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Supplemental Data

Identification of *KLHL41* Mutations Implicates BTB-Kelch-Mediated Ubiquitination as an Alternate Pathway to Myofibrillar Disruption in Nemaline Myopathy

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Supplemental Inventory

Supplemental Figures and Tables

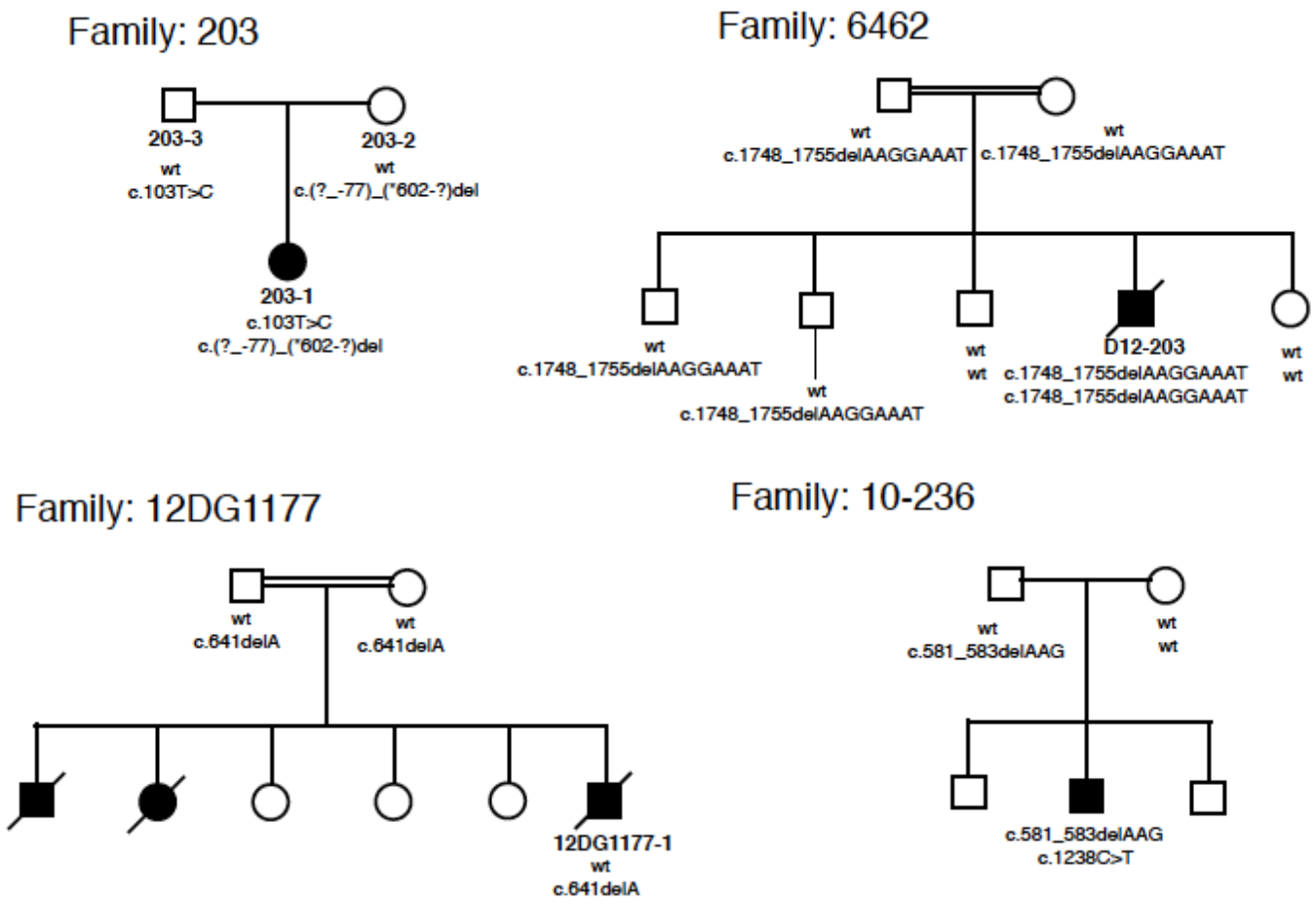

Figure S1. Pedigrees of affected individuals with *KLHL41* mutations. The segregation of *KLHL41* mutations in affected families is consistent with an autosomal-recessive mode of inheritance. "+" indicates presence of the variant. "-" indicates presence of the wild type reference sequence.

