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Mislocalization of prelamin A Tyr646Phe mutant to the nuclear pore complex in human embryonic kidney 293 cells

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Abstract

Mature lamin A is formed after post-translational processing of prelamin A, which includes prenylation and carboxymethylation of cysteine 661 in the CaaX motif, followed by two proteolytic cleavages by zinc metalloprotease (ZMPSTE24). We expressed several prelamin A mutants, C661S (defective in prenylation), Y646F (designed to undergo prenylation but not second proteolytic cleavage), double mutant, Y646F/C661S and Y646X (mature lamin A), and the wild-type construct in human embryonic kidney (HEK-293) cells. Only the Y646F mutant co-localized with nuclear pore complex proteins, including Nup53 and Nup98, whereas the other mutants localized to the nuclear envelope rim. The cells expressing Y646F mutant also revealed abnormal nuclear morphology which was partially rescued with the farnesyl transferase inhibitors. These data suggest that the unprenylated prelamin A is not toxic to the cells. The toxicity of prenylated prelamin A may be due to its association and/or accumulation at the nuclear pore complex which could be partially reversed by farnesyl transferase inhibitors.

Keywords: Lamin A/C; Progeria; Nuclear pore complex protein; CaaX motif protein; Lipodystrophy; Farnesyl transferase inhibitors; Prenylation; Zinc metalloprotease (ZMPSTE24)

Mutations in *LMNA* gene have now been associated with several diseases including lipodystrophies, muscular dystrophies, cardiomyopathy, mandibuloacral dysplasia, neuropathy, restrictive dermopathy and progeria [1–3]. The mutated proteins, lamins A and C, mostly affect the tissues originating from mesenchymal cells, such as adipose tissue, skeletal muscle or bone. The lamins A and C are intermediate filament proteins formed by alternative splicing from the same *LMNA* gene, and these proteins consists of two α-helical rod-domains and a globular domain which assumes an immunoglobulin fold [4,5]. In addition, the prelamin A (precursor of the mature lamin A) has a CaaX-motif at its carboxy-terminus, and this cysteine is prenylated during post-translational processing (Fig. 1A). Prenylated prelamin A undergoes proteolytic cleavage by a zinc metalloprotease (ZMPSTE24), first the removal of three amino acids—aaX, then carboxy-methylation of the prenylated cysteine, and finally the excision of the next 15 amino acids at the carboxy-terminus, again by ZMPSTE24 [6]. The mature lamin A is then translocated to the nucleus where it participates in formation of the nuclear lamina.

Most disease causing mutations reported in *LMNA* gene are missense, and a few involve small deletions. Recently, Gly608Gly and Gly608Ser mutations in *LMNA* gene were reported in patients with Hutchinson-Gilford progeria syndrome (HGPS) [7,8]. These mutations activate cryptic splice sites, such that they cause in-frame deletion of 50 residues, including the second proteolytic site for ZMPSTE24 in the carboxy-terminus. The truncated protein, named progerin, retains the prenylated lipid due to the lack of second proteolytic site recognized by ZMPSTE24 [7]. The toxicity of prenylated progerin suggests that it is the presence of the prenylated moiety, rather than the lack of a second proteolytic cleavage that renders these lamin A mutations

