SYMPLEKIN AND TRANSFORMING ACIDIC COILED-COIL CONTAINING PROTEIN 3 SUPPORT THE CANCER CELL MITOTIC SPINDLE

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ABSTRACT

KATHRYN CAPPELL: Symplekin and Transforming Acidic Coiled-Coil Containing Protein 3 Support the Cancer Cell Mitotic Spindle
(Under the direction of Dr. Angelique Whitehurst)

An increased rate of proliferation in cancer cells, combined with abnormalities in spindle architecture, places tumors under increased mitotic stress. Previously, our laboratory performed a genome-wide paclitaxel chemosensitizer screen to identify genes whose depletion sensitizes non-small cell lung cancer (NSCLC) cells to mitotic stress induced by paclitaxel treatment. This screen uncovered a cohort of genes that are required for viability only in the presence of paclitaxel. Two genes uncovered in this screen were the polyadenylation scaffold symplekin and the gametogenic protein transforming acidic coiled-coil containing protein 3 (TACC3).

Herein, we examine the impact of polyadenylation and gametogenesis on the tumor cell mitotic spindle. First, we demonstrate that depletion of SYMPK and other polyadenylation components sensitizes many NSCLC cells, but not normal immortalized lines, to paclitaxel by inducing mitotic errors and leading to abnormal mitotic progression. Second, we demonstrate that multiple gametogenic genes are required for normal microtubule dynamics and mitotic spindle formation in the presence of paclitaxel. Additionally, we show that the gametogenic protein TACC3 is uniquely required for mitosis only in transformed cell lines but not normal immortalized cell lines and that this unique dependency can be targeted in vitro with a small molecule.

These studies reveal an unanticipated dependence of the cancer cell mitotic spindle on polyadenylation and gametogenic genes. We propose that, faced with mitotic stress, cancer cells develop *conditional dependencies* on processes such as polyadenylation that occur in all cells and *emergent dependencies* on gametogenic genes that are overexpressed in tumor cells.

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LIST OF ABBREVIATIONS AND SYMBOLS

3’ UTR	3’ untranslated region
ACRBP	Acrosin binding protein
APC	Anaphase promoting complex
ARNT	Aryl hydrocarbon receptor nuclear translocator
AURKA	Aurora kinase A
BLI	Bioluminescent imaging
BRDT	Bromodomain testis specific
Bub3	Budding uninhibited by benzimidazoles 3
BubR1	Budding uninhibited by benzimidazoles R1
cdc20	Cell division cycle 20
cDNA	Complementary DNA
CKAP5	Cytoskeleton associated protein 5
CNTRL	Control
CPE	Cytoplasmic polyadenylation element
CPEB	Cytoplasmic polyadenylation element binding protein
CPSF1-6	Cleavage and polyadenylation specific factor 1-6
CSTF1-3	Cleavage stimulation factor 1-3
Ct	Cycle threshold
CT-antigen	Cancer-testis antigen
DAC	5-aza-2’-deoxycytidine
DAPI	4’-6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DSE	Downstream element
EGFR	Epidermal growth factor receptor

EGTA	Ethylene glycol tetraacetic acid
FATE1	Fetal and adult testis expressed 1
FDA	Food and Drug Administration
FMR1NB	Fragile X mental retardation neighbor 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GFP-H2B	Green fluorescent protein conjugated to histone 2B
HBEC	Human bronchial epithelial cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
hTERT	Human telomerase reverse transcriptase
MCAK	Kinesin family member 2C
MPS1	TTK protein kinase
NA	Nuclear aperature
NSCLC	Non-small cell lung cancer
NuMa	Nuclear mitotic apparatus protein 1
NXF2	Nuclear RNA export factor 2
PABP	Poly(A) binding protein
Pac	Paclitaxel
PAP	Poly(A) polymerase
PARP	Poly(ADP-ribose) polymerase 1
PAS	Polyadenylation signal
PCR	Polymerase chain reaction
PLK4	Polo-like kinase 4
PolII CTD	RNA polymerase II C-terminal domain

RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SAC	Spindle assembly checkpoint
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
STARD6	StAR-related lipid transfer (START) domain containing 6
SYMPK	Symplekin
TACC3	Transforming acidic coiled coil containing protein 3

CHAPTER I

INTRODUCTION

Hallmarks of cancer and the stress phenotype of cancer cells

An increased proliferation rate in cancer cells, coupled with dysregulation of normal controls on cellular growth, drives several hallmarks of tumors. These hallmarks were originally elegantly proposed by Hanahan and Weinberg and include self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, and metastasis (1). Over the past several decades, scientists have unraveled the signaling networks driving the hallmarks of cancer and found many hallmarks derive from activation of oncogenes or loss of tumor suppressors. Knowledge of the signaling pathways driving the hallmarks of cancer has led to significant improvements in cancer treatment using drugs that target oncogenic signaling networks. However, researchers have also realized new complexities in cancer including a tremendous degree of genetic heterogeneity between tumors, the existence of subpopulations within a single tumor that may respond differently to drugs, the propensity for tumors to develop resistance to targeted therapeutics, and a strong influence of stress pathways on the tumor cell phenotype. These complexities demand new directions for scientific inquiry but also represent potential inroads for the development of novel therapeutics.

Stress phenotypes in cancer cells – One recent development in cancer biology has been increased recognition of the importance of stress phenotypes in modulating tumor cell growth. The stress phenotypes commonly observed in cancer include immune, metabolic, proteotoxic, oxidative, DNA damage, and mitotic stress (2). Stress phenotypes are so common in cancers that it has been proposed these represent additional hallmarks of the tumor cell (2). These stress phenotypes arise from the increased proliferation rate and aberrant signaling networks found in cancer cells. For example, DNA damage stress can arise from alterations in DNA repair signaling networks that limit the ability of a cancer cell to fix damaged DNA. Interestingly, stress phenotypes can enhance cancer cell growth but also represent a unique vulnerability of the tumor cell. This is exemplified by BRCA mutant breast cancers, which are markedly deficient in DNA repair. Mutations in BRCA correlate with a poor prognosis and increased tumor incidence.

However, BRCA mutant breast cancers have markedly increased sensitivity to PARP inhibitors that target DNA damage repair (3). Thus, a second perturbation of the DNA damage repair pathway in BRCA mutant breast cancers can greatly increase tumor cell killing. The stress phenotypes therefore represent a pressure point in the cancer cell that can be therapeutically exploited.

While the idea that these stress phenotypes represent additional hallmarks of cancer cells is new, stress pathways have in actuality been indirectly targeted in cancer treatment for decades. Two notable examples of this are cytotoxic agents that induce DNA damage and mitotic stress in tumor cells. DNA damage stress is commonly targeted in cancers using platinum compounds or radiation, both of which damage the DNA. Mitotic stress is targeted with agents such as the Vinca alkaloids and paclitaxel, which damage the tumor cell mitotic spindle. These cytotoxic therapies represent the mainstream of cancer treatment today. Although cytotoxic therapies activate stress pathways in tumor cells, potent effects on normal tissues limit the usefulness of cytotoxic therapies. These off-target effects are likely due to the fact that cytotoxic drugs target components that are present in both tumor and normal cells, in contrast to agents such as PARP inhibitors that preferentially affect BRCA mutant cancer cells. A better understanding of how cytotoxic drugs activate stress pathways in cancer may lead to more directed targeting of stress phenotypes unique to cancer cells, leading to improved efficacy and less effects on normal tissues.

Using a genome-wide synthetic lethal paclitaxel chemosensitizer screening strategy our laboratory has uncovered a cohort of gene products that sensitize cells to the mitotic stress induced by paclitaxel treatment (4). This dissertation will focus specifically on two groups of genes uncovered in this genome-wide screen; those involved in polyadenylation and those involved in gametogenesis. Herein, we demonstrate an unappreciated link between these genes and the response of non-small cell lung cancer (NSCLC) lines to mitotic stress. Therefore, a deeper discussion of the mitotic stress phenotype of cancer cells is warranted.

Mitotic stress in cancer

Mitotic stress is widely observed in cancer cells and likely accounts for the clinical success of numerous anti-mitotic therapies including paclitaxel and the Vinca alkaloids. Mitotic stress arises from an increased rate of proliferation in combination with structural changes in the cancer cell including aneuploidy, supernumerary centrosomes, and altered microtubule stability. These three structural alterations are interdependent since development of any one of these alterations can drive development of the others. Importantly, these structural alterations in the tumor cell mitotic spindle represent unique vulnerabilities that could provide a method to more specifically target mitotic stress in tumor cells.

Aneuploidy and the spindle assembly checkpoint - Aneuploidy describes cells that have an uneven number of chromosomes that is either more or less then the normal diploid chromosomal complement. Greater then 90% of human solid tumors are aneuploid (5). The widespread occurrence of aneuploidy in solid tumors is somewhat of a paradox because aneuploidy consistently reduces proliferative capacity both at the organismal (6, 7) and the cellular level (8) yet cancers grow at an extremely high rate. This paradox can be partially explained by aneuploidy leading to increased expression of oncogenes or loss of tumor suppressors (9). For example, changes in chromosomal composition can lead to amplification of the oncogene ERBB2 (10) or loss of the tumor suppressor PTEN (11). These alterations in expression of oncogenic genes could provide a proliferative advantage to the tumor cell and account for the widespread occurrence of aneuploidy in tumors.

