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Parc: A Cytoplasmic Anchor for p53

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Summary

Nuclear localization of p53 is essential for its tumor suppressor function. Here, we have identified Parc, a Parkin-like ubiquitin ligase, as a cytoplasmic anchor protein in p53-associated protein complexes. Parc directly interacts and forms a ~1 MDa complex with p53 in the cytoplasm of unstressed cells. In the absence of stress, inactivation of Parc induces nuclear localization of endogenous p53 and activates p53-dependent apoptosis. Overexpression of Parc promotes cytoplasmic sequestration of ectopic p53. Furthermore, abnormal cytoplasmic localization of p53 was observed in a number of neuroblastoma cell lines; RNAi-mediated reduction of endogenous Parc significantly sensitizes these neuroblastoma cells in the DNA damage response. These results reveal that Parc is a critical regulator in controlling p53 subcellular localization and subsequent function.

Introduction

The p53 protein acting as a bona fide tumor suppressor can induce cell growth arrest, apoptosis, and aging/cell senescence in response to various types of stress (Vogelstein et al., 2000; Sharpless and DePinho, 2002). p53 mutations have been well documented in more than half of all human tumors (Hollstein et al., 1999), and in the cells that retain wild-type p53, other defects in the p53 pathway also play an important role in tumorigeneisis. p53 promotes tumor suppression through its ability to bind specific DNA sequences and function as a transcription factor (El-Deiry et al., 1992). The importance of p53-mediated transcriptional activation is underscored by the fact that the vast majority of tumor-associated p53 mutations occur within the domain responsible for sequence-specific DNA binding (Hollstein et al., 1999).

Tight regulation of p53 is essential for maintaining normal cell growth and for its effect on tumorigenesis. Wild-type p53 is a short-lived protein that is maintained as a latent form in unstressed cells. The precise mechanism by which p53 is activated by cellular stress is not completely understood. Although both posttranslation modifications (phosphorylation and acetylation) and stabilization (ubiquitination) of p53 are well accepted as key events in the p53-mediated stress response, subcellular localization also appears to play a critical role in the regulation of p53 function (Vousden, 2002; Jimenez et al., 1999). p53 is diffusely distributed in normal unstressed cells and, in response to DNA damage and other types of stress, p53 translocates to the nucleus where it activates endogenous target genes. Thus, nuclear localization of p53 is essential for its function as a transcription factor. Indeed, wild-type p53 is functionally inactivated by abnormal cytoplasmic sequestration in many tumor types including inflammatory breast carcinoma, undifferentiated neuroblastoma, colorectal carcinoma, and retinoblastoma; constitutive cytoplasmic localization of p53 in these tumors has been linked with poor response to chemotherapy, tumor metastasis, and poor long-term patient survival (Moll et al., 1992, 1996; Bosari et al., 1995; Sun et al., 1992; Schlamp et al., 1997; Ueda et al., 1995).

The p53 protein contains three lysine-rich nuclear localization signals (NLSs) in the C terminus (Liang et al., 1998; Shaulsky et al., 1990). NLS I (aa 305–322) has been implicated as the primary NLS, and NLS II (aa 369–375) and NLS III (aa 379–384) appear to increase the efficiency of nuclear import mediated by NLS I (Shaulsky et al., 1990). Many NLS-containing transcriptional factors, such as c-Myc, Max, and c-Fos, are exclusively present in nuclei. However, since subcellular localization of the p53 protein is a dynamic process and the levels of p53 distributed in the cytoplasm and nucleus vary with different cell types and cellular conditions (Jimenez et al., 1999), cellular factors/pathways that specifically regulate both nuclear import and export of p53 are likely to exist.

A number of studies have shown that nuclear export of p53 is specifically regulated by Mdm2 (Tao and Levine, 1999; Geyer et al., 2000; Boyd et al., 2000). p53 contains a leucine-rich, rev-like nuclear export signal (NES) at the C terminus (Stommel et al., 1999; Freedman and Levine, 1998; Middeler et al., 1997), and recently, a second NES in the p53 transactivation domain has also been identified (Zhang and Xiong, 2001). Mdm2 can significantly enhance nuclear export of p53 through the C-terminal NES (Lohrum et al., 2001; Gu et al., 2001). In addition to its p53 binding domain, the ubiquitin ligase activity of Mdm2 is also critical for its ability to promote nuclear export of p53 (Geyer et al., 2000; Boyd et al., 2000). Notably, the C-terminal lysine residues of p53 that serve as both acetylation sites (Li et al., 2002b) and the sites of Mdm2-mediated ubiquitination are also required for the enhancement of p53 nuclear export by Mdm2 (Lohrum et al., 2001; Gu et al., 2001).

The mechanism by which p53 is diffusely distributed in normal unstressed cells remains unknown. Since the Mdm2 protein is often undetectable in unstressed cells, cellular factors other than Mdm2 are likely involved in retaining the p53 protein in the cytoplasm under these

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conditions. A number of proteins have been proposed to serve as cytoplasmic anchor proteins to block nuclear localization of p53, including ribosomal proteins (Fontoura et al., 1997; Abou Elela and Nazar, 1997), Hsc70 (Gannon and Lane, 1991), vimentin (Klotzsche et al., 1998), tubulin (Giannakakou et al., 2000), and F-actin (Metcalfe et al., 1999). As each of these identified proteins is highly abundant and the specificity of their interactions with p53 needs further verification, none of them has received wide acceptation as the bona fide cytoplasmic anchor of p53. Nevertheless, these studies indicate that a fraction of p53 resides in the cytoplasm and the levels of cytoplasmic p53 proteins may be regulated by cytoplasmic factors.

To reevaluate the hypothesis that certain cytoplasmic factors may directly affect the subcellular localization of p53, we have biochemically purified p53-containing protein complexes from the cytoplasm of unstressed cells and identified a protein, Parc (p53-associated, Parkin-like cytoplasmic protein), as a key component of these complexes. This discovery reveals an important regulatory pathway that controls the subcellular localization of p53 and its subsequent biological function.

Results

Affinity Purification of p53-Containing Protein Complexes from the Cytoplasm of Unstressed Human Cells

To identify the key cytoplasmic binding partner for p53, we used an epitope-tagging strategy to isolate p53-containing protein complexes from cells. Similar approaches have been used successfully in our lab and others to purify bona fide protein complexes such as the SMCC/TRAP and HDAC1 complexes (Gu et al., 1999; Luo et al., 2000). However, since expression of wild-type p53 strongly induces apoptosis in human cells, it is very difficult to obtain a sufficient quantity of cell extract for further protein purification. Also, a predominantly nuclear pattern of p53 localization is usually obtained when wild-type p53 is ectopically expressed in human cells (Geyer et al., 2000; Boyd et al., 2000; also see Figure 6). Taking these factors into consideration, we generated a derivative of the human lung carcinoma p53 null H1299 cell line that stably expresses a tumor-derived human p53 mutant [p53(175)] containing an N-terminal FLAG epitope (Flag-p53[175]) (Figure 1A). Importantly, a significant proportion of the ectopic p53 protein was present in the cytoplasm of this stably transfected cell line (data not shown; also see Figures 1B and 1C).

To isolate protein complexes containing epitope-tagged p53, cytoplasmic extracts from Flag-p53(175)-expressing H1299 cells and from control cells (parental H1299) were subjected to affinity chromatography on M2 (Flag antibody) agarose. As shown in Figure 1B, a major protein band of ~270 kDa specifically copurified with cytoplasmic p53 from Flag-p53(175)-expressing H1299 cells (lane 3), but not from parental H1299 cells (lane 2). Peptide sequencing of this band by mass spectrometry revealed three peptide sequences, all of which matched a single, partial cDNA clone (GeneBank accession number Gi4558043, also known as KIAA0708).

