Supporting Information

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SI Materials and Methods

Protein Expression and purification. The coding sequences of ankyrin-B ZZUD (residues 966–1535) were PCR amplified from the full-length human 220-kDa ankyrin-B, which is a generous gift from Vann Bennett (Duke University) and cloned into the modified pET32a vector. The N-terminal His_6-tagged ZZUD was expressed in *Escherichia coli* BL21(DE3) and purified by Ni^{2+}-NTA affinity chromatography followed by size-exclusion chromatography in the buffer containing 50 mM Tris, 100 mM NaCl, 1 mM EDTA and 1 mM DTT at pH 7.5. The ZZUD mutant used for crystallization with the nine-residue deletion (^{1211}SDVMLNGFG^{1219}) was created using the standard PCR-based mutagenesis method. All point mutations including ankyrin-B ZZUD with residues substituted in the domain interfaces and disease-causing mutations were created using the Quick Change site-directed mutagenesis kit and confirmed by DNA sequencing. The HRV 3C protease-cleavable ZZUD was constructed by replacing the "^{1436}IDMTSEKN^{1443}" fragment of the protein with the protease recognition sequence "LEVLFQGP." The mutant proteins were purified using the procedure identical to that used for the wild-type ZZUD. The coding sequence of human β-spectrin repeats 13–15 (residues 1583–1906) was PCR amplified from a human cDNA library and cloned into the modified pET32a vector. The β-spectrin repeats 13–15 and repeats 14–15 (residues 1686–1906) were purified using the same procedure as that described for the purification of ZZUD.

Analytical Gel Filtration Chromatography. Analytical gel filtration chromatography was carried out on an AKTA FPLC system (GE Healthcare). Proteins were loaded onto a Superose 12 10/300 GL column (GE Healthcare) equilibrated with a buffer containing 50 mM Tris, 100 mM NaCl, 1 mM EDTA and 1 mM DTT at pH 7.5.

Isothermal Titration Calorimetry Assay. Isothermal titration calorimetry (ITC) measurements were carried out on a VP-ITC Microcal calorimeter (Microcal) at 25 °C. All proteins were in 50 mM Tris buffer containing 100 mM NaCl, 1 mM EDTA and 1 mM DTT at pH 7.5. Each titration point was performed by injecting a 10 μL aliquot of β-spectrin R14-15 into various ankyrin protein samples in the cell at a time interval of 120 seconds to ensure that the titration peak returned to the baseline. The titration data were analyzed using the program Origin7.0 and fitted by the one-site binding model.

Analytical Ultracentrifugation. Equilibrium sedimentation experiments were performed using a Beckman proteomelab XL-I ultracentrifuge equipped with a 50Ti rotor. Each equilibrium sedimentation was lasted for 72 h with absorption scanning taken at every 12-h interval. Buffer corrected data were analyzed by Sedfit and Sedphat (http://www.analyticalultracentrifugation.com/default.htm).

Circular Dichroism. CD spectra of WT ankyrin-B ZZUD and its various mutations were measured on a JASCO J-815 CD spectropolarimeter at room temperature using a cell path length of 1 mm. Each spectrum was collected with three scans spanning a spectral window of 200–250 nm. The samples were dissolved in 25 mM Tris buffer containing 50mM NaCl, 0.5 mM EDTA and 0.5 mM DTT at pH 7.5 with the increasing concentrations of urea in the same buffer. The protein concentration used in the CD experiment was 10 μM.

Crystallography. Crystals of ankyrin-B ZZUD were obtained by hanging drop vapour diffusion method at 16 °C. To set up a hanging drop, 1 μL of ZZUD (approximately 12 mg/mL) was mixed with 1 μL of crystallization solution with 18% w/v PEG3350 and 0.2 M ammonium acetate. The diffraction qualities of crystals were improved by adding 5% w/v n-Octyl-β-D-glucoside to the crystallization buffer. The diffraction data were collected at Shanghai Synchrotron Radiation Facility and were processed and scaled using HKL2000 (1).

The initial phase was determined by molecular replacement using the modified structure models of the first ZU5 domain of Ankyrin-R (PDB ID code 3F59), the ZU5 domain of UNC5b (PDB ID code 3G5B), and the death domain of Ankyrin-R (PDB ID code 2YVI). Each asymmetric unit contains one ankyrin-B ZZUD molecule. The phase was improved by density modifications with RESOLVE (2). An incomplete structure model was built manually based on the improved phase. The model was refined in Refmac5 (3) and PHENIX (4). COOT was used for model rebuilding and adjustments (5). In the final stage, an additional TLS refinement was performed in PHENIX. The final refinement statistics are listed in Table S1. All structure figures were prepared by PyMOL (http://www.pymol.org/).

