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Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival

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We have used mouse embryonic fibroblasts (MEFs) devoid of Ras proteins to illustrate that they are essential for proliferation and migration, but not for survival, at least in these cells. These properties are unique to the Ras subfamily of proteins because ectopic expression of other Ras-like small GTPases, even when constitutively active, could not compensate for the absence of Ras proteins. Only constitutive activation of components of the Raf/Mek/Erk pathway was sufficient to sustain normal proliferation and migration of MEFs devoid of Ras proteins. Activation of the phosphatidylinositol 3-kinase (PI3K)/PTEN/Akt and Ral guanine exchange factor (RalGEF)/Ral pathways, either alone or in combination, failed to induce proliferation or migration of Rasless cells, although they cooperated with Raf/Mek/Erk signalling to reproduce the full response mediated by Ras signalling. In contrast to current hypotheses, Ras signalling did not induce proliferation by inducing expression of D-type Cyclins. Rasless MEFs had normal levels of Cyclin D1/Cdk4 and Cyclin E/Cdk2. However, these complexes were inactive. Inactivation of the pocket proteins or knock down of pRb relieved MEFs from their dependence on Ras signalling to proliferate.

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Introduction

The Ras family of small GTPases, H-Ras, N-Ras and the two K-Ras isoforms, K-Ras4A and K-Ras4B, are some of the most studied proteins in signal transduction because of their central role in mediating mitogenic signalling and, when mutated, in human cancer. These studies have illustrated how Ras proteins become activated by growth factor receptors and have unveiled those pathways implicated in mediating downstream signalling to their ultimate effectors (reviewed in Malumbres and Barbacid, 2003). Among these pathways, the Raf/Mek/Erk, the phosphatidylinositol 3-kinase (PI3K)/PTEN/Akt and the Ral guanine exchange factor (RalGEF)/Ral pathways have been the best characterized. Accumulated evidence strongly implicates the Raf kinases and their effectors, the Mek and Erk kinases, in mediating cell proliferation or cell differentiation depending on the intensity and duration of the signal (reviewed in Marshall, 1995). The PI3K/Pdk/Akt pathway is supposed to mediate survival signals (reviewed in Cully *et al*, 2006; Engelman *et al*, 2006). However, the role of this pathway in mediating Ras signalling is less well characterized, mainly because of the fact that PI3K can be activated by other growth factor receptors through Ras-independent mechanisms. Another group of well-characterized Ras effectors involve the GDP/GTP exchange factors such as Tiam1 and RalGDS. Tiam1 is responsible for activating the Rho/Rac families of small GTPases involved in regulating cell polarity, motility and adhesion (reviewed in Malliri and Collard, 2003). On the other hand, RalGDS is responsible for activation of the Ral proteins primarily involved in membrane trafficking (reviewed in Camonis and White, 2005). Additional downstream effectors include PLCε whose activation ultimately results in activation of PKC and release of Ca^{2+} (reviewed in Bunney and Katan, 2006), AF6 that may also interact with cytoskeletal structures (reviewed in Kooistra *et al*, 2007) and, more surprisingly, certain proteins that have tumour suppressor activities such as Rin1 (reviewed in Milstein *et al*, 2007) and RASSF (reviewed in van der Weyden and Adams, 2007). These proteins may be part of feedback loops responsible for downregulation of Ras signalling.

Genetic analysis of *Ras* signalling pathways has been primarily carried out in lower organisms. In *Caenorhabditis elegans* and *Drosophila melanogaster* most phenotypes associated with Ras activity are mediated by the Raf/Mek/Erk pathway (Perrimon, 1994; Rommel and Hafen, 1998; Sternberg and Han, 1998). However, the complexity of Ras signalling may have increased in higher organisms such as mammals. Although endogenous Ras is not necessary for PI3K activity in *D. melanogaster* (Prober and Edgar, 2001), expression of a PI3K p110α subunit that cannot interact with Ras proteins in mice results in extensive perinatal death because of defective lymphatic vasculature (Gupta *et al*, 2007). Unfortunately, most genetic data involving Ras proteins are limited to knock out strains. These studies have showed that only K-Ras is essential for embryonic development (Johnson *et al*, 1997; Koera *et al*, 1997; Esteban *et al*, 2001). However, these observations are unlikely to be a consequence of unique signalling properties by K-Ras

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because expression of H-Ras from the K-*Ras* locus also leads to normal development (Potenza *et al*, 2005).

Germ line ablation of Raf kinases has not provided relevant information regarding their role as Ras effectors. C-Raf knockout mice are not viable because of early vascular defects and increased apoptosis without a major effect on cell proliferation (Mikula *et al*, 2001). B-*Raf* is only essential for extraembryonic development. Moreover, mouse embryonic fibroblasts (MEFs) lacking B-Raf only show a marginal decrease in cell proliferation (Galabova-Kovacs *et al*, 2006). Finally, ablation of A-*Raf* results in postnatal neurological and intestinal abnormalities depending on the genetic background (Pritchard *et al*, 1996), but has no consequences for cell proliferation (Mercer *et al*, 2005). The lack of effect on cell proliferation may be a consequence of compensatory activities among the Raf kinases. Indeed, double knockout embryos lacking C-Raf and A-Raf kinases show decreased proliferation rates in spite of sustaining normal levels of Erk phosphorylation (Mercer *et al*, 2005). The full range of consequences resulting from eliminating the three Raf kinases remains to be determined. Germ line ablation of Mek and Erk kinases has also failed to provide a functional link between these kinases and Ras proteins (Pagès *et al*, 1999; Bélanger *et al*, 2003; Fischer *et al*, 2005; Bissonauth *et al*, 2006).

In this study, we have analysed the relative contributions of the main Ras signalling pathways to cell proliferation, motility and survival by a genetic approach. To this end, we have generated MEFs carrying *null* H-*Ras* and N-*Ras* alleles along with a floxed K-*Ras* locus and a knocked-in inducible Cre recombinase (Esteban *et al*, 2001; Guerra *et al*, 2003). These MEFs can be rendered Rasless—devoid of Ras proteins—by exposure to 4-hydroxytamoxifen (4OHT) or by infection with adenoviruses expressing a Cre recombinase. Availability of Rasless MEFs has allowed us to analyse the contribution of the main Ras effectors to cell proliferation, motility and survival in the absence of upstream Ras signalling.

Results

Redundant activity of Ras proteins

To determine whether individual *Ras* genes could drive cell proliferation, primary H-Ras$^{+/+}$;N-Ras$^{-/-}$;K-*Ras*$^{lox/lox}$ and H-Ras$^{-/-}$;N-Ras$^{+/+}$;K-*Ras*$^{lox/lox}$ MEFs were infected with Adeno-Cre viruses to excise the K-*Ras*^{lox} alleles. Elimination of the floxed sequences resulted in loss of both K-Ras4A and K-Ras4B protein isoforms. Primary H-Ras$^{-/-}$;N-Ras$^{-/-}$;K-*Ras*$^{lox/lox}$ MEFs were used for cells expressing only K-Ras proteins. To control for any effects caused by adenoviral infection, H-Ras$^{-/-}$;N-Ras$^{-/-}$;K-*Ras*$^{lox/lox}$ MEFs were infected with control Adeno-GFP whereas wild-type MEFs were infected with Adeno-Cre. No effect on cell proliferation was observed in either case (data not shown). Expression of individual Ras proteins was verified by western blot analysis using pan-Ras and H-Ras-specific antibodies (Figure 1A). As illustrated in Figure 1B, expression of individual H-Ras, N-Ras or K-Ras proteins was sufficient to sustain proliferation of primary MEFs. However, K-Ras was considerably more efficient than H-Ras or N-Ras, leading to proliferation rates similar to that of wild-type MEFs. These differences were not because of changes in the levels of expression of the individual Ras proteins because they remained constant independently of the expression of the other isoforms (Figure 1A).

