Regulation of p53 tetramerization and nuclear export by ARC

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Inactivation of the transcription factor p53 is central to carcinogenesis. Yet only approximately one-half of cancers have p53 loss-of-function mutations. Here, we demonstrate a mechanism for p53 inactivation by apoptosis repressor with caspase recruitment domain (ARC), a protein induced in multiple cancer cells. The direct binding in the nucleus of ARC to the p53 tetramerization domain inhibits p53 tetramerization. This exposes a nuclear export signal in p53, triggering Crm1-dependent relocation of p53 to the cytoplasm. Knockdown of endogenous ARC in breast cancer cells results in spontaneous tetramerization of endogenous p53, accumulation of p53 in the nucleus, and activation of endogenous p53 target genes. In primary human breast cancers with nuclear ARC, p53 is almost always WT. Conversely, nearly all breast cancers with mutant p53 lack nuclear ARC. We conclude that nuclear ARC is induced in cancer cells and negatively regulates p53.

apoptosis | breast cancer

The tumor suppressor p53 is critical in the prevention of neoplasia through its activation of programs that promote genomic stability, cell cycle arrest, and apoptosis (1). Although some p53 effects may involve nontranscriptional mechanisms, many are mediated through its function as a transcription factor (2). Inactivation of p53 signaling is essential for carcinogenesis (3). This is achieved through mutations in the p53 protein itself, most often in the DNA binding domain. Such mutations occur, however, in only ≈50% of tumors (4). In the remainder in which p53 is WT, the protein is degraded or relocated from the nucleus (5–7).

Apoptosis repressor with caspase recruitment domain (ARC) is an endogenous inhibitor of apoptosis that is expressed primarily in terminally differentiated cells such as cardiac and skeletal myocytes and neurons (8). ARC resides in both the nucleus and cytoplasm (9, 10). Whereas cytoplasmic ARC inhibits both the death receptor and mitochondrial apoptosis pathways through direct interactions with Fas, Fas-associated death domain (FADD), and Bax (11), the function of nuclear ARC is unknown. Recently, ARC has been noted to be upregulated in a wide variety of cancer cell lines and primary human breast cancers (9, 10).

In this study, we demonstrate an unexpected direct interaction in the nucleus between endogenous ARC and endogenous p53. This interaction, which involves the tetramerization domain of p53, disrupts p53 tetramerization. This, in turn, exposes a nuclear export signal in p53 that stimulates Crm1-dependent exclusion of p53 from the nucleus. The physiological significance of this mechanism is underscored by knockdown of endogenous ARC in cancer cells, which induces endogenous p53 to tetramerize, relocate to the nucleus, and transactivate its endogenous target genes. Furthermore, the observation in primary human breast cancers that nuclear ARC is almost always accompanied by WT p53, and conversely, that nuclear ARC is absent when p53 is mutant suggests that ARC serves to inactivate WT p53.

Results

Endogenous ARC Interacts Directly with Endogenous p53 and Inhibits p53-Dependent Cell Death and Transcription. Given that p53 is an activator of apoptosis, that disruption of the p53 axis is involved in almost all tumors, and that ARC is an inhibitor of apoptosis that is induced in cancer cells, we hypothesized a link between ARC and p53. By using MCF7 breast cancer cells, which contain abundant ARC and WT p53, we found that endogenous ARC interacts with endogenous p53 (Fig. 1*A*), an interaction that was detected in both nuclear and cytoplasmic compartments (data not shown). We determined that the binding between ARC and p53 is direct by using radiolabeled *in vitro*-transcribed translated proteins [see supporting information (SI) Fig. 5] and purified, bacterially expressed proteins (Fig. 2).

We next assessed the effect of ARC on p53 function. By using HEK293 cells, which have low levels of endogenous ARC, we observed that ectopic expression of ARC markedly inhibits p53-induced cell death (Fig. 1*B*). Because p53 is a transcription factor and ARC interacts with p53 in the nucleus, we tested the ability of ARC to inhibit p53-dependent transcription. By using a p53 reporter gene in ARC-deficient U2OS cells, we found that expression of ARC interfered with transcription driven by both endogenous (Fig. 1*C*, lanes 4 and 5) and overexpressed (Fig. 1*C*, lanes 6–10) p53 in a dose-dependent manner. ARC also abrogated doxorubicin-induced expression of endogenous mdm2 and p21CIP1/WAF1, well established p53 target genes (Fig. 1*D*).

