

# Necessary and Sufficient Role for a Mitosis Skip in Senescence Induction

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## SUMMARY

Senescence is a state of permanent growth arrest and is a pivotal part of the antitumorigenic barrier *in vivo*. Although the tumor suppressor activities of p53 and pRb family proteins are essential for the induction of senescence, molecular mechanisms by which these proteins induce senescence are still not clear. Using time-lapse live-cell imaging, we demonstrate here that normal human diploid fibroblasts (HDFs) exposed to various senescence-inducing stimuli undergo a mitosis skip before entry into permanent cell-cycle arrest. This mitosis skip is mediated by both p53-dependent premature activation of APC/C<sup>Cdh1</sup> and pRb family protein-dependent transcriptional suppression of mitotic regulators. Importantly, mitotic skipping is necessary and sufficient for senescence induction. p16 is only required for maintenance of senescence. Analysis of human nevi also suggested the role of mitosis skip in *in vivo* senescence. Our findings provide decisive evidence for the molecular basis underlying the induction and maintenance of cellular senescence.

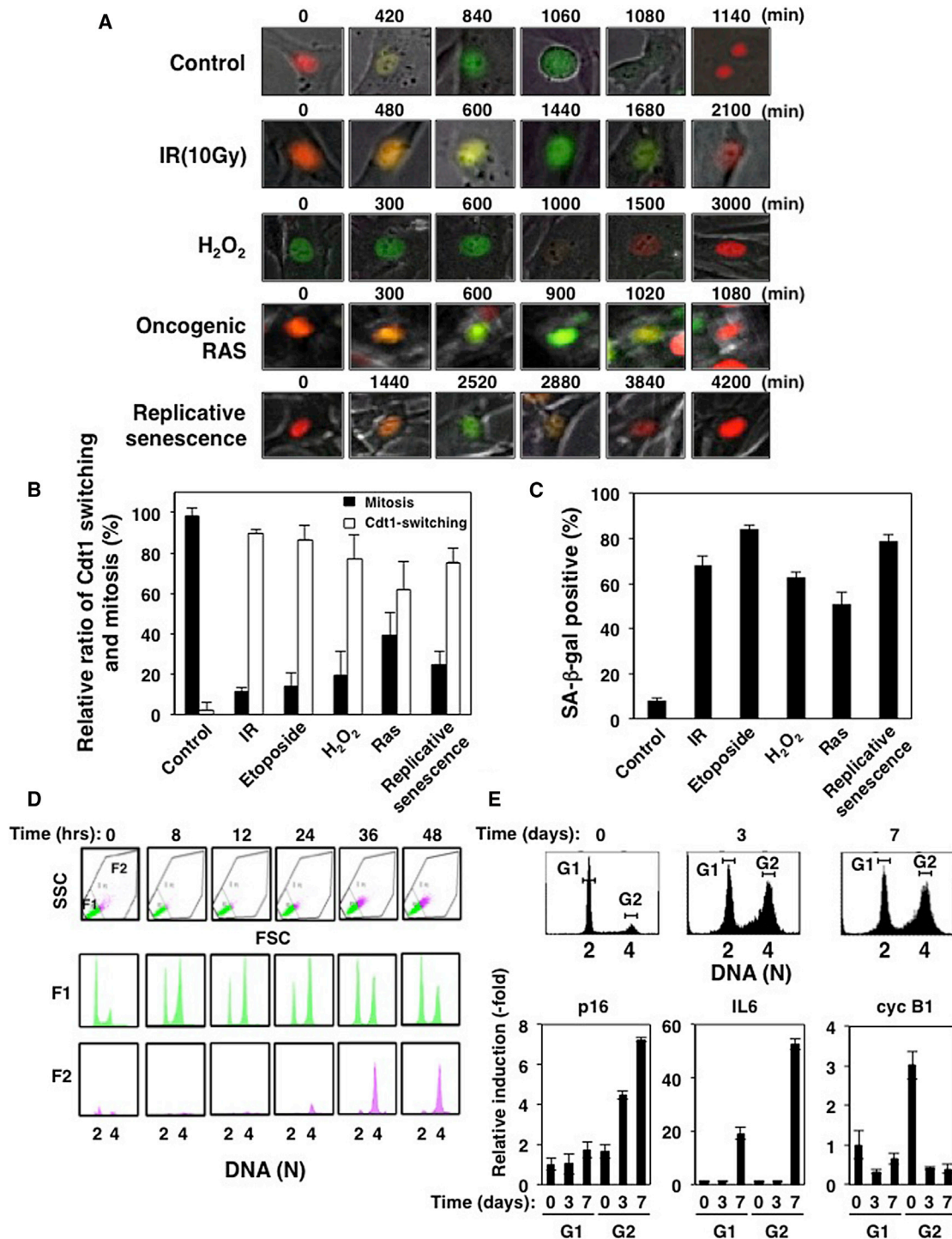
## INTRODUCTION

The inability of cultured human cells to proliferate indefinitely, ending in cellular senescence, was first described by Hayflick and Moorhead (1961). Subsequently, several lines of evidence revealed that cellular senescence was also triggered by diverse genotoxic stimuli, including telomere dysfunction, activated oncogenes, reactive oxygen species (ROS), and DNA damage (Kuilman et al., 2010). Senescence is now believed to play a critical role in suppression of tumorigenesis as well as in aging-related changes in various organs resulting from a permanent loss of proliferation capacity (Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008).

Cellular senescence requires functional p53 and pRb family proteins, both of which regulate growth signaling. This may explain why these genes are often mutated in a vast majority of human cancers (Burkhardt and Sage, 2008; Levine and Oren, 2009). This is supported by the fact that viral oncoproteins that can inhibit either p53 or pRb family proteins allow cells to bypass cellular senescence (Shay et al., 1991). Although the precise roles of these tumor suppressors in the senescence process are incompletely understood, various models of senescence induction have been proposed (Adams, 2009; Courtois-Cox et al., 2008; Ruffini et al., 2013). One such proposal is that senescence-inducing stimuli ultimately trigger a DNA damage response (DDR) that in turn activates p53. p21 (*CDKN1A*), a p53-target gene, is expressed and arrests cells at the G1 phase of the cell cycle by preventing phosphorylation and inactivation of pRb through inhibition of G1 and S phase Cdk activities (Cobrinik, 2005). pRb phosphorylation is also suppressed by another Cdk inhibitor, p16 (*CDKN2A*), that is upregulated during the senescence process (Rayess et al., 2012). Hypophosphorylated pRb suppresses transcription of canonical E2F (E2F1–E2F3) target genes to arrest the cell cycle at G1 (Rowland and Bernards, 2006).

In contrast, the accumulation of G2 phase cells during replicative senescence has also been reported, arguing against the senescence model described above (Mao et al., 2012; Ye et al., 2013). In addition, p21-mediated inhibition of Cdk1 and Cdk2 was proposed to prematurely activate APC/C<sup>Cdh1</sup> to destroy various APC/C substrates, resulting in long-term growth arrest at G2 in response to genotoxic stress (Baus et al., 2003; Wiebusch and Hagemeier, 2010). Thus, the fundamental basis for senescence induction and the phases at which senescent cells exit the cell cycle remain controversial. The factors that determine whether cells will undergo senescence (terminal growth arrest) versus transient cell-cycle arrest, also remain largely elusive.

In this study, we have analyzed the senescence process induced by various stimuli using time-lapse live-cell imaging. We found that the majority of cells underwent a mitosis skip before permanently exiting the cell cycle. This mitotic skipping appears to be necessary and sufficient for the induction of senescence both *in vitro* and *in vivo*.



**Figure 1. Senescent Cells Are Mononucleated Tetraploid G1 Cells Triggered by a Mitosis Skip**

(A) Young (PD 8) and near senescent (PD > 60) HCA2 cells (FUCCI-HCA2 cells) were infected with FUCCI lentiviruses expressing mKO2-hCdt1 (red) and mAG-hGeminin (green). Young cells were untreated (Control) or treated with IR (10 Gy), H<sub>2</sub>O<sub>2</sub> (50 μM) for 48 hr or etoposide (200 nM) for 48 hr. Expression of oncogenic RAS was induced by the addition of doxycycline to young cells expressing Tet-on 3×Flag-H-Ras<sup>val12</sup>. Replicative senescence was analyzed using near senescent cells. The resulting cells were imaged 3 days after treatment. Representative images at the indicated times are shown.

