

RESEARCH PAPER

LMNA missense mutations causing familial partial lipodystrophy do not lead to an accumulation of prelamin A

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ABSTRACT

A variety of missense mutations in *LMNA* (the gene for lamin C and prelamin A) cause familial partial lipodystrophy (FPLD), a disease associated with reduced adipose tissue, particularly in the limbs. Several studies have reported that fibroblasts from FPLD subjects have an accumulation of prelamin A. Those findings were intriguing but also perplexing because many of the *LMNA* missense mutations associated with lipodystrophy are located in sequences distant from the sequences required for the farnesylation of prelamin A and ZMPSTE24-mediated conversion of prelamin A to mature lamin A. Here, we revisited the issue of prelamin A accumulation in the setting of FPLD mutations. We used western blots with lamin A/C antibodies and prelamin A-specific monoclonal antibodies to assess prelamin A levels in wild-type fibroblasts and fibroblasts carrying *LMNA* mutations associated with lipodystrophy (R482W, I299V, C591F, T528M). None of the mutant fibroblasts exhibited an accumulation of prelamin A. Also, the amount of prelamin A accumulation in response to lopinavir (an inhibitor of ZMPSTE24) was similar in wild-type and mutant fibroblasts. Thus, the *LMNA* lipodystrophy mutations that we examined did not lead to prelamin A accumulation, nor did they render those cells more susceptible to prelamin A accumulation when ZMPSTE24 was inhibited by lopinavir.

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Introduction

A variety of *LMNA* missense mutations cause familial partial lipodystrophy (FPLD2 or FPLD, Dunnigan Type), a disease associated with partial loss of adipose tissue from the limbs and trunk. Cao and Hegele¹ reported in 2000 that a heterozygous *LMNA* mutation, R482Q, causes FPLD2 and predisposes to hyperlipidemia, diabetes mellitus, and atherosclerotic heart disease. In the same year, Shackleton *et al.*² showed that R482W and R482L mutations cause FPLD2. Speckman *et al.*³ studied 15 FPLD families and found the R482W mutation in 7 families, a R482Q mutation in 5 families, and a G465D mutation in one family. Later, other *LMNA* mutations were identified in association with lipodystrophy (e.g., D230N, R399C, I299V, D47Y, L92F, L387V, R399H, L421P).^{4–6} Most of the mutations are located in sequences shared by lamin C and lamin A.

Loss of adipose tissue clearly occurs in the setting of “lamin A related” progeroid syndromes. Children with Hutchinson-Gilford progeria syndrome

(HGPS) exhibit a striking loss of subcutaneous adipose tissue.⁷ HGPS is caused by a mutation that alters *LMNA* splicing and results in an in-frame deletion of 50 amino acids in prelamin A.⁸ This deletion eliminates the ZMPSTE24 cleavage site and therefore prevents the processing step that would normally convert farnesyl-prelamin A to mature lamin A;⁹ consequently, a large amount of an internally truncated farnesyl-prelamin A (progerin) accumulates in cells. Progerin is toxic to cells and is responsible for all HGPS disease phenotypes, including the loss of adipose tissue. More recently, Wang *et al.*¹⁰ uncovered a subject with a *LMNA* missense mutation (L647R) that eliminates prelamin A's ZMPSTE24 cleavage site and thus causes an accumulation of farnesyl-prelamin A; that subject had multiple progeria-like disease phenotypes, including reduced adipose tissue. ZMPSTE24 missense mutations that reduce ZMPSTE24 catalytic activity also cause an accumulation of farnesyl-prelamin A,

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resulting in a progeroid syndrome with reduced adipose tissue.^{11,12} In the setting of these progeroid syndromes, the prelamin A that accumulates is farnesylated, as judged by metabolic labeling experiments.¹³⁻¹⁵

Farnesyl–prelamin A accumulates in fibroblasts treated with lopinavir, an HIV-protease inhibitor that binds to ZMPSTE24 and blocks its catalytic activity.^{14,16} The inhibition of ZMPSTE24 by lopinavir might be relevant to the loss of adipose tissue that occurs in lopinavir-treated HIV patients.¹⁷⁻¹⁹ Of note, the farnesyl–prelamin A that accumulates in lopinavir-treated cells is distinct from the prelamin A that accumulates with a protein farnesyltransferase inhibitor or an inhibitor of HMG-CoA reductase (*i.e.*, a statin); those drugs block protein farnesylation and lead to an accumulation of nonfarnesylated prelamin A.^{20,21}

