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Abstract

The INK4a/ARF locus which is frequently inactivated in human tumours encodes two different tumour suppressive proteins, $p16^{INK4a}$ and ARF. $p16^{INK4a}$ is a major component of the RB pathway. ARF is part of an ARF-mdm2-p53 network that exerts a negative control on hyperproliferative signals emanating from oncogenic stimuli. Among these is the transcription factor E2F1, a final effector of the RB pathway, that induces *ARF* expression. Recent data suggest that ARF function is not restricted to the p53 pathway. However, ARF target(s) implicated in this p53-independent function remains to be identified. We show that ARF is able to inhibit the proliferation of human cell lines independently of their p53 status. In this context, we demonstrate that ARF interacts physically with E2F1 and inhibits its transcriptional activity. Moreover, we show that mdm2 is required for the modulation of E2F1 activity by ARF. Beside the well-known p53 and mdm2 partners, these results identify E2F1 as a new ARF target. Thus, ARF can be viewed as a dual-acting tumour suppressor protein in both the p53 and RB pathways, further emphasizing its role in tumour surveillance.

Introduction

The INK4a/ARF locus encodes two gene products, $p16^{INK4a}$ and ARF, both of which are crucial for cell growth regulation and senescence (Quelle et al. 1995; Chin et al 1998). $p16^{INK4a}$ is a major component of the RB pathway (Serrano et al 1993). ARF has been principally ascribed to exert a negative control on abnormal proliferative signals including ras (Palmero et al 1998), c-myc (Zindy et al 1998), E1A (De Stanchina et al 1998), Abl (Radfar et al 1998), and E2F1 (Bates et al 1998) by participating to a well defined ARF/mdm2/p53 pathway (Kamijo et al 1998; Pomerantz et al 1998; Stott et al 1998; Zhang et al 1998). Because ARF loss in mice facilitates spontaneous tumour development (Kamijo et al 1997), it has been proposed to be most important in tumour surveillance (Sherr et al 1998). ARF increases p53 activity by interacting with the mdm2 protein. Mdm2 is encoded by a p53-responsive gene and antagonizes p53 function by inhibiting its transcriptional activity, catalyzing its ubiquitination through its E3 ligase activity, and triggering its nuclear export and degradation in cytoplasmic proteasomes (for review see Levine 1997; Prives 1998). ARF is able to sequester mdm2 in nucleoli and to inhibit its nuclear export (Weber et al 1999; Tao et al 1999). This prevents the mdm2-mediated p53 degradation and thus stabilizes a transcriptionally active p53 in the nucleoplasm.

Some recent data have shown that ARF is not implicated only in this ARF/mdm2/p53 pathway. Carnero et al. reported that $p19^{ARF}$ (murine ARF) is able to suppress colony formation in p53 -/- cells by affecting the Rb pathway (Carnero et al 2000). Weber et al. provided evidence that reintroduction of $p19^{ARF}$ in mouse embryo fibroblasts (MEFs) lacking p53, mdm2 and $p19^{ARF}$ stops cell proliferation (Weber et al 2000). In addition, mice lacking both p53 and ARF develop a more diverse spectrum of tumours than animals lacking either gene alone (Weber et al 2000). Altogether these observations demonstrated that the antiproliferative functions of ARF are not restricted

to the p53 pathway. However, the relevant ARF target protein(s) implicated in this p53-independent pathway remains unknown.

E2F1, one of the upstream transcriptional activators of the *ARF* gene (Bates et al 1998), is a major component of the RB pathway. It was the first identified member of a transcription factors family generically referred to as E2F (Nevins 1992). E2F1 plays a critical role in G1/S transition by coordinating early cell cycle events with the transcription of genes required for S phase entry (De Gregori et al 1995). Its activity is tightly regulated by RB. Binding with an hypophosphorylated form of RB sequesters E2F1 into inactive repressive complexes and inhibits its transcriptional capacity (Flegmington et al 1993). The phosphorylation of RB by cyclinD-cdk4/6 complexes in late G1 allows the release of a free active form of E2F1 (Helin et al 1993). Accordingly, impairing the RB function leads to abnormal proliferation related to the deregulation of E2F1.

We previously observed that a significant proportion of high grade neuroendocrine human lung tumours displayed inactivation of both *p53* and *ARF* genes (Gazzeri et al 1998). This led us to propose that ARF could play a role independently of p53. Indeed, we show here that ARF is able to arrest cell-growth of human cell lines in a p53 independent manner. More interestingly, we demonstrate that human ARF displays physical and functional interaction with E2F1 and that mdm2 is required for the modulation of E2F1 activity by ARF. Overall, these data clearly identify E2F1 as a new direct ARF target and provide for the first time a candidate for the growth regulatory function of ARF at the interface of both p53 and RB pathways.

Results

Human ARF inhibits cell growth independently of p53

To test the hypothesis that ARF might display p53-independent functions we transfected an ARF expression vector in Saos2 (human osteosarcoma) and H358 (human bronchioloalveolar carcinoma) p53-null cell lines. After 2 weeks of drug selection, resistant cells were counted. As shown in Figure 1, a high decrease in the number of cells was observed after ARF transfection in both cell lines when compared to cells transfected with an empty vector. Same results were obtained when the cells were transfected with a mutant ARF expression vector containing the exon 1β (referred to here as GST-ARF$^{1-65}$). In contrast, the exon 2 of ARF (GST-ARF $^{66-132}$) had no effect. Since we never detected apoptosis in ARF-transfected cells (data not shown), these data indicated that ARF was able to inhibit cell growth in the absence of wild-type p53.