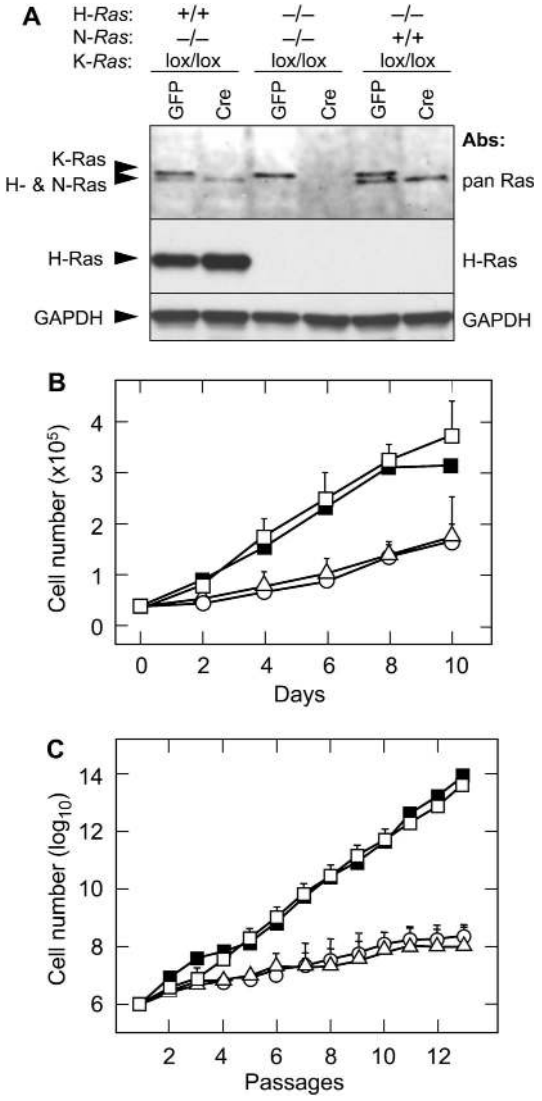

Figure 1 Proliferative properties of MEFs expressing single *Ras* loci. (**A**) Western blot analysis of H-*Ras*$^{-/-}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$, H-*Ras*$^{+/+}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$, and H-*Ras*$^{-/-}$;N-*Ras*$^{+/+}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs 3 days after infection with Adeno-GFP or Adeno-Cre. The pan-Ras antibody recognizes all Ras proteins. GAPDH expression was used as a loading control. (**B**) Growth curve of primary (passage 1) wild type (solid squares), H-*Ras*$^{-/-}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs infected with Adeno-GFP (open squares) and H-*Ras*$^{+/+}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs (open triangles) as well as H-*Ras*$^{-/-}$;N-*Ras*$^{+/+}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs (open circles) infected with Adeno-Cre to remove the conditional K-*Ras*^{lox} alleles. Ras mutant MEFs were derived from two independent embryos. Error bars indicate standard deviation. (**C**) Immortalization of wild type (solid squares), H-*Ras*$^{-/-}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs (open squares), H-*Ras*$^{+/+}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs (open triangles) and H-*Ras*$^{-/-}$;N-*Ras*$^{+/+}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs (open circles) following a 3T3 protocol. H-*Ras*$^{+/+}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ and H-*Ras*$^{-/-}$;N-*Ras*$^{+/+}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs were maintained in the presence of 4OHT to achieve excision of the K-*Ras*^{lox} alleles. Ras mutant MEFs were derived from two independent embryos. Error bars indicate standard deviation.

We also examined whether individual Ras proteins could bypass senescence induced by adaptation to culture

conditions. When MEFs expressing individual Ras proteins were submitted to a standard 3T3 protocol, all cultures became immortalized albeit with different kinetics (Figure 1C). Although MEFs expressing K-Ras proteins overcame senescence rapidly with the same kinetics as wild-type MEFs, cells expressing either H-Ras or N-Ras alone required additional passages until they acquired an immortal phenotype. These observations indicate that although each member of the Ras family is able to sustain cell proliferation, K-Ras proteins elicit a more robust mitogenic response than H-Ras or N-Ras, at least in MEFs.

Ras proteins are essential for cell proliferation

Next, we examined whether MEFs could proliferate in the absence of all Ras proteins. Primary H-*Ras*$^{-/-}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERTn$^{ert/ert}$ MEFs, from now on designated as K-Ras^{lox} MEFs, were exposed to either Adeno-GFP or Adeno-Cre viruses. Excision of the K-*Ras*^{lox} alleles resulted in complete growth inhibition, indicating that primary MEFs devoid of Ras proteins cannot proliferate (Supplementary Figure 1). Immortal K-Ras^{lox} MEFs also ceased proliferation in the absence of Ras proteins (Figure 2A). In this case, we used 4OHT to activate the resident CreERT2 recombinase present in these cells (Guerra *et al*, 2003). As illustrated in Figure 2A (inset), the conditional K-*Ras*^{lox} alleles became fully excised after 6–9 days in the presence of 4OHT, leading to complete loss of K-Ras expression (Figure 2B). The resulting H-*Ras*$^{-/-}$;N-*Ras*$^{-/-}$;K-*Ras*$^{-/-}$;RERTn$^{ert/ert}$ cells will be designated from now on as Rasless MEFs.

Rasless MEFs showed significant morphologic alterations including a flat shape with an overall appearance reminiscent of senescent cells (Figure 2C, top). However, Rasless MEFs did not express senescence markers (data not shown), indicating that they remained in a non-proliferative state distinct from senescence. Moreover, Rasless MEFs did not undergo overt apoptosis and could be maintained in culture for several weeks without significant decrease in cell numbers. These observations indicate that Ras proteins are essential for cell proliferation but not survival. Furthermore, they show that none of the other members of the Ras superfamily of proteins can compensate for the lack of Ras signalling to drive cell proliferation.

Ras signalling is essential for cell migration

Loss of Ras proteins severely restricts cell motility and migration. As illustrated in Supplementary Video 1, incubation of K-Ras^{lox} MEFs for 5 to 7.5 days in the presence of 4OHT almost completely eliminated mitotic events and prevented cells from migrating around the plate. During this time (60 h), cells appeared to increase in size as a consequence of acquiring a flatter shape. Nevertheless, they retained significant membrane plasticity and showed considerable membrane ruffling activity (Supplementary Video 1). Complete elimination of Ras proteins significantly reduced membrane plasticity and abolished cell migration (Supplementary Video 2). To provide a more quantitative measurement of cell migration, movement of individual MEFs was imaged as indicated in 'Materials and methods'. Although K-Ras^{lox} cells migrated in a random manner, Rasless MEFs were completely unable to migrate (Figure 2D; Supplementary Video 2).

Rasless MEFs also showed major alterations in cytoskeletal structures, such as microtubules and stress fibres (Figure 2C). Moreover, they showed a dramatic increase in the number of focal adhesions as determined by immunostaining for Paxillin, a typical component of focal adhesion structures (Figure 2C). In contrast to normal cells in which focal adhesions are subject to constant reorganization, those present in Rasless MEFs were highly static (Supplementary Video 3). This defect is likely to be the primary cause for the lack of migratory activity of Rasless MEFs. These observations suggest that activation of small Ras-like GTPases involved in membrane ruffling and cell migration may be dependent on active Ras signalling.

The 'Rasless' state is reversible

To determine whether the proliferative arrest of Rasless cells was reversible, we generated Rasless MEFs ectopically expressing a K-*Ras* cDNA driven by a Tet-Off system. Cells became fully arrested within 2–4 days after addition of doxycycline (Figure 3A) and acquired the same morphologic properties as Rasless MEFs (Figure 3B). Removal of doxycycline after 10 days or longer resulted in rapid induction of DNA synthesis within 24 h, followed by acquisition of normal morphology and restoration of proliferative and migratory properties (Figure 3B). Similar experiments carried out with an oncogenic K-*Ras*^{G12V} cDNA resulted in a more robust recovery of cell proliferation even before complete restoration of K-Ras^{G12V} expression (Figure 3A). These cells also recovered their normal morphology including a reversal of all their cytoskeletal defects and migrated normally on withdrawal from doxycycline (Figure 3C). These findings indicate that the cell cycle arrest, as well as the morphologic and migratory changes induced by loss of Ras signalling, is fully reversible.

A simple assay to identify that sustain cell proliferation in the absence of Ras proteins

We next developed a simple assay to interrogate whether other signalling molecules could sustain cell proliferation in the absence of Ras proteins. As schematically shown in Figure 4A, K-Ras^{lox} MEFs were infected with retroviruses encoding the effector molecule of interest, selected in the presence of the corresponding antibiotic and seeded at limiting dilution ($5–10 \times 10^3$ cells per 10 cm plate) in the presence or absence of 4OHT. After 2 weeks in culture, proliferating colonies were scored taken as reference parallel cultures not exposed to 4OHT (K-Ras^{lox} cells). Colonies $>2–3$ mm in diameter were isolated to verify loss of Ras expression as well as expression of the effector molecule. Control plates infected with empty retroviruses did not show any colonies, thus, illustrating the robustness of the assay (Figure 4B). These plates contained small groups of cells (<50 cells) that resulted from initial proliferation of K-Ras^{lox} MEFs before the activation of the resident Cre recombinase (Figure 4B, upper right).

As expected, K-Ras^{lox} cells infected with retroviruses encoding H-Ras, N-Ras and K-Ras4B led to the formation of colonies in the presence of 4OHT (Supplementary Figure 2; Figure 4B, lower right). Nevertheless, the number of colonies observed never exceeded 30–40% of those expressing the endogenous K-Ras protein (Figure 4B; Supplementary Figure 2A). In contrast, their oncogenic counterparts resulted in colony forming efficiencies of 80–90% of those present in

