Lamina-associated polypeptide 2α regulates cell cycle progression and differentiation via the retinoblastoma–E2F pathway

Daniela Dorner,[1] Sylvia Vlcek,[1] Nicole Foeger,[1] Andreas Gajewski,[1] Christian Makolm,[1] Josef Gotzmann,[1] Christopher J. Hutchison,[2] and Roland Foisner[1]

[1]Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University of Vienna, A-1030 Vienna, Austria
[2]Department of Biological Sciences, University of Durham, Durham DH1 3HP, United Kingdom

Lamina-associated polypeptide (LAP) 2α is a non-membrane-bound LAP2 isoform that forms complexes with nucleoplasmic A-type lamins. In this study, we show that the overexpression of LAP2α in fibroblasts reduced proliferation and delayed entry into the cell cycle from a G0 arrest. In contrast, stable down-regulation of LAP2α by RNA interference accelerated proliferation and interfered with cell cycle exit upon serum starvation. The LAP2α-linked cell cycle phenotype is mediated by the retinoblastoma (Rb) protein because the LAP2α COOH terminus directly bound Rb, and overexpressed LAP2α inhibited E2F/Rb-dependent reporter gene activity in G1 phase in an Rb-dependent manner. Furthermore, LAP2α associated with promoter sequences in endogenous E2F/Rb-dependent target genes in vivo and negatively affected their expression. In addition, the expression of LAP2α in proliferating preadipocytes caused the accumulation of hypophosphorylated Rb, which is reminiscent of noncycling cells, and initiated partial differentiation into adipocytes. The effects of LAP2α on cell cycle progression and differentiation may be highly relevant for the cell- and tissue-specific phenotypes observed in laminopathic diseases.

Introduction

The nuclear lamina is part of the nuclear envelope in multicellular eukaryotes opposing the inner nuclear membrane (Hutchison and Worman, 2004; Gruenbaum et al., 2005). The major components of the nuclear lamina are type V intermediate filament proteins referred to as lamins (Hutchison, 2002; Shumaker et al., 2003) and various lamin-binding proteins (Burke and Stewart, 2002). In mammals, at least one of the B-type lamins, encoded by *LMNB1* and *LMNB2*, is constitutively expressed and essential for cell viability. The A-type lamins A and C, encoded by *LMNA*, are expressed in most terminally differentiated somatic cells but are absent in early embryos and some undifferentiated cells. Interestingly, lamins A and C are also found in the nucleoplasm as discrete foci, filamentous structures, or are uniformly distributed throughout the nucleus (Dechat et al., 2000) and have been implicated in chromatin organization (Gruenbaum et al., 2005), DNA replication (Moir et al., 2000), RNA Pol II–dependent transcription (Spann et al., 2002), and control of gene expression (Gruenbaum et al., 2005).

Aside from lamins, numerous lamin-binding proteins are also considered to be important components of the nuclear lamina and of nucleoplasmic lamin structures (Foisner, 2001; Burke and Stewart, 2002). Among the best-studied lamin-binding proteins are the LAP2 family members. Of the six alternatively spliced LAP2 isoforms, four—LAP2β, -γ, -δ, and -ε—are type II membrane proteins in the inner nuclear membrane (Harris et al., 1994; Berger et al., 1996) and bind lamin B (Foisner and Gerace, 1993; Furukawa et al., 1998). LAP2α is structurally and functionally different. It shares only the NH$_2$ terminus with the other isoforms and contains a unique COOH terminus. LAP2α is localized throughout the nucleus (Dechat et al., 1998; Vlcek et al., 1999) and has been identified as a specific binding partner of nucleoplasmic A-type lamins (Dechat et al., 2000).

All LAP2 isoforms as well as the inner nuclear membrane proteins emerin and MAN1 share a common structural motif called the LAP2–emerin–MAN1 (LEM) domain (Lin et al., 2000), which mediates binding to BAF (barrier to autointegration factor), an essential, highly conserved DNA-binding protein
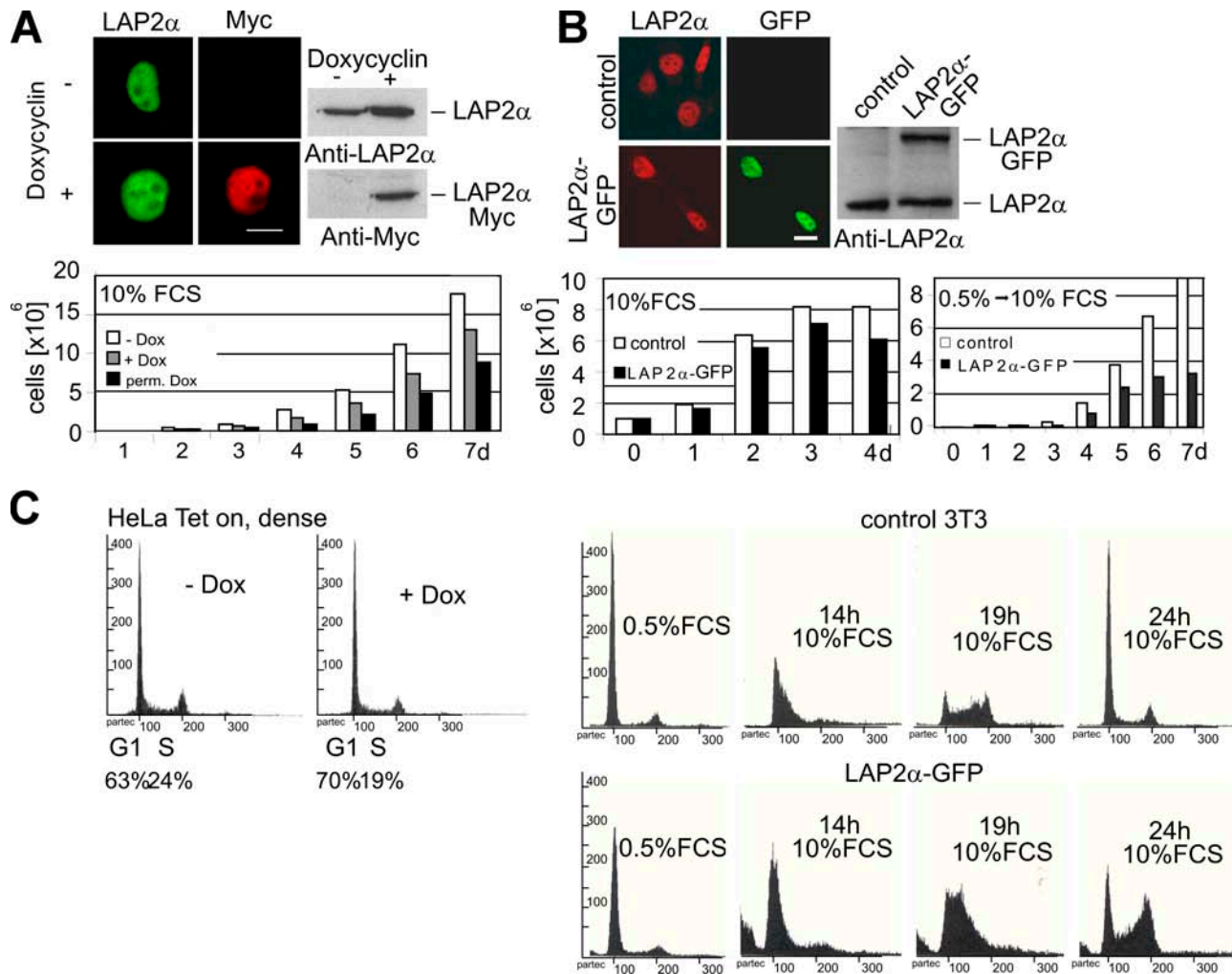
Figure 1. **Overexpression of LAP2α negatively affects cell cycle progression.** (A) Stable HeLa Tet-on clones expressing myc-tagged LAP2α under the control of a doxycyclin-dependent promoter were grown in the absence or presence of doxycyclin and processed for immunofluorescence microscopy and immunoblot analyses of cell lysates. Cells grown permanently in the absence (−Dox) or presence (perm Dox) of doxycyclin or cells transferred to doxycyclin medium upon splitting at day 0 were seeded into plates, and cumulative cell numbers were determined. (B) Stable 3T3 cells expressing a LAP2α-GFP fusion or untransfected control cells were analyzed as in A. Cumulative cell numbers were analyzed in cultures grown in complete medium (10% FCS) or upon serum starvation in 0.5% FCS for 7 d before the addition of complete medium (0.5→10%). Graphs show one representative dataset from at least three independent experiments. Bars, 10 μm. (C) DNA flow cytometry of dense LAP2α-myc HeLa tet-on cultures grown in the absence or presence of doxycyclin and of 3T3 and LAP2α-GFP cells after 7 d of serum starvation (0.5% FCS) and at different time points after restimulation (10% FCS). Numbers at the bottom indicate percentages of cells in G1 and S phase.

in eukaryotes (Segura-Totten and Wilson, 2004). Based on these interactions, LEM domain proteins in the nuclear lamina and the nuclear interior have been implicated in chromatin organization (Dechat et al., 2004; Segura-Totten and Wilson, 2004).