(B) The relative ratio of Cdt1-switching cells versus total cells changing from green to red color was determined by counting at least 100 cells treated as in (A). Data are presented as means ±SD of at least three independent experiments.

(C) SA-β-gal-positive cells were identified using cells 6 days after treatment as in (A). Data are presented as means ±SD of at least three independent experiments.

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## RESULTS

### Normal Human Diploid Fibroblasts Skip Mitosis in Response to Various Senescence-Inducing Stimuli before Growth Arrest Occurs

In order to directly determine from where senescent cells exit the cell cycle, we performed time-lapse live-cell imaging of asynchronously growing normal human diploid fibroblast (HDF) HCA2 cells transduced with lentiviruses expressing Cdt1 fragment-fused mCherry and geminin fragment-fused AmCyan proteins (Sakaue-Sawano et al., 2008). Imaging was conducted over a 3 day period and included control cells that divide normally (Figure 1A). In contrast to the control cells, 70% of the cells treated with senescence-inducing agents degraded geminin and accumulated Cdt1 without entry into mitosis (Cdt1 switching) (Figure 1B). Imaging allowed us to determine that there were changes in the nucleus that indicated entry into mitosis, such as mitotic rounding and nuclear envelope breakdown. These cells ceased cycling in the 3 day observation time. The rate was somewhat lower in cells treated with oncogenic Ras, most likely because induction of senescence via this stimulus is less effective, as evaluated by means of the senescence marker, senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity (Figure 1C) (Dimri et al., 1995). Given that Cdt1 switching per se does not infer that cells have entered into G1, we examined changes in the expression of various mitotic regulators as well as known senescence markers. All mitotic regulators tested were decreased and phosphorylation of histone H3 at serine 10 (H3-P-S10), a mitotic marker, was lost after exposure to senescence-inducing stimuli, and there was a marked increase in the population of tetraploid cells, suggesting that the cells had skipped mitosis before its initiation (see Figures S1A and S1B, available online). Although loss of mitotic regulators is a common feature of senescence, kinetics showing their loss prior to senescence induction indicated that this loss is a cause, but not a consequence, of senescence (Figure S1C). We then examined whether ectopic expression of the constitutively active form of cyclin B1-Cdk1 (cyclin B1-Cdk1AF fusion protein) or SV40 large T antigen in cells treated with IR resulted in entry of cells into mitosis or rereplication of DNA, respectively. In this case, one can expect that if cells are still in G2, then active cyclin B1-Cdk1 and SV40 large T antigen enforce the entry into mitosis but fail to initiate DNA rereplication, respectively. In contrast, if cells are in G1, then active cyclin B1-Cdk1 and SV40 large T antigen fail to force cells into mitosis but initiate DNA rereplication, respectively. We found that ectopic expression of cyclin B1-Cdk1AF fusion protein, which was used as a constitutively active form of cyclinB1-Cdk1 complex (Katsuno et al., 2009), enforced entry into mitosis in cells at 24 hr after treatment with IR, but not in cells at 48 hr (Figure S1D), by which time mitotic regulators had almost completely disappeared (completion of mitotic skipping) (Figure S1B). Expression of SV40 large T anti-

gen resulted in DNA rereplication in cells at 48 hr after treatment, but not at 24 hr, generating an octaploid population (Figure S1E). Taken together, the results clearly indicated that a mitosis skip took place between 24 to 48 hr after IR treatment. Here, we considered that a mitotic skip had occurred when a cell exhibited both Cdt1 switching and loss of mitotic regulators without entry into mitosis. A similar cellular response was also observed in other HDFs, such as BJ and IMR90 cells, and in normal human retinal pigment epithelial cells (data not shown), indicating that this phenomenon is not cell type specific.

Senescent cells show very distinctive changes in morphology, such as assuming a flattened and enlarged shape. Although enlarged senescent cells were hardly detectable by FACScan until 36 hr after IR irradiation, they accumulated thereafter (Figure 1D). The vast majority of these large cells (F2) had a tetraploid DNA content, whereas many cells of normal size (F1) showed a diploid DNA content. These tetraploid cells were geminin negative and Cdt1 positive as well as SA- $\beta$ -gal positive. Tetraploid fractions (G2) sorted by Hoechst intensities as G1 and G2 phase cells by FACScan at 3 and 7 days after DNA damage showed a marked increase in the expression of p16 and IL6, a senescence-associated cytokine, when compared with the diploid fraction (G1) (Figure 1E). In contrast, the expression of cyclin B1 was drastically decreased at 3 and 7 days specifically in G2 fractions. Furthermore, G2 fractions demonstrated a dramatic accumulation of p16 protein as well as SA- $\beta$ -gal-positive cells at 7 days or later (Figures S1F and S1G); however, G1 fractions did not, even at 10 days after IR treatment. Taken together, cells in the tetraploid fraction predominantly appeared to undergo senescence.

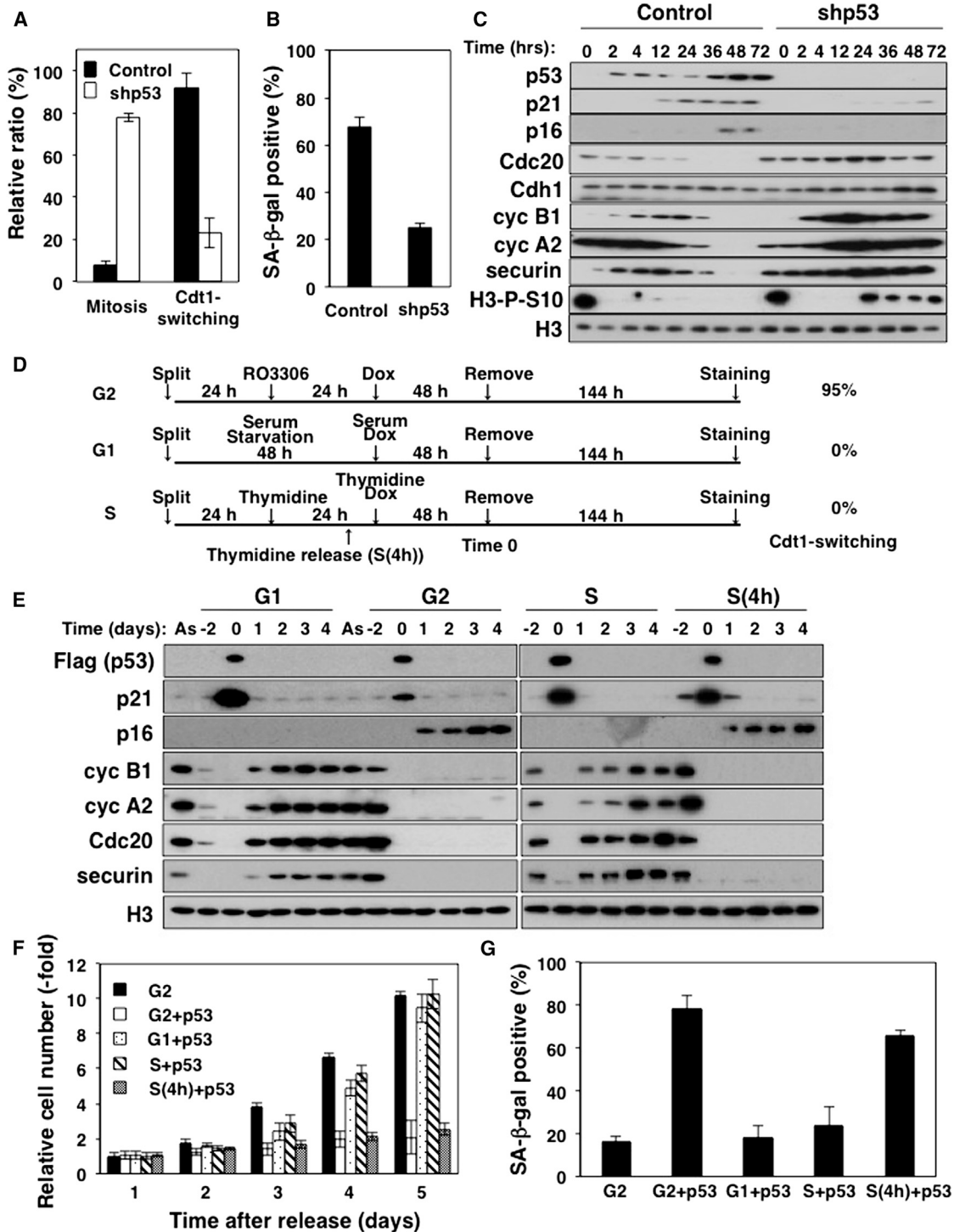
### Activation of p53 at G2 Is Necessary and Sufficient for Induction of Senescence

We then uncovered the molecular basis underlying mitotic skipping and determined whether it played a central role in senescence induction. p53 is known to be an essential factor for senescence induction (Rufini et al., 2013) and is activated in response to all senescence-inducing stimuli tested. p53 depletion markedly compromised Cdt1 switching and induced abnormal mitoses (Figure 2A). The number of cells that were SA- $\beta$ -gal positive was reduced in p53-depleted cells (Figure 2B). Expression of mitotic regulators was almost completely lost in control senescent cells, whereas their expression was slightly induced in p53-depleted cells (Figure 2C).