The fact that “progeroid syndrome” mutations that block the processing of farnesyl–prelamin A are accompanied by reduced adipose tissue inevitably raised the question of whether *LMNA* missense mutations causing FPLD also result in prelamin A accumulation. In recent years, several groups performed protein gel blots on FPLD fibroblasts with commercial antibodies and concluded that FPLD mutations (including the classic R482W mutation) are associated with an accumulation of prelamin A.^{6,22-24} These reports were intriguing, but we reasoned that caution was warranted. First, it was unclear why the *LMNA* mutations causing FPLD would affect prelamin A processing, given that the responsible amino acid substitutions are widely dispersed within lamin A/C and most are not located in sequences important for prelamin A processing. Second, we have learned to be cautious about commercial antibodies against lamin A/C and prelamin A because some lots of those antibodies bind non-specifically to other proteins. For that reason, we generated 2 monoclonal antibodies against prelamin A; both are specific and sensitive in western blot and immunocytochemistry studies.²⁵⁻²⁸

In the current studies, we used a polyclonal antibody against lamin A/C and the prelamin A monoclonal antibodies to determine if prelamin A accumulates in FPLD fibroblasts. We also tested whether FPLD fibroblasts would manifest an exaggerated accumulation of prelamin A in the presence of lopinavir.

Methods

Fibroblasts from FPLD subjects

Fibroblasts were isolated by 2 of the authors (D.A.-V. and S.S.-I.) from skin biopsies of normal subjects and FPLD subjects with one of 4 *LMNA* mutations (R482W, C591F, T528M, I299V). This study was approved by the Ethics Review Panel of the Conselleria de Sanidade (Xunta de Galicia, Spain) and carried out according to the ethical guidelines of the Declaration of Helsinki. All of the patients provided informed consent for participation in the study and publication of their clinical, and genetic information. The identity of the fibroblasts was confirmed at UCLA by DNA sequencing. Fibroblasts were cultured in 5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium (Gibco, Life Technologies) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM glutamine, 10 units/ml penicillin, and 10 µg/ml streptomycin (Gibco). Most studies were performed on fibroblasts that had been seeded into 12-well plates. In some studies, the cells were treated with a protein farnesyltransferase inhibitor (FTI) (lonafarnib, 0.5 µM final concentration); an HIV protease inhibitor (lopinavir, 20 to 30 µM final concentration); or vehicle (DMSO) alone at 37°C for 72 h. Lopinavir was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program; lonafarnib was provided by Schering-Plough, Kenilworth, NJ. In some studies, fibroblasts were grown in medium containing lopinavir (25 µM) and in medium containing from 0 to 50% FBS. In other studies, fibroblasts were grown in medium containing bovine serum albumin (BSA; 1–30 mg/ml), 3% FBS, and either an FTI (0.5 µM) or lopinavir (25 µM).

Western blots

Urea-soluble extracts of fibroblasts were prepared as described.²⁹ Proteins were size-fractionated on 4–12% gradient polyacrylamide Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane. Western blots were performed with a goat polyclonal antibody against lamin A/C (1:400; sc-6215, Santa Cruz Biotechnology); a goat polyclonal antibody against actin (1:2,000; sc-1616, Santa Cruz Biotechnology); a rat monoclonal antibody (7G11) that binds to nonfarnesylated prelamin A; or a rat monoclonal antibody (3C8) that binds to both farnesylated and nonfarnesylated prelamin A. Antibody 3C8 was labeled directly

with an infrared dye with the DyLight Antibody Labeling Kit 53026 (Thermo Scientific). Binding of polyclonal antibodies and antibody 7G11 was detected with a secondary antibody labeled with an infrared dye (Rockland Immunochemicals). The intensity of prelamin A bands, relative to lamin C or actin, was quantified with an Odyssey infrared scanner (Li-Cor).

We also assessed the effects of lopinavir on prelamin A accumulation in primary embryonic fibroblasts from wild-type mice and 2 littermates that were heterozygous for a knockout mutation in *Zmpste24* (*Zmpste24*^{+/-}).

