

Asymmetric activation of ANKRD1-CAND1-Atrogin-1 signaling in mature myofibers promotes adolescent idiopathic scoliosis progression by inducing asymmetric paravertebral muscle atrophy

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Research Article

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Abstract

Background

Adolescent idiopathic scoliosis (AIS) represents one of the primary three-dimensional spinal malformations in youth and teenagers, though its etiology is currently inadequately elucidated. Growing data suggests that non-uniform modification of the paraspinal muscles drives curvature advancement; the specific factors triggering this imbalance, nevertheless, are still unidentified.

Methods

Paired paraspinal muscle samples from the convex and concave regions of individuals suffering from AIS underwent histological analysis, single-nucleus RNA sequencing, and molecular evaluations to identify regulators of asymmetric muscular reshaping. The functional impact of ankyrin repeat domain protein 1 (ANKRD1) on myofibrillar wasting and protein degradation was investigated in vitro. Protein association and domain-localization studies delineated the manner in which ANKRD1 interacts with CAND1 and how this binding influences the Atrogin-1 ubiquitin ligase assembly. A bipedal mouse model was used to assess the pathogenic function of ANKRD1 and the medicinal efficacy of the proteasome inhibitor bortezomib.

Results

ANKRD1 was selectively and aberrantly upregulated in mature myofibers of the concave-side paraspinal tissues in AIS, which was associated with regional muscle wasting. Functionally, ANKRD1 interacted with a CAND1 structural interface using the N-terminal arch via its ankyrin repeat region, promoted the formation of the Atrogin-1 aggregate, and amplified ubiquitin-dependent degradation of vital muscle proteins. Experimentally, ANKRD1 activation shrank myotubes, and inhibition of proteasomal turnover mitigated this impact. In vivo, unilateral elevation of ANKRD1 signaling generated asymmetric paraspinal muscle atrophy, diminished muscle strength, and facilitated scoliotic progression. Bortezomib alleviated ANKRD1 triggered muscle wasting and impairment and significantly slowed deformity advancement.

Conclusions

This study reveals the mechanism of abnormal protein homeostasis regulation mediated by the ANKRD1-CAND1-Atrogin-1 axis, which promotes the onset and progression of scoliosis by driving asymmetric atrophy of the paraspinal muscles. It provides a new theoretical basis for research into the etiology of AIS and for precision intervention strategies targeting muscle protection.

Background

AIS represents a sophisticated three-dimensional spinal malformation developing during early youth, having an international incidence of approximately 1–4% [1, 2]. Besides its mechanical and emotional toll, profound angulations may impair respiratory and cardiac health and potentially pose a life-endangering risk [3]. The precise physiological mechanisms remain unclear. Elucidating etiology thus possesses significant medical importance.

Etiological investigations encompass various tiers [4–6]. Yet, a specific domain has traditionally been overlooked regarding rigorous examination: the paraspinal muscles, which serve as terminal effectors where diverse pathogenic elements converge upon the vertebral column. These muscles form a proactive stabilizing framework that ensures spinal equilibrium, distributes spinal burdens, and maintains proper force vectors; their operational stability is a fundamental requirement for spinal homeostasis [7, 8]. Clinical radiographic analyses [9, 10], biomechanical evaluations [11, 12], and histological investigations [13–15] uniformly demonstrate significant structural and functional disparity between the bilateral paraspinal muscles in AIS, alongside substantial metabolic restructuring. Since these muscles maintain spinal dynamic equilibrium, the strength asymmetry generated by such remodeling can perturb the mechanical equilibrium on both sides of the vertebral column. Hueter–Volkmann's principle posits that bone development follows mechanical stress: relatively stronger convex side paraspinal muscle diminishes local loading and promotes growth, while weakened concave-side muscle increases local loading and inhibits it. Sustained bilateral imbalance of this nature finally triggers and exacerbates spinal curvature [16, 17].

A defining feature of pathological paraspinal muscular remodeling in AIS involves asymmetrical atrophy of bilateral muscle groups [18, 19]. At the microscopic scale, such atrophy represents a disruption in regulatory pathways controlling protein catabolism within adult muscle fibers. The volume of skeletal muscle relies upon a fluid equilibrium between biosynthesis and proteolysis; whenever that equilibrium shifts toward degradation, myofibrils degenerate [20]. Within the frameworks maintaining muscle proteostasis, the ubiquitin–proteasome system (UPS) serves as a pivotal pathway for selective protein degradation. E3 ubiquitin ligases label particular substrates with ubiquitin, which is subsequently digested by the proteasome—a process fundamental to muscle growth, recovery from trauma, energy modulation, and volume preservation [21]. Overwhelming UPS engagement represents a confirmed pathological foundation for muscle-wasting disorders: prolonged activity excessively degrades muscular proteins and results in fibers being shrunken and non-functional [22–24]. Genomic and proteomic analyses during the past few years have demonstrated significant expression disparities between concave and convex side paraspinal muscles in AIS, indicating a protein homeostasis disturbance in asymmetrical muscle loss [25–28]. Which elements are at the heart of this mechanism remains uncertain. Pinpointing the primary molecules that link aberrant UPS activation with asymmetrical paraspinal muscular atrophy in AIS would enhance our understanding of the molecular mechanisms underlying disease initiation and development.

ANKRD1 (ankyrin repeat domain 1) represents a stress regulated protein that responds to diverse microenvironmental shifts. Previous research focused primarily on its roles in cardiac pathology and

malignancies [29, 30], whereas recent data link it to stress adaptation and tissue reconstruction following trauma [31]. Its specific biology within skeletal muscle tissue has thus far received limited scientific attention. This investigation analyzed ANKRD1 expression during pathological remodeling in paraspinal muscles of individuals with AIS. ANKRD1 exhibited significant elevation in mature myofibers on the concave side of these muscles in AIS patients. By modulating the functional state of the Atrogin-1 complex, ANKRD1 promoted accelerated proteolysis of skeletal muscle proteins and resulted in localized muscle wasting. In vivo, adeno associated virus (AAV) mediated unilateral overexpression of ANKRD1 in paraspinal muscles was sufficient to trigger a scoliotic phenotype in bipedal mice. Simultaneous delivery of bortezomib, a Food and Drug Administration (FDA) approved proteasome inhibitor, to the AAV transduced side successfully slowed down vertebral deformity progression. These observations establish a direct link between impaired skeletal muscle proteostasis and the evolution of AIS, providing a conceptual framework for targeted therapeutic approaches that maintain muscular stability and restore protein equilibrium.

Methods

Clinical sample collection

Individuals diagnosed with AIS requiring posterior corrective operations were recruited. Identification adhered to the standards set by the Scoliosis Research Society (SRS). Exclusion criteria included congenital vertebral anomalies, neuromuscular pathologies, connective tissue syndromes, and previous spinal interventions. Age-matched subjects with congenital scoliosis (CS) and non-scoliotic participants (NS) served as controls.

Intraoperatively collected paraspinal muscle was obtained from the apical vertebral site in scoliosis patients, or from a similar level in non-scoliotic subjects, following standardized procedures [32–34]. Upon immediate retrieval, every tissue sample was divided based on subsequent objectives. One segment was encapsulated in optimal cutting temperature (OCT) medium for histological examination, another was preserved in 2.5% glutaraldehyde for transmission electron microscopy, and specimens for genetic evaluations were flash-frozen in liquid nitrogen and kept at -80°C . A further segment was set aside uniquely for nuclear extraction in samples intended for single-nucleus RNA sequencing.

Animals

All vertebrate trials used 3-week-old male C57BL/6J mice obtained from Changsha Tianqin Biotechnology Co., Ltd. The mice were kept in a specific pathogen-free environment at $22 \pm 1^{\circ}\text{C}$ under a 12 h light/12 h dark regimen, with sterilized food and water available ad libitum.

A bipedal mouse model was developed to mimic the vertical stance and longitudinal spinal loading of people, as documented earlier [35]. Specifically, 3 week old males were sedated, and both forelimbs and the tail were surgically removed. Throughout subsequent recovery periods, nourishment and liquid

vessels were elevated progressively, instructing the rodents to assume and maintain an upright bipedal posture while feeding and drinking.

To trigger scoliosis via unilateral ANKRD1 upregulation, a recombinant AAV9 vector containing the murine Ankrd1 open reading frame was synthesized. The Ankrd1 gene (NCBI Reference Sequence: NM_013468.3) was inserted into the AAV vector CMV-bGlobin-mAnkrd1-EGFP-3FLAG-WPRE-hGH polyA; the null control vector was CMV-bGlobin-EGFP-3FLAG-WPRE-hGH polyA. GeneChem executed viral assembly within the AAV9 capsid framework, resulting in a titer of 1×10^{12} vector genomes (vg)/mL. Utilizing a microsyringe (Hamilton Company, model 1701 RN), virus was delivered intramuscularly across various locations along the longitudinal axis of the paraspinal tissues on one side, 5×10^{10} vg per animal in total; the opposite side was injected with an equivalent volume of blank-vector control at identical sites. Rodents were kept for a further 3 weeks following injection. In vivo gene delivery was evaluated through small animal fluorescence imaging, and ANKRD1 enhancement in the paraspinal muscles was confirmed by tissue immunofluorescence and Western blot analysis.

For pharmacological treatment, bortezomib (MedChemExpress, Cat. No. HY-10227), a proteasome inhibitor, was administered locally within the paraspinal tissues on the ANKRD1 overexpressed side 4 weeks following induction of asymmetric ANKRD1 expression. Bortezomib was dissolved to the working solution in DMSO (Solarbio, Cat. No. P1010) and delivered via local intramuscular injection at 0.25 mg/kg once every 3.5 days over 2 consecutive weeks, 100 μ L per side. Control animals were injected with an equivalent volume of DMSO at the identical site. Spinal alignment and paraspinal muscle performance were assessed 2 weeks following the ultimate dose.

Histological staining

New paraspinal tissue was washed carefully in chilled phosphate-buffered saline to remove external blood, sliced along the muscle fibers, and arranged vertically within embedding molds containing OCT medium (Sakura Finetek, Cat. No. 4583). Specimens were swiftly frozen in liquid nitrogen; after the medium had frozen solidly, they were stabilized in a cryostat for 30 minutes. Consecutive 7 μ m cryosections were prepared using a CryoStar NX50 cryostat (Thermo Scientific) and affixed onto adhesion slides (Thermo Fisher Scientific, Cat. No. 22-037-246).

Frozen sections underwent hematoxylin and eosin, picrosirius red, and Oil Red O staining procedures. Specimens were equilibrated at ambient temperature for 10 min and preserved using eco-friendly GD muscle fixative (Servicebio, Cat. No. G1111) for 15 min. Following three 5 min PBS wash cycles, HE staining used an HE Staining Kit (Beyotime, Cat. No. C0105S), picrosirius red employed a Picrosirius Red Stain Kit (Beyotime, Cat. No. C0190S), and Oil Red O used an Oil Red O Stain Kit (Beyotime, Cat. No. C0158S), all performed according to the manufacturer's instructions. Dye-stained sections were sealed with glycerol gelatin aqueous mounting medium (Applygen, Cat. No. C1213) for microscopic examination.

Immunofluorescence staining

Tissue slices and cell cultures were preserved in 4% paraformaldehyde (Sigma-Aldrich, Cat. No. P6148) for 15 minutes at ambient temperature, rinsed three times with PBS (5 minutes per session), and then permeabilized using 0.3% Triton X-100 (Sigma-Aldrich, Cat. No. T8787) for 10 minutes, followed by an additional rinse. To prevent nonspecific interactions, samples were incubated with 5% bovine serum albumin (Sigma-Aldrich, Cat. No. A9647) for 1 hour at room temperature. Primary antibodies were then applied overnight at 4°C: anti-dystrophin (Santa Cruz Biotechnology, Cat. No. sc-73592; 1:200), anti-myosin heavy chain (MyHC; Developmental Studies Hybridoma Bank, Cat. No. MF20; 1:200), anti-ANKRD1 (Santa Cruz Biotechnology, Cat. No. sc-365056; 1:200), and anti-CAND1 (Abcam, Cat. No. ab101987; 1:200). Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen, Cat. No. A-11034; 1:500) or goat anti-mouse IgG (Invitrogen, Cat. No. A-11008; 1:500) was subsequently added for 1 hour at room temperature under dark conditions. Following PBS washes, the specimens were sealed with antifade reagent containing DAPI (Beyotime, Cat. No. P0131) for microscopic analysis.

Transmission electron microscopy

The newly excised paraspinal muscle was promptly immersed in electron microscopy fixative (Servicebio, Cat. No. G1102) and preserved at 4°C for 12 h. The specimen was then washed three times with PBS (15 min per session), followed by secondary fixation using 1% osmium tetroxide (Electron Microscopy Sciences, Cat. No. 19150) for 1 h, and subsequently rinsed, dehydrated via a descending ethanol sequence, and underwent acetone exchange. Infiltration was carried out using acetone:epoxy resin blends (Electron Microscopy Sciences, Cat. No. 14120) at 2:1, 1:1, and 1:2 ratios, followed by pure resin overnight. The sample was thereafter embedded in fresh resin and hardened at 60°C for 48 h. Ultrathin sections (~70 nm) were prepared on an EM UC7 ultramicrotome (Leica Microsystems) and mounted onto copper grids. The sections were stained with 2% uranyl acetate (Electron Microscopy Sciences, Cat. No. 22400) for 15 min, rinsed with distilled water, and further stained with lead citrate (Electron Microscopy Sciences, Cat. No. 17810) for 5 min. The ultrastructure was analyzed using a JEM-1400Flash transmission electron microscope.

