The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53

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Summary

Gankyrin is an ankyrin repeat oncoprotein commonly overexpressed in hepatocellular carcinomas. Gankyrin interacts with the S6 proteasomal ATPase and accelerates the degradation of the tumor suppressor Rb. We show here that gankyrin has an antiapoptotic activity in cells exposed to DNA damaging agents. Downregulation of gankyrin induces apoptosis in cells with wild-type p53. In vitro and in vivo experiments revealed that gankyrin binds to Mdm2, facilitating p53-Mdm2 binding, and increases ubiquitylation and degradation of p53. Gankyrin also enhances Mdm2 autoubiquitylation in the absence of p53. Downregulation of gankyrin reduced amounts of Mdm2 and p53 associated with the 26S proteasome. Thus, gankyrin is a cofactor that increases the activities of Mdm2 on p53 and probably targets polyubiquitylated p53 into the 26S proteasome.

Introduction

The ability of p53 to exert antiproliferative effects, including cell cycle arrest and apoptosis, is strongly associated with its tumor suppressor function (Hanahan and Weinberg, 2001; Vogelstein et al., 2000). The ability of p53 to bind DNA and activate transcription is required for p53-dependent growth arrest. p53 can mediate apoptosis by activating transcription of proapoptotic genes and repressing transcription of antiapoptotic genes (Johnstone et al., 2002; Vousden and Lu, 2002; Villunger et al., 2003). p53 also controls apoptosis through transcription-independent mechanisms (Mihara et al., 2003). Tight regulation of p53 activity is imperative for maintaining normal cell growth and preventing tumorigenesis, with too much or too little p53 contributing to diseases.

The degradation of p53 is regulated by the ubiquitin-proteasome system (Brooks and Gu, 2003; Yang et al., 2004). In this pathway, the first step in ubiquitin conjugation involves the ATP-dependent activation of ubiquitin by a ubiquitin-activating enzyme (E1) (Ciechanover et al., 2000; Pickart, 2001). The activated ubiquitin is then transferred from the E1 to a ubiquitin-conjugating enzyme (E2). A ubiquitin-protein ligase (E3) then facilitates transfer of ubiquitin from an E2 to the substrate. Polyubiquitylated substrates are the preferred substrates of the 26S proteasome, whereas proteins modified by single or only a few ubiquitin molecules appear to be long-lived in vivo or are subject to alternative degradation pathways (Pickart, 2001). Efficient polyubiquitylation has been suggested to require an additional conjugating factor, named E4 (Koegl et al., 1999).

At least 5%–10% of all human tumors possess inappropriate overexpression of Mdm2 (also called HDM2 in humans), and in many of them, the *p53* gene remains in its wild-type configuration (Michael and Oren, 2003). Binding of Mdm2 to p53 reduces the transcriptional activity of p53. Mdm2 is an E3 for p53 and enhances its degradation (Yang et al., 2004). Mdm2 also promotes conjugation of NEDD8 to p53 and represses its transactivating activity (Xirodimas et al., 2004). Mdm2 itself is the product of a p53-inducible gene, and this dependency creates an autoregulatory feedback loop in which both the activity of p53 and the expression of Mdm2 are regulated (Chene, 2003). In vitro, Mdm2 catalyzes the addition of single ubiquitin moieties to a cluster of six C-terminal lysines in p53 (Lai et

SIGNIFICANCE

Gankyrin is overexpressed in most hepatocellular carcinomas. We show here that gankyrin is an antiapoptotic oncoprotein and increases the degradation of p53 as well as Rb. Gankyrin binds to Mdm2, an E3 ubiquitin ligase for p53, and potentiates the ubiquitylating activity. Gankyrin inhibits major tumor suppressors, and the attenuation of gankyrin expression induces apoptosis in tumor cells. Therefore, gankyrin could be a good target for developing therapeutic and preventive strategies against human hepatocelluar carcinomas.

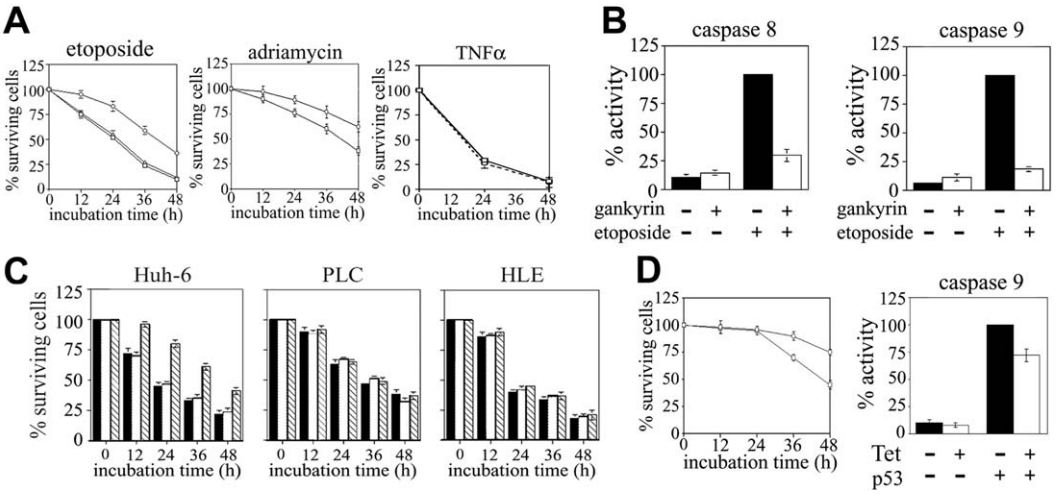

Figure 1. Antiapoptotic acitivity of gankyrin

A: Increased survival after exposure to etoposide (left) or adriamycin (middle), but not to TNF-α with cycloheximide (right). Three clones each of U-2 OS cells stably expressing HA-gankyrin (○), HA-gankyrin isoform 2 (△), or HA alone (□) were counted at the indicated times after treatment. Data represent mean ± SD of triplicates.

B: Decreased activation of caspase 8 and caspase 9. Caspase activities were determined in U-2 OS cells expressing HA-gankyrin or HA alone 4 hr after treatment with etoposide as indicated. Data represent mean ± SD of triplicates.

C: Effects on survival of Huh-6 (left), PLC (middle), and HLE (right) HCC cells. Surviving cells expressing HA-gankyrin (striped bars), HA-gankyrin isoform 2 (white bars), or HA alone (black bars) were counted at the indicated times after etoposide treatment. Data represent mean ± SD of triplicates.

D: Effects on p53-induced apoptosis in H1299 cells that express gankyrin in the presence of doxycycline (Tet). After transfection with p53 expression plasmids, cells were cultured in the presence (○) or absence (□) of Tet, and the survival (left) and caspase 9 activity (right) were assayed. Data represent mean ± SD of triplicates.

al., 2001; Rodriguez et al., 2000). Mdm2 does not efficiently polyubiquitylate p53 under usual in vitro conditions, and the E4 activity of p300 is required in addition to Mdm2 for p53 polyubiquitylation (Grossman et al., 2003). The activity of Mdm2 toward p53 is regulated by several mechanisms (Michael and Oren, 2003). For example, phosphorylation of p53 reduces the avidity of p53 for Mdm2 and thereby inhibits subsequent ubiquitylation and degradation. Alternatively, covalent modification of Mdm2 reduces its ability to promote the ubiquitylation and degradation of p53 (Maya et al., 2001). The tumor suppressor ARF binds to and functionally inactivates Mdm2 (Michael and Oren, 2003).

Gankyrin (also known as PSMD10, p28, and Nas6p) is an oncoprotein overexpressed in most hepatocellular carcinomas (HCCs), and consists of seven ankyrin repeats (Higashitsuji et al., 2000; Krzywda et al., 2004). Gankyrin binds to the retinoblastoma gene product (Rb) and the S6 ATPase subunit of the 26S proteasome, and increases degradation of Rb in vitro and in vivo (Higashitsuji et al., 2000; Dawson et al., 2002). Gankyrin also binds to Cdk4 and counteracts the inhibitory function of the tumor suppressors $p16^{INK4A}$ and $p18^{INK4C}$ (Dawson et al., 2002; Li and Tsai, 2002). In a rodent model of hepatocarcinogenesis, gankyrin is overexpressed from the earliest stage of tumor development (Park et al., 2001). These findings suggest that gankyrin is a major player in cell cycle control and tumorigenesis in HCCs. Here, we have found that gankyrin, an interactor with a protesomal ATPase, can also control the ubiquitin ligase activity of Mdm2 on p53.

Results

Gankyrin increases resistance to apoptosis induced by DNA damaging agents

Overexpression of gankyrin increases the tumorigenic potential of immortalized NIH/3T3 cells (Higashitsuji et al., 2000). To confirm the transforming activity of gankyrin, we transfected primary rat embryonic fibroblasts (REFs) with plasmids expressing gankyrin in conjunction with activated Ras (Finlay, 1993). 10 to 14 days after transfection, multiple dense transformed foci appeared in the dishes that received Ras and gankyrin, whereas Ras or gankyrin alone did not induce a significant number of foci (Supplemental Figure S1 and Supplemental Table S1). Although the number and size of foci were smaller compared with those induced by Ras and adenovirus E1A, gankyrin cooperates with ras and transforms normal diploid fibroblasts.

