Multipolar mitosis of tetraploid cells: inhibition by p53 and dependency on Mos

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Tetraploidy can constitute a metastable intermediate between normal diploidy and oncogenic aneuploidy. Here, we show that the absence of p53 is not only permissive for the survival but also for multipolar asymmetric divisions of tetraploid cells, which lead to the generation of aneuploid cells with a near-to-diploid chromosome content. Multipolar mitoses (which reduce the tetraploid genome to a sub-tetraploid state) are more frequent when p53 is down-regulated and the product of the *Mos* oncogene is upregulated. *Mos* inhibits the coalescence of supernumerary centrosomes that allow for normal bipolar mitoses of tetraploid cells. In the absence of p53, *Mos* knockdown prevents multipolar mitoses and exerts genome-stabilizing effects. These results elucidate the mechanisms through which asymmetric cell division drives chromosomal instability in tetraploid cells.

The EMBO Journal (2010) 29, 1272–1284. doi:10.1038/emboj.2010.11; Published online 25 February 2010

Subject Categories: genome stability & dynamics

Keywords: aneuploidy; apoptosis; centrosome; colon carcinoma; mitotic catastrophe

Introduction

Tetraploid cells are detected in some precancerous lesions such as Barrett's oesophagus and cervical dysplasia, where their presence coexists with the loss of functional p53 (Heselmeyer *et al*, 1996; Maley *et al*, 2004). Owing to the increase in the number of chromosomes, perhaps coupled to changes in the geometry of the mitotic machinery (Storchova *et al*, 2006; Storchova and Kuffer, 2008), tetraploid cells

frequently activate the DNA damage response and become genomically unstable. Thus, tetraploidy may be considered as a metastable state that links normal diploidy to cancer-associated aneuploidy (Storchova and Pellman, 2004; Fujiwara *et al*, 2005; Margolis, 2005).

Numerous tumour suppressor genes including *p53* (Margolis, 2005), *BRCA1* (Schlegel *et al*, 2003), *LATS2* (Aylon *et al*, 2006) and *APC* (Tighe *et al*, 2004) actively repress tetraploidy, meaning that their removal can either stimulate the spontaneous tetraploidization of cells or facilitate the survival of tetraploid cells generated upon cytokinesis or karyokinesis inhibition. The former has been shown for the knockdown of *APC* in cultured cells and for the conditional knockout of *APC* in small intestine epithelia *in vivo* (Caldwell *et al*, 2007). The latter has been demonstrated in cultured cancer cell lines that were depleted from p53 by gene knock-out or RNA interference (Cross *et al*, 1995; Andreassen *et al*, 2001; Castedo *et al*, 2006a, 2006b), as well as in *p53*^{−/−} primary mouse mammary epithelial cells (Fujiwara *et al*, 2005; Senovilla *et al*, 2009). Moreover, the expression of some oncogenes including *Myc* (Yin *et al*, 1999), *Aurora-A* (Wang *et al*, 2006) and human papillomavirus (HPV)-encoded *E6* (Incassati *et al*, 2006) can stimulate tetraploidization.

The mechanisms through which tetraploidy favours oncogenesis are complex and have not yet been entirely elucidated. One single tetraploid cell can undergo multipolar mitosis, which often leads to the generation of three or more daughter cells (Storchova and Pellman, 2004). This process causes the near-to-stochastic distribution of chromosomes and hence is lethal for most daughter cells. Nullisomy (the total absence of one particular chromosome) and polysomy (the presence of extra copies of one chromosome), indeed, result in major genetic defects involving the incorrect assembly of multiprotein complexes and fatal linkage disequilibria, which are rarely compatible with cell survival (Zhivotovsky and Kroemer, 2004; Roumier *et al*, 2005; Ganem *et al*, 2007). Moreover, during mitosis, the presence of more than two centrosomes in tetraploid cells can also favour merotelic chromosome attachments and hence chromosomal lagging, which may favour chromosome loss or asymmetric distribution among daughter cells, even when the division is bipolar (Ganem *et al*, 2009).

Multipolar and asymmetric cell division, as they can result from tetraploidy (Levine *et al*, 1991; Ganem *et al*, 2007), are commonly observed in malignant lesions and have been suspected to contribute to oncogenesis for over a century (Boveri, 2008). Supernumerary centrosomes, as they are detected in malignant cells (Levine *et al*, 1991; D'Assoro *et al*, 2002), can be induced experimentally, and this reportedly suffices to trigger oncogenesis (Basto *et al*, 2008; Gergely and Basto, 2008). Moreover, 'anisocytosis' and 'anisokaryosis' (heterogeneity in cell size and nuclear size, respectively), which presumably result from asymmetric divisions, are well-established histological hallmarks of malignancy (Boveri, 2008; Holland and Cleveland, 2009). It is interesting

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Received: 19 August 2009; accepted: 22 January 2010; published online: 25 February 2010

to note that in some cancer types (e.g., non-small cell lung cancer), these morphological traits of malignancy correlate with the expression of one particular oncogene, *Mos* (Gorgoulis *et al*, 2001).

Mos (also called *c-Mos*) is the first human oncogene cloned, and has been identified as the cellular homologue of the viral oncogene *v-Mos*, which is encoded by the Moloney murine sarcoma virus (Oskarsson *et al*, 1980; Watson *et al*, 1982). The $p39^{\text{Mos}}$ protein (hereafter referred to as *Mos*) has been shown to stimulate the transformation of murine fibroblasts *in vitro* (Okazaki and Sagata, 1995; Fukasawa and Vande Woude, 1997). Moreover, transfection-enforced overexpression of *Mos* reportedly inhibits mitotic progression (Wang *et al*, 1994) and causes the generation of binucleated cells due to the inhibition of cytokinesis (Okazaki *et al*, 1992; Fukasawa and Vande Woude, 1995). Apparently, *Mos* can stabilize another oncogene product, c-Fos, (Okazaki and Sagata, 1995) and enhance the expression of cyclins (Rhodes *et al*, 1997), thereby stimulating cell proliferation. The genetic invalidation of *Mos* has no obvious phenotypic consequences in mice (Colledge *et al*, 1994; Hashimoto *et al*, 1994). However, although *Mos*^{-/-} male mice exhibit normal reproduction rates, *Mos*^{-/-} female are nearly infertile (Colledge *et al*, 1994; Hashimoto *et al*, 1994), in line with the fact that *Mos* is strictly necessary for the first meiotic division of oocytes (Sagata *et al*, 1989a), and then exerts a critical checkpoint function during metaphase II (Sagata *et al*, 1989b). Both the meiosis-regulatory and the transforming effects of *Mos* require its serine-threonine kinase activity (Haccard *et al*, 1993; Okazaki and Sagata, 1995). Known *Mos* substrates include cyclin B2, tubulin and MEK1 (Roy *et al*, 1990; Zhou *et al*, 1991; Sagata, 1997), and the meiotic checkpoint function of *Mos* depends on its capacity to activate the mitogen-activated protein kinase (MAPK) pathway (Haccard *et al*, 1993). Thus, very little is known about the role of *Mos* in somatic cells and on the mechanisms by which *Mos* can act as an oncogene.

