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Report

p53 represses the polyploidization of primary mammary epithelial cells by activating apoptosis

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Abbreviations: Cyt *c*, cytochrome *c*; CytD, cytochalasin D; DCB, dihydrocytochalasin B; MMECs, mouse mammary epithelial cells; MOMP, mitochondrial outer membrane permeabilization; Noco, nocodazole; WT, wild type

Key words: cancer, chemoresistance, cytochalasin D, dihydrocytochalasin B, mitochondrial outer membrane permeabilization, mitoxantrone, nocodazole

Tetraploidy may constitute a metastable state leading to numeric and structural chromosome abnormalities that are associated with cancer. Here, we show that cultured primary *p53*$^{-/-}$ (but not wild type, WT) mouse mammary epithelial cells (MMECs) accumulate a tetraploid sub-population in vitro. This occurs spontaneously, yet can be exacerbated by the addition of microtubule inhibitors as well as of inhibitors of cytokinesis. As compared to WT cells, tetraploid *p53*$^{-/-}$ MMECs contain supernumerary centrosomes and exhibit a reduced propensity to initiate the mitochondrial pathway of apoptosis. Moreover, tetraploid *p53*$^{-/-}$ MMECs are more resistant against anthracyclin-induced cell killing than their diploid counterparts. Altogether, these data indicate that p53 normally suppresses the generation of tetraploid cells, presumably by activating the intrinsic pathway of apoptosis. In the absence of p53, tetraploid cells accumulate as a result of inhibited apoptosis, which contributes to the acquisition of chemotherapy resistance.

Introduction

Tetraploid cells are found in some precancerous lesions including Barrett's esophagus and cervical carcinoma, where their presence accompanies the loss of functional p53.[1,2] Due to the increase in the number of chromosomes, perhaps coupled to changes in the geometry of the mitotic machinery, tetraploid cells tend to spontaneously activate the DNA damage response and to be genomically unstable. In this sense, tetraploidy may be considered as a metastable state that links normal diploidy to cancer-associated aneuploidy.[3,4]

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When non-transformed cells are treated with inhibitors of cytokinesis (such as cytochalasins), they arrest proliferation at a binucleate stage. In contrast, cells transformed by the SV40 virus (which neutralizes the tumor suppressors p53 and Rb) do not arrest their cell cycle upon cytochalasin administration, and rather proliferate at a polyploid stage.[5] Thus, a "tetraploidy checkpoint", which was shown to be effectively controlled by p53,[6] might avoid the proliferation of tetraploid cells. Accordingly, one efficient strategy for engineered carcinogenesis consists in the treatment of p53-deficient mouse mammary epithelial cells (MMECs) with cytochalasins followed by mutagens.[7]

Many authors have investigated the role of p53 in the cell cycle arrest induced by tetraploidizing agents including cytochalasins (which suppress cytokinesis) and microtubule inhibitors (which block karyokinesis and induce "mitotic slippage", a reversion from metaphase to a tetraploid G_1-like state) (reviewed in refs. 3 and 4). In the past, our group has shown that p53-proficient human colon carcinoma HCT-116 cells treated with cytochalasin D (CytD) or nocodazole (Noco) die as they reach a tetraploid state, while p53-deficient cells fail to do so,[8,9] indicating that the so-called "tetraploidy checkpoint" might consist in the selective apoptotic abortion of tetraploid cells. In favor of this hypothesis, we found that the absence of Bax (which is a pro-apoptotic effector activated by p53) mimics the permissive effect of p53 deficiency on tetraploidization in vitro.[9] Moreover, p53-proficient tetraploid cells (which can be generated at a low frequency) tended to spontaneously undergo delayed apoptosis, and their survival strongly depended on the activity of checkpoint kinases (in particular of Chk1).[10] Thus, the inhibition of Chk1 was shown to kill tetraploid cells through a pathway that involves the p38^{MAPK}-dependent activation of p53,[11] followed by p53-dependent activation of Bax-/Bak-dependent mitochondrial outer membrane permeabilization (MOMP).[12-15]

Because the aforementioned results were obtained in transformed cells, they cannot be extrapolated to the physiology of

primary non-transformed cells, in which the suppression of tetraploidy ensured by apoptosis might constitute a barrier against oncogenesis. Therefore, we decided to reinvestigate the contribution of p53 and apoptosis to the inhibition of tetraploidization in primary epithelial cells from the mammary gland. As shown here, a p53-dependent apoptotic pathway is ignited in primary cells as they become tetraploid.