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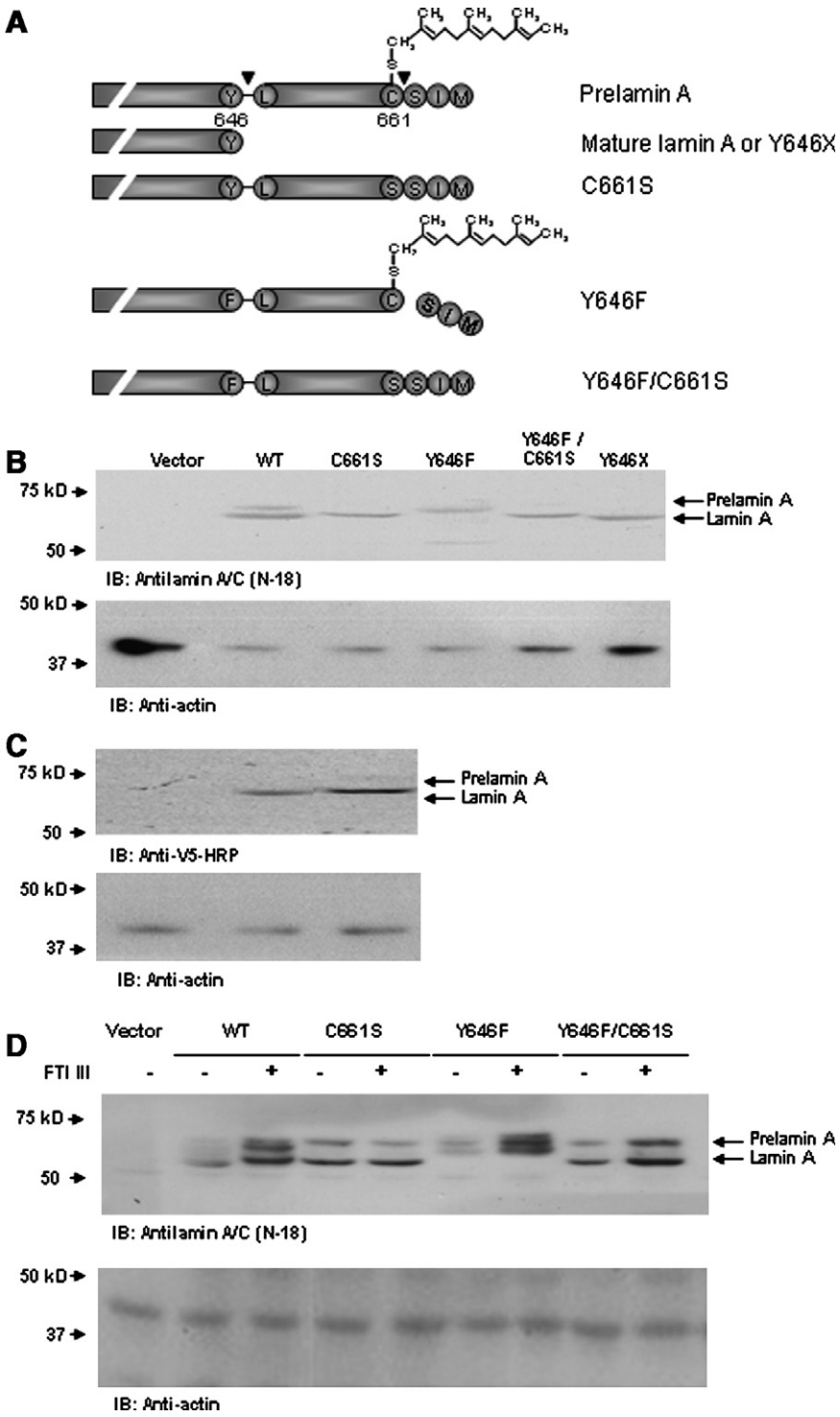

Fig. 1. Expression of wild-type and prelamin A mutations in human embryonic kidney 293 (HEK-293) cells. (A) Schematic representation of wild-type prelamin A, mature lamin A or mutations (Y646X), and C661S, Y646F and Y646F/C661S mutations. The two endoprotease proteolytic sites are represented by filled triangles, and prenylation of cysteine 661 is shown. In mutation Y646F, the three amino acids, SIM, are shown as removed from the expressed protein. (B) Representative Western blots of cell lysates obtained from transiently transfected cells (48 h) probed with amino terminal specific anti-lamin A/C antibody, (N-18), showing a major species corresponding to mostly mature lamin A in all cases, except for mutant Y646F. Similar observations were made at 24 or 72 h of incubation (data not shown). (C) Western blot of cell lysates after transfection of the V5 epitope-tagged prelamin A (wild type) and mutation C661S, showing similar results as in (B). (D) Western blot of cell lysates obtained after transfection of mutants C661S, Y646F and Y646F/C661S in the absence (−) or presence (+) of FTI III (20 μM). Same blots in (B–D) were stripped and reprobed with antibody to actin.

cytotoxic. To dissociate the influence of prenylation and proteolysis on cytotoxicity, we designed lamin A mutations that selectively interfere with one or both of these processes. We expressed mutations, C661S, Y646F, Y646X and double mutation Y646F/C661S and wild type lamin A construct in human embryonic kidney 293 (HEK-293) cells.