When p53 was transiently expressed for 48 hr by the addition of doxycycline to cells synchronized at G2 by RO3306, time-lapse imaging revealed that Cdt1 switching occurred in 95% of cells, whereas cells synchronized at G1 or S phases were unaffected (Figure 2D). Levels of mitotic regulators in cells transiently expressing p53 at G2 were severely decreased, whereas those at G1 or S phases were readily detectable (Figure 2E). p16 was induced in cells transiently expressing p53 at G2 phase, but

(D) HCA2 cells at the indicated times after IR treatment (10 Gy) were stained with Hoechst 33342 and sorted by FSC and SSC as F1 and F2 and analyzed by FACScan.

(E) HCA2 cells at 0, 3, and 7 days after IR treatment (10 Gy) were stained with Hoechst 33342 and sorted by intensity as G1 and G2 phase cells by FACScan. The relative induction of p16, IL6, and cyc B1 transcripts were examined by qPCR analysis using the sorted fractions indicated as G1 and G2. Data are presented as means  $\pm$ SD of at least three independent experiments. See also Figures S1A–S1G.



**Figure 2. p53 Activation at G2 Phase Is Sufficient for the Induction of Senescence**

(A and B) Fucci-HCA2 cells expressing Tet-on sh-luciferase (control) or Tet-on sh-p53 (shp53) were treated with doxycycline (1  $\mu$ g/ml). The treated cells were imaged by time-lapse microscopy after IR treatment (10 Gy). The relative ratio of Cdt1-switching cells was determined as in Figure 1B (A). The SA- $\beta$ -gal-positive cells were identified as in Figure 1C (B). Data are presented as means  $\pm$ SD of at least three independent experiments.

(C) Cell lysates of cells treated with sh-luciferase (control) or sh-p53 lentiviruses at the indicated time after IR treatment were subjected to immunoblotting using the indicated antibodies.

(D) Experimental outlines of cell-cycle-specific and transient expression of p53. Asynchronous Fucci-HCA2 cells expressing Tet-on 3 $\times$ Flag-p53 (As) were synchronized at G2, G1, or S phases by RO3306 (9  $\mu$ M) for 24 hr, serum starvation for 48 hr, or thymidine (2 mM) for 24 hr, respectively. In some experiments, cells

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not in those at G1 or S phases. p53 was only induced at day 0 in both cells. Intriguingly, cells transiently expressing p53 at G2 phase failed to proliferate, whereas those at G1 or S phase grew effectively (Figure 2F). The majority of cells transiently expressing p53 at G2 phase were SA- $\beta$ -gal positive, whereas those at G1 or S phase were not (Figure 2G). Transient expression of p53 in cells released to G2 phase for 4 hr from a thymidine block resulted in loss of mitotic regulators, cessation of cell proliferation, and an increase in the SA- $\beta$ -gal-positive fraction (Figures 2E–2G). Longer exposure of RO3306 per se did not induce Cdt1 switching, loss of mitotic regulators, and consequent induction of senescence (Figures S2A and 2B), although it might predispose cells to senescence upon p53 activation. These results clearly indicated that p53 activation at G2 was sufficient for senescence induction. Transient expression of p53 at G2 or G1 did not appear to induce DNA damage, eliminating the possibility that the mitosis skip and subsequent senescence induction by transient expression of p53 were not the result of a DDR (Figure S2C). Similar results were also observed in experiments using Nutlin 3a, a mdm2 inhibitor that activates endogenous p53 without causing DNA damage (Figure S2D). During the process of senescence induction in response to oxidative stress or oncogene activation, similar requirements for p53, p21, and Cdh1 in Cdt1 switching; its timing from S phase entry (please see below); loss of mitotic regulators; and senescence induction were observed when compared with IR-induced senescence (Figures S2E and S2F). The induction of senescence in p21-depleted cells appeared to be due to the fact that a majority of p21-depleted cells underwent cytokinesis failure, leading to eventual cell death within 6 days after treatment, and the remaining cells skipped mitosis, leading to senescence (see below). These results suggest that senescence induction in response to H<sub>2</sub>O<sub>2</sub> or oncogenic Ras likely requires a p53-dependent mitosis skip.

#### Premature Activation of APC/C<sup>Cdh1</sup> by the p53-p21 Axis Is Insufficient for a Mitosis Skip and Senescence Induction

p21 is a major downstream target of p53 (Levine and Oren, 2009). Time-lapse imaging revealed that upon DNA damage, many cells lacking p21 entered into mitosis but failed to complete cytokinesis and eventually died (Figures S3A and S3B). Transient expression of p21 at G2 phase failed to induce cessation of cell proliferation and senescence and strongly suppressed the expression of mitotic regulators, indicating that p21 induction is insufficient for senescence induction (Figures S3C–S3E).

p21 induction after various senescence-inducing stimuli resulted in premature activation of APC/C<sup>Cdh1</sup>, showing a complex formation between Cdh1 and Cdc27 around 24 hr after treatment, indicating that it is a general feature of senescence induc-

tion (Takahashi et al., 2012) (Figure S3F). We then examined whether premature activation of APC/C<sup>Cdh1</sup> by sequential inhibition of Cdk1 (RO3306) and Cdk2 (SU9516) was sufficient for the mitosis skip and subsequent senescence induction. Sequential inhibition of Cdk1 and Cdk2 resulted in Cdt1 switching, whereas inhibition of Cdk1 alone had no effect (Figure 3A, upper panel). Intriguingly, however, these Cdt1-positive cells rapidly entered into mitosis when the inhibitors were removed by washing (Figure S3G). These cells were not SA- $\beta$ -gal positive, although treatment with Nutlin 3a at G2 effectively induced senescence (Figure 3A, lower panel). Mitotic regulators were still detectable after removal of the inhibitors, even in cells sequentially treated with Cdk1 and Cdk2 inhibitors or expressing p21, although significant reductions in the amounts of these proteins were observed (Figure 3B). These results suggested that premature activation of APC/C<sup>Cdh1</sup> is insufficient for complete loss of mitotic regulators, indicating the existence of an alternative pathway(s) for suppressing the expression of mitotic regulators. Cdh1 depletion resulted in only a slight reduction in senescence induction (Figure 3C). It also resulted in an ultimate Cdt1 switching, presumably due to nonspecific degradation of geminifused fluorescence protein (Figure 3D). However, loss of Cdh1 markedly delayed the timing of Cdt1 switching, implicating its function in this process, because loss of Cdh1 did not affect S phase progression in the absence of DNA damage (Figure 3E). Thus, loss of mitotic regulators was still observed in cells lacking Cdh1 after DNA damage, although the timing of the disappearance of these proteins was markedly delayed (Figure 3F). Importantly, mitotic regulators were lost after DNA damage, even in the presence of the proteasome inhibitor MG132, although the effect of this reagent on the stabilization of mitotic regulators was significant at early time points (Figure 3G).