Results and Discussion

p53 deficiency is permissive for the polyploidization of primary epithelial cells. Mouse mammary epithelial cells (MMECs) from wild type (WT) or $p53^{-/-}$ animals were obtained by enzymatic dissociation of virgin mammary glands, followed by in vitro culture for one week, during which fibroblasts were eliminated by selective trypsinization. MMECs (which stained positively with a pan-cytokeratin antibody, not shown) were then cultured for further 48 h in the absence or in the presence of nocodazole (Noco, a reversible microtubule depolymerizing agent that arrests karyokinesis) or either of two chemically related inhibitors of cytokinesis, namely cytochalasin D (CytD) or dihydrocytochalasin B (DCB), and finally stained with the chromatinophilic dye Hoechst 33342 for the cytofluorometric assessment of their ploidy. We observed that $p53^{-/-}$ MMEC cultures contained a higher percentage of polyploid (>4n)—mostly tetraploid—cells than their WT counterparts. This difference was exacerbated upon treatment with Noco, CytD or DCB (Fig. 1A and B), indicating that the absence of p53 is permissive for the polyploidization of MMECs. In parallel experiments, WT and $p53^{-/-}$ MMECs were stained with the vital dye propidium iodide (PI) and the mitochondrial transmembrane potential ($\Delta\Psi_m$)-sensitive dye $DiOC_6(3)$,[16,17] to determine the percentage of dead (PI^+) and dying ($PI^-/DiOC_6(3)^{low}$) cells. $p53^{-/-}$ MMEC cultures exhibited lower levels of dying/dead cells than their WT counterparts, in all conditions tested (irrespective of the presence of Noco, CytD or DCB) (Fig. 1C and D), suggesting a possible link between apoptosis resistance and enhanced susceptibility to polyploidization.

p53-deficient tetraploid cells contain supernumerary centrosomes and are resistant to apoptosis. Intrigued by the aforementioned observations, we decided to further explore the specific features of tetraploid $p53^{-/-}$ MMECs. In contrast to their WT counterparts, tetraploid $p53^{-/-}$ MMECs often contained supernumerary (>4) centrosomes (Fig. 2A and B). This finding is in line with the intrinsic genomic instability of $p53^{-/-}$ cells, which has previously been described.[7] We also investigated whether freshly formed tetraploid cells exhibit the mitochondrial release of cytochrome *c* (Cyt *c*) and hence manifest MOMP, which ignites the intrinsic pathway of apoptosis.[18,19] To this aim, WT or $p53^{-/-}$ MMECs were exposed to DCB, fixed, permeabilized and stained to visualize the subcellular distribution of Cyt *c* by immunofluorescence (Fig. 2C). Upon DCB exposure, a sizeable fraction (>50%) of WT cells exhibited a diffuse (as opposed to punctuate) Cyt *c* staining, which is indicative of the mitochondrio-cytosolic translocation of Cyt *c*. Such a DCB-triggered cell death response of WT MMECs was more pronounced among tetraploid cells than among their diploid counterparts. $p53^{-/-}$ MMECs were less prone to undergo MOMP than WT MMECs, irrespective of their ploidy (Fig. 2C and D). Morevoer, tetraploid $p53^{-/-}$ MMECs did not manifest increased mitochondrial release of Cyt *c* over diploid $p53^{-/-}$ MMECs (Fig. 2C and D). These results suggest that $p53^{-/-}$ MMECs (and in particular tetraploid $p53^{-/-}$ MMECs) are more resistant against apoptosis induction than WT cells. We therefore FACS-purified diploid and tetraploid MMECs obtained upon DCB treatment of primary WT or $p53^{-/-}$ MMECs (Fig. 2E), and determined their fate in vitro. In culture, tetraploid WT MMECs spontaneously manifested signs of apoptosis at a higher frequency than tetraploid $p53^{-/-}$ MMECs (Fig. 2F), confirming that the presence of p53 predisposes newly generated tetraploid cells to undergo apoptotic cell death. Accordingly, upon prolonged culture, $p53^{-/-}$ MMECs progressively replicated and outnumbered WT MMECs, which—on the other hand—ultimately died (Fig. 2G). We and others have previously observed that tetraploid tumor cells are more resistant against DNA damaging agents than their diploid parental cells.[9,20] We therefore investigated whether tetraploid $p53^{-/-}$ MMECs are intrinsically resistant against apoptosis induction by anthracyclins, which constitute the first line chemotherapeutic agents for the treatment of breast carcinomas.[21] We found that freshly isolated tetraploid $p53^{-/-}$ MMECs are significantly less prone to die in response to the anthracyclin mitoxantrone than their diploid precursors (Fig. 2H), further corroborating the notion that tetraploidy can confer chemotherapy resistance.

