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Pre-Lamin A Processing Is Linked to Heterochromatin Organization

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Abstract Pre-lamin A undergoes subsequent steps of post-translational modification at its C-terminus, including farnesylation, methylation, and cleavage by ZMPSTE24 metalloprotease. Here, we show that accumulation of different intermediates of pre-lamin A processing in nuclei, induced by expression of mutated pre-lamin A, differentially affected chromatin organization in human fibroblasts. Unprocessed (non-farnesylated) pre-lamin A accumulated in intranuclear foci, caused the redistribution of LAP2alpha and of the heterochromatin markers HP1alpha and trimethyl-K9-histone 3, and triggered heterochromatin localization in the nuclear interior. In contrast, the farnesylated and carboxymethylated lamin A precursor accumulated at the nuclear periphery and caused loss of heterochromatin markers and Lap2alpha in enlarged nuclei. Interestingly, pre-lamin A bound both HP1alpha and LAP2alpha in vivo, but the farnesylated form showed reduced affinity for HP1alpha. Our data show a link between pre-lamin A processing and heterochromatin remodeling and have major implications for understanding molecular mechanisms of human diseases linked to mutations in lamins. J. Cell. Biochem. 102: 1149–1159, 2007.

Key words: lamin A; pre-lamin A; heterochromatin organization; HP1α, LAP2α

Lamins are structural proteins of the nuclear envelope in multicellular metazoa. In mammalian cells, B-type lamins are expressed in all cells and are essential for cell viability, while A-type lamins are expressed in differentiated cells and have important functions in tissue homeostasis [Mattout et al., 2006]. Lamin A, encoded by the *LMNA* gene, is produced as a precursor protein which undergoes four steps of post- translational modifications, including farnesylation, double cleavage by ZMPSTE 24 endoprotease [Corrigan et al., 2005] and methylation by the cysteine methyltransferase Icmt [Rusinol and Sinensky, 2006]. Pre-lamin A contains a C-terminal CaaX motif, a sequence shared by farnesylated proteins, in which C is cysteine, a stands for any aliphatic amino acid, and X for the amino acid that specifies whether a farnesyl (X = serine, alanine, methionine, or glutamine) or a geranyl (X = leucine) group will be covalently attached to the cysteine [Barton and Worman, 1999]. In human pre-lamin A, X is a methionine and the cysteine is modified by a 15 Carbon farnesyl residue. Following farnesylation, the aaX tripeptide is cleaved by ZMPSTE24 and the C-terminal cysteine carboxymethylated, followed by a second ZMPSTE 24-mediated cleavage of 15 amino acids upstream of the cysteine, thus removing the farnesyl residue [Corrigan et al., 2005]. It has been recently

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proposed that RCE1, another CaaX endoprotease may also accomplish the first cleavage step of pre-lamin A processing [Rusinol and Sinensky, 2006]. Transient farnesylation of lamin A is supposed to favor its targeting to the nuclear membrane, but the specific role for the sequential processing is unclear. Narf, a nuclear protein of unknown function, was found to selectively bind the farnesylated sequence [Barton and Worman, 1999]. Sasseville and Raymond [1995] showed accumulation of pre-lamin A in intranuclear foci and at the nuclear lamina upon drug-mediated inhibition of isoprenylation, and they hypothesized about a functional link between the lamin A precursor and chromatin organization. We previously showed that treatment of C2C12 mouse myoblasts with a farnesylation inhibitor caused accumulation of pre-lamin A in intranuclear foci and triggered formation of large heterochromatin areas protruding into the nuclear interior [Maraldi et al., 2005]. In line with the hypothesis of a functional link between lamin A and chromatin, lamins have been shown to bind to polynucleosomes [Yuan et al., 1991], histones [Goldberg et al., 1999; Mattout et al., 2007], and DNA without sequence specificity [Stierlé et al., 2003], indicating a more general role of lamins in chromatin dynamics.

Recent evidence shows that pre-lamin A processing is altered in a group of *LMNA*-linked diseases, including Hutchinson-Gilford progeria [Eriksson et al., 2003], mandibuloacral dysplasia (MAD) [Capanni et al., 2005; Filesi et al., 2005], familial partial lipodystrophy [Capanni et al., 2005], Werner syndrome [Capanni et al., 2005], and restrictive dermopathy (RD) [Navarro et al., 2005]. Moreover, mice deficient for the pre-lamin A endoprotease ZMPSTE24 develop a disease resembling MAD phenotype [Pendas et al., 2002]. All these laminopathies are characterized, at the cellular level, by anomalous accumulation of the lamin A precursor, as well as by gross alterations of heterochromatin organization, ranging from focal loss or clumping of heterochromatin to a complete absence of peripheral heterochromatin [Capanni et al., 2003; Fong et al., 2004; Goldman et al., 2004; Columbaro et al., 2005; Filesi et al., 2005; Liu et al., 2005; Scaffidi and Misteli, 2005]. Pre-lamin A accumulation also causes loss of trimethylated H3 histone (lysine 9) (tri-H3K9), a marker for pericentric constitutive heterochromatin, in progeria cells [Columbaro et al., 2005; Scaffidi and Misteli, 2005], and mis-localization of H3K9 and of heterochromatin-associated protein HP1 in MAD cell lines [Filesi et al., 2005]. Drugs impairing accumulation of lamin A precursor could rescue heterochromatin organization [Columbaro et al., 2005], indicating a direct link between pre-lamin A and heterochromatin.