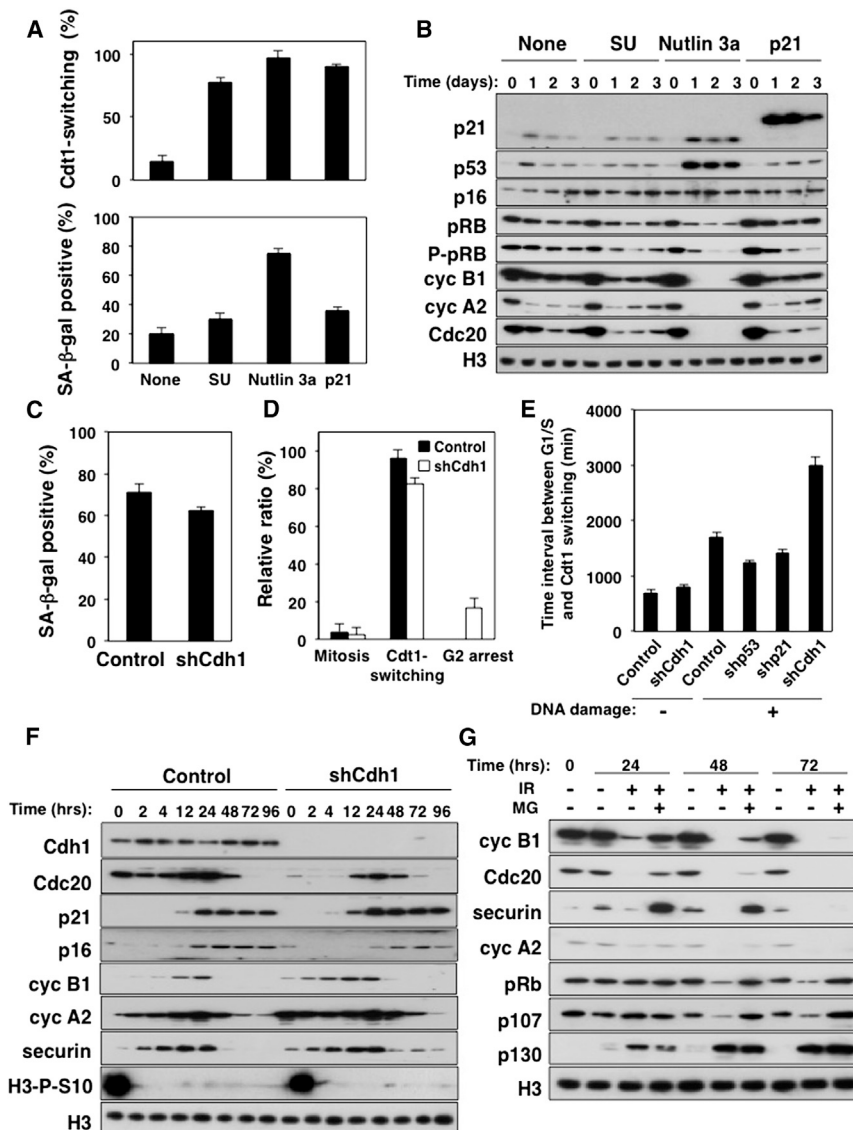
#### pRb Family Pocket Protein-Dependent Transcriptional Repression of Mitotic Regulators Is Required for a Mitosis Skip

Transcription of mitotic regulators was rapidly suppressed after treatment with Nutlin 3a (Figure 4A). This transcriptional repression was also observed in cells treated with H<sub>2</sub>O<sub>2</sub> or expressing oncogenic Ras, indicating that this is also a general feature of senescence induction (Figure S4A). This suppression was completely dependent on the functional p53 but was only partly on the presence of p21 (Figure S4B). This transcriptional repression, as well as those of cyclin A2 and cyclin E1 (Figure S4C), was severely compromised when pRb, p107, and p130 were depleted (Figure 4B). Single or double depletion of the pocket proteins did not appear to affect the transcription of these genes (Figures S4D and S4E; data now shown). Thus, mitotic regulators were readily detectable, and their levels increased at later time points in cells lacking pRb, p107, and p130 when treated with IR (Figure 4C). Mitotic skipping was severely suppressed by

were released to G2 phase by removing thymidine 4 hr before addition of doxycycline (S(4h)). Cells were then treated with doxycycline (1  $\mu$ g/ml) in the absence of RO3306 (G2), in the presence of 15% serum (G1) and thymidine (S), and released into fresh medium (Time 0). The relative ratios of Cdt1-switching cells were determined as in (A) and are shown in the figure.

(E and F) (E) Cell lysates of cells treated as in (D) at the indicated times were subjected to immunoblotting using the indicated antibodies. Time 0 is shown in (D). The relative number of cells treated as in (E) was determined as a multiple of those at day 1 (F). SA  $\beta$ -gal-positive cells were determined as in (B).

(G) Data are presented as means  $\pm$ SD of at least three independent experiments. See also Figures S2A–S2F.



**Figure 3. Induction of p21 at G2 Is Insufficient for Senescence Induction**

(A) Fucci-HCA2 cells expressing Tet-on 3×Flag p21 were synchronized at G2 by RO3306. The synchronized cells were untreated (None) or treated with SU9516 (SU; 10 μM), Nutlin 3a (5 μM), or doxycycline (1 μg/ml). The treated cells were then analyzed by time-lapse microscopy. The relative ratios of Cdt1-switching cells (upper panel) or SA-β-gal cells at 6 days after the treatments (bottom panel) were determined as in Figures 1B and 1C. Data are presented as means ±SD of at least three independent experiments.

(B) Cell lysates of cells treated as in (A) at the indicated times were subjected to immunoblotting analyses. Time 0 represents the day on which cells were treated.

(C and D) Fucci-HCA2 cells expressing Tet-on sh-luciferase (control) or Tet-on sh-Cdh1 (shCdh1) were treated with doxycycline (1 μg/ml). Cells were then treated with IR (10 Gy) and subjected to SA-β-gal staining as in Figure 1C (C) or time-lapse microscopy for determining the relative ratio of Cdt1-switching cells as in Figure 1B (D). Data are presented as means ±SD of at least three independent experiments.

(E) Time intervals for cells treated as in (C) between G<sub>1</sub>/S (change from red to green color) and Cdt1 switching (change from green to red color) with or without DNA damage were determined by examining at least 100 cells.

(F) Cell lysates from (C) at the indicated times after IR were subjected to immunoblotting using the indicated antibodies.

(G) Cell lysates from HCA2 cells treated with or without IR (10 Gy) at the indicated times in the presence or absence of MG132 (MG; 10 μg/ml) were subjected to immunoblotting using the indicated antibodies. See also Figures S3A–S3G.

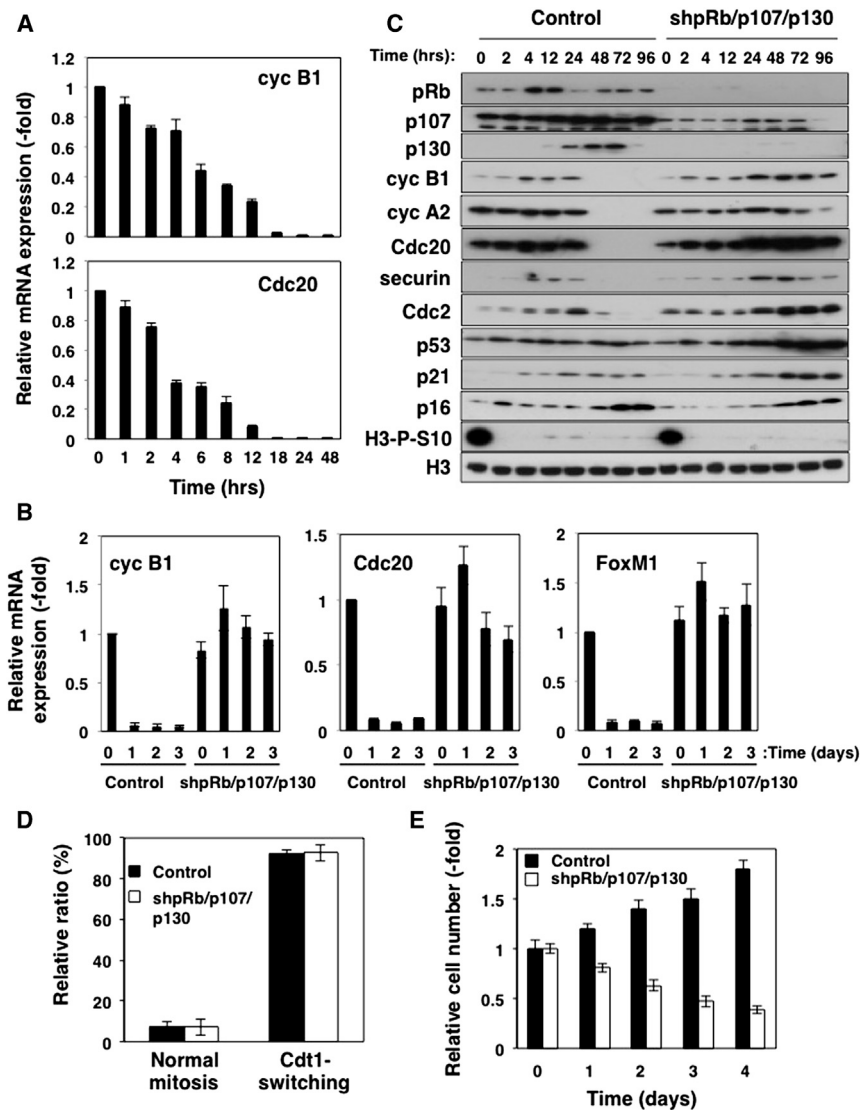
triple knockdown of pRb family pocket genes, although the appearance of Cdt1 switching cells was apparently unaffected (Figure 4D). Senescence induction in cells lacking all three pRb family proteins could not be analyzed, because these cells were not healthy in the presence of DNA damage after 6 days of culture (Figure 4E).

