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Nucleoplasmic LAP2α–lamin A complexes are required to maintain a proliferative state in human fibroblasts

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Introduction

The nuclear envelope (NE) is composed of inner and outer nuclear membranes that are perforated by nuclear pore complexes and supported by the nuclear lamina (Hutchison, 2002). Proteins of the inner nuclear membrane have been implicated in organization of nuclear architecture and cell cycle control. One such family of inner nuclear membrane proteins is lamina-associated polypeptide 2 (LAP2), generated by alternative splicing from a single gene (Foisner and Gerace, 1993; Harris et al., 1994; Berger et al., 1996). LAPs have only been found in vertebrates; up to six isoforms exist in humans and mice (α, β, γ, δ, ε, and ζ).

Most LAP2 isoforms have a closely related N-terminal nucleoplasmic domain of variable length with a nuclear localization signal sequence, a single-membrane spanning region, and a short luminal domain at their C terminus. LAP2α is structurally and functionally unique. It shares the first N-terminal 187 residues with all other LAPs but contains a unique C-terminal domain of 506 residues, which lacks a membrane spanning domain. As a result, LAP2α is distributed diffusely throughout the interphase nucleus except for nucleoli (Dechat et al., 1998).

Overexpression of C-terminal fragments of LAP2α dominantly inhibits assembly of endogenous LAP2α, nuclear membranes, and A-type lamins in vitro nuclear assembly assays and causes a cell cycle arrest in interphase, indicating a role for LAP2α in cell cycle progression (Vlcek et al., 2002).

In human diploid fibroblasts (HDFs), expression of lamina-associated polypeptide 2 α (LAP2α) upon entry and exit from G0 is tightly correlated with phosphorylation and subnuclear localization of retinoblastoma protein (Rb). Phosphoisoforms of Rb and LAP2α are down-regulated in G0. Although RbS780 phosphoform and LAP2α are up-regulated upon reentry into G1 and colocalize in the nucleoplasm, RbS795 migrates between nucleoplasmic and speckle compartments. In HDFs, which are null for lamins A/C, LAP2α is mislocalized within nuclear aggregates, and this is correlated with cell cycle arrest and accumulation of Rb within speckles. Nuclear retention of nucleoplasmic Rb during G1 phase but not of speckle-associated Rb depends on lamin A/C. siRNA knock down of LAP2α or lamin A/C in HDFs leads to accumulation of Rb in speckles and G1 arrest, probably because of activation of a cell cycle checkpoint. Our results suggest that LAP2α and lamin A/C are involved in controlling Rb localization and phosphorylation, and a lack or mislocalization of either protein leads to cell cycle arrest in HDFs.

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Abbreviations used in this paper: HDF, human diploid fibroblast; LAP2, lamina-associated polypeptide 2; MEF, mouse embryonic fibroblast; NCS, newborn calf serum; NE, nuclear envelope; Rb, retinoblastoma protein.

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LAP2α is found in stable complexes with the type V intermediate filament proteins lamins A/C in the nucleoplasm (Dechat et al., 2000). The LAP2α-specific C terminus associates with the C terminus of lamins A/C (aa 319–566) in vivo and in vitro. Dominant-negative lamin mutants, which cause aggregation of lamins A/C, also cause LAP2α to redistribute to the same aggregates, indicating a functional association between LAP2α and lamin A/C (Dechat et al., 2000). Mutations in the gene encoding lamins A/C (LMNA) cause a spectra of human diseases (termed laminopathies), including muscular dystrophies, lipodystrophies, cardiomyopathies, neuropathies, dermatopaties, and premature aging syndromes (Hutchison and Worman, 2004). It has been suggested that altered lamin A/C–LAP2α associations might occur in laminopathies (Goldman et al., 2004) because LAP2α binds to a region of lamin A/C where many different mutations have been found. In support of this hypothesis, a mutation in the LAP2 gene causing cardiomyopathy and affecting the C-terminal domain of LAP2α alters LAP2α interaction with lamins A/C (Taylor et al., 2005).