Quantitative RT-PCR

RNA was isolated with RNeasy Mini Kit (QIAGEN) and treated with DNase I (Ambion, Life Technologies); it was then reverse transcribed with oligo(dT), random primers, and SuperScript III (Invitrogen). Quantitative RT-PCR (qPCR) reactions were performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems) with SYBR Green PCR Master Mix (Bioline). Transcript levels were determined by the comparative cycle threshold method and normalized to *GAPDH* for human samples and cyclophilin A for mouse samples using the 2- $\Delta\Delta$ CT method.³⁰ Primers for human *LMNA* were 5'-ATGAGGATGGAGATGACCTGC-3' and 5'-AGGCAGAAGAGCCAGAGGAGA-3' (exons 10 and 11, respectively); primers for human *ZMPSTE24* were 5'-GGAAGTTGGGACATACAGTCAAA-3' and 5'-TCCTTTCGACCAATTAATACAGC-3' (exons 8 and 9, respectively); another set of primers for human *ZMPSTE24* was 5'-TATGAGATCACTCAGTCCCTGGT-3' and 5'-AGTCTGTTGATTGAAGCCATGTT-3' (exons 3/4 and 4/5, respectively). Primers for mouse *Lmna* were 5'-GGTTGAGGACAATGAGGATGA-3' and 5'-TGAGCGCAGGTTGTACTCAG-3' (exons 10 and 11, respectively). Primers for mouse *Zmpste24* were 5'-ACTGGAAGTTGGGACACACAGTA-3' and 5'-TTCCCTTCGACC AATTAATACAG-3' (exons 8 and 9, respectively).

Results

We examined prelamin A levels in wild-type fibroblasts and fibroblasts from FPLD subjects carrying one of 4 *LMNA* mutations (R482W, C591F, T528M, I299V). The R482W mutation is associated with classic FPLD2 and has been encountered by multiple investigators.^{2,3,22,31} The C591F mutation causes an

atypical FPLD accompanied by hypertrophic cardiomyopathy.³² A T528M mutation was encountered in 2 sisters with typical FPLD2; this mutation was identified previously in FPLD subjects (compound heterozygotes who also carried an S583L mutation)³³ and in a subject with a progeroid syndrome (a compound heterozygote who also carried an M540T mutation).³⁴ The I299V mutation was identified in an atypical FPLD subject (severe insulin resistance, an absence of adipose tissue in limbs and buttocks but increased subcutaneous adipose tissue and increased adipose tissue in the trunk).⁵

After verifying the identity of R482W, I299V, C591F, and T528M fibroblasts by DNA sequencing (Fig. 1), we used protein gel blots to determine if fibroblasts from 2 R482W FPLD subjects (L8, L160) contained more prelamin A than fibroblasts from a wild-type subject (C9). In the absence of lopinavir or a farnesyltransferase inhibitor (FTI; lonafarnib), prelamin A could not be detected in either wild-type or R482W fibroblasts, as judged by western blots with a polyclonal lamin A/C antibody (Fig. 2A–B) or 2 different prelamin A-specific monoclonal antibodies (3C8, 7G11) (Fig. 2C–D). When lopinavir was added to the medium, the amount of farnesyl-prelamin A was similar in wild-type and R482W fibroblasts (Fig. 2A–C). Antibody 7G11 is specific for nonfarnesylated prelamin A; consequently, it did not bind to prelamin A in lopinavir-treated fibroblasts (Fig. 2D). As expected, the electrophoretic mobility of the farnesyl-prelamin A in lopinavir-treated cells was slightly faster than that of nonfarnesylated prelamin A in FTI-treated cells (Fig. 2A–C).

Similar results were observed in T528M and C591F fibroblasts (Fig. 3). In the absence of an FTI or lopinavir (Fig. 3), no prelamin A was found in T528M and C591F fibroblasts. Also, adding lopinavir to the medium did not lead to exaggerated accumulation of prelamin A (Fig. 3). Similar findings were observed in I299V fibroblasts (Fig. 4).

A recent study pointed out that lopinavir is protein-bound and that the amount of prelamin A accumulation in peripheral blood mononuclear cells is reduced when the cells are cultured in a medium with high concentrations of protein.³⁵ We obtained similar results in fibroblasts; increased amounts of fetal bovine serum in the medium reduced the amount of prelamin A accumulation during lopinavir treatment (Fig. S1). Adding bovine serum albumin (BSA) to the

