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ORIGINAL ARTICLE

A p27^{Kip1} mutant that does not inhibit CDK activity promotes centrosome amplification and micronucleation

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Mitotic catastrophe occurs when cells enter mitosis with damaged DNA or excess centrosomes. Cells overexpressing the centrosome protein CP110 or depleted of cyclin F, which targets CP110 for destruction, have more than two centrosomes and undergo mitotic catastrophe. Our studies show centrosome reduplication and mitotic catastrophe in osteosarcoma cells inducibly expressing a p27^{Kip1} mutant (termed p27K) that binds cyclins but not cyclin-dependent kinases (CDKs). p27K inhibited cell proliferation but not CDK activity or cell cycle progression. It did not induce apoptosis; however, cells expressing p27K had more than two centrosomes and, indicative of mitotic catastrophe, irregularly shaped nuclei or multiple micronuclei. p27K interacted with cyclin F *in vivo* (as did endogenous p27^{Kip1}) and displaced cyclin F from CP110. Depletion of CP110 rescued p27K-expressing cells from centrosome reduplication and mitotic catastrophe. Collectively, our data show that p27^{Kip1} can perturb mitosis and suggest that it does so by sequestering cyclin F, which prevents its interaction with and the subsequent degradation of CP110, ultimately resulting in centrosome reduplication, mitotic catastrophe and abrogation of cell proliferation.
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Introduction

The canonical function of p27^{Kip1} is inhibition of cyclin-dependent kinase (CDK) activity. Its targets are CDK4, CDK6, CDK2 and CDK1. All are nuclear serine-threonine kinases; all orchestrate the passage of cells through the cell cycle. CDK4 and CDK6 (with cyclin D), and CDK2 (with cyclin E) promote S phase entry; CDK2 (with cyclin A) and CDK1 (with cyclin B) propel cells through S/G2 and into mitosis, respectively (Toyoshima and Hunter, 1994; Morgan, 1995). p27^{Kip1} interacts with both cyclins and CDKs; the cyclin-binding site and the CDK-binding site are in close proximity in the N terminus of p27^{Kip1} (Russo *et al*., 1996).

When CDKs are inactive, cells typically arrest in G0/G1. CDKs become inactive when p27^{Kip1} accumulates in cells in response to growth inhibitory signals (for example, mitogen deprivation and cell-cell contact). When conditions become favorable for growth, p27^{Kip1} degrades, and CDK-escorted cell cycle progression resumes. Two ubiquitin E3 ligase systems target p27^{Kip1} for destruction in the proteasome: KPC (p27^{Kip1} ubiquitination–promoting complex) in G0/G1 and SCF^{Skp2} (SCF: Skp1-Cul1-F box) in S and G2 (Carrano *et al*., 1999; Tsvetkov *et al*., 1999; Malek *et al*., 2001; Kamura *et al*., 2004). Other events contributing to CDK activation include transcriptional repression, translational inhibition and cytoplasmic sequestration of p27^{Kip1} (Agrawal *et al*., 1996; Hengst and Reed, 1996; Viglietto *et al*., 2002; Bagui *et al*., 2009; Khattar and Kumar, 2010).

p27^{Kip1} also has CDK-independent functions. Most notable is the modulation of cell migration. Cytoplasmic p27^{Kip1} binds RhoA and stathmin via its C terminus: interaction with the GTPase RhoA enhances motility; interaction with the microtubule-destabilizing protein stathmin inhibits motility (Nagahara *et al*., 1998; Besson *et al*., 2004; Baldassarre *et al*., 2005; Wu *et al*., 2006). p27^{Kip1} also stabilizes the neuronal differentiation-promoting protein neurogenin by a mechanism that requires its N terminus but not its cyclin-CDK binding domain (Nguyen *et al*., 2006), and prevents premature DNA replication by sequestering the replication licensing factor MCM7 (Nallamshetty *et al*., 2005). Also of interest are data showing amplification of retinal and bronchioalveolar stem cell populations in knock-in mice expressing a p27^{Kip1} mutant (p27CK) that does not bind cyclins or CDKs: amplification in these mice was greater than in p27^{Kip1}-null mice (Besson *et al*., 2007).

The multifunctionality of p27^{Kip1} presumably explains its paradoxical actions in tumorigenesis. On one hand, p27^{Kip1} is a tumor suppressor: p27^{Kip1}-null mice spontaneously develop pituitary adenomas (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996), and p27^{Kip1} deficiency correlates with poor prognosis (Catzavelos *et al*., 1997; Loda *et al*., 1997; Slingerland and Pagano, 2000). On the other hand, it is an oncogene: p27$^{+/-}$ mice form tumors more readily in cancer-promoting backgrounds than do p27$^{-/-}$ mice (Muraoka *et al*., 2002; Gao *et al*., 2004), and tumors in p27CK

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knock-in mice arise in multiple tissues and not just in the pituitary (Besson *et al*., 2007). $p27^{Kip1}$ is underexpressed in many tumors, typically due to accelerated degradation, but $p27^{Kip1}$ gene deletions or mutations are rare (Larrea *et al*., 2009).

To uncover additional functions of $p27^{Kip1}$, we asked whether $p27^{Kip1}$ mutants that do not bind cyclins, CDKs or both affect phenotype, and if so, how they do so. We show that a $p27^{Kip1}$ mutant (p27K) that binds cyclins but not CDKs inhibits cell proliferation, but not CDK activity or cell cycle progression. Cells expressing this mutant were multinucleated, indicative of mitotic catastrophe, and contained more than two centrosomes. We identify cyclin F (an atypical cyclin that does not bind CDKs) as a $p27^{Kip1}$-binding partner and propose a mechanism in which sequestration of cyclin F by $p27^{Kip1}$ promotes centrosome reduplication.

Results

p27K and p27CK do not inhibit CDK activity

We prepared human osteosarcoma (U2OS) cell lines that express hemagglutinin (HA)-tagged wild-type (p27WT) or mutant (p27C, p27K and p27CK) $p27^{Kip1}$ in the presence of doxycycline (Dox). p27C does not bind cyclins, p27K does not bind CDKs, and p27CK does not bind cyclins or CDKs. p27C and p27K associate with cyclin/CDK complexes (via their CDK- and cyclin-binding sites, respectively); p27CK does not. These properties were determined by Vlach *et al*. (Vlach *et al*., 1997) using recombinant proteins.

