

## SHORT COMMUNICATION

**A functional link between the tumour suppressors ARF and p33ING1**L González<sup>1</sup>, JMP Freije<sup>2</sup>, S Cal<sup>2</sup>, C López-Otín<sup>2</sup>, M Serrano<sup>3</sup> and I Palmero<sup>1</sup><sup>1</sup>Institute of Biomedical Research, CSIC-UAM, Arturo Duperier 4, Madrid, Spain; <sup>2</sup>Department of Biochemistry and Molecular Biology, Institute of Oncology, University of Oviedo, Oviedo, Spain and <sup>3</sup>Spanish National Cancer Center (CNIO), Melchor Fernandez Almagro 3, Madrid, Spain

**The ARF tumour suppressor protein plays a critical role in the activation of p53 in response to oncogenic stress. ARF can activate p53 through nucleolar sequestration of Mdm2. However, several lines of evidence indicate that this is not the only way of action of ARF, and alternative mechanisms must exist. p33ING1 is a putative tumour suppressor, which induces cell-cycle arrest and apoptosis in a p53-dependent manner. Here, we describe that ARF and p33ING1 can interact *in vivo*. We also show that the subcellular localization of ING1 can be modulated by ARF protein levels, causing a displacement from nuclear to nucleolar localization. Finally, the ability of p33ING1 to cause cell-cycle arrest and induction of p21CIP1, or Mdm2, is impaired in ARF-deficient primary mouse fibroblasts. Based on these observations, we propose that the interaction with p33ING1 represents a novel mechanism for the tumour suppression function of ARF.**

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Activation of the tumour suppressor protein p53 is a critical event in many cellular responses against potentially oncogenic stimuli (Vogelstein *et al.*, 2000). The ARF protein (p14ARF in humans and p19ARF in mice) is encoded in the INK4a/ARF locus, together with p16INK4a (Sherr, 2001). ARF exerts its tumor suppressor action through the activation of p53, in response to a variety of stress signals (Sherr, 2001; Lowe and Sherr, 2003). ARF can counteract Mdm2, a negative regulator of p53, through sequestration of Mdm2 in the nucleolus (Weber *et al.*, 2000). However, ARF can also cause cell-cycle arrest without relocation of Mdm2, and retains part of its antiproliferative effect in cells lacking Mdm2 and p53 (Weber *et al.*, 2000; Llanos *et al.*, 2001; Korgaonkar *et al.*, 2002; Yarbrough *et al.*, 2002; Kuo *et al.*, 2003). These observations clearly suggest the existence of alternative mechanisms for ARF tumour suppressor function. The Inhibitor of Growth (ING)

