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Retinoblastoma-independent regulation of cell proliferation and senescence by the p53–p21 axis in lamin A/C-depleted cells

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Summary

The expression of A-type lamin is downregulated in several cancers, and lamin defects are the cause of several diseases including a form of accelerated aging. We report that depletion of lamin A/C expression in normal human cells leads to a dramatic downregulation of the Rb family of tumor suppressors and a defect in cell proliferation. Lamin A/C-depleted cells exhibited a flat morphology and accumulated markers of cellular senescence. This senescent phenotype was accompanied by engagement of the p53 tumor suppressor and induction of the p53 target gene p21 and was prevented by small hairpin RNAs against p53, p21, or by the oncoprotein Mdm2. The expression of E2F target genes, normally required for cell cycle progression, was downregulated after lamin A/C depletion but restored after the inactivation of p53. A similar senescence response was observed in myoblasts from a patient with a lamin A mutation causing muscular dystrophy. We thus reveal a previously unnoticed mechanism of controlling cell cycle genes expression, which depends on p53 but does not require the retinoblastoma family of tumor suppressors and that can be relevant to understand the pathogenesis of laminopathies and perhaps aging.

Key words: E2F; lamin A; muscular dystrophy; p21; p53; senescence.

Introduction

Lamins are intermediate filament proteins that form the nuclear lamina, a lattice structure in intimate contact with the inner membrane of the nuclear envelope that is reversibly disassembled during cell division (Gerace & Blobel, 1980; Gruenbaum *et al.*, 2003). They are also located in internal regions of the nucleus during the G1 phase of the cell cycle in proliferating cells in culture or in regenerating tissues *in vivo* (Bridger *et al.*, 1993; Dorner *et al.*, 2007). There are two kinds of lamins, the B-type, essential for viability, and the A-type (primarily lamins A and C, which are the products of alternative splicing of the *LMNA* gene) (Gruenbaum *et al.*, 2003; Hutchison & Worman, 2004). Embryos of up to 8 days of gestation contain only B-type lamin (Stewart & Burke, 1987) while A-type lamin expression correlates with cell differentiation (Rober *et al.*, 1989). Mice disabled for the expression of A-type lamins develop normally, but their postnatal growth is impaired by a severe muscular dystrophy (Sullivan *et al.*, 1999). At the cellular level, the absence of A-type lamins confers deformability properties to the cell nucleus (Broers *et al.*, 2004; Lammerding *et al.*, 2004). This nuclear trait can also be observed in embryonic stem cells that do not express lamin A/C or in epithelial cells after knockdown of lamin A/C (Pajerowski *et al.*, 2007).

Mutations in A-type lamins are the underlying cause to a variety of diseases, including the accelerated aging condition known as Hutchinson-Gilford progeria syndrome (HGPS) (De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003; Gruenbaum *et al.*, 2003). Because lamins are part of the scaffold of the nuclear envelope, it has been proposed that defects in this structure lead to cellular stress and eventually a compromise in tissue functions (Liu *et al.*, 2005). Consistent with this idea, *Lmna*$^{-/-}$ cells or fibroblasts from patients having lamin A mutations have nuclear envelope deformations (Eriksson *et al.*, 2003; Mounkes *et al.*, 2003; Goldman *et al.*, 2004).

A-type lamins associate with the tumor suppressor protein retinoblastoma (Rb) (Ozaki *et al.*, 1994; Markiewicz *et al.*, 2002) protecting it and the related protein p107 from proteasome-dependent degradation (Johnson *et al.*, 2004). Rb and p107 interact with E2F family members and maintain cells in the G1 phase of the cell cycle through repression of E2F target genes (Dyson, 1998). Therefore, *Lmna*$^{-/-}$ cells are expected to have altered cell cycle dynamics, an observation confirmed in immortalized fibroblasts (Johnson *et al.*, 2004; Nitta *et al.*, 2006). In addition, Rb controls the formation of heterochromatin (Nielsen *et al.*, 2001; Narita *et al.*, 2003; Gonzalo *et al.*, 2005) perhaps explaining why cells with defects in lamin A also display a reduction in several heterochromatin marks (Scaffidi & Misteli, 2005). The expression of A-type lamins is reduced in several cancers (Prokocimer *et al.*, 2006). For instance, in leukemias and lymphomas, the A-type lamin promoter is silenced by hypermethylation and this event is associated with poor prognosis (Agrelo *et al.*, 2005). Taking together, these data suggest that A-type lamin expression is important for proper control of cell proliferation, and its loss could promote tumorigenesis. Here, we report that the loss of A-type lamin expression in normal cells triggers a p53- and p21-dependent senescence checkpoint and a Rb-independent mechanism of repression of E2F target genes.

Results

Depletion of lamin A in primary cells does not increase cell proliferation but triggers a senescent cell cycle arrest

Several reports have linked lamin A expression to cell cycle progression. Lamin A controls Rb stability, and in its absence, Rb is degraded by the proteasome (Johnson *et al.*, 2004). Similarly, Rb levels are decreased in the liver of mice that accumulate progerin because of genetic inactivation of the Zmpste24 protease (Varela *et al.*, 2005). In the absence of Rb, elevated E2F activity could drive an aberrant proliferative response and stimulate cell transformation. Consistently, A-type lamin-depleted tumor cells are resistant to the antiproliferative activities of the INK4 family of CDK

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inhibitors, which require Rb for their actions (Nitta *et al.*, 2006). Also, fibroblasts from patients with HGPS are characterized by a hyperproliferative phenotype that is accompanied by increased cell death (Bridger & Kill, 2004). In contrast to these studies, it has been shown that depletion of lamin A/C, or its partner in the nucleoplasm LAP2α, leads to G1 cell cycle arrest in normal human fibroblasts (Pekovic *et al.*, 2007). Since to date there is no explanation for these discrepancies, we decided to investigate the response of normal human fibroblasts to A-type lamin depletion using shRNAs.