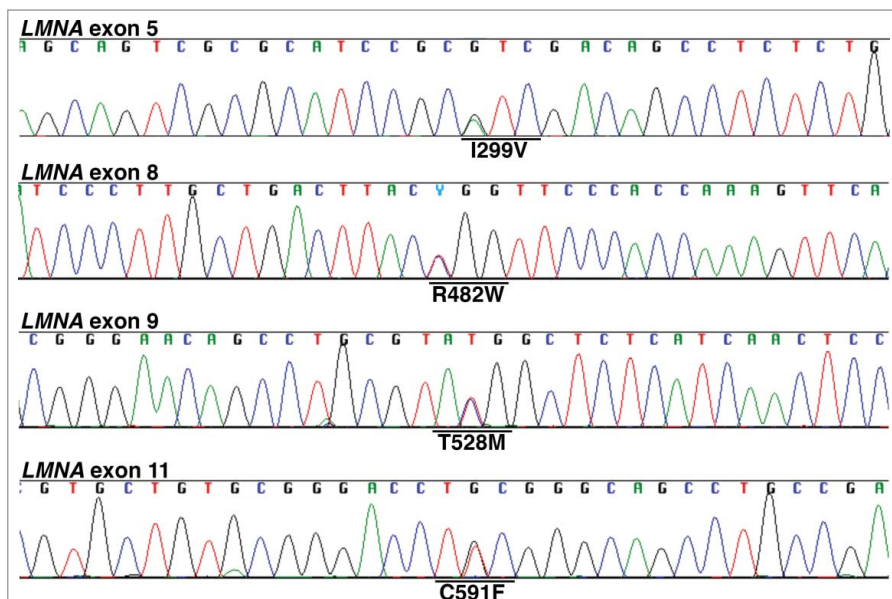


Figure 1. DNA sequencing chromatograms to verify the identity of R482W, T528M, C591F, and I299V fibroblasts. Sequencing chromatograms were obtained on cDNA amplicons generated from fibroblast RNA. All fibroblasts were heterozygous for the indicated *LMNA* mutation.

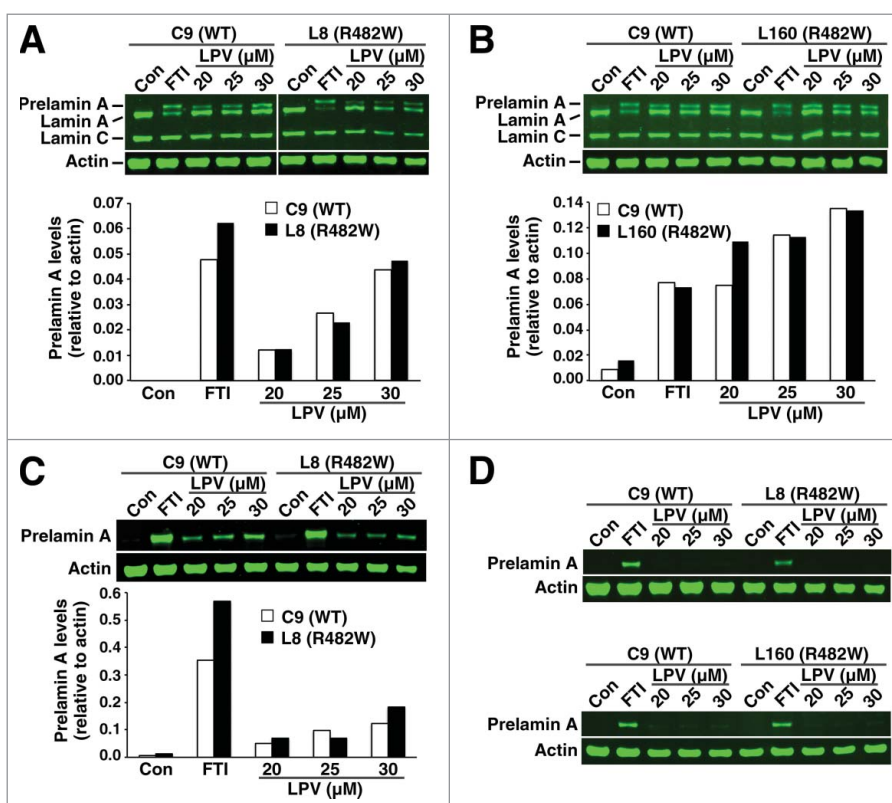


Figure 2. Western blots of cell extracts from wild-type (C9) and R482W fibroblasts (L8 and L160) with laminin A/C- and prelamin A-specific antibodies. Fibroblasts were grown in the absence of a drug; with a protein farnesyltransferase inhibitor (FTI); or with lopinavir (LPV), an HIV protease inhibitor that binds to ZMPSTE24 and inhibits catalytic activity. (A–B) Western blots with a polyclonal laminin A/C antibody. Bar graphs show the amount of prelamin A, relative to actin, as judged by an Odyssey infrared scanner. (C) Western blot with a prelamin A monoclonal antibody, 3C8, that recognizes both farnesylated and nonfarnesylated prelamin A. (D) Blot with a prelamin A monoclonal antibody, 7G11, that recognizes only nonfarnesylated prelamin A.