The p53–ARC Interaction Is Mediated by the Tetramerization Domain of p53. To gain insight into the mechanism by which ARC inhibits the induction of p53- responsive genes, we mapped the domains of ARC and p53 that mediate their interaction. Deletion of ARC residues 125–175 (ARCΔ125–175), which are within the proline-glutamic acid-rich domain, disrupts the interaction of ARC with endogenous p53 in HEK293 cells (see SI Fig. 6). In contrast, a deletion in the ARC caspase recruitment domain (CARD) does not interfere with p53 binding, which is notable because previously demonstrated ARC interactions with Fas, FADD, and Bax have involved the ARC CARD (11). To map the ARC-interacting domain in p53, purified recombinant p53 fragments were mixed with purified recombinant ARC (Fig. 2). Our results indicate that p53 residues 323–356, which represent the tet-

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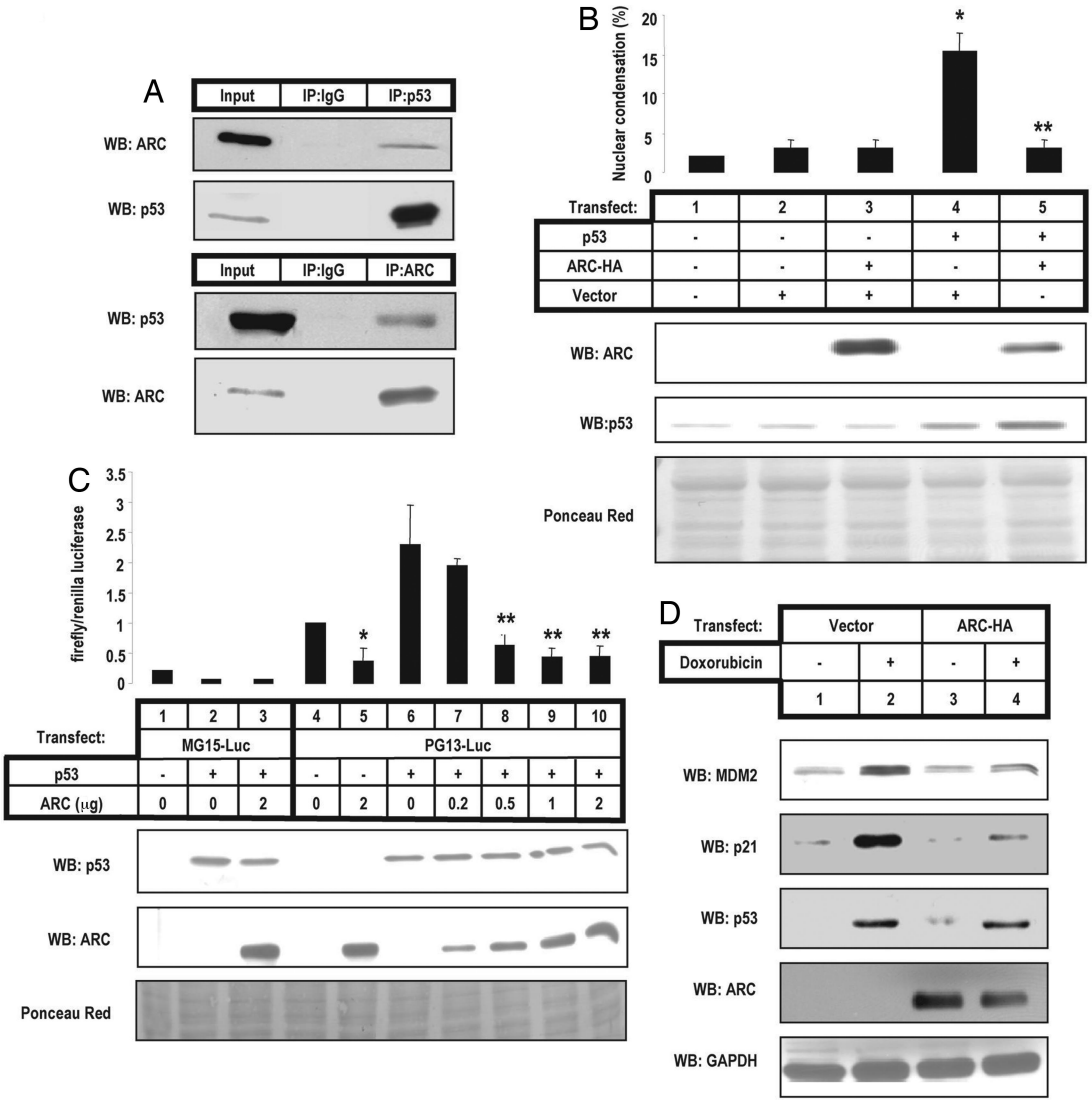