Depletion of lamin A/C led to a reduction in Rb and p107 (Fig. 1A) as previously reported (Johnson *et al.*, 2004; Nitta *et al.*, 2006). We also observed a reduction in the Rb-related pocket p130 by immunofluorescence in the nuclear compartment (Fig. 1B), but we could not clearly visualize this protein in immunoblots using two different antibodies. Despite the marked downregulation of the Rb family of proteins, cells expressing shRNA against lamin A/C exhibited a pronounced proliferation defect (Fig. 1C). This proliferation defect could be the result of an intrinsic inability of lamin A/C-depleted cells to proliferate. Alternatively, cells can sense lamin defects and activate a checkpoint pathway as it happens after DNA damage.

We noticed that a significant fraction of cells expressing shRNA against A-type lamins developed a flat morphology (Fig. 2A) typical of cellular senescence, which is a cellular programmed response to short telomeres, oncogene activation, or persistent DNA damage (Mallette & Ferbeyre, 2007; Yaswen & Campisi, 2007). Consistent with the appearance of this phenotype, lamin A/C knockdown led to the accumulation of senescence markers such as the senescence-associated β-galactosidase (SA-β-Gal) (Fig. 2A) and the CDK inhibitor p21 (Fig. 2B). Another marker of the senescence state, the accumulation of Promyelocytic leukemia (PML) bodies (Ferbeyre *et al.*, 2000), was also observed in lamin A/C-depleted cells (Fig. 2C). Intriguingly, some of these cells displayed abnormal PML body distribution, with some PML bodies colocalizing with the nuclear envelope or clustered in the nucleus (Fig. 2C). These atypical PML bodies are reminiscent of those observed in embryonic stem cells (Butler *et al.*, 2009) where lamin A/C expression is also low (Pajerowski *et al.*, 2007). Taken together, the data suggest lamin A/C knockdown leads to a proliferation arrest with features of cellular senescence in the absence of Rb and p107 and a reduction in the levels of p130 in the nucleus. An shRNA against p130 (Chicas *et al.*, 2010) did not rescue the proliferation defect and senescent traits induced by lamin A/C depletion (Fig. S1A–C). We thus conclude that lamin A/C depletion leads to cell cycle arrest and senescence in cells with a significant deficiency in the Rb family.

To further characterize the mechanisms by which lamin A/C knockdown leads to cell cycle arrest, we looked at additional markers of activation of the p53 tumor suppressor. Intriguingly, p53 levels, p53 phosphorylation at serine 15 (Fig. 2B), and many other p53 targets (BTG2, GADD45α, DAPK1, PMP22, MDM2, and SESN1) were not upregulated (Fig. 2B,D). Nevertheless, the increase in p21 protein and mRNA (Fig. 2B,D) and the decrease in BIRC5 mRNA (Fig. 2D), a p53 repression target (Hoffman *et al.*, 2002), pointed to a unique profile of p53 target gene regulation in cells with lamin A/C depletion. One explanation for the lack of p53 phosphorylation and expression of some p53 targets could be the reduction in the expression of SOCS1 (Fig. 2B), an adaptor required for p53 phosphorylation by ATM (Calabrese *et al.*, 2009). Next, we looked at other features that characterize cell senescence in cells bearing oncogenes or short telomeres. DNA containing foci reminiscent of the senescence-associated heterochromatin foci (SAHF) (Narita *et al.*, 2003) were readily visualized in lamin A/C-depleted cells after staining the DNA with 4,6-diamidino-2-phenylindole (DAPI) (Fig. 2C) or propidium iodide (PI) (Fig. 2E) or after staining the heterochromatin with an antibody specific for the heterochromatin protein HP1γ (Fig. 2E). Finally, lamin A/C depletion leads to a mitochondrial dysfunction phenotype (Passos *et al.*, 2007; Moiseeva *et al.*, 2009) characterized by high levels of ROS (reactive oxygen species) production (Fig. 2F,G) and a compensatory increase in mitochondrial mass (Fig. 2H). Key aspects of the senescence cell cycle arrest induced by our shRNA against lamin A/C were also confirmed by knocking down lamin A/C with another shRNA (Fig. S1D–F).

Rb-independent regulation of E2F target genes in lamin A/C-depleted cells

Senescence is commonly characterized by the inhibition of E2F gene expression associated with the activation of the Rb family of repressors (Narita *et al.*, 2003; Chicas *et al.*, 2010). However, the absence of expression of the Rb family in lamin A/C-depleted cells raised questions about the status of E2F target genes in these cells. Intriguingly, the mRNAs of E2F1, E2F2, and several E2F target genes were downregulated in lamin A/C-depleted cells (Fig. 3A). This downregulation was confirmed at the protein level for E2F1 and Mcm6 using immunoblots (Fig. 3B) and for E2F1 and 2 using immunofluorescence (Fig. 3C,D). Strikingly, the levels of E2F3 were not downregulated in lamin A/C-depleted cells (Fig. 3A,B), but the E2F3 protein was found confined to the nucleolus away from its target promoters (Fig. 3E). This sequestration of E2F3 in the nucleolus was also observed in senescence induced by oncogenic *ras*, the tumor suppressor PML, or short telomeres (replicative senescence), indicating that it may be a general mechanism to control E2F3 activity during senescence (Fig. S2). It has been reported that the absence of E2F1–3 can be compensated by the Myc transcription factor (Chen *et al.*, 2009).