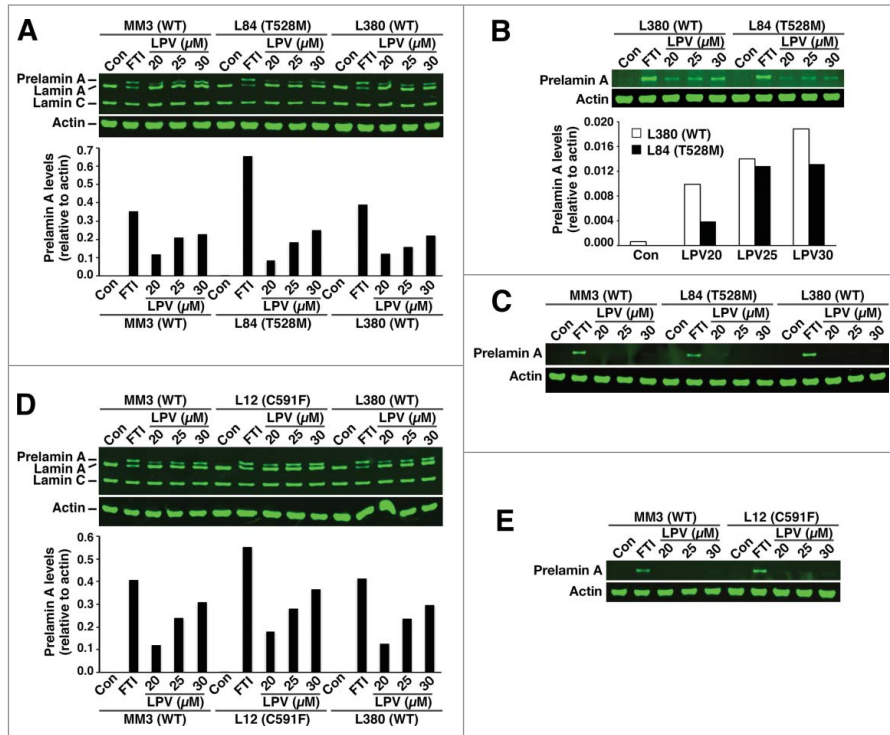


Figure 3. Western blots of cell extracts from wild-type fibroblasts (MM3 and L380) and FPLD fibroblasts T528M (L84) and C591F (L12). (A–C) Wild-type and T528M fibroblasts were grown without a drug or in the presence of a protein farnesyltransferase inhibitor (FTI) or lopinavir (LPV). (A) Western blot of fibroblast extracts with the lamin A/C polyclonal antibody. Bar graph shows the amount of prelamin A, relative to actin, as judged by an Odyssey infrared scanner. (B) Western blot of fibroblast extracts with the prelamin A-specific antibody 3C8. (C) Western blot with the prelamin A-specific antibody 7G11. (D–E) Wild-type and C591F fibroblasts were grown without a drug or in the presence of an FTI or LPV. Western blots of cell extracts were performed with the lamin A/C polyclonal antibody (D) or antibody 7G11 (E).

cell culture medium also reduced lopinavir-induced prelamin A accumulation (Fig. S2). In contrast, BSA had no effect on the accumulation of nonfarnesylated prelamin A in FTI-treated cells (Fig. S2).

The fact that prelamin A accumulation in response to lopinavir was similar in FPLD and wild-type fibroblasts implies that ZMPSTE24 activity is also similar in wild-type and mutant fibroblasts. If ZMPSTE24