We wanted to evaluate the effect of different lamin A precursor molecules, including non-farnesylated or farnesylated and carboxymethylated pre-lamin A, on heterochromatin organization. Cells expressing different pre-lamin A forms were used. Here, we show that pre-lamin A forms localized differently in the nuclei depending on their modifications and differently affected heterochromatin organization. Moreover, in vivo binding of pre-lamin A to the heterochromatin protein HP1α was detected within a protein complex including LAP2alpha, a finding that represents a hint for the understanding of chromatin changes associated with pre-lamin A accumulation.

MATERIALS AND METHODS

Cell Cultures and Transfection

Skin fibroblast cultures were obtained from skin biopsies of healthy patients (mean age 24) undergoing orthopedic surgery, following a written consent. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The experiments were performed at passages 3–12. HEK 293 cells were used for the immuno-precipitation study. The C661M and L647R point mutations were introduced into the full-length lamin A cDNA [Kumaran et al., 2002] using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and sequences were confirmed by DNA sequencing (MWG Biotech, Germany). Expression vectors encoding full-length FLAG-tagged rat pre-lamin A (FLAG-LA) and the mutated constructs LA-C661M and LA-L647R were transiently transfected into cycling fibroblasts or HEK 293 cells using the Amaxa Nucleofector or the FuGene reagent (Roche) according to the manufacturers' instructions. Biochemical and immunofluorescence analyses were performed 24 h after transfection.

Antibodies

Antibodies employed were: anti-LAP2α, rabbit polyclonal [Dechat et al., 2004]; anti-HP1α, rabbit polyclonal (Novus Biologicals); anti-HP1α, mouse monoclonal (Upstate); anti- tri-H3K9, rabbit polyclonal (Upstate); anti-tri-H3K27, mouse monoclonal (Abcam); anti-tri-H4K20, rabbit polyclonal (Upstate); anti-FLAG, mouse monoclonal (Sigma, M2); anti-FLAG, rabbit polyclonal (Sigma).

Western Blot and Immunoprecipitation Study

Western blot analysis was done as follows. Cells were lysed in Ripa buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, and protease inhibitors. Cell lysates were diluted in Laemmli buffer, subjected to SDS–PAGE (6–20%) and transferred to nitrocellulose membrane. Membranes were saturated with 4% BSA and incubated with primary antibodies for 1 h at room temperature. Immunoblotted bands were revealed by the Amersham ECL detection system. The immunoprecipitation study was done as previously reported [Dorner et al., 2006]. Briefly, HEK 293 cells expressing wild-type FLAG-tagged pre-lamin A and pre-lamin A mutants were fixed in 1% formaldehyde in PBS for 10 min at room temperature, and then lysed in buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0 plus protease inhibitors. Cells were lysed by glass beads (Sigma) and thereafter by sonication. Immunoprecipitation was performed overnight at 4°C with 1 μl of anti-FLAG monoclonal antibody. Control immunoprecipitations were performed in the absence of anti-FLAG antibody. After saturation with 1 μg/ml of herring sperm DNA (Roche), 40 μl/ml of protein A/G (Santa Cruz) were added for 90 min at 4°C. Immunoprecipitated protein complexes were washed and subjected to Western blot analysis.

Immunofluorescence and Image Processing

Human fibroblasts grown on coverslips were fixed in 4% paraformaldehyde at 4°C for 10 min and permeabilized with 0.15% Triton X-100 for 5 min. Samples were incubated with PBS containing 4% BSA and incubated with primary antibodies overnight at 4°C, and with secondary antibodies for 1 h at room temperature. Propidium iodide (PI) staining was performed with 0.5 μg/ml PI for 20 min, following 30 min incubation with RNase (1 mg/ml) at 37°C. Slides were post-fixed in 2% paraformaldehyde at 4°C for 10 min, rinsed and mounted with an anti-fade reagent in glycerol. The confocal imaging was performed on a TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Heidelberg). The confocal unit was attached to an inverted microscope (Leica DM IRE2) equipped with a HCX Plan-Apo 63x oil immersion/1.40 NA objective for *Z*-stack imaging. The detection was performed with an argon and a helium–neon laser to simultaneously excite FITC (488 nm line laser) and TRITC/Cy3 (543 nm line laser) fluorescence. The pinholes used for confocal imaging were set to ~1 Airy Unit. Optical sections were obtained at increments of 0.3 μm in the Z-axis and were digitalized with a scanning mode format of 512×512 pixels and 256 gray levels. Image processing and volume rendering was carried out with ImageSpace software (Molecular Dynamics, CA), running on a Silicon Graphics workstation Indigo2 (Mountain View, CA). The *Z*-stack images were digitally filtered (3D Gaussian filter $3 \times 3 \times 3$) to reduce the background noise. The confocal image reconstructions were obtained using the four or five optical sections passing through the mid-section of the nucleus. PI confocal images were displayed in pseudo-color with a scale ranged from 0 to 255 U to enhance the contrast of the fluorescence intensity.

