Cancer-associated centrosomal transforming acidic coiled coil (TACC) proteins are involved in mitotic spindle function. By employing gene targeting, we have recently described a non-redundant and essential role of TACC3 in regulating cell proliferation. In this study, we used an inducible RNA interference approach to characterize the molecular function of TACC3 and its role in mitotic progression and cell survival. Our data demonstrate that a TACC3 knockdown arrests G1 checkpoint-compromised HeLa cells prior to anaphase with aberrant spindle morphology and severely misaligned chromosomes. Interestingly, TACC3-depleted cells fail to accumulate the mitotic kinase Aurora B and the checkpoint protein BubR1 to normal levels at kinetochores. Moreover, localization of the structural protein Ndc80 at outer kinetochores is reduced, indicating a defective kinetochore-microtubule attachment in TACC3-deficient cells. As a consequence of prolonged TACC3 depletion, cells undergo caspase-dependent cell death that relies on a spindle checkpoint-dependent mitotic arrest. TACC3 knockdown cells that escape from this arrest by mitotic slippage become highly polyploid and accumulate supernumerary centrosomes. Similarly, deficiency of the post-mitotic cell cycle inhibitor p21WAF exacerbates the effects of TACC3 depletion. Our findings therefore point to an essential role of TACC3 in spindle assembly and cellular survival and identify TACC3 as a potential therapeutic target in cancer cells.

The centrosome organizes the bipolar mitotic spindle to ensure faithful separation of chromosomes during mitosis (1). Spindle poles, kinetochores, and various microtubule-associated proteins are involved in the regulation of microtubule dynamics (2). The assembly of the mitotic spindle is a highly dynamic process and tightly controlled by the cell cycle. On the other hand, alterations in centrosome and mitotic spindle architecture have profound consequences for cell cycle progression and lead to chromosomal instability, aneuploidy, and cell death (1, 3–6). The finding that many cancer cells have genetic instability and centrosomal abnormalities has generated much interest in studying the role of chromosomal mis-segregation and aneuploidy for tumorigenesis.

Members of the transforming acidic coiled-coil (TACC) family as important structural components of the centrosome/spindle apparatus (7). TACC proteins are evolutionarily conserved and share a 200-amino acid coiled coil motif at their C terminus but have only limited homology outside this domain (8). TACC proteins interact with the microtubule-stabilizing protein ch-TOG/Msps/XMAP215 (9) that is important for centrosome integrity, centrosome-dependent assembly of microtubules, and spindle stability (10–12). Moreover, the Xenopus TACC homologue Maskin is involved in translational mRNA regulation during oocyte development (13), a TACC function so far not observed in mammals.

Although there is only one TACC gene in Drosophila and Xenopus, the mammalian TACC family consists of three genes. Interestingly, in humans all TACC homologues map closely to chromosomal translocation breakpoints that are associated with certain cancers, including mammary tumors and multiple myeloma (14, 15). Overexpression of TACC1 promotes cellular transformation in vitro (14) and mammary tumorigenesis in vivo (16). In contrast, the putative role of TACC2 as a tumor suppressor (17) could not be confirmed so far, because TACC2-deficient mice show neither increased tumor formation nor signs of genomic instability nor any other obvious phenotype (18). Finally, several recent reports link TACC3, the third member of the mammalian TACC family, to human cancer. This

3 The abbreviations used are: TACC, transforming acidic coiled-coil; cLSM, confocal laser scanning microscopy; DAPI, 4,6 diamidino-2-phenylindole; DOX, doxycycline; SAC, spindle assembly checkpoint; siRNA, short interfering RNA; RNAL, RNA interference; FACS, fluorescence-activated cell sorter; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; Q-VD-OPH, N-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxo)-methyl ketone; ch-TOG, colonic and hepatic tumor overexpressed protein.
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includes its identification as a novel prognostic marker in non-small cell lung cancer (19) and the association of TACC3 aberrations with ovarian cancer (20).

We have knocked out the TACC3 gene in mice resulting in embryonic lethality at mid to late gestation (21). TACC3-deficient mice showed chromosomal instability, greatly reduced cell numbers, and widespread apoptotic cell death. In particular, we found that TACC3 is required for progenitor cell expansion and hematopoiesis, suggesting an essential and nonredundant role of TACC3 for embryonic development especially in highly proliferating cells. The critical and gene dosage-dependent function of TACC3 in development is further underlined by the impaired chondrocyte differentiation and skeletal malformations in homozygous mutant mice carrying a hypomorphic TACC3 allele (22). Moreover, in the absence of TACC3, murine NIH3T3 fibroblasts enter a G1 and G2 cell cycle arrest (23).