Small animal micro-computed tomography

Upon reaching the experimental conclusion, animals were humanely euthanized while maintaining head, spine, and extremities, followed by total immersion in 4% paraformaldehyde for a night at 4°C. Every subject was subsequently positioned vertically within the scanning device with head, shoulders, and hindlimbs held in a natural alignment. Imaging was conducted using a high resolution micro-CT apparatus (SkyScan 1272, Bruker) at 60 kV and 200 μ A, yielding ~10 μ m resolution with 360° rotation; all cohorts underwent imaging under the same conditions. Tomographic datasets were processed via NRecon software (Bruker), and subsequent three-dimensional spinal modeling and morphological evaluation were executed through CTAn software.

Ex vivo muscle contractility testing

The bilateral paraspinal muscle tissue was excised and promptly submerged in cooled Krebs–Ringer solution (Solarbio, Cat. No. G0430) that was constantly gassed with 95% O₂ and 5% CO₂. Myogenic activity was evaluated using an isolated muscle mechanics apparatus (Aurora Scientific, model 1300A). Specimens were stabilized for 15 minutes, and the ideal resting length was determined through stepwise modification. Tetanic responses were triggered by successive electrical pulses at 10, 20, 40, 60, 80, 100, 120, and 150 Hz, with each burst lasting 300 ms and a 2 minute gap separating the pulses. Maximum tension at every frequency was measured, and the force–frequency graph was generated. Endurance capacity was examined via a repetitive tetanic regimen: triggering at a constant rate for 300 ms every 5 seconds for 5 minutes, with tension values recorded for each cycle. The fatigue ratio was calculated as the tension at the conclusion of the session as a fraction of the initial peak. Following experimentation, the muscles were weighed, and the cross-sectional area was calculated from the muscle length and mass to compute absolute force relative to cross-sectional area, thus providing specific force for group comparisons.

Single-nucleus RNA sequencing

Paraspinal muscle intended for single-nucleus RNA sequencing (snRNA-seq) was kept immediately following harvesting and underwent nucleus separation. Nuclear isolation, quality evaluation, library creation, and sequencing were executed by Novogene (Beijing, China) as a specialized single-nucleus transcriptomic service. Briefly, samples were physically fragmented under chilled conditions and membranes were lysed to discharge nuclei; particles and clusters were removed, and the nuclear solutions were cleaned and re-suspended for library development. Every preparation was verified for quality, clumping, and particulate contamination prior to library construction.

Suitable nuclear samples were mounted on the 10x Genomics Chromium system, whereby single cells were isolated into gel beads-in-emulsions (GEMs) and labeled. Reverse transcription, cDNA enrichment, and 3' transcriptomic library preparation were executed according to the 10x Genomics protocol. Following library quality assessment, sequencing was performed on an Illumina NovaSeq instrument using a paired-end 150 bp (PE150) approach.

Initial sequencing reads were quality-controlled using fastp v0.20.0 and analyzed via Cell Ranger v7.0.0 (10x Genomics). Sequences were mapped to the human genome, and expression datasets were constructed based on barcode and UMI tags. Since these samples originated from single nuclei, all exon and intron sequences were preserved to evaluate the levels of nuclear gene activity.

For downstream investigation, Seurat version 4.3.0 was used within R. Genes identified in less than three nuclei and cells containing fewer than 200 expressed genes were discarded. Sample-specific quality metrics excluded barcodes exhibiting abnormally elevated gene counts, disproportionate mitochondrial transcript ratios, or elevated hemoglobin gene expression. Potential doublets were detected and eliminated using DoubletFinder. Following quality control, normalized and integrated expression matrices were generated for dimensionality reduction and clustering.

Genes exhibiting high variability were inputted into principal component analysis (PCA); a shared nearest neighbor graph derived from these components facilitated unsupervised clustering, while uniform manifold approximation and projection (UMAP) illustrated the nuclear populations. Clusters were labeled by canonical marker genes, with mature myonuclear subtypes designated based on recognized skeletal muscle fiber type markers. Cluster specific markers and differentially expressed genes were identified using the Wilcoxon rank-sum test. Functional enrichment of differentially expressed genes used clusterProfiler v3.14.0; an adjusted $P < 0.05$ determined statistical significance.

Cell culture and myogenic differentiation

The murine C2C12 myoblast cell line originated from Sunncell Wuhan ShangEn Biotechnology Co., Ltd. These cells were cultured in high glucose Dulbecco's modified Eagle's medium (Gibco, Cat. No. 11965118) enriched with 10% fetal bovine serum (Gibco, Cat. No. 26010074) and 1% penicillin streptomycin (Solarbio, Cat. No. P1400) within a 37°C incubator under humidified 5% CO₂. Upon reaching approximately 80% confluence, the growth medium was replaced with differentiation medium consisting of 2% horse serum (Gibco, Cat. No. 16050122) to initiate myogenic maturation, with frequent medium exchanges every 48 h.

Quantification of myofiber cross-sectional area and myotube morphology

A minimum of three distinct, non-redundant fields were examined for every specimen. On cryosections of paraspinal muscles, dystrophin immunofluorescence was used to define the sarcolemmal limits of individual myofibers for calculating cross-sectional area (CSA). For differentiated C2C12 cells, MyHC immunofluorescence was employed to identify mature myotubes for assessing their dimensions and fusion status. Every image was captured using consistent microscopic parameters and illumination levels, and analysis was conducted using ImageJ. Concerning CSA, the perimeters of myofibers completely surrounded by dystrophin-positive signals were drawn manually and the internal regions were quantified. For C2C12 myotubes, diameters were recorded at the central point of each MyHC-positive fiber; nuclei inside MyHC-positive multi-nucleated myotubes and overall nuclei were enumerated, and the fusion index was calculated as the proportion of total nuclei located within MyHC-positive multi-nucleated myotubes.

Cell viability assay

C2C12 cells were cultured within 96-well plates (Corning, Cat. No. CLS3992) at a density of approximately 1×10^4 cells per well in 100 μ L of complete medium and subjected to the specified treatments or conditions as outlined in the research plan. Post treatment, 10 μ L of CCK-8 solution (Beyotime, Cat. No. C0038) was added to each well and the plates were incubated for 2 h at room temperature under dark conditions. The absorbance at 450 nm was measured using a BioTek Synergy H1 microplate reader. Five replicate wells were established per group across three distinct trials, and the cell viability was represented relative to the optical density of the control group.

Cell transfection

ANKRD1 overexpressing lentiviruses, shRNA vectors targeting ANKRD1, siRNA sequences for CAND1, ANKRD1 deletion mutant plasmids, and CAND1 deletion or truncated vectors were integrated into C2C12 cells according to the supplier's protocols (GeneChem, Shanghai, China). All lentiviral systems, siRNA tags, full-length clones, and deletion/truncation vectors were designed, synthesized, and provided by GeneChem, which additionally conducted lentivirus production, purification, and titer assessment. The ANKRD1 expression plasmid was Ubi-mAnkrd1-3FLAG-SV40-puromycin, with Ubi-3FLAG-SV40-puromycin serving as the null control; the shANKRD1 construct was hU6-shANKRD1-CMV-puromycin, with hU6-CMV-puromycin as the mock control.

shRNA target sequences against Ankrd1:

shAnkrd1-1: 5'-GTTTCAGAAATGGGAGAATAT-3'

shAnkrd1-2: 5'-GGCGATCGTGGAGAAGTTAAT-3'

shAnkrd1-3: 5'-GAGCATGCTTAGAAGGACATA-3'

siRNA target sequences against Cand1:

siCand1-1: 5'-AACUGGGCAA AUGUUUCAU-3'

siCand1-2: 5'-GCAAGUACCUCAAGAAUAU-3'

siCand1-3: 5'-GGAAGCUUCUUC AAGGAU-3'

To delineate the structural framework for the ANKRD1–CAND1 complex, complete length, deletion deficient, and truncated vectors were derived from the murine Ankrd1 and Cand1 genomic sequences. Each vector was inserted into pcDNA3.1(+)-CMV (Invitrogen, USA). ANKRD1 vectors featured an N-terminal 3×FLAG epitope fused by a GGGGS spacer: full length ANKRD1 (ANKRD1-FL, residues 1–319) along with five deletion variants— Δ PEST1 (omitting residues 1–95), Δ Coiled-coil (omitting residues 20–90), Δ NLS (omitting residues 120–150), Δ PEST2 (omitting residues 150–190), and Δ ANK repeats (omitting residues 190–319). CAND1 vectors included a C-terminal 3×FLAG epitope with identical linker: full length CAND1 (CAND1-FL, residues 1–1230) alongside three deletion variants— Δ N-terminal arch (omitting residues 1–400), Δ Central hinge (omitting residues 401–650), and Δ C-terminal arch (omitting residues 651–1230). Independent N-terminal arch (residues 1–400) and C-terminal arch (residues 651–1230) vectors were also synthesized.

The viral transduction efficacy within C2C12 cells was initially evaluated via GFP imaging, and persistently modified lines were isolated using puromycin (Sigma-Aldrich, Cat. No. P8833). These expression vectors were introduced into the cells using Lipofectamine 2000 (Invitrogen, Cat. No. 12566014).

RNA extraction and reverse-transcription quantitative PCR

Total RNA was isolated from tissues or C2C12 cells using the TRIzol reagent (Invitrogen, Cat. No. 15596018CN). Tissue samples were finely ground under liquid nitrogen, while cells were lysed immediately within TRIzol. Following a 5 min incubation at room temperature, chloroform (Sigma-Aldrich, Cat. No. C2432) was introduced, and the mixture was vigorously agitated and spun at 12,000 × g for 15 min at 4°C. The aqueous fraction was gathered and combined with an equivalent volume of isopropanol (Sigma-Aldrich, Cat. No. 563935) for RNA precipitation. Pellets were rinsed with 75% ethanol, reconstituted in RNase-free water, and assessed for concentration and purity using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

cDNA was generated from 1 µg total RNA using the PrimeScript RT Reagent Kit (Takara Bio, Cat. No. RR037A) according to the manufacturer’s guidelines. The quantitative PCR was performed with TB Green Premix Ex Taq II (Takara Bio, Cat. No. RR820A) on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). All primer sequences are detailed in Table 1.

Table 1. Primer sequences used for RT–qPCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Mouse Ankrd1	AGCCTGGTCAATGTTGTGCT	TGGTGACGTTGTAGTAGCGG
Human ANKRD1	TGCCAGATTTTCAGAAGGTGATG	GGTCGTTTCCATCCTCCTTG
Mouse Myod1	CCACTCCGGGACATAGACTTG	AAAAGCGCAGGTCTGGTGAG
Mouse Myog	CAACCAGCGGCTGCCTAAAGT	GCATTCACTGGGCACCATGG
Mouse Myh1	TGAGGAACAATCCAACGCCAT	CGGTCCTCCTTGTCGTTGAT
Mouse Fbx32	CTTTCAACAGACTGGACTTCTCG	CAGCTCCAACAGCCTTACTAC
Mouse Trim63	ACCTGCTGGTGGAAAACATC	TGCTGTTCTCTCAAGGCTC
Mouse Cand1	GAGGAGCTGGAGATCGTGAA	TCCACAGGTAGGTGGTCTTG
Mouse Cul1	CTGGTGGACATCGAACTGGT	GCTTCTTGGTGATGTCGGTG
Mouse eIF3f	GCTGCTGAGATGGAGGAGAA	TGGTCTTGTCCTTGGTGGTG
Mouse calcineurin A	AGGAGGACCTGAAGATGGTG	TTGGTCCAGGTAGCGTTGTT
Mouse Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Human GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Protein extraction and Western blotting

Tissue samples and C2C12 cells were disrupted in RIPA lysis buffer (Beyotime, Cat. No. P0013B) supplemented with a protease inhibitor cocktail (Roche, Cat. No. 05892970001). Tissues were

homogenized on ice, while cells were lysed on ice for 30 min prior to centrifugation at $12,000 \times g$ for 15 min at 4°C. The supernatants were harvested, and protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. No. 23225). Proteins were separated via 10% or 12% SDS-PAGE gels and electrotransferred onto polyvinylidene fluoride membranes (Millipore, Cat. No. IPFL00010) using a wet transfer apparatus. The membranes were activated with methanol and then blocked with 5% bovine serum albumin for 1 h at room temperature before overnight incubation at 4°C with the following primary antibodies: anti-ANKRD1 (Santa Cruz Biotechnology, Cat. No. sc-365056; 1:1,000); anti-Atrogin-1 (Proteintech, Cat. No. 67172-1-Ig; 1:1,000); anti-MuRF1 (Proteintech, Cat. No. 55456-1-AP; 1:1,000); anti-MyoD (Santa Cruz Biotechnology, Cat. No. sc-32758; 1:1,000); anti-eIF3f (Invitrogen, Cat. No. A303-005A; 1:2,000); anti-calcineurin A (Cell Signaling Technology, Cat. No. 2614; 1:1,000); anti-polyubiquitin (Proteintech, Cat. No. 10201-2-AP; 1:1,000); anti-CAND1 (Abcam, Cat. No. ab101987; 1:2,000); anti-CUL1 (Cell Signaling Technology, Cat. No. 4995; 1:1,000); anti-FLAG (Proteintech, Cat. No. 66008-4-Ig; 1:5,000); anti-GAPDH (Proteintech, Cat. No. 60004-1-Ig; 1:5,000); anti- β -tubulin (Proteintech, Cat. No. 10094-1-AP; 1:5,000); and anti- β -actin (Proteintech, Cat. No. 66009-1-Ig; 1:20,000). The following morning, the membranes were washed three times with TBST (5 min each) and incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG (Proteintech, Cat. No. RGAR001; 1:10,000) or goat anti-mouse IgG (Proteintech, Cat. No. RGAM001; 1:10,000). Signal detection was performed using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Cat. No. 34580) and captured on a ChemiDoc MP system (Bio-Rad). The relative intensity of each band was normalized to the corresponding loading control (GAPDH, β -tubulin, or β -actin, as specified).