Fig. 1. Endogenous ARC interacts with an endogenous p53 and regulates p53-dependent cell death and transcription. (*A*) Endogenous ARC interacts with endogenous p53 in MCF7 cells. Immunoprecipitations (IPs) were performed, resolved by SDS/PAGE, and analyzed by Western blotting (WB). Input lanes contained 3% of the lysate used for immunoprecipitations. (*B*) ARC inhibits p53-induced apoptosis. HEK293 cells, which have low levels of ARC, were transfected with the indicated plasmids, and cell death was assessed by the percentage of cells with nuclear condensation (mean ± SEM). *, $P < 0.01$, lane 4 vs. lane 2. **, $P < 0.01$, lane 5 vs. lane 4 (Student's *t* test). Expression of ARC and p53 was confirmed by WB. (*C*) ARC inhibits transcription of a p53-dependent reporter gene. U2OS cells, which are deficient in ARC, were transfected with the indicated plasmids. PG13-Luc is a p53-dependent firefly luciferase reporter gene containing 13 p53-response elements, whereas MG15-Luc is a control containing 15 mutated p53-response elements. In addition, a constitutively driven *Renilla* luciferase reporter gene was included for normalization. p53-dependent transcription is indicated by the ratio of firefly/*Renilla* luciferase activities. *, $P < 0.01$, lane 5 vs. lane 4. **, $P < 0.01$, lanes 8–10 vs. lane 6 (Student's *t* test). Expression of p53 and ARC was confirmed by WB. (*D*) ARC inhibits activation of endogenous p53-dependent genes. U2OS cells were transfected with the indicated plasmids. Thirty-six hours later, cells were treated with 1 μM doxorubicin (dox) for 4 h, after which WB was performed.

ramerization domain, are sufficient for binding to full-length ARC. Interestingly, p53 fragments encompassing all three nuclear localization signals (NLSs) (12, 13), as well as those encompassing the transactivation and sequence-specific DNA binding domains, fail to bind ARC (data not shown).

ARC Disrupts p53 Tetramerization and Stimulates p53 Nuclear Hyperexport. Because ARC binds the p53 tetramerization domain, we asked whether ARC modulates p53 tetramerization. Saos2 cells, which are deficient in both p53 and ARC, were cotransfected with p53 and WT ARC, the interaction defective mutant ARCΔ125–175, or EGFP. Immunoblotting of glutaraldehyde-cross-linked lysates demonstrated that ARC, but not ARCΔ125–175 (data not shown) or EGFP, results in the absence of p53 tetramers (Fig. 3*A*).

The p53 tetramerization domain is known to contain a functional nuclear export signal (NES) (14). Structural analyses have shown that this NES is hidden when p53 is tetrameric (15–17). Because ARC inhibits p53 tetramerization, we next assessed whether ARC promotes p53 cytoplasmic localization by nuclear export. To address this question, we first used a GFP-tagged p53 C-terminal fragment (318–393) that includes the tetramerization domain (GFP-p53CT). When transfected into Saos2 cells, GFP-p53CT localizes to the nucleus as previously reported (18) (Fig. 3*B*, first row). Coexpression of ARC, which itself resides in both nucleus and cytoplasm, relocates GFP-p53CT to the cytoplasm (Fig. 3*B*, second row). To dissect the relocation mechanism, we