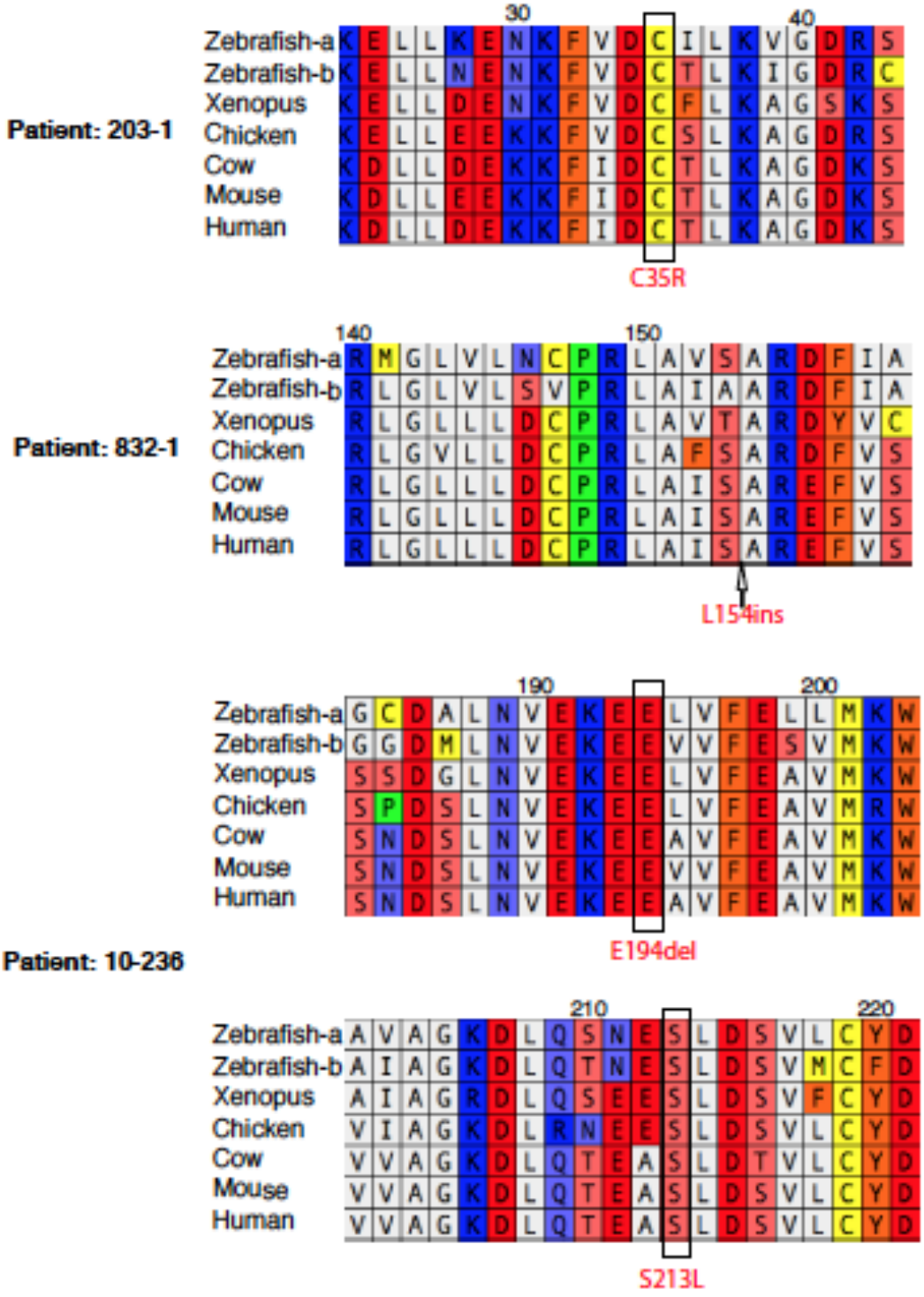

Figure S2. Conservation of substituted residues and the surrounding regions in vertebrates.
KLHL41 sequences were aligned using ClustalW. The missense changes affected amino acid residues that are highly conserved across species (203-1 and D10-236). The regions surrounding the single amino acid insertion and deletion are also highly conserved in all vertebrates (832-1 and D10-236). All species contain one KLHL41 gene, whereas two duplicated copies are present in the zebrafish genome.

