ACCELERATED PUBLICATION

Alternative reading frame protein (ARF)-independent function of CARF (collaborator of ARF) involves its interactions with p53: evidence for a novel p53-activation pathway and its negative feedback control

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CARF, a collaborator of ARF (alternative reading frame protein), was cloned as a novel ARF-binding protein from a yeast-interaction screen. It potentiated ARF-mediated p53 function, and also caused a moderate increase in p53 activity in the absence of ARF. We herein report the molecular mechanism of ARF-independent function of CARF. By employing a variety of approaches, including overexpression of CARF, its suppression by small interfering RNA and use of protease inhibitors, we demonstrate that: (i) CARF directly interacts with wild-type p53, causing its stabilization and functional activation; and (ii) CARF and p53 levels show an inverse relationship that is instigated by a negative-feedback control via a proteasome-mediated degradation pathway.

Key words: ARF (alternative reading frame protein), CARF (collaborator of ARF), HDM2 (human double minute-2 oncoprotein), p53, RNAi (RNA interference).

INTRODUCTION

The *ARF–INK4a* locus on human chromosome 9p21 (where ARF represents 'alternative reading frame protein') plays a key role in tumour suppression. It encodes two distinct proteins, $p16^{INK4A}$ and ARF [1–4]. $p16^{INK4A}$ protein blocks the phosphorylation of the retinoblastoma gene product, and ARF acts upstream of p53 and enhances its function by sequestering the p53 antagonist, HDM2 (human double minute-2 oncoprotein), into the nucleolus [5–7]. It was shown that nucleolar localization of ARF is not essential, although it may enhance the availability of ARF to inhibit HDM2 [8,9]. Furthermore, the function of ARF did not fully correlate with HDM2 import into the nucleolus, suggesting that ARF function requires other events [9]. In addition to ARF homo-oligomers, a number of ARF-binding partners were shown to regulate the ARF/p53 pathway. These include E2F family members, spinophilin, topoisomerase I, MdmX, Pex19p, CARF (collaborator of ARF), cyclin G_1 and p120 (E4F) (reviewed in [10]). We have previously shown that an ARF-binding partner, CARF, potentiates stabilization of p53 and its transcriptional activation function [11]. In addition, CARF caused a moderate stabilization and activation of p53 in the absence of ARF [11,12]. In the present study, we report that the ARF-independent action of CARF involves direct interaction with p53. In turn, CARF encounters negative feedback regulation via a proteasome-mediated degradation pathway.

EXPERIMENTAL

Plasmid constructions, cell culture and transfections

Full-length CARF was cloned into the mammalian expression vectors pcDNA3.1/V5, pcDNA4/HisMax, pEGFPN1 and pIND/V5, and the bacterial expression vector pQE30 (Qiagen) [11]. p53 was cloned into the bacterial expression plasmid pGEX-6P (Pharmacia Biotech) to encode a GST (glutathione S-transferase)–p53 fusion protein.

Cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10 % (v/v) fetal bovine serum and induced for protein expression, as described below. Transfections were performed using lipofectAMINE™ Plus (Invitrogen). Typically, 3 μg of plasmid DNA was used per 80 % confluent 6-cm dish culture.

p53 activity and cell growth assays

p53 activity was assayed by p53-dependent reporter assays. Cell growth was monitored by cell counting and colony-forming assays. Cells were fixed in methanol, stained with 0.1 % (v/v) Crystal Violet solution, photographed and counted.

Co-immunoprecipitation, pull-down assay and Western blot analysis

Cell lysates were incubated at 4 °C for 1–2 h with an antibody, as described in the Figure legends. Immunocomplexes were separated by incubation with Protein-A/G–Sepharose, and Western blotting was performed as described below. For immunodepletion assays, lysates were sequentially incubated with anti-CARF, anti-p53 (positive control) or an unrelated antibody (negative control). Immunoprecipitated proteins were pelleted by incubation with Protein-A/G–Sepharose, and the supernatant was again incubated with the respective antibodies. The amount of p53 protein remaining in the supernatant was visualized by Western blotting with anti-p53 antibody. For pull-down assays, approx. 125 ng of

Abbreviations used: ARF, alternative reading frame protein; CARF, collaborator of ARF; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S-transferase; HDM2, human double minute-2 oncoprotein; HM, His Max; Ni-NTA, Ni^{2+}-nitrilotriacetate; RNAi, RNA interference; RT-PCR, reverse transcriptase-PCR; siRNA, small interfering RNA.