The mechanism by which tumor cells become aneuploid likely involves some type of bypass of the spindle assembly checkpoint (SAC) (Figure 1.1). The SAC is the major mitotic checkpoint that monitors for proper attachment of chromosomes at the kinetechore to microtubules of the mitotic spindle. In the absence of proper attachments, the SAC delays entry into anaphase by preventing activation of the E3 ligase CDC20-APC. This prevents the polyubiquitination and destruction of substrates such as securin and cyclin B1 that is needed for

Figure 1.1

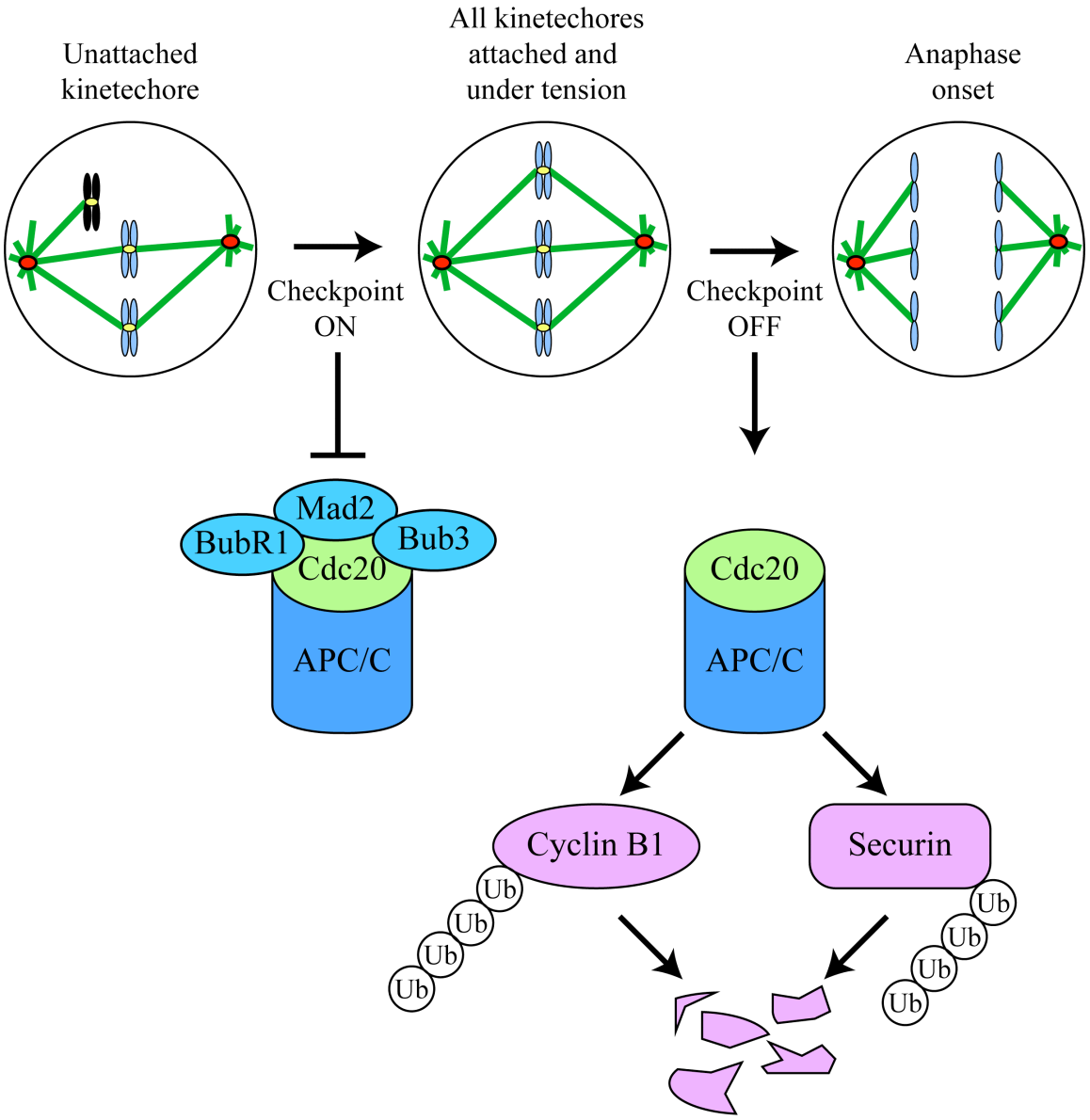


Figure 1.1 The spindle assembly checkpoint

Cartoon depicting the core components and function of the SAC. The presence of an unattached kinetechore triggers activation of the SAC and recruitment of checkpoint proteins such as MAD2, BubR1 and Bub3 to CDC20 of the anaphase promoting complex (APC), inhibiting APC activity. When all kinetechores make the proper attachments and spindle tension is established, the checkpoint is turned off and CDC20-APC becomes active. This allows for ubiquitination and subsequent degradation of CDC20-APC substrates, including cyclin B1 and securin, and allows for anaphase onset.

anaphase onset (12). Activation of this checkpoint protects the cell from uneven segregation of chromosomes during mitosis and therefore acts as a fail-safe to avoid the development of aneuploidy. Thus, for a cell to develop aneuploidy, it must bypass the SAC.

There are several possible mechanisms by which aneuploid cells may avoid detection by the SAC including mutations in checkpoint genes, altered expression of checkpoint proteins, aberrant spindle attachments, or the generation of multi-polar spindles (13). Originally, it was thought that tumor cells must exhibit widespread mutation or loss of checkpoint genes to allow for bypass of the SAC. However, mutations in checkpoint proteins were later shown to be relatively rare (14-16), which is inconsistent with the widespread incidence of aneuploidy in tumor cells (13). In fact, checkpoint proteins are often over-expressed in tumor cells (17, 18) and overexpression of checkpoint proteins has been shown to drive the development of aneuploidy (18, 19). The mechanism by which overexpression of checkpoint proteins drives aneuploidy may involve an increased mitotic delay leading to nondisjunction of sister chromatids and tetraploidy (18). In addition to alterations in the SAC components themselves, tumor cells may also become aneuploid by developing spindle attachments that are not detected by the SAC. Two examples of these types of defects are changes in chromatid cohesion or merotelic attachments. Altered chromatid cohesion occurs when the two sister chromatids adhere to each other abnormally during mitosis, allowing for improper segregation of an additional chromatid to one daughter cell without activation of the SAC (13, 20, 21). Merotelic attachments, which occur when a single kinetechore attaches to both spindle poles, are also not detected by the SAC and could lead to aneuploidy (22, 23). Finally, the ability of some cancer cells to undergo multipolar mitoses, as discussed below, could also account for aneuploidy development (13). It is currently unclear which of these mechanisms is the major driver of aneuploidy in tumor cells.

The necessity for aneuploid cells to avoid activation of the SAC during each round of replication undoubtedly places tumor cells under increased mitotic stress as compared to normal cells (13, 24). It may be possible to exploit this increased mitotic stress by developing drugs

that specifically target aneuploid cells. As the vast majority of normal cells in the human body are euploid, aneuploidy-specific compounds could have greatly reduced off-target effects on normal tissues. A recent study has identified compounds that specifically kill aneuploid as compared to euploid cells in culture (25). Future studies to examine the impact of these drugs in vivo may uncover a more specific method for targeting mitotic stress in tumors.

Supernumerary centrosomes and multipolar mitoses - An additional structural barrier to mitosis in tumor cells is abnormal expression of greater then two centrosomes in a single mitotic cell. The presence of these supernumerary centrosomes in tumor cells is common and may correlate with tumor aggressiveness (26-29). Supernumerary centrosomes can arise through a number of mechanisms including fusion of two cells, failed cytokinesis, over-duplication, and following activation of oncogenic signaling pathways (27, 30-32). The presence of additional centrosomes in a single mitotic cell can lead to nucleation of more then two spindle poles and therefore places the cell at risk for a multipolar mitosis (33). Multipolar mitoses result in abnormal segregation of chromosomes into three or more daughter cells and can foster the development of aneuploidy (33, 34). However, it is unclear whether this is a significant source for aneuploidy in tumors since the daughter cells from a multipolar division are often inviable (33, 35, 36). Regardless of whether additional centrosomes drive the development of aneuploidy, it is clear that supernumerary centrosomes represent a source of mitotic stress in the cancer cell.

To overcome this mitotic stress, tumor cells must find a way to achieve a bipolar mitosis even in the presence of additional centrosomes. A cell with supernumerary centrosomes may achieve a bipolar mitosis by either inactivating (27), removing (27, 37) or clustering (38-40) the additional centrosomes. Of these options, centrosome inactivation and removal is relatively rare in human tumors while centrosome clustering is much more common (27). Centrosomal clustering occurs when the mitotic cell creates a bipolar spindle by grouping the redundant centrosomes into two poles. This can allow the cell to undergo a bipolar mitosis even in the

presence of additional centrosomes. Centrosome clustering may represent either a unique adaptation of tumor cells to the extra centrosome or an additional dependence of tumors on centrosomal clustering pathways that exist in all cells (27). Since normal human cells rarely express redundant centrosomes, the pathways guiding centrosome clustering may be uniquely required in tumor cells and represent tumor-specific drug targets. The requirement for clustering in cancer cells has spurred efforts to identify genes specifically required for this process, including a recent genome-wide screen (41). Importantly, both CKAP5 (41, 42) and TACC3 (42), discussed in Chapters 2 and 3 of this work, have been implicated in centrosomal clustering.

Changes in microtubule stability and composition - A final mitotic stress phenotype often observed in tumor cells is profound changes in the stability and composition of the microtubule network. Microtubules are structures composed of heterodimers of alpha and beta tubulin. There are six isotypes of alpha and 7 isotypes of beta tubulin (43). Microtubules are dynamic structures that continually cycle between a state of growth and shrinkage. This cycling is called microtubule dynamicity and is vital for rapid formation of the mitotic spindle. In tumor cells, this equilibrium is shifted such that tumor cell microtubules are more stable then those in normal immortalized cells (44-46). Increased microtubule stability in tumor cells has been postulated to drive the development of aneuploidy because kinetechore-microtubule attachments that are excessively stable increase the likelihood of improper segregation of the chromosomes between the daughter cells (44-46). The enhanced microtubule stability in tumor cells may partially explain the success of chemotherapeutic drugs targeting the microtubules.

Several mechanisms may account for the increased stability of microtubules in tumor cells. First, altered microtubule stability observed in tumor lines could be due to changes in oncogenic signaling, which can have important effects on the microtubule network (47-51). Second, increased stability could derive from changes in the composition of microtubule fibers in tumor cells. Different cell types can utilize distinct tubulin isotypes and this can have important impacts on the efficacy of microtubule-targeted drugs (52). These changes in isotype

composition could likewise influence stability of microtubules in tumor lines. Third, changes in microtubule stability could develop in tumor cells due to altered expression of microtubule stabilizing proteins. For example, the microtubule stabilizing proteins HEC1 (53, 54), TACC3 (55) and CKAP5 (56, 57) are all highly over-expressed in cancer cells. This overexpression may drive increased microtubule stability in tumor cells. An important future direction of study is determining the factors uniquely supporting microtubule stability in tumor cells and understanding how these influence the development of aneuploidy.

Overall, tumor cells face profound mitotic stress due to aneuploidy, the presence of supernumerary centrosomes, and altered microtubule stability. Identifying the molecular mechanisms by which these stresses interface with each other and with tumorigenic phenotypes is an important future direction of study. In particular, these stresses may have a strong influence on the response of tumor cells to anti-mitotic therapies.