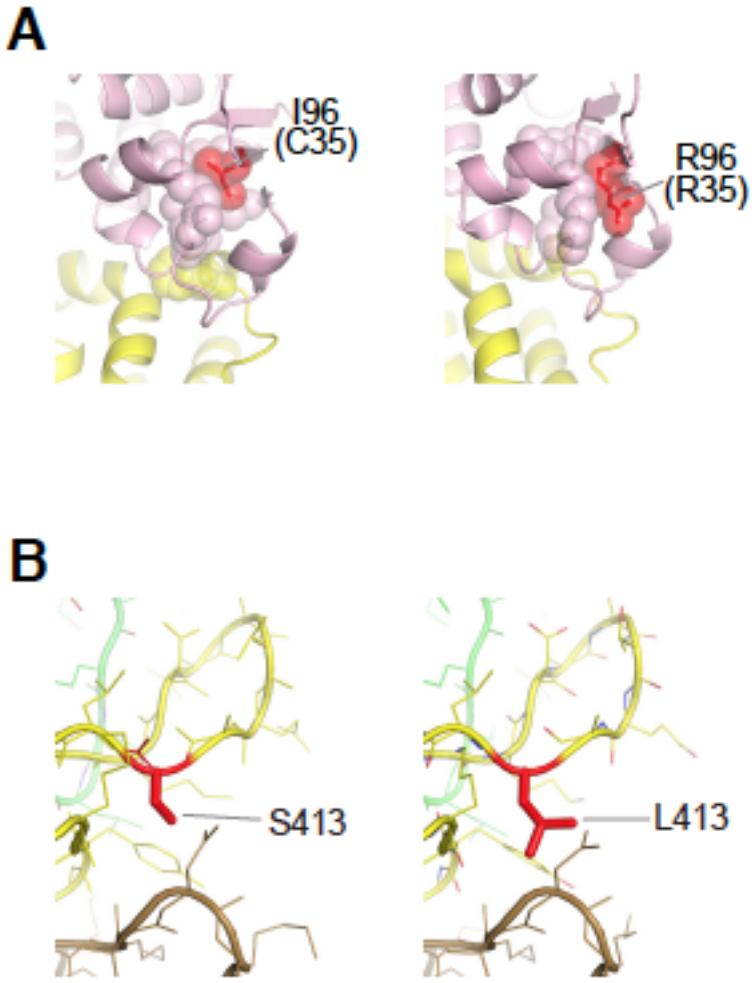

Figure S3. Modeled structures of the substituted BTB-BACK and Kelch domains. (A and B) The wild-type (left) and mutated (right) structures of the BTB-BACK domain of human KLHL11 in complex with Cul3 (PDB code 4AP2) (A) and the Kelch domain of rat KLHL41 (PDB code 2WOZ) (B). α-helices, β-strands and loops are drawn as ribbons, arrows and threads, respectively. The point-mutated structures were constructed based on their wild-type structures using FoldX software. Colors are the same as Figure 1.