Experimental procedures

Cloning of human prelamin A and site-directed mutagenesis. The human cDNA for prelamin A was previously amplified from clone # IMAGE: 4863480 (Invitrogen, Carlsbad, CA) and was cloned in yeast expression vector pYcDE (pYcDE-hprelaminA, unpublished). For cloning in mammalian expression vector, the human prelamin A was amplified from pYcDE-hprelaminA by PCR using the primers (sense: 5′-T AAT CAT ***GAA TTC*** ATG GAG ACC CCG TCC CAG-3′ and anti-sense: 5′-T TAT ATG ***CTC GAG*** TTA CAT GAT GCT GCA GTT CTG G-3′) containing the cloning sites *Eco*RI and *Xho*I shown in bold. The PCR products were initially cloned in pDrive (Qiagen, Valencia, CA) and sequenced to assure lack of PCR errors. The human prelamin A was subsequently released from the plasmid by restriction digestion with *Eco*RI and *Xho*I, gel purified and subsequently cloned in pcDNA3.1(+) vector (Invitrogen). The pcDNA-prelaminA (wild type) plasmid was used as template to generate the mutations, C661S and Y646F, using the following primers and QuickChange Mutagenesis protocol obtained from Stratagene (C661S mutant: sense: 5′-G AGC CCC CAG AAC-***TCC***-AGC ATC ATG TAA C-3′ and anti-sense: 5′-G TTA CAT GAT GCT-***GGA***-GTT CTG GGG GCT C-3′; Y646F mutant: sense: 5′-G GTC ACC CGC TCC-***TTC***-CTC CTG GGC AAC-3′ and anti-sense: 5′-GTT GCC CAG GAG-***GAA***-GGA GCG GGT GAC C-3′), the mutated nucleotides are in bold. The double mutant, Y646F/C661S, was generated using pcDNA prelaminA-C661S as template and the same primer pair as for mutant Y646F. The mature lamin A (Y646X) construct was created by PCR amplification using primers (sense: 5′-T AAT CAT ***GAA TTC*** ATG GAG ACC CCG TCC CAG-3′ and anti-sense: 5′-CCG ***CTC GAG*** TTA GTA GGA GCG GGT GAC CAG ATT GTC-3′).The cloning strategy was the same as described for the wild-type prelamin A. These mutant plasmids were sequenced to assure that only the desired mutations were introduced.

Cloning and construction of V5 tagged human prelamin A constructs. To create V5 epitope tagged human prelamin A wild-type and C661S mutant, the previous plasmid construct were again amplified using the primers (sense: 5′-ATG **GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG** GAG GAG ACC CCG TCC CAG CGG CGC GCC AC-3′ and anti-sense: 5′-TTA CAT GAT GCT GCA GTT CTG GGG GCT C-3′) where the V5 epitope is shown in bold and cloned in pDrive (Qiagen). The V5 tagged prelamin A was excised with *Bam*HI and *Xho*I and cloned at the same site in pcDNA3.1(+).

Cell culture and transfection. The HEK-293 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 1× penicillin/streptomycin and 2 mM L-glutamine at 37 °C in 5% CO_2. Transient transfections were carried using Lipofectamine 2000 (Invitrogen) as per the manufacturer's recommendations. Briefly, approximately 30,000 cells were seeded in each well (6-well plate) and transfected with 2 μg/well of each construct using a DNA: lipofectamine ratio of 1:2.5.

Farnesyl transferase inhibitors (FTI) treatment. In some experiments, HEK-293 cells were transfected in the presence of a single dose of 10 or 20 μM of FTI inhibitor III, 20 mM stock solution in water, a competitive inhibitor that mimics farnesyl pyrophosphate (Calbiochem, EMD Biosciences, La Jolla, CA) or a single dose of 1 μM FTI 277, 1 mM stock solution in DMSO, a peptidomimetic inhibitor based on the CaaX motif (Calbiochem) [9,10].

