

LMNA variants cause cytoplasmic distribution of nuclear pore proteins in *Drosophila* and human muscle

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Mutations in the human *LMNA* gene, encoding A-type lamins, give rise to laminopathies, which include several types of muscular dystrophy. Here, heterozygous sequence variants in *LMNA*, which result in single amino-acid substitutions, were identified in patients exhibiting muscle weakness. To assess whether the substitutions altered lamin function, we performed *in vivo* analyses using a *Drosophila* model. Stocks were generated that expressed mutant forms of the *Drosophila* A-type lamin modeled after each variant. Larvae were used for motility assays and histochemical staining of the body-wall muscle. In parallel, immunohistochemical analyses were performed on human muscle biopsy samples from the patients. In control flies, muscle-specific expression of the wild-type A-type lamin had no apparent effect. In contrast, expression of the mutant A-type lamins caused dominant larval muscle defects and semi-lethality at the pupal stage. Histochemical staining of larval body wall muscle revealed that the mutant A-type lamin, B-type lamins, the Sad1p, UNC-84 domain protein Klaroid and nuclear pore complex proteins were mislocalized to the cytoplasm. In addition, cytoplasmic actin filaments were disorganized, suggesting links between the nuclear lamina and the cytoskeleton were disrupted. Muscle biopsies from the patients showed dystrophic histopathology and architectural abnormalities similar to the *Drosophila* larvae, including cytoplasmic distribution of nuclear envelope proteins. These data provide evidence that the *Drosophila* model can be used to assess the function of novel *LMNA* mutations and support the idea that loss of cellular compartmentalization of nuclear proteins contributes to muscle disease pathogenesis.

INTRODUCTION

Laminopathies comprise a class of human diseases that typically affect one or more tissues, resulting in cardiomyopathies, premature ageing syndromes, lipodystrophies, neuropathies, dermatopathies or muscular dystrophies (1). Many genetic subtypes of muscular dystrophy cause progressive wasting and degeneration of skeletal muscle (2), including autosomal-dominant Emery–Dreifuss muscular dystrophy (AD-EDMD) (3). More than 250 distinct mutations in the ubiquitously expressed *LMNA* have been linked to AD-EDMD, yet a

general understanding of the cause and progression of the disease remains elusive (3,4).

Lamin genes are found in all metazoans, but are absent in plants and unicellular organisms (5,6). Lamins are classified into A and B types, based on their biophysical properties and expression profiles (7–12). In mammals, A-type lamins (lamins A, C, A Δ 10 and C2) are the products of *LMNA* transcripts that are alternatively spliced (13–15), whereas B-type lamins (lamins B1 and B2–B3) are encoded by the *LMNB1* and *LMNB2* genes, respectively (16,17). In *Drosophila*, A- and B-type lamins are encoded by two genes, *Lamin C*

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and lamin *Dm₀*, respectively (11). The A-type lamins are expressed upon differentiation in a wide variety of cell types (18); in contrast, the B-type lamins are expressed ubiquitously throughout development (19–21).

Lamins play a structural role by assembling into the lamina network that underlies the inner nuclear membrane of the nuclear envelope (NE). Lamins participate in nuclear migration and positioning, nuclear shape maintenance, nuclear pore spacing, chromatin organization, chromosome segregation, gene expression, signal transduction and cytoskeletal organization (13,22–29). In addition to their peripheral localization, lamins are components of supramolecular complexes residing at the nucleus interior (30), where they play a role in retinoblastoma-mediated cell proliferation (31,32) and the initiation of DNA replication (33). Given that the A-type lamins are nearly ubiquitously expressed and participate in so many different cellular processes, it is challenging to explain the cellular and molecular mechanisms by which mutant forms contribute to tissue-specific pathology. The literature describes several non-mutually exclusive models to explain this paradox (34,35). (1) The ‘mechanical stress’ hypothesis proposes that mutations in *LMNA* give rise to a weakened nuclear envelope, predisposed to damage (35). (2) The ‘gene regulation’ hypothesis proposes that disruptions in the nuclear lamina prevent proper associations with chromatin, causing misregulation of gene expression (35). (3) A newly emerging hypothesis proposes that the A-type lamins regulate tissue homeostasis (36). In this model, the disease-causing mutants in the A-type lamins are suggested to perturb the balance between proliferation and differentiation in adult stem cells, thereby compromising tissue regeneration (37,38). All three models are mutually compatible, which might explain why it has been challenging to determine the exact mechanism by which the mutant lamins cause muscle disease.

This study focused on three non-related pediatric patients exhibiting muscle weakness characteristic of muscular dystrophy. Each patient harbored a heterozygous nucleotide substitution in *LMNA* that resulted in an amino-acid substitution within the A-type lamin. However, it was unclear whether these substitutions were polymorphisms or pathogenic. To test the hypothesis that these variants disrupt lamin function and to reveal potential disease mechanisms, we modeled the substitutions (and a known AD-EDMD-causing substitution) in the *Drosophila* lamin. Our studies revealed novel molecular defects that occurred upon expression of mutant lamins, which were then confirmed using human muscle biopsy tissue.

RESULTS

Three patients with muscular weakness harbor amino-acid substitutions in the Ig-fold domain of the A-type lamin

Previous studies described amino-acid substitutions within the Lamin A/C Ig-fold domain that resulted in muscular dystrophy (3,4). We identified three patients with muscle atrophy that harbored heterozygous variant alleles of *LMNA* (Table 1). Each variant was predicted to cause a novel amino-acid substitution in the Ig-fold domain (G449V, L489P and W514R; see Table 1). These point mutations were not reported in the

Table 1. Genetic lesions in *LMNA* and age of onset for muscle atrophy

Nucleotide change	Exon	Amino-acid change	Age of onset
1072G>A ^a	6	N456I	9
1346G>T	7	G449V	3
1466T>C	8	L489P	7
1540T>C	9	W514R	5

^aAn AD-EDMD mutation described in Ref. (42).

Leiden Muscular Dystrophy Database (<http://www.dmd.nl/>), which catalogs *LMNA* mutations associated with muscular dystrophy. Whether these variants were silent or pathogenic was unknown, and how they might alter the Ig-fold structure and lamin function was not obvious. There was no test to assay the function of lamin variants *in vivo*. To determine whether the variants we identified might be pathogenic mutations causing *LMNA*-associated muscular dystrophy, we used a *Drosophila* model (39,40).

Drosophila is the only widely used invertebrate model that expresses endogenous A- and B-type lamins homologous to those in humans. Furthermore, *Drosophila* express a single A-type lamin—Lamin C. Two of the substitutions (G449 and L489) discovered in the patients were in residues conserved between human Lamin A/C and *Drosophila* Lamin C (G489 and V528); the third amino acid (W514) is not conserved in *Drosophila* Lamin C (M553), however, it resides within a conserved stretch of amino acids (Fig. 1A). The position of each amino acid is indicated in the known structure of the Ig-fold (41) (Fig. 1B). In addition, we modeled N496I (N456I), a known AD-EDMD-causing substitution as a ‘positive control’ (42). We generated transgenic *Drosophila* stocks expressing versions of the *Drosophila* A-type lamin that were homologous to the patient Lamin A/C variants and the known disease-causing mutant under control of the Gal4/UAS system (43).