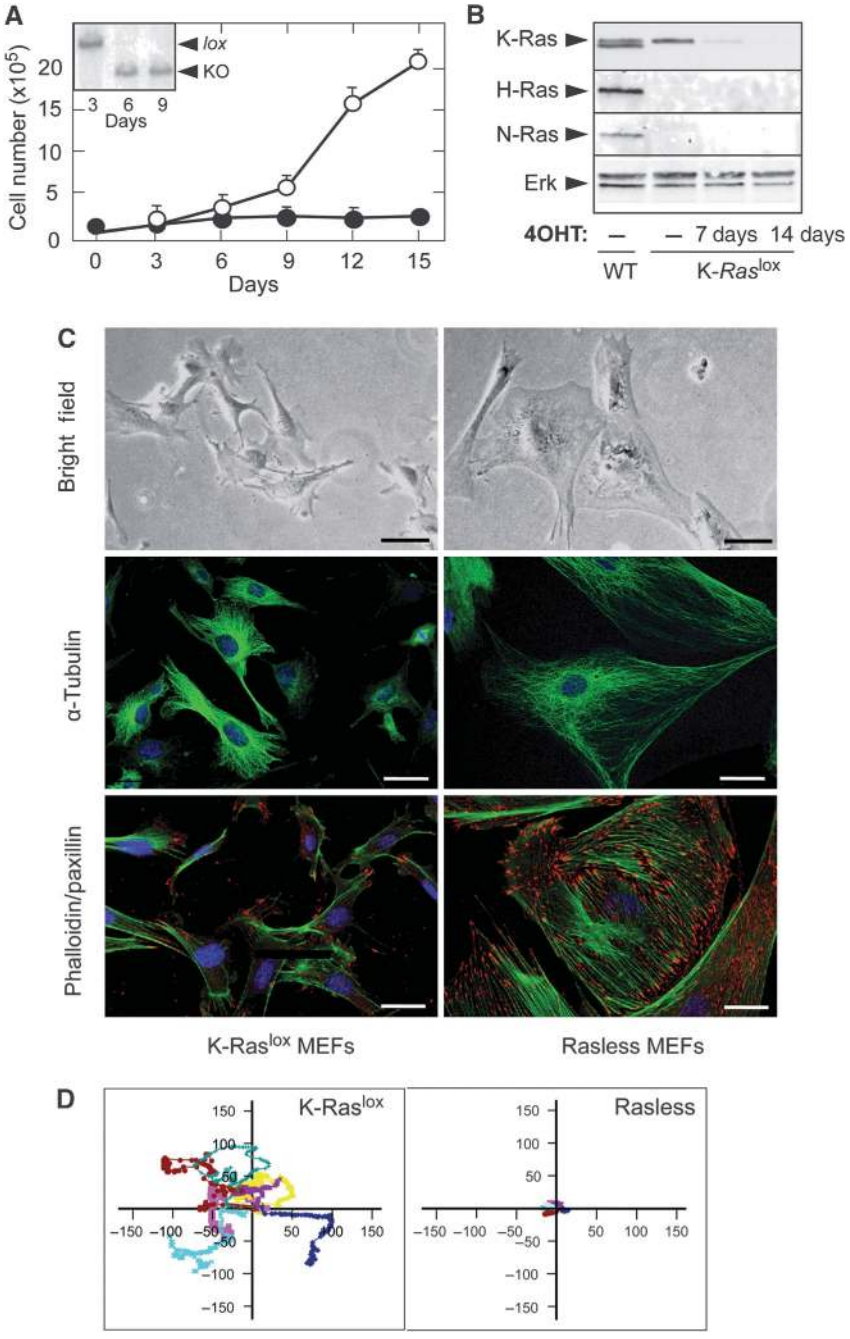

Figure 2 Ras proteins are essential for cell proliferation and migration. (**A**) Proliferation of immortal K-Ras^{lox} MEFs left untreated (open circles) or exposed to 4OHT for the indicated time to eliminate the conditional K-*Ras*^{lox} alleles (solid circles). Error bars indicate standard deviation. Inset depicts a Southern blot analysis showing excision of the conditional K-*Ras*^{lox} alleles (lox) in the presence of 4OHT (knock out, KO). (**B**) Western blot analysis showing Ras protein expression in K-Ras^{lox} cells either left untreated or exposed to 4OHT for 7 or 14 days. Wild-type (WT) MEFs were used as control. Migration of the indicated proteins is shown by arrowheads. Total Erk proteins were used as loading control. (**C**) Top: bright field microscopic images of K-Ras^{lox} MEFs left untreated (K-Ras^{lox}) and treated with 4OHT for 14 days (Rasless). Scale bar represents 100 μm. Middle: confocal microscopy images of microtubule organization in K-Ras^{lox} MEFs left untreated (K-Ras^{lox}) or 14 days after treatment with 4OHT (Rasless) as determined by α-tubulin staining (green) and DAPI counterstaining (blue). Scale bar represents 50 μm. Bottom: confocal microscopy images of actin stress fibres and focal adhesions in K-Ras^{lox} MEFs left untreated (K-Ras^{lox}) or exposed to 4OHT for 14 days (Rasless) as determined by Phalloidin (green) and Paxillin staining (red). DAPI counterstaining (blue) indicates nuclei. Scale bar represents 50 μm. (**D**) Migration tracks of individual K-Ras^{lox} MEFs left untreated (K-Ras^{lox}) (left) or 14 days after treatment with 4OHT (Rasless) (right) as determined by time-lapse microscopy. Migration was tracked for 14 h ($n = 8$).

K-Ras^{lox} cells (Supplementary Figure 2A). The K-Ras4A isoform, a protein not required for mouse development or homeostasis (Plowman *et al*, 2003), also sustained proliferation of Rasless cells. However, the efficiency of its oncogenic version, K-Ras4A^{G12V}, was only half of that obtained with the K-Ras4B^{G12V} isoform (Supplementary Figure 2A) in spite of similar levels of expression (Supplementary Figures 2B and 2C). Cells derived from colonies ectopically expressing Ras

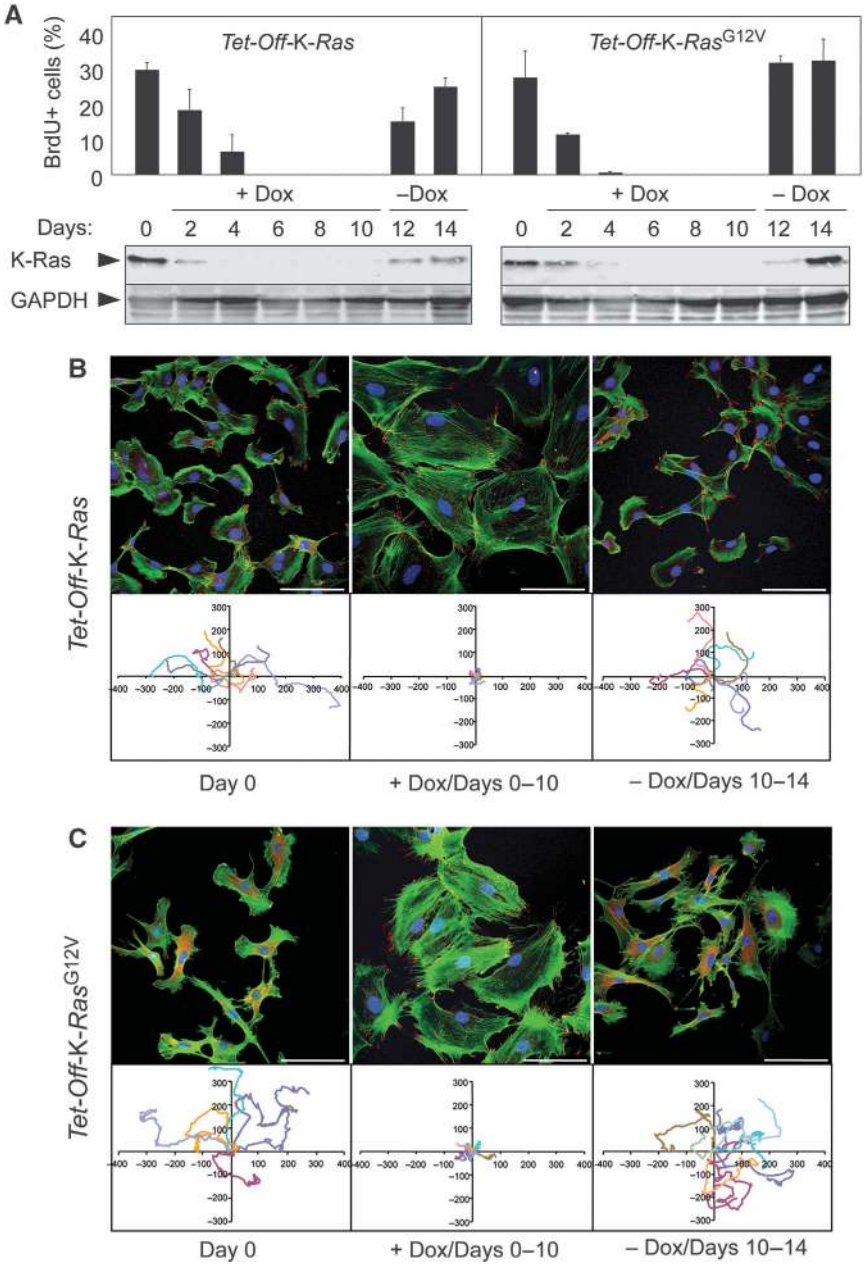

Figure 3 The proliferation and migration defects of Rasless MEFs are reversible. (**A**) Upper panel: percentages of cells in S phase as indicated by BrdU incorporation in (left) *Tet-Off*-K-*Ras* and (right) *Tet-Off*-K-*Ras*^{G12V} cells after addition and subsequent withdrawal of doxycyline at the indicated time points. Error bars indicate standard deviation. Lower panel: western blot analysis of K-Ras expression at the indicated times. GAPDH expression served as loading control. (**B**, **C**) Upper panels: migration defects and cytoskeletal alterations in (**B**) *Tet-Off*-K-*Ras* and (**C**) *Tet-Off*-K-*Ras*^{G12V} cells before addition of doxycycline (Day 0), 10 days after treatment with doxycycline (+Dox/Days 0–10) and four additional days after doxycyline withdrawal (−Dox/Days 10–14) as determined by Phalloidin (green) and Paxillin staining (red). DAPI counterstaining (blue) indicates nuclei. Scale bar represents 75 μm. Lower panels: migration tracks from individual (**B**) *Tet-Off*-K-*Ras* and (**C**) *Tet-Off*-K-*Ras*^{G12V} cells as determined by time-lapse microscopy. Migration was tracked for 14 h ($n = 8$).

proteins had the same proliferative and morphologic and migratory properties as wild type or K-Ras^{lox} MEFs (data not shown).

