Ras induces elevation of E2F-1 mRNA levels.

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Abstract

Both E2F-1 and Ras play pivotal roles in regulation of cell proliferation and, in some biological settings, they collaborate in cell transformation. We show here that activated Ras induces an increase in E2F-1 mRNA and protein levels. This Ras-induced increase in E2F-1 levels is dependent on both MEK and PKB and it is RB-independent. The effect of Ras on up-regulation of E2F-1 mRNA is at the level of mRNA stability. Our data describe a novel functional link between Ras and the RB/E2F pathway. Furthermore, we suggest that one of the molecular mechanisms underlying the collaboration between Ras and E2F-1 involves a Ras-induced elevation of transcriptionaly active E2F-1 levels.
Introduction

The E2F transcription factors control cell cycle-dependent expression of genes that are essential for cell proliferation (for review see (1,2)). The DNA binding complex named E2F is a heterodimeric complex consisting of an E2F component and a dimerization partner, DP. To date, six E2F genes and two DP genes have been cloned (2). E2F-1, -2 and -3 represent a subgroup of the E2F family, and they are specifically regulated by RB and not by the RB related proteins, p107 and p130.

In agreement with the regulation of many growth related genes by E2F, overexpression of E2F-1, -2, or -3 is sufficient to induce quiescent cells to enter S-phase (3-7).

The Ras gene family encodes small GTP-binding proteins that play a critical role in cell growth control as pivotal mediators of mitogenic signals from tyrosine kinase receptors (Reviewed by (8)). Mutations in Ras genes which result in constitutively activated Ras proteins are frequent in human tumors (reviewed by (9)).

Co-expression of activated Ras together with E2F-1 and its heterodimeric partner DP-1 leads to the formation of morphologically transformed foci in primary rat embryo fibroblasts and these cells induce tumor formation in nude mice (10). Furthermore, double transgenic animals over-expressing E2F-1 and activated Ras in their epidermis develop skin tumors (11). The molecular mechanisms underlying the co-operation between Ras and E2F-1 in cell transformation are currently not fully understood. When expressed alone, either deregulated E2F-1 or constitutively active Ras transform immortal rodent cells (12,13), but not primary cells. In fact, expression of either E2F-1 or activated Ras in primary cells leads to cell cycle arrest resembling premature senescence (14-16). In both cases induction of the senescence-like phenotype involves up-regulation of the expression of p19ARF which neutralizes MDM2 and thereby stabilizes p53 (6,17).

E2F activity is tightly regulated by a number of mechanisms during cell cycle progression. E2F/DP heterodimer formation facilitates binding to and negative regulation by the product of the retinoblastoma gene, RB and its related proteins p107 and p130, collectively referred to as the pocket proteins. Indeed, complexes of unphosphorylated RB and E2F/DP act as transcriptional repressors, which contribute to RB-dependent cell cycle arrest in G1. Complex formation is cell cycle regulated via phosphorylation of the pocket proteins by Cdk4/cyclin D.
and Cdk2/cyclin E heterodimers. These phosphorylations lead to dissociation of E2F/pocket protein complexes, resulting in free, transcriptionally active E2F/DP heterodimers. The combination of cessation of repression of some E2F-regulated genes and activation of others by the now activated transcription factor(s) is a major step in promoting G1 exit. Additional controls of E2F-1 activity include up-regulation of its DNA binding activity by acetylation (18) and down-regulation of this activity via phosphorylation of DP-1 by Cdk2/cyclin A (19-21). E2F-1 mRNA and protein levels are also tightly regulated. It is subjected to cell cycle dependent transcriptional control and its mRNA level peak in late G1. In addition, E2F-1 is a short-lived protein and is degraded by the proteasome pathway (22-24).