The retinoblastoma protein (Rb) has functions in muscle and fat cell differentiation and in coordinating proliferation and differentiation during muscle regeneration (Smith and Kudlow, 2005). The C-terminal domain of Rb is involved in its nuclear tethering, which is essential for its growth-suppressing activity (Mittnacht, 1998). In early G1, hypophosphorylated Rb is tethered in the nucleus and is capable of binding transcription factor E2F-1, which prevents the transcriptional activation of S phase–specific genes and traverse to S phase. Hyperphosphorylation of Rb during late G1 phase releases it from nuclear tethers, which in turn releases and derepresses E2F and leads to passage through S phase. A-type lamins (Mancini et al., 1994; Ozaki et al., 1994) and LAP2α (Markiewicz et al., 2002; Dorner et al., 2006) both interact with the C-terminal nuclear anchorage domain of Rb. In line with an Rb tethering function of LAP2α–lamins A/C complexes, LAP2α has recently been shown to be involved in an Rb-mediated negative cell cycle control (Dorner et al., 2006).

One model for laminopathies proposes that mutations in LMNA impair the control of cell proliferation, particularly, Rb-mediated regulatory mechanisms controlling the exit and reentry into the cell cycle (Gotzmann and Foisner, 2006). In support of this model, embryonic fibroblasts from a Lmna−/− mouse show cell cycle defects because of reduced levels of Rb (Johnson et al., 2004). Interestingly, despite the proposed growth-suppressive function of LAP2α (Dorner et al., 2006), its expression is dramatically down-regulated upon cell cycle exit in human diploid fibroblasts (HDFs) and conversely up-regulated during reentry into the cell cycle (Markiewicz et al., 2002). Therefore, we tested the hypothesis that in HDFs, LAP2α expression is required for cell cycle progression via direct regulation of Rb. Here, we report that expression and lamin A–dependent organization of LAP2α is indeed required for maintaining HDFs in a proliferative state by promoting uniform nucleoplasmic localization of Rb. Our findings support the “cell cycle–proliferating–aging model” for laminopathies and provide a novel biological function for LAP2α in cell cycle regulation of human nontransformed adult cells.

Results

Changes in expression, solubility, and distribution of lamins, LAPs, and pRb isoforms in quiescent human fibroblasts

We previously showed that the expression of LAP2α is downregulated as HDFs progress from a proliferating to a G0 state after serum starvation (Markiewicz et al., 2002). To further investigate LAP2α responsiveness to factors that induce quiescence in HDFs, we grew cultures to confluence and used immunoblotting to investigate the expression of proteins of interest. The level of expression of LAP2α declined dramatically as cultures reached confluence and the protein was undetectable in postconfluent cultures. In contrast, no changes in the level of expression of LAP2β, lamin A/C, or lamin B2 were detected in confluent or postconfluent cultures (Fig. S1 a, available at http://www.jcb.org/cgi/content/full/jcb.200606139/DC1). The level of expression of hyperphosphorylated Rb and, in particular, the RbS780 isoform also declined progressively in confluent and postconfluent cultures and correlated precisely with changes in the expression of LAP2α (Fig. S1, a and b). These data confirm that loss of expression of LAP2α occurs as a direct consequence of entry into G0, and is closely correlated with dephosphorylation of pRb at serine 780.

To further investigate the correlation between changes in expression of LAP2α and phosphorylation of pRb, we performed double immunofluorescence microscopy to investigate both the expression and distribution of total Rb and forms phosphorylated at serines 780 and 785. In cultures induced to enter G0 through confluence, LAP2α as well as total Rb (Ab2) and RbS780 were barely detected in a majority of cells (Fig. 1, a–c). Cells still expressing higher levels of LAP2α also expressed relatively high levels of RbS780 and RbS795 (Fig. 1, a and c). In contrast to RbS780, RbS795 was still detectable in cells with low levels of LAP2α and distributed in a small number of nucleoplasmic foci (Fig. 1 c, arrows).