Human ARF interacts with E2F1

E2F1, an upstream activator of *ARF*, is required for cell cycle progression (Helin et al 1998). In an effort to identify proteins that could be involved in the p53-independent growth arrest induced by ARF, we investigated a putative regulation of E2F1 by ARF. The E2F1 protein levels were analyzed by direct immunoblot of crude extracts of Saos2 and H358 cells transfected with ARF. No difference was observed in the steady state levels of the protein after overexpression of ARF (Figure 2A, left). Nonetheless, the presence of complexes between ARF and E2F1 was detected by immunoprecipitation using antibodies to each protein (Figure 2A, middle and right). Identical results were obtained from cells cotransfected with both ARF and E2F1 (Figure 2B). In order to demonstrate that ARF/E2F1 complexes were not artefacts generated by overexpression of the proteins, immunoprecipitation experiments were repeated in 293

cells (human kidney cells transformed with Adenovirus 5 DNA) that contain relatively high levels of endogenous ARF. When ARF immunoprecipitates from 293 cells were immunoblotted with antibody against E2F1, co-precipitation of both proteins was detected again indicating that the interaction occurred also between the two endogenous proteins (Figure 2C). ARF-E2F1 binding was further investigated in a cell-free system using a glutathione S-transferase (GST)-ARF fusion protein. In this direct binding assay, in vitro translated E2F1 co-precipitated efficiently with the recombinant ARF protein demonstrating that both proteins had the ability to physically interact (Figure 2D). This result was confirmed by examining extracts of sf9 cells infected with ARF and E2F1-baculovirus (data not shown). Taken together, these data indicate that ARF can form stable complexes in vivo with either endogenous or exogenous E2F1. In addition, the results of the in vitro assays strongly suggest that this association occurs even in the absence of other partners such as p53 and mdm2.

ARF inhibits the transcriptional activity of E2F1

E2F1 controls the expression of a number of genes needed for DNA synthesis and progression into S phase (De Gregori et al 1995). The finding of a direct interaction between human ARF and E2F1 prompted us to examine potential effect of ARF on E2F1-dependent transcription. In this respect, H358 cells were co-transfected with ARF and E2F1 expression vectors and a plasmid encoding the Chloramphenicol-acetyl-transferase (Cat) reporter gene under the control of a promoter bearing E2F1 elements. Cat-assays repeatedly indicated that ARF significantly inhibited E2F1 transactivating activity in a dose-dependent fashion (Figure 3A and 3B). In contrast, the activity of an E2F1-independent reporter construct (PCAT3) was unaffected by ARF overexpression (Figure 3A) and unrelated PCMV lacZ and PCMV luc control plasmids did not modulate the

activity of the E2F1-dependent reporter construct (Figure 3B). Taken together these results indicated that downregulation of E2F1CAT by ARF was not the result of unspecific trancriptional inhibition. Furthermore, this inhibition was confirmed in several cell types including normal cells (Figure 3C).

Finally, we further investigated the ARF ability to inhibit endogenous E2F1 transcriptional activity in Saos2 cells that abundantly express E2F1. In these cells, we found that the activity of an E2F1-dependent reporter plasmid cyclinE-Luc but not of a control plasmid PGLuc was downregulated by ARF (Figure 3D). From these data we concluded that ARF had also the ability to inhibit endogenous E2F1 transcriptional activity. Since Saos2 cells lack functional RB, these data also indicate that ARF inhibition of E2F1 trancriptional function does not require the presence of RB.

Exon1 β of ARF is necessary and sufficient to interact with E2F1 and to mediate inhibition of its transcriptional activity.

We next looked for sequences implicated in the ARF/E2F1 interaction and inhibition of E2F1 trancriptional activity. To this end we constructed a set of different ARF-GST fusion proteins and used them in a pull-down assay after incubation with in vitro translated E2F1. The study of E2F1 sequences implicated in ARF binding was performed using two truncated E2F1 proteins translated and radiolabelled in vitro: the first one bore the 1-374 N-terminal residues ($E2F1^{1-374}$) including sequences involved in DNA binding capacity of E2F1 and interaction with its cofactor DP-1; the second one contained the carboxy-terminal residues 323 to 437 that encode E2F1 transactivation domain ($E2F1^{323-437}$). A GST pull-down assay using a wild type ARF-GST fusion protein showed that E2F1 residues 1-374 were implicated in ARF binding whereas the 323-437

C-terminal sequence of E2F1 was not (Figure 4A). These data indicate that E2F1 interacts with ARF through its N-terminal domain.

The same approach was repeated with in vitro translated wild type E2F1 and mutant GST-ARF fusion proteins. We found that the N-terminal domain of ARF (referred to here as GST-ARF$^{1-65}$) was sufficient to bind E2F1 (Figure 4B) and to inhibit its transcriptional activity in a Cat assay (Figure 4C). In contrast, the C-terminal part (GST-ARF $^{66-132}$) did not react with E2F1 (Figure 4B) and was not able anymore to inhibit E2F1 transcriptional function (Figure 4C). Strikingly, the HA-ARF$^{1-65}$ protein exhibited a higher reducing transcription capacity than its wild type counterpart. The same results were obtained when these ARF mutants proteins were tested for their ability to inhibit the transcriptional activity of endogenous E2F1 using cyclinE-Luc reporter vector (data not shown).

The nucleolar accumulation of ARF has been reported to govern its activity towards its mdm2 and p53 effectors (Weber et al 1999; Tao et al 1999). Two sequences necessary for nucleolar ARF localization have been identified in exon1β (residues 1-29) and exon2 (residues 83-101) of human ARF [Weber et al 1999; Zhang et al 1999; Rizos et al 2000)]. Implication of ARF nucleolar localization in the inhibition of E2F1 was tested using a myc-tagged ARF deletion mutant (referred to here as myc-ARF$^{1-82}$) that lack the 50 C-terminal residues of exon2. As expected, myc-ARF$^{1-82}$ diffused throughout both the cytoplasm and the nucleus of the majority of transfected cells (data not shown and see also Zhang et al 1999) and was still able to interact with E2F1 (data not shown). In these conditions, inhibition of E2F1-dependent transcription was maintained (Figure 4C). Taken together, these data demonstrate that exon1β of ARF is necessary and sufficient to

mediate E2F1 transcriptional inhibition but that nucleolar localization of ARF is not implicated in this process.