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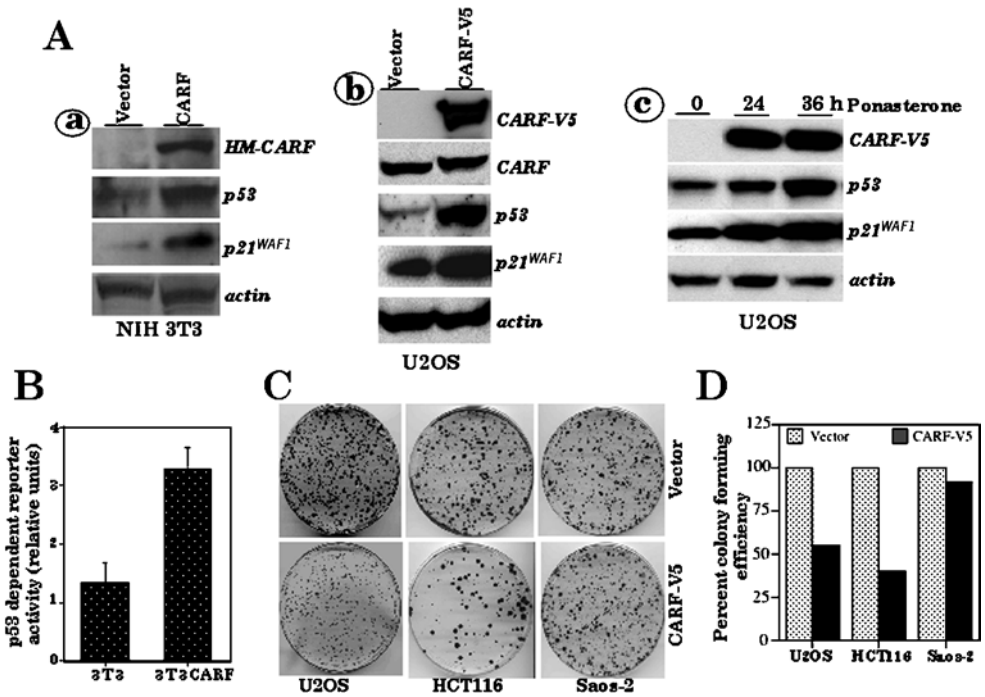

Figure 1 ARF-independent activation of p53 function by CARF

(**A**) NIH-3T3 cells stably transfected with vector or HM-tagged CARF-encoding expression plasmid were analysed for expression of p53 and $p21^{WAF1}$ by Western blotting. Exogenous expression of CARF was detected by anti-Express antibody. The membrane was probed with anti-actin antibody as a loading control. Cells overexpressing CARF showed high levels of p53 and $p21^{WAF1}$. Similar to NIH-3T3 cells, CARF-overexpressing derivatives of U2OS cells showed high levels of p53 and $p21^{WAF1}$. Ponasterone (5 μM)-inducible expression of CARF-V5 in U2OS cells led to a parallel increase in p53 and $p21^{WAF1}$ levels. (**B**) p53-dependent reporter assay in control and CARF-overexpressing NIH-3T3 transfectants. A higher level of transcriptional activation of p53 was detected in the CARF derivatives. (**C** and **D**) Colony-forming efficiency of U2OS, HCT116 and Saos-2 cells transfected with vector or CARF-V5-encoding expression plasmid. CARF-overexpressing cells showed a reduction in colony-forming efficiency in U2OS (45 %) and HCT116 (60 %) cells. Saos-2 cells did not show any significant effect.

bacterially expressed His_6-CARF protein and GST–p53 protein were mixed in Nonidet P40 lysis buffer (100 μl) in the presence of 200 ng of BSA as a competitor. His_6-CARF was pulled-down with Ni-NTA (Ni^{2+}-nitrilotriacetate) agarose (20 μl), and the co-precipitation of GST–p53 was detected by Western blotting with anti-GST antibody. For proteasome-inhibitor assays, cells were treated with MG132 (20 μM; Calbiochem), leptomycin B (10 ng/μl; Sigma) and lactacystin (20 μM; Calbiochem) for 12–24 h. For RNAi (RNA interference) assays, CARF-specific siRNA (small interfering RNA) was used to target CARF expression, as described previously [11].

