ERK1/2 MAP kinases promote cell cycle entry by rapid, kinase-independent disruption of retinoblastoma–lamin A complexes

Javier Rodríguez,[1] Fernando Calvo,[1] José M. González,[2] Berta Casar,[1] Vicente Andrés,[2] and Piero Crespo[1]

[1]Instituto de Biomedicina y Biotecnología de Cantabria, Consejo Superior de Investigaciones Científicas, Investigación Desarrollo e Innovación Cantabria, Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria, 39011 Santander, Spain
[2]Laboratoria de Patofisiología Cardiovascular Molecular y Genética, Centro Nacional de Investigaciones Cardiovasculares, 28029 Madrid, Spain

As orchestrators of essential cellular processes like proliferation, ERK1/2 mitogen-activated protein kinase signals impact on cell cycle regulation. A-type lamins are major constituents of the nuclear matrix that also control the cell cycle machinery by largely unknown mechanisms. In this paper, we disclose a functional liaison between ERK1/2 and lamin A whereby cell cycle progression is regulated. We demonstrate that lamin A serves as a mutually exclusive dock for ERK1/2 and the retinoblastoma (Rb) protein. Our results reveal that, immediately after their postactivation entrance in the nucleus, ERK1/2 dislodge Rb from its interaction with lamin A, thereby facilitating its rapid phosphorylation and consequently promoting E2F activation and cell cycle entry. Interestingly, these effects are independent of ERK1/2 kinase activity. We also show that cellular transformation and tumor cell proliferation are dependent on the balance between lamin A and nuclear ERK1/2 levels, which determines Rb accessibility for phosphorylation/inactivation.

Introduction

ERK1/2 MAPKs (ERKs) are cytoplasmic serine/threonine kinases that participate in the transduction of signals from the surface to the interior of the cell. ERKs play an essential role in the conveyance of extracellular stimuli that orchestrate cellular proliferation, differentiation, and survival (Raman et al., 2007). A major event in these processes is the regulation of cell cycle entry and progression. ERKs serve in such a task by different mechanisms (for review see Chambard et al., 2007). For example, ERKs play a fundamental role in the transit from G_0/G_1 to S phase, being required for the transcriptional induction of D-type cyclins (Lavoie et al., 1996). Active ERKs translocate to the nucleus, where they phosphorylate preexisting transcription factors, such as Elk-1, that in turn induce the transcription of immediate early genes like *Fos* (Gille et al., 1995). Prolonged ERK activity results in c-Fos phosphorylation, which stabilizes this transcription factor, thereby enabling the induction of cyclin D expression several hours after stimulation (Weber et al., 1997; Balmanno and Cook, 1999). ERKs also phosphorylate and stabilize c-Myc, which directly participates in the transcriptional induction of D-type cyclins (Seth et al., 1991; Daksis et al., 1994).

Newly synthesized cyclin D forms active complexes with the existing Cdks CDK4/6, which undertake the phosphorylation of the retinoblastoma (Rb) pocket protein, a key step for G_1/S transition. In its hypophosphorylated state, Rb is bound to E2F family transcription factors. Sequential phosphorylation of Rb by cyclin D–CDK4/6 and cyclin E-A–CDK2 complexes triggers its inactivation and the release of E2F, ensuring cell cycle progression and, ultimately, DNA replication (Weinberg, 1995). Activated ERKs also contribute to Rb phosphorylation by regulating CDK2 nuclear translocation by yet unknown mechanisms (Keenan et al., 2001). In addition, during mid-G_1, ERKs promote Rb phosphorylation by cyclin E–CDK2 by down-regulating the levels of its inhibitor $p27^{Kip1}$ (Kerkhoff and Rapp, 1997) and titrating it away by promoting the formation

Correspondence to Piero Crespo: crespop@unican.es

Abbreviations used in this paper: DK, kinase-deficient mutant; DMEM, Dulbecco's minimum essential medium; ERK, ERK1/2 MAPK; ERNF, extraction-resistant nuclear fraction; FRET, fluorescence resonance energy transfer; GDI, guanine dissociation inhibitor; MEF, mouse embryo fibroblast; Rb, retinoblastoma; SNF, soluble nuclear fraction; wt, wild type.

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of cyclin D1–CDK4 complexes (Cheng et al., 1998). ERKs also regulate the levels of the inhibitor p21 by diverse mechanisms (for review see Chambard et al., 2007). Interestingly, the necessity for ERKs during G_0 exit and G_1/S transition is abolished in the absence of Rb: Rb-null mouse embryo fibroblasts (MEFs) can leave quiescence, progress through the cell cycle, and proliferate even when ERK signaling is inhibited (D'Abaco et al., 2002).

In addition to the classical control of Rb through phosphorylation, some nuclear structural proteins have been shown to impact Rb functions in diverse ways. A-type lamins are major constituents of the mammalian nuclear lamina, nucleoskeleton, and nucleoplasm. They have been shown to regulate key events in health and disease through interplay with signaling molecules, transcription factors, and chromatin-associated proteins. Interest in A-type lamins has progressively increased since the discovery that *LMNA* mutations cause severe human diseases, which are termed laminopathies (for reviews see Vlcek and Foisner, 2007; Andrés and González, 2009). Lamin A–deficient cells show an aberrant cell cycle (Boban et al., 2010), but the underlying causes are not fully understood. Lamin A binds to Rb (Mancini et al., 1994; Ozaki et al., 1994), an association that orchestrates Rb subnuclear organization, prevents its proteasomal degradation (Johnson et al., 2004), and facilitates cell cycle arrest by the inhibitor INK4A (Nitta et al., 2006). Rb also complexes with the lamin A/C–binding protein LAP2α (Markiewicz et al., 2002). Rb/E2F-dependent gene expression and cell cycle progression are regulated by LAP2α expression levels (Dorner et al., 2006; Naetar et al., 2008), which may be related to the role of LAP2α in maintaining Rb in an adequate subnuclear compartment (Pekovic et al., 2007), apparently an important requisite for Rb functionality.

Upon stimulation, ERKs translocate to the nucleus within minutes (Lenormand et al., 1993). Intriguingly, according to the data currently available, it takes hours before their effects on cell cycle become evident through transcriptional events such as the induction of c-Fos and cyclin D expression. The question remains whether ERKs can also regulate cell cycle entry by other, yet undisclosed, mechanisms occurring immediately after their entrance in the nucleus. In line with this notion, we have previously demonstrated that A-type lamins regulate AP-1 transcriptional activity by sequestering preexisting c-Fos at the nuclear envelope (Ivorra et al., 2006) in a reversible manner modulated by ERKs (González et al., 2008). We found that ERKs also interact with A-type lamins and colocalize with lamin A and c-Fos at the nuclear envelope. Serum stimulation brings about the rapid release of c-Fos from its complex with lamin A in response to ERK-dependent phosphorylation, thereby leading to fast c-Fos activation in advance of de novo c-Fos synthesis (González et al., 2008). These findings demonstrate that processes relevant for cell cycle progression can be triggered soon after ERK nuclear entry before any transcriptional event.

Herein, we describe a novel mechanism whereby ERKs directly stimulate cell cycle entry immediately after they penetrate in the nucleus. We present data showing that, in a fashion independent of their kinase activity, ERKs displace Rb from its association to lamin A, thereby promoting its release to the soluble nucleoplasm, where it is rapidly phosphorylated, leading to E2F-dependent transcription and cell cycle entry. We also demonstrate that the proliferation of tumor cell lines harboring high levels of nuclear ERKs and, subsequently, high levels of phosphorylated Rb can be attenuated by the overexpression of lamin A, which reduces the levels of phosphorylated Rb. Our results disclose a critical role for lamin A in cell cycle control through its interaction with signaling intermediaries, such as ERKs, and cell cycle regulators, such as Rb.

Results

ERK1/2 disrupt Rb binding to lamin A

In a former study, we described that, upon entry in the nucleus after activation, a significant amount of ERKs associated to the nuclear envelope by directly binding to lamin A. We also identified the region within lamin A responsible for binding to ERKs, which corresponded to a segment spanning amino acids 247–355 (González et al., 2008). Most interestingly, the same fragment had been previously characterized as the region through which lamin A bound to the pocked protein Rb (Ozaki et al., 1994). Thus, the possibility existed that ERKs and Rb competed for association with lamin A. To test this hypothesis, we initially looked at how mitogenic stimulation affected endogenous ERKs and Rb segregation between the extraction-resistant nuclear fraction (ERNF), containing insoluble lamin A and largely associated to the nucleoskeleton and the nuclear envelope, and the soluble nuclear fraction (SNF), mainly representing nucleoplasmic lamin A. In NIH3T3 cells, almost all Rb was found associated to the ERNF under quiescence, but it underwent an abrupt redistribution after PDGF stimulation, relocalizing to the SNF concomitantly with the incorporation of ERK2 to the ERNF. Noticeably, Rb displacement in response to PDGF was fully dependent on ERK activation because pretreatment with the MAPK ERK kinase (MEK) inhibitor UO126 completely abolished its reallocation (Fig. 1 A). The proportion of Rb that shuttled between the ERNFs and SNFs was approximately a third of the total cellular levels (Fig. S1, A and B).