Homology Modeling. The structural model of ankyrin-G ZZUD was built and assessed by SWISS-MODEL (6) using the ankyrin-B ZZUD structure as the template. Due to the high sequence identity (approximately 76%) between the two isoforms, the output model shows high structural similarity (overall rmsd <1.0 Å) with the ankyrin-B ZZUD tandem.

1. Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
2. Terwilliger TC (2000) Maximum-likelihood density modification. *Acta Crystallogr D Biol Crystallogr* 56:965–972.
3. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53:240–255.
4. Adams PD, et al. (2002) PHENIX: Building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* 58:1948–1954.
5. Emsley P, Cowtan K (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126–2132.
6. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* 22:195–201.

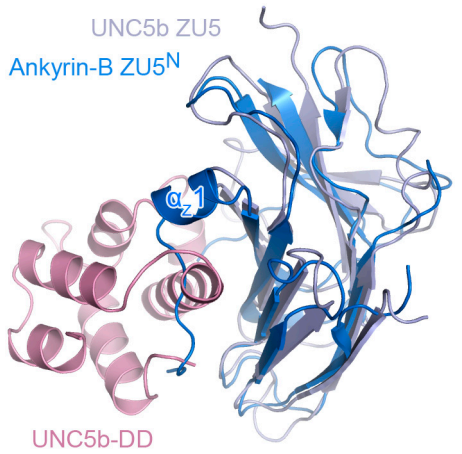

Fig. S1. Superposition of ankyrin-B ZU5^N with UNC5b ZU5 structures showing that the α_Z1 helix together with the following loop prevent the potential interaction between ZU5^N and DD in ankyrin-B.