Immunofluorescence microscopy. The HEK-293 cells were grown on cover slips (in 6 well plates) and transfected with various prelamin A constructs. The transfected cells were fixed with methanol (−20 °C), permeabilized with 0.1% Triton X-100, and blocked with 5% fetal calf serum and 0.03% bovine serum albumin in PBS. The permeabilized cells were incubated with an anti-lamin A/C antibody at 1:200 dilution (amino terminus, N-18, Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by Alexa Fluor 568 donkey anti-goat IgG antibody at 1:200 dilution or in some experiments labeled with Alexa Fluor 488 (Molecular Probes/Invitrogen). To detect nuclear pore protein Nup53, the cells were incubated with rabbit anti-Nup53 antibody at 1:1000 dilution (NOVUS Biologicals, Littleton, CO), followed by Alexa Fluor 488 goat anti-rabbit IgG at 1:200 dilution (Invitrogen) at 37 °C for 1 h, respectively. Similarly, the transfected cells were stained with anti-Nup98 (1:400 dilution, sc-30112, Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by Alex Fluor 568 donkey anti-goat IgG antibody at 1:500 dilution. The transfected cells were also incubated with MAb414 (monoclonal antibody recognizing several nuclear pore complex proteins; MMS-120P; 1:200 dilution, Covance, Berkely, CA) followed by Alexa Fluor 568 rabbit anti-mouse IgG antibody at 1:500 dilution. The slides then were incubated with 4′,6-diamidino-2-phenylindole, dihydrochloride (1 μg/ml) for 1 min. Cell imaging was performed by Zeiss Axiovert 100 M [11]. The digital images were adjusted for contrast and brightness and prepared using Adobe Photoshop (Adobe Systems, San Jose, CA).

Western blotting. HEK-293 cell extracts were prepared by harvesting cells in lysis buffer consisting of 1% SDS, 20 mM NaF and protease inhibitors (protease inhibitor cocktail from Roche, Indianapolis, IN). The cell lysates were centrifuged at 13,000*g* for 15 min at room temperature and protein concentrations were determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Ten micrograms of total proteins were denatured by heating the samples at 100 °C for 10 min and resolved on 20 × 20 cm, 7.5% SDS–PAGE gels. Resolved proteins were transferred onto PDVF (Immobilon membranes, Millipore, Billerica, MA) and blocked in 5% non-fat milk overnight at 4 °C. The blots were probed with goat anti-lamin A/C (N-18) at a dilution of 1:2500. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies detected with the ECL Plus chemiluminescence system (Amersham Pharmacia, Buckinghamshire, UK) and exposed to X-ray film. Immunoblot for V5 tagged proteins was probed with anti V5 antibody conjugated to horseradish alkaline phosphatase at the dilution of 1:5000 (Invitrogen). The same blots were stripped using the restore Western blot stripping buffer (Pierce, Rockford, IL) according to the manufacture protocol, re-probed with anti-actin antibody at a dilution of 1:1000 (catlog #A 2066, Sigma, St. Louis, MO) and detected with goat anti-rabbit IgG conjugated with horseradish peroxidase at a dilution of 1:2500.

Results

Expression of prelamin A and mutant constructs in HEK-293 cells

Transient over expression of wild-type prelamin A in HEK-293 cells yields mostly mature lamin A, as shown by immunoblot with an amino terminus antibody (N-18) to prelamin A (Fig. 1B). Unexpectedly, prelamin A mutation C661S, which lacks the prenylated cysteine residue still yields a major species of approximately the same size as mature lamin A, which indicates that proteolytic processing of prelamin A occurs even in the absence of prenylation. Expression of prelamin A mutation Y646F, in which the second proteolytic cleavage site for ZMPSTE24 is mutated, yields prelamin A and improperly processed fragments rather than mature lamin A. As expected the control construct Y646X (mature lamin A) yielded the protein band corresponding to mature lamin A. However, the double mutation, Y646F/C661S, yielded a major band of the same size as mutation C661S. To confirm the processing of prelamin A mutation C661S to mature lamin A, we expressed wild-type prelamin A and mutation C661S with a V5 epitope tagged at the amino terminus in HEK-293 cells. When probed with antibody specific for the V5 epitope, both wild-type prelamin A and mutation C661S yielded predominantly a species indistinguishable from mature lamin A (Fig. 1C), suggesting that prelamin A is

