Suppression of Centrosome Amplification after DNA Damage Depends on p27 Accumulation

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Abstract

The centrosome plays a fundamental role in cell division, cell polarity, and cell cycle progression. Centrosome duplication is mainly controlled by cyclin-dependent kinase 2 (CDK2)/cyclin E and cyclin A complexes, which are inhibited by the CDK inhibitors p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\). It is thought that abnormal activation of CDK2 induces centrosome amplification that is frequently observed in a wide range of aggressive tumors. We previously reported that overexpression of the oncogene MYCN leads to centrosome amplification after DNA damage in neuroblastoma cells. We here show that centrosome amplification after \(\gamma\)-irradiation was caused by suppression of p27 expression in MYCN-overexpressing cells. We further show that p27\(^{-/-}\) and p27\(^{+/+}\) mouse embryonic fibroblasts and p27-silenced human cells exhibited a significant increase in centrosome amplification after DNA damage. Moreover, abnormal mitotic cells with amplified centrosomes were frequently observed in p27-silenced cells. In response to DNA damage, the level of p27 gradually increased in normal cells independently of the ataxia telangiectasia mutated/p53 pathway, whereas Skp2, an F-box protein component of an SCF ubiquitin ligase complex that targets p27, was reduced. Additionally, p27 levels in MYCN-overexpressing cells were restored by treatment with Skp2 small interfering RNA, indicating that down-regulation of p27 by MYCN was due to high expression of Skp2. These results suggest that the accumulation of p27 after DNA damage is required for suppression of centrosome amplification, thereby preventing chromosomal instability. (Cancer Res 2006; 66(8): 4020-9)

Introduction

The centrosome is the major microtubule organizing center of animal cells. Each centrosome consists of a pair of centrioles and a surrounding protein matrix referred to as pericentriolar materials (1). The centrosome duplicates once every cell cycle, which starts during the G1-S transition, coincident with the onset of DNA replication. During mitosis, duplicated centrosomes direct formation of bipolar spindles, which is critical for accurate segregation of chromosomes and cytokinesis (2). Abrogation of regulatory mechanisms governing centrosome duplication leads to generation of amplified centrosomes (more than two centrosomes), which in turn leads to mitotic aberration (i.e., multipolar spindles and unequal segregation of chromosomes (2). Centrosome amplification and chromosomal instability have been found in a wide range of aggressive tumors (3-6). Possible mechanisms for centrosome amplification include multiple rounds of centrosome duplication within a single cell cycle, uncontrolled splitting of a centriole pair, and centrosome reduplication following cytokinesis failure or DNA endoreplication (1).

A number of tumor-related proteins involved in numeral homeostasis of centrosomes have been reported. For instance, mouse embryonic fibroblasts (MEF) lacking tumor suppressor genes, such as p53 or promyelocytic leukemia gene 3 (PML3), display centrosome amplification and a significant increase in cyclin-dependent kinase 2 (CDK2) activity (7, 8). Previous studies have shown that CDK2/cyclin E and cyclin A complexes are required for initiation of centrosome duplication (9, 10). Phosphorylation of several proteins by CDK2, including nucleophosmin/B23, Mps1, and CP110, is implicated in the initiation and promotion of centrosome duplication (11-13). It has been shown that in Chinese hamster ovary cells treated with DNA synthesis inhibitor, such as hydroxyurea, centrosomes continue to duplicate without DNA synthesis (10, 14). However, expression of the CDK inhibitors p21\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\) suppresses centrosome overduplication in hydroxyurea-treated cells (10, 14). p27 was initially identified as a negative regulator of G1 progression in growth-arrested cells (15-17). p27 directly binds to CDK2/cyclin complexes and thereby regulates cell cycle progression (18). The levels of p27 are mainly regulated by the ubiquitin-proteasome system during the cell cycle. Skp2, an F-box protein component of the SCF ubiquitin ligase complex, recognizes p27 and induces its degradation during S phase and G2 (19, 20). Furthermore, the recently identified KPC1/KPC2 ubiquitin ligase complex leads to p27 degradation at G1 (21).