One of the hallmarks of the cancer cell phenotype is evasion of apoptosis (Hanahan and Weinberg, 2001). When gankyrin is overexpressed in U-2 OS cells, the number of cells surviving after exposure to etoposide or adriamycin was increased (Figure 1A). In contrast, no effect of gankyrin was observed in cells treated with TNF-α and cycloheximide. DNA damage induces p53-dependent apoptosis (Fridman and Lowe, 2003), whereas TNF-α triggers apoptosis p53 independently (Varfolomeev and Ashkenazi, 2004). In gankyrin-expressing U-2 OS cells, etoposide-induced cell surface expression of annexin V and activation of caspase 8 and caspase 9 were inhibited (Figure 1B and data not shown), indicating that the increased survival was due to the decreased apoptosis. We observed similar effects of

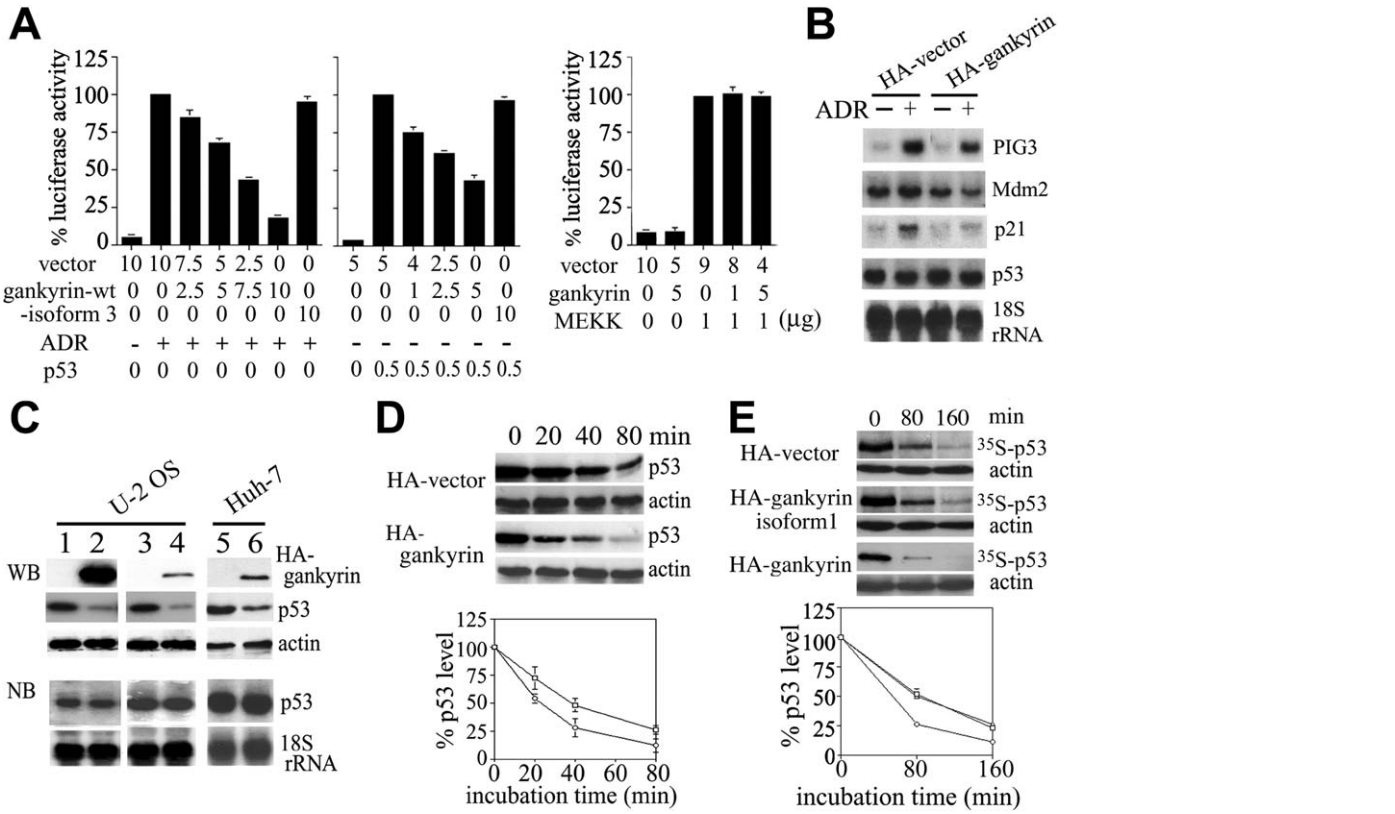

Figure 2. Effects of gankyrin on activity and protein level of p53

A: Reduced transcriptional activity of p53. Left, WI-38 cells were cotransfected with a p53-responsive luciferase reporter, pRL-TK, and plasmids expressing HA-gankyrin wild-type (wt), HA-gankyrin isoform 3, or HA vector. After exposure to adriamycin (ADR), luciferase activity was assayed. Middle, H1299 cells were cotransfected and analyzed as above, except that plasmids expressing p53 were used instead of adriamycin. Right, WI-38 cells were cotransfected and analyzed as above except that an AP-1-responsive luciferase reporter was used instead of a p53-responsive reporter. pFC-MEKK served as a positive control. The results were normalized to *Renilla* luciferase activity, and represent the mean ± SD of triplicates.

B: Reduced induction of p53-inducible genes. U-2 OS cells were transfected with plasmids expressing HA-gankyrin or HA alone. After incubation with (+) or without (−) ADR, gene expression was analyzed by Northern blotting using the indicated cDNA probes.

C: Effects on p53 levels. U-2 OS cells stably expressing HA-gankyrin (lane 2) or HA alone (lane 1), and U-2 OS cells (lanes 3 and 4) and Huh-7 cells (lanes 5 and 6) transiently transfected with plasmids expressing HA-gankyrin (lanes 4 and 6) or vector alone (lanes 3 and 5) were analyzed for p53 expression by Western blotting (WB, upper) and Northern blotting (NB, lower) using indicated antibodies and cDNA probes.

D: Enhanced p53 degradation in vivo. 3 stable clones each of U-2 OS cells expressing HA-gankyrin (○) or HA alone (□) were treated with cycloheximide, and harvested at the indicated time points. Lysates were analyzed by WB (upper panels). The intensity of the bands was quantified (bottom). Data represent mean ± SD of triplicates.

E: Pulse chase experiment. H1299 cells cotransfected with plasmids expressing HA-gankyrin (○), HA-gankyrin isoform1 (△), or HA alone (□) and p53 were pulse labeled with ^{35}S-methionine. At indicated times, p53-immunoprecipitates were analyzed by SDS-PAGE and fluorography. The intensity of the bands was quantified (bottom). As a loading control, 5% lysates were analyzed by Western blotting using anti-actin antibody. Data represent mean ± SD of triplicates.

gankyrin in adriamycin-treated cells (data not shown). The anti-apoptotic effect of gankyrin was observed in etoposide-treated Huh-6 HCC cells expressing wild-type p53, but not in PLC and HLE HCC cells expressing mutant p53 (Figure 1C). In p53 null H1299 cells, introduction of p53 induced apoptosis and activation of caspase 9, which were inhibited by overexpression of gankyrin (Figure 1D). These results suggest that gankyrin suppresses the proapoptotic signaling pathway mediated by p53.

Gankyrin suppresses transcriptional activity of p53

To probe the functional relationship between gankyrin and p53 in apoptosis, we first analyzed the effects of gankyrin on the transcriptional activity of p53. We transfected normal diploid WI-38 cells with a luciferase p53-*cis* reporter plasmid. Adriamycin induced luciferase activity, which was suppressed dose dependently by gankyrin (Figure 2A). In the p53 null H1299 and Saos-2 cells that undergo apoptosis after introduction of p53 as well as adriamycin- or etoposide-treated U-2 OS cells, expression of gankyrin suppressed the reporter activity (Figure 2A and data not shown). The inhibition was specific to the p53 transactivation, since gankyrin did not affect a luciferase AP-1-*cis* reporter activity. In U-2 OS cells exposed to adriamycin, the mRNA levels of p53-inducible genes *p21* and *Mdm2*, and those involved in apoptosis, such as *Noxa*, *PIG3*, and *Bax*, but not *Perp*, *Killer*, and *Fas*, were decreased by gankyrin (Figure 2B and data not shown).

The p53 protein level was decreased by gankyrin in U-2 OS and Huh-7 cells expressing only wild-type and mutant p53, re-

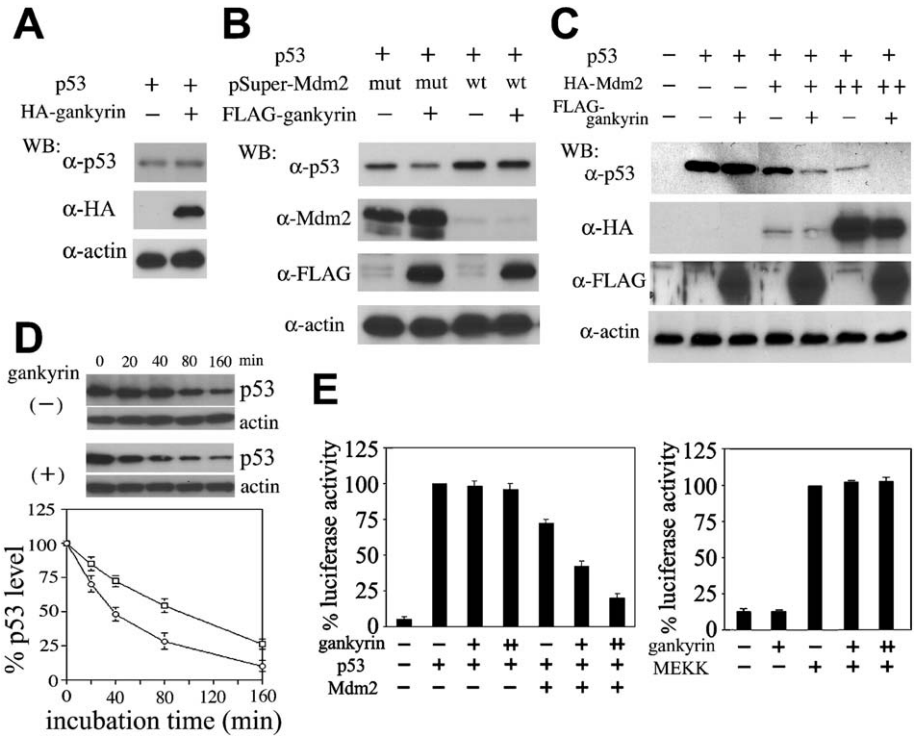

Figure 3. Mdm2-dependent effects of gankyrin on p53

A: Effects on p53 protein level in HeLa cells. HeLa cells were transiently transfected with plasmids expressing p53 and HA-gankyrin (lane 2) or vector alone (lane 1) as indicated. Cell lysates were analyzed by Western blotting (WB).

