Structural basis for high-affinity actin binding revealed by a β-III-spectrin SCA5 missense mutation

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Spinocerebellar ataxia type 5 (SCA5) is a neurodegenerative disease caused by mutations in the cytoskeletal protein β-III-spectrin. Previously, a SCA5 mutation resulting in a leucine-to-proline substitution (L253P) in the actin-binding domain (ABD) was shown to cause a 1000-fold increase in actin-binding affinity. However, the structural basis for this increase is unknown. Here, we report a 6.9 Å cryo-EM structure of F-actin complexed with the L253P ABD. This structure, along with co-sedimentation and pulsed-EPR measurements, demonstrates that high-affinity binding caused by the CH2-localized mutation is due to opening of the two CH domains. This enables CH1 to bind actin aided by an unstructured N-terminal region that becomes α-helical upon binding. This helix is required for association with actin as truncation eliminates binding. Collectively, these results shed light on the mechanism by which β-III-spectrin, and likely similar actin-binding proteins, interact with actin, and how this mechanism can be perturbed to cause disease.
Spinocerebellar ataxia type 5 (SCA5) is a neurodegenerative disease that stems from autosomal dominant mutations in the cytoskeletal protein β-III-spectrin. SCA5 pathogenesis results from a functional deficit in Purkinje cells, in which the expression of β-III-spectrin is required for normal cerebellar control of motor coordination. β-III-spectrin is thought to form a heterotetrameric complex with α-II-spectrin, and to cross-link actin filaments to form a cytoskeleton localizing to the shafts and spines of Purkinje cell dendrites. β-III-spectrin is required for normal dendrite structure and synaptic transmission. Recently, our group reported that a SCA5 missense mutation, L253P, localized to the N-terminal actin-binding domain (ABD), causes a ~1000-fold increase in actin-binding affinity. The actin subdomains (SD1, SD2, SD3, and SD4) have been labeled on one actin subunit, while SD1 (cyan) and the green) and the L253P mutation is localized to the second α-helix. Extensive contacts are made between CH1 and CH2, suggesting a tendency to exist in a "closed" conformation in the absence of actin. A cryo-EM structure of the fimbrin ABD shows that it associates with actin in a closed structural state. In contrast, cryo-EM showed that α-actinin associates with actin in an "open" structural state in which only a single CH domain is bound to the filament and the second domain is structurally disordered on account of it being dissociated from the interacting CH domain. A similar conclusion was reached for filamin, another member of the spectrin superfamily. Binding studies suggested that the CH1 domain of α-actinin has greater intrinsic affinity for actin in isolation and this suggested that it was CH1 bound in the cryo-EM structure. This led to the hypothesis that the CH2 domain functions to regulate the actin-binding function of CH1 through steric hindrance when the two domains are associated. Consistent with this, many mutations in the CH2 domains of both α-actinin and filamin impart modest gains in ABD affinity for actin. Collectively, these studies suggest that the L253P mutation of β-III-spectrin, which is similarly localized to CH2, causes high-affinity actin binding by disrupting a regulatory mechanism that shifts the ABD structural equilibrium from a closed to more open binding-competent state. Here, we report cryo-EM, co-sedimentation, and pulsed electron paramagnetic resonance (EPR) data consistent with such a mechanism.