Here, we developed a cellular model of aneuploidization in which *p53*^{-/-} cells were driven into tetraploidy, which was followed by multipolar mitosis and re-acquisition of a near-to-diploid chromosome content. We found that *Mos* was upregulated in *p53*-deficient tetraploid cells and that it was strictly required for the occurrence of multipolar divisions, presumably because *Mos* acts as an inhibitor of centrosome coalescence.

Results and discussion

Generation of sub-tetraploid derivatives from *p53*-deficient tetraploid cells

Shortly (2 days) after a 48 h-long treatment with the microtubule poison nocodazole or the cytokinesis inhibitor cytochalasin D, *p53*^{-/-} human colon carcinoma HCT 116 cell cultures contained a higher fraction of polyploid cells (with a $\geq 4n$ DNA content), yet a lower amount of dying and dead cells (exhibiting the dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the breakdown of plasma membranes, respectively) (Kroemer *et al*, 2007; Galluzzi *et al*, 2009) than their *p53*-proficient counterparts (Figure 1A and Supplementary Figure S1). Moreover, fluorescence-activated cell sorter (FACS)-purified cells with an $\sim 8n$ DNA content formed colonies more efficiently when they did not express *p53* than when they did so (Figure 1B), in line with the notion

that *p53* deficiency is permissive for the generation and survival of tetraploid cells (Castedo *et al*, 2006b). Cultures derived from FACS-purified cells with an $\sim 8n$ DNA content were characterized by a $4n$ DNA content in the G_1 phase of the cell cycle and by an $8n$ DNA content in the G_2 and M phases, thereby exhibiting *bona fide* traits of tetraploidy. However, after several passages, *p53*^{-/-} (but neither *p21*^{-/-} nor *Bax*^{-/-}) cultures progressively accumulated a population of cells with a $\sim 2n$ DNA content (Figure 1C and D). This was observed in six independent experiments in which the initial contamination with $\sim 2n$ cells (measured immediately after FACS purification) was undetectable. To understand the origin of such $\sim 2n$ population, we followed the fate of tetraploid cells 1 week after their generation by videomicroscopy, and found that *p53*^{-/-} cells underwent multipolar (mostly tri- or tetrapolar) divisions—which are associated with Y- and X-shaped metaphases (Figure 2A)—much more frequently than *p53*^{+/+} control cells (Figure 2B and Supplementary Videos 1 and 2). These results suggest that cells with a $\sim 2n$ DNA content (hereafter referred to as ‘sub-tetraploid’) appearing at significant frequencies ($\geq 10\%$), as early as 15 days after tetraploidization, might result from a peculiar process of multipolar division. A significant percentage of daughter cells that originated from *p53*^{-/-} tetraploid cells by multipolar mitosis could enter and terminate normal bipolar divisions (Figure 2C and D, and Supplementary Video 3), suggesting that such sub-tetraploid cells can give rise to a new lineage. The reduction of the chromosomal content was significantly more frequent among *p53*^{-/-} tetraploid cells than among control ones (Figures 1 and 2), and, in another cell line, was exacerbated by pharmacological inhibition of *p53* by cyclic pifithrin- α (Komarov *et al*, 1999) (Supplementary Figure S2). Thus, the tumour suppressor *p53* reduces the probability of sub-tetraploidy.

Sub-tetraploidy is linked to centrosome defects and aneuploidy

Next, we generated multiple tetraploid clones from isogenic HCT 116 cells. As compared with wild type (WT), *Bax*^{-/-}, *p21*^{-/-} or apoptosis-resistant (owing to the expression of the caspase inhibitor p35 from Baculovirus) tetraploid cells, *p53*^{-/-} tetraploid clones displayed some difficulties in maintaining a stable tetraploid genome. Four weeks after cloning, indeed, only one-third of *p53*^{-/-} tetraploid clones still exhibited a clean tetraploid DNA content profile, whereas the other two-thirds accumulated viable sub-tetraploid populations (Figure 3A and B), first as rather broad shoulders (‘phase 1’) and later as sharper peaks (‘phase 2’) of sub-tetraploid cells. Such sharper peaks presumably arise in cultures that are dominated by one or few viable sub-clones with a comparable sub-tetraploid DNA content. Phase 1 and phase 2 unstable sub-tetraploid populations also arose after repeated transfection (approximately once every 5 days) with a small interfering RNA (siRNA) targeting *p53* (Supplementary Figure S2). Chromosome counting confirmed that phase 2 unstable *p53*^{-/-} tetraploid cells frequently contained a roughly diploid number of chromosomes (Figure 3C and D). Phase 1 unstable *p53*^{-/-} tetraploid cells were characterized by a high frequency of aberrant mitoses that were either monopolar, bipolar characterized by lagging chromosomes or multipolar linked to supernumerary centrosomes. Multipolar mitoses were also more frequent among phase 2 unstable

Figure 1 Effect of p53 on the survival and on the genomic stability of tetraploid HCT 116 cells. (**A**, **B**) The absence of p53 increases the clonogenic survival of freshly generated polyploid cells. Wild type (WT), $p53^{-/-}$, $Bax^{-/-}$ and $p21^{-/-}$ diploid human colon carcinoma HCT 116 cells and HCT 116 cells stably transfected with a plasmid encoding the baculoviral inhibitor of caspases p35 (p35) were left untreated or treated with 100 nM nocodazole (Noco) for 48 h. After washing, cells were cultured for additional 48 h in drug-free culture medium, then stained with Hoechst 33342, followed by fluorescence-activated cell sorter (FACS) purification of diploid (2n, white and grey symbols for untreated and nocodazole-treated cells, respectively) or polyploid ($>4n$, black symbols) cell populations. In **A**, representative cell cycle distributions of WT and $p53^{-/-}$ cells from one out of three independent experiments are shown. X-axis = Hoechst 33342 fluorescence (DNA content); Y-axis = cell number per channel (counts). In **B**, the results of clonogenic survival assays carried out on such FACS-purified cells are reported. The upper part shows representative pictures of colonies formed by WT and $p53^{-/-}$ cells as observed upon crystal violet staining 10 days after FACS purification. Columns depict the survival fraction (mean $\pm$ s.e.m., $n = 3$ parallel wells, normalized for plating efficiency and to control diploid cells) of the diploid and polyploid cell populations with the indicated genotype and corresponding to the FACS-purified populations represented in **A**. Asterisks indicate statistically significant differences as compared to WT tetraploid cells (Student's t -test, $P < 0.05$). (**C**, **D**) Genomic instability of viable polyploid cells. Diploid HCT 116 cells with the indicated genotype were treated for 48 h with nocodazole and tetraploid populations were FACS-purified as in **A** ($>4n$, black symbols). Cells then were cultured for the indicated number of days and analyzed for DNA content. Representative cell cycle profiles of WT and $p53^{-/-}$ tetraploid cells are shown in **C**, whereas quantitative data are reported in **D** (mean $\pm$ s.e.m., $n = 6$ independent determinations). In **C**, the percentages of cells with a sub-tetraploid DNA content (indicating chromosomal loss) are indicated. Statistical significance resulting from the comparison to WT cells is highlighted by asterisks (Student's t -test, $P < 0.05$).

tetraploids as compared to WT or stable $p53^{-/-}$ tetraploids (Figure 3E). As compared to phase 1 cells, phase 2 cells proliferated more quickly, and among them the sub-tetraploid population had shorter duplication times and shorter mitoses than the tetraploid one (Supplementary Figure S3). This explains the outgrowth of sub-tetraploid cells over their

tetraploid counterparts. When such sub-tetraploid cells were exposed to nocodazole, they could again tetraploidize and then revert once more to sub-tetraploidy, but this process of reversion was not accelerated (Supplementary Figure S4).