B: Effects of decreased Mdm2. H1299 cells were cotransfected with plasmids expressing RNAi specific to wild-type (wt) or mutant (mut) *Mdm2* mRNA (pSuper-Mdm2), p53, and FLAG-gankyrin as indicated. Cell lysates were analyzed by WB.

C: Dependence on Mdm2. DKO-MEFs were transfected with plasmids expressing human p53, HA-Mdm2, and FLAG-gankyrin as indicated. Levels of p53 were analyzed by WB. p53:Mdm2 molar ratios were 4:1 in lanes 4 and 5, and 1:1 in lanes 6 and 7.

D: Effects on p53 stability. DKO-MEFs were transfected with plasmids expressing human p53 and Mdm2 (1:1 molar ratio) with (+, ○) or without (–, □) gankyrin, treated with cycloheximide, and harvested at the indicated times. Lysates were analyzed by WB using anti-p53 and anti-actin antibodies (upper panels). The intensity of the bands was quantified (bottom). Data represent mean ± SD of triplicates.

E: Effects on p53 and AP-1 transcriptional activities. DKO-MEFs were transfected with a p53- (left) or AP-1- (right) responsive luciferase reporter, pRL-TK, and plasmids expressing human p53 and Mdm2 (p53:Mdm2, 1:1 molar ratio) and HA-gankyrin (+ and ++, a low and high dose, respectively) as indicated, and luciferase activity was assayed. pFC-MEKK served as a positive control. The results were normalized to *Renilla* luciferase activity, and represent the mean ± SD of triplicates.

spectively (Figure 2C). The level of *p53* mRNA was not reduced by gankyrin, suggesting that gankyrin affects the rate of synthesis and/or the stability of p53 protein.

Gankyrin accelerates the Mdm2-dependent degradation of p53

In U-2 OS cells stably overexpressing gankyrin, the stability of endogenous p53 protein was decreased (Figure 2D). Pulse-chase experiments confirmed that the half-life of ectopically expressed p53 was shortened by gankyrin (Figure 2E). These results demonstrate that gankyrin accelerates degradation of p53 in vivo, and suggests that the decreased reporter activity in the presence of gankyrin (Figure 2A) is due, at least partly, to this effect.

The degradation of p53 is mainly mediated by Mdm2 (Yang et al., 2004). In HeLa cells, however, p53 is predominantly degraded through the human papillomavirus (HPV)18 E6 pathway (Hengstermann et al., 2001). Gankyrin did not decrease the p53 protein level in HeLa cells (Figure 3A). When p53-deficient H1299 cells were transfected with plasmids that express *Mdm2*-specific RNAi together with plasmids expressing p53 and gankyrin, the endogenous Mdm2 protein level was decreased and the suppressive effect of gankyrin on the p53 protein level was ameliorated (Figure 3B). These results suggest that gankyrin promotes p53 degradation via Mdm2. To further investigate this possibility, we utilized embryonic fibroblasts from *p53/mdm2* double-knockout mice (DKO-MEFs). As shown in Figure 3C, the protein level of ectopically expressed p53 was not affected by overexpression of gankyrin in the absence of Mdm2. Mdm2 decreased the p53 level, which was further decreased by coexpression of gankyrin. The decrease in the p53 level was due to an increase in the degradation rate (Figure 3D). To analyze the effects on p53 transactivating activity, we next transfected DKO-MEFs with plasmids expressing p53-luciferase reporter, p53, gankyrin, and Mdm2. Luciferase activity was decreased by gankyrin in the presence, but not in the absence, of Mdm2 (Figure 3E). No effect of gankyrin was observed on AP-1-luciferase reporter activity. These results demonstrate that gankyrin accelerates degradation of p53 and reduces its transcriptional activity in an Mdm2-dependent manner.

Specific interaction of gankyrin with Mdm2

When we transfected U-2 OS cells with plasmids that expressed HA-tagged Mdm2 and FLAG-tagged gankyrin, Mdm2 coimmunoprecipitated with gankyrin (Figure 4A). The interaction of FLAG-gankyrin and HA-Mdm2 was also observed in DKO-MEFs (Figure 4B), indicating that the binding was not mediated by p53. Gankyrin was unable to coimmunoprecipitate with MdmX, E6-AP, COP1, or Pirh2 (data not shown). Endogenous gankyrin was also coimmunoprecipitated with Mdm2 from WI-38 cell lysates (Figure 4C). Notably, gankyrin was immunoprecipitated with anti-p53 antibody. GST pulldown assay demonstrated a physical interaction of gankyrin with Mdm2, but not p53, in vitro (Figure 4D).

By using a series of gankyrin deletion mutants and isoforms lacking one or more ankyrin repeats (Hiroaki Higashitsuji and Jun Fujita, unpublished data) in coimmunoprecipitation and Western blot analysis, full-length gankyrin was found to be necessary for binding to Mdm2 (Figure 4E). Similarly, the region of Mdm2 necessary for binding to gankyrin was determined to be the amino acid residues 412 to 437, which is adjacent to

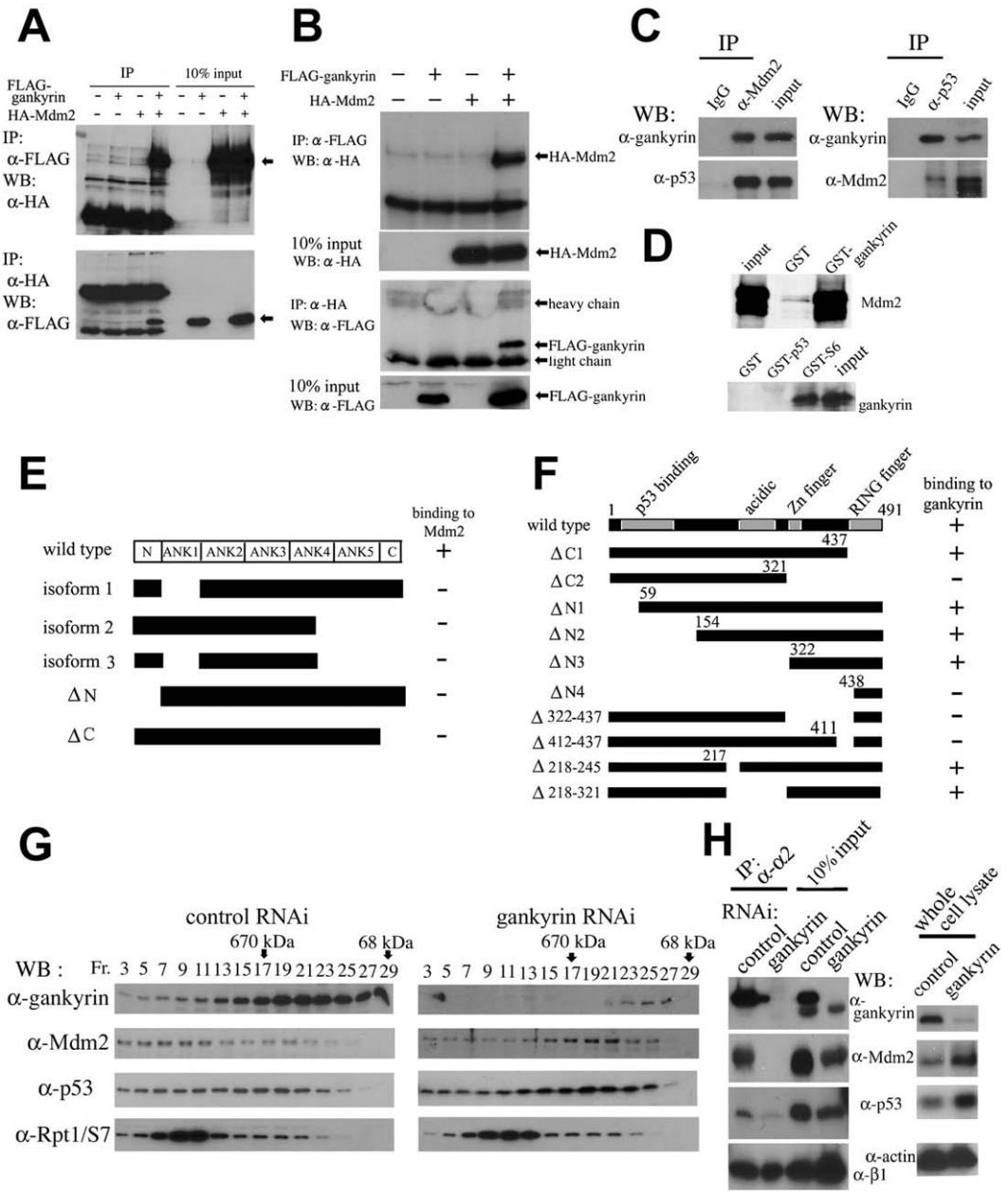

Figure 4. Interaction of gankyrin with Mdm2

A: Coimmunoprecipitation (IP) of exogenous proteins. U-2 OS cells were cotransfected with plasmids expressing FLAG-gankyrin and HA-Mdm2 as indicated. Cell lysates (10% input) and immunoprecipitates prepared by IP with indicated antibodies were analyzed by Western blotting (WB). Arrows indicate mobilities of HA-Mdm2 (upper) and FLAG-gankyrin (lower).