The diagram in Figure 1a shows the locations of the point mutations in p27C, p27K and p27CK. Data in the top panels of Figures 1b, d and e show Dox induction of p27WT, p27C, p27K and p27CK in our cell lines. Amounts of p27K and p27CK were similar to or greater than amounts of p27WT and p27C, due at least in part to enhanced stability (data not shown) (Besson *et al*., 2006). p27C and p27CK migrated more slowly than did p27WT and p27K, most likely because of post-translational modifications. We note that U2OS cells express relatively little endogenous $p27^{Kip1}$ compared with other tumor cell lines.

We used two methods to monitor CDK activity in control and Dox-treated cells: western blotting of cell extracts with an antibody to Rb (retinoblastoma protein) phosphorylated at a CDK4/6-specific site (serine 780) (Kitagawa *et al*., 1996) and *in vitro* kinase assay of cyclin E, cyclin A, cyclin B1 and HA-$p27^{Kip1}$ immunoprecipitates using histone H1 as substrate.

p27C inhibited CDK4/6 activity (Figure 1b) and cyclin E-associated activity (Figure 1c) as efficiently as did p27WT. It apparently did so by directly inactivating CDKs. First, amounts of cyclins D1 and E, and CDKs 4 and 2 were similar in Dox-treated p27C and p27WT cells (Figure 1d, left panels). Thus, the loss of activity does not reflect loss of protein. Second, p27C bound cyclin-associated CDKs: anti-HA antibody coprecipitated both CDK4 and cyclin D1, and both CDK2 and cyclin E from p27C-expressing cells (Figure 1d, right panels). Binding of p27C to cyclin-free CDKs would be inconsequential because cyclin-free CDKs are inactive. p27C also effectively suppressed cyclin A- and cyclin B1-associated activity. Whether inhibition is direct or results from reduced expression of cyclins A and B1 (Figure 1d, left panels) cannot be ascertained from this experiment. Indicative of lack of associated CDK activity, anti-HA immunoprecipitates of p27WT- and p27C-expressing cells did not phosphorylate histone H1 in *in vitro* kinase assays (Figure 1e).

p27K did not inhibit CDK4/6 or cyclin E-, A- or B1-associated activity (Figures 1b and c). Anti-HA antibody did not coprecipitate cyclin D1 or CDK4 from p27K-expressing cells (Figure 1d, right panels); thus, p27K does not bind (and therefore cannot inactivate) cyclin D1/CDK4 complexes. Anti-HA antibody did, however, coprecipitate cyclins E, A and B1 and their cyclin partners (CDK2 and CDK1) from p27K-expressing cells; thus, p27K binds these complexes in a non-inhibitory manner. Consistent with this premise, anti-HA immunoprecipitates of p27K-expressing cells contained histone H1-phosphorylating activity, presumably a combination of CDK2 and CDK1 activity (Figure 1e). We note that p27K interacted with cyclin D1 and cyclin D1/CDK4 complexes *in vitro* (Vlach *et al*., 1997). Why it did not do so *in vivo* is unclear.

p27CK did not bind cyclins (with the exception noted below) or CDKs (Figure 1d, right panels) or inhibit CDK activity (Figures 1b and c), and anti-HA immunoprecipitates of p27CK-expressing cells lacked histone H1-phosphorylating activity (Figure 1e). For unclear reasons, p27CK interacted with cyclin E to a limited extent. In summary, our data show that p27WT and p27C inhibit CDK activity, whereas p27K and p27CK do not.

p27K inhibits cell doubling but not cell cycle progression

Consistent with CDK inactivation, p27WT and p27C blocked cell cycle progression and cell proliferation. The percentage of S phase cells in Dox-treated populations was $<10\%$ as compared with $>40\%$ in untreated populations (Figure 2a). Dox-treated p27WT and p27C cells accumulated in G0/G1 (Figure 2b). This finding dovetails with the limited expression of cyclin A and cyclin B1 in these cells: cyclins A and B are expressed in S/G2 and mitosis, respectively. Dox-treated p27WT and p27C cells did not double in number over a 3-day period, whereas untreated cells doubled approximately three times (Figure 2c).

p27K and p27CK did not inhibit cell cycle progression. Percentages of cells incorporating bromodeoxyuridine (BrdU) were similar in the presence and absence of Dox (Figure 2a). Dox-treated p27K and p27CK cells were distributed throughout the cell cycle, as were untreated cells (Figure 2b). They were not static (that is, blocked at multiple points in the cell cycle): BrdU-tagged cells progressed from S to G2/M to G1 (Figure 2d). As expected, p27CK cells proliferated in Dox-containing medium; p27K cells (clones 2 and 8), however, did not (Figure 2c and data not shown). Thus, although they exhibit CDK activity and are cycling, cells expressing p27K do not increase appreciably in number.