The impaired proliferation and increased apoptosis in the absence of TACC3 presumably results from improper kinetochore attachment and chromosome alignment. In the presence of unattached chromosomes cells activate the spindle assembly checkpoint (SAC) to prevent aneuploidy. During metaphase, the SAC senses misaligned chromosomes and stabilizes cyclin B, whose degradation by the anaphase-promoting complex is necessary for mitotic progression (24, 25). Essential components of the SAC are Mad2 and BubR1, whose initial localization to kinetochores is dependent on the chromosomal passenger complex protein Aurora B (26, 27). Although activation of the SAC arrests cells in metaphase by preventing the anaphase transition, like all checkpoints the SAC does not arrest cells permanently. Cells rather gradually degrade cyclin B1 and escape from this block by mitotic slippage and potentially exit mitosis without cytokinesis (28, 29). These cells will then arrest in a tetraploid G1-like state through a post-mitotic checkpoint, which is p53- and p21WAF-dependent (30–32), or through an additional checkpoint in the next G2 phase (33).

It is well established that activation of p53 results not only in p21WAF-dependent cell cycle arrest but also in apoptosis, depending on the cellular context and stress stimulus (34). Interestingly, the embryonic lethality caused by TACC3 deficiency was partially rescued in mice with a complete loss or reduced levels of p53 (21). This observation indicates that TACC3 deficiency leads to cellular defects that are sensed post-mitotically by the p53/p21WAF-dependent pathway. In contrast, cell death that directly originates from mitosis is presumably p53-independent and mediated through caspase-dependent pathways (35). It is unknown to what extent mitotic cell death contributes to the phenotype observed upon TACC3 deficiency.

Unlike in TACC3-deficient cells from gene-targeted mice, it was previously shown that down-regulation of TACC3 in HeLa cells using a transient oligonucleotide-based RNAi approach did not result in dramatic damage of mitotic spindles during the first round of cell division, where cells performed a relatively normal although delayed mitosis (10). In that study, TACC3-knockdown cells revealed mostly well organized spindles with only partial defects in microtubule stability and chromosome attachment. In contrast, depletion of ch-TOG, the binding partner of TACC3, had much stronger effects and resulted in increased multinucleation, disorganized spindles, and multipolar spindle formation, indicating that ch-TOG but not TACC3 plays a major role in spindle pole organization. Down-regulation of neither TACC3 nor ch-TOG, however, was reported to induce cell death (10). In this study, we used a stable drug-controllable RNA interference approach to elucidate the cellular effects of a persistent TACC3 gene silencing. Our results clearly demonstrate that long term TACC3 depletion disturbs chromosomal alignment and a proper spindle and kinetochores architecture. We also demonstrate that an impaired post-mitotic G1 checkpoint that often occurs in tumor cells strongly accelerates mitotic defects and results in increased apoptotic cell death. Thus, our data indicate an essential role of TACC3 in spindle assembly and mitotic cell survival.

EXPERIMENTAL PROCEDURES

Cloning of Lentiviral Constructs for Conditional Gene Suppression of TACC3—The original lentiviral vectors for siRNA expression (pLVTH) and the method for generation of tTR-KRAB transrepressor-positive target cells lines (pLV-tTRKRAB-dsRed) have been published (36). Several siRNA sequences of 19 nucleotides targeting human TACC3 were selected by a siRNA prediction software (Whitehead Institute for Biomedical Research, Boston). Complementary synthetic short hairpin RNA oligonucleotides consisting of sense-loop-antisense sequences (Operon, Cologne, Germany) were cloned into the pLVTH vector downstream of the H1 promoter (36). The cDNAs and RNAi sequences used are indicated in the supplemental Table 1S.

Cell Culture, Lentiviral Transductions, and Isolation of Stably Transduced Subclones—293T and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HCT116 cell lines were cultured under the same conditions using McCoy’s medium (Genaxxon, Biberach, Germany). Recombinant lentiviruses were produced, and target cells were transduced essentially as described (37). In brief, lentiviruses were generated by transient transfection of 293T cells plated at a density of 4.5 × 10⁶ cells per 10-cm dish. The following day, cells were co-transfected with 10 μg each of lentivector pLVTH, pCMV-dR8.74psPAX2, and pCR-VSVG using the calcium phosphate precipitation method. Lentivirus production was enhanced by the addition of 10 mM sodium butyrate to the culture medium for 6 h. Virus-containing supernatants were supplied 24 h later with 7 μg/ml Polybrene (Sigma) and directly used to transduce target cells at a density of 3.5 × 10⁶ cells per 3.5-cm plate for a period of 24 h. Puromycin-selected HeLa cell lines stably expressing the tTR-KRAB transrepressor were used to generate lines expressing the different siRNAs upon treatment with doxycycline (DOX, 5 μg/ml; MP Biomedicals, Eschwege, Germany). These cell lines were further subcloned by single cell cloning, and for each siRNA various subclones were analyzed throughout this study.