Western blotting was performed with anti-V5 tag (Invitrogen), anti-CARF antibody [11], anti-p53 (DO-1 and FL-393; Santa Cruz Biotechnology), anti-p21 (C-19; Santa Cruz Biotechnology), anti-GST (B-14; Santa Cruz Biotechnology), anti-GFP (Clontech) and anti-actin (Boehringer Mannheim) antibodies, as described previously [11].

Immunostaining

Cells were stained with anti-CARF [11], anti-p53 (DO-1) and anti-nucleolin (sc-8031; Santa Cruz Biotechnology) antibodies, as described in [11]. Alexa-488-conjugated goat anti-mouse and Alexa-594-conjugated goat anti-rabbit (Molecular Probes) antibodies were used as secondary antibodies. The cells were examined either on an Olympus microscope and images were captured using the EM CCD system (Andor Technology Ltd, Tokyo, Japan) with CCD Chip (Texas Instruments) or on a Carl Zeiss microscope. The extent to which the two proteins overlapped was assessed by combining the two images using Metamorph software.

RT-PCR (reverse transcriptase-PCR) analysis

Total RNA (2 μg) was used to amplify the CARF fragment by RT-PCR (Gibco BRL) using CARF-specific primers (sense: 5′-AGC TCA GAG ATC GAG GTG CC-3′ and antisense: 5′-CTC TCA CTT GAC TGT GAA GT-3′) for 40 cycles (95 °C for 5 min; 94 °C for 1 min; 57 °C for 1 min; and 72 °C for 1 min). For amplification of 1.0 kb of the p53 transcript from control and CARF-overexpressing cells, p53-specific primers (sense: 5′-ATG ACT GCC ATG GAG GAG TC-3′ and antisense: 5′-CCT GCT GTC TCC AGA CTC CTC-3′) were used for 20 cycles (95 °C for 5 min; 94 °C for 1 min; 54 °C for 1 min; and 72 °C for 1 min). The amplified products were visualized on a 1 % (w/v) agarose gel. Control PCRs were performed with GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-specific primers.

RESULTS AND DISCUSSION

Mouse immortal (NIH-3T3) and human osteosarcoma (U2OS) cells that lack endogenous $p19^{ARF}$ and $p14^{ARF}$ were stably transfected with expression plasmids encoding HM (His Max)- or V5-tagged CARF proteins respectively. As compared with the vector-transfected controls, the CARF-overexpressing transfectants both in NIH-3T3 (Figure 1A, panel a) and U2OS (Figure 1A, panels b and c) cells had an increased level of p53 protein. Furthermore, p53 activity, as detected by the p53-downstream effector

protein $p21^{WAF1}$ (Figure 1A) and p53-dependent reporter assay (Figure 1B), was also increased in the transfectants. The transfectants showed flattened morphology and slow growth (results not shown) as compared with the vector-transfected controls, suggesting that CARF can activate p53 function in the absence of ARF. Overexpression of CARF in U2OS (osteosarcoma) and HCT116 (human colon carcinoma) cells with wild-type p53 function caused an approx. 45–60 % reduction in the colony number in two independent experiments. Saos-2 cells lacking p53 function showed only a 6–10 % reduction (Figures 1C and 1D). No apparent signs of apoptosis were observed. These data demonstrated that, in addition to the activation of ARF-dependent p53 function [11], CARF could activate ARF-independent p53 function, leading to the growth arrest of cells. This constitutes a novel pathway for p53 activation.

We next investigated whether the activation of p53 by CARF involves an interaction between p53 and CARF. *In vitro* and *in vivo* immunoprecipitation assays were performed. p53 was immunoprecipitated from CARF-V5-transfected COS-7 cells by a p53-specific polyclonal antibody. Co-immunoprecipitation of exogenously expressed CARF-V5 was detected by Western blotting of the p53 immunocomplexes with anti-V5 antibody (Figure 2A panel a, lane 2). Immunoprecipitation of endogenous CARF resulted in co-immunoprecipitation of p53 in U2OS cells (Figure 2A panel b, lane 2). Since multiple antibodies raised against CARF are not available at present, immunoprecipitation and Western detection of the proteins were performed with the same antibody. Thus the detection of IgG on Western blots was unavoidable, resulting in a rather poor resolution of the p53 and CARF bands (Figure 2A, panel b). To circumvent this limitation, we performed sequential immunodepletion of CARF by an anti-CARF antibody, and detected the level of p53 in the CARF-immunodepleted lysates. CARF immunodepletion from cell lysates resulted in a decreased level of p53 protein in the supernatant (Figure 2B, panels a and b). These assays strongly supported the notion that CARF and p53 interact *in vivo*, and that the interaction does not require the presence of ARF.

