

Report

Preferential killing of tetraploid tumor cells by targeting the mitotic kinesin Eg5

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Abbreviations: DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; DIMEN, dimethylnastron; PI, propidium iodide; siRNA, small interfering RNA; KSP, kinesin spindle protein; H2B, histone 2B

Key words: apoptosis, mitotic catastrophe, monoastral mitosis, dimethylnastron, kinesin spindle protein

Tetraploid cells may constitute a metastable intermediate between normal euploidy and cancer-associated aneuploidy. Tetraploid cells are relatively more resistant against DNA damaging agents and are genetically unstable, due to their tendency towards multipolar, asymmetric division. Therefore, it is important to develop strategies for the selective removal of tetraploid cells. Here, we show that targeting the mitotic kinesin Eg5 (also known as kinesin spindle protein, KSP) by a small interfering RNA (siRNA) or by the pharmacological inhibitor dimethylnastron (DIMEN) kills tetraploid tumor cells more efficiently than their diploid precursors. Cell death occurs after an attempt of monoastral mitosis that, in diploid cells, is followed by a prolonged mitotic arrest and morphological reversion to the interphase, with a 4n DNA content. In contrast, DIMEN-treated tetraploid cells exhibit a shorter mitotic arrest, bipolar or multipolar karyokinesis, followed by apoptosis of the daughter cells, as assessed by fluorescence videomicroscopy of cells that express a histone 2B-GFP fusion construct to monitor their chromosomes. Cell death occurred with hallmarks of apoptosis, namely loss of the mitochondrial transmembrane potential and terminal chromatin compaction. In conclusion, tetraploid cells are particularly vulnerable to undergo mitotic catastrophe after genetic or pharmacological inhibition of Eg5.

Introduction

Polyploidization constitutes an evolutionary strategy in speciation, especially in plants, yet can also affect animal cells in specific tissues. In humans, polyploidy (that is accumulation of more than two sets of chromosomes) can result from cell fusion (for example in the syncytiotrophoblast, striated muscle cells and osteoclasts),^{1,2} in which case terminally differentiated cells contain several nuclei in an irreversibly arrested G₁ phase. Alternatively, polyploidy can result from endoreplication (that is chromosomal duplication without nuclear division), as this occurs in differentiating megakaryocytes,^{3,4} and from karyokinesis without cytokinesis (as it occurs physiologically in a fraction of hepatocytes).⁵ In pathological circumstances, macrophages (which are terminally differentiated cells) can fuse among each other to form giant polyploid cells in inflamed tissues.⁶ Inflammation and irradiation have also been reported to give rise to cell-to-cell fusion in other tissues, for instance between hematopoietic and neuronal cells.^{7,8} Several viruses, in particular the human immunodeficiency virus (HIV) encode fusogenic protein that can trigger the generation of syncytia in lymphoid tissues as well as in the central nervous system, and such syncytia then succumb to apoptosis.^{9,10}

Aberrant tetraploidization events have been implicated in the pathogenesis of cancer. Thus, abnormal cell-to-cell fusion, as can be induced by some oncogenic viruses has been accused to participate in malignant transformation.^{11,12} Aberrant fusion between cancer cells and leukocytes, perhaps triggered by inflammatory processes, has also been suggested to participate in tumor progression and metastasis.¹³ Tetraploidization can also arise in tumor cell precursors, through a variety of mechanisms not involving cell-to-cell fusion. First, chromosome non-disjunction, an error that occurs when both copies of a chromosome segregate to the same daughter cell, can promote cleavage furrow regression and

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subsequent tetraploidy.¹⁴ Second, cells can become tetraploid after prolonged arrest by the activation of the spindle assembly checkpoint. The spindle assembly checkpoint arrests cells in mitosis if normal kinetochore–microtubule attachment is impaired, and upon prolonged activation of the checkpoint, cells can adapt and re-enter G_1 as tetraploids, a phenomenon that is referred to as “mitotic slippage”.¹⁵

Tetraploid cells are commonly found in some oncogenic lesions, for instance in the Barrett’s esophagus, a condition that culminates in the development of carcinoma.¹⁶ Similarly, some environmental agents such as specific bacteria contained in the intestinal flora can induce tetraploidization,¹⁷ and this can be speculatively linked to carcinogenesis. Experiments that are specifically designed to induce tetraploidy in mouse mammary gland cells can lead to the formation of cancers, provided that the cells are p53-deficient.¹⁸ Tetraploid cells tend to be genomically unstable, perhaps due to aberrations in the centrosome cycle (which can cause asymmetric division) or due to geometric constraints on the chromosomal duplication, repair and division machineries (that increase the probability of aneuploidization events).^{19,20} In synthesis, it appears that polyploidization (including its simplest manifestation, tetraploidization) can participate in cancer-related aneuploidization processes. Therefore, there is considerable interest in developing strategies for the selective killing of polyploid cells.