Electron Microscopy

Fibroblasts to be examined by electron microscopy were transfected using the Amaxa Nucleofector device, according to manufacturer instructions. To perform immunogold labeling of transfected fibroblasts, cells grown as monolayers were fixed in 1% glutaraldehyde, while post-fixation in OsO_4 was omitted. Samples were then dehydrated in an ethanol series and embedded in Epon resin. After saturation with 4% BSA plus 1% normal goat serum, monoclonal anti-FLAG antibody was applied to ultrathin sections overnight at 1:300 dilution. Gold-conjugated anti-mouse IgG was applied for 1 h at room temperature.

RESULTS

Different Pre-Lamin A Forms Differently Affect Chromatin Organization

Wild-type pre-lamin A, which was processed to mature lamin A (LA-WT), was detected at

the nuclear rim of transfected cells using an anti-FLAG antibody (Fig. 1A), as shown before [Kumaran et al., 2002; Capanni et al., 2005]. Ectopic expression of the C661M-lamin A mutant (Fig. 1B), in which the farnesylation target site is mutated, is expected to accumulate as a non-farnesylated pre-lamin A [Holtz et al., 1989; Hennekes and Nigg, 1994]. The mutated protein localized mainly to intranuclear aggregates which were observed at all the focal planes (Fig. 1B). The nuclear shape was not affected by overexpression of non-farnesylable pre-lamin A in the majority of nuclei (Fig. 1B).

Overexpression of the uncleavable L647R pre-lamin A mutant, missing the second ZMPSTE24 cleavage site, is expected to yield a permanently farnesylated and carboxymethylated protein (Fig. 1C). LA-L647R was found at the nuclear envelope and partly also in nuclear invaginations (Fig. 1C, white arrow and asterisk in the electron microscopy image). Ten percent of LA-L647R expressing nuclei were also misshapen (Fig. 1C). Taking together, our results show that non-farnesylated pre-lamin A efficiently localized to nucleoplasmic aggregates, while farnesylated pre-lamin A polypeptides predominantly accumulated at the nuclear envelope and may affect nuclear organization. To analyze chromatin reorganization in pre-lamin A expressing cells in more detail, we performed pseudocolor analyses of PI-stained nuclei to visualize the densely packed heterochromatic regions (PI pseudocolor Fig. 1A,B,C). Moreover, transfected cells were analyzed by electron microscopy. Major chromatin changes were not observed in wild-type lamin A-expressing cells, except that the electron density of peripheral heterochromatin areas