Discussion

In this paper, we provide evidence suggesting that p53 is required for the apoptotic abortion of freshly formed tetraploid cells. In the absence of p53, primary epithelial cells exposed to inhibitors of cytokinesis or karyokinesis fail to die as they become tetraploid. Conversely, p53 guarantees that primary epithelial cells undergo apoptosis as they are forced into tetraploidy.

Transformed cells that are compelled to become polyploid also tend to undergo apoptosis, especially when they are p53-proficient. This has previously been shown by our group for CytD-, Noco- and cell fusion-induced polyploidization.[9] In this latter case, p53 activation represents the final step of a complex signal transduction cascade that necessarily implicates cyclin-dependent kinases (in particular Cdk1),[22] elements of the DNA damage response (such as ATM and PML),[23,24] as well as stress kinases (such as $p38^{MAPK}$),[13] which are the actual p53-phosphorylating enzymes. The transactivation of p53 target genes including those coding for Bax and for the BH3-only protein Puma contributes to MOMP induction, which is followed by Cyt *c* release and activation of the caspase cascade.[18,25] Bax and Puma are also upregulated in tetraploid cancer cell clones.[9] While it is tempting to speculate that this pathway is also activated in MMECs, the molecular mechanisms accounting for p53 activation and p53-mediated apoptosis remain to be characterized in the context of primary cells that are submitted to tetraploidizing regimens.

Irrespective of these incognita, the present data indicate that p53 controls the apoptotic demise of freshly formed tetraploid cells, including those derived from primary tissues. It is tempting to assume that this novel property of p53 might contribute to its oncosuppressive activity.