Loss of p16 did not appear to affect mitosis skip (Figures 5A and 5B). However, consistent with a previous report (Beauséjour et al., 2003), conditional knockdown of p16 by addition of doxycycline to cells treated with Nutlin 3a at G2 phase (Figure 5C) caused the reversal of the senescent state and allowed entry into S phase (Figure 5D), cell proliferation (Figure 5E), and re-expression of mitotic regulators (Figure S5). Interestingly, fluorescence-activated cell sorting (FACS) analysis revealed the appearance of octaploid cells after depletion of p16, further supporting the idea that senescent cells are tetraploid G1 cells (Figure 5F). Taken together, the present results suggest a general

mechanism of senescence induction (Figure 5G). Activation of p53 at G2 in response to senescence-inducing stimulus leads to induction of p21 that suppresses both Cdk1 and Cdk2 activities. This suppression results in the premature activation of APC/C<sup>Cdh1</sup> that degrades various mitotic regulators, leading to the Cdt1 switching. Activated p53 also enhances functions of pRb family pocket proteins through unknown mechanisms other than p21-dependent suppression of Cdk activities (Figure S4B) and consequently suppresses transcription of mitotic regulators. Both pathways cooperatively ensure mitosis skipping, leading to senescence induction.

**Transient Expression of Cdh1-4A and pRb7LP at G2 Is Sufficient for Senescence Induction**

In order to confirm our above model of senescence induction, we first examined whether transient expression at G2 of a constitutively active pRb (pRb7LP), in which all Cdk phosphorylation sites were substituted (Angus et al., 2003), would be sufficient for senescence induction under premature activation of APC/C<sup>Cdh1</sup>. Transient expression (24 hr) of pRb7LP by the addition of



#### Figure 4. pRb Family Proteins Are Essential for Transcriptional Repression of Mitotic Regulators

(A and B) The relative expression of the indicated transcripts were determined by qPCR analysis using total RNA from HCA2 cells at the indicated times after Nutlin 3a (A) or HCA2 cells expressing Tet-on sh-luciferase (control) or Tet-on sh-pRb, sh-p107, and sh-p130 (shpRb/p107/p130) in the presence of doxycycline (1  $\mu$ g/ml) at the indicated times after IR treatment (10 Gy) (B). The results are presented as a multiple of those without treatment. Data are presented as means  $\pm$ SD of at least three independent experiments.

(C–E) (C) Cell lysates from (B) at the indicated times after IR treatment were subjected to immunoblotting using the indicated antibodies. Fucci-HCA2 cells expressing Tet-on sh-luciferase (control) or Tet-on sh-pRb, sh-p107, and sh-p130 (shpRb/p107/p130) were treated with doxycycline (1  $\mu$ g/ml) for 24 hr. The treated cells were subjected to time-lapse microscopy after IR treatment (10 Gy). The relative ratio of Cdt1-switching cells was determined as in Figure 1B (D). The relative cell number was determined and expressed as a multiple of those without treatment (E). Data are presented as means  $\pm$ SD of at least three independent experiments. See also Figures S4A–S4E.

doxycycline to G2 cells, in the presence of a Cdk2 inhibitor to prematurely activate Cdh1 (Figure 6A), resulted in impaired cell proliferation (Figure 6B) and an increase in the population of cells staining positive for SA- $\beta$  gal (Figure 6C). The expression of mitotic regulators was also greatly decreased in these cells (Figure 6D). Under these conditions, treatment with a Cdk2 inhibitor did not affect the transcriptional suppression of mitotic regulators, although it markedly enhanced loss of mitotic regulators (Figures S6A and S6B). Transient expression (24 hr) of pRb7LP at G2 in the absence of a Cdk2 inhibitor was far less effective in inducing senescence than when the inhibitor was present, suggesting a role for prematurely activated Cdh1 in mitosis skip (Figure S6C). However, longer expression (72 hr) of pRb7LP at G2 ultimately induced senescence, supporting the dispensability of Cdh1 for senescence induction (Figures 3C–3F).

We therefore asked whether transient expression (24 hr) of both a constitutively active mutant of Cdh1 (Cdh1-4A) (Lukas

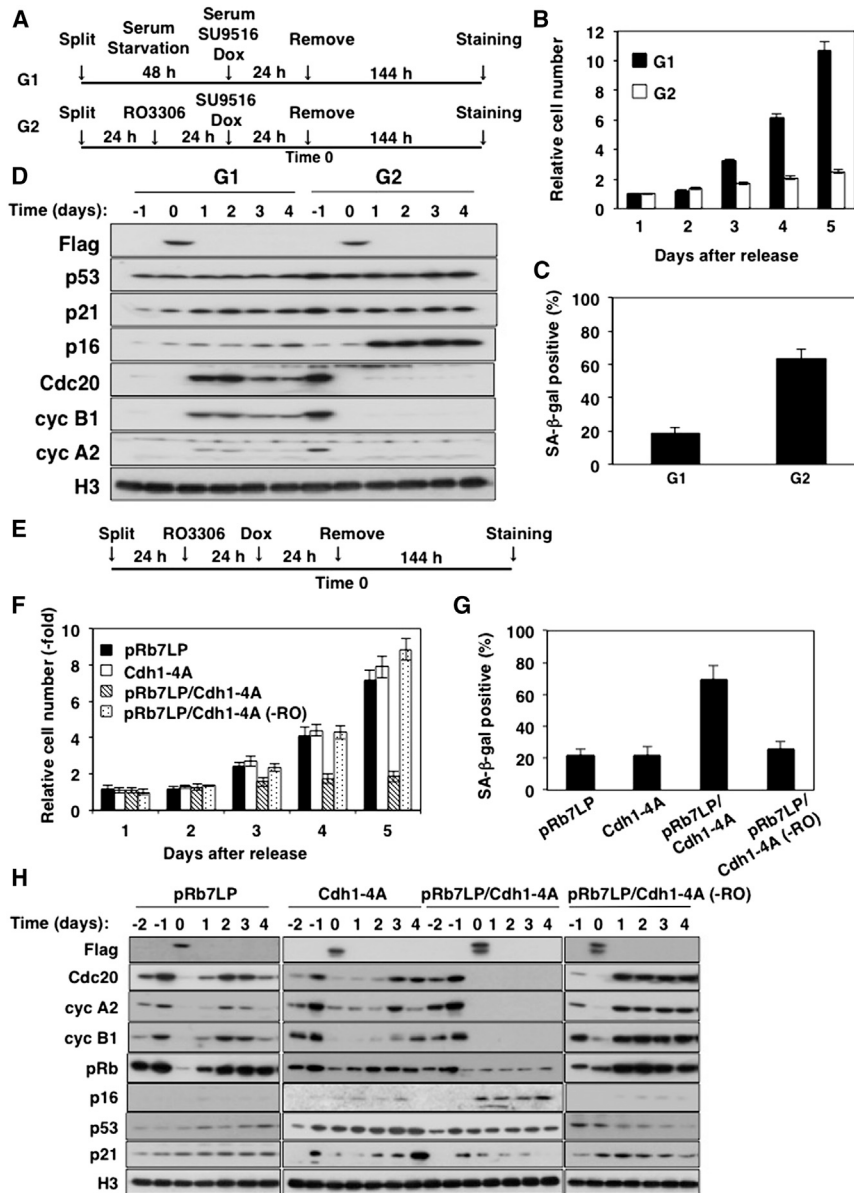
et al., 1999) and pRb7LP is sufficient for a mitosis skip and senescence induction. When both proteins were transiently expressed in synchronized G2 cells (Figure 6E), cessation of cell proliferation (Figure 6F), an increase in the population of SA- $\beta$ -gal-positive cells (Figure 6G), and a loss of mitotic regulators were observed (Figure 6H), whereas expression of either one alone failed to have the same effect. Expression of both proteins in asynchronous cells also failed to induce senescence, further confirming that a mitosis skip is necessary and sufficient for the induction of cellular senescence. The resulting senescence phenotype of the cells was further confirmed by the induction of p16 and senescence-associated cytokines such as IL-6 and IL-8 (Figure S6D). Transient expression of both proteins did not induce DNA damage, eliminating the possibility that senescence induction was an indirect consequence of an activated DDR (Figure S6E).

