A progeria mutation reveals functions for lamin A in nuclear assembly, architecture, and chromosome organization

Pekka Taimen1, Katrin Pfleghaar2, Takeshi Shimi3, Dorothee Möller4, Kfir Ben-Harush5, Michael R. Erdos6, Stephen A. Adam7, Harald Hermann3, Ohad Medali4, Francis S. Collins8,2, Anne E. Goldman9, and Robert D. Goldman10,2

1Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611; 2Division of Molecular Genetics, German Cancer Research Center, D-69120 Heidelberg, Germany; Department of Life Sciences, Ben Gurion University and the NIBN, Beer-Sheva 84120 Israel; and 3Genome Technology Branch, National Human Genome Research Institute, Bethesda, MD 20892

Contributed by Francis S. Collins, October 15, 2009 (sent for review September 10, 2009)

Numerous mutations in the human A-type lamin gene (LMNA) cause the premature aging disease, progeria. Some of these are located in the α-helical central rod domain required for the polymerization of the nuclear lamins into higher order structures. Patient cells with a mutation in this domain, 433G>A (E145K) show severely lobulated nuclei, a separation of the A- and B-type lamins, alterations in pericentric heterochromatin, abnormally clustered centromeres, and mislocalized telomeres. The induction of lobulations and the clustering of centromeres originate during postmitotic nuclear assembly in daughter cells and this early G1 configuration of chromosomes is retained throughout interphase. In vitro analyses of E145K-lamin A show severe defects in the assembly of protofilaments into higher order lamin structures. The results show that this central rod domain mutation affects nuclear architecture in a fashion distinctly different from the changes found in the most common form of progeria caused by the expression of LAΔ50/ progerin. The study also emphasizes the importance of lamins in nuclear assembly and chromatin organization.

Results

Dermal fibroblasts from an E145K progeria patient (E145K cells) contain severely lobulated nuclei (Fig. L4 and Fig. S1 A). Approximately 32% (n = 300) of the nuclei showed three or more lobules (multilobulated) by passage (p) 9, and by p13 this increased to approximately 69% (n = 300) (Fig. S1 B). Normal fibroblasts (control cells) were essentially free of lobules (~1% (n = 300), p15) (Fig. S1 B). Contour ratio analyses confirmed that E145K nuclei had very different shapes compared to controls (Fig. S1 C). Furthermore, the lobulated nuclei in E145K cells did not regain normal nuclear shapes following 48-72 h of treatment with the farnesyl transferase inhibitor FTI-277 (Fig. S2), which is distinctly different from cells expressing LAΔ50/progerin (8, 9).

Immunofluorescence revealed that LA/C were concentrated in the lamina with lower levels in the nucleoplasm in both E145K nuclei and controls (Fig. L4 A). However, in multilobulated E145K nuclei there was an overall reduction in LB1 and LB2, which, by p13 this decreased to approximately 69% (n = 300) (Fig. S1 B). Contour ratio analyses confirmed that E145K nuclei had very different shapes compared to controls (Fig. S1 C). Furthermore, the lobulated nuclei in E145K cells did not regain normal nuclear shapes following 48-72 h of treatment with the farnesyl transferase inhibitor FTI-277 (Fig. S2), which is distinctly different from cells expressing LAΔ50/progerin (8, 9).

The authors declare no conflict of interest.

FREELY AVAILABLE ONLINE THROUGH THE PNAS OPEN ACCESS OPTION.

This article contains supporting information online at www.pnas.org/cgi/content/full/0911895106/DCSupplemental.
were enriched in the proximal regions of the lobules frequently in close association with heterochromatin, where little or no LA/C could be detected (Fig. 1A). Approximately 15% (n = 300) of the multilobulated E145K nuclei also had smaller nuclear envelope (NE) “blebs” enriched in LA/C, but devoid of LB1/2 (Fig. 1A) (10, 11). Immunoblotting confirmed a significant reduction in the amount of LB1, and to a lesser extent LB2, in E145K cells relative to controls (Fig. 1B). Emerin, an inner nuclear membrane protein, and nuclear pore complexes (NPCs), were located in all regions of the nuclear surface in the multilobulated E145K nuclei, extending into the clefts formed by adjacent lobules (Fig. S3 A and B).