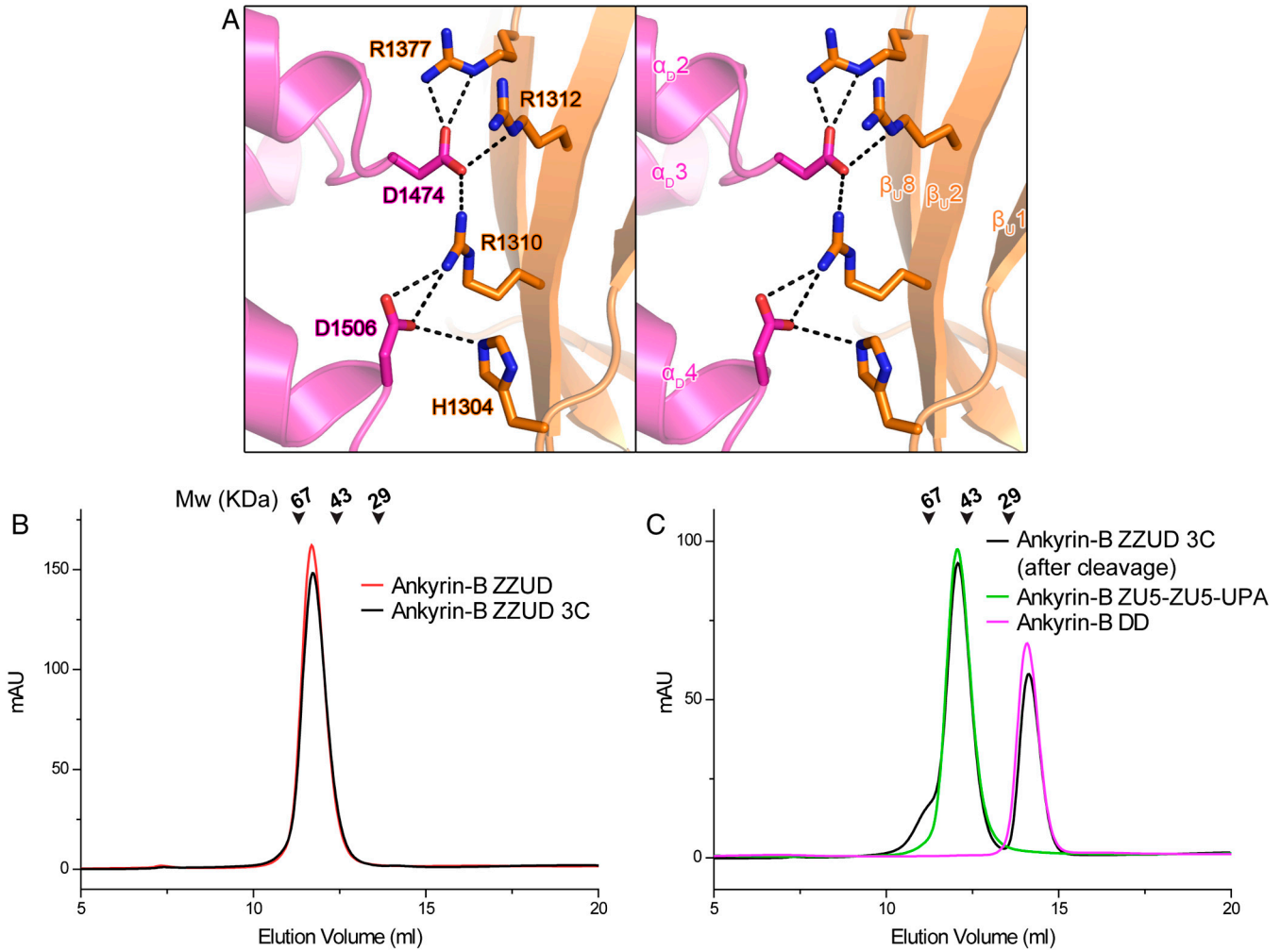

Fig. S2. DD does not associate with the ZZU tandem in ankyrin-B. (*A*) Stereo view showing the UPA/DD interface in the crystal structure of the ankyrin-B ZZUD. The salt bridges are indicated with the dashed lines. (*B*) The engineered insertion of a HRV 3C protease cutting site between UPA and DD did not alter the overall structure of ankyrin-B ZZUD as shown by gel filtration chromatography. (*C*) Gel filtration profile of the HRV 3C protease cleaved ankyrin-B ZZUD 3C mutant showing that DD does not associate with ZZU in solution.

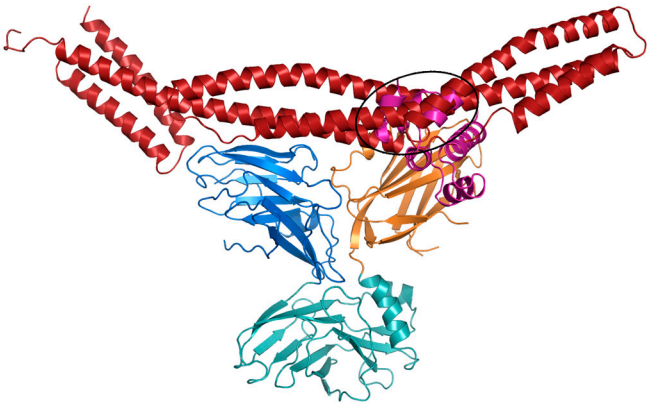

Fig. S3. The ankyrin-B_ZZUD/β-spectrin complex structure model shown in Fig. 4. In this model, the UPA/DD interaction found in the crystal would create a steric hindrance (highlighted by a circle) for the binding of β-spectrin to the ankyrin-B ZZU tandem.

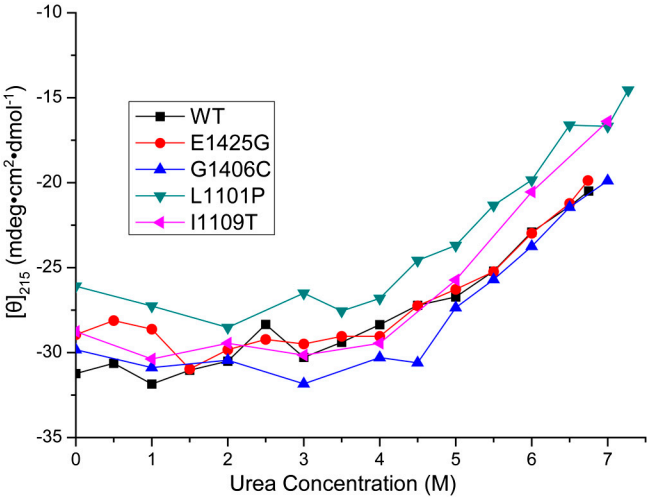

Fig. S4. Urea denaturation-based assay of the stabilities of the WT ankyrin-B ZZUD protein and its four mutants. In this assay, the molar ellipticity values of circular dichroism signals at 215 nm are plotted as a function of urea concentration in the denaturation buffer.