Inhibition of E2F1 transcriptional activity by ARF is dependent of mdm2

Mdm2 has been shown to contribute to ARF function through both the p53 and the RB pathways (Carnera et al 2000). Indeed, in H358 and Saos2 cells transfected with ARF, endogenous mdm2 was found to coprecipitate with ARF or E2F1 (data not shown) suggesting that it might play a role in ARF-mediated E2F1 activity. To get further insight into the potential implication of mdm2 in our system, we cotransfected $mdm2^{-/-}/p53^{-/-}$ (double-null) mouse embryonic fibroblasts (MEFs) with ARF and E2F1 (since we did not detect endogenous E2F1 in these cells). Consistent with the data presented in figure 2D, E2F1 coprecipitated with ARF in the absence of mdm2 (Figure 5A). Nevertheless, in these conditions wild type ARF had lost its ability to inhibit the transcriptional activity of E2F1 (Figure 5B). In contrast, after reintroduction of mdm2 product we partially restored ARF ability to inhibit E2F1 transcriptional function (Figure 5C). Taken together, these results demonstrate that mdm2 is implicated in the modulation of E2F1 activity by ARF.

Discussion

Until recently, ARF was principally ascribed to be part of a three-partners ARF-mdm2-p53 pathway that exerts a negative control on abnormal proliferative signals. This effect was achieved through ARF-mediated neutralization of mdm2 that resulted in the stabilization of a trancriptionally active p53 (Kamijo et al 1998, Pomerantz et al 1998; Stott et al 1998; Zhang et al 1998). However, recent data reported that murine ARF was able to arrest cell growth independently of p53 implying that $p19^{ARF}$ may functionally

interact with proteins other than p53 (Carnero et al 2000; Weber et al 2000). Our present results provide evidence that human ARF is also able to arrest cell growth in human p53-null cell lines and identify E2F1 as a new target for ARF.

E2F1 has the ability to promote cell proliferation and/or apoptosis depending on the cellular context (Dyson et al 1998). In p53 expressing cells, E2F1 potentiates cell death probably through its capacity to transactivate *ARF* leaving p53 free for promoving apoptotic process (Bates et al 1998). In contrast, in p53-null cells, E2F1-induced apoptosis does not require an intact E2F1 transactivation domain (Hsieh et al 1997; Philipps et al 1997). Since our data show that ARF inhibits E2F1 transactivating activity, it is unlikely that ARF affects the E2F1 pro-apoptotic function. In contrast, ARF could modulate the ability of E2F1 to induce cell proliferation which is greatly dependent on its capacity to transactivate the expression of several genes implicated in G1/S transition (Dyson et al 1998). Consistent with this, $p19^{ARF}$ has been recently reported to induce a G1 arrest in p53-null cells by preventing an unknown target to mediate G1 exit (Weber et al 2000). In addition, Carnero et al demonstrated that strong expression of E2F1 was sufficient to overcome the arrest induced by $p19^{ARF}$ in p53-/- cells (Carnero et al 2000) suggesting that $p19^{ARF}$ might ultimately act by regulating E2F1. Our finding that ARF can interact with E2F1 and inhibit its transcriptional activity clearly identifies E2F1 as an effector by which ARF can inhibit cell growth in a p53 independent context. In this respect, ARF can be viewed as a dual-regulatory component of both the p53 and RB pathways. Additionally, E2F1 can directly activate *ARF* transcription (Bates et al 1998) as well as its own expression (Johnson et al 1994). Thus, a direct ARF/E2F1 interaction could also allow ARF to negatively regulate its own expression as well as E2F1 expression. Consistent with this view, we recently observed that loss of ARF expression was frequently associated with elevated levels of E2F1 product (Eymin et al., submitted).

Overall, modulation of E2F1 transcriptional activity by ARF makes sense with the direct implication of ARF in oncogenic processes as inactivation of ARF would relieve a block in cell cycle progression by deregulating E2F1 activity.

We found that the inhibition of E2F1 transcription activity induced by ARF was lost in p53-/-, mdm2-/- MEFs. However, in our hands, reintroduction of mdm2 in these cells restored some repression of E2F1 transcriptional capacity. These data indicate that mdm2 protein is required for the ARF-induced inhibition of E2F1 transcriptional function to take place. In contrast, Weber et al. recently demonstrated that mdm2 antagonized ARF-mediated G1 arrest in MEF TKO (Triple knock-out for p53, ARF and mdm2)(Weber et al 2000). While we have no clear explanation for this discrepancy, Carnero et al. reported data also obtained in murine system, that demonstrated mdm2 cooperation in the activity of ARF in the p53 as well as in the RB pathways (Carnero et al 2000). As a whole, our results are more consistent with those of Carnero and indicate that direct interactions between ARF, mdm2 and E2F1 might constitute a pivotal structure in cell growth regulation processes. Mdm2 has been reported to stimulate the transactivatory capacity of E2F1 (Martin et al 1995). Paradoxically, we found that the inhibition of E2F1 transcriptional capacity induced by ARF was dependent on mdm2 expression. One hypothesis to this apparent discrepancy could be that binding of ARF to the mdm2-E2F1 complex would reverse the positive activity of mdm2 on E2F1 into inhibitory activity. As ARF 1-65 residues have a significant higher binding affinity for mdm2 compared to wild type ARF (Rizos et al 2000), this could explain why this mutant ARF construct inhibits E2F1 transcription better than its wild type counterpart. In addition, these data would also be consistent with the finding that the exon2 C-terminal sequence of ARF which does not bind mdm2 (our data not shown and see also Midgley et al 2000) does not inhibit E2F1 transcription.

Overall, our data identify E2F1 as a potential mediator of ARF functions independently of p53. An important consequence for human oncogenesis is that the so-called mutual exclusion resulting from the participation of p53 and ARF proteins to the same and unique pathway cannot be maintained anymore. In keeping with this situation, we and others have shown that in human lung tumours, coexistence of inactivated p53 and ARF proteins is not a rare event (Gazzeri et al 1998; Sanchez-Cespedes et al 1999) suggesting that a growth advantage is gained by ARF invalidation in p53 mutant clones.