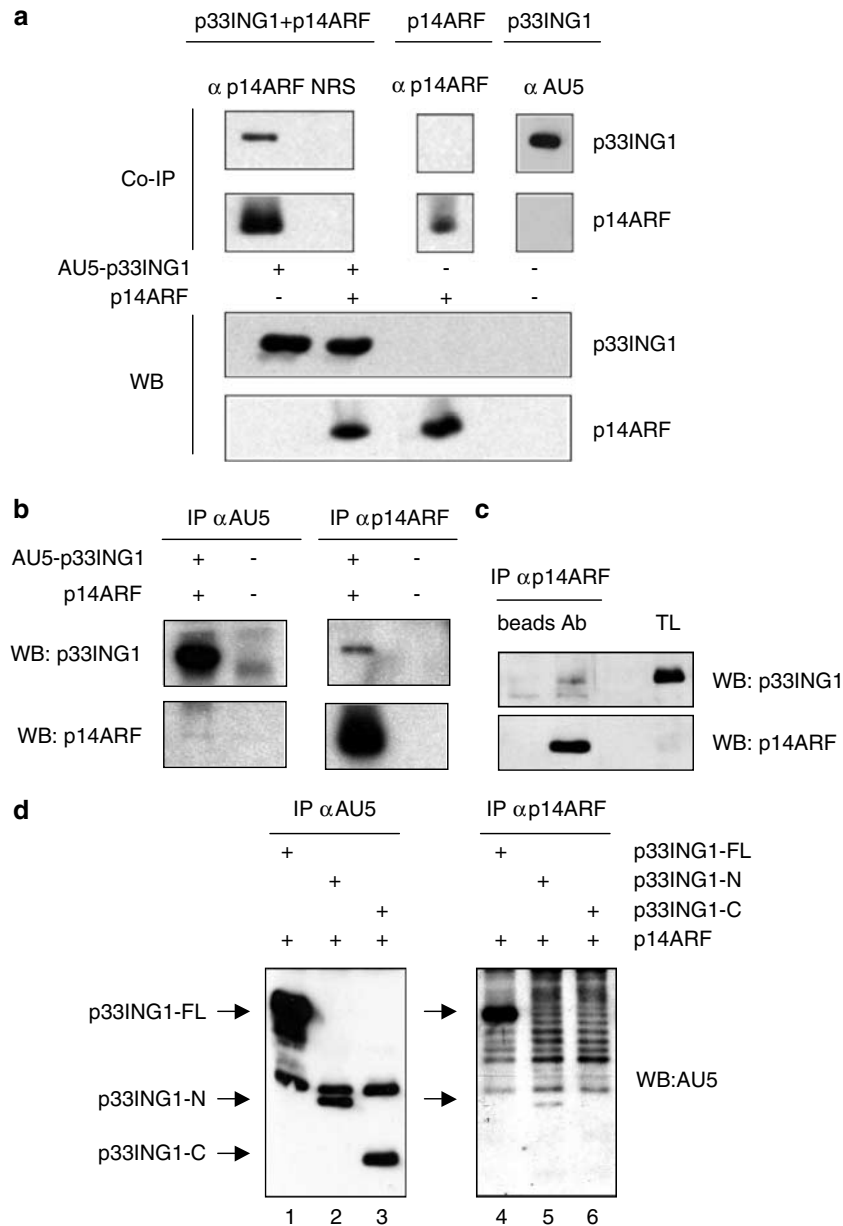
family of proteins includes several sequence-related proteins, encoded by at least five different loci in humans (reviewed by Feng *et al.*, 2002; Campos *et al.*, 2004; Gong *et al.*, 2005). ING proteins have been linked functionally to the p53 pathway at different levels, including the control of p53 protein stability, as transcriptional cofactors, or through post-translational modifications of p53 (Nagashima *et al.*, 2001; Nourani *et al.*, 2001; Gozani *et al.*, 2003). ING proteins also participate in chromatin remodelling through their association with histone deacetylases and histone acetyltransferases (Nourani *et al.*, 2001; Feng *et al.*, 2002; Kuzmichev *et al.*, 2002; Vieyra *et al.*, 2002; Xin *et al.*, 2004; Goeman *et al.*, 2005). The most extensively studied member of the ING family is p33ING1, one of the products of the ING1 locus (also referred to as p33ING1b, or ING1b elsewhere; Feng *et al.*, 2002). Interestingly, the ING1 locus is located in a region of frequent loss of heterozygosity, and p33ING1 is mutated, deleted, silenced or mislocalized in a large number of human tumours (reviewed by Nouman *et al.*, 2003; Gong *et al.*, 2005). Given the fact that ARF and p33ING1 are putative tumour suppressors linked to p53, we decided to explore the possibility that these two proteins could be functionally linked.

As an initial step to investigate possible links between ARF and p33ING1, we looked for interactions between these two proteins *in vivo*. To this end, we transiently transfected 293T human epithelial cells with vectors directing the expression of p14ARF, and an AU5-tagged version of human p33ING1. The association between these two proteins was studied by immunoprecipitation followed by Western blotting, using total protein extracts from cells transfected with both constructs. When a specific antibody against p14ARF was used for immunoprecipitation, we were able to detect both p14ARF and p33ING1 in the immunocomplex, indicating that the two proteins can associate in the cell (Figure 1a). The interaction was specific, as shown by the use of a non-related serum. The association could also be observed in a reverse experiment, using the anti-AU5 antibody for immunoprecipitation, although in this case, the signal obtained after Western blotting with an anti-p14ARF antibody was much weaker (Figure 1b). To confirm whether the observed interaction occurred between the endogenous proteins, we carried out immunoprecipitation experiments with an antibody against p14ARF in untransfected 293T cells