Paclitaxel and genome-wide screen to identify modulators of chemosensitivity

Discovery of paclitaxel - Paclitaxel is an anti-mitotic chemotherapy that targets microtubules in dividing cells (43). Paclitaxel was originally isolated from the bark of the Pacific Yew tree and later shown to have potent efficacy in killing tumor cells (58). The earliest clinical trials of paclitaxel were hugely successful with efficacy seen in some cancers, such as ovarian carcinoma, which previously had few treatments available. Early trials in ovarian carcinoma demonstrated a 30% overall response rate and some patients exhibited complete remissions (59). At the time, this level of response rate in ovarian carcinoma was outstanding. Since then, paclitaxel has become widely utilized to treat a range of cancers including breast, ovarian, lung, head and neck cancers, and Kaposi sarcoma, and is now an important part of the clinical armamentarium (43).

The history of discovery of paclitaxel demonstrates the hurdles encountered in developing a clinically useful drug. First, paclitaxel was difficult to isolate from the bark of the

Pacific Yew. Researchers attempted to isolate paclitaxel from almost every part of the Yew tree but were unable to derive a sufficient quantity of drug to satisfy the appetites of researchers, clinicians, and patients (60). Large quantities of trees had to be cut down to obtain enough paclitaxel to meet demand. There was so much demand, however, that there were fears the Pacific Yew would be driven to extinction (61). Fortunately, a synthetic method to make paclitaxel was developed, compromising one of the most difficult chemical syntheses to date (62). Second, paclitaxel had poor solubility, which made it difficult to administer the drug to patients. This almost led researchers to abandon paclitaxel but was somewhat solved by the use of the agent Cremophor, a derivative of castor oil, to resuspend paclitaxel (60). Third, there was an initial concern that paclitaxel had too much toxicity on normal cells. However, further research indicated there was a sufficient therapeutic window to allow paclitaxel to become a clinically useful drug. The fact that paclitaxel, now one of the most widely utilized chemotherapies, had to overcome many significant hurdles demonstrates the challenges faced in cancer drug discovery.

Problems of paclitaxel treatment – The widespread clinical usage of paclitaxel demonstrates its success as a chemotherapeutic agent. However, paclitaxel treatment still has two major problems: drug resistance and drug side effects. First, resistance to paclitaxel treatment is common. Resistance to paclitaxel can be primary, occurring in chemonaïve patients, and secondary, developing in patients after several cycles of treatment. The biological mechanisms of paclitaxel drug resistance are the object of intense study and are discussed in more detail below (52). Second, paclitaxel treatment can lead to several side effects including allergic reactions, alopecia, nausea and vomiting, dose-related immunosuppression, and peripheral neuropathy (63). These effects are related both to paclitaxel itself and to the Cremophor delivery vehicle. In particular, Cremophor is believed responsible for many of the allergic reactions that occur with treatment and potentially could also account for peripheral neuropathies (60, 64). Researchers are currently attempting several new methods of delivering paclitaxel including nanoalbumin-bound

or nab-paclitaxel. Nab-paclitaxel (Abraxane or ABI-007) is a Cremophor-free formulation that uses albumin-stabilized nanoparticles to deliver paclitaxel and was recently approved by the FDA for the treatment of breast cancer (65) and has shown efficacy in NSCLC (66). Many of the other side effects seen with paclitaxel are observed with other cytotoxic chemotherapeutic agents and stem from the ability of paclitaxel to kill rapidly dividing cells of the gut, bone marrow, and hair follicle. New formulations of paclitaxel that allow for increased concentration of paclitaxel within tumors may reduce effects on normal tissues by allowing for decreased drug dosage (67, 68). Alternatively, it may be possible to combine paclitaxel treatment with other agents to avoid several of the dose-related side effects of paclitaxel (4). Future efforts to address the problems of drug resistance and side effects related to paclitaxel treatment could have important impacts on patient care.

Biological mechanism of paclitaxel - Shortly after the discovery of the efficacy of paclitaxel in killing cancer cells (58), paclitaxel treatment was shown to alter mitotic spindle function by increasing microtubule stability (69, 70). This was the first link of paclitaxel to the microtubule network and it was later shown that paclitaxel binds directly to the beta tubulin subunit (71). Since paclitaxel binds the beta tubulin subunit to increase microtubule stability, it is classically taught in pharmacology courses that paclitaxel functions by stabilizing microtubules, in contrast to other agents, such as nocodazole, that destabilize microtubules (72). Although the division of microtubule-targeted agents into those that stabilize or destabilize microtubules is conceptually simple and consistent with the impact of these drugs on microtubules in vitro, the reality of how paclitaxel functions in a living cancer cell is somewhat more complex. In particular, paclitaxel has potent effects on both mitotic progression and cancer cell death at concentrations 10-1000 fold below the dose required to produce polymerized microtubules (43, 73, 74). Therefore, it is thought that the biological effects of paclitaxel derive not from it causing massive microtubule polymerization in tumor cells but rather from effects on microtubule dynamics. In a dividing cell, microtubules are constantly cycling between a state of growth and a

state of shrinkage or catastrophe, a process called microtubule dynamicity (75). Since paclitaxel has effects on microtubule dynamicity at a much lower dose then that needed to induce outright polymerization, the efficacy of paclitaxel likely derives from its ability to disrupt microtubule dynamicity (43). The disruption of microtubule dynamics in paclitaxel-treated cells impairs the ability of the mitotic spindle to make proper kinetechore-microtubule attachments. These improper attachments lead to activation of the spindle assembly checkpoint (SAC), mitotic delay, and triggering of cell death pathways (63). In summary, paclitaxel functions by binding to the beta tubulin subunit and inducing alterations in microtubule dynamics, which causes impaired mitotic progression and cell death.

Heterogeneity in response to paclitaxel – Ideally paclitaxel treatment causes death of the cancer cell in mitosis as described above. Unfortunately, there is much more complexity in the ways in which both patients and tissue culture cells respond to paclitaxel. In particular, while some patients demonstrate a marked response to paclitaxel, others show no response at all (59). This heterogeneity of response is paralleled in tissue culture cells where there is great variation in cell fate after paclitaxel treatment both between and within tumor lines (35, 76-80). In tissue culture cells, paclitaxel treatment can lead to a range of responses (Figure 1.2). These responses include dying directly in mitosis, exiting from mitosis, returning to interphase, or exiting as a micronucleated cell that may either die or continue to cycle. This heterogeneity of response in vitro may explain the heterogeneity of response to paclitaxel treatment in patients.

Whether a mitotic cell exposed to paclitaxel dies during mitosis likely relates to how the cell responds to activation of the spindle assembly checkpoint (SAC). Although the term SAC suggests a checkpoint that cells presumably must satisfy before proceeding through mitosis, the SAC is better thought of as a "check-pause". This means that cells can sometimes slip past the checkpoint without forming the proper kinetechore-microtubule attachments. This ability to slip past the checkpoint is due to a slow degradation of mitotic cyclin B1 following prolonged mitotic arrest (76, 81). Slippage past the SAC is problematic because once the cell has slipped out of

Figure 1.2

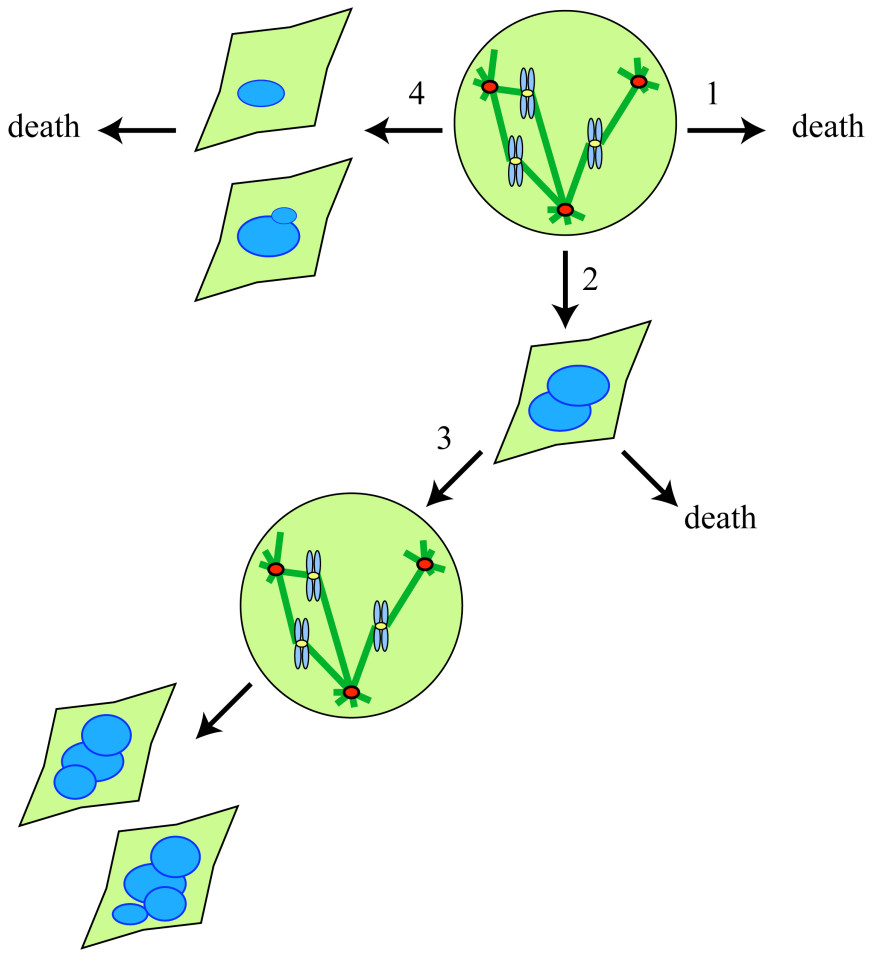


Figure 1.2 Variation in response of mitotic cells to anti-mitotic therapies

Depiction of fates observed in cancer cells following treatment with anti-mitotic agents such as paclitaxel. These fates include (1) dying in mitosis (2) exiting as a micronucleated cell that can either continue to cycle (3) or die and (4) exiting as two daughter cells that die later in interphase.

mitosis, paclitaxel treatment is not effective until the next round of replication. Therefore, researchers have attempted to understand what guides the decision of a cell to die in mitosis after treatment with paclitaxel. The most effective studies have used single-cell time-lapse imaging to follow cells treated with paclitaxel and map the fate of individual treated cells (35, 76-80). The prevailing theory suggests that whether a cell dies in mitosis after treatment with paclitaxel relates to a balance between two competing networks: the rate of degradation of cyclin B1 and the activation rate of apoptotic pathways (63). In this model, cyclin B1 is slowly degraded in the SAC-arrested cell by a proteasome-dependent mechanism (35, 76). Concurrently, activation of the SAC triggers apoptotic pathways that are designed to detect mitotic cells that cannot form the proper kinetechore-microtubule attachments. When the rate of activation of apoptotic pathways exceeds the rate of cyclin B1 destruction, the cell dies in mitosis. In contrast, if the rate of cyclin B1 destruction exceeds the rate of apoptotic pathway activation, the cell slips through the checkpoint. The heterogeneity observed in response of different tumor lines to paclitaxel may therefore derive from variation in apoptotic factors between individual cells and cell lines (35, 79, 80, 82). Therefore, agents that allow for prolonged activation of the SAC may have increased efficacy in killing cancer cells during mitosis (82).