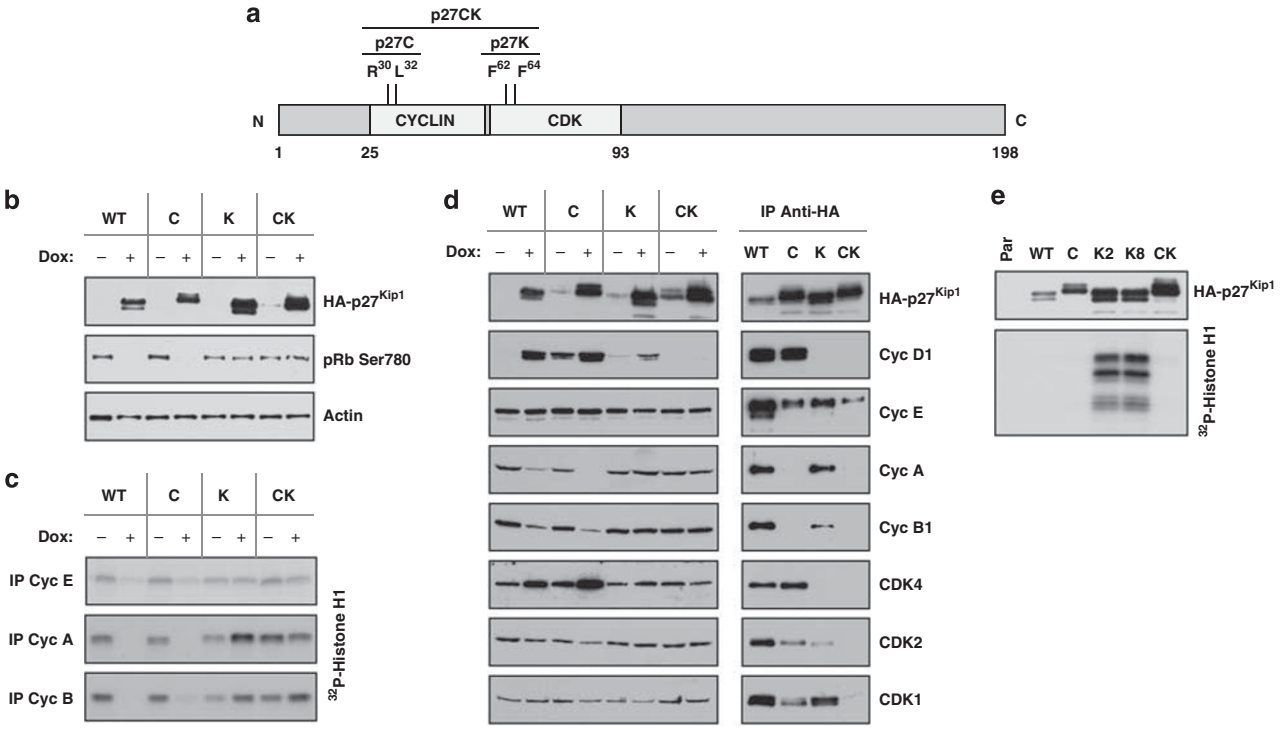

Figure 1 Interaction of p27^{Kip1} mutants with cyclins and CDKs and effects on CDK activity. (**a**) Diagram showing the sites mutated in the cyclin- and CDK-binding domains of p27^{Kip1}. Arginine 30, leucine 32, and phenylalanines 62 and 64 were replaced with alanine. (**b**–**d**) p27WT, p27C, p27K and p27CK cells received 1 μg/ml Dox for 24 h. (**b**) Cell extracts were immunoblotted with antibody to Rb phosphorylated at serine 780, a CDK4/6-specific site. Actin is shown as a loading control. (**c**) Cell extracts were immunoprecipitated with antibody to cyclin E, cyclin A or cyclin B1. Immunoprecipitates were assayed for kinase activity using histone H1 as substrate. (**d**) Cell extracts were immunoblotted with antibodies to the indicated proteins (left panels). Anti-HA immunoprecipitates were immunoblotted with the indicated antibodies (right panels). The results shown in panels (**c**) and (**d**) are from the same experiment. (**e**) Parental (Par), p27WT, p27C, p27K and p27CK cells received Dox for 24 h. Anti-HA immunoprecipitates were immunoblotted with antibody to HA (top panel) or assayed for kinase activity using histone H1 as substrate (bottom panel). Two p27K clones (K2 and K8) were examined.

p27K induces centrosome reduplication and mitotic catastrophe

The paradoxical behavior of p27K-expressing cells—cell cycling in the absence of cell doubling—may be explained by a balance between cell division and cell death. To determine whether p27K induces apoptotic cell death, cells were incubated with annexin V, which detects externalized phosphatidylserines; phosphatidylserines flip from the inside to the outside of the plasma membrane in apoptotic cells (Fadok *et al*., 1992; Koopman *et al*., 1994). Effects of p27K on apoptosis were modest at best and were not unique: p27K increased the percentage of annexin-positive cells from approximately 2 to 12%, as did p27WT and p27C (although not p27CK) (Figure 3a). We suggest that apoptosis does not account or accounts only in part for the impaired proliferation of p27K-expressing cells.

Rather than apoptosis, p27K-expressing cells may undergo mitotic catastrophe, a form of cell death that occurs during or after aberrant mitosis (Castedo *et al*., 2004; Portugal *et al*., 2010). We observed striking morphological changes indicative of mitotic catastrophe in two clones of Dox-treated p27K cells co-stained with antibodies to p27^{Kip1} and α-tubulin (Figure 3b, top panel) or γ-tubulin (middle panel): approximately 20% of cells had irregularly shaped nuclei or micronuclei (clone 2) or completely fragmented nuclei (clone 8). Nuclei of Dox-treated p27WT, p27C and p27CK cells were normal in shape and number. All four forms of HA-tagged p27^{Kip1} were predominantly nuclear. We note that anti-p27^{Kip1} antibody did not detect endogenous p27^{Kip1} in parental cells under the conditions used (Figure 3b, middle panel); thus, endogenous p27^{Kip1} does not contribute to the immunostaining seen in cells expressing ectopic p27^{Kip1}.

One cause of mitotic catastrophe is centrosome reduplication, which leads to multipolar spindle formation and chromosome missegregation (Dodson *et al*., 2007). Approximately 20% of Dox-treated p27K cells had more than two centrosomes, as determined by staining of cells with antibody to γ-tubulin, a centrosomal marker (Figure 3b, middle and bottom panels, 3c). The photographs in Figure 3b (bottom panels) show the presence of clumps of centrosomes in two clones of p27K cells. Less than 3% of Dox-treated parental, p27WT, p27C and p27CK cells had more than two centrosomes. These findings suggest that p27K induces centrosome reduplication, which results in mitotic catastrophe.