Cell Synchronization—For arresting cells in prometaphase, cells were seeded at a density of 8 × 10⁵/cm² and kept continuously under DOX treatment. Two days later cells were exposed to 100 nM nocodazole for 16 h. Mitotic cells were har-
vected by mechanical shake off. Cells were then washed three times with PBS, released into nocodazole-free medium, and analyzed at given time points. When stated, cells were supplied upon release with 20 μM of the pan-caspase inhibitor N-(2-quinoIyl)valyl-aspartyl-(2,6-difluorophenoxo)-methylketone (Q-VD-OPH; MP Biomedicals).

Flow Cytometry—Cell cycle analysis was performed on a FACSCalibur (BD Biosciences) using CellQuest software. For determination of cell cycle distribution, cells were stained with propidium iodide (50 μg/ml in 0.1% sodium citrate, 0.1% Triton X-100) and treated with 40 μg/ml RNase on ice. DNA content and the percentage of cells in different cell cycle phases (G1/S/G2/M) were quantified in a linear mode (FL2-A) using winMDI software. The percentage of cells undergoing apoptotic DNA fragmentation (DNA content < 2N, sub-G1) or polyploidization (DNA content > 4N) was determined in a logarithmic mode (FL3-H). Alternatively, apoptosis was assessed with the apoptosis detection kit I (BD Biosciences) using phycoerythrin-coupled annexin V and 7-aminoactinomycin D. For cyclin B1 staining, cells were incubated with anti-cyclin B1 (1:50; Ab3; Labvision, Freemont, CA) and fluorescein isothiocyanate-conjugated secondary antibodies (1:40; Dako, Glostrup, Denmark). DNA was counterstained with propidium iodide (20 μg/ml in 0.5% bovine serum albumin/PBS containing 40 μg/ml RNase). Cyclin B1-positive cells were assessed in a logarithmic mode (FL1-H).

Confocal Laser Scanning Microscopy—HeLa cells were seeded at densities of 8 × 10^3 cells/cm^2 on coverslips and grown in media containing 5 μg/ml DOX. For the analysis of microtubule stability after cold treatment, cells were placed at 4°C for 2 h and re-transferred to 37°C for 25 min (10). After the indicated time, cells were fixed with ice-cold methanol/acetic acid (1:1) for 20 min at −20°C and subsequently incubated in IF buffer (4% bovine serum albumin, 0.05% saponin in PBS) for 1 h. Cells were stained in IF buffer with the following primary antibodies at the indicated dilutions: anti-α-tubulin (DM1a, 1:500, Sigma, or YOL1/34, Acris Antibodies, Hidenhausen, Germany); anti-TACC3 (H300, Santa Cruz Biotechnology); anti-γ-tubulin (GTU-88, 1:100, Sigma); anti-pericentrin and anti-Ndc80 (9G3, 1:500, Abcam, Cambridge, UK); anti-centromere serum (CREST serum, 1:500, Immunovision, Springdale, AR); anti-cyclin B1 (H-433, 1:200, Santa Cruz Biotechnology, Heidelberg, Germany); anti-phosphohistone 3 (Ser-10; 1:100; Beckman Coulter, Krefeld, Germany); anti-Aurora B (1:100; BD Biosciences); anti-BubR1 (1:1000; kindly provided by Stephen S. Taylor, University of Manchester, UK). Thereafter, cells were washed and labeled with secondary antibodies (Alexa488-, Alexa568-, Alexa594-, or Alexa633-coupled anti-mouse, anti-rabbit, anti-rat, or anti-human antibodies; Molecular Probes, 1:400). DNA was detected using 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma). Analyses were performed with a Leica TCS SP2/AOBS microscope equipped with a HCX PL APO ×63 immersion objective and excitation wavelengths of 405, 488, 594, and 633 nm. Confocal pictures shown in Figs. 3 and 4 are single optical slices of 0.9–1.3 μm thickness. For live cell imaging analysis, cells were grown in CO2-independent medium (Invitrogen) and maintained in a heating chamber at 37°C. Images were collected at a single focal plane at 3-min intervals on an LSM510-Meta confocal microscope with a ×40/1.3 immersion objective.

Reverse Transcriptase-PCR Expression Analysis—Total cellular RNA was isolated using the Qiazol RNA isolation kit (Qiagen, Hilden, Germany). First strand cDNAs were generated from 2 μg of RNA in a total volume of 20 μl by oligo(dT) priming using Omniscript reverse transcriptase (Qiagen). Primer sets and PCR conditions used to amplify fragments of individual human TACC isoforms have been published previously for TACC1 (38), TACC2 (39), and TACC3 (40).