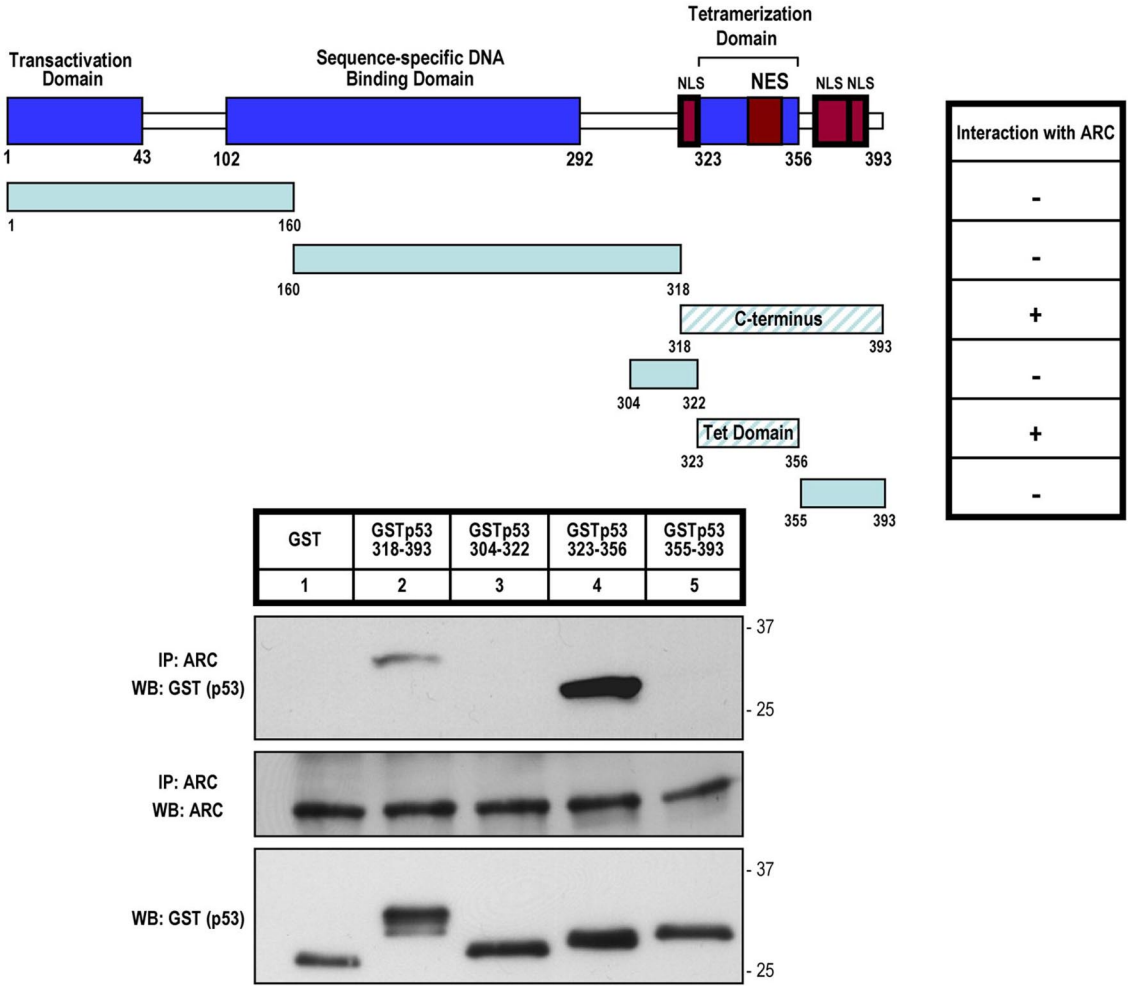

Fig. 2. The tetramerization domain of p53 binds ARC directly. Purified, recombinant GST-p53 peptides were mixed with purified, recombinant His full-length ARC and analyzed by immunoprecipitation (IP)–Western blotting (WB) as indicated.

used ARC-NLS, containing three NLSs fused to ARC and which is almost exclusively nuclear (Fig. 3*B*, third row). Consistent with an effect originating within the nucleus, ARC-NLS once again relocates GFP-p53CT to the cytoplasm (Fig. 3*B*, third row). To assess whether nuclear export is, in fact, the mechanism, cells were treated with leptomycin B (LMB), which specifically inhibits the binding of Crm1, an essential part of the p53 nuclear export machinery, to the NES (19). We found that LMB abrogates ARC-NLS-induced cytoplasmic relocation of GFP-p53CT (Fig. 3*B*, fourth row). Similarly, full-length p53 is relocated to the cytoplasm by ARC and ARC-NLS, and this relocation is reversed by LMB (Fig. 3*C*). These results indicate that ARC stimulates the nuclear export of p53 through a Crm1-dependent mechanism. Taken together with the structural data showing that the p53 NES is masked in the tetramer and that ARC inhibits p53 tetramerization (Fig. 3*A*), we conclude that ARC drives p53 nuclear hyperexport by interfering with p53 tetramerization.

Nuclear hyperexport of p53 would be expected to provide an explanation for the inhibition by ARC of p53-dependent transcription. However, the fact that the p53 tetramer binds its DNA target sequences with more affinity than the dimer or monomer and transactivates target genes more efficiently (20, 21) raises a second, nonmutually exclusive, mechanism. Specifically, inhibition of p53-dependent transcription may result directly from interference by ARC of p53 tetramerization, independently of effects of ARC on p53 abundance in the nucleus. To test this possibility, we assessed the ability of ARC-NLS to antagonize doxorubicin-induced mdm2 expression when nuclear export was inhibited. We found that increases in mdm2 expression were suppressed in the presence, as well as the absence, of LMB (Fig. 3*D*, compare lanes 5 and 6). These data reveal that inhibition by ARC of p53-dependent gene expression may result not only from increased nuclear export but also from interference with the ability of p53 to function as a transcription factor when tetramerization is inhibited.