Mutations in the *LMNA* gene have been shown to cause a variety of inherited human diseases (laminopathies) that affect different tissues, including skeletal muscle, heart, adipose tissue, peripheral nerves, and skin, or cause premature aging (Burke and Stewart, 2002; Hutchison and Worman, 2004; Mounkes and Stewart, 2004). The molecular basis of these diseases is still unclear. Besides structural defects in lamin complexes, it has also been suggested that disease-causing mutations in *LMNA* may interfere with the proposed gene regulatory functions of lamins (Hutchison and Worman, 2004). Among several reported interactions of lamina proteins with transcriptional activators or repressors (Gruenbaum et al., 2005), the interaction

of A-type lamins (Ozaki et al., 1994) and of nucleoplasmic lamin A/C–LAP2α complexes (Markiewicz et al., 2002) with the retinoblastoma (Rb) protein may also regulate gene expression via influencing Rb activity. Rb regulates progression through the cell cycle at the G1→S-phase transition by inhibiting the activity of E2F-type transcription factors in a phosphorylation-dependent manner and by mediating epigenetic changes on the promoter region of E2F/Rb target genes (Frolov and Dyson, 2004). It has been shown that Rb is required for the terminal differentiation of many tissues, including adipose and muscle tissue (Hansen et al., 2004; Huh et al., 2004), which are also affected in laminopathies. Based on these data, an intriguing laminopathy disease model has recently been raised, arguing that lamin A/C complexes may cooperate with Rb in controlling cell cycle progression and differentiation of mesenchymal stem cells during tissue regeneration and turnover. Disease-causing

mutations in *LMNA* or a lack of lamin A/C may affect the balance between proliferation and differentiation in stem cells, leading to a defect in tissue regeneration (Gotzmann and Foisner, 2005). Intriguingly, mutations in the gene encoding the nucleoplasmic lamin A/C–interacting protein LAP2α has recently been linked to dilated cardiomyopathy in humans, clinically resembling lamin A–linked disease phenotypes (Taylor et al., 2005). As LAP2α is only expressed at very low levels in differentiated muscle, it is hard to imagine how mutations in LAP2α can lead to the disease phenotype in fully differentiated heart muscle cells. Therefore, it is tempting to speculate that LAP2α, which has previously been shown to mediate the nuclear retention of Rb (Markiewicz et al., 2002), may also be involved in Rb-mediated control of cell cycle progression and differentiation in muscle precursor cells. Deregulation of this function in patients expressing the disease variant of LAP2α may then lead to impaired tissue turnover.

In this study, we investigate whether LAP2α can affect cell cycle progression and differentiation. We found that LAP2α inhibits progression from G1 to S phase and initiates early stages of differentiation in an in vitro adipocyte differentiation culture model. We further show that the cell cycle and differentiation regulatory function of LAP2α requires Rb and involves regulation of the activity of E2F transcription factors.

Results

LAP2α expression levels affect cell cycle progression

To test the influence of LAP2α expression levels on cell cycle progression, we used two different cell models. First, we generated stable HeLa cell clones expressing a myc-tagged LAP2α under the control of a doxycyclin-dependent promoter, which allowed analysis of the cell cycle phenotypes in a single cell clone expressing different levels of LAP2α. Although HeLa cells are transformed by human papilloma virus E7 oncogene (Helt and Galloway, 2003), they retain a certain level of cell cycle control, as shown by their growth-inhibitory response to serum starvation (see serum starvation data in Fig. 2). LAP2α-myc HeLa cells grown in the absence of doxycyclin expressed exclusively endogenous LAP2α, which is shown by immunofluorescence microscopy and immunoblot analyses of total cell lysates. The addition of doxycyclin to the culture medium induced the expression of myc-tagged LAP2α in the nucleoplasm (Fig. 1 A), giving rise to a total LAP2α level twice as high as that in uninduced cells. Cells grown in the absence of doxycyclin proliferated significantly faster than the cells grown permanently in doxycyclin-containing medium (18- vs. 22-h doubling times; Fig. 1 A). When doxycyclin was added to cells only upon seeding into culture dishes, they showed intermediate growth rates (∼20-h doubling time; Fig. 1 A). The addition of doxycyclin to untransfected cells had no influence on cell growth (Vlcek et al., 1999). Interestingly, the cell proliferation-inhibitory effect of LAP2α was most prominent in dense cultures. DNA flow cytometry analyses in dense cultures grown in the absence or presence of doxycyclin (Fig. 1 C) showed a slight increase in the relative number of cells in G1 phase (63→70%) and a
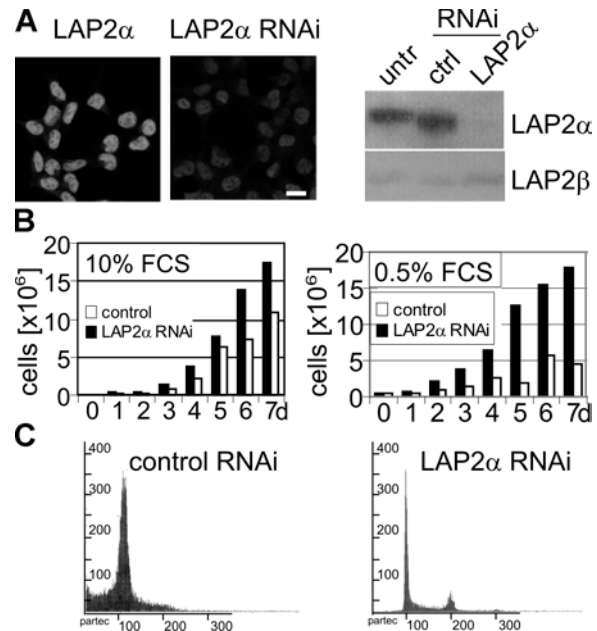

Figure 2. **Reduced LAP2α levels favor cell cycle progression.** (A) HeLa cells were stably transfected with a LAP2α-specific shRNA vector construct (LAP2α RNAi) or with a construct targeting firefly luciferase (ctrl). Cells were analyzed by immunofluorescence microscopy using monoclonal anti-LAP2α antibody and by immunoblot analyses of total cell lysates using anti-LAP2 antibody (detecting both LAP2α and LAP2β). Bar, 10 μm. Note that the LAP2α RNAi immunofluorescence image was overexposed to detect cells. (B) Cumulative cell numbers were determined within 7 d of culture in complete medium (10% FCS) or in low serum medium (0.5% FCS). Graphs show the dataset of one of three independent clones showing the same phenotype. (C) DNA flow cytometry of control and LAP2α-deficient (LAP2α RNAi) cells grown in low serum medium for 4 d.

decrease in S-phase cells (24→19%) upon doxycyclin addition. In addition, we showed previously by BrdU incorporation assays that transient overexpression of LAP2α leads to a reduction in cells in S phase (Vlcek et al., 2002). Therefore, we concluded that increased LAP2α protein levels negatively affect G1→S-phase transition.

To confirm this result in a cell system with an intact cell cycle checkpoint control mechanism, we stably expressed a LAP2α-GFP fusion protein in mouse 3T3 fibroblasts. As shown by immunoblot analyses of cell lysates and by immunofluorescence microscopy, stable 3T3 cell clones expressed roughly equal amounts of ectopic LAP2α-GFP and endogenous LAP2α, both of which localized to the nucleoplasm (Fig. 1 B). In three independent clones of LAP2α-GFP–expressing cells, we found a subtle but reproducible reduction in their cell proliferation rate compared with that of untransfected or GFP-expressing control cells under optimal culture conditions (10% FCS). DNA flow cytometry also revealed a slight increase in G1-phase cells upon LAP2α-GFP expression (not depicted), indicating an inhibitory role of LAP2α on G1→S-phase progression. However, because both control cells and LAP2α-GFP–expressing cells showed an efficient cell cycle arrest in dense cultures (Fig. 1 B), the proliferation-inhibitory role of LAP2α may not be detectable as clearly as in dense HeLa cells, which have a compromised, although not completely inhibited, density-mediated growth control.

Therefore, we used an alternative approach and forced 3T3 cells into cell cycle arrest by serum starvation (0.5% FCS) for 7 d before we induced cell cycle entry by the addition of complete medium. DNA flow cytometry revealed that LAP2α-GFP–expressing cells showed a significantly delayed reentry into S phase (Fig. 1 C) and a persistent growth reduction within 7 d after induction (Fig. 1 B) when compared with untransfected or GFP-expressing cell clones. We concluded that increased LAP2α protein levels only weakly affected cell proliferation in growing 3T3 cells but interfered with pathways regulating G0/G1 phase to S-phase transition.

Having shown that the overexpression of LAP2α negatively affects proliferation and cell cycle entry, we wondered whether reduced levels of LAP2α would have opposing effects. Although stable RNAi-mediated down-regulation of LAP2α was not achieved in 3T3 fibroblasts, stable HeLa cell clones with reduced levels of LAP2α were generated successfully. Immunoblot analyses of total cell lysates showed that unlike an unrelated control RNAi construct (targeting firefly luciferase), stable transfection with a LAP2α-specific short hairpin RNA (shRNA) construct reduced endogenous LAP2α to <10% of the protein level in control cells, whereas the expression of LAP2β remained unchanged (Fig. 2 A). In addition, immuno-fluorescence microscopy of LAP2α knockdown versus control cells revealed a significant down-regulation of LAP2α protein. As expected, the proliferation rate of LAP2α knockdown cell clones was increased significantly compared with that of control cells even in optimal growth conditions (Fig. 2 B, 10% FCS).