Immunoblotting—Total cell lysates were prepared in lysis buffer containing 1% Nonidet P-40, 50 mM Tris/HCl, pH 8.0,
150 mM NaCl, and 2 mM EDTA. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond C, GE Healthcare). Blots were probed with the following primary antibodies at a final concentration of 1 μg/ml or according to the manufacturer’s instructions: anti-β-actin (C4; MP Biomedicals), anti-TACC3 (H300, Santa Cruz Biotechnology), anti-caspase 3 (R & D Systems, Bad Nauheim, Germany), anti-PARP (Ab3; Labvision); anti-Ndc80, anti-Aurora B (BD Biosciences), and anti-BubR1. Specific signals were visualized with the ECL detection system (GE Healthcare) following incubation with horseradish peroxidase-coupled secondary antibodies (Cell Systems, St. Katharinen, Germany).

**Statistical Analysis**—To evaluate statistical significance, we have performed Student’s t tests. Results are given as means ± S.D. p values below 0.05 were considered significant.

**RESULTS**

**Efficient Down-regulation of Endogenous TACC3 Expression Using Drug-inducible Production of siRNAs**—Recent studies indicate an important function of TACC3 in mouse development and centrosome-dependent microtubule assembly (21, 41). To further study the cellular role of TACC3, we developed a controllable siRNA-based method for the down-regulation of TACC3 expression. We took advantage of a lentivirus-based transduction system followed by stable siRNA expression under the control of the doxycycline (DOX)-responsive tTR-KRAB transrepressor (36) (supplemental Fig. 1S, A). In HeLa cells stably transduced with tTR-KRAB, expression of a siRNA directed against TACC3 (siRNA2h) led to an almost complete depletion of TACC3 protein levels by day 2 of continuous DOX treatment (Fig. 1A). In contrast, TACC3 protein levels did not change in cells transduced with a nonfunctional siRNA (siRNA2m) throughout this study. Importantly, there was no significant influence of any of the siRNAs on the mRNA expression of the other two mammalian TACC family members, TACC1 and TACC2 (supplemental Fig. 1S, B). Thus, we established an effective lentivirus-based method permitting the specific and conditional down-regulation of TACC3 expression.

**HeLa Cells Progressively Arrest Prior to Anaphase upon Prolonged TACC3 Depletion**—Genetic inactivation of the mitotically expressed TACC3 gene in mice leads to embryonic lethality, hypocellularity, and cellular apoptosis (21, 22). Therefore, we investigated the molecular mechanisms and consequences of inducible TACC3 gene silencing on proliferation and cell division.

**FIGURE 2. Impaired cell division following TACC3 down-regulation.** A, determination of mitotic progression by intracellular cyclin B staining and FACS analysis. Nocodazole-arrested cells were collected by mitotic shake off, washed, and replated into nocodazole-free medium. Cyclin B expression and DNA content (2N, rectangles; 4N, circles) were analyzed by flow cytometry at the indicated time points following nocodazole release. B, quantification of the percentages of cyclin B1-positive mitotic cells and cells that entered the next G1 phase following nocodazole release. Values shown are means ± S.D. (*, p < 0.05; **, p < 0.005) from at least three experiments analyzing at least 10,000 cells per time point by flow cytometry. C, quantification of the percentage of mitotic cells undergoing cell division within a period of 5 h following release from a nocodazole arrest as determined by live cell imaging. The total number of cells scored is indicated.
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A control and TACC3-depleted HeLa cells were stained using TACC3 (green) and α-tubulin-specific antibodies (red) and analyzed by confocal laser scanning microscopy (cLSM). A, assay of spindle stability by cold treatment. Control and TACC3 siRNA-expressing cells were transferred to 4 °C for 2 h and then allowed to recover at 37 °C for 25 min. Cells were stained using α-tubulin antibodies (green) and analyzed by cLSM. Note that already on day 1 of DOX treatment TACC3-depleted cells failed to efficiently regrow their spindle microtubules following cold treatment as compared with control cells. DNA in A and C was visualized by DAPI staining (blue). Representative experiments are shown.

Survival using HeLa cells as a model system. To characterize the primary cellular defects resulting from lack of TACC3 function in greater detail, we performed confocal laser scanning microscopy (cLSM) analysis using combined α-tubulin/cyclin B1/DNA staining. The mitotic marker cyclin B1 identifies cells from late G2 to metaphase and is degraded prior to anaphase (25). Typically, on day 3 of DOX treatment, TACC3-depleted cells exhibited a 4-fold increase in their mitotic index as compared with control cells. This was determined by flow cytometry (phosphohistone 3 staining) and cyclin B1 staining (Fig. 1B and data not shown). In contrast, cyclin A levels (typical for S/G2) were not significantly altered in TACC3-depleted cells (data not shown). Upon prolonged DOX treatment, TACC3-depleted cells rapidly accumulated in prometaphase with a concomitant decrease of cells in later mitotic stages, i.e. metaphase, anaphase, and telophase (Fig. 1C). Also, the percentage of cells undergoing cytokinesis decreased progressively upon TACC3 knockdown as compared with control cultures.