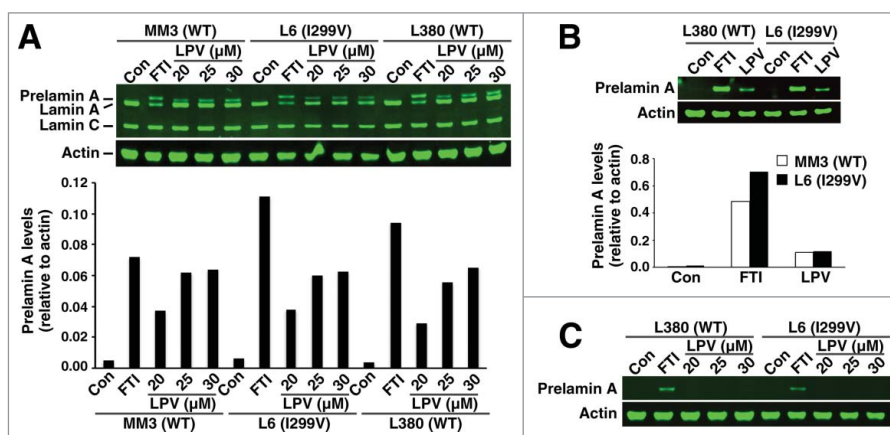


Figure 4. Western blots of cell extracts from wild-type fibroblasts (MM3 and L380) and I299V fibroblasts (L6). Wild-type and I299V fibroblasts were grown without a drug or in the presence of a protein farnesyltransferase inhibitor (FTI) or lopinavir (LPV). (A) Western blot of fibroblast extracts with the lamin A/C polyclonal antibody. Bar graph shows the amounts of prelamin A, relative to actin, as judged by an Odyssey infrared scanner. (B) Western blot of fibroblast extracts with antibody 3C8. The LPV concentration was 25 μM . Bar graph shows the amounts of prelamin A, relative to actin. (C) Western blot of fibroblast extracts with antibody 7G11.

expression had been reduced in FPLD fibroblasts, more prelamin A would have accumulated during lopinavir treatment. In control studies, we found that half-normal amounts of ZMPSTE24 expression in *Zmpste24*^{+/-} fibroblasts is accompanied by twofold greater accumulation of prelamin A during lopinavir treatment (Fig. 5A–D). As expected, *Zmpste24* transcripts were reduced by 50% in *Zmpste24*^{+/-} fibroblasts (Fig. 5E). We also measured ZMPSTE24 transcript levels by qRT-PCR in wild-type and FPLD fibroblasts with 2 different oligonucleotide primer pairs; we observed no evidence for reduced ZMPSTE24 expression in FPLD fibroblasts (Fig. 6A–B). Similarly, there appeared to be no effect of the FPLD mutations on prelamin A transcript levels (Fig. 6C).

Discussion

Rare mutations underlying progeroid syndromes, for example the *LMNA* mutation causing HGPS and *ZMPSTE24* missense mutations that impair *ZMPSTE24* activity, interfere directly with the conversion of farnesyl–prelamin A to lamin A.^{7,10–12} In those cases, the mechanisms for prelamin A accumulation are straightforward and make perfect sense. Several publications have suggested that the *LMNA* mutations causing FPLD, including the classic R482W mutation, also cause an accumulation of prelamin A—and that this accumulation is evident in cultured fibroblasts.^{6,22–24} Prelamin A accumulation in the setting of FPLD mutations is more difficult to understand, given