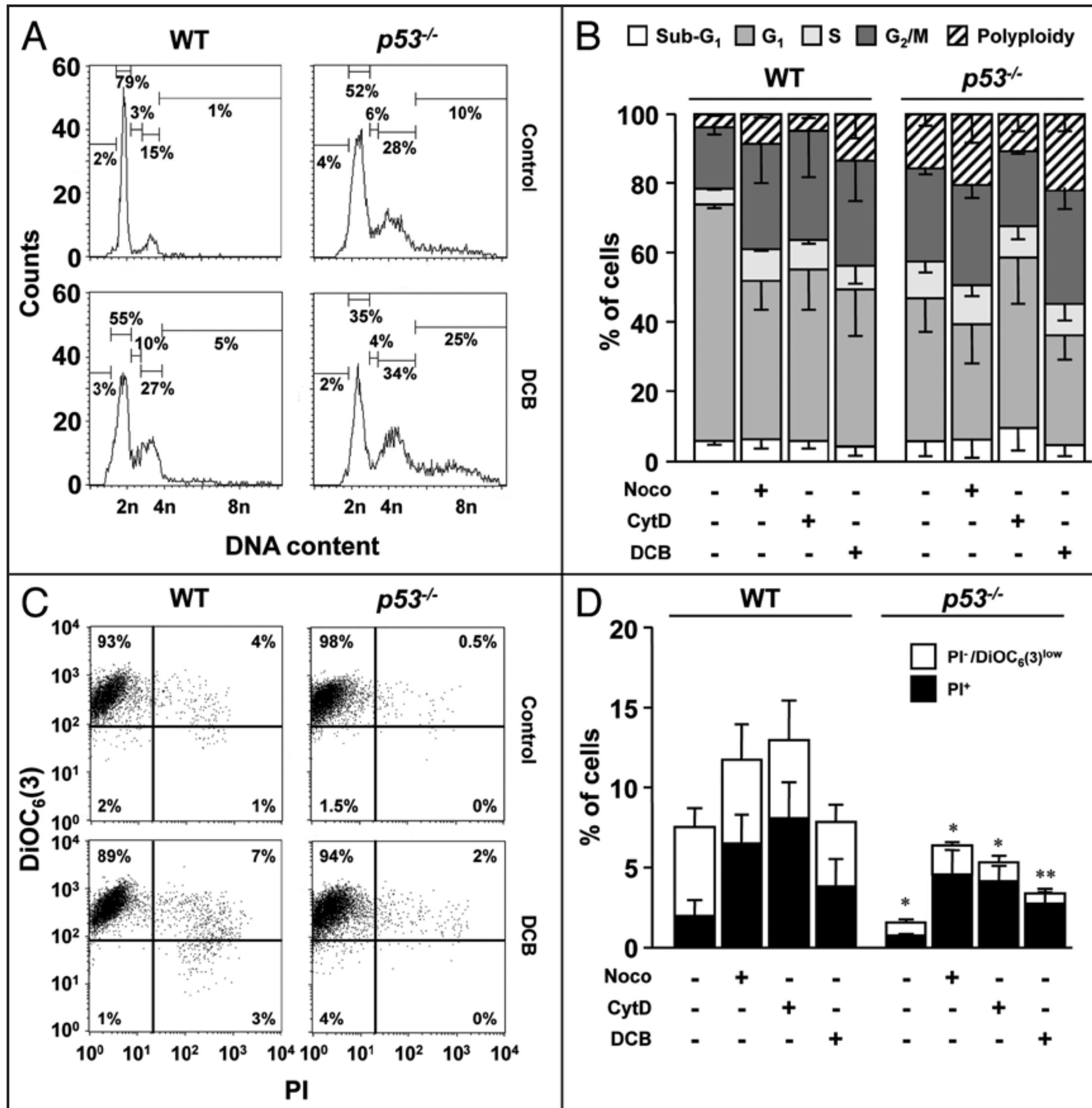

Figure 1. p53 modulates the mortality of mouse mammary epithelial cells (MMECs) induced by tetraploidization-inducing agents. MMECs from $p53^{+/+}$ (wild type, WT) and $p53^{-/-}$ animals were cultured for 48 h in the absence or in the presence of 100 nM nocodazole (Noco), 1 μM cytochalasin D (CytD), or 2 μM dihydrocytochalasin B (DCB), followed by FACS-assisted analysis of DNA content (A and B). In (A), representative cell cycle distributions are shown. From left to right, numbers indicate the percentage of cells characterized by sub-G_1, G_1, S, G_2/M and polyploid DNA content. (B) illustrates quantitative data (mean ± SEM, n = 5). As an alternative, MMECs were treated for 24 h with 100 nM Noco, 1 μM CytD or 2 μM DCB, and then stained with the vital dye propidium iodide (PI) and the mitochondrial transmembrane potential ($\Delta\Psi_m$)-sensitive fluorochrome $DiOC_6(3)$, to measure apoptosis-associated parameters (C and D). (C) illustrates representative dot plots, as obtained upon incubation of WT and $p53^{-/-}$ cells with DCB. Numbers indicate the percentage of cells found in each quadrant. In (D), black and white columns illustrate the percentage of dead (PI^+) an dying ($PI^-/DiOC_6(3)^{low}$) cells, respectively. Asterisks indicate statistically significant difference between WT and $p53^{-/-}$ cells (paired Student's t test, *$p < 0.05$; **$p < 0.01$).