Endogenous ARC Inhibits Endogenous p53 Tetramerization, Nuclear Localization, and p53-Dependent Gene Expression. To test the physiological importance of inhibition by ARC of p53 tetramerization, we knocked down endogenous ARC in MCF7 cells with siRNA (Fig. 4). Controls included inverted (Fig. 4) or scrambled (data not shown) siRNAs. We found that ARC knockdown triggers spontaneous dimerization and tetramerization of endogenous p53 (Fig. 4*A*) and accumulation of endogenous p53 in the nucleus [Fig. 4 *B* ($P < 0.0002$) and *C*, and additional controls in SI Fig. 7], despite no change in the abundance of total cellular p53 (Fig. 4*D*). Similar results were obtained with a second, independent ARC siRNA (data not shown). The end effect of ARC knockdown is induction in the expression of endogenous p53-dependent genes (Fig. 4*D*).

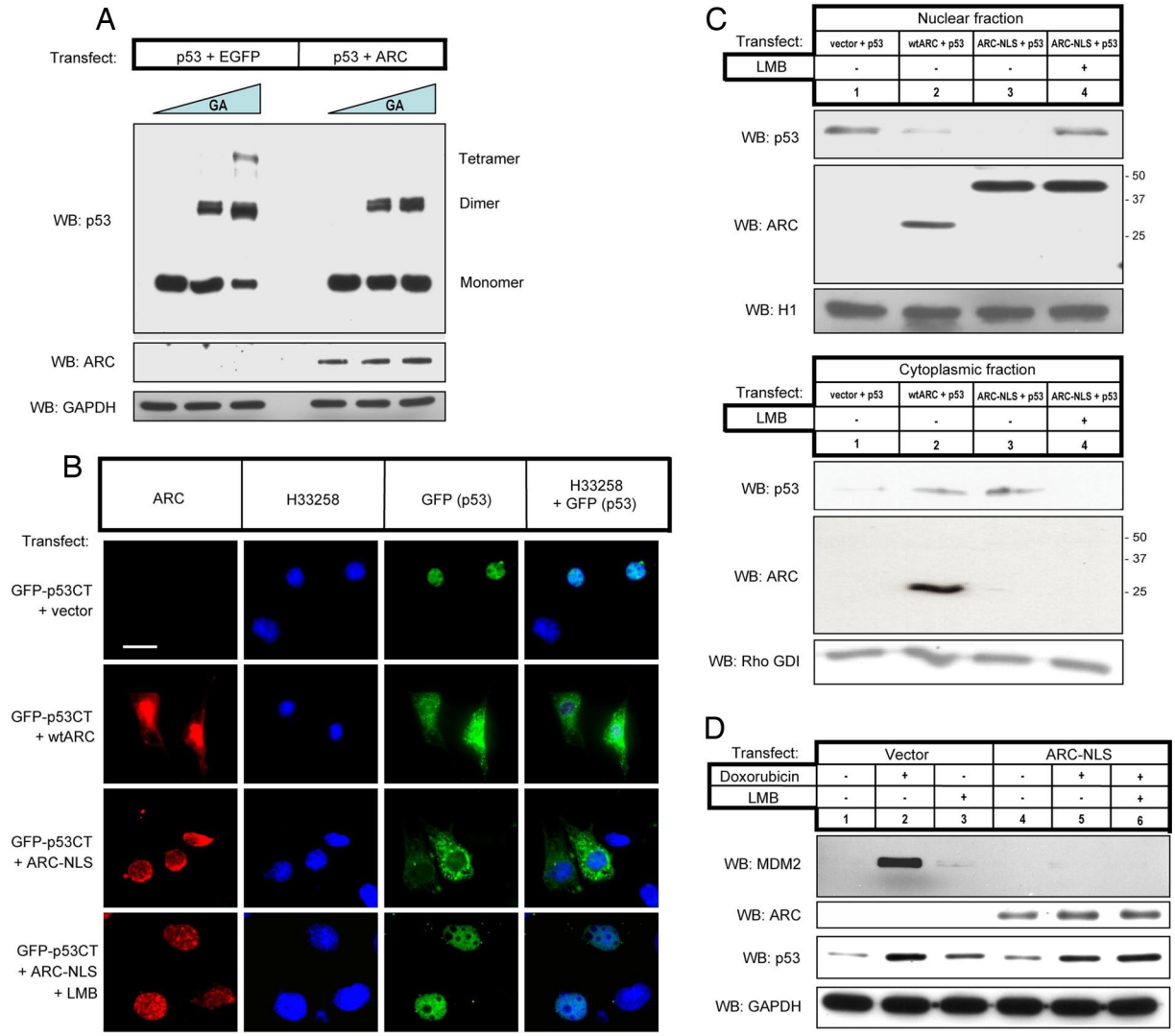

Fig. 3. ARC disrupts p53 tetramerization, stimulates p53 nuclear hyperexport, and independently inhibits p53-dependent transcription. (*A*) ARC inhibits p53 tetramerization. Saos2 cells were transfected as indicated, and lysates were treated with increasing concentrations of glutaraldehyde (see *Materials and Methods*), resolved by SDS/PAGE, and analyzed by Western blotting (WB). (*B*) WT and nuclear-localized ARC stimulate nuclear export of a p53 C-terminal fragment (GFP-p53CT) in a Crm1-dependent manner. Saos2 cells were transfected with a GFP-p53CT (containing the tetramerization domain) and vector (row 1), WT ARC (row 2), or nuclear-localized ARC (rows 3 and 4). At 36 h after transfection, cells were treated or not treated with LMB (10 ng/ml) for 6 h and analyzed by GFP fluorescence, ARC immunofluorescence, and Hoechst 33258 counterstaining. (Scale bar, 20 μm.) (*C*) WT and nuclear-localized ARC stimulate nuclear export of full-length p53 in a Crm1-dependent manner. Saos2 cells were transfected as noted. At 36 h after transfection, cells were then treated or not treated with LMB for 6 h and fractionated. Nuclear (*Upper*) and cytoplasmic (*Lower*) fractions were analyzed by