In response to DNA damage, normal cells immediately activate DNA damage checkpoints (i.e., at G1, intra-S, and G2) that induce cell cycle arrest; thereafter, the DNA damage is repaired, if possible, to maintain genomic integrity (22). Because coordination between the centrosome duplication cycle and the DNA replication cycle is strictly regulated, DNA damage-induced cell cycle arrest should be accompanied by centrosome duplication arrest. Several reports have shown that colon cancer and breast cancer cells lacking p53 and its targets, p21 and Kruppel-like factor 4 (KLF4), exhibit centrosome amplification after DNA damage (23-25) and that centrosome amplification after DNA damage induced by a Rad51...
deletion in chicken DT40 cells involves ataxia telangiectasia mutated (ATM; ref. 26). Contrast to our understanding of the regulatory mechanisms of cell cycle arrest after DNA damage, the regulation of the centrosome duplication cycle following DNA damage is not well understood.

We have previously shown that enhanced expression of MYCN leads to centrosome amplification after DNA damage in neuroblastoma cells (27). MYCN is a member of the MYC family and is often amplified and overexpressed in malignant neuroblastoma cells (28). Clarifying the molecular mechanism of centrosome amplification by MYCN overexpression after DNA damage may contribute to understanding the mechanism of tumor development in MYC-overexpressing neuroblastomas and to identifying additional mechanisms of the regulation of centrosome duplication following DNA damage. In this study, we overexpressed MYCN in neuroblastoma cells and found that centrosome amplification after DNA damage was caused by suppression of p27 expression through increased expression of Skp2. We further found that centrosome amplification after DNA damage was significantly increased in p27−/− and p27+/− MEFs and p27-silenced human cells. After DNA damage, the level of p27 is gradually increases independently of the ATM/p53 signaling pathway, whereas the level of the Skp2 is reduced. In addition, p27 can interact with CDK2/cyclin E after DNA damage. These results suggest that p27 plays a crucial role in suppressing centrosome amplification after DNA damage.

Materials and Methods

Cell lines. The human neuroblastoma cell line SHEP and SHEP cells transfected with retroviral control vector or retroviral vector containing MYCN (pGCDNsamRES-EGFP) have been previously described (27). Wild-type, p27−/−, p27+/− and p53−/− MEFs and p27-silenced human cells. The cells were first incubated with blocking buffer, consisting of 5% nonfat dry milk in TBS with 0.05% Tween 20 (TBS-T), and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). The blots were incubated with horseradish peroxidase–conjugated secondary antibody (Sigma) for 1 hour at room temperature. The antibody-antigen complex was visualized with enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's protocol.

Immunoprecipitation. The cells were lysed on ice for 30 minutes with a lysis buffer, consisting of 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% NP40, 0.1% Triton X-100, 1 mmol/L NaF, 1 mmol/L Na3VO4, and proteinase inhibitors (proteinase inhibitor cocktail, Roche Diagnostics). The lysates were centrifuged at 20,000 × g for 1 hour at 4°C. The supernatants were diluted with SDS sample buffer, consisting of 62.5 mmol/L Tris (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. The samples were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The blots were incubated for 1 hour at room temperature in blocking buffer, consisting of 5% nonfat dry milk in TBS with 0.05% Tween 20 (TBS-T), and then with the diluted primary antibodies overnight at 4°C. The blots were incubated with horseradish peroxidase–conjugated secondary antibody (Sigma) for 1 hour at room temperature. The antibody-antigen complex was visualized with enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's protocol.

DNA transfection and small interfering RNA experiments. The pCDN-HA-tagged human p27 and mutant p27 (W60G) vectors were transfected into cells with LipofectAMINE Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The sequences of the small interfering RNAs (siRNA) were as follows: p27-600, 5'-GGAGAGACUCUGCA-GAGACAUCAA-3'; p27-632, 5'-GGCGCGCAGUGGAAUUCGGAU-3'; p27-910, 5'-GCGAGAUAGAGGAGACUCUGCAA-3' (stall RNA interference, purchased from Invitrogen); Skp2, 5'-GAGUGUUAUCGCUAGCTT-3'; Luciferase (GL-2), 5'-CGUAUGCAGGAAUUCUGATT-3' (Japan BioService, Asaka, Japan). The siRNAs were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol.