Transmission electron microscopy (TEM) revealed that the lobules of E145K nuclei contained a distinct lamina and a significant reduction in peripheral heterochromatin compared to controls (Fig. S3C) (10). In agreement with the fluorescence observations, the heterochromatin in the multilobulated nuclei was more pronounced in regions where the deep invaginations of adjacent lobules appeared to merge (Fig. S3C).

The reduction in heterochromatin in lobules suggested a loss of epigenetic marks as previously described in patient cells expressing LAΔ50/progerin (12). Immunofluorescence revealed that histone H3 trimethylated at lysine 9 (H3K9me3), a mark for pericentric constitutive heterochromatin and repressed euchromatic, was concentrated in the same central region that stained intensely with Hoechst (Fig. 2A). This is different from normally shaped E145K nuclei or nuclei in normal cells where this mark is enriched in the nuclear periphery and perinucleolar region (Fig. 2A). Staining for H3K27me3, a mark for facultative heterochromatin, showed no obvious differences between multilobulated and normally shaped nuclei in E145K or control cells (Fig. 2A).

Immunostaining with CREST antibody (13) revealed that approximately 80% (n = 100) of multilobulated E145K nuclei contained abnormally clustered centromeres in the region of intense Hoechst staining (Fig. 2B). Similar centromere clustering occurred in only approximately 6% (n = 100) of normally shaped E145K nuclei and approximately 3% (n = 200) of control nuclei. The majority of normally shaped nuclei had centromeres distributed throughout the nucleoplasm, with some closely associated with the lamina (Fig. 2B). We also determined telomere positioning relative to centromeres with a telomeric FISH probe and immunostaining with CREST and LA antibodies. In normally shaped E145K and control cell nuclei, telomeres and centromeres were found interspersed throughout the nucleoplasm (Fig. 2C). In multilobulated E145K nuclei, many telomeres were no longer interspersed with centromeres, but were found at varying distances from the clustered centromeres and were frequently associated with the lamina (Fig. 2C).

To determine if the changes in nuclear architecture are due directly to E145K-LA, FLAG-tagged E145K-LA (FLAG-E145K-LA) was expressed in HeLa cells (Fig. 3A). After 24 h, 2.7 ± 1.4% of the transfected cells were multilobulated, increasing to 18 ± 3% and 37 ± 9% at 48 and 72 h posttransfection (p.t.), respectively. Control cells expressing wild-type LA (FLAG-WT-LA) were rarely lobulated at 72 h p.t. (2.4 ± 0.8%) (Fig. 3A). The multilobulated HeLa nuclei expressing FLAG-E145K-LA also contained centrally clustered centromeres as indicated by CREST staining (Fig. 3A), and few telomeres were interspersed with the centromeres (Fig. 3B). To verify this, the distribution of the telomeres was quantified in Z-stacks of confocal images revealing that 16 ± 3 telomeres (n = 14) were associated with the lamina region in FLAG-WT-LA expressing HeLa cells, whereas in FLAG-E145K-LA expressing cells, an average of 35 ± 6 telomeres (n = 10 nuclei) were associated with the lamina. These results demonstrate that the expression of E145K-LA has a profound effect on the organization of chromatin.

To test whether this atypical chromatin organization affects DNA replication, double replication labeling of E145K patient cells was used (11, 14). The number of E145K cells with both early and late replication foci (~9%, n = 538) was significantly decreased compared to controls (~27%, n = 528) at p13. Furthermore, the distribution of early and late replication foci was altered in E145K cells. In controls, early replication foci were distributed throughout the nucleoplasm, while mid-to-late replication took place both in the lamina region and in large nucleoplasmic foci. Many of the latter coagulated with centromeres (Fig. 4). In contrast, the majority of the early replicating foci were in lobulated regions in E145K nuclei, while many late replicating foci were concentrated in the central region containing clustered centromeres (Fig. 4). These data suggest that although chromatin is disorganized, the overall timing of early and late replication was not affected. Analysis of other proliferation markers revealed that approximately 6% of E145K cells (p13, n = 300) and approximately 26% of control cells (p13, n = 300) showed typical S phase PCNA patterns (15); while approximately 9% of E145K (p13, n = 200) and approximately 42% of control (p15, n = 200) cells were positive for the Ki-67 antigen (16), suggesting that the mutant cells had reduced proliferative capacity.