In view of the ability of both Ras and E2F-1 to play a role in cellular transformation on the one hand and to induce premature cell senescence on the other hand, we studied possible functional relationships between Ras and E2F-1. We report here that activated Ras up-regulates both E2F-1 mRNA and protein levels. This constitutes a novel mechanism of regulating E2F-1 levels.

**Experimental Procedures**

**Cell Culture.**
Rat-1a-MT-wtE2F-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and G418 (500µg/ml, GibcoBRL). 293 and 293T cells were grown in DMEM supplemented with 10% fetal calf serum (FCS). H1299 cells were grown in RPMI supplemented with 10% FCS. Swiss 3T3, NIH 3T3 and 3T3 fibroblasts derived from RB-/- mice were grown in DMEM supplemented with 10% bovine calf serum (BCS). All cells were maintained at 37°C in a humidified 8% CO2 - containing atmosphere. For Actinomycin D treatment: After infection of Rat-1a-MT-wtE2F-1 cells they were selected with 2µg/ml puromycin for 24hr. Then Actinomycin D (10µg/ml, Sigma A-9415) was added for the indicated times. For cyclohexamide treatment: 24hr after transfection of 293 cells, 10µg/ml of cyclohexamide (Sigma C0934) were added to the plates for the indicated times and then cells were harvested. For MG-132 treatment: 24hr after transfection of 293 cells, 50µM of MG-132 (Calbiochem 474790) were added to the plates for 2hr. and then cells were harvested.

**Plasmids.**
The following plasmids have been previously described: pcDNAI-E2F-1, pcDNAI-HA-DP-1 and pcDNAI-E2F-1A18 (25). pReCMV-HA-E2F-2 and pReCMV-HA-E2F-3 (12). pBABE-RasN17, pCMV-HA-RasV12 (26). pCMV-HA-E2F4, pCMV-β-Gal (27). E1β-luciferase (17). pECEmyrΔ4-129Akt (28). pCMV-ΔN-EE-MEK (29). pSV-ψ-E-MLV (30).
pcDNAIII-HA-E2F-1(381-1311) was generated by PCR using pcDNAI-E2F-1 as template and oligonucleotides GTTGGATCCGCCATGTATGAGACCTCACTGACC and GCCGAATTCTCAGAAATCCAGGGGGGTGAG. pcDNAIII-HA-E2F-1(1-1089) was generated from pRcCMV-HA-E2F1(1-363) (22) by digestion with HindIII and partial digestion with BglII followed by subcloning of the fragment containing bases 1-1089 of E2F-1 coding region into pcDNAIII.

**Retroviral infection, transfections and reporter assay.**

Cells of the packaging cell line 293T (2x10^6 cells) were co-transfected with 10µg of ψ ecotropic packaging plasmid, pSV-ψ-E-MLV, providing packaging helper function and 10µg of the relevant plasmid using the calcium phosphate method. Chloroquin (25µM final concentration, Sigma C6628) was added to the transfection medium. After 8hr the transfection medium was replaced with fresh DMEM supplemented with 10% FCS, and 5ml of medium containing retroviruses were collected at 6hr intervals. Five collections were pooled together and frozen in aliquots. For infection of Swiss 3T3 or Rat1a, cells were incubated for 5hr at 37°C in 3 ml of retroviral supernatant, supplemented with 8µg/ml of polybrene (Sigma H9268). Then, 7 ml of DMEM, containing 10% FCS were added, and after 24hr the medium was replaced with fresh medium containing 10% serum and 2µg/ml of puromycin (Sigma P7130).

Transfections - H1299 and 293 cells were transfected by a calcium phosphate method. NIH 3T3 cells and 3T3 fibroblasts derived from RB⁻/⁻ mice were transfected using lipofectAMINE reagent (GIBCO BRL). Cells were harvested 24-40hr following transfection. Cell lysis, β-Gal, and luciferase assays were performed essentially as described (27).