Materials and methods

Transfection and growth suppression assay

Transient transfections were carried out using Fugène 6 (Roche Diagnostic) according to the manufacturer's protocol. The total amount of DNA used for transfection was held constant by adding empty vector DNA to the transfection mixture. Cells were harvested 48-72h after transfection. In the growth suppression assay, G418 was added to the media 48h post transfection and cells were maintained for 12 days to generate stable transfectants. Trypan blue excluding cells were counted.

Western blot analysis

Western blots were performed by using standard procedures with antibodies directed against ARF (Della Valle et al 1997), E2F-1 (Ab-4, Neomarkers), myc (Ab-2, Neomarkers) or Haemagglutinin (HA) (3F10, Roche Diagnostic).

Immunoprecipitations

Mammalian cell pellets were lysed with 120mM NaCl, 50mM Tris pH=7.4, 0.5% NP40, 1mM EDTA and incubated for 30 min on ice. The supernatants were cleared by centrifugation. Equal amounts of protein (200μg) were precleared using protein A/protein G agarose beads and immunoprecipitated by using standard procedures. Antibodies used were anti-ARF (Della Valle et al 1997) for ARF, KH95 (Pharmingen) for E2F1, myc Ab-1 (Neomarkers) for myc-ARF$^{1-82}$ and clone 3F10 (Roche Diagnostic) for HA Tag.

GST pull-down assay

Beads coated with GST, GST-ARF, GST-ARF$^{1-65}$ or GST-ARF$^{66-132}$ fusion proteins were prepared according to the manufacturer's protocol (Bulk GST Purification module, Pharmacia Biotech). Beads were incubated for 45 min at room temperature with equivalent amounts of in vitro translated ^{35}S-methionine-labelled E2F1 proteins (E2F1 1-437; E2F1 1-374; E2F1 323-437; TNT SP6/T7 system, Promega) in a final volume of 150μl binding buffer (25mM Hepes pH 7.6, 12.5 mM $MgCL_2$, 150mM KCL, 0.1% NP40, 20% glycerol) containing 0.2mg/ml BSA. Beads were washed three times with NETN buffer (100mM NaCL, 1mM EDTA, 0.5% NP40, 20mM Tris pH 8.0), once with PBS and then analyzed by 10% SDS-PAGE.

Cat and luciferase assays

$2x10^5$ cells were transfected in a 6-cm dish. In CAT assays, transcriptional activity of E2F1 was analyzed by measuring CAT activity using CAT ELISA (Roche diagnostic). For Luciferase assays, cells were lysed at 24h after transfection in 300µl lysis buffer (Passive lysis buffer from Promega). The cell debris were removed by centrifugation at 14 000 r.p.m for 2 min and luciferase activity was measured on a 20 µl aliquot in a luminometer using the luciferase kit from Promega.

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Legends to figures

Figure. 1: ARF inhibits cell growth in p53-null cell lines. Saos2 and H358 cells were transfected with either pcDNA (empty bars) or pcDNA-ARF (filled bars) or pcDNA-

HA-ARF$^{1-65}$ (left slanting hatched bars) or pcDNA-myc-ARF$^{66-132}$ (right slanting hatched bars) expression vectors. At the end of 2 weeks of G418 selection, the remaining adherent cells were counted. In each case, the number of cells transfected with empty vector was normalized to 100%. Results of ARF or mutant ARF-transfected cells were expressed relative to this. Results are the means of 5 independent experiments performed in duplicate. Bars, SD

Figure. 2: ARF interacts with E2F1. **A** and **B**: E2F1 and ARF can immunoprecipitate each other. Saos2 cells were transiently transfected with pcDNA or pcDNA-ARF (**A** left) or both pcDNA-ARF and pCMV-E2F1 (**B** left). Whole-cell extracts were immunoprecipitated with anti-ARF serum (**A** and **B** middle) or anti-E2F1 (**A** and **B** right) or control (IgG) antibodies and immunoblotted with anti-E2F1 (**A** and **B** middle) or anti-ARF antibodies (**A** and **B** right). **C**: Endogenous E2F1 and ARF can interact. 293 cell extracts were immunoprecipitated by anti-ARF serum or control IgG and blotted with anti-E2F1 antibody. In all experiments, input represented loading of 40μg of whole cell extracts. **D**: Recombinant ARF binds to E2F1 in vitro. Labelled E2F1 was subjected to a GST-pull down assay using beads covered with GST or GST-ARF fusion proteins.

Figure. 3 ARF inhibits the transactivating function of E2F1. **A**: H358 cells were transiently transfected with either 1μg of 4xE2F1-CAT reporter vector (filled bars) with or without 0.05μg pCMV-E2F1 expression vector or 1μg of pCAT3 E2F1-independent promoter (hatched bars). Various amount of pcDNA-ARF expression vector (0.1 to 0.5μg) was added as indicated. Promoter activity of the E2F1-CAT reporter in the presence of E2F1 expression vector was normalized to 1.0 and the activities of the remaining transfection reactions were expressed relative to this. Results are the means of

3 independent experiments performed in duplicate. Bars, SD. **B**: H358 cells were transiently transfected with 1μg E2F1-CAT reporter vector, 0.05μg pCMV-E2F1 expression vector in the absence (empty bars) or presence of increasing amounts (0.1μg, 0.25μg, 0.5μg) of pCDNA-ARF (filled bars), pCMV-LacZ (speckled bars) or pCMV-Luc (hatched bars) expression vectors. As above, promoter activity of the E2F1-CAT reporter in the presence of E2F1 expression vector was normalized to 1.0 (empty bars). **C**: ARF inhibits E2F1 transcriptional activity in several cell types. Indicated cell types were transfected with 1μg of 4xE2F1-CAT reporter vector (filled bars) and 0.05μg pCMV-E2F1 with or without 0.5μg pCDNA-ARF. For each cell type, promoter activity of the E2F1-CAT reporter in the presence of E2F1 expression vector was normalized to 1.0 and the activity in the presence of ARF expression vector was expressed relative to this CAT activity. **D:** ARF inhibits the transcriptional activity of endogenous E2F1. Saos2 cells were transiently transfected with 1μg of either PGLuc E2F1-independent promoter (hatched bars) or cyclinE-Luc reporter vector (filled bars) with or without 0.5μg pcDNA-ARF expression vector. Luciferase activity obtained with cyclinE-Luc or PGLuc in the absence of ARF constructs was normalized to 1 and the activity in the presence of ARF expression vector was expressed relative to this luciferase activity. Results are the mean of 3 independent experiments performed in duplicate.