Flow cytometry. The cells were fixed in 70% ethanol for 1 hour at 4°C. The fixed cells were incubated with PBS with 300 μg/ml RNase A at room temperature and stained with 50 μg/ml propidium iodide. Flow cytometric analysis was done with FACS.Popup and Cell Quest software (Becton Dickinson, San Jose, CA).

γ-Irradiation. The cells were irradiated at room temperature using a 137Cs source γ-irradiator Gamma Cell 40 (Atomic Energy of Canada Ltd, Ontario, Canada).

Semiquantitative reverse transcription-PCR. Total RNAs were isolated with the TRIZOL reagent (Invitrogen), and cDNAs were synthesized from 2 μg of each RNA preparation using the Super Script RT III (Invitrogen) according to the manufacturer's protocol. To allow semiquantitative comparisons among cDNAs developed from identical reactions, the reverse transcription-PCR exponential phase was determined for each primer set. All reactions involved an initial denaturation at 95°C for 5 minutes followed by 30 cycles for p27 (forward primer, 5'-TGGAGAACAGCTGAGACG-3'; reverse primer, 5'-GCGGCTGCCGAGGTTAGCC-3'), 25 cycles for p21 (forward primer, 5'-ACTGTGACGCTTAATGCG-3'; reverse primer, 5'-AATGGGCTTCTCGTGAGTCC-3'), and 25 cycles for GAPDH (forward primer, 5'-CAACACCTCAGATCATA-3'; reverse primer, 5'-AGGTTCCAC-CAGTACAGTCT-3') for 95°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute using a Gene Amp PCR system 9600 (Perkin-Elmer, Norwalk, CT). The PCR products were separated by PAGE on a 3% gel.

Statistical analysis. Differences between two groups were compared using the two-tailed unpaired Student's t test. P < 0.05 was considered statistically significant.

Results

Down-regulation of p27 levels in MYCN-overexpressing cells leads to centrosome amplification after γ-irradiation. To explore the molecular mechanism of centrosome amplification

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after DNA damage in cells overexpressing MYCN, we examined a neuroblastoma cell line (SHEP) transfected with MYCN (SHEP/MYCN) or empty retroviral vector (SHEP/vector; ref. 27). The parental SHEP cells have neither amplified MYCN nor p53 mutations (32). Centrosome amplification, as indicated by more than two dot signals of γ-tubulin staining under a fluorescence microscope, was frequently seen in SHEP/MYCN cells 48 hours after 10 Gy of γ-irradiation compared with vector-transfected cells (Fig. 1A). Among the proteins involved in regulation of centrosome duplication that we tested, we found that p27 alone increased in SHEP/vector cells after γ-irradiation but did not increase in SHEP/MYCN cells (Fig. 1B). Although higher expression of p53 was seen in SHEP/MYCN cells both before and after γ-irradiation compared with vector-transfected cells, both p21 and KLF4, targets of p53, were present at similar levels in both SHEP/MYCN and control cells. CDK2, cyclin E, and cyclin A also were expressed at similar levels in both cells. To test whether ectopic expression of p27 could suppress centrosome amplification after γ-irradiation in SHEP/MYCN, the cells were also transfected with HA-tagged wild-type (WT) p27 or mutant p27 (W60G: Trp60 → Gly), which is unable to interact with CDK2, cyclin E, and cyclin A (ref. 33; Fig. 1C). After 24 hours, the cells were irradiated. Centrosome amplification at 48 hours after γ-irradiation was significantly suppressed in SHEP/MYCN cells that were also transfected with HA-p27 vector (8.5%) compared with cells transfected with mock (39.9%) or mutant HA-W60G vector (26.7%; Fig. 1D and E). These results suggest that centrosome amplification after γ-irradiation in cells overexpressing MYCN is caused by down-regulation of p27 levels.