Co-immunoprecipitation and ubiquitination assays

Co-IP was performed using a proprietary commercial kit (Proteintech, Cat. No. PK10007). Cells were rinsed with ice-cold PBS and subsequently lysed; a portion of the lysate was kept as input, while the rest was subjected to incubation with the specific antibody for precipitation. Antibodies used for immunoprecipitation included anti-FLAG (Proteintech, Cat. No. 66008-4-Ig; 1 μ g/mg lysate), anti-ANKRD1 (Santa Cruz Biotechnology, Cat. No. sc-365056; 2 μ g/mg lysate), anti-CAND1 (Abcam, Cat. No. ab101987; 2 μ g/mg lysate), anti-CUL1 (Cell Signaling Technology, Cat. No. 4995; 1:50), and anti-Atrogin-1 (Proteintech, Cat. No. 67172-1-Ig; 1:50), alongside isotype-matched IgG (Proteintech, Cat. No. 30000-0-AP; 1 μ g/mg lysate) as a negative control. Immunoprecipitates underwent washing, elution, and subsequent evaluation via Western blotting.

Ubiquitination experiments evaluated the impact of ANKRD1 on Atrogin-1 substrate polyubiquitination. Prior to collection, cells were incubated for 6 hours using 10 μ M MG132 (MedChemExpress, Cat. No. HY-13259). The cell lysates were subsequently subjected to immunoprecipitation as previously outlined. Anti-MyoD (Santa Cruz Biotechnology, Cat. No. sc-32758; 2 μ g/mg lysate), anti-eIF3f (Invitrogen, Cat. No. A303-005A; 2 μ g/mg lysate), and anti-calcineurin A (Cell Signaling Technology, Cat. No. 2614; 1:50) were used to precipitate the respective targets, and their polyubiquitination levels were determined via Western blot analysis. Isotype-specific IgG was employed as a negative control.

Immunoprecipitation mass spectrometry

C2C12 cells were infected with LV-FLAG-ANKRD1 and cultured to approximately 1×10^8 cells. FLAG-tagged ANKRD1 was isolated through immunoprecipitation, and the resulting samples were purified. Mass spectrometry was carried out by Wuhan Igenebook Biotechnology Co., Ltd.

To proceed, bead-bound specimens were heated to 95°C for 10 minutes within a reaction mixture including 1% sodium deoxycholate, 100 mM Tris-HCl (pH 8.5), 10 mM tris(2-carboxyethyl)phosphine, and 40 mM chloroacetamide to unfold proteins and modify and alkylate cysteines. The supernatant was mixed with an equivalent volume of water, followed by the addition of trypsin at a 1:50 (w/w) ratio for digestion overnight at 37°C. This digestion was halted the subsequent day by acidifying the sample to pH 6.0 with trifluoroacetic acid. Upon centrifugation at $12,000 \times g$ for 15 minutes, peptides were refined via a styrene-divinylbenzene desalting column, and the fraction was vacuum-evaporated and kept at -20°C prior to analysis.

For liquid chromatography-tandem mass spectrometry, peptides were fractionated on a NanoElute ultrahigh-performance system operating at nanoliter flow. Mobile phase A consisted of 0.1% formic acid in water; mobile phase B comprised 0.1% formic acid in 100% acetonitrile. Samples were injected onto an analytical column (IonOpticks, Australia; 15 cm $\times$ 75 μ m, 1.6- μ m C18 particles) maintained at 50°C within an integrated column oven. Loading volume was 200 ng, flow rate 500 nL/min, and total duration was 20 min: 0–17 min, 5–25% B; 17–18 min, 25–40% B; 18–19 min, 40–95% B; and 19–20 min, 95% B.

Mass spectrometry datasets were analyzed using DIA-NN v1.8.1 (library-free mode) relative to the UniProtKB mouse proteome (uniprotkb_proteome_UP000000589_mouse_54910_20240528.fasta; 54,910 entries). DIA-NN generated the spectral library via deep learning while enabling match-between-runs, and this generated library was used to reprocess the identical dataset. Peptide precursor and protein recognitions were screened at 1% false discovery rate. Distinct peptides were used for protein determination and quantification, and refined data entered the following bioinformatic evaluations directly.

GST pull-down assay

The entire coding region of the mouse ANKRD1 was inserted into pGEX-4T-1 to generate pGEX-4T-1-GST-ANKRD1 for GST-ANKRD1 fusion protein expression; GST derived from the blank vector acted as a negative control. Full length mouse CAND1 was inserted into a His-tagged prokaryotic vector to produce the His-CAND1 expression construct. GeneChem Co., Ltd. synthesized and sequenced the plasmids for recombinant protein expression and purification. The purity of GST, GST-ANKRD1, and His-CAND1 was verified via SDS-PAGE stained with Coomassie Brilliant Blue, while tagged expression was validated by Western blot analysis.

For the pull-down step, equivalent quantities of GST or GST-ANKRD1 were mixed with Glutathione Sepharose 4B beads (Cytiva/GE Healthcare, Cat. No. 17-0756-01) at 4°C under gentle agitation to

capture the fusion constructs. The beads were washed extensively with chilled binding buffer to eliminate nonspecifically bound proteins, followed by incubation with equivalent volumes of His-CAND1 overnight at 4°C with rotation. The beads were gathered by centrifugation at 4°C and washed four times with chilled washing buffer to eliminate both unbound and nonspecifically bound His-CAND1. Captured proteins were eluted in 1× SDS loading buffer by heating at 95°C for 5 min, separated via SDS-PAGE, and examined by Western blotting.

Molecular docking

The docking used structural models of ANKRD1 (AlphaFold Protein Structure Database ID Q9CR42) and CAND1 (ID Q6ZQ38). For rigid-body docking, the GRAMM Docking Web Server was employed with CAND1 as the receptor and ANKRD1 as the ligand. The configuration exhibiting the minimal calculated binding energy was chosen as the best, and the protein–protein interaction surfaces were displayed using PyMOL.

Statistical analysis

Data processing used GraphPad Prism v8.3.0. Results are displayed as mean ± standard error of the mean (SEM). Two distinct groups were evaluated using the unpaired Student's t-test. Linked samples, such as bilateral paraspinal tissues from one subject or animal, were examined via the paired t-test. Three or more categories were assessed through one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For force–frequency and fatigue plots containing paired bilateral data, paired t-tests were performed at the specified frequencies or time intervals, as detailed within the figure legends. All analyses were two-sided, with $P < 0.05$ determining statistical significance. Sample size (n) and the specific test employed for every trial are provided in the relevant figure legends.

Use of large language models

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to enhance the linguistic flow and clarity of the document. Following its application, the authors reviewed and edited the material as necessary and take full responsibility for the content of this article.

Results

ANKRD1 is overexpressed within developed myofibers of concave side paraspinal muscles in individuals suffering from AIS.

To investigate the role of paraspinal muscles in AIS development, bilateral paraspinal muscle samples were obtained from age matched patients with AIS during corrective procedures. Myofiber cross-sectional area was notably diminished on the concave side compared to the convex side (Fig. 1B, D), while fat infiltration and fibrosis remained unchanged between sides (Fig. 1A, C). Transmission electron microscopy provided additional detailed evidence: concave side fibers exhibited widespread myofibrillar

degradation, sarcomeric disruption, and disoriented myofibrillar organization (Fig. 1E), suggesting significant myofiber impairment within the concave side paraspinal muscles.

Subsequently, we analyzed the cellular architecture and genetic characteristics of these bilateral muscles via single-nucleus RNA sequencing (snRNA-seq) to identify specific genes linked to pathological modifications. Unsupervised clustering identified 10 unique nuclear clusters, and representative expression profiles of conventional markers for every subtype are displayed in Fig. 2B. UMAP dimensionality reduction followed by cell-type labeling revealed that the primary clusters were generally similar between concave and convex sides, although their ratios varied somewhat (Fig. 2A, C). Focus subsequently shifted toward the mature myonuclei. Using fiber type specific indicators, mature myonuclei were categorized into type I slow twitch (MYH7), type IIa fast oxidative (MYH2), and type IIx fast glycolytic (MYH1) myonuclei [36]. These nuclei constitute the primary group involved in skeletal muscle energy metabolism, contractile modulation, and tissue reconstruction, thus genetic transitions within them directly reflect the pathological condition of the corresponding myofibers [37]. Proportion evaluation demonstrated a substantially higher prevalence of type IIx myonuclei in concave side muscles compared to convex side muscles, with comparatively smaller numbers of type I and type IIa myonuclei (Fig. S2A, B). GO enrichment analysis throughout the entire mature myonuclear region then highlighted distinct functional disparities between both sides. Among the mechanisms most prominently elevated on the concave side were those regulating ubiquitin mediated protein degradation (Fig. 2D). Subtype specific analysis confirmed this trend: type I, type IIa, and type IIx myonuclei from the concave side consistently exhibited marked enrichment of ubiquitination and protein degradation pathways (Fig. S2C). This consistency across fiber types suggests that accelerated ubiquitin dependent proteolysis is a common molecular hallmark of concave side mature myofibers in AIS. Differential expression assessment then highlighted ANKRD1, which was markedly upregulated in all mature myonuclear subtypes on the concave side (Fig. 2E, F; Fig. S2D, E). Other nuclear populations expressed ANKRD1 at minimal levels, with no concave–convex distinction (Fig. 2G, H), situating the dysregulated expression exclusively within mature myofibers.

Since myonuclei represent the primary nuclear type within paraspinal muscles, we verified these results via RT-qPCR on matched concave and convex samples. ANKRD1 mRNA was notably elevated in the concave side tissues of individuals with AIS (Fig. S1A), while immunoblotting demonstrated a substantial protein rise (Fig. S1F, G). No comparable asymmetrical expression emerged in paraspinal muscles from subjects with congenital scoliosis (CS) or from non-scoliotic controls (NS) (Fig. S1A).

To conclude, ANKRD1 levels are distinctly imbalanced within developed myonuclei of paraspinal muscles in AIS, where its increased concave side expression correlates with microscopic injury and muscle atrophy.

ANKRD1 represents a prospective pathogenic element which triggers paraspinal muscle wasting and spinal deformity.

To elucidate the role of ANKRD1 in muscle tissue, we initially overexpressed or silenced ANKRD1 within C2C12 myoblasts via lentiviral constructs, using a null-vector virus as a control; persistent clones were isolated by puromycin. Differentiation was triggered in a 2% horse serum medium. On days 4 and 8 throughout maturation, myotube width and fusion efficiency were measured using ImageJ. Overexpression of ANKRD1 notably decreased both myotube size and fusion efficiency at both periods (Fig. 3A, B). Conversely, shRNA mediated silencing of ANKRD1 increased both metrics markedly (Fig. S3A, B).

We subsequently inquired if the non-uniform activation of ANKRD1 within paraspinal tissues alters spinal alignment in living subjects. A bipedal rodent framework was developed in 3 week old male C57BL/6J mice. A recombinant AAV9 vector containing the ANKRD1 coding sequence driven by a CMV promoter including an EGFP marker was produced, and the experimental schedule is depicted in Fig. 3C. A microsyringe infused 5×10^{10} vg of AAV9-ANKRD1 into multiple locations in the paraspinal region on one side, whereas the identical volume of null-vector reference virus was injected into equivalent sites on the opposite side. Three weeks thereafter, in vivo fluorescence detection verified transgene delivery (Fig. S3C), and three-dimensional micro-CT scanning evaluated spinal geometry. Rodents with unilateral ANKRD1 augmentation demonstrated more severe misalignment in both coronal and sagittal views compared to animals receiving bilateral PBS controls (Fig. 3D–F). Following sacrifice, bilateral paraspinal tissues were collected. GFP-positive fibers constituted more than 90% of total fibers on the treated side, validating effective viral infection (Fig. S3D). Myofiber cross-sectional dimensions were reduced on the ANKRD1 expressing side compared to the contralateral side (Fig. 3G, H). Ex vivo contractile assays additionally demonstrated that ANKRD1 upregulation decreased tetanic strength, shifted the force–frequency graph rightward, and impaired fatigue endurance (Fig. 3I).

ANKRD1 triggers muscle fiber wasting through facilitating the Atrogin-1 assembly driven polyubiquitination and turnover of target proteins.

Given that ANKRD1 modifies myotube dimensions, we initially ruled out its impact on myogenic specification. RT-qPCR failed to identify any substantial shift in the mRNA expressions of the primary myogenic factors MYOD, MYOG, or MYH1 following ANKRD1 overexpression or silencing (Fig. S4A). ANKRD1 thus seems unnecessary for lineage specification, and its effect on myotube morphology probably functions via muscle protein equilibrium or proteolytic degradation. ANKRD1 has been suggested as a modulator of E3 ubiquitin ligases during renal ischemia–reperfusion damage [38], although its role in skeletal muscle remained unexplored. Our specific enrichment analysis confirmed this trend: ubiquitin–proteasome pathway activity was intensified in mature myonuclei extracted from the concave side paraspinal muscles of individuals with AIS. We consequently posited that ANKRD1 controls myofiber dimensions by regulating ubiquitin dependent protein turnover.

Differentiation day 7 derived LV-ANKRD1 stable cells were used to evaluate total protein levels of atrophy related E3 ligases and their specific substrates. Western blot analysis demonstrated that while ANKRD1 overexpression maintained the protein concentrations of muscle specific E3 ligases MuRF1 and Atrogin-

1 nearly constant, it notably diminished the levels of established Atrogin-1 targets MyoD, eIF3-f, and calcineurin A (Fig. 4A, B). Combining co-immunoprecipitation with ubiquitination assays revealed that ANKRD1 overexpression substantially enhanced the polyubiquitination state of these target proteins (Fig. 4C, D); conversely, ANKRD1 knockdown caused substrate accumulation (Fig. S4B, C) and decreased ubiquitination (Fig. 4C, D). In bilateral paravertebral samples from individuals with AIS, all three Atrogin-1 substrates were notably reduced on the concave side (Fig. S1B–E), and the general quantity of ubiquitinated proteins was significantly elevated (Fig. S1H, I).