Fluorescence *in situ* hybridization (FISH) carried out during interphase proved that a large portion of sub-tetraploid

Figure 2 Multipolar divisions of *p53*-deficient tetraploid cells. Isogenic wild type (WT) and *p53*^{-/-} diploid HCT 116 cells expressing a histone 2B-green fluorescent protein (H2B-GFP) chimera were treated for 48 h with nocodazole and then cultured in drug-free medium for additional 48 h. Polyploid cells were fluorescence-activated cell sorter (FACS)-sorted as in Figure 1A (>4n, black symbols) and monitored by fluorescence videomicroscopy for 48 h. In **A** snapshots taken at the indicated time points exemplify the appearance of tripolar and tetrapolar mitosis, thus exhibiting the representative Y- and X-shaped metaphase, respectively. Full-length movies proving the completion of cell division are available as Supplementary Videos S1 and S2. **B** reports the percentage of cell cycle arrest, apoptosis induction and various mitotic aberrations as quantified among 150 to 200 mitoses for each genotype (mean ± s.e.m., *n* = 3 independent experiments). In **C** representative images captured at the indicated time points show that some daughter cells that originated from *p53*^{-/-} tetraploid HCT 116 cells by multipolar mitosis can enter and terminate normal bipolar divisions (red arrows), whereas others undergo apoptosis (yellow arrows). A full-length movie that demonstrates the completion of a normal cell division by such a daughter cell is available as Supplementary Video S3. **D** reports the percentage of these cells (arisen from *p53*^{-/-} tetraploid HCT 116 cells by multipolar mitosis) that underwent the indicated fate by the end of the experiment (as quantified among 150-200 daughter cells, mean ± s.e.m., *n* = 3 independent experiments). Note that only cells entering multipolar mitosis within the first 18 h of the assays were tracked for the subsequent 30 h. A full-colour version of this figure is available at *The EMBO Journal* Online.

cells (which were FACS-purified from unstable *p53*^{-/-} tetraploid clones) was aneuploid (Figure 4A-C). Thus, especially in phase 1 cultures, nullisomies (which are always lethal) were frequently detected (Figure 4C). Accordingly, most (>99%) of such phase 1 sub-tetraploid cells failed to form stable offspring in clonogenic assays and died (Figure 4D). In phase 2 cultures, the frequency of aneuploid cells was

lower, and nullisomies were infrequent (Figure 4C), presumably because viable cells (which efficiently form clones, Figure 4D) had been positively selected. To further explore the behaviour of sub-tetraploid cells, we generated phase 1 and phase 2 clones from *p53*^{-/-} tetraploid cells expressing histone H2B fused to the N-terminus of green fluorescent protein (H2B-GFP, which labels chromatin in green and

Figure 3 Chromosome instability and centrosome amplification in $p53^{-/-}$ tetraploid HCT 116 clones. (A, B) $p53$ deficiency increases the percentage of unstable tetraploid clones. Tetraploid HCT 116 clones were generated from wild type (WT), $p53^{-/-}$, $Bax^{-/-}$, $p21^{-/-}$ and $p35$ -expressing parental cells as described in Supplementary Data. Cell cycle distribution and apoptosis-related parameters were evaluated 4 weeks after cloning by multiparametric cytofluorometry upon staining with Hoechst 33342 (which measures DNA content), the mitochondrial transmembrane potential ($\Delta\Psi_m$)-sensitive dye 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3)) and propidium iodide (PI, an exclusion dye that only stains dead cells). Representative plots are shown in A, the inserts therein showing positive controls for $\Delta\Psi_m$ dissipation (as obtained by treating the cells for 30 min with 100 μ M protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, FCCP) and plasma membrane permeabilization (as resulting from a 2 min-long incubation in 0.5% (w/v) saponin (Sapo)). Tetraploid clones were classified according to genomic instability (as evaluated by the quantification of viable sub-tetraploid populations) in stable (sub-tetraploid cells < 10%) and unstable (sub-tetraploid cells > 10%). Unstable clones were subdivided into 'phase 1' and 'phase 2' clones, characterized by broad and sharp sub-tetraploid peaks, respectively. B reports the frequency of unstable clones generated from parental tetraploid cells of the indicated genotype. Frequency was calculated among 50–100 clones for each genotype (mean $\pm$ s.e.m., $n = 3$ independent series of clones). (C, D) Chromosome count in tetraploid clones. Representative examples of 4',6 diamidino-2-phenylindole (DAPI)-stained metaphase spreads with the corresponding number of chromosomes are shown in C. For WT and $p53^{-/-}$ clones of the indicated type, D reports the percentage of cells containing 40–60, 61–80, 81–100 or > 101 chromosomes, as quantified among 100 metaphases per condition (mean $\pm$ s.e.m., $n = 4$ different clones in independent assessments) (E) Centrosome amplification correlates with genomic instability. WT and $p53^{-/-}$ clones of the indicated class were cultured on glass coverslips and subjected to immunofluorescence detection of mitotic spindles (β -tubulin staining, green fluorescence) and centrosomes (γ -tubulin staining, red fluorescence). Nuclei were counterstained with Hoechst 33342 (emitting in blue). According to the number of centrosomes, interphase cells were divided in normal (1 or 2 centrosomes) and abnormal (more than 2 centrosomes, either congressed in a single pole or not), whereas metaphases were classified as monopolar (2 congressed centrosomes), normal and abnormal bipolar (2 centrosomes, the latter exhibiting the misalignment of one or more chromosomes), and multipolar (tripolar, tetrapolar or of higher-order polarity, characterized by 3, 4 or more centrosomes, respectively). Representative immunofluorescence microscopy images of each category are shown. The percentage of occurrence of each category is reported, as quantified among 150 to 200 cells for WT and $p53^{-/-}$ clones of the indicated type (mean $\pm$ s.e.m., $n = 4$ distinct clones). A full-colour version of this figure is available at *The EMBO Journal Online*.

hence allows for monitoring chromosome movement), and followed the fate of their sub-tetraploid derivatives after FACS purification (Figure 4E, F and Supplementary Figure S5, and Supplementary Videos 4, 5 and 6). Although phase 1

sub-tetraploid cells rarely ($\sim 3\%$) engaged in subsequent cycles of division, phase 2 sub-tetraploid cells did so much more frequently ($\sim 60\%$). Nonetheless, cell cycle blockade, mitosis without cytokinesis and cell death occurred more