Figure. 4 ARF exon1β sequence is implicated in E2F1 binding and inhibition of its transcriptional capacity. **A**: Involvement of E2F1 N-terminal sequence in binding to ARF. The $E2F1^{1-374}$ and $E2F1^{323-437}$ mutants proteins translated and radiolabelled in vitro were subjected to a GST pull-down assay using GST-ARF fusion protein. **B**: E2F1

protein interacts with exon1β of ARF. In vitro translated ^{35}S-labelled E2F1 protein was subjected to GST pull-down analysis using ARF mutants expressed as GST fusion proteins. **C:** Exon1β of ARF is sufficient for the inhibition of E2F1 transcriptional activity. H358 cells were transfected with 1μg E2F1-CAT reporter vector, 0.05μg pCMV-E2F1 and either 0.5μg pcDNA (empty bars) or pCDNA-ARF (filled bars) or pcDNA-HA-ARF $^{1-65}$ (left slanting hatched bars) or pcDNA-myc-ARF $^{66-132}$ (right slanting hatched bars) or pcDNA-myc-ARF $^{1-82}$ (speckled bars) expression vectors as described. The promoter activity of the E2F1-CAT reporter in the presence of E2F1 expression vector was normalized to 1.0 and the activity in the presence of ARF expression vectors was expressed relative to this CAT activity. Results are the mean of 3 independent experiments performed in duplicate.

Figure. 5 Mdm2 is not necessary for interaction between E2F1 and ARF but is implicated in ARF-induced inhibition of E2F1 transcriptional activity. **A**: ARF interacts with E2F1 in the absence of mdm2. Crude extracts of MEFs mdm2$^{-/-}$/p53$^{-/-}$ transfected with either pcDNA or

pcDNA-ARF and pCMV-E2F1 expression vectors were immunoprecipitated with ARF anti-serum or IgG control and immunoblotted using anti-E2F1 antibody. **B,C:** mdm2 is implicated in the modulation of E2F1 activity by ARF. MEFs mdm2$^{-/-}$/p53$^{-/-}$ were co-transfected with either 1 μg PGLuc alone (hatched bars) or 1 μg cyclin E-luc (filled bars) and 0.05 μg pCMV-E2F1, and various amounts (0.1 to 0.5μg) of pCDNA-ARF as indicated, in the absence (**B**) or presence (**C**) of 5μg pCMV-mdm2 expression vector. Luciferase activity obtained with cyclin-E luc reporter in the presence of pCMV-E2F1 expression vector or with PGLuc alone was normalized to 1 and the activities of the

remaining transfection reactions were expressed relative to this. 5C is representative of an experiment performed with 5μg pCMV-mdm2. Other experiments with 0.25μg pCMV-mdm2 expression vector gave the same result.

Figure 1

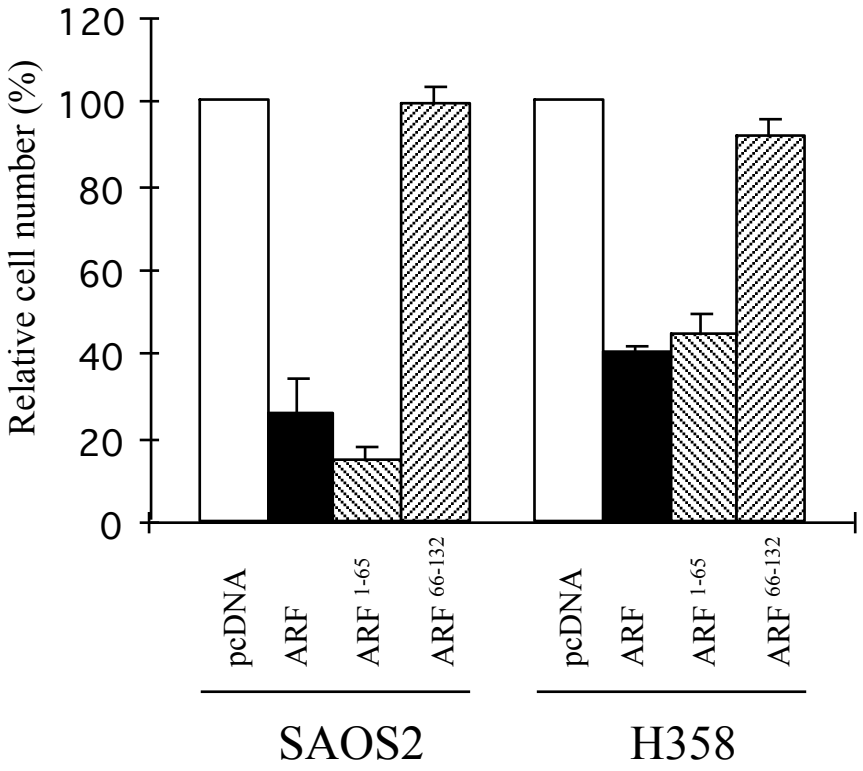

Relative cell number (%)
120
100
80
60
40
20
0
pcDNA
ARF
ARF 1-65
ARF 66-132
pcDNA
ARF
ARF 1-65
ARF 66-132
SAOS2
H358