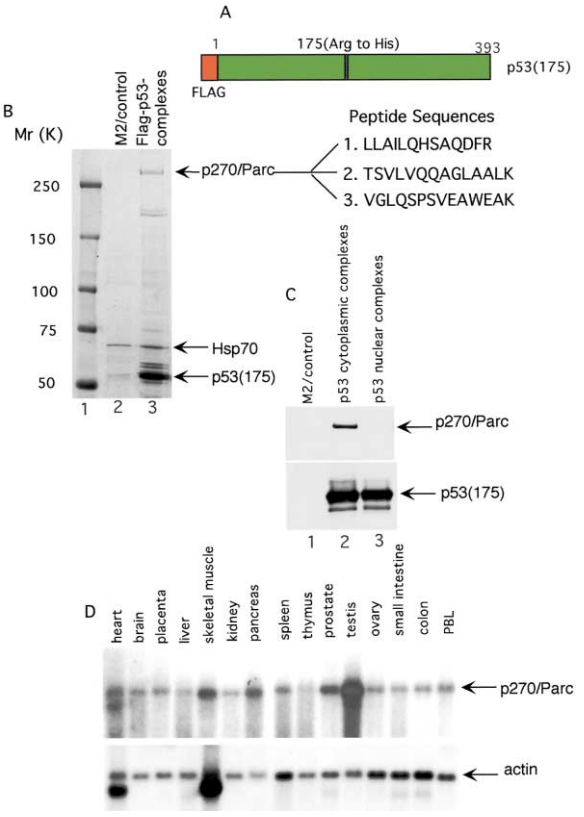

Figure 1. Identification of p270/Parc as a Cytoplasmic p53-Interacting Protein

(A) Schematic representation of the Flag-p53(175) protein used for affinity purification of p53-interacting proteins. The arginine residue of the DNA binding domain is replaced with histidine (aa 175).

(B) Collodial-blue staining of the protein marker (lane 1), a control eluate from M2 beads loaded with parental H1299 cytoplasmic cell extract (lane 2), and affinity-purified Flag-p53(175) complexes from a cytoplasmic extract of the Flag-p53(175)/H1299 stable cell line (lane 3). Specific p53-interacting protein bands were peptide sequenced by mass spectrometry, and the p270/Parc peptide sequences are presented.

(C) p270/Parc is present in cytoplasmic, but not nuclear, p53-containing complexes. Western blot analysis of M2 immunoprecipitates from parental H1299 cytoplasmic extract (lane 1), Flag-p53(175)/H1299 cytoplasmic (lane 2), and nuclear (lane 3) extracts by immunoblotting with p270/Parc-specific antibody (upper) or p53-specific DO-1 antibody (lower).

(D) p270/Parc mRNA is ubiquitously expressed. A multiple tissue Northern filter was hybridized with p270/Parc (upper) or actin (lower) cDNA probes.

Cloning and Initial Characterization of Parc (p53-Associated, Parkin-Like Cytoplasmic Protein)

A full-length human p270 cDNA was assembled by exon prediction from genomic sequences, RACE (rapid amplification of cDNA ends), and homology alignment with the partial sequence of KIAA0708. The full-length cDNA encodes a 2517 amino acid protein (about 800 amino acids longer than the KIAA0708 sequences, Figure 2B). Northern blot analysis showed that the p270 mRNA is highly expressed in testis but very low in thymus (Figure 1D). Nevertheless, it is ubiquitously expressed in all different tissues.

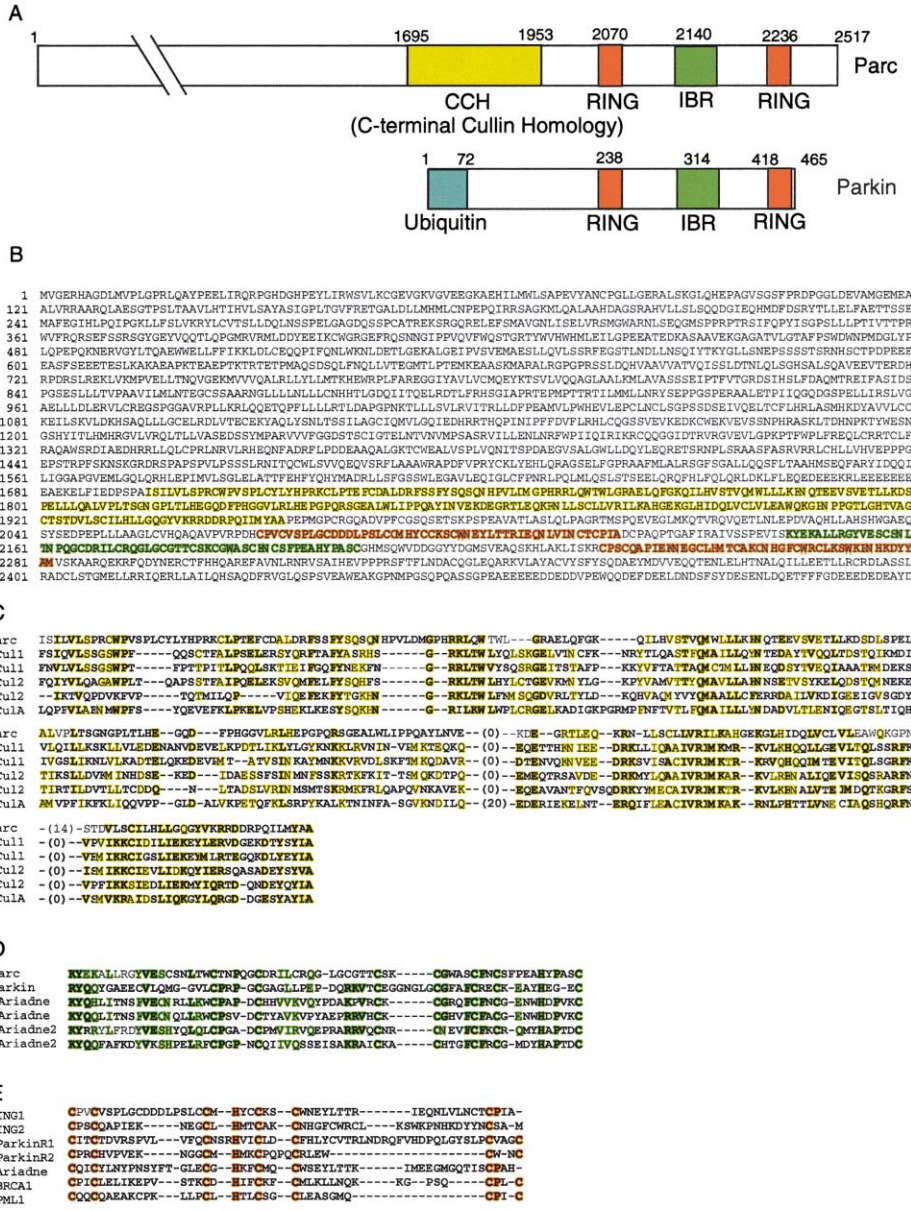

Figure 2. Parc Contains the Ring-IBR-Ring Motif and a C-Terminal Cullin Homology Domain (CCH)

(A) Schematic representation of the Parc and Parkin polypepetides.

(B) The amino acid sequence of Parc, with the CCH, RING, and IBR domains highlighted in colors.

(C) An alignment of the CCH domain of Parc with those of human cullin1 (hCul1) and cullin2 (hCul2), *C. elegans* cullin1 (cCul1) and cullin2 (cCul2), and yeast cullinA (yCulA). Homologous amino acid residues are highlighted in colors.

(D) An alignment of the IBR domain of Parc with those of Parkin, human ariadne (hAriadne) and ariadne2 (hAriadne2), and *D. melanogaster* ariadne (dAriadne) and ariadne2 (dAriadne2). Homologous amino acid residues are highlighted in colors.

(E) An alignment of the RING domains of Parc (RING1, RING2) with those of Parkin (hParkinR1, hParkinR2), human Ariadne (hAriadne), human BRCA1 (hBRCA1), and human PML (hPML1) proteins. Homologous amino acid residues are highlighted in colors.

