Mechanisms Promoting Escape from Mitotic Stress–Induced Tumor Cell Death

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Abstract

Non–small cell lung cancer (NSCLC) is notorious for its paltry responses to first-line therapeutic regimens. In contrast to acquired chemoresistance, little is known about the molecular underpinnings of the intrinsic resistance of chemo-naïve NSCLC. Here we report that intrinsic resistance to paclitaxel in NSCLC occurs at a cell-autonomous level because of the uncoupling of mitotic defects from apoptosis. To identify components that permit escape from mitotic stress–induced death, we used a genome-wide RNAi-based strategy, which combines a high-throughput toxicity screen with a live-cell imaging platform to measure mitotic fate. This strategy revealed that prolonging mitotic arrest with a small molecule inhibitor of the APC/cyclosome could sensitize otherwise paclitaxel-resistant NSCLC. We also defined novel roles for CASC1 and TRIM69 in supporting resistance to spindle poisons. CASC1, which is frequently co-amplified with KRAS in lung tumors, is essential for microtubule polymerization and satisfaction of the spindle assembly checkpoint. TRIM69, which associates with spindle poles and promotes centrosomal clustering, is essential for formation of a bipolar spindle. Notably, RNAi-mediated attenuation of CASC1 or TRIM69 was sufficient to inhibit tumor growth in vivo. On the basis of our results, we hypothesize that tumor evolution selects for a permissive mitotic checkpoint, which may promote survival despite chromosome segregation errors. Attacking this adaptation may restore the apoptotic consequences of mitotic damage to permit the therapeutic eradication of drug-resistant cancer cells. Cancer Res; 74(14); 3857–69. ©2014 AACR.

Introduction

Paclitaxel is a first-line chemotherapeutic agent that inhibits the dynamic instability of microtubules, thus preventing bi-orientation of chromosomes during mitosis (1). Although the reduction of breast and ovarian tumor burden following taxane-based therapies demonstrates efficacy in these settings, responses in non–small cell lung cancer (NSCLC) are rarely curative, as only 30% of patients exhibit a partial response at best, indicating a widespread intrinsic resistance to antimitotic agents (2, 3). Thus, given the promise, yet limitation, of current antimitotic therapies, the identification of mechanisms supporting intrinsic resistance to paclitaxel in NSCLC is essential. Our goal here was to identify the cell autonomous components that permit escape from mitotic stress–induced cell death in a paclitaxel-resistant NSCLC setting.

Efficacy of paclitaxel and other antimitotic agents hinges on the coupling of mitotic defects to cell death. By inhibiting the dynamic instability of microtubules, paclitaxel disrupts chromosome alignment, thereby preventing satisfaction of the spindle assembly checkpoint (SAC). The SAC is composed of sentinel proteins, including MAD2 and BUBR1, which, in the absence of proper microtubule-kinetochore attachments, inhibit the activity of the anaphase promoting complex/cyclosome (APC/C; refs. 4 and 5). Nearly all tumor cells are sensitive to paclitaxel-induced mitotic defects and engage the SAC. However, live-cell imaging studies have revealed that the length and outcome of this mitotic arrest is variable within and among tumor cell lines. For example, apoptosis may be activated directly from mitotic arrest. Otherwise, arrested cells undergo mitotic slippage, defined as an aberrant exit in the presence of misaligned chromosomes thereby forming micronucleated cells that can either die, arrest, or reenter a subsequent division cycle (6–8). This slippage from an SAC-mediated mitotic arrest has been implicated as a survival mechanism, as delaying mitotic exit, either by inhibiting an activator of the APC/C, CDC20, or overexpressing cyclin B1, can increase mitotic dwell time and cell death during or following mitosis (6, 9). The prolonged mitotic arrest may allow for accumulation of...
At single cell resolution, we investigated the molecular components supporting sensitivity of NSCLC cells to paclitaxel (12). Here, we use this platform to investigate the molecular components supporting resistance to paclitaxel in a NSCLC system that exhibits no resistance to paclitaxel. Suppression of a cohort of these chemosensitizers induced a protracted mitotic arrest, which we find is essential for postmitotic cell death. Functional elaboration of these chemosensitizers reveals that prolonging mitotic arrest can be accomplished either by direct inhibition of the APC/C, or through collateral spindle damage, because of the depletion of CASC1 or TRIM69, which we reveal here are novel regulators of mitotic spindle assembly in tumor cells. Thus, prolonging mitotic arrest, which can be achieved through multiple avenues, is a dominant mechanism to sensitize otherwise paclitaxel-resistant NSCLC cells.