**Western blot analysis.**

For western blot analysis 30µg of protein from each lysate, as determined by Bradford assay, or 20µl from the luciferase assay extracts were loaded, resolved by electrophoresis on an SDS 10% polyacrylamide gel and transferred to filters (Protran, BA 85, S&S). Filters were incubated with the indicated antibodies over night in PBS with 0.05% Tween and 5% dry milk. Binding of the primary antibodies was detected using an enhanced chemiluminescence kit (ECL, Amersham).

**Northern blot analysis.**

RNA was extracted from cells using the Tri Reagent method (MRC, TR-118). 30µg of total RNA from each sample were separated on a formaldehyde gel and then blotted to GeneScreen Plus membrane (DU PONT) according to manufacturer’s instructions.
Results

Oncogenic Ras elicits an increase in E2F-1 levels

The effect of Ras on E2F-1 was studied initially using co-transfection experiments. Co-expression of E2F-1 and oncogenic Ras (H-RasV12) in 293 cells led to a significant increase of E2F-1 protein level. This increase in the levels of E2F-1 did not depend on co-expression of its heterodimeric partner, DP-1, since it was detected both in the absence and presence of DP-1 (Fig 1A). Similar results were obtained using the human lung carcinoma cell line H1299 (data not shown). Levels of E2F-2 and E2F-3 were similarly elevated upon co-expression of oncogenic Ras (Fig. 1B). However, this Ras-induced increased of E2F levels was not shared by all E2Fs and, as previously reported (31), activated Ras did not cause a significant change in the levels of another E2F family member, E2F-4. This suggests that the effect of H-RasV12 on E2F level is specific to the E2F-1, -2, -3 sub-family. Additional studies focused on the effect of oncogenic Ras on E2F-1 levels.

In certain settings E2F-1 can induce apoptosis (4,32,33) and Ras can inhibit apoptosis (34). Therefore, we tested whether the differences we detect in E2F-1 protein level are due to changes in cell viability. H1299 cells were transfected with a GFP expression vector together with E2F-1 either alone or with H-RasV12 and then viable transfectants were counted. No apoptotic cells were detected among the GFP positive cells. In the presence of E2F-1, 7.15% of all viable cells were GFP positive, while in the presence of both E2F-1 and H-RasV12 6.3% of the viable cells were GFP positive. These similar values indicate that, under these experimental settings, inhibition of E2F-1-induced apoptosis by H-RasV12 is not the cause for the elevated E2F-1 protein levels. Immunostaining for E2F-1 demonstrated that, as reported (27), it is a nuclear protein. More importantly, the Ras induced increase in E2F-1 levels did not affect the sub-cellular localization of E2F-1 (data not shown), ruling out changes in sub-cellular localization as the reason for changes in protein levels.

The effect of Ras on E2F-1 protein levels was not limited to exogenous E2F-1. Expression of endogenous E2F-1 is cell cycle-dependent; in cells emerging from growth arrest, it peaks during late G1 (35,36). We analyzed endogenous E2F-1 levels in H1299 cells infected with either a retrovirus expressing H-RasV12, H-RasN17 or an empty retroviral vector. As expected, E2F-1 levels increased upon serum stimulation of starved cells. Interestingly, H-RasV12 augmented the increase of endogenous E2F-1 protein level in serum-stimulated cells (Fig. 1C). Moreover, endogenous Ras activity was required for the cell cycle-dependent induction of endogenous E2F-1 since dominant negative H-RasN17 interfered with this
induction (Fig. 1C). Similar results were obtained using Swiss 3T3 murine fibroblasts (data not shown). These results corroborate our data with exogenous E2F-1 and further implicate Ras in the physiological regulation of E2F-1 levels.