Remarkably, the C-terminal sequences of p270 possess a signature motif, the Ring-IBR-Ring domain, that was first identified in the C terminus of Parkin (Figures 2A, 2D, and 2E), the protein implicated in an autosomal recessive form of Parkinson's disease (PD) (Kitada et al., 1998; Shimura et al., 2000). Accordingly, we designated p270 as the p53-associated, Parkin-like cytoplasmic protein Parc. Biochemical studies have shown that the Ring-IBR-Ring is responsible for the intrinsic ubiquitin ligase activity of Parkin (Shimura et al., 2000). Parkin also contains an ubiquitin-homology domain at the N terminus that is absent from Parc. Interestingly however, Parc has a unique motif that is highly homologous to the C terminus of the Cullin proteins (here designated as the C-terminal Cullin Homology [CCH] domain) (Figure 2C). The Cullins, including Cul1 and Cul2, are critical

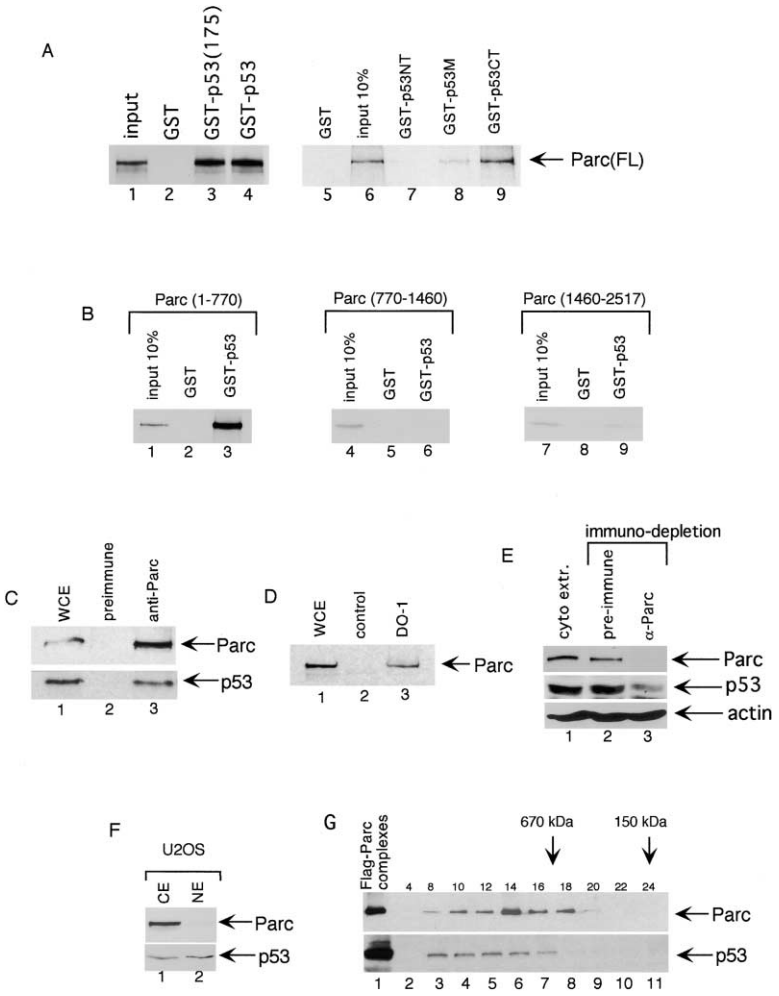

Figure 3. The Interaction between p53 and Parc In Vitro and In Vivo

(A) Direct interactions of Parc with GST-p53. The wild-type GST-p53 full-length protein (GST-p53) (lane 4), the mutant GST-p53 full-length protein (GST-p53[175]) (lane 3), the N terminus of p53 protein (1-73) (lane 7), the middle part of p53 (100-290) (lane 8), the C terminus of p53 (290-393) (lane 9), or GST alone (lanes 2 and 5) were used in a GST pull-down assay with in vitro translated ^{35}S-labeled full-length Parc.

(B) The N terminus of Parc directly interacts with GST-p53. The GST-p53 full-length protein (GST-p53) (lanes 3, 6, and 9) or GST alone (lanes 2, 5, and 8) were used in a pull-down assay with the in vitro translated ^{35}S-labeled N terminus of Parc (1–770) (lanes 1–3), the middle part of Parc (770–1460) (lanes 4–6), and the C terminus of Parc (1460–2517) (lanes 7–9).

(C) Coimmunoprecipitation of p53 with Parc from U2OS cells. Western blot analysis of indicated whole-cell extract (WCE) (lane 1) and immunoprecipitates with a Parc-specific antibody (lane 3) or preimmune serum (lane 2) by anti-p53 monoclonal antibody DO-1 (lower) or anti-Parc antibody (top).

(D) Coimmunoprecipitation of Parc with p53 from U2OS cells. Western blot analysis of whole-cell extract (WCE) (lane 1) or immunoprecipitates with anti-p53 monoclonal antibody DO-1 (lane 3) or control immunoprecipitates with anti-Ras antibody (lane 2) by a Parc-specific antibody.

(E) Endogenous p53 was codepleted with endogenous Parc from U2OS cytoplasmic extracts by anti-Parc antibody. U2OS cytoplasmic extracts were incubated with preimmune serum or α-Parc antibody in the presence of protein A/G beads. Preimmune and α-Parc immunoprecipitation flowthroughs (lanes 2 and 3) and U2OS cytoplasmic extract (lane 1) were resolved in 8% SDS PAGE and immunoblotted with anti-Parc (upper) or anti-p53 monoclonal antibody DO-1 (middle) or actin antibody (AC-15, bottom).

(F) Endogenous Parc is found in cytoplasmic, but not nuclear, extracts of U2OS cells. Western blot analysis of cytoplasmic (lane 1) and nuclear (lane 2) extracts from U2OS cells with Parc-specific (upper) or p53-specific (DO-1) antibodies.

(G) Parc forms a ~1 Mda complex with p53 in the cytoplasm of U2OS cells. Chromatographic fractions (lanes 2–11) generated by the gel filtration (Superose 6, SMART system) of Flag-Parc-containing complexes (lane 1) from the cytoplasmic extract of Flag-Parc/U2OS stable cells were immunoblotted with anti-Parc (upper) and anti-p53 (DO-1) (lower) antibodies.

components of several ubiquitin ligase complexes, such as the anaphase-promoting complex (APC/C) and the Skp-Cullin-F box complex (SCF) (Pickart, 2001).

Parc Interacts with p53 Both In Vitro and In Vivo

To confirm the physical interaction between p53 and Parc, we first tested whether Parc binds to p53 in vitro. As shown in Figure 3A, ^{35}S-labeled in-vitro-translated Parc bound to immobilized GST-p53 (wild-type) and the p53 mutant form GST-p53(175) (lanes 3 and 4), but not to GST alone (lane 2); Parc bound the C terminus of p53 (GST-p53CT) (lane 9) but showed no binding to the N-terminal domain of p53 (GST-p53NT) (lane 7). A similar strategy was used to map the p53-interacting sequences of Parc. As shown in Figure 3B, p53 was associated with the N terminus of Parc. Neither the central region nor C-terminal region that encompasses the CCH domain and Ring-IBR-Ring motif showed strong binding with p53 (lane 3 versus lanes 6 and 9). Thus, our data indicate that the N terminus of Parc interacts with the C terminus of p53.

To confirm the interaction between p53 and Parc in vivo, we developed an affinity-purified polyclonal antiserum against the C-terminal 100 residues of Parc (amino acids 2417–2517), a region that shows no apparent homology with any known protein (Figure 2A). Upon Western blot analysis, this antibody specifically detects Parc proteins in human cell extracts (lane 1, Figure 3C). Next, we used this antibody to investigate whether the endogenous Parc and p53 polypeptides interact in vivo. Cell extracts from human U2OS cells, which express wild-type p53, were immunopreciptated with α-Parc or with the corresponding preimmune serum. As expected, Western analysis revealed that this antibody immunoprecipitated endogenous Parc (lane 3, top, Figure 3C). More importantly, p53 was clearly detected in the immunoprecipitations obtained with the α-Parc antiserum (lane 3), but not the preimmune serum (lane 2, bottom,

Figure 3C). Conversely, endogenous Parc was readily immunoprecipated with the p53-specific monoclonal antibody DO-1 (lane 3, Figure 3D), but not with a control antibody (anti-Ras monoclonal antibody) (lane 2, Figure 3D). Similar results were also obtained in other cell lines, such as human lung carcinoma H460 cells (see Supplemental Figure S1 online at http://www.cell.com/cgi/content/full/112/1/29/DC1). Taken together, these data indicate that p53 and Parc interact both in vitro and in vivo.