Materials and Methods

Cells and reagents

Human bronchial epithelial cells (HBEC), NSCLC, 293, and HeLa cells were a gift from J. Minna and M. White (University of Texas Southwestern Medical Center, Dallas, TX). HeLa, H1299, HCC4017, HCC1171, H1299, H1155, and HBEC lines were validated by short tandem repeat analysis. NSCLC cell lines were maintained in RPMI medium (Gibco) with 5% fetal bovine serum (FBS). HBEC cell lines were maintained in keratinocyte medium plus supplements (Gibco). HeLa and 293 cells were maintained in DMEM with 10% FBS. Paclitaxel (Sigma or Tocris), proTAME (Boston Biochem), and Nocodazole (Sigma) were dissolved in dimethyl sulfoxide (DMSO). Cell-Titer Glo (CTG) and APO-ONE were obtained from Promega. Dharmafect transfection reagents were obtained from Thermo-Fisher. Control siRNA transfections were performed with either non-targeting siRNA pool (Dharmacon) or a pool targeting DLNB14 (12).

Paclitaxel dose curves

Cell lines were plated in 96-well plates to reach 50% confluence at 48 hours, when they were exposed to paclitaxel for 48 hours. Cell viability assessed using the Cell-Titer Glo assay.

Immunoblotting

Cells were lysed in boiling 2× Laemmli sample buffer and resolved by SDS-PAGE. Antibodies used as follows: actin, ANAPC5, CASC1, GAPDH, MCL-1, PDE3B, Myc-A14, Myc-9E10, GST (Santa Cruz); cleaved caspase-3 (Epitomics); MAD2L1 (Covance); TRIM69, β-tubulin (Sigma); pericentrin (Abcam); phospho-histone-H3 (Millipore).

Immunofluorescence

Cells were grown on glass coverslips, fixed in 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and blocked with PBS, 0.1% Tween-20, 10 mg/mL bovine serum albumin (BSA). Slips were incubated with primary antibodies in PBTA and, following PBTA washes, incubated with Alexa Fluor–conjugated secondary antibodies (Invitrogen). For BUBR1 staining, cells were fixed in cold methanol. For microtubule preservation in Fig. 3C, cells were extracted with BRB80 (80 mmol/L PIPES pH 6.8, 1 mmol/L MgCl2, 5 mmol/L EGTA) and 0.5% Triton X-100 for 30 seconds followed by a 0.5% glutaraldehyde fix and 0.1% NaH2O2 quench. Slides were imaged on an Axioiager upright microscope (Zeiss) equipped with a charge-coupled device camera or a Leica CTR5500 upright scope with a monochrome digital camera (Leica DFC340 FX). Quantitation of fluorescence was performed with ImageJ software and normalized to cytoplasmic fluorescence.

Stable cell line production

Cell lines stably expressing green fluorescent protein-histone H2B (GFP-H2B) or myc-TRIM69A were generated by retroviral transduction. Retrovirus was produced by transfection of 293GP cells with vesicular stomatitis virus G protein (SVS-G) and either pCLNCX-GFP-H2B (gift from Gray Pearson) or pLPCX-myc-TRIM69A. Virus was harvested 48 hours after transfection and transduced cells were selected with genetin (pCLNCX-GFP-H2B) or puromycin (pLPCX-myc-TRIM69A).

High-content live-cell imaging

Cell lines stably expressing GFP-H2B were transfected in a 96-well plate and exposed to vehicle or paclitaxel 48 hours after transfection and, unless otherwise indicated, imaged immediately following the addition of paclitaxel. Imaging was performed on a BD Pathway 855 Biomager using a 40× or a 20× high-numerical aperture objective. Images were taken every 20 minutes for 48 to 72 hours. Image sequences were generated using ImageJ and manually quantified.

siRNA screen

A 2-condition, triplicate analysis screen was performed in a 96-well plate format using a Thermo-Fisher library that targets 21,127 unique genes (12). HCC366 cells were transfected with 40 nmol/L siRNAs for 48 hours, followed by exposure to vehicle or 10 nmol/L paclitaxel for an additional 48 hours. Subsequently, CTG was added and values were read on an EnVision Plate Reader. The screening protocol was identical to that previously described with the following exceptions: HCC366 cells were seeded at 1 × 104, transfected with Dharmafect 2 complexed siRNAs in RPMI medium, and cells were treated at 48 hours after transfection with vehicle or paclitaxel and a final PBS concentration of 10% (12). High interest chemosensitizers were ranked by z-score (Supplementary Methods).

siRNA transfections

siRNA transfections were performed using Thermo-Fisher or Sigma siRNA pools. Pools were used at a final concentration of 50 nmol/L for indicated times.
Quantitative reverse transcription PCR

Total RNA was collected using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). Two micrograms of total RNA was used in subsequent reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcription PCR (qRT-PCR) was performed with TaqMan gene expression assays and ribosomal protein L27 (RPL27) was the endogenous control.