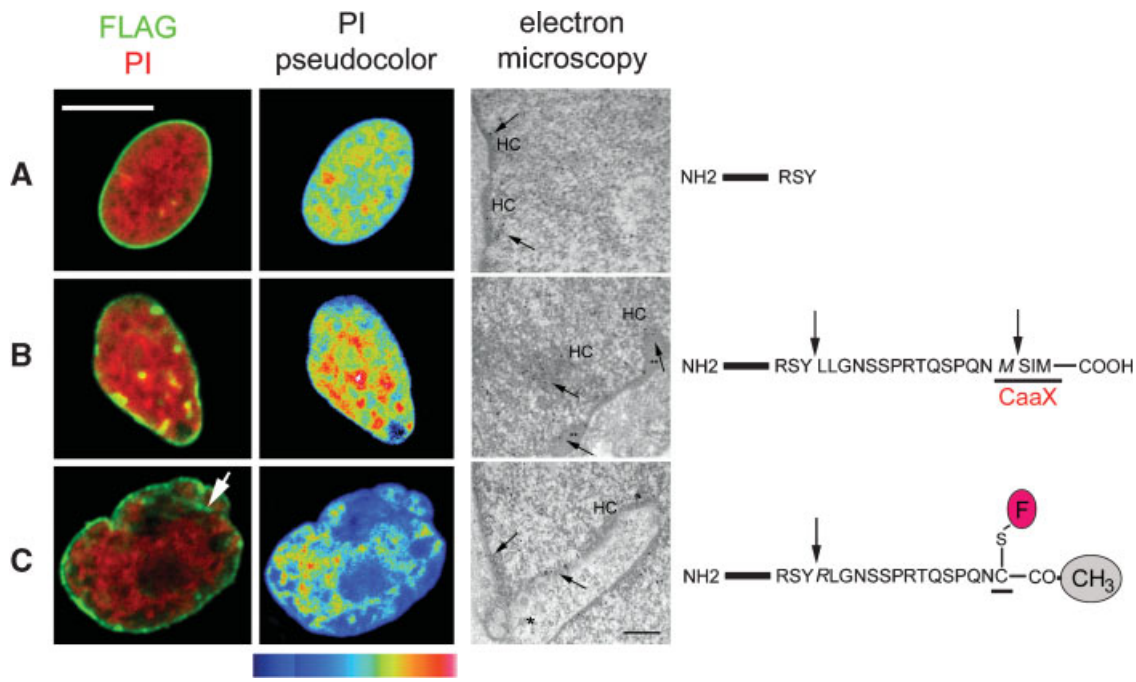

Fig. 1. Different pre-lamin A forms affect nuclear lamina and chromatin organization. Localization of FLAG-tagged pre-lamin A by confocal microscopy 24 h after transfection of cycling human skin fibroblasts with plasmids encoding wild-type lamin A (LA-WT) (**A**), C661M-mutated pre-lamin A (LA-C661M) (**B**), and L647R-mutated pre-lamin A (LA-L647R) (**C**). FLAG-tagged pre-lamin A was detected by anti-FLAG antibody (green). PI staining (red) is merged with FLAG labeling. A reconstruction of four confocal planes passing through the mid-section of the nucleus is shown. The pseudo-coloring of PI pictures obtained by using the ImageSpace software reveals the distribution of the heterochromatin domains (yellow to red). The intensity of fluorescence is represented on a pseudocolor scale (palette bar in panel C). Data are representative of at least six different experiments. White bar, 10μm. A representative picture of the electron microscopy analysis of each transfected cell line is shown in the central column. Cells were labeled by a post-embedding technique with anti-FLAG monoclonal antibody and labeling was revealed by gold-conjugated anti-mouse IgG. Gold particles (10 nm) are indicated by arrows. HC, heterochromatin. The asterisk in C indicates an invagination of the nuclear envelope. Black bar, 500 nm. The C-terminus sequence of the pre-lamin A form expressed in each sample is reported to the right side of each panel. Mutated amino acid residue is written in italics. The farnesyl residue is represented as a pink circle labeled "F." The methyl residue is represented as a gray circle labeled "CH_3." The CaaX residue at the C terminus end is indicated in red. The cleavage sites are indicated by arrows.

appeared slightly reduced at the ultrastructural analysis (Fig. 1A, electron microscopy). Focal thickening of the nuclear lamina (double asterisks in Fig. 1B) and clustering of pre-lamin A labeling close to the heterochromatin areas were detectable in LA-C661M- expressing fibroblasts (Fig. 1B, electron microscopy). Neither focal thickening of the lamina, nor clustering of pre-lamin A labeling in heterochromatin areas was observed with LA-WT or LA-L647R constructs. Instead, expression of LA-L647R determined an overall decondensation of chromatin in transfected cells as well as formation of nuclear envelope invaginations surrounded by thin heterochromatin layers (Fig. 1C, electron microscopy). Apoptotic nuclei [Columbaro et al., 2001] were not observed in transfected cells. We conclude that the distribution of heterochromatin regions differently changes upon expression of pre-lamin A mutants, indicating reorganization of heterochromatin.

Pre-Lamin A Affects the Distribution of Heterochromatin-Associated Proteins

To analyze the effect of the accumulation of pre-lamin A polypeptides on heterochromatin proteins HP1α and tri-H3K9, we performed Western blot analysis of total cell lysates 24 h after transfection with LA-WT, LA-C661M, and LA-L647R. A strong 70 kDa band representing fully processed ectopic lamin A was detected in fibroblasts and HEK-293 cells overexpressing LA-WT using antibodies to the tag (Fig. 2). The 74 kDa band representing lamin A precursors was faintly detected in these cells, indicating that processing of the ectopic protein is efficient. In contrast, only lamin A precursor proteins of 74 kDa, were detected in samples overexpressing the mutated proteins (Fig. 2), showing that their processing is severely impaired. The amount of HP1α and tri-H3K9 were not changed 24 h after transfection (Fig. 2). Localization of HP1α and tri-H3K9 was affected in pre-lamin A-accumulating cells. Only non-farnesylated intranuclear pre-lamin A forms (LA-C661M) caused reorganization of HP1α to bright fluorescent intranuclear foci in part overlapping with pre-lamin A aggregates (Fig. 3A). Carboxy-methylated pre-lamin A accumulated in LA-L647R-transfected cells caused loss of HP1α foci (Fig. 3A). Tri-H3K9, a marker for constitutive, pericentric heterochromatin, was observed in fluorescent foci throughout the