Ras proteins are essential for tyrosine protein kinase receptors signalling

It is generally accepted that Ras proteins are key mediators of tyrosine protein kinase receptor signalling. To verify this concept, K-Ras^{lox} MEFs were infected with retorviruses expressing the EGF receptor, the related ErbB2 receptor along with its transforming rat isoform, NeuT (Olayioye *et al*, 2000), and Trk5, a transforming allele of TrkA, the NGF receptor (Coulier *et al*, 1990). NeuT and Trk5 efficiently transformed NIH3T3 cells. However, they were unable to induce proliferation of K-Ras^{lox} MEFs in the presence of 4OHT, indicating that they require Ras proteins to mediate their mitogenic signalling (data not shown). K-Ras^{lox} MEFs expressing the wild-type EGFR and ErbB2 receptors also ceased proliferation on ablation of the conditional K-*Ras* locus in the presence of 4OHT. These observations validate

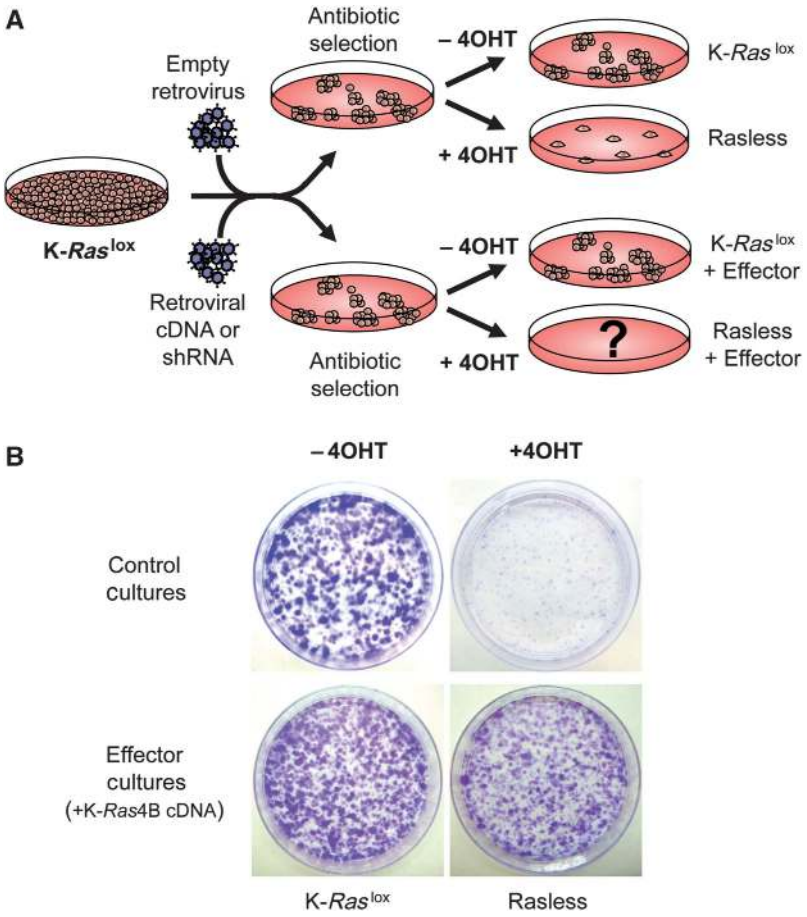

Figure 4 (**A**) Schematic representation of the assay used to identify genes capable of inducing cell proliferation in the absence of Ras proteins. (**B**) Representative plates from colony formation assays. K-Ras^{lox} MEFs were infected with empty retroviruses (control cultures) or retroviruses expressing a K-*Ras*4B cDNA (effector cultures). After antibiotic selection, cells were plated in the absence (−) or presence (+) of 4OHT and allowed to form colonies for 14 days. The tiny colonies observed in the control Rasless plate consist of small groups of cells (8–32 cells on average) resulting from limited cell proliferation until the K-*Ras*^{lox} alleles are completely excised. None of these cells was able to proliferate for extended periods of time nor on transfer to other plates. Cells were fixed with glutaraldehyde and stained with crystal violet.

previous studies indicating that tyrosine protein kinase receptors signal through Ras proteins. Moreover, they further illustrate that there are no alternative mechanisms that can compensate for the absence of Ras proteins, at least in MEFs.

Ras-related GTPases cannot compensate for the absence of Ras signalling

We also analysed whether constitutively active forms of Ras-related proteins including R-Ras^{G38V}, R-Ras2^{G23V} (also known as TC21), R-Ras3^{G22V} (also known as M-Ras) and E-Ras, a constitutively active Ras-like protein expressed in ES cells, could sustain cell proliferation in the absence of Ras proteins (Movilla *et al*, 1999; Self *et al*, 2001; Takahashi *et al*, 2003). None of these proteins were able to induce proliferation of Rasless cells in spite of robust expression (Figure 5A and B). Similar results were obtained with Rac1^{N115I} and RhoA^{Q63L} (Khosravi-Far *et al*, 1995), two representative members of the Rho/Rac/Cdc42 protein family (data not shown). Next, we examined whether R-Ras^{G38V}, R-Ras2^{G23V}, R-Ras3^{G22V} and E-Ras proteins could activate the Mek/Erk and PI3K/Akt signalling pathways in Rasless MEFs. As shown in Figure 5C, none of these constitutively active small GTPases induced phosphorylation of either Mek or Akt in the absence of Ras. As expected, ectopic expression of H-Ras^{G12V} induced robust phosphorylation of both downstream kinases. However, these proteins, with the possible exception of E-Ras, induced Mek phosphorylation in the absence of serum, as long as the cells expressed endogenous K-Ras (Figure 5C). Moreover, R-Ras2^{G23V} and E-Ras proteins, but not R-Ras^{G38V} or R-Ras3^{G22V}, efficiently induced phosphorylation of Akt under the same experimental conditions. These observations suggest that constitutively active Ras-like proteins may require the presence of endogenous Ras to exert their biological activity.

Ras downstream effectors: the Raf kinases

Next, we infected K-Ras^{lox} MEFs with H-Ras^{G12V} effector mutants known to selectively activate downstream signalling pathways (White *et al*, 1995; Rodriguez-Viciana *et al*, 1997). These mutants preferentially activate the Raf/Mek/Erk pathway (D38E mutation), the PI3K/Akt pathway (Y40C) and the RalGDS (E37G) pathway. Only H-Ras$^{G12V/D38E}$ sustained cell proliferation in the absence of endogenous Ras proteins albeit with rather low efficiency, about 5% of that observed with H-Ras^{G12V} (Figure 6A and B). Similar results were obtained with the corresponding K-Ras4B^{G12V} mutants (data not shown).

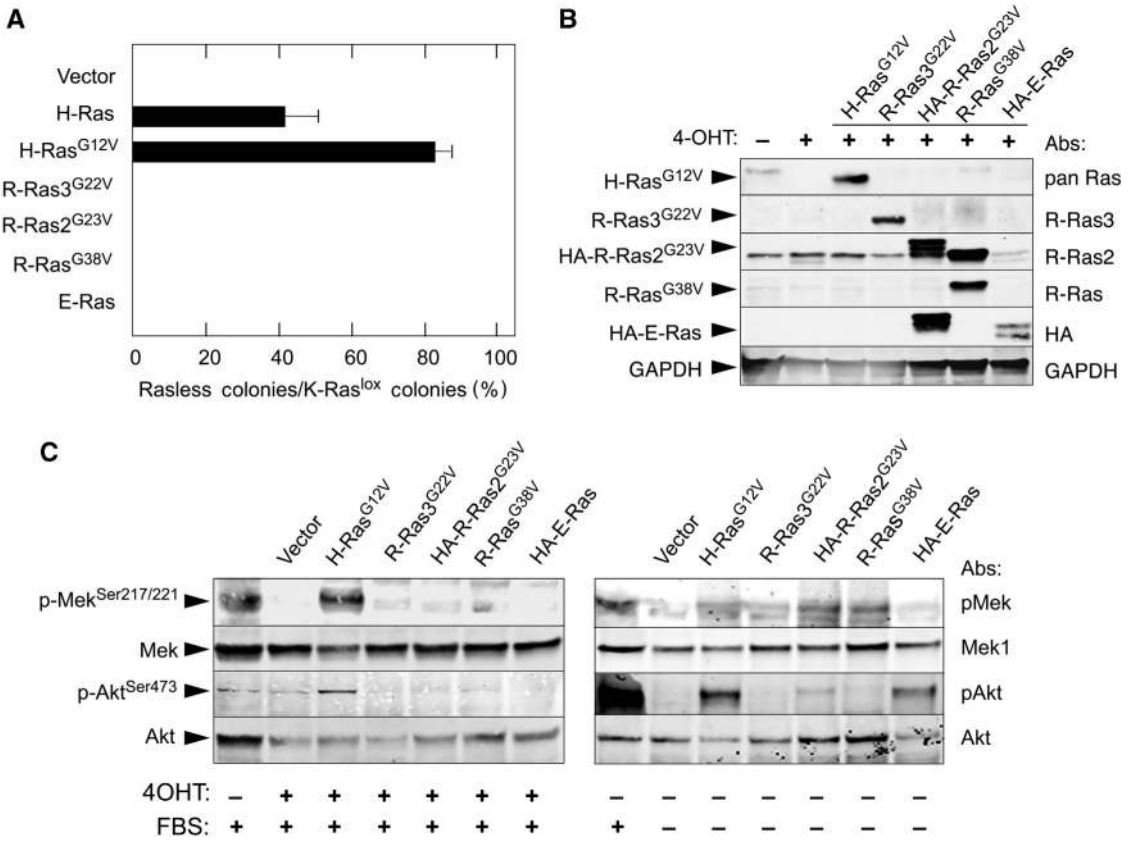

Figure 5 Expression of constitutively active Ras-related small GTPases do not sustain proliferation of Rasless MEFs. (**A**) Colony-formation assay using K-Ras^{lox} MEFs infected with retroviruses expressing the indicated cDNAs. Error bars indicate standard deviation. (**B**) Western blot analysis showing levels of expression of the proteins encoded by the corresponding cDNAs indicated in (**A**). GAPDH expression served as a loading control. Migration of the indicated proteins is shown by arrowheads. (**C**) Western blot analysis of Mek and Akt phosphorylation in K-Ras^{lox} MEFs infected with retroviruses encoding the indicated Ras-related proteins incubated in the presence (+) or absence (−) of 4OHT. Indicated cultures were either maintained in the presence of 10% FBS (+) or subjected to serum withdrawal (0.1% FBS for 24 h) (−). Migration of the indicated proteins is shown by arrowheads. Total Mek1 and Akt proteins served as loading controls.