Colony formation assays

HCC366 cells were transfected in a 24-well format and exposed to 0 and 10 nmol/L paclitaxel 48 hours after transfection. Ninety-six hours after transfection, 2 × 10^5 viable cells were replated in a 6-well format. Cells were fed biweekly for up to 3 weeks before formaldehyde fixation and Geimsa staining. Colonies were counted manually.

Microtubule regrowth assay

Cells were exposed to 11 nmol/L nocodazole for 1 hour, 72 hours after transfection. Cells were then rinsed with PHEM buffer (60 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA, 2 mmol/L MgCl₂, 1 μmol/L paclitaxel) and placed in fresh media to recover for the time indicated. Cells were then permeabilized with 0.2% Triton X-100 for 1 minute then fixed and immunostained.

In vivo polymerized tubulin assay

Seventy-two hours after transfection, cells were lysed in microtubule stabilizing buffer (100 mmol/L PIPES, 2 mol/L glycerol, 0.1 M MgCl₂, 2 mmol/L EGTA, 0.5% Triton X-100, 5 μmol/L paclitaxel). Subsequently, whole-cell lysates samples were collected and centrifuged for 30 minutes at 4°C and 16,000 × g. The supernatant (monomeric tubulin) was collected and pellet (polymerized tubulin) resuspended in microtubule stabilizing buffer. Fractions were resolved by SDS-PAGE.

cDNA expression and plasmids


Tumor xenografts

pLKO.1 vectors expressing short hairpin RNA (shRNA) were used to generate lentivirus to infect HCC366 cells. Target knockdown was assessed 72 hours after infection. Cells were harvested 96 hours after infection and 2 × 10^5 cells were injected into the flank of female NSG (NOD.Cg-Prkdcre<sup>−/−</sup> Il2rg<sup>−/−</sup>/Jax) mice. The protocol used 10 week old mice weighing 15–20 grams were used for paclitaxel exposure, revealed that the majority (92%) of these cells continued to survive for 40 hours and successfully underwent a subsequent division cycle (Fig. 1E and Supplementary Fig. S1). Replating assays revealed that 40% to 50% of treated cells were capable of colony formation, indicating that these cells, despite mitotic damage, maintain the capacity to proliferate (Supplementary Fig. S1C). In contrast, a representative sensitive cell line, H1155, undergoes a dramatic mitotic arrest resolved by either cell death or micronucleation at 100 nmol/L (Supplementary Fig. S1D). The HCC366 mitotic slippage response was also recapitulated in vivo, as tumors grown subcutaneously in immune-deficient mice exposed to paclitaxel were composed of micronucleated cells (Fig. 1F). This finding suggests that HCC366 cells harbor a regulatory context in which mitotic stress is uncoupled from tumor cell death, leading to paclitaxel resistance and continued tumor cell proliferation.

To comprehensively investigate the molecular basis for detection of paclitaxel-induced death, we performed a pan-genomic, siRNA-based loss of function screen in the presence and absence of 10 nmol/L paclitaxel in HCC366 cells (Supplementary Fig. S1E and S1F). This analysis returned 49 candidate
Figure 1. A, paclitaxel dose curves in indicated NSCLC cell lines. Each point is the mean ± standard error of the mean (SEM) for three independent experiments. B, whole-cell lysates from HCC366 and H1155 exposed to paclitaxel for 48 hours were immunoblotted as indicated. C, immunostaining of HCC366 cells treated for 24 hours. Arrowheads, micronucleated cells. Scale bars, 5 or 15 μm (right). D, single-cell lineage tracing of HCC366 cells stably expressing GFP-H2B (HCC366-GFP-H2B) for 48 hours post-paclitaxel exposure. Each circle represents a single cell. Bar is mean mitotic transit time, which is also indicated. Right, cells were transfected with indicated siRNAs for 48 hours before drug exposure. E, quantitation of single-cell lineage tracing of HCC366-GFP-H2B micronucleates after exposure to 10 nmol/L paclitaxel. Bars represent mean ± range for 100 cells over two experiments. F, H&E staining of HCC366 subcutaneous tumor xenografts treated as indicated. Arrowheads, micronucleated cells. Scale bars, 40 μm. Zoomed images are of white-boxed area.
siRNA pools with a z-score of $<-2.5$ that correspond to annotated genes in the human GENE database (see Supplementary Methods, Table S1 and S2).