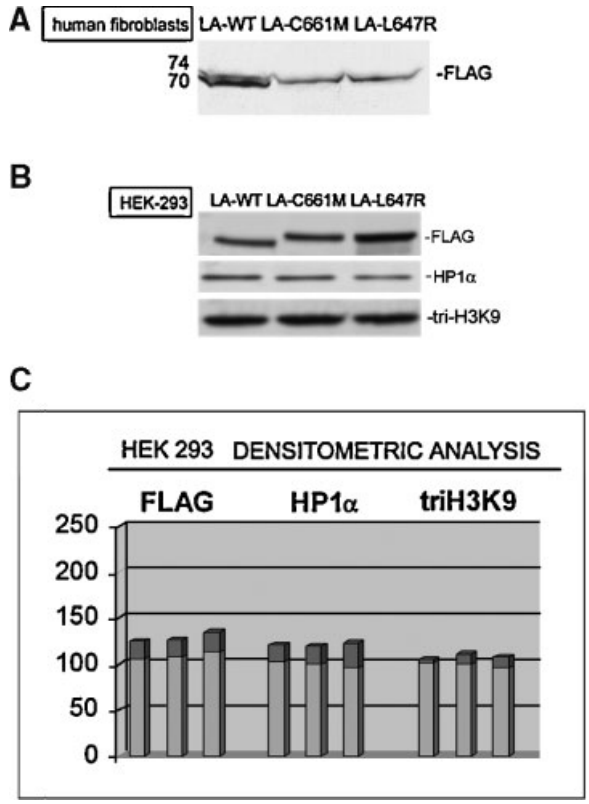

Fig. 2. Accumulation of FLAG-tagged pre-lamin A in fibroblasts and HEK293 cells. **A**: Western blot analysis of FLAG-tagged pre-lamin A in human skin fibroblasts. **B**: Western blot analysis of FLAG-tagged pre-lamin A and heterochromatin constituents HP1alpha and tri-H3K9 in HEK293 cells 24 h after transfection. Molecular weight markers are reported in kDa. **C**: Densitometric analysis of immunoblotted bands shown in panel B (data are means of three different Western blot analyses ±standard error of the mean and are expressed in arbitrary units).

nucleus in cells overexpressing LA-WT (Fig. 3B). Overexpression of non-farnesylable pre-lamin A (LA-C661M) caused slight increase of tri-H3K9 fluorescent foci, while overexpression of farnesylated LA-L647R pre-lamin A in fibroblasts dramatically affected tri-H3K9 organization (Fig. 3B). In 15% of LA-L647R expressing nuclei, the level of tri-H3K9 fluorescence was reduced to almost undetectable levels (Fig. 3B). We further investigated the effects of pre-lamin A forms on the expression and localization of tri-H4K20, another known marker of pericentric heterochromatin and tri-H3K27, a marker of facultative heterochromatin which is highly enriched in the inactive X chromosome of female cells [Shumaker et al., 2006]. The typical staining pattern of tri-H4K20 observed in fibroblasts overexpressing wild-type pre-lamin A consisted of a

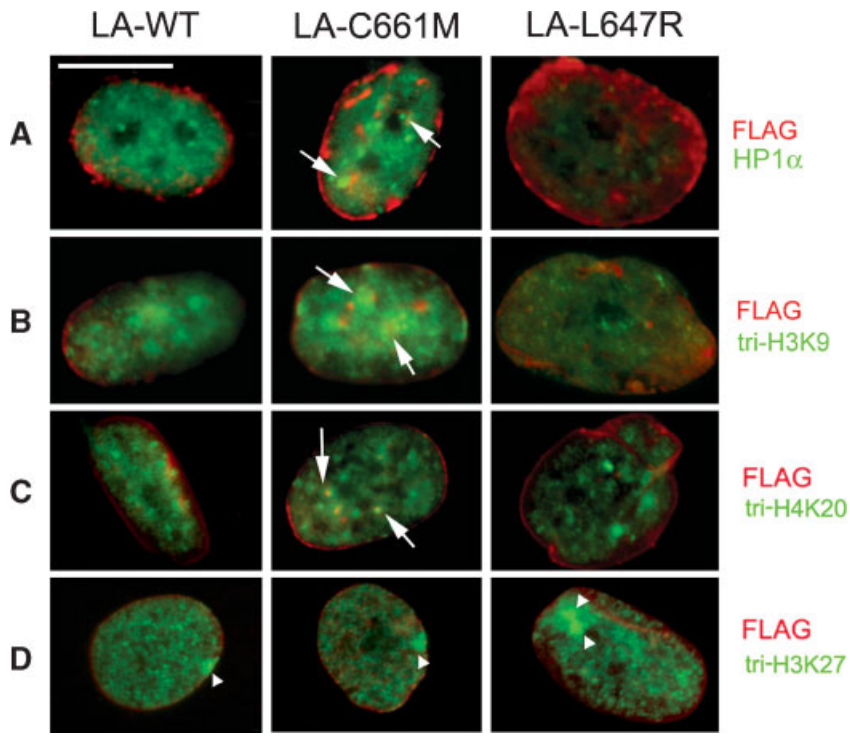

Fig. 3. Heterochromatin markers are affected by pre-lamin A. Fibroblasts transfected with LA-WT, LA-C661M or LA-L647R were subjected to immunofluorescence staining. **A**: Detection of HP1α and FLAG-pre-lamin A. **B**: Detection of tri-H3K9 and FLAG-pre-lamin A. **C**: Immunofluorescence localization of tri-H4K20 and FLAG-pre-lamin A. **D**: Immuno-fluorescence localization of tri-H3K27 and pre-lamin A. Inactive X chromatin stained by tri-H3K27 is indicated by arrowheads. Pre-lamin A was detected by Cy3-conjugated anti-FLAG antibody (red). Heterochromatin markers HP1α, tri-H3K9, tri-H4K20, tri-H3K27, detected by specific antibodies, are visualized by FITC-conjugated secondary antibodies (green). Arrows indicate aggregates of HP1α, tri-H3K9, or tri-H4K20 in close proximity to non-farnesylated (LA-C661M) pre-lamin A aggregates. A reconstruction of four confocal planes passing through the mid-section of the nucleus is shown. Bar, 10 μm.