A paclitaxel-treated cell that successfully slips through the SAC without dying may subsequently die during interphase or continue to cycle (Figure 1.2). The factors governing fate of these post-mitotic cells are less clear. In particular, it is largely unknown what factors allow some micronucleated cells to re-enter the cell cycle while other micronucleated cells die or senesce. Several studies have documented a p53-dependent arrest of micronucleated cells in G1 following abnormal mitotic exit (83-85). This suggests the presence of a post-mitotic checkpoint that restrains proliferation of damaged cells. In addition, the degree of mitotic damage does not appear to correlate with post-mitotic fate because the duration of the preceding mitotic arrest does not influence the rate of post-mitotic death in interphase (35). Since prolonged mitotic arrest usually indicates a greater degree of mitotic damage, this finding suggests the likelihood of cell

death following a micronucleated exit is independent of the degree of mitotic damage. Therefore, heterogeneity in propensity for micronucleated cell death may depend on variations in activation of apoptotic pathways between individual cells and tumor cell lines. A more complete understanding of the factors governing post-mitotic cell fate may help in developing better anti-mitotic therapies.

Resistance to paclitaxel treatment – Paclitaxel treatment is limited by significant drug resistance that can be due to several factors including mutations in beta tubulin, upregulation of drug efflux pumps, changes in expression of beta tubulin isotypes, and changes in cell death pathways. First, mutations in beta tubulin that may impair paclitaxel binding could drive resistance. However, mutations in beta tubulin are rare and unlikely to account for the widespread incidence of drug resistance (52, 86). Second, upregulation of drug efflux pumps can drive drug resistance. Drug efflux pumps result in a decreased concentration of paclitaxel in the cell and therefore less cancer cell death with drug treatment. Upregulation of drug efflux pumps has been documented with paclitaxel therapy (87). Third, changes in expression of tubulin isotypes can lead to paclitaxel resistance. Changes in tubulin isotypes have been documented in paclitaxel-resistant ovarian carcinomas (88). Most commonly, increased expression of beta-3 tubulin has been shown to drive paclitaxel resistance (52). Finally, altered apoptotic signaling networks could also influence the development of resistance (89-91). Cells with a decreased propensity to die in mitosis may therefore exhibit increased resistance to paclitaxel therapy. Overall, it is clear that multiple factors can determine the development of resistance to paclitaxel.

Our own laboratory has performed genome-wide paclitaxel chemosensitizer screens in two NSCLC cell lines: paclitaxel-sensitive H1155 cells (4) and paclitaxel-resistant HCC366 cells (unpublished). Interestingly, the genes identified as hits in the H1155 screen had very little overlap with the genes identified in the HCC366 screen. Moreover, depleting genes identified in the H1155 screen in HCC366 cells had minimal effects on HCC366 viability. This suggests that the lack of overlap from the screens derives not from false negatives but from intrinsic

differences between resistant and sensitive cells. Therefore, very different factors may govern the response to paclitaxel in primarily sensitive as opposed to resistant cells.

Genome-wide screen to identify modulators of paclitaxel sensitivity - Given the significant limitations of paclitaxel, coupled with the widespread usage of this drug in the clinic, identification of new drug targets that synergize with paclitaxel could have a significant impact. Additionally, identification of such targets could reveal important biology about how tumor cells respond to mitotic stress. To address these questions, our lab performed the first genome-wide siRNA chemosensitizer screen to identify genes whose depletion significantly increases response of the NSCLC line H1155 to paclitaxel (4). For the screen, the H1155 cell line was transfected in 96-well plates in a one-well one-gene format with siRNAs targeting greater then 21,000 genes. After two days of gene knockdown, the transfected cells were treated with either no paclitaxel or a dose of paclitaxel that has minimal effects on cell viability (10 nM). After an additional two days of growth in the presence of paclitaxel, cell viability was determined using a luminescence-based CellTiter Glo viability assay. This screen returned two types of hits; monogenic lethal hits, in which siRNA knockdown alone reduces viability, and synthetic lethal hits in which gene knockdown only reduces viability in the presence of paclitaxel. Importantly, the screen returned genes whose knockdown was previously shown to synergize with paclitaxel, identified novel drug targets, and also uncovered nearly every component of the gamma-tubulin ring complex, a structure needed for nucleation of spindle microtubules (4). This ability of the screen to uncover both novel genes and genes which have already been shown to influence paclitaxel sensitivity validates the screening approach and suggests the completion of a successful screen.

Several relevant points should be made about this screen. First, the screen hit list was significantly enriched for genes previously shown to impact the mitotic spindle. This enrichment likely derives from the fact that the screen was performed using paclitaxel to induce mitotic stress. Second, as expected with any genome-wide screen, this screen pulled many hits that had no previous function associated with them or no logical reason by which their knockdown would

synergize with paclitaxel. Given the identification of multiple mitotic proteins in the screen, it is likely that many of these unknown hits impact mitotic spindle formation. This has proved to be the case with several hits, including symplekin (SYMPK), the subject of Chapter 2 of this dissertation (92). Third, the screen pulled several members of a gene family called the cancer-testis antigens (CT-antigens) including ACRBP, FATE1, FMR1NB and NXF2 (4, 93). The CT-antigens are a family of genes that share a common expression pattern showing upregulation in tissues of gametogenesis and cancer cells with minimal expression in normal adult tissues (93). The CT-antigens are discussed in more detail below but their identification in a screen for genes affecting tumor cell viability suggests these genes may have an unrecognized role in supporting tumor cell phenotypes. The CT-antigens and a related protein, TACC3, is the subject of Chapter 3 of this dissertation. Fourth, as necessary with any genome-wide screen extensive validation has been performed to reduce the potential that any observation is due to an off-target effect of the siRNA. Most commonly, screen hits have been validated by deconvolution of the siRNA pool both at the level of viability and on relevant phenotypes. As an additional control for these effects, the parallel system of shRNA to mediate gene knockdown has also been effective. Finally, follow-up of hits from this genome-wide screen has revealed important information about the mitotic spindle, response of tumor cells to mitotic stress, and uncovered new drug targets. Next, I will discuss the functions of the screen hit symplekin.

Role of symplekin in polyadenylation and tumorigenesis

Discovery of SYMPK at the tight junction and in polyadenylation - Symplekin (SYMPK) was originally discovered as a component of the *zona occludens* plaques found in tight junctions of epithelial cells (94). It was given the name symplekin, meaning "to tie together" in Greek, for its localization to these important sites of cellular contact. Subsequent studies demonstrated that SYMPK interacts with the Y-box transcription factor ZONAB at the tight junction and can modulate expression of ZONAB target genes (95). In particular, symplekin

increases transcription of the target genes cyclin D1 (95) and claudin-2 (96) but decreases expression of the transcription factor AML1/Runx1 (97). Functionally, these effects of SYMPK at the tight junction drive proliferation while reducing cellular differentiation.

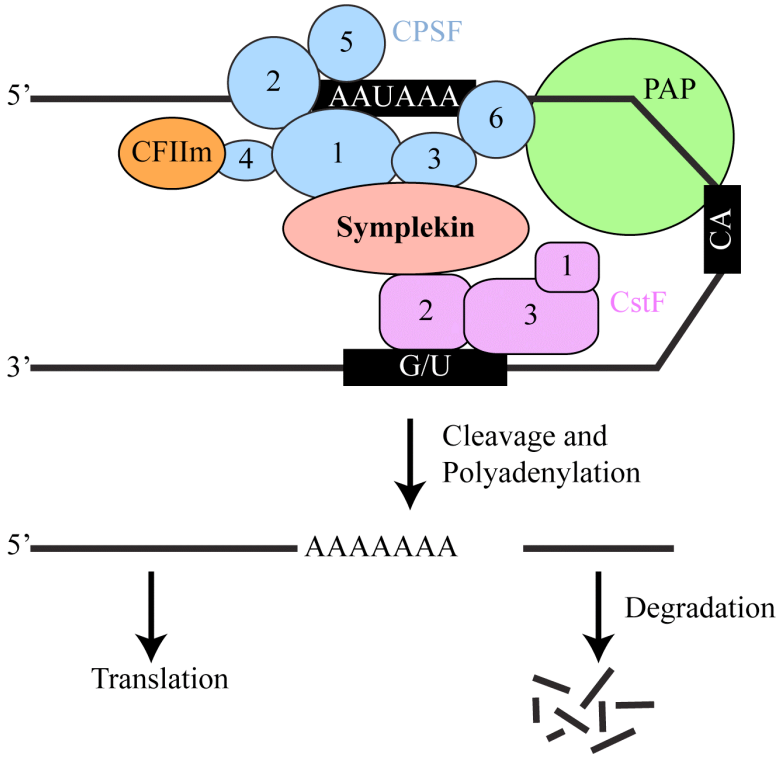
The authors who discovered SYMPK at the tight junction also observed strong SYMPK expression in the nucleoplasm of a range of cells that lack tight junctions (94). The function of SYMPK in this cellular compartment was not elucidated until several years later when a group identified SYMPK as a component of the polyadenylation machinery (98). In particular, it was found that SYMPK interacts directly with the polyadenylation protein CSTF2, demonstrates some homology to the yeast polyadenylation component PTA1 (99), and exists in a complex with multiple polyadenylation factors. Therefore, while SYMPK regulates transcription at the tight junction it also has a role in controlling polyadenylation.