Materials and Methods

Mice cross-breeding and genotyping. C57/Bl6 mice with a heterozygous deletion in the *p53* gene[26] (CNRS UMR6218, Orleans, France) were cross-bred to obtain $p53^{+/+}$ (wild type, WT) and $p53^{-/-}$ animals. F_1 mice were genotyped by standard PCR procedures[26] based on the following primers: p53-fwd (5'-ACA GCG TGG TGG TAC CTT AT-3'), p53-rev (5'-TAT ACT CAG AGC CGG CCT-3') and pNeo-fwd (5'-CTA TCA GGA CAT AGC GTT GG-3'). Mice were maintained in pathogen-free conditions and all experiments followed the FELASA guidelines.

Chemicals, isolation of primary cells and culture conditions. Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, USA), while culture media and supplements for cell culture from Gibco-Invitrogen (Carlsbad, USA). The isolation of mammary gland primary epithelial cells was performed on 8–9 week old female mice, as described by Fujiwara et al.[7] with slight modifications. Quickly after sacrifice, inguinal mammary glands were surgically removed and washed in DMEM/F12 medium supplemented with 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate and 100 μg/mL gentamycin sulfate. Thereafter, they were dissociated by incubation (30 min with

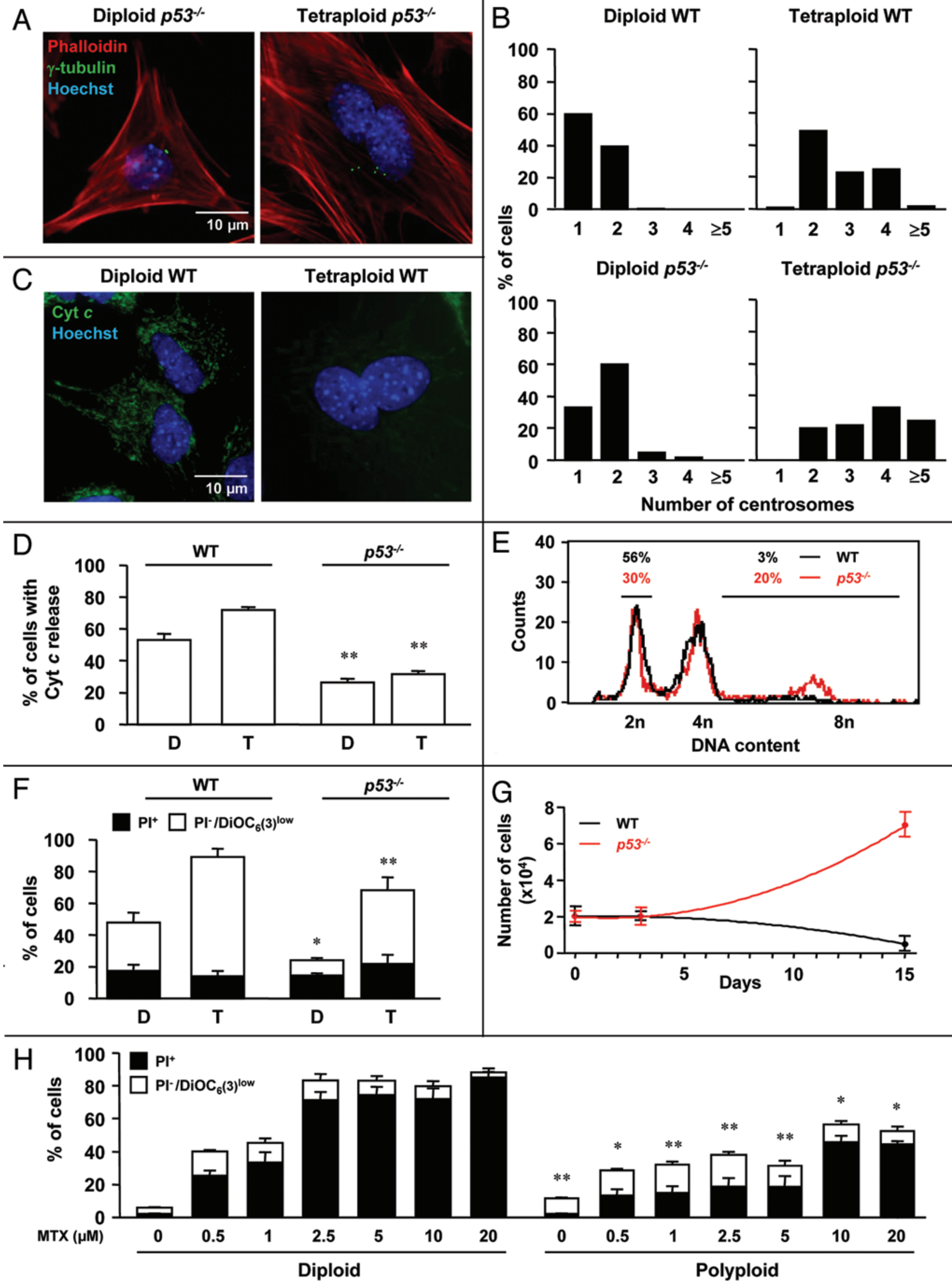
A
Diploid p53-/-
Tetraploid p53-/-
Phalloidin
γ-tubulin
Hoechst
10 μm
B
Diploid WT
Tetraploid WT
Diploid p53-/-
Tetraploid p53-/-
% of cells
Number of centrosomes
C
Diploid WT
Tetraploid WT
Cyt c
Hoechst
10 μm
D
WT
p53-/-
% of cells with Cyt c release
D
T
E
56%
30%
3%
20%
WT
p53-/-
Counts
2n
4n
8n
DNA content
F
WT
p53-/-
PI+
PI-/DiOC6(3)low
% of cells
G
WT
p53-/-
Number of cells (x10⁴)
Days
H
PI+
PI-/DiOC6(3)low
% of cells
MTX (μM)
Diploid
Polyploid