To determine whether CARF and p53 interact directly, *in vitro* pull-down assays of the recombinant proteins were performed. Bacterially expressed epitope-tagged proteins were mixed in the presence of BSA as a competitor. His_6-CARF was precipitated from the mixture with Ni-NTA agarose. The precipitant was examined for the presence of GST–p53 by Western blotting with anti-p53 antibody. Indeed, GST–p53 was also pulled down, along with His-CARF, by precipitation with Ni-NTA agarose (Figure 2A panel c, lane 4); Ni-NTA agarose did not precipitate GST–p53 directly (lane 2). The degraded forms of GST–p53 (Figure 2A, panel c, lane 1) were not precipitated, confirming that the pull-down of GST–p53 with His_6-CARF is not due to non-specific aggregation of the proteins. Similarly, a pull-down of GST–p53 with glutathione–Sepharose 4B beads also precipitated His_6-CARF (results not shown). On the basis of these data, it was concluded that CARF and p53 proteins interact directly. Results from the co-immunostaining experiments revealed that the two proteins co-localized in the nucleoplasm (Figure 2C, panels a and b). CARF was excluded from the nucleolus in U2OS cells (Figure 2C, panels c). Taken together, the results revealed that CARF interacts with p53 in the nucleoplasm, and activates its transcriptional activation function in the absence of $p19^{ARF}$.

We next addressed the physiological relevance of the p53-activation function of CARF. The level of CARF protein was examined in normal and immortalized cells with wild-type or functionally compromised p53. Notably, we found that the cells with wild-type p53 function (U2OS and MCF-7) have a low level of CARF expression as compared with those with compromised p53 function (Saos-2 and HeLa; Figure 3A). Consistent with the present data, CARF expression was most significantly up-regulated in WET cells [WI-38 cells transformed with E6E7 and hTERT (human telomerase reverse transcriptase)] and MRC5–simian virus (SV)40 cells (transformed human embryonic fibroblasts with compromised p53 function) as compared with their parent normal cells, WI-38 and MRC-5 respectively (Figure 3A). Since CARF activates wild-type p53 function leading to growth arrest of cells, such a reciprocal regulation of CARF and p53 expression is physiologically relevant, and would allow cells with wild-type p53 function to progress through the cell cycle. It is notable that CARF-overexpressing derivatives of Saos-2 cells did not show significant retardation in growth or colony formation (Figure 1C). These data clearly suggested that cells compromised for p53 function can tolerate a high level of CARF and continue to divide. This is similar to the relationship between ARF and p53 [13].

Since an overactivation of p53 function by CARF would be unfavourable for progression through the cell cycle, we hypothesized that the high level of wild-type p53 might down-regulate CARF for cell cycle progression. The level of wild-type p53 is determined by its synthesis and degradation, and varies with cell cycle progression. Therefore we examined the relationship of CARF and p53 at the single-cell level. In an unsynchronized U2OS cell culture that has cells at different phases of the cell cycle, and thus different levels of expression of p53, variations in the level of CARF were observed. Notably, cells with a high level of p53 expression showed faint CARF staining, and vice versa (Figure 3B). Such a variation in the level of expression of CARF, and its inverse relationship with p53, is consistent with its p53-activating and growth-retarding properties. The data supported the inverse relationship of CARF and p53 observed in different cell lines (Figure 3A).

We examined this inverse regulation of CARF and p53 expression in tumour cell lines (Saos-2tetp53 and H1299tsp53) with inducible expression of p53. Indeed, an induction of wild-type p53 in H1299 cells by a temperature-shift to 32 °C, or in p53-inducible Saos-2 cells by incubation with doxycycline, caused a reduction in the level of CARF expression (Figures 3C and 3D). In a continuation of our previous study that demonstrated the interaction of CARF and $p19^{ARF}$, their co-regulation and collaboration in p53-activation function [11], we have shown in the present study that: (i) CARF interacts with p53 directly and enhances its transcriptional activation function, resulting in growth arrest of cells; and (ii) CARF expression is inversely related to that of wild-type p53, suggesting a negative feedback regulatory circuit.