B: Co-IP in the absence of p53. DKO-MEFs were cotransfected with plasmids expressing FLAG-gankyrin and HA-Mdm2 as indicated. Lysates (10% input) and immunoprecipitates prepared by IP with anti-HA or agarose-immobilized anti-FLAG antibody were analyzed by WB using the indicated antibodies.

C: Co-IP of endogenous proteins. Lysates from WI-38 cells (10% input) and immunoprecipitates prepared by IP with agarose-immobilized anti-Mdm2 (left) or anti-p53 (right) antibody or normal mouse IgG were analyzed by WB using the indicated antibodies.

D: GST pulldown assays. GST-gankyrin fusion protein or GST was incubated with in vitro translated ^{35}S-labeled Mdm2 (upper). GST-p53, GST-S6 (positive control), or GST (negative control) was incubated with in vitro translated ^{35}S-labeled gankyrin (lower). Bound proteins and 25% input were analyzed by SDS-PAGE and fluorography.

E: Region of gankyrin necessary for binding to Mdm2. Gankyrin mutants and isoforms as indicated were expressed in U-2 OS cells, and assayed for co-IP with HA-tagged full-length Mdm2. (+) and (−) indicate presence and absence, respectively, of binding. Top row, schematic structure of wild-type gankyrin. N and C, the first and last ankyrin repeats, respectively. ANK1 to ANK5, the second to sixth ankyrin repeats.

F: Region of Mdm2 necessary for binding to gankyrin. Various Mdm2 mutants were expressed in U-2 OS cells, and assayed for co-IP with HA-tagged full-length gankyrin. (+) and (−) indicate presence and absence, respectively, of binding. Top row, schematic structure of wild-type Mdm2 (modified from Michael and Oren, 2003).

G: Cosedimentation of gankyrin, Mdm2, p53, and the 26S proteasome. The fractions generated by 10%–40% glycerol gradient centrifugation of lysates from U-2 OS cells transfected with *gankyrin* RNAi (right) or control RNAi (left) were analyzed by WB using the indicated antibodies. Positions of markers are shown on the top.

H: Co-IP of the proteasome subunits with gankyrin, Mdm2, and p53. IP with agarose-immobilized monoclonal anti-proteasomal α2 subunit antibody of proteins in gradient fractions 7–15 from U-2 OS cells transfected with indicated RNAi. Precipitated proteins, 5% input, and whole cell lysates were separated by SDS-PAGE and analyzed by WB using the indicated antibodies.

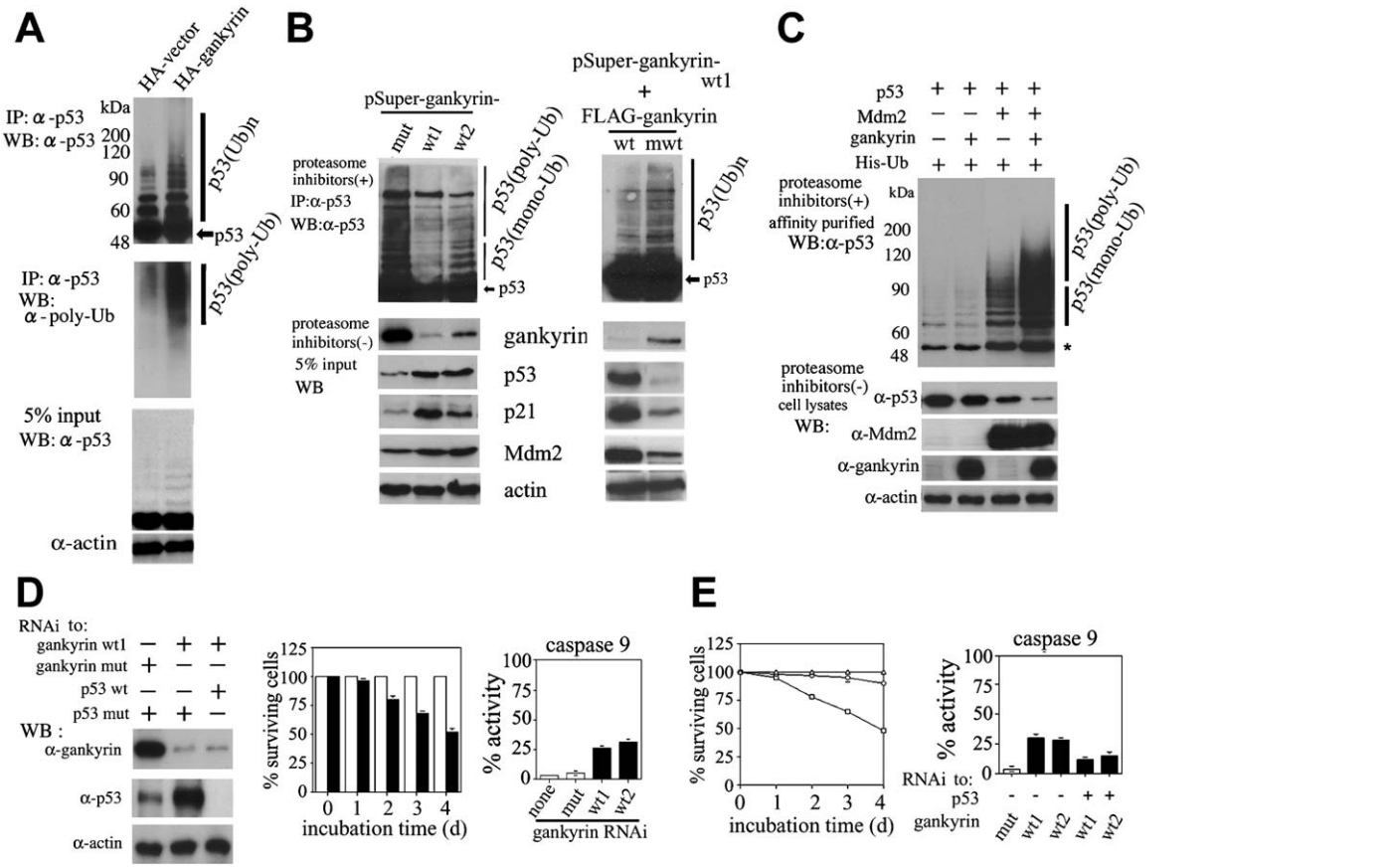

Figure 5. Effects of gankyrin on p53 and apoptosis

A: Effects on p53 ubiquitylation. U-2 OS transfectants stably expressing HA-gankyrin or HA-vector alone were treated with MG132 for 6 hr. Cell lysates (5% input) and immunoprecipitates prepared by immunoprecipitation (IP) with anti-p53 antibody were analyzed by Western blotting (WB) using the indicated antibodies. p53(Ub)n indicates the mobility of ubiquitylated p53.

B: Effects of decreased gankyrin expression. Left, U-2 OS cells were transfected with pSuper-gankyrin that produces RNAi exactly matching (-wt1 and -wt2) or 3-base-mismatching (-mut) to the *gankyrin* mRNA nucleotide sequence. Right, U-2 OS cells were transfected with pSuper-gankyrin-wt1 together with plasmids expressing normal gankyrin from wild-type cDNA (wt) or 3-base-mutated cDNA (mwt). Cells were cultured in the presence or absence of MG-132 and analyzed as in **A**.

C: Mdm2-dependent effect of gankyrin. DKO-MEFs were cotransfected with plasmids expressing p53, Mdm2, FLAG-gankyrin, and His-ubiquitin and cultured in the presence (+) or absence (−) of MG132 as indicated. Cell lysates and those affinity-purified using Ni-NTA-agarose beads were analyzed by WB using the indicated antibodies. Asterisk indicates nonspecific bands.

D: Apoptosis induced by decreased gankyrin expression. Left, Huh-6 cells were transfected with RNAi specific to wild-type (wt1 and wt2) gankyrin, mutated (mut) gankyrin, wild-type (wt) p53, or mutated (mut) p53 mRNA. Cell lysates were prepared 2 days after transfection, and analyzed by WB using indicated antibodies. Middle, Huh-6 cells were transfected with pSuper-gankyrin-wt1 (black columns) or -mut as a control (white columns). The cell numbers were analyzed and expressed as relative to the control. Right, Lysates from Huh-6 cells transfected with indicated RNAi were assayed for caspase 9 activity. The activity was expressed as relative to that induced by etoposide alone. Data represent mean ± SD of triplicates.

E: p53 dependency of apoptosis. Huh-6 cells were transfected with gankyrin-wt1 RNAi (□), gankyrin-wt1 RNAi plus p53 RNAi (△), gankyrin-mut RNAi (○), or indicated RNAi. Cell numbers were counted at indicated times (left). Caspase 9 activity was assayed as in **D** (right). Data represent mean ± SD of triplicates.

the RING finger domain and different from the region for p53 binding (Figure 4F).

In U-2 OS cells, cosedimentation of endogenous gankyrin with p53, Mdm2, and the 26S proteasome was observed during glycerol gradient centrifugation of cell lysates (Figure 4G). Furthermore, Western blot analysis of proteins coimmunoprecipitated from the fractions 7–15 with agarose-immobilized monoclonal anti-proteasomal α2 subunit antibody revealed the presence of gankyrin, p53, and Mdm2 (Figure 4H). Interestingly, when gankyrin was knocked down by RNAi, both Mdm2 and p53 moved to lighter fractions during centrifugation (Figure 4G), and much lower amounts of Mdm2 and p53 were coimmunoprecipitated with the proteasome, although the total cellular amounts of Mdm2 and p53 were increased (Figure 4H). These results indicate a direct interaction of gankyrin with Mdm2 and suggest a gankyrin-Mdm2-mediated interaction of p53 with the 26S proteasome.