#### Role of the Mitosis Skip in the Induction of Senescence In Vivo

Finally, we asked whether mitotic skipping plays a role in in vivo senescence. Human nevi are benign tumors of melanocytes with frequent mutations in BRAF, a protein kinase and downstream effector of Ras, and were reported to be invariably positive for SA- $\beta$ -gal staining, suggesting that nevus cells are oncogene-induced senescent cells in vivo (Michaloglou et al., 2005). Therefore, we examined whether human nevus cells were tetraploid







**Figure 6. Expression of pRb7LP and Cdh1-4A at G2 Is Sufficient for the Induction of Senescence**

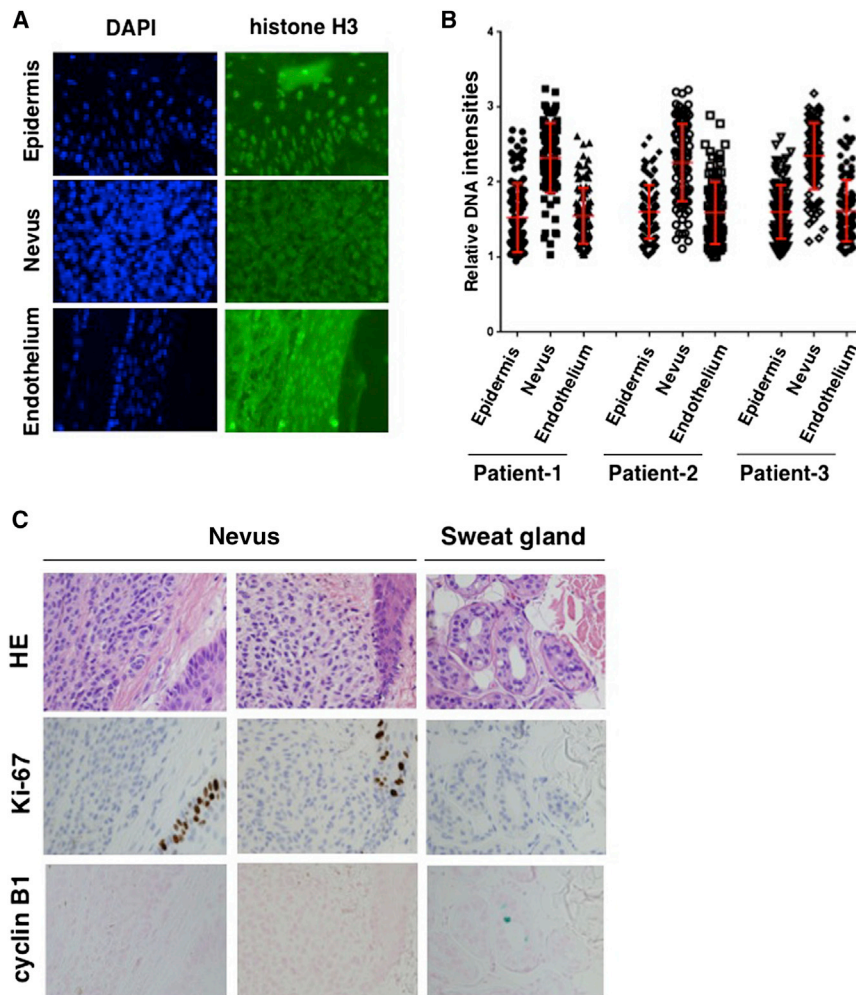
(A–C) (A) The experimental outline of the cell-cycle-specific and transient expression of Tet-on 3×Flag-pRb7LP. HCA2 cells expressing Tet-on 3×Flag-pRb7LP were synchronized at G0 or G2 by serum starvation or RO3306 (9 μM), respectively. The synchronized cells were treated with SU9516 (10 μM) and doxycycline (1 μg/ml) and then released into fresh medium (Time 0). The relative cell number of cells treated as in (A) was determined (B). The relative ratio of SA-β-gal-positive cells treated as in (A) was determined as in Figure 1C (C). Data are presented as means ±SD of at least three independent experiments. (D) Cell lysates treated as in (A) were subjected to immunoblotting using the indicated antibodies. (E) The experimental outline of the transient expression of Tet-on 3×Flag-pRb7LP and Tet-on 3×Flag-Cdh1-4A at G2. HCA2 cells expressing Tet-on 3×Flag-pRb7LP and/or 3×Flag-Cdh1-4A were synchronized at G2 with RO3306 (9 μM), treated with doxycycline (1 μg/ml), and then released into fresh medium (Time 0). The relative number of cells treated as in (E) was determined (F). –RO; without RO3306 treatment. The relative ratio of SA-β-gal-positive cells treated as in (E) was determined as in (C). (G) Data are presented as means ±SD of at least three independent experiments. (H) Cell lysates from cells treated as in (E) were subjected to immunoblotting using the indicated antibodies. –RO; without RO3306 treatment. See also Figures S6A–S6E.

telomere shortening (Mao et al., 2012; Ye et al., 2013). Senescent mitotic skipping is a form of cell-cycle arrest that is distinct from mitotic slippage or a postmitotic checkpoint that results from incomplete mitosis or impaired cytokinesis, because the latter form is induced after entry into mitosis (Andreassen et al., 2001; Lanni and Jacks, 1998). In addition, it should be noted that mammalian cells were reported not to possess checkpoints for tetraploidy or an aberrant centrosome number (Uetake and Sluder, 2004; Wong and Stearns, 2005).

Many signals and genes are reported to be involved in the induction of senescence, depending on the experimental context, demonstrating the complex nature of the phenotype (Campisi and d’Adda di Fagagna, 2007; Courtois-Cox et al., 2008). However, all senescent signals ultimately lead to p53 and pRb family pocket proteins, both of which are essential for

senescence induction (Dannenberg et al., 2000; Rufini et al., 2013; Sage et al., 2000). Although it is widely believed that the mere activation of pRb and p53 is not sufficient to trigger senescence, we found that transient activation of p53, specifically at G2, was enough to trigger senescence through induction of a mitosis skip (Figures 2D–2G). This signal transcriptionally induces p21, which in turn inhibits cyclin B1/Cdk1 and prematurely activates APC/C<sup>Cdh1</sup> (Figure S3F) (Baus et al., 2003; Wiebusch and Hagemeier, 2010; Takahashi et al., 2012). p21 was also shown to function in the regulation of Cdk2 activity at normal mitosis, making the proliferation-quiescence decision (Spencer et al., 2013). Therefore, the level of p21 at mitosis might be a key determining factor to decide whether cells undergo senescence, quiescence, or proliferation.

Upon persistent telomere damage, a distinct form of mitosis skip in p53-deficient cells was also reported to generate polyploidy by premature activation of APC/C<sup>Cdh1</sup>, the key result of Chk1-dependent suppression of Cdk1 (Davoli et al., 2010). However, activation of APC/C<sup>Cdh1</sup> at G2 was insufficient for a mitosis skip during senescent induction (Figures 3A and 3B). Deprotected telomeres that remain in a fusion-resistant intermediate



**Figure 7. Human Melanocytic Nevus Cells Are Ki-67 and Cyclin B1 Negative, but Possess Almost Twice the DNA Content of Control Cells**

(A) Paraffin-embedded sections of human nevi were subjected to immunohistochemical staining with anti-histone H3 antibody (green) along with DAPI staining for DNA (blue). The representative images for epidermis, nevus, and endothelium are shown.