E2F-1 is a short-lived protein and is degraded through the ubiquitin-proteasome pathway (22,24). Therefore, a plausible mechanism explaining the Ras-induced increase in E2F-1 protein levels involves interfering with E2F-1 degradation. However, this is not the case since analysis of E2F-1 protein stability by measurement of its half-life time indicated that the E2F1 protein stability was not significantly altered in the presence of activated Ras (Fig 2A, B). Inhibition of proteasome activity further supported the notion that activated Ras does not have a notable effect on E2F-1 degradation. As expected, treating cells with a proteasome inhibitor, MG132, resulted in an increase in E2F-1 protein levels. In cells co-expressing E2F-1 and Ras, E2F-1 protein level was significantly higher prior to MG132 addition but it was further increased, to a similar extent, upon MG132 addition (Fig 2C). Thus, the Ras-induced increase in E2F-1 protein levels is most probably not due to inhibition of E2F-1 protein degradation.

Next, we studied the effect of Ras on E2F-1 levels using a Rat-1a-derived cell line containing a stably integrated Zinc-inducible E2F-1 (Rat-1a-MT-wtE2F-1) (4). Infection of these cells with a retrovirus harboring H-RasV12 resulted in a significant increase in E2F-1 protein after addition of Zinc (Fig. 3A). This increase was accompanied by an elevation in E2F-1 mRNA (Fig. 3B). A similar elevation was detected in endogenous E2F-1 mRNA levels upon infection of Swiss 3T3 cells with a retrovirus harboring H-RasV12. Hence, Ras elevates E2F-1 mRNA levels. Ras-dependent transcriptional regulation of the E2F-1 gene is probably not the underlying mechanism since the increase in E2F-1 was detected also when E2F-1 expression was driven by heterologous promoters (Figures 1A, 3A). Therefore, we next studied the effect of Ras on the half-life of E2F-1 mRNA. As can be seen in figure 3C exogenous E2F-1 mRNA was easily detectable in Rat-1a-MT-wtE2F-1 cells, however, its levels were reduced below detection level 3 hours after addition of actinomycin D. In the presence of activated Ras E2F-1 mRNA levels were not significantly changed after 3 hours and only slightly reduced after 5 hours of the same treatment. These data indicate that activated Ras leads to a significant increase in E2F-1 mRNA stability.

The Ras-induced increase in E2F-1 mRNA levels was detected when oncogenic Ras was co-expressed with an E2F-1 expression plasmid containing the full E2F-1 coding region and no 3’ or 5’ non-coding sequences (i.e. bases 1-1311 of the coding region). A similar Ras-induced
increase was detected when using an E2F expression plasmid containing bases 1-1089 of the coding region (encoding amino acids 1-363). In contrast, co-expression of oncogenic Ras did not elevate the mRNA levels of a truncated E2F-1 containing only bases 381-1311 of its coding region (Fig 3D). These data indicate that the effect of oncogenic Ras on E2F-1 mRNA stability is mediated by an element within bases 1-381 of the E2F-1 coding sequence.

The Ras induced increase in E2F-1 levels is mediated by MEK and PKB and it is RB independent

Since Ras activates a number of signal transduction pathways, we tested which of these pathways plays a role in controlling E2F-1 protein levels. Co-expression of either activated PKB or activated MEK together with E2F-1 resulted in elevated E2F-1 protein levels (Figure 4A,B), indicating that both the PI3K/PKB pathway and the MAPK/MEK pathway can contribute to the Ras-dependent increase in E2F-1 levels. The Ras-induced increase in E2F-1 protein levels was not blocked by either the PI3K inhibitor Wortmanin alone or by the MEK inhibitor PD-098059 alone (Fig. 4C). However, it was diminished upon simultaneous treatment of cells with both inhibitors (Fig. 4C), further supporting the notion that both PKB and MEK play a role in the Ras-induced increase of E2F-1 levels.