Gankyrin enhances the Mdm2-dependent ubiquitylation of p53

Because p53 is degraded through the ubiquitin-proteasome pathway, we assessed the effect of gankyrin on ubiquitylation of p53 in U-2 OS cells treated with a proteasome inhibitor. In Western blot analysis of p53, a ladder of slowly migrating ubiq-

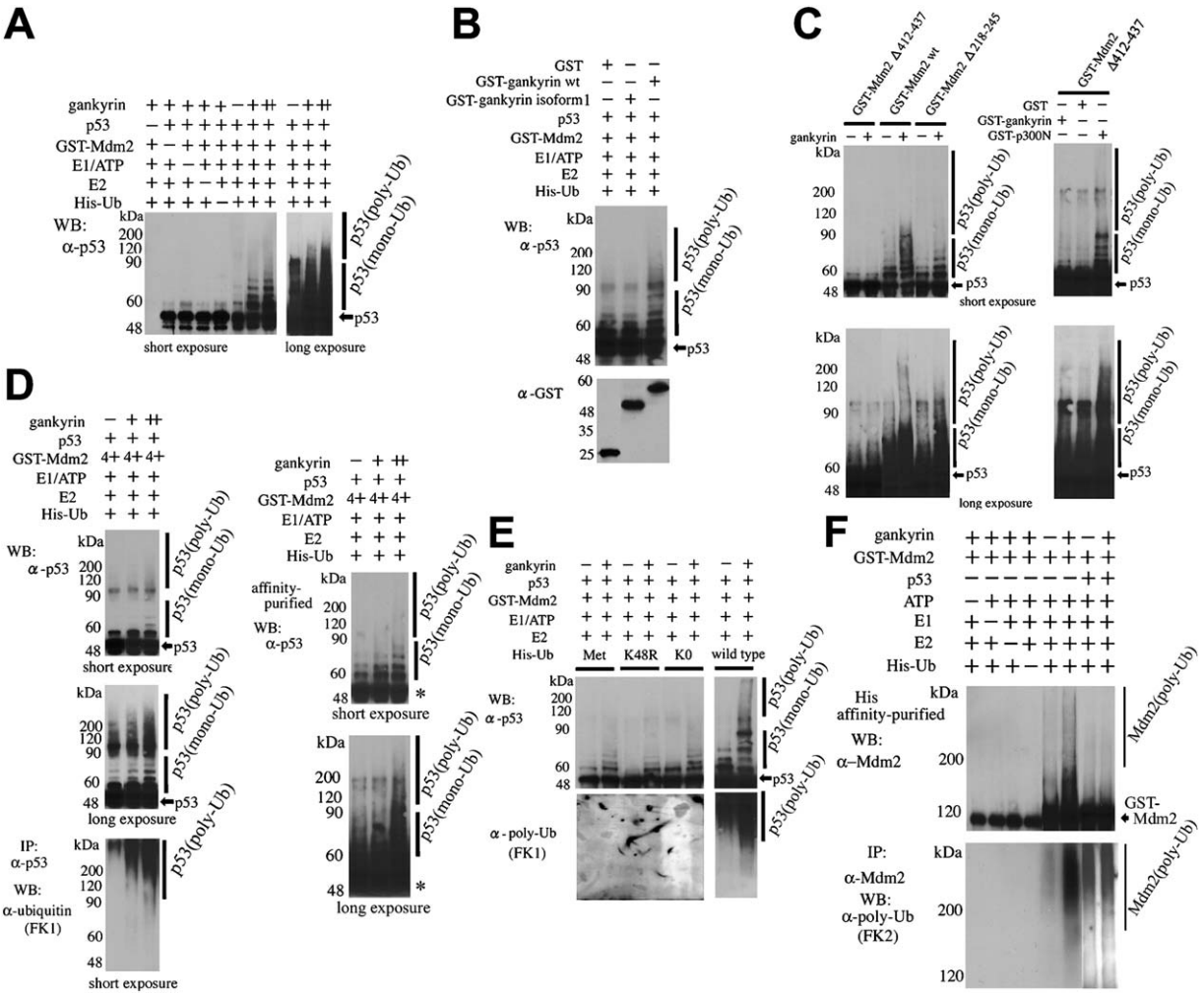

Figure 6. Effects of gankyrin on in vitro ubiquitylation

A: Effects on mono- and polyubiquitylating activities of Mdm2. In vitro ubiquitylation assay was performed using gankyrin (low or high dose), p53, GST-Mdm2, E1, E2, and His-ubiquitin as indicated. The products were analyzed by Western blotting (WB) using antibodies against p53. Short and long exposures are shown. Arrow indicates the mobility of p53.

B: Effects of gankyrin isoform1 defective in binding to Mdm2. In vitro ubiquitylation assay was performed as in **A** and the products were analyzed by WB.

C: Effects of gankyrin in the presence of mutant Mdm2. In vitro ubiquitylation assay was performed as in **A** using GST-Mdm2 mutants instead of GST-Mdm2 wild-type (wt) in the presence (+) or absence (−) of GST-gankyrin or GST-p300N as indicated. The products were analyzed by WB using anti-p53 antibody. Mdm2Δ412–437 and Mdm2Δ218–245 lack gankyrin binding activity and p300 binding activity, respectively.

D: Effects on p53 at high Mdm2:p53 ratio. In vitro ubiquitylation assay and WB were performed as in **A** using the Mdm2:p53 ratio of 4:1 instead of 1:1. The reaction products were also immunoprecipitated (IP) with anti-p53 antibody or affinity-purified with Ni-NTA-agarose beads and analyzed by WB as indicated. Asterisks indicate nonspecific bands.

E: Effects on p53-monoubiquitylating activity of Mdm2. Using methylated ubiquitin (Met), K48R, or UbK0 (right) instead of ubiquitin, in vitro ubiquitylation assay was performed as in **A**. The products were analyzed by WB using anti-p53 and anti-ubiquitin antibodies as indicated.

F: Effects on autoubiquitylating activity of Mdm2. In vitro ubiquitylation assay was performed using gankyrin, GST-Mdm2, E1, E2, and His-ubiquitin in the presence or absence of p53 as indicated. The products were affinity-purified with Ni-NTA-agarose beads or IP with anti-Mdm2 antibody, and analyzed by WB using anti-Mdm2 antibody or polyubiquitin-specific anti-ubiquitin antibody, respectively.

uitylated forms was more prominent in anti-p53 immunoprecipitates from gankyrin-expressing cells than those from control cells, although the levels of endogenous p53 were comparable (Figure 5A). Similar results were obtained when WI-38 cells were cotransfected to express FLAG-gankyrin and His-ubiquitin (Supplemental Figure S2). We next ablated endogenous gankyrin expression and assessed its effects on p53 ubiquitylation. When *gankyrin*-specific RNAi was expressed in U-2 OS cells, the levels of endogenous gankyrin and ubiquitylated p53 were decreased, whereas those of p53, Mdm2, and p21 were increased (Figure 5B). Essentially similar results were obtained in WI-38, A549, A2780, and 293 cells, and the level of ubiquitylated p53 was decreased in both the cytoplasm and nucleus in U-2 OS cells treated with *gankyrin*-specific RNAi (data not shown). Gankyrin expressed from mutated mRNA such that the *gankyrin*-specific RNAi cannot knockdown its expression abrogated the effect of RNAi on p53 ubiquitylation (Figure 5B, right), suggesting that the effect on p53 is not due