Next, we analyzed whether the fluctuations of Rb and ERK2 at the ERNF corresponded with their association to lamin A. Significant amounts of Rb coimmunoprecipitated with lamin A in quiescent NIH3T3, but its levels were progressively reduced after PDGF stimulation, in parallel to the formation of ERK2–lamin A complexes. The release from lamin A was specific for Rb because the levels of LAP2α bound to lamin A (Markiewicz et al., 2002) were unchanged in response to PDGF (Fig. 1 B and Fig. S1, C–F). Strikingly, concomitant with Rb release from its interaction with lamin A, phosphorylated Rb appeared in the total lysates at stages in which neither D-, E-, nor A-type cyclins were detectable (Fig. 1 C). These results demonstrated that mitogenic stimulation causes ERK-dependent removal of Rb from its complex with lamin A at the ERNF.

To study in further depth the mechanism whereby ERKs disrupted Rb–lamin A interaction, we generated constructs aimed at bolstering ERK's constitutive accumulation in the nucleus. To this end, we added an SV40 NLS to the C terminus of wild-type (wt) ERK2 and several of its mutant forms, which enabled

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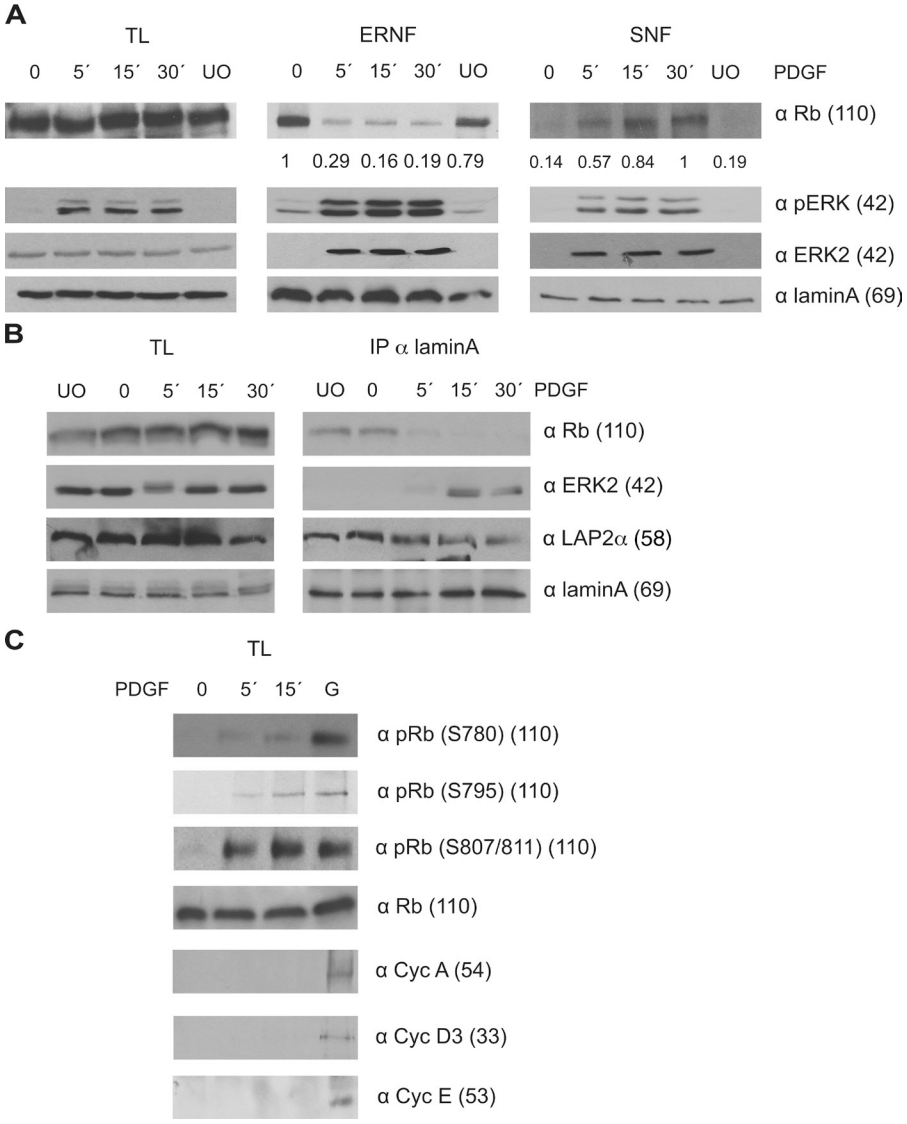

Figure 1. **Mitogenic stimulation releases Rb from lamin A complexes in an ERK-dependent fashion.** (A) Agonist stimulation provokes Rb release from the nuclear envelope. NIH3T3 cells were serum starved for 18 h and subsequently stimulated with 20 ng/ml PDGF for the indicated periods. Where shown (UO), cells were pretreated for 30 min with 10 μM UO126 before 30-min stimulation with PDGF. ERNFs and SNFs were obtained, and equal amounts of fractions were fractionated by SDS-PAGE. Total lysates (TL) from parallel plates are also shown. The indicated proteins were identified by immunoblotting (α protein of interest). Numbers show Rb levels quantified relative to the highest signal. (B) Mitogenic stimulation breaks lamin A–Rb interaction. Cells were stimulated as in A. At the shown time points, cellular lysates were immunoprecipitated for lamin A. Immunoprecipitates (IP) and the corresponding total lysates were probed for the indicated proteins by immunoblotting. Immunoblots are representative of three independent experiments. (C) Mitogenic stimulation induces rapid Rb phosphorylation. Total lysates from cells treated as in A and B were probed for the indicated proteins by immunoblotting. A total lysate from exponentially growing cells (G) was used as a control. Molecular masses (given in kilodaltons) are shown in parentheses after the protein name.

a strict nuclear localization for all of these recombinant proteins (Fig. 2 A). We then examined the capability of ERK2 to break lamin A–Rb complexes in quiescent NIH3T3 cells. Interestingly, Rb was displaced from its association to lamin A by ERK2-NLS wt just as efficiently as by a kinase-deficient mutant (DK) K52R (Ajenjo et al., 2000) and by an unphosphorylatable form (AEF; Wolf et al., 2001). Contrarily, a deletion mutant lacking the insert region (ΔINS), which we had previously identified as responsible for ERK2 binding to lamin A (González et al., 2008), was impaired for dislodging Rb from lamin A. These results were ascertained further by fluorescence resonance energy transfer (FRET) experiments, which allow the measurement of protein–protein interactions at a molecular resolution of 1–10 nm (Kenworthy, 2001). U2OS cells were transfected with CFP–lamin A and either YFP-Rb or YFP as a negative control. FRET efficiency was prominent in cells expressing YFP-Rb, but it was markedly reduced in cells cotransfected with ERK2-NLS wt or DK, though not in those cotransfected with the ΔINS mutant (Fig. 2 C), in full consonance with the results obtained by biochemical means. We wanted to know whether ERK1 was also able to disrupt Rb–lamin A interaction. To this end, we generated an ERK1-NLS construct, which was capable of displacing Rb from its complex with lamin A as effectively as ERK2 (Fig. 2 D).

It was important to find out whether the rupture of Rb–lamin A interaction occurred as a consequence of direct competition between Rb and ERKs for binding to a common site. For this purpose, we took an in vitro approach: GST–lamin 247–355, the fragment previously determined to bind both Rb and ERK2 (Ozaki et al., 1994; González et al., 2008), was loaded with Rb and incubated with purified, bacterially produced ERK2. It was found that increasing concentrations of ERK2 gradually displaced Rb from its complex with lamin A (Fig. 2 E). These results demonstrated that Rb and ERKs bound to lamin A in a mutually exclusive fashion and that ERKs can directly displace Rb from lamin A.