Next we verified that HeLa cells were arrested prior to anaphase transition upon TACC3 depletion. We synchronized cells by nocodazole treatment and monitored their mitotic progression after release in nocodazole-free medium using intracellular cyclin B1 staining and FACS analysis. Within the first 2 h of release, TACC3-depleted cells failed to degrade cyclin B1 and hence to proceed into anaphase, whereas the majority of control cells completed mitosis and progressed into the next G1 phase (Fig. 2, A and B). These findings were further confirmed by live cell imaging analysis. TACC3-depleted HeLa cells, which eventually entered anaphase, often failed to complete cytokinesis (data not shown), thus yielding binucleated cells. Moreover, ~90% of TACC3-depleted cells released from a nocodazole arrest did not divide within an observation period of 5 h (Fig. 2C). During the same time, nearly 60% of control cells performed normal cell division. These findings therefore indicate that TACC3 knockdown inhibits cell proliferation by inducing an arrest prior to anaphase.

TACC3 Function Is Required for Proper Chromosome Alignment and Spindle Assembly—Mitotic arrest is mediated through the SAC, which senses unattached kinetochores and the resulting misalignment of chromosomes (28, 29). Therefore, we visualized the organization of mitotic spindles upon TACC3 knockdown using α-tubulin staining. In contrast to control cells, the chromosomal arrangement in TACC3-depleted cells was clearly perturbed in (pro)metaphase and often accompanied by an aberrant spindle morphology and multipolar spindles (Fig. 3A). An abnormal spindle morphology was already evident in ~40% of TACC3-depleted cells at day 1 of DOX treatment as assessed by a combined analysis of spindle architecture and chromosomal alignment. The percentage of cells with affected spindles increased to ~70% by day 3 of TACC3 siRNA expression (Fig. 3B). Moreover, cells with two spindle poles, as detected by γ-tubulin staining, displayed numerous chromosomes that failed to align on the spindle midplate (data not shown). In addition, TACC3-depleted cells, as compared with control cells, were not capable of reassembling functional mitotic spindles at 37 °C after microtubule polymerization through cold treatment (Fig. 3C). Again, this microtubule destabilization was already prevalent at day 1 of TACC3 siRNA expression. These findings indicate that TACC3-regulated spindle function is essential for proper chromosomal alignment and hence mitotic progression beyond metaphase.

TACC3 Depletion Affects the Localization of Structural and Checkpoint Proteins at Kinetochores—In a next step we asked whether the absence of TACC3 expression affects spindle-dependent kinetochore protein assembly and function. Aurora B, a component of the chromosomal passenger complex, is typi-
TACC3 is present at centromeres in (pro)metaphase and at the midplate in anaphase cells. However, as indicated in Fig. 4A, Aurora B failed to accumulate to normal levels at these target structures in TACC3-depleted cells. The reduction of Aurora B signal intensity at centromeres became detectable following day 2 of DOX-dependent TACC3 down-regulation. By day 4 of DOX treatment, the majority of mitotically arrested TACC3 knockdown cells had strongly reduced Aurora B signals at centromeres (data not shown). Concomitantly, we observed a reduced kinetochore staining of BubR1, an important regulatory protein of the SAC (Fig. 4A), whose kinetochore localization is dependent on Aurora B to control chromosomal alignment (27). The localization of Ndc80, a structural protein at the outer kinetochore crucial for the attachment of microtubules to chromosomes (42), was also affected by TACC3 depletion. By day 3 of DOX treatment, the Ndc80 signal was remarkably weaker at kinetochores of TACC3-depleted (pro)metaphase cells as compared with control cells (Fig. 4B). This phenotype was already observed in bipolar cells with few misaligned chromosomes.

Immunoblot analysis revealed that Aurora B and phosphorylated BubR1 were expressed in TACC3-depleted mitotic HeLa cells at levels comparable with controls. Interestingly, the total protein levels of Ndc80 were diminished both in mitotic and nonmitotic TACC3-depleted cells (Fig. 4C). This finding and the obvious reduction of Ndc80, Aurora B, and BubR1 at kinetochores/centromeres by day 3 of TACC3 depletion, combined with the observed chromosomal misalignment, indicate a progressive deterioration of the spindle and kinetochore architecture in the course of TACC3 down-regulation.