SYMPK and polyadenylation - Polyadenylation is a post-transcriptional process that is required for the maturation of most mammalian pre-mRNAs, excluding histones, and controls mRNA nuclear export, stability, and translation (100). A longer poly(A) tail typically leads to more translation of the mRNA while a shorter tail decreases translation of the mRNA. Polyadenylation occurs simultaneously with cleavage of the pre-mRNA and is directed by a large complex of polyadenylation and cleavage factors (Figure 1.3A) (100). These factors include five cleavage and polyadenylation specificity factors (CPSF1-5 and hFip1), three cleavage stimulation factors (CSTF1-3), cleavage factors I_m and II_m, poly (A) polymerase (PAP), poly(A) binding protein (PABP) and symplekin (Table 1.1) (100). While all these factors, with the exception of PABP, are required for the in vitro cleavage reaction, only a subset (the CPSFs, PAP and PABP) are needed for in vitro polyadenylation (101). Several of these factors were identified in our genome-wide paclitaxel screens in the NSCLC lines H1155 and HCC366 (Figure 1.3B) (4). This suggests an important role for the polyadenylation machinery and symplekin in controlling chemosensitivity.

The process of polyadenylation and cleavage begins with recognition of three primary

Figure 1.3

A.



B.

	Cell line	
siRNA	**H1155**	**HCC366**
SYMPK		
CSTF1		
CSTF2		
CSTF3		
CPSF1		
CPSF2		
CPSF3		
CPSF4		
hFip1		
CFIm-68		
CFIm-25		
hPcf11		
hClp1		
PAP		
PABII		

Viability ratio in paclitaxel	
>0.7	
0.7-0.75	
0.75-0.8	
0.8-0.85	
0.85-0.9	
0.9-0.95	
0.95-1.0	
Monogenic lethal	

Figure 1.3 Protein factors involved in cleavage and polyadenylation and impact of their depletion in two genome-wide screens

(A) Simplified cartoon depicting the poladenylation machinery assembled on a pre-mRNA transcript with canonical primary sequence elements represented in black boxes. Components of the CPSF are shown in blue while components of the CstF are in purple. SYMPK acts as a scaffold to link the CPSF to the CstF machinery. (B) Data from genome-wide screens in two NSCLC lines, H1155 and H1299. Viability ratio in paclitaxel (siGENE 10 nM/siCONTROL 10 nM) is represented in blue with darker colors indicating a stronger synthetic lethal hit. Genes identified as monogenic lethals are indicated in red.

Table 1.1 Characteristics of mammalian cleavage and polyadenylation factors

Group	Subunits	Alternative names	Step in processing	Interacts with:	Function
CstF	CSTF1	CstF-50	Cleavage	CSTF3, PolII CTD	Binds PolII/CTD complex
	CSTF2	CsfF-64		SYMPK, CSTF3	Binds RNA through G/U rich sequence
	CSTF3	CstF-77		CPSF1, CSTF2, CSTF1	Scaffolding function
CPSF	CPSF1	CPSF-160	Cleavage and poly(A) addition	CSTF3, PolII CTD, PAP, hFip1, SYMPK	Binds RNA at PAS site
	CPSF2	CPSF-100		CPSF3, SYMPK	Binds U-rich RNA
	CPSF3	CPSF-73		CPSF2, SYMPK	Cleavage endonuclease
	CPSF4	CPSF-30		HFip1	Binds U-rich RNA
	hFip1	FIPIL1		PAP, CPSF1, CPSF4, CSTF3	Binds U-rich RNA and positions PAP at polyadenylation site
CFIm	CF Im-68	CPSF6	Polyadenylation	CF Im-25	Aids in PAS site recognition
	CF Im-25	CPSF5		CFIm-68 and PAP	Aids in PAS site recognition
CFIIm	hPCF11	PCF11	Unknown	Unknown	Unknown
	hCLp1	HEAB		CFIm and CPSF	Unknown
-	Symplekin	SYMPK	Cleavage and poly(A) addition	CSTF2, CPSF1, CPSF2, CPSF3	Scaffolding function, links CPSF to CSTF
-	PAP	PAPOLA	Polyadenylation	hFip1, CFIm, CPSF1	Catalyzes addition of the poly(A) tail
RNA Polymerase II	Pol II CTD	-	Transcriptional termination	hPcf11, CPSF1, CSTF1, CSTF3	Terminates transcription
-	PABII	PABN1	Polyadenylation	CPSF4	Lengthens and stabilizes poly(A) tail

Information in this table is derived from data in the following sources: (100, 101)

sequence elements in the pre-mRNA by components of the polyadenylation and cleavage complex. The primary sequence elements include the polyadenylation signal (PAS), the cleavage site, and the G/U-rich downstream element (DSE) (100). The PAS usually has the sequence AAUAAA (102) and is recognized and bound by CPSF1 (103). The cleavage site is recognized by the cleavage stimulation factor CSTF2 and does not have a conserved sequence (104) but most often consists of the nucleotide sequence CA with cleavage occurring after the cytosine (100). The DSE can have several sequences but is typically either GU-rich or U-rich (105, 106), is located downstream of the PAS and cleavage sites, and binds CSTF2 (107). Together these sequence elements in the pre-mRNA direct the assembly of the polyadenylation and cleavage factors and lead to simultaneous addition of the poly(A) tail and mRNA cleavage. SYMPK is believed to act as a molecular scaffold in this process by binding to a number of proteins in the complex including CSTF2, CPSF1, CPSF2, and CPSF3 (98, 108, 109). Therefore, activity of SYMPK and the polyadenylation components is essential for polyadenylation and expression of mRNAs.

Additionally, SYMPK and components of the polyadenylation and cleavage machinery also participate in the maturation of the 3' end of histone mRNAs (110, 111). Histone mRNAs are the only eukaryotic mRNAs that are not polyadenylated. Rather, histone mRNAs undergo a 3' maturation process which combines cleavage with addition of a stem-loop structure at the 3' end of the mRNA (112). Many of the same components that mediate the polyadenylation and cleavage reaction, including SYMPK, also direct maturation of histone mRNAs (112). Therefore, SYMPK mediates histone maturation and polyadenylation.

SYMPK and cytoplasmic polyadenylation - In most mammalian cells, polyadenylation is a nuclear process. However, in *Xenopus laevis* oocytes (113-115) and in a subset of mammalian cell types (116-118) polyadenylation can also occur in the cytoplasm. Cytoplasmic polyadenylation is an important method of post-transcriptional control of gene expression during meiotic cell divisions in *Xenopus*. In *Xenopus* oocytes, many meiotic transcripts, including cyclin

B1, encode a cytoplasmic polyadenylation element (CPE) with the sequence UUUUUAU (114). These CPE-containing transcripts exist in the unstimulated oocyte as dormant transcripts with a very short poly(A) tail (119-122). Upon stimulation of the oocyte to proceed through meiosis, the CPE is bound by the cytoplasmic polyadenylation element binding protein (CPEB) and a poly(A) tail is added (123). Addition of the poly(A) tail drives expression of the transcripts and meiotic progression. Therefore, cytoplasmic polyadenylation allows for precise temporal control of gene expression during meiosis.

The machinery controlling cytoplasmic polyadenylation shares many players in common with the nuclear polyadenylation machinery, including SYMPK (108, 124). Two unique components of the cytoplasmic polyadenylation machinery with relevance to this work include the cytoplasmic polyadenylation element binding protein (CPEB) and the protein Maskin. The CPEB functions to bind the CPE in dormant transcripts and direct the assembly of the cytoplasmic polyadenylation machinery (123). Importantly, SYMPK has been shown to interact with the CPEB (124). Additionally, the protein Maskin is a component of the cytoplasmic polyadenylation machinery that represses cytoplasmic polyadenylation by simultaneously binding the CPEB and the cap-binding factor eIF4E to prevent proper assembly of the cytoplasmic polyadenylation machinery on transcripts such as cyclin B1 (120, 125, 126). The human TACC proteins, subject of Chapter 3 of this dissertation, demonstrate significant homology to *Xenopus* Maskin (127). The identification of both TACC3 and SYMPK in our genome-wide paclitaxel chemosensitizer screen, coupled with the important role of cytoplasmic polyadenylation in controlling meiotic cell division, suggests that SYMPK may have an important role in mammalian cell division.

SYMPK and polyadenylation in tumorigenesis - Emerging evidence has implicated both SYMPK and alterations in polyadenylation in cancer development. First, SYMPK becomes upregulated as lung cancer cells progress from normal to malignant cells (128). The upregulation of SYMPK in tumor cells could indicate an important role for SYMPK in supporting tumorigenic

phenotypes. Second, a recent study demonstrated a decrease in colorectal tumor formation following SYMPK depletion (96). This phenotype could be due to either the effects of SYMPK on transcription at the tight junction or on polyadenylation. This study therefore directly implicates SYMPK in colorectal tumor growth. Third, the process of polyadenylation in general is frequently corrupted in tumor cells through a process called alternative polyadenylation. Alternative polyadenylation occurs when pre-mRNAs are polyadenylated at a site upstream of the canonical AAUAAA PAS sequence, resulting in a shorter 3'UTR (129, 130). Alternative polyadenylation can occur in more then 50% of human genes (131) and use of these sites can alter mRNA export, stability and translation (129). Usage of alternative polyadenylation sites is common in cancer cells and can activate oncogene expression (132). This activation of oncogenes is likely due to a shorter 3'UTR that may impair binding of regulatory elements like microRNAs (132). Fourth, polyadenylation can be dysregulated in tumor cells in response to DNA damage (133), through interaction with tumor suppressors (134), and by upregulated expression of polyadenylation components (135). These corrupted polyadenylation networks in tumors may account for the efficacy of the adenosine analog and non-specific polyadenylation inhibitor cordycepin in hematologic malignancies (136-141). Together, this research demonstrates that changes in symplekin expression or polyadenylation can profoundly alter the tumorigenic state.

The cancer-testis antigens

Expression pattern of the cancer-testis antigens - The cancer-testis (CT) antigens are a diverse group of proteins that share a common pattern of expression showing enrichment in the organs of gametogenesis (including the testis, ovary and placental tissue), limited to no expression in normal adult tissues, and aberrant upregulation in tumor tissue (93). To date, there are over 130 genes classified as CT-antigens and the features of these genes have been compiled at CTpedia (http://www.cta.lncc.br), a publically accessible database (142, 143). Many, but not all, of the CT-antigens are encoded on the X chromosome. In fact, up to 10% of X-chromosome

encoded genes are CT-antigens (144). The common expression of the CT-antigens in tumor cells suggests a functional role for these proteins in tumorigenesis.