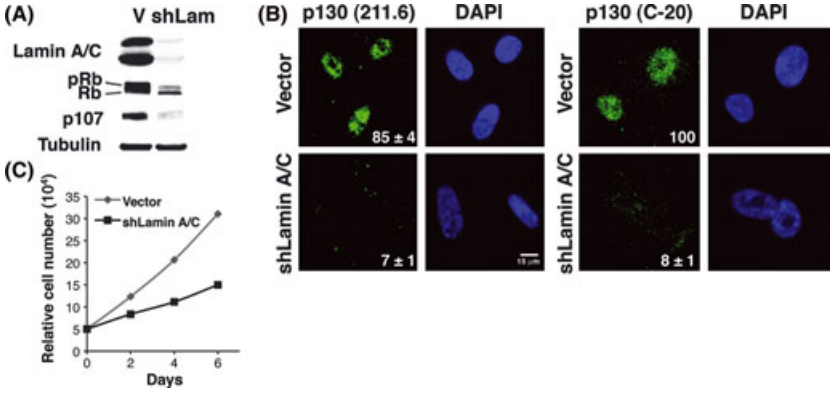

Fig. 1 Lamin A/C knockdown in primary human fibroblasts leads to a decrease in pocket protein levels. (A) Immunoblots from cells expressing an shRNA against lamin A/C (shLam) or control vector expressing a nontargeting shRNA (shRNA against Green Fluorescence Protein (GFP) (V). Protein extracts were prepared at day seven after infection with the indicated retroviral vectors. (B) Immunofluorescence for p130 from cells as in (A), using two different anti-p130 antibodies in cells fixed 7 days postinfection. (C) Growth curves of cell populations expressing an shRNA against lamin A/C or an shRNA against GFP (vector control).

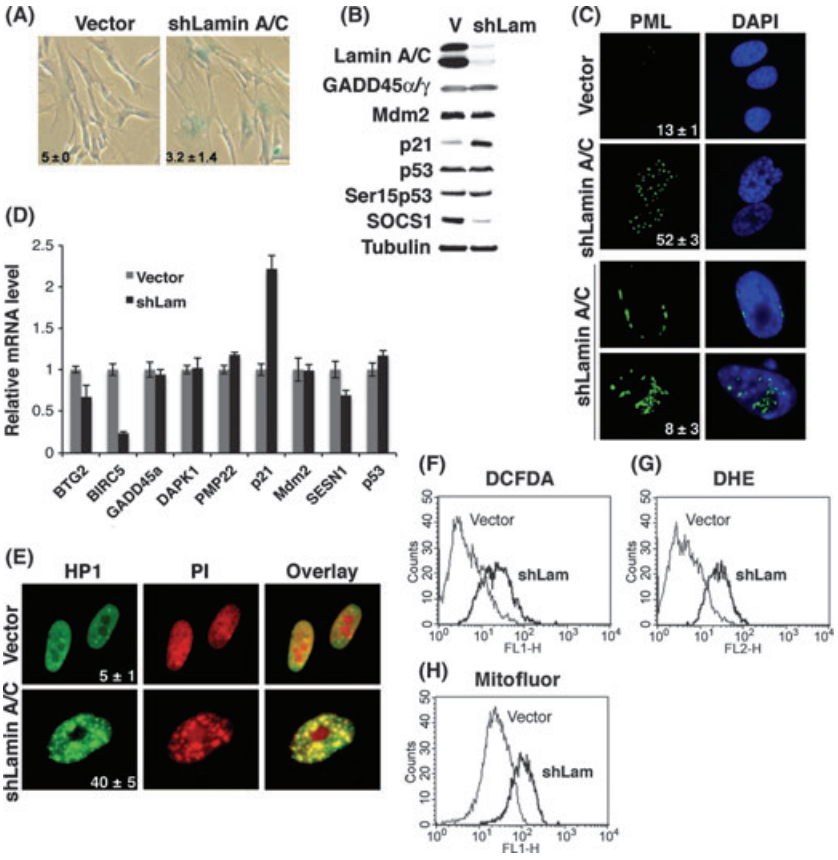

Fig. 2 Lamin A/C knockdown in primary human fibroblasts leads to a senescent cell cycle arrest. (A) Senescence-associated β-galactosidase from cells fixed 7 days after infection with a retroviral vector expressing sh Lamin A/C (shLam) or an shRNA against GFP (V). The average percent and standard deviation of the number of positive cells is indicated in the bottom left of each panel. Quantitation was made with three independent replicas counting 200 cells in three field of the plate each time. (B) Immunoblots with antibodies against the indicated proteins from cell extracts taken 7 days after infection with shLam or control vector (V). (C) PML bodies visualized by immunofluorescence with a specific anti-PML antibody in cells fixed as in Fig. 1B. We quantified the number of cells with numerous PML (more than five/cell). In the bottom panel, the number of cells with aberrant distribution of PML bodies is indicated. (D) p53 target gene expression in lamin A/C-depleted cells as measured using qPCR. (E) Immunofluorescence imaging of the heterochromatin protein HP1 and propidium iodide (PI) staining in cells fixed as in Fig. 1B. The percent of cells with distinctive HP1/PI foci (SAHFs) is indicated at the bottom right of the HP1 panels. (F–H) Fluorescence intensity of cells analyzed by flow cytometry and stained with dichlorodihydrofluorescein diacetate (DCFDA), dihydroethidium (DHE), or Mitofluor Green (MitoFluor) as indicated.