Modeling human lamin mutations in *Drosophila* revealed muscle defects

The amino-acid substitutions, which appeared to have a dominant effect in the patients, were tested for a dominant effect in a *Drosophila* model system. The three variant forms of Lamin C, the ‘positive control’ and wild-type Lamin C were expressed in different tissues of transgenic flies in an otherwise wild-type genetic background. Tissue-specific expression was achieved using the Gal4/UAS system and a series of ‘driver’ stocks that express Gal4 in specific tissues (44). Thus, the various forms of Lamin C could be expressed ubiquitously, or specifically in eye discs (negative control), or muscle tissue of embryos, larvae and adults (Table 2).

Wild-type Lamin C, expressed by any of the Gal4 drivers tested, did not decrease viability or results in any visible phenotype (Table 2). Likewise, no effects were observed upon expressing that altered forms of A-type lamin in non-muscle tissue such as eye discs. No effects on viability were seen upon expression in adult muscle; however, a thorough analysis of adult muscle function was not undertaken. In

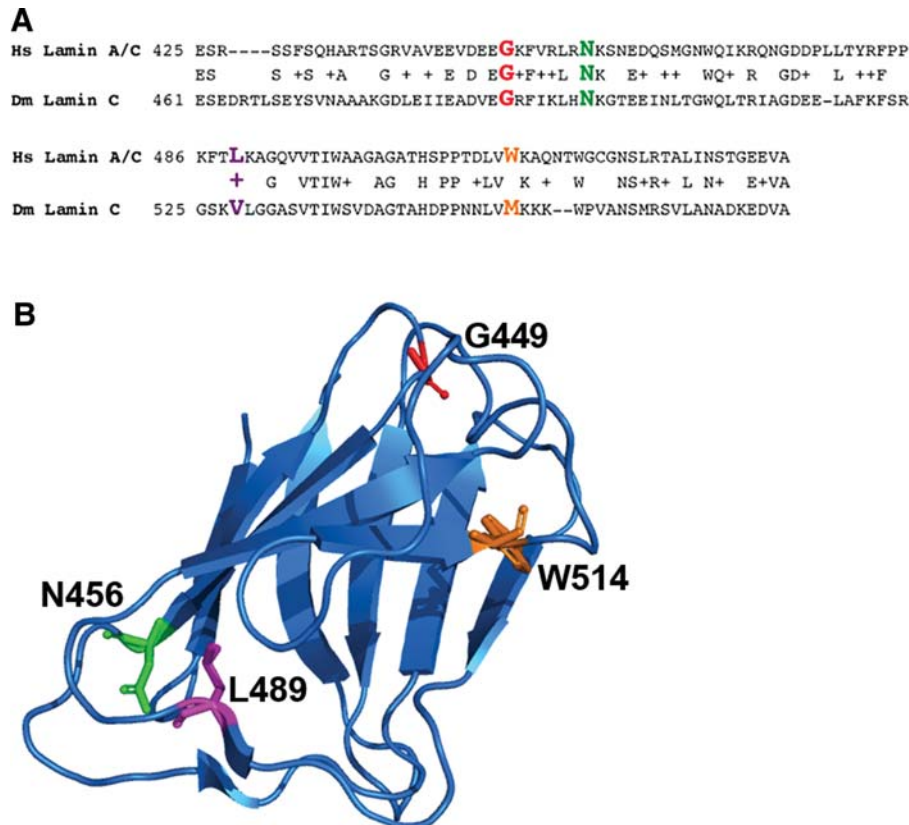


Figure 1. Ig-fold domain of human and *Drosophila* A-type lamins. (A) Alignment of the Ig-fold domain of human Lamin A/C (Hs) and *Drosophila* Lamin C (Dm), using BLAST (bl2seq, NIH). One of the amino acids affected in the patient cases (G449) is identical between the two species; the second amino acid is similar (L489) and the third amino acid is not conserved (W514) but is flanked by conserved residues. Amino acid N456 is known to cause AD-EDMD when substituted with I. (+) Conserved residue based on similar amino-acid structure; (–) Gap in amino-acid sequence. (B) Ribbon diagram of the human Lamin A/C Ig-like fold (PDB: 1IFR) showing the relative position of the amino acids affected in patient cases studied here.

stark contrast, viability dropped dramatically when any of the three variants were expressed in muscle of late embryos and larvae (using either the C57 or MEF2 promoters). Approximately 70–95% of the expected progeny died at the late pupal stage. The percent lethality depended upon which mutant was expressed; N496I gave the least amount of death, and G489V caused complete lethality. Examination of the developing pupae revealed mutant phenotypes that included short, kinked legs and a gas bubble that remained at the bottom of the pupal case—phenotypes exhibited by known muscle mutants (45) (Table 2). The ‘escaper’ adults that managed to live past the pupal stage showed adult leg defects: the last tarsal segments were twisted and shorter—a phenotype nearly identical to that observed in flies expressing a ‘headless’ Lamin C (44). We confirmed that these mutant phenotypes were not simply due to over-expression of the A-type lamin variants, as western analysis showed that the wild-type Lamin C (which had no effect) and mutant variants were expressed at similar levels (Supplementary Material, Fig. S1).

The decreased viability, pupal defects and late pupal death caused by expressing the lamin variants in late embryonic/larval muscle suggested that the amino acids altered are important for larval muscle function. To directly test muscle

function, we performed locomotion assays in which larval crawling was recorded over 2-min intervals (Fig. 2). Wandering, third-instar larvae were placed at the center of a Petri dish sectioned into concentric zones and their movement measured after 2 min. Larvae expressing wild-type Lamin C crawled to zone 3, whereas the larvae expressing the mutant forms remained in zone 1. These assays clearly show that *Drosophila* expressing the A-type lamins characteristic of human muscle disease have reduced mobility (Fig. 2).

Amino-acid substitutions in the Ig-fold domain of A-type lamins cause both A- and B-type lamins to mislocalize

These locomotion defects prompted us to examine the muscle tissue in the transgenic larvae at the cellular level. Using indirect immunofluorescence, the larval body wall muscle was stained with antibodies recognizing Lamin C and lamin Dm₀ (the *Drosophila* B-type lamin). In larvae expressing wild-type lamin, staining showed myonuclei localized in an evenly spaced pattern at the periphery of the fiber (Fig. 3A). Myonuclei also appeared normal in *Drosophila* expressing the N456I Lamin C mutant that is homologous to the A-type lamin previously linked to AD-EDMD (Fig. 3A and B). In contrast, ~30% of the

Table 2. Tissue-specific expression of wild-type and mutant forms of Lamin C

Lamin	Eyeless (eye discs)	C57 (embryo/larval muscle)	Mef2 (embryo/larval muscle)	MHC (adult muscle)
wt	Viable	Viable	Viable	Viable
G489V (G449V)	Viable	Semi-lethal	Lethal	Viable
N496I (N456I)	Viable	Semi-lethal ^a , LD, PD	Lethal	Viable
V528P (L489P)	Viable	Semi-lethal ^a , LD, PD	Lethal	Viable
M553R (W514R)	Viable	Semi-lethal ^a , LD, PD	Semi-lethal ^a , PD	Viable

Human residues in parentheses. LD, leg defect; PD, pupal defects.

^aSemi-lethal: 70–95% lethality.