These observations suggested that the Raf/Mek/Erk pathway might be the primary driver of cell proliferation.

To examine this possibility we expressed individual components the Raf/Mek/Erk pathway in Rasless cells. Expression of unmodified A-Raf, B-Raf or C-Raf proteins failed to induce proliferation of MEFs in the absence of Ras proteins (Figure 6A). These results were not unexpected as one of the main roles proposed for Ras proteins is to bring Raf kinases to the plasma membrane (Wellbrock *et al*, 2004). To bypass this requirement, we added the K-Ras4B carboxy-terminal domain, including the CAAX motif, to each of the Raf kinases (Leevers *et al*, 1994). As illustrated in Supplementary Figure 3, these modified proteins activated the Erk pathway with different efficiencies, as determined by their ability to phosphorylate their downstream effectors Mek1 and Erk1/2. Although C-Raf^{CAAX} was as efficient as K-Ras^{G12V} in phosphorylating Mek1 and Erk1/2, A-Raf^{CAAX} and B-Baf^{CAAX} failed to induce significant changes in their phosphorylation levels (Supplementary Figure 3).

However, K-Ras^{lox} MEFs expressing either A-Raf^{CAAX} or B-Baf^{CAAX} reproducibly yielded proliferating colonies of Rasless cells (Figure 6A). These colonies, when expanded, showed the same proliferative properties as K-Ras^{lox} or wild-type MEFs in spite of lacking Ras proteins (data not shown). These observations indicate that the A-Raf and B-Raf kinases are sufficient to activate those Ras signalling pathways required for cell proliferation in spite of their limited ability to phosphorylate Mek1 and Erk1/2. Moreover, these results further support the hypothesis that the main role of Ras proteins in activating Raf kinases is to bring them to the appropriate location within the plasma membrane. However, expression of A-Raf^{CAAX} or B-Raf^{CAAX} proteins did not fully reproduce the results observed with Ras oncoproteins. First of all, the percentage of proliferating colonies generated by A-Raf^{CAAX} or B-Raf^{CAAX} was only 25% of those observed with exogenous H-Ras^{G12V} or K-Ras4B^{G12V} (Figure 6A). As their expression levels were similar, these observations suggest that only a percentage of MEFs can proliferate in response to A-Raf^{CAAX} or B-Raf^{CAAX}. Moreover, when B-Raf^{CAAX} was co-expressed with the E1A oncogene, we did not observe foci of transformed cells (data not shown). These results are in agreement with the limited ability of A-Raf^{CAAX} and B-Raf^{CAAX} to transform NIH3T3 cells (Supplementary Figure 3).

Surprisingly, expression of C-Raf^{CAAX} failed to induce proliferation of Rasless cells (Figure 6A). As C-Raf^{CAAX} induced high levels of Mek1 and Erk1/2 phosphorylation and was at least 100-fold more efficient than A-Raf^{CAAX} or B-Raf^{CAAX} in transforming NIH3T3 cells (Supplementary Figure 3), we suspected that C-Raf^{CAAX} may induce a stress response that prevented proliferation of Rasless cells.

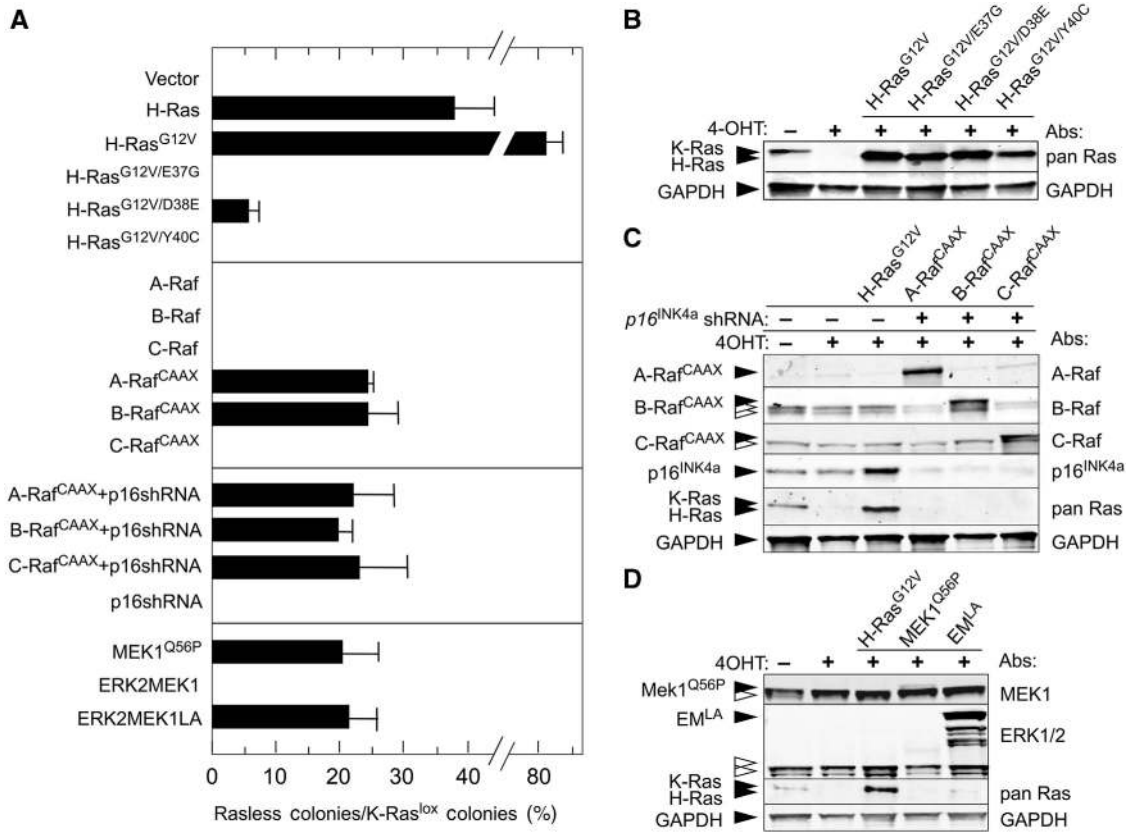

Figure 6 Constitutive activation of the Raf/Mek/Erk pathway drives proliferation of Rasless MEFs. (**A**) Colony-formation assay using K-Ras^{lox} MEFs infected with retroviruses expressing the indicated cDNAs. p16 shRNA indicates a retrovirus encoding an shRNA directed against p16^{INK4a}-specific sequences. Error bars indicate standard deviation. (**B**) Western blot analysis showing levels of expression of the indicated H-Ras effector mutant proteins probed with a pan-Ras antibody. Expression of the corresponding H-Ras or K-Ras proteins is indicated by arrowheads. GAPDH expression served as a loading control. (**C**) Western blot analysis showing expression of the indicated H-Ras^{G12V} or Raf^{CAAX} proteins in K-Ras^{lox} MEFs co-infected with a retrovirus encoding an shRNA directed against p16^{INK4a}-specific sequences (+). Expression of p16^{INK4a} is also indicated. Cells were left untreated (−) or exposed to 4OHT (+). Probing antibodies are indicated in the right panel. Migration of the corresponding proteins is indicated by solid arrowheads. Open arrowheads indicate migration of the endogenous B-Raf^{CAAX} and C-Raf^{CAAX} proteins. GAPDH expression served as loading control. (**D**) Western blot analysis showing expression of H-Ras^{G12V}, Mek1^{Q56P} and ERK2MEK1LA (EM^{LA}) in K-Ras^{lox} MEFs left untreated (−) or exposed (+) to 4OHT. Probing antibodies are indicated in the right panel. Migration of the corresponding proteins is indicated by solid arrowheads. Open arrowheads indicate migration of endogenous Mek1 and Erk1/2 proteins. GAPDH expression served as loading control.