Figure 2. (A–D) Fate of tetraploid cells. Wild type (WT) and *p53*$^{-/-}$ mouse mammary epithelial cells (MMECs) were treated with 2 μM dihydrocytochalasin B (DCB) for 48 h and then processed for the immunofluorescence microscopy-assisted observation of centrosomes (with an antibody that recognizes γ-tubulin, in green) and actin filaments (with fluorescent phalloidin, in red) (A and B). As an alternative, cells were processed to visualize chromatin (with Hoechst 33342, emitting in blue) and to evaluate the subcellular distribution of cytochrome *c* (Cyt *c*, green fluorescence) (C and D). (A and C) report representative microphotographs. White bars indicate picture scale (10 μm). In (B), columns depict the percentage of cells characterized by the indicated number of centrosomes (mean, n = 200 cells/condition). In (D), columns depict the percentage of cells (mean ± SEM, n = 3) exhibiting a diffuse (as opposed to punctuate) Cyt *c* signal, which is indicative of mitochondrial outer membrane permeabilization. Asterisks indicate significant differences between WT and *p53*$^{-/-}$ cells (paired Student's t test, $^{**}p < 0.01$). (E–G) Long-term effect of p53 on the survival of tetraploid cells. Cells with the indicated genotype were cultured for 48 h in the presence of DCB, followed by chromatin staining with Hoechst 33342, FACS purification of diploid (2n) and tetraploid (>4n) populations (E), and culture for 15 days in standard culture conditions (F and G). In (E), numbers indicate the percentage of WT and *p53*$^{-/-}$ cells that were characterized by the indicated ploidy (as depicted by the horizontal bars) at the moment of sorting. In (F), black and white columns illustrate the percentage of dead (PI^+) an dying ($PI^-/DiOC_6(3)^{low}$) cells, respectively. Asterisks depict statistical significant differences between WT and *p53*$^{-/-}$ cells (paired Student's t test, $^{**}p < 0.01$). (G) illustrates the progression of WT and *p53*$^{-/-}$ cells as extrapolated from the number (mean ± SEM, n = 3) of viable (capable of excluding trypan blue) cells found in the cultures on the 3rd and 15th day after FACS-assisted purification. (H) Selective resistance of *p53*$^{-/-}$ tetraploid cells to mitoxantrone (MTX). Diploid or tetraploid *p53*$^{-/-}$ cells were treated for 48 h with the indicated dose of MTX, and then labeled with the vital dye propidium iodide (PI) plus the mitochondrial transmembrane potential ($\Delta\Psi_m$)-sensitive fluorochrome $DiOC_6(3)$. Black and white columns depict the percentage of dead (PI^+) an dying ($PI^-/DiOC_6(3)^{low}$) cells, respectively. Asterisks indicate statistically significant difference between WT and *p53*$^{-/-}$ cells (paired Student's t test, $^{*}p < 0.05$; $^{**}p < 0.01$).