Several studies have examined the expression pattern of CT-antigens in human cancers and led to some broad conclusions (143, 145, 146). First, CT-antigen expression can be detected in many tumor types but appears most commonly in melanoma and carcinomas of the bladder, lung, ovary, and liver (93). Second, upregulation of the CT-antigens is often sporadic and not all CT-antigens are enriched in all tumors of a specific type (143, 145, 146). For example, the CT-antigen FMR1NB was detected in 5 out of 19 lung carcinomas (147). This sporadic expression pattern has made it difficult to study the functional role of the CT-antigens since not all antigens are present in all cell lines. However, there are cases where many tumors of the same type express a particular antigen. This is exemplified by the CT-antigen ACRBP, which is expressed in over 70% of ovarian cancers (148). Third, in tumors that express CT-antigens, expression of the antigen is often confined to a subpopulation of cells within the tumor (149). This has led some to propose that the CT-antigens may have a role in conferring stem-cell like properties (93). Fourth, some CT-antigens are expressed at a low-level in a subset of adult tissues, particularly in brain tissue (143). This suggests parallels between the networks controlling expression in brain and those controlling expression in tumors. Finally, expression of many CT-antigens appears to be driven at least partially by promoter demethylation (146, 150-152). Therefore, treatment with demethylating agents such as 5-aza-2'-deoxycytidine (DAC) can induce expression of CT-antigens (146, 150). However, hypomethylation appears to be necessary but not sufficient for CT-antigen expression since DAC treatment only induces expression of a subset of these genes (146). Overall, expression of CT-antigens is sporadic both between and within tumors, is generally confined to tumors or gametogenic tissues, and is driven by promoter demethylation.

Immunogenic properties of the CT-antigens - In addition to sharing a common pattern of gene expression, many, but not all, of the CT-antigens also share immunogenic properties. The ability to elicit an immune response has not been assessed for all CT-antigens and is not a

criterion for the CT antigen classification (93, 143). However, the immunogenicity and unique expression pattern of the CT-antigens has made them tractable drug targets for cancer vaccines. For example, a vaccine targeting the CT-antigen MAGEA5 has caused significant tumor regressions in 7 out of 25 patients in a recent clinical trial (153). This vaccine, developed by GlaxoSmithKline, has currently advanced to a larger phase III clinical trial in patients with NSCLC (154). Importantly, CT-antigen targeted vaccines work by eliciting an immune response against tumor cells that express the CT-antigens. Therefore, the efficacy of these vaccines does not necessarily derive from inhibition of cellular function of the CT-antigen.

Functions of the CT-antigens - While sharing a similar expression pattern, the CT-antigens have diverse cellular functions in everything from modulating protein-protein interactions to regulating transcription (93). However, a function for the majority of the CT-antigens has not been assigned (93). It is therefore unclear whether most of the CT-antigens are simply byproducts of dysregulated gene expression in tumor cells or whether they have a functional role in supporting tumorigenic phenotypes. This is an important distinction therapeutically because CT-antigens with no functional role are likely mainly useful as vaccine targets while CT-antigens with a functional role in supporting tumor phenotypes could be targeted with small molecules. Recent work demonstrated that the CT-antigen ACRBP functionally supports ovarian tumorigenesis by modulating expression of the microtubule-associated protein NuMa (155). Additionally, other CT-antigens have demonstrated functional relevance to tumor cell phenotypes (156, 157). This data suggests a functional role of at least some CT-antigens in tumorigenesis but the role of the vast majority of CT-antigens remains a black box. The known functions of the CT-antigens and gametogenic genes discussed in Chapter 3 of this dissertation are detailed in Table 3.1.

Due to the limitations of studying the CT-antigens, stemming from limited knowledge of their function and their sporadic expression pattern, this dissertation also focuses on a gametogenic gene called transforming acidic coiled-coil containing protein 3 (TACC3). TACC3

is strongly upregulated during gametogenesis and in tumor tissue but is also present in normal adult tissues, albeit at a lower level. This expression pattern and the rich functional knowledge about TACC3 compelled us to utilize it as a model for studying the gametogenic genes in cancer.

Transforming acidic coiled-coil containing protein 3

The TACC family - Transforming acidic coiled-coil containing protein 3 (TACC3) is a member of the TACC protein family. This family shares a common TACC domain at the C-terminus that associates members of the family with microtubules and allows them to modulate microtubule stability (158, 159). There are three TACC proteins in humans (TACC1-3) but only one TACC protein in mouse (AINT), *Drosophila* (D-TACC), and *Xenopus* (Maskin) (160). All the human TACC genes are located in chromosomal regions that are commonly rearranged in cancer (55, 160, 161) but only TACC1 is directly transforming (161). Outside of the TACC domain, members of this family have distinct N-terminal signaling domains that allow TACC proteins to impact a range of processes including transcription and polyadenylation (160).

TACC3 expression pattern - TACC3 demonstrates a pattern of expression that suggests a role in gametogenesis, embryonic development, and tumorigenesis. In particular, TACC3 is highly expressed in the testis and during embryogenesis but is expressed at much lower levels in normal adult tissues with the exception of the hematopoietic system (55, 127, 162-165). The finding that TACC3 knockout mice exhibit embryonic lethality accompanied by massive mitotic defects supports a role for TACC3 in development (164). In cancer cells, TACC3 expression is dramatically upregulated in both tumor cell lines (55) and tissues (166, 167). The exception to this consistent upregulation of TACC3 in cancer lines may be in ovarian cancer, where two studies have documented increased expression while another found decreased TACC3 expression (168-170). The upregulation of TACC3 in tumor tissue may derive somewhat from its pattern of cell cycle expression, which is significantly increased in G2/M of the cell cycle (164, 171). Therefore, TACC3 expression is increased during gametogenesis and tumorigenesis and may be

controlled in a cell-cycle specific manner.

Role of TACC3 in mitosis - During mitosis TACC3, which is expressed in the nucleus of interphase cells, is phosphorylated by aurora kinase A (AURKA) at three consensus AURKA phosphorylation sites (160, 172, 173). Phosphorylation at these sites directs TACC3 to a diffuse region around the centrosome in mitotic cells (158, 174). Mutation of the AURKA phosphorylation sites or treatment with the AURKA inhibitor VX-680 impairs TACC3 phosphorylation and inhibits its localization to the centrosome (172, 175). Once at the centrosome, TACC3 recruits and binds to the microtubule stabilizing protein CKAP5 (TOGp) (176). CKAP5 functions by opposing the depolymerizing activity of the protein kinesin family member 2C (MCAK) to allow for increased microtubule growth and stability (177, 178). This important role in stabilizing microtubules probably accounts for the synergy previously observed by other groups between TACC3 knockdown and paclitaxel treatment (179, 180). Therefore, TACC3 functions as a microtubule stabilizing protein and loss of TACC3 leads to decreased microtubule stability in vitro (176).

Role of TACC3 outside of mitosis - Aside from its most well studied role in mitosis, TACC3 can influence a number of other processes including several that are relevant to this dissertation: cytoplasmic polyadenylation, differentiation, and response to hypoxia. First, human TACC3 demonstrates 36% homology to *Xenopus* Maskin, which mediates cytoplasmic polyadenylation (127). Cytoplasmic polyadenylation, as discussed above, is an important method of post-transcriptional control of gene expression during *Xenopus* oocyte development. Maskin acts as a negative regulator of cytoplasmic polyadenylation of meiotic transcripts by binding to CPEB, which interacts with SYMPK, and inhibiting assembly of the polyadenylation complex on the pre-mRNA (120, 125, 126). Importantly, human TACC3 lacks the domain in Maskin that interacts with CPEB (160). An interaction between TACC3 and SYMPK by co-immunoprecipitation can be detected but a functional role for the interaction is unclear (Figure 4.2). However, this interaction suggests that the function of human TACC3 may impinge on

polyadenylation, similarly to that observed in *Xenopus*. Second, TACC3 has been shown to have a role in driving cellular differentiation, potentially by controlling activity of transcription factors (163, 165, 181). This has relevance to this dissertation since a screen to identify small molecules that promote neuronal differentiation uncovered a TACC3 inhibitor that is used in Chapter 3 (182). Finally, the mouse TACC3 protein, AINT, may regulate the cellular response to hypoxia by binding to and altering the localization of the Ah receptor nuclear translocator protein (ARNT) (183). ARNT heterodimerizes with HIF-1alpha in the presence of low oxygen tension to induce transcription from hypoxia response element containing genes (183). This is relevant to this dissertation because the TACC3 inhibitor used in Chapter 3 likewise appears to influence response to hypoxia. Therefore, TACC3 can influence several important processes outside of its role in mitosis.

Thesis Summary

This dissertation will cover two processes that may impact responsiveness of tumor cells to mitotic stress: polyadenylation and gametogenesis. Both these projects arose from findings in genome-wide paclitaxel chemosensitizer screen (4). In particular, Chapter 2 will focus on the potent screen hit SYMPK while Chapter 3 will focus on the screen hit TACC3.

In Chapter 2 "Symplekin is required for appropriate microtubule function and mitosis" we investigate the role of the polyadenylation scaffold symplekin in impacting mammalian mitosis. This work uncovers critical links between the polyadenylation machinery, paclitaxel responsiveness and mitosis. In particular, in this chapter we show that symplekin depletion sensitizes NSCLC cells to paclitaxel by inducing mitotic errors, leads to altered microtubule dynamics, and causes loss of the microtubule stabilizing protein CKAP5. Additionally, we demonstrate that other members of the polyadenylation machinery have similar impacts on mitosis, providing one of the first links between polyadenylation and mammalian mitosis. Finally, data in this chapter also demonstrates that the symplekin depletion may preferentially

affect tumor, as opposed to normal cell lines, suggesting a unique dependence of tumor lines on polyadenylation for mitotic progression.

In Chapter 3 "TACC3 and multiple gametogenic genes support the cancer cell mitotic spindle" we study the links between several gametogenic genes and mitosis. We begin this work by depleting a panel of cancer-testis antigens and gametogenic genes from the NSCLC line H1155 in the presence of paclitaxel and showing how this impacts mitotic progression. Next, we focus on the gametogenic gene TACC3 and demonstrate TACC3 is uniquely required for mitosis in tumor cell lines. In particular, we perform immunofluorescence and live-cell imaging studies in a NSCLC progression model to determine the point during tumor evolution when TACC3 becomes required for mitosis. Finally, we use a TACC3 inhibitor to demonstrate that gametogenic genes can be targeted with small molecules and that doing so can increase the efficacy of current anti-mitotic drugs.

In Chapter 4, I discuss the impact of these findings on the factors supporting tumorigenesis, response to chemotherapy, and mitosis. In particular, I discuss how polyadenylation and the gametogenic machinery may interface with mitotic stress in tumor cells. I additionally discuss the clinical implications of this work and potential directions for future study.