Parc Forms a ~1 MD Complex with p53 in the Cytoplasm

Initial purification and identification of Parc showed that Parc is associated with p53 in cytoplasmic complexes (Figure 1B). To demonstrate that Parc is a cytoplasmic-specific p53 binding protein, we tested whether p53 is present exclusively in p53-associated cytoplasmic complexes by Western analysis with anti-Parc antibody. As indicated in Figure 1C, Parc is completely undetectable in p53-associated complexes obtained from nuclear extracts of the Flag-p53(175) stable cell line (top, lane 3 versus lane 2), although similar levels of p53 were readily immunopreciptitated from both the cytoplasmic and nuclear fractions of these cells (bottom). Moreover, Western analysis of cellular fractions from native U2OS cells showed that the endogenous Parc protein is present only in cytoplasmic, but not nuclear, extracts (top, Figure 3F). In accord with these findings, we also observed a predominantly cytoplasmic pattern of immunofluorescent staining in human lung carcinoma H1299 cells transiently transfected with a Flag-tagged Parc polypeptide (Figure 4A).

Strikingly, through immunodepletion of the Parc protein from cytoplasmic extracts, we found that more than 70% of cytoplasmic p53 was also codepleted from the extract, indicating that the majority of cytoplasmic p53 is associated with endogenous Parc (lane 3 versus lanes 1 and 2, Figure 3E). To further evaluate whether Parc can form a cytoplasmic complex with endogenous p53 in unstressed cells, we stably transformed U2OS cells with Flag-tagged Parc. To isolate Parc-containing complexes, the cytoplasmic extract was subjected to affinity chromatography for the Parc-containing complexes. As expected, Western analysis showed that p53 is present in the Parc-containing complexes (lane 1, Figure 3G). Moreover, when these complexes were subjected to gel-filtration chromatography on Superose 6 (SMART system) as described previously (Gu et al., 1999), p53 and Parc coeluted in fractions 8–16 with an apparent size of ~1 MDa (Figure 3G). Since coelution of purified protein complexes on gel-filtration chromatography requires a very stable interaction, our data demonstrate that Parc strongly interacts with p53 in the cytoplasm of unstressed human cells.

Parc Has an Ubiquitin Ligase Activity but Fails to Directly Induce p53 Degradation

Like Parkin, Parc contains the Ring-IBR-Ring motif (Figure 2A). Since this motif is required for Parkin-mediated ubiquitination (Shimura et al., 2000), we asked whether Parc also contains an intrinsic ubiquitin ligase activity. As indicated in Figure 4B, in vitro self-ubiquitination of Parc occurred in the presence of E1, E2 (UBCH7), and ubiquitin (lane 2). Furthermore, highly ubiquitinated species of Parc were readily detected in human cells that coexpressed Flag-tagged Parc and HA-tagged ubiquitin (lane 3, Figure 4C).

To explore the functional relationship between p53 and Parc, we tested whether Parc directly induces p53 ubiquitination. As indicated in Figure 4D, a high level of ubiquitinated p53 was found in cells cotransfected with Mdm2 (lane 2) (Li et al., 2002a); however, Parc expression failed to induce significant ubiquitination of p53 (lane 3 versus lane 2). Likewise, using an in vitro assay, we detected strong ubiquitination of p53 by Mdm2, but not by Parc (lane 3 versus lane 2, Figure 4E). Moreover, while Mdm2 expression strongly induced p53 degradation, Parc had no effect on the steady-state levels of p53 (Figure 4F). Notably, we also failed to detect a significant effect of Parc on the cellular levels of p53mNLS (Figure 4G), an NLS mutant of p53 that, like Parc, resides exclusively in the cytoplasm (data not shown). Thus, Parc is a potential ubiquitin ligase but fails to directly induce p53 degradation.

RNAi Ablation of Parc Induces Nuclear Localization of p53 and p53-Dependent Apoptosis

Since we did not observe a significant effect of Parc on p53 ubiquitination, it is likely that Parc may regulate p53-mediated function through other mechanisms. To elucidate the physiological significance of the Parc-p53 interaction, we examined the functional consequences of RNAi ablation of endogenous Parc. For this purpose, U2OS cells, which express both Parc and wild-type p53 proteins (Figure 3F), were transfected with either a Parc-specific RNA oligonucleotide (Parc-RNAi) or a control oligonucleotide (control-RNAi). As shown in Figure 5A, endogenous Parc polypeptides were nearly undetectable after three consecutive oligofectamine-mediated transfections (top, lane 2 versus lane 1), while the level of control protein (actin) remained the same (bottom). Strikingly, ablation of Parc expression strongly induced the expression of p21, one of the key transcriptional targets of p53, despite the fact that the steady-state levels of p53 were unchanged (middle, lane 2 versus lane 1, Figure 5A).

Next, we examined the effect of Parc on p53-mediated apoptosis. As shown in Figure 5C, Parc-RNAi treated cells were susceptible to programmed cell death, with about 49.3% of the cells apoptotic (II) while the control transfected U2OS cells (control-RNAi) showed no significant apoptosis under the same conditions (I). Similar results were also obtained with the TUNEL assay (data not shown). Furthermore, the same experiments were also performed in the human p53 null cell line H1299. As shown in Figure 5A, although endogenous Parc expression was successfully abrogated by RNAi, neither p21 activation nor significant apoptosis was detected in these cells (lanes 3 and 4, Figure 5A; III and IV, Figure 5C). These results indicate that ablation of endogenous Parc expression significantly induces p53-mediated transcriptional activation and activates p53-dependent apoposis.

To explore the molecular mechanism by which Parc affects p53 function, we tested whether Parc directly