A well-established effect of activated Ras on the RB/E2F pathway involves Ras-induced accumulation of cyclin D1. This accumulated cyclin D1 complexes with Cdk4, resulting in phosphorylation of RB and release of active E2Fs. Indeed, the presence of functional RB was shown to be essential for some of the effects of Ras on cell proliferation (26,37). However, this effect of Ras on the cyclin D/Cdk4/RB/E2F pathway does not lead to an increase in E2F-1 levels. To test whether RB is required for the increase that we observe, we studied the effect of H-RasV12 on the levels of an E2F-1 mutant lacking the RB binding domain, E2F-1Δ18. As can be seen in figure 5A levels of both wtE2F-1 and E2F-1Δ18 were similarly increased upon co-expression of activated Ras suggesting that the effect of Ras is RB-independent. Furthermore, co-expression of H-RasV12 and E2F-1 in 3T3 fibroblasts derived from RB<sup>−/−</sup> mice led to an increase in E2F-1 protein levels similar to that detected in wt 3T3 cells (Figure 5B). Thus, RB is dispensable for this increase.

The Ras-induced increase in E2F-1 levels is accompanied by an increase in the promoter activity of an E2F-regulated gene suggesting that the Ras-induced E2F-1 is transcriptionally active (fig. 6).
Overall, the data presented here demonstrate that activated Ras leads to an increase in E2F-1 mRNA and protein levels that results in enhanced E2F transcriptional activity.

Discussion

Both Ras and E2F-1 play pivotal roles in the control of cell proliferation and they collaborate in cell transformation both in tissue culture experiments and in transgenic mice. However, the molecular mechanism(s) by which these two regulators of cell growth cooperate are not fully understood. This collaboration may involve initiation of distinct cascades of events that synergies downstream to E2F-1 and Ras, though an effect of one of these two proteins on the activity of the other may also contribute to such a collaboration. We demonstrate here that oncogenic Ras induces an increase in the levels of E2F-1 mRNA and protein levels. Moreover, the dominant negative RasN17 abolishes the induction of E2F-1 in quiescent cells upon serum stimulation, indicating that endogenous Ras plays a role in the regulation of endogenous E2F-1 levels.

High levels of transcriptionaly active E2F-1 were shown to induce S-phase entry in quiescent immortal cells (3,4,38). Ras brings about the accumulation of transcriptionaly active E2F-1 (Fig. 6) and therefore, this ability of Ras to up-regulate E2F-1 levels may be an important factor in the biological activities of E2F-1. Furthermore, it may play an important role in the collaboration between Ras and E2F-1 in controlling cell growth, although additional molecular mechanisms are, most probably, involved. For example, Ras and E2F-1 might collaborate on the activation of the phosphatase Cdc25A that is a known E2F target gene (7) and is directly phosphorylated and activated by the Ras/Raf pathway (39). Another possible point of convergence of E2F-1 and Ras activities may be the control of the kinase activity of the cyclin E/Cdk2 complex. Both E2F-1 and Ras affect its activity since cyclin E is an E2F target gene (40,41) while Ras plays a central role in the control of protein levels of the Cdk2 inhibitor, p27kip1 (39,42).

The ras-dependent increase in E2F-1 levels, described here, is reminiscent of the recently reported Ras-dependent increase in the levels of another critical regulator of cell proliferation, c-myc (31). Both c-myc and E2F-1 are transcription factors that regulate cell growth and cooperate with Ras in cell transformation. Ras activity results in an increase in the protein levels and the transcriptional activity of both c-myc and E2F-1 ((31) and this
work). However, our data suggests that the molecular mechanisms underlying these increases are different. While in the case of c-myc Sears and colleagues clearly demonstrated that Ras induces an increase in the c-myc protein stability (31), we did not detect a significant change in the half-life of the E2F-1 protein in the presence of constitutively active Ras (Fig 2). Instead we observed a Ras-induced increase in the levels of E2F-1 mRNA (Fig. 3B and 3D). This increase in E2F-1 mRNA levels was seen when E2F-1 expression was driven by different promoters, making a specific Ras dependent transcriptional effect unlikely. The mechanism underlying this Ras-induced increase in E2F-1 mRNA level is enhancement of mRNA stability. This is evident from the Ras-induced increase in E2F-1 mRNA levels in the presence of Actinomycin D (Fig. 3C). The first 381 bases of E2F-1 coding sequence mediate the response to Ras-induced signals since the mRNA levels of E2F-1 lacking these 381 are not affected by co-expression of oncogenic Ras (Fig 3D). Effects of Ras on mRNA stability have been documented for other mRNA molecules such as VEGF, fibronectin, NF1 and Ornithine Decarboxylase (43-46).