If the ubiquitin–proteasome system mediates this atrophy, proteasome inhibition ought to correct it. C2C12 cells persistently overexpressing ANKRD1 were incubated with 1 μ M MG132 during the 6th day of differentiation and maintained for further 48 h. MG132 significantly reversed the myotube wasting triggered by ANKRD1 (Fig. 4E, F) and recovered the substrate proteins diminished by overexpression (Fig. 4G, H). ANKRD1 consequently triggers myotube wasting by enhancing ubiquitin–proteasome mediated degradation of Atrogin-1 targets.

ANKRD1 physically interacts with CAND1

To ascertain how ANKRD1 interacts with the ubiquitin–proteasome system, we started by examining its domain architecture. ANKRD1 possesses multiple functional motifs—a C-terminal regulatory region, an N-terminal signal peptide, a nuclear localization sequence, and ankyrin repeat modules—that enable it to perform various signaling functions [31]. FLAG-tagged ANKRD1 was transiently transduced into C2C12 cells, immunoprecipitated using an anti-FLAG antibody, and assessed via mass spectrometry (Co-IP–MS; Fig. 5A) to identify potential binding partners and delineate how ANKRD1 could regulate the ubiquitination and degradation of Atrogin-1 targets. Using a threshold of 2, we discovered 137 putative interacting molecules (Fig. 5B). Considering the established functional link between ANKRD1 and the ubiquitin–proteasome system, CAND1, which binds CUL1 and regulates the disassembly, reassembly, and substrate-receptor exchange of SCF complexes, capturing their assembly efficiency and dynamics [39–41], garnered our specific interest. Immunofluorescence demonstrated significant ANKRD1–CAND1 co-localization within both cytoplasmic and nuclear compartments (Fig. 5C). Co-immunoprecipitation subsequently validated this association: endogenous CAND1 was consistently identified in FLAG immunoprecipitates derived from FLAG–ANKRD1 transfected cells yet absent in IgG controls (Fig. S5A), while reciprocal Co-IP isolated endogenous ANKRD1 from FLAG–CAND1 cells (Fig. S5B). Direct binding was evaluated with purified proteins: GST-ANKRD1 pulled down His-CAND1, whereas GST alone yielded no signal (Fig. 5D), confirming this interaction at the molecular level in vitro.

The structural basis underlying this interaction was subsequently investigated. Protein architectures of ANKRD1, CUL1, and CAND1 were forecasted via the AlphaFold platform; the GRAMM docking software executed rigid-body docking, PDBePISA pinpointed prospective interface residues, and PyMOL generated the visual models (Fig. 5E; Fig. S5C). Docking suggested a robust ANKRD1–CAND1 complex stabilized primarily by hydrogen bonds. This anticipated network links CAND1 residues Asp19, Asp21, Ser3, Lys14, Asp104, Gly115, Thr112, Ser121, Arg188, Lys193, Gln236, Tyr274, Glu1188, Glu1190, Gln1216, and

Lys1217 with ANKRD1 residues Lys97, Lys100, Thr102, Val106, Ser145, Cys149, Asp150, Lys153, Arg195, Lys217, Thr229, Tyr265, Asn282, Cys283, and His294, collectively forming a stable binding interface. On the ANKRD1 side, these critical residues reside primarily within the C-terminal ANK repeat segment—tandem ankyrin motifs folded into a distinct solenoid-like protein binding surface that provides significant interaction potential [42]. On the CAND1 side, interface residues are distributed across the N- and C-terminal segments, which are rich in HEAT-like repeats presenting numerous exposed α -helical surfaces that function as binding platforms [43]. The predicted CUL1 binding interface, conversely, occupies the central section of CAND1 (amino acids 409–931) and demonstrates minimal overlap with the anticipated ANKRD1 binding regions. ANKRD1 thus seems to interact with the terminal HEAT-like structures of CAND1 via its ANK repeat domain and to influence the stability of the CAND1–CUL1 complex rather than compete with CUL1 for the identical site.

To evaluate these hypotheses empirically, domain mapping was performed. A collection of ANKRD1 deletion variants (Fig. 5F) were evaluated for intrinsic CAND1 association through Co-IP (Fig. 5G). Sole removal of the ANK repeat segment (Δ 190–319) eliminated the link; the other variants exhibited binding similar to full-length ANKRD1. This interaction relies primarily upon the C-terminal ANK repeat region of ANKRD1. We subsequently developed CAND1 deletion and truncation proteins (Fig. 5F) to delineate the reverse surface. The detached C-terminal arch (651–1230) could not bind ANKRD1, whereas the N-terminal arch deletion variant (Δ 1–400) plus the detached N-terminal arch (1–400) showed only faint binding, and the Δ 401–650 and Δ 651–1230 mutants retained measurable interactions (Fig. 5G). ANKRD1 thus fails to identify a unique linear portion of CAND1; its interaction is better described by a combined, conformational interface supported by the complete three-dimensional architecture of CAND1. The N-terminal arch participates in identification but fails to maintain robust binding independently, and the isolated C-terminal arch is similarly inadequate—stable engagement demands a conformational interface preserved collectively by the terminal parts of CAND1.

Docking simulations and Co-IP results support a single framework: ANKRD1 binds CAND1 mainly via its C-terminal ANK repeat motif, whereas the CAND1 interface relies on a stable structure collectively stabilized by its N- and C-terminal HEAT-like elements. Since CUL1 resides within the central portion of CAND1, ANKRD1 probably manages the longevity of the CAND1–CUL1 association by modifying CAND1's structural configuration rather than by displacing CUL1 from its binding position.

ANKRD1 augments Atrogin-1 related E3 ubiquitin protein ligase triggered target ubiquitylation and proteolysis via its binding to CAND1.

Atrogin-1, a muscle specific F-box protein, forms an SCF type E3 ubiquitin ligase assembly with SKP1, CUL1, and RBX1. The ubiquitination potential of this structure relies on the levels of its primary constituents as well as its assembly effectiveness and structural configuration [44]. Since we demonstrated that ANKRD1 interacts with CAND1, we suggested that ANKRD1 modulates the Atrogin-1 complex assembly via CAND1 and consequently controls substrate ubiquitination and turnover. Co-immunoprecipitation using CUL1 and Atrogin-1 antibodies examined this hypothesis. ANKRD1

overexpressing C2C12 cells produced greater endogenous Atrogin-1 co-precipitating with CUL1 compared to negative control cells, and ANKRD1 silencing yielded the reverse outcome (Fig. 6A, B). ANKRD1 therefore facilitates the binding of Atrogin-1 to CUL1 and enhances the assembly of the Atrogin-1 related E3 ligase complex.

CAND1 reliance was evaluated through knockdown-rescue experiments. siRNAs targeting CAND1 were introduced into LV-ANKRD1 transgenic cells, and successful depletion was verified via immunoblotting. Loss of CAND1 significantly inhibited the ANKRD1 mediated rise in Atrogin-1 complex assembly (Fig. 6C, D) and notably restored the diminished ubiquitination and increased levels of Atrogin-1 substrates (Fig. 6E–G). ANKRD1 consequently enhances the formation and biological potency of the Atrogin-1 related E3 ubiquitin ligase complex in a CAND1 reliant manner.

Bortezomib attenuates ANKRD1 induced skeletal muscle atrophy and slows scoliosis progression

Considering the critical role of ANKRD1 in concave side paraspinal muscle wasting within AIS, we identified pharmacological compounds capable of suppressing muscle atrophy and mitigating disease advancement. From an FDA licensed compound collection, we selected bortezomib, a reversible 26S proteasome inhibitor used for treating multiple myeloma and mantle cell lymphoma [45, 46]. Bortezomib transiently binds to the chymotrypsin-like active site of the 26S proteasome $\beta 5$ subunit, thereby preventing the breakdown of ubiquitinated targets [47, 48]. Various preclinical investigations suggest that it improves pathologic muscle alterations in models of skeletal muscle atrophy or deterioration [49–51].

Experiments were performed to evaluate in vitro efficacy initially. C2C12 myoblasts were exposed to increasing concentrations of bortezomib (0, 1, 5, 10, 20, 50, 100, and 150 nM), and cellular viability and toxicity were monitored using CCK-8 at 24, 48, and 72 h (Fig. S6A, B). Toxicity became apparent above 20 nM, aligning with previous findings [49–51], thus 10 nM was used for further trials. On day 6 of differentiation, C2C12 cells stably expressing ANKRD1 were incubated with 10 nM bortezomib or an equivalent amount of dimethyl sulfoxide (DMSO) for 48 h. Bortezomib significantly counteracted the myotube wasting triggered by ANKRD1 (Fig. 7A, B). Simultaneously, ubiquitination of Atrogin-1 targets MyoD, eIF3f, and calcineurin A decreased while their protein abundances increased (Fig. S6C–E), suggesting that proteasome suppression halted the excessive proteolytic degradation driven by ANKRD1.

The in vivo effectiveness was subsequently evaluated. Vertebral deformity was generated by administering rAAV9-ANKRD1 into the ipsilateral paraspinal tissues, following the aforementioned protocol. Animals in the therapy group ($n = 6$) were given bortezomib injections into the affected paraspinal sites at 0.25 mg/kg every 3.5 days for 2 weeks, receiving an equivalent DMSO volume in the opposite side; the sham group ($n = 6$) received bilateral DMSO following the identical timeline. Subjects were sacrificed 2 weeks post last injection, and bilateral paraspinal samples were harvested for

assessment (Fig. 7C). Myofiber cross-sectional dimensions on the concave region were notably greater in bortezomib administered mice compared to the sham group (Fig. 7F, G). Ex vivo tension testing demonstrated that bortezomib restored the tetanic power, the rightward slope of the force–frequency plot, and the diminished fatigue tolerance caused by ANKRD1 overexpression, confirming alleviated muscular impairment (Fig. 7H). Crucially, three-dimensional micro-CT modeling showed remarkably enhanced spinal posture following bortezomib therapy, with substantial decreases in both coronal and sagittal malalignment (Fig. 7D, E). Bortezomib thus hinders the advancement of spinal curvature and emerges as a potential therapeutic intervention for AIS.

Discussion

The etiology of AIS is still poorly defined, and focus has progressively shifted toward paraspinal muscles as participants in curve onset and development [13, 15, 52]. Clinical investigations highlight muscular atrophy as a significant pathological hallmark of concave side paraspinal muscles in AIS [18, 19], however whether such transition simply follows a solid deformity or actively propels disease remains largely uncertain. This research establishes a clear connection between asymmetrical paraspinal muscle atrophy and AIS evolution: skeletal muscle wasting caused by abnormal ANKRD1 activation within mature myofibers on one side of paraspinal muscles was adequate to foster scoliosis emergence. Precisely, we showed that (1) ANKRD1 was specifically upregulated in mature myofibers of concave side paraspinal muscles in patients with AIS, whereas no equivalent shift occurred in subjects with CS or NS; (2) ANKRD1 directly engaged with CAND1, facilitated assembly of the Atrogin-1 related complex, and boosted the ubiquitination and turnover of essential substrate proteins, thus triggering skeletal muscle wasting; and (3) solitary ANKRD1 overexpression in the paraspinal muscles was enough to trigger a scoliosis pattern in a bipedal mouse model, whereas the proteasome inhibitor bortezomib partially mitigated these pathological shifts and delayed scoliosis advancement.

The ANKRD1 locus is located on human chromosome 10q23.31, showing significant conservation across mammalian species and producing a 319 amino acid protein [53]. Initially characterized within cardiac tissue, this gene reacts to mechanical tension [54], oxidative pressure [38], inflammatory signals, and cellular damage [55]. Within normal muscle tissue, ANKRD1 is primarily distributed in the I band of sarcomeres, interacting with structural proteins like titin; during mechanical activation, it detaches from sarcomeres and migrates into the nucleus to modulate stress-sensitive gene expression [56, 57]. ANKRD1 has traditionally functioned as a critical link between external mechanical conditions and internal signaling pathways. Its functions in cardiovascular illnesses are extensively documented [58], yet its specific impact on skeletal muscle pathologies—along with any potential connection to AIS—remained unexplored.

A primary discovery in this study pertains to the pathologic specificity of ANKRD1 modulation. Our snRNA-seq and computational evaluations demonstrated significantly heightened ANKRD1 levels within concave paraspinal tissues of AIS subjects, whereas no analogous shift occurred in CS patients. Unilateral ANKRD1 upregulation induced scoliotic curves in bipedal mice, and suppressing downstream

proteasomal degradation delayed deformity advancement. Consequently, abnormal ANKRD1 induction engages in a unique AIS pathology rather than functioning as a nonspecific cellular stress reaction triggered by malformation. This aberrant activation of ANKRD1 exhibited marked cell-type specificity. Single-nucleus transcriptomic analysis showed that differential ANKRD1 expression was almost exclusively confined to mature myonuclear populations, with no significant changes detected in satellite cells, fibroblasts, or immune cells. This finding suggests that ANKRD1 activation is unlikely to result from a systemic inflammatory response or tissue-repair process. Likewise, ANKRD1 associated concave side paraspinal muscle atrophy does not appear to arise primarily from impaired regenerative capacity, but is more likely attributable to proteostatic disruption caused by enhanced protein degradation within mature myofibers. This interpretation is consistent with the established function of ANKRD1 as a mechanosensitive protein. Based on these observations, we propose that ANKRD1 acts as a pathological amplifier during AIS progression by converting physiological mechanical stimuli into sustained asymmetric protein degradation signals, thereby driving asymmetric paraspinal muscle atrophy and the progression of spinal deformity. Under physiological conditions, mechanical stimuli may induce only transient ANKRD1 activation and promote adaptive tissue remodeling. However, in individuals predisposed to AIS, genetic background, epigenetic regulation, or local microenvironmental abnormalities may alter the response threshold of ANKRD1 to mechanical stress, resulting in persistent and excessive asymmetric activation.