To examine this possibility, we co-infected K-Ras^{lox} MEFs with retroviruses encoding C-Raf^{CAAX} and an shRNA against *p16*^{INK4a}, a tumour suppressor known to be implicated in stress responses after C-Raf activation (Lin *et al*, 1998; Zhu *et al*, 1998). As illustrated in Figure 6A, C-Raf^{CAAX} was as efficient as A-Raf^{CAAX} or B-Raf^{CAAX} in inducing proliferation of Rasless cells providing that expression of the tumour suppressor p16^{INK4a} was inhibited. Knock down of *p16*^{INK4a} alone was not sufficient to allow proliferation of Rasless cells (Figure 6A). Moreover, it had no effect on the ability of A-Raf^{CAAX} or B-Raf^{CAAX} to sustain proliferation of Rasless cells in spite of the fact that the three modified Raf proteins induced similar levels of p16^{INK4a} expression (Supplementary Figure 3A). Thus, it is likely that the stress response induced by C-Raf^{CAAX} might be mediated by selective downstream effectors, although it can be ultimately bypassed by knocking down of p16^{INK4a} expression.

Ras downstream effectors: the Mek and Erk kinases

We also examined whether constitutively active forms of the Mek1 and Erk2 kinases elicited cell proliferation in the absence of upstream Ras proteins. K-Ras^{lox} cells were infected with a mutant form of human MEK1 in which the Glu^{56} residue was replaced by proline (Bottorff *et al*, 1995). Rasless MEFs expressing Mek1^{Q56P} formed colonies of proliferating cells with similar efficiency as those expressing the B-Raf^{CAAX} and A-Raf^{CAAX} kinases (Figure 6A). Likewise, ERK2MEK1LA (EM^{LA}), a constitutively active Erk2 kinase, also allowed proliferation of Rasless cells with an efficiency similar to Mek1^{Q56P} (Figure 6A). ERK2MEK1LA is a fusion protein between the rat Erk2 and the human Mek1 kinases in which the four lysine residues of the Mek1 nuclear export sequences were mutated to alanine, resulting in constitutive nuclear expression and activation (Robinson *et al*, 1998). No cell proliferation was observed when we used a similar fusion protein, ERK2MEK1, in which the nuclear export sequences were not mutated (Figure 6A) (Robinson *et al*, 1998). These observations indicate that all kinases of the Raf/Mek/Erk pathway, if properly activated, can drive cell proliferation in the absence of Ras proteins. Moreover, Rasless MEFs expressing Raf^{CAAX} proteins, Mek1^{Q56P} or EM^{LA} showed normal morphologic features and had migratory properties

indistinguishable from those of wild type or K-Ras^{lox} MEFs (data not shown).

Other Ras downstream effectors: the RalGEF and PI3K pathways

To determine whether activation of the RalGEF and/or the PI3K pathways could contribute to cell proliferation in the absence of Ras proteins, we infected K-Ras^{lox} MEFs with retroviruses encoding RalGDS and Rlf, two independent RalGEFs (Wolthuis *et al*, 1997; Matsubara *et al*, 1999). Neither of these exchange factors, even when they they carried a modified carboxy-terminal domain (RalGDS^{CAAX} and Rlf^{CAAX}), endowed proliferative properties to Rasless MEFs (Figure 7A). Similar results were obtained with their cognate substrate, the small GTPase protein RalA. Neither the wild type nor a constitutively activated isoform (RalA^{G23V}) induced colonies in Rasless cells (Figure 7A). These observations suggest that the RalGEF pathway is not primarily involved in mediating cell proliferation induced by Ras proteins.

We also infected K-Ras^{lox} cells with three distinct versions of the p110α subunit of PI3K. They included p110α^{myr}, the wild-type protein carrying a myristoylation signal at its amino-terminus to facilitate membrane anchoring (Link *et al*, 2005), a mutated isoform frequently present in human tumours, p110α^{H1047R} (Samuels *et al*, 2005), and p110α$^{myr/H1047R}$, a protein that carried both modifications. None of these proteins was able to confer Rasless MEFs the ability to proliferate (Figure 7A). Unfortunately, lack of reliable antibodies prevented us to show expression of these p110α protein isoforms in Rasless cells. However, ectopic expression of each of these proteins resulted in significant increases in phosphorylation of Akt in Ser^{473} (Supplementary Figure 4).

To avoid this limitation, we knocked down the tumour suppressor PTEN, a lipid phosphatase that antagonizes PI3K activity (Cully *et al*, 2006). We used a PTEN shRNA that efficiently inhibited PTEN expression >90% and resulted in robust phosphorylation of Akt (Figure 7B; Supplementary Figure 4). However, K-Ras^{lox} MEFs infected with this PTEN shRNA failed to proliferate in the presence of 4OHT (Figure 7A). Thus, indicating that constitutive activation of the PI3K/PTEN/Akt pathway cannot sustain proliferation of MEFs in the absence of Ras proteins.

As illustrated in Supplementary Figure 4, activation of the PI3K/PTEN/Akt pathway also resulted in increased expression of the p16^{INK4a} tumour suppressor. Thus, we examined whether the failure of this pathway as well as the RalGDS/RalA pathway to sustain cell proliferation in the absence for Ras proteins might be due to activation of a p16^{INK4a}-mediated stress response. Ectopic expression of PTEN shRNA and Rlf^{CAAX}, with the same p16^{INK4a} shRNA used above to license C-Raf^{CAAX} activity, also failed to induce colonies of Rasless cells (Supplementary Figure 5). Similar results were obtained by expressing two representative Ras-like small GTPases, R-Ras2^{G23V} and E-Ras (Supplementary Figure 5).

Cooperation between Ras signalling pathways

To identify potential synergisms between the Raf/Mek/Erk, PI3K/Akt and RalGEF pathways, K-Ras^{lox} MEFs were

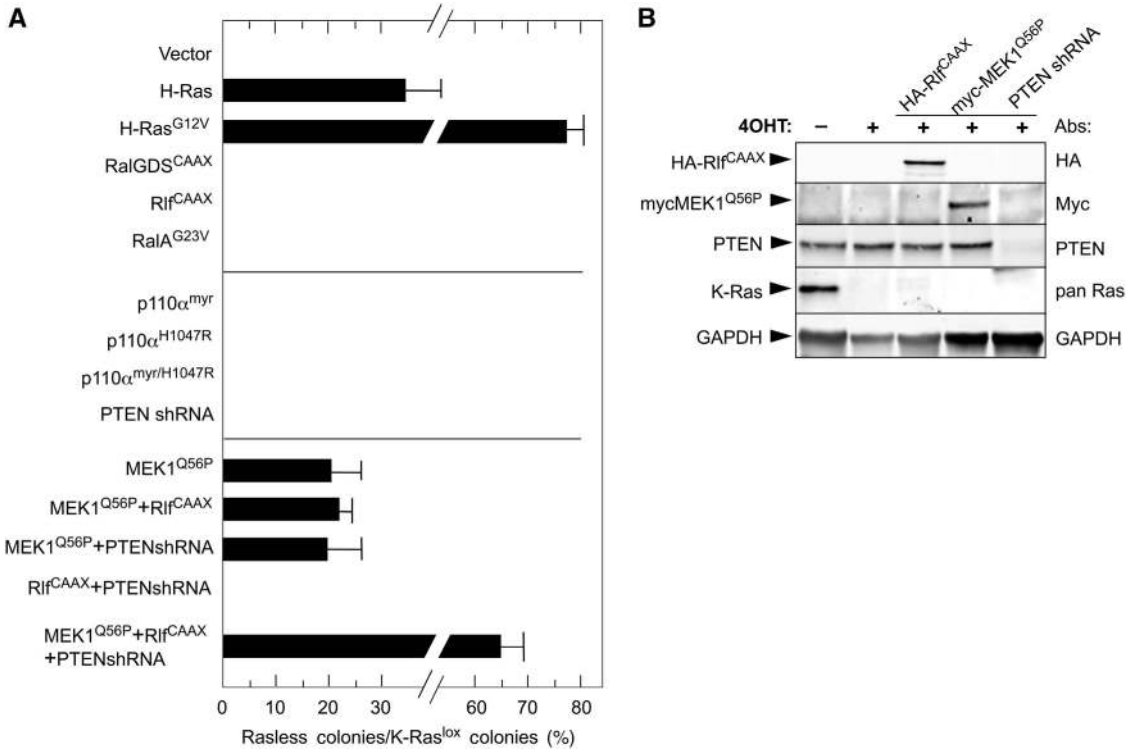

Figure 7 Constitutive activation of the RalGDS and PI3K pathways fails to confer proliferative properties to Rasless cells. (**A**) Colony-formation assay using K-Ras^{lox} MEFs infected with retroviruses expressing the indicated cDNAs or an shRNA directed against PTEN-specific sequences. Error bars indicate standard deviation. (**B**) Western blot analysis of K-Ras^{lox} MEFs infected with the indicated retroviruses. Cells were left untreated (−) or exposed (+) to 4OHT. Probing antibodies are listed in the right panel. Migration of the corresponding proteins is indicated by solid arrowheads. GAPDH expression served as loading control.

co-infected with retroviruses encoding Rlf^{CAAX} and PTEN shRNA. No colony formation could be observed on exposure to 4OHT, indicating that concomitant activation of the RalGDS and PI3K pathways cannot compensate for the lack of activation of the Raf/Mek/Erk kinases (Figure 7A). Next, we interrogated whether these pathways could cooperate with the Raf/Mek/Erk pathway. K-Ras^{lox} MEFs were co-infected with retroviruses encoding $Mek1^{Q56P}$ with either Rlf^{CAAX} or PTEN shRNA. No significant cooperation could be observed (Figure 7A). However, when we co-expressed $Mek1^{Q56P}$, Rlf^{CAAX} and PTEN shRNA, the percentage of proliferating Rasless colonies were significantly increased to levels similar to those observed with retroviruses encoding the constitutively activated H-Ras^{G12V} oncoprotein (Figure 7).

