Therefore, pair distance distributions accounting for one of these two sites are unlikely to be detected in the experimental data.

Table S4. Summary of Binding Affinities of α-Actinin Complexes (Dissociation Constants), Related to Figure 3

α-actinin-2 constructs	**Ligand**	**K_d (μM, ±SD)**	**Method**
WT	PIP2-C16*	2.96±0.26[a, (i)]	MST[1]
WT (+Tween 20)	Zr-7	2.90±0.12[a, (ii)]	MST
WT+PIP2-C16* (+Tween 20)	Zr-7	0.48±0.13[a(iii)]	MST
WT+PIP2-C16*	Zr-7	0.38±0.06[a, (ii)]	MST
PIP2mut (+Tween 20)	Zr-7	2.76±0.34[a, (ii)]	MST
PIP2mut+PIP2-C16*	Zr-7	1.60±0.22[a, (ii)]	MST
NEECK (+Tween 20)	Zr-7	0.92±0.02[a, (ii)]	MST
EF1-4 (+Tween 20)	Zr-7	0.24±0.04[a, (ii)]	MST
EF1-4	Zr-7	0.19±0.01[b], 0.10[c]	ITC[2]
EF1-4	Zr-7	0.10-0.30	CD[3]
EF1-4	α-actinin-2 ABD-NECK-SR1	0.57±0.03[b]	ITC

[a]Measured in this study, [b](Young and Gautel, 2000), [c](Joseph et al., 2001).

[1]Microscale Thermophoresis, [2]Isothermal Titration Calorimetry, [3]Circular Dichroism.

[(i)],[(ii),] Values are derived from binding isotherms shown in the **Figure 3D-F**.

[(iii),] The CMC of PIP2-C16* determined under our experimental conditions is ≈ 100 μM, addition of Tween-20 (0.05% v/v) decreased the CMC significantly to 10-15 μM. It is possible that in the Tween-20/PIP2-C16* mixture, non-micellar PIP2-C16* concentration is reduced for interaction with α-actinin-2. Nevertheless, we still observe a positive effect of the Tween-20 (0.05% v/v) /PIP2-C16* (80 μM) mixture on α-actinin-2 binding to titin Zr-7 when compared to α-actinin-2 in presence of Tween-20 alone. PIP2-C16* (≈ 40 μM) alone showed the same activation of titin Zr-7 binding as seen in samples with addition Tween-20 + 80 μM PIP2-C16*. Control experiments using the PIP2 mutant evaluated the selectivity of this PIP2-C16* regulation and confirmed that PIP2 mutant has lower affinity for PIP2-C16* (**Figure 3D**), reducing the activating effect on Zr-7 binding (**Figures 3 and 5F**). In essence, these results show that the addition of Tween-20 does not affect the major regulatory function of PIP2- C16* on α-actinin-2 activation.

*Fluorescently labelled Bodipy-TMR-PIP2-C16 (chemical structure is shown in **Figure S2A**)

Table S5. Overall Structural Parameters from SAXS and SEC-MALLS Data, Related to Figure 4

Parameters*	**Wild-type α-actinin-2**	**NEECK mutant**
	SAXS	
MM_{calc} (kDa)	211.3	211.3
MM (kDa)	210±15	190±15
Rg^{XT} (Å)	114	114
R_g (Å)	118±3	120±3
D_{max} (Å)	370±10	390±10
χ^{XT}	2.35	2.67
χ^{RB}	1.25	1.14
	SEC-MALLS	
MM (kDa)	205.3±5.5	204.6±4.7
R_s (Å)	61.3±0.2	60.5±0.1
V_{el} (ml)	9.64±0.01	9.49±0.03

MM, R_g, D_{max}, R_s, V_{el} denote the molecular mass, radius of gyration, maximum size, Stokes' radius and elution volume, respectively. Parameters without superscripts are experimental values; superscripts RB, and XT refer to rigid-body fitted models and the crystal structure, respectively. MM_{calc} is the theoretical MM of α-actinin-2 dimer computed from the protein sequence. The discrepancy between experimental data and those computed from models is defined by χ^{XT} and χ^{RB} values.

*Data are represented as mean values ± SD when applicable