Skeletal muscle wasting essentially manifests as a disruption of the equilibrium between protein synthesis and catabolism [20], and the UPS serves as a major pathway for the degradation of muscle proteins [21]. Atrogin-1, a muscle specific E3 ubiquitin ligase, plays a pivotal role in muscular atrophy during denervation [59], disuse [60], infection [61], and cachexia [62]. Prior investigations focused on the transcriptional regulation of Atrogin-1 by FoxO factors [63]. Our observations introduce a post-translational dimension: ANKRD1 maintained Atrogin-1 expression and transcriptional potential constant while boosting functional efficacy by facilitating the formation of the Atrogin-1 linked E3 ligase assembly. CAND1, a well characterized modulator of the cullin–RING E3 ligase cycle, is progressively acknowledged as an exchange factor that maintains SCF complexes cycling; by assisting the turnover of F-box proteins, it consistently reconfigures the SCF network to align with fluctuating substrate landscapes [39–41]. In this current research, ANKRD1 interacted with CAND1 directly and improved Atrogin-1 complex construction in a CAND1 reliant fashion, classifying ANKRD1 as a previously unidentified auxiliary modulator of the SCF cycle with a function in skeletal muscle proteostasis regulation.

Our specific domain evaluations elucidated the manner in which ANKRD1 potentially manages CAND1 activity. The fundamental architectural characteristic of ANKRD1 involves its consecutive ankyrin repeat sequences located in the C-terminal area; recognized associations with cytoskeletal elements [42], transcriptional controllers [30], and ubiquitination linked proteins [38] all rely on this region, although its role in proteostasis modulation during pathological muscular states was previously unconfirmed. We discovered that the interaction between ANKRD1 and CAND1 occurs primarily via the ankyrin repeat section of ANKRD1, whereas the complementary surface on CAND1 involves its N-terminal arch

containing zone. This arch represents a central component of the CAND1 superhelix and assists in cullin identification; architectural analyses demonstrate that the N- and C-terminal portions form an arched configuration which encircles CUL1 to produce a robust structure regulating the SCF cycle [43]. Our findings suggest that the N-terminal arch of CAND1 contributes to ANKRD1 identification but cannot ensure steady attachment solely, suggesting that ANKRD1 is not simply a passive interaction partner but an energetic regulator of CAND1 structure. Since the anticipated ANKRD1 binding site does not completely match the primary CUL1 binding area, ANKRD1 probably affects the longevity of the CAND1–CUL1 assembly by reshaping CAND1 structure rather than by competing for the CUL1 position. Within this framework, ANKRD1 interacts with the N-terminal arch containing structural interface of CAND1 through its ankyrin repeat region, promotes CAND1 driven SCF turnover and F-box protein replacement, and consequently enhances the rejuvenation efficacy and substrate detection potential of the Atrogin-1 linked complex. Establishing accurately how ANKRD1 binding modifies the morphology and switching behavior of CAND1 necessitates additional structural and biochemical investigation.

AIS has traditionally been viewed as a condition characterized by significant biomechanical traits. The Hueter–Volkmann principle suggests that uneven mechanical pressure produces growth-rate variances between spinal segments and drives deformity advancement [16, 17]. How bilateral asymmetrical modifications in paraspinal muscles contribute to this cycle at a molecular scale has stayed largely unexplored. Our research identifies ANKRD1 as a pivotal modulator connecting local stress reactions within paraspinal tissue to asymmetric wasting. We suggest the subsequent pathway: in AIS vulnerable subjects, bilateral paraspinal muscles vary in their vulnerability to stress; throughout the swift maturation of puberty and under enduring alterations in the regional microenvironment, ANKRD1 gets continuously and abnormally triggered in mature myofibers on one spinal half. Via its ankyrin repeat motif, ANKRD1 interacts with the N-terminal area of CAND1, bolsters the function of the Atrogin-1 linked ensemble, and initiates the breakdown of vital muscle components. The ensuing asymmetric wasting intensifies the biomechanical disparity between both sides of the spine and finally maintains curve evolution. These discoveries highlight the significance of muscular tissue in AIS pathology and offer a molecular foundation for subsequent etiology-focused therapeutic approaches.

Existing approaches for treating AIS primarily depend on bracing and surgical interventions [1]. This identified molecular mechanism might provide a more accurate and practical therapeutic plan. Bortezomib, an FDA licensed medication, significantly improved ANKRD1 induced muscular wasting and postponed spinal curvature advancement. Extended usage of proteasome inhibitors is limited by general toxicity [64], however, our findings indicate the proteostasis network as a viable medicinal goal within AIS. Particular blockers of the ANKRD1–CAND1 binding site might eventually provide more focused therapy while bypassing the wide ranging impacts of whole body proteasome suppression.

Although the ANKRD1–CAND1–Atrogin-1 axis has been recognized as vital for AIS, numerous uncertainties persist. Primarily, the specific molecular mechanisms underlying the increased ANKRD1 vulnerability in AIS remain unelucidated; it remains to be determined if genetic polymorphisms, epigenetic modifications, or adolescent specific developmental triggers are the primary contributors.

Furthermore, it is currently undetermined if ANKRD1 interacts with additional atrophy related pathways, such as the autophagy–lysosomal network or mitochondrial homeostasis. Moreover, this research focused on mature myofibers; the potential involvement of alternative cell types in ANKRD1 induced pathological remodeling via paracrine mechanisms also demands further exploration.

Conclusions

To summarize, this research discovers, for the initial time, a pathologic function for ANKRD1 within AIS and suggests that ANKRD1 functions as a potent enhancer of dysregulated stress responsive signaling in the condition. By using its ankyrin repeat motif, ANKRD1 interacts with the structural interface comprising the N-terminal arch of CAND1, strengthening the formation and proteolytic capacity of the Atrogin-1 related complex. Consequently, the ensuing compromise of proteostasis in the paraspinal tissues causes muscular atrophy and facilitates the emergence and advancement of spinal deformity. Such discoveries provide a novel conceptual architecture for interpreting AIS pathogenesis and a basis for precise medical approaches addressing pathological modifications in the paraspinal muscles.

Abbreviations

AIS: Adolescent idiopathic scoliosis ANKRD1: Ankyrin repeat domain 1 CAND1: Cullin-associated and neddylation-dissociated 1 CUL1: Cullin 1 SCF: Skp1-Cullin-F-box UPS: Ubiquitin-proteasome system Atrogin-1: F-box protein 32 (FBXO32) MuRF1: Muscle RING finger 1 CSA: Cross-sectional area snRNA-seq: Single-nucleus RNA sequencing UMAP: Uniform manifold approximation and projection AAV: Adeno-associated virus CMV: Cytomegalovirus EGFP: Enhanced green fluorescent protein GAPDH: Glyceraldehyde-3-phosphate dehydrogenase DAPI: 4',6-Diamidino-2-phenylindole DMSO: Dimethyl sulfoxide OCT: Optimal cutting temperature PBS: Phosphate-buffered saline RIPA: Radioimmunoprecipitation assay SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis PVDF: Polyvinylidene fluoride HRP: Horseradish peroxidase Co-IP: Co-immunoprecipitation GST: Glutathione S-transferase RT-qPCR: Reverse-transcription quantitative polymerase chain reaction cDNA: Complementary DNA mRNA: Messenger RNA MYH: Myosin heavy chain MyHC: Myosin heavy chain GO: Gene Ontology DEGs: Differentially expressed genes PCA: Principal component analysis SEM: Standard error of the mean ANOVA: Analysis of variance FDA: Food and Drug Administration NCBI: National Center for Biotechnology Information GEO: Gene Expression Omnibus SRS: Scoliosis Research Society CS: Congenital scoliosis NS: Non-scoliotic TEM: Transmission electron microscopy micro-CT: Micro-computed tomography UMI: Unique molecular identifier FAPs: Fibro-adipogenic progenitors ECs: Endothelial cells SMCs: Smooth muscle cells MuSCs: Muscle stem cells eIF3f: Eukaryotic translation initiation factor 3 subunit f CaN: Calcineurin A

Declarations

Ethics approval and consent to participate

The research involving human subjects was authorized by the Ethics Committee of Affiliated Rehabilitation Hospital of Nanchang University (approval No. SFYLL-KY-PJ-2026-040) and carried out following pertinent institutional protocols and moral standards. Formal written informed consent was acquired from each individual participant and their authorized guardian prior to specimen procurement. The C2C12 cell line used within this project was commercially sourced and managed under institutional biosafety protocols. All animal protocols were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Nanchang University (approval No. CDYFY-IACUC-202607GR011) and executed according to institutional guidelines for the welfare and utilization of laboratory animals.

Consent for publication

Each co-author has reviewed and endorsed the ultimate version of this paper and agrees to its release.

Availability of data and materials

The single-nucleus RNA sequencing datasets derived from this research have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) with accession codes GSM9966091, GSM9966092, GSM9966093, GSM9966094, GSM9966095, and GSM9966096. The Co-IP mass spectrometry information and additional data related to the results are accessible within this paper and its Supplementary Information or from the primary author following a reasonable inquiry.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XY and KC designed the study and conceived the experiments. XY, YL, FM, QW, KC, GW, and JX performed the experiments and data collection. XY, YL, FM, QW, and KC analyzed and interpreted the data. ZZ and JZ supervised the project and provided critical revision of the manuscript. KC conceived the

project, acquired funding, and supervised the study. XY drafted the manuscript. All authors read and approved the final manuscript.

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Figures

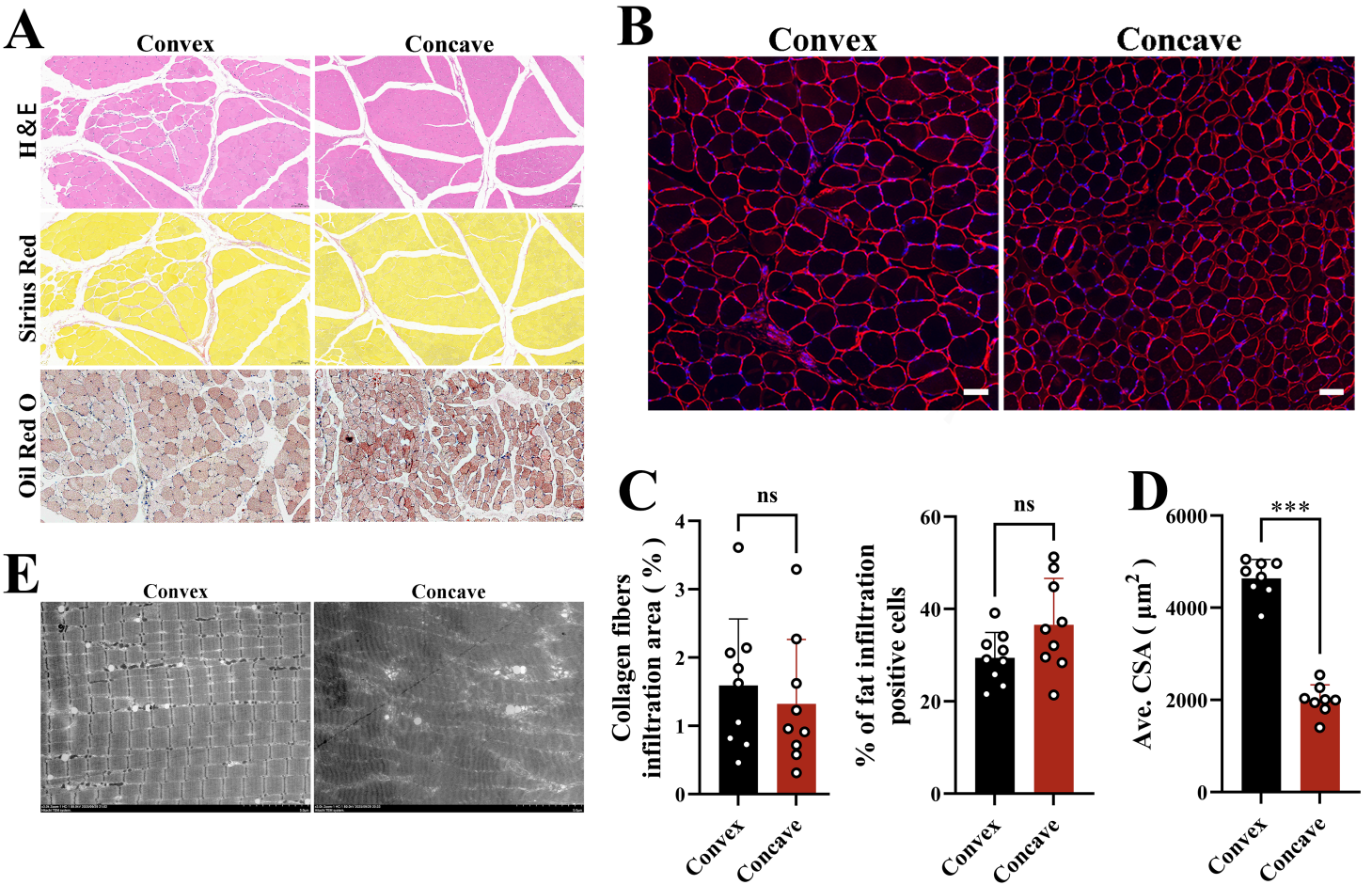


Figure 1

Significant asymmetric alterations are present in the bilateral paraspinal muscles of patients with adolescent idiopathic scoliosis (AIS). (A) Representative histological images of paraspinal muscles from patients with AIS. Frozen sections were prepared from bilateral paraspinal muscle samples, and histochemical staining included hematoxylin and eosin (H&E), Sirius Red, and Oil Red O. Scale bar: 100 μ m. (B) Representative dystrophin immunofluorescence staining of bilateral paraspinal muscle tissues. Dystrophin (red) delineated myofiber boundaries, and DAPI (blue) labeled nuclei. Scale bar: 100 μ m. (C)

Quantification of the Sirius Red-positive area and the proportion of Oil Red O-positive cells in (A), comparing fibrosis and fatty infiltration between concave and convex paraspinal muscles, n = 8. Paired Student's t-test, ns = not significant. (D) Myofiber cross-sectional area (CSA) measured from the dystrophin staining in (B), comparing myofiber size between bilateral paraspinal muscles, n = 8. Paired Student's t-test, ***P < 0.001. (E) Transmission electron microscopy (TEM) of the ultrastructure of bilateral paraspinal muscles. Disorganized myofibrillar arrangement, disrupted sarcomeric structures, and myofiber dissolution were observed in concave paraspinal muscles, whereas convex side myofiber structure remained relatively intact. Scale bar: 5 μ m.