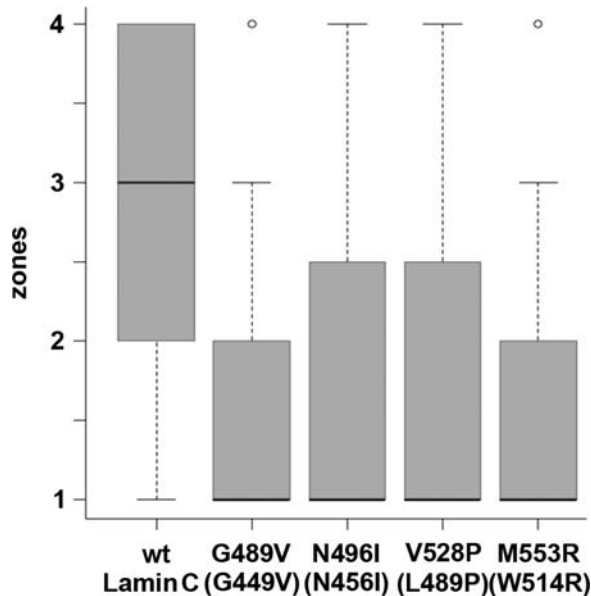


Figure 2. Mutant forms of Lamin C cause locomotion defects. The results from locomotion assays of larvae expressing Lamin C transgenes by the C57 Gal4/UAS driver are shown. Three independent assays were performed using eight similarly staged larvae each. Box plots display 25–75 percentile range of the data and the thick black line indicates the median value. The Y-axis represents zones outlined in a Petri dish containing agarose. The X-axis shows the mutant form of Lamin C expressed in larvae.

muscle fibers in larvae expressing one of the three new A-type lamin mutants showed the myonuclei aggregated. The nuclei were comparable in size to those in larvae that expressed wild-type Lamin C. The average diameter of 30 nuclei expressing wild-type Lamin C was $7.8 \pm 0.5 \mu\text{m}$, similar to $7.2 \pm 0.9 \mu\text{m}$ obtained for nuclei expressing mutant Lamin C. Both A- and B-type lamins remained exclusively nuclear in the wild-type control and N496I larvae (Fig. 3A and B). In contrast, the three Lamin C mutants modeled after our patients showed A- and B-type lamins localized in a punctate, non-nuclear pattern in ~30% of the myofibers (Fig. 3A and B). These defects did not appear due to developmental abnormalities. We analyzed the larval body wall muscle of first and second instar larvae and found no such defects (data not shown). Thus, the mislocalization is likely the result of accumulation of mutant Lamin C and/or increased mechanical stress experienced during the third instar stage.

Amino-acid substitutions in the Ig-fold domain cause non-nuclear localization of SUN domain and nuclear pore proteins

To determine whether the localization defects were restricted to lamins, we examined the localization of Klaroid (46). Klaroid is a Sad1p, UNC-84 (SUN) domain-containing protein that is required for proper nuclear migration and localization of Klarsicht [a Klarsicht, ANC-1, Syne homology domain-containing protein] to the nuclear envelope (46). In larvae expressing wild-type Lamin C or the N496I mutant, Klaroid staining exhibited the expected rim-like pattern around the myonuclei (Fig. 4A). In contrast, expression of G489V, V528P and M553R showed cytoplasmic localization, with apparent enrichment immediately outside the nucleus (Fig. 4). Thus, A-type lamin Ig-fold substitutions lead to cytoplasmic mislocalization of an inner nuclear envelope protein.

To determine whether mislocalization was confined to inner nuclear envelope proteins or also affected other proteins associated with the nuclear envelope, the localization of two different types of nuclear pore components (NPCs) was determined. Antibodies that recognize FG-repeat-containing nucleoporins (47) stained the nuclear periphery in the wild-type control and N496I mutant. In contrast, the other Ig-fold amino-acid substitutions caused the FG-repeat-containing proteins to be distributed broadly throughout the cytoplasm of a myofiber (Fig. 5A). A nearly identical pattern was observed with antibodies to GP210, a *trans*-membrane NPC (Figs 4B and 5B) (48). These data suggest that the amino-acid substitutions found in our patients alter the lamin network, causing miscompartmentalization of NPC protein.

To determine whether the unusual cytoplasmic distribution of proteins was limited to those of the nuclear envelope or also affected chromatin proteins, antibodies to Heterochromatin Protein 1a (HP1a) were tested (49). HP1 proteins are enriched in heterochromatic regions, which are juxtaposed to the nuclear envelope, and display dynamic association with chromatin (50–54). In all larvae tested, staining with antibodies against HP1a revealed normal heterochromatin organization (Supplementary Material, Fig. S2), suggesting that not all nuclear proteins were affected.

Patient muscle biopsies and transgenic larvae share similar myofiber and nuclear defects

Muscle from transgenic *Drosophila* larvae was compared with skeletal muscle biopsies from the patients in whom the *LMNA* mutations were originally identified (Table 1). Cross-sections