It has been demonstrated that Rb also associates with LAP2α, a protein that binds to nucleoplasmic lamin A (Markiewicz et al., 2002). Thus, it was of interest to find whether ERKs were also capable of interfering with Rb binding to LAP2α. As expected, endogenous Rb and LAP2α coimmunoprecipitated in NIH3T3

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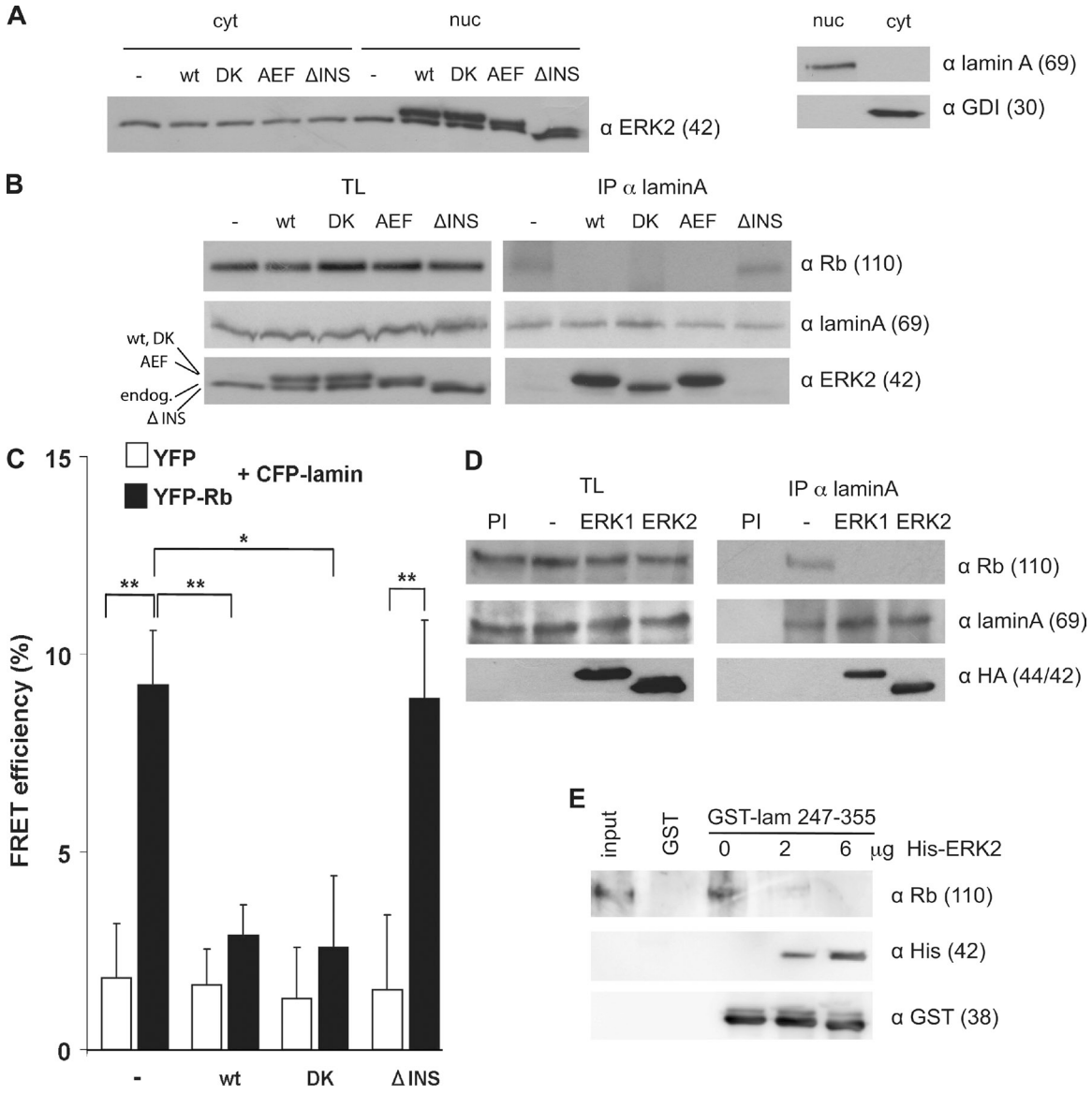

Figure 2. **Nuclear ERKs dislodge Rb from lamin A complexes.** (A, left) Nuclear localization of the different HA-tagged ERK2-NLS forms as determined in nuclear (nuc) and cytoplasmic (cyt) fractions of 293T cells transfected with 1 μg of the indicated constructs: control cells transfected with vector DNA (−), ERK2 wt (wt), ERK2 DK (DK), ERK2 unphosphorylatable mutant (AEF), and ERK2 insert region deletion mutant (ΔINS). (right) The purity of the fractions was ascertained using lamin A and Rho GDI as nuclear and cytoplasmic markers, respectively. (B) Nuclear ERK2 disrupts lamin A–Rb interaction. NIH3T3 cells were transfected with 1 μg each of the indicated ERK2-NLS constructs, grown until confluence, and kept in 0.5% CS for 18 h. Cellular lysates were immunoprecipitated for lamin A, and immunoprecipitates (IP) and the corresponding total lysates (TL) were probed by immunoblotting for the indicated proteins (α protein of interest). (C) Effects of nuclear ERK2 on lamin A–Rb interaction analyzed by FRET in U2OS cells using the acceptor-photobleaching method. Cells were transiently cotransfected with 5 μg ECFP–lamin A plus 5 μg each of the different plasmids as indicated. Data show quantification of protein–protein interactions calculated as the percentage of CFP fluorescence increments after YFP photobleaching in 30–50 cells. *, $P < 0.05$ and **, $P < 0.01$. Results show means ± SEM. (D) ERK1 disrupts lamin A–Rb interaction as effectively as ERK2. NIH3T3 cells were transfected with 1 μg each of HA-tagged ERK1-NLS and ERK2-NLS and processed as in B. Cellular lysates were immunoprecipitated with an antibody against lamin A or with preimmune serum (PI). Immunoprecipitates and the corresponding total lysates were probed by immunoblotting for the indicated proteins. (E) ERK2 displaces Rb from lamin A in vitro. GST and GST–lamin 247–355 were loaded with YFP-Rb from transfected U2OS cell extracts, incubated with the indicated amounts of purified His-tagged ERK2 for 1 h, and tested for their interaction by Western blotting. Molecular masses (given in kilodaltons) are shown in parentheses after the protein name.

cells, but the expression of ERK2-NLS, wt, and DK did not affect such interaction whatsoever (Fig. 3 A). Moreover, neither of these ERK2 forms could be detected in anti-LAP2α immunoprecipitates, indicating that ERKs do not bind to LAP2α. By the same approach, we also investigated whether ERKs could impact the interaction between lamin A and LAP2α. Our results showed that the amount of LAP2α coimmunoprecipitating with lamin A was unchanged in the presence of the nuclear ERK2 forms (Fig. 3 B). Overall, these results demonstrated that ERKs can directly displace Rb from its interaction with the fraction of lamin A present in the ERNF without affecting Rb or lamin A binding to other proteins such as LAP2α. Importantly, they also disclose that ERK phosphorylation and kinase activity are not required for such a purpose.

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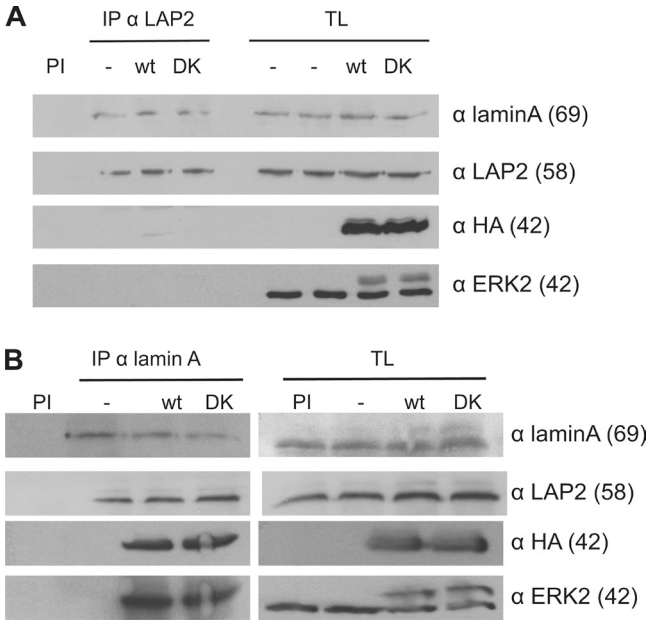

Figure 3. **ERK2 does not affect other lamin A and Rb interactions.** (A) ERK2 does not affect LAP2α–Rb interaction. NIH3T3 cells were transfected with 1 μg each of vector (−), HA-ERK2-NLS-wt (wt), and HA-ERK2-DK (DK), grown until confluence, and kept in 0.5% CS for 18 h. Cellular lysates were immunoprecipitated with an antibody against LAP2α or with preimmune serum (PI), and immunoprecipitates (IP) and total lysates (TL) were probed for the indicated proteins (α protein of interest). (B) ERK2 does not affect LAP2α–lamin A interaction. The experiment was performed as in A, but cellular lysates were immunoprecipitated for lamin A. Molecular masses (given in kilodaltons) are shown in parentheses after the protein name.