To see how the distribution and phosphorylation of Rb is correlated with expression of LAP2α, G0 cultures were restimulated to reenter the cell cycle. 12 h after serum restimulation, LAP2α was detected in the nuclei of all cells and was distributed uniformly within the nucleoplasm, excluding nucleoli. Total and RbS780 were also expressed in nearly all cells and had a distribution that was very similar to that of LAP2α (Fig. 1, a and b). In contrast, RbS795 was located in a restricted number of nucleoplasmic foci (Fig. 1 c). 18 h after serum restimulation, the intensity of LAP2α, Rb, and RbS780 had increased but remained uniformly distributed throughout the nucleoplasm, whereas RbS795 remained within nucleoplasmic foci. Only at 24 h after serum restimulation, when the staining intensity of LAP2α, Rb, RbS780, and RbS795 was maximal, were all four proteins distributed uniformly throughout the nucleoplasm.

Next, we investigated the solubility properties of lamins, LAPs, and Rb in HDFs using a nuclear matrix extraction procedure. Using immunoblotting, we found that lamins A/C and lamin B2 were relatively more soluble in proliferating HDFs than in quiescent HDFs. In contrast, both LAP2α and LAP2β displayed increased solubility properties in quiescent HDFs.
compared with proliferating HDFs. Similarly, Rb was more soluble in quiescent HDFs than in proliferating HDFs (Fig. S1 c).

To investigate the solubility properties of the total Rb and the two Rb phosphoisoforms more closely, G₀ or serum-restimulated HDFs were extracted in situ. In most HDFs induced to enter G₀, total Rb, RbS780, and RbS795 were barely detectable (Fig. 1, d–f). Between 12 and 18 h after serum restimulation, all three forms of Rb were detectable and resistant to extraction. However, although Rb and RbS780 showed a more uniform staining throughout the nucleoplasm (Fig. 1, d and e), RbS795 was located in brightly stained nucleoplasmic foci (Fig. 1 f). 24 h after serum restimulation, when cells enter into S phase, all isoforms were relatively more soluble after extraction with Triton X-100 (Fig. 1, e and f).

Collectively, our data suggest that progression of HDFs from G₀ to a proliferating state (and vice versa) leads to a substantial remodeling of lamina proteins correlated with a more complex pattern of Rb nuclear anchorage than had previously been thought. In HDFs entering or exiting a quiescent state, Rb appears to be present in at least two different compartments depending on its phosphorylation status. Only when cells enter S phase (24 h after serum restimulation) are all forms of Rb detected in an apparently uniform distribution and largely soluble.

**Rb795 associates with splicing speckle compartments during cell cycle exit and entry**

The nature of the foci detected by antibodies against RbS795 was examined by colocalization with a range of antibodies previously reported to detect nuclear foci. There was no substantial colocalization between RbS795 and dense chromatin compartments (detected by DAPI; Fig. S2 a, available at http://www.jcb.org/cgi/content/full/jcb.200606139/DC1), phosphohistone H2A.X, or PML bodies (Fig. S2, c and d). In contrast, there was almost complete colocalization of RbS795 foci with splicing factor SC-35, which resides within splicing speckle compartments (Fig. S2 b), indicating that in cells entering G₀, this form of Rb is localized in splicing speckle compartments.

To further investigate the distribution and nuclear anchorage of RbS795, we performed double immunofluorescence and confocal microscopy on HDFs that had been restimulated from G₀, before and after nuclear matrix extraction. Between 6 and 12 h after serum restimulation, Rb795 colocalized with
splicing speckle compartments and remained insoluble within this compartment. However, after 18 h, colocalization between RbS795 and SC-35 was lost, but RbS795 was still resistant to extraction. Only upon entry into S phase (24 h) did RbS795 become completely soluble (Fig. 2). Thus, associations of Rb with the splicing speckles are both growth dependent and related to the expression of LAP2α. HDFs just entering the cell cycle and expressing low levels of LAP2α have RbS795 associated with splicing speckles. As LAP2α levels increase, RbS795 is no longer associated with speckle compartments but codistributes with LAP2α and other Rb forms within the nucleoplasm.