The kinesin Eg5, also called kinesin spindle protein (KSP), Hs Eg5, kinesin-5 or KIF11, is a molecular motor that participates in mitosis, by separating the microtubules that are attached to the two centrosomes, thus contributing to the bipolar arrangement of the spindle.²¹ Overexpression of Eg5 causes the disruption of normal spindles (which are replaced by monopolar or multipolar spindles), and its expression as a transgene causes the formation of tetraploid cells in mice, as well as genomic instability and tumor formation.²² Pharmacological inhibition of Eg5 causes monoastral mitoses,^{23,24} a phenomenon that explains the name of the first Eg5 inhibitor, monastrol.²⁵ A series of improved Eg5 inhibitors constitute a novel class of antimitotics that are being evaluated as anticancer agents.^{23,26–28} For instance, the monastrol derivative dimethylenastron (DIMEN) has been used to prevent the growth of xenografted human pancreatic cancers in mice.²⁹

Based on these premises, we speculated that Eg5 inhibition might have a selective effect on tetraploid cells. Here, we demonstrate that this is the case and reveal that Eg5 inhibition has profoundly different effects on the cell cycle progression of diploid and tetraploid cells.

Results and Discussion

Preferential killing of tetraploid cells after inhibition of Eg5. Among our collection of diploid and tetraploid HCT116 clones, we randomly picked two diploid (A5, W5) and two tetraploid (F6, F11) HCT116 clones, and determined their response to the knockdown (via siRNA) of the Eg5 motor kinesin (Fig. 1A), while assessing well-established signs of imminent and complete cell death, namely the loss of the mitochondrial transmembrane potential [$\Delta\Psi_m$, determined by means of the cationic lipophilic dye 3,3'-dihexyloxycarbocyanine iodide, DiOC₆(3)]

and the permeabilization of the plasma membrane to the vital dye propidium iodide (PI).³⁰ Depletion of Eg5 was more toxic for tetraploid than for diploid cancer cells (Fig. 1B and C), and this result could be duplicated in a series of clones derived from another colon cancer cell line, RKO (data not shown). Next, we determined whether the Eg5-specific chemical inhibitor, dimethylenastron would also kill tetraploid cells more efficiently. Indeed, tetraploid cells were more susceptible to killing by 1 μ M DIMEN than their diploid precursors (Fig. 1D). It should be noted that the preference in tetraploid cell death induction was more pronounced when Eg5 was depleted (with several distinct siRNA, not shown) than when Eg5 was inhibited with DIMEN, a poorly characterized agent with possible pro-apoptotic off-target effects. However, this difference was statistically significant in both cases.

Eg5 inhibition triggers abnormal mitoses of tetraploid cells. Diploid and tetraploid cells exhibited a clear difference in their cell cycle when they were treated with DIMEN. While diploid cells accumulated in G_2/M in response to DIMEN, tetraploid cells were less affected in their cell cycle (Fig. 2A and B). Similar results were obtained when the advancement of the cell cycle was monitored after Eg5 knockdown with specific siRNA. After Eg5 depletion, diploid cells were arrested in G_2/M , while tetraploid cells exhibited an apparently unperturbed cell cycle (Fig. 2C). Next, we took advantage of HCT116 cells that stably express a histone 2B-GFP fusion protein, allowing for monitoring chromosomes *in vivo* by fluorescence videomicroscopy. Again, we compared diploid and tetraploid clones, in a systematic fashion. There was no difference in the capacity of DIMEN to induce monoastral metaphases in diploid and tetraploid cells. Virtually all mitoses exhibited a perturbed morphology with a ring-like distribution of chromosomes during the metaphase. However, there were marked differences in the behavior of diploid and tetraploid cells exposed to DIMEN (Fig. 2D–H, Suppl. Material). Diploid cells exhibited a prolonged metaphase arrest (mean 8.1 hours), which was often (in approximately 63% of the cases), followed by decondensation of chromosomes and reversion to the interphase. In contrast, in tetraploid cells this metaphase arrest was shorter (mean 4.8 hours) and was followed in the majority of the cases (around 52%) by a bipolar, sometimes asymmetric, division, which then led to apoptosis of one or both of the daughter cells (Fig. 2D–H). In conclusion, diploid and tetraploid cells exhibit a fundamental difference in their response to Eg5 inhibition, that is reversion of arrested metaphases to the interphase in the case of diploid cells, but advancement to abnormal karyokinesis and cytokinesis in the case of tetraploid cells.