Rb release from lamin A complexes promotes its inactivation

It was essential to understand the functional consequences of the disruption of Rb–lamin A interaction in response to the presence of ERKs in the nucleus. A hallmark of Rb function is its inactivation by phosphorylation leading to the release of E2F transcription factors (Weinberg, 1995). Thus, we performed immunoblotting using antibodies specific for phosphorylated Rb to analyze its activation status in lysates from quiescent NIH3T3 cells transfected with the different ERK2-NLS constructs. We found that the levels of phosphorylated Rb were markedly incremented in cells expressing such constructs, with the exception of those harboring ΔINS, at a time in which the expression of A, D, and E cyclins was undetected (Fig. 4 A). We sought to determine whether the rise in Rb phosphorylation was related to its release from the nuclear lamina. As such, we isolated ERNFs and SNFs and found that phosphorylated Rb was found exclusively in the SNF from cells transfected with ERK2-NLS wt, AEF, and DK, in which Rb had been displaced from the ERNF. Noticeably, Rb phosphorylation was most prominent at residues S807 and S811. Contrarily, in cells expressing ΔINS, in which Rb remained at the ERNF, phosphorylated Rb was undetectable in the soluble fraction. In all instances, the levels of phosphorylated Rb in the ERNF were minimal (Fig. 4 B).

In light of these results, we tested whether ERK-induced release of Rb from insoluble lamin A and its subsequent phosphorylation also resulted in an increment on E2F transcriptional activity. To this end, we cotransfected wt MEFs and counterparts deficient for Rb (Zalvide and DeCaprio, 1995) with an E2F-responsive luciferase reporter plus the ERK2-NLS constructs previously mentioned. With the exception of the ΔINS mutant, all the ERK2-NLS forms induced a potent E2F-dependent transcriptional activity in wt MEFs (Fig. 4 C). Contrarily, in Rb-null MEFs that, expectedly, displayed much higher basal E2F activity, E2F was insensitive to the overexpression of the nuclear ERK constructs. These results demonstrate that E2F response to nuclear ERKs is completely dependent on Rb inactivation and that the sole presence of ERKs at the nucleus, even when deprived of kinase activity, is sufficient to induce Rb inactivation by phosphorylation and E2F transcriptional activity.

Nuclear ERKs induce cell cycle entry in an Rb- and lamin A–dependent fashion

Having determined that the presence of ERKs at the nucleus was sufficient to induce a fast disruption of lamin A–Rb complexes, Rb phosphorylation, and E2F-mediated transcription, we proceeded to examine whether these events were accompanied by an augmented cell cycle entry. As such, we monitored cell cycle progression in MEFs transiently transfected with ERK1-NLS and the different ERK2-NLS forms, which were synchronized by an overnight aphidicolin block. Remarkably, even immediately after the release from the aphidicolin block, the cells expressing ERK1 and ERK2, wt, DK, and AEF exhibited an enhanced cell cycle entrance, with ∼15% of the cells in G2/S phases versus ∼6% detected in control cells (Fig. 5 A, top) and in cells expressing the ΔINS mutant (not depicted). Such an effect was exacerbated after 16 h in culture with 0.5% serum, a stimulus insufficient to induce cell cycle progression in control cells but enough to drive into G2/S phases ∼30% of the cells transfected with either wt, DK, or AEF ERK2. Similar results were obtained in NIH3T3 cells (Fig. S2). In agreement, the proliferation rate of NIH3T3 cells stably expressing wt ERK2-NLS was significantly enhanced in comparison to parental cells (Fig. 5 B). Importantly, cell cycle progression induced by ERKs was dependent on lamin A because its overexpression was sufficient to suppress the growth advantage conferred by ectopic nuclear ERK2 (Fig. 5 A, top and bottom). Furthermore, nuclear ERK's capacity to promote cell cycle transit was dependent on Rb, as it did not significantly alter the percentage of cycling Rb-null MEFs, both immediately after the release from the aphidicolin block and 16 h after stimulation. Moreover, the check posed by lamin A overexpression on nuclear ERK2-induced cell cycle entry in normal MEFs was completely lost in Rb-null MEFs (Fig. 5 A), indicating that the limiting effect exerted by lamin A on cell cycle progression was mediated by its interplay with Rb. Accordingly, the cell cycle profile in *LMNA*-null MEFs (Sullivan et al., 1999) was indistinguishable between control cells and those transfected with the ERK2-NLS constructs, further endorsing the notion that the ability of nuclear ERKs to promote advancement through the cell cycle is regulated by lamin A.

Next, we examined whether nuclear ERKs, capable of inducing Rb phosphorylation and cell cycle entry under our experimental conditions, were affecting other cell cycle regulators previously described to be under the influence of their signals at later stages of

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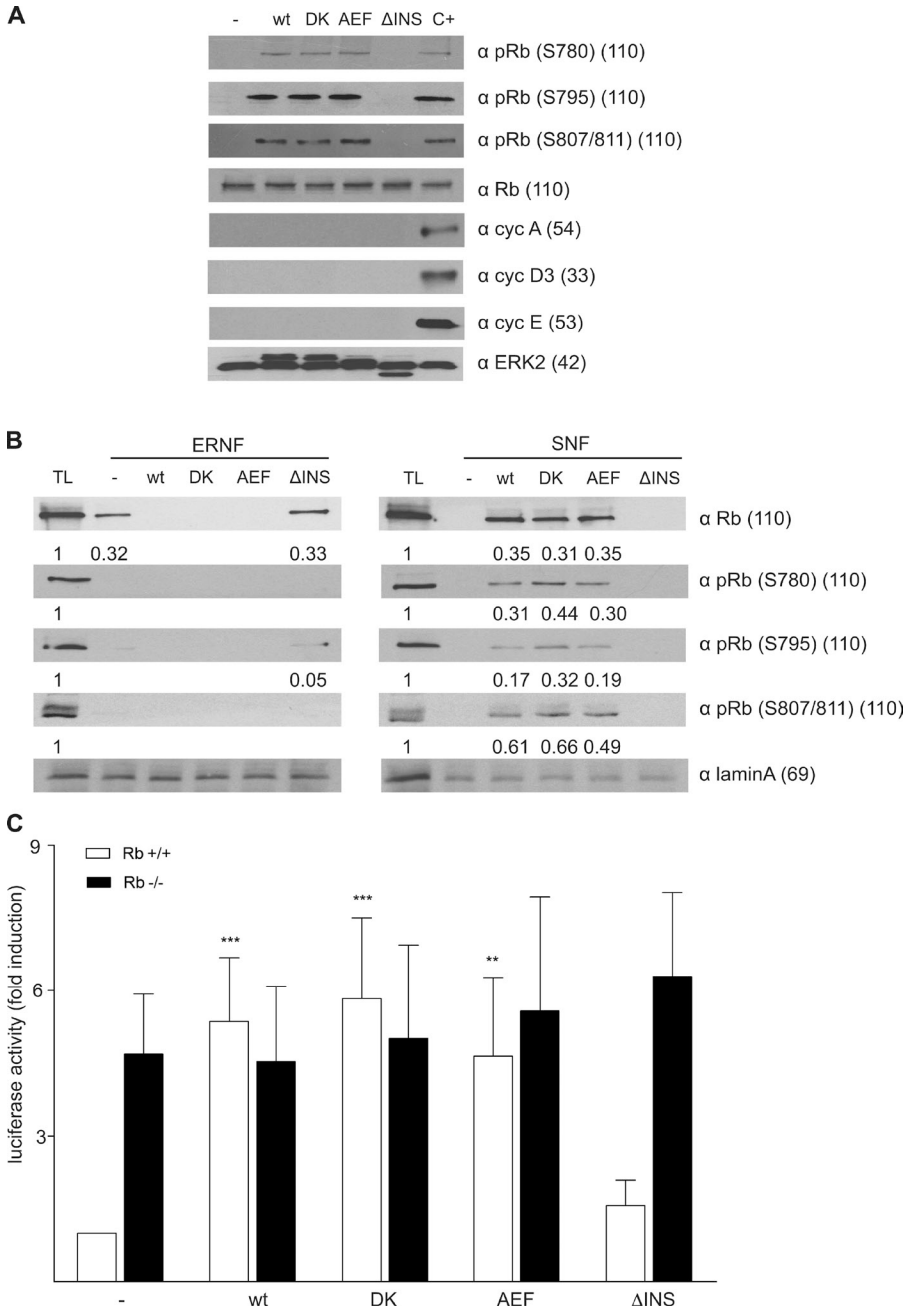