Absence of lamin A/C in HDFs correlates with a mislocalization of LAP2α, cell cycle arrest, and accumulation of pRb in speckles

To investigate whether lamins A/C directly influence the distribution of Rb within speckles, we performed double immunofluorescence on HDFs from a patient with lethal fetal akinesia, which harbors a homozygous mutation Y259X in the LMNA gene and is null for lamins A/C (Muchir et al., 2003). When grown for 2–4 d, an increasing number of Y259X HDFs (20–40%) displayed an abnormal accumulation of LAP2α have RbS795 associated with splicing speckles. As LAP2α levels increase, RbS795 is no longer associated with speckle compartments but codistributes with LAP2α and other Rb forms within the nucleoplasm.

To investigate the influence of lamins A/C on nuclear anchorage of the different Rb isoforms, control or Y259X HDFs were subjected to extraction with detergents before staining with different Rb antibodies. In exponentially dividing control HDFs, the nuclei of most cells stained positively for total Rb and RbS780 before extraction. After extraction, ~40% cells had greatly reduced or absent staining for total Rb and RbS780, whereas ~60% remained strongly positive (Fig. 4, a and b), reflecting the different cell cycle stages. RbS795 was uniformly distributed in the nucleus of most cells before extraction, whereas it was found in speckles in ~60% of cells and greatly reduced or absent in ~40% of cells after extraction (Fig. 4 c). In contrast to control cells, the majority of Y259X HDFs were negative or only weakly positive for total Rb and RbS780 before extraction, and nearly 100% of cells were negative for Rb and RbS780 after extraction (Fig. 4, d and e), whereas ~90% of Y259X HDFs still contained RbS795 restricted entirely to speckles after extraction (Fig. 4 f).

These data suggest that nuclear anchorage of RbS780 was lamin A/C dependent. To test this hypothesis, Y259X HDFs were transfected with either GFP–lamin A or GFP–lamin C and prepared for immunofluorescence before or after detergent extraction. RbS780 was strongly retained in most cells that expressed GFP–lamin A after extraction (Fig. 4 g). Surprisingly, RbS780 was not retained in cells expressing GFP–lamin C (Fig. 4 h). Our data suggest that anchorage of nucleoplasmic forms of Rb is dependent on expression of lamin A, whereas anchorage of forms of Rb located in speckles is independent of lamins A and C.

siRNA knock down of lamin A/C and LAP2α leads to cell cycle arrest and accumulation of Rb in speckles

In Y259X HDFs, aggregation of LAP2α and cell cycle arrest occurs in only 40% of cells, suggesting that a compensatory mechanism might be abrogating the effects of loss of lamin A/C.
in culture. To investigate the consequences of loss of lamin A/C or LAP2α more directly, we used siRNA to knock down each protein independently. HDFs were transfected with siRNA targeted to LAP2α or lamins A/C or as a control, scrambled siRNA. We observed a ~70% reduction in the level of LAP2α or lamins A/C expression 48 h after transfection with specific siRNA as compared with control siRNA, whereas expression of LAP2β was unaffected. Surprisingly, lamin A expression decreased by ~30% in cells transfected with LAP2α RNAi, and LAP2α decreased similarly in lamin A/C RNAi-treated cells. The total amount of Rb and, more dramatically (by ~70%), the levels of RbS780 were reduced in both LAP2α and lamins A/C RNAi cultures (Fig. 5 a).

To investigate the effects of siRNA knock down of LAP2α or lamins A/C on cell proliferation, we tested the expression of Ki67. After transfection with scrambled siRNA, only 10% of cells were negative for Ki67. In contrast, ~70% of cells transfected with LAP2α-specific siRNA or lamin A/C–specific siRNA, respectively, were negative for Ki67 (Fig. 5 b). We concluded that knock down of LAP2α or lamins A/C in HDFs leads to cell cycle arrest.