(B) Fluorescence intensities of DAPI and anti-histone H3 signals in (A) were quantified with Image J software. The quantified DAPI intensity of each cell was normalized by the corresponding anti-histone H3 intensity, and the results are shown as relative DNA intensities. Nevi, epidermis, and endothelia from three patients were analyzed. Data are averages  $\pm$ SD ( $n = 100$ ).

(C) Paraffin-embedded sections of human nevi and sweat gland were subjected to hematoxylin (HE) and eosin staining (HE) and immunohistochemical staining using anti-Ki-67 antibody (brown) or anti-cyclin B1 antibody (green). See also Figure S7.

state were also shown to trigger a cell-cycle exit from G1. This intermediate telomere erosion activates unique DDRs that fail to activate G2/M checkpoint, leading to a p53-dependent G1 arrest (Cesare et al., 2013). However, actual involvement of this cell-cycle exit in senescence induction is unclear, because Chk1- and Chk2-dependent G2/M checkpoint activation is evident in telomere-initiated senescence (d'Adda di Fagagna et al., 2003).

The most striking observation in the present study was senescence induction by transient expression of both Cdh1-4A and pRb7LP in cells at G2, but not at other phases of the cell cycle (Figures 6E–6H). Although the unique and nonredundant role of pRb in the transcriptional repression of a certain set of E2F target genes during senescence was reported (Chicas et al., 2010), a single or double depletion of pRb family pocket proteins did not appear affect the mitosis skip and senescence induction (Figures S4D and S4E; data not shown), indicating that their function is at least in part redundant. E2F7 is a p53 target and has been proposed to compensate for the loss of pRb in repressing mitotic regulators (Aksoy et al., 2012). However, under our experimental conditions, the presence of E2F7 was not sufficient for suppressing the expression of mitotic regulators in the

absence of all pRb family pocket proteins (Figure 4C), although our results do not exclude the possibility that it plays a role in the maintenance of senescence. Another surprising observation was the dispensable role of p16 in mitotic skipping and senescence induction (Figures 5A and 5B), although it has been reported to be essential for the maintenance of senescence (Figures 5D–5F) (Beauséjour et al., 2003). It should be noted that induction of p16 consistently appeared to be a later event than that of p53 and

p21 (Figure 2C). Intriguingly, a loss of p16 in senescent cells caused reentry into S phase, resulting in an increased octaploid population.

Accumulating evidence has suggested that senescence plays a pivotal role in antitumorogenesis and aging-related changes in vivo (Campisi and d'Adda di Fagagna, 2007; Halazonetis et al., 2008). For example, human melanocytic nevi with BRAF mutations show typical hallmarks of senescence, suggesting that oncogene-induced senescence is a bona fide physiological process (Michaloglou et al., 2005). Importantly, nevus cells were Ki-67 negative and cyclin B1 negative but possessed almost twice the DNA content of control cells, suggesting that oncogene-induced senescent cells are tetraploid G1 cells in vivo (Figures 7A–7C and S7). Therefore, these results are consistent with in vitro observations that the mitosis skip plays a necessary and sufficient role in the induction of senescence.

## EXPERIMENTAL PROCEDURES

### Plasmid Construction

To generate lentivirus-based shRNA constructs, a 19–21 base shRNA-coding fragment with a 5'-ACGTGTGCTGTCGGT-3' loop was introduced into

pENTR4-H1 digested with *AgeI*/*EcoRI*. To insert the H1tetOx1-shRNA into a lentivirus vector, we mixed the resulting pENTR4-H1-shRNA vector and CS-RfA-ETBsd, CS-RfA-ETHygro, or CS-RfA-ETPuro vector with Gateway LR clonase (Invitrogen). All the target sequences for lentivirus-based sh-RNAs are summarized in Table S1.

To construct Tet-on inducible lentivirus constructs, the *BamHI/NotI* fragment PCR containing cDNA for human p16, p21, p53, pRb7LP (Angus et al., 2003), Cdh1-4A (Lukas et al., 1999), or H-RAS<sup>val12</sup> (Barradas et al., 2009) was inserted into a pENTR-1A vector (Invitrogen) containing 3×Flag epitope digested with *BamHI/NotI*. The resultant plasmid was mixed with CS-IV-TRE-RfA-UbC-Puro vector, or CS-IV-TRE-RfA-UbC-Hygro vector, and reacted with Gateway LR clonase to generate the lentivirus plasmid.

#### Immunoblotting Analyses

Immunoblotting was performed as previously described (Katsuno et al., 2009). All antibodies used in this study are listed in Table S2.

#### Cell Culture

Early passage normal HDFs, HCA2 (Nakanishi et al., 1995), BJ (ATCC), IMR90 (ATCC), IMR90-ER:RAS<sup>val12</sup> (Barradas et al., 2009), and HEK293T cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For the serum starvation experiment, cells were cultured with DMEM supplemented with 0.5% FBS for 48 hr. Cells were synchronized at early S phase by treatment with 2 mM of thymidine (Sigma-Aldrich) for 24 hr or at G2 phase by treatment with 9 μM of RO3306 (Roche) for 24 hr. Nutlin-3a (Sigma-Aldrich), SU9516 (Merck Biosciences), MG132 (Sigma-Aldrich), or NU7026 (Sigma-Aldrich) was used at a concentration of 5 μM, 10 μM, 10 μg/ml, or 10 μM, respectively, for the indicated interval. Treatments with IR at 10 Gy, Etoposide (Sigma-Aldrich) at 200 nM for 24 hr and H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) at 50 μM for 24 hr were used to generate IR-induced, DNA damage-induced, and oxidative stress-induced senescent cells, respectively. Senescent cells treated with IR at the 10 Gy dose were analyzed 6 days after treatment and were evaluated by SA-β-gal staining. Oncogene-induced senescent cells were generated by the expression of ER-H-Ras<sup>val12</sup> or 3×Flag-H-Ras<sup>val12</sup> in the presence of 4-OHT for 8 days. Replicative senescent cells were generated by the culture of near senescent HDFs (PD > 60).

#### Virus Generation and Infection

Lentiviruses expressing the respective shRNAs or genes were generated by cotransfection of 293T cells with pCMV-VSV-G-RSV-Rev, pCAG-HIVgp, and the respective CS-RfA-ETBsd, CS-RfA-ETHygro, CS-RfA-ETPuro, CS-IV-TRE-RfA-UbC-Puro, or CS-IV-TRE-RfA-UbC-Hygro using the calcium phosphate coprecipitation method. Cells infected with the indicated viruses were treated with 10 μg/ml of blasticidin (Invitrogen), 200 ng/ml of hygromycin (Sigma-Aldrich), and/or 2 μg/ml of puromycin (Sigma-Aldrich) for 2–3 days. Doxycycline (Sigma-Aldrich) was added to the medium at a concentration of 1 μg/ml for inducible expression of the respective shRNAs or genes.

#### Time Lapse Microscopy

HCA2 or IMR90-ER:RAS<sup>val12</sup> cells expressing FUCCI 2.1 indicators (pMXs-AmCyan-hGeminin [1/110] and pMXs-IP-mCherry-hCdt1 [30/120]) were cultured on a glass-based dish (Iwaki), placed on the stage of a BZ-9000 (Keyence) equipped with an environmental chamber (Keyence), providing an adequate temperature, humidity, and CO<sub>2</sub> control. Time-lapse images were captured every 20 min for 72 hr with a set of green 494/20 and 536/40 emission filters. Images were analyzed using BZ-9000 software.

#### SA-β-Gal Staining

SA-β-gal staining was performed as previously described (Dimri et al., 1995).

#### Quantitative RT-PCR

Total RNA was extracted using ISOGEN II (Wako) according to the manufacturer's instructions. For quantitative RT-PCR analysis, cDNAs were synthesized using a SuperScript II cDNA synthesis kit (Invitrogen). Real-time PCR amplifications were performed in 96-well optical reaction plates with Power SYBR Green PCR Master Mix (Applied Biosystems). The relative expression

values of each gene were determined by normalization to GAPDH expression for each sample. Primer sequences are available upon request.