Interestingly, in low serum conditions (0.5% FCS), control cells exhibited a very low proliferation rate (45-h doubling time), whereas proliferation of LAP2α knockdown cells was similar to that in complete medium (25-h doubling time). DNA flow cytometry confirmed that LAP2α-deficient cells were not as efficiently arrested in G1 phase as control cells in low serum medium (Fig. 2 C). Altogether, we concluded that LAP2α is involved in cell cycle entry/exit checkpoints and/or in G1→S-phase transition.

LAP2α interacts with Rb in the COOH terminus and associates with E2F/Rb promoter sequences in vivo

Next, we wanted to gain more insights into the molecular basis of LAP2α's effect on the cell cycle. Our previous data revealed that LAP2α bound to hypophosphorylated Rb (Markiewicz et al., 2002), which is the predominant Rb form in G1 phase of proliferating cells or in resting cells in G0 phase. Hypophosphorylated Rb blocks transition into S phase by inhibiting the activity of E2F transcription factors that are essential for the expression of S phase–specific genes (Classon and Harlow, 2002). Therefore, we reasoned that overexpressed LAP2α may bind Rb and, in turn, affect its E2F-repressing activity. To analyze the interaction of LAP2α and Rb in more detail, we narrowed down the Rb interaction domain in LAP2α. In vitro–translated and [^{35}S]methionine-labeled Rb was overlaid onto transblotted fragments of LAP2α. Full-length protein (aa 1–693) as well as NH$_2$-terminally truncated LAP2α fragments missing either the

Figure 3. **LAP2α binds to Rb at its COOH terminus and associates with Rb, lamin A/C, and E2F promoter sequences in vivo.** (A) Schematic drawing of LAP2α and LAP2α fragments used for binding studies and localization of molecular domains and interaction regions with Rb and lamins A/C in the polypeptide. Numbers denote amino acid positions; +, interaction; −, no interaction of respective LAP2α fragments with Rb. Light gray boxes denote the constant LAP2 regions, including LEM-like and LEM domains (hatched). Medium gray denotes the α-specific region, whereas dark gray indicates the chromatin interaction domain. On the right, in vitro–translated ^{35}S-labeled Rb was overlaid onto transblotted recombinant LAP2α fragments. A Ponceau S stain of the respective blot and an autoradiogram of the overlay are shown. Numbers on the right indicate molecular masses in kD. (B) HeLa cells stably expressing LAP2α-GFP were fixed in formaldehyde, mechanically lysed by sonication, and LAP2α was immunoprecipitated using monoclonal (m) or polyclonal (p) antibody to LAP2α. Control precipitations were performed with antibody-free medium (ctrl) and with preimmune serum (PI). Immunoblots of immunoprecipitates and respective inputs (IP, diluted 1:10) with antiserum to LAP2α, Rb, or monoclonal antibody to lamin A/C are shown. (C) Chromatin immunoprecipitation was performed from HeLa and 3T3 cells using monoclonal and polyclonal antibody to LAP2α, preimmune serum, or anti-acetylhistone H4 (H4), and E2F-dependent promoter sequences in the cyclin D1 and thymidine kinase (TK) genes were amplified by PCR. As a negative control, the presence of histone H4 or E-cadherin promoter sequences was tested. Ethidium bromide–stained DNA fragments in agarose gels are shown. IP, 1% input.
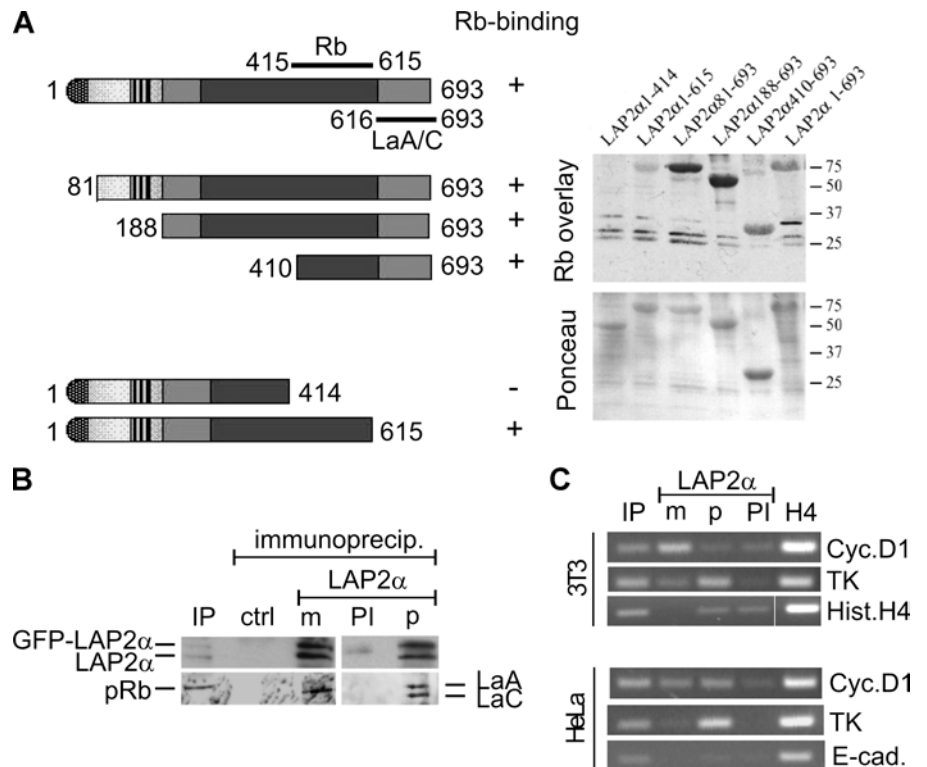
LEM-like domain (aa 81–693) or the LAP2 common domain (including the LEM-like and LEM domain; aa 188–693) strongly interacted with Rb (Fig. 3 A). Because a COOH-terminal LAP2α fragment (aa 410–693) and a fragment missing the last COOH-terminal 78 residues (aa 1–615) also bound Rb, although less strongly than the other construct, we concluded that the Rb interaction domain of LAP2α is located in LAP2α's unique COOH-terminal domain between aa 415–615, but residues downstream of aa 615 may also contribute to the interaction (Fig. 3 A). Intriguingly, the Rb interaction domain is located next to the previously identified lamin A/C interaction domain (Fig. 3 A; Dechat et al., 2000). Thus, it may well be possible that LAP2α, lamin A/C, and Rb form a trimeric complex in cells, as previously suggested based on coimmunoprecipitation of these proteins from lysates of untransformed human dermal fibroblasts (Markiewicz et al., 2002). To test whether this complex can also be formed in transformed HeLa cells, in which at least part of Rb is bound to E6 and E7 oncoproteins, we immunoprecipitated LAP2α complexes from LAP2α-GFP–expressing HeLa cells and analyzed samples by immunoblotting. As shown in Fig. 3 B, LAP2α/LAP2α-GFP immunoprecipitates obtained with a monoclonal antibody (m) or a polyclonal serum to LAP2α (p) contained Rb and lamin A/C. Control precipitations with mouse IgG (Fig. 3 B, ctrl) or preimmune serum (Fig. 3 B, PI) did not pull down any of these proteins. Thus, LAP2α–lamin A/C–Rb complexes also exist in transformed HeLa cells and could potentially be involved in the aforementioned cell cycle regulatory function of LAP2α (Figs. 1 and 2).

We were unable to detect E6, E7, and E2F proteins in cell lysates and immunoprecipitates most likely as a result of their low concentrations in the samples. Therefore, to test the association of LAP2α with E2F complexes, we performed chromatin immunoprecipitation with monoclonal and polyclonal LAP2α antibodies and tested the association of LAP2α with endogenous E2F-dependent promoters (Fig. 3 C). Intriguingly, unlike the preimmune serum (Fig. 3 C, PI), both antibodies pulled down DNA fragments containing E2F-dependent promoter sequences of the thymidine kinase (Fig. 3 C, TK) genes in HeLa and 3T3 cells. Furthermore, the E2F-dependent promoter sequence of cyclin D1 was efficiently precipitated by monoclonal LAP2α antibody (Fig. 3 C, m) in 3T3 and HeLa cells and by LAP2α antiserum (Fig. 3 C, p) in HeLa cells, whereas promoter sequences of the E2F-independent genes histone H4 and E-cadherin were not detected in the immunoprecipitates over background level. In contrast, antibodies to acetylated histone H4 as a positive control pulled down all DNA sequences tested. Thus LAP2α complexes specifically bind to E2F-dependent promoter sequences in vivo.