We next investigated the molecular basis of the inverse relationship of CARF and p53. RT-PCR analysis of CARF in cells with variable p53 functions revealed a comparable level of CARF expression, suggesting that the inverse relationship of CARF and p53 is not regulated at the transcriptional level (Figure 4A). Notably, cells induced for CARF-V5 expression by ponasterone (Figure 4B, panel a) showed no difference in the level of expression of the p53 transcript (Figure 4B, panel b). The data clearly showed that CARF does not up-regulate p53 protein at the transcriptional level; instead, it may function by stabilizing p53.

p53 is known to undergo degradation by HDM2, a ubiquitin ligase that negatively regulates proteins (including itself and p53) via ubiquitination and targeting for proteasomal degradation [14,15]. We next used inhibitors of proteasome-mediated degradation, leptomycin (which inhibits nuclear-cytoplasmic export) and lactacystin and MG132 (which inhibit the proteasome-degradation machinery), and examined the following two possibilities, i.e. (i) whether p53 acts as a transcriptional repressor of CARF or (ii) whether p53 drives CARF to proteasome-mediated

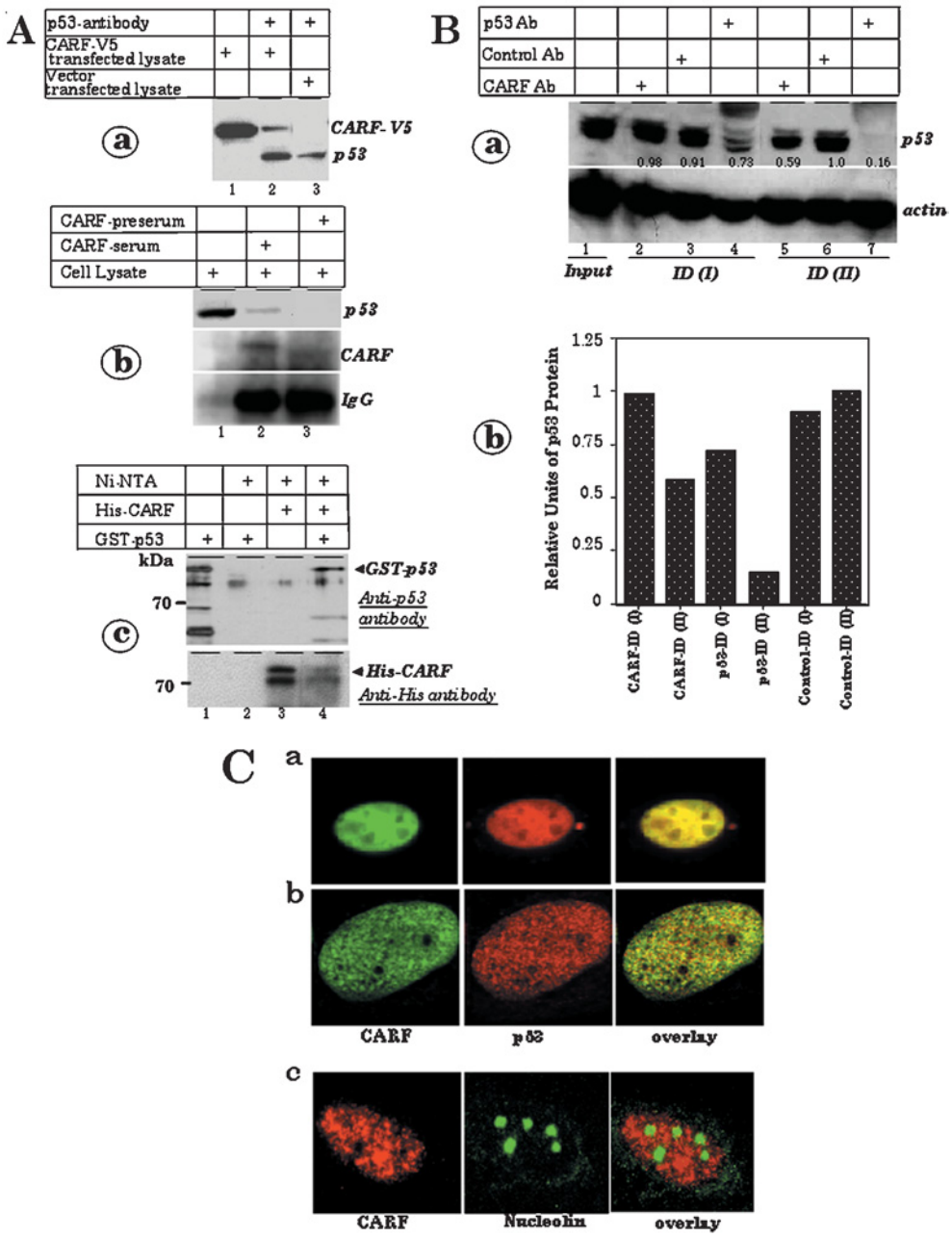

Figure 2 CARF directly interacts with p53

(**A** and **B**) Co-immunoprecipitation of CARF and p53. (**A**, **a**) p53 was immunoprecipitated from cells transfected either with the vector (lane 3) or with CARF-V5 expression plasmid (lane 2). An anti-p53 polyclonal antibody was used for immunoprecipitation. Precipitated p53 was detected by Western blotting with an anti-p53 monoclonal antibody. Co-immunoprecipitated CARF-V5 was detected by Western blotting with anti-V5 antibody. (**A**, **b**) CARF-V5 immunoprecipitated with endogenous p53. Endogenous CARF was immunoprecipitated from U2OS cells by anti-CARF antibody, and CARF immunocomplexes were examined for the presence of p53 by Western blotting with anti-p53 antibody. p53 was detected in CARF complexes. Immunoprecipitated CARF was also detected by Western blotting with anti-CARF antibody, which was used for