Concluding remarks. Although the sole difference between tetraploid and diploid cells is that the former contain twice as many chromosomes than the latter, there are major ploidy-related differences that impact on the biological consequences of polyploidization. We and others have found that tetraploid cells are relatively resistant against DNA damaging agents including ionizing irradiation, UV, cisplatin, oxaliplatin, etoposide and anthracyclins,^{31,32} a difference that was due to the overactivation of p53-induced antioxidant and DNA repair enzymes.³¹ In

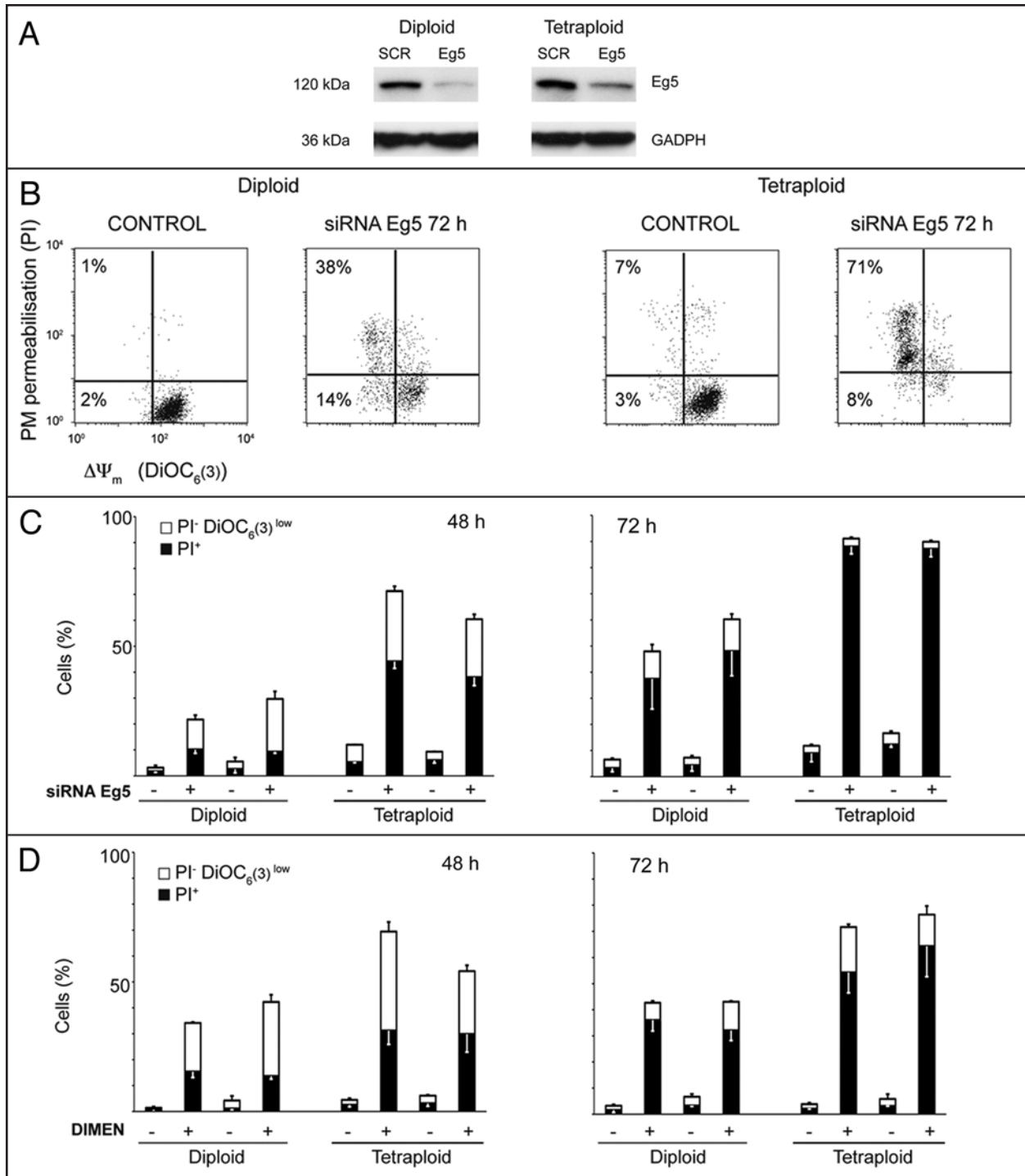


Figure 1. Selective killing of tetraploid cells after genetic or pharmacological inhibition of Eg5. (A) Depletion of Eg5 by means of a specific siRNA. Diploid or tetraploid HCT116 cells (clones A5 and F6) were transfected with the indicated siRNAs, and 48 h later cell lysates were subjected to immunoblot detection of Eg5 and GAPDH as a loading control. (B and C) Cell death induction in diploid and tetraploid cancer cells after Eg5 knockdown. Forty-eight or 72 h after transfection, the cells were stained with DiOC₆(3) plus PI and subjected to cytofluorometric analysis. Representative pictograms, as obtained after 72 h for clones A5 and F6 are shown in (B), where percentages refer to the number of cells in each quadrant. Mean results, as obtained for two distinct, randomly picked, diploid clones (A5, W5) and two tetraploid clones (F6, F11) are given in (C). Results are means ± standard deviation (SD) for triplicate determinations. (D) Cell death of diploid and tetraploid cancer cells after treatment with the Eg5 inhibitor dimethylnastron (DIMEN). Cells were left untreated or treated with 1 μM DIMEN and then analyzed as in (B and C). Results are representative for three independent experiments.