Figure 4 Characterization and fate of the sub-tetraploid offspring of $p53^{-/-}$ tetraploid cells. (A–C) An important fraction of sub-tetraploid cells are aneuploid. Sub-tetraploid cells derived from phase 1 and 2 unstable $p53^{-/-}$ tetraploid HCT 116 clones or the 2n population of the parental $p53^{-/-}$ diploid cell line were fluorescence-activated cell sorter (FACS)-purified as indicated in A. Thereafter, nuclei from the sorted populations were subjected to fluorescence *in situ* hybridization (FISH) with probes specific for the centromeric region of chromosome 8, 10 and 18 (green, red and blue fluorescence, respectively). Representative immunofluorescence microphotographs are shown in B. In C the percentage of wild type (WT) diploid or $p53^{-/-}$ sub-tetraploid cells characterized by the indicated chromosomal setup is reported, as scored among ≥ 100 metaphases for each cell type (mean $\pm$ s.e.m., $n = 3$ independent determinations). Alternatively, FACS-purified cells obtained as in A were subjected to clonogenic survival assays (D). Columns depict the survival fraction (mean $\pm$ s.e.m., $n = 3$ independent experiments carried out in triplicate with at least three distinct clones for each type) of WT diploid or sub-tetraploid cells derived from $p53^{-/-}$ tetraploid clones of the indicated class. (E, F) Videomicroscopy of sub-tetraploid cells. $p53^{-/-}$ diploid cells and tetraploid HCT 116 clones expressing a histone 2B-green fluorescent protein chimera (H2B-GFP) were subjected to FACS-purification of diploid or sub-tetraploid populations as in A. Purified cells with a $\sim 2n$ DNA content were then monitored by fluorescence videomicroscopy for 40 h. Representative snapshots taken at the indicated time points illustrate sub-tetraploid cells that undergo two consecutive rounds of symmetric division (example 1), divide into two daughter cells that die by apoptosis (example 2), or undergo mitosis followed by incomplete cytokinesis, resulting in increased ploidy (example 3). Full-length movies can be found in Supplementary Videos S4–S6. F provides a graphic representation and quantitative data (% , mean $\pm$ s.e.m., $n = 3$ independent experiment, 100–150 cells analyzed) about the fate of WT diploid or $p53^{-/-}$ sub-tetraploid cells derived from clones of the indicated type. Only cells entering mitosis within the first 10 h were tracked for subsequent 30 h. Please note that cytokinesis failure (leading to an increase in ploidy as well as in cell volume) is depicted with a duplication in bar thickness. The entire catalogue of the observed cell fates is available in Supplementary Figure S5.

frequently among sub-tetraploid cells than among their diploid progenitors (Figure 4E, F and Supplementary Figure S5, and Supplementary Videos 4, 5 and 6).

It is important to note that upon inoculation into immunodeficient (*nu/nu*) mice, tetraploid cells FACS-purified from unstable tetraploid clones (and in particular established phase 2 cultures) formed tumours more rapidly than stable

($p53^{-/-}$ and $p53^{+/+}$) tetraploid cells (Figure 5A), corroborating the idea that multipolar divisions might favour the selection of aggressive tumour variants. After *in vivo* selection, sub-tetraploid tumour cells could be recovered at similar frequencies as after an equivalent period of *in vitro* culture (Figure 5B and C) and were particularly frequent among tumours that arose from unstable phase 2 clones. Thus,

Figure 5 Phase 2 unstable $p53^{-/-}$ tetraploid clones form aggressive tumours. (A) Nude mice were subcutaneously xenografted with similar numbers of wild type (WT) or tetraploid cells that were fluorescence-activated cell sorter (FACS)-purified from $p53^{-/-}$ tetraploid clones of the indicated type (stable, phase 1 or 2 unstable). Tumour volumes (mean $\pm$ s.e.m., $n = 10$ mice for each clone type) were monitored over time. (B, C) Tumour cells were recovered from mice (*in vivo*) and the DNA-content was assessed by propidium iodide (PI) staining and cytofluorometry. The same was done on aliquots of the FACS-purified parental cells that were kept frozen or that were cultured *in vitro* for the same time as tumours proliferated *in vivo*. B reports representative cell cycle distributions of tumour cells of the indicated type upon recovery from mice. Percentages refer to the sub-tetraploid populations. C depicts the percentage of sub-tetraploid cells (mean $\pm$ s.e.m., $n = 3$ independent assessments) observed for the indicated cell type.

it appears that the tetraploidization of $p53^{-/-}$ cells can accelerate tumour progression in the context of the emergence of a sub-tetraploid cancer cell population arising from multipolar mitoses.

Obligate contribution of Mos to the generation of sub-tetraploid cells

Tetraploidization has been associated with the overexpression of meiosis-specific proteins, in particular Mos (Kalejs *et al*, 2006). Accordingly, we observed an increase in Mos protein levels that was particularly pronounced in unstable

clones (as compared with stable ones) (Figure 6A). We then assessed whether Mos overexpression (as induced by transfection with a polycistronic vector encoding Mos and GFP) might modify the behaviour of stable tetraploid cells. Although Mos-transfected $p53^{+/+}$ were unable to proliferate (Fukasawa and Vande Woude, 1997; Kalejs *et al*, 2006), $p53^{-/-}$ tetraploid cells survived Mos overexpression while exhibiting an unstable phenotype, associated with the gradual increase of sub-tetraploid cells in the cultures (Figure 6B and Supplementary Figure S6). Accordingly, depletion of Mos with three different siRNAs had the opposite effect on unstable tetraploid cells and reduced their propensity to generate sub-tetraploid offspring (Figures 6C and Supplementary Figure S6). Such Mos-specific siRNAs had no anti-proliferative effects, ruling out a trivial explanation for their tetraploidy-stabilizing activity (Supplementary Figure S7). Moreover, retransfection of unstable tetraploid cells with a polycistronic construct coding for a non-interferable Mos mutant and GFP annihilated the genome-stabilizing effect of one of the Mos-specific siRNAs, formally excluding off-target effects of this siRNA (Figure 6D). These results could be recapitulated in a completely different experimental system, namely WT HCT 116 cells subjected to repetitive transient transfections with a $p53$ -specific siRNA alone or in combination with a Mos-specific siRNA. In this setting, the emergence of a sub-tetraploid population resulting from recurring depletion of $p53$ was prevented when Mos was concomitantly downregulated (Figure 6E).