limited number of brightly fluorescent spots (Fig. 3C). Interestingly, in LA-C661M expressing nuclei, tri-H4K20 spots in part co-localized with intra-nuclear pre-lamin A foci (Fig. 3C), supporting the association of pre-lamin A with constitutive heterochromatin. The number of fluorescent spots was not significantly changed in the enlarged nuclei accumulating farnesylated pre-lamin A by LA-L647R transfection (Fig. 3C). Using female fibroblasts as a cellular model we could detect a clear tri-H3K27 staining of inactive X chromatin (Fig. 3D). Expression of lamin A mutants did not significantly alter tri-H3K27 labeling (Fig. 3D). However, in LA-L647R-transfected cells, double or triple fluorescent spots were observed in 18% of nuclei (Fig. 3D), suggesting a transition state [Shumaker et al., 2006]. Thus, HP1α and tri-H3K9 were reorganized to newly formed heterochromatic foci adjacent to intranuclear aggregates of non-farnesylated pre-lamin A. In contrast, farnesylated and carboxy-methylated pre-lamin A caused loss of heterochromatin and diffuse localization of the heterochromatic proteins HP1α and trimethylated H3K9.

Pre-Lamin A Forms a Protein Complex With HP1α and LAP2α In Vivo

In order to identify a molecular interaction of pre-lamin A capable of interfering with chromatin organization, we overexpressed pre-lamin A mutants in HEK293 cells and immunoprecipitated pre-lamin A by anti-FLAG antibody. Lamin A was efficiently recovered both in its fully processed form (LA-WT) and in its non-farnesylable (LA-C661M) or stably farnesylated (LA-L647R) unprocessed form (Fig. 4A). Both HP1α and the known lamin A-binding protein LAP2α were recovered in the protein complex formed by the fully processed lamin A or by non-farnesylable pre-lamin A (Fig. 4A). However, HP1α was hardly detectable in the immunoprecipitated complex from cells

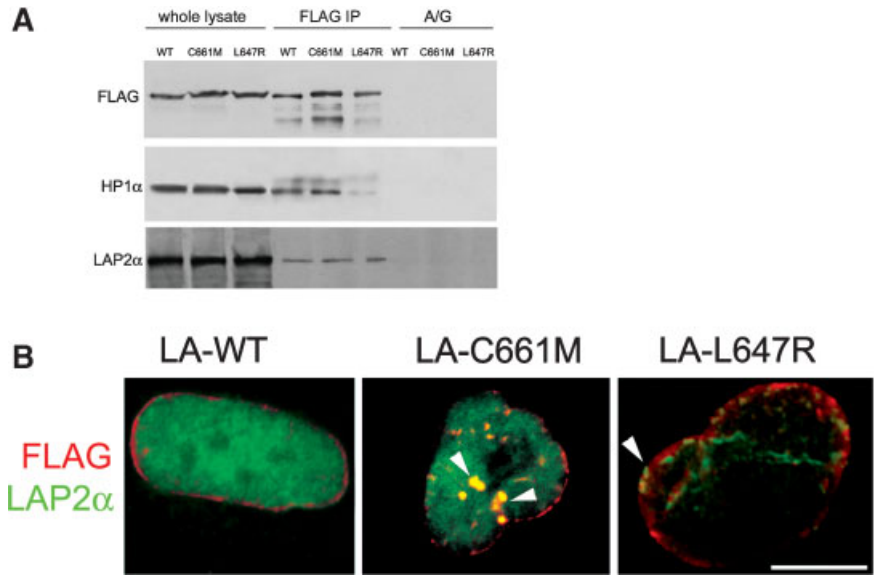

Fig. 4. Pre-lamin A is bound to HP1alpha and LAP2alpha in vivo and affects LAP2alpha localization. **A**: HEK 293 cells expressing FLAG-tagged pre-lamin A forms were fixed in formaldehyde, mechanically lysed by sonication, and pre-lamin A was immunoprecipitated using monoclonal anti-FLAG antibody. Control precipitations were performed with antibody-free medium (A/G). Western blots of immunoprecipitates (pre-lamin A IP) and whole cellular lysates immunolabeled with antibodies to FLAG, HP1α, or LAP2α are shown. **B**: Double labeling of LAP2α (green) and FLAG-tagged pre-lamin A (detected by anti-FLAG antibody, red). The images are reconstruction of four equatorial sections passing through the mid-section of the nucleus, obtained by confocal microscopy analysis. Arrowheads indicate areas of co-localization in LA-C661M and LA-L647R expressing nuclei. Bar, 10 μm.