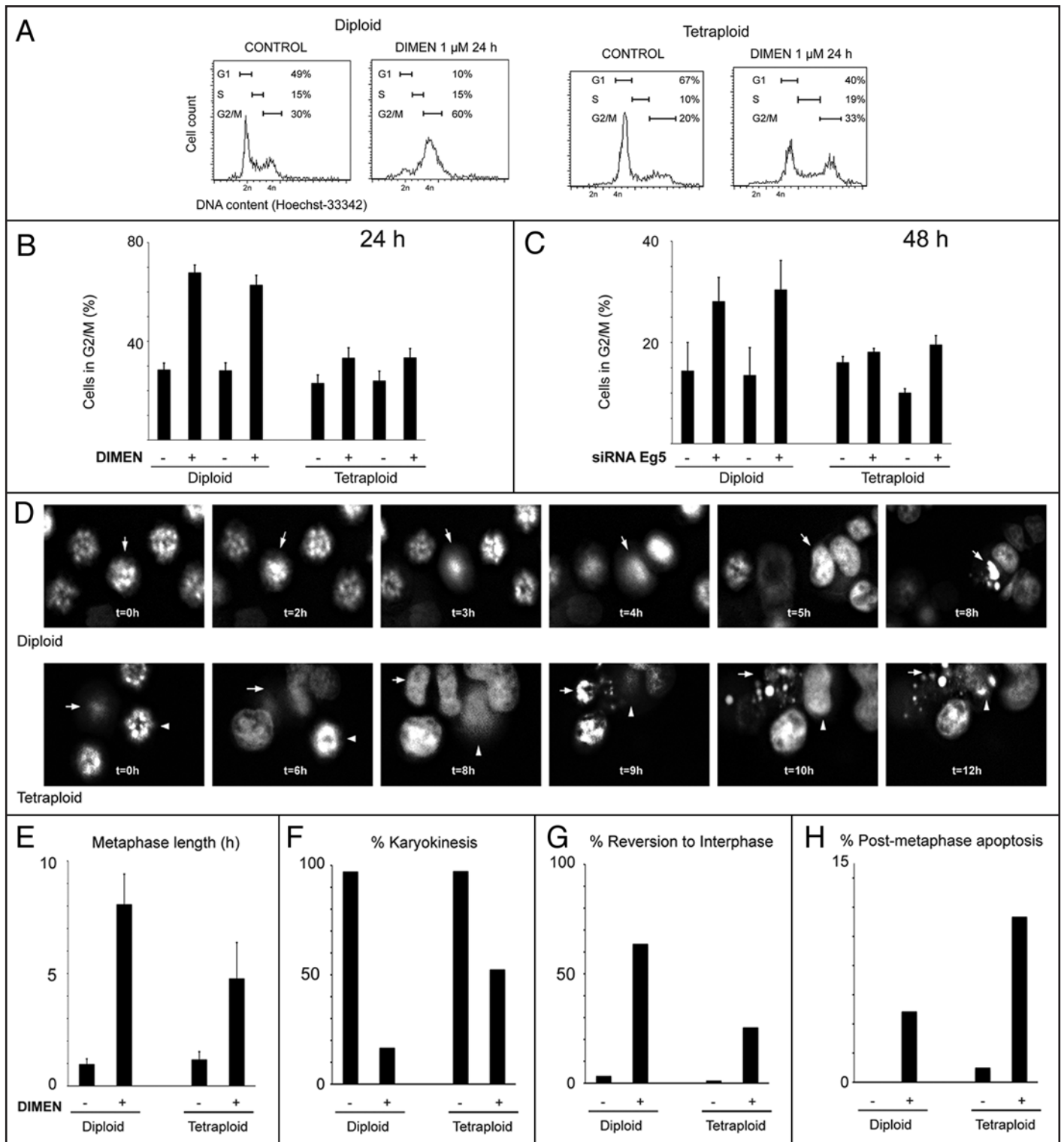


Figure 2. For figure legend, see page 1034.

an attempt to identify strategies for selectively killing tetraploid cells, we found that inhibition of Chk1 would selectively kill tetraploid (and less so diploid) HCT116 and RKO colon cancer cells,³³ likewise through an increase in p38 MAPK activation³⁴ that culminates in the p53-dependent transactivation of proapoptotic

genes.³³ Similarly, depletion of Chk1 accelerates the spontaneous apoptosis of polyploid cells resulting from aberrant cell fusion.³⁵ We also found that the knockdown of endonuclease G killed tetraploid colon cancer cells, a result that could be recapitulated by eliminating endonuclease G from the genome of tetraploid yeast cells.³⁶

Figure 2. Cell cycle effects of Eg5 inhibition in diploid and tetraploid tumor cells. (A and B) DNA content analysis after treatment with DIMEN. Diploid and tetraploid clones were cultured for 24 h in the absence or presence of 1 μ M DIMEN, and the cells were stained with Hoechst-33342 and subjected to cytofluorometric analysis. Representative staining profiles are shown in A (for clones A5 and F6) and the means (\pm SD, triplicates) of randomly picked clones (W5 and W4, diploid and F6 and W43, tetraploid clones) are shown in (B). (C) DNA content analysis after knockdown of Eg5. Forty-eight hours after transfection with the control siRNA or the Eg5-specific siRNA, cell cycle analyses were performed as in (A and B). (D–H) Fluorescence videomicroscopic analyses of diploid and tetraploid HCT116 clones, stably transfected with H2B-GFP, after treatment with DIMEN. Representative videomicroscopic images are shown for one diploid and one tetraploid cell starting approximately 18 h after addition of 1 μ M DIMEN in (D). Diploid cells remain arrested in monoastrial metaphase for several hours and then revert to the interphase (without prior division) that shortly precedes apoptosis (D, upper, arrow). Tetraploid cells divide asymmetrically after a shorter mitotic arrest and both daughter cells finally succumb to apoptosis (D, lower, arrow). Alternatively, tetraploid cells behave like diploid cells in the sense that they undergo a protracted metaphase arrest, then recover the interphase morphology and finally undergo apoptosis (D, lower, arrowhead). Quantitative analyses are provided in (E–H) for a minimum of 100 metaphases each. These analyses include the mean length of metaphases before karyokinesis or reversion to the interphase (E), the percentage of metaphases that proceed to full karyokinesis (F), the percentage of metaphases that revert to a interphase-like appearance (G) and the percentage of post-metaphase events leading to apoptosis (H). This experiment has been repeated twice, yielding similar results.