In line with reports indicating that Mos can associate with centrosomes (Wang *et al*, 1994; Doxsey *et al*, 2005; Nogales-Cadenas *et al*, 2009), we found that a Mos-GFP fusion protein localized to centrosomes in HCT 116 cells (Figure 7A and Supplementary Figure S8). To control specificity, we demonstrated that the expression of Mos-GFP (but not of GFP alone) was abolished by a Mos-specific (but not by a control) siRNA (Supplementary Figure S9). Therefore, we investigated whether Mos might influence centrosome dynamics, and in particular whether Mos might affect centrosome clustering, one mechanism relying on the molecular motor HSET that allows for the maintenance of tetraploid genomes (Kwon *et al*, 2008). Upon siRNA-mediated knockdown of HSET (Supplementary Figure S10), stable $p53^{-/-}$ tetraploid cell cultures progressively accumulated a sub-tetraploid population and this effect was not counteracted by the simultaneous knockdown of Mos (Figure 7B). In phase 1 unstable $p53^{-/-}$ tetraploid cells, Mos depletion limited the generation of a sub-tetraploid offspring (Figure 7B) while resulting in the conversion of multipolar to bipolar mitoses, in which supernumerary centrosomes either congressed to two centrosome pairs or lost their connection to the β -tubulin network and were inactivated (Figure 7C, D and Supplementary Figure S8). In this setting, HSET depletion lead to an increase in multipolar divisions paralleled by a decrease in bipolar mitoses characterized by centrosome coalescence (Figure 7D), correlating with a further increase in the generation of sub-tetraploid cells (Figure 7B). These effects were reduced by the simultaneous depletion of Mos (Figure 7B and D), in line with the hypothesis that Mos modulates centrosome dynamics, thereby affecting the frequency of multipolar mitoses in tetraploid cells. To directly evaluate the putative effects of Mos on centrosome clustering, we transiently transfected a (phase 2) unstable $p53^{-/-}$ tetraploid

Figure 6 Correlation between Mos expression and genomic instability in p53^{-/-} tetraploid clones. (A) Mos is upregulated in unstable tetraploid clones. Protein extracts obtained from wild type (WT) or p53^{-/-} clones of the indicated type (S = stable, P1 = phase 1, P2 = phase 2), were subjected to immunoblotting with antibodies that specifically recognize Mos or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, monitored as a loading control). (B) Mos overexpression triggers genomic instability in stable tetraploid cells. Stable p53^{-/-} HCT 116 cells were transfected for 48 h with a polycystronic plasmid expressing green fluorescent protein (GFP) alone or co-expressing Mos and GFP. The GFP⁺ populations were fluorescence-activated cell sorter (FACS)-purified, cultured for 48 h, and then subjected to subsequent rounds of transfections with the same constructs at intervals of ~5 days. Columns depict the percentage of sub-tetraploid cells (mean ± s.e.m., n = 3 independent experiments) as assessed after one (I) or multiple (II, III) rounds of transfection. Representative cell cycle distributions as observed after three rounds of transfection are illustrated in Supplementary Figure S6. (C) Mos depletion stabilizes unstable p53^{-/-} tetraploid clones. Phase 1 unstable p53^{-/-} tetraploid HCT 116 cells were left untreated (—) or were transfected with a control (UNR) or Mos-silencing siRNAs for 72 h, followed by DNA content analysis. Representative cell cycle distributions are illustrated in Supplementary Figure S6. The percentage of sub-tetraploid cells (mean ± s.e.m., n = 5 independent experiments) was determined. The efficacy of the small interfering RNA (siRNA)-mediated downregulation of Mos was confirmed by immunoblotting (insert). (D) A non-interferable Mos variant restores genomic instability of originally unstable p53^{-/-} tetraploid cells stabilized by transfection with siRNAs that deplete endogenous Mos. Phase 1 unstable p53^{-/-} tetraploid HCT 116 cells were transfected with a control (UNR) or a Mos-depleting siRNA (Mos_1) for 24 h, followed by further transfection with a control vector encoding green fluorescent protein (GFP) alone or with constructs for the co-expression of GFP and wild type (Mos-GFP) or mutant non-interferable Mos (Mos^{mut}-GFP). After 48 h, Mos expression was controlled by immunoblotting (insert), and 24 h later the percentage of sub-tetraploid cells was quantified (mean ± s.e.m., n = 3 independent assessments). (E) Mos depletion avoids genomic instability caused by p53 knockdown. Wild type (WT) tetraploid HCT 116 cells were repeatedly (6 times) transfected with a control (UNR), a p53-specific (p53_2) and a Mos-specific (Mos_1) siRNA alone or in combination for 72 h, followed by DNA content analysis and quantification of sub-tetraploid cells. The histogram summarizes data (mean ± s.e.m.) from five independent experiments.

clone that had previously been engineered for the expression of GFP-H2B with a centrin-DsRed fusion-encoding construct, allowing for the simultaneous monitoring of chromosomal and centrosomal dynamics by videomicroscopy (Figure 7E). Cells that were left untreated or were transfected with a control siRNA frequently underwent multipolar divisions, which were characterized by the presence of more than two centrin-DsRed-positive dots. However, upon transfection with a Mos-specific siRNA the frequency of multipolar (or failed) divisions dropped (Figure 7E and F). In line with immunofluorescence microscopy results (Figure 7C, D and Supplementary Figure S8), we observed an increase in the incidence of bipolar divisions that exhibited centrosome

coalescence. Taken together, these data strongly suggest that the knockdown of Mos favours centrosome clustering, correlating with the suppression of multipolar mitosis.

Concluding remarks

On theoretical grounds, tetraploid cells may generate an aneuploid offspring by two non-exclusive mechanisms. First, as tetraploid cells contain supernumerary centrosomes, they may enter multipolar mitosis, thereby dividing into more than two (usually three or four) daughter cells provided with randomly distributed chromosomes. Most of these cells would be non-viable and would die during the subsequent interphase or after one additional round of (failing) mitosis.

Figure 7 Effects of Mos on centrosome coalescence in $p53^{-/-}$ tetraploid clones. (A) Mos localizes to centrosomes. Immunofluorescence staining for the detection of β - or γ -tubulin (both in red) in $p53^{-/-}$ tetraploid cells transfected with a plasmid for the expression of a Mos-green fluorescent protein (Mos-GFP) chimera. Nuclei were counterstained with Hoechst 33342 (emitting in blue). Yellow dots that result from the overlap of green and red fluorescence signals suggest that Mos and γ -tubulin colocalizes at centrosomes (arrows). Representative immunofluorescence microphotographs are shown for cells in interphase. Single-channel microphotographs are provided in Supplementary Figure S8. (B) Mos depletion partially reverts genomic instability induced by HSET downregulation. Stable and unstable $p53^{-/-}$ tetraploid HCT 116 clones were transfected with a control (UNR), a Mos-specific (Mos_1) or a HSET-specific (HSET_1) small interfering RNA (siRNA) alone or in combination for 72 h, followed by cytofluorometric analysis of DNA content and quantification of sub-tetraploid cells (mean $\pm$ s.e.m., $n = 3$ independent experiments). (C, D) Increased coalescence and inactivation of supernumerary centrosomes upon Mos depletion. Unstable $p53^{-/-}$ tetraploid HCT 116 cells were co-transfected with the indicated siRNA combinations for 72 h, and then subjected to immunofluorescence staining for the detection of β - and γ -tubulin (green and red fluorescence, respectively). Hoechst 33342 was employed for nuclear counterstaining. C shows representative immunofluorescence microphotographs of supernumerary centrosomes coalescing or being inactivated (c_i), which allows for the bipolar organization of spindles. Single-channel microphotographs are provided in Supplementary Figure S8. D depicts the percentage of metaphases (mean $\pm$ s.e.m., $n = 3$ independent experiments) characterized by the indicated mitotic spindle arrangements, as scored among at least 100 cells per for each condition. All experiments have been performed on at least three distinct clones of each type. (E, F) Live-cell imaging of the Mos effects on centrosomal and chromosomal dynamics. A (phase 2) unstable tetraploid $p53^{-/-}$ GFP-H2B-expressing clone was transiently transfected with a Mos-specific or a control siRNA (UNR) together with a construct coding for centrin-DsRed (E). E shows representative snapshots. Please note that bipolar divisions are characterized by the coalescence of DsRed-positive centrosomes in two clusters. Full-length movies can be found in Supplementary Videos S7 and S8. Hatched lines indicate the position of daughter cells arising from the observed mitoses. The frequency of multipolar divisions and failed cytokineses observed by live-cell imaging is reported in F (mean $\pm$ s.e.m., $n = 3$ independent experiments).