WB. (*D*) Nuclear-localized ARC inhibits p53-dependent transcription independently of its stimulation of p53 nuclear export. U2OS cells were transfected as indicated. Cells were then treated or not treated with LMB (starting 36 h after transfection until harvest at 46 h) and/or dox (starting 42 h after transfection until harvest). Lysates were analyzed by WB.

Inverse Correlation Between Nuclear ARC and Mutant p53 in Primary Human Breast Cancers. To ascertain the relevance of p53 inactivation by nuclear ARC in cancer, we analyzed the relationship between the presence of nuclear ARC and p53 genotype by using a primary human breast cancer tissue microarray (22) comprised of tumors in which we also sequenced p53 (Table 1; and see SI Fig. 8 and SI Table 2). Functionally important genetic changes in cancer are usually nonredundant. Consistent with this, p53 was WT in 30 of 32 primary human breast cancers that contain nuclear ARC. Conversely, nuclear ARC was undetectable in 9 of 11 breast cancers with mutant p53. Thus, a statistically significant relationship exists between the presence of nuclear ARC and p53 genotype ($P < 0.05$). Moreover, in those breast tumors with WT p53, nuclear ARC was present in more than one-half (30 of 54). In addition, 32 of 32 tumors with nuclear ARC were devoid of p53 protein in the nucleus. These data suggest a role for ARC in inactivating p53 in breast cancers with WT p53.

Discussion

The experiments herein identify ARC as a negative regulator of p53 in cancer cells and delineate a mechanism by which this inhibition is mediated: ARC binds p53 and inhibits p53 tetramerization. Inhibition of p53 tetramerization disables p53 function as a transcription factor and exposes an NES in p53 that triggers its Crm1-dependent nuclear export.

The relocation of WT p53 to the cytoplasm in cancer cells has long been recognized (5), but the underlying mechanisms remain incompletely understood. In addition to the ARC-dependent mechanism delineated here, monoubiquitination of p53 by Mdm2 has been described to stimulate p53 nuclear export in