We further stratified these siRNAs using a secondary screening strategy to measure caspase-3/-7 activity and mitotic transit time for 23 candidates selected from diverse functional categories (Fig. 2A; Supplementary Fig. S2A and S2B, and Supplementary Table S1). Target mRNA knockdown was also evaluated (Supplementary Fig. S2C). This counter screen returned 9 targets whose inhibition activated apoptotic signaling, 4 of which increased mitotic transit time at least 20%. Colony formation assays revealed a 50% decrease in replate efficiency, indicating that the cell death observed was sufficient to reduce survival of the population (Fig. 2B). This finding indicated that even in slippage prone NSCLC, a number of molecular mechanisms exist to enhance SAC robustness. Thus, we focused on this cohort of ANAPC5, PDE3B, CASC1, and TRIM69 for further mechanistic elaboration. All 4 of these siRNA pools met our off-target validation criteria of 2 independent siRNAs or nonredundant pools reproducing the apoptotic phenotype (Supplementary Fig. S2D).

Single-cell lineage tracing revealed that the prolonged mitotic arrest in target-depleted cells resolved as mitotic slippage to generate micronucleated progeny; 40% to 50% of which subsequently died as measured by cell blebbing and a loss of movement (Fig. 2C). Indicative of a functional conservation of these genes across multiple genetic backgrounds, their inhibition enhanced paclitaxel-induced mitotic arrest in HCC1171, HCC515, and H2887, cell lines that also exhibit a deflection of mitotic stress-induced death (Supplementary Fig. S2E). Thus, prolonging mitotic arrest induced a postmitotic apoptotic response.

Restoring paclitaxel sensitivity requires a protracted mitotic arrest

Of these validated genes, ANAPC5 is the best characterized as encoding a scaffold in the multiprotein APC/C complex, whose inhibition attenuates the proteolytic cascades required for anaphase onset (13). Although roles for CASC1, TRIM69, and PDE3B in mitosis have not been described, their identification in a cohort with ANAPC5 suggests that they may contribute to APC/C inhibition. Indeed, codepletion of CASC1, TRIM69, or PDE3B with MAD2 significantly reduced mitotic transit time in the presence of paclitaxel (Supplementary Fig. S3A). Codepletion also rescued micronucleated cell death as assessed by live-cell imaging and cleaved caspase-3 protein levels (Fig. 2D and Supplementary Fig. S3B).

The prosurvival protein, MCL-1, is degraded during a prolonged mitosis and has been implicated as a regulator of apoptosis following exposure to antimitotic therapeutics (10, 11). Depletion of CASC1 or TRIM69 leads to a reduction in MCL-1 levels in a paclitaxel-dependent manner whereas PDE3B inhibition led to a reduction in MCL-1 even in the absence of paclitaxel. Consistent with previous reports indicating that MCL-1 is degraded by residual APC/C activity during mitosis, siANAPC5 did not decrease MCL-1 levels (Fig. 2E; ref. 10). Cell death despite MCL-1 stabilization in ANAPC5-depleted cells indicates existence of MCL-1-independent apoptotic mechanisms. Taken together, these findings suggest that enhancing mitotic arrest by 20% to 50% in the presence of paclitaxel can be sufficient to engage a programmed cell death response in chemoresistant NSCLC.

Inhibition of APC/C is synthetic lethal with paclitaxel in chemorefractory NSCLC

Findings thus far indicate that, in principle, prolonging mitotic arrest by inhibiting the APC/C may be a therapeutic strategy to restore paclitaxel sensitivity in NSCLC. Importantly, suppression of ANAPC5 in nontumorigenic cells (HBEC3KT) did not enhance the impact of paclitaxel, suggesting that tumor cells may have an enhanced sensitivity to APC/C inhibition (Fig. 2F). The difference in response is unlikely because of cell-cycle kinetics as the HBEC3KT and HCC366 cells proliferate at approximately the same rate (Supplementary Fig. S3C).