Figure 4. **ERK-induced Rb release from lamin A complexes facilitates Rb phosphorylation.** (A) Nuclear ERK2 promotes Rb phosphorylation. NIH3T3 cells were transfected with 1 µg each of vector (−) or the indicated ERK2-NLS constructs, grown until confluence, and kept in 0.5% CS for 18 h. The indicated proteins (α protein of interest) were identified in total lysates by immunoblotting. (B) Phosphorylated Rb appears at the SNF in response to nuclear ERKs. Cells were treated as in A. ERNFs and SNFs were separated, and the indicated proteins were identified by immunoblotting. Numbers show Rb and phosphorylated Rb levels quantified relative to the levels detected in a total cell extract (TL) run alongside. (C) Nuclear ERK2 promotes E2F transactivation. E2F transactivation was examined in wt and Rb-null MEFs transfected with 1 µg 3x-wt-E2F-luc plus 1 µg of the indicated ERK2-NLS constructs. Results show means ± SEM of three independent experiments relative to vector-transfected cells (−). **, P < 0.005 and ***, P < 0.001 relative to levels in control cells. Molecular masses (given in kilodaltons) are shown in parentheses after the protein name.

the cell cycle (for review see Chambard et al., 2007). It was found that the levels of cyclin D3 and of the inhibitors p21 and p27 were unchanged by the expression of ERK2-NLS DK both in wt and in *LMNA*$^{-/-}$ MEFs (Fig. 5 C). Conversely, in wt MEFs, phosphorylated Rb was markedly higher in those expressing ERK2-NLS DK compared with vector-transfected controls, a difference that could not be detected in *LMNA*$^{-/-}$ MEFs. These results demonstrated that under our experimental settings, the induction of cell cycle progression by nuclear ERKs is caused by their capacity to facilitate Rb phosphorylation in a fashion dependent on lamin A.

Lamin A restrains transformation and proliferation of tumor cells

Finally, we evaluated how the balance among ERKs, lamin A, and Rb impacted biological processes such as transformation and tumor cell proliferation. To this aim, we conducted focus formation assays in NIH3T3 fibroblasts, as induced by the oncogene *v-Src* with or without ERK2-NLS wt. We found that the presence of nuclear ERK2 markedly enhanced *v-Src* transforming potential, an effect that could be reverted by the cotransfection of lamin A (Fig. 6 A). This observation was substantiated further under more physiological conditions. The MCF7 cell line is derived from a breast ductal carcinoma and displays highly tumorogenic and metastatic characteristics (Soule et al., 1973). MCF10A cells are also derived from mammary epithelium but are nontumorogenic and retain many features reminiscent of normal cells (Soule et al., 1990). Interestingly, we found that MCF7 cells expressed much more ERKs, particularly ERK1, than MCF10A (Fig. 6 B), most of which was present at the nucleus (Fig. 6 C). Moreover, in

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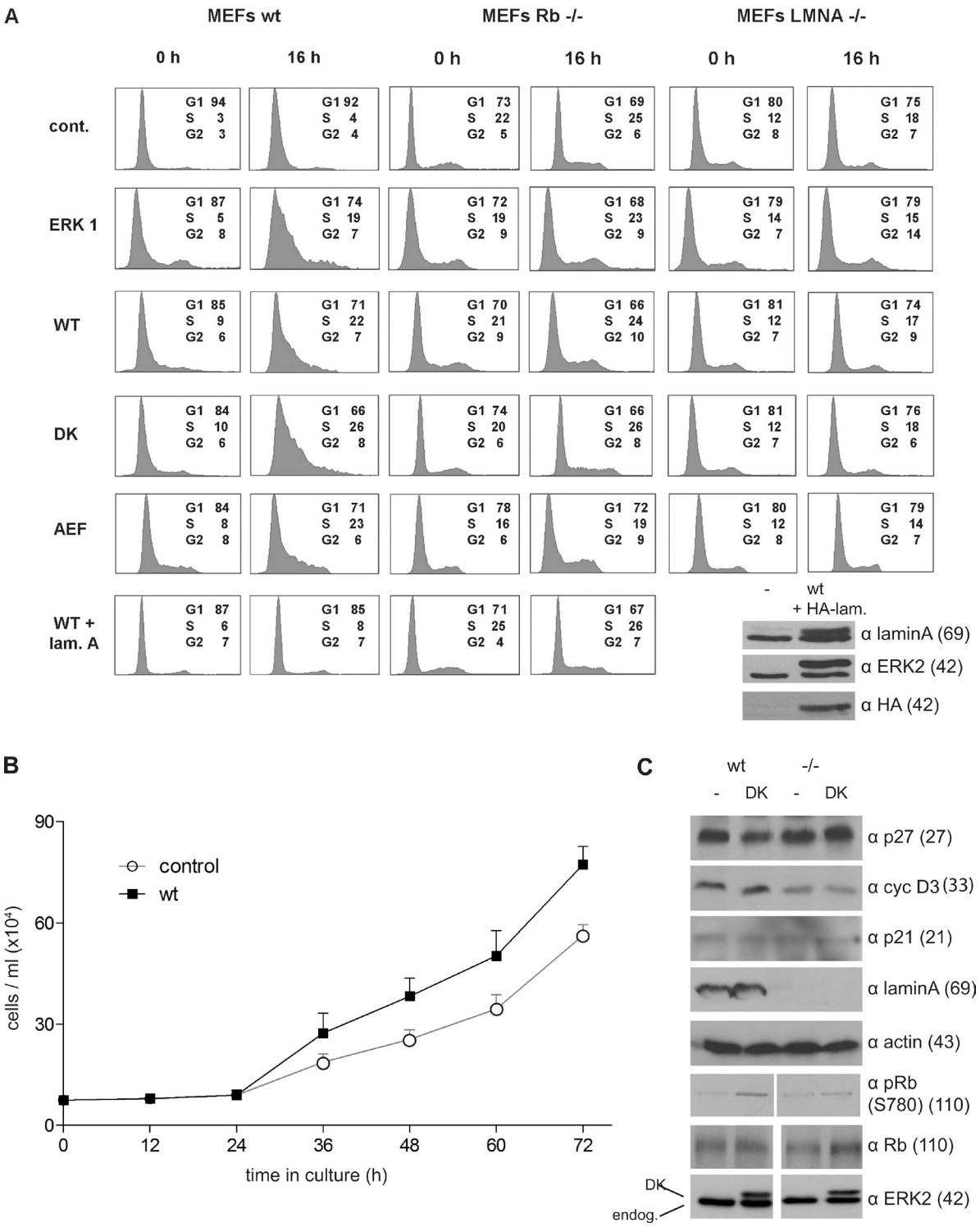

Figure 5. **Rb- and lamin A–dependent cell cycle progression induced by nuclear ERKs.** (A, top) Nuclear ERK2 enhances cell cycle progression. MEFs (wt, $Rb^{-/-}$, and $LMNA^{-/-}$) were transfected as shown with 1 μg ERK1-NLS and ERK2-NLS forms (wt, dead kinase [DK], unphosphorylatable mutant [AEF], and HA–lamin A [lam. A]) as indicated. Cells were synchronized by aphidicolin block, and cell cycle phases were analyzed by flow cytometry immediately after the block release and after 16 h of stimulation with 0.5% FCS. Graphs show a representative experiment out of three independent events. (bottom) Expression levels of cotransfected lamin A and ERK2-NLS wt. (B) Nuclear ERK2 enhances proliferation rate. Proliferation kinetics of NIH3T3 cell lines: parental (control) and stably expressing ERK2-NLS wt (wt). Results show means ± SEM of three independent experiments. (C) Nuclear ERK2 does not affect other cell cycle regulators. MEFs, wt, and $LMNA^{-/-}$ (−/−) and parental (−) or stably expressing ERK2-NLS DK (DK) were analyzed for the expression of the indicated cell cycle regulators by immunoblotting. For p21 and p27, total cell lysates from starved cells are shown. For the rest, lysates correspond to cells grown in 5% FCS. Molecular masses (given in kilodaltons) are shown in parentheses after the protein name.