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**Figure 1** Physical interaction between p14ARF and p33ING1. **(a)** 293T cells were transiently transfected with vectors expressing p14ARF and AU5-tagged p33ING1, using a standard calcium phosphate protocol. Total protein lysates were prepared as previously described (Pantoja and Serrano, 1999; Palmero *et al.*, 2002). For immunoprecipitation, lysates containing 1 mg of protein were incubated with 1  $\mu$ l of an antibody against p14ARF (DP40, a generous gift of David Parry, DNAX Research Institute, 1:500 dilution), against the AU5 tag (MMS-135R, BabCO, 1:1000 dilution), or a non-related serum (NRS), overnight at 4°C, with constant rotation. Thirty microlitres of a slurry containing Protein A beads (Pharmacia), preblocked with 3% powder milk in lysis buffer for 1 h at 4°C, was added to the mix and incubated for 1 h in the same conditions. Beads were washed four times with ice-cold lysis buffer and incubated with 2  $\times$  SDS loading buffer (20  $\mu$ l per immunoprecipitation) for 5 min at 90°C to release bound proteins. The presence of each protein in the immunocomplex was analysed by Western blotting with antibodies against p14ARF or the AU5 tag. Controls with cells transfected with p14ARF alone or p33ING1 alone are also shown. The bottom panel shows a Western blot analysis of the total lysates used in the above-described immunoprecipitation. **(b)** 293T cells were transfected as indicated and analysed by immunoprecipitation as in **(a)**, using antibodies against the AU5 tag and against p14ARF for immunoprecipitation. Ectopic p33ING1 was detected by Western blotting with an antibody against the AU5 tag. **(c)** Non-transfected 293T cells were analysed by immunoprecipitation as in **(a)**, using an antibody against p14ARF for immunoprecipitation. Endogenous p33ING1 was detected with the LG1 antiserum. **(d)** 293T cells were transfected with vectors for p14ARF and the indicated AU5-p33ING1 deletion constructs, and analysed by immunoprecipitation followed by Western blotting, as described in **(a)**. Note that the exposure shown in the right-hand panel is about threefold longer than in the left-hand panel.

(Figure 1c). Western blotting with an antiserum against the C-terminus of p33ING1 (LG1) revealed a specific band comigrating with p33ING1 in the p14ARF