Our data indicate that E2F-1 levels are affected both by the PI3 kinase/PKB pathway and by the Raf/MEK/ERK pathway. In support of this notion, the combined action of Worthmanin and PD-098059, inhibitors of PI3K and MEK respectively, is required to significantly diminish the Ras-mediated increase in E2F-1 levels. Interestingly, the Raf/ERK and the PI3K/PKB pathways are involved also in the Ras-induced stabilization of c-myc (47). In addition, a similar involvement of two independent Ras effectors has been observed in a number of other Ras-induced phenomena, including induction of parathyroid hormone-related peptide (PTHRP), repression of the homeobox gene product TTF-1 and prevention of caspase-3 activation (48-50).

The Ras-dependent regulation of E2F-1 levels is a novel functional link between Ras and the RB/E2F pathway. It comes in addition to the well-established effect of Ras on the RB/E2F pathway, namely its ability to elicit an increase in the levels of cyclin D1. Ras stimulates cyclin D1 accumulation via different mechanisms including induction of cyclin D1 gene expression (42,51-54), increased translation of cyclin D1 mRNA (55) and stabilization of the cyclin D1 protein (56,57). Complexing of the accumulated cyclin D1 with Cdk4 results in phosphorylation of RB and release of an active E2F from the RB/E2F complex. Indeed, expression of the dominant negative RasN17 was shown to prevent both RB phosphorylation and the concomitant increase in E2F activity in quiescent cells.
stimulated by serum addition (37). The effect of Ras on cyclin D results in an RB dependent effect on E2F activity, and it leads to increased E2F activity without significant changes in E2F levels. In contrast, the Ras-induced increase in E2F-1 levels described here is RB independent and it occurs in the absence of RB and also in the absence of the RB binding domain in the E2F-1 molecule.

The Ras-induced increase in E2F-1 levels and activity, reported here, constitutes a novel functional link between Ras and E2F-1 and it may be part of the explanation for their ability to collaborate in affecting cell growth.

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Figure Legends:

Figure 1:
**Ras increases protein levels of both exogenous and endogenous E2F-1.**

**A.** Ras increases protein levels of exogenous E2F-1.

293 cells were transfected with the indicated combinations of expression vectors of E2F-1, DP-1 and H-RasV12. An expression plasmid for β-Gal was included in all transfections. Cell extracts were prepared 24hr after transfection and used for β-Gal assay. Extracts containing equal β-Gal activity were used for western blot analysis with an anti E2F-1 monoclonal antibody (SC-251, Santa-Cruz). A molecular size marker in kilodaltons is shown on the left. None – cells transfected only with an expression plasmid for β-Gal.

**B.** Ras increases protein levels of exogenous E2F-1, -2, and 3 but not E2F-4.

293 cells were transfected with the expression vectors of HA-E2F-1, HA-E2F-2, HA-E2F-3 and HA-E2F-4 either alone or with H-RasV12. An expression plasmid for β-Gal was included in all transfections. Cell extracts were prepared 24hr after transfection and used for β-
Gal assay. Extracts containing equal β−Gal activity were used for western blot analysis with an anti HA monoclonal antibody (MMS-101R, BABCO). A molecular size marker in kilodaltons is shown on the left.