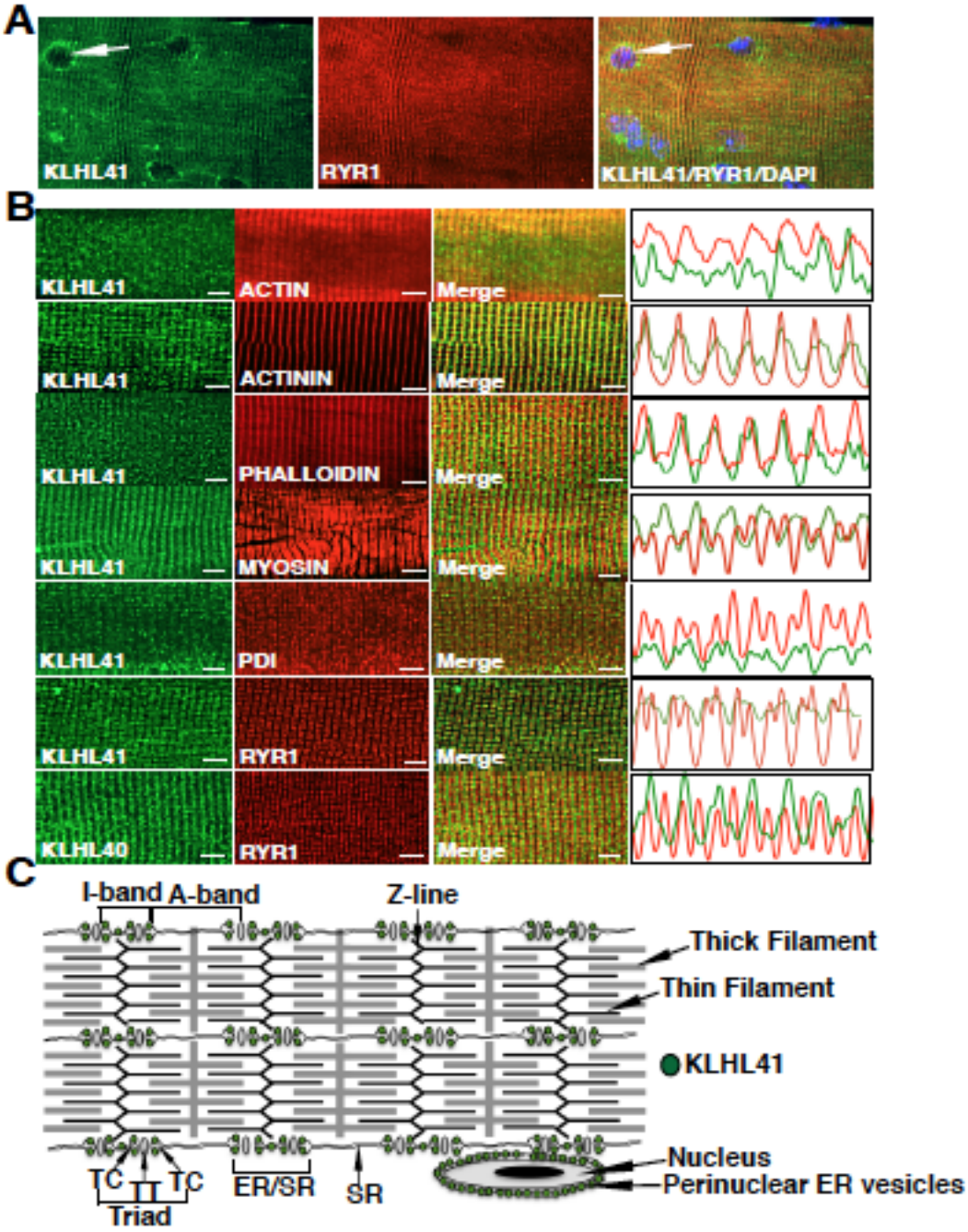

Figure S4. KLHL41 is primarily localized in sarcompasmic reticulum-endoplasmic-reticulum membranes (SR-ER) in cultured myofibers. Cultured murine FDB myofibers were co-immunostained with KLHL41 (green) and skeletal muscle markers (red), visualized by confocal microscopy and Z-stacks were obtained. (A) Confocal Z-stack of a myofiber showed localization of KLHL41 in perinuclear area (arrows) and in a striated pattern primarily co-localizing with RYR1. (B) Single frames in the middle of myofibers show that most of the KLHL41 appears co-localized with α-actinin (Sigma, A7811, clone EA-53) and actin (Sigma, alexa fluor 546 phalloidin, A22283) over I-bands. Immunostaining with RYR1 (Sigma, R129 clone 34C) and protein disulfide isomerase (Abcam, ab2792) show localization of KLHL41 in the SR-ER domains. At right are single channel desitometry tracings from seven representative sarcomeres with colors corresponding to staining patterns at left. (C) Schematic diagram depicting localization of KLHL41 in relation to the contractile apparatus and nuclei. KLHL41 (shown as green balls) localizes to perinuclear regions where ER is present, and between myofibrils at the triadic regions and longitudinal vesicles of the SR spanning the I-band, corresponding to ER microdomains within the SR of myofibers. Scale bar = 5 μm.