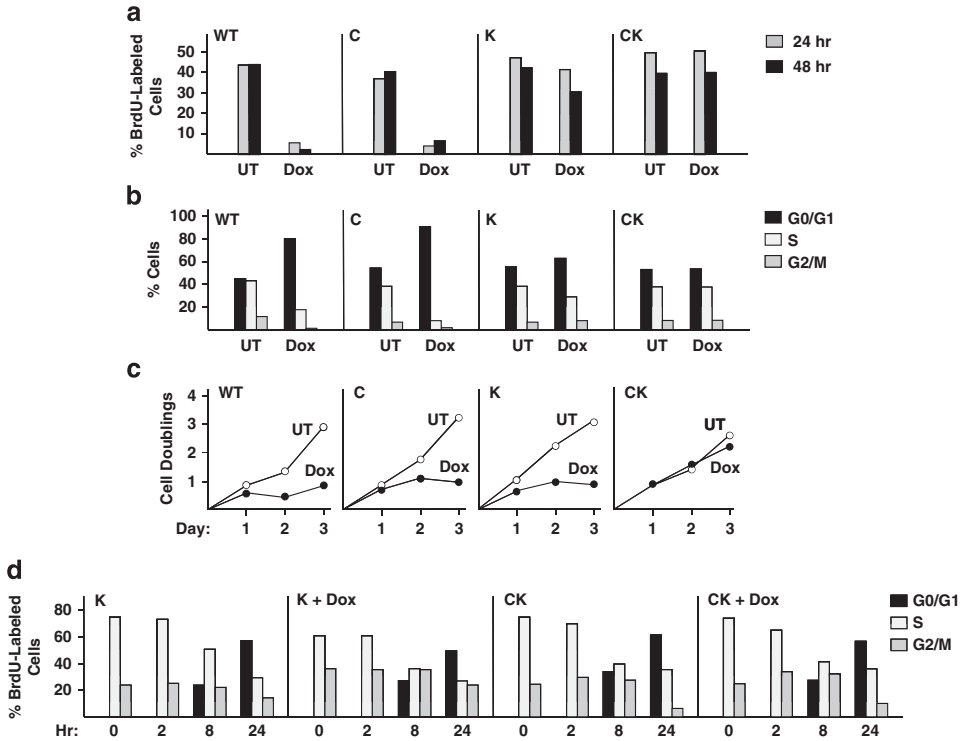

Figure 2 Effects of the p27Kip1 mutants on cell cycle progression and proliferation. (**a**) p27WT, p27C, p27K and p27CK cells received Dox for 24 or 48 h, or were left untreated (UT). BrdU (20 μM) was added to cells 1.5 h before harvest. Percent BrdU-labeled cells was determined by flow cytometry. (**b**) Cells received Dox for 48 h. Cell cycle position was determined by flow cytometry of propidium iodide-stained cells. (**c**) Cells received Dox 24 h after seeding. Cell number was determined 1, 2 and 3 days after Dox addition by hemacytometer counting. (**d**) p27K and p27CK cells received Dox for 48 h. Cells were pulsed with BrdU for 1.5 h and refed with medium containing 100 μM deoxythymidine. Cells were harvested 2, 8 and 24 h after BrdU removal for determination of percent BrdU-labeled cells.

p27K interacts with cyclin F

p27K may perturb centrosome duplication by interacting with other proteins. To address this possibility, we identified proteins present in anti-HA immunoprecipitates of Dox-treated p27K cells by mass spectrometry. Such proteins included known p27Kip1-binding partners (for example, cyclins E, A and B). They also included cyclin F, whose association with p27Kip1 has not been reported previously. Cyclin F is an atypical cyclin that lacks a CDK partner (Bai *et al*., 1994, 1996). It mediates protein degradation rather than CDK activation; it is an F-box protein and a component of SCF ubiquitin ligase complexes (Winston *et al*., 1999).

Amounts of cyclin F were similar in p27K and p2CK cells in the presence and absence of Dox (Figure 4a). Cyclin F was less abundant in Dox-treated than in untreated p27WT and p27C cells, presumably because of G0/G1 arrest of Dox-treated cells: previous studies have shown that cyclin F is not appreciably expressed in G0/G1 (Bai *et al*., 1994). Anti-HA antibody coprecipitated cyclin F from cells expressing p27WT or p27K but not from cells expressing p27C or p27CK, which lack intact cyclin-binding sites (Figures 4b and c). Consistent with amounts of total cyclin F (Figure 4a), amounts of coprecipitated cyclin F (Figure 4c) were lower in p27WT-expressing cells than in p27K-expressing cells. These data validate the results of the mass spectrometry analysis and indicate that p27Kip1 associates with cyclin F via its cyclin-binding site.

We were unable to detect the interaction of endogenous p27Kip1 with endogenous cyclin F in U2OS cells, presumably because of negligible amounts of p27Kip1 in these cells. To enhance detection, we used human embryonic kidney (HEK) 293T cells, which express more p27Kip1 than do U2OS cells, and we depleted them of Skp2, an F box protein that targets p27Kip1 for destruction in the proteasome (Carrano *et al*., 1999; Tsvetkov *et al*., 1999). Cells receiving Skp2 short interfering RNA (siRNA) expressed much less Skp2 and much more p27Kip1 than did cells receiving non-targeting siRNA (Figure 4d). Antibody to p27Kip1 coprecipitated cyclin F from Skp2-depleted cells and, to a lesser extent, from mock-depleted cells. Preimmune serum did not appreciably precipitate p27Kip1 or cyclin F. Thus, endogenous cyclin F associates with endogenous p27Kip1 *in vivo*. Of interest are studies showing that Skp2-null cells contain more than two centrosomes (Nakayama *et al*., 2000), as do p27K cells.

p27K disrupts the interaction of cyclin F with CP110

Cyclin F associates with the centrosomal protein CP110 in G2/M and promotes its proteasome-mediated destruction (Chen *et al*., 2002; D'Angiolella *et al*., 2010). Cells overexpressing a non-degradable CP110 mutant have