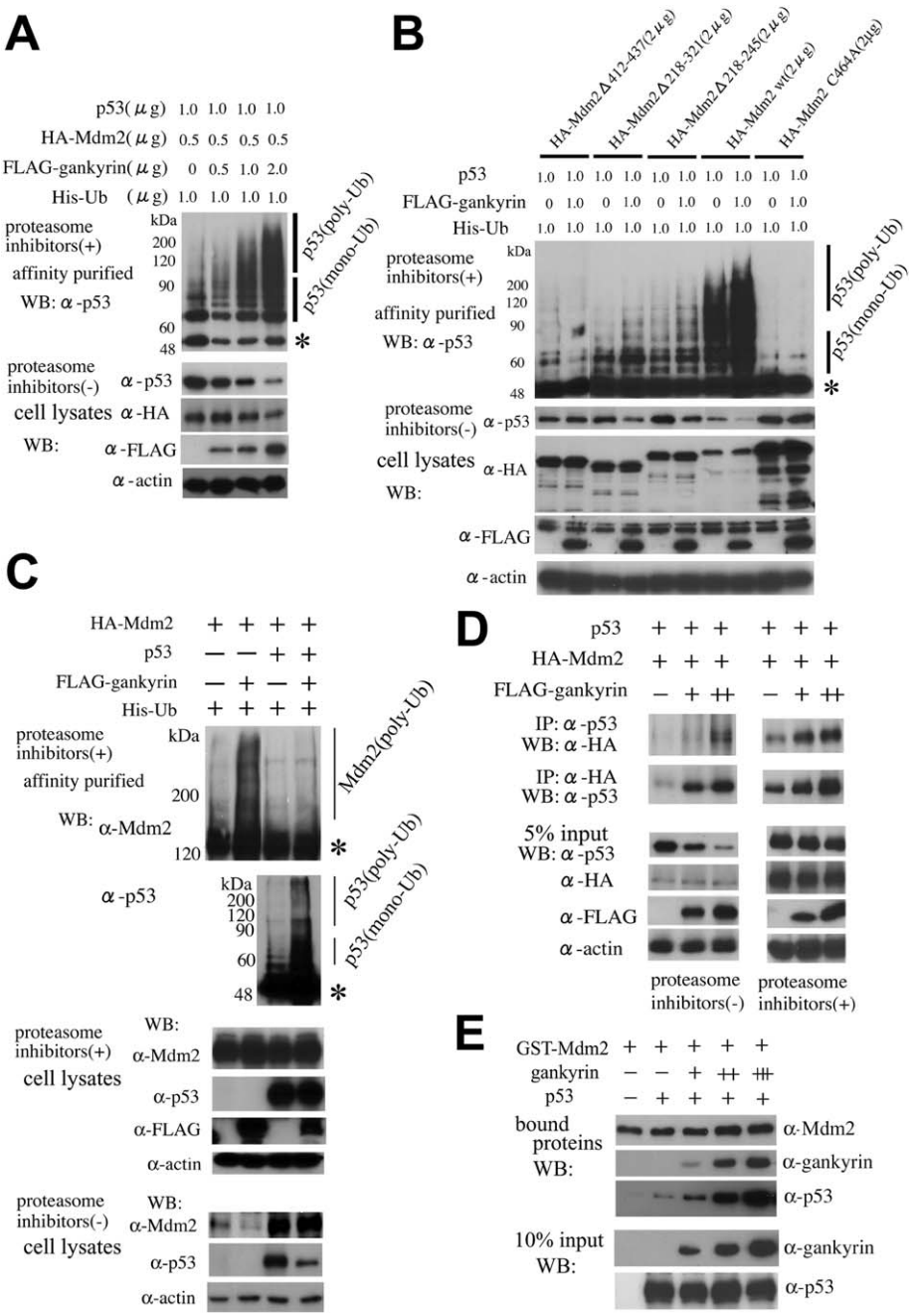

Figure 7. In vivo effects of gankyrin on p53-ubiquitylating and binding activities of Mdm2

A: Effects on mono- and polyubiquitylation of p53. DKO-MEFs were cotransfected with plasmids expressing proteins as indicated and cultured in the presence (+) or absence (−) of MG132. Cell lysates (10% input) and those affinity-purified using Ni-NTA-agarose beads were analyzed by Western blotting (WB) using the indicated antibodies. Asterisk indicates nonspecific bands.

B: Effects of mutation in Mdm2. DKO-MEFs were cotransfected with plasmids expressing indicated proteins and analyzed as in **A**. HA-Mdm2 C464A has no E3 activity. Asterisk indicates nonspecific bands.

C: Effects on the level of Mdm2. DKO-MEFs were cotransfected with plasmids expressing indicated proteins and analyzed as in **A**.

D: Effects on the amount of p53 bound to Mdm2. DKO-MEFs were cotransfected with plasmids expressing p53, HA-Mdm2, and FLAG-gankyrin as indicated. Cells were cultured in the presence or absence of MG132. Cell lysates were prepared and immunoprecipitated with anti-p53 or anti-HA antibody. Immunoprecipitates and cell lysates (10% input) were analyzed by WB.

E: GST pulldown assay. GST-Mdm2 was incubated with p53 in the presence of increasing amounts of gankyrin as indicated. Proteins bound to glutathione-Sepharose as well as 10% of recombinant proteins incubated with GST-Mdm2 (10% input) were analyzed by WB.

to the off-target effects of RNAi. The observed effects of gankyrin were dependent on Mdm2, since ubiquitylation of exogenous p53 was increased by gankyrin only in the presence of Mdm2 in DKO-MEFs (Figure 5C).

Gankyrin is commonly overexpressed in HCCs (Higashitsuji et al., 2000). Therefore, we examined the effects of *gankyrin* RNAi in HCC cell lines Huh-6 and Huh-7, expressing wild-type and mutant p53, respectively. In Huh-6 cells, *gankyrin* RNAi decreased the gankyrin expression, increased the p53 protein level, and induced apoptosis with activation of caspase 9 (Figure 5D). This proapoptotic activity was abrogated by concomitant suppression of p53 expression (Figure 5E), indicating its dependence on p53. No proapoptotic effect was observed in Huh-7 cells (data not shown). *Gankyrin* RNAi induced apoptosis in U-2 OS cells and A549 cells with wild-type p53, but not in p53 null H1299 cells or HeLa cells (data not shown). These results suggest that overexpression of gankyrin in tumor cells decreases the p53 protein level by enhancing its ubiquitylation and degradation by Mdm2, resulting in inhibition of p53-dependent apoptosis.

Gankyrin enhances the E3 activity of Mdm2

We next investigated whether gankyrin has intrinsic E3 ubiquitin ligase activity by an in vitro ubiquitylation assay. When

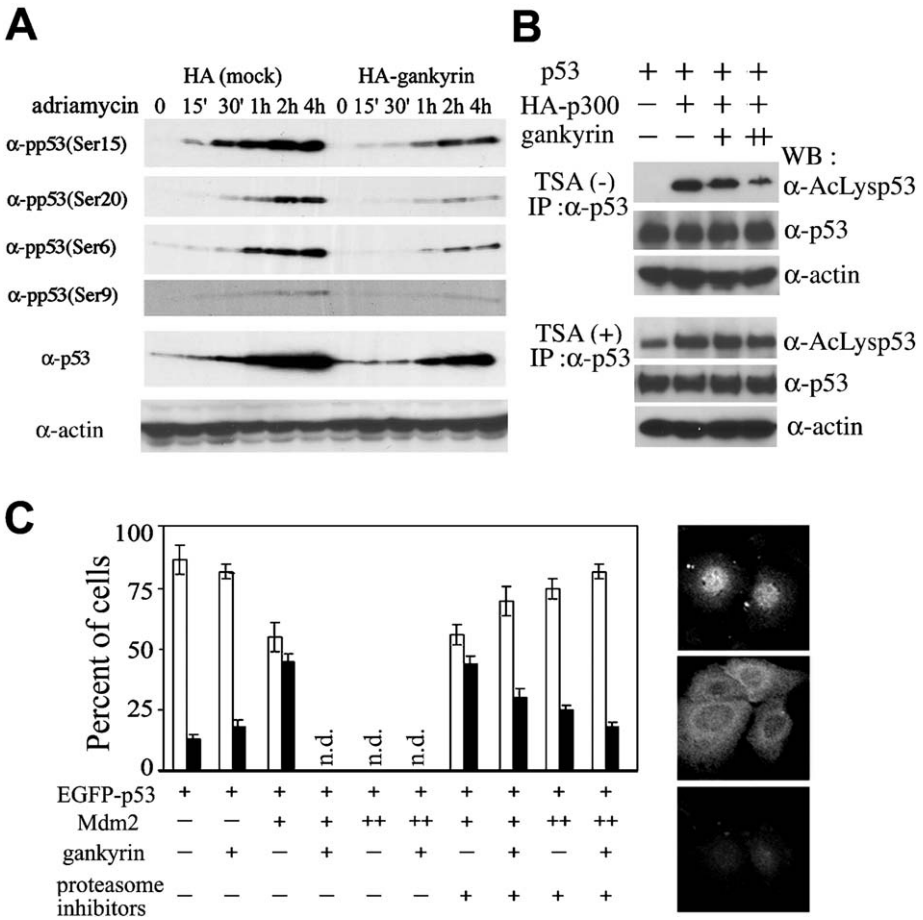

Figure 8. Effects of gankyrin on posttranslational modifications and subcellular localization of p53

A: Effects on DNA damage-induced phosphorylation of p53. U-2 OS cells stably overexpressing HA-gankyrin or HA alone (mock) were treated with adriamycin, and cell lysates were analyzed by Western blotting (WB) using antibodies as indicated.

B: Suppression of p300-induced acetylation of p53. REFs were cotransfected with plasmids expressing human p53, HA-p300, and FLAG-gankyrin, cultured, and treated with trichostatin A (TSA) (+) or vehicle alone (−) in the presence of MG132. Cell lysates were immunoprecipitated with agarose-immobilized antibody specific to human p53 and analyzed by WB.

C: Effects on localization of p53. Left, DKO-MEFs were transfected with plasmids expressing EGFP-p53, HA-Mdm2 (low and high doses), and FLAG-gankyrin in the presence (+) or absence (−) of MG132 as indicated. Localization of EGFP-p53 fluorescence was determined in 300 cells for each culture. Ordinate shows the percentage of cells with localization of EGFP-p53 mainly in the nucleus (white columns) or cytoplasm (black columns). nd, no EGFP-p53 detected. Data represent mean ± SD of triplicates. Right, photographs of representative cells transfected with EGFP-p53 alone (nuclear localization, top), in combination with low-dose Mdm2 (cytoplasmic, middle) or low-dose Mdm2 plus gankyrin (nd, bottom) observed under confocal microscope.

affinity-purified GST-gankyrin was incubated in ubiquitin reaction buffer that contained recombinant E1, E2 (UbcH5a or UbcH5b), and ubiquitin, no autoubiquitylation of gankyrin was observed (data not shown). When we incubated gankyrin with p53 in ubiquitin reaction buffer, no detectable change in p53 migration was observed (Figure 6A). However, gankyrin dose dependently increased the formation of mono- and polyubiquitylated p53 in the presence of Mdm2. Gankyrin isoform 1 lacking Mdm2 binding activity showed no effect on ubiquitylation of p53 (Figure 6B). Ubiquitylation of p53 induced by mutant Mdm2 lacking its gankyrin binding region (Mdm2Δ412–437) was increased in the presence of p300, but not gankyrin (Figure 6C). These results indicate that gankyrin has no intrinsic E4 activity and that binding to Mdm2 is necessary for gankyrin to increase ubiquitylation of p53. Since mutant Mdm2, which was unable to bind p300 but able to bind gankyrin (Mdm2Δ218–245), polyubiquitylated p53 in the presence of gankyrin (Figure 6C), the E4 activity of p300 was unlikely to be involved in the observed effect of gankyrin. These in vitro assays were performed using the Mdm2:p53 molar ratio of 1:1 (low Mdm2). At 4:1 molar ratio (high Mdm2), polyubiquitylation of p53 was observed in the absence of gankyrin, and it was further increased by gankyrin (Figure 6D).