To confirm these findings, we performed DNA flow cytometry on HDFs. Cells were arrested in G0 by serum starvation, transfected with siRNA, restimulated by addition of complete serum medium, and harvested 0, 24, 48, or 72 h after transfection (Fig. 5 c). After 24 h, a large proportion of cells had entered S phase, and there was no difference in the cell cycle profiles between any of the transfected cultures. 48 h after transfection (the time at which knock down of LAP2α and lamins A/C were detected), control RNAi cultures proceeded normally through G1/M and displayed a cell cycle profile similar to asynchronously dividing fibroblasts. In contrast, cultures in which LAP2α or lamin A/C had been knocked down had a greater proportion of cells in S phase and an abnormally high proportion of cells in G2/M phase. In view of previous data, showing a negative effect of LAP2α on the G1–S phase transition (Dorner et al., 2006), our result may indicate that loss of LAP2α or lamins A/C promotes more rapid progression through G1 and premature entry into S phase, leading to a checkpoint arrest in G2. However, 72 h after transfection, the LAP2α RNAi–treated cultures appeared to accumulate in G1 phase of the cell cycle. At the same time point, many cells in lamin A/C knockdown cultures had accumulated in G1, but some remained arrested in G2. Control cultures were dividing asynchronously at this time point (Fig. 5 c). These data are consistent with a cell cycle arrest of LAP2α- and lamin A/C–deficient cells in the G1 phase, presumably by activating a checkpoint to overcome defects due to a premature S phase entry in the previous cell cycle round.

We previously reported that down-regulation of LAP2α after cell cycle arrest during myoblast differentiation is correlated with relocation of lamins A/C from the nucleoplasm to the NE (Markiewicz et al., 2005). To investigate the effects of siRNA knock down of LAP2α on lamins A/C distribution, fibroblasts were costained with antibodies against LAP2α and either lamin A or lamin C. In cells in which LAP2α was reduced or absent, both lamin A (Fig. 6 a) and lamin C (not depicted) concentrated at the NE. In contrast, in control fibroblasts or in those fibroblasts in knockdown cultures that still expressed LAP2α, both lamins were found in the nucleoplasm and at the NE.

To investigate the influence of LAP2α on the nuclear distribution of Rb, control and LAP2α siRNA–transfected cells were costained with antibodies against LAP2α and Ki67 (a), LAP2α and Ab2 (b), LAP2α and RbS780 (c), or LAP2α and RbS795 (d). All cultures were stained with DAPI (blue fluorescence) and investigated by confocal microscopy. Micrographs are projected as individual black-and-white images or three-color merged images with LAP2α fluorescence in red and Ki67 or Rb fluorescence in green. Bar, 10 μm.

Figure 3. Organization of LAP2α and expression of Rb isoforms in lamin A/C–null human fibroblasts. (a) HDFs with Y259X LMNA mutation were grown in culture for 2 or 4 d and costained with antibodies against LAP2α and Ki67 (a), LAP2α and Ab2 (b), LAP2α and RbS780 (c), or LAP2α and RbS795 (d). All cultures were stained with DAPI (blue fluorescence) and investigated by confocal microscopy. Micrographs are projected as individual black-and-white images or three-color merged images with LAP2α fluorescence in red and Ki67 or Rb fluorescence in green. Bar, 10 μm.
restricted to nuclear speckles (Fig. 6 d), whereas it had more uniform distribution in LAP2α-expressing cells. Our data suggest that knock down of LAP2α or lamin A/C expression in HDFs leads to cell cycle arrest, dephosphorylation, and migration of Rb into nuclear speckles.