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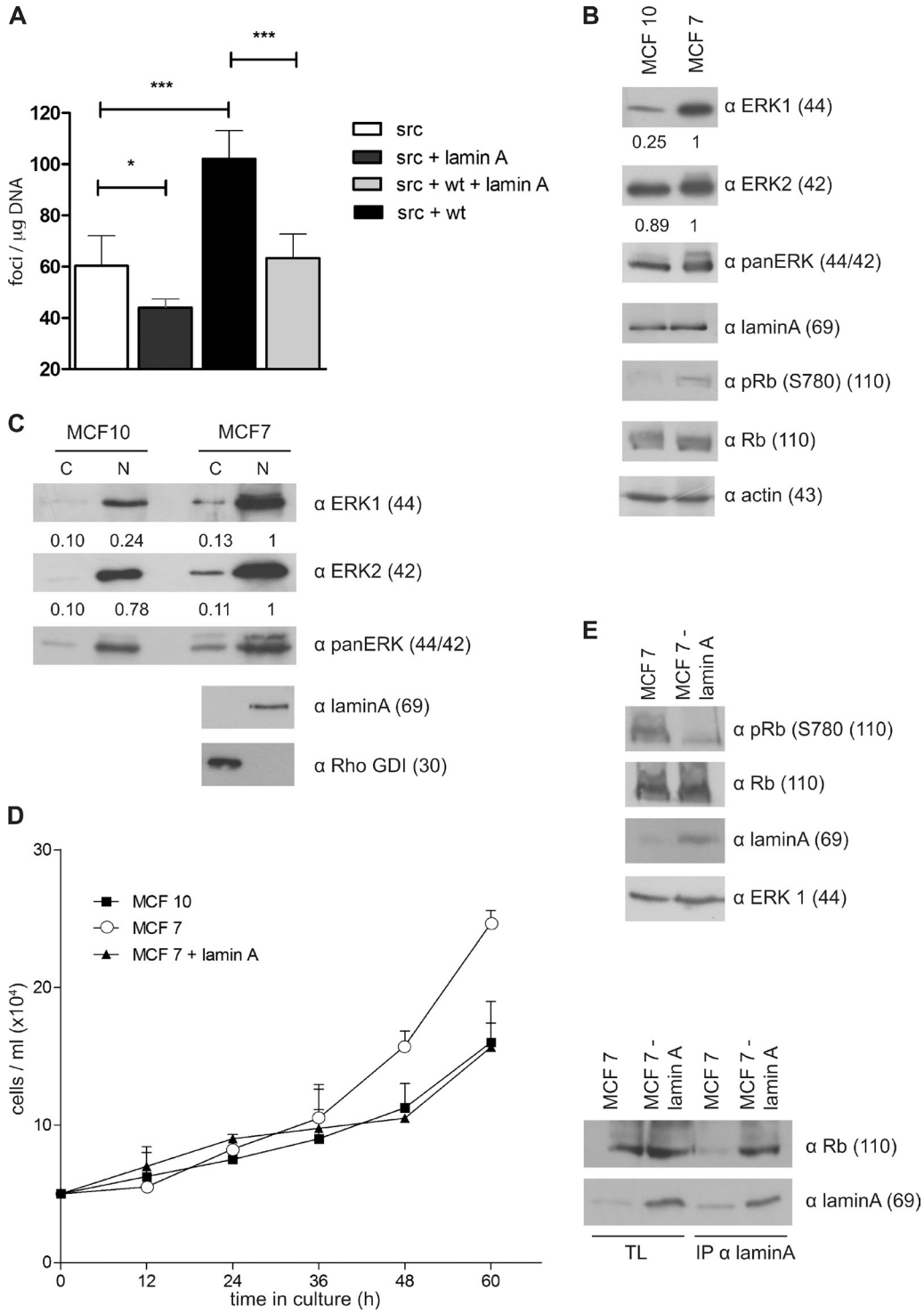

Figure 6. **Biological effects of ERK/Rb–lamin A balance.** (A) Nuclear ERK2 promotes and lamin A hampers transformation. Focus formation assays in NIH3T3 cells transfected with 0.25 µg *v-Src* plus 1 µg each ERK2-NLS wt with or without lamin A. ***, $P < 0.005$ and *, $P < 0.05$. (B) Relative levels of ERKs, phospho-Rb, and lamin A in MCF7 and MCF10A cells. Total lysates were analyzed for the expression of the indicated proteins (α protein of interest) by immunoblotting. (C) MCF7 cells exhibit high levels of nuclear ERKs. Nuclear (N) and cytoplasmic (C) extracts from MCF7 and MCF10A cells were analyzed for their content on ERKs by immunoblotting. The purity of the fractions was ascertained using lamin A and Rho GDI as nuclear and cytoplasmic markers, respectively. (B and C) Numbers show ERK1 and ERK2 expression levels quantified relative to the highest signal. (D) Lamin A attenuates the proliferation rate of tumor cells. Proliferation kinetics of the cell lines MCF10A, MCF7, and MCF7 overexpressing lamin A. (E) Lamin A down-regulates phospho-Rb levels in tumor cells. (top) Total lysates (TL) from MCF7 cells, parental and overexpressing lamin A, were analyzed for the expression of the indicated proteins by immunoblotting. (bottom) Enhanced Rb–lamin A association in MCF7 cells overexpressing lamin A, as determined in anti–lamin A immunoprecipitates (IP). Molecular masses (given in kilodaltons) are shown in parentheses after the protein name. Results show means ± SEM of three independent experiments.

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agreement with the aforementioned findings, MCF7 cells exhibited much higher levels of phosphorylated Rb than MCF10A cells (Fig. 6 B). Consequently, the proliferation rate of MCF7 cells was ∼70% higher than that for MCF10A (Fig. 6 D). We then generated a line of MCF7 cells stably overexpressing lamin A. These cells exhibited much lower levels of phosphorylated Rb compared with parental MCF7 cells (Fig. 6 E, top) and, consequently, an augmented association of Rb to lamin A (Fig. 6 E, bottom). These alterations, resulting from lamin A overexpression, coincided with a reduction in the proliferation rate of MCF7–lamin A cells to levels similar to those found in MCF10A cells (Fig. 6 D). Overall, these results suggest that biological outputs such as transformation and proliferation are highly dependent on the balance between lamin A and the levels of nuclear ERKs, which determine the amount of Rb accessible for phosphorylation and, subsequently, regulate cell cycle entry and cellular proliferation.

Discussion

A previous study from our laboratories demonstrated that, upon nuclear entry in response to stimulation, ERKs bind to lamin A/C and, in so doing, trigger immediate pretranscriptional events such as rapid c-Fos activation (González et al., 2008). Following this line of research, herein, we report that ERK interaction with lamin A also orchestrates a novel mechanism for cell cycle regulation by facilitating a fast phosphorylation of Rb, preceding other transcriptional events whereby ERKs are known to control the cell cycle machinery (for review see Chambard et al., 2007). We noticed that ERKs and Rb bind to lamin A through the same region (Ozaki et al., 1994; González et al., 2008). We now demonstrate that they do so in a competitive fashion with remarkable functional consequences. In an unprecedented fashion, ERKs physically dislodge Rb from its interaction with insoluble lamin A, releasing Rb to the SNF, thereby making possible its inactivation by phosphorylation, the subsequent activation of E2F transcription factors, and, ultimately, cell cycle progression. These data suggest that lamin A interaction with Rb prevents its inactivation, either by inhibiting its phosphorylation or by favoring its dephosphorylation. Accordingly, the rupture of Rb–lamin A complexes, either by competitive binding, as we show here for ERKs, or by the absence of lamin A as previously demonstrated (Van Berlo et al., 2005), increases the levels of phosphorylated Rb.

Our data indicate that Rb phosphorylation occurs rapidly after ERK nuclear influx, long before the assembly of cyclin D–CDK4/6 or cyclin E-A–CDK2 complexes, the main inactivators of Rb (Weinberg, 1995). This indicates that some other kinase must be phosphorylating Rb at very early stages of the cell cycle. This notion could be inferred from a previous study showing that, in MEFs lacking all interphase Cdks (CDK2, CDK3, CDK4, and CDK6), Rb is phosphorylated, and progression through the cell cycle is achieved (Santamaría et al., 2007). Evidently, a major goal will be to identify the responsible kinase, the transcriptional Cdks (Malumbres and Barbacid, 2009) being attractive candidates. Irrespective of its identity, our results unveil that such an Rb kinase is not regulated by ERK kinase activity, as mutant forms devoid of catalytic function can trigger Rb phosphorylation under serum deprivation conditions by virtue of their ability to disrupt the Rb–lamin A complexes. However, we cannot discard that the unknown Rb kinase is regulated by ERKs in a kinase-independent fashion. Indeed, ERK2 substrates such as PARP-1 (Cohen-Armon et al., 2007), topoisomerase II (Shapiro et al., 1999), and MKP3 (Camps et al., 1998) are activated by direct interaction with ERK2 without involving its kinase activity.