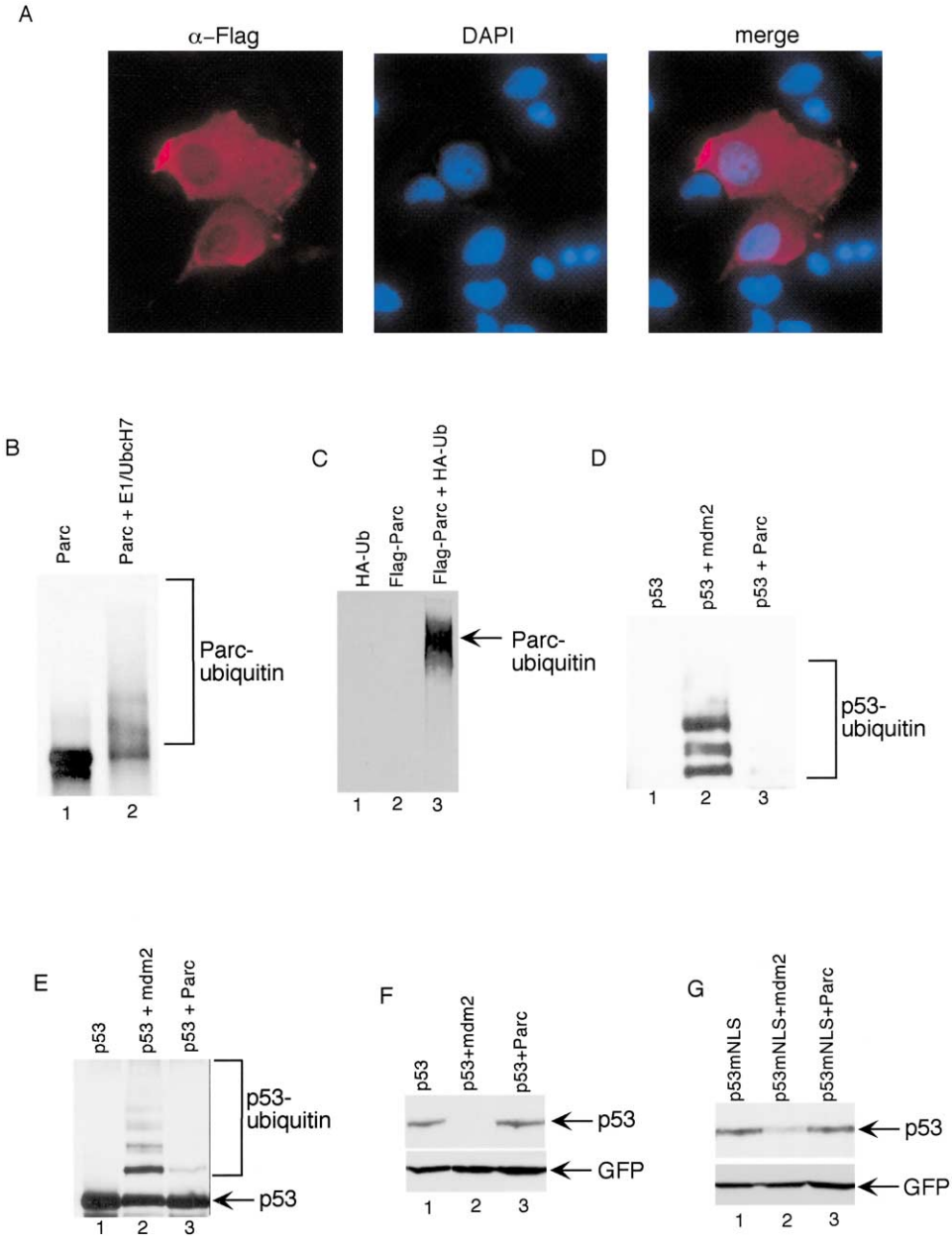

Figure 4. Parc Is a Cytoplasmic Ubiquitin Ligase

(A) Ectopically expressed Parc is diffusely localized in the cytoplasm of Flag-Parc-transfected H1299 cells. These cells were stained with anti-Flag monoclonal antibody to detect Parc.

(B) Self-ubiquitination of Parc in vitro. In vitro translated ^{35}S labeled Parc was incubated with E1, E2 (GST-UbcH7), and His-ubiquitin.

(C) Ubiquitination of Parc in vivo. Immunoprecipitations from H1299 cells transfected with HA-Ub (lane 1), Flag-Parc (lane 2), or both (lane 3) by the M2 (Flag) antibody, were immunoblotted with anti-HA monoclonal antibody.

(D) Parc does not ubiquitinate p53 effectively in vivo. M2 immunoprecipitations from H1299 cells cotransfected with Flag-ubiquitin and either p53 alone (lane 1), or p53 and mdm2 (lane 2), or with p53 and Parc (lane 3) were immunoblotted with anti-p53 monoclonal antibody (DO-1).

(E) Parc does not ubiquitinate p53 effectively in vitro. In vitro ubiquitination reactions containing GST-p53 incubated either alone (lane 1), with GST-mdm2/GST-UbcH5 (lane 2), or Flag-Parc/GST-UbcH7 (lane 3) were immunoblotted with anti-p53 monoclonal antibody (DO-1). Mammalian E1 was included in all three reactions (lanes 1–3).

(F) Parc does not promote degradation of wild-type p53. H1299 cell extracts transfected with CMV-p53/CMV-GFP (lane 1), or CMV-53/CMV-GFP and CMV-mdm2 (lane 2), or CMV-p53/CMV-GFP and CMV-Parc (lane 3) were immunoblotted with anti-p53 antibody (DO-1) (upper) or anti-GFP monoclonal antibody (lower).

(G) Parc does not promote degradation of the p53-mNLS mutant (K319A, K320A, K321A). H1299 transfections and immunoblotting were done as described in (F).

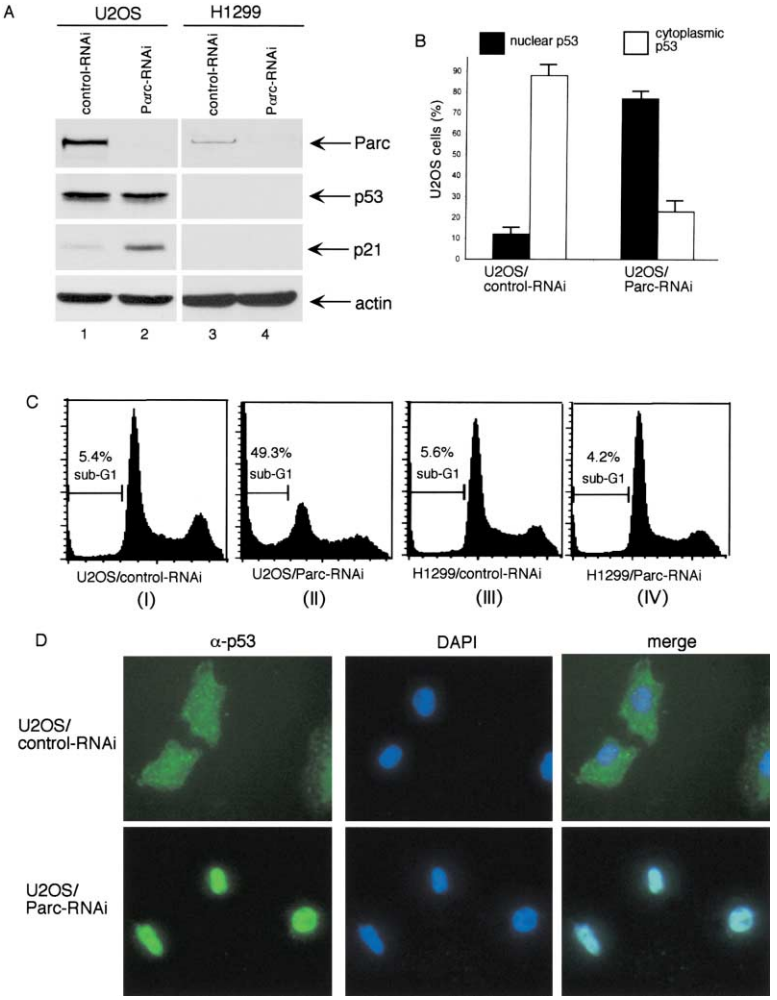

Figure 5. RNAi Ablation of Endogenous Parc Induces Nuclear Localization of p53 and p53-Dependent Apoptosis

(A) RNAi ablation of endogenous Parc expression in U2OS and H1299 cells. Whole-cell extracts from Parc-RNAi or control-RNAi treated cells (U2OS and H1299), were immunoblotted with anti-Parc, anti-p53 (DO-1), anti-p21 (C19) and anti-actin (AC-15) antibodies.

(B) Quantitation of subcellular p53 localization in U2OS cells transfected with Parc-RNAi or control oligonucleotide. The data represent the average of three experiments with standard deviations indicated.

(C) p53-dependent induction of apoptotic response by Parc RNAi ablation. U2OS and H1299 cells transfected with either Parc-RNAi and control oligonucleotides were analyzed for apoptotic cells (sub-G1) according to DNA content (PI staining).

(D) Subcellular localization of p53 in U2OS cells transfected with Parc-RNAi or control oligonucleotides. The transfected cells were immunostained with anti-p53 antibody (1801) (visualized by green fluorescence) and counterstained with DAPI to visualize the nuclei.

controls subcellular localization of p53. Under normal conditions, p53 is diffusely distributed in the cytoplasm of U2OS cells, as shown by immunofluorescence staining with a p53-specific monoclonal antibody (1801) (top, Figure 5D). Strikingly however, after RNAi ablation of endogenous Parc, p53 was predominantly relocalized to the nucleus of these cells (bottom, Figure 5D); the exclusive nuclear staining of p53 was found in as many as 80% of total Parc-RNAi-treated cells compared with only 12% of control cells (Figure 5B). Similar results were also obtained in the cells expressing mutated p53 proteins (see Supplemental Figure S2 online at http://www.cell.com/cgi/content/full/112/1/29/DC1). These results indicate that RNAi ablation of Parc expression activates p53-mediated function by regulating its subcellular localization.

Overexpression of Parc Induces Cytoplasmic Sequestration of p53

To elucidate the mechanism by which Parc affects subcellular localization of p53, we investigated whether Parc overexpression directly promotes cytoplasmic retention of p53. As indicated in Figure 6A, ectopic expression of wild-type p53 yields predominantly nuclear staining (top), with less than 10% of cells showing clear cytoplasmic localization (Geyer et al., 2000; Boyd et al., 2000). However, overexpression of full-length Parc strongly induced cytoplasmic retention of p53, with more than 87% of cells showing cytoplasmic p53 localization (bottom, Figures 6A and 6C). In contrast, Parc had no effect on another nuclear protein, c-Myc (Figure 6B), indicating that Parc-mediated cytoplasmic sequestration is specific for p53.