Figure 2

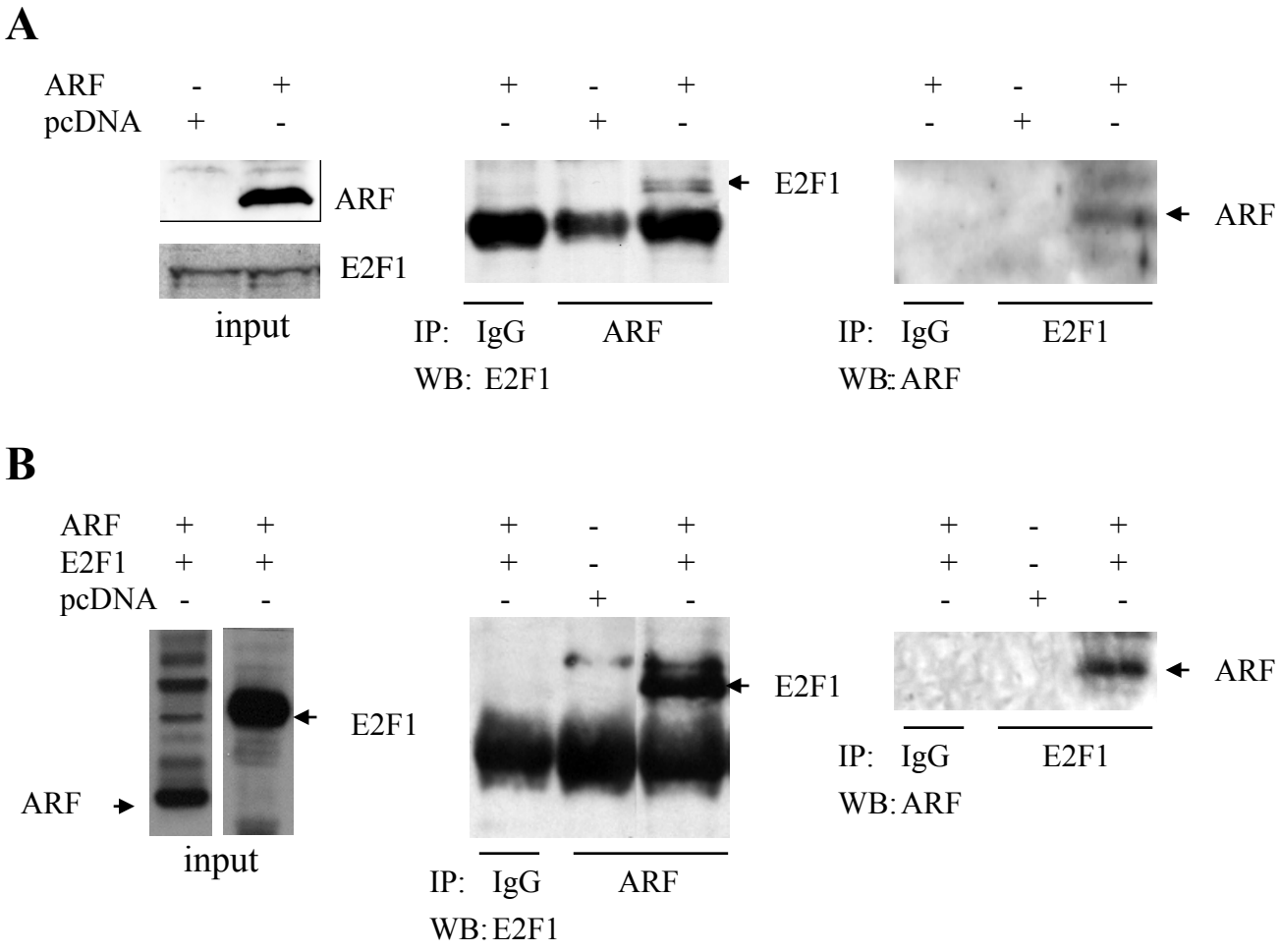

A
ARF - +
pcDNA + -
ARF
E2F1
input
+ - +
- + -
E2F1
IP: IgG ARF
WB: E2F1
+ - +
- + -
ARF
IP: IgG E2F1
WB: ARF
B
ARF + +
E2F1 + +
pcDNA - -
E2F1
ARF
input
+ - +
+ - +
- + -
E2F1
IP: IgG ARF
WB: E2F1
+ - +
+ - +
- + -
ARF
IP: IgG E2F1
WB: ARF
C
input
IP: IgG ARF
E2F1
WB: E2F1
D
input (100%)
GST
GST-ARF
E2F1