The E145K cells also senesce prematurely compared to controls as assayed by senescence-associated β-galactosidase (17). While approximately 8% (n = 620) of control cells were positive for β-galactosidase at p17, approximately 23% (n = 743) of E145K cells were positive at p14. There was also a strong correlation between senescence and nuclear lobulation. This was supported by the findings that approximately 4% (n = 208) of E145K cells with normally shaped nuclei, approximately 12% with one or two nuclear lobules (n = 163), and approximately 39% with multilobulated nuclei (n = 372) were positive for β-galactosidase (Fig. 5A).

To determine when multilobulated nuclei with clustered centromeres form, we carried out live cell imaging studies in HeLa cells stably expressing YFP-tagged centromere protein A (YFP-CENP A). When these cells were cotransfected with mCherry-WT-LA and either FLAG-E145K-LA or FLAG-WT-LA, approximately 99% of the mCherry-WT-LA positive cells also expressed FLAG-tagged LA at 72 h p.t. This multiple transfection was necessary as EGFP-E145K-LA did not induce the
nuclear phenotype seen with FLAG-E145K-LA. The reason for this difference is unknown, but may be attributable to the larger size of the EGFP tag relative to FLAG. Live imaging of interphase and mitotic cells revealed no differences between the FLAG-WT-LA and FLAG-E145K-LA-transfected cells through early telophase (Fig. 5, Fig. S5, and Movies S1 and S2). However, approximately 59% (n/11005) of the FLAG-E145K-LA expressing cells entering mitosis with normally shaped nuclei, formed

Fig. 2. Mislocalization of heterochromatin, centromeres, and telomeres in E145K cells. (A) E145K (p11) and control cells (p11) were stained with either anti-H3K9me3 or anti-H3K27me3 and Hoechst. Mid-plane confocal sections show enrichment of H3K9me3 in the central region of the nucleus in E145K cells (arrowhead), while it is enriched in the nuclear periphery and in perinucleolar regions in controls (arrowheads). H3K27me3 is distributed throughout the nucleus in both cells and enriched in the Xi in female control cells (arrowhead). (Scale bars, 5 μm.) (B) E145K (p13) and control cells (p15) were stained with anti-LA, CREST antiserum, and Hoechst. Maximum projections of series of z-sections spanning the entire nucleus and side projections are shown. Centromeres are clustered in the central region of the nucleus in E145K cells, while they are either closely associated with the peripheral lamina region or elsewhere in the nucleoplasm in control cells. Inset shows an example of the close association of one centromere with the lamina in a single confocal section. (Scale bars, 5 μm.) (C) E145K and control cells were stained with anti-LA/C, CREST antiserum, and Hoechst. A FISH probe was used to visualize telomeres. Maximum projections of series of z-sections spanning the entire nucleus and side projections from merged images are shown. Centromeres and telomeres are distributed throughout the nuclei in control cells. In E145K cells centromeres are clustered in the middle of one region of the nucleus, while telomeres are more frequently associated with the peripheral lamina region. (Scale bars, 5 μm.)

Fig. 3. Expression of FLAG-E145K-LA in HeLa cells induces nuclear lobulation and mislocalization of centromeres and telomeres. (A) HeLa cells were transfected with either FLAG-WT-LA or FLAG-E145K-LA for 72 h and stained with anti-FLAG and CREST antiserum. Maximum projections of series of z-sections spanning the entire daughter cell nuclei are shown. Note the extensive lobulation of the lamina and clustering of centromeres in FLAG-E145K-LA-expressing cells. (Scale bars, 5 μm.) (B) HeLa cells were transfected with either FLAG-WT-LA or FLAG-E145K-LA for 72 h, fixed, and stained with anti-FLAG, CREST antiserum, Hoechst, and a FISH probe to visualize telomeres. Maximum projections of z-sections and side projections are shown as above. Centromeres and telomeres are distributed throughout the nuclei in FLAG-WT-LA-expressing cells. In FLAG-E145K-LA-expressing cells centromeres and telomeres are less interspersed, and telomeres are more likely to be associated with the peripheral lamina region. (Scale bars, 5 μm.)