**Discussion**

**LAP2α function is required for cell proliferation in human fibroblasts**

Here, we show that entry of HDFs into G0 is correlated with loss of expression of LAP2α. Furthermore, in HDFs that are null for lamins A/C, LAP2α accumulates in aggregates, which is correlated with cell cycle arrest. Finally, siRNA knock down of LAP2α or lamin A/C in HDFs results in cell cycle arrest, and this is correlated with accumulation of Rb into speckles. Our data suggests that the expression and normal distribution of LAP2α allows proper regulation of Rb and thus maintains a proliferative state in HDFs. Our findings are consistent with other reports that show that HDFs lacking lamins A/C grow very slowly in culture (Muchir et al., 2003) and that HDFs harboring mutant lamins enter a senescent state prematurely (Goldman et al., 2004). Other papers, however, appear to partly contradict these findings. Embryonic fibroblasts from a Lmna−/− mouse (−/− mouse embryonic fibroblasts [MEFs]) display a rapid growth phenotype with characteristics of Rb-null fibroblasts resulting from proteosomal degradation of Rb (Johnson et al., 2004). A second paper found that Lmna−/− MEFs displayed a rapid growth phenotype but suggested that this resulted from inhibition of TGFβ signaling, leading to increased phosphorylation of Rb (Van Berlo et al., 2005). We have shown that in mouse fibroblasts and adipocytes, LAP2α represses E2F activity via an Rb-dependent pathway and that knock down of LAP2α in HeLa cells leads to rapid growth (Dorner et al., 2006).

Although there are discrepancies between each paper as to the precise mechanism involved, all three agree that a loss of lamin A/C or LAP2α function leads to rapid proliferation through pathways involving Rb.

The apparent discrepancies between the findings reported here and those reported previously (Johnson et al., 2004; Van Berlo et al., 2005; Dorner et al., 2006) probably reflect fundamental differences between the model systems used. Our study exclusively made use of HDFs, whereas the previous papers used either MEFs or transformed human cell lines. HDFs are able to respond to stimuli, such as genotoxic stresses, by inducing a checkpoint arrest transiently in G2 and eventual G1 arrest. This checkpoint is nonfunctional in MEFs as well as in transformed human cell lines with inactivated pocket proteins, which continue to divide or enter apoptosis after exposure to genotoxic agents (Baus et al., 2003; Jackson et al., 2005). Interestingly, FACS analysis of LAP2α and lamin A/C knockdown HDFs reveals that, initially, cells accumulate in G2 before entering a G1
arrest. Therefore, it appears that HDFs respond to a loss of LAP2α or lamin A/C function as if they were treated with a genotoxic stress. Alternatively, the loss of Rb repressor activity upon LAP2α or lamin A/C down-regulation may prematurely drive the cells into S phase, causing activation of an incomplete S phase cell cycle arrest initially in G2 and eventually in G1.

Therefore, the reason that previous studies have not found that a loss of either lamins A/C or LAP2α leads to cell cycle arrest is most likely because these studies used cell lines in which this checkpoint pathway was abrogated.

**LAP2α maintains pRb within a nucleoplasmic compartment**

It has been shown that Rb binds to lamin A in vitro and associates with intermediate filament-like structures in the nucleus (Mancini et al., 1994; Ozaki et al., 1994). As Rb is no longer anchored in the nucleus of Lmna−/− MEFs (Johnson et al., 2004), these findings are widely interpreted as evidence that anchorage of Rb within the nucleoplasm is dependent on lamins A/C. Our current data suggest that anchorage of Rb within the nucleoplasm depends on both lamin A and LAP2α. We show that nucleoplasmic forms of Rb are entirely absent from cells that contain either no LAP2α or in which LAP2α is entirely restricted to aggregates. These findings are consistent with our previous observations that Rb is anchored within the nucleoplasm via its C-terminal pocket C domain, which also binds to LAP2α in vitro (Markiewicz et al., 2002). Therefore, our results suggest that functional complexes of lamin A and LAP2α are required for anchorage of Rb within the nucleoplasm of HDFs.

We also show that absence of LAP2α or its accumulation in aggregates is correlated with the preferential association of Rb with speckle compartments. Previous studies have suggested that Rb can bind speckle-associated protein p84 via sequences within its N-terminal domain (Durfee et al., 1994). These data are entirely consistent with our current findings because the N-terminal domain of Rb does not bind to lamins A/C or LAP2α (Markiewicz et al., 2002), and we show that Rb anchorage in speckles is independent of lamins A/C or LAP2α.

We propose that Rb is distributed predominantly within a nucleoplasmic compartment, through an association of pocket C with LAP2α and lamin A. However, Rb can also associate with speckle compartments via its N-terminal domain. Although in principle these two modes of association are noncompetitive,
we propose that in practice Rb only associates with speckles when LAP2α expression is down-regulated.