Figure 1. Suppression of p27 expression and centrosome amplification after γ-irradiation in MYCN-overexpressing cells. A, SHEP cells transfected with control vector (SHEP/vector) or MYCN (SHEP/MYCN) were immunostained with a γ-tubulin antibody (red) at 48 hours after 10 Gy of γ-irradiation. Cells were also counterstained with Hoechst 33342 (blue). Bar, 5 μm. B, cell lysates from SHEP/vector (left) and SHEP/MYCN cells (right) at each time point after γ-irradiation (γ-IR) were subjected to immunoblot analysis with antibodies to the indicated proteins. α-Tubulin was examined as a loading control. C, vector pcDNA3-HA-p27 or pcDNA3-HA-W60G was transfected into 293 cells for 48 hours. Cells were then lysed and immunoprecipitated with anti-HA antibody. The resulting precipitates were subjected to immunoblot analysis with antibodies to the indicated proteins. Control, total cell lysates from untransfected 293 cells. D, SHEP/MYCN cells were transfected with a vector encoding HA-p27 or HA-W60G for 24 hours. The cells were then irradiated and communostained with anti-γ-tubulin (green) and anti-HA (red) antibodies at 48 hours after γ-irradiation. Cells were also counterstained with Hoechst 33342 (blue). Bar, 5 μm. E, quantitative analysis of centrosome amplification in SHEP/MYCN cells transfected with a vector encoding HA-p27 or HA-W60G at 48 hours after γ-irradiation. The cells were communostained with anti-γ-tubulin and anti-HA antibodies, and the number of spots of γ-tubulin staining in HA-positive cells was counted. More than 200 cells were examined during the analysis. Columns, mean of three independent experiments; bars, SE. *, P < 0.05, compared with mock-transfected cells.
Loss of p27 results in centrosome amplification after DNA damage in MEFs. To clarify whether p27 plays a key role in suppression of centrosome amplification after DNA damage, we examined MEFs derived from WT, p27+/−, and p27−/− mice. p53−/− MEFs were also examined because a defect in p53 is known to cause centrosome amplification after DNA damage (23, 24). Centrosome amplification was frequently observed in p27−/− MEFs 48 hours after γ-irradiation (Fig. 2A). Although centrosome amplification was increased in all MEFs after γ-irradiation, the frequencies of centrosome amplification in the p27−/− (60.2%), p27+/− (68.5%), and p53−/− (73.5%) MEFs were significantly higher than WT MEFs (34.1%) at 48 hours after γ-irradiation (Fig. 2B, left). WT and p27−/− MEFs were also treated with another DNA-damaging agent, doxorubicin, a topoisomerase II inhibitor. The percentage of cells showing centrosome amplification was significantly higher in p27−/− MEFs (64.4%) than WT MEFs (36.6%) after 48 hours of doxorubicin treatment (Fig. 2B, right). Additionally, each dot signal of γ-tubulin staining included two spots of the centriole component centrin 3 in p27−/− MEFs at 48 hours after γ-irradiation, showing that each amplified centrosome had a pair of centrioles (Fig. 2C). Furthermore, fluorescence-activated cell sorting analysis showed that the percentage of p27−/− MEFs with DNA content of >4N to 8N was not significantly increased (5.3-9.7%) from 0 to 48 hours after γ-irradiation compared with WT MEFs (6.4-8.0%; Fig. 2D). Thus, centrosome amplification in p27−/− MEFs after DNA damage was not due to dysregulation of splitting centrioles, cytokinesis failure, or DNA endoreplication. These findings further suggest that p27 plays an
important role in the suppression of centrosome amplification after DNA damage in MEFs.