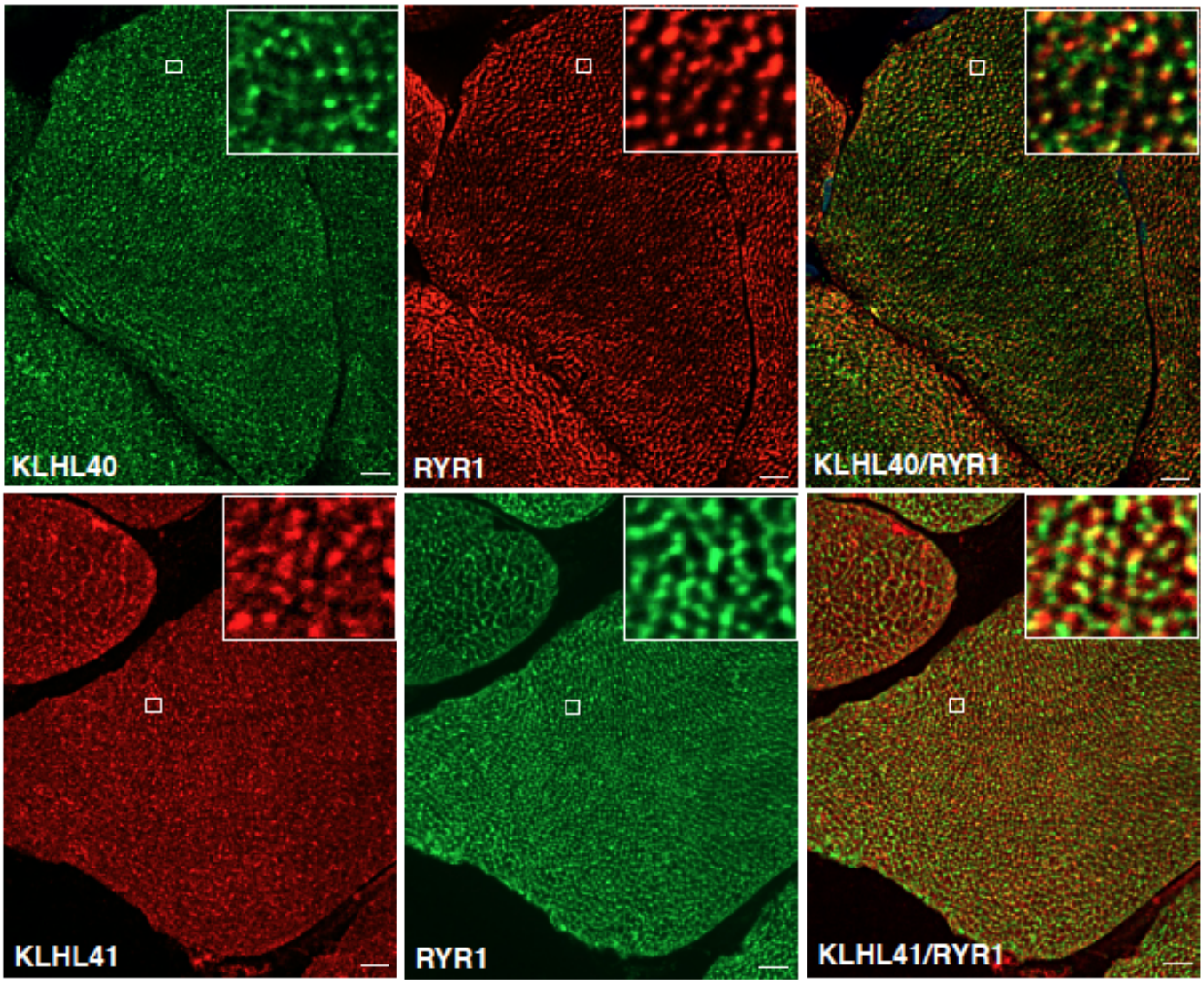

Figure S5. KLHL40 and KLHL41 are is localized in sarcompasmic reticulum-endoplasmic-reticulum membranes in human skeletal muscle. Frozen transverse sections of control human skeletal muscles were immunostained with KLHL40 (Sigma, HPA024463) or KLHL41 (Sigma, AV38732) with RYR1 antibodies, visualized by confocal microscopy and Z-stacks were obtained. Single frames in the middle of the section shows that KLHL40 and KLHL41 are co-localized with RYR1. Scale bar = 10 μm.