LAP2α affects E2F-dependent transcription

To test whether LAP2α may affect E2F-dependent promoter activity, we tested the effect of overexpressed LAP2α in an E2F-dependent luciferase reporter assay. Proliferating 3T3 cultures exhibited a basal reporter activity, which was induced threefold upon the transient expression of E2F-1. Coexpression of E2F-1 and Rb reduced reporter activity to basal levels as expected because Rb can sequester and inactivate free active E2F-1 (Fig. 4 A). Importantly, the coexpression of LAP2α with
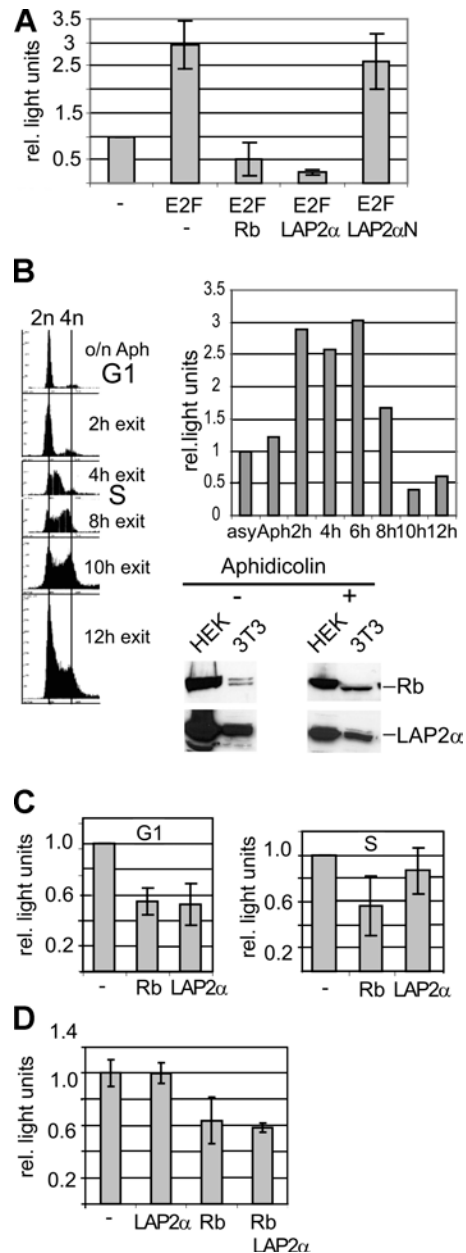

Figure 4. **LAP2α reduces E2F transcription activity in an Rb-dependent manner.** Asynchronous 3T3 cells (A), 3T3 cells synchronized by an overnight aphidicolin block and release from the block for different time periods (B), aphidicolin-arrested cells (G1 phase) and 4-h released cells (S phase; C), or TKO mouse fibroblasts lacking pocket proteins Rb, p107, and p130 (D) were used for E2F-reporter assays. Cells were transfected with an E2F-dependent luciferase reporter and a β-galactosidase expression construct (for normalization of transfection efficiencies) together with empty vectors (−) or expression vectors encoding E2F-1, Rb, LAP2α, and LAP2α N terminus (LAP2α-N). Reporter activity was measured 24 h after transfection. Relative activities were calculated by referring measured light units to that of the respective controls (reporter plasmids with empty vectors). Data represent mean values of at least three independent datasets, and SDs (error bars) are shown. DNA flow cytometry profiles of synchronized 3T3 cells at different stages of the cell cycle and immunoblot analysis of total cell lysates of untreated (−) or aphidicolin-treated 3T3 cells and of transformed HEK cells are shown in B.

E2F-1 also decreased the reporter activity as effectively as Rb, whereas an NH2-terminal LAP2α fragment (LAP2α-N) that did not bind Rb (Fig. 3 A) and did not effect cell cycle progression

in HeLa cells (Vlcek et al., 2002) had no significant influence on E2F-dependent activity. Thus, full-length LAP2α efficiently inhibited exogenous E2F-dependent activity, which is consistent with the negative effect of LAP2α on cell proliferation and cell cycle reentry.

So far, we have shown that LAP2α interferes with the activity of overexpressed ectopic E2F-1. To confirm this result under more physiological conditions, we wanted to analyze the effect of LAP2α on the activity of endogenous E2F. As expected, endogenous E2F-dependent activity was low in aphidicolin-treated cells, which were arrested at the G1→S-phase transition (shown by DNA flow cytometry; Fig. 4 B). Aphidicolin-arrested 3T3 cells also contained predominantly hypophosphorylated Rb that migrates faster on SDS PAGE, whereas proliferating 3T3 cells revealed an Rb double band, reflecting hyper- and hypophosphorylated forms. Thus, unlike transformed HEK cells (shown as a control in Fig. 4 B), aphidicolin-treated 3T3 cells were efficiently arrested at the G1–S-phase boundary. Upon the release of cells from the block, they synchronously proceeded through S phase, and E2F-dependent activity was increased 2–6 h after the release, whereas it declined again after 8–10 h when cells were in late S/G2 phase (Fig. 4 B). We measured the effect of overexpressed LAP2α on endogenous activity at two different cell cycle stages: in late G1 phase (aphidicolin arrested), when E2F-dependent activity is low because of the expression of hypophosphorylated Rb, and in S phase (4 h after release), when E2F-dependent activity is high. In G1 phase, a low basal E2F-dependent activity was further reduced (twofold) upon the expression of exogenous Rb or of LAP2α (Fig. 4 C). In contrast, in S-phase cells with a higher basal E2F-dependent activity, LAP2α expression was unable to repress E2F-dependent activity, whereas overexpressed Rb still reduced it up to twofold (Fig. 4 C). These results imply that LAP2α can repress E2F-dependent activity through hypophosphorylated Rb in G1 phase, which is consistent with our previous data that LAP2α binds preferentially to the G1 phase–specific hypophosphorylated Rb (Markiewicz et al., 2002). Overall, our studies strongly support a role of LAP2α in regulating E2F-dependent activity in an Rb-dependent manner.

Having shown that LAP2α binds to promoter sequences of endogenous E2F target genes, we wondered whether overexpressed LAP2α also affects the expression of these genes. As E2F target genes are differentially expressed during the cell cycle, we arrested 3T3 control cells and LAP2α-GFP–expressing 3T3 cells in G0 phase by serum starvation. After reentry into the cell cycle by serum addition, we analyzed mRNA levels of three known E2F target genes (cyclin D1, thymidine kinase, and cyclin E) and of one E2F-independent control gene (actin) by semiquantitative RT-PCR. As expected, unlike actin mRNA, the mRNA levels of all three E2F target genes in control 3T3 cells increased between 10 and 14 h after serum addition (Fig. 5), when cells proceeded to the G1–S-phase boundary (for DNA flow cytometry, see Fig. 1 C). In LAP2α-GFP–expressing 3T3 cells, the increase in E2F target gene mRNAs was delayed by ~5 h compared with the control (Fig. 5). These findings are consistent with an inhibitory function of LAP2α repressing endogenous E2F target gene expression.

LAP2α function in cell cycle progression depends on Rb

Our findings could be explained if LAP2α regulates cell cycle progression by binding to hypophosphorylated Rb and, as a consequence, increases Rb's repressor function or delays its deactivation during G1→S-phase transition. However, we cannot fully exclude the possibility that LAP2α inhibits E2F activity via an Rb-independent function. To address this question directly, we performed E2F reporter assays in triple knockout (TKO) mouse embryonic fibroblasts (MEFs) in which all three pocket proteins (Rb, p107, and p130) were knocked out (Sage et al., 2000). These cells express normal levels of LAP2α and lamins A/C compared with wild-type fibroblasts (unpublished data). Endogenous E2F activity was not affected by the overexpression of LAP2α in TKO MEFs (Fig. 4 D). In contrast, the expression of Rb alone or in combination with LAP2α reduced E2F activity to a similar level (approximately twofold), although the coexpression of Rb and LAP2α was slightly more effective than Rb alone. The high level of Rb expression in these transient transfection assays is most likely sufficient to effectively repress E2F activity so that the additional expression of LAP2α may not significantly increase this activity. Overall, we concluded that LAP2α's effect on E2F activity is strictly dependent on the presence of the pocket proteins Rb, p107, or p130.
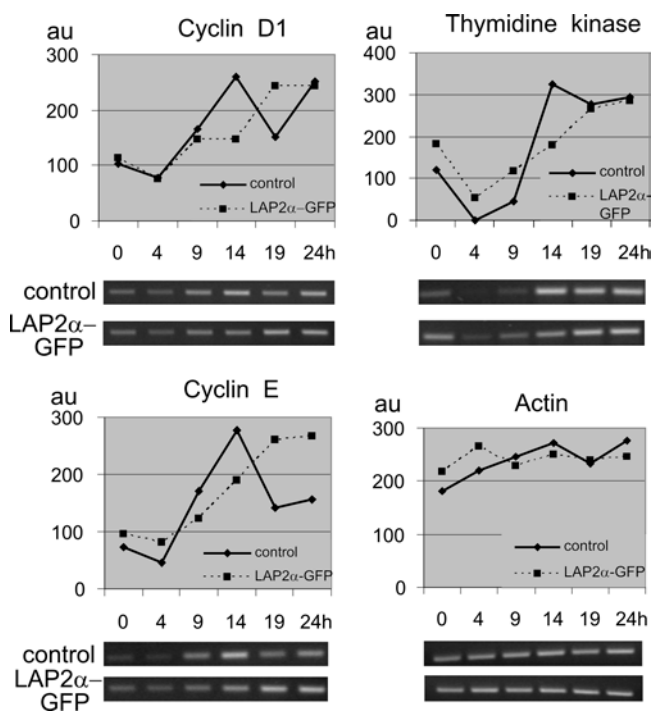
Figure 5. **Overexpressed LAP2α affects the expression of endogenous E2F target genes.** mRNA levels of the E2F target genes cyclin D1, thymidine kinase, and cyclin E and of actin as a control were determined by semiquantitative RT-PCR from control 3T3 cells (solid lines) or LAP2α-GFP–expressing 3T3 cells (dotted lines) harvested 0–24 h after serum addition to serum-starved cultures. Ethidium bromide–stained DNA fragments in agarose gels (bottom) were quantified by QuantiScan and plotted against incubation times (top).