cleaved to a product indistinguishable from mature lamin A on SDS–PAGE even in the absence of prenylation. However, these data do not directly demonstrate which peptide bond is cleaved or whether the cleavage is mediated by ZMPSTE24. Treatment with FTI III, increased the accumulation of wild-type prelamin A, due to inefficient processing, relative to mature lamin A as expected (Fig. 1D). FTI III treatment did not alter the relative amounts of mature and unprocessed lamin A for the unprenylated mutations C661S and Y646F/C661S, but treatment did increase the accumulation of unprocessed and unprenylated fragment for mutation Y646F, consistent with prenylation of Y646F without FTI III treatment (Fig. 1D).

Nuclear morphology in HEK-293 cells expressing wild-type prelamin A or C661S, Y646F, Y646X, and Y646F/C661S mutations

The subcellular localization of these over-expressed prelamin A species was studied by indirect immunofluorescence microscopy. Under these conditions, the HEK-293 cells expressed negligible amounts of endogenous lamin A or C (not shown). Over expressed wild-type prelamin A localized uniformly to the nuclear periphery (Fig. 2). As expected mature lamin A and Y646X, also localized to the nuclear periphery. Prelamin A mutations C661S and Y646F/C661S both localized to the nuclear envelop rim, consistent with their processing to mature lamin A or species functionally similar to mature lamin A, as observed on the Western blot analysis. In contrast, prelamin A mutation Y646F, which did not produce mature lamin A, localized in patches to the nuclear membrane (Fig. 2). This patchy, peripheral pattern is reminiscent of nuclear pore localization, so co-localization studies with nuclear pore complex protein, Nup53, were pursued.

Co-localization of prelamin A mutation Y646F and nuclear pore complex proteins

To determine if prelamin A mutation Y646F is localized to the nuclear pore, in transiently transfected HEK-293 cells, immunofluorescence studies were performed with antibodies specific to lamin A and C and Nup53. In cells expressing prelamin A Y646F mutant, Nup53 also localized to patches on the nuclear membrane (Fig. 3). Merged images showed that prelamin A Y646F mutant and Nup53 co-localized in these cells. The co-localization of the mutant prelamin A, Y646F, was also observed with two additional antibodies against the nuclear pore complex proteins, Nup98 and MAb414 (Fig. 3). The co-localization suggests that prelamin A species retaining prenylated cysteine residue elicits the nuclear pore staining patterns observed in Fig. 3, and perhaps creates nuclear dysfunction.

To examine the role of prenylation, these experiments were also conducted in the presence of FTIs. Transfected cells expressing prelamin A mutation Y646F were incubated with FTI III or FTI-277, and lamin A and Nup53 were localized by immunofluorescence 48 h after transfection (Fig. 4A). In the presence of both the inhibitors, wild-type lamin A localized to the nuclear periphery. Both FTIs corrected the mislocalization of prelamin A mutation Y646F (Fig. 4A), suggesting that the prenylation is required for nuclear pore mislocalization. The rescue of nuclear morphology was observed in about 50% of the cells (Fig. 4B).