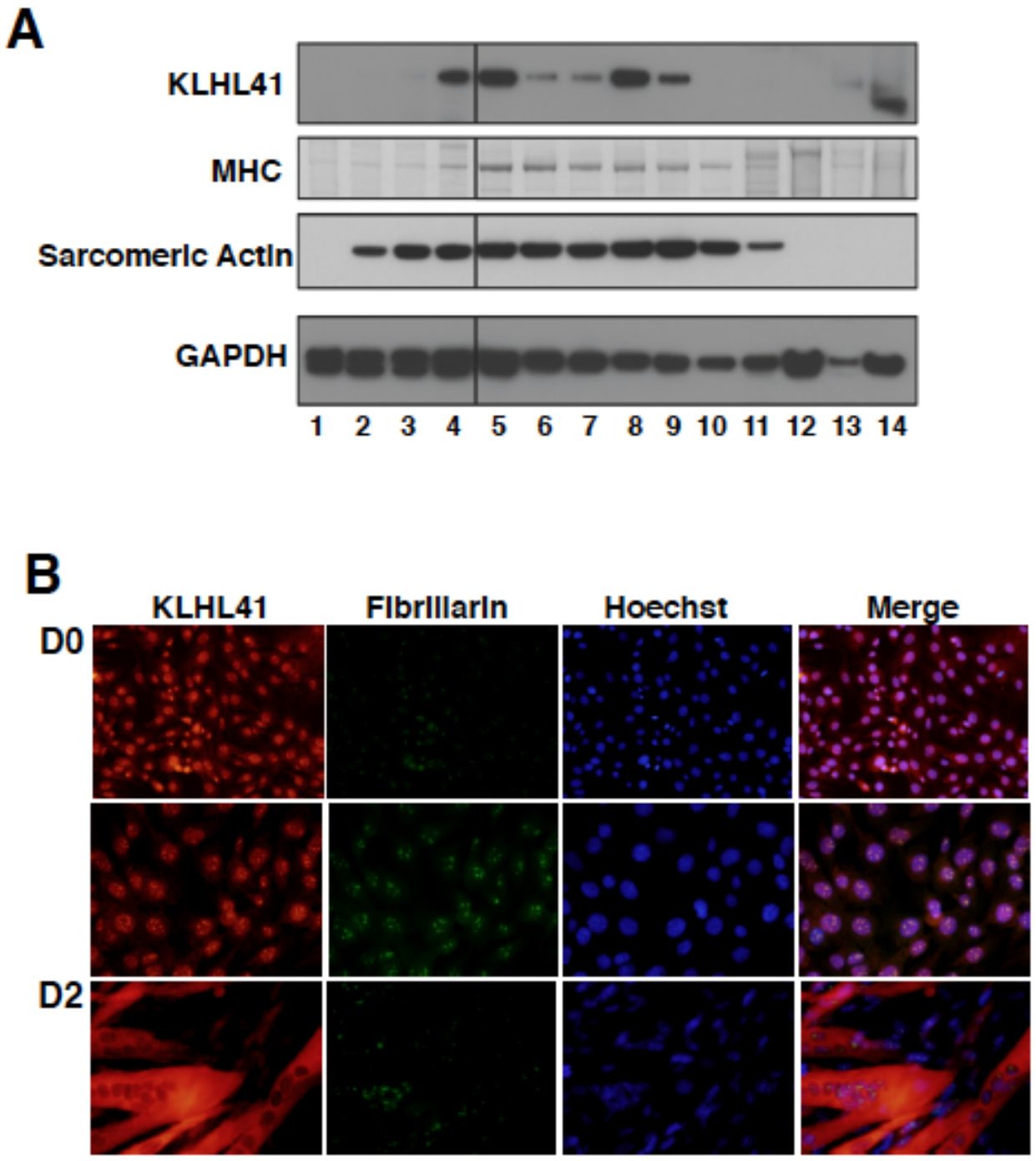

Figure S6. **Expression of KLHL41 in myoblasts and mouse tissues.** (A) Western blot analysis of KLHL41 in murine tissues showed high protein levels in skeletal muscle and diaphragm but not other tissues. GAPDH and sarcomeric actin mmunoblotting were used as the loading controls. Lanes: (1) C2C12 D0 myoblasts, (2) C2C12 D2 myotubes (3) C2C12 D4 myotubes (4) C2C12 D6 myotubes. (5) Post natal day 2 C57BL/6 gastroc, (6) 8 week C57BL/6 gastroc (7) 8 week C57BL/6 EDL (8) 8 week C57BL/6 soleus (9) 8 week C57BL/6 diaphragm (10) 8 week C57BL/6 heart (11) 8 week C57BL/6 masseter (12) 8 week C57BL/6 brain (13) 8 week C57BL/6 lung (14) 8 week C57BL/6 liver. (B) Immunofluorescence shows KLHL41 is present in myoblasts (nuclear) as well as myotubes (cytoplasmic).