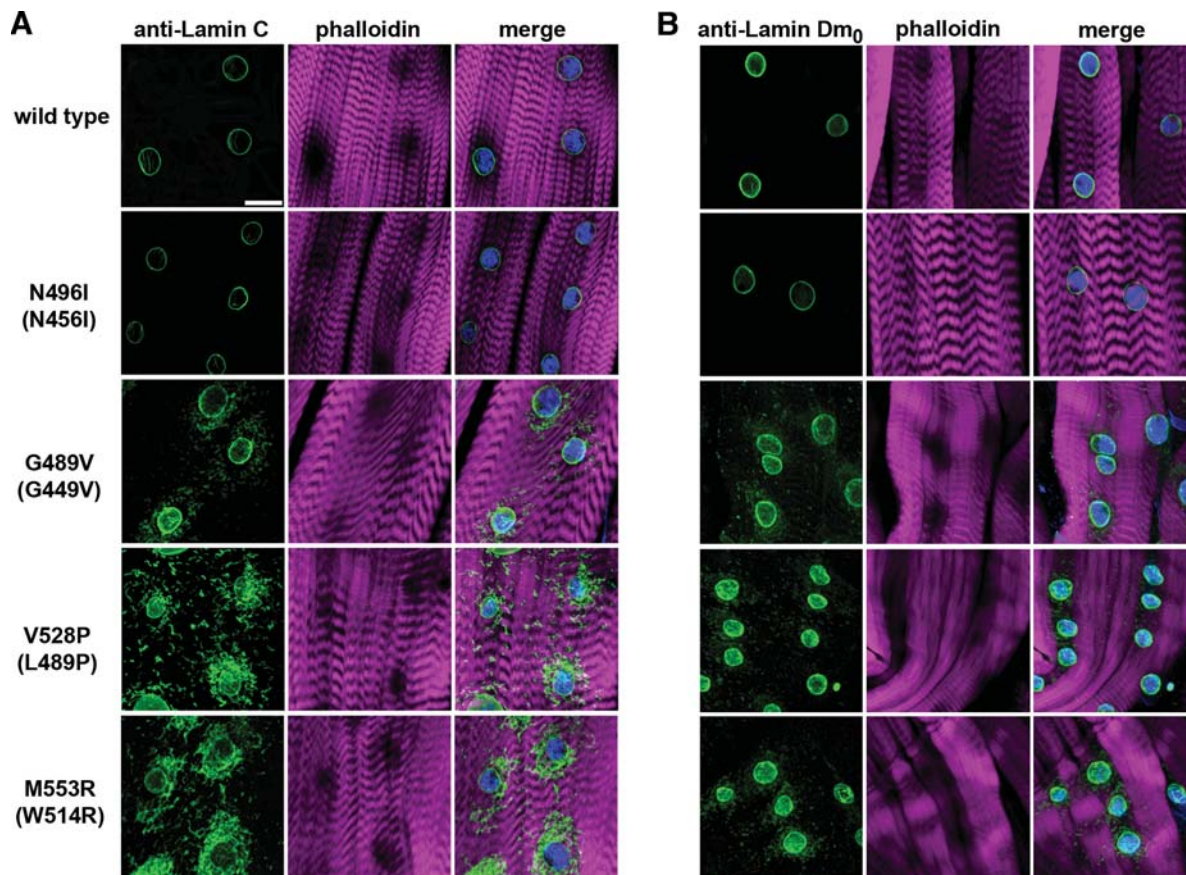


Figure 3. Expression of mutant forms of Lamin C in larval muscle causes mislocalization of A- and B-type lamins, nuclear and cytoskeletal defects. Staining of third instar larval body wall muscle fillets with anti-Lamin C (A, green), anti-lamin Dm₀ (B, green), phalloidin (A and B, purple) and DAPI (A and B/merge, blue) from larvae expressing wild-type Lamin C (wild-type) or mutant Lamin C transgenes by the muscle-specific driver C57. *Drosophila* amino-acid numbers are shown with human amino acid numbering in parentheses. Bar, 10 μ m.

of muscle biopsy tissue were stained by indirect immunofluorescence using antibodies against Lamin A/C, Lamin B1, dystrophin (a marker of the muscle cell membrane) and 4',6-diamidino-2-phenylindole (DAPI, which stains DNA). In muscle biopsies from healthy controls as well as patients, staining for both A- and B-type lamins decorated the periphery of myofiber nuclei (Fig. 6A and B). Dystrophin immunofluorescence illustrates the normal variation in muscle fiber diameters in controls and the wide variation in diameters, ranging from atrophic to hypertrophic, characteristic of muscular dystrophies in the patient samples (Fig. 6A and B). In addition, control biopsies show myonuclei localized in the normal, eccentric, subsarcolemmal position. In contrast, patient samples reveal some muscle fibers with internally placed myonuclei (Fig. 6A and B), which is commonly seen in myofibers that have undergone necrosis and regeneration.

As the transgenic *Drosophila* displayed myonuclear defects, the myonuclei were examined in patient biopsies. In myonuclei from healthy individuals, SUN2 localized at the nuclear periphery (Fig. 7A, control); but in patient biopsies, a fraction of the myofibers ($\sim 0.5\%$) contained SUN2 aggregating at non-nuclear sites (Fig. 7A). Likewise, control samples showed that the FG-repeat NPCs localized at the nuclear periphery. However, in up to 0.5% of the muscle fibers examined from patient tissue, anti-nuclear

pore protein staining was also cytoplasmic, similar to what had been observed in the *Drosophila* muscle (Fig. 7B). The NPC protein formed foci of various sizes and fluorescent intensity (Fig. 7B).

To our knowledge, this unusual localization of NPCs has not been reported for *LMNA*-related muscular dystrophies. We wondered whether cytoplasmic NPC staining could be due to myofiber damage and cell necrosis. This hypothesis was tested in human biopsies by simultaneously staining with antibodies against NPCs and dystrophin (Fig. 8). The analysis revealed that dystrophin staining in myofibers with cytoplasmic NPCs (average of 10 fibers gave 240 ± 8 arbitrary units) was similar to that of fibers without cytoplasmic NPCs (average of 30 fibers gave 247 ± 3 arbitrary units). Fiber integrity was also tested in the *Drosophila* muscle by staining larval body wall muscle from transgenic flies expressing wild-type and mutant Lamin C with Evans Blue dye, which only penetrates cells in which the membrane is disrupted. Muscle cells of both genotypes showed no blue staining, indicating that the muscle fibers were intact, whereas injured control cells showed intense blue staining (data not shown). Thus, analysis of biopsies from patient muscle revealed that SUN domain and NPCs were miscompartmentalized in intact muscle fibers, a remarkably similar observation to that observed in the *Drosophila* model.

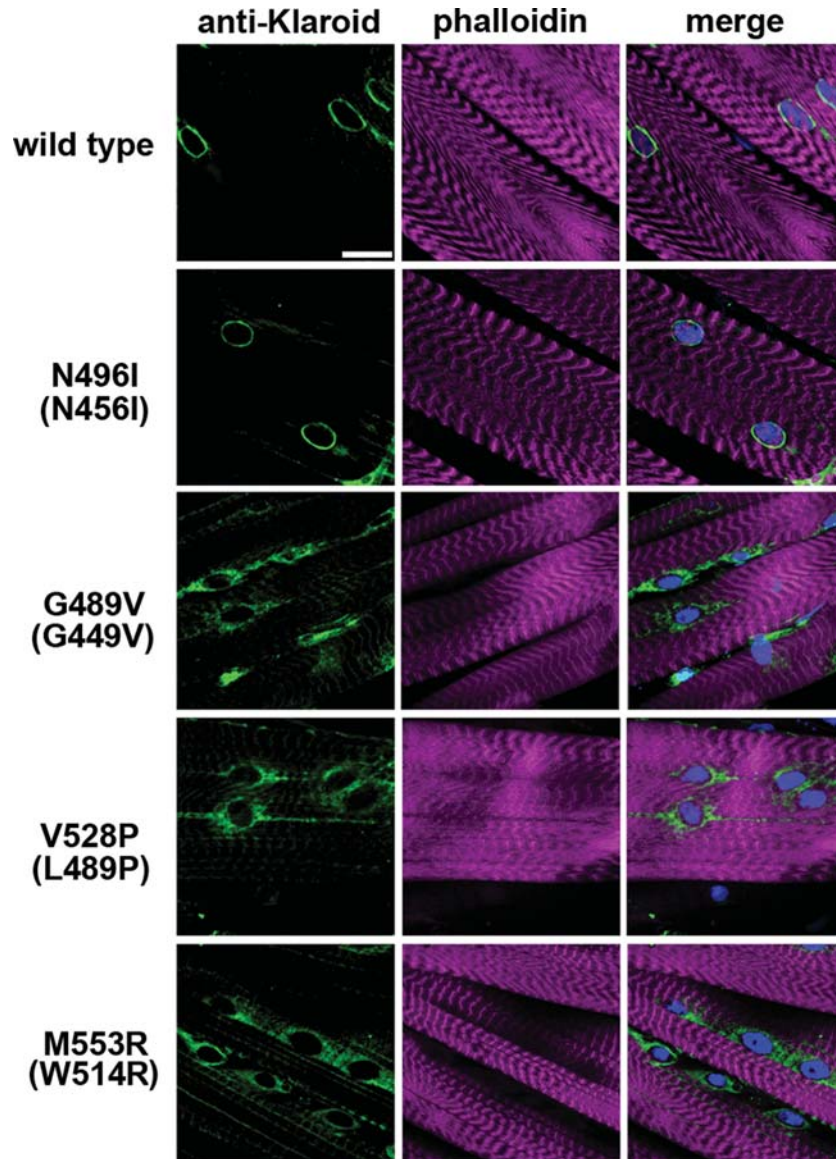


Figure 4. Expression of mutant forms of Lamin C in larval muscle causes mislocalization of Klaroid. Staining of third instar larval body wall muscle fillets with anti-Klaroid (green), phalloidin (purple) and DAPI (merge, blue) from larvae expressing wild-type Lamin C (wild-type) or mutant Lamin C transgenes by the muscle-specific driver C57. *Drosophila* amino-acid numbers are shown with human numbers in parentheses. Bar, 10 μ m.