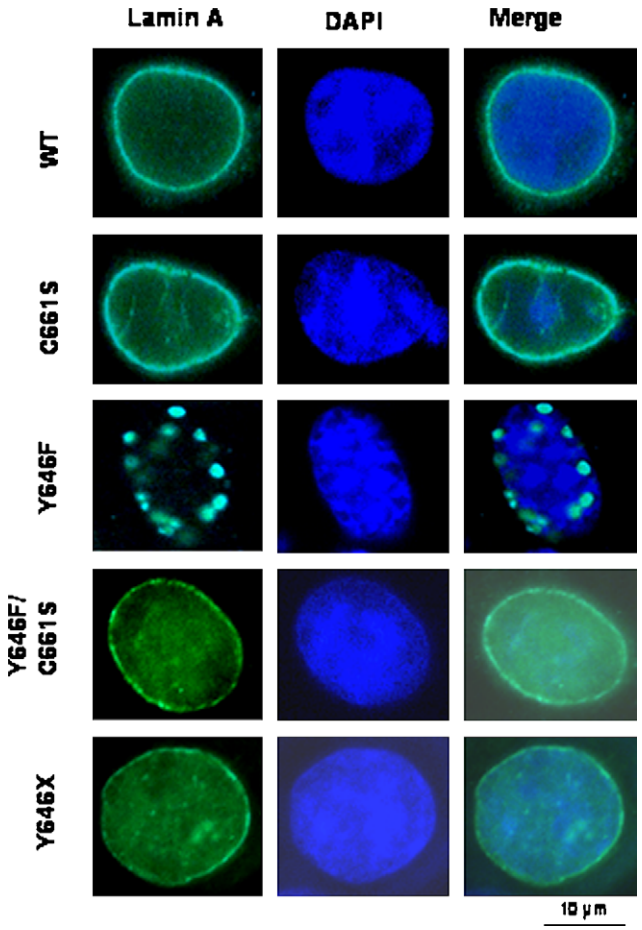

Fig. 2. Immunofluorescence images of transiently transfected HEK-293 cells over expressing wild-type lamin A, mature lamin A (Y646X) and prelamin A mutations C661S, Y646F, and Y646F/C661S. This being a transient transfection, the nuclear morphology of only those cells was examined in which fluorescence was detectable. The cells were fixed after 48 h of expression and stained for lamin A (green fluorescence) and nuclear DNA (blue fluorescence). Wild-type prelamin A, mature lamin A (Y646X) and mutations C661S and Y646F/C661S localize to the nuclear periphery; in contrast, prelamin A mutation Y646F shows patchy aggregates on the nuclear membrane. Magnification 63×. Shown is the scale bar in micrometers.

Discussion

In this study, we determined the consequences of mutations at two residues thought to be essential for prelamin A processing. Mutation C661S precludes prenylation of cysteine 661, yet this mutation is cleaved to a species corresponding to mature lamin A in HEK-293 cells (Fig. 1B).

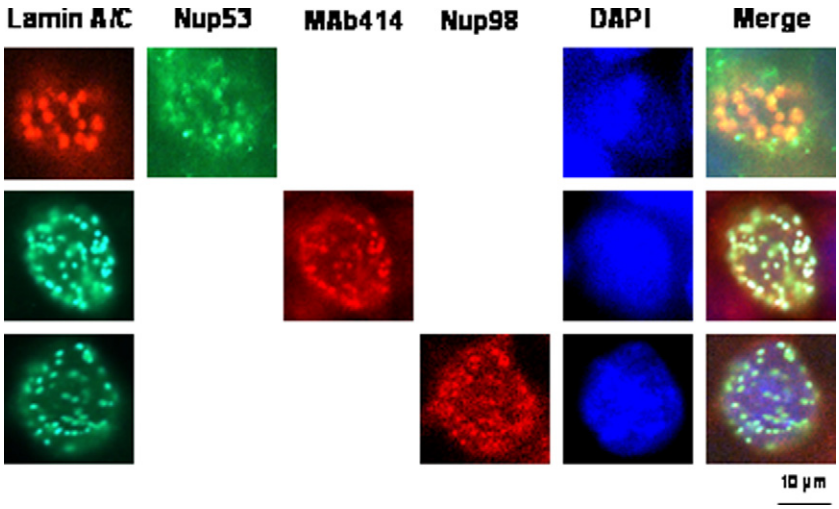

Fig. 3. Co-localization of prelamin A mutation Y646F with nuclear pore complex marker Nup53, MAb414, and Nup98 in HEK-293 cells. Immunofluorescence image of the nucleus stained for lamin A (red fluorescence or green fluorescence), Nup53 (green fluorescence) and MAb414 and Nup98 (red fluorescence), nuclear DNA (blue fluorescence). The merged images illustrate the co-localization of lamin A and Nup53, MAb414, and Nup98 in three independent experiments. Magnification 63×. Shown is the scale bar in micrometers.