**TACC3 Depletion Leads to Caspase-dependent Cell Death during Mitosis—TACC3 deficiency in the mouse is associated with embryonic lethality and greatly reduced cell numbers (21). In addition, TACC3 siRNA-expressing HeLa cells could only be maintained in cell culture for a few days (data not shown). Consistent with this, the observed deterioration of the mitotic spindle apparatus upon TACC3 depletion was accompanied by a rapidly increasing cell death rate. This was evident through the accumulation of cells with hypodiploid and fragmented DNA.**
Together with a progressive loss of cells in 2N (G1) (Fig. 5A and supplemental Fig. 2S, A). Furthermore, TACC3 depletion resulted in the processing and activation of caspase-3 as well as in the cleavage of its substrate PARP (Fig. 5B). The apoptotic death of TACC3 siRNA-expressing cells was further confirmed by flow cytometric staining with annexin V and 7-aminoactinomycin D (supplemental Fig. 2S, B and C), which was almost completely abolished by the pan-caspase inhibitory peptide Q-VD-OPH (Fig. 5C).

Next, we asked whether activation of cell death upon TACC3 knockdown originates during mitosis or is a post-mitotic event. We isolated TACC3-depleted HeLa cells following mitotic synchronization through nocodazole treatment and released them into fresh medium with or without Q-VD-OPH for a period of 4 h. In the presence of the caspase inhibitor, significantly less cells underwent apoptosis from mitosis. By DNA content analysis we detected an increased percentage of viable cyclin B1-positive cells in the 4N gate and a concomitantly decreased fraction of cells with fragmented DNA in sub-G1 (<2N; squares) as compared with control cultures without Q-VD-OPH treatment. Thus, these data indicate that TACC3 down-regulation initiates caspase-dependent cell death in HeLa cells already during mitosis.

Requirement of the Spindle Assembly Checkpoint for Induction of Cell Death upon TACC3 Depletion—The mitotic proteins BubR1 and Mad2 are essential components of the SAC complex and required to arrest cells in mitosis when chromosomes are unattached to microtubules (29). Considering that these regulators possibly determine the cellular outcome following depletion of TACC3, we down-regulated TACC3 expression in the human colon carcinoma cell line HCT116 and its isogenic derivate HCT116-Mad2−/−. Because of haploinsufficiency, the Mad2−/− cells are characterized by a defective SAC (43). As indicated in Fig. 6, HCT116 wild-type cells underwent cell death upon TACC3 depletion as measured by an increased fraction of cells with DNA fragmentation in sub-G1 at day 4 of DOX treatment. In contrast, the absence of a functional spindle checkpoint in HCT116-Mad2−/− cells significantly protected against cell death following TACC3 knockdown. These findings point to a requirement of a functional SAC to induce cell death upon TACC3 depletion.

Mitotic Checkpoint Slippage of TACC3-depleted Cells Is Associated with Micronuclei Formation, Polyploidization, and Supernumerary Centrosomes—Mitotic cells arrested by the SAC can aberrantly escape from this block and subsequent apoptosis by a process termed mitotic slippage (28, 29). This slippage is often accompanied by the formation of micronuclei in G1, which are derived from misaligned chromosomes (44). Indeed, as compared with control siRNA-expressing cells, micronuclei were already prominent in TACC3-depleted monoclonal interphase cells at day 1 of DOX treatment. The incidence of micronuclei further increased by day 3 of
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TACC3 depletion and affected both mono- and multinucleated interphase cells (supplemental Fig. 3S). In addition, during prolonged TACC3 depletion not only apoptotic cells resulting from mitotic arrest but also polyploid cells (DNA content > 4N; Fig. 4A) became increasingly detectable in HeLa cultures. As indicated in supplemental Figs. 3S and 4S, down-regulation of TACC3 was associated with a profound multinucleation and the occurrence of giant cells. Within 1 day of DOX treatment, the percentage of bi- or polynucleated cells was already 2.2-fold higher in TACC3 siRNA-expressing cultures as compared with control cells. This difference increased progressively to 7.6-fold on day 4 of DOX treatment (data not shown). Furthermore, at day 3 of DOX treatment, TACC3-depleted and multinucleated cells displayed supernumerary centrosomes, as visualized by staining and detection of the centrosome/pericentriolar material/pericentriolar-specific markers γ-tubulin, pericentrin, and centrin-2, respectively (supplemental Fig. 4S). Because supernumerary centrosomes (23.4 ± 6.3% of TACC3-depleted cells with >2 centrosomes/4.6 ± 4.1% of controls; mean ± S.D. at day 3 of DOX treatment; see supplemental Fig. 4S) were predominantly found in multinuclear cells, these findings point to cell division failures as the likely mechanism for the accumulation of centrosomes upon TACC depletion.