Figure 3

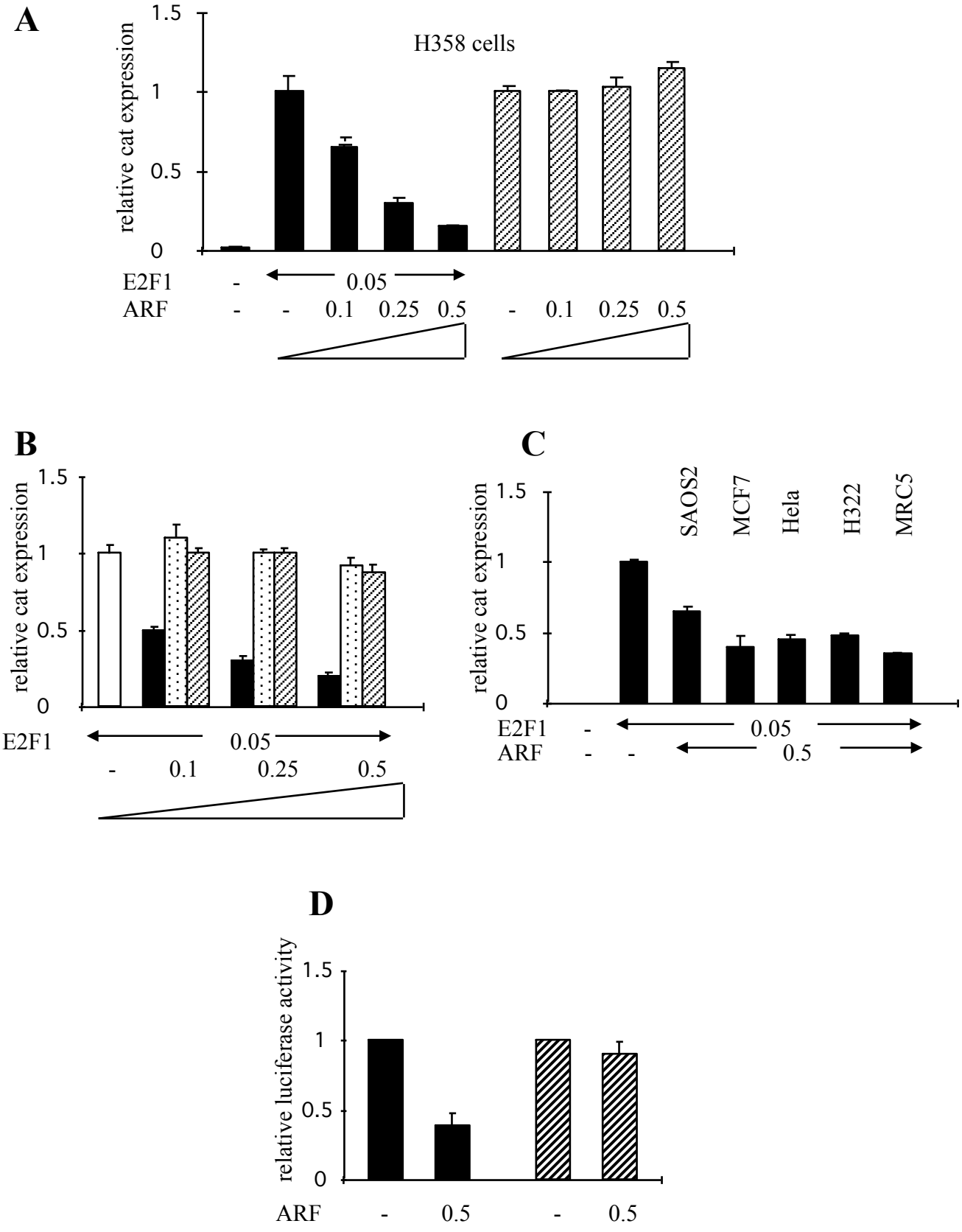

A
H358 cells
relative cat expression
1.5
1
0.5
0
E2F1 - 0.05
ARF - - 0.1 0.25 0.5 - 0.1 0.25 0.5
B
relative cat expression
1.5
1
0.5
0
E2F1 0.05
- 0.1 0.25 0.5
C
relative cat expression
SAOS2
MCF7
Hela
H322
MRC5
E2F1 - 0.05
ARF - - 0.5
D
relative luciferase activity
1.5
1
0.5
0
ARF - 0.5 - 0.5