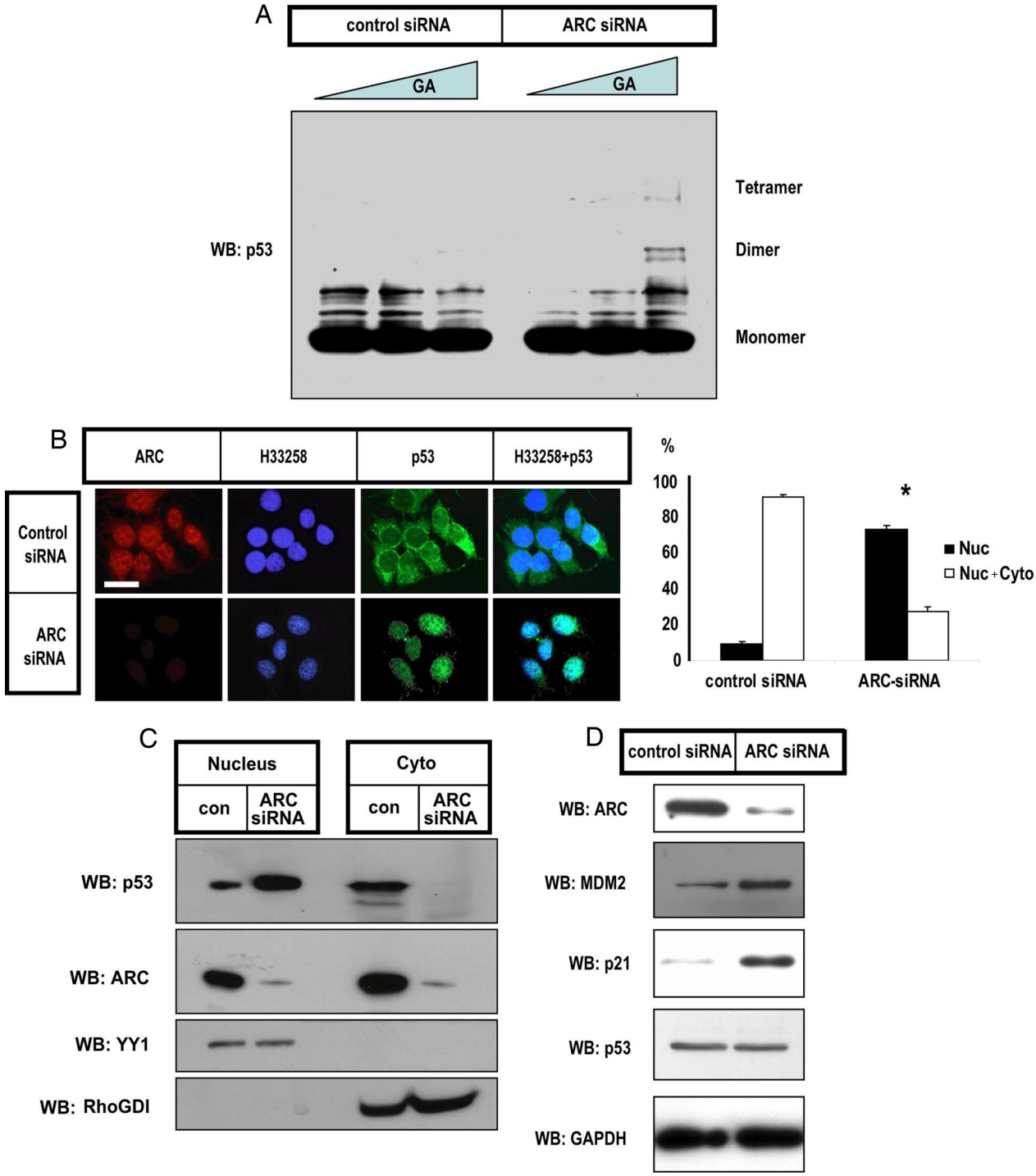

Fig. 4. Endogenous ARC inhibits endogenous p53 tetramerization, nuclear localization, and p53-dependent gene expression. (*A*) Knockdown of endogenous ARC in MCF7 cells stimulates spontaneous dimerization and tetramerization of endogenous p53. MCF7 cells were transfected with control or ARC siRNA, and p53 tetramerization was assessed as described. (*B* and *C*) Knockdown of endogenous ARC relocates endogenous p53 to the nucleus in MCF7 cells. MCF7 cells were transfected with control or ARC siRNA. Analysis by ARC and p53 immunofluorescence and Hoechst 33258 counterstaining at 48 h after transfection. (Scale bar, 20 μm.) (*B*) Graph shows mean $\pm$ SEM. Results are the average of three independent experiments, with 300–400 cells scored. *, $P < 0.0002$ for ARC siRNA vs. control siRNA (Student's *t* test). (*C*) Analysis by Western blotting (WB) of nuclear and cytoplasmic fractions. (*D*) Knockdown of endogenous ARC in MCF7 cells activates endogenous p53-dependent gene expression. MCF7 cells were transfected with control or ARC siRNA. Lysates obtained 48 h after transfection were analyzed by WB.

some cancer cells (23–25). ARC-dependent nuclear export appears distinct from that mediated by Mdm2, however, in that GFP-p53CT, which is exported by ARC, lacks the N-terminal Mdm2 binding site that is required for Mdm2-mediated p53 nuclear export (23). Noncanonical ubiquitination by Ubc13 has also been shown to stimulate p53 nuclear export, although this mechanism may involve interference with p53 tetramerization similar to ARC (26). Relocation of p53 to the cytoplasm is

Table 1. Inverse correlation between nuclear ARC and mutant p53 in primary human breast cancers

p53 genotype	Nuclear ARC positive	Nuclear ARC negative
Wild type	30	24
Mutant	2	9

Specimens were scored positive for nuclear ARC if >10% of the nuclei exhibited ARC immunostaining. Scoring for nuclear ARC was performed blinded to p53 genotype. $P < 0.05$ (Fisher's exact test).

brought about in some cancer cells by cytoplasmic retention rather than nuclear hyperexport. For example, Parc is a cytoplasmic protein that binds and retains p53 (27). Notably, Parc is expressed at very low levels in MCF7 cells (data not shown) where there is abundant ARC (Fig. 1*A*). Conversely, Parc levels are high in U2OS cells (27), which contain little ARC (Fig. 1 *C* and *D*). These observations suggest complementarity between different p53-inactivating mechanisms.