Results

Structure of L253P β-III-spectrin ABD bound to actin. To begin testing our hypothesis, we first performed cryo-EM on the β-III-spectrin ABD bound to actin filaments. The actin-binding affinity of the wild-type (WT) β-III-spectrin ABD is low ($K_d = 75 \mu M$), resulting in poorly decorated actin filaments that were of insufficient quality for analysis (Supplementary Fig. 1a). The L253P ABD yielded high-quality complexes of decorated filaments (Supplementary Fig. 1b, c), enabling a three-dimensional reconstruction (Fig. 1a) of the mutant ABD–actin complex at 6.9 Å resolution. The map (left, gray transparent surface) has been fit with a model for actin (cyan) and the β-III-spectrin ABD (magenta). The actin subdomains (SD1, SD2, SD3, and SD4) have been labeled on one actin subunit, while SD1′ and SD2′ are labeled on a different subunit. The actin subdomains are labeled with respect to the CH domain of β-III-spectrin, with the closest related atomic structures of N-terminal ABDs from the spectrin superfamily, including α-actinin, dystrophin and utrophin, invariably showing extensive contacts between CH1 and CH2, suggesting a tendency to exist in a "closed" conformation in the absence of actin. A cryo-EM structure of the fimbrin ABD shows that it associates with actin in a closed structural state. In contrast, cryo-EM showed that α-actinin associates with actin in an "open" structural state in which only a single CH domain is bound to the filament and the second domain is structurally disordered on account of it being dissociated from the interacting CH domain. A similar conclusion was reached for filamin, another member of the spectrin superfamily. Binding studies suggested that the CH1 domain of α-actinin has greater intrinsic affinity for actin in isolation and this suggested that it was CH1 bound in the cryo-EM structure. This led to the hypothesis that the CH2 domain functions to regulate the actin-binding function of CH1 through steric hindrance when the two domains are associated. Consistent with this, many mutations in the CH2 domains of both α-actinin and filamin impart modest gains in ABD affinity for actin. Collectively, these studies suggest that the L253P mutation of β-III-spectrin, which is similarly localized to CH2, causes high-affinity actin binding by disrupting a regulatory mechanism that shifts the ABD structural equilibrium from a closed to more open binding-competent state. Here, we report cryo-EM, co-sedimentation, and pulsed electron paramagnetic resonance (EPR) data consistent with such a mechanism.

Fig. 1 Cryo-EM map and model of L253P β-III-spectrin ABD bound to actin. a The map (left, gray transparent surface) has been fit with a model for actin (cyan) and the β-III-spectrin ABD (magenta). On the right, the surface of the reconstruction has been color coded for the two actin strands (blue and green) and the β-III-spectrin ABD (magenta). b Close-up view of a showing that the CH1 domain has an additional N-terminal helix (red) interacting with F-actin. The actin subdomains (SD1, SD2, SD3, and SD4) have been labeled on one actin subunit, while SD1' and SD2' are labeled on a different subunit.
Fig. 2 The β-III-spectrin N-terminus is required for actin binding. a Coomassie blue stained gel of purified WT ABD or WT ABD without the N-terminal 51 amino acids (A52). b F-actin co-sedimentation assays showing that the N-terminal truncation abolishes actin affinity. c CD spectra demonstrating α-helical absorption profiles. The A52 ABD has a statistically significant increase in helicity (n = 3). d CD denaturation at 222 nm. The A52 ABD has a statistically significant increase in T_m (n = 3)

Å resolution (Supplementary Fig. 2). This represents a substantial improvement over previous ABD–actin reconstructions13, 14, 19, the best of which was 12 Å. The reconstruction and resulting atomic model provide several mechanistic insights. First, the density map reveals that only a single CH domain is bound to actin, as observed previously for the α-actinin ABD. Second, the bound CH domain has an additional N-terminal helix that is tightly associated with actin (Fig. 1b, red). This helix was not identified in other ABD–actin cryo-EM complexes. However, a reexamination of the α-actinin–actin reconstruction14 suggests that extra density is present, consistent with such an N-terminal helical extension. By comparison, the higher resolution fimbrin–actin reconstruction19, containing closed CH domains, shows no extra density. The presence of this contiguous N-terminal helix unambiguously identifies the bound domain as CH1. Thus, high-affinity actin binding, caused by the L253P mutation in the CH2 domain, is mediated through the CH1 domain. The L253P mutation does not expose or generate a de novo high-affinity actin binding site in the CH2 domain, as has been suggested previously20.

All N-terminal ABDs contain amino acid sequences of variable length and composition preceding the conserved CH1 domain. However, a structured N-terminal region preceding the globular fold of a CH domain has not been previously observed in most reported ABD crystal structures. This reflects either disorder in this region or the intentional truncation of the region based on predicted intrinsic disorder21. However, when calmodulin was crystallized with the plectin ABD, calmodulin was bound to the N-terminal region which had become α-helical21. Solution studies confirmed that in the absence of calmodulin, the plectin N-terminal region is unstructured. The β-III-spectrin CH1 domain with the extended N-terminal helix built into the cryo-EM map superimposes very well with the corresponding plectin CH1 domain with calmodulin (Supplementary Fig. 3), and shows that calmodulin would be involved in massive clashes with actin. As proposed this explains how calmodulin, in the presence of Ca^2+^, dissociates the plectin ABD from actin since the binding of actin and calmodulin is competitive22.