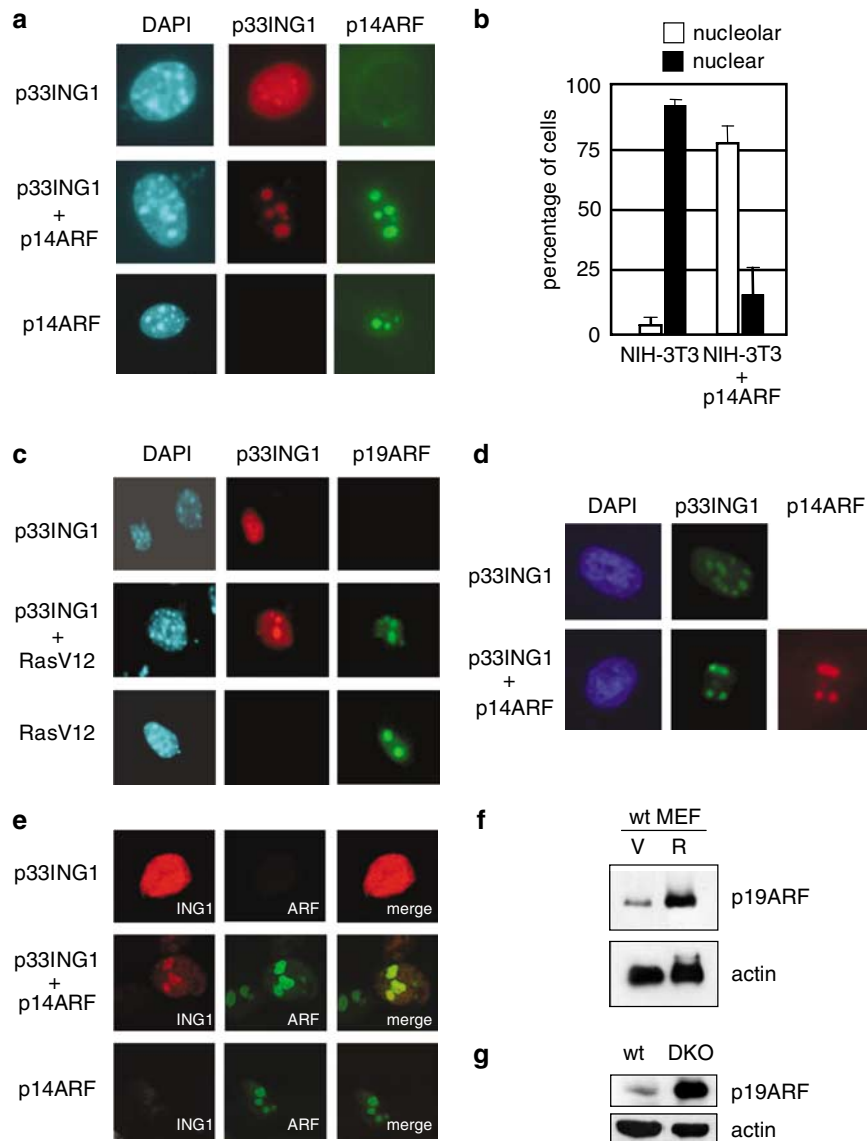
immunoprecipitate, indicative of interaction between the endogenous proteins. It should be noted that 293T cells express moderate levels of p33ING1, but low levels

of p14ARF. Similar results have been obtained using H1299 cells (data not shown). Next, we sought to define the p33ING1 domains involved in this interaction. We have recently defined two functional motifs, in the N- and C-terminus of p33ING1, which are important for its activity (Goeman *et al.*, 2005). After co-transfection of p14ARF with specific deletion constructs for p33ING1 in 293T cells, we were able to observe co-precipitation between p14ARF and a mutant protein containing residues 1–171 of p33ING1 (p33ING1-N), whereas no interaction was observed with a C-terminal construct containing residues 172–279 (p33ING1-C), indicating that the N-terminal domain of p33ING1 is sufficient for binding to p14ARF (Figure 1d).

The ARF protein localizes predominantly in the nucleolus (Sherr, 2001). It is well established that ARF can provoke the displacement to the nucleolus of proteins with which it interacts, such as Mdm2 (Weber *et al.*, 1999). The functional relevance of this ARF-mediated nucleolar sequestration is a matter of debate (Llanos *et al.*, 2001; Korgaonkar *et al.*, 2002), but, nevertheless, it is a hallmark of all the known bona fide interactions with ARF. Accordingly, we decided to analyse whether ARF protein levels had any effect on the subcellular localization of p33ING1. To this end, we used NIH-3T3 mouse fibroblasts, which carry a deletion in the entire *INK4a/ARF* locus, and analysed the cellular localization of ectopically expressed p33ING1 by indirect immunofluorescence. Consistent with previous reports (Scott *et al.*, 2001a, b), we observed a uniform nuclear staining for p33ING1 in these *ARF*-null cells (Figure 2a, top panel). However, when we restored ARF function to these cells, by co-transfection with a vector expressing p14ARF, a dramatic change in p33ING1 localization was observed, resulting in accumulation of the p33ING1 signal in the nucleoli (Figure 2a, middle panel; Figure 2b). To validate this observation in a more physiological setting, we wished to know whether changes in the level of endogenous ARF protein could have a similar effect on p33ING1 localization. With this purpose, early passage, wild-type mouse embryo fibroblasts (MEFs) were retrovirally transduced with a vector expressing the oncogenic form of human Ha-Ras (RasV12) and subsequently transfected with a vector expressing AU5-p33ING1. ARF is a critical mediator of the antiproliferative response of primary mouse fibroblasts to RasV12, and exposure to this oncogenic stress results in a dramatic induction of p19ARF in these cells, as shown in Figure 2f (Serrano *et al.*, 1997; Palmero *et al.*, 1998). In a similar manner to that observed in *ARF*-null NIH-3T3, the increase in endogenous p19ARF levels caused by RasV12 expression was accompanied in a large majority of cells by a shift in the staining pattern of p33ING1 to a predominantly nucleolar localization (Figure 2c). This shift in localization of ectopic p33ING1 was also observed in MEFs doubly deficient for p53 and Mdm2, indicating that both proteins are dispensable for this effect (Figure 2d). Note that the p53/Mdm2-null MEFs showed a tendency to accumulate p33ING1 in nucleoli in the absence of ectopic p14ARF, which most likely