Ras signalling and the cell cycle

It is widely accepted that one of the ultimate targets of Ras signalling is to induce expression of D-type Cyclins to activate Cdk4 and Cdk6. In turn, these complexes phosphorylate and inactivate the pocket proteins to license initiation of the cell cycle (Aktas *et al*, 1997; Coleman *et al*, 2004). Availability of Rasless cells has allowed us to test this hypothesis by genetic means. Surprisingly, Rasless cells are not devoid of D-type Cyclins. Instead, they showed increased levels of Cyclin D1, the main D-type Cyclin expressed in MEFs (Figure 8A). Cyclin E1, another Cyclin presumably involved in the early phases of the cell cycle, was still present in Rasless MEFs (Figure 8A). In addition, Cyclin D1/Cdk4 and Cyclin E1/Cdk2 complexes were present at normal levels in Rasless MEFs (Figure 8B). However, these complexes are not capable of phosphorylating pRb at their cognate residues Ser^{807} and Ser^{811} (Figure 8C). Analysis of the *in vitro* kinase activities of these complexes obtained from Rasless cells showed that they were less efficient in phosphorylating recombinant pRb protein *in vitro* than the corresponding complexes obtained from proliferating K-Ras^{lox} MEFs cells than in control cells (Supplementary Figure 6). Other cell cycle-related complexes were not taken into consideration as Cdk1 is absent from Rasless MEFs (Supplementary Figure 6). These observations indicate that Ras proteins are dispensable for the regulation of Cyclin D1 and Cyclin E1 expression. However, the Cyclin D1/Cdk4 and Cyclin E1/Cdk2 complexes present in these cells are not active and, thus, cannot inactivate the pocket proteins. Failure to inactivate the pocket proteins, and hence

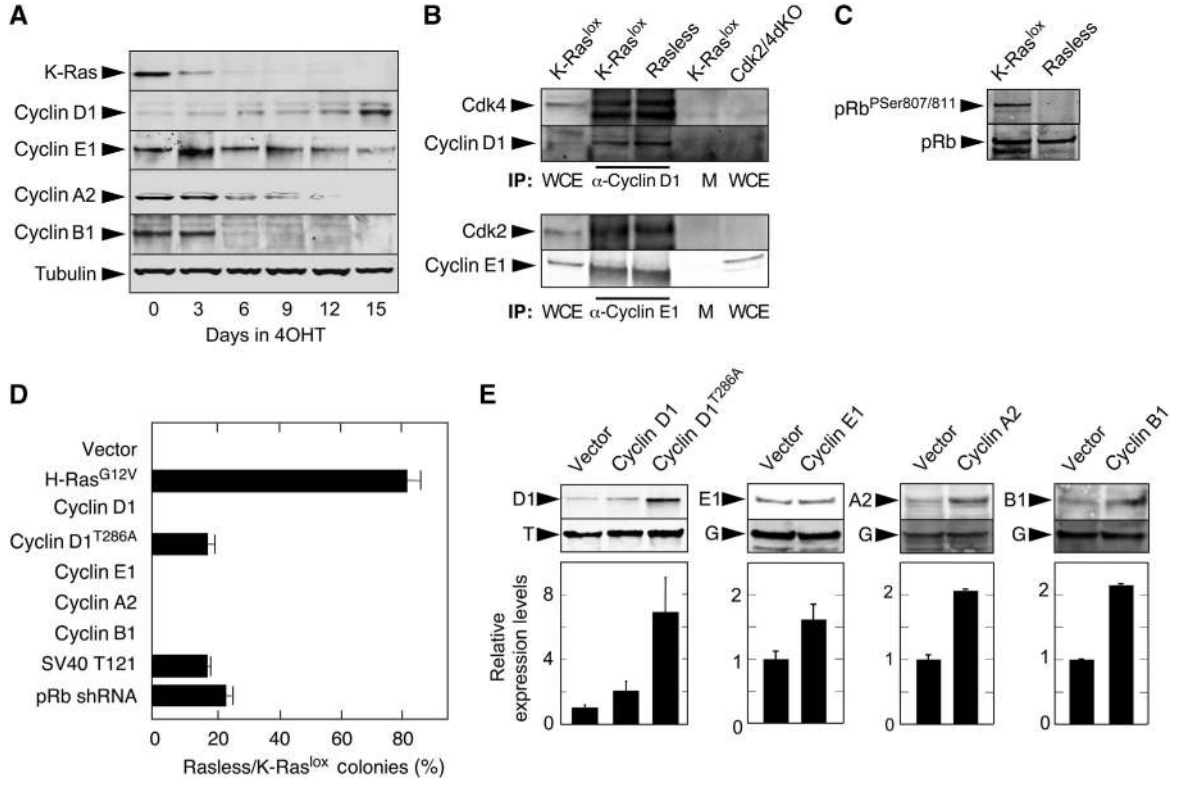

Figure 8 Role of cell cycle regulators on the proliferative properties of Rasless cells. (**A**) Expression levels of cell cycle Cyclins in K-Ras^{lox} MEFs exposed to 4OHT for the indicated times as determined by western blot analysis. Loss of K-Ras expression is illustrated in the upper panel. α-Tubulin expression served as loading control. (**B**) Identification of Cyclin D1/Cdk4 (upper panel) and Cyclin E1/Cdk2 (lower panel) complexes by immunoprecipitation with antibodies against Cyclin D1 (upper panel) and Cyclin E (lower panel) in K-Ras^{lox} and Rasless cells; 1/16th of the whole cell extract (WCE) used for immunoprecipitation of K-Ras^{lox} MEFs was loaded as a control. Additional controls included mock (M) immunoprecipitates obtained from K-Ras^{lox} MEFs by incubating without primary antibody and 1/16th of WCE from $Cdk2^{-/-}$; $Cdk4^{-/-}$ MEFs (Cdk2/4dKO). (**C**) Analysis of pRb phosphorylation in K-Ras^{lox} and Rasless cells by western blot analysis using antibodies against PSer807/811-phosphorylated pRb. Total pRb was used as a loading control. (**D**) Colony-formation assay using K-Ras^{lox} MEFs infected with retroviruses expressing the indicated cDNAs or an shRNA directed against pRb-specific sequences. Error bars indicate standard deviation. (**E**) Upper panels: western blot analysis of Cyclin D1, Cyclin E1, Cyclin A2 and Cyclin B1 expression in K-Ras^{lox} MEFs infected with an empty vector or with retroviruses encoding wild-type Cyclin D1, Cyclin $D1^{T286A}$, Cyclin E1, Cyclin A2 and Cyclin B1. Migration of the indicated proteins is shown by arrowheads. α-Tubulin (T) or GAPDH (G) expression served as loading controls. Lower panels: quantification of protein expression levels from at least two independent experiments.

to drive cells through G1 explains the lack of expression of Cyclin A2, Cyclin B1 and their cognate partner Cdk1 in Rasless cells (Figure 8A; Supplementary Figure 6).

To further test whether inhibition of the pocket proteins is one of the ultimate outcomes of Ras signalling to drive cell proliferation, we inactivated the pocket proteins in these cells by two independent approaches. First, we expressed a fragment of the SV40 large T antigen (T121) known to inhibit all three members of the Rb family (Saenz Robles *et al*, 1994). As shown in Figure 8D, Rasless cells expressing T121 proliferated efficiently. Similar results were obtained using an shRNA specific for the pRb protein (Figure 8D). These observations indicated that MEFs can proliferate in the absence fo Rass proteins providing that the pocket proteins are inhibited. Moreover, they suggest that in these cells pRb is the main effector of Ras signalling (Coleman *et al*, 2004). Nevertheless, the levels of proliferating colonies in Rasless cells expressing T121 and pRb shRNA were considerable lower than in wild-type cells or in Rasless cells ectopically expressing the constitutively activated H-Ras^{G12V} oncoprotein. Thus, indicating that other Ras-mediated signalling pathways are likely to contribute to drive the cell cycle.

Ectopic expression of Cyclin D1 resulting in a two-fold increase in its levels of expression also failed to sustain proliferation of Rasless cells (Figure 8D and E). Similar results were obtained with the G2/M Cyclins, Cyclin A2 and Cyclin B1, suggesting that Rasless cells are blocked in G1 (Figure 8D and E). This hypothesis was further supported by expressing a non-degradable mutant of Cyclin D1, Cyclin D1^{T286A} (Diehl *et al*, 1998). Overexpression (7–8-fold) of this protein in Rasless cells allowed proliferation of these cells with the same efficiency as inactivation/knockdown of pRb (Figure 8D and E; Supplementary Figure 6). Thus, suggesting that overloading the cell nucleus with large amounts of Cyclin D1 could activate the resident Cdk4/6 kinases, leading to phosphorylation and inactivation of pRb.

Discussion

Selective ablation of the three *Ras* loci in MEFs has shown that each Ras protein is capable of sustaining proliferation of MEFs in culture in the absence of the other two. Moreover, MEFs expressing individual *Ras* genes became immortal on continuous passage in culture. Nevertheless, K-Ras proteins (K-Ras4B and K-Ras4A) were more efficient to drive cell proliferation and immortalization than H-Ras or N-Ras. Indeed, cells expressing K-Ras proteins alone proliferated and bypassed senescence as efficiently as wild-type MEFs. These differences are not because of changes in expression levels as ablation of individual Ras proteins did not affect expression of the other members of the family, thus suggesting that they are independently regulated. Expression of chimaeric Ras proteins carrying distinct combinations of effector and carboxy-terminal domains in Rasless cells should provide relevant information as of whether the differential properties of the K-Ras proteins are due to their interaction with downstream effectors or to their distinct subcellular localization (Buday and Downward, 2008; Omerovic and Prior, 2009).