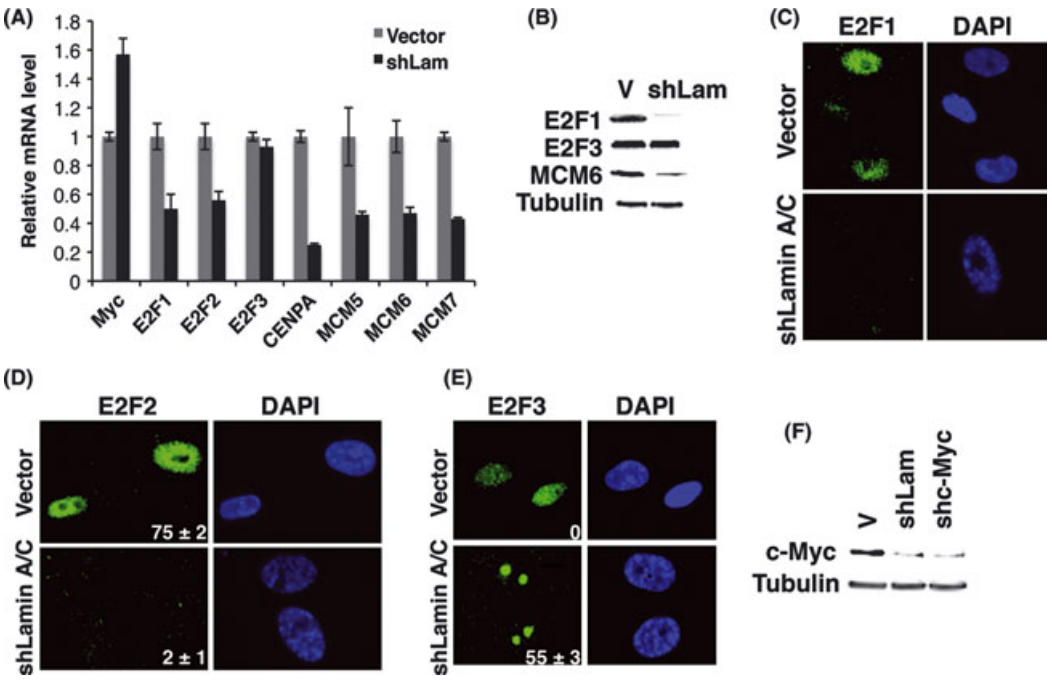

Fig. 3 Lamin A/C knockdown in primary human fibroblasts leads to the downregulation of transcription factors implicated in cell proliferation. All measurements were taken with cells obtained 7 days postinfection with shLam or a vector control. (A) qPCR for Myc, the E2Fs 1, 2, and 3 and several E2F target genes. (B) Immunoblots for the indicated proteins. (C–E) Immunostaining for E2F1–3. Cells in panel C are representative of most cells in the population. In D, we quantified the percent of nuclei with high E2F2 signals. In E, we quantified the percent of nuclei with nucleolar E2F3. (F) Myc immunoblot in cells expressing the indicated vectors.

However, in lamin A/C-depleted cells, Myc protein was also downregulated (Fig. 3F) although its mRNA was slightly induced (Fig. 3A). The reduction in Myc levels could be the result of a proteasome-dependent degradation process, as reported for Rb (Johnson *et al.*, 2004), and suggests that Myc does not compensate for the E2F loss in lamin A/C-depleted cells. Hence, lamin A/C knockdown induces an Rb family-independent senescence that involves downregulation of Myc, E2F1, and E2F2, nucleolar sequestration of E2F3, and reduced expression of E2F target genes.

Lamin A/C depletion inhibits E2F functions via p53

In normal cells, cellular senescence is controlled by the p53 and Rb tumor suppressor pathways. However, in lamin A/C-depleted cells, the Rb family is poorly expressed and therefore unlikely to mediate the process. Intriguingly, after lamin A/C depletion, p53 seems to be engaged in a very particular way because only few of the targets analyzed were found modulated. To investigate whether p53 was required for the senescent cell cycle arrest induced after the depletion of lamin A/C, we used an shRNA against p53 used previously to bypass oncogene-induced senescence (Voorhoeve & Agami, 2003; Moiseeva *et al.*, 2009). p53-depleted cells did not show growth arrest (Fig. 4A) and SA-β-Gal (Fig. 4B) or p21 induction (Fig. 4C) after knocking down lamin A/C. The accumulation of PML bodies or phospho-ATM foci seen in senescent cells after lamin A/C depletion was also inhibited by p53 inactivation (Fig. S3A,B). Of note, the inactivation of p53 did not rescue the nuclear morphology alterations associated with lamin A/C depletion (Fig. S3C). This suggests that p53 activation is downstream the nuclear envelope alterations caused by lamin A/C depletion but upstream DNA damage and the DNA damage response in this context.

p53 activation and the senescence phenotype of lamin A/C-depleted cells could be the outcome of Rb family downregulation because loss of the Rb family increases E2F activity, and high E2F activity induces senescence via a DNA damage response (Mallette *et al.*, 2007). Because lamin A/C depletion actually decreases E2F levels and activity, it is unlikely that this is the pathway leading to the observed p53-dependent senescent phenotype. Nevertheless, to further investigate this possibility, we knocked down Rb using a previously validated shRNA (Narita *et al.*, 2003). Cells where Rb was depleted senesced with high E2F levels and a pattern of p53 activation different from what we observed in lamin A/C-depleted cells (Fig. S4). We thus conclude that senescence after lamin A/C depletion is not simply the consequence of Rb depletion.

Next, we used an Mdm2 expression vector to investigate whether Mdm2-dependent p53 degradation is also sufficient to rescue cells from the senescent cell cycle arrest that follows lamin A/C depletion. Mdm2 overexpression reduced moderately the proliferation of normal cells but completely rescued the proliferation defect of cells expressing sh Lamin A/C (Fig. 4D), efficiently blocking senescence (Fig. 4E) and p21 expression (Fig. 4F). Mdm2 clearly restored the expression of the E2F target gene *MCM6* (Fig. 4F). Altogether, these results suggest the E2F activity can be regulated by p53-dependent but Rb-independent mechanisms.