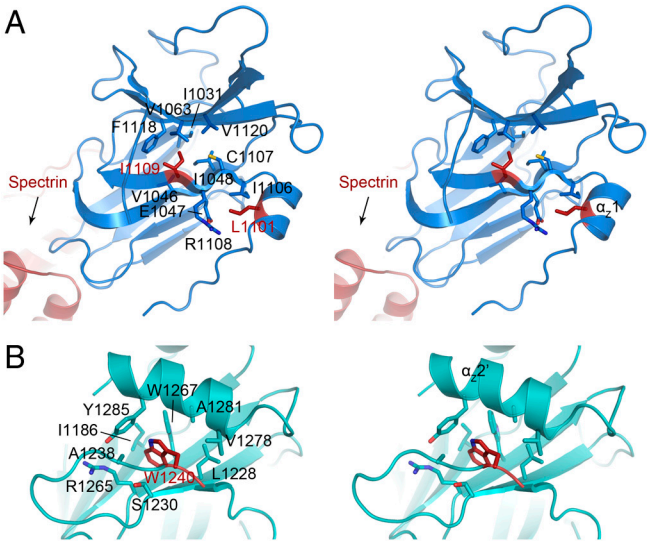

Fig. S5. The combined ribbon and stick representations showing how the three disease-causing mutation residues (I1109, L1101, and W1240) in the ZU5^N (*A*) or ZU5^C (*B*) of the ankyrin-B ZZUD structure.