How do lamin A and LAP2α influence cell proliferation?

The cell cycle arrest caused by down-regulation of LAP2α and lamin A/C in HDFs is correlated with rapid dephosphorylation of pRb. In a related study, we have shown that the introduction of dominant-negative lamin A mutants into C2C12 myoblasts causes loss of expression of LAP2α, which is also correlated with the absence of RbS780 (Markiewicz et al., 2005). Dephosphorylated forms of Rb bind to and inhibit the transcription factor E2F, thereby suppressing growth in both normally dividing cells and during differentiation of several mesenchymal cell types (for review see Mittnacht, 1998). Therefore, it seems likely that the growth arrest caused by knock down of LAP2α results from dephosphorylation of Rb. Whether association of Rb into speckle domains is also a prerequisite for growth arrest is unclear. Certainly, the N-terminal region of Rb, through which Rb can associate with speckle-associated protein p84, is crucial for both terminal differentiation and growth suppression (Riley et al., 1997). We propose that functional LAP2α–lamin A nucleoplasmic complexes might be required to anchor Rb in a nucleoplasmic compartment. This may allow correct regulation of Rb by cyclin-dependent kinases and protein phosphatases, which in turn makes cells responsive to environmental stresses, such as genotoxic agents. In conclusion, the corollary of this hypothesis is that a loss of function of lamin A or LAP2α, in cells with functional checkpoint pathways, might lead to irreversible cell cycle arrest and possibly cellular senescence because Rb can no longer be maintained in a phosphorylated state within the nucleoplasm and instead enters speckle compartments by default. This could in turn explain why mutations in lamins A/C and LAP2α both cause diseases associated with premature aging (Hutchison and Worman, 2004).

Figure 6. siRNA knock down of LAP2α causes changes in subnuclear distribution of lamins A/C and pRb expression patterns. HDFs were transfected with either control or LAP2α RNAi. (a) After 72 h in culture, transfected cells were prepared for immunofluorescence and contain with antibodies against lamin A and LAP2α (a), LAP2α and Ab2 (b), LAP2α and RbS780 (c), or LAP2α and RbS795 (d). All cultures were counterstained with DAPI and viewed by confocal microscopy. Micrographs are presented as individual black-and-white images. Bar, 10 μm.

Materials and methods

Cell culture and media

HDFs from a needle biopsy of the forearm were cultured in DME (Invitrogen) supplemented with 10% newborn calf serum (NCS) and 10 U/ml penicillin plus 50 μg/ml streptomycin, at 37°C in humidified incubators containing 5% CO₂. HDFs from a patient with a homozygous Y259X LMNA mutation were obtained as an autopsy sample after an informed consent. Cultures were grown to 70–80% confluence and subcultured thereafter at a seeding density of 3 × 10⁵ cells per 75 cm² flasks. In this study, control and Y259X HDFs were used between passages 8 and 12.

Serum starvation, serum restimulation, and contact inhibition

To induce quiescence by serum starvation, control HDFs were grown for 3 d in complete medium and maintained in starvation medium (0.5% NCS) for 5 d. To induce cell cycle reentry, quiescent HDFs were serum restimulated (10% NCS) for 6, 12, 18, or 24 h and prepared for immunofluorescence microscopy. To induce quiescence by contact inhibition, HDFs were grown in complete medium (10% serum) for 7 d (confluent stage) or for 10 d (postconfluent stage).

Immunofluorescence and confocal microscopy

Immunofluorescence was performed according to established laboratory procedures (Markiewicz et al., 2002). The primary antibodies used and their dilutions are described in Table I. Secondary antibodies were donkey anti–mouse and anti–rabbit IgG conjugated to rhodamine (TRITC) or fluorescein (FITC; Strata-Tech) and, for viewing, DNA in cell coverslips were mounted in DAPI. For imaging cells, a confocal microscope imaging system (LSM 510 META; Carl Zeiss Microimaging, Inc.) or multitrack mode (Carl Zeiss Microimaging, Inc.) was used. Any brightness adjustments were performed in Photoshop (Adobe).