Earlier studies using anti-Ras antibodies (Mulcahy *et al*, 1985) and Ras dominant negative mutants (Feig and Cooper, 1988) have suggested that Ras proteins are essential for cell proliferation. Our results, involving ablation of the three *Ras* genes provide definitive genetic proof for these earlier observations. Our observations indicate that the cell cycle arrest because of ablation of Ras proteins is reversible and does not elicit an apoptotic or a senescence response. In addition, our results showed that loss of Ras proteins induced major alterations in cytoskeletal structures, such as microtubules and stress fibres resulting in a significant increase in the number of focal adhesions along with a reduced turnover. As a consequence, Rasless MEFs showed a very flat morphology, have reduced membrane plasticity and are not able to migrate. Thus, Ras signalling is also essential for cell migration, at least in rodent fibroblasts.

Our results provide genetic evidence that none of the members of the large Ras superfamily of small GTPases can compensate for the absence of Ras proteins. Ectopic expression of constitutively activated small GTPases known to induce malignant transformation of rodent fibroblasts, such as the R-Ras family and E-Ras, also failed to induce proliferation in the absence of Ras proteins. Interestingly, these constitutively active GTPases required the presence of Ras proteins to activate the Raf/Mek/Erk pathway. This requirement is unlikely to be mediated by Ras-dependent activation of exchange factors, such as Tiam1 or RalGDS, as the small GTPases are already constitutively active. It is possible that Ras proteins are required to allow specific effectors for the R-Ras/E-Ras GTPases to either phosphorylate Mek and Erk kinases or prevent their dephosphorylation by negative regulators (Rodriguez-Viciana *et al*, 2006).

Among the best-characterized Ras effector pathways, only the Ras/Mek/Erk pathway was capable of sustaining cell proliferation in the absence of Ras proteins. Interestingly, the only requirement for the Raf kinases to induce proliferation of Rasless cells was to be attached to the plasma membrane. These observations provide further support to the concept that the main role of Ras proteins to activate the Ras/Mek/Erk pathway is to bring the Raf kinases to the membrane (Wellbrock *et al*, 2004). Each of the Raf kinases induced proliferation of Rasless cells to similar levels, independently of their potential to activate Mek kinases or to transform NIH3T3 cells. However, C-Raf^{CAAX}, the most efficient Raf isoform in activating Mek and Erk proteins and in inducing tranformation of NIH3T3 cells, elicited a stress response that could be overcome by knocking down p16^{INK4a} expression. Interestingly, all Raf^{CAAX} proteins, as well as the H-Ras^{G12V} and K-Ras^{G12V} oncoproteins, induced similar levels of p16^{INK4a} expression. Thus, suggesting that the stress response induced by C-Raf^{CAAX} is mediated by selective downstream effectors.

Constitutively, active forms of Mek1 and Erk2 kinases were as efficient as the membrane-bound Raf kinases to induce cell proliferation in the absence of Ras proteins. These observations provide further support to the concept that the Raf/Mek/Erk pathway is primarily a linear signalling pathway in which activation of each kinase family has similar biological readouts (Roberts and Der, 2007). Thus, the evolutionary requirement for the linear activation of these families of kinases must stem from activities other than basic cell proliferation or by inducing proliferation in other cell types.

Genetic interrogation of the PI3K and RalGEF pathways in Rasless cells, either alone or in combination, indicates that

they cannot sustain cell proliferation in the absence of Ras proteins, at least in rodent fibroblasts. Whether these pathways could sustain proliferation of other cell types in the absence of Raf/Mek/Erk signalling is currently under investigation (Gupta *et al*, 2007). However, activation of the PI3K and RalGEF pathways complemented Mek1-mediated signalling to levels similar to those obtained expressing constitutively active Ras proteins. Thus, reconstitution of the full proliferative response mediated by Ras signalling requires cooperation by the three main Ras effector pathways, PI3K, RalGEF and Raf/Mek/Erk. Interrogation of other cellular functions beyond cell proliferation should help to better ascertain the individual contributions of each of these pathways in mediating Ras signalling.

The growth arrest characteristic of Rasless MEFs is fully reversible. To what extent the cell cycle arrest induced by loss of Ras protein expression in the presence of serum is similar to the quiescence (G_0) reached by the absence of serum is currently unknown. Downregulation of a single pocket protein, pRb, was sufficient to confer proliferative properties to cells devoid of Ras proteins. Thus, our results support the concept that the ultimate readout of Ras signalling is inactivation of the pocket proteins (Coleman *et al*, 2004). However, our results do not support the widely accepted hypothesis that Ras proteins induce initiation of the cell cycle by inducing expression of D-type Cyclins. Indeed, growth-arrested Rasless cells have even increased levels of Cyclin D1 and normal levels of Cyclin D-Cdk4/6 or Cyclin E-Cdk2 complexes. However, these complexes do not have kinase activity *in vitro* and are unable to phosphorylate pRb *in vivo*. Thus, Ras signalling results in initiation of the cell cycle by activating pre-existing Cyclin D-Cdk4/6 or Cyclin E-Cdk2 complexes rather than by inducing expression of D-type Cyclins.

It is likely that the lack of activity of these Cyclin/Cdk complexes is due to the presence of a cell cycle inhibitor rather than to activation of other effectors. The ability of a non-degradable mutant of Cyclin D1 to induced proliferation of Rasless cells supports this concept. However, such inhibitor is unlikely to be the $p16^{INK4a}$ tumour suppressor as downregulation of $p16^{INK4a}$ did not restore proliferation of Rasless MEFs. Recent results from our laboratory suggest that the cell cycle inhibitor $p21^{Cip1}$ might be involved in preventing cell proliferation in the absence of Ras proteins (unpublished observations).

In summary, genetic interrogation of Ras signalling has confirmed some of the basic tenants of Ras biology. However, our observations have raised important issues about other widely accepted hypotheses regarding Ras signalling such as their role in inducing Cyclin D expression or in sustaining cell survival. Ablation of Ras proteins in more specialized cell types such as keratinocytes, haematopoietic cells or neurons should provide a better and wider picture of the biological role of these key signalling molecules in mammalian cells.

Materials and methods

Cell culture assays

Wild type, H-*Ras*$^{-/-}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$, H-*Ras*$^{+/+}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$, and H-*Ras*$^{-/-}$;N-*Ras*$^{+/+}$;K-*Ras*$^{lox/lox}$;RERT$^{ert/ert}$ MEFs were isolated from E13. 5 embryos of the corresponding genotype and frozen as primary cultures or immortalized following a standard 3T3 protocol, as described earlier (Malumbres *et al*, 2004). Other cell lines including NIH3T3 cells and the retroviral packaging Phoenix-Eco cells (ATCC) have been described (Martín *et al*, 2005). Low passage (p12–15), immortal H-*Ras*$^{-/-}$;N-*Ras*$^{-/-}$;K-*Ras*$^{lox/lox}$,RERT$^{ert/ert}$ MEFs were infected with retroviral supernatants and selected with the appropriate antibiotic as described earlier (Martín *et al*, 2005). Resistant cells were seeded in equal cell numbers ($5–10 \times 10^3$) in the absence or presence of 4OHT (Sigma, 600 nM). Cells were allowed to form colonies for 2 weeks. Plates were fixed with 1% glutaraldehyde (Sigma), stained with crystal violet (Merck) and colonies >2 mm in diameter scored. When needed, representative colonies were picked and expanded for further analysis.

Protein analysis

For western blot analysis, cells were lysed as described earlier (Sotillo *et al*, 2001). Usually, 40 μg of total protein extract was resolved by SDS–PAGE and transferred to nitrocellulose membranes. Probing antibodies are described in Supplementary data. For immunoprecipitation, antibodies against Cyclin D1 (Ab-4, Neomarkers) or Cyclin E1 (Abcam) were used. Kinase assays were performed as described (Malumbres *et al*, 2004). Recombinant pRb used as a substrate in kinase assays was obtained from Santa Cruz Biotechnology (Rb 769).

Cell migration assay and time-lapse videomicroscopy

To trace the movement of individual MEFs, cells were seeded on fibronectin-coated plates and imaged with a DeltaVision Microscope (Stress Photonics) for 14 h at 1 frame/5 min and manually tracked with Metamorph (Molecular Devices). For focal adhesion dynamics, cells were transfected with a plasmid encoding DsRed-Zyxin using lipofectamin (Invitrogen) and imaged with an SP5-MP confocal microscope (Leica) for 45 min at 2 frames/min. To visualize the cell cycle arrest in live MEFs, cells were initially treated with 4OHT for 5 days and then imaged with an SP2 confocal microscope (Leica) for 60 h at 1 frame/5 min.

Confocal microscopy

For confocal microscopy, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Primary antibodies for Cyclin D1 (Clone DCS-6, Neomarkers), α-tubulin (DM1a, Sigma) and Paxillin (clone 5H11, Upstate) were incubated in PBS with 3% BSA. To stain actin fibres, Alexa Fluor 488 Phalloidin (Invitrogen) was directly added to the primary antibody mixture. Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Invitrogen) were used to detect the indicated proteins. Cells were counterstained with DAPI (Sigma).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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