We have demonstrated that ERK nuclear influx causes the immediate dislodgement of Rb from its association to lamin A, thereby triggering cell cycle entry. This result unveils a previously undocumented role for ERKs in the regulation of the initial steps of the cell cycle and complements previous studies that addressed ERK's importance at later stages, such as mid-G_1 and G_1/S transition, but had not examined events previous to 1 h after stimulation (Jones and Kazlauskas, 2001; Yamamoto et al., 2006; Villanueva et al., 2007), as we have herein. It is noteworthy that, in agreement with our findings, the importance of ERKs for the onset of cell cycle had been noticed before (Jones and Kazlauskas, 2001). Furthermore, we show that cells deficient for Rb are refractory to the increments on E2F-mediated transcription and on cell cycle progression induced by nuclear ERKs, in full consonance with previous experiments (D'Abaco et al., 2002). One interpretation could be that ERK's mission to disrupt Rb–lamin A interaction would be unnecessary in the absence of Rb. In support of this notion, we show that (a) lamin A's inhibitory effect on ERK-induced cell cycle transit is lost in $Rb^{-/-}$ cells and (b) cells devoid of lamin A are insensitive to the stimulatory effects of ERKs on cell cycle entry. These data clearly suggest that lamin A's regulatory effect on cell cycle is based, to a significant extent, on its role as a mutually exclusive dock for ERKs and Rb.

The nuclear lamina and the nucleoskeleton are thought to play important roles in gene regulation (Schneider and Grosschedl, 2007). It has been demonstrated that gene-rich chromatin regions associate with nuclear structures rich in lamin A (Shimi et al., 2008) and that lamin A can act as a transcriptional repressor at different promoters (Lee et al., 2009; Ottaviani et al., 2009). In addition, it is well known that Rb is bound to E2F-regulated promoters in resting cells (Weintraub et al., 1995). Based on these previous notions and on our present results, we propose the model depicted in Fig. 7: under quiescence, Rb would be bound to insoluble lamin A, which is present at the nuclear lamina and other structures of the nucleoskeleton. At the same time, Rb would also be bound to E2F-regulated promoters via E2F. In response to mitogenic stimulation, ERKs will enter the nucleus and immediately displace Rb from its complex with insoluble lamin A, releasing it to the soluble nucleoplasm, where it will be available for phosphorylation by some yet unidentified Rb kinase, thereby triggering Rb inactivation, E2F release, and transcriptional activation of the target promoters.

It is worth noticing that the disruption of lamin A–Rb interaction by ERKs seems to be quite specific, as we found that the association between LAP2α and Rb is unaffected. Moreover, lamin A interaction with LAP2α is also insensible to nuclear ERKs. Intriguingly, if ERKs bind to lamin A and lamin A binds

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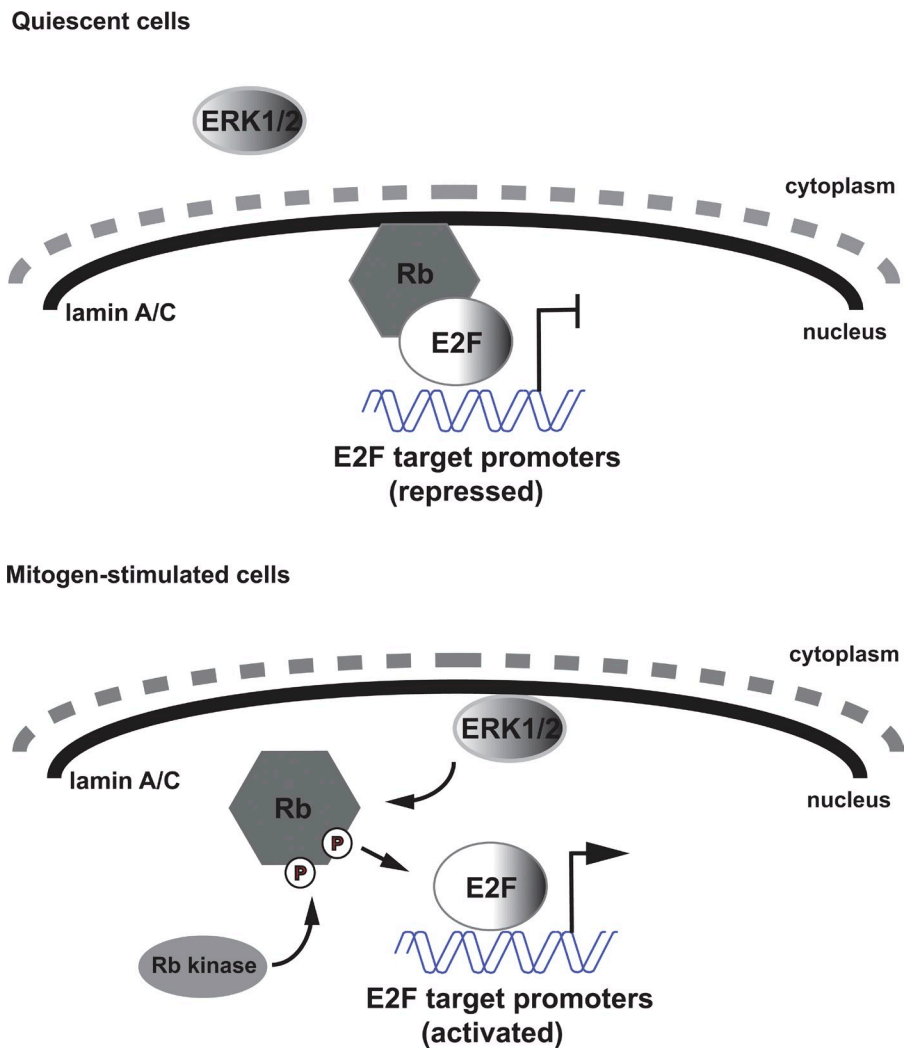

Figure 7. **A model for ERK/lamin A–mediated regulation of Rb function.** In quiescent cells, ERKs reside at the cytoplasm. At the nucleus, Rb is bound to lamin A, which maintains it in an active state bound to E2F and E2F-regulated promoters. After mitogenic stimulation, phosphorylated ERKs enter the nucleus and disrupt Rb–lamin A interaction. Rb is released to the nucleoplasm, where it will be phosphorylated/inactivated by an unidentified Rb kinase, liberating E2F and setting in motion the cell cycle machinery.

to LAP2α, ERKs should be detected in LAP2α immunoprecipitates, but we have found that this is not the case. This could have a technical explanation: the macrocomplex is not stable enough to resist the immunoprecipitation conditions. However, we hypothesize a second possibility. We have shown that ERKs bind to insoluble lamin A, whereas LAP2α associates with nucleoplasmic lamin A (Dorner et al., 2006; Naetar et al., 2008). As such, ERKs and LAP2α would not be bound to the same pool of lamin A. This opens the possibility that two distinct populations of lamin A may be orchestrating cell cycle progression through the interaction with Rb and two different regulators, ERKs versus LAP2α, at two different stages, immediate G_0 exit, as we show here, versus G_1 (Dorner et al., 2006; Naetar et al., 2008). It is also possible that these events may take place at different sublocalizations within the nucleus, for example, the nuclear periphery/lamina versus internal nuclear zones. These notions will require further studies.

Finally, we have investigated how ERK/lamin A–Rb interplay impacts biological processes relevant to carcinogenesis, such as cellular transformation and tumor cell proliferation. We demonstrate that in physiological settings, a direct correlation exists among ERK nuclear levels, Rb phosphorylation, and cellular proliferation. Noticeably, Rb phosphorylation and proliferation can be attenuated by the expression of ectopic lamin A, which can also forestall cellular transformation as induced by the potent oncogene *v-Src*. It has been shown that cell lines deficient for lamin A/C proliferate faster (Johnson et al., 2004; Van Berlo et al., 2005; Ivorra et al., 2006; Nitta et al., 2006). It is also known that lamin A levels inversely correlate with proliferation rates in different tissues (Broers et al., 1997) and that expression of A-type lamins is a marker of tumor cell differentiation (Foster et al., 2010). Our present results disclose a molecular mechanism whereby lamin A could be exerting the aforementioned antitumoral effects through the regulation of cell cycle entry by virtue of its capacity to prevent Rb inactivation immediately unleashed by ERK entry into the nucleus after mitogenic stimulation. A major goal will be to determine whether defects on such a mechanism underlie the pathogenesis of some severe medical conditions that result from mutations in A-type lamins.

Materials and methods

Plasmids

ERK2 DK, AEF, ΔINS, and ECFP–lamin A have been previously described (Ajenjo et al., 2000; Wolf et al., 2001; Ivorra et al., 2006; Casar et al., 2007). To generate nuclear-targeted HA-ERK2-NLS forms, the SV40 T antigen nuclear localization signal (PKKKRKV) was introduced by PCR directly downstream of ERK2's last codon. An identical strategy was used to generate HA-ERK1-NLS. pEYFP-Rb was generated by subcloning the full-length

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human Rb1 into pEYFP-C1 (Takara Bio Inc.). pCEFL HA–lamin A was a gift from J.M.P. Freije and C. Lopez-Otín (University of Oviedo, Oviedo, Spain).