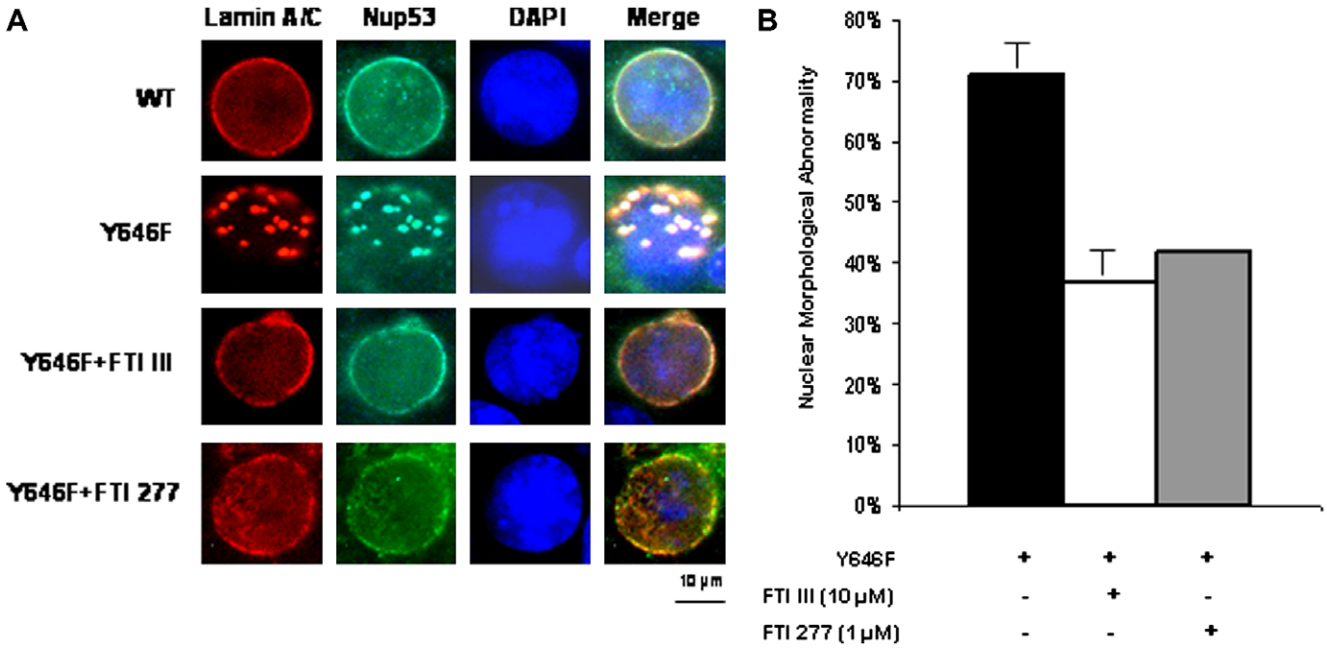

Fig. 4. Farnesyl transferase inhibitors (FTIs) rescue nuclear morphology of HEK-293 cells expressing prelamin A mutation Y646F. Nearly 300 abnormal nuclei were examined for the nuclear morphology in the presence of FTI III and FTI-277 (A) Cells transfected with the prelamin A mutation Y646F were incubated with a single dose of FTI III (10 μM) and FT-277 (1 μM) at the start of the incubation . After 48 h, the cells were stained for lamin A (red fluorescence), Nup53 (green fluorescence) and nuclear DNA (blue fluorescence). The merged images of lamin A and Nup53 demonstrate the patchy co-localization of the two proteins to the nuclear pore complex in the control experiments and peripheral co-localization in the presence of either FTase inhibitors. Magnification 63×. Shown is the scale bar in micrometer. (B) Percent of nuclear abnormality rescued in the presence of (FTIs). Only 50% improvement in nuclear morphology was observed at 48 h, with similar results at 72 h with FTI III (data not shown). Shown are the mean ± SEM from three independent experiments in the presence or absence of FTI III. Data for FTI-277 is from one experiment, which essentially corroborated the FTI III experiment. For each experiment more than 50 transfected nuclei were examined.