Taimen et al. PNAS Early Edition
daughter cells with multilobulated nuclei and clustered centromeres (Fig. 5 and Movie S1). Centromere clustering was maintained for the entire period of observation up to 10 h following cytokinesis (Fig. 5). In contrast, newly divided cells expressing FLAG-WT-LA always formed normally shaped nuclei with a normal distribution of centromeres within 1-2 h into G1 (n = 12) (Fig. S5 and Movie S2). The multilobulated nuclei induced by FLAG-E145K-LA never reverted to normal shapes following mitosis. In fact, some cells with lobulated nuclei divided to form daughter cells with lobulated nuclei and clustered centromeres (Fig. S6A). This observation was supported by flow cytometric DNA content analysis, which showed no differences in the distribution of cells in different phases of the cell cycle between FLAG-WT-LA and FLAG-E145K-LA expressing cells at 72 h p.t. (Fig. S7). Further confirmation that the nuclear shape changes induced by FLAG-E145K-LA require cell division was obtained by arresting cells in early S phase with aphidicolin (18). This treatment completely inhibited the formation of multilobulated nuclei (Fig. S6B).

The location of the E145K mutation in the central rod domain of LA/C suggested that a disruption of normal lamin assembly might be responsible for the changes in nuclear architecture. In vitro analyses of LA complex formation by analytical ultracentrifugation (AUC) (19, 20) showed that both E145K-LA and LAΔ50/progerin form dimers that are indistinguishable from WT-LA (Fig. S8A). To initiate assembly of higher order structures, solutions of dimers were diluted into assembly buffer, and samples were prepared at different times for TEM analysis. Both WT-LA and LAΔ50/progerin formed extended arrays of filaments of varying diameter after 10 min (Fig. 6). In contrast, E145K-LA formed interconnected fibrous strands, which aggregated into globular disorganized assemblies. The structures formed by WT-LA and LAΔ50/progerin at 10 min were transient states leading to larger and more ordered arrays after 30 min (Fig. S8B). These structures exhibited a characteristic 24-nm axial repeat reflecting the lateral anti-parallel association of head-to-tail chains of dimers. In contrast, some thin E145K-LA filaments appeared after 30 min, but no axial repeats could be detected (Fig. S8B).

The end point of the in vitro assembly of lamins is the formation of PCs. Due to the thickness of PCs, we determined their structure in unfixed preparations embedded in amorphous ice by using cryo-electron tomography (cryo-ET) and 3-D image processing (21). Slices (x-y) of 60-nm-thick sections through reconstructed volumes of typical WT-LA and LAΔ50/progerin PCs showed regular arrays of protofilaments exhibiting the expected 24-nm repeats of dimeric tail domains along the ordered structures (Fig. 6). In contrast, E145K-LA formed atypical PCs with no obvious longitudinal repeat patterns demonstrating that the lateral association of protofilaments was abnormal (Fig. 6).

Since previous studies showed that LAΔ50/progerin is less soluble than WT-LA (22), the solubility of E145K-LA was also tested. Sequential extraction of FLAG-E145K-LA and FLAG-WT-LA expressing HeLa cells, however, showed no differences in the solubilities of WT-LA and E145K-LA, suggesting that both assembled into stable structures under these conditions (Fig. S9).

Discussion
The E145K progeria mutation in LA/C alters lamin structure and assembly, inducing profound changes in nuclear architecture, a reduction in B-type lamin expression, and premature senescence. In contrast to the more common LAΔ50/progerin mutation, the E145K mutation does not alter the posttranslational processing of the C terminus, which explains why its impact on nuclear shape is not reversed by FTTI treatment (6, 7). The E145K mutation also causes the clustering of centromeres and their associated H3K9me3, but it does not alter the distribution of H3K27me3 heterochromatic mark. In contrast, LAΔ50/progerin alters both of these epigenetic marks, and the association between H3K9me3 and some centromeres is lost (12). In addition, the E145K mutation has no obvious impact on the distribution of NPCs, which is in contrast to the abnormal distribution of NPCs in LAΔ50/progerin expressing cells (10). Therefore, distinctively different cellular phenotypes are induced by these two mutations causing premature aging.