**Down-regulation of p27 in human cells results in centrosome amplification after γ-irradiation.** To test whether p27 functions to regulate centrosome duplication after DNA damage in human cells, we first designed three p27-specific siRNAs (p27-600, 632, and 910) and examined their effects on p27. The result showed that p27-910 was the most effective in reducing the level of p27 (Fig. 3A). Therefore, we used p27-910 as a p27 siRNA to down-regulate p27, whereas p27-632, for which the effect was equivalent to mock-treated cells, was used as a control siRNA. Normal human fibroblasts (NB1RGB) and SHEP cells were transfected with control or p27 siRNA. The level of p27 was significantly reduced by p27 siRNA in NB1RGB and SHEP cells before and after γ-irradiation, whereas the control siRNA had no effect (Fig. 3B). Consistent with the results obtained in p27−/− MEFs, centrosome amplification was more frequently observed at 48 hours after γ-irradiation in both NB1RGB and SHEP cells transfected with p27 siRNA than in cells with control siRNA (Fig. 3C). For p27 siRNA-transfected NB1RGB and SHEP cells, there was a significant increase in the number of cells with centrosome amplification at 48 hours after γ-irradiation: from 4.4% to 24.5% and from 4.7% to 37.9%, respectively. In contrast, NB1RGB and SHEP cells transfected with control siRNA showed only slight increases, from 4.8% to 10.9% and from 4.3% to 11.3%, respectively (Fig. 3D). Thus, as in mouse cells, p27 plays an important role in suppression of centrosome amplification after DNA damage in human cells.

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**Figure 3.** Centrosome amplification in p27 siRNA-transfected human cells after γ-irradiation. A, 293 cells were transfected with siRNAs (p27-600, 632, and 910). The cells were lysed and subjected to immunoblot analysis with antibodies against p27 and α-tubulin. α-Tubulin was examined as a loading control. B, NB1RGB and SHEP cells were transfected with control or p27 siRNA for 24 hours and then irradiated (10 Gy). Cell lysates at 0 and 48 hours after γ-irradiation (γ-IR) were subjected to immunoblot analysis with anti-p27 and anti-α-tubulin antibodies. C, NB1RGB and SHEP cells transfected with control or p27 siRNA were immunostained with anti-γ-tubulin antibody (green) at 48 hours after γ-irradiation. The cells were also counterstained with Hoechst 33342 (blue). Bar, 5 μm. D, quantitative analysis of centrosome amplification in NB1RGB and SHEP cells transfected with control or p27 siRNA at 0 and 48 hours after γ-irradiation. For the analysis, the number of spots of γ-tubulin staining was counted in ≥200 cells. Columns, mean of three independent experiments; bars, SE. *P < 0.05, compared with control siRNA-transfected cells 48 hours after γ-irradiation.
Down-regulation of p27 leads to multipolar mitosis after γ-irradiation. Cells with amplified centrosomes frequently form multipolar spindles, a mitotic aberration that leads to unequal segregation of chromosomes, resulting in alteration of chromosomal compositions in daughter cells (1). To clarify the effect of amplified centrosomes caused by down-regulation of p27 after DNA damage in mitosis, we examined p27-silenced cells. Interestingly, whereas we found that the number of mitotic cells, as scored by the mitotic marker phospho-histone H3, among the cells transfected with control siRNA fell from 4.0% to 0.1% at 48 hours after γ-irradiation, the number of mitotic cells among cells transfected with p27 siRNA decreased only slightly (4.2–3.6%) after γ-irradiation (Fig. 4A). This result suggests that p27 may be involved in regulating entry into mitosis after DNA damage. Multipolar mitotic cells with amplified centrosomes were frequently observed in cells transfected with p27 siRNA at 48 hours after γ-irradiation (Fig. 4B). Quantitative analysis showed that the fraction of mitotic cells with amplified centrosomes increased substantially (from 1.9% at 0 hours to 89.1% at 48 hours after γ-irradiation) in cells transfected with p27 siRNA (Fig. 4C). Micronuclei and amplified centrosomes are related to radiation-induced mitotic abnormalities (34, 35). Notably, p27-silenced cells with micronuclei were substantially increased (0.4–22.1%) compared with control cells (0.4–1.85%; Fig. 4D and E). These findings suggest that after DNA damage, p27 plays an important role in suppressing mitosis in cells with amplified centrosomes, thereby helping to prevent chromosomal instability.