The Post-mitotic Checkpoint Proteins p53 and p21WAF Are Induced Upon TACC3 Depletion—Polyploidization and centrosomal amplification are counteracted by the p53 pathway, which senses an aberrant mitosis and arrests cells post-mitotically in G1 (45). Depending on the cellular context, p53 inhibits cell cycle progression through its transcriptional target p21WAF or induces cell death (34). HeLa cells display only a very weak induction of p53 and p21WAF upon TACC3 knockdown (data not shown), which is consistent with their known failure to activate a functional p53 response (46). Therefore, we addressed the functional role of p53 and p21WAF in cell survival by targeting TACC3 in the HCT116 cell line and its p53- or p21WAF-deficient mutants. As demonstrated in Fig. 7, the percentage of apoptotic cells with fragmented DNA (sub-G1) was significantly higher in TACC3-depleted wild-type HCT116 cells as compared with p53−/− cells. In contrast, a p21WAF-deficient background strongly enhanced polyploidization and apoptosis following TACC3 depletion, as indicated by the lack of viability of TACC3 siRNA-expressing HCT116 p21WAF−/− cells beyond day 5 (Fig. 7 and data not shown). These data therefore suggest that p21WAF, which is up-regulated in a p53-dependent manner upon TACC3 depletion (supplemental Fig. 5S and data not shown), exerts an anti-apoptotic role in the absence of TACC3 function by inducing a post-mitotic cell cycle arrest. Thus, cell death through TACC3 depletion in HCT116 cells is augmented by p53 function and highly exacerbated by p21WAF deficiency.

DISCUSSION

TACC proteins contribute to the dynamics of the mitotic spindle apparatus (47). Mechanistically, they stabilize centrosomal microtubules and are required to recruit ch-TOG/XMAP215/Msps to the centrosome in an Aurora A-regulated manner, thereby counteracting the microtubule-destabilizing activity of the kinesin MCAK (41, 48). Similar to the phenotypes of TACC3 gene deletion and hypomorphic allele expression in the mouse (21, 22), loss of TACC3 in Drosophila melanogaster and Caenorhabditis elegans resulted in dose-dependent effects in embryogenesis, which was mainly attributed to the occurrence of shorter and weaker microtubules (49–52).

Role of TACC3 in Normal Spindle Function and Chromosome Alignment—In this study, we characterized the long term effects of TACC3 gene silencing on mitotic progression and cellular survival in cells with functional versus compromised mitotic and postmitotic checkpoints. Mitotic spindle components are validated targets in cancer treatment. Spindle poisons like taxol induce mitotic cell death or trigger tetraploidy, which results in a G1 arrest or p53-dependent cell death (30). From these viewpoints, we addressed the question whether TACC3 deficiency is sufficient for mitotic checkpoint activation and induction of cell death. Our data show that this is indeed the case. TACC3 is important for both spindle stability and kinetochore-microtubule interactions and thereby regulates mitotic survival.

Our results are in contrast to a previous study showing that down-regulation of TACC3 in HeLa cells by a transient oligonucleotide-based RNAi approach led to only a mild immediate response, which was characterized by partially destabilized microtubules and a few lagging chromosomes but no overt prometaphase arrest (10). In contrast, depletion of ch-TOG in the same study resulted in a much stronger phenotype, including a block in prometaphase, severely disorganized spindles, and increased multinucleation (10). In both cases, cells were eventually able to complete mitosis, and cell death from mitosis was not reported. It is conceivable that the differences between our
study and the previous study (10) are based on the duration and efficiency of TACC3 depletion, since we used a stable and inducible RNAi approach. Consistent with the work of Gergely et al. (10), we observed a reduced ability of spindles to recover from cold stress already after day 1 of TACC3 siRNA expression. However, the long term effects of TACC3 depletion in our work were rather comparable with the phenotype of ch-TOG-depleted cells (10). In particular, prolonged TACC3 depletion resulted in a progressive accumulation of cells prior to anaphase with misaligned and disorganized chromosomes. Furthermore, this was accompanied by excessive apoptotic cell death from mitosis, which apparently resulted from the failure of TACC3-depleted cells to assemble a functional mitotic spindle apparatus. Thus, our data clearly indicate that depletion of TACC3 to sufficiently low levels has cellular effects comparable with those of ch-TOG down-regulation. This conclusion is supported by the occurrence of distinct mitotic abnormalities in primary fibroblasts from mice expressing a hypomorphic TACC3 allele (22).

Our data provide evidence for a strongly impaired microtubule-kinetochore interaction in TACC3-depleted cells. First, the progressive spindle disorganization was accompanied by a reduction of the scaffold protein Ndc80 at kinetochores. Interestingly, down-regulation of Ndc80 was reported to result in impaired spindle assembly, activation of the SAC, and mitotic cell death (53). Second, the mitotic kinase Aurora B and the checkpoint protein BubR1 failed to localize to normal levels at kinetochores. Mechanistically, Aurora B directs BubR1 and Mad2, essential components of the SAC, to kinetochores during prometaphase (26, 27). Consistent with our findings is a report demonstrating that a damaged kinetochore architecture caused by depletion of components of the Mis12 complex can also provoke chromosome misalignment, delocalization of BubR1, and a sustained metaphase arrest (54). Thus, a disturbed localization of different kinetochore- and centromere-associated regulators, i.e. Ndc80, Aurora B, and BubR1, might hallmark the deterioration of the kinetochore structure and impairment of microtubule attachment.