LAP2α overexpression initiates the adipocyte differentiation program

The Rb–E2F pathway is essential for the differentiation of several cell types, including skeletal muscle (Huh et al., 2004) and adipocytes (Hansen et al., 2004). To test whether LAP2α-mediated regulation of the Rb–E2F pathway can also influence differentiation, we used the 3T3 F442A clone. These cells proliferate in normal culture medium and can be considered as preadipocytes, whereas the addition of insulin and triiodothyronine (T3) to the culture medium induces cell cycle arrest and differentiation into mature adipocytes within 10 d, as seen in the phase-contrast microscope by a morphological change from spindlelike preadipocytes to rounded adipocytes with numerous fat droplets (Fig. 6 A). Immunoblot analyses of total cell lysates revealed a switch from the high molecular weight hyperphosphorylated Rb in proliferating cells to the low molecular weight hypophosphorylated form in adipocytes, reflecting cell cycle arrest in G0 phase (Fig. 6 B). Furthermore, LAP2α appeared as a double band in proliferating cells, representing mitotic hyperphosphorylated and interphase hypophosphorylated LAP2α (Dechat et al., 1998), whereas the hypophosphorylated form accumulated in differentiated adipocytes because of the lack of mitotic cells. Finally, the adipocyte-specific differentiation marker peroxisome proliferator–activated receptor γ (PPARγ; Fajas et al., 2002b) was expressed exclusively in differentiated cells, and lamins A/C were slightly up-regulated in differentiated cells (Fig. 6 B). Overall, these data showed that in differentiation medium, preadipocytes enter G0 phase and differentiate to adipocytes in vitro.

To test the effect of overexpressed LAP2α on differentiation, we used clones of the 3T3 F442A cells stably expressing LAP2α-GFP, which showed a delayed response in cell cycle entry upon serum starvation and restimulation (Fig. 1 B). Cell cycle arrest and morphological changes during differentiation as detected by phase-contrast microscopy did not differ significantly in several independent clones of LAP2α-GFP–expressing versus control cells (untransfected or GFP expressing). However, we did detect significant differences by immunoblot analyses of total cell lysates. Hypophosphorylated Rb, LAP2α, and PPARγ, all of which are early markers of adipogenesis, clearly accumulated in proliferating LAP2α-GFP preadipocytes compared with control cells, suggesting that the overexpression of LAP2α initiated early events in the differentiation pathway, accompanying a partial cell cycle arrest.

Upon insulin/T3 stimulation of LAP2α-GFP cells, hypophosphorylated Rb and LAP2α accumulated similarly to the control cells. In contrast, the expression level of PPARγ did not reach the levels observed in the control (Fig. 6 B), indicating that differentiation was incomplete. To test this possibility, we stained for differentiation-specific lipid droplets using oil red. As shown in Fig. 6 C, LAP2α-GFP–expressing cells had significantly fewer oil red–positive droplets than control cells. Thus, although the overexpression of LAP2α-GFP initiated differentiation events in the absence of insulin and T3, further differentiation did not occur even in the presence of insulin/T3.

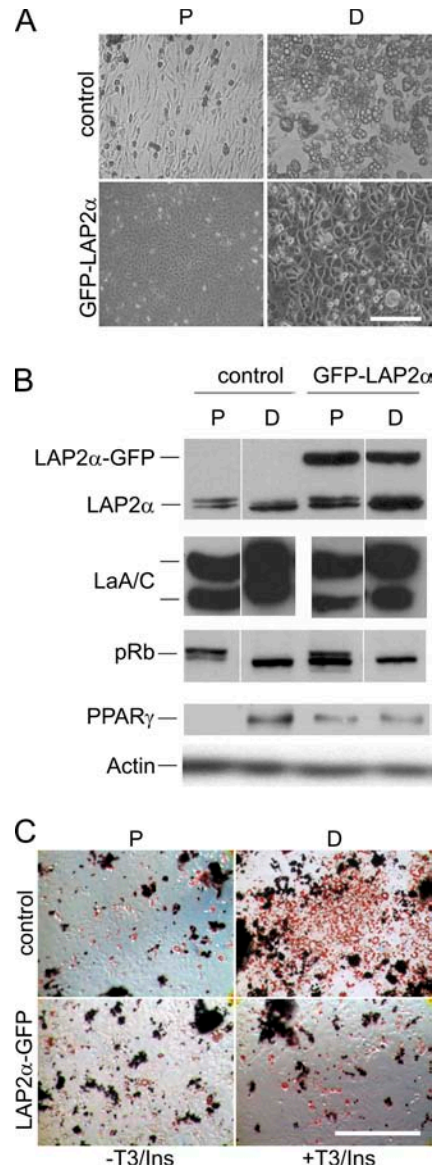Overall, our studies show that LAP2α–lamin A/C complexes regulate G1→S-phase transition via the Rb–E2F

Figure 6. **LAP2α affects adipocyte differentiation.** 3T3 F442A cells (control) or 3T3 F442A cells expressing LAP2α-GFP were grown in proliferation medium (P) or in differentiation medium containing insulin and T3 (D) for 10 d and were analyzed by phase-contrast microscopy (A), immunoblot analyses of total cell lysates (B), and oil red staining (C). White lines indicate that intervening lanes have been spliced out. Bars, 40 μm.

pathway and control the balance between cell proliferation and differentiation.

Discussion

In this study, we analyzed the effects of LAP2α on cell cycle progression and differentiation and showed that decreased LAP2α levels negatively affected the growth arrest response to serum starvation, whereas increased levels delayed the transition from G0 to S phase. We further showed that this function of LAP2α is mediated by pocket proteins.

What are the molecular mechanisms of the Rb-mediated cell cycle control by LAP2α complexes? Rb regulates the

activity of E2F transcription factors, which control the expression of cell cycle regulatory genes (Classon and Harlow, 2002; Frolov and Dyson, 2004). In noncycling cells, Rb is hypophosphorylated and is active as a transcriptional repressor. Hypophosphorylated Rb is bound to E2F transcription factors, blocks its transactivation domain, inhibits the recruitment of preinitiation complexes, and represses genes directly by recruiting chromatin-modifying proteins, including histone deacetylase HDAC1 (Brehm et al., 1998), methyl transferase Suv39h, and heterochromatin protein HP1 (Nielsen et al., 2001; Ait-Si-Ali et al., 2004). Through sequential phosphorylation of Rb by cyclin D and E–dependent kinases (Mittnacht, 1998), Rb is released from E2F, causing its activation as a transcription factor. Based on our previous findings (Dechat et al., 2000; Markiewicz et al., 2002; Vlcek et al., 2002) and results reported here, we postulate that nucleoplasmic LAP2α–lamin A/C complexes bind hypophosphorylated Rb, thereby delaying its deactivation and maintaining E2F in a repressed state.

The molecular mechanisms as to how LAP2α–lamin A/C complexes exert their regulatory function on Rb are unknown. However, several possibilities can be envisaged: first, LAP2α may regulate Rb phosphorylation. LAP2α preferentially bound to Rb phosphorylated on serine 780, which is the first serine to be targeted at the G1→S-phase transition (Lundberg and Weinberg, 1998). Recruitment of Rb-S780 to LAP2α–lamin A/C may delay the phosphorylation of downstream sites. Alternatively, LAP2α–lamin A/C complexes may favor the dephosphorylation of Rb, leading to its deactivation. In line with the latter hypothesis, Van Berlo et al. (2005) have recently shown that in *Lmna* knockout fibroblasts, TGFβ-mediated cell cycle arrest is impaired through a less efficient phosphatase PP2A-mediated dephosphorylation of Rb. Second, LAP2α–lamin A/C may recruit regulatory proteins to the Rb complex. Third, LAP2α–lamin A/C may recruit Rb–E2F complexes to subnuclear compartments or chromatin regions. Our findings that LAP2α complexes associate with E2F-dependent promoter regions imply that the cell cycle regulatory activity of LAP2α acts directly on the promoter of E2F target genes. Fourth, in view of the reported destabilization of Rb in *Lmna*$^{-/-}$ fibroblasts by proteasomal degradation (Johnson et al., 2004), the interaction of Rb with LAP2α–lamin A/C complexes may stabilize the proteins. However, this would imply that proteasomal degradation also contributes to the deactivation of Rb repressor activity at the G1→S-phase transition, although no direct evidence for this has been reported so far. Furthermore, we did not see any changes in Rb protein level upon the overexpression or knockdown of LAP2α. Finally, it should be mentioned that another LAP2 isoform, LAPß, was also found to down-regulate E2F activity, although the mechanisms are most likely different from that of LAP2α, involving the binding of LAP2β to the protein germ cell less (Nili et al., 2001) and to histone deacetylase 3 (Somech et al., 2005).