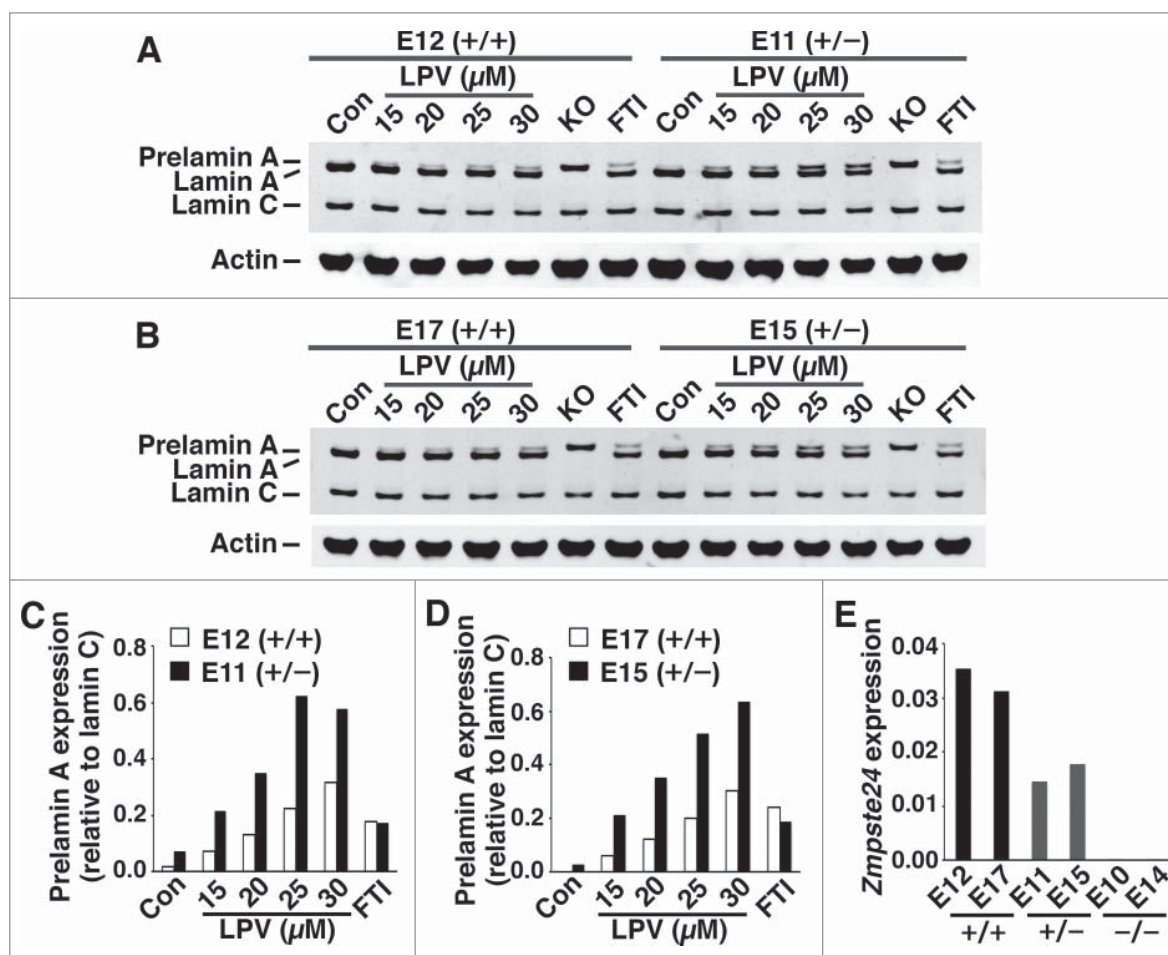


Figure 5. Prelamin A accumulation in wild-type mouse fibroblasts (*Zmpste24*^{+/+}) and heterozygous *Zmpste24* knockout fibroblasts (*Zmpste24*^{+/-}) in response to lopinavir treatment. (A–B) Western blots, using a lamin A/C polyclonal antibody, of extracts from *Zmpste24*^{+/+} fibroblasts (E12, E17) and *Zmpste24*^{+/-} fibroblasts (E11, E15) after lopinavir treatment. In these studies, *Zmpste24*^{-/-} fibroblasts were included as controls (KO). (C–D) Bar graphs show amounts of prelamin A, relative to lamin C, in fibroblasts E12 and E11 (C) and in fibroblasts E17 and E15 (D). (E) Half-normal levels of *Zmpste24* transcripts in *Zmpste24*^{+/-} fibroblasts (E11, E15), compared with *Zmpste24*^{+/+} fibroblasts (E12, E17). As controls, we included 2 different *Zmpste24*^{-/-} fibroblast cell lines (E10, E14).