Figure 4

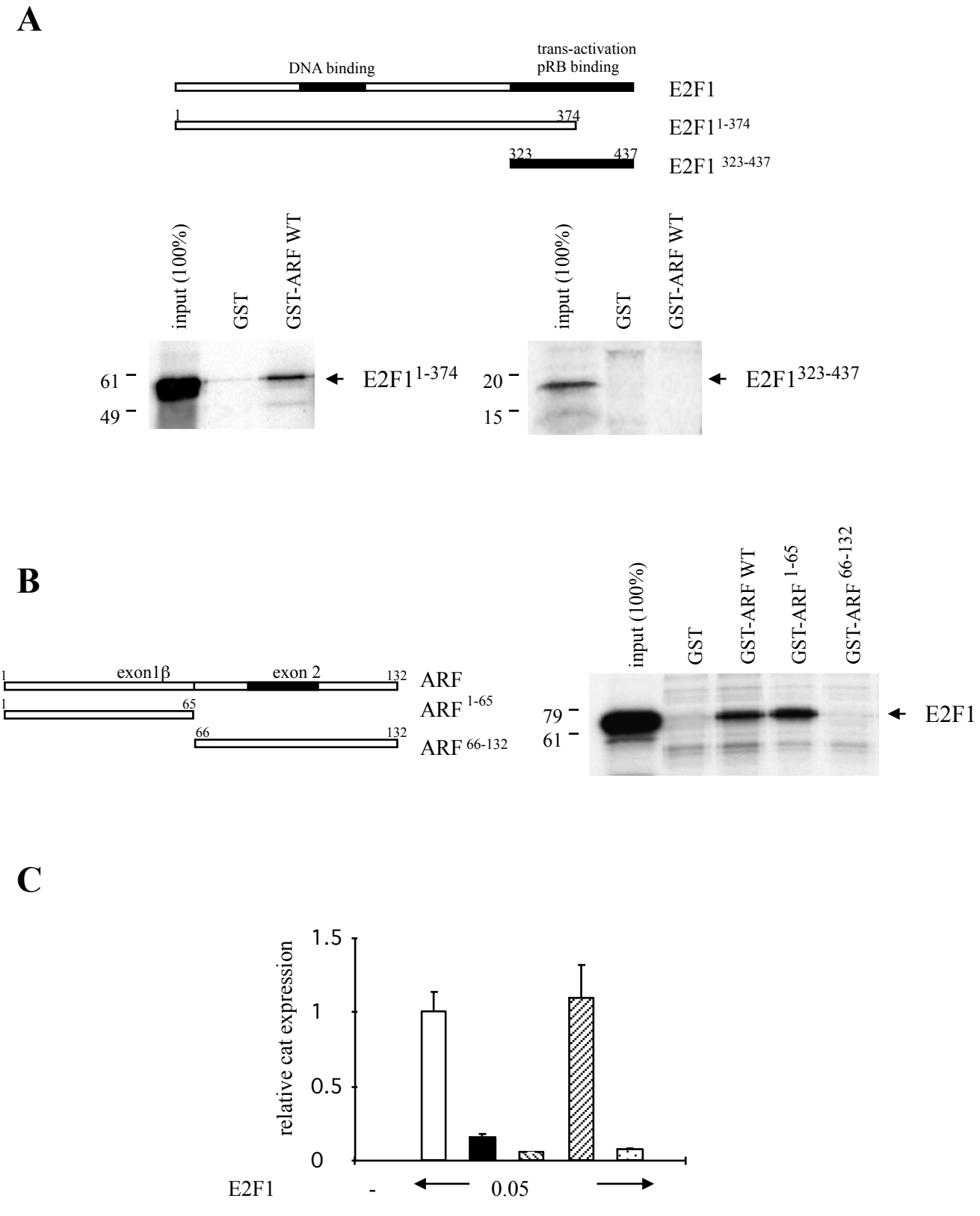
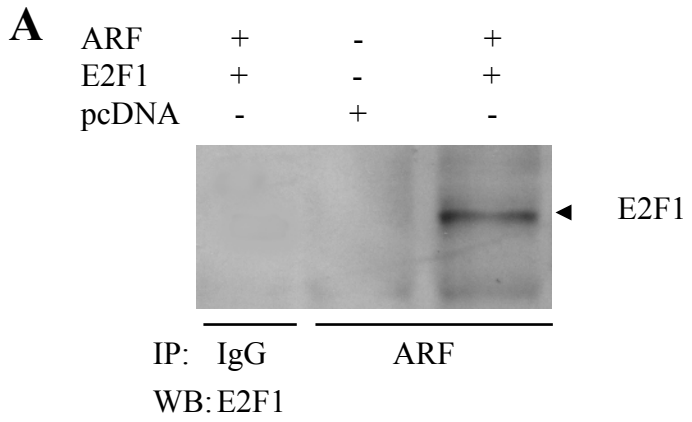
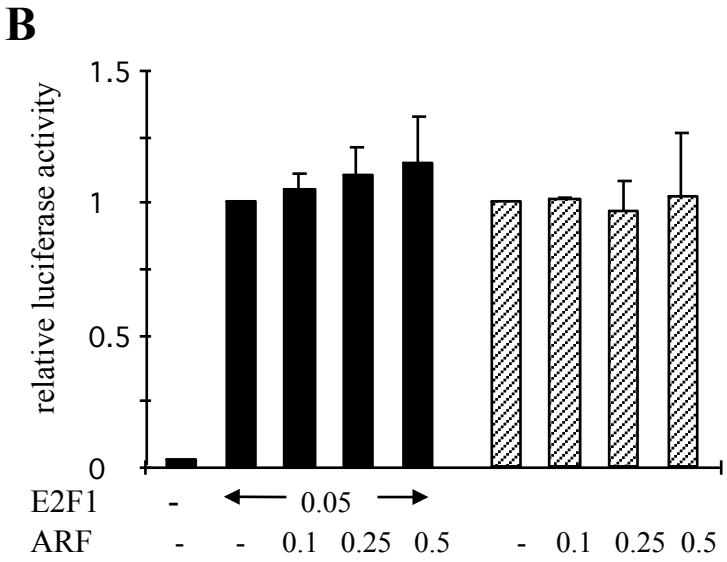
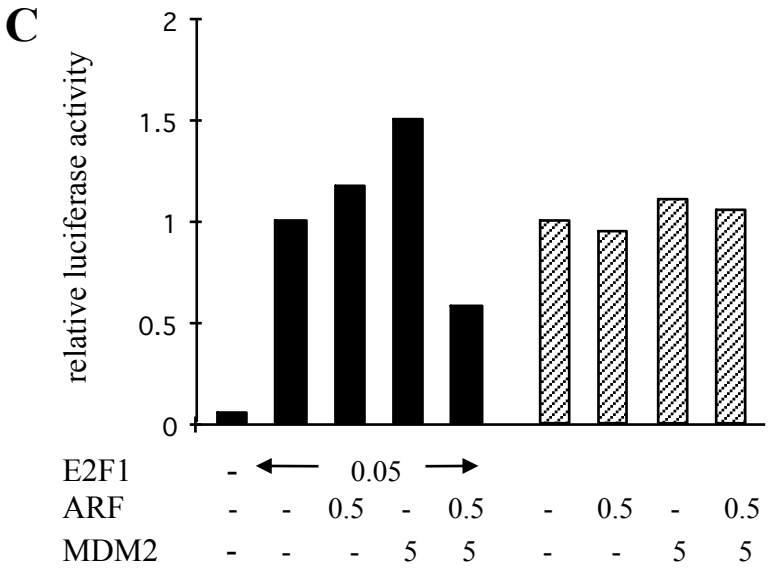

A
DNA binding
trans-activation
pRB binding
E2F1
1
374
E2F1 1-374
323
437
E2F1 323-437
input (100%)
GST
GST-ARF WT
61
49
E2F1 1-374
input (100%)
GST
GST-ARF WT
20
15
E2F1 323-437
B
exon1β
exon 2
1
132
ARF
1
65
ARF 1-65
66
132
ARF 66-132
input (100%)
GST
GST-ARF WT
GST-ARF 1-65
GST-ARF 66-132
79
61
E2F1
C
relative cat expression
1.5
1
0.5
0
E2F1
-
0.05

Figure 5


A
ARF + - +
E2F1 + - +
pcDNA - + -
E2F1
IP: IgG ARF
WB: E2F1


B
relative luciferase activity
1.5
1
0.5
0
E2F1 - 0.05
ARF - - 0.1 0.25 0.5 - 0.1 0.25 0.5


C
relative luciferase activity
2
1.5
1
0.5
0
E2F1 - 0.05
ARF - - 0.5 - 0.5 - 0.5 - 0.5
MDM2 - - - 5 5 - - 5 5