Accumulation of p27 after DNA damage is independent of the ATM/p53 signaling pathway in normal cells. We found that...
the p27 is up-regulated after γ-irradiation in SHEP/vector and in SHEP and NB1RGB cells transfected with control siRNA (Figs. 1B and 3B). To analyze p27 regulation after DNA damage in detail, NB1RGB cells were irradiated or treated with doxorubicin. We found that p27 levels gradually increased following γ-irradiation as well as doxorubicin treatment in NB1RGB cells and that Skp2 levels gradually declined (Fig. 5A). The KPC1/KPC2 complex showed no change after γ-irradiation or doxorubicin treatment. Levels of p53 and its target, p21, which are known to be up-regulated after DNA damage, increased more rapidly than p27 after γ-irradiation and doxorubicin treatment. Additionally, in contrast to the substantial increase in p21 mRNA levels, we found that p27 mRNA levels in NB1RGB cells showed a slight increase during 24 to 48 hours after γ-irradiation (Fig. 5B). To clarify whether p27 accumulation after DNA damage depends on the p53 and/or ATM-mediated signaling pathway, a well-known DNA damage checkpoint pathway, we examined the expression of p27 in p53−/− MEFs, and NB1RGB cells treated with caffeine, a potent ATM inhibitor. We found that p27 increased equally in both p53−/− MEFs and WT MEFs after γ-irradiation (Fig. 5C). Furthermore, addition of caffeine had no effect on the levels of p27 in γ-irradiated NB1RGB cells, whereas expression of p53 and p21, downstream effectors of ATM, was reduced in γ-irradiated, caffeine-treated cells (Fig. 5D). These findings suggest that, after DNA damage, p27 accumulates due to a reduction of the ubiquitin ligase complex component, Skp2, and to a slight increase in p27 transcription independent of the ATM-p53 signaling pathway.

The interaction between p27 and CDK2/cyclin E or cyclin A is important for regulation of CDK2 activity (18). To clarify whether p27 could bind to CDK2/cyclin E or cyclin A after DNA damage, the lysates prepared from NB1RGB cell before and after γ-irradiation were subjected to immunoprecipitation using anti-p27 antibody.

Figure 5. Molecular analysis of p27 regulation after DNA damage. A, NB1RGB cells were irradiated (10 Gy), treated with doxorubicin (0.1 μg/mL), or serum starved for 48 hours. Serum starvation was done as a control for p27 accumulation. NB1RGB cell lysates at each time point were subjected to immunoblot analysis with antibodies to the indicated proteins. *, nonspecific band. α-Tubulin was examined as a loading control. B, total RNAs isolated from NB1RGB cells at each time point after γ-irradiation (γ-IR) were subjected to semiquantitative RT-PCR using p27- or p21-specific primers. Expression of the gene GAPDH was examined as a loading control. C, cell lysates from WT and p53−/− MEFs at 0 and 30 hours after γ-irradiation were subjected to immunoblot analysis with antibodies to the indicated proteins. *, nonspecific band. D, NB1RGB cells were irradiated and treated with 2 mmol/L caffeine simultaneously. NB1RGB cell lysates at 0 or 24 hours after γ-irradiation were subjected to immunoblot analysis with antibodies to the indicated proteins. E, cell lysates from NB1RGB cells at 0 or 24 hours after γ-irradiation were subjected to immunoprecipitation with anti-p27 antibody, and the resulting precipitates were subjected to immunoblot analysis (right lanes) with antibodies to the indicated proteins. A portion of the input lysates was also subjected to immunoblot analysis with the same antibodies (left lanes).
We found that γ-irradiation did not affect the extent of cyclin E and CDK2 coimmunoprecipitation with p27 (Fig. 5E). The cyclin A level, however, decreased significantly following γ-irradiation; thus, it was not possible to assess its binding to p27. These results suggest that, after DNA damage, higher levels of p27 promote its interaction with CDK2/cyclin E, contributing to the inhibition of CDK2 activity and the concomitant suppression of centrosome amplification.