When methylated ubiquitin was used instead of ubiquitin in an in vitro ubiquitylation assay, the ladder of monoubiquitylated p53 species was increased in the presence of gankyrin (Figure 6E). Similar results were obtained using ubiquitin-K48R or UbK0 in which all seven lysine residues were replaced by arginine. These results demonstrate that gankyrin enhances mono- and polyubiquitylating activities of Mdm2 toward p53. Interestingly, gankyrin increased autoubiquitylating activity of Mdm2 in the absence, but not in the presence, of p53 (Figure 6F).

Essentially similar findings were made in DKO-MEFs. When Mdm2 plasmids were transfected at a low dose, gankyrin caused an increase in polyubiquitylation of p53 dose dependently (Figure 7A). When Mdm2 plasmids were transfected at a high dose, mono- and polyubiquitylation of p53 were observed, which were further increased by gankyrin (Figure 7B). Gankyrin enhanced the p53-ubiquitylating activity of Mdm2Δ218–245, but not Mdm2Δ412–437 lacking the ability to bind gankyrin. Although the acidic domain of Mdm2 has been shown to be critical in the ubiquitylation of p53 (Meulmeester et al., 2003; Kawai et al., 2003), gankyrin increased the level of ubiquitylated p53 induced by Mdm2Δ218–321.

In DKO-MEFs, the exogenous Mdm2 level was not affected by gankyrin in the presence of p53 (Figure 7C). In the absence of p53, however, ubiquitylation of Mdm2 was increased and the Mdm2 protein level was decreased.

When gankyrin was overexpressed in DKO-MEFs, the p53 level decreased, but significantly more Mdm2 was coimmunoprecipitated with p53 compared with those not transfected with gankyrin-expressing plasmids (Figure 7D). When the deg-

radation of p53 was inhibited by proteasome inhibitors, more Mdm2 and p53 were coimmunoprecipitated in cells expressing gankyrin. In vitro GST pulldown assay definitely demonstrated that gankyrin facilitates binding of Mdm2 to p53 (Figure 7E).

Effects of gankyrin on posttranslational modification of p53

The stability and activity of p53 are affected by posttranslational modifications (Brooks and Gu, 2003). Phosphorylation of multiple serine residues of p53 has been proposed to interfere with the ability of Mdm2 to negatively regulate p53. Gankyrin did not affect phosphorylation of these residues (Figure 8A). Gankyrin did not affect phosphorylation of Mdm2 at Ser166 and Ser186 either (data not shown), although these modifications are known to affect the activity of Mdm2 (Michael and Oren, 2003).

p53 is specifically acetylated at multiple lysine residues of the C-terminal regulatory domain by p300/CBP, and the acetylation levels of p53 correlate well with its activation and stabilization induced by stress (Brooks and Gu, 2003; Ito et al., 2002). Mdm2 negatively regulates the p300-induced p53 acetylation (Chene, 2003). Gankyrin also reduced the p300-dependent p53 acetylation in REFs (Figure 8B). The effect was reversed by a pan-histone deacetylase inhibitor trichostatin A, suggesting that active deacetylation is involved in the decrease in the level of acetylated p53.

A number of studies have shown that nuclear export of p53 is specifically regulated by Mdm2 (Chene, 2003). As shown in Figure 8C, ectopic expression of wild-type p53 fused to EGFP yielded predominantly nuclear localization of p53 in DKO-MEFs. Gankyrin alone had no effect on the localization. Consistent with the report by Li et al. (2003), low Mdm2 increased cytoplasmic localization of p53-EGFP, and at high Mdm2, no EGFP signal was detected (Figure 8C). In the presence of gankyrin, however, p53-EGFP was not detected even with low Mdm2. Taken together, the present study has demonstrated that gankyrin is an antiapoptotic oncoprotein that binds to and enhances activities of Mdm2, resulting in suppression of p53.

Discussion

We have found that the oncoprotein gankyrin reduces the level of the p53 protein by increasing its ubiquitylation and degradation. Several E3 ubiquitin ligases are known for p53, including Mdm2, COP1, Pirh2, and p300 (Grossman et al., 2003; Leng et al., 2003; Dornan et al., 2004). Experiments using Mdm2 null DKO-MEFs demonstrated that the effects of gankyrin are Mdm2-dependent. Gankyrin directly binds to Mdm2, and enhances its ubiquitylating activity toward p53. The activities of Mdm2 are regulated by several mechanisms positively or negatively (Yang et al., 2004; Michael and Oren, 2003; Wu et al., 2004). Mdm2-interacting proteins such as ARF, YY1, hHR23A, MdmX, MTBP, and Cul4A can affect the Mdm2-mediated degradation of p53 (Michael and Oren, 2003; Brignone et al., 2004; Nag et al., 2004; Brady et al., 2005). Gankyrin did not affect the phosphorylation, ubiquitylation, or protein level of Mdm2 in the presence of p53. There is now evidence that trans- and autoubiquitylating activity of Mdm2 can be differentially regulated through its interaction with other proteins. The tumor suppressor ARF binds to Mdm2 and preferentially inhibits its p53-ubiquitylating activity (Xirodimas et al., 2001). Gankyrin may increase Mdm2 function by displacing ARF. However, this is unlikely, because gankyrin did not affect the binding of ARF to Mdm2 (Hiroaki Higashitsuji and Jun Fujita, unpublished data) and the ARF binding region of Mdm2 (Michael and Oren, 2003) is different from that for gankyrin. Recently, YY1 has been suggested as a cofactor for Mdm2, facilitating Mdm2-p53 interaction, p53 polyubiquitylation, and degradation (Sui et al., 2004; Gronroos et al., 2004). In contrast to gankyrin, YY1 directly binds to p53 and ARF as well.

Mdm2 efficiently induces monoubiquitylation, but not polyubiquitylation, of p53 in vitro (Lai et al., 2001). p300 binds to Mdm2 and shows the E4 activity, promoting the polyubiquitylation of p53 (Grossman et al., 2003). The E4 activity has been suggested to represent a specialized type of E3 activity (Hatakeyama et al., 2001), and p300 indeed has an autoubiquitylating E3 activity (Grossman et al., 2003). Gankyrin promoted both mono- and polyubiquitylation of p53 by Mdm2 in a p300-independent manner, but showed no E3 or E4 activity. Direct interaction between gankyrin and Mdm2 was necessary to enhance p53 ubiquitylation, and gankyrin facilitated the binding of Mdm2 to p53 in vivo and in vitro, suggesting that gankyrin causes a conformational change(s) in bound Mdm2. Since gankyrin also enhanced Mdm2 autoubiquitylation and degradation in the absence of p53, gankyrin probably has some direct effect on the E3 activity of Mdm2, the exact nature of which remains to be resolved.

Gankyrin also seems to exert a postubiquitylation effect on p53. Previously, evidence has been presented for the binding to the 26S proteasome of ubiquitin ligases and proteins with ubiquitin-like domains, which may in turn bind to ubiquitylated proteins (Brignone et al., 2004; Hartmann-Petersen et al., 2003). Forming a complex with the 26S proteasome, endogenous gankyrin cosedimented with p53 and Mdm2 during glycerol centrifugation of U-2 OS cell lysates (Figure 4G). Furthermore, downregulation of gankyrin resulted in dissociation of most Mdm2 and p53 from the 26S proteasome (Figure 4H). Although little of the cellular gankyrin was found in complex with Mdm2, these results suggest that gankyrin assists in the targeting of an E3 ubiquitin ligase (Mdm2) and its ubiquitylated substrate (p53) directly to a proteasomal ATPase (S6), to ensure efficient unfolding of the protein target and degradation.

Recently, low Mdm2 has been shown to induce monoubiquitylation and nuclear export of p53, whereas high Mdm2 promotes p53's polyubiquitylation and nuclear degradation (Li et al., 2003). In DKO-MEFs, we found that in the presence of gankyrin, low Mdm2 degrades p53 like high Mdm2 (Figure 8C). Acetylation of the C-terminal lysine residues of p53 prevents Mdm2-dependent ubiquitylation (Michael and Oren, 2003), and Mdm2 reduces p300/CBP-mediated p53 acetylation, either by directly interfering with activities of p300/CBP or through recruitment of the histone deacetylase HDAC1 (Ito et al., 2002). We found that active deacetylation is involved in the suppression of p300-induced acetylation of p53 by gankyrin (Figure 8B). However, whether it is also due to enhanced activity of Mdm2 is presently unknown. Also unknown is a possible effect of gankyrin on Rb-ubiquitylating activity of Mdm2 (Uchida et al., 2005).

Viral oncoproteins such as HPV E7 and adenovirus E1A block the function of Rb, which results in the production of the ARF, leading to stabilization of p53 (Vousden and Lu, 2002; Michael and Oren, 2003). The viruses counteract these cellular

defenses by producing proteins that inhibit the function of p53, such as HPV E6 and adenovirus E1B, leading finally to tumor formation (Yang et al., 2004). Gankyrin binds to and inhibits Rb (Higashitsuji et al., 2000). Gankyrin also inhibited p53 and functioned as a transforming gene. Furthermore, reducing gankyrin expression induced apoptosis in HCC cells with wild-type p53. p53 mutation is not frequent in HCCs, especially in low-grade or low-stage HCCs, compared with other types of tumors (Konishi et al., 1993). Considering its function and common overexpression in HCCs, gankyrin must play important roles in hepatocarcinogenesis, and blocking gankyrin may prove to be a valuable therapeutic and/or preventive strategy against human HCCs.