Together these data suggest that this combinatorial approach may exhibit a therapeutic window. Indeed, combining a sublethal dose of the APC/C inhibitor, proTAME, with paclitaxel in HCC366 cells enhanced the accumulation of mitotic cells and induced cleaved caspase-3/-7 above levels detected for either agent alone (Fig. 2G and Supplementary Fig. S3D; ref. 14).

To further examine molecular mechanisms that contribute to the duration of mitotic arrest, we focused on CASC1 and TRIM69, whose inhibition displayed minimal, if any, single-agent activity, but which collaborated with paclitaxel to both increase mitotic transit time and reduce MCL-1 levels.

CASC1 regulates microtubule function

Although limited information is available on the function of human CASC1, a polymorphism in the murine Cascl gene at codon 60 [AGT (Ser) $\rightarrow$ AAT (Asp)] is associated with susceptibility to urethane-induced lung carcinogenesis in mouse models (15, 16). The significance of these polymorphisms has not been realized in human cancer as none of the 5 human CASC1 isoforms harbor Asn or Ser at residue 60, and other polymorphisms in CASC1 have not been linked to NSCLC susceptibility or progression (17). However, TCGA analysis revealed an amplification of CASC1 in 15%, 11%, and 7% of lung (adenoma and squamous), ovarian, and breast tumors, respectively (Supplementary Fig. S4A: 18). This amplification typically co-occurs with KRAS, which is adjacent to CASC1 on chromosome 12. Although the function of human CASC1 has not been documented, returning this gene as a top paclitaxel sensitizer in a genome-wide screen implies it may have essential roles in supporting cell division. We assessed the consequences of CASC1 depletion alone and in the presence of an otherwise innocuous dose of paclitaxel in a panel of NSCLC lines with differing paclitaxel sensitivity. In resistant cell lines, where we detected mitotic arrest phenotypes, we found a dependence on CASC1 for viability alone or in the presence of paclitaxel (Fig. 3A). In addition, siCASC1 is monogenic lethal or synthetic lethal with paclitaxel in 5 additional NSCLC cell lines, a defect we did not observe in an immortalized human bronchial epithelial (HBEC3KT) cell line (Fig. 3A). Thus, CASC1 seems to be essential in both resistant and sensitive tumor settings, indicating that tumor cells develop a dependence on...
Figure 2. A, graph of relative mitotic transit time (y) vs. relative caspase-3/-7 activity (APO-ONE) in HCC366 cells exposed to paclitaxel. A subset of siRNA gene targets is indicated. B, colony formation assay in HCC366s exposed to 10 nmol/L paclitaxel for 48 hours. Bars represent mean ± SEM from n = 3 independent experiments. C, HCC366-GFP-H2B cells were transfected with indicated siRNAs for 48 hours followed by exposure to 10 nmol/L paclitaxel. Live-cell imaging commenced with paclitaxel treatment and single-cell lineage traces of 50 cells from two independent experiments were measured. D, as in C, but in the presence of siMAD2. E, whole-cell lysates from HCC366 cells transfected for 48 hours followed by exposure to vehicle or 10 nmol/L paclitaxel for 48 hours were immunoblotted with indicated antibodies. F, whole-cell lysates from nonneurogenic HBEC3KT (N) and HCC366 (T) cells treated as in 2E were immunoblotted with indicated antibodies. G, left, relative cleaved caspase-3/-7 activity (APO-ONE) in HCC366s treated with vehicle, 10 nmol/L paclitaxel, or 2.5 μmol/L proTAME for 24 hours. Bars represent mean ± SEM for n = 9 independent experiments. ***, P < 0.001 and ****, P < 0.0001 (unpaired 2-tailed Student t test). Right, immunoblot of WCLs of HCC366s treated as in the left.
this protein for mitotic progression. Examination of individual cells indicated that CASC1 is essential for mitotic spindle integrity as inhibition led to (i) exacerbation of the frequency of spindles with >2 pericentrin positive foci in HCC366 cells, (ii) generation of multipolar or miniature spindles in H1299 cells, and (iii) induction of aberrant mitotic spindles in the presence of an otherwise innocuous dose of paclitaxel in H1155 cells (Fig. 3B and Supplementary Fig. S4B). Live-cell imaging of