immunoprecipitation resulting in detection of dense IgG bands. (**A**, **c**) *In vitro* pull-down assays of CARF and p53. His_6-CARF and GST–p53 were mixed in the presence of BSA as a competitor. His_6-CARF was pulled down by Ni-NTA beads, and co-precipitation of GST–p53 was detected by Western blotting with anti-p53 antibody. The multiple bands seem in lane 1 represent degraded forms of GST–p53 (with the uppermost band being the full-length GST–p53 protein) that are detected by anti-p53 antibody. GST-p53 (lane 4) was pulled down with His_6-CARF, but not with Ni-NTA directly (lane 2). (**B**) Cell lysates were immunodepleted with anti-CARF, anti-p53 or unrelated control antibody. The amount of p53 left in the supernatant (immunodepleted for CARF) was determined by Western blotting with anti-p53 antibody. Two rounds of immunodepletion were performed, and the relative amounts of p53 in the supernatant before and after immunodepletion of CARF were calculated by quantification of the signals using imaging software. Quantification of p53 protein in the supernatant after immunodepletion of CARF is shown in the histogram (**B**, **b**). CARF immunodepletion led to an approx. 42 % decrease in p53 in two rounds, and there was no change in p53 protein in the supernatant after immunodepletion of an unrelated protein. As expected, immunodepletion with p53 caused an 80 % decrease in p53 in the supernatant. (**C**) Co-immunostaining of endogenous p53 and CARF in U2OS cells. Low- (**a**) and high (**b**)-resolution images are shown, along with double immunostaining of CARF and nucleolin (**c**) to demonstrate the exclusion of CARF from the nucleolus. CARF and p53 co-localized to a considerable extent in the nucleoplasm.

degradation. In the first scenario, an increased amount of p53 resulting from inhibition of its degradation would result in an enhanced transcriptional repression of endogenous CARF. In the second scenario, the inhibition of p53 degradation would also inhibit degradation of CARF, causing an increase in the levels of both CARF and p53. We found that all the three inhibitors