To investigate the role of p53 on the repression of E2F target genes seen after lamin A/C depletion, we measured the expression of E2F1–3

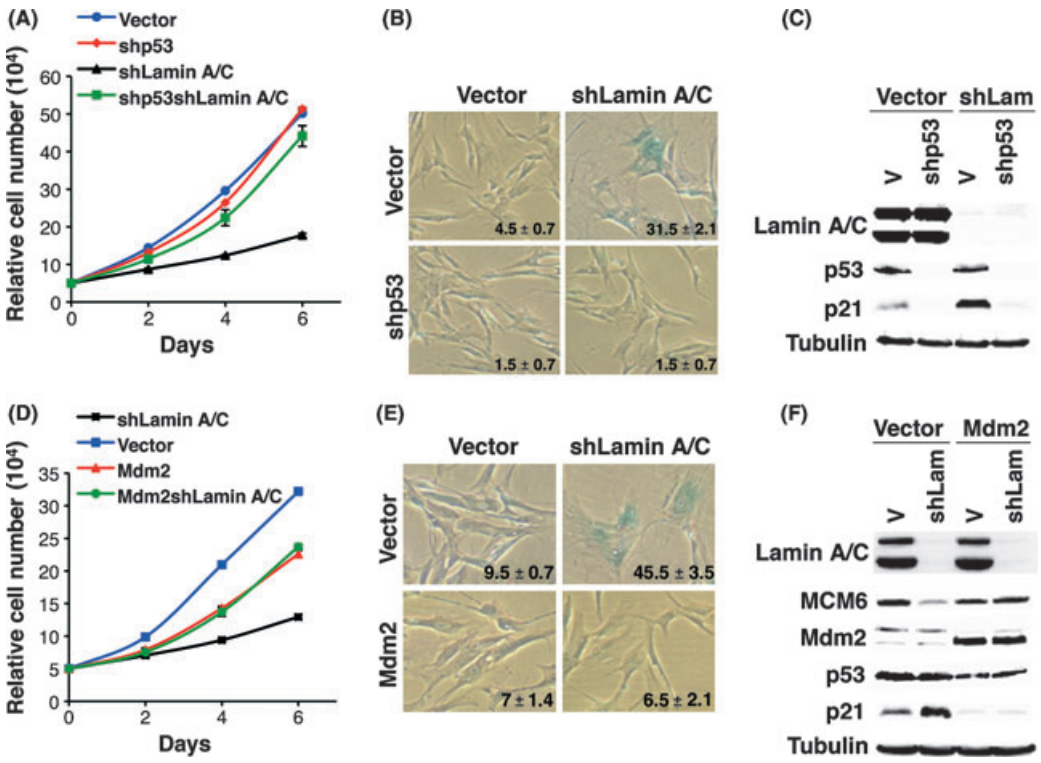

Fig. 4 Inhibition of p53 bypasses the senescence response to lamin A/C depletion in normal human fibroblasts. (A, D) Growth curves. (B, E) Senescence-associated β-galactosidase using cells fixed 7 days after infection with the indicated vectors. (D, F) Immunoblots with antibodies against the indicated proteins using cell extracts from cells as before. In A–C, p53 was inhibited with shRNA against p53 and in D–F by overexpression of Mdm2. Note that we enforced the expression of Mdm2 (mouse protein), and that the endogenous version is the human protein MDM2 also known as HDM2.

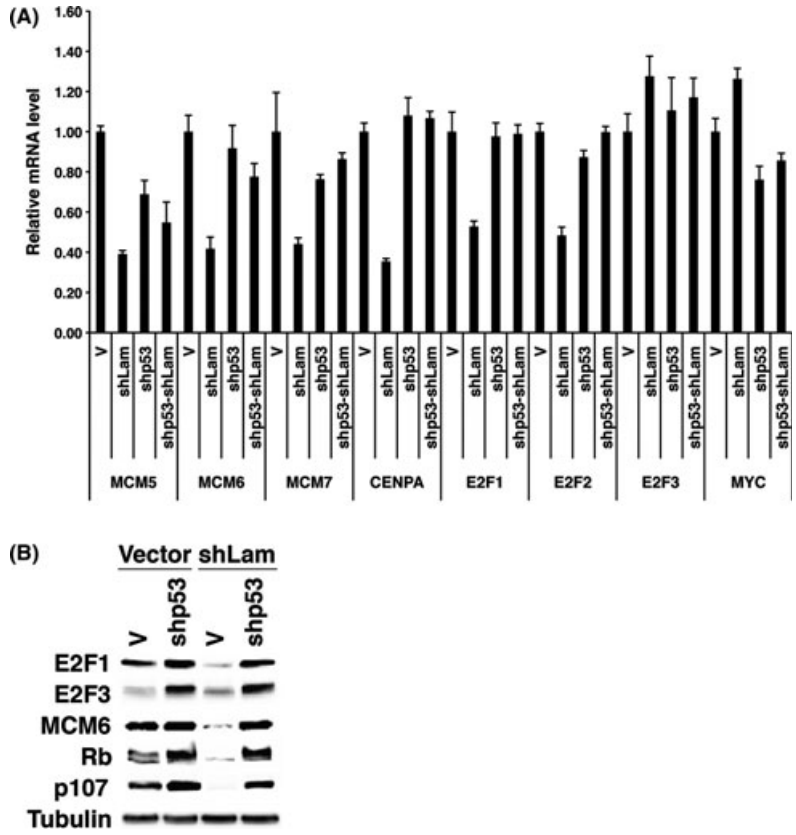

Fig. 5 p53-dependent repression of E2F target genes in lamin A/C-depleted normal human fibroblasts. (A) qPCR for several E2F target genes, the E2Fs 1, 2, and 3 and Myc using RNA from cells lysed 7 days postinfection. (B) Immunoblots for the indicated proteins.