Experimental procedures

Cell culture and apoptosis assays

Mouse NIH/3T3 cells, DKO-MEFs (kindly provided by D.P. Lane), REFs, human U-2 OS, Saos-2, HeLa, H1299, A549, A2780, T24, adenovirus-transformed human embryonic kidney 293 cells, WI-38 normal diploid embryonic fibroblasts, and HCC cell lines (p53 status as reported by Jia et al., 1997), Huh-6 (wild-type p53), Huh-7 (Y220C p53), PLC (R249S p53), and HLE (V272M p53) were maintained in Dulbecco's modified Eagle medium or RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Induction and analysis of apoptosis, including assays for caspase 8 and 9 activities, transfection, isolation of stable clones, and immunofluorescence staining were performed as described (Higashitsuji et al., 2000; Higashitsuji et al., 2002; Nagao et al., 2003). For evaluation of p53 localization, 300 cells were scored for each condition under a fluorescence microscope.

Reporter gene assay

Luciferase reporter plasmids containing p53- or AP-1 binding sites (p53-Luc or pAP-1-Luc, respectively, Stratagene) and pRL-TK (Promega) were cotransfected with increasing amounts of HA-gankyrin or its isoform cDNA in expression vector pCMV4-3HA or vetor alone as described (Higashitsuji et al., 2002). 24 hr later, cells were assayed for luciferase activity or treated with etoposide or adriamycin and assayed 12 hr later. Luciferase activity was measured by the Luciferase Assay System (Promega) according to the manufacturer's protocol. In some experiments, plasmids expressing p53 and/or Mdm2 were cotransfected as well. pFC-MEKK plasmid (Stratagene) served as a positive control for the AP-1 reporter assay.

Analyses of gene expression and protein-protein interactions

RNA extraction, Northern blot analysis, immunoprecipitation, Western blot analysis, and immunofluorescence staining of cells were performed as described (Higashitsuji et al., 2002; Nagao et al., 2003). For immunoprecipitation, anti-p53 antibody (DO-1 and FL-393, Santa Cruz), anti-HA antibody (12CA5, Roche), anti-FLAG antibody (M2, Sigma), agarose-immobilized monoclonal anti-proteasomal α2 subunit antibody (Affiniti Research Products), agarose-immobilized anti-p53 antibody (DO-1, Santa Cruz), agarose-immobilized anti-FLAG antibody (M2, Sigma), and agarose-immobilized anti-Mdm2 antibody (SMP14, Santa Cruz) were used. Western blot analysis was performed using the antibody against p53 (DO-1, Santa Cruz; DO-7, PharMingen), gankyrin (Santa Cruz), β1 and Rpt1/S7 (Affiniti Research Products), HA, FLAG, β-actin (C4, Chemicon), ubiquitin (FL-76, Santa Cruz), Mdm2 (SMP14, Santa Cruz), acetylated lysine (Cell Signaling Technology), and acetylated p53 (Lys 320, Lys 373/382) (Upstate). The anti-ubiquitin antibody FK2 (MBL, Nagoya, Japan) recognizes polyubiquitin, but not monoubiquitin.

Glycerol gradient centrifugation of cell lysates was performed as described (Dawson et al., 2002). For GST pulldown assays, full-length gankyrin cDNA and Mdm2 cDNA was cloned into the expression vector pGEX-4T (Amersham Pharmacia) and expressed in *E. coli*. GST-gankyrin fusion protein and GST were immobilized on glutathione-Sepharose and incubated with in vitro translated ^{35}S-labeled Mdm2 proteins at 4°C for 60 min. Bound proteins were analyzed by SDS-PAGE and fluorography as described (Higashitsuji et al., 2000). GST-Mdm2 was incubated with recombinant p53 and gankyrin at a molar ratio of 1:1:0, 1:1:0.5, 1:1:1, or 1:1:2. After short incubation, proteins bound to glutathione-Sepharose were recovered and directly analyzed by Western blotting.

To determine the regions of gankyrin necessary for binding to Mdm2, plasmids expressing various deletion mutants tagged with FLAG were cotransfected with plasmids expressing HA-tagged full-length Mdm2 onto U-2 OS cells. Lysates were prepared from them, immunoprecipitated with anti-HA antibody, and analyzed by Western blotting using anti-FLAG antibody. The regions of Mdm2 necessary for binding to gankyrin were determined similarly.

Analysis of p53 stability

For the in vivo p53 degradation assay, 80%–90% confluent cultures of U-2 OS cells stably expressing HA-gankyrin or HA alone (3 clones each) were incubated with cycloheximide (100 μg/ml). After 20, 40, and 80 min, the cells were harvested and subjected to Western blot analysis using the anti-p53 antibody. The intensity of the bands was quantified by densitometry. To study the stability of exogenous p53 protein, DKO-MEFs were cotransfected with plasmids expressing p53 and Mdm2 with or without gankyrin, treated with cycloheximide, and analyzed as above.

For pulse chase analysis, H1299 cells were cotransfected with plasmids expressing p53 with or without gankyrin-expressing plasmids. After 18 hr, the transfected cells were metabolically labeled with ^{35}S-methionine/cysteine for 30 min. Subsequently, cells were washed and chased in nonradioactive medium for various periods of times. The cell lysates containing the same amount of trichloroacetic acid-insoluble radioactivity were immunoprecipitated with anti-p53 antibody (FL-393), and analyzed by SDS-PAGE and fluorography.

In vitro and in vivo ubiquitylation assays

In vitro ubiquitylation assays were carried out in ubiquitin reaction buffer (50 mM Tris-HCl, 2 mM ATP, 5 mM $MgCl_2$, 2 μM DTT) with rabbit E1 (200 ng, Boston Biochem), UbcH5a or UbcH5b (100–200 ng, Boston Biochem), and His-tagged ubiquitin, methylated ubiquitin, ubiquitin-K48R, or ubiquitinK0 (10 μg, Boston Biochem) in the presence or absence of ubiquitin aldehyde (25 ng/ml, Affinity Research Products). The cDNAs for p53, gankyrin, Mdm2, and their mutants were cloned into pGEX6P-1 (Amersham Pharmacia Biotech) and expressed as GST-fusion proteins in *Escherichia coli* strain BL21. Where indicated, the GST was cleaved using the PreScission protease. The Mdm2:p53 molar ratio used was 1:1, 2:1, or 4:1. The reactions were incubated at 30°C for 30–90 min. The reactions were stopped with 2× SDS loading buffer and heated to 95°C for 5 min, and the proteins were separated on a 5%–10% SDS-PAGE. In some experiments, the reaction products were immunoprecipitated with anti-p53 antibody (FL393) or affinity-purified with Ni-NTA-agarose beads (Xirodimas et al., 2001). Ubiquitylated proteins were visualized by Western blot analysis. To assess the effect of gankyrin or p300 on E3 activity of Mdm2, Mdm2 was incubated with GST-gankyrin or GST-p300N proteins for 1 hr at 4°C prior to incubation with the ubiquitin reaction buffer.

For in vivo ubiquitylation assay, H1299 cells or DKO-MEFs (in 60 mm dish) were cotransfected with plasmids expressing p53 (1.0 μg), FLAG-gankyrin (0.5, 1.0, or 2.0 μg), HA-Mdm2 or its mutant (0.5, 1.0, or 2.0 μg), and His-tagged ubiquitin in various combinations. Prior to collection after 48 hr, cells were treated with MG132 (25 μM) for 6 hr. Then, cells were lysed in modified RIPA buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.025% SDS, and proteinase inhibitors), and lysates were immunoprecipitated with anti-p53 antibody (FL393) or affinity-purified with Ni-NTA-agarose beads (Xirodimas et al., 2001) and analyzed by Western blotting.

In vivo acetylation assay

Cells were lysed in buffer (20 mM Tris-HCl [pH 7.6], 170 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 μM DTT) supplemented with 5 μM trichostatin A and proteinase inhibitors. To detect acetylated human p53, equal amounts of cell lysates (250–500 μg proteins) were incubated with 1 μg of agarose-immobilized antibody specific to human p53 (DO-1) for 4 hr at 4°C. Precipitated proteins were analyzed by Western blotting.

Inhibition of endogenous gene expression by RNAi

For production of RNAi within the cells, we used the pSuper vector as described (Higashitsuji et al., 2002). pSuper-gankyrin-wt1 and pSuper-gank-

yrin-wt2 contained the 19 nucletides (nt) derived from the gankyrin cDNA (nt number 456 to 474, and 205 to 223, respectively; GenBank accession number D83197) as the target sequence. The control pSuper-gankyrin-mut contained mutations at gankyrin cDNA nt numbers 461 (C to T), 462 (A to T), and 468 (A to T). Double-stranded RNAs corresponding to those expressed by pSuper-gankyrin-mut, pSuper-gankyrin-wt1, and pSuper-gankyrin-wt2 (gankyrin-mut RNAi, gankyrin-wt1 RNAi, and gankyrin-wt2 RNAi, respectively) were synthesized and transfected using the SiPORT Amine Transfection Agent (Ambion). Double-stranded RNA containing scrambled 19 nt was used as a control. To avoid inhibition by pSuper-gankyrin-wt1 but express normal gankyrin protein, 3 point mutations were introduced into nt numbers 471, 473, and 474 of gankyrin cDNA and cloned into expression vector. pSuper-Mdm2-wt and pSuper-p53 contained the 19 nt derived from the Mdm2 cDNA (nt numbers 402 to 420, GenBank accession number Z12020) and the p53 cDNA (nt numbers 989 to 1007, GenBank accession number K03199), respectively, as the target sequence. The control pSuper-p53-mut contained mutations at p53 cDNA nt numbers 993 (C to T), 994 (C to T), and 1001 (A to T). Double-stranded RNAs p53 RNAi and p53 mut RNAi corresponding to those expressed by pSuper-p53 and pSuper-p53-mut, respectively, were also synthesized and used. The control pSuper-Mdm2-mut contained mutations at Mdm2 cDNA nt numbers 404 (G to A) and 413 (T to G). Transfections of pSuper plasmids were performed using Fugene6 Reagent (Roche).

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/8/1/75/DC1/.

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