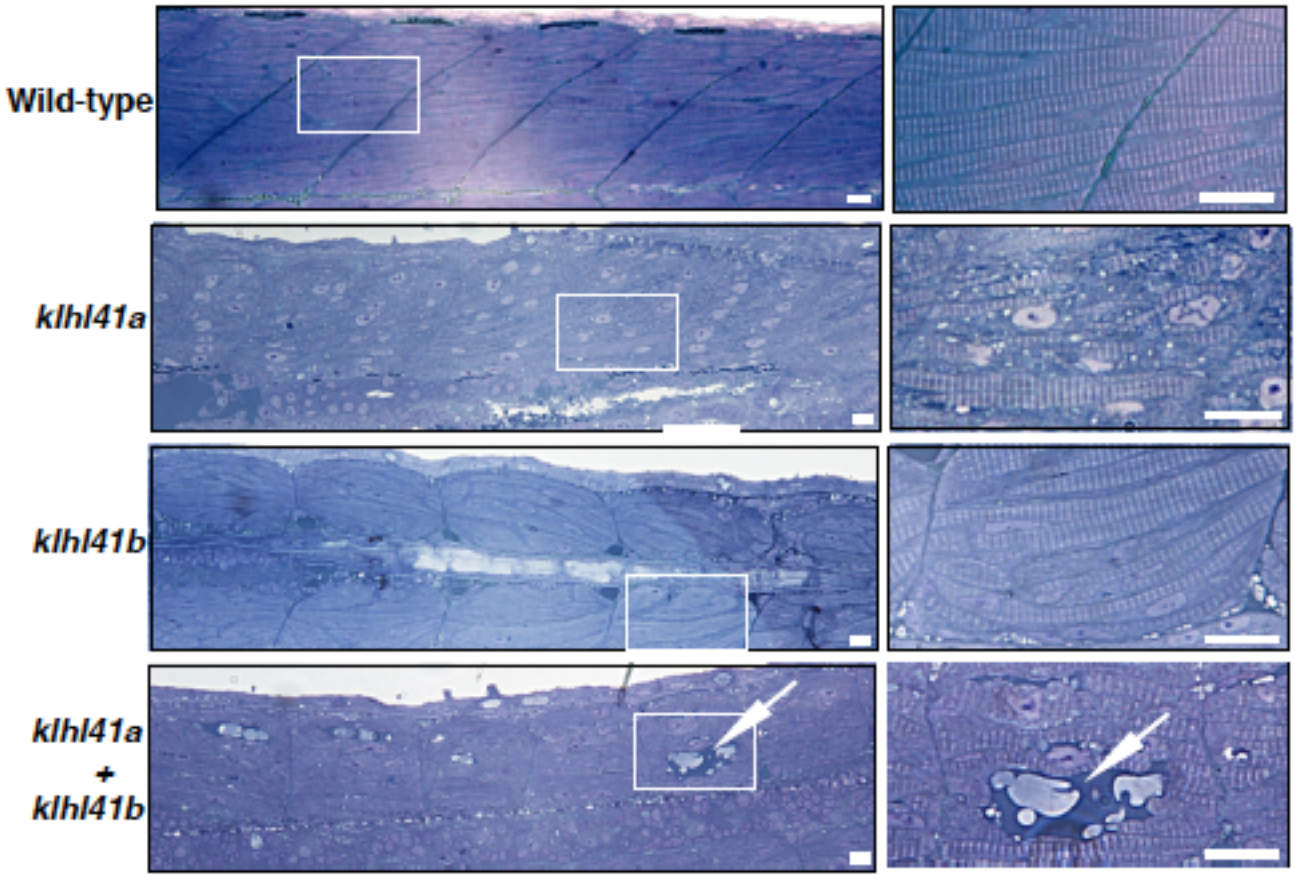

Figure S7. Skeletal muscle histology of *klhl41* morphant fish at 3 dpf. Toluidine blue staining of semithin sections (1 µm) of wild-type and *klhl41* morphant fish. *Klhl41a* morphant fish showed extensive sarcomeric disorganization. *Klhl41b* morphant skeletal muscle is less severely affected in comparison to *klhl41a* fish but still shows areas that lacked the level of sarcomeric organization seen in wild-type muscles. *Klhla* and klhl*41b* double morphant fish exhibit greater myofibrillar disarray. In addition, several core like areas lacking any sarcomeric components were also observed in double knock down fish (white arrow). Scale bar = 20 µm.