To identify the sequences of Parc that are responsible for cytoplasmic retention of p53, the same assay was used to evaluate selected segments of the Parc polypeptide. As summarized in Figure 6C, the subcellular localization of p53 was not altered by overexpression of individual Parc segments corresponding to either the CCH domain (residues 1695–1953), the Ring-IBR-Ring motif (residues 2070–2282), or two N-terminal regions (residues 1–770 or 770–1600). In contrast, however, a segment encompassing both the N-terminal sequences and the CCH domain (residues 1–1960) readily induced cytoplasmic relocalization of p53. These data also indicate that the Ring-IBR-Ring motif is not required for Parc-mediated sequestration of p53.

Reduction of Endogenous Parc in Neuroblastoma Cells Also Induces p53 Nuclear Localization

Many neuroblastoma cell lines display predominantly cytoplasmic localization of wild-type p53 (Moll et al.,

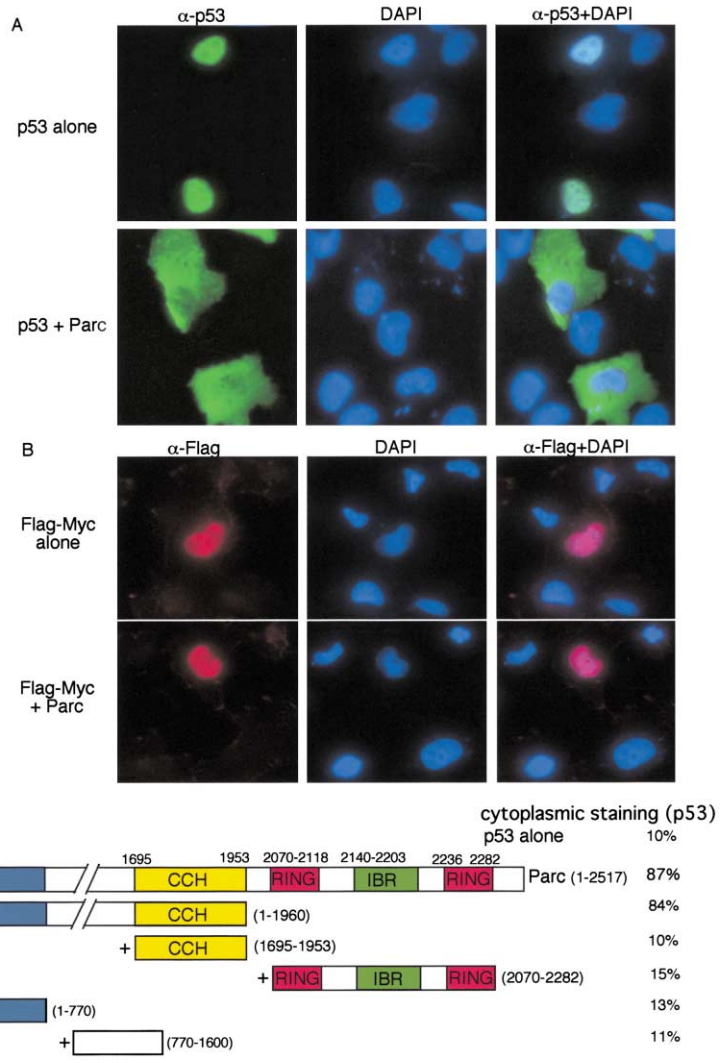

Figure 6. Overexpression of Parc Promotes Cytoplasmic Sequestration of Ectopic p53

(A) Subcellular localization of ectopic p53 in H1299 cells transfected with CMV-p53 alone or with CMV-p53 and CMV-Parc. The transfected cells were immunostained with anti-p53 antibody (visualized by green fluorescence) and counterstained with DAPI to visualize the nuclei.

(B) Subcellular localization of ectopic Myc in H1299 cells transfected with CMV-Flag-Myc alone or CMV-Flag-Myc and CMV-Parc. The transfected cells were immunostained with anti-Flag monoclonal antibody (M2) (visualized by red fluorescence) and counterstained with DAPI to visualize the nuclei.

(C) The Ring-IBR-Ring domain of Parc is not required for Parc-mediated cytoplasmic sequestration of ectopic p53. H1299 cells were transfected with CMV-p53 alone, with CMV-p53 and CMV-Parc (full-length), or with CMV-p53 and various CMV-Parc deletion constructs. The transfected cells were immunostained as described in (A) and the p53-positive cells were quantitated for subcellular p53 localization.

1996; Sengupta et al., 2000; Stommel et al., 1999; Zaika et al., 1999). The mechanisms by which p53 is sequestered in the cytoplasm of these cells are not completely understood. Interestingly, as indicated in Figures 7A and 7B, the Parc protein is highly expressed in the neuroblastoma cell lines that we tested, including IMR32, KCNR, SK-N-AS, and LAN5, compared to either normal human brain tissues or human glioma cell lines.

To explore the role of Parc in this phenomenon, we tested whether inactivation of endogenous Parc expression by RNAi modulates the subcellular localization of p53 in the SK-N-AS and IMR32 neuroblastoma lines. As indicated in Figure 7D, the protein levels of endogenous Parc in these cells were significantly decreased by Parc-RNAi treatment (top), although the reduction was less than that obtained by the same technique in U2OS cells (Figure 5A). This presumably reflects the higher steady-state levels of endogenous Parc and lower transfection efficiency of neuroblastoma cells. Nevertheless, RNAi-mediated reduction of endogenous Parc also leads to nuclear localization of p53 in both SK-N-AS and IMR32 cells; the typical punctuated pattern of cytoplasmic p53 staining was converted to a predominantly nuclear staining pattern in about 35% of cells, compared to less than 10% in the control cells (Figures 7C and 7E). Furthermore, expression of p21, a major transcriptional target of p53, was significantly induced despite the fact that the total level of p53 polypeptides was unchanged by Parc-specific RNAi treatment (middle, Figure 7D).

RNAi-Mediated Parc Reduction Restores a Strong p53-Dependent Stress Response in Neuroblastoma Cells

Neuroblastoma cells and other types of tumor cells that show constitutive cytoplasmic localization of p53 often exhibit an impaired response to low levels of DNA damage treatment (Moll et al., 1996). Therefore, we tested whether a normal DNA damage response could be restored in neuroblastoma cells by RNAi-mediated inhibition of Parc function. As indicated in Figure 8, native SK-N-AS neuroblastoma cells responded poorly to treatment with low levels of the genotoxic drug etoposide; only 20% of cells showed nuclear localization of p53 (II, Figure 8A) and less than 15% became apoptotic (II, Figure 8B). However, after treatment with Parc-RNAi, almost 70% of the cells showed nuclear staining of p53

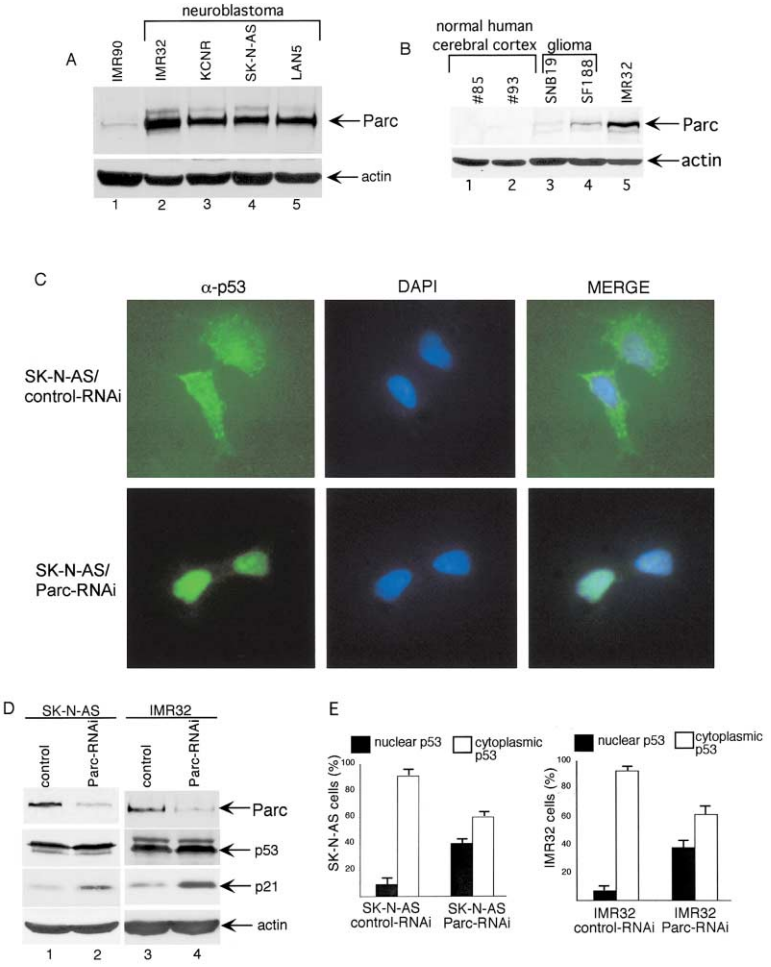

Figure 7. RNAi-Mediated Reduction of Parc Induces p21 Activation and p53 Nuclear Localization in Neuroblastoma Cells

(A) Parc is highly expressed in neuroblastoma cell lines. Whole-cell extracts of primary fibroblast (IMR90) (lane 1) and neuroblastoma cell lines (IMR32, KCNR, SK-N-AS, LAN-5) (lanes 2–5) were immunoblotted with anti-Parc antibody (upper) and anti-actin antibody (lower).