N-terminal ABD residues are essential for actin binding. The β-III-spectrin cryo-EM structure showing the N-terminal helix bound to actin suggests that the helix must contribute to binding affinity. To test this, we measured affinity of WT ABD with and without the first 51 amino acids (A52). Strikingly, truncation of the N-terminal sequence abolished binding of the ABD to actin (Fig. 2a, b). Circular dichroism (CD) indicates that this loss in binding is not due to misfolding (Fig. 2c, d). On the contrary, the A52 ABD showed small but reproducible increases in helicity and stability, suggesting that the N-terminal residues contain intrinsic disorder, which we verified by CD (Supplementary Fig. 4). Collectively, these data sets, combined with the cryo-EM
L253P mutation promotes structural opening of CH domains. Previously we demonstrated that the L253P mutation substantially destabilizes the ABD ($\Delta T_m = -14.8 \, ^\circ \text{C}$). If the open structural state of the ABD is responsible for high-affinity actin binding, then decreased stability may facilitate opening of the CH domains from a closed state. In our previous structural homology model of the $\beta$-III-spectrin ABD in the closed structural state, L253 is positioned at the CH domain interface (Supplementary Fig. 5), suggesting that the L253P mutation could also perturb CH1–CH2 interactions that stabilize the closed structural state. To test this hypothesis, we used double electron–electron resonance (DEER) to measure inter-CH domain distance with and without the L253P mutation. We exploited the native cysteine residues at positions 76 and 231 for irreversible attachment of spin labels.

For WT ABD in the absence of actin, clear oscillations were present in the echo amplitude decay (Fig. 3). Analysis (Supplementary Fig. 6) revealed an inter-probe distance centered at 4.8 nm, consistent with the distance predicted in the homology model of the closed state shown in Supplementary Fig. 5. Upon introduction of the L253P mutation, the distance distribution undergoes a shift to populate a longer inter-probe distance, visible as a shoulder to the right of the 4.8 nm peak, consistent with structural opening of the ABD.

Discussion
SCA5 pathology is characterized by atrophy of the cerebellum, likely reflecting degeneration of dendritic arbors extended by Purkinje cells. Within dendrites, $\beta$-III-spectrin binds to actin filaments to form a spectrin-actin skeleton underlying the plasma membrane. The low affinity of WT $\beta$-III-spectrin for actin suggests that normal membrane function requires a dynamic spectrin-actin cytoskeleton in which spectrin-actin linkages form and dissociate. We suggest that the high affinity of L253P $\beta$-III-spectrin for actin decreases dynamics of spectrin-actin linkages, resulting in reduced plasticity of the spectrin-actin cytoskeleton. We speculate that spectrin-actin cytoskeleton plasticity is important for the cytoskeleton to expand or retract within structurally dynamic regions of the dendritic arbor, such as growing or remodeling dendrites and spines. Recent work has highlighted the role of $\beta$-III-spectrin in support dynamic spine structure and post-synaptic signaling. In addition, disrupted microtubule-based transport has been reported for the L253P mutation, and these transport defects may disrupt arborization and contribute to SCA5 pathogenesis. Disrupted transport may be secondary to defects in microtubule tracts that are organized by the spectrin-actin cytoskeleton, and/or result from the direct impact of high-affinity binding of L253P $\beta$-III-spectrin to the actin-related protein, ARP1, a component of the dynactin complex that facilitates cargo transport by microtubule motor proteins.

Collectively, the 6.9 Å cryo-EM structure, binding studies, and DEER distance distributions converge on a structural mechanism for disease. The CH2 domain-localized L253P mutation perturbs a closed-open structural equilibrium in $\beta$-III-spectrin’s ABD by lowering the energetic barrier between structural states. The ABD is then relieved of its regulatory mechanism allowing for the CH1 domain to interact with actin filaments, aided by an additional N-terminal unstructured region that becomes helical upon binding actin.