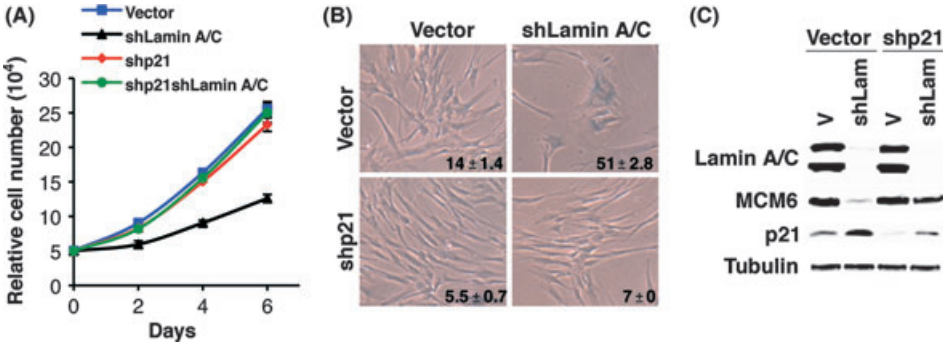

Fig. 6 Knockdown of p21 inhibits the senescence response to lamin A/C depletion. (A) Growth curves of cell populations expressing the indicated shRNAs. (B) Senescence-associated β-galactosidase. (C) Immunoblots with antibodies against the indicated proteins.

and several of their targets using qPCR. The expression of E2F1, E2F2, MCM5, MCM6, MCM7, and CENPA but not of E2F3 and Myc was downregulated by shLamin, and their expression restored when p53 was inhibited by shp53 (Fig. 5A). We confirmed this observation at the protein level for MCM6 (Figs 4F and 5B) and E2F1 (Fig. 5B). In addition, knocking down p53 also restored the total levels of Rb and p107 (Fig. 5B), indicating a previously unnoticed role of p53 in the control of the Rb family protein stability acting in the context of lamin A/C depletion.

p53-mediated gene repression can be the consequence of multiple mechanisms (Barsotti & Prives, 2010). However, it has been reported that p21 is necessary and sufficient to mediate the negative effects of p53 on gene expression (Lohr *et al.*, 2003). We tested a validated shRNA against p21 (Chicas *et al.*, 2010) to rescue the senescence phenotype triggered by lamin A/C knockdown. We found that shp21 reduced p21 expression and efficiently inhibited the growth arrest and senescence induced by shLamin (Fig. 6A–C), indicating that in this context, p21 was essential for the effects of p53 after lamin A/C knockdown.

Senescence in myoblasts with a mutation in lamin A

Lamin mutations are linked to muscular dystrophies both in humans and mouse models (Bonne *et al.*, 1999; Sullivan *et al.*, 1999). Mutations of the lamin-interacting protein emerin also cause muscular dystrophy (Bione *et al.*, 1994). We thus first studied the localization of emerin in lamin A/C-depleted fibroblasts and found an abnormal localization pattern or a downregulation of emerin as described in *Lmna* null mice

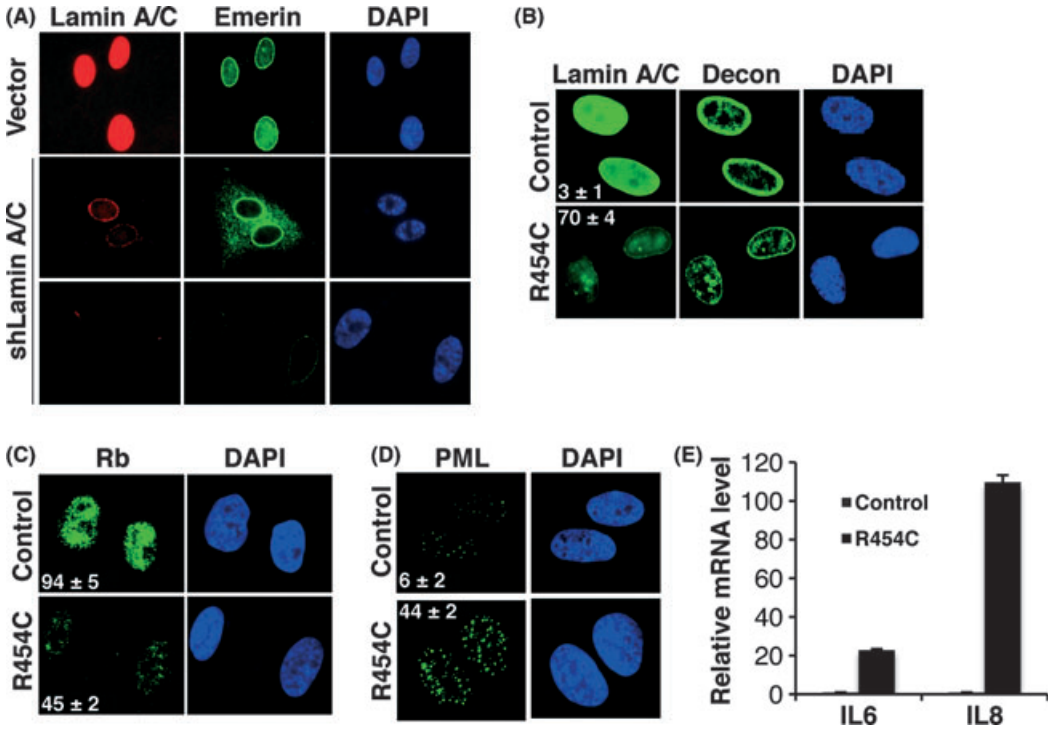

Fig. 7 Lamin A/C knockdown leads to altered emerin localization in fibroblasts while lamin A R545C myoblasts display markers of cell senescence. (A) Emerin localization in lamin A/C-depleted normal human fibroblasts. (B–D) Immunofluorescence for the indicated proteins in control myoblasts and myoblasts from a patient with the R545C lamin A mutation. In (B), the percent of cells with low levels of lamin A in nuclear lamina is quantified and indicated in the left panel. In (C), the percent of cells with high levels of Rb is quantified. In (D), the percent of cells with abundant PML bodies is quantified. (E) qPCR for IL-6 and IL-8 using RNA from control myoblasts and myoblasts from a patient with the lamin A mutation R545C.