Figure 3. A, whole-cell lysates of NSCLC cells immunoblotted with indicated antibodies following siRNA transfection for 48 hours and subsequent treatment as indicated for 48 hours. siPLK1 was used as a positive control for cleaved caspase-3 detection in HBEC3KT cells. B, as in A, except paclitaxel treatment was 24 hours before immunostaining. Scale bars, 5 μm. C, top, H1299 cells were transfected for 72 hours with indicated siRNAs, then fixed in glutaraldehyde and immunostained. Scale bars, 15 μm. Bottom, immunoblots of H1299 cells following an in vivo polymerized tubulin assay. D, in vivo polymerized tubulin assay in H1299 cells transfected for 72 hours. Lysates were immunoblotted with indicated antibodies. E, HCC366 cells, treated as in B, were fixed in cold methanol and immunostained as indicated. Scale bars, 5 μm. BUBR1 foci were counted by manual inspection in 60 cells per experiment in three independent experiments. Bar indicates mean. ***P < 0.001, Mann–Whitney t test.
H1155 cells indicated that depletion of CASC1 prolonged mitotic arrest, which resolved as either aberrant mitotic exit or cell death in the presence of paclitaxel (Supplementary Fig. S4C).

Previous reports indicate that murine and chlamydomonas CASC1 (IC97) orthologues can bind microtubules (15, 19). Given that human and mouse CASC1 proteins are 66.7% identical and 92% identical in the microtubule binding region, we reasoned that the mitotic defects observed following CASC1 depletion could be because of a microtubule defect. We examined the microtubule network in H1299 cells, where siCASC1 alone had a potent impact on mitotic spindle formation and cell viability, siCASC1 diminished the polymerized tubulin network in interphase cells and attenuated microtubule regrowth after nocodazole washout (Fig. 3C and D). Recently, SAC signaling has been correlated with levels of unattached kinetochores. Thus, increasing the number of unattached kinetochores only slightly could prolong mitotic arrest (20–22). An increase of BUBR1 positive kinetochores in HCC366 cells exposed to paclitaxel and depleted of CASC1 suggest that CASC1 may be supporting mitotic slippage by promoting microtubule–kinetochore attachments and reducing SAC strength (Fig. 3E).

We further examined the impact of siCASC1 on microtubules in HBECK3T alone, or following stable depletion of p53 in the absence (HBECK3T-p53) and presence of oncogenic K-RAS (HBECK3T-p53RASV12; ref. 23). Here, we found that CASC1 was essential for microtubule polymerization in all settings, but only essential for viability following depletion of p53, thus suggesting the tumor-specific dependency on CASC1 for viability can be driven by a loss of tumor suppressor activity (Supplementary Fig. S4D and S4E). Taken together, these findings suggest that combinatorial regimens that incorporate multiple antitubulin agents may enhance or restore sensitivity to NSCLC.

**TRIM69 is a centrosomal and microtubule-associated protein essential for mitotic spindle formation**

TRIM69 is a member of the TRIPartite Motif (TRIM) containing protein family, which contains a RING E3 ligase domain, B box, and coiled coil domains (24). Two isoforms exist, A and B, with only the TRIM69A isoform containing a RING domain (Supplementary Fig. S5A). Indicative that TRIM69A is an E3 ligase, we detected autoubiquitination of TRIM69A in an *in vitro* ubiquitination assay (Supplementary Fig. S5B). We found that TRIM69 depletion, similar to CASC1, activated cell death signaling alone or in the presence of paclitaxel in multiple sensitive and resistant NSCLC backgrounds, with the exception of H2887. This defect was not observed in normal HBECK3T cells (Fig. 4A), thus implicating a dependency on TRIM69 for tumor cell survival. At the single cell level, we observed a number of mitotic defects, including (i) micronucleation (A549), (ii) paclitaxel-enhanced micronucleation (H1299), and (iii) multipolar spindles in the presence of paclitaxel (HCC366 and H1299; Fig. 4B and Supplementary Fig. S5C). Depletion of TRIM69 also increased BUBR1 occupancy at kinetochores in paclitaxel-treated HCC366 cells (Fig. 4C). Thus, we conclude that TRIM69 is essential for mitotic fidelity and may be essential for the proper attachment of microtubules to kinetochores.