#### FACS Analysis and FACS Sorting

Cell-cycle profiles were analyzed by a standard procedure using a FACS CANT2 (BD Biosciences). Diploid and tetraploid fractions were isolated by FACS sorting using a BD FACS Aria-2 cell sorter (BD Biosciences) after incubation with 10 μM Hoechst 33342 (Wako) for 10 min.

#### Immunohistochemistry

Human pathological sections containing nevi were fixed in 4% formaldehyde overnight and embedded in paraffin. Paraffin sections were deparaffinized, rehydrated, and incubated with peroxidase blocking reagent (DAKO). The tissue was incubated with the primary antibodies 96C10 for histone H3 (Cell Signaling), MIB-1 for Ki-67 (DAKO), and GNS1 for cyclin B1 (Santa Cruz). Primary antibody binding was detected using a FITC-linked secondary antibody or a HRP-linked antibody and revealed by conventional immunostaining using DAB (DAKO) or HistoGreen (ABC Scientific) as a substrate. This study was approved by the Ethics Committee of Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan. All subjects provided written informed consent.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2014.05.003>.

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#### REFERENCES

- Adams, P.D. (2009). Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. *Mol. Cell* 36, 2–14.
- Aksoy, O., Chicas, A., Zeng, T., Zhao, Z., McCurrach, M., Wang, X., and Lowe, S.W. (2012). The atypical E2F family member E2F7 couples the p53 and RB pathways during cellular senescence. *Genes Dev.* 26, 1546–1557.
- Andreassen, P.R., Lohez, O.D., Lacroix, F.B., and Margolis, R.L. (2001). Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. *Mol. Biol. Cell* 12, 1315–1328.
- Angus, S.P., Solomon, D.A., Kuschel, L., Hennigan, R.F., and Knudsen, E.S. (2003). Retinoblastoma tumor suppressor: analyses of dynamic behavior in living cells reveal multiple modes of regulation. *Mol. Cell. Biol.* 23, 8172–8188.
- Barradas, M., Anderton, E., Acosta, J.C., Li, S., Banito, A., Rodriguez-Niedenführ, M., Maertens, G., Banck, M., Zhou, M.M., Walsh, M.J., et al. (2009). Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. *Genes Dev.* 23, 1177–1182.

- Baus, F., Gire, V., Fisher, D., Piette, J., and Dulic, V. (2003). Permanent cell cycle exit in G2 phase after DNA damage in normal human fibroblasts. *EMBO J.* *22*, 3992–4002.
- Beauséjour, C.M., Krtolica, A., Galimi, F., Narita, M., Lowe, S.W., Yaswen, P., and Campisi, J. (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.* *22*, 4212–4222.
- Burkhardt, D.L., and Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat. Rev. Cancer* *8*, 671–682.
- Campisi, J., and d'Adda di Fagnana, F. (2007). Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol. Cell Biol.* *8*, 729–740.
- Cesare, A.J., Hayashi, M.T., Crabbe, L., and Karlseder, J. (2013). The telomere deprotection response is functionally distinct from the genomic DNA damage response. *Mol. Cell* *51*, 141–155.
- Chicas, A., Wang, X., Zhang, C., McCurrach, M., Zhao, Z., Mert, O., Dickins, R.A., Narita, M., Zhang, M., and Lowe, S.W. (2010). Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer Cell* *17*, 376–387.
- Cobrinik, D. (2005). Pocket proteins and cell cycle control. *Oncogene* *24*, 2796–2809.
- Courtois-Cox, S., Jones, S.L., and Cichowski, K. (2008). Many roads lead to oncogene-induced senescence. *Oncogene* *27*, 2801–2809.
- d'Adda di Fagnana, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., and Jackson, S.P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature* *426*, 194–198.
- Dannenberger, J.H., van Rossum, A., Schuijff, L., and te Riele, H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev.* *14*, 3051–3064.
- Davoli, T., Denchi, E.L., and de Lange, T. (2010). Persistent telomere damage induces bypass of mitosis and tetraploidy. *Cell* *141*, 81–93.
- Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* *92*, 9363–9367.
- Giacinti, C., and Giordano, A. (2006). RB and cell cycle progression. *Oncogene* *25*, 5220–5227.
- Halazonetis, T.D., Gorgoulis, V.G., and Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. *Science* *319*, 1352–1355.
- Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell Res.* *25*, 585–621.
- Katsuno, Y., Suzuki, A., Sugimura, K., Okumura, K., Zineldien, D.H., Shimada, M., Niida, H., Mizuno, T., Hanaoka, F., and Nakanishi, M. (2009). Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. *Proc. Natl. Acad. Sci. USA* *106*, 3184–3189.
- Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. *Genes Dev.* *24*, 2463–2479.
- Lanni, J.S., and Jacks, T. (1998). Characterization of the p53-dependent post-mitotic checkpoint following spindle disruption. *Mol. Cell Biol.* *18*, 1055–1064.
- Levine, A.J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. *Nat. Rev. Cancer* *9*, 749–758.
- Lukas, C., Sørensen, C.S., Kramer, E., Santoni-Rugiu, E., Lindeneg, C., Peters, J.M., Bartek, J., and Lukas, J. (1999). Accumulation of cyclin B1 requires E2F and cyclin-A-dependent rearrangement of the anaphase-promoting complex. *Nature* *401*, 815–818.
- Mao, Z., Ke, Z., Gorbunova, V., and Seluanov, A. (2012). Replicatively senescent cells are arrested in G1 and G2 phases. *Aging (Albany, N.Y. Online)* *4*, 431–435.
- Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* *436*, 720–724.
- Nakanishi, M., Robortory, R.S., Adami, G.R., Pereira-Smith, O.M., and Smith, J.R. (1995). Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. *EMBO J.* *14*, 555–563.
- Rayess, H., Wang, M.B., and Srivatsan, E.S. (2012). Cellular senescence and tumor suppressor gene p16. *Int. J. Cancer* *130*, 1715–1725.
- Rowland, B.D., and Bernards, R. (2006). Re-evaluating cell-cycle regulation by E2Fs. *Cell* *127*, 871–874.
- Rufini, A., Tucci, P., Celardo, I., and Melino, G. (2013). Senescence and aging: the critical roles of p53. *Oncogene* *32*, 5129–5143.
- Sage, J., Mulligan, G.J., Attardi, L.D., Miller, A., Chen, S., Williams, B., Theodorou, E., and Jacks, T. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev.* *14*, 3037–3050.
- Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K., Miyata, T., Miyoshi, H., et al. (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* *132*, 487–498.
- Shay, J.W., Pereira-Smith, O.M., and Wright, W.E. (1991). A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.* *196*, 33–39.
- Spencer, S.L., Cappell, S.D., Tsai, F.C., Overton, K.W., Wang, C.L., and Meyer, T. (2013). The proliferation-quiescence decision is controlled by a bifurcation in CDK2 activity at mitotic exit. *Cell* *155*, 369–383.
- Takahashi, A., Imai, Y., Yamakoshi, K., Kuninaka, S., Ohtani, N., Yoshimoto, S., Hori, S., Tachibana, M., Anderton, E., Takeuchi, T., et al. (2012). DNA damage signaling triggers degradation of histone methyltransferases through APC/C(Cdh1) in senescent cells. *Mol. Cell* *45*, 123–131.
- Uetake, Y., and Sluder, G. (2004). Cell cycle progression after cleavage failure: mammalian somatic cells do not possess a “tetraploidy checkpoint”. *J. Cell Biol.* *165*, 609–615.
- Vodermaier, H.C. (2004). APC/C and SCF: controlling each other and the cell cycle. *Curr. Biol.* *14*, R787–R796.
- Wiebusch, L., and Hagemeier, C. (2010). p53- and p21-dependent premature APC/C-Cdh1 activation in G2 is part of the long-term response to genotoxic stress. *Oncogene* *29*, 3477–3489.
- Wong, C., and Stearns, T. (2005). Mammalian cells lack checkpoints for tetraploidy, aberrant centrosome number, and cytokinesis failure. *BMC Cell Biol.* *6*, 6.
- Ye, C., Zhang, X., Wan, J., Chang, L., Hu, W., Bing, Z., Zhang, S., Li, J., He, J., Wang, J., and Zhou, G. (2013). Radiation-induced cellular senescence results from a slippage of long-term G2 arrested cells into G1 phase. *Cell Cycle* *12*, 1424–1432.