What is the physiological relevance of LAP2α–lamin A/C complexes in cell cycle control? In cycling cells, these proteins may provide additional checkpoint mechanisms, preventing premature entry into S phase. However, we consider it more likely that LAP2α–lamin A/C complexes are involved in controlling cell cycle exit during the differentiation of adult stem cells. Rb has been found to be essential for the differentiation of various tissues, including skeletal muscle and adipocytes (Hansen et al., 2004; Huh et al., 2004). Inactivation of the *Rb* gene in proliferating myoblasts and in differentiated muscle fibers in mice revealed that Rb is required for the initiation of myogenic differentiation but not for the maintenance of the terminal differentiation state (Huh et al., 2004). Also in adipocyte differentiation, Rb facilitates the initiation of differentiation by inducing cell cycle arrest (Fajas et al., 2002a). Thus, Rb seems to be involved in maintaining a balance between proliferation and differentiation in adult stem cells (Classon et al., 2000). Adult stem cells do not proliferate but enter the cell cycle upon specific signals and self propagate to maintain a stable population of stem cells in the tissue. At the same time, they differentiate to regenerate damaged tissue or maintain a basic turnover of differentiated cells (Wagers and Weissman, 2004). Our data suggest that LAP2α–lamin A/C complexes are essential cofactors of Rb in maintaining the balance between proliferation and differentiation in adult stem cells. Similar to Rb, LAP2α's critical role would be in transit amplifier cells rather than in terminally differentiated cells. Consistent with this hypothesis, the highest levels of LAP2α expression during the differentiation of skeletal muscle satellite cells is just before entry into a postmitotic state (Markiewicz et al., 2005). Moreover, by artificially maintaining high levels of LAP2α expression in preadipocytes, early events in differentiation were enhanced but terminal differentiation was inhibited (Fig. 6). Therefore, we propose that the LAP2α–lamin A/C complex is critical for Rb-mediated entry of adult stem cells into a postmitotic state but is not required for terminal differentiation.

During the last 10 yr, geneticists have identified mutations in the human *LMNA* gene that give rise to pathological phenotypes in muscle, adipose, bone, neuronal, and skin tissue or cause premature ageing (Burke and Stewart, 2002; Mounkes and Stewart, 2004). Mice lacking lamin A/C or expressing mutated lamin A/C show similar phenotypes (Sullivan et al., 1999; Mounkes et al., 2003; Arimura et al., 2005). The molecular basis of these diseases is unclear. It may involve structural defects in mutated lamins, leading to nuclear fragility, or deregulated functions of lamins in gene expression (Hutchison and Worman, 2004). As LAP2α is involved in targeting a subfraction of lamin A/C to the nucleoplasm (Dechat et al., 2000, 2004), it may be relevant for the nucleoplasmic functions of lamin A/C. If nucleoplasmic lamins together with LAP2α control Rb in cell cycle progression and differentiation, disease-causing mutations in lamin A/C may cause differentiation defects. In support of this model, the expression of a disease-linked lamin A mutant in C2C12 myoblasts (Favreau et al., 2004) inhibited the in vitro differentiation into myotubes, and the expression of wild-type and mutated lamin A inhibited the differentiation of 3T3 cells into adipocytes (Boguslavsky et al., 2006). We propose that disease-causing mutations in A-type lamins perturb the balance between proliferation and differentiation in adult stem cells, leading to less efficient tissue regeneration. This model would help to explain the late onset of the disease during

childhood or adulthood, an age when tissue regeneration becomes relevant, as well as progressive muscle weakness, which may be caused indirectly by a defect in regeneration rather than by direct degeneration (Gotzmann and Foisner, 2005). Intriguingly, mutations in the gene encoding LAP2α have recently been linked to laminopathy-like cardiomyopathy (Taylor et al., 2005). Disease-causing LAP2α mutants bind less efficiently to lamin A/C, supporting the hypothesis that nucleoplasmic LAP2α–lamin A/C complexes fulfill important functions.

Materials and methods

Cell culture
3T3-F442A and HeLa cells were cultured at 37°C and 8% CO_2 in high glucose DME medium supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.2 mM glutamine (Invitrogen), 0.8 μg/ml biotin, and 0.4 μg/ml pantothenic acid (Sigma-Aldrich) for 3T3-F442A cells. Confluent 3T3-F442A cells were differentiated with 17 nM insulin and 2 nM T3 (Sigma-Aldrich) for 7–10 d. Lipid drops were stained for 1 h with 0.3% Oil Red O (Sigma-Aldrich) after fixation in 3.7% formaldehyde (Merck) in PBS for 2 min. For DNA flow cytometry, 5×10^5 cells in 1 ml PBS were mixed with 4 ml 85% ethanol at −20°C. Cell pellets were briefly treated with 0.05% aqueous pepsin, stained with 2 μg/ml DAPI and 15 μg/ml sulforhodamine (Sigma-Aldrich) in 100 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, and 0.1% Triton X-100, and analyzed with a flow cytometer (PAS II; Partec). For cell proliferation assays, 5×10^4 cells were seeded onto 100-mm dishes (Nunc), and cell numbers were determined within 7 d using a coulter counter Z2 system (Scharfe System). For serum starvation, cells were grown in medium with 0.5% FCS for 5 d.

Transfections and reporter assays
LAP2α-myc HeLa Tet-on cells (Vlcek et al., 1999) were grown in medium with 200 μg/ml hygromycin and induced with 2 μg/ml doxycyclin (Invitrogen). pTD35 encoding LAP2α-GFP was generated by cloning LAP2α cDNA via NheI–XhoI from pTD15 into a modified pEGFP-N3 vector (CLONTECH Laboratories, Inc.) as described previously (Vlcek et al., 2002) and was used for stable transfection of 3T3 cells with LipofectAMINE 2000 (Invitrogen). Stable clones were selected in medium with 700 μg/ml geneticin.

For reporter assays, cells were cotransfected with 2 μg luciferase reporter p3xE2F-Luc (provided by H. Rotheneder, Medical University of Vienna, Vienna, Austria; Krek et al., 1993) and 2 μg β-galactosidase control reporter plasmid pAD-CMV1-βgal (Eger et al., 2000) together with various combinations of the following plasmids (1 μg each): pCI-neo-HA-E2F1 (Rotheneder et al., 1999), pCMV-Rb (provided by M. Busslinger, Research Institute of Molecular Pathology, Vienna, Austria; Hinds et al., 1992), pSV61 (full-length LAP2α in pcDNA3.1), pSV62 (LAP2 common in pcDNA3.1), and pcDNA3.1 (Invitrogen). Luciferase activity was measured with the lumat system (Berthold) and normalized for β-galactosidase activity. Relative luciferase activities were calculated as the light units relative to the reporter plasmid controls. Data were obtained from at least three independent experiments, and mean values and standard errors were calculated.

RNAi
pSHAG-1 vector containing a human U6 promoter fragment (−265 to 1) was derived from pTOPO-ENTR/D (Invitrogen) as described previously (Paddison et al., 2002; http://katahdin.cshl.org:9331/RNAi_web/scripts/main2.pl). Human LAP2α-specific oligonucleotides encoding a shRNA were designed as described (http://katahdin.cshl.org:9331/homepage/portal/scripts/main2.pl?link=protocols&content=protocols.html): 5′-ACTGAT-CAATTCTCTTCTGGAAGCTTGCAGAAGAGAATTGATCAGTCTTTTTT-3′ and 5′-GATCAAAAAAGACTGATCAATTCTCTTCTGCAAGCTTCCAGA-AGAGAATTGATCAGTCG-3′. Annealed oligonucleotides were cloned into pSHAG-1 via BseRI–BamHI. As a control, we used pSHAG-FF1 encoding a shRNA targeting firefly luciferase (gift of Greg Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Paddison et al., 2002). Using the Gateway system (Invitrogen), shRNA expression cassettes in pSHAG-1 were shuttled into pTRACER-EF/Bsd plasmids in which a Gateway-compatible conversion cassette had been introduced in a reverse orientation via EcoRV. The resulting RNAi vectors (pJG118 targeting LAP2α and pJG121 control vector targeting luciferase)

were transfected into HeLa cells, and stable clones were selected with 20 μg/ml blasticidin (Invitrogen).