The nuclear phenotypes induced by E145K-LA and LAΔ50/progerin suggest that these mutations may cause progeria by altering the structure of the lamina, albeit by different mechanisms. In support of this, the E145K mutation has a deleterious effect on LA polymerization into higher order structures in vitro;
whereas the polymerization of LAΔ50/progerin, whose amino acid sequence is significantly different from both WT-LA and E145K-LA, appears normal. Thus, it is likely that it is the abnormal association and accumulation of permanently farnesylated LAΔ50/progerin at the NE membrane that disrupts the normal functions of the lamin in the LAΔ50/progerin patients (6–10, 12), whereas in the case of E145K patients, it is the abnormal polymerization of LA that prevents the assembly of a normal lamina structure. Ultimately such differences could help to explain the different phenotypes seen in progeria, as well as other laminopathy patients bearing different mutations in LMNA.

The impact of alterations in the E145 residue are most likely attributable to its location in subdomain 1B of the central rod domain of LAC/C. Similarly, mutations in the central rod domain of cytoskeletal IF proteins, such as desmin, keratins, and vimentin, have severe consequences for filament structure and function and cause variety of diseases including skeletal and cardiac myopathies (23), skin blistering diseases (24), and cataracts (25). The E145 residue is in an e position of one of the six heptads within the 42-amino acid sequence that is unique to lamin, and it is predicted to be solvent exposed rather than residing in the coiled-coil interface. Although substituting lysine for glutamic acid at residue 145 does not alter dimer assembly as shown by AUC analysis, it does alter the normal alignment of protofilaments into filaments and PCs (2). In further support of this, a mutation in the corresponding site of Caenorhabditis elegans lamin (lmm-1), Q159K, alters the structure of in vitro assembled filaments (21, 26).

As we previously observed in LAΔ50/progerin expressing patient fibroblasts (10), nuclear shape defects accumulate as a function of passage number in E145K cells suggesting a dominant negative effect of this mutant LAC/C protein. In addition, live imaging of HeLa cells expressing FLAG-E145K-LA and treatment with aphidicolin demonstrate that lobulation is established during postmitotic nuclear assembly. Therefore, it is likely that the assembly of the lamina during late telophase/early G1 is severely affected once the relative amount of the E145K mutant lamin reaches a critical level. It is also relevant to note that the E145K mutation is present in both LA and LC, whereas the 1824C>T (LAΔ50/progerin) mutation does not affect the sequence of LC.

Within interphase nuclei, chromosomes are organized into “territories,” with gene-rich chromosomes more likely to be internally located and gene-poor chromosomes more likely to be at the nuclear periphery (27, 28). Individual chromosomes are typically oriented with their centromeres situated in the nuclear periphery (29), while telomeres are distributed throughout the nucleoplasm (30). In the multilobulated E145K, nuclei are organized with their centromeres clustered in one region. This is reminiscent of the "Rab1 configuration," a polarized chromosome configuration found in the interphase nuclei of Drosophila and certain plant cells (31). The Rab1 configuration originates in anaphase, when sister centromeres separate as chromosomes move to the spindle poles, and it is maintained throughout interphase until the next mitosis (32). The polarized configuration of chromosomes in E145K cells is also established during and immediately following mitosis and persists throughout the G1 and S phases of the cell cycle (Fig. S10).

Therefore, the expression of LAΔ50/progerin inhibits the normal reorganization of interphase chromosome territories in daughter cells in early G1, most likely due to the formation of an abnormal lamina structure. In support of this, it has been shown that lamins interact with mitotic chromosomes (32–34) and that their assembly at the surface of chromosomes is crucial for postmitotic nuclear formation (35, 36). It is also possible that the E145K mutation interferes with the nuclear lamina binding to matrix attachment regions of DNA (37) or interactions with other chromatin associated lamin-binding proteins such as histones, LAP2α, BAF (33), and/or SUN2 (38). Even though we do not know the exact mechanisms responsible for the impact of the E145K mutation, the results of this study provide important insights into the role of lamins in the spatial and temporal organization of chromosomes throughout the cell cycle.