(Sullivan *et al.*, 1999) (Fig. 7A). Then, we obtained myoblasts from a patient with the lamin A mutation R545C, which is associated with muscular dystrophy and premature myoblasts senescence (Kandert *et al.*, 2009). We found that the organization of lamin A was altered in myoblasts with lamin A545C mutation (Fig. 7B) and a reduction in the levels of the Rb protein (Fig. 7C). In addition, these mutant myoblasts accumulated PML bodies (Fig. 7D) and expressed high levels of the inflammatory cytokines IL-6 and IL-8 (Fig. 7E), as described in senescent cells (Rodier *et al.*, 2009). However, the mutation R545C did not reduce lamin A levels in the nuclear envelope as our shRNAs explaining why the myoblasts expressing Lamin R545C still conserved some ability to grow in culture and why the reduction in Rb and the accumulation of PML bodies do not affect every cell in the population albeit a significant fraction of them (Fig. 7).

Discussion

We report here that inhibition of lamin A/C expression leads to downregulation of the Rb/E2F family of transcriptional regulators, growth arrest, and senescence. The growth arrest and senescent phenotype were rescued by inactivation of the tumor suppressor p53 or its target p21. These observations are reminiscent of the process of oncogene-induced senescence, which normally prevents tumor formation by cells that accidentally acquire oncogenic mutations. By analogy, the depletion of lamin A/C can be potentially oncogenic. First, lamin A/C downregulation is characteristic of stem cells (Pajerowski *et al.*, 2007), and it is currently believed that the process of tumorigenesis is driven by a minor population of tumor-initiating cells with stem cells properties. In addition, it is known that p53 and its target p21 oppose to the reprogramming of normal somatic cells into stem cells, and this function may be important for its tumor suppressor activities (Li *et al.*, 2009; Banito & Gil, 2010). Together, our results clearly indicate that the p53 tumor suppressor provides a proliferative barrier to cells with disabled expression of lamin A/C and explain why previous results using immortalized cell lines with mutant p53 concluded that lack of lamin A expression led to increased cell proliferation.

The activation of the p53 pathway in lamin A/C-depleted fibroblasts was not similar to what has been reported during oncogene-induced senescence or replicative senescence (Ferbeyre *et al.*, 2000) because p53 levels and phosphorylation at serine 15 were not affected. This could be explained by the altered DNA damage response observed in *Lmna*$^{-/-}$ fibroblasts and by the poor expression of mediators of p53 activation such as p53BP1 (Gonzalez-Suarez *et al.*, 2009) and SOCS1, which normally facilitate p53 phosphorylation by ATM/ATR (Calabrese *et al.*, 2009). We thus propose that defects in cells depleted for lamin A/C lead to an altered p53 response but still sufficient to mediate cellular senescence. *Lmna*-knockout fibroblasts display telomeres abnormalities with a shift in the localization of telomeres toward the nuclear periphery, telomere shortening, a decrease in H4K20 methylation and telomere noncoding RNAs (TERRAs) (Gonzalez-Suarez *et al.*, 2009). Lamin A also has intranuclear functions relevant for controlling Rb and the cell cycle (Naetar & Foisner, 2009). It is thus possible that altered telomeres or a dysfunctional chromatin because of defects in lamin function in the nuclear envelope and/or the nucleoplasm provides the signal for the activation of the p53-dependent senescence program in lamin A/C-depleted cells.

E2F target genes are repressed during cellular senescence by the Rb family (Narita *et al.*, 2003; Chicas *et al.*, 2010) in a process that involves PML bodies and heterochromatin (Vernier *et al.*, 2011). Inhibition of E2F activity is sufficient to trigger senescence, proliferation defects, and p53

activation (Maehara *et al.*, 2005; Timmers *et al.*, 2007). However, because lamin A/C depletion leads to downregulation of the Rb family, E2F target gene repression has to occur via an Rb-independent mechanism. It has been reported that p53 can negatively regulate several E2F target genes. The authors noticed that the promoter of one of these genes (*MCM7*) contained a Myc-binding site close to the E2F-binding site that is targeted by a p53-dependent repression mechanism (Scian *et al.*, 2008). We examined the promoters of several E2F targets downregulated in lamin A/C-depleted cells and found that they also possess Myc-binding sites close to E2F sites (Fig. S5A). It is thus possible that p53 or a protein modulated by p53 represses genes containing close Myc/E2F sites. However, we could not detect p53 binding to the Mcm7 promoter in our system (Fig. S5B), suggesting that in the context of lamin A/C depletion, p53 is not the direct regulator of E2F target genes.

In the studies of global gene expression analysis, p53-mediated gene repression is as important as gene activation. However, whether repression is a consequence of direct p53 action at promoters or the effect of p53 targets is still debatable (Barsotti & Prives, 2010). The CDK inhibitor p21 has emerged as an important mediator of p53-mediated repression, and its mechanism of action has been mainly explained by its ability to repress CDKs preventing the inactivation of the Rb family (Barsotti & Prives, 2010). However, p21 can also bind to some promoters to mediate repression (Vigneron *et al.*, 2006). It is also known that depletion of the SWI2/SNF2 chromatin–remodeling protein p400 triggers a p53–p21-dependent senescence process that involves repression of E2F targets and SAHF formation (Chan *et al.*, 2005). So far, there are no connections reported between lamin A and p400. In agreement with the latter study, we found that p21 was required for the senescence response after lamin A/C depletion, suggesting that p21 mediates repression of E2F target genes in the absence of the Rb family, a phenomenon that will require further studies.