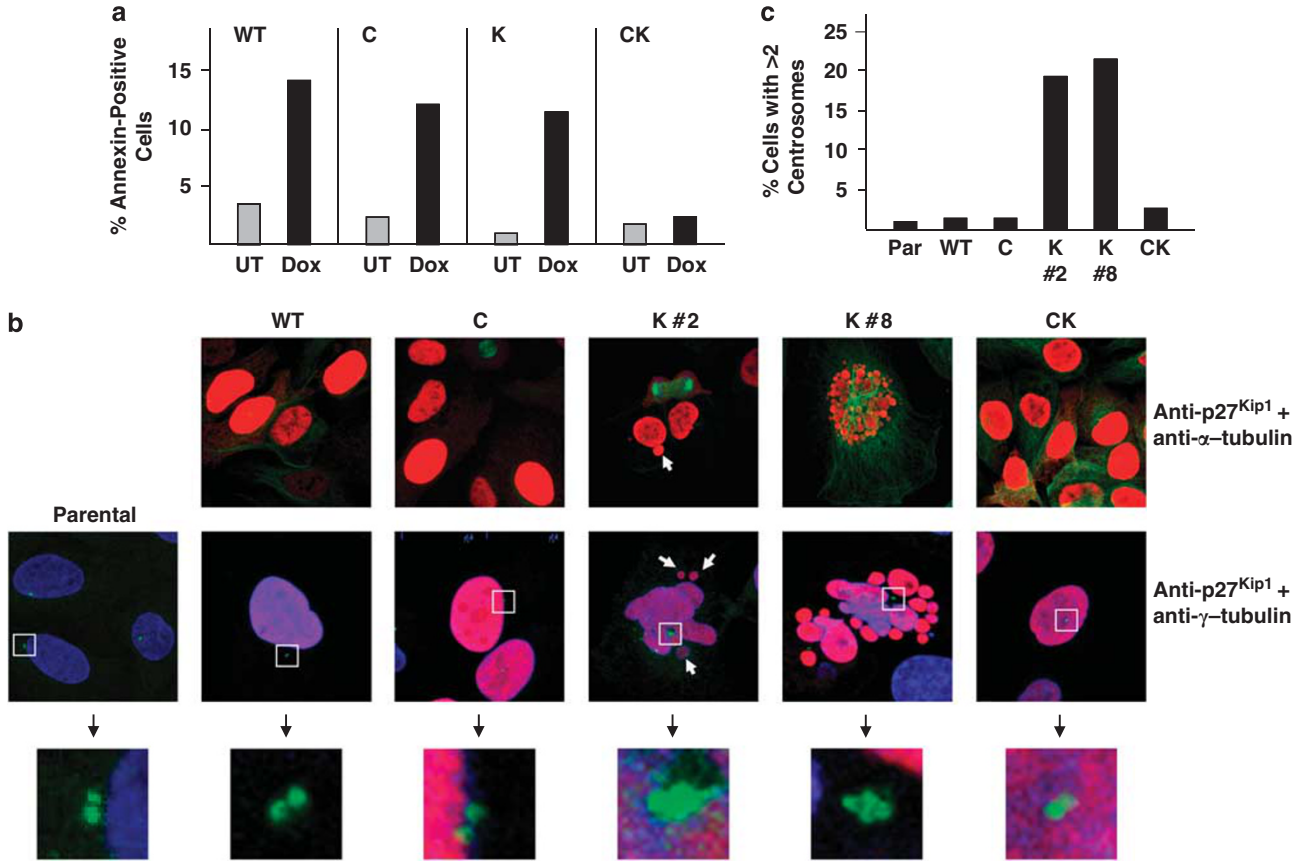

Figure 3 p27K induces mitotic catastrophe and centrosome reduplication. (**a**) p27WT, p27C, p27K and p27CK cells received Dox for 72 h or were left untreated (UT). Percent annexin-positive cells was determined by flow cytometry. (**b**) Top panels: p27WT, p27C, p27K and p27CK cells received Dox for 60 h. Cells were immunostained with antibodies to $p27^{Kip1}$ (red) and α-tubulin (green). Middle and bottom panels: Parental (Par), p27WT, p27C, p27K and p27CK cells received Dox for 30 h. Cells were immunostained with antibodies to $p27^{Kip1}$ (red) and γ-tubulin (green). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (blue). Micrographs were taken with a Leica TCS SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Germany) through a 100 × /1.40 NA Plan Apochromat oil immersion objective lens. Areas in the boxes are enlarged below. White arrows denote micronuclei. (**c**) Centrosome numbers per cell were determined. Middle panels of (**b**) and data in (**c**) are from the same experiment. At least 200 cells per sample were examined with an Axio Imager Z1, Carl Zeiss upright fluorescent microscope at 63 × /1.40 NA oil objective lens. Binucleated cells were not counted.

more than two centrosomes, as do cells depleted of cyclin F, and contain multiple micronuclei (D'Angiolella *et al*., 2010). $p27^{Kip1}$ and CP110 interact with their respective cyclins via their RXL motifs (Schulman *et al*., 1998; D'Angiolella *et al*., 2010); thus, p27K may promote centrosome reduplication (and consequent micronucleation) by occupying CP110-binding sites on cyclin F.

In support of this hypothesis, we show that CP110 does not efficiently associate with cyclin F in the presence of p27WT or p27K. In this experiment, HEK 293T cells were co-transfected with Flag-HA-cyclin F, and either p27WT, p27K, p27CK or empty vector. Anti-Flag antibody coprecipitated equal amounts of ectopic cyclin F from all four populations; however, it coprecipitated much less CP110 from cells expressing p27WT or p27K than from cells expressing p27CK or empty vector (Figure 5a, western blots). Similar to results for endogenous cyclin F, ectopic cyclin F interacted with p27WT and p27K but not p27CK. Suggestive of abortive degradation, CP110 was reproducibly more abundant in cells expressing p27WT or p27K than in cells expressing p27CK or empty vector. HEK 293T cells are highly transformed and continue to cycle when supplied with p27WT: the bar graphs in Figure 5b show similar cell cycle distribution profiles for p27WT, p27CK and vector-only populations. Thus, antagonistic effects of p27WT on cyclin F/CP110 interaction are not due to cell cycle arrest.

p27K requires CP110 to effectively elicit centrosome reduplication and mitotic catastrophe