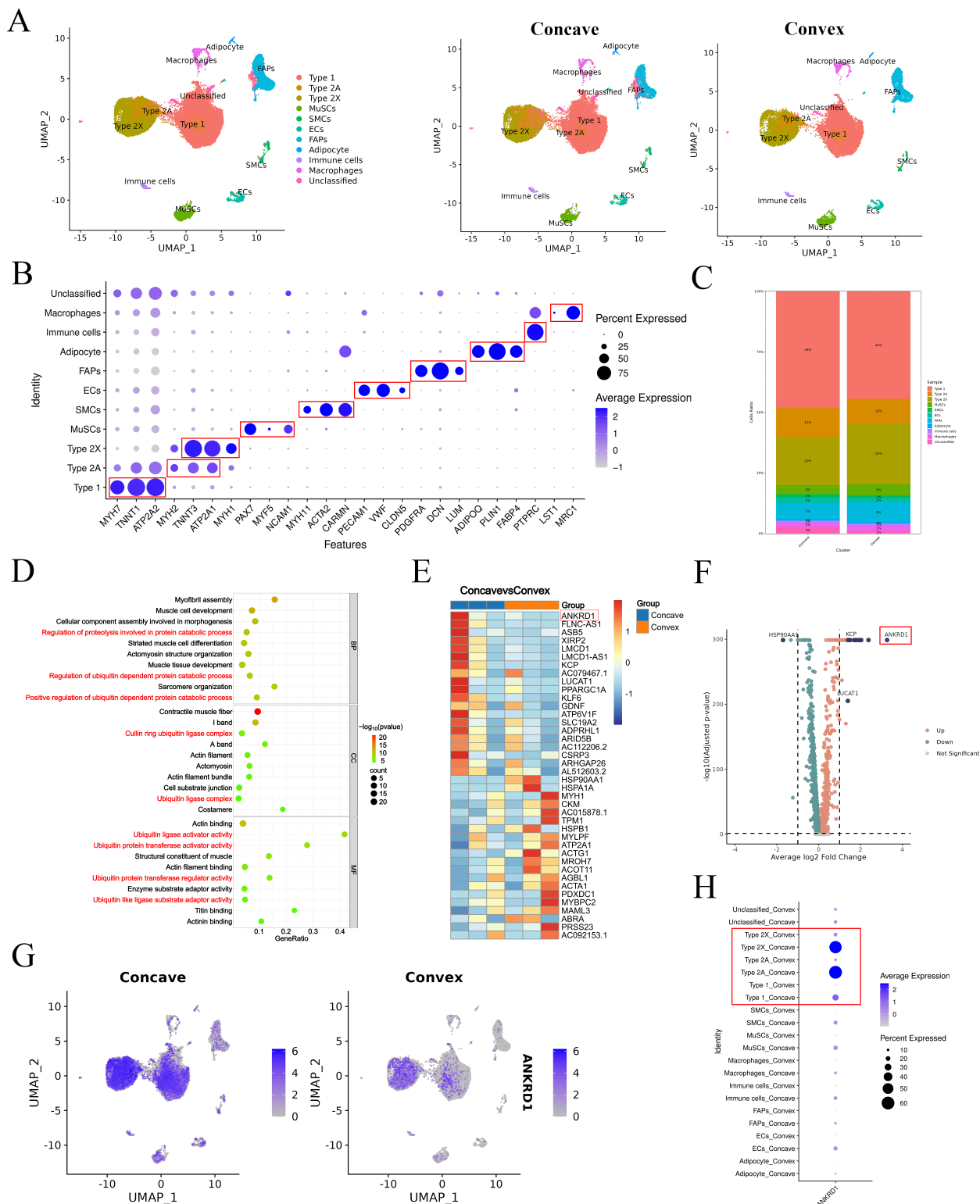


Figure 2

Asymmetric activation of ANKRD1 in mature myonuclei of the paraspinal muscles. (A) UMAP plot showing the major nuclear populations in concave and convex side paraspinal muscles of patients with AIS. FAPs, fibro-adipogenic progenitors; ECs, endothelial cells; SMCs, smooth muscle cells; MuSCs, muscle stem cells. (B) Dot plot of representative marker gene expression across the major nuclear populations. (C) Relative proportions of each nuclear population in concave and convex side paraspinal

muscles. (D) Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in mature myonuclei between the concave and convex sides. (E) Hierarchical clustering heatmap of DEGs in mature myonuclei between sides, with ANKRD1 highlighted by a red box. (F) Volcano plot of DEGs in mature myonuclei between sides, with ANKRD1 highlighted by a red box. (G) UMAP feature plot showing ANKRD1 expression across nuclear populations in the paraspinal muscles. (H) Dot plot of ANKRD1 expression across nuclear populations.

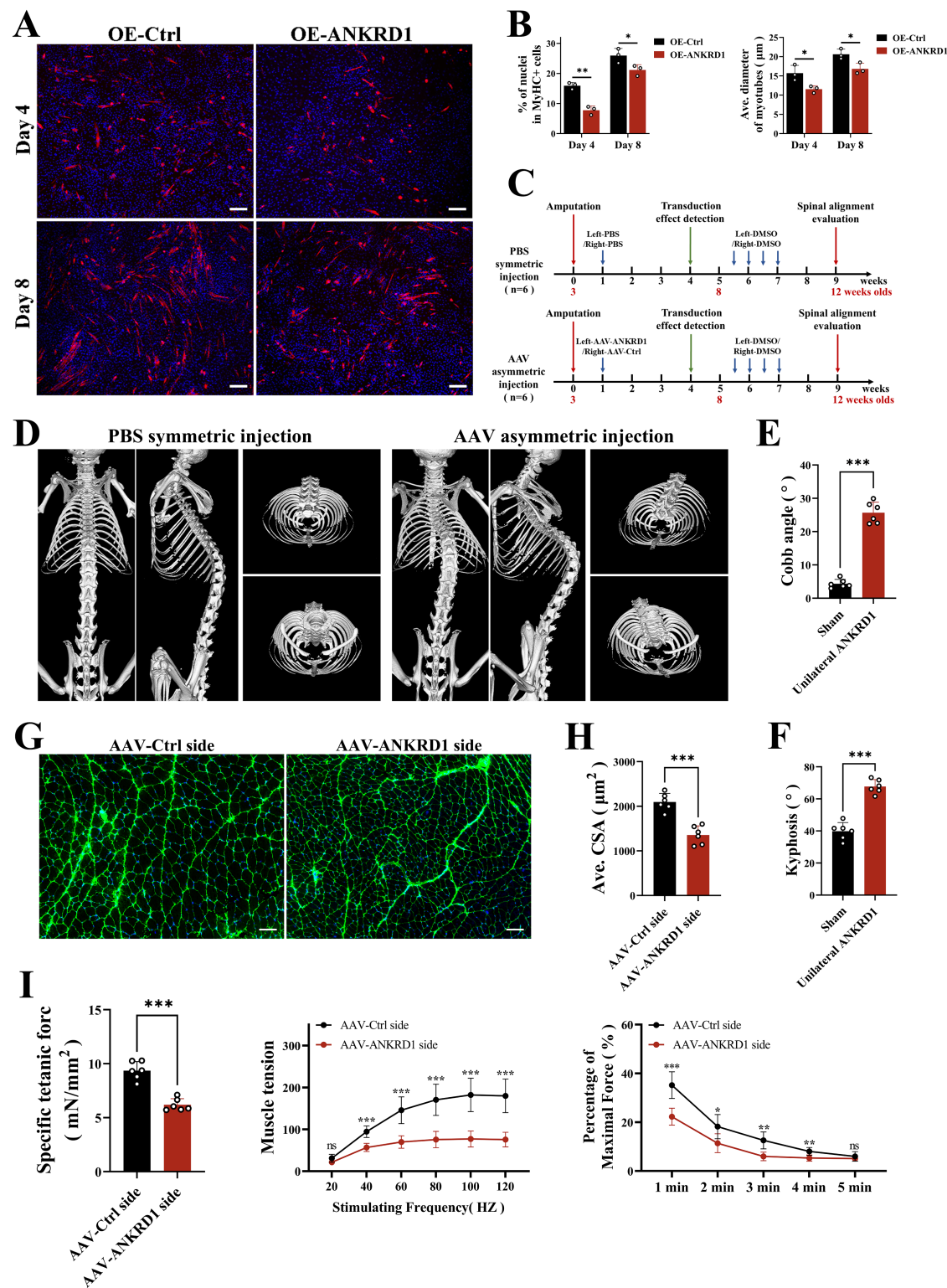


Figure 3

ANKRD1 induces skeletal muscle atrophy, and its asymmetric expression in bilateral paraspinal muscles drives scoliosis formation in bipedal mice. (A) C2C12 cells stably overexpressing ANKRD1 were subjected to MyHC immunofluorescence on days 4 and 8 of differentiation. MyHC (red) labeled mature myotubes, and DAPI (blue) labeled nuclei. Scale bar: 100 μ m. (B) Quantification of mean myotube diameter and fusion index from the MyHC staining in (A), $n = 3$. Unpaired Student's t-test, $*P < 0.05$, $**P < 0.01$. (C) Schematic of the experimental procedure in bipedal mice. 4 week old bipedal mice received AAV-ANKRD1 injection into the left paraspinal muscle and AAV-Ctrl into the corresponding site of the right paraspinal muscle to establish asymmetric ANKRD1 expression in bilateral paraspinal muscles. Spinal alignment and paraspinal muscle structure and function were evaluated 8 weeks after adeno-associated virus injection. (D) Representative micro-CT three-dimensional reconstructed images of the bilateral symmetric PBS injection group and the asymmetric AAV-ANKRD1/AAV-Ctrl injection group. The AAV-ANKRD1/AAV-Ctrl group displayed evident scoliosis and thoracic asymmetry. (E) Coronal Cobb angle measured from the micro-CT images in (D), comparing the bilateral symmetric PBS group with the asymmetric AAV-ANKRD1/AAV-Ctrl group, $n = 6$. Unpaired Student's t-test, $***P < 0.001$. (F) Sagittal spinal kyphosis angle measured from the micro-CT images in (D), compared between the two groups, $n = 6$. Unpaired Student's t-test, $***P < 0.001$. (G) Representative dystrophin immunofluorescence staining of bilateral paraspinal muscles in the asymmetric AAV-ANKRD1/AAV-Ctrl injection group. Dystrophin (green) delineated myofiber boundaries, and DAPI (blue) labeled nuclei. Scale bar: 100 μ m. (H) CSA measured from the dystrophin staining in (G), comparing myofiber size between bilateral paraspinal muscles, $n = 6$. Paired Student's t-test, $***P < 0.001$. (I) Ex vivo contractile function analysis of bilateral paraspinal muscles in the asymmetric AAV-ANKRD1/AAV-Ctrl injection group. Maximal tetanic force of bilateral paraspinal muscles was measured and compared, $n = 6$. Paired Student's t-test, $***P < 0.001$. Contractile tension at different stimulation frequencies (20, 40, 60, 80, 100, and 120 Hz) was measured to generate force–frequency curves, $n = 6$. Paired Student's t-test, ns = not significant, $***P < 0.001$. The decline in contractile force during continuous stimulation over 1–5 min was measured to generate fatigue curves and evaluate contractile function and fatigue resistance of bilateral paraspinal muscles, $n = 6$. Paired Student's t-test, ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