occasional vigorous shaking, 37°C) in wash medium further supplemented with 0.15% type A collagenase (Roche Diagnostics GmbH, Mannheim, Germany). Dissociated cells were washed 4 times in PBS containing 5% fetal bovine serum (FBS), and eventually seeded in culture flasks that had been pre-coated with 0.1% gelatin (4 h, 37°C). Mammary gland primary epithelial cells were maintained in culture (37°C, 5% CO_2) for no more than 6 passages, in DMEM/F12 medium supplemented with 2% FBS, 50 μg/mL gentamycin sulfate, 100 μg/mL insulin and 5 ng/mL epithelial growth factor (EGF). Tetraploidization was obtained by cultivating cells for 24–48 h in the presence of either 100 nM nocodazole (Noco), 1 μM cytochalasin D (Cyt D), or 2 μM dihydrocytochalasin B (DCB), as previously reported.[9] To induce apoptosis, cells were treated for 48 h with 0.5–20 μM mitoxantrone (MTX).

Immunofluorescence microscopy. Immunofluorescence microscopy determinations were performed as previously reported.[10,27] In brief, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% sodium dodecylsulfate and then stained with Alexa Fluor® 568-conjugated phalloidin (Molecular Probes-Invitrogen), which binds to actin, or with antibodies that specifically recognize cytochrome *c* (mouse monoclonal, #556432, BD Pharmingen, San Diego, USA) or γ-tubulin (rabbit polyclonal, #T3559, Sigma-Aldrich), according to the manufacturers' instructions. Primary antibodies were revealed with anti-mouse and anti-rabbit secondary antibodies coupled to the Alexa Fluor® 488 fluorochrome (Molecular Probes-Invitrogen). Nuclear counter-staining was achieved with 10 μM Hoechst 33342 (Molecular Probes-Invitrogen), and slides were observed on a DMIR2 inverted fluorescence microscope equipped with a CoolSnap EZ CCD camera (Roper Scientific GmbH, Friedland, Germany) and MetaVue™ software (Molecular Devices, Downingtown, USA).

Cytofluorometry and cell sorting. For cell cycle analysis, cells were fixed in 80% ice-cold ethanol and labeled with propidium iodide (PI, 50 μg/mL) in the presence of 500 μg/mL RNAse.[28,29] To measure apoptosis, unfixed cells were co-stained with 40 nM 3,3'dihexiloxalocarbocyanine iodide ($DiOC_6(3)$, from Molecular Probes-Invitrogen), which quantifies the mitochondrial transmembrane potential ($\Delta\Psi_m$), plus 1 μg/mL PI, for the identification of plasma membrane breakdown.[16,17,30] Cytofluorometric determinations were performed by means of a FACScan cytofluorometer (Becton Dickinson, San Jose, USA), equipped with the analytical software Cell Quest. As an alternative, living cells were labeled with 10 μM Hoechst 33342 (Molecular Probes-Invitrogen) for 40 min at 37°C, and then sorted into two distinct populations according to DNA content (2n vs. >4n), by means of a FACS Vantage cell sorter (Becton Dickinson).

Statistical procedures. Experiments were performed at least three times. The result section reports data from all experiments of the same kind (mean ± SEM, n = 3–9). Data were analyzed on Microsoft Excel (Microsoft Co., Redmond, USA), and statistical significance was assessed by means of the Student's t test.

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