accumulating the farnesylated pre-lamin A mutant (Fig. 4A). Non-specific precipitation of proteins was never detected in the absence of primary antibody (Fig. 4A). These results show the existence of a complex formed by LAP2α and pre-lamin A which interacts with HP1α. Pre-lamin A farnesylation might affect the stability of this complex. Since we had detected a stable interaction between pre-lamin A and LAP2α, we analyzed LAP2α localization in fibroblasts expressing different pre-lamin A forms. LAP2α was uniformly distributed in the nucleus of cells expressing LA-WT, while it partially co-localized with intra-nuclear pre-lamin A aggregates in LA-C661M-transfected nuclei (Fig. 4B). Enlarged mis-shapen nuclei frequently observed in LA-L647R-transfected cells showed LAP2α staining at the nuclear periphery, and significantly reduced LAP2α fluorescence in the nuclear interior (Fig. 4B). We speculate that these might be cells that have exited the cell cycle.

DISCUSSION

Our studies show that different intermediates of the lamin A precursor processing pathway differently affect chromatin organization and cause re-localization of heterochromatin-associated proteins and LAP2α. The effects involve a protein complex which includes LAP2α and HP1α and suggest a role of pre-lamin A in higher order chromatin dynamics.

All the overexpressed proteins were efficiently localized to the nuclear envelope or to intranuclear sites, indicating that neither the tag nor the mutated sequence interfere with the nuclear import of the protein. Although the expression level of transfected proteins slightly varied from one nucleus to the other, only the nuclei with an expression level comparable to drug-induced pre-lamin A accumulation levels were considered. The overall evaluation of the data reported in our study shows that pre-lamin A post-translational modifications differently affect protein localization. Non-farnesylated pre-lamin A, the fully unprocessed lamin A precursor harboring the CaaX sequence [Holtz et al., 1989; Hennekes and Nigg, 1994], localized predominantly in internal nuclear foci, while carboxymethylated–farnesylated pre-lamin A was detected at the nuclear envelope and in nuclear invaginations. While the localization of

non-farnesylated pre-lamin A in the nuclear interior and of the farnesylated pre-lamin A forms at the periphery has been reported before [Hennekes and Nigg, 1994; Sasseville and Raymond, 1995; Rusinol and Sinensky, 2006], a thorough comparison of the properties of the different lamin A precursors has not been reported yet. Formation of pre-lamin A-containing nuclear aggregates had been reported both in cells overexpressing wild-type lamin A and in cells overexpressing pathogenetic *LMNA* mutants [Hubner et al., 2006a]. Since we have observed nuclear aggregates in fibroblasts expressing non-farnesylated C661M-lamin A only, we can suggest that the nuclear aggregates described in that study [Hubner et al., 2006a] must contain an amount of non-farnesylated lamin A precursor. The presence of non-farnesylated pre-lamin A in cells from laminopathic patients affected by MADA or lipodystrophy showing nuclear aggregates [Capanni et al., 2003, 2005; Hubner et al., 2006b] represents an important issue to be addressed. However, unlike the data reported by Hubner et al., the appearance of nuclear aggregates in wild-type lamin A-transfected cells was an extremely rare event in our experimental model and it was restricted to the nuclei with very high amount of overexpressed protein. It should be noted that high levels of overexpressed nuclear proteins have been proposed to cause self-aggregation of excess protein that is not used [Misteli, 2005]. In our experimental model, nuclear pre-lamin A aggregates were observed with LA-C661M non-fanesylable pre-lamin A, irrespective of the expression levels, indicating an effect specific of that molecule.

In the C661M-lamin A expressing nuclei the pre-lamin A aggregates were localized both at the periphery and at the nuclear interior and they were formed by a proteinaceus constituent positive to FLAG labeling, as determined by immuno-electron microscopy analysis. A role of pre-lamin A aggregates in nuclear function was suggested by Hubner et al., who showed recruitment of several nuclear factors such as Rb1 and SREBP1 at the aggregates, possibly leading to the downstream regulation of some transcriptional activities [Hubner et al., 2006a]. Along this line, we had shown that binding of pre-lamin A to SREBP1 regulates the transcriptional activation of adipocyte-specific genes [Capanni et al., 2005]. Our present study adds the knowledge of a molecular interplay between pre-lamin A and chromatin-regulating factors including LAP2α and HP1α.