Chromatin immunoprecipitation and RT-PCR
Chromatin immunoprecipitation was performed as described previously (Hauser et al., 2002) with a few modifications. Cross-linking with formaldehyde was performed for 15 min, and the soluble chromatin fraction was diluted 1:10 in 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, and 0.01% SDS and was precipitated with monoclonal antibody 15 or antiserum 245 to LAP2α or rabbit preimmune serum as a control. Chromatin–antibody complexes were isolated with 30 μl of protein A/G–Sepharose beads and solubilized in 2% SDS, 0.1 M $NaHCO_3$, 10 mM DTT, and 250 mM NaCl overnight at 65°C. Samples were analyzed by immunoblotting, or DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in water. The immunoprecipitated DNA was analyzed for cyclin D1, thymidine kinase, E-cadherin, and histone H4 promoter sequences by PCR using a thermocycler (PTC-200; MJ Research) and PuReTaq Ready-To-Go PCR beads (GE Healthcare). The linear range for each primer pair was determined empirically using different amounts of genomic DNA. PCR products were resolved on 2% agarose gels. Primers used are as follows: 5′-GACCCTGGCCAGGATAAAC-3′ and 5′-AGACGAGCCCTAAGCTCTC-3′ for mouse cyclin D1; 5′-GACC-GACTGGTCAAGGTAGG-3′ and 5′-GTTTCATTCCGGCGCACAGG-3′ for human cyclin D1; 5′-AGACCCCGCACCTGAATCTG-3′ and 5′-TTCACGT-AGCTGAGAGGTGG-3′ for mouse and 5′-CGATCAGCCACGTCCATC-3′ and 5′-CGCCGACCGCTTTAAACC-3′ for human thymidine kinase; 5′-GA-CACCGCATGAAAAGAATAGCTG-3′ and 5′-CTTTCCCAAGGCCTTTACC-ACC 3′ for mouse histone H4; and 5′-AACTCCAGGCTAGAGGGTCA-3′ and 5′-GGGCTGGAGTCTGAACTGA-3′ for human E-cadherin.

For RT-PCR, poly(A)$^+$ mRNA was extracted from cells with an mRNA Isolation Kit and reverse transcribed using the First Strand cDNA Synthesis Kit (both from Roche). Aliquots of the resulting products were used as templates for specific PCR amplifications using PuReTaq Ready-To-Go PCR beads (GE Healthcare). The conditions for PCR reactions were optimized for each primer pair. All cDNAs were normalized according to actin expression levels. Primers used are as follows: 5′-AGTGCGTGCAGAAGGA-GATT-3′ and 5′-CACAACTTCTCGGCAGTCAA-3′ for cyclin D1; 5′-ATGAGCTACATCAATCTGCCC-3′ and 5′-TTCCGATCATGTGTGGAG-AA-3′ for thymidine kinase; 5′-CAAAGCCCAAGCAAAGAAAG-3′ and 5′-CCACTGTCTTTGGAGGCAAT-3′ for cyclin E; and 5′-ATCTGGCACCA-CACCTTCTAC-3′ and 5′-CAGCCAGGTCCAGACGCAGG-3′ for actin. PCR reactions were quantified with QuantiScan software (Biosoft).

Immunofluorescence microscopy
Immunofluorescence microscopy and immunoblotting were performed as described previously (Vlcek et al., 2002; Dechat et al., 2004). For microscopy, samples were mounted in mowiol and analyzed using a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) equipped with a confocal laser-scanning unit (LSM510 META; Carl Zeiss MicroImaging, Inc.) and α–plan-Fluar 100× NA 1.45 oil (a = 0.11 mm) and plan-Apochromat 63× NA 1.40 oil differential contrast MC27 (a = 0.19 mm) objectives (Carl Zeiss MicroImaging, Inc.). Phase-contrast images were taken on a microscope (Axiovert 40C; Carl Zeiss MicroImaging, Inc.) equipped with a digital camera (PowerShot G5; Canon) and plan-Neofluar 10× NA 0.30 (a = 5.6 mm) and Achromat 20× NA 0.4 objectives. Images were prepared with Adobe Photoshop software.

Immunoblotting and blot overlay assays
Total protein lysates of cells for immunoblotting were prepared by dissolving cells of one 10-cm dish in 500 μl of 2× SDS-PAGE sample buffer. For detection of blotted proteins, the Super Signal ECL system (Pierce Chemical Co.) was used. The preparation of recombinant LAP2α and blot overlay assays was described previously (Dechat et al., 2000).

Antibodies
Primary antibodies used were hybridoma supernatants of antibodies to LAP2 and LAP2α (Dechat et al., 1998), rabbit antiserum to LAP2α (Immu-Quest; Vlcek et al., 2002), monoclonal myc 1-9E10.2 antibody (American Type Culture Collection), monoclonal antilamins A/C antibody 1E4 (a gift of F. McKeon, Harvard Medical School, Boston, MA; Loewinger and McKeon, 1988), monoclonal anti-Rb G3-245 (BD Biosciences), monoclonal anti-PPARγ (Santa Cruz Biotechnology, Inc.), polyclonal anti-actin (Sigma-Aldrich), and anti-acetylhistone H4 (Upstate Biotechnology). Secondary antibodies were goat anti–mouse IgG and goat anti–rabbit IgG conjugated to Texas red (Jackson ImmunoResearch Laboratories), goat

anti–rabbit IgG conjugated to AlexaFluor488 (Invitrogen), and goat anti–mouse IgG and goat anti–rabbit IgG conjugated to peroxidase (Jackson ImmunoResearch Laboratories).

References

Ait-Si-Ali, S., V. Guasconi, L. Fritsch, H. Yahi, R. Sekhri, I. Naguibneva, P. Robin, F. Cabon, A. Polesskaya, and A. Harel-Bellan. 2004. A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. *EMBO J.* 23:605–615.

Arimura, T., A. Helbling-Leclerc, C. Massart, S. Varnous, F. Niel, E. Lacene, Y. Fromes, M. Toussaint, A.M. Mura, D.I. Keller, et al. 2005. Mouse model carrying H222P-Lmna mutation develops muscular dystrophy and dilated cardiomyopathy similar to human striated muscle laminopathies. *Hum. Mol. Genet.* 14:155–169.

Berger, R., L. Theodor, J. Shoham, E. Gokkel, F. Brok-Simoni, K.B. Avraham, N.G. Copeland, N.A. Jenkins, G. Rechavi, and A.J. Simon. 1996. The characterization and localization of the mouse thymopoietin/lamina-associated polypeptide 2 gene and its alternatively spliced products. *Genome Res.* 6:361–370.

Boguslavsky, R.L., C.L. Stewart, and H.J. Worman. 2006. Nuclear lamin A inhibits adipocyte differentiation: implications for Dunnigan-type familial partial lipodystrophy. *Hum. Mol. Genet.* 15:653–663.

Brehm, A., E.A. Miska, D.J. McCance, J.L. Reid, A.J. Bannister, and T. Kouzarides. 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*. 391:597–601.

Burke, B., and C.L. Stewart. 2002. Life at the edge: the nuclear envelope and human disease. *Nat. Rev. Mol. Cell Biol.* 3:575–585.

Classon, M., and E. Harlow. 2002. The retinoblastoma tumour suppressor in development and cancer. *Nat. Rev. Cancer*. 2:910–917.

Classon, M., B.K. Kennedy, R. Mulloy, and E. Harlow. 2000. Opposing roles of pRB and p107 in adipocyte differentiation. *Proc. Natl. Acad. Sci. USA*. 97:10826–10831.

Dechat, T., J. Gotzmann, A. Stockinger, C.A. Harris, M.A. Talle, J.J. Siekierka, and R. Foisner. 1998. Detergent-salt resistance of LAP2alpha in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics. *EMBO J.* 17:4887–4902.

Dechat, T., B. Korbei, O.A. Vaughan, S. Vlcek, C.J. Hutchison, and R. Foisner. 2000. Lamina-associated polypeptide 2alpha binds intranuclear A-type lamins. *J. Cell Sci.* 113:3473–3484.

Dechat, T., A. Gajewski, B. Korbei, D. Gerlich, N. Daigle, T. Haraguchi, K. Furukawa, J. Ellenberg, and R. Foisner. 2004. LAP2alpha and BAF transiently localize to telomeres and specific regions on chromatin during nuclear assembly. *J. Cell Sci.* 117:6117–6128.

Eger, A., A. Stockinger, B. Schaffhauser, H. Beug, and R. Foisner. 2000. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of β-catenin and upregulation of β-catenin/lymphoid enhancer binding factor-1 transcriptional activity. *J. Cell Biol.* 148:173–188.

Fajas, L., V. Egler, R. Reiter, J. Hansen, K. Kristiansen, M.B. Debril, S. Miard, and J. Auwerx. 2002a. The retinoblastoma-histone deacetylase 3 complex inhibits PPARgamma and adipocyte differentiation. *Dev. Cell*. 3:903–910.

Fajas, L., R.L. Landsberg, Y. Huss-Garcia, C. Sardet, J.A. Lees, and J. Auwerx. 2002b. E2Fs regulate adipocyte differentiation. *Dev. Cell*. 3:39–49.

Favreau, C., D. Higuet, J.C. Courvalin, and B. Buendia. 2004. Expression of a mutant lamin A that causes Emery-Dreifuss muscular dystrophy inhibits in vitro differentiation of C2C12 myoblasts. *Mol. Cell. Biol.* 24:1481–1492.