If CP110 mediates the effects of p27K on centrosome reduplication and micronucleation, its removal from cells should rescue p27K-expressing cells. To test this premise, parental and p27K cells (clones 2 and 8) were transfected with CP110 siRNA or non-targeting siRNA, and subsequently exposed to Dox. Western blots show near elimination of CP110 from cells receiving CP110 siRNA and confirm expression of p27K in Dox-treated p27K cells (Figure 6a). Most parental cells had one or two centrosomes (Figure 6b) and normal mitotic figures (Figure 6c) in the presence or absence of CP110 siRNA. p27K-expressing cells (18–33%) receiving non-targeting siRNA had more than two centrosomes and abnormal

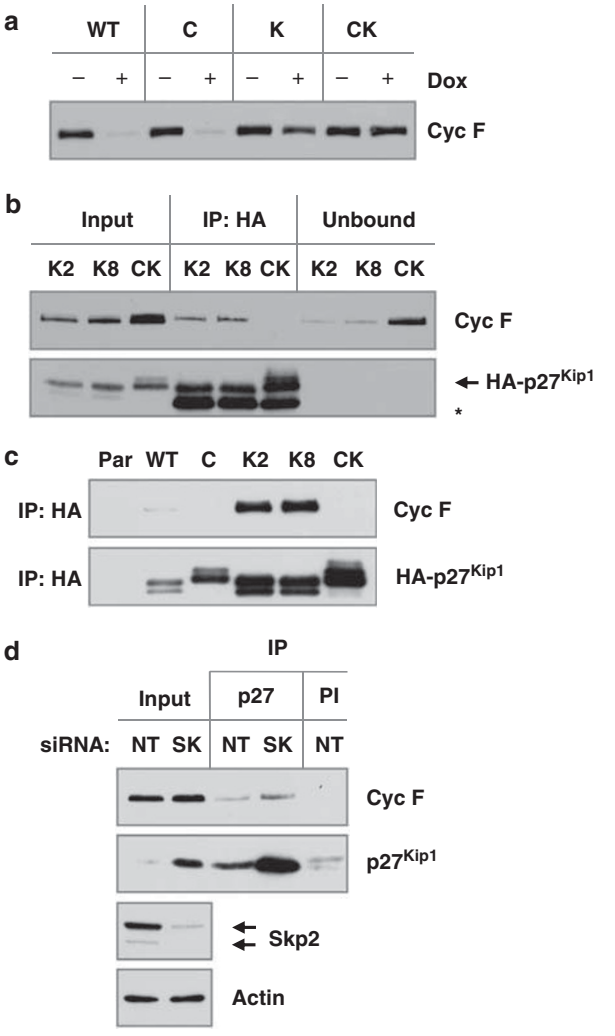

Figure 4 p27Kip1 Interacts with cyclin F. (**a**) Cells received Dox for 24 h. Cell extracts were immunoblotted with antibody to cyclin F. (**b**) p27K (clones 2 and 8) and p27CK cells received Dox for 24 h. Anti-HA immunoprecipitates were immunoblotted with antibody to cyclin F or HA. Input was 0.6% of total protein used for immunoprecipitation. Asterisk denotes non-specific band. (**c**) Parental (Par), p27WT, p27C, p27K and p27CK cells received Dox for 24 h. Anti-HA immunoprecipitates were immunoblotted with antibody to cyclin F. (**d**) Skp2 siRNA (SK) or non-targeting (scrambled) siRNA (NT) (40 nM, Thermo Scientific Dharmacon, Lafayette, CO, USA) was introduced into HEK 293T cells using Dharmafect I (Dharmacon). Cells were harvested 48 h after transfection. Cell extracts were incubated with preimmune serum (PI) or antibody to p27Kip1, and precipitated material was immunoblotted with antibody to cyclin F, Skp2 or p27Kip1. Input was 0.4% of total protein used for immunoprecipitation. Actin is shown as a loading control.

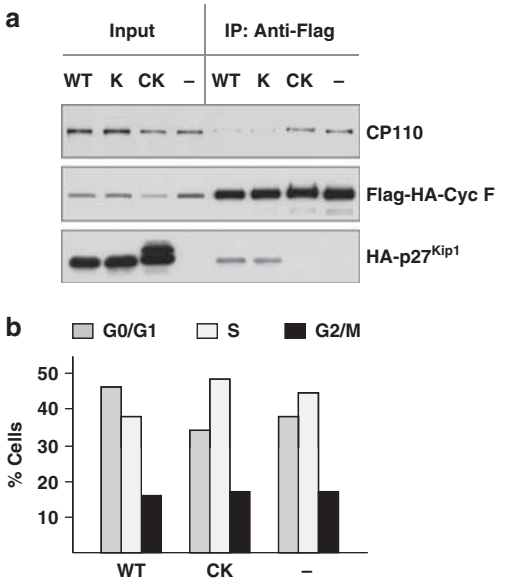

Figure 5 p27K blocks the association of cyclin F with CP110. (**a**, **b**) HEK 293T cells were transfected with Flag-HA-cyclin F and either p27WT, p27K, p27CK or empty vector (pcDNA3, denoted by minus sign) using Fugene HD (Roche, Mannheim, Germany). Cells were harvested 48 h after transfection. (**a**) Anti-Flag immunoprecipitates were immunoblotted with antibody to CP110 or HA. Input was 0.75% of total protein used for immunoprecipitation. (**b**) Cell cycle position was determined by flow cytometry of propidium-stained cells.

mitotic figures. Indicative of CP110 dependence, these numbers declined in p27K-expressing cells receiving CP110 siRNA. Rescue from mitotic catastrophe by CP110 depletion was nearly complete and statistically significant; rescue from centrosome reduplication was partial but statistically significant (clone 2) or suggestive (clone 8). This distinction suggests that additional p27K-induced events may contribute to mitotic catastrophe. On the other hand, we note that centrosomes in CP110-depleted cells (both parental and p27K-expressing) had abnormal γ-tubulin-staining fibrous structures (data not shown), presumably centrioles, which confounded the counting. Microtubular extensions of distal ends of centrioles in CP110-depleted cells have been described previously (Schmidt *et al*., 2009; Korzeniewski *et al*., 2010).