To determine how general NPC mislocalization was in cases of *LMNA*-related muscular dystrophy, we examined tissues from additional AD-EDMD patients in which mutations affected the rod domain (R541K) or the Ig-fold (E358K, R541P and R453W). To determine whether NPC mislocalization was specific for *LMNA*-related muscular dystrophies, we examined tissues from patients with muscle disease not due to mutations in *LMNA*, namely Duchenne muscular dystrophy (DMD) and facioscapulohumeral muscular dystrophy (FSHD). Muscle tissues were stained with antibodies to FG-repeat-containing NPCs, phalloidin and DAPI, and 600–1000 muscle fibers per sample were examined. Again, control samples showed normal localization of the nuclear pore proteins; no detectable signal appeared outside the myonuclei of the muscle fibers (Fig. 9). In contrast, both *LMNA*-mutated samples (E358K and R541P, Lamin A/C

mutations) contained a small percentage (up to 0.5%) of myofibers with cytoplasmic NPCs, similar to our three new patient mutations. Both non-*LMNA*-associated muscular dystrophies did not show this phenotype. Thus, we propose that the NPC localization defect is not confined to amino-acid substitutions within the Ig-fold and might be a general feature of *LMNA*-associated muscular dystrophies.

DISCUSSION

Drosophila as a model for testing novel *LMNA* mutations

We have developed a *Drosophila* model for the study of human laminopathies. This model is possible in part, because *Drosophila* (unlike other invertebrate model organisms) possess the A- and B-type lamins found in humans

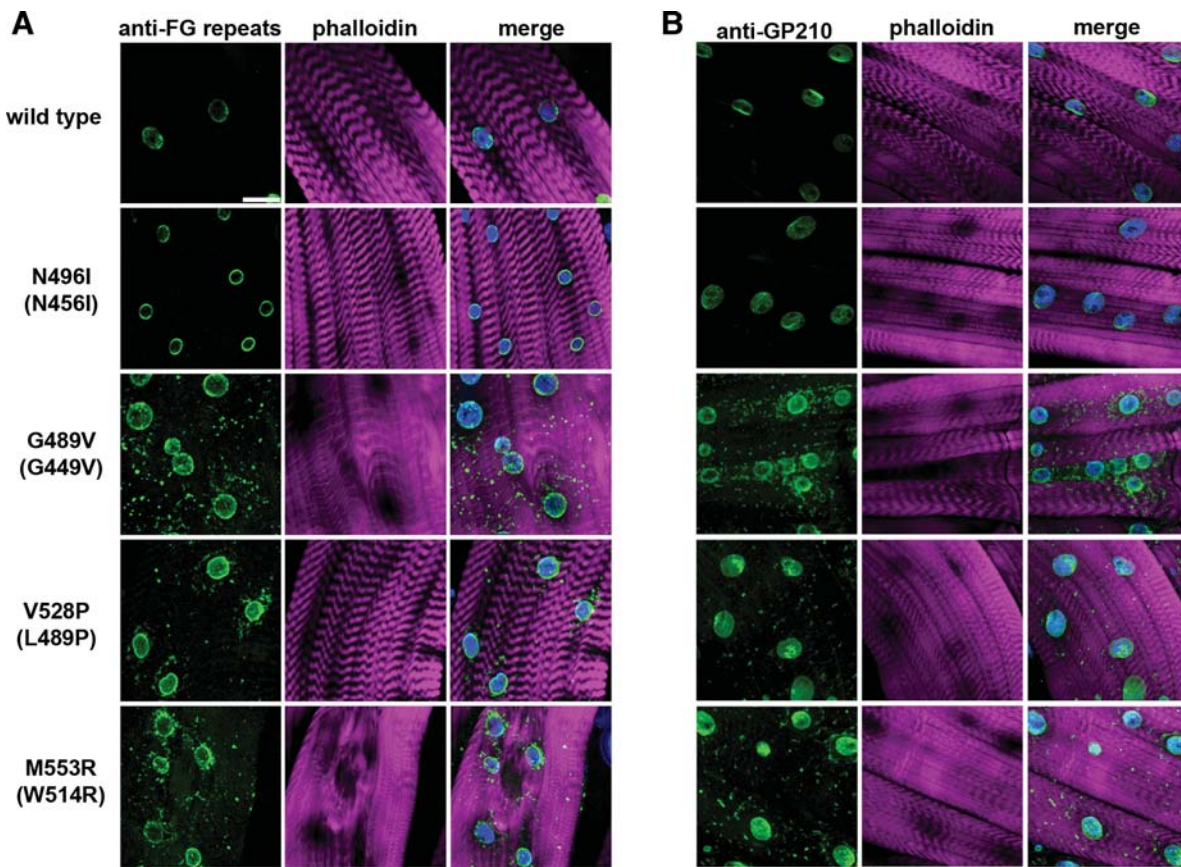


Figure 5. Expression of mutant forms of Lamin C in larval muscle causes mislocalization of nuclear pore proteins. Staining of third instar larval body wall muscle fillets with an antibody that recognizes nuclear pores possessing an FG-repeat (A, green), and the nuclear pore protein GP210 (B, green), phalloidin (A and B, purple) and DAPI (A and B/merge, blue) from larvae expressing wild-type Lamin C (wild-type) or mutant Lamin C transgenes by the muscle-specific driver C57. *Drosophila* amino-acid numbers are shown with human numbering in parentheses. Bar, 10 μ m.

(11,39,55). Moreover, 70% of the amino-acid residues altered in laminopathies affect residues conserved in the *Drosophila* A-type lamin. All of the Ig-fold variants tested here caused dominant muscle defects, similar to laminopathies in humans, suggesting that *Drosophila* can be used to assay the function of novel sequence variants for their pathogenicity.

The *Drosophila* model can also be used to provide insights on the mysteries surrounding the molecular defects caused by mutant lamins. The availability of larval muscle allows for relatively quick and easy cytological and biochemical analyses of candidate proteins. For example, a collection of antibodies could be used to assay for protein mislocalization, and the key findings validated using the limited human tissue. This approach rapidly led us to the identification of SUN2 and nuclear pore proteins in the cytoplasm of the patients, which might be useful as a histochemical marker for *LMNA*-associated muscle disease (Fig. 7).

It is unclear as to how mutant lamins cause muscle disease. The Ig-fold mutant N496I (known to cause AD-EDMD) had relatively little effect on nuclear shape, location and nuclear envelope protein cellular compartmentalization (Figs 3–5), yet it caused locomotion defects and lethality at the pupal stage (Fig. 2 and Table 2). Expression of the Ig-fold variants discovered in the three patients caused nearly an identical locomotion defect and stage of death, yet had gross cellular

defects in \sim 30% of muscle fibers (Figs 3–5). These data suggest that different substitutions exhibit a range of phenotypes, yet all converge on lethality, and that the *Drosophila* expression system might provide a means of classifying newly isolated substitutions.