CHAPTER II

SYMPLEKIN IS REQUIRED FOR APPROPRIATE MICROTUBULE FUNCTION AND MITOSIS

Elements of the work referenced in this chapter have been published in:

> Cappell KM, Larson B, Sciaky N, and Whitehurst AW. (2010). Symplekin specifies mitotic fidelity by supporting microtubule dynamics. Mol. Cell Biol. 30(21), 5135-44.

Summary

Using a pangenomic loss of function screening strategy, we have previously identified 76 potent modulators of paclitaxel responsiveness in non-small cell lung cancer. The top hit isolated from this screen, symplekin, is a well-established component of the mRNA polyadenylation machinery. Here, we performed studies to reveal the mechanistic underpinnings by which symplekin depletion synergizes with paclitaxel. We find that symplekin supports faithful mitosis by contributing to the formation of a bipolar spindle apparatus. Depletion of symplekin attenuates microtubule polymerization activity as well as expression of the critical microtubule polymerization protein, CKAP5 (TOGp). Depletion of additional members of the polyadenylation complex results in similar phenotypes, suggesting that the polyadenylation machinery is coupled to microtubule function and mitotic spindle formation. These results demonstrate a critical connection between the polyadenylation machinery and mitosis and suggest the polydenylation machinery may be a useful target in combination with current anti-mitotic therapies.

Introduction

Pangenomic loss of function screening is emerging as an effective tool for revealing the components that support core biological processes including viral infection, DNA repair, chemotherapeutic responsiveness, melanogenesis and endocytosis (4, 184-188). A number of screening efforts have focused on identifying those gene products that are required for mitotic progression in both the normal and tumorigenic setting (38, 41, 189-191). These screens have successfully returned validated mitotic participants but also have isolated a diverse set of unanticipated genes whose encoded proteins have no previously described role in mitotic progression but instead have well established roles in processes such as transcription (189-191), RNA splicing and translation (190, 191) and vesicle transport (190); thereby revealing an unexpected diversity in the compendium of gene products supporting mitosis.

We have recently applied a genome-wide loss of function paclitaxel synthetic lethal strategy to identify genes that modulate chemoresponsiveness in non-small cell lung cancer cells (NSCLC) (4). This strategy returned a diverse set of gene products, including symplekin, whose depletion was the most potent for sensitizing NSCLC to a dose of paclitaxel that has no detectable impact on cell viability. Symplekin is a scaffold protein that supports the assembly of polyadenylation machinery on pre-mRNA transcripts; however no role for symplekin in drug sensitivity or mitosis has been reported (124). Polyadenylation is essential for the maturation of most pre-mRNAs and regulates mRNA nuclear export, stability and translation (100). In *Xenopus laevis* oocytes, the polyadenylation of specific meiotic transcripts is regulated such that their activation only occurs following meiotic maturation signals (120, 192, 193). In mammalian cells, the poly(A) tail length of specific transcripts changes in a cell cycle dependent manner (194), suggesting that cytoplasmic polyadenylation is a conserved mechanism for exerting translational regulation of gene expression prior to and during cell division.

Here, we evaluate the contribution of the polyadenylation machinery to mitotic control in

the tumor cells. We find that symplekin is required to support bipolar spindle formation in multiple NSCLC derived tumor cells and that symplekin depletion impairs proliferation of NSCLC cells in vivo. The basis of symplekin's contribution to mitotic progression appears to be at the level of microtubule function and expression of a critical component of the microtubule polymerization machinery, CKAP5 (TOGp). Depletion of other polyadenylation components causes similar alterations in CKAP5 expression and mitotic progression. Therefore, our results demonstrate that mitosis is acutely sensitive to perturbations of the polyadenylation machinery and suggest inhibition of polyadenylation may synergize with current anti-mitotic agents.

Results

Symplekin is required for mitotic spindle integrity - Symplekin was originally identified as a statistically significant modulator of paclitaxel sensitivity using a high-throughput chemosensitizer screening platform. Thus, we first validated symplekin for off-target consequences by verifying that multiple independent siRNA sequences were capable of inducing synthetic lethality (Figure 2.1A). In addition, we validated that symplekin protein levels were depleted (Figure 2.1A). Because we have previously observed that RNAi paclitaxel sensitizer screens can return components whose depletion induces a mitotic arrest (4), we evaluated the integrity of the mitotic spindle in symplekin depleted H1155 cells treated with 10 nM paclitaxel. As observed for other targets identified by our whole genome screening effort, symplekin depletion led to an increase in the accumulation of mitotic figures (Figure 2.1B). This accumulation was also detected at the population level by flow cytometry which revealed a marked increase in 4N DNA content of symplekin depleted cells exposed to 10 nM paclitaxel as compared to control transfected cells (Figure 2.1C). Immunoblot analysis revealed elevated levels of cyclin B1, a substrate of the Anaphase Promoting Complex (APC), the E3 ligase whose activity is restricted in the presence of improper chromosome alignment (Figure 2.1D). Thus, the observed accumulation of mitotic cells in the symplekin-depleted samples could indicate an

Figure 2.1

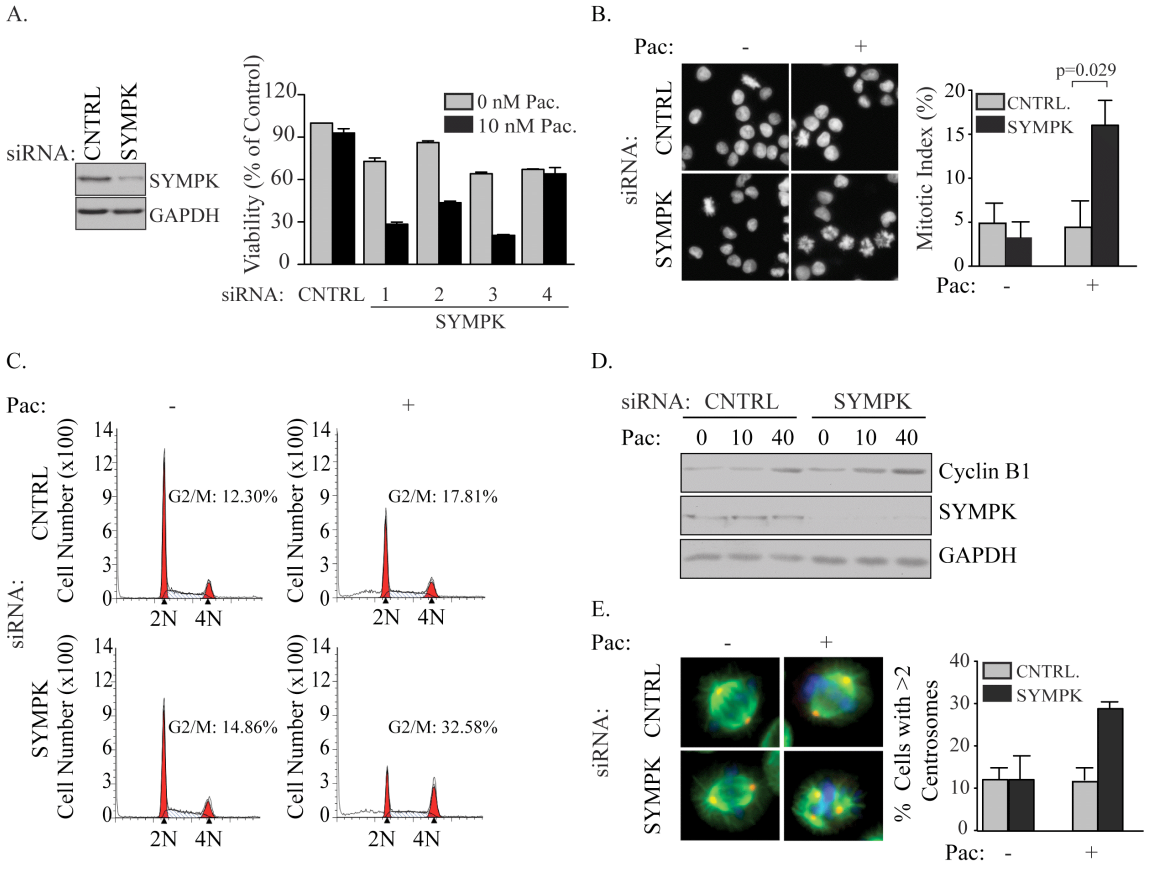


Figure 2.1 SYMPK is required for spindle integrity after exposure to paclitaxel

(A) Whole cell extracts of H1155 cells transfected with indicated siRNAs were immunoblotted to detect endogenous SYMPK (left panel). Viability assay in H1155 cells transfected with control or independent siRNAs targeting SYMPK. Error bars represent Standard Error of the Mean (SEM), n=2. (B) H1155 cells transfected with the indicated siRNAs and exposed to paclitaxel for 24 hours were stained with DAPI to detect mitotic cells. Error bars represent Standard Deviation (SD), n=2. (C) H1155 cells were transfected with indicated siRNAs and at 48 hours post-transfection, cells were exposed to paclitaxel for 24 hours. Cells were then fixed and stained with propidium iodide. A representative flow cytometry profile is shown. (D) Whole cell lysates of H1155 cells transfected with indicated siRNAs for 48 hours then exposed to paclitaxel for an additional 24 hours were immunblotted for cyclin B1. (E) H1155 cells transfected with indicated siRNAs for 48 hours then exposed to paclitaxel for 24 hours were fixed and stained with β-tubulin (green), pericentrin (red) and DAPI. Cells were scored as having > 2 centrosomes if there were more than 2 pericentrin positive poles during mitosis. Error bars represent SD, n=3. All p-values were calculated using a Student's t-test.

aberrant mitosis. Indeed, symplekin depleted H1155 cells exposed to 10 nM paclitaxel displayed a high frequency of multipolar spindles characterized by disorganized tubulin and multiple centrosomes as compared to control-transfected cells (Figure 2.1E). These data suggest that symplekin function is directly coupled to the ability of cells to form a normal bipolar spindle.

Symplekin is required for high fidelity mitosis - Given our observations that symplekin contributes to bipolar spindle formation, we directly assessed the consequence of symplekin depletion on mitotic progression in real-time by live imaging of H1155 cells stably expressing the chromatin marker GFP-H2B. By performing single-cell lineage tracing, we measured both the length and outcome of mitosis in symplekin and control siRNA transfected cells (Figure 2.2A). As expected, control or symplekin depletion alone had little effect on either mitotic fate or mitotic timing (Figure 2.2A and 2.2B). However, symplekin depleted cells exposed to 10 nM paclitaxel exhibited a significantly prolonged mitosis as compared to control transfected and paclitaxel treated cells (Figure 2.2B). The outcome of this prolonged mitosis was aberrant in 75% of the individual cells studied. In particular, instead of the formation of 2 daughter cells, symplekin depleted samples underwent either apoptosis, micronucleation or a multipolar mitosis following mitotic arrest (Figure 2.2A and 2.2C). Taken together, these observations suggest that symplekin supports mitotic spindle formation and mitotic progression in NSCLC.