Here, we developed a novel strategy for the removal of tetraploid cells, namely by the inhibition of Eg5. It is tempting to draw a parallelism between the selective killing of tetraploid cells by Chk1 and Eg5 inhibition. Chk1 inhibition results in an unrestricted advancement of the cell cycle, which in tetraploid cells apparently relies more heavily on intact checkpoints than in diploid cells, and this may explain why tetraploid cells are killed more efficiently by Chk1 inhibitors than diploid control cells. As shown here, Eg5 inhibition causes a prolonged metaphase arrest in diploid cells that is followed by reversion from the metaphase to a G_1 -like state. However, tetraploid cells respond to Eg5 inhibition in a completely different fashion. The length of the metaphase arrest is significantly shortened (by near-to 50%), and the outcome is different. Instead of a reversion to G_1 , the cells advance to karyokinesis, resulting in later apoptosis of presumably aneuploid daughter cells. Hence, in both cases (with Chk1 inhibitors as well as with Eg5 inhibitors), tetraploid cells are killed preferentially due to specific changes in their cell cycle control that have to be explored in greater detail in the future. We anticipate that the in-depth comprehension of the cell biology of tetraploidy will furnish invaluable information on how pre-malignant cells can be targeted.

Materials and Methods

Cell lines and culture conditions. Different diploid (A5, W5, W4) and tetraploid (W43, F6, F11) monoclonal lines derived from HCT116 human colon carcinoma cells were used. Tetraploid cells were generated from diploid HCT116, as previously described.^{31,37} Cells were routinely maintained in McCoy's 5A medium supplemented with 10% FCS (at 37°C in a 5% CO₂ atmosphere) and seeded onto the appropriate support (6-, 12-, 24- or 96-well microtiter plates, 35 or 100 mm \varnothing Petri dishes) 24 h before the beginning of the experiment. Media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, USA), whereas plastics were obtained from Corning B.V. Life Sciences (Schiphol-Rijk, Netherlands).

Eg5 inhibition induction. The knockdown of Eg5 protein was performed with previously validated, specific siRNA purchased from Invitrogen (siRNA Eg5): sense 5'-CUG AAG ACC UGA AGA CAA UdT dT-3'.³⁸ As a control, a siRNA with an unrelated, scrambled sequence was employed (SCR): sense 5'-GCC GGU AUG CCG GUU AAG UdT dT-3'.³⁹ Diploid and tetraploid HCT116 cells were cultured in 12-well plates and transfected

at 30–40% confluence by means of the HiPerFect transfection reagent (Qiagen, Hilden, Germany) as previously described.⁴⁰ 72 h later, the transfection efficiency was determined by immunoblot. The compound dimethylnastron (Eg5 Inhibitor III, DIMEN) was purchased from Calbiochem-Merck (Darmstadt, Germany). Stock solutions in DMSO (Sigma-Aldrich, Saint Louis, USA) were prepared following the manufacturer's recommendations.

Western blotting. HCT116 cells were 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.^{41,42} Thereafter, protein extracts (80 mg/lane) were subjected to standard separation on SDS-PAGE, followed by immunoblotting with mouse monoclonal antibodies specific for Eg5 from AbCam (Cambridge, UK) and GAPDH (Millipore, Billerica, USA), which was monitored as loading control.

Cytofluorometric studies. For the simultaneous quantification of DNA content, mitochondrial transmembrane potential ($\Delta\Psi_m$) and plasma membrane integrity, live cells were collected and stained with 10 μ g/ml Hoechst-33342 (from Molecular Probes-Invitrogen), 40 nM of the $\Delta\Psi_m$ -sensitive dye 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3), Molecular Probes-Invitrogen) and 1 μ g/ml propidium iodide (PI, which only incorporates into dead cells, from Sigma-Aldrich) for 30 min at 37°C.^{43,44} Cytofluorometric acquisition of blue (Hoechst-33342), green (DiOC₆(3)) and red (PI) fluorescence was carried out by means of a FACSCalibur (BD Biosciences, San Jose, USA) equipped with a 70 μ m nozzle. Data were statistically evaluated using CellQuest™ software (BD Biosciences). Only the events characterized by normal forward scatter (FSC) and side scatter (SSC) parameters were included in subsequent analyses.

Videomicroscopic analysis. Histone 2B conjugated with green fluorescent protein (H2B-GFP) expressing HCT116 cells (diploid and tetraploid lines) were seeded in 96-well Black/Clear Imaging Plates (BD Falcon) treated with DIMEN approximately 4 h before beginning video recording using a BD Pathway™ 855 workstation (BD Biosciences). Images were captured each 12 min using the appropriate settings for GFP fluorescence with a x40 objective. Processing of videos was performed using BD AttoVison™ (BD Biosciences) and ImageJ software.³³

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/RelloVaronaCC8-7-Sup.pdf

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