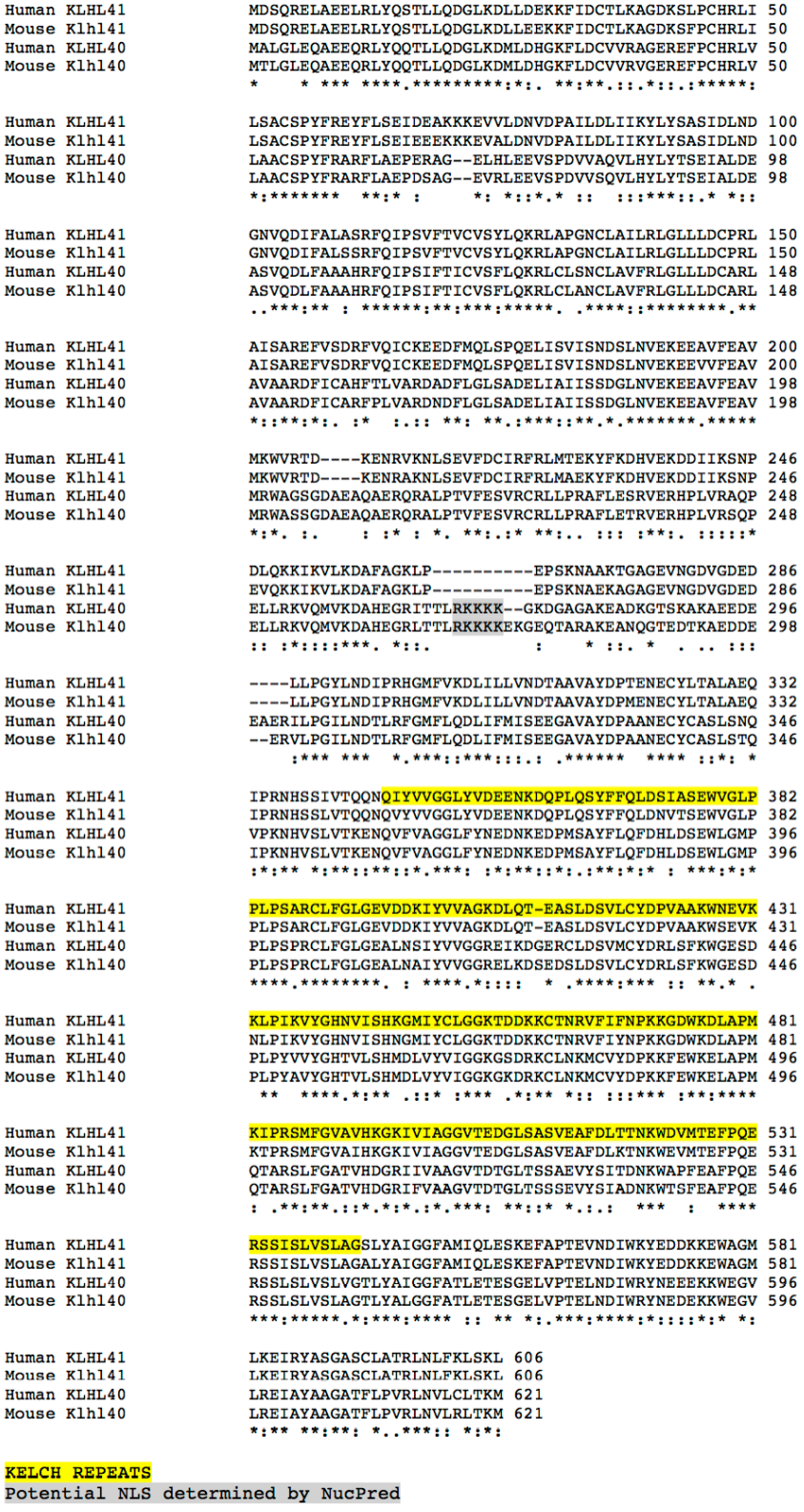

Figure S8. Protein sequence alignment of human and mouse KLHL40 and KLHL41. Protein sequence alignment of human and mouse KLHL40 and KLHL41 was performed using ClustalW. Upstream of Kelch repeats (highlighted in yellow), KLHL40 contains a nuclear localization sequence (NLS, highlighted in grey) that is absent in KLHL41.

Table S1: Primer sequences for cloning zebrafish *in-situ hybridization* probes.

Gene	Primer sequences
klhl41a	Forward primer: 5'CCCCCTCGAGGAGGAGGACAAGAAGCAGTGG3' Reverse primer: 5'CCCCGGATCCTGATGAATGCAGGAGAGATGG3'
klhl41b	Forward primer: 5'CCCCCTCGAGGGAGCGCAGCTCTGTGAAT3' Reverse primer: 5'CCCCGGATCCTACCTTATCTTGCATACGTGGTTC3'

Table S2: Morpholino sequences targeting zebrafish *klhl40 and klhl41 genes*.

Gene	Primer sequences
klhl41a	Translational start site: 5' CTCTTTGACACTCATTGGTTCCAT 3' Exon2-intron2 junction: 5' ATGTGGTAGAAACTTACTCGACATC 3'
klhl41b	Translational start site: 5' GATCCATGATGTTTCGTCTCAAAGT3' Exon2-intron2 junction: 5' TCAAAGATGAACTCATACTCGGTGT 3'