Foisner, R. 2001. Inner nuclear membrane proteins and the nuclear lamina. *J. Cell Sci.* 114:3791–3792.

Foisner, R., and L. Gerace. 1993. Integral membrane proteins of the nuclear envelope interact with lamins and chromosomes, and binding is modulated by mitotic phosphorylation. *Cell*. 73:1267–1279.

Frolov, M.V., and N.J. Dyson. 2004. Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. *J. Cell Sci.* 117:2173–2181.

Furukawa, K., C.E. Fritze, and L. Gerace. 1998. The major nuclear envelope targeting domain of LAP2 coincides with its lamin binding region but is distinct from its chromatin interaction domain. *J. Biol. Chem.* 273:4213–4219.

Gotzmann, J., and R. Foisner. 2005. A-type lamin complexes and regenerative potential: a step towards understanding laminopathic diseases? *Histochem. Cell Biol.* 125:33–41.

Gruenbaum, Y., A. Margalit, R.D. Goldman, D.K. Shumaker, and K.L. Wilson. 2005. The nuclear lamina comes of age. *Nat. Rev. Mol. Cell Biol.* 6:21–31.

Hansen, J.B., C. Jorgensen, R.K. Petersen, P. Hallenborg, R. De Matteis, H.A. Boye, N. Petrovic, S. Enerback, J. Nedergaard, S. Cinti, et al. 2004. Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. *Proc. Natl. Acad. Sci. USA*. 101:4112–4117.

Harris, C.A., P.J. Andryuk, S. Cline, H.K. Chan, A. Natarajan, J.J. Siekierka, and G. Goldstein. 1994. Three distinct human thymopoietins are derived from alternatively spliced mRNAs. *Proc. Natl. Acad. Sci. USA*. 91:6283–6287.

Hauser, C., B. Schuettengruber, S. Bartl, G. Lagger, and C. Seiser. 2002. Activation of the mouse histone deacetylase 1 gene by cooperative histone phosphorylation and acetylation. *Mol. Cell. Biol.* 22:7820–7830.

Helt, A.M., and D.A. Galloway. 2003. Mechanisms by which DNA tumor virus oncoproteins target the Rb family of pocket proteins. *Carcinogenesis*. 24:159–169.

Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell*. 70:993–1006.

Huh, M.S., M.H. Parker, A. Scime, R. Parks, and M.A. Rudnicki. 2004. Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. *J. Cell Biol.* 166:865–876.

Hutchison, C.J. 2002. Lamins: building blocks or regulators of gene expression? *Nat. Rev. Mol. Cell Biol.* 3:848–858.

Hutchison, C.J., and H.J. Worman. 2004. A-type lamins: guardians of the soma? *Nat. Cell Biol.* 6:1062–1067.

Johnson, B.R., R.T. Nitta, R.L. Frock, L. Mounkes, D.A. Barbie, C.L. Stewart, E. Harlow, and B.K. Kennedy. 2004. A-type lamins regulate retinoblastoma protein function by promoting subnuclear localization and preventing proteasomal degradation. *Proc. Natl. Acad. Sci. USA*. 101:9677–9682.

Krek, W., D.M. Livingston, and S. Shirodkar. 1993. Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. *Science*. 262:1557–1560.

Lin, F., D.L. Blake, I. Callebaut, I.S. Skerjanc, L. Holmer, M.W. McBurney, M. Paulin-Levasseur, and H.J. Worman. 2000. MAN1, an inner nuclear membrane protein that shares the LEM domain with lamina-associated polypeptide 2 and emerin. *J. Biol. Chem.* 275:4840–4847.

Loewinger, L., and F. McKeon. 1988. Mutations in the nuclear lamin proteins resulting in their aberrant assembly in the cytoplasm. *EMBO J.* 7:2301–2309.

Lundberg, A.S., and R.A. Weinberg. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol. Cell. Biol.* 18:753–761.

Markiewicz, E., T. Dechat, R. Foisner, R.A. Quinlan, and C.J. Hutchison. 2002. Lamin A/C binding protein LAP2alpha is required for nuclear anchorage of retinoblastoma protein. *Mol. Biol. Cell*. 13:4401–4413.

Markiewicz, E., M. Ledran, and C.J. Hutchison. 2005. Remodelling of the nuclear lamina and nucleoskeleton is required for skeletal muscle differentiation in vitro. *J. Cell Sci.* 118:409–420.

Mittnacht, S. 1998. Control of pRB phosphorylation. *Curr. Opin. Genet. Dev.* 8:21–27.

Moir, R.D., T.P. Spann, H. Herrmann, and R.D. Goldman. 2000. Disruption of nuclear lamin organization blocks the elongation phase of DNA replication. *J. Cell Biol.* 149:1179–1192.

Mounkes, L.C., and C.L. Stewart. 2004. Aging and nuclear organization: lamins and progeria. *Curr. Opin. Cell Biol.* 16:322–327.

Mounkes, L.C., S. Kozlov, L. Hernandez, T. Sullivan, and C.L. Stewart. 2003. A progeroid syndrome in mice is caused by defects in A-type lamins. *Nature*. 423:298–301.

Nielsen, S.J., R. Schneider, U.M. Bauer, A.J. Bannister, A. Morrison, D. O'Carroll, R. Firestein, M. Cleary, T. Jenuwein, R.E. Herrera, and T. Kouzarides. 2001. Rb targets histone H3 methylation and HP1 to promoters. *Nature*. 412:561–565.

Nili, E., G.S. Cojocaru, Y. Kalma, D. Ginsberg, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, R. Berger, S. Shaklai, N. Amariglio, et al. 2001. Nuclear membrane protein LAP2beta mediates transcriptional repression alone

and together with its binding partner GCL (germ-cell-less). *J. Cell Sci.* 114:3297–3307.

Ozaki, T., M. Saijo, K. Murakami, H. Enomoto, Y. Taya, and S. Sakiyama. 1994. Complex formation between lamin A and the retinoblastoma gene product: identification of the domain on lamin A required for its interaction. *Oncogene.* 9:2649–2653.

Paddison, P.J., A.A. Caudy, E. Bernstein, G.J. Hannon, and D.S. Conklin. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16:948–958.

Rotheneder, H., S. Geymayer, and E. Haidweger. 1999. Transcription factors of the Sp1 family: interaction with E2F and regulation of the murine thymidine kinase promoter. *J. Mol. Biol.* 293:1005–1015.

Sage, J., G.J. Mulligan, L.D. Attardi, A. Miller, S. Chen, B. Williams, E. Theodorou, and T. Jacks. 2000. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev.* 14:3037–3050.

Segura-Totten, M., and K.L. Wilson. 2004. BAF: roles in chromatin, nuclear structure and retrovirus integration. *Trends Cell Biol.* 14:261–266.

Shumaker, D.K., E.R. Kuczmarski, and R.D. Goldman. 2003. The nucleoskeleton: lamins and actin are major players in essential nuclear functions. *Curr. Opin. Cell Biol.* 15:358–366.

Somech, R., S. Shaklai, O. Geller, N. Amariglio, A.J. Simon, G. Rechavi, and E.N. Gal-Yam. 2005. The nuclear-envelope protein and transcriptional repressor LAP2beta interacts with HDAC3 at the nuclear periphery, and induces histone H4 deacetylation. *J. Cell Sci.* 118:4017–4025.

Spann, T.P., A.E. Goldman, C. Wang, S. Huang, and R.D. Goldman. 2002. Alteration of nuclear lamin organization inhibits RNA polymerase II-dependent transcription. *J. Cell Biol.* 156:603–608.

Sullivan, T., D. Escalante-Alcalde, H. Bhatt, M. Anver, N. Bhat, K. Nagashima, C.L. Stewart, and B. Burke. 1999. Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *J. Cell Biol.* 147:913–920.

Taylor, M.R., D. Slavov, A. Gajewski, S. Vlcek, L. Ku, P.R. Fain, E. Carniel, A. Di Lenarda, G. Sinagra, M.M. Boucek, et al. 2005. Thymopoietin (lamina-associated polypeptide 2) gene mutation associated with dilated cardiomyopathy. *Hum. Mutat.* 26:566–574.

Van Berlo, J.H., J.W. Voncken, N. Kubben, J.L. Broers, R. Duisters, R.E. van Leeuwen, H.J. Crijns, F.C. Ramaekers, C.J. Hutchison, and Y.M. Pinto. 2005. A-type lamins are essential for TGF-beta1 induced PP2A to dephosphorylate transcription factors. *Hum. Mol. Genet.* 14:2839–2849.

Vlcek, S., H. Just, T. Dechat, and R. Foisner. 1999. Functional diversity of LAP2alpha and LAP2beta in postmitotic chromosome association is caused by an alpha-specific nuclear targeting domain. *EMBO J.* 18:6370–6384.

Vlcek, S., B. Korbei, and R. Foisner. 2002. Distinct functions of the unique C-terminus of LAP2alpha in cell proliferation and nuclear assembly. *J. Biol. Chem.* 277:18898–18907.

Wagers, A.J., and I.L. Weissman. 2004. Plasticity of adult stem cells. *Cell.* 116:639–648.