Contributions of the Ig-fold to *LMNA*-associated pathology

Lamins play critical roles in nuclear structure, shape, mechanical stability and chromatin organization (56,57) and in doing so, they interact with numerous proteins in the nuclear envelope and nucleoplasm (58). Proteins within the inner membrane appear to rely on the lamin network for stable association (59–62). The three pathogenic amino-acid substitutions we identified in Lamin A/C—G449V, L489P and W514R—are located in AB, C'E and EF loops of the Ig-fold, respectively (41) (Fig. 1B). L489 contacts residues of the two main β sheets, stabilizing the β sandwich and the positioning the BC and C'E loops. The W514 residue, along with other residues (W520 and W498), likely plays a role in positioning of the loop β 7 β 8 (41). G449 lies within the AB loop, the most flexible loop region that is the least well defined within the Ig-fold structure. Replacement of G449 by the bulkier and hydrophobic V might destabilize the Ig-fold structure. This idea is supported by the fact that the

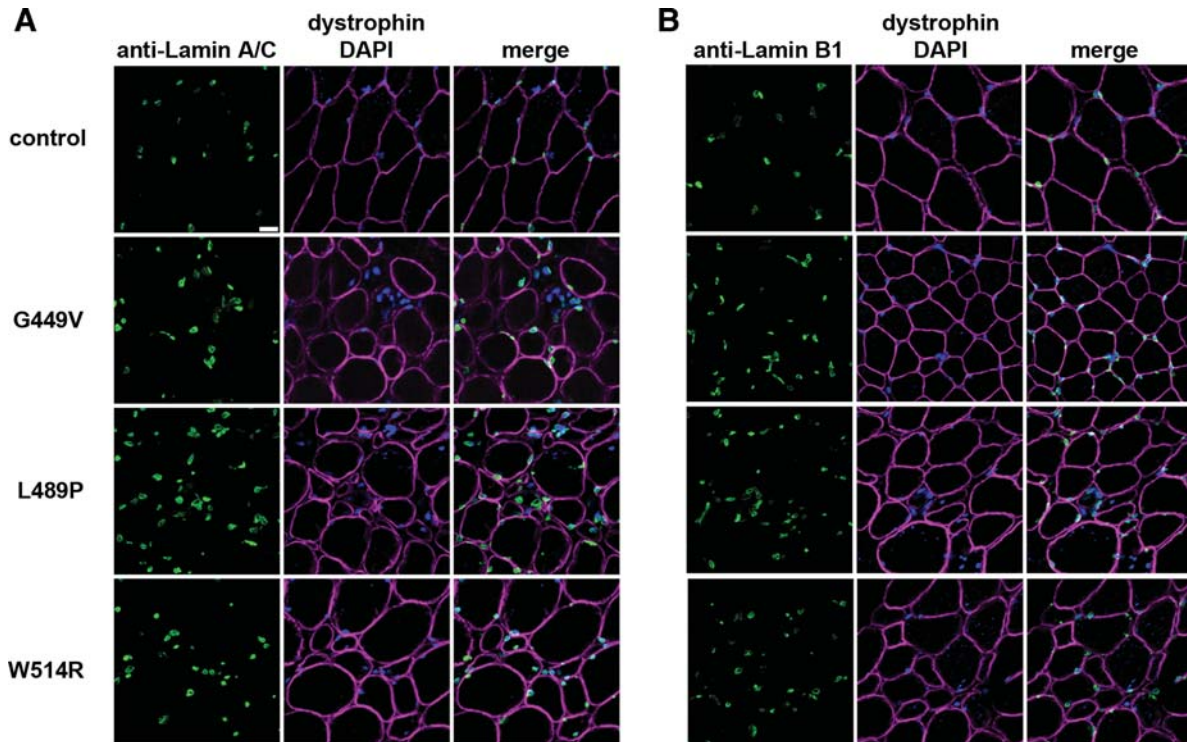


Figure 6. Dystrophic features in human patient skeletal muscle biopsies. Staining of human muscle biopsies from healthy individuals (control) or novel *LMNA* mutation patients (G449V, L489P, W514R) with anti-Lamin A/C (**A**, green), anti-Lamin B1 (**B**, green), anti-dystrophin (**A** and **B**, purple) and DAPI (**A** and **B**, blue). Bar, 10 μ m.

apparent melting temperature of this Ig-fold variant is dramatically less than that of the wild-type Ig-fold (O. Shresthna and L.L.W., unpublished). As the Ig-fold is known to interact with other proteins, amino-acid substitutions at these residues might affect the Ig-fold structure in such a way so as to disrupt crucial interactions with these proteins.

The new mutations we identified might disrupt (a) polymerization or association/dissociation kinetics, (b) post-translational modifications of A-type lamins or (c) protein–protein interactions. It is worth pointing out that the Ig-fold domain is the site of many partner associations (63). The C-terminal SUN domain of both Sun1 and Sun2 project into the perinuclear space, whereas their N-terminal region is nucleoplasmic and interacts directly with the Ig-fold of A- and B-type lamins (64–68). Lamins also interact with Nup88 and Nup153 through a region containing the Ig-fold (69,70). Connections with the Ig-fold might play a role in anchoring these proteins or complexes containing these proteins.

Altered cellular compartmentalization as a disease mechanism

Loss of cellular compartmentalization was recently observed in primary fibroblasts from patients with laminopathies (71). In these cases, transcription factors normally enriched in the cytoplasm or the nucleus under defined conditions were observed in the incorrect cellular compartment. Improper localization appeared to be caused by transient, non-lethal,

rupture of the nuclear envelope. Imaging data in the literature support the notion of nuclear envelope rupture in conjunction with mutant lamins. Electron microscopy (EM) images of tissue from patients with muscle-specific laminopathies show dilated perinuclear space, regional absence of lamina and extrusion of chromatin from the nucleus (71–73). In addition, EM images of *Drosophila Lamin C* null larvae show similar results including regional discontinuity between the inner and outer nuclear membranes and large ‘holes’ in the nuclear envelope (39). Therefore, rupture of the nuclear envelope could explain the appearance of SUN domain proteins and nuclear pores within the cytoplasm, as released fragments of the NE or miscompartmentalization of the proteins during membrane rupture.

MATERIALS AND METHODS

Site-directed mutagenesis

Constructs encoding the mutant forms of *Drosophila Lamin C* were generated using the Quick Change II Site Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The sequences of the PCR primers used for mutagenesis were as follows:

G489V forward, 5'-GGCGGACGTTGAGGTACGCTTCA TCAAGCTGC-3'; G489V reverse, 5'-GCAGCTTGATGAA GCGTACCTCAACGTCCGCC-3'; N496I forward, 5'-GCTT CATCAAGCTGCACATCAAGGGAACCGAGGAG-3'; N496I reverse, 5'-CTCCTCGGTTCCCTTGATGTGCAGCTT