In fact, the most striking results reported in our study are the different effects of pre-lamin A forms on the re-organization of heterochromatin domains. Confocal and electron microscopy analysis of cells accumulating non-farnesylated pre-lamin A, showed heterochromatin areas in the nuclear interior, at sites where both LAP2α, tri-H4K20, and HP1α accumulated as well. This observation is in strong agreement with the finding here reported that a molecular complex including LAP2α, HP1α, and pre-lamin A is formed in cells accumulating non-farnesylated pre-lamin A. That complex could bring about recruitment (and possibly remodeling) of heterochromatin domains in the nuclear interior. This aspect requires deep studies since complex mechanisms triggered by HP1 in association or not with tri-methylated H3K9 are involved in the recruitment and remodeling of heterochromatin domains [Verschure et al., 2005]. On the other hand, the effect elicited by carboxymethylated pre-lamin A was the overall decondensation of heterochromatin domains. The underlying mechanism may involve relocalization of heterochromatin areas into the nuclear interior, as indicated by the presence of multiple nuclear envelope invaginations surrounded by thin heterochromatin layers or lacking heterochromatin completely. Loss of heterochromatin was accompanied by dispersion of HP1α and tri-H3K9 in the nucleoplasm. Interestingly, a mechanism leading to decondensation of heterochromatin domains, involving HP1α redistribution in the nucleus has also been described in trichostatin A-treated cells [Taddei et al., 2001; Bartova et al., 2005], further supporting the hypothesis that pre-lamin A processing is linked to epigenetic mechanisms in higher order chromatin organization.

Importantly, HP1α was co-immunoprecipitated with pre-lamin A in a LAP2α—containing complex, strongly suggesting an involvement of LAP2α in pre-lamin A-mediated chromatin dynamics. The effects of lamin A precursors on LAP2α localization support this hypothesis. We suggest that partial co-localization is due to a dynamic interaction between pre-lamin A and LAP2α which is likely regulated by the presence of other molecular partners. In previous studies, LAP2α has been reported to

associate with chromatin during nuclear assembly and to be involved in cell cycle dependent chromatin reorganization [Dechat et al., 2004]. It is noteworthy that the amount of HP1α in the LAP2α-pre-lamin A complex was dramatically reduced when farnesylated pre-lamin A was expressed. In a set of experiments also different stoichiometry of LAP2α was observed in the immunoprecipitated complex for different pre-lamin A forms (not shown). These results indicate that the stability of the protein complex could be regulated by pre-lamin A farnesylation. In this context, we speculate that cell cycle- and/or tissue-specific interactions of pre-lamin A with LAP2α could affect the regulation of differentiation-related heterochromatin remodeling. Interestingly, pre-lamin A-LAP2α binding is disrupted by LAP2α mutations causing cardiomyopathy [Taylor et al., 2005], suggesting a key and possibly tissue-specific role of their interaction.

Other lamin A binding partners potentially involved in the pre-lamin A-mediated nuclear changes are B-type lamins. A recent work shows co-polymerization of progerin, which is a carboxymethylated farnesylated pre-lamin A form, and lamin B1, supporting the notion that an altered nuclear lamina composition could contribute to the nuclear effects elicited by carboxymethylated–farnesylated pre-lamin A accumulation [Delbarre et al., 2006]. The functional relationship between BAF, which is a key player of lamin-mediated chromatin effects [Margalit et al., 2005], and pre-lamin A accumulation remains an intriguing question. A possible cooperative role of other lamin A partners and nuclear envelope proteins such as the lamin B receptor and emerin in pre-lamin A-linked heterochromatin remodeling appears likely. It is noteworthy that LBR localization is altered in MAD cells accumulating pre-lamin A [Filesi et al., 2005]. Moreover, an interplay between LBR and HP1 and nucleosomal DNA has been demonstrated [Ye et al., 1997].

A functional link between the processing of lamin A precursors and the remodeling of chromatin can also be seen in laminopathies, as heterochromatin re-organization consisting of progressive loss of condensed chromatin areas, and of tri-H3K9 and HP1α staining is observed in those laminopathic cells that accumulate farnesylated pre-lamin A forms [Capanni et al., 2003; Columbaro et al., 2005; Filesi et al., 2005; Navarro et al., 2005; Scaffidi and Misteli, 2005] including lipodystrophy or progeroid disorders, while less severe alterations are observed in other laminopathies [reviewed in Reference Maraldi et al., 2006]. Interestingly, also the mislocalization and downregulation of LAP2α observed in the nuclei accumulating carboxy-methylated pre-lamin A has been found in RD and HGPS nuclei (our unpublished results). In MAD cells, HP1β solubility was found to be dramatically increased, and heterochromatin domains reduced [Filesi et al., 2005]. On the other hand, complete loss of tri-H3K27 staining was not observed in cells expressing pre-lamin A mutants, while it was reported in HGPS cells at high passage number [Shumaker et al., 2006 and our unpublished results]. This suggests that the passage number in cultured cells influences the loss of facultative heterochromatin domains. In conclusion, our results indicate a key role of pre-lamin A in chromatin dynamics, and suggest that the processing rate and hence the level of each processing intermediate must be properly regulated to avoid toxic heterochromatin changes. On the other hand, transient accumulation of pre-lamin A may allow dynamic reorganization of heterochromatin areas during differentiation. Sustained accumulation of unprocessed protein as detected in some laminopathies clearly causes nuclear disorganization and improper chromatin organization.

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