Increased levels of Skp2 in MYCN-overexpressing cells lead to p27 down-regulation and centrosome amplification after γ-irradiation. Given that p27 down-regulation was observed in MYCN-overexpressing cells (Fig. 1B), we tested whether this down-regulation is due to the ubiquitin-proteasome pathway. We found that, after treatment with a proteasome inhibitor MG132, p27 levels in SHEP/MYCN cells restored to the level similar to SHEP/vector cells (Fig. 6A). Furthermore, higher levels of Skp2 were observed in SHEP/MYCN cells before and after γ-irradiation compared with SHEP/vector cells (Fig. 6B). To prove our hypothesis that increased expression of Skp2 in SHEP/MYCN causes p27 down-regulation and centrosome amplification after DNA damage, we transfected with control or Skp2 siRNA into SHEP/MYCN cells. p27 levels substantially increased in SHEP/MYCN cells transfected with Skp2 siRNA (Fig. 6C). Next, we did immunostaining for γ-tubulin in these cells after DNA damage and found that SHEP/MYCN cells transfected with Skp2 siRNA showed a significant reduction in centrosome amplification at 48 hours after γ-irradiation (12.4%) compared with cells transfected with control siRNA (32.6%; Fig. 6D). These results suggest that Skp2 plays as a mediator of MYCN for p27 down-regulation and centrosome amplification after DNA damage.

Discussion
In this study, we showed that accumulation of p27 after DNA damage is a novel general mechanism to suppress centrosome amplification. p27 is up-regulated under several conditions, including transforming growth factor-β stimulation, contact inhibition, and serum starvation, to arrest the cell cycle at G0-G1 (18). However, to date, p27 regulation after DNA damage has not
been well understood. Our data show that in normal fibroblasts after γ-irradiation or doxorubicin treatment, p27 levels gradually increase and Skp2 levels decline. Additionally, the p27 mRNA level increases slightly after DNA damage. These results suggest that, after DNA damage, reduction of p27 ubiquitination due to the down-regulation of Skp2 and transactivation of p27 synergistically lead to accumulation of p27. Recent reports have suggested that, during the cell cycle, Skp2 is regulated through transactivation by both a GA-binding protein and the ubiquitin-proteasome pathway via the ubiquitin ligase anaphase-promoting complex/cyclosome and its activator Cdh1 (APC/C/Cdh1; refs. 36–38). Therefore, these molecules might be involved in a reduction of Skp2 after DNA damage, resulting in up-regulation of p27. Furthermore, it has been reported that a Forkhead transcription factor, Foxo4, trans-activates p27 in response to oxidative stress that induces DNA damage (39). Thus, Foxo4 may also be involved in transactivation of p27 after γ-irradiation. The ATM-mediated signaling pathway is a well-established cellular response to DNA damage caused by γ-irradiation, which leads to activation of downstream effectors that execute checkpoint responses (22). p53 is one of the effectors in this pathway and transactivates the target genes p21 and KLF4 to suppress centrosome amplification after DNA damage (23–25). However, both p53+/− MEFs and NB1RGB cells treated with an ATM inhibitor revealed that, after γ-irradiation, p27 accumulation was not dependent on p53 or the ATM-mediated signaling pathway. Taken together, these results indicate that DNA damage mediates the induction of p27, and that this acts as a key effector in the suppression of centrosome amplification independently of the ATM/p53 pathway (Fig. 6E).

The CDK2/cyclin E and cyclin A complexes are thought to initiate and promote centrosome duplication (2). The CDK inhibitors p21 and p27 interact with these complexes and thereby inhibit CDK2 activity (18). Our results show that, after DNA damage, p27 physically interacted with CDK2/cyclin E, whereas levels of cyclin A were substantially reduced after DNA damage. Furthermore, the ectopic expression of WT p27 suppressed centrosome amplification, whereas mutant p27 was unable to bind to CDK2/cyclin E and did not suppress centrosome amplification in MYCN-transfected cells after γ-irradiation. Therefore, p27 may play a critical role in direct inhibiting CDK2/cyclin E activity after DNA damage and thereby suppresses centrosome amplification. Our results are supported by a report showing that silencing of Skp2 by siRNA leads to p27 accumulation, resulting in reduction of CDK2 activity and suppression of centrosome amplification in lung cancer cells (40). The other CDK inhibitor, p21, also contributes to suppressing CDK2 activity and centrosome amplification after DNA damage (23, 24). Thus, cooperation of both CDK inhibitors might be important for suppression of centrosome amplification after DNA damage. Recent studies have reported that CDK2 knockout mice unexpectedly display normal cell cycle progression and normal centrosome duplication (41, 42), and that CDK2 is dispensable for cell cycle inhibition by p27 (43, 44). In addition, these reports suggest that CDK1 compensates for the role of CDK2. However, Duensing et al. have argued that CDK2 is at least required for the multiple rounds of centrosome duplication (centrosome amplification; ref. 45). They have shown that a potential CDK2 inhibitor and CDK2 siRNA do not suppress normal centrosome duplication but suppress centrosome amplification associated with expression of human papillomavirus type 16 E7 oncprotein. Consistent with this result, we observed that a number of SHEP/MYCN cells transfected with HA-p27 vector had two centrosomes after DNA damage, suggesting that p27 could suppress centrosome amplification but not normal centrosome duplication (data not shown). Therefore, normal centrosome duplication and aberrant centrosome amplification may have different mechanisms, and given that CDK1 is not required for centrosome amplification by hydroxyurea treatment (10), CDK2 might mainly control centrosome amplification. Thus, after DNA damage, p27 may target CDK2 and thereby suppresses CDK2-controlled centrosome amplification.