Discussion

Our studies uncover a novel function of p27Kip1 (interaction with cyclin F) and novel events associated with inappropriate expression of p27Kip1 (centrosome amplification and mitotic catastrophe). p27K, a p27Kip1 mutant with a defective CDK-binding site, did not inhibit CDK activity or cell cycle progression or induce apoptosis when inducibly expressed in U2OS cells. Cells expressing p27K, however, had more than two centrosomes and characteristics of mitotic catastrophe (irregularly shaped nuclei, micronucleation and nuclear fragmentation). Mitotic catastrophe can lead to non-apoptotic cell death (Castedo *et al*., 2004; Portugal *et al*., 2010) and thus apparently accounts for the failure of p27K-expressing cells to increase in number.

We suggest that p27K triggers centrosome reduplication and consequent mitotic catastrophe by antagonizing the cyclin F-mediated degradation of the centrosome component CP110. First, p27K bound cyclin F *in vivo*.

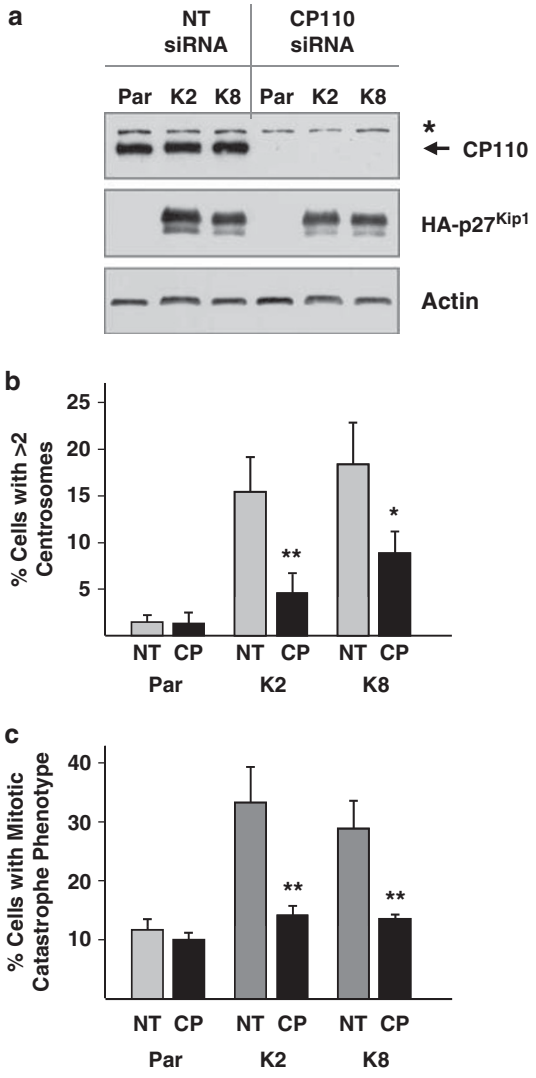

Figure 6 CP110 depletion suppresses p27K effects on centrosome reduplication and micronucleation. (**a**) Parental (Par) and p27K (clones 2 and 8) cells were transfected with 40 nM non-targeting (NT) or CP110 (CP) siRNA using Dharmafect 1. Cells received Dox for 32–36 h after transfection. Cell extracts were immunoblotted with antibody to CP110, HA or actin (loading control). (**b**, **c**) Cells on coverslips were transfected with siRNAs and treated with Dox as in panel (**a**) and stained with DAPI and immunostained with antibody to γ-tubulin. Number of cells with more than two centrosomes (**b**) or with a phenotype indicative of mitotic catastrophe (**c**) was determined. Three independent experiments were performed, and at least 100 cells were examined per sample with an Axio Imager Z1, Carl Zeiss upright fluorescent microscope at 63× magnification. Error bars indicate standard deviation. Statistical significance was evaluated with a two-tailed, unpaired Student's *t* test (assuming unequal variances) using Microsoft's Excel 2003 software. *P* values <0.05 (denoted by double asterisks) are considered significant; *P* values between 0.05 and 0.1 (denoted by single asterisk) are considered suggestive. *P* values for the data in panel (**b**) are: 0.845 (parental), 0.02 (clone 2) and 0.046 (clone 8). *P* values for the data in panel (**c**) are: 0.378 (parental), 0.033 (clone 2) and 0.032 (clone 8).

Second, p27K displaced CP110 from cyclin F and increased CP110 abundance. Third, p27K did not effectively induce centrosome reduplication or mitotic catastrophe in cells depleted of CP110. Others have shown aborted CP110 degradation, centrosome reduplication and mitotic catastrophe in cells lacking cyclin F (D'Angiolella *et al*., 2010). p27WT also interacted with cyclin F, as did endogenous $p27^{Kip1}$, and disrupted cyclin F/CP110 complexes.

Of note are studies showing centrosome amplification and micronucleation in conditions known to increase $p27^{Kip1}$ abundance: knockout of the ubiquitin ligase components Skp2, Cul4 and DDB1, and knockdown of the transcription factor FoxM1 (Nakayama *et al*., 2000; Wang *et al*., 2005; Wonsey and Follettie, 2005). Importantly, centrosome amplification and micronucleation in Skp2-null cells required $p27^{Kip1}$; these abnormalities were not seen in cells lacking both Skp2 and $p27^{Kip1}$ (Nakayama *et al*., 2004). Thus, both p27K and $p27^{Kip1}$ can elicit these events.

Wild-type $p27^{Kip1}$ inhibits CDK activity, and previous reports suggest that centrosomes cannot re-replicate in the absence of CDK2 activity. As examples, the HPV E7 oncoprotein induced centrosome reduplication in wild-type but not CDK2-null MEFs (Duensing *et al*., 2006), and hydroxyurea induced centrosome reduplication in CHO cells in the absence but not presence of dominant-negative CDK2 (Meraldi *et al*., 1999). Overexpression of $p27^{Kip1}$ also prevented reduplication in several systems (Hinchcliffe *et al*., 1999; Meraldi *et al*., 1999; Sugihara *et al*., 2006). Thus, the reduplication-inducing activity of $p27^{Kip1}$ requires its uncoupling from its CDK2 inhibitory activity. p27K did not inhibit CDK activity (or cell cycle progression) nor did silencing of Skp2, despite consequent accumulation of $p27^{Kip1}$ (Nakayama *et al*., 2000). These situations, therefore, unmasked the deleterious effects of $p27^{Kip1}$ on centrosome duplication.