To further elucidate the mitotic function of TRIM69, we examined the localization of both isoforms of TRIM69 during the cell cycle. Examination of cells overexpressing myc-TRIM69A revealed localization to microtubules during interphase and centrosomes during mitosis (Fig. 5A). Overexpressed myc-TRIM69A also induced microtubule bundling, rendering microtubules resistant to nocodazole and leading to the generation of micronucleated cells (Fig. 5A and Supplementary Fig. S5D). The localization of myc-TRIM69A to microtubules and centrosomes was dependent upon an intact RING domain as mutation of canonical cysteines in the zinc finger of TRIM69A abrogated microtubule and centrosomal localization of the overexpressed construct, indicating that the localization of TRIM69A is dependent upon E3-ligase activity and/or interaction partners (Fig. 5A). Consistent with this finding, myc-TRIM69B, which lacks the RING domain, was dispersed throughout the cytoplasm during the cell cycle and did not display microtubule localization or induce alterations in microtubule stability (Fig. 5A and Supplementary Fig. S5D). These data suggest that TRIM69 may play a critical role in supporting mitotic fidelity.

We next studied the dynamics of myc-TRIM69A localization during the cell cycle, using H1299 cells with stable expression of myc-TRIM69A. In this setting, we found that myc-TRIM69A is recruited to centrosomes during prometaphase where it remains through telophase (Fig. 5B and Supplementary Fig. S5E). myc-TRIM69A also associated with endogenous pericentrin in cells arrested in mitosis (Supplementary Fig. S5F). The centrosomal localization of TRIM69A was further bolstered by proteomics datasets that indicate interactions between TRIM69 and a number of mitotic proteins, including centrosomal and cytoskeletal components (Supplementary Fig. 5G; refs. 25–36). Indeed, depletion of 2 centrosomal proteins that putatively interact with TRIM69, MYPT1, and GNAI3, attenuate centrosomal localization of myc-TRIM69A during mitosis (Fig. 5C).

Tumor cells frequently amplify centrosomes, which can be induced by tumor suppressors and/or oncogenes. As multiple centrosomes can lead to multipolar mitosis, mitotic defects, and cell death, tumor cells depend upon mechanisms that promote centrosomal clustering for cell survival (37). Proteomic studies suggest that TRIM69 interacts with the 8-member Augmin complex (also known as HAUS), which is essential for centrosomal clustering (31, 37). To evaluate a functional interaction between TRIM69 and the HAUS complex, we depleted HAUS1 in H1299 cells in the presence and absence of myc-TRIM69A. We find that spindle multipolarity induced by inhibition of HAUS1 can be reversed by overexpression of myc-TRIM69A, indicating that TRIM69A can promote centrosomal clustering (Fig. 5D). Thus, inhibition of TRIM69A may enhance the vulnerability of tumor cells to paclitaxel by attenuating centrosome clustering, which may prolong mitotic arrest and allow for the reengagement of cell death signaling.

**CASC1 and TRIM69 are required for in vivo tumor growth**

We next determined whether CASC1 and TRIM69 were essential for growth of tumors in vivo. Specifically, to achieve stable depletion of CASC1 and TRIM69, we introduced shRNAs...
targeting GFP, CASC1, or TRIM69 into HCC366 cells (Fig. 6A). These cells were subcutaneously injected into immunodeficient mice, and we observed attenuation in growth of tumors expressing shTRIM69 and shCASC1 (Fig. 6B and C), indicating that the depletion of CASC1 or TRIM69 in vivo was sufficient to reduce mitotic fidelity, hematoxylin and eosin (H&E) staining on excised tumors revealed extensive micronucleation, and aberrant mitotic figures (Fig. 6D). Furthermore, TUNEL staining on tissue sections revealed enhanced cell death (Fig. 6E). Taken together, these findings indicate that CASC1 and TRIM69 suppression is sufficient to affect mitotic fidelity and viability in vivo.