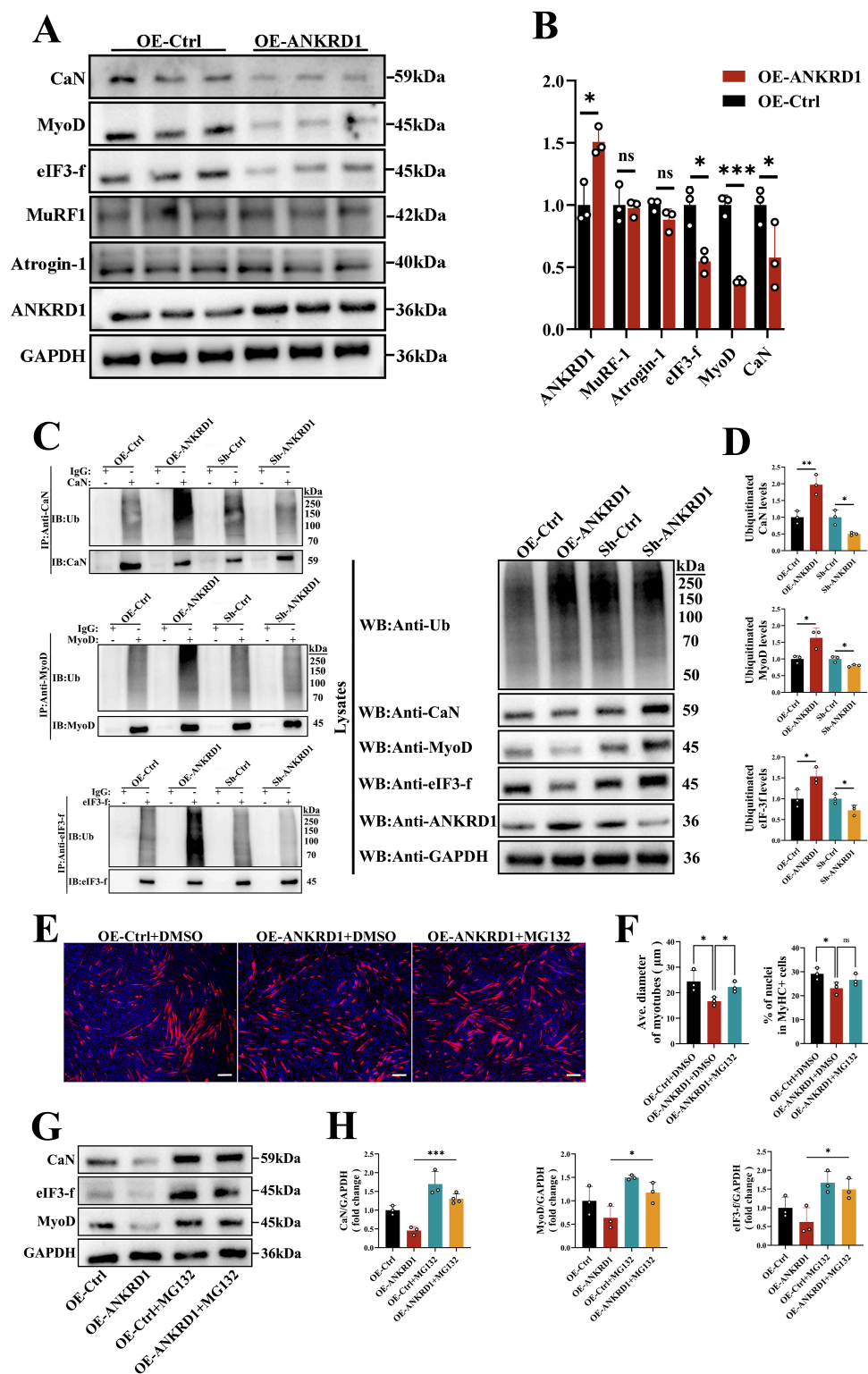


Figure 4

ANKRD1 promotes ubiquitination and degradation of Atrogin-1 substrate proteins through the ubiquitin-proteasome pathway and induces myotube atrophy. (A) C2C12 cells stably overexpressing ANKRD1 were established by lentiviral transduction. Representative Western blots showing ANKRD1, Atrogin-1, MuRF1, eIF3-f, MyoD, and CaN protein levels in OE-Ctrl and OE-ANKRD1 cells. (B) Densitometric quantification of the blots in (A). In each independent experiment, densitometric values of three

technical replicates per group were averaged before between group statistical analysis, $n = 3$. Unpaired Student's t-test, ns = not significant, $*P < 0.05$, $***P < 0.001$. (C) Detection of endogenous ubiquitination of CaN, MyoD, and eIF3-f after ANKRD1 overexpression. OE-Ctrl and OE-ANKRD1 C2C12 lysates were immunoprecipitated with IgG or antibodies against CaN, MyoD, or eIF3-f, followed by detection of ubiquitination with a ubiquitin antibody. Immunoprecipitated proteins were detected with antibodies against CaN, MyoD, and eIF3-f. Expression of CaN, MyoD, eIF3-f, and ubiquitin in whole cell lysates was also examined. (D) Quantification of relative ubiquitination levels of CaN, MyoD, and eIF3-f in (C), $n = 3$. Unpaired Student's t-test, $*P < 0.05$, $**P < 0.01$. (E) After stable ANKRD1 overexpression in C2C12 cells, 1 μ M MG132 was added on differentiation day 6, and MyHC immunofluorescence was performed on day 8. MyHC (red) labeled mature myotubes, and DAPI (blue) labeled nuclei. Scale bar: 100 μ m. (F) Quantification of mean myotube diameter and fusion index from the MyHC staining in (E), $n = 3$. One-way ANOVA followed by Tukey's post hoc test, ns = not significant, $*P < 0.05$. (G) Western blot analysis of lysates from each group on differentiation day 8 in (E), detecting CaN, MyoD, and eIF3-f. (H) Densitometric quantification of CaN, MyoD, and eIF3-f protein levels in (G), $n = 3$. One-way ANOVA followed by Tukey's post hoc test, $*P < 0.05$, $***P < 0.001$.

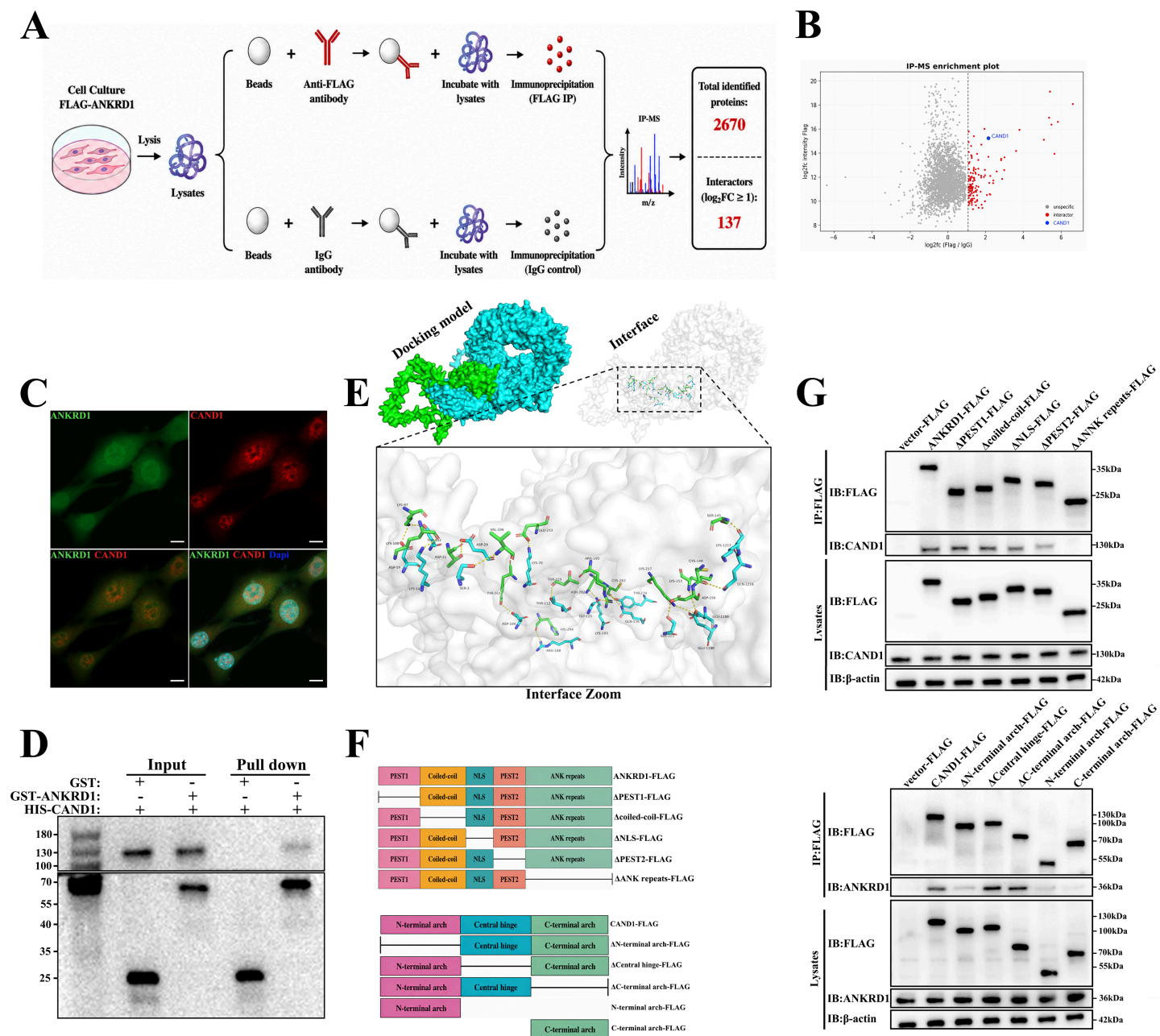


Figure 5

ANKRD1 directly interacts with CAND1, and binding is mediated by specific domains. (A) Schematic of the ANKRD1 immunoprecipitation mass spectrometry (IP-MS) procedure. (B) Volcano plot of the IP-MS results identifying CAND1 among candidate ANKRD1 interacting proteins. (C) Subcellular localization and colocalization of ANKRD1 and CAND1 in C2C12 cells. ANKRD1 is shown in green, CAND1 in red, and DAPI labeled nuclei in blue. Merged images show evident colocalization in cytoplasmic and nuclear regions. Scale bar: 20 μ m. (D) GST pull-down assay validating direct ANKRD1–CAND1 interaction. Purified GST or GST-ANKRD1 recombinant proteins immobilized on glutathione agarose beads were incubated with His-CAND1 recombinant protein, and bound His-CAND1 was detected by Western blotting. GST served as negative control. (E) Prediction of the ANKRD1–CAND1 interaction by molecular docking, with CAND1 as receptor and ANKRD1 as ligand. Predicted hydrogen bonds are marked by

yellow dashed lines. (F) Schematics of mouse ANKRD1 domain deletion mutants and mouse CAND1 deletion mutants and truncations. (G) Co-IP assays mapping the domains required for ANKRD1–CAND1 interaction. FLAG-tagged full length ANKRD1 or domain deletion mutants were transfected separately into C2C12 cells. Immunoprecipitation used a FLAG antibody, and endogenous CAND1 bound to the different ANKRD1 mutants was detected with a CAND1 antibody. FLAG and CAND1 antibodies also detected the corresponding proteins in immunoprecipitates and lysates. Similarly, FLAG-tagged full length CAND1, deletion mutants, or truncations were transfected separately into C2C12 cells; immunoprecipitation used a FLAG antibody, and endogenous ANKRD1 bound to the different CAND1 mutants was detected with an ANKRD1 antibody. FLAG and ANKRD1 antibodies also detected the corresponding proteins in immunoprecipitates and lysates.

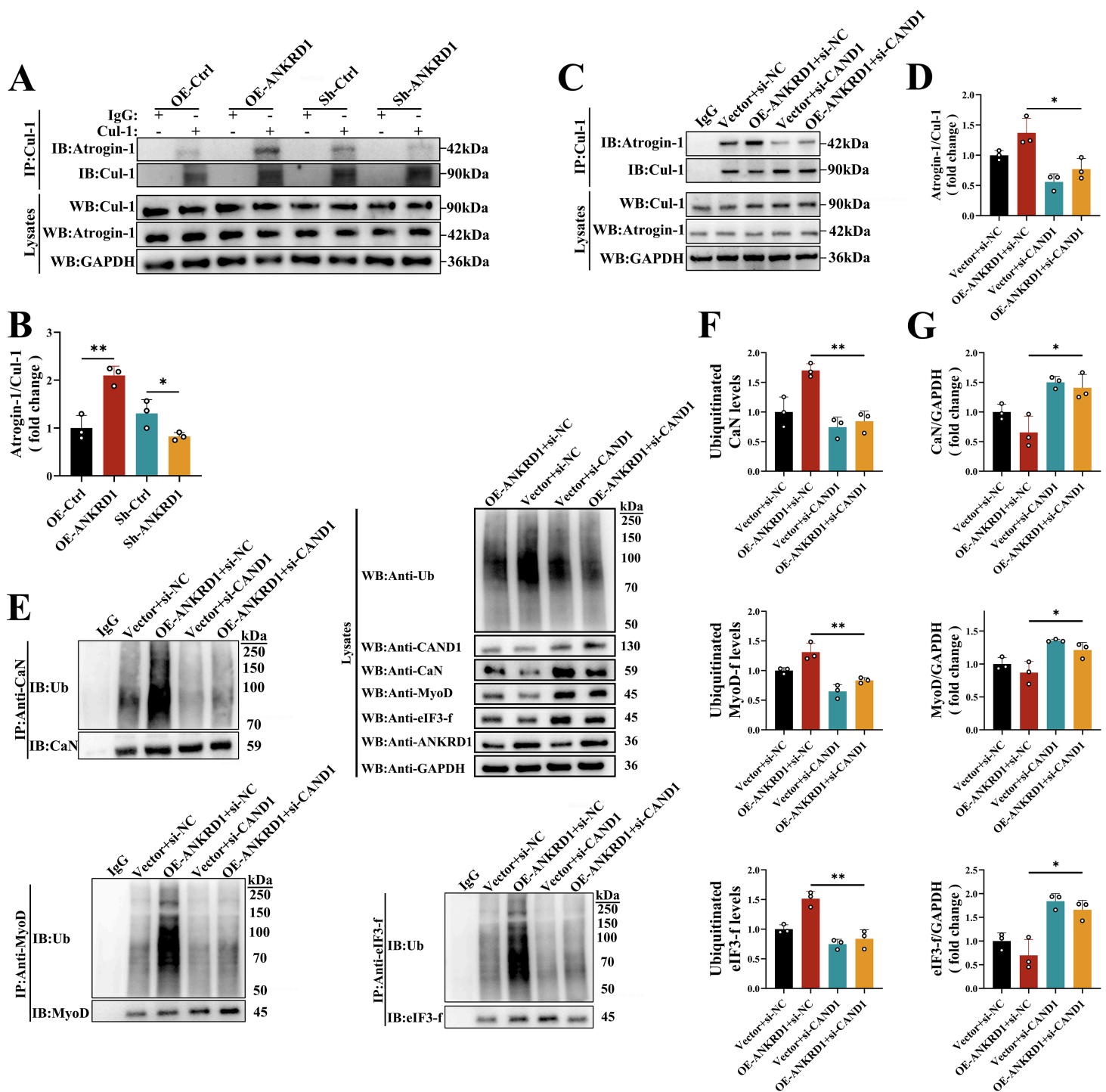


Figure 6

ANKRD1 promotes Atrogin-1 complex assembly through CAND1 and enhances ubiquitination and degradation of its substrate proteins. (A) Endogenous CUL1–Atrogin-1 interaction detected after ANKRD1 overexpression or knockdown. Lysates of OE-Ctrl, OE-ANKRD1, sh-Ctrl, and sh-ANKRD1 C2C12 cells were immunoprecipitated with IgG or a CUL1 antibody, and CUL1-bound Atrogin-1 was detected with an Atrogin-1 antibody. A CUL1 antibody verified immunoprecipitation efficiency, and CUL1 and Atrogin-1 levels in total lysates were also examined. (B) Quantification of Atrogin-1 endogenously bound to CUL1 in (A), $n = 3$. Unpaired Student's t-test, * $P < 0.05$, ** $P < 0.01$. (C) Effect of CAND1 knockdown on