However, in exceptional circumstances, the overall genome composition of one daughter cell may be compatible with survival, and rare, particularly fit cells would then out compete their tetraploid parent, as it has been observed in cell cultures and in tumours. Alternatively, tetraploid cells

might lose single (or few) chromosomes because of their merotelic attachment, which results from a 'multipolar spindle intermediate'. In this case, the presence of supernumerary centrosomes enforces an initial multipolar metaphase followed by the clustering of all centrosomes in two poles,

which results in a bipolar division, whereby the chromosome that is merotelically attached lags during anaphase and eventually is either lost or distributed into the wrong daughter cell. This mechanism might give rise to a gradual, progressive loss of chromosomes until the achievement of a sub-tetraploid stage.

Irrespective of the molecular details underlying the aneuploidization of tetraploid cells, it appears that centrosome clustering constitutes a prime mechanism for the maintenance of tetraploid genomes. Supernumerary centrosomes found in tetraploid cells would indeed cause multipolar division or merotelic chromosome attachment (and hence chromosome loss), unless they aggregate in two discrete pools and form two microtubule-organizing centres. This aggregation is under the control of molecular motors including HSET, and HSET depletion indeed compromises the stability of tetraploid cells (Kwon *et al*, 2008). As shown here, overexpression of Mos that occurs in *p53*^{-/-} tetraploid cells also inhibits centrosome clustering, thereby favouring multipolar divisions and resulting in the reduction of the tetraploid genome to a sub-tetraploid one.

Little information is available on the cross talk between p53 and Mos. In samples from non-small cell lung carcinoma patients, p53 alterations were shown to correlate with high Mos expression, aneuploidy and tumour aggressiveness (Gorgoulis *et al*, 2001). As a possible scenario, the inactivation of p53 would favour the presence of supernumerary centrosomes (Fukasawa *et al*, 1996; Carroll *et al*, 1999) while augmenting the probability of cells to survive the tetraploidization process (Castedo *et al*, 2006a; Senovilla *et al*, 2009). Tetraploid cells that lack an intact p53 system would then upregulate Mos, in turn favouring aneuploidization. Beyond such an oncogenic cooperation at the functional level, additional interactions between p53 and Mos may be envisioned. Reportedly, p53-deficient cells tolerate higher levels of Mos than their p53-proficient counterparts (Fukasawa and Vande Woude, 1997; Kalejs *et al*, 2006), suggesting that the 'oncogenic stress' mediated by ectopic expression of this meiosis-restricted protein may kill cells or arrest their cycle if the p53 system is intact. Thus, it is conceivable that the absence of p53 may be permissive for the upregulation of Mos (Fukasawa and Vande Woude, 1997; Gorgoulis *et al*, 2001; Kalejs *et al*, 2006), which must occur at the post-transcriptional level, as Mos mRNA levels were not increased in tetraploid cells (data not shown). However, the exact molecular mechanisms explaining the unscheduled expression of Mos in tumour cells remain elusive.

The precise oncogenic mode of action of Mos is an ongoing conundrum. Enforced Mos expression in fibroblasts reportedly causes centrosome amplification (Saavedra *et al*, 1999). However, although siRNA-mediated knockdown of Mos in tetraploid cells apparently did not decrease the number of centrosomes, Mos expression inversely correlated with the coalescence of supernumerary centrosomes, as if Mos acted as an inhibitor of centrosome clustering. Accordingly, the depletion of Mos favoured centrosome coalescence while switching multipolar to bipolar mitoses, and this effect was inhibited by the simultaneous knockdown of the kinesin HSET, which is indispensable for centrosome clustering. Based on these results, we conclude that Mos may exert its oncogenic activity, at least in part, through the induction of multipolar, asymmetric cell division. It remains to be

determined whether the oncogenic activity of Mos is mediated by its direct interaction with centrosomes or by hitherto unknown effects on cellular morphology/dynamics that indirectly affect centrosome clustering.

Materials and methods

Unless otherwise indicated, media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, CA, USA), plasticware from Corning B.V. Life Sciences (Schiphol-Rijk, The Netherlands), and chemicals from Sigma-Aldrich (St Louis, MO, USA).

Cell lines, culture conditions and chemicals

Wild type, *p53*^{-/-}, *Bax*^{-/-} and *p21*^{-/-} diploid human colon carcinoma HCT 116 cells (kindly provided by Bert Vogelstein), diploid HCT 116 cells stably transfected with a vector encoding the baculoviral caspase inhibitor p35 (Zhang *et al*, 2006), WT and *p53*^{-/-} diploid HCT 116 cells transfected with a cDNA coding for the fusion between histone H2B and green fluorescent protein (H2B-GFP, PharMingen, San Diego, CA, USA), as well as WT diploid human colon carcinoma RKO cells were routinely maintained in McCoy's 5A medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% foetal calf serum (FCS) at 37 °C in a 5% CO₂ atmosphere. H2B-GFP-expressing cells were cultured in the presence of 20 µg/ml blasticidin. Cells were seeded onto the appropriate supports (6-, 12-, 24- or 96-well plates, 35 or 100 mm Ø Petri dishes) 24 h before the beginning of the experiment. To generate tetraploid clones, parental diploid cells were treated for 48 h with 0.6 µg/ml cytochalasin D or 100 nM nocodazole and then cultured for 2 weeks in drug-free culture medium, followed by staining with Hoechst 33342 (2 µM; Molecular Probes-Invitrogen, Eugene, OR, USA) and cloning of cells characterized by an ~8n DNA content on a FACS Vantage cell sorter (BD Biosciences, San Jose, CA, USA), as previously described (Castedo *et al*, 2006b). Alternatively, tetraploid clones were isolated by the limiting dilution technique (Castedo *et al*, 2006b). To prevent drifts in cell composition, clones were split well before complete confluence. For each experiment, at least three different clones for each genotype and/or phenotype were used.