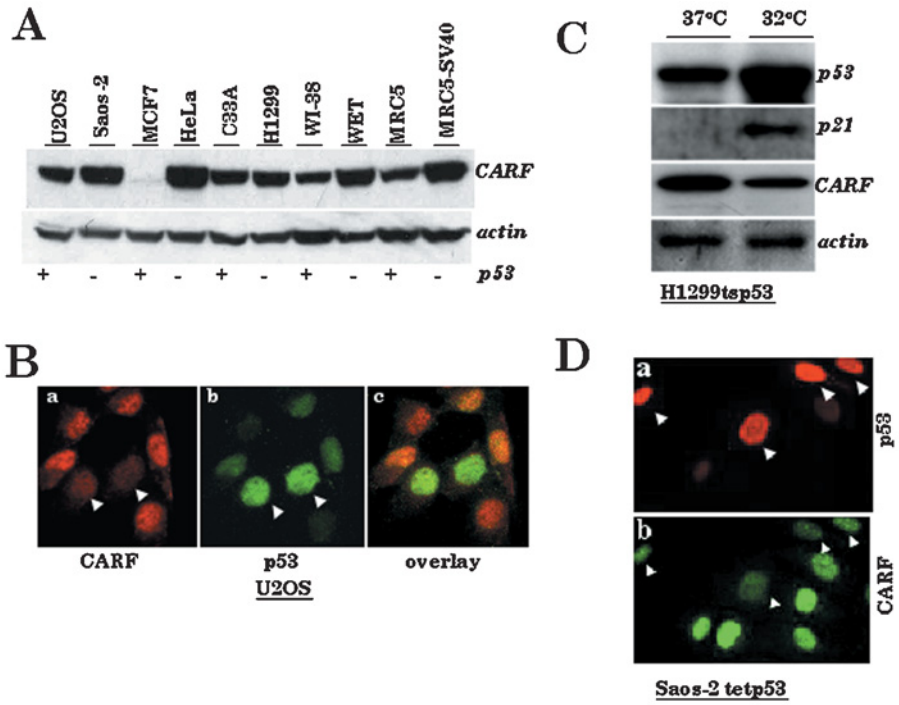

Figure 3 CARF and p53 are inversely regulated

(**A**) Expression level of CARF protein in various cells. Full wild-type p53 function is indicated by the plus signs, and minus signs signify its partial or complete functional loss. The level of CARF protein was high in cells that have compromised p53 function. (**B**) Comparison of CARF and p53 expression at the single-cell level in an unsynchronized culture of U2OS cells. Cells with high level of p53 showed a low level of CARF fluorescence, and vice versa. (**C**) Induction of wild-type p53 in H1299 cells resulted in a reduction of CARF expression, as detected by Western blotting. (**D**) Induction of wild-type p53 in Saos-2 cells resulted in reduction of CARF, as detected by immunofluorescence. Note that the cells lacking p53 expression maintained a high level of CARF expression.

[leptomycin, lactacystin (results not shown) and MG132] resulted in stabilization of p53 as well as CARF in both mouse (Figure 4C) and human (Figure 4D) cells with wild-type p53. Most noticeably, the level of CARF expression did not increase in response to treatment with the proteasome inhibitor MG132 in Saos-2 cells that lack p53 (Figure 4D, lanes 5 and 6). In the same cells, when p53 was expressed exogenously, MG132 treatment caused an increase in CARF protein (Figure 4D, lanes 7 and 8). The data strongly supported the second scenario, i.e. CARF is negatively regulated via a proteasomal-degradation pathway that involves p53. Involvement of HDM2, one of the downstream effectors of p53, in proteasomal degradation of p53 is well established. It is possible that the CARF–p53 complex is degraded by a similar mechanism. This would also provide an explanation as to how p53 activity may impinge on CARF expression. It remains to be elucidated whether CARF has a role in the proteasomal degradation machinery driven by HDM2.

We next performed siRNA-mediated suppression of CARF expression, and examined the consequences of this on p53 levels. Cells targeted for CARF expression did not show any change in the level of p53 (Figure 4E). It is noteworthy that, in ARF-expressing cells, targeting of CARF resulted in decreased ARF and p53 proteins [11]. These data demonstrated that the increased level of CARF protein results in increased stability and activity of p53 in the presence [11] or absence (Figure 1) of ARF. However, its suppression affects only ARF-mediated p53 function [11]. Taken together with the above data (Figures 3 and 4), we may conclude that: (i) an overexpression of CARF resulted in up-regulation of ARF-dependent [11] and -independent p53 function; (ii) p53 executes a negative feedback control on CARF; and (iii) whereas knockdown of CARF compromised both the level of ARF and p53 activity [11], it did not affect the p53 level, suggesting that, in the absence of ARF, a low level of CARF expression may be sufficient for p53 stability (Figure 4F).

ARF-mediated activation of p53 function has been implicated in replicative and premature senescence-associated growth arrest of cells [1,2,4,16–18], and in many aspects of the cell's response to its environment [19]. Thus understanding the factors that regulate its activity is critically important. Different pathways for ARF function involving HDM2, p53, cyclin G_1 or the E2F family of transcription factors have been suggested [5,7,11,17,20,21]. CARF was cloned as a novel binding partner of ARF that activates p53 function [11]. In a cellular background lacking ARF, but with intact wild-type p53 function (both in mouse and human cells), CARF overexpression resulted in growth arrest with no apparent signs of apoptosis. Those data suggested that overexpression of CARF supports p53-mediated growth arrest of cells independently from that of ARF protein.