In this study, we observed that centrosome amplification after DNA damage was increased to some extent even in WT MEFs and normal human fibroblasts. Although the precise mechanism is unclear, this observation may be consistent with centrosome amplification during G2 arrest by ATM after DNA damage (26). Indeed, the number of WT MEFs with DNA content of 4N (cells in G2-M) increased after DNA damage. However, we showed that, in p27-deficient cells, further centrosome amplification occurred after DNA damage. Additionally, our data show that centrosome amplification in p27-deficient cells after DNA damage is not due to dysregulation of centriole pairing, cytokinesis failure, or DNA endoreplication. These findings suggest that multiple rounds of centrosome duplication during cell cycle arrest might occur in p27-deficient cells after DNA damage. Centrosome amplification is also frequently observed in Skp2+/− cells (46); however, the mechanism in this case likely differs from that of p27+/− MEFS after DNA damage because Skp2−/− cells show centrosome reduplication together with DNA endoreplication (47).

Chromosomal instability is a hallmark of cancer cells and is thought to be partially caused by centrosome amplification. Amplified centrosomes frequently form multipolar spindles during mitosis, resulting in unequal segregation of chromosomes. We observed that, following DNA damage, p27−/− silenced cells having amplified centrosomes entered mitosis and formed multipolar spindles. Furthermore, micronuclei formation was frequently observed in p27−/− silenced cells after γ-irradiation. These results suggest that, following DNA damage, p27 plays an important role not only in suppression of centrosome amplification but also in preventing premature entry into mitosis. A correlation between defects in p27 and tumor development after DNA damage has been suggested by the fact that, after γ-irradiation, p27+/− and p27−/− mice develop multiple tumors in the intestine, lung, ovary, uterus, and adrenal gland (48). This indicates that p27 is haploinsufficient for suppression of DNA damage-induced tumors. In the present study, centrosome amplification in p27+/− and p27+/− MEFS increased significantly after γ-irradiation. This finding raises the possibility that reduced levels of p27 in p27+/− and p27−/− MEFS after DNA damage lead to centrosome amplification and chromosomal instability, resulting in enhanced tumor development in these mice. Indeed, low levels of p27 are frequently observed in a wide range of human tumors in colon, breast, prostate, lung, ovary, and brain and in lymphoma (18). Furthermore, it is reported that Skp2 is overexpressed in human cancers (49). In neuroblastoma cells overexpressing MYCN, we observed down-regulation of p27 through up-regulation of Skp2. Additionally, p27 levels were restored by a proteasome inhibitor. These results are consistent, in part, with a report that MYCN down-regulates p27 levels through a Skp2-dependent ubiquitin-proteasome pathway in neuroblastoma cells (50). Therefore, p27 down-regulation by Skp2 is a key mechanism of centrosome amplification after DNA damage in MYCN-overexpressing neuroblastoma cells (Fig. 6F). We believe that our findings of p27 function in the regulation of centrosome duplication after DNA
damage provide a new insight into tumorigenesis and/or tumor development in cells lacking p27 function.

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References

Suppression of Centrosome Amplification after DNA Damage Depends on p27 Accumulation

Eiji Sugihara, Masayuki Kanai, Soichiro Saito, et al.


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