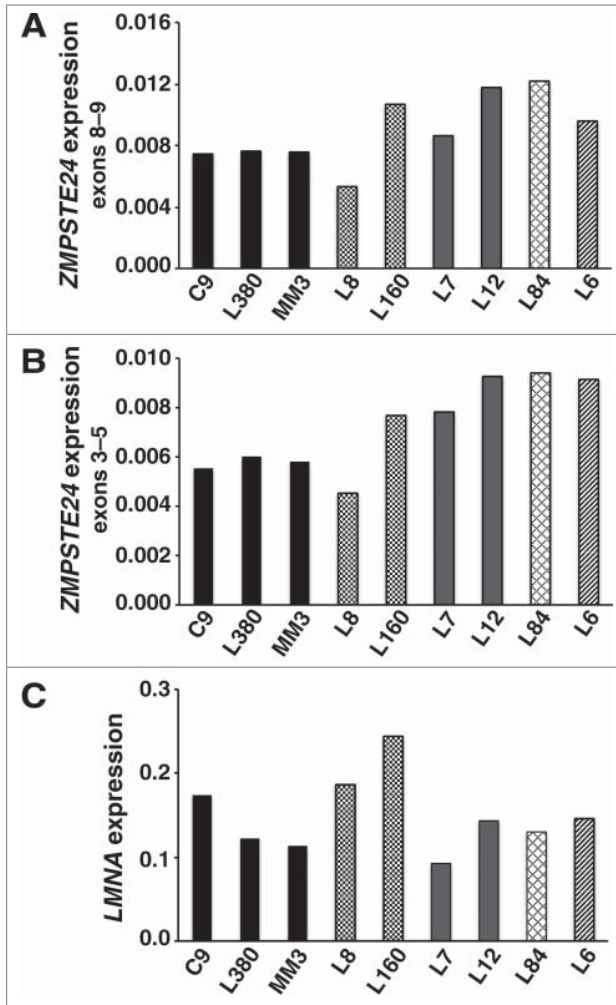


Figure 6. Transcript levels in wild-type fibroblasts and fibroblasts from FPLD subjects. (A–B) *ZMPSTE24* transcript levels, as judged by qRT-PCR with 2 primer pairs (exons 8–9 and exons 3–5). (C) *LMNA* transcript levels with a primer pair that detects prelamin A transcripts. Wild-type fibroblasts: C9, L380, MM3. R482W fibroblasts: L8, L160. C591F fibroblasts: L7, L12. T528M fibroblasts: L84. I299V fibroblasts: L6.

that most of those mutations are located in sequences distant from those important for prelamin A processing. In the current study, we revisited whether FPLD mutations lead to prelamin A accumulation in fibroblasts. We examined fibroblasts from subjects with 4 *LMNA* mutations associated with lipodystrophy, including the classic R482W mutation, and found no evidence for prelamin A accumulation. Our conclusions were based on protein gel blots with a polyclonal lamin A/C antibody and a pair of prelamin A-specific monoclonal antibodies. The western blots were conducted with methods that allowed us to discern differences in the mobilities of nonfarnesylated and farnesylated prelamin A.^{14,16}

We do not understand why earlier studies reached the conclusion that FPLD fibroblasts, including R482W fibroblasts, had an accumulation of prelamin A. One possibility is nonspecific binding of antibodies from commercial vendors. Additional formal possibilities include differences in the site of the skin biopsy, differences in cell passage, and differences in the components of the medium, although one would have to propose that those differences selectively affected FPLD fibroblasts. In the current studies, we utilized both lamin A/C polyclonal antibodies and 2 different prelamin A-specific monoclonal antibodies.

An earlier study suggested that FPLD cells have reduced *ZMPSTE24* expression.²³ That finding was intriguing, but it was unclear why lamin A/C missense mutations would influence *ZMPSTE24* expression. In the current study, we found no evidence for reduced *ZMPSTE24* expression in FPLD fibroblasts. The levels of *ZMPSTE24* transcripts were not reduced in FPLD fibroblasts, nor was prelamin A accumulation exaggerated during lopinavir treatment. Had *ZMPSTE24* activity been reduced in FPLD fibroblasts, we would have observed an exaggerated response to lopinavir. When *Zmpste24*^{+/-} fibroblasts were treated with lopinavir, they exhibited twofold more prelamin A accumulation.

In earlier studies finding prelamin A accumulation in FPLD cells, the assumption was that the prelamin A was farnesylated.^{22,23} The prelamin A that accumulates in the setting of HGPS or restrictive dermopathy (*ZMPSTE24* deficiency) is definitely farnesylated, as judged by metabolic labeling.¹³⁻¹⁵ Of note, progeroid disorders similar to HGPS can be caused by *LMNA* missense mutations that do not disrupt prelamin A processing.^{34,36} For example, R644C and E578V mutations in *LMNA* cause progeroid syndromes without causing prelamin A accumulation.^{36,37} In the case of R644C fibroblasts, lopinavir treatment did not lead to an exaggerated accumulation of prelamin A (not shown). Also, it is interesting that a FPLD syndrome occurs in the setting of a prelamin A truncation mutation that eliminates any possibility of prelamin A farnesylation.³⁸

One limitation of our study is that we examined cultured fibroblasts and not adipose tissue from human subjects. Another is that the FPLD cell lines described in the literature were not available to us for study. In any case, we are confident in our findings with 4 different *LMNA* mutations linked to lipodystrophy, and we hope that our

observations prompt additional studies. If future studies were to uncover a FPLD mutation causing substantial prelamin A accumulation, it would be very interesting to define the underlying mechanisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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