Plasmid transfection and RNA interference

A cDNA encoding for centrin derived from a human testis mRNA library (GenBank accession number BC029515.1) was amplified in a pCMV-sport6 vector (Invitrogen) and then cloned into a pDsRed2-C1 plasmid (Clontech Laboratories, Mountain View, CA, USA) as a *Bgl*III restriction fragment, generating a construct for the expression of a centrin-DsRed chimera. The human Mos cDNA (Image Clone 40016104) was purchased from Geneservice (Nottingham, UK) within a pCR-bluntII-TOPO plasmid (Invitrogen). The Mos sequence was then transferred either to the polycistronic expression vector pIRES-hrGFP2 (Agilent Technologies, Santa Clara, CA, USA) as a *Bam*HI-*Not*I restriction fragment, or to the pEGFP-C3 plasmid (Clontech Laboratories) as a *Xho*I-*Bam*HI restriction fragment upon PCR amplification (primers 5'-TATACTCGAGCCCTCGCCCCTGGCCC-3' and 5'-TATAGGATCCTCAGCCGAGTTTCAGCTTCA-3', restriction sites are underlined). In order to generate a non-interferable but functional Mos mutant, the Mos sequence was further modified from within the pIRES-hrGFP2 vector to introduce the silent mutations ATCATA at position 619 (Ile207) and TTGCTA at position 622 (Leu208). Site-directed mutagenesis was performed with the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the primers 5'-GGACCTGAAGCCCGCAACATACTAATCAGTGAGCAGGATGTC-3' and 5'-GACATCCTGCTCACTGATAGTATGTTTCGCGGGCTTCAGGTCC-3' (mutated nucleotides are underlined), according to the manufacturer's instructions. Briefly, PCR was employed to produce several copies of the entire plasmid including the desired mutations. The reaction mix was then incubated for 1 h at 37 °C with the *Dpn*I restriction enzyme, which cleaves only methylated DNA (in this case, the parental, non-mutated molecules). Uncleaved (mutated) DNA was then used for transformation of *Escherichia coli* XL1-blue competent cells (Stratagene).

Custom-designed siRNAs duplexes targeting Mos (Mos_1 sense 5'-CCCCGGAACAUCUUGAUCdTdT-3'; Mos_2 sense 5'-GCCUAA

AGCCGACAUUUUAUdTdT-3') and p53 (p53_1 sense 5'-GUGAGCGC UUCGAGAUGUdTdT-3'; p53_2 sense 5'-GACUCCAGUGGUAU UCUACdTdT-3') (Gu *et al*, 2004) were purchased from Sigma-Proligo (The Woodlands, TX, USA). Alternatively, siRNA duplexes for the downregulation of Mos (Mos_3) and HSET (HSET_1) (siGENOME Smart-pool M-003859 and L-004958, respectively) were purchased from Dharmacon (Chicago, IL, USA). As a control, a non-targeting siRNA with an sequence unrelated to both human and murine genomes was used (UNR, sense 5'-GCCGGUAUGCCG GUUAAGUdTdT-3'). HCT 116 cells in 12-well plates were transfected with plasmids at 80% confluence by means of the Attractene[®] transfection reagent (Qiagen, Hilden, Germany) as recommended by the manufacturer, or with siRNAs at 30–40% confluence by means of the HiPerFect[®] transfection reagent (Qiagen), as previously described (Hoffmann *et al*, 2008). After 72 h, transfection efficiency was determined by immunoblotting (see below). When required, cells were transfected first with siRNAs and 24 h later with plasmids.

Cytofluorometric studies

For the simultaneous quantification of DNA content (cell cycle profiling), plasma membrane integrity and mitochondrial transmembrane potential ($\Delta\Psi_m$), live cells were collected and stained with 2 μ M Hoechst 33342 (Molecular Probes–Invitrogen), 1 μ g/ml propidium iodide (PI, which only incorporates into dead cells) and 40 nM of the $\Delta\Psi_m$ -sensitive dye 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3), from Molecular Probes–Invitrogen) (Castedo *et al*, 2002; Galluzzi *et al*, 2009). To assess cell cycle distribution, cells were collected, fixed by gentle vortexing in ice-cold 80% (v/v) ethanol (Carlo Erba Reagents, Milano, Italy) and stained with 50 μ g/ml PI in 0.1% (w/v) D-glucose in PBS supplemented with 1 μ g/ml (w/v) RNase A (Mouhamad *et al*, 2007; Mondragon *et al*, 2009). For the cell count assay, the same amount of FITC-labeled beads (Becton Dickinson) was added to each sample and cells were counted until 5000 beads had passed through the FACS. Cell proliferation was determined by counting the cells every 24 h for 4 days upon the seeding. Cytofluorometric acquisition of blue (Hoechst 33342), red (PI) and green (DiOC₆(3)) fluorescence were carried out by means of a FACSCalibur or a FACScan cytofluorometer (BD Biosciences) equipped with a 70 μ m nozzle. Statistical analysis was carried out by using the CellQuest[™] software (BD Biosciences), upon gating on the events characterized by normal forward scatter and side-scatter parameters.

Videomicroscopy

For videomicroscopy, HCT 116 cells stably transfected with the cDNA encoding for H2B–GFP were grown in 96-well imaging plates (BD Biosciences) under standard culture conditions (37 °C, 5% CO₂) and subjected to pulsed observations (every 10 min for up to 48 h) with a BD pathway 855 automated live cell microscope (BD Biosciences). Images were analyzed with the open source software Image J (freely available from the National Institute of Health, Bethesda, MD, USA at the address <http://rsb.info.nih.gov/ij/>).

Cell proliferation assays

Cell proliferation was measured by the fluorescein-based dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. Briefly, 1×10^6 cells were incubated with 2.5 μ M CFSE in PBS for 10 min at 37 °C. The labelling reaction was stopped by adding an equal volume of FCS and 5×10^4 cells were seeded in 12-well plates. After 24 h, cells were transfected with siRNAs and grown in culture for additional 3–5 days. Cell proliferation was monitored daily on a FACSCalibur cytofluorometer (BD Biosciences) upon excitation at 488 nm and acquisition in the FL1 (green) channel, followed by data analysis by means of the CellQuest software (BD Biosciences).

Immunofluorescence microscopy

Immunofluorescence microscopy determinations were performed as previously described (Vitale *et al*, 2007, 2008). Briefly, cells were fixed in 4% (w/v) paraformaldehyde in PBS, permeabilized with 0.1% SDS and immunostained with antibodies specific for γ - and β -tubulin (both from Sigma-Aldrich). Slides were then incubated with the appropriate Alexa Fluor[®] conjugates (Molecular Probes–Invitrogen) in the presence of 10 μ M Hoechst 33342 (Molecular Probes–Invitrogen), which was used for nuclear counterstaining. Fluorescence and confocal fluorescence images were captured using

an IRE2 microscope equipped with a DC300F camera (both from Leica Microsystems GmbH, Wetzlar, Germany) and a TSC-SPE microscope (Leica Microsystems GmbH) equipped with a 63X/1.15 objective (Olympus America, Center Valley, PA, USA), respectively. Signals from different probes were acquired in sequential scan mode and image analysis was performed with the open source software Image J (National Institutes of Health).