C. Ras augments the serum-induced expression of endogenous E2F-1. H1299 cells expressing an ecotropic retrovirus receptor were infected with a retrovirus expressing either H-RasV12 (Ras), H-RasN17 (RasN17) or a retroviral vector (Vector). 20hr post infection puromycin was added to the cultures for 24hr and then cells were kept in medium containing 0.5% serum for additional 24hr. At this point cells were either collected (-) or kept for additional 15hr in medium containing 15% serum and then collected (+). Equal amounts of cell extracts (determined by Bradford assay) were used for western blot analysis with an anti E2F-1 monoclonal antibody (SC-251, upper panel). The blot was re-blotted with an anti Ras monoclonal antibody (R02120 from transduction laboratories, lower panel). In vitro transcribed/translated E2F-1 (TNT E2F-1) is in the most right lane.

Figure 2:
Ras does not stabilize E2F-1 protein.
A. 293 cells were transfected with the expression vector of E2F-1 either alone (left panel) or with RasV12 (right panel). Protein synthesis was blocked by cycloheximide (10µg/ml). Cell extracts were prepared at different time points after cycloheximide addition (indicated at the top of each lane) and analyzed by Western blot with an anti-E2F-1 monoclonal antibody (SQ-41). Molecular size markers in kilodaltons are shown on the left.
B. E2F-1 and E2F-1/RasV12 cyclohexamide chase shown in A. were quantified using NIH image densitometry software. The background of the relevant blot was calculated from an equivalent lane area and subtracted from each value of E2F-1 O.D.. Zero time was set to 100% and all other time points were plotted on the graph as a percentage of Zero time (Y axis) cross indexed with the relevant time point (X axis). Linear correlation curves were calculated using Microsoft Excel software.
C. 293 cells were transfected with the expression vector of E2F-1 either alone or with RasV12. 24hr after transfection the cells were subjected to DMSO (-) or 50µM of MG-132 (+) for 2hr. Cell extracts were prepared 24hr after transfection and used for western blot analysis with an anti-E2F-1 monoclonal antibody (SQ-41). Molecular size markers in kilodaltons are shown on the left.

Figure 3:
Ras increases levels of E2F-1 mRNA.
A. Ras causes an increase of inducible E2F-1 protein.
Rat-1a-MT-wtE2F-1 cells were infected with a retroviral vector (Vector) or a retrovirus harboring H-RasV12 (Ras). 20hr post infection puromycin was added to the cultures for 24hr, then the cells were kept with (+) or without (-) 100µM ZnCl₂ for 16hr. Cell extracts were prepared and equal protein amounts (determined by Bradford assay) were used for western blot analysis with an anti E2F-1 monoclonal antibody (SQ-41). In vitro transcribed/translated E2F-1 (TNT E2F-1) is in the most right lane.

B. E2F-1 mRNA levels are up-regulated by Ras.
Left panel: Rat-1a-MT-wtE2F-1 (Rat1wtE2F-1) cells were infected and treated as in A. Total RNA was prepared from the cells, and equal amounts of RNA were subjected to northern analysis using an E2F-1 probe. A probe for Acidic Ribosomal Protein (ARPP PO) was used as a loading control. Right panel: Swiss fibroblasts were infected with a retroviral vector (Vector) or a retrovirus harboring H-RasV12 (Ras). 20hr post infection puromycin was added to the cultures for 24hr, then the cells were kept in medium containing 0.5%BCS for 25hr. Total RNA was prepared from the cells, and equal amounts of RNA were subjected to northern analysis using an E2F-1 probe.

C. Ras stabilizes E2F-1 mRNA.
Upper panel: Rat-1a-MT-wtE2F-1 cells were infected with an empty retroviral vector (E2F-1) or a retrovirus harboring H-RasV12 (E2F-1/rasV12). 20hr post infection puromycin was added to the cultures for 24hr, then the cells were washed and kept with 100µM ZnCl₂ for 12hr before adding Actinomycin D (10µg/ml, Sigma). Total RNA was extracted at the indicated time after Actinomycin D addition and assayed by Northern blotting with an E2F-1 probe. Lower panel: a photo of the gel before transfer. rRNA levels are used as a loading control.