(B) Parc expression levels in human cerebral cortex, glioma, and neuroblastoma cell lines. Western blot analysis of whole-cell extracts of human cerebral cortex from two healthy individuals (# 85, #93) (lanes 1 and 2), SNB19 and SF188 human glioma cell lines (lanes 3 and 4), and neuroblastoma cell line (IMR32) (lane 5) with anti-Parc antibody (upper) and anti-actin antibody (lower).

(C) Subcellular localization of p53 in neuroblastoma cells (SK-N-AS) transfected with Parc-RNAi or control oligonucleotide. The transfected cells were immunostained with anti-p53 antibody (1801) (visualized by green fluorescence) and counterstained with DAPI to visualize the nuclei.

(D) RNAi-mediated reduction of Parc in SK-N-AS and IMR32 neuroblastoma cell lines. Whole-cell extracts from Parc-RNAi or control oligonucleotide transfected cells, were immunoblotted with anti-Parc, anti-p53 (DO-1), anti-p21 (C19), and anti-actin (AC-15) antibodies.

(E) Quantization of subcellular p53 localization (cytoplasmic and nuclear) in SK-N-AS and IMR32 neuroblastoma cells transfected with Parc-RNAi or control oligonucleotide. The data represent the average of three experiments with standard deviations indicated.

(III, Figure 8A) and more than half (56.4%) became apoptotic with the same dose of etoposide (V versus II, Figure 8B), whereas Parc-RNAi treatment alone only induced apoptosis in 17.1% of cells (IV, Figure 8B). Thus, the combination of RNAi-mediated Parc reduction and DNA damage restores a strong p53-mediated apoptotic response in neuroblastoma cells.

Discussion

The present data reveal the existence of a key cytoplasmic protein Parc (p53-associated, Parkin-like cytoplasmic protein) that is critically involved in the regulation of p53 subcellular localization and subsequent function. As a transcription factor, nuclear localization of p53 is essential for its role in tumor suppression (Vousden, 2002; Jimenez et al., 1999). However, p53 is diffusely distributed in the cytoplasm of normal unstressed cells; many types of tumors cells, including neuroblastoma cells, have abnormal cytoplasmic localization of p53 and an impaired p53-dependent stress response despite the fact that they express wild-type p53 proteins (reviewed in Jimenez et al., 1999; Moll et al., 1992, 1995, 1996). We show that the majority of cytoplasmic p53 is tightly associated with endogenous Parc. In the absence of stress, inactivation of endogenous Parc leads p53 activation through inducing p53 nuclear localization. Furthermore, our results also indicate that the levels of Parc proteins are relatively high in neuroblastoma cells. It is conceivable, therefore, that the high levels of Parc in these cells may prevent nuclear translocation of p53, even in the presence of genotoxic stress although other mechanisms may also contribute to this phenomenon (Stommel et al., 1999). In consistence with this notion, we have also shown that RNAi-mediated reduction of Parc protein levels can restore a strong p53-dependent stress response in these neuroblastoma cells. As such, the Parc-mediated pathway of p53 regulation may prove to be a potential target for cancer therapy. In particular, agents that down-regulate Parc protein levels or abrogate the Parc-p53 interaction may sensitize tumor cells to p53-dependent apoptosis.

Parc Plays a Critical Role in the Regulation of Subcellular Localization of p53

In unstressed cells, p53 is diffusely distributed, and p53-mediated function appears to be severely inhibited. While no obvious effect on the total p53 protein levels by RNAi-mediated ablation of endogenous Parc, the p53 polypeptides are relocated to the nucleus, and p53-mediated functions are also strongly activated in the absence of stress. Conversely, overexpression of Parc

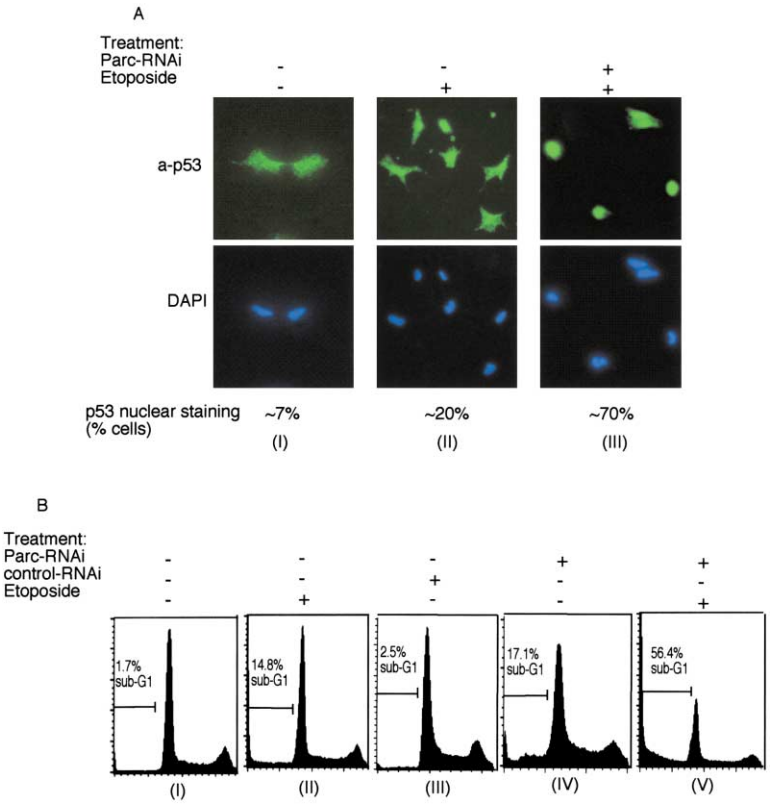

Figure 8. The Combination of Parc Reduction and Genotoxic Stress Strongly Activates p53-Mediated Apoptosis in Neuroblastoma Cells

(A) Subcellular localization of p53 in neuroblastoma cells (SK-N-AS) transfected with Parc-RNAi or control-RNAi and treated with 0.25 μM etoposide.

(B) Enhancement of the DNA damage-induced apoptotic response in neuroblastoma cells by RNAi-mediated Parc reduction. Parc-RNAi or control oligonucleotide transfected SK-N-AS cells were treated with 0.25 μM etoposide and analyzed for apoptotic cells (sub-G1) according to DNA content (PI staining).

induces cytoplasmic sequestration of p53. Therefore, we propose that Parc serves as an anchor protein that tethers p53 in the cytoplasm and thereby regulates p53 subcellular localization.

Parc is a constitutive cytoplasmic protein that strongly interacts with the C-terminal domain of p53. Since this domain harbors the three known NLS sequences of p53, it is conceivable that Parc blocks nuclear import of p53 by concealing its C-terminal NLS motifs. Interestingly, overexpression of the p53 binding domain of Parc (residues 1–770) alone is not sufficient to induce cytoplasmic retention of p53 (Figure 6). Thus, it is likely that additional Parc sequences such as the CCH domain facilitate cytoplasmic sequestration of p53, perhaps by linking the Parc protein to stable cytoplasmic complexes/or structures. In any case, latent p53 is tightly associated with Parc in the cytoplasm of unstressed cells. However, in response to DNA damage and other types of stress, p53 is rapidly stabilized and translocated into the nucleus while no significant effect on Parc subcellular localization was observed (see Supplemental Figures S3 and S4 online at http://www.cell.com/cgi/content/full/112/1/29/DC1). As such, regulation of the Parc-p53 interaction in response to stress is an extremely important issue that warrants further investigation. Since p53 is subjected to posttranslational modifications in stressed cells, it is possible that phosphorylation and/or acetylation of the p53 protein may regulate its interaction with Parc.