Methods
Protein purification. For cryo-EM analyses, the WT or L253P human $\beta$-III-spectrin ABD coding sequences were amplified using a HiTrap Q 5 ml ion-exchange column, followed by a Superdex 200 size exclusion column (GE Healthcare Life Sciences). Elution fractions of the Superdex 200 column containing pure ABD proteins as assessed by SDS-PAGE were pooled and concentrated (Amicon Ultra-4 Centrifugal Filter, 10 K MWCO). A Bradford assay (Biorad) was then used to determine protein concentrations which equaled 44.0 and 40.6 $\mu$g/mI for WT and L253P, respectively. ABD proteins were stored on ice until preparation of ABD–F actin complexes.

To test the contribution of the N-terminus to actin-binding affinity, the coding sequences for WT ABD (amino acids 1–284) or truncated WT ABD (amino acids 52–284) were PCR amplified using the forward primer AAACACCTGCAGGAAGAAG GTATGACGAACGACGCTGTCACCC or AAAACACCTGCAAAAGTGGTGCAGAT GAAAGGAAUGCTGTCGTCGTCGCCATGTAATGGTAGTAAG. PCR products were digested with AarI and Bxb1 restriction enzymes and ligated into the BsaI site of pE-SUMOpro (LifeSensors) containing His and SUMO tags. The final constructs pE-SUMO-ABD WT and pE-SUMO-A52-ABD WT were sequence verified and transformed into E. coli BL21 (DE3) pilayerS (Agilent). Transformed bacteria were incubated with...
rotation at 27 °C in flasks containing 1 L LB media with 100 µg per ml ampicillin and 50 µg per ml chloramphenicol until an absorbance of 0.5 at 550 nm was reached. Then flasks were placed in ice for 10 min before addition of IPTG to 0.5 mM final.

The flakes were then incubated with rotation for 4 h in a 22 °C water bath. Bacteria were harvested at 5000 × g and pellets stored at −20 °C. Bacteria were lysed by addition of lysozyme (Sigma) for 1 h at 4 °C in buffer containing 50 mM Tris, pH 7.5, 300 mM NaCl, and 150 mM imidazole. Fractions eluted from the Ni-NTA agarose column were pooled and loaded into a Slide-a-Lyzer, 10 K MWCO, dialysis cassette (ThermoScientific), and dialysis performed at 4 °C in buffer containing 25 mM Tris, pH 7.5, 150 mM NaCl and 5 mM β-mercaptoethanol. To cleave off the SUMO tag, Ulp1 SUMO protease was added to dialyzed ABD proteins at a 1:14 (protease:ABD) mass ratio, and digests incubated for 1.5 h at 4 °C. To separate ABD proteins from the cleaved His-tagged SUMO tag and His-tagged SUMO protease, ABD proteins were loaded onto a Poly-Prep chromatography column containing 0.5 mL Ni-NTA agarose equilibrated in 25 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM β-mercaptoethanol. Elution fractions containing ABD proteins were collected and then loaded onto a gel filtration column (Superdex S100, GE) equilibrated in buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM MgCl2, and 1 mM DTT at 4 °C. The images were dose-fractionated into seven chunks (Supplementary Table 1). The MolProbity scores were used to evaluate the quality of the model (Supplementary Table 1). The MolProbity score for the actin-spectrin filament models compare favorably (99th percentile) with structures of similar resolution.

Although segments were sorted to exclude naked actin, Phoxen refinement of the actin-spectrin reconstruction clearly shows that the occupancy by spectrin is not 100%, and the actual occupancy is ~75%. Therefore, the threshold chosen for the filament needed to show the full volume for spectrin shows a somewhat larger and lower resolution actin.

Co-sedimentation assays. Actin was purified from acetonite powder derived from the poas muscle of New Zealand white rabbit (Oryctolagus cuniculus). Acetonite was hydrated in 4 mM water for 30 min to extract actin. The resultant slurry was passed through a Whatman filter paper and 30 mM KCl was added to the filtrate to polymerize actin for a period of 1 h at room temperature. Filamentous actin was then pelleted by 30 min centrifugation at 80,000 rpm in a TLA 100.3 rotor. The actin pellet was resuspended in buffer containing 5 mM Tris, pH 7.6, 0.5 mM MgCl2, and 1 mM DTT. This appeared to arise from incomplete occupation. We therefore used atomic

where Y equals fraction ABD bound and X equals free F-actin concentration.