Activation of p53 in response to stress stimuli elicits cell cycle arrest or apoptosis (28). In this study, we show that ARC antagonizes p53-induced apoptosis. Some stress stimuli that activate p53, however, also trigger ubiquitin-mediated degradation of ARC, and decreases in ARC levels are required for these stimuli to kill (29). Whether the machinery required for ARC degradation operates in cancer cells remains to be determined.

Knockdown of endogenous ARC in breast cancer cells, by itself, stimulates endogenous p53 to tetramerize, relocate to the nucleus, and activate endogenous p53 target genes. These data indicate that physiological levels of ARC are adequate to inhibit p53. Moreover, genetic analysis of human breast cancers shows that the presence of nuclear ARC is almost invariably associated with WT p53, and conversely, the presence of mutant p53 is associated with the absence of nuclear ARC. These data suggest an important role for ARC in the inactivation of WT p53 in human cancer.

Materials and Methods

Plasmids, Recombinant Proteins, and siRNA. ARC (11) and p53 (30) constructs are as described or were modified by using PCR and standard cloning techniques. All constructs were verified by DNA sequencing. Radiolabeled *in vitro*-transcribed translated ARC and p53 proteins were produced as described (TNT kit; Promega). Recombinant ARC and p53 proteins were produced in BL21 *Escherichia coli* as His- and GST-fusions, respectively, and purified on Ni-nitrilotriacetic acid agarose (Invitrogen) or glutathione Sepharose (Amersham Biosciences) columns. ARC siRNA duplex corresponding to the human ARC 3′-UTR (5′-GGCGCUCUAUACAUAUUAU-3′) and containing a 3′-dTdT overhang was synthesized (Dharmacon) (used in Fig. 4). A second human ARC siRNA duplex (5′-CUAUGACCCUCCAUGCCCAUU-3′) was also used in experiments (data not shown). Controls included the same sequence in the inverted orientation and scrambled sequence (siCONTROL; Dharmacon). MCF7 cells stably transfected with pol III-driven p53 siRNA or scrambled siRNA are as described in ref. 31.

Antibodies. ARC rabbit polyclonal antiserum (Cayman) was used for immunoprecipitation, Western blotting, immunofluorescence, and immunohistochemistry. p53 mouse monoclonal antibodies were used for immunoprecipitation and Western blotting (DO1; Santa Cruz), and immunofluorescence (1801; Santa Cruz). p21 (Calbiochem), Mdm2 (Calbiochem), GST (Neomarkers), H1 (Neomarkers), and GAPDH (Abcam) mouse monoclonal antibodies and HA (Santa Cruz) rabbit polyclonal antiserum were used for Western blotting.

Transfection, Subcellular Fractionation, Immunostaining, Immunoprecipitation, and Western Blotting. Plasmids were transfected by using Effectene (Qiagen) and siRNA by using Oligofectamine (Invitrogen). Subcellular fractionation (32), immunostaining (27), and immunoprecipitation and Western blotting (11) were performed as described.

Death Assay. HEK293 cells were transfected as indicated and nuclear condensation scored 48 h later as described in ref. 11.

Luciferase Assay. U2OS cells were transfected with p53 (0.2 μg), and/or ARC (amounts in Fig. 1C), PG13-Luc or MG15-Luc (0.093 μg), phRL-TK (0.007 μg), and empty vector to total 2.3 μg. Cell lysates harvested 30 h later were assayed for firefly and *Renilla* luciferase activities by using Dual-Glo Luciferase Reporter Assay (Promega).

***In Vivo* Tetramerization Assay.** Saos2 cells were transfected with p53 and EGFP, ARC, or ARCΔ125–175. Cell lysates were harvested 30–36 h later and treated or not treated for 5 min with glutaraldehyde at a final concentration of 0.016 or 0.08%, after which samples were analyzed by SDS/PAGE (18).

Analysis of p53 Mutations and the Presence of Nuclear ARC in Primary Human Breast Cancers. Fresh frozen pieces of tumor from surgical resection of 65 primary human breast cancers were collected with institutional review board approval at Nottingham City Hospital and used to isolate genomic DNA. Exons 2–11 of p53 were amplified (including exon–intron boundaries), and PCR products used for direct sequencing of both strands. Mutations were confirmed in a second independent PCR. Matched paraffin-embedded blocks from the same cancers were used to construct a tissue microarray as described in ref. 22, and sections were used for ARC and p53 immunostaining.

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