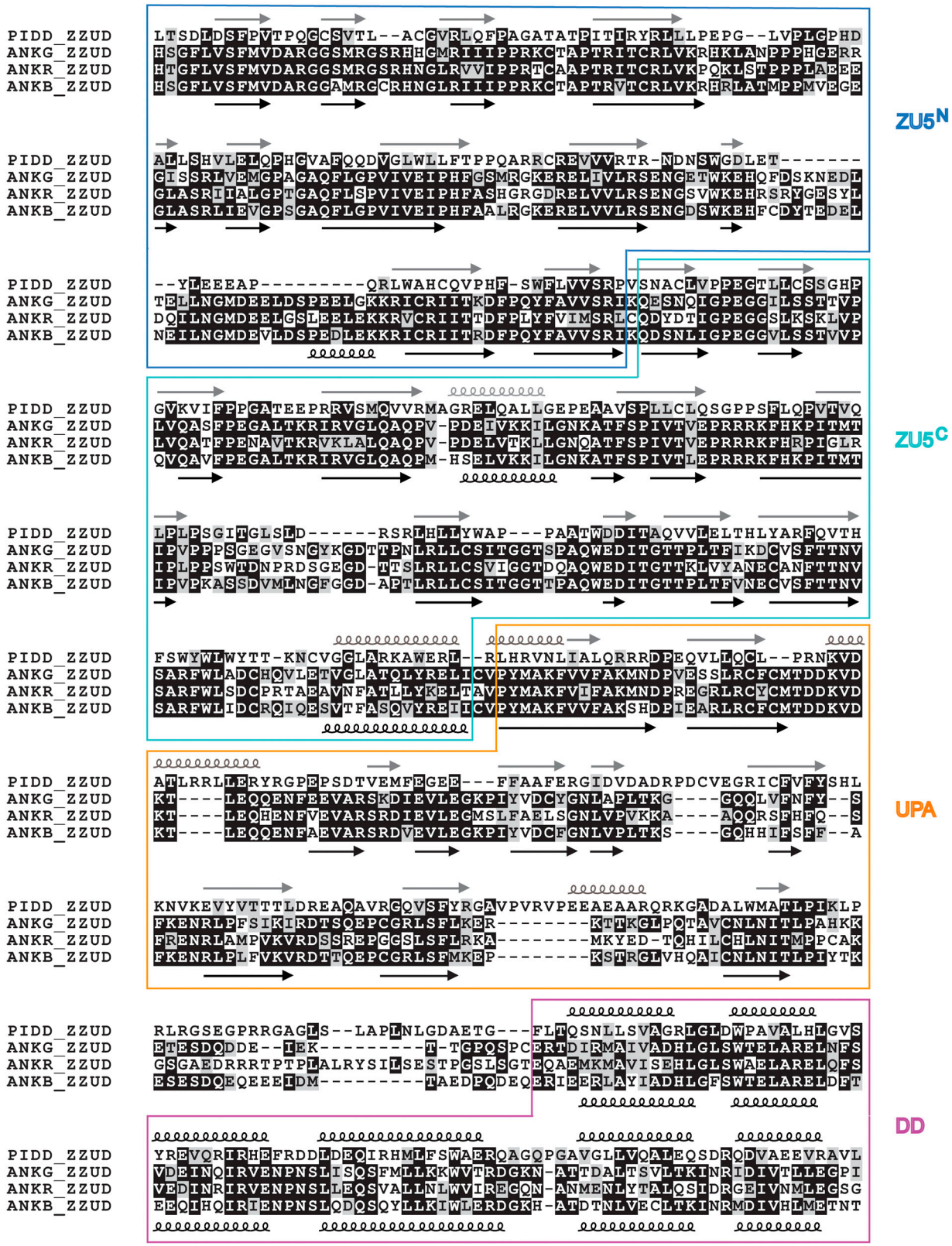

Fig. S6. Amino acid sequence alignment of the ZZUD tandems of human ankyrins and PIDD. The secondary structure elements of PIDD and Ankyrin-B are labeled above and below the alignment, respectively. The secondary structures of the PIDD ZZU tandem are predicted by JPred (http://www.compbio.dundee.ac.uk/www-jpred/) and colored in gray.