reflects their higher p19ARF basal levels relative to wild-type MEFs (Figure 2g). The impact of p14ARF levels in p33ING1 localization was further studied using the fluorescent fusion proteins dsRed-p33ING1 and GFP-p14ARF (Figure 2e). When these constructs were ectopically expressed in 293T cells, either individually or together, the pattern of subcellular localization observed for dsRed-p33ING1 was very similar to that described in Figure 2a and c. 293T cells are refractory to the antiproliferative effect of p14ARF, because of the expression of SV40 large T antigen in these cells (Pomerantz *et al.*, 1998). Therefore, these results also suggest that the observed shift in p33ING1 localization is a direct consequence of its interaction with p14ARF, and not due to changes in cell-cycle profiles. In support of this notion, we have also observed that *ARF*-null NIH-3T3 cells made quiescent by serum starvation retain a uniform nuclear distribution of ectopic p33ING1 (data not shown). The characteristic nucleolar localization of p14ARF or p19ARF was not affected by the levels of p33ING1 in any case (compare middle and bottom panels in Figure 2a, c and e). It is worth mentioning that a similar accumulation of p33ING1 in nucleoli has been described to occur in human diploid fibroblasts upon exposure to high doses of UV radiation (Scott *et al.*, 2001a). In that study, mutant versions of the p33ING1 protein that did not relocalize in the nucleolus showed a reduced ability to induce apoptosis. These observations, together with our data, strongly indicate that relocation to the nucleolus plays an important role in p33ING1 function, and suggest a relevant role for ARF in this process.

Overexpression of p33ING1 has been shown to induce cell-cycle arrest and/or apoptosis in several cell types. In all cases, these responses require the presence of functional p53 (Shinoura *et al.*, 1999; Nagashima *et al.*, 2001). Prompted by the connections between ARF and p33ING1 described above, we decided to investigate whether ARF was required for the activation of p53-mediated responses by p33ING1. To this end, early-passage MEFs of wild-type or *p19ARF*<sup>-/-</sup> genotypes were retrovirally transduced with a vector expressing p33ING1. *p53/Mdm2*<sup>-/-</sup> MEFs were used as controls. As expected, p33ING1 expression in wild-type MEFs resulted in a clear reduction in the number of 5-bromodeoxyuridine (BrdU)-positive cells (Figure 3a). On the contrary, *ARF*-null MEFs were largely refractile to the antiproliferative effects of ING1 and retained a normal proliferation rate, evidenced by the similar percentage of BrdU-positive cells in vector- and p33ING1-infected cells. The proliferation rate of *p53/Mdm2*<sup>-/-</sup> MEFs was not affected by p33ING1 expression, in accordance with the reported requirement of p53 for p33ING1 function. Similar results were obtained when the rate of thymidine incorporation was measured (data not shown). Retroviral ectopic expression of p33ING1 in MEFs of either genotype did not cause a significant increase in apoptosis (Supplementary Figure 1). The induction of the p53 target, p21CIP1, is the most robust readout of ING-dependent p53 activation (Nagashima *et al.*, 2001). p21CIP1 contributes