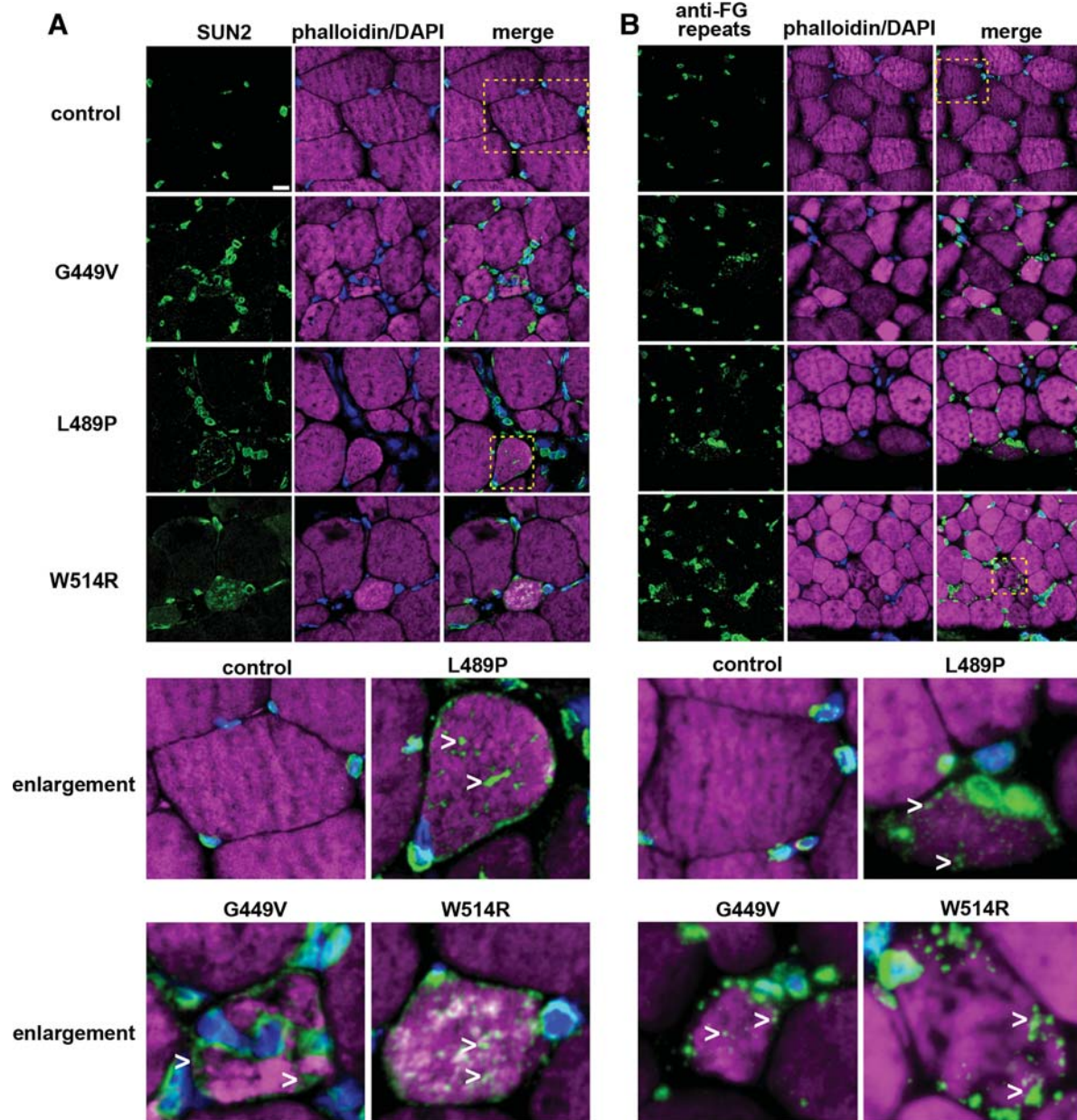


Figure 7. Mislocalization of SUN2 and nuclear pore proteins in human patient skeletal muscle biopsies. Staining of human muscle biopsies from healthy individuals (control) or novel *LMNA* mutation patients (G449V, L489P, W514R) with anti-SUN2 (A, green), antibodies to nuclear pore proteins possessing FG repeats (B, green), phalloidin (A and B, purple) and DAPI (A and B, blue). Areas boxed in yellow are shown as enlargements below. Arrowheads indicate cytoplasmic localization of SUN2 and FG-repeat containing proteins in skeletal muscle fibers. Bar, 10 μ m.

GATGAAGC-3'; V528P forward, 5'-CGCGCGGATCGAAG CCACTCGGCGGTGCTAGTG-3'; V528P reverse, 5'-CAC TAGCACC GCCGAGTGGCTTCGATCCGCGCG-3'; M553R forward, 5'-CCCAACAATCGGTGCGGAAGAAGAAGTGG CCG-3'; M553R reverse, 5'-CGGCCACTTCTTCTCCG CACCAGATTGTTGGG-3'.

The primers contain nucleotide substitutions that result in single amino-acid substitutions within Lamin C. The mutated *Lamin C* sequences were cloned into the P-element transformation vector pUAST (74) and used to generate transgenic stocks.

Drosophila stocks and genetic analyses

Drosophila stocks were raised at room temperature on standard sucrose and cornmeal medium (75). Generation of transgenic stocks expressing wild-type and Lamin C Δ N was previously reported (39,40). The GAL4-UAS system (43,74) was used to drive expression of wild-type and mutant forms of Lamin C in specific tissues. The GAL4 driver stocks used in this study were obtained from the Bloomington Stock Center (Act5C, Eyeless, MEF2) and a gift from W. Maddox (MD Anderson Cancer Center) (MHC) and a gift from

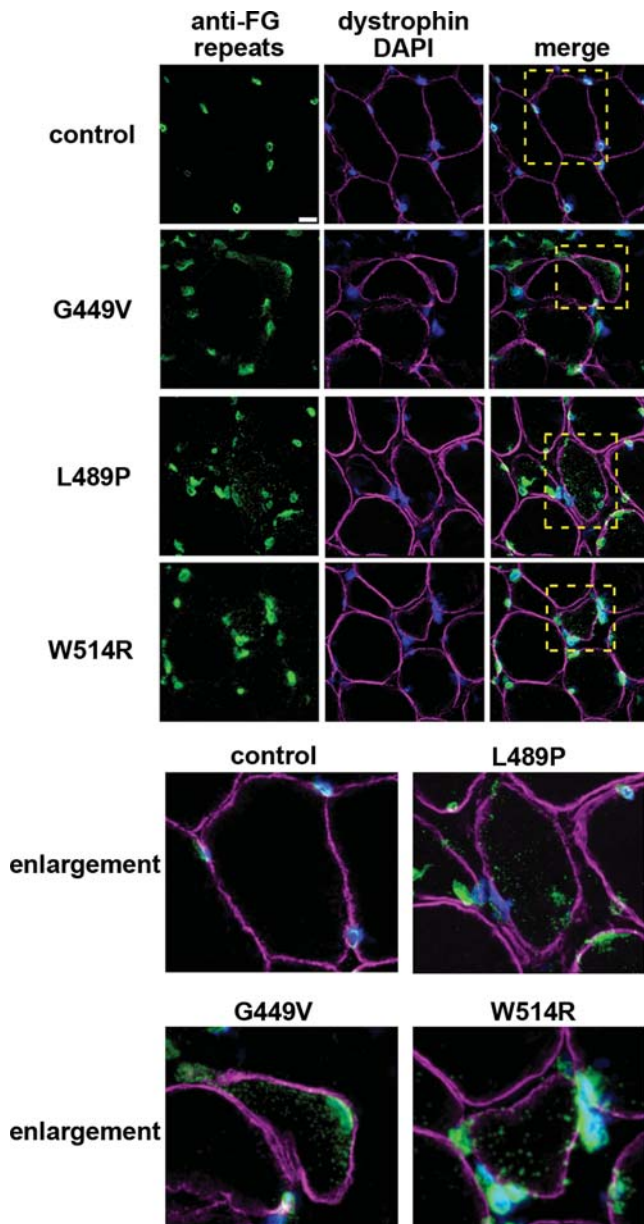


Figure 8. Intact myofibers showing mislocalization of nuclear pore complexes in human patient skeletal muscle biopsies. Staining of human muscle biopsies from healthy individuals (control) or novel *LMNA* mutation patients (G449V, L489P, W514R) with antibodies that recognize nuclear pore proteins possessing FG repeats (green), anti-dystrophin (purple) and DAPI (blue). Areas boxed in yellow are enlarged below. Asterisks indicate cytoplasmic localization of NPCs in skeletal muscle fibers. Bar, 10 μ m.