Discussion

Although genomic studies have begun to isolate cohorts of patients that may be selectively sensitive to precision therapeutics, the majority of patients with NSCLC are administered standard chemotherapeutic regimens that are comprised of DNA damage agents and antimitotics. These regimens are noncurative and in 70% of cases fail to reduce tumor burden (2, 3). The underlying basis for the intrinsic resistance of NSCLC to standard chemotherapy is poorly understood. Here, we use a genome-wide RNAi approach to define the molecular components that deflect cell death in the presence of mitotic damage. This work resulted in a number of new principles about chemoresistance and mitosis in tumor cells. First, a common feature to uncouple tumor cell death from an insurmountable spindle defect is mitotic slippage. Second, inhibition of specific proteins can increase mitotic transit time >20%, which is sufficient to activate cell death. Third, CASC1 is a novel regulator of microtubule dynamics that is essential for tumor, which is sufficient to enhance sensitivity in NSCLC cells resistant to paclitaxel (12). These findings suggest a new therapeutic strategy for chemoresistant NSCLC: combining antimitotic agents, such as paclitaxel, with agents, such as small molecule inhibitors of the APC/C may enhance the potency of these first-line agents with limited impacts on normal tissues.
Many tumor cells exhibit a brief SAC-dependent arrest and mitotic escape following exposure to spindle poisons in vitro and in vivo (6, 38). The frequency of this behavior suggests that tumor evolution may generally select for variants that are capable of bypassing prolonged mitotic arrest. Oncogenic changes such as p53 loss, EGFR activation, or K-RAS mutation can induce mitotic spindle and centrosomal alterations, which could conceivably disrupt mitotic progression and lead to cell death in pre-tumorigenic lesions (39–41). Thus, those preneoplastic variants that bypass mitotic arrest could have a selective advantage, if they retain fecundity. A collateral consequence of this relationship would be an intrinsic resistance to...
antimitotic chemotherapeutics. Our work supports the hypothesis that re-engaging a prolonged mitotic arrest can enhance sensitivity to antimitotics (6, 9). We find that elevation of mitotic transit time by 20% to 50% is sufficient to switch a paclitaxel-resistant setting to a sensitive one. However, frank inhibition of the APC/C, through depletion of CDC20, is highly lethal in tumor cells and likely normal cells, given the requirement for CDC20 to activate the APC/C (9). Thus, single agent targeting of the APC/C could have a narrow or nonexistent therapeutic window. Our finding that ANAPC5 depletion can exhibit a synthetic lethal interaction with paclitaxel, suggests that tumor cells may have an enhanced sensitivity to APC/C inhibition that may be exploited by combining sublethal inhibition of APC/C with low-dose paclitaxel to produce a synthetic phenotype.

We also define a mitotic and microtubule role for the human CASC1 protein in tumor cells. Although this gene has been linked with murine lung tumorigenesis, any functional role in human tumorigenesis has not been demonstrated. We find that tumor cells require CASC1 for microtubule polymerization and inhibition of CASC1 potentially impacts microtubule availability for the alignment of chromosomes, thereby increasing the number of unattached kinetochores and prolonging the SAC. The heightened dependency on tubulin regulation in tumor cells could be driven by oncogenic stress that may increase centrosomal clustering defects and

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We also define a mitotic and microtubule role for the human CASC1 protein in tumor cells. Although this gene has been linked with murine lung tumorigenesis, any functional role in human tumorigenesis has not been demonstrated. We find that tumor cells require CASC1 for microtubule polymerization and inhibition of CASC1 potentially impacts microtubule availability for the alignment of chromosomes, thereby increasing the number of unattached kinetochores and prolonging the SAC. The heightened dependency on tubulin regulation in tumor cells could be driven by oncogenic stress that may increase centrosomal clustering defects and
attachment errors, thus creating an environment in which mild disruptions to the microtubule network can wreak havoc on chromosome segregation in tumor cells. As a recent report suggests that mitosis is compromised in K-RAS backgrounds, the genetic link between CASC1 and K-RAS may allow for mitigation of transformation induced mitotic defects by increasing the expression of a microtubule regulatory protein in concert with an oncogene (40). Importantly, these findings suggest that one mechanism for prolonging the SAC in tumor cells may be to compromise microtubule function in parallel, but independent, manners by combining antitubulin agents.

TRIM69A is an E3 ligase with only limited characterized function, which we implicate as critical for mitotic fidelity. Specifically, TRIM69A seems to promote centrosomal clustering, which is an essential process to prevent multilobar spindles and decreased mitotic fidelity (37, 42, 43). Thus, interfering with centrosomal clustering may be a route to sensitize resistance cells to paclitaxel. Furthermore, the dependency of TRIM69 on MYPT1 and GNAI3 for centrosomal localization implicates TRIM69 in the regulation of phosphatase and receptor-independent G(ialpha) signaling during mitosis. MYPT1, which is localized to the spindle, kinetochores and centrosomes, is thought to regulate SAC checkpoint silencing though dephosphorylation of Aurora B and BUB1 (44–46). GNAI3 and other G(ialpha) subunits have been localized to the centrosome and depletion of GNAI3 induces cytokinesis defects, whereas overexpression increases mitotic transit time (47). Thus, TRIM69A activity may be essential for coordinating multiple molecular events that promote bipolar spindle formation and allow for rapid checkpoint silencing. Investigation into the role of TRIM69A regulation may reveal additional mechanisms governing spindle alignment and centrosome clustering.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Mechanisms Promoting Escape from Mitotic Stress–Induced Tumor Cell Death

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