Cell culture

MEFs and MCF7 cells were grown in Dulbecco's minimum essential medium (DMEM) and 10% FCS. NIH3T3 was grown in DMEM and 10% CS. MCF10A was grown in DMEM F12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, and 10 µg/ml insulin. Cells were transfected with Lipofectamine reagent (Invitrogen). ERK2-NLS stable cell lines were generated by pooling 10 clones previously tested for stable expression. UO126 was obtained from Promega, and PDGF was purchased from Invitrogen. $Rb^{-/-}$ MEFs were provided by J. Zalvide (University of Santiago, Santiago, Spain). $Lamin^{-/-}$ MEFs were provided by O. Meucci (Drexel University, Philadelphia, PA).

Antibodies

The mouse monoclonals used were anti-HA, anti-ERK2, phospho-ERK, and phospho-Lap2α. The rabbit polyclonals used were anti–lamin A, anti-Rb, anti-panERK, anti–Rho guanine dissociation inhibitor (GDI), anti–Elk-1, anti–cyclin D3, anti-p21, and anti-p27. The goat polyclonal used was anti-actin. All were obtained from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-ERK1 was obtained from BD. Rabbit polyclonal anti–phospo-Rb (S780, S795, and S807/811) was purchased from Cell Signaling Technology. Mouse monoclonal anti–lamin A/C Manlac was provided by G.E. Morris (MRIC Biotechnology Group, Wrexham, England, UK).

Lamin A coimmunoprecipitation assays

Cells were washed in ice-cold PBS and lysed in 20 mM Hepes, pH 7.5, 10 mM EGTA, 40 mM glycerophosphate, 1.5% NP-40 (Fluka), 2.5 mM $MgCl_2$, 2 mM orthovanadate, 1 mM DTT, 1 mM PMSF, and 10 µg/ml aprotinin/leupeptin. After 30 min on ice, lysates were cleared by centrifugation, 0.1 vol was separated to be loaded as the total lysate, and the rest were incubated with antibody rocking at 4°C for 4 h. Protein G–Sepharose was added and agitated at 4°C for 12 h. Beads were collected and washed three times with NP-40/PBS. Pellets were resuspended in 5× Laemmli buffer, boiled for 5 min, and fractionated by SDS-PAGE.

Immunoblotting

Total lysates and affinity precipitates were fractionated by SDS-PAGE and transferred onto nitrocellulose filters. Immunocomplexes were visualized by enhanced chemiluminescence detection (GE Healthcare) with horseradish peroxidase–conjugated secondary antibodies (Bio-Rad Laboratories).

In vitro pull-down assays

Bacterially produced His-ERK2 was purified according to the manufacturer's instructions (Protino Ni-IDA 150 packed columns; Macherey-Nagel). GST and GST–lamin A 247–355 bound to glutathione–Sepharose 4B beads were incubated in cell lysates from U2OS cells transfected with YFP-RB1. After incubation for 2 h at 4°C, the beads were collected and washed twice with cold PBS and 1% NP-40/PBS and twice with lysis buffer. Where indicated, different amounts of His-ERK2 were added, and, after incubation for 2 h at 4°C, the beads were collected and washed as before. Pellets were resuspended in 2× Laemmli buffer, boiled for 5 min, and separated by 10% SDS-PAGE.

Focus-forming assays

Assays were performed basically as previously described (Arozarena et al., 2000). In brief, subconfluent NIH3T3 cultured in DMEM and 10% CS were transfected with the indicated constructs using Lipofectamine reagent according to the manufacturer's instructions. After 10–15 d in culture, plates were stained in 5% GIEMSA, and foci were scored.

Nucleocytoplasmic fractionation

Nucleocytoplasmic fractionation was performed in 20 mM Hepes buffer, pH 7.4, basically as described previously (Casar et al., 2007). In brief, cells were collected in 50 mM β-glycerophosphate, pH 7.3, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT, centrifuged, and lysed in 40 mM Hepes, pH 7.5, 5 mM EGTA, 0.1% NP-40, 5 mM $MgCl_2$, 1 mM DTT, 1 mM VO_4, and 1 mM benzinamide. The lysate was vortexed vigorously and centrifuged to obtain the cytoplasmic fraction as supernatant. Nuclei were resuspended in 50 mM β-glycerophosphate, pH 7.3, 0.2 mM EDTA, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 25% glycerol, sonicated briefly on ice, vortexed, and centrifuged, and the precipitated cell debris was discarded.

Subnuclear fractionation

ERNFs and SNFs were separated as briefly described: cells were washed, scraped into TEN buffer (150 mM NaCl, 1 mM EDTA, and 40 mM Tris, pH 7.4), collected by centrifugation, and resuspended in lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF). After 15 min on ice, NP-40 was added to a final concentration of 0.5%. Lysates were centrifuged, and the nuclear pellet was collected, resuspended in 20 mM of ice-cold Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF, sonicated for 1 min, and vigorously vortexed at 4°C for 15 min. The nuclear lysate was centrifuged at 15,000 *g* for 45 min at 4°C to obtain the supernatant (SNF) and the pellet (ERNF) that were resuspended in lysis buffer plus NP-40 (1% to a volume equal to that of the SNF). In the experiments in which total lysates are run alongside SNFs and ERNFs, these were collected from parallel plates using the lamin A immunoprecipitation protocol described in Lamin A coimmunoprecipitation assays.

FRET

U2OS cells were cotransfected with calcium phosphate (Sigma-Aldrich) using 5 µg ECFP–lamin A plus 5 µg each of the different combinations of plasmids as indicated in Fig. 2 C. Images were acquired on a confocal microscope (TCS/SP5; Leica) with a 63× NA 1.4 oil immersion objective at room temperature using glycerol as an imaging medium. An argon laser line of 458 nm was used to excite CFP (photomultiplier tube window of 465–505 nm), and a 514-nm line (10% laser intensity for acquisition and 100% for every five frames for photobleaching) was used to excite YFP (photomultiplier tube window of 525–600 nm). FRET experiments were performed in 4% paraformaldehyde-fixed cells using the acceptor-photobleaching method (Kenworthy, 2001) in which FRET efficiency is calculated as the relative increase in total intensity as a result of the reduction or elimination of energy transfer when the acceptor is photobleached. Specifically, the percentage of donor total intensity (area multiplied by mean intensity) that increases its fluorescence after acceptor photobleaching was quantified in the nucleus using the following equation: $FRET = (C_{after} - C_{before})/C_{after} \times 100$, in which C_{before} and C_{after} are the total fluorescence intensity of the CFP channel before and after photobleaching, respectively. Image quantification was performed using MetaMorph software (MDS Analytical Technologies).

Luciferase assays

Luciferase assays were performed in MEFs basically as described previously (Sanz-Moreno et al., 2003) using a 3×-wt-E2F-luc reporter plasmid that contains three wt E2F binding sites upstream of the luciferase gene (Krek et al., 1993) provided by A. Zubiaga (University of Bilbao, Bilbao, Spain). In brief, cells were transfected using Lipofectamine reagent with a β-galactosidase reporter and the E2F reporter plasmid. The total amount of DNA for each transfection was kept constant at 5 µg using pCDNA3. The luciferase activities were determined using a commercial kit (Dual Luciferase reporter assay kit; Promega) according to the manufacturer's instructions and normalized by dividing by the β-galactosidase activity.

Cellular proliferation assays

Cellular proliferation assays were performed exactly as previously described (Matallanas et al., 2006). In brief, cells were plated at low density in 6-well plates and cultured for different time intervals under standard conditions. Cells were detached and scored by standard cell-counting techniques at the indicated intervals.

Cell cycle analyses

To be synchronized, cells were treated for 24 h with 1 µM aphidicolin (Sigma-Aldrich) in DMEM. Subsequently, cells were washed twice with PBS and stimulated with 0.5% FBS for 16 h. Cells were trypsinized, washed three times in PBS, fixed in 70% ethanol for 30 min at 4°C, resuspended in 500 µl of staining solution (PBS, 100 µg/ml RNase A, and 75 µg/ml propidium iodide), incubated for 2 h at 4°C in a light-proof container, and subjected to cytometric analysis. Cells were analyzed with a flow cytometer (FACSCanto II; BD) using the DiVa 6.1.1 software.

Statistical analyses

Statistical analyses were performed using Prism 4.0 software (GraphPad Software, Inc.). Results show analysis of variance performed by Bonferroni multiple comparisons test.

Online supplemental material

Fig. S1 shows the specificity of the anti–lamin A immunoprecipitations, the quantification of the Rb levels that fluctuate between the lamin A soluble and insoluble fractions, the down-regulation of Lap2α levels, and the specificity

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of the Rb antibodies. Fig. S2 shows the cell cycle progression induced by nuclear ERKs in NIH3T3 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201004067/DC1.

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