Immunoblotting

HCT 116 cells were washed with cold PBS and lysed in a buffer containing 1% NP40, 20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 1 mM dithiothreitol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin, as previously described (Zermati *et al*, 2007). Thereafter, protein extracts (50 μ g per lane) were separated according to molecular weight on precast 4–12% SDS–PAGE gels (Invitrogen), followed by electrotransfer to Immobilon[™] membranes (Sigma-Aldrich) and immunoblotting with antibodies specific for HSET and Mos (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies that specifically recognize glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β -actin (both from Millipore-Chemicon International, Temecula, CA, USA) were used as loading control. Finally membranes were incubated with appropriate goat IgG conjugated to horseradish peroxidase (Southern Biotech, Birmingham, AL, USA), followed by chemiluminescence detection with the SuperSignal West Pico[®] reagent and CL-XPosure[®] X-ray films (both from Thermo Scientific–Pierce, Rockford, IL, USA).

Cytogenetic analysis and FISH

Chromosome spreads were prepared by conventional procedures (Mouhamad *et al*, 2007). Briefly, HCT 116 clones were treated with 100 nM nocodazole for 8 h to enrich mitotic cells, then collected and subjected to hypotonic lysis by incubation in 75 μ M KCl for 10 min at 37 °C. After removal of hypotonic solution, cells were fixed in freshly prepared Carnoy solution (3:1 methanol:acetic acid) and stored at –20 °C. Fixed cells were then dropped onto pre-cooled Superfrost Plus glass microscope slides (Thermo Fisher Scientific, Waltham, MA, USA) and dried at room temperature. Chromosomes were stained with 100 ng/ml 4',6-diamidino-2-phenylindole (DAPI, from Molecular Probes–Invitrogen) and mounted with glass coverslips in Vectashield H-1000 mounting medium (Vector Laboratories, Burlingame, CA, USA). Finally, fluorescence images were visualized and captured using an IRE2 microscope equipped with a DC300F camera (both from Leica Microsystems GmbH). For each experimental condition, 100 cells from three independent experiments were analyzed. For FISH, freshly FACS-purified diploid and sub-tetraploid cells were dropped onto Superfrost Plus polylysine-coated glass microscope slides (Thermo Fisher Scientific) and fixed *in situ* with 9:1 methanol:acetic acid for 5 min. Thereafter, cells were air-dried overnight and hybridized with a commercial mixture of three probes (Abbott Laboratories, Abbott Park, IL, USA) that detect the centromeric region of chromosome 8 (labelled with FITC–green colour), chromosome 10 (labelled with rhodamine–red colour) and chromosome 18 (labelled with Aqua–blue colour). For each experimental conditions, 100–300 nuclei were surveyed.

Clonogenic survival assays

To evaluate clonogenic survival, freshly generated tetraploid and sub-tetraploid cells were stained with 2 μ M Hoechst 33342 (Molecular Probes–Invitrogen), FACS-purified on a FACSVantage cell sorter (BD Biosciences), seeded at different concentrations (from 1 to 50×10^3 for well) in 6-well plates, and cultured for up to 10 days under normal conditions. Colonies were then fixed/stained with an aqueous solution containing 0.25% (w/v) crystal violet, 70% (v/v) methanol and 3% (v/v) formaldehyde (Carlo Erba Reagents) and counted (Zhang *et al*, 2006). Only colonies made of ≥ 30 cells were included in the quantification. For each treatment, the surviving fraction (SF) was estimated according to the formula: $SF = (\text{number of colonies formed} / \text{number of cells seeded}) \times \text{plating efficiency}$ (defined as the ratio between the number of colonies formed and the number of cells seeded in control conditions).

In vivo xenograft model

Athymic nu/nu female mice (age = 42 days, body weight = 20 g, provided by the Institut Gustave Roussy (IGR) in-house animal

facility) were used throughout this study in strict compliance with widely accepted ethical guidelines for animal experimentation. Mice were kept in Makrolon[®] type III wire mesh laboratory cages (Charles River, Boston, MA, USA), under poor germ conditions at 24°C and 50–60% humidity, and were allowed for food and water *ad libitum*. Light cycle was artificially controlled to provide 14 h of light (from 0630 h to 2030 h). After 4 days of acclimation period, mice were subcutaneously xenografted with 2×10^6 WT or p53^{-/-} tetraploid HCT 116 cells, as previously described (Vitale et al, 2007). Tumour growth was then assessed every 2–4 days with a standard caliper. For ethical reasons, mice were killed before tumours reached a volume of 3400 mm³ or a surface of 250 mm². Statistical analysis was carried out by means of the SigmaStat package (Systat Software, San Jose, CA, USA). One-way analysis of variance was carried out and statistical significance was determined by means of two-tailed Student's *t*-test (**P* < 0.05) in the context of a pairwise comparison procedure.

Tumour recovery

To recover tumour cells, mice were killed by cervical dislocation following the FELASA guidelines. Quickly after killing, tumour tissue was surgically removed and washed in RPMI 1640 culture medium supplemented with 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulphate and 10% FCS. Thereafter, a tissue fragment of ~125 mm³ was cut into pieces of ~1 mm³, which were dissociated by incubation with 0.05% trypsin for 45 min at 37°C. Every 15 min the disaggregation of cells was mechanically carried out. Finally, dissociated cells were washed once with RPMI 1640 supplemented with 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulphate and 10% FCS, and seeded into 25 cm² flasks. Recovered tumour cells were routinely maintained under standard culture conditions (37°C, 5% CO₂) in McCoy's 5A medium supplemented with 100 U/ml penicillin G sodium, 100 µg/ml

streptomycin sulphate, 100 mM HEPES buffer, 1 mM sodium pyruvate and 10% FCS.

Statistical procedures

Unless otherwise specified, all experiments were carried out in triplicate parallel instances and independently repeated at least three times. Data were analyzed with Microsoft Excel (Microsoft, Redmond, WA, USA) and statistical significance was assessed by means of two-tailed Student's *t*-test (**P* < 0.05).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

GK is supported by the Ligue Nationale contre le Cancer (Equipe labellisée), Agence Nationale pour la Recherche, European Commission (Apo-Sys, ChemoRes, ApopTrain), Fondation pour la Recherche Médicale, Institut National du Cancer, Cancéropôle Ile-de-France. MC is supported by the Association pour la Recherche sur le Cancer (ARC). LS is supported by ApopTrain Marie Curie training network of the European Union and FRM, MM and SR-V by FRM, LG by the Apo-Sys consortium of the European Union, OK by EMBO. IV, LS, MJ, MM, OK, LN, AC, SRV, GM, DM, SV, NT, NJ, AV and MC carried out the experiments presented in this article. IV, LG, MC and GK prepared the figures and wrote/edited the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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