Interactions of ARF and HDM2 in the nucleolus, leading to functional inactivation of HDM2 by its sequestration in the nucleolus, which results in activation of p53 function have been described. The p53-activating function of ARF, independent of the import of HDM2 to the nucleolus and involving other proteins, has been suggested by other studies (e.g. [13]). We cloned CARF as a novel ARF-binding protein and, together with our previous studies [11,12], it is concluded that: (i) CARF interacts with ARF in the nucleolus, causes its stabilization and activation of p53 function [11]; (ii) in the absence of ARF, CARF interacts with p53 in the nucleoplasm, causing its stabilization and functional activation; and (iii) CARF undergoes proteasomal-mediated degradation that involves p53. Taken together, a novel pathway of CARF-mediated p53 activation and its feedback regulation is demonstrated.

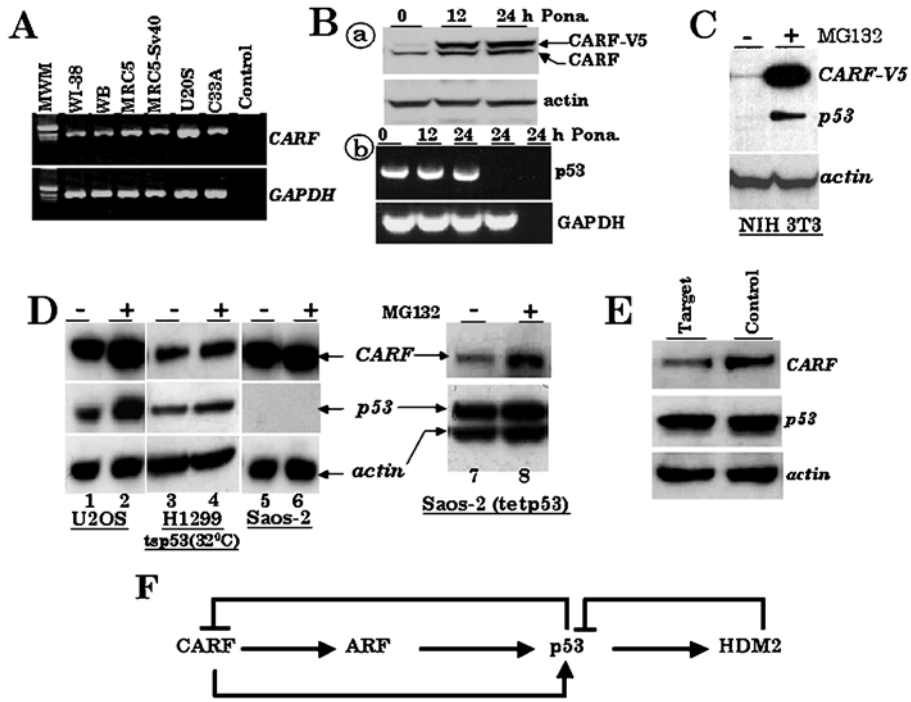

Figure 4 Feedback regulation of CARF by p53 involves degradation

(**A**) RT-PCR analysis of CARF in different cell lines. GAPDH was used as an internal control. (**B**) Western blotting for CARF (**a**) and RT-PCR for p53 (**b**) in U2OS cells induced for CARF expression with ponasterone (Pona.) where lane 4 from the left shows RT-PCR without p53 primers and lane 5 is a water control. CARF-induced cells showed no change in the expression of p53 at the transcriptional level. (**C**) Elevated p53 and CARF proteins following treatment of mouse cells with the proteasome inhibitor, MG132 (20 μM). (**D**) Human osteosarcoma (U2OS) and lung carcinoma with temperature-sensitive p53 (H1299tsp53) cells showed high levels of p53 and CARF expression following treatment with MG132 (20 μM). Actin was used as a loading control. An MG132-induced increase in CARF expression was observed in Saos-2 cells only when they were induced for p53 expression (lanes 7 and 8 as compared with lanes 5 and 6). (**E**) Effect of CARF siRNA on p53 levels in U2OS cells. Suppression of CARF expression by siRNA (target) did not cause any change in the p53 level. The control lane shows the protein levels in cells transfected with the control oligonucleotides. (**F**) Schematic representation of the involvement of CARF in the p53 pathway, and its feedback regulation.

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