In situ nuclear matrix extraction

In situ nuclear matrix extractions using sequential treatment with detergents, nucleases, and salt were performed as described by Dyer et al. (1997). After extraction, cells were prepared for immunofluorescence microscopy.

Preparation of whole cell extracts

HDF cell pellets were washed with ice-cold PBS and lysed in 0.1 ml of ice-cold hypotonic buffer per 10⁵ cells (10 mM Tris, pH 7.4, 10 mM KCl,
LAP2 reveals two LEM-domain structures: one binds BAF and the other bind DNA.


Gel electrophoresis and immunoblotting

1D SDS-PAGE was performed according to Laemmli (1970). For immunoblotting, proteins separated on gels were electrotransferred onto nitrocellulose membranes (Schleicher & Schuell) using the Mini Trans-Blot system (Bio-Rad Laboratories) and processed according to standard protocols (Markiewicz et al., 2002). Secondary antibodies were donkey anti–mouse or donkey anti–rabbit IgG conjugated to HRP (Jackson Immuno-Research Laboratories). For the immunodetection of proteins, membranes were incubated in ECL reagents (GE Healthcare) and visualized using either LAS-1000 intelligent dark box (FujiFilm) or autoradiography.

Selection of siRNA sequences, transfection of siRNAs, and determination of transfection efficiency

LAP2α- and lamin A/C–specific siRNA duplexes were obtained from Ambion. The sequences were selected from the open reading frames to obtain 21-nt sense and 21-nt antisense strand with symmetric 2-nt 3′ overhangs of identical sequence. The sequences of each strand of siRNA oligos were as follows: LAP2 sense, 5′-GCUGAAGAAAAGCUAUCUCAU-3′; LAP2 anti-sense, 5′-UAAGUAGUAUCUCCUUGACGTCG-3′; lamin A/C sense, 5′-UCUGACUUGCAGAAAGCAAT3′; and lamin A/C anti-sense, 5′-UCUGACUUGCAGAAAGCAAT-3′. RNAi transfection procedure was modified from Harborth et al. (2001). On the day of transfection, cells were seeded at 5 × 10⁶ cells/well in 6-well plates in the presence of 10% NCS and no antibiotics and transfected in tandem with specific or control (scrambled) siRNAs using Oligofectamine reagent (Life Technologies). 24 h after transfection, medium was replaced by fresh medium [10% NCS] without antibiotics. Cells were assayed 48–72 h after transfection. Transfection efficiency was determined by immunofluorescence microscopy and immunoblotting. Specific silencing of LAP2α or lamin A/C was confirmed by four independent experiments.

Flow cytometry

HDFs were synchronized by serum starvation and restimulation. Cultures were trypsinized, counted, and transfected with LAP2α, lamin A/C, or control siRNA. Transfected cells were harvested by trypsinization after 0, 24, 48, or 72 h and resuspended in PBS and methanol prechilled at −20°C (1:9 ratio). Subsequently, washed cell pellets were incubated in PBS containing 100 μg/ml RNase and 25 μg/ml propidium iodide, washed in PBS, centrifuged, and diluted in PBS for cell cycle analysis on a FACSCaliber flow cytometer (Becton Dickinson). Data were collected as DNA histograms from 5,000 single-cell events, and cell cycle phase distribution (percentage of G1, S, and G2/M cells) was determined by the Deau/jett/Fox model using FlowJo software.

Online supplemental material

Fig. S1 shows expression and solubility properties of confluent HDFs. Fig. S2 shows colocalization of RbS795 foci with splicing speckles in confluent HDFs. Fig. S3 shows colocalization of RbS795 foci with splicing speckles in Y259X HDFs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200606139/DC1.

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References


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IF, immunofluorescence; IB, immunoblotting; m, monoclonal; p, polyclonal.
chromosomes early in nuclear assembly implies functions in nuclear structure dynamics. EMBO J. 17:4887–4902.