Circular dichroism. ABD proteins were purified at 100,000 × g for 20 min at 4 °C. A Bradford assay was performed to determine ABD protein concentrations, and ABD proteins were diluted to 250 µg/ml in buffer containing 10 mM Tris, pH 7.5, 130 mM NaCl, 2 mM MgCl2, 1 mM DTT. CD spectra were acquired in a Jasco J-815 Spectropolarimeter equipped with a Peltier temperature controller. Immediately before analysis, the instrument was baseline-corrected using ABD protein buffer. For secondary structure analyses, CD spectra were measured from 200 and 260 nm at 25 °C. Thermal unfolding of the ABD protein sample was analyzed by recording CD spectra at 222 nm over the temperature range of 20–85 °C. CD analyses were performed three times for each protein. Non-linear regression analysis was performed in Prism 5 (GraphPad Software, Inc.) to determine the melting temperature using the following equation for a two-state transition, reported previously:
proteins. The reconstituted peptide was then diluted to a concentration of 99 ng per µl and subsequently scanned over the same wavelength range described above. In the case of both ABDS and peptide, raw ellipticity was normalized to each sample’s respective concentrations according to the following equation:

\[ \text{MRE} = \left[ \theta \text{[MW]} / (N - 1) / l \right] / l, \]

where \( \theta \) represents the raw ellipticity, MW represents the protein molecular weight, \( N \) is the number of amino acids, \( l \) is the path length, and \( c \) is the concentration in milligrams per milliliter.

**Statistical analyses.** Unpaired, two-tailed \( t \)-tests were performed in Prism 5 software to determine whether significant differences existed in ABDS protein melting temperatures or 222/208 absorbance ratios determined by CD. The value was equal to three in all cases.

**Spin labeling.** In the β-III-spectrin ABDS constructs, 500 µM of the spin label 4-maleimido-TEMPO (MSL, 4-maleimido-2,2,6,6-tetramethyl-1-piperidinylxylene) Sigma-Aldrich) was added to 25 µM protein and equilibrated on a rocker for 3 h at 4 °C. Prior to addition of MSL, the protein solution had been run over a Zeba desalting column pre-equilibrated with 10 mM Tris, pH 7.5, and 150 mM NaCl to remove most of the 1 mM DTT left over from size exclusion. After the spin-label incubation period, the protein was once again subjected to a Zeba desalting column to remove any unreacted spin label. To ensure complete removal, however, the spin-labeled protein was then subjected to three 4 h rounds of dialysis in 4 L solutions containing 10 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM DTT. MSL was ultimately chosen over the more commonly used (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTSSL) because spin labeling of the β-III-spectrin ABDS constructs was incomplete, requiring inclusion of DTT reducing agent post-labeling to prevent undesired ABDS cross-linking. Incubation of the ABDS constructs with spin label for periods longer than 3 h resulted in significant protein loss due to precipitation. The spin-labeled WT and L253P β-III-spectrin ABDS constructs were concentrated down to 230 and 175 µM, respectively, prior to spin counting and DEER sample preparation.

**EPR spectroscopy.** To verify labeling, a continuous wave EPR spectrum was acquired with sample temperature of 296 K on the E500 Bruker EPR spectrometer operating at X-band (9.5 GHz) and equipped with a SHQ cavity. The derivative spectrum acquired with sample temperature of 296 K on the E500 Bruker EPR spectrometer was equal to three in all cases.

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Author contributions
A.W.A. and T.S.H. initiated the project. A.W.A. and M.E.F. in consultation with D.D.T. and T.S.H. designed the experiments, excluding the cryo-EM and structural modeling that were conducted by F.W., A.O., and E.H.E. A.W.A. and M.E.F. expressed, purified, and characterized all protein samples. A.W.A., M.E.F., A.R.T, and D.D.T. analyzed DEER data. The manuscript was prepared by A.W.A. and M.E.F. with edits by D.D.T., E.H.E., and T.S.H.

Additional information
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