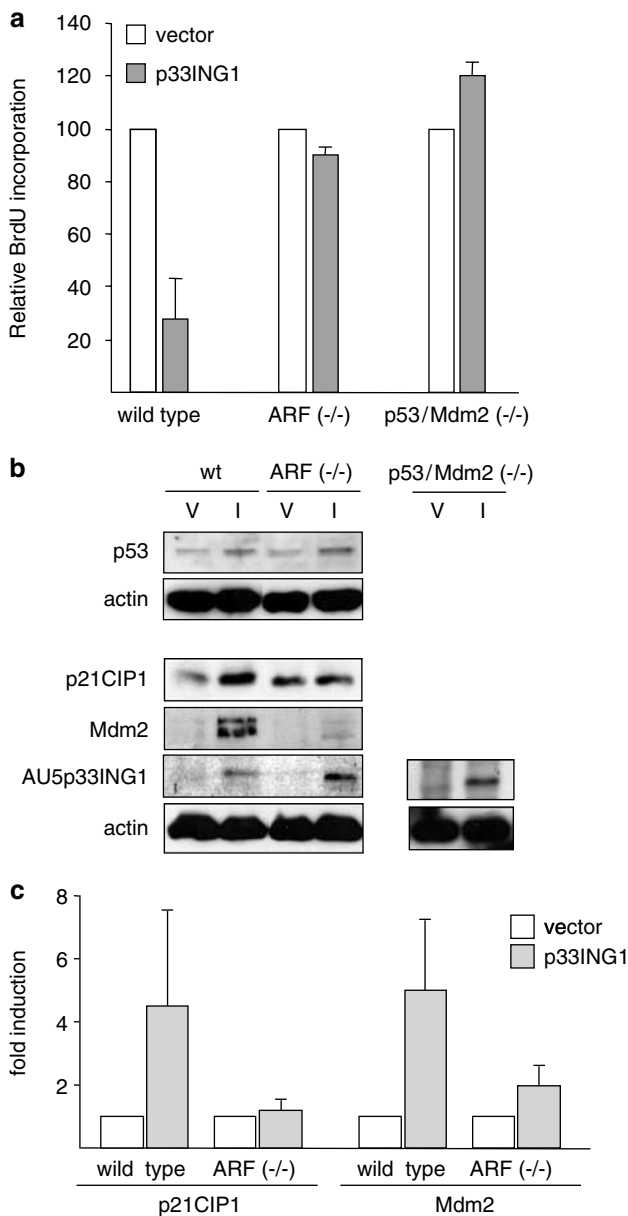


**Figure 2** Effects of ARF levels on the subcellular localization of p33ING1. **(a)** NIH-3T3 immortal murine fibroblasts growing on glass coverslips (LabTek) were transiently transfected with vectors encoding AU5-p33ING1, p14ARF or both as indicated, using Fugene 6 (Roche, Mannheim, Germany). Twenty-four hours later, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS and incubated in blocking solution (PBS containing 2% bovine serum albumin), overnight at 4°C. Cells were then incubated with the relevant antibodies in blocking solution for 60 min at room temperature, and washed with PBS (3–4 washes, 15 min each) before incubation with fluorochrome-conjugated secondary antibodies, and washing in the same conditions used with the primary antibodies. Cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4,6-diamidino-2-phenylindole. **(b)** Percentage of cells showing staining for p33ING1 predominantly in nucleoli (*nucleolar*) or evenly distributed in the nucleus (*nuclear*), in the absence or presence of p14ARF. The data shown are the average of two experiments. At least 200 cells per experiment were scored for each point. **(c)** Wild-type, early-passage mouse embryo fibroblasts (MEFs) were retrovirally transduced with a vector encoding RasV12, or an empty vector, and subsequently transfected with a vector expressing AU5-p33ING1. The subcellular localization of each protein was analysed as described in **(a)**. To detect human p14ARF, we used a 1:200 dilution of rabbit polyclonal DP40; mouse p19ARF was detected with rabbit polyclonal R562 (Abcam, Cambridge, UK, 1:200 dilution) and AU5-tagged p33ING1 protein was detected with anti-AU5 mouse monoclonal MMS-135R from Babco, Berkeley, CA, USA, at a 1:1000 dilution. As secondary fluorochrome-conjugated antibodies, we used Cy3-conjugated goat anti-mouse (Jackson Laboratories, West Grove, PA, USA, 1:800 dilution) and Alexa 488-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR, USA, 1:400 dilution). **(d)** Mouse embryo fibroblasts doubly deficient for p53 and Mdm2 were transiently transfected with vectors encoding AU5-p33ING1, p14ARF or both as indicated, and analysed by immunofluorescence as described in **(a)**. **(e)** 293T cells were transfected with vectors encoding DsRed-p33ING1 or GFP-p14ARF, and processed as described in **(a)**, except for the absence of primary and secondary antibodies. **(f)** Western blot analysis showing the changes in p19ARF protein levels after RasV12 retroviral infection of wild-type MEFs. **(g)** Western blot analysis of basal levels of p19ARF in the wild-type and p53/Mdm2-null MEFs used for immunofluorescence.