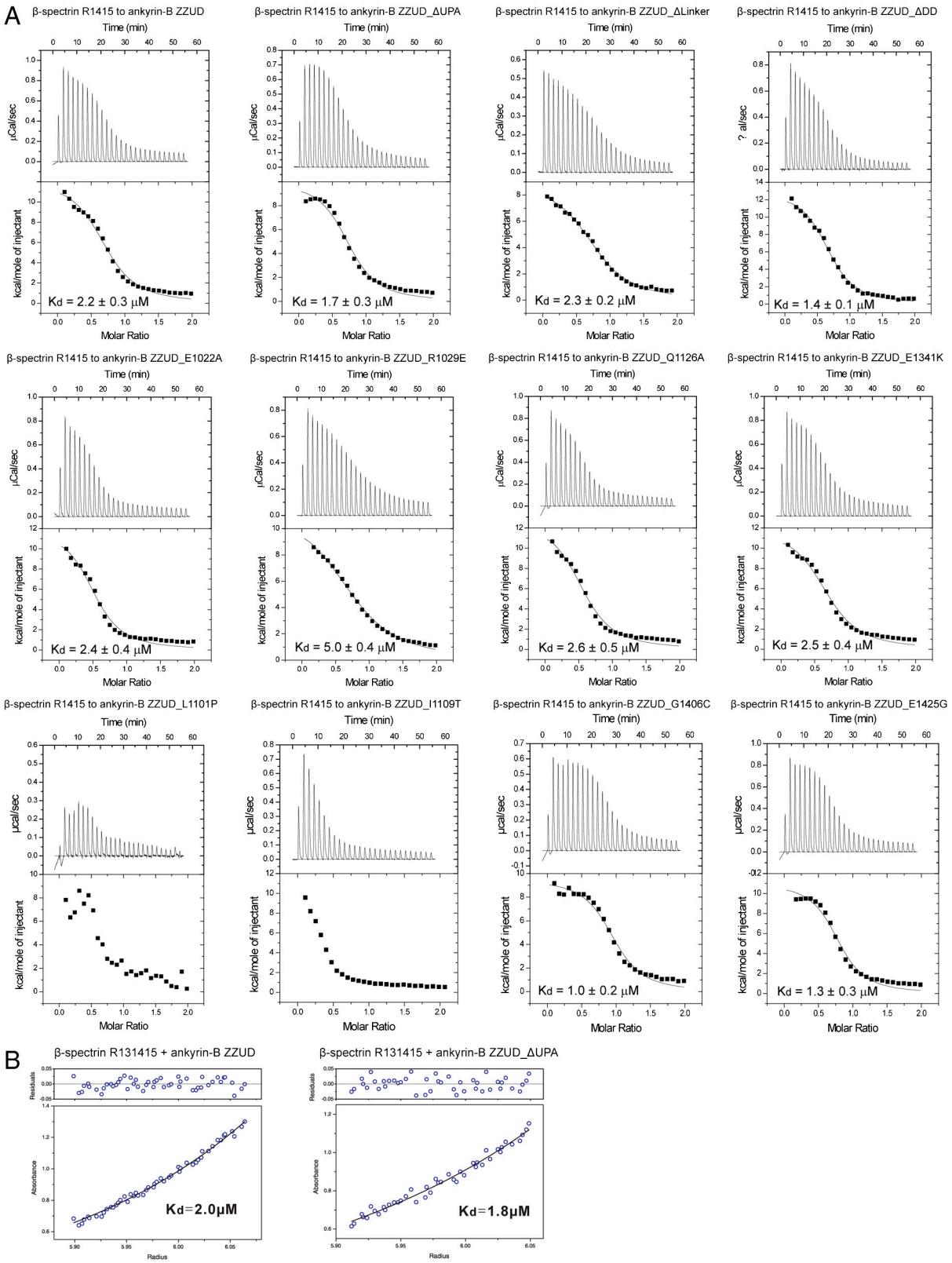

Fig. S7. (*A*) The ITC titration curves for calculating the dissociation constants showing in Table S2. In these binding reactions, the protein concentrations in syringe and in the cell were 0.25 mM and 0.025 mM, respectively. The ITC curves of the L1101P and the I1109T mutants of ankyrin-B ZZUD cannot be reliably fitted, and thus no Kd values are provided in the figure. (*B*) Sedimentation equilibrium analysis of the β-spectrin/ankyrin-B complex. The samples were prepared by mixing β-spectrin R13–15 with ankyrin-B ZZUD or ZZUD_ΔUPA_at 1:1 ratio at a concentration of approximately 10 μM and were centrifuged at 8,700 rpm (ZZUD) or 9,500 rpm (ZZUD_ΔUPA). The curves were fitted using a heterodimer association model and a simulated annealing algorithm.

Table S1. Statistics of data collection and model refinement

Data collection			
Space group		P212121	
Unit cell parameters (Å)		$a = 75.7$, $b = 80.1$, $c = 95.0$	
Resolution range (Å)		50–2.2 (2.24–2.2)	
No. of unique reflections		29,634 (1,458)	
Redundancy		6.8 (7.3)	
I/σ		21.5 (4.3)	
Completeness (%)		99.4 (100.0)	
R_{merge} (%)*		8.5 (48.3)	
Structure refinement			
Resolution (Å)		50–2.2 (2.28–2.2)	
R_{cryst}/R_{free} (%)†	20.8 (25.4) / 24.5 (29.9)	rmsd bonds (Å)/angles (°)	0.005/0.87
No. of reflections		No. of atoms	
working set	26,945	protein atoms	3,989
test set	1,457	water molecules	101
Average B-factor (Å2)		Ramachandran plot regions‡	
main chain	54.7	favored regions (%)	96.6
side chain	60.3	allowed regions (%)	100.0
water	47.1	outliner (%)	0.0

Numbers in parentheses represent the value for the highest resolution shell.

*$R_{merge} = \sum |I_i\text{-}I_m| / \sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry related reflections.

†$R_{cryst} = \sum ||F_{obs}|\text{-}|F_{calc}|| / \sum |F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated structure factors. $R_{free} = \sum_T ||F_{obs}|\text{-}|F_{calc}|| / \sum_T |F_{obs}|$, where T is a test dataset of about 5% of the total reflections randomly chosen and set aside prior to refinement.

‡Defined by MolProbity (1).

1. Davis IW, et al. (2007) MolProbity: All-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res* 35:W375–383.

Table S2. Dissociation constants of bindings between various forms of ankyrin-B ZZUD and β-spectrin

	K_d-ITC (μM)*	K_d-AUC (μM)*
ZZUD	2.2 ± 0.3	2.0
ΔUPA	1.7 ± 0.3	1.8
ΔDD	1.4 ± 0.1	n.d.
Δlinker	2.3 ± 0.2	n.d.
E1022A	2.4 ± 0.4	n.d.
R1029E	5.0 ± 0.4	n.d.
Q1126A	2.6 ± 0.5	n.d.
E1341K	2.5 ± 0.4	n.d.
L1101P	n.d.†	n.d.
I1109T	n.d.†	n.d.
G1406C	1.0 ± 0.2	n.d.
E1425G	1.3 ± 0.3	n.d.

*The K_d values are calculated based on the ITC and AUC curves shown in Fig. S7.

†The ITC data cannot be used to fit a reliable K_d value.