Subcellular Localization and p53 Degradation

Based on the observations that p53 can be stabilized by blocking the nuclear export (Freedman and Levine, 1998; Stommel et al., 1999), several earlier studies proposed that additional cytoplasmic factors may be required for complete degradation of p53 in the cytoplasm—although more recent studies reported that degradation of p53 can also be carried out, to some extent, by nuclear proteasomes (Xirodimas et al., 2001). Although Mdm2 is a potent ubiquitin ligase, it was reported that Mdm2 more efficiently induces monoubiquitination, but not polyubiquitination of p53 (Honda et al., 1997; Lai et al., 2001). Since polyubiquitination is generally required for proteasome-mediated degradation (Pickart, 2001), it is possible that there is another ubiquitin ligase (E3) or multiubiquitin chain assembling enzyme (E4) resides in the cytoplasm and contributes to the efficiency of p53 degradation.

In several respects, the Parc protein is an appropriate candidate for such a factor. Parc can tether p53 in the cytoplasm and like Parkin, it contains a signature ubiquitin ligase motif. Moreover, we have found that Parc has an intrinsic ubiquitin ligase activity and can ubiquitinate itself very efficiently. However, Parc fails to induce direct ubiquitination of p53 to a significant degree in vivo or in vitro. Moreover, ablation of endogenous Parc expression strongly induced p53 nuclear localization, but did not significantly affect p53 protein levels. Thus, our study indicates that a primary function of Parc is to control the subcellular localization of p53. We can not, however, exclude the possibility that Parc is also involved in the regulation of p53 ubiquitination in the presence of other cofactors. Very recently, the CHIP protein, which contains a multiubiquitin chain assembling enzyme E4-like activity, was shown to interact functionally

and physically with the Ring-IBR-Ring domain of Parkin (Imai et al., 2002). Since Parc also contains the Ring-IBR-Ring domain, it is possible that Parc may recruit the CHIP-mediated E4 activity to induce polyubiquitination of p53 for degradation.

The relationship between Parc- and Mdm2-mediated negative regulations on p53 needs to be further elucidated. Although both proteins are p53-associated ubiquitin ligases, they apparently regulate p53 function through different mechanisms. In fact, Mdm2 also plays a critical role in promoting nuclear export of p53 in addition to ubiquitination-mediated degradation of p53 (Geyer et al., 2000; Boyd et al., 2000). Thus, it is most likely that Parc and Mdm2 cooperatively regulate subcellular localization and stability of p53 and more effectively keep p53 under control.

Experimental Procedures

Plasmids and Antibodies

To construct Parc expresson constructs, the full-length Parc cDNA or deletion mutants were amplified by PCR from Marathon-Ready HeLa cDNA (Clontech, BD) and subcloned into pcDNA3.1/V5-His-Topo vector (Invitrogen). The Flag sequence was introduced to the N terminus of Parc by PCR and subcloned into pcDNA3.1/V5-His-Topo vector (Invitrogen). To prepare the Parc antiserum, DNA sequences corresponding to the C-terminal 100 amino acids of Parc (residues 2417–2517) were amplified by PCR (2417–2517) and subcloned into pGEX-2T (Luo et al., 2001). α-Parc antiserum was raised in rabbits against the purified GST-Parc (2417–2517) fusion protein (Covance) and further affinity-purified on the antigen column.

Purification of p53-Interacting Proteins

The epitope-tagging strategy to isolate protein complexes has been described previously (Gu et al., 1999; Luo et al., 2000). To obtain Flag-p53 expressing cell line, we transfected p53 null H1299 cells with pCIN4-Flag-p53(175) and selected for 2 weeks on 1 mg/ml G418 (GIBCO). The p53-expressing colonies were expanded and used for cytoplasmic and nuclear extract preparations essentially as described before (Dignam et al., 1983). For cytoplasmic extract preparation, cells were incubated in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and fresh proteinase inhibitors [Sigma]) for 15 min, NP40 (CALBIOCHEM) was added to 0.5%, and the samples were centrifuged for 5 min at 3000 rpm. Supernatants were filtered with 0.45 μm syringe filters (NALGENE). NaCl was added to the supernatants at the final concentration of 200 mM and resulting samples were used as cytoplasmic extracts for M2 immunoprecipitations. Pellets were vortexed for 15 min in buffer C (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and fresh proteinase inhibitors (Sigma), centrifuged for 10 min at 15,000 rpm, and filtered with 0.45 μm syringe filters (NALGENE). Supernatants were diluted with buffer D (20 mM HEPES [pH 7.9], 1 mM EDTA, and 1 mM EGTA) to the 200 mM final NaCl concentration and used as nuclear extracts for M2 immunoprecipitations. Affinity chromatography on M2 beads was used to purify Flag-p53(175) complexes. The binding proteins where eluted from the column with Flag peptide and resolved by SDS-PAGE in a 5%–15% gradient gel (Novex). Specific bands were cut out from the gel and subjected to mass-spectrometry peptide sequencing.

Ablation of Endogenous Parc by RNAi

H1299, U2OS, IMR32, and SK-N-AS cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. The RNAi-mediated ablation of endogenous Parc was performed essentially decribed as previously (Elbashir et al., 2001). A 21-nucleotide siRNA duplex with 3′dTdT overhangs corresponding to Parc mRNA (AAG CUUUCCUCGAGAUCCAGG) was synthesized (Dharmacon). The same sequence in the inverted orientation (AAGGACCUAGAGCUC CUUUCG) was used as a nonspecific RNAi control. RNAi transfection controls also included Parc sense DNA oligonucleotides and regular plasmids. RNAi transfections were performed using Oligofectamine Reagent (Invitrogen). 24 hr before transfection, about 1 million cells were plated on a 10 cm dish. Cells were transfected using manufacturer's protocol (Invitrogen) for three times with 24–48 hr intervals. After three consecutive transfections, cells were harvested for the Western blot analysis, flow cytometry analysis, or immunostaining.

DNA Damage Response and Immunofluorescent Staining

The assay for the DNA damage response was performed essentially as described previously (Luo et al., 2001). Neuroblastoma cells were treated with 0.25 μM etoposide for 8 hr, washed twice with PBS, and supplemented with fresh DMEM with 10% FBS. 36 hr after treatment, cells were stained with PI and analyzed by flow cytometry for apoptotic cells (subG1) according to DNA content. For immunofluorescent staining, cells were grown on 8-well polylysine slides essentially as described previously (Guo et al., 2000). For the p53 cytoplasmic sequestration assay, cells were transfected with 30 ng/well CMV-p53 and 150 ng/well CMV-Parc. 24 hr posttransfection, cells were fixed with 4% paraformaldehyde for 20 min on ice, rehydrated for 5 min in serum-free DMEM, and permeabilized with 0.2% Triton X-100 (Fisher) for 10 min on ice. Cells were incubated in 1% bovine serum albumin (BSA) (Sigma)/phosphate buffered salt solution (PBS) (Cellgro) for 30 min. Primary p53-specific monoclonal (for endogenous p53 immunostaining) (DO-1, 1801, Santa Cruz) or polyclonal (for transfected p53 immunostaining) (FL-393, Santa Cruz) antibodies or anti-Flag monoclonal antibody (for transfected Flag-Parc and Flag-Myc immunostaining) (Santa Cruz) were added in 1% BSA/PBS for 45 min at room temperature. After washing with 1% BSA/PBS, Alexa-488 (or Alexa-568) conjugated anti-mouse (or anti-rabbit for polyclonal primary antibody) (Molecular Probes) antibody was added and incubated for 30 min at room temperature. Finally, cells were counterstained with DAPI to visualize the nuclei essentially as described before (Guo et al., 2000).

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GenBank Accession Number

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