significantly, although not exclusively, to the antiproliferative effects of p53 (Brugarolas *et al.*, 1995; Deng *et al.*, 1995; Pantoja and Serrano, 1999). Therefore, p21CIP1 protein levels were analysed as a measure of the effectiveness of ING1 to trigger p53-dependent responses in these cells. Wild-type MEFs showed a clear induction of p21 upon retroviral infection with p33ING1 (Figure 3b and c). In accordance with the lack of antiproliferative effect of p33ING1 in *ARF*-null MEFs, p21CIP1 levels did not change significantly in these cells upon introduction of p33ING1 (Figure 3b and c). p21CIP1 was undetectable in *p53/Mdm2*<sup>-/-</sup> MEFs infected with p33ING1 or empty vector (data not shown). To determine whether the presence of functional ARF had a general impact on p53-dependent gene expression triggered by p33ING1, we extended our analysis to Mdm2, a p53 target whose expression can be

modulated by p33ING1 (Shimada *et al.*, 2002). Mdm2 levels were significantly increased in p33ING1-infected wild-type MEFs, but a much-reduced effect was observed in p33ING1-infected *ARF*-null MEFs (Figure 3b and c). It has been suggested that ING proteins can control p53 protein stability, possibly by interfering with Mdm2-mediated degradation of p53 (Leung *et al.*, 2002). Given the well-established role for ARF in the control of p53 protein stability, we wished to investigate a possible effect of ARF at this level. As shown in Figure 3b, ectopic expression of p33ING1 resulted in a modest but reproducible increase in p53 protein levels, both in wild-type and *ARF*-null MEFs, arguing against the participation of ARF in p33ING1-mediated p53 protein stabilization. We have also observed that deletions including either the N-terminus or the C-terminus of p33ING1 are unable to trigger cell-cycle arrest to the same extent as the full-length protein, this effect being more obvious for the C-terminal construct. This is in agreement with previous observations in human diploid fibroblasts (Goeman *et al.*, 2005). Interestingly, both deletion constructs showed an impaired capacity to increase p53 levels, and accordingly p21CIP1 and Mdm2 (Supplementary Figure 2). From these data, we conclude that stabilization of p53 and interaction with ARF appear to be separable functions of p33ING1. These observations clearly suggest that ARF is required for the induction by p33ING1 of cell-cycle arrest and p53-mediated transactivation, and highlight the functional relevance of the interaction between ARF and p33ING1.