In summary, the E145K mutation causes changes in lamin polymerization and assembly, resulting in a cellular phenotype that is distinctly different from LAΔ50/progerin expressing cells. Common features shared by these two very different mutations are that they both result in the down-regulation of B-type lamins (39) and premature replicative senescence (40). These factors may represent common denominators in the etiology of the different forms of premature aging disease. Interestingly, the expression of LAΔ50/progerin in human adult mesenchymal...
stem cells also alters their differentiation pathways (41), which may ultimately explain why mesenchymal tissues are those most affected in HGPS. Whether E145K-LA also leads to a similar failure in stem cell differentiation remains to be determined.

Materials and Methods

Cell Culture. Fibroblasts from a male progeria patient with the 433G>A (E145K) mutation (AG10677) and from a healthy donor (AG08470; Coriell), HeLa cells, and a HA cell line expressing YFP-CENP A (from D.R. Foltz) were cultured as described in ref. 10. Transfections were carried out with the TransIT-HeLa MONSTER kit (Mirus). A farnesytransferase inhibitor FTI-277 (Sigma) was used at a concentration of 5 or 10 μM for 48–72 h. Aplidocilin (Sigma) was used at a concentration of 5 μg/mL.

Plasmids. pEGFP-C1-E145K-LA was prepared by RT-PCR amplification of the E145K-LA sequence from RNA prepared from the AG10677 cell line and ligation into pEGFP-C1; pFLAG-CMV2-E145K-LA was subcloned into pEGFP-myc-hLMNA (32) and pFLAG-CMV2-LA-WT was derived from pFLAG-CMV2-E145K-LA by using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). To construct a mCherry-myc-hLMNA vector, the LA fragment was cut from pEGFP-myc-hLMNA (32) and inserted into pm-Cherry-C1 (42).

Microscopy. Immunofluorescence and Hoechst staining were performed and preparations analyzed as in (11). Mouse anti-V-A4-C (Lc2; Chemicon), LB2 (LN43; Abcam), emerin (Novocastra), PCNA (PC10; Santa Cruz Biotechnology), FLAG (Sigma), NPC (MAbA14; Covance), and rabbit anti-LA (7), LB1 (43), H3K9me3 and H3K27me3 (from T. Jenuwein), Ki-67 (DAKO), and human anti-CREST (from B. Brinkley) were used. Secondary antibodies were goat anti-mouse, anti-rabbit, and anti-human IgG-Alexa Fluor 488, 568, and 633 (Molecular Probes). Double replication labeling was carried out as in (11). Centromere and telomere localization was analyzed in z-stacks of nuclei by using Zeiss LSM software. In transfection studies, nuclear shape in 300 cells in three experiments was analyzed (mean ± SD). Live imaging was performed as in (11). Telomere FISH was carried out as described in ref. 44. For TEM, cultured cells were processed as in (45) and for negative staining as in (46). Cyo-ET and structural analysis was carried out as in (21).

Immunoblotting. Equal amounts of protein from whole cell lysates were analyzed by immunoblotting as in (11). Mouse anti-a-actin (Sigma) was used as a loading control.

In Vitro Assembly. Details are provided in SI Text.

ACKNOWLEDGMENTS. We thank D.R. Foltz (University of Virginia, Charlottesville, VA) for the YFP-CENP A HeLa line and D. Parry for a helpful discussion. R.D.G. was supported by the National Institute on Aging and the Ellison Foundation; H.H. by the European Commission; O.M. by the Fritz-Thysen Stiftung; H.H. and O.M. by the German-Israel Foundation; P.T. by the Sigrid Jusélius Foundation, Orion-Farmos Research Foundation, Cancer Society of Southwestern Finland, and Finnish Cultural Foundation; and K.P. by the Deutsche Forschungsgemeinschaft.