In addition to the cancer relevance of our study, the findings presented here are significant to understand the pathogenesis of laminopathies specially those where lamin A levels are reduced as well. In myoblasts from a patient with the lamin A mutation R545C, we found traits of a senescent phenotype that included accumulation of PML bodies and high expression of inflammatory cytokines. In relation to aging and age-related diseases, the ability of senescent cells to secrete inflammatory cytokines and not their terminal arrest could be the most important pathogenic factor (Campisi, 2005; Mallette *et al.*, 2010). Noteworthy, patients with other forms of muscular dystrophy display high levels of IL-6 (Chahbouni *et al.*, 2010), and their myoblasts senesce prematurely in culture (Wright, 1985). Moreover, fibroblasts from patients with HGPS that express mutant lamin A also senesce in culture in association with telomeric DNA damage signals and activation of both p53 and Rb (Liu *et al.*, 2006; Benson *et al.*, 2010). In addition, mice that accumulate progerin because of genetic inactivation of the protease Zmpste24 exhibit nuclear envelope alterations, p53 activation, and a premature aging phenotype (Varela *et al.*, 2005). Hence, progerin accumulation or lamin depletion triggers distinct nuclear damage signals that ultimately converge into a common senescence program. This cellular stage may then act as an active mediator and/or amplifier in the pathophysiology of laminopathies and perhaps normal aging.

Experimental procedures

Cells, reagents, and plasmids

IMR90 cells (normal human fibroblasts) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Wisent, St-Bruno, QC, Canada) and 1% penicillin G/streptomycin sulfate (Wisent). Myoblasts were obtained from the Muscle Tissue Culture Collection (MTCC) of the Friedrich-Baur-Institute. They were cultured in HAM F-10 (Sigma, Oakville, ON, Canada) supplemented with 15% FBS (Wisent), 1% chick embryo extract (US Biological, Swampscott, MA, USA), 400 ng mL^{-1} dexamethasone (Sigma), and 1% penicillin G/streptomycin sulfate (Wisent). Retroviral-mediated gene transfer, immunoblotting, and chromatin immunoprecipitation were performed as previously described in (Calabrese *et al.*, 2009). qPCR techniques were described in (Vernier *et al.*, 2011), and qPCR primers, vectors, and shRNAs can be found in Data S1 (Supporting information).

Cell proliferation and senescence determination

To determine cell proliferation rates, we estimated cell counts at different times after plating using a crystal violet retention assay (Growth curves) (Ferbeyre *et al.*, 2000). Senescence-associated β-galactosidase (SA-β-gal) activity was assayed as described (Ferbeyre *et al.*, 2000). Data were quantified from 100 cells counts in triplicate in at least two independent experiments.

Mitochondrial fluorescence probes

To measure hydrogen peroxide cells, we used 2 μM dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, OR, USA). To measure superoxide, we used 1 μM dihydroethidium (DHE; Molecular Probes). To measure mitochondrial mass, we used MitoFluor Green (M-7502; Molecular Probes) as described in the manufacturer's protocol, and the intensity of labeling was measured by FACS.

Fluorescence microscopy

Cells were plated on coverslips at least 24 h prior of fixation with 4% paraformaldehyde in PBS for 15 min at room temperature (RT). After washing with PBS, cells were permeabilized for 5 min at RT with 0.2% Triton X-100. Then, cells were washed with 3% BSA in PBS (PBS/BSA) and incubated overnight with the following primary antibodies diluted in PBS/BSA: anti-E2F1 (KH95, 1/50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-E2F2 (C-20, 1/200; Santa Cruz Biotechnology), anti-E2F3 (C-18, 1/200; Santa Cruz Biotechnology), anti-Rb (#G3245, 1/200; BD Pharmingen, San Diego, CA, USA), anti-PML (PG-M3, 1:200; Santa Cruz Biotechnology), anti-PML rabbit polyclonal produced in our laboratory (1:400) (Vernier *et al.*, 2011), anti-phopho-$ATM^{Ser1981}$ (#9298, 1/200; Cell Signaling, Pickering, ON, Canada), anti-Lamin A/C (636, 1/200, Santa Cruz Biotechnology), anti-emerin (FL-254, 1/100; Santa Cruz Biotechnology), anti-p130 (C-20 or 211.6 1/200; Santa Cruz Biotechnology), anti-p107 (#GTX73049, 1/200; GeneTex, Irvine, CA, USA), anti-HP1γ (#MAB3450, 1/100; Millipore).

After primary antibodies, cells were washed three times in PBS/BSA and incubated with the appropriate secondary antibody combination (1/4000, AlexaFluor 488 goat anti-mouse, AlexaFluor 488 goat anti-rabbit, AlexaFluor 568 goat anti-mouse or AlexaFluor 568 goat anti-rabbit, Molecular Probes) for 1 h at room temperature. Finally, cells were rinsed three times with PBS alone and once with PBS containing 300 nM DAPI for 10 min when needed. Propidium iodide (Molecular Probes) staining was performed according to manufacturer's instructions. Images were captured Olympus FV300 Point scanner confocal laser microscope

(Markham, ON, Canada) and processed with the software Metamorph (Sunnyvale, CA).

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Author contributions

O. Moiseeva contributed Figs 1–7and Figs S2 and S3 (Supporting information). V. Bourdeau contributed the qPCRs to Figs 2, 3, and 5. M. Vernier contributed the Fig. S2 (Supporting information). M.-C. Dabauvalle contributed the myoblast model of lamin R545C in Fig. 7. G. Ferbeyre supervised the work and wrote the manuscript with O. Moiseeva, V. Bourdeau, and M.-C. Dabauvalle.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Lamin A/C knockdown with a second shRNA leads to a senescent cell cycle arrest in primary fibroblasts.

Fig. S2 Localization of E2F3 in the nucleolus during cellular senescence induced by different stimuli.

Fig. S3 Effects of p53 knockdown on the senescence response to lamin A/C depletion.

Fig. S4 Rb knockdown leads to p53 dependent cellular senescence with high E2F levels.

Fig. S5 E2F promoters potentially regulated by p53/p21 in lamin A/C depleted cells.

Data S1 Materials and methods.

Table S1 qPCR primers.

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