This result suggests that prenylation is not required for proteolytic processing of the prelamin A carboxy-terminus and incorporation of the mature protein into the nuclear lamina of HEK-293 cells (Fig. 2).

We do not know if the cleavage of prelamin A mutation C661S is mediated by ZMPSTE24 or if the same peptide bonds are cleaved in the processing of mutation C661S as in wild-type prelamin A. It is possible that mutation C661S renders the carboxy-terminus of this prelamin A mutation a substrate for other proteases. The observation that the double mutation Y646F/C661S yields major species of the same size further suggests that ZMPSTE24 does

not mediate this processing of these unprenylated proteins. The two mutation, C661S and Y646F/C661S, both localize to the nuclear periphery, suggesting that unprenylated prelamin A is able to integrate into the nuclear lamina, suggesting that the exact site of cleavage may not be essential for function.

As expected, mutation Y646F precludes both processing of prelamin A to mature lamin A and alternate cleavage pathway followed by mutation C661S and Y646F/C661S (Fig. 1B). Unlike wild-type prelamin A and C661S mutant, Y646F mutant is not incorporated into the nuclear lamina but instead accumulates in patches on the nuclear membrane (Figs. 2 and 3). Immunofluorescence microscopy demonstrates that mutation Y646F co-localizes with Nup53, which is a component of the nuclear pore complex (NPC) [12]. Furthermore, the Y646F mutant co-localizes with Nup98 (this antibody recognizes Nup98, in the Nup155 complex consisting of Nup155, Nup98, and Nup170) [13]. Likewise, antibody, MAb414, which also recognizes four nuclear pore complex proteins [14], was found to co-localize with the mutant Y646F protein. The farnesyl transferase inhibitors, FTI III and FTI-277, both rescue the nuclear morphology and subcellular localization of about half the cells expressing the prelamin A mutation Y646F (Fig. 4A and B). Taken together, these results suggest that unprocessed prelamin A, which includes the prenylated C661, accumulates in the NPC, and that the prenylation itself participates in this mislocalization process.

Our results are consistent with studies of the prelamin A mutation progerin found in patients with HGPS [15–18]. Immunofluorescence studies with a carboxy-terminus antibody showed that nuclear dysmorphology accompanies progerin accumulation in HeLa cells or fibroblasts from HGPS subjects [17]. Treatment with a FTI, however, ameliorates the nuclear dysmorphology despite continued progerin expression [17]. These experiments and our results with FTIs suggest that the prenylation process itself is intimately involved in the nuclear toxicity of these *LMNA* mutations. Similar results are obtained using fibroblasts from $Lmna^{HG}$ mice, which express only progerin and no normal, mature lamin A [18].

The co-localization of prelamin A mutation Y646F with Nup53 also suggests a mechanism for the cytotoxicity of prenylated lamin A species. Although, not directly demonstrated whether mutation Y646F retains the isoprenoid lipid moiety, previous studies would strongly favor its retention on the protein, due to the loss of ZMPSTE24 proteolytic cleavage site. Nup53 contains an RNA recognition motif, and this protein is believed to participate in mRNA export into the cytoplasm [19,20]. Retention of the prenyl moiety results in the adherence of incompletely processed lamin A to the nuclear pore complex, and this association may initiate NPC dysfunction, resulting in damage to essential cellular functions. This model of NPC dysfunction may also apply to other prelamin A mutations that disrupt processing such as the T623S mutation reported in a Japanese patient with mild progeria [21]. However, this model does not explain why cytotoxicity is limited to certain mesenchymal tissues. The selectivity of tissue damage from prelamin A mutations might derive from heterogeneity in NPC components in different tissues, as well as the vulnerability of specific cells to NPC damage and/or existence of compensatory mechanism [22]. Nevertheless, the importance of prenylation in some laminopathies suggests that FTIs may be useful agents to mitigate the toxic effects of *LMNA* mutations [23].

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