ANKRD1 mediated CUL1–Atrogin-1 complex assembly. si-CAND1 or negative-control siRNA was transfected into C2C12 cells stably overexpressing ANKRD1. Lysates from Vector + si-NC, OE-ANKRD1 + si-NC, Vector + si-CAND1, and OE-ANKRD1 + si-CAND1 groups were immunoprecipitated with IgG or a CUL1 antibody; Atrogin-1 bound to CUL1 was detected with an Atrogin-1 antibody, a CUL1 antibody verified the precipitated protein, and CUL1 and Atrogin-1 levels in total lysates were also examined. (D) Quantification of Atrogin-1 endogenously bound to CUL1 in (C), $n = 3$. One-way ANOVA followed by Tukey's post hoc test, $*P < 0.05$. (E) Effect of CAND1 knockdown on ANKRD1 mediated ubiquitination of Atrogin-1 substrate proteins. Lysates from the four groups described in (C) were immunoprecipitated with IgG or antibodies against CaN, MyoD, or eIF3-f; a ubiquitin antibody detected ubiquitination of the corresponding proteins, and CaN, MyoD, and eIF3-f antibodies verified the precipitated proteins. Expression of CaN, MyoD, eIF3-f, CAND1, ANKRD1, and total ubiquitinated proteins in total lysates was also examined. (F) Quantification of relative ubiquitination levels of CaN, MyoD, and eIF3-f in (E), $n = 3$. One-way ANOVA followed by Tukey's post hoc test, $**P < 0.01$. (G) Quantification of relative protein levels of CaN, MyoD, and eIF3-f in the lysates of each group in (D), $n = 3$. One-way ANOVA followed by Tukey's post hoc test, $*P < 0.05$.

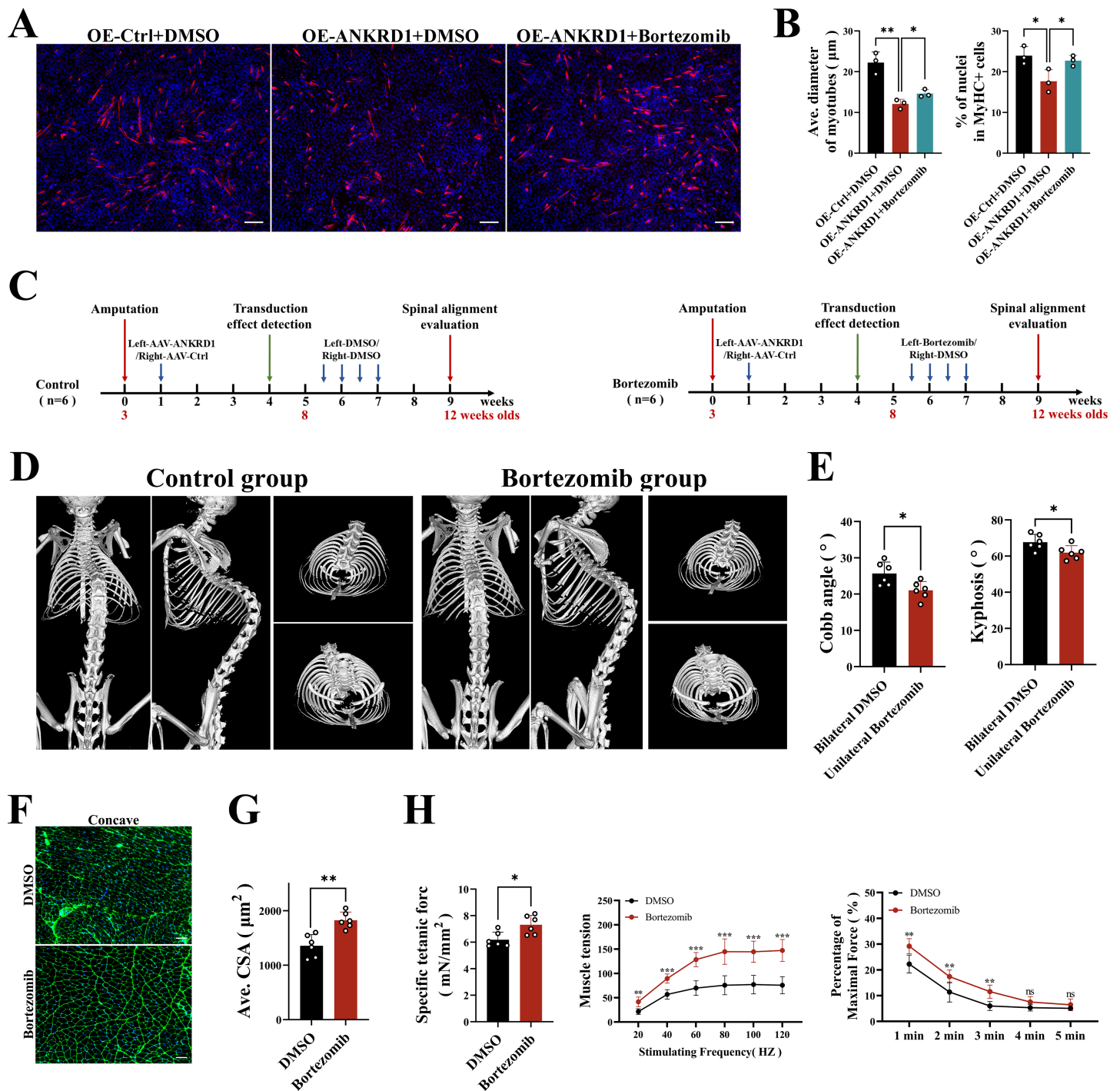


Figure 7

Bortezomib ameliorates ANKRD1 induced skeletal muscle atrophy and delays scoliosis progression caused by asymmetric ANKRD1 expression in the paraspinal muscles. (A) After stable ANKRD1 overexpression in C2C12 cells, 10 nM bortezomib was added on differentiation day 6, and MyHC immunofluorescence was performed on day 8. MyHC (red) labeled mature myotubes, and DAPI (blue) labeled nuclei. Scale bar: 100 μm . (B) Quantification of mean myotube diameter and fusion index from the MyHC staining in (A), $n = 3$. One-way ANOVA followed by Tukey's post hoc test, * $P < 0.05$, ** $P < 0.01$.

(C) Schematic of the local paraspinal muscle bortezomib intervention in bipedal mice. 4 week old bipedal mice received AAV-ANKRD1 injection into the left paraspinal muscle and AAV-Ctrl at the corresponding site on the right. Four weeks after adeno associated virus injection, bortezomib was administered at the left AAV-ANKRD1 injection site, with an equal volume of DMSO at the matching site on the right. Spinal alignment and paraspinal muscle structure and contractile function were evaluated 4 weeks after the start of bortezomib treatment. (D) Representative micro-CT three-dimensional reconstructed images of the bilateral DMSO group and the local bortezomib treatment group on the AAV-ANKRD1 injected side. Relative to the bilateral DMSO group, scoliosis progression and thoracic asymmetry were inhibited in the local bortezomib treatment group. (E) Coronal Cobb angle and sagittal kyphosis angle measured from the micro-CT images in (D), comparing the bilateral DMSO group with the local bortezomib treatment group on the AAV-ANKRD1 injected side, $n = 6$. Unpaired Student's t-test, $*P < 0.05$. (F) Representative dystrophin immunofluorescence staining of AAV-ANKRD1 injected side paraspinal muscles in the bilateral DMSO group and the local bortezomib treatment group. Dystrophin (green) delineated myofiber boundaries, and DAPI (blue) labeled nuclei. Scale bar: 100 μm . (G) CSA measured from the dystrophin staining in (F), comparing myofiber size of AAV-ANKRD1 injected side paraspinal muscles between the bilateral DMSO group and the local bortezomib treatment group, $n = 6$. Unpaired Student's t-test, $***P < 0.001$. (H) Ex vivo contractile function analysis of AAV-ANKRD1-injected side paraspinal muscles in the bilateral DMSO group and the local bortezomib treatment group. Maximal tetanic force was measured and compared between groups, $n = 6$. Unpaired Student's t-test, $*P < 0.05$. Contractile tension at different stimulation frequencies (20, 40, 60, 80, 100, and 120 Hz) was measured to generate force–frequency curves, $n = 6$. Unpaired Student's t-test, $**P < 0.01$, $***P < 0.001$. The decline in contractile force during continuous stimulation over 1–5 min was recorded to generate fatigue curves and evaluate contractile capacity and fatigue resistance.

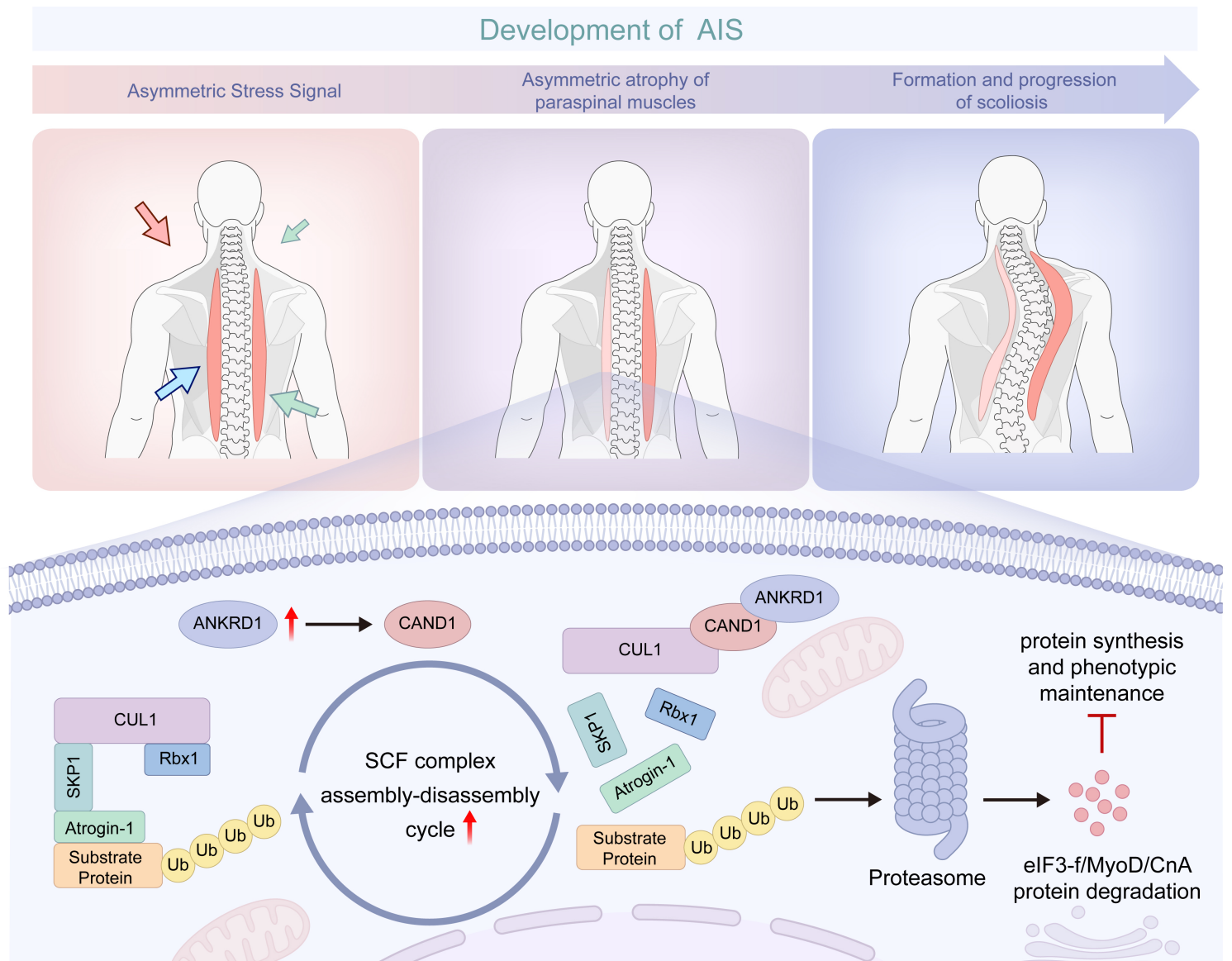


Figure 8

Schematic illustration showing that asymmetric activation of ANKRD1–CAND1–Atrogin-1 signaling in mature myofibers leads to paraspinal muscle atrophy and AIS progression.

Supplementary Files

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