Induction of mitotic catastrophe suppresses tumorigenesis. Indeed, it is the primary means by which radiation kills tumor cells (Dodson *et al*., 2007). On the other hand, if cells with more than two centrosomes escape mitotic catastrophe, they may become aneuploid, a hallmark of genetic instability and cancer. Cells with tripolar spindles, for example, divide and are aneuploid and viable (Fukasawa, 2008). Aneuploidy can also result from centrosomes that nucleate but fail to position on a bipolar axis during centrosome clustering. We note that morphology compatible with mitotic catastrophe has been observed in pleomorphic giant cell carcinomas, which are highly aggressive malignant tumors (Caruso *et al*., 2011). Thus, the contribution of $p27^{Kip1}$-mediated centrosome reduplication and mitotic catastrophe to tumorigenesis requires further investigation.

Cyclin F may have functions in addition to implementing CP110 degradation (Kong *et al*., 2000), and $p27^{Kip1}$ may affect these processes as well. The reverse may also be true; cyclin F may titrate $p27^{Kip1}$. This would conceivably promote proliferation, and in this regard it is interesting that cyclin F-null mouse embryo fibroblasts enter S phase with delayed kinetics (Tetzlaff *et al*., 2004) and that lung carcinoma cells overexpress cyclin F mRNA (Singhal *et al*., 2003).

Materials and methods

Generation of stable cell lines
The human wild-type $p27^{Kip1}$ coding sequence in pcDNA was fused at its C terminus to a sequence encoding an HA epitope. The tagged sequence was cloned into pTRE2_Hyg (Clontech, Mountain View, CA, USA) and used as a template for site-directed mutagenesis to generate p27C, p27K and p27CK. Mutations were verified by DNA sequencing. Constructs were introduced into U2OS Tet ON cells (Clontech) using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Hygromycin-resistant clones were selected (200 μg/ml hygromycin for 3 weeks), expanded and screened for expression of HA-tagged proteins using anti-HA antibody. Clones showing 50-fold more tagged protein in the presence than in the absence of Dox (1 μg/ml) were chosen for further analysis.

Immunoprecipitations and immunoblotting
Cells were lysed for 45 min in immunoprecipitation buffer (50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.2, 150 mM NaCl, 0.7–1.0% nonyl phenoxypolyethoxylethanol-40 (NP-40), 1 mM ethylene diamine tetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1:100 dilution of protease inhibitor cocktail set III, 1:100 dilution of phosphatase inhibitor cocktail set I, 1 mM orthovanadate, 1 mM NaF, 0.5 mM dithiothreitol). Cleared lysates were incubated with antibody/antiserum or anti-HA- or anti-Flag-agarose beads (Sigma-Aldrich, St Louis, MO, USA) for 5–16 h and subsequently with protein A- or protein G-agarose beads (Invitrogen) for 2 h. Immune complexes were washed 3 × with immunoprecipitation buffer and applied to 10% SDS–polyacrylamide gels. Immunoblotting was performed as described previously (Bagui *et al*., 2000).

In vitro *kinase assays*
Immune complexes were washed twice with lysis buffer and once with 2 × kinase reaction buffer (100 mM HEPES, pH 7.5, 20 mM MgCl, 10 mM MnCl, 20 mM dithiothreitol). Washed complexes were resuspended in 1 × kinase reaction buffer containing 10 microcuries of (γ-^{32}P)adenosine triphosphate, 10 mM adenosine triphosphate and 100 μg/ml histone H1, and incubated for 30 min at 30 °C. Reactions were stopped by boiling in Laemmli buffer, and proteins were resolved by SDS–polyacrylamide gel electrophoresis. Phosphoproteins were visualized by autoradiography.

Immunostaining
Cells on glass coverslips were fixed in 4% paraformaldehyde for 10 min at room temperature or, for detection of centrosomes, in methanol for 15 min at −20 °C. Fixed cells were permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 for 5 min at room temperature, incubated in blocking buffer (phosphate-buffered saline containing 0.1% Triton X-100, 3% BSA and 10% goat serum) for 1 h at room temperature, and immunostained overnight at 4 °C with primary antibody in blocking buffer. Primary antibody staining was developed with secondary antibodies conjugated to Alexa fluor 488 and 594 and mounted with Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were analyzed with a Leica TCS SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) through a 100 × /1.40 NA Plan Apochromat oil immersion objective lens.

Antibody sources
BD Transduction Laboratories (Lexington, KY, USA) provided CDK2, CDK4 and $p27^{Kip1}$ monoclonal antibodies. Bethyl Laboratories (Montomery, TX, USA) provided CP110 (A301-343A). HA and pRb Ser780 were obtained from Cell Signaling, cyclin E from BD Pharmingen (San Diego, CA, USA), anti-rabbit and anti-mouse IgG-HRP from GE Healthcare (Buckinghamshire, UK), IgG native detection reagent-HRP from Pierce (Rockford, IL, USA), CDK1 (H-297), cyclin A (H-432), cyclin B1 (GNS1), cyclin D1 (A12), cyclin F (C-20), goat anti-α-actin-HRP (I-19), $p27^{Kip1}$ (C-19) and Skp2 (H-435) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and α-tubulin (T5168) and γ-tubulin (GTU88) from Sigma. We also used a $p27^{Kip1}$ rabbit polyclonal antibody described previously (Bagui *et al*., 2000).

Annexin V binding
Cells were detached from plates with trypsin-EDTA and combined with floating cells. Cells were stained with Annexin V-fluorescein isothiocyanate and propidium iodide (PI)/7-amino-actinomycin D (7-AAD) (BD Pharmingen), and analyzed by FACS.

Conflict of interest

The authors declare no conflict of interest.

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