How TACC3 depletion disturbs the kinetochore structure is currently unclear. TACC3 might interact with essential kinetochore proteins. Alternatively, the disorganized spindle architecture could result from the fact that TACC3 depletion impairs the localization of ch-TOG at centrosomes and the mitotic spindle, thereby permitting a stronger recruitment of MCAK toward the centrosome. The essential microtubule-stabilizing function of ch-TOG is required to counteract the microtubule destabilizing activity of MCAK (55). Indeed, in preliminary experiments we detected an increased centrosomal MCAK localization in TACC3-depleted HeLa cells. The observation that kinetochore localization of BubR1 was still detectable in ch-TOG-depleted HeLa cells (56) as opposed to TACC3-depleted cells, however, also argues for critical interactions of TACC3 with additional proteins, which have to be investigated in future studies.

4 L. Schneider and R. Piekorz, unpublished results.
TACC3 in Mitotic Progression and Survival

Role of a Functional SAC and p53 Pathway in TACC3 Depletion-induced Cell Death—The mitotic defects caused by TACC3 depletion in HeLa cells likely evoke a prolonged activation of the SAC, which prevents degradation of cyclin B1 and anaphase transition (57). An increasing amount of evidence indicates a linkage between SAC activation and cell death from mitosis (29). For instance, induction of apoptosis following pharmacological inhibition of the mitotic kinesin KSP has been reported to depend on SAC (58). To investigate the requirement of the SAC for apoptosis, we down-regulated TACC3 expression in wild-type HCT116 cells and their mitotic checkpoint-impaired counterpart, Mad2 heterozygous HCT116 cells (43). Although wild-type cells succumbed to cell death following TACC3 knockdown, Mad2<sup>+/−</sup> cells were significantly protected. Thus, a functional SAC sensitizes cells to cell death from mitosis following prolonged TACC3 depletion.

Because of a slow but continuous degradation of cyclin B1, the SAC is not permanent and allows mitotic slippage of cells with relatively minor defects into a tetraploid G<sub>1</sub> state. These cells are typically characterized by the appearance of interphase micronuclei resulting from lagging chromosomes (28). Interestingly, micronuclei are often found in p53-deficient cells after sustained stress to the mitotic spindle (59). Indeed, we observed an increased percentage of interphase cells with micronuclei and nuclear buds in p53-compromised HeLa cells following TACC3 depletion. However, the number of cells with micronuclei did not strongly increase upon long term TACC3 depletion, because the disturbed spindle function very soon precluded further cell divisions. Instead, we observed a progressive mitotic cell death as well as an increasing polyploidization of the surviving cells with multiple nuclei and supernumerary centrosomes. Because TACC3-depleted HeLa cells failed to maintain long term mitotic arrest in the continuous presence of nocodazole as compared with untreated control cells, these findings further argue for an adaptation to overcome the SAC-induced arrest upon TACC3 depletion.

The slippage from prolonged and aberrant mitosis normally activates the p53-p21<sup>WAF</sup> pathway, which can induce post-mitotic arrest, leading to the inhibition of re-replication and polyploidization (30). Our studies of TACC3 depletion in murine cells confirm the importance of p21<sup>WAF</sup> in preventing severe mitotic abnormalities (23). The p53/p21<sup>WAF</sup> and retinoblastoma-dependent G<sub>1</sub> checkpoint is compromised in HeLa cells (46), whereas both are functional in the HCT116 cells. Like HeLa cells, also p21<sup>WAF</sup>-deficient HCT116 cells underwent rapid cell death and polyploidization within a few days, again arguing for a crucial survival of the post-mitotic G<sub>1</sub> arrest upon TACC3 down-regulation. In contrast, HCT116 p53<sup>−/−</sup> cells were partially protected from apoptosis, but instead displayed increased polyploidization upon TACC3 depletion (data not shown). However, although HCT116 p53<sup>−/−</sup> cells fail to induce p21<sup>WAF</sup> upon TACC3 down-regulation (supplemental Fig. 5S), they are still capable of inducing a retinoblastoma-dependent G<sub>1</sub> arrest (60). In conclusion, our data indicate that a compromised p53 G<sub>1</sub> checkpoint renders cells highly susceptible to prolonged TACC3 withdrawal. Consistent with this, VX-680, a pharmaceutical inhibitor of Aurora kinases, has been reported to induce apoptosis preferentially in G<sub>1</sub> checkpoint-compromised cells (61). Because the p53-dependent G<sub>1</sub> checkpoint is often affected in tumor cells, TACC3 might therefore represent an attractive antitumor target.

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