V. Budnik (Department of Neurobiology, University of Massachusetts, Worcester, MA, USA) (76).

Immunostaining of muscles

Larval muscle preparations were performed according to the published procedures (77). After fixation, the dissected body wall preparations were blocked in PBS2+ (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 10 mM ethylene glycol tetraacetic acid, 0.1% Triton-X 100) containing 0.1% bovine serum

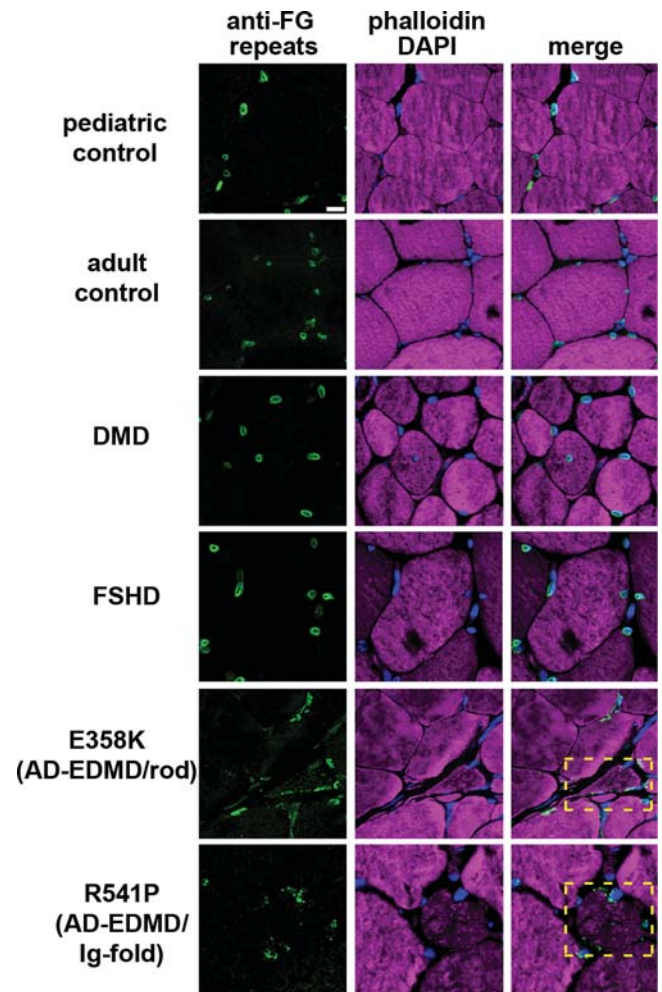


Figure 9. Mislocalization of nuclear pore proteins in AD-EDMD, but not other types of MD patients. Staining of human muscle biopsies from healthy individuals (pediatric and adult control) or patients with *LMNA* mutations causing AD-EDMD (E358K/rod, R511P/Ig-fold), LGMD (R453W/Ig-fold), DMD or FSHD with antibodies that recognize nuclear pores possessing FG repeats (green), phalloidin (purple) and DAPI (blue). Area boxed in yellow shows cytoplasmic distribution of nuclear pore proteins. Bar, 10 μ m.

albumin (BSA) for 1 h. Muscle preparations were stained with 66 nM fluorescent Phalloidin (Alexa Fluor 488 from Molecular Probes) in PBS2+ containing 0.1% BSA, rat anti-tyrosinated tubulin (1:50 dilution). Peripheral nuclear pore proteins were detected using MAb414 (anti-mouse, 1:3000 dilution; Covance; recognizes the FXFG repeat sequence in nucleoporins, a related family of NPC proteins including p62, p152 and p90) and transmembrane nuclear pore protein GP210 was detected using AGP26.10 (anti-mouse, 1:50 dilution) obtained from the University of Iowa Hybridoma Core facility. Klaroid was detected using rat anti-Klaroid (1:20 dilution; anti-Koi, a generous gift from J. Fischer, University of Texas, Austin, TX, USA). *Drosophila* Lamin C was detected using LC28.26 (anti-mouse, 1:400 dilution), *Drosophila* lamin Dm₀ using ADL84.12 (anti-mouse, 1:400 dilution) and *Drosophila* HP1a using C1A9 (anti-mouse, 1:100 dilution) obtained from the University of Iowa Hybridoma Core facility. Imaging was performed on a Bio-Rad MRC 1024 confocal

microscope (Center for Microscopy, University of Iowa, IA, USA).

For immunohistochemistry of human tissue, quadriceps muscle biopsies were performed. Each patient sample showed evidence of necrotizing myopathy: scattered muscle fibers undergoing myonecrosis or regeneration without lymphocytic inflammatory infiltrates. A mixture of atrophic and hypertrophic fibers was present. Immunofluorescence studies for the dystrophin–glycoprotein complex and merosin were normal in all biopsies. Collagen VI was normal in the patients with L489P and W514R variants. A mild basement membrane reduction in collagen VI immunostaining was detected in the biopsy from the patient with the G449V variant. However, evaluation of fibroblast cultures from this patient revealed normal collagen VI synthesis and secretion, and no mutations in the *COL6* gene.

Western analysis

To determine the expression levels of *Drosophila* Lamin C, proteins were extracted from third instar larvae (78) and separated by size on 10–12% polyacrylamide gels, transferred to nitrocellulose membrane and incubated with anti-Lamin C LC28.26 anti-mouse IgG (11) used at 1:5000–1:8000 dilution or anti-alpha tubulin anti-mouse IgG1 [Sigma (St. Louis) no. T5168] used at 1:400 000 dilution. A horseradish peroxidase-conjugated anti-mouse IgG [Pierce (Rockford, IL, USA) no. 31446] used at a 1:20 000 dilution served as a secondary antibody. Detection was carried out using the SuperSignal West Pico chemiluminescent substrate (Pierce no. 34080). Signal from the membranes was collected from an Epi Chemi II dark-room unit fitted with a CCD camera (UVP, San Gabriel, CA, USA) and the resulting data were quantified using LabWorks Image Acquisition software (UVP) and/or Image J software (<http://rsb.info.nih.gov/ij/>). Three independent protein isolations were performed for each genotype.

Larval motility assays

Flies expressing wild-type or mutant forms of Lamin C were crossed to the C57 driver (76) and the resulting third instar larvae were used for analysis. Eight larvae per experiment were rinsed with ddH₂O and allowed to acclimate on a 1.5% agarose Petri plate for 10 min. The larvae were placed at the center of a 1.5% agarose Petri plate with four 1-in. concentric circles representing four zones and allowed to wander. After 2 min, total larvae in each circle were recorded. Data are displayed in a box plot using the computer program 'R' (79).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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