D. The effect of Ras on E2F-1 mRNA is mediated by bases 1-381 of E2F-1 coding sequence.
293 cells were transfected with pCMV-β-Gal together with either an expression vector of E2F-1 or the indicated E2F-1 mutants. E2F-1(1-1089) and E2F-1(381-1311) contain the indicated bases of E2F-1 coding region. An expression vector of H-RasV12 was added where indicated (+). 24hr post transfection proteins and total RNA were extracted from the cells. Protein extracts were used for β-Gal assay. RNA amounts, after normalization for β-Gal activity, were subjected to northern analysis using an E2F-1 probe.

Figure 4:
Both PKB and MEK increase E2F-1 protein levels.
A. 293 cells were transfected with the indicated combinations of expression vectors of E2F-1, PKB (PKB) and H-RasV12 (Ras). Cell extracts were prepared 24hr after transfection and used for western blot analysis with an anti E2F-1 monoclonal antibody (SQ-41). Molecular size markers in kilodaltons are shown on the left. In vitro transcribed/translated E2F-1 (TNT E2F-1) is in the most right lane. None – untransfected cells.

B. 293 cells were transfected with the indicated combinations of expression vectors of E2F-1, H-RasV12 (Ras) and ΔN-EE-MEK (MEK) and processed as in A.
C. H1299 cells were transfected with expression vector of E2F-1 either alone or together with H-RasV12 (Ras). 2.5hr before harvesting 10⁻⁷M Wortmanin or 25μM PD-098059 or both were added as indicated (+). Cell extracts were prepared 40hr after transfection and processed as in A.

Figure 5:
Ras-induced increase in E2F-1 protein levels is Rb-independent.

A. 293 cells were transfected with the indicated combinations of expression vectors of E2F-1, E2F-1Δ18 and H-RasV12 (Ras). Cell extracts were prepared 24hr after transfection and used for western blot analysis with an anti E2F-1 monoclonal antibody (SQ-41). Molecular size markers in kilodaltons are shown on the left. None – untransfected cells.

B. 3T3 mouse fibroblasts from Rb⁺/⁺ and Rb⁻/⁻ genotype were transfected with the indicated combinations of expression vectors of E2F-1 and H-RasV12 (Ras). An expression plasmid for β-Gal was included in all trasfections. Cell extracts were prepared 40hr after transfection and used for β-Gal assay. Extracts containing equal β-Gal activity were subjected to western blot analysis with an anti E2F-1 monoclonal antibody (SQ-41, upper panel). The blot was re-blotted with anti Rb monoclonal antibody (Pharmingen 14001A, middle panel), and then further re-blotted with an anti Ras monoclonal antibody (R02120 from transduction laboratories, lower panel). Molecular size markers in kilodaltons are shown on the left.

Figure 6:
Ras-induced E2F-1 is transcriptionaly active.

293 cells were transfected with the reporter plasmids E1β-Luc and pCMV-β-Gal together with increasing amounts of the expression vector of E2F-1 either alone or together with H-RasV12 (300ng) (Ras). Cell extracts were prepared 24hr after transfection and used for β-Gal assay, Luciferase assay and western blot analysis with an anti E2F-1 monoclonal antibody (SQ-41). Fold of activation in the luciferase assay, after normalization for β-Gal activity, is depicted in the bar graph (upper panel). None – cells transfected only with reporter plasmids.
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A.

E2F-1

Time (hr.): 0 1 3 5

E2F-1/Ras

Time (hr.): 0 1 3 5

B.

% O.D. of Zero Time

120

100

80

60

40

20

0

0 2 4 6

Time (hr)

E2F-1

E2F-1/Ras

C.

MG-132:

- + - +

E2F-1

E2F-1/Ras

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