In this report, we have presented evidence that indicates that the tumour suppressors ARF and p33ING1 are functionally linked. We show that both proteins can interact, that the subcellular location of p33ING1 can be modulated by ARF and that the ability of p33ING1 to provoke cell-cycle arrest and trigger p53-dependent transactivation requires functional ARF. There are several possible mechanisms by which ARF



**Figure 3** Requirement of ARF function for cell-cycle arrest and gene activation by p33ING1. **(a)** The proliferation rates of wild-type, *ARF*-null and *p53/Mdm2*-null early-passage mouse embryo fibroblasts (MEFs) infected with a retrovirus encoding AU5-p33ING1 or an empty vector were estimated by measuring their rate of 5-bromodeoxyuridine (BrdU) incorporation. Infected MEFs were plated on glass coverslips, and 24 h later, were incubated with BrdU (10  $\mu$ M final concentration, 2 h). Cells that were positive for BrdU were detected by indirect immunofluorescence with antibody BP40250 from Megabase Research Products, Lincoln, NE, USA (1:1000 dilution). The data are the average of three independent experiments. A total of 100–250 cells were counted for each point. **(b)** Western blotting analysis of the levels of p53, p21CIP1, Mdm2 and AU5p33ING1 in MEFs of the indicated genotypes, infected with empty vector (V) or pLPCAU5p33ING1 (I). The following antibodies were used: anti-p53 (CM5p, Novocastra, Newcastle, UK, 1:500), anti-p21 (C19, Santa Cruz, Santa Cruz, CA, USA, 1:500), anti-Mdm2 (2A10, a gift of Jesus Gil, Cancer Research UK, 1:250), anti-AU5 (MMS-135R, Babco, 1:1000) and anti- $\beta$ -actin (AC-15, Sigma, St Louis, MO, USA, 1:10000). **(c)** For quantitation, the signal for p21CIP1 or Mdm2 from four independent experiments was normalized by the signal for actin for each sample, and the value for the empty vector-infected cells was taken as reference.

and p33ING1 might cooperate. On the one hand, it has been suggested that ING proteins can control p53 protein stability, in connection with Mdm2 (Zeremski *et al.*, 1999; Leung *et al.*, 2002). Despite the important role of ARF in the control of p53 protein stability in other settings, our data indicate that p53 stabilization by p33ING1 does not require ARF, in agreement with the suggestion of competitive binding of Mdm2 and p33ING1 to p53 (Leung *et al.*, 2002). Furthermore, the analysis of deletion mutants indicates that p53 stabilization and ARF binding do not correlate. Other alternative levels of functional link must therefore be contemplated. In this regard, recent reports show that ARF plays an important role in ribosomal biogenesis, in the nucleolus (Itahana *et al.*, 2003; Sugimoto *et al.*, 2003; Bertwistle *et al.*, 2004). As p33ING1 can also accumulate in the nucleolus (Figure 2; see also Scott *et al.*, 2001a), a possible functional connection at this level is possible. More importantly, there is ample evidence, both in mammals and yeast, showing that ING proteins can form complexes with histone deacetylases and histone acetyltransferases. Based on this, it has been suggested that ING proteins could play a role in transcriptional control, through chromatin remodelling (Nourani *et al.*, 2001; Skowyrza *et al.*, 2001; Feng *et al.*, 2002; Kuzmichev *et al.*, 2002; Vieyra *et al.*, 2002; Xin *et al.*, 2004; Goeman *et al.*, 2005). An increasing number of evidences indicate that ARF can have a direct role in

transcriptional control (Kamijo *et al.*, 1998; Rocha *et al.*, 2003, 2005; Calabro *et al.*, 2004; D'Amico *et al.*, 2004; Datta *et al.*, 2004; Kalinichenko *et al.*, 2004; Qi *et al.*, 2004) and, therefore, it is feasible that ARF might cooperate with p33ING1 in transcriptional regulation, through chromatin remodelling processes. Our data showing a clear effect of ARF on p33ING1-dependent transactivation, in the absence of effects on p53 protein levels, are consistent with this hypothesis. In this regard, it is important to note that the N-terminal domain of p33ING1, which is sufficient for binding to p14ARF, is implicated in histone deacetylase-mediated transcriptional repression (Goeman *et al.*, 2005). Further understanding of the functional connection between ARF and p33ING1 should provide important information about the mechanisms underlying the role of these two proteins in tumour suppression.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)