Symplekin is necessary for mitosis in diverse NSCLC genetic settings - To determine if the impacts of symplekin depletion can be observed across diverse genetic backgrounds, we assayed mitotic defects following symplekin depletion in H1299 cells, which were derived from a NSCLC lymph node metastasis. H1299 cells undergo a mitotic arrest followed by bypass of an activated APC and form micronucleated daughter cells when exposed to doses of paclitaxel 10 nM and greater. Similar to the synthetic lethality seen in H1155 cells, H1299 cells depleted of symplekin and exposed to 10 nM paclitaxel displayed an increase in the frequency of micro- and multi-nucleated cells as compared to control (Figure 2.3A). We do not observe the same effects in the normal-tissue derived human bronchial epithelial cell line, HBEC3 (Figure 2.3B).

Figure 2.2

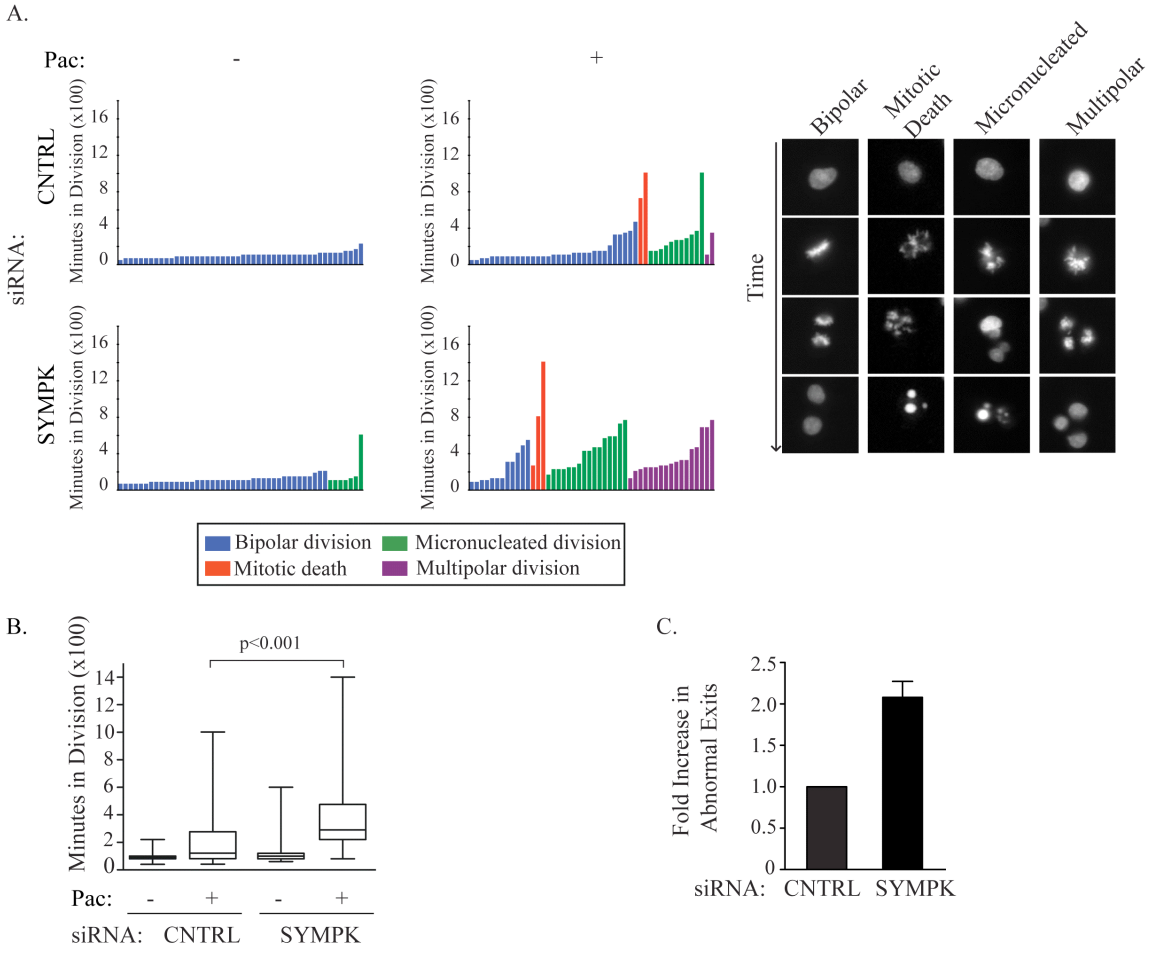


Figure 2.2 SYMPK is required for normal mitotic progression in tumor cells

(A) H1155 cells expressing GFP-Histone H2B were transfected with indicated siRNAs. At 48 hours post-transfection, cells were exposed to 10 nM paclitaxel or carrier and imaged by live time-lapse microscopy from 24-72 hours post-paclitaxel treatment. Single-cell lineage tracing was performed to identify mitotic exit phenotype for a total of 48 cells per condition. (B) Quartile ranges for time in division as calculated from chromosome condensation to anaphase onset in 48 cells under the conditions described in A. P-values were calculated with the Mann-Whitney test. (C) Fold increase in abnormal mitotic exits in 10 nM paclitaxel for control or SYMPK-depleted cells across 3 independent live cell imaging experiments. The data represents the fate of at least 145 individual cells for each treatment. Error bars represent SD.

Figure 2.3

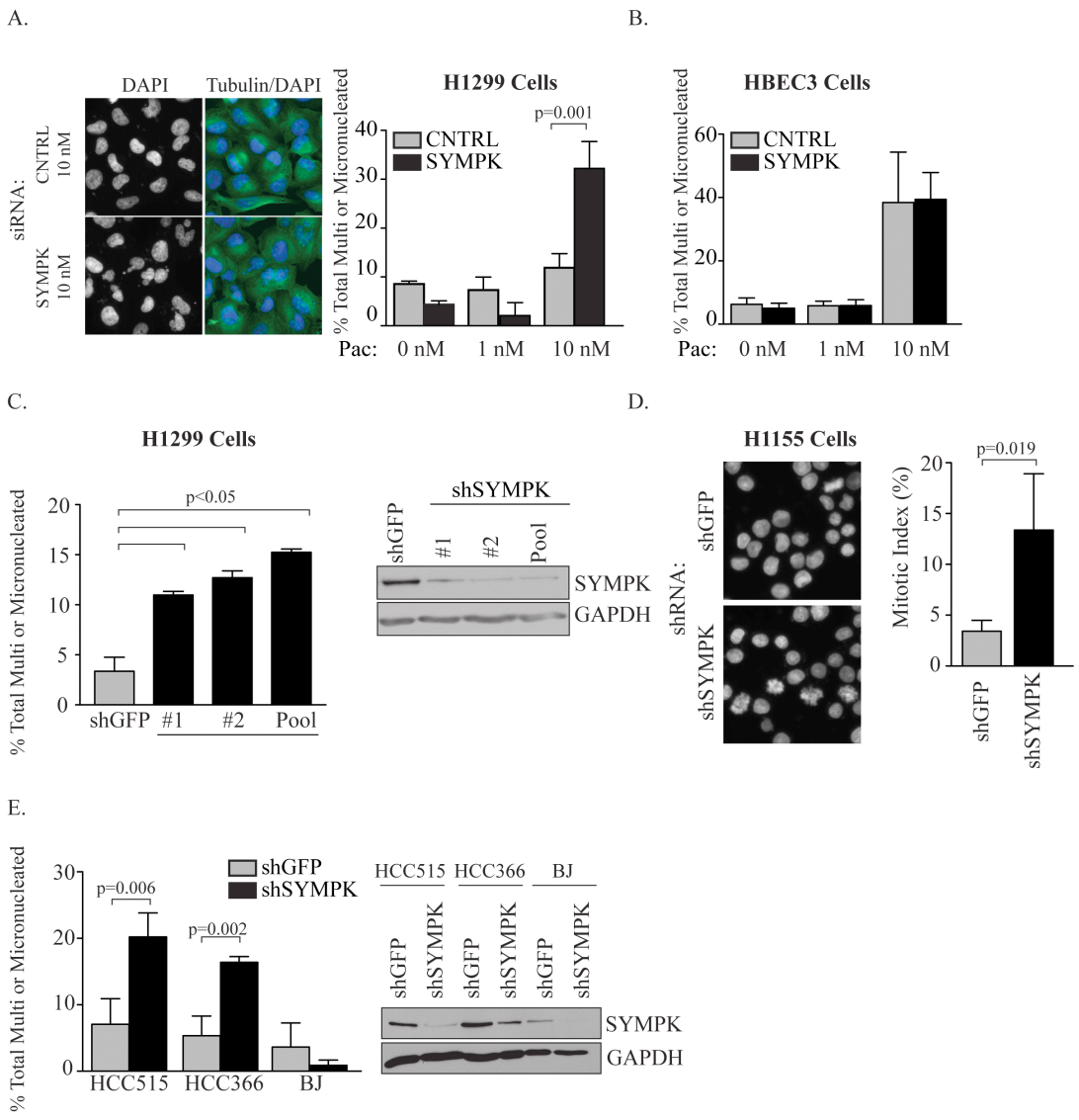

A.
DAPI
Tubulin/DAPI
siRNA:
CNTRL 10 nM
SYMPK 10 nM
H1299 Cells
% Total Multi or Micronucleated
CNTRL
SYMPK
p=0.001
Pac: 0 nM 1 nM 10 nM
B.
HBEC3 Cells
% Total Multi or Micronucleated
CNTRL
SYMPK
Pac: 0 nM 1 nM 10 nM
C.
H1299 Cells
% Total Multi or Micronucleated
p<0.05
shGFP #1 #2 Pool
shSYMPK
shGFP #1 #2 Pool
SYMPK
GAPDH
D.
H1155 Cells
shRNA:
shGFP
shSYMPK
Mitotic Index (%)
p=0.019
shGFP
shSYMPK
E.
% Total Multi or Micronucleated
shGFP
shSYMPK
p=0.006
p=0.002
HCC515 HCC366 BJ
HCC515 HCC366 BJ
shGFP shSYMPK shGFP shSYMPK shGFP shSYMPK
SYMPK
GAPDH

Figure 2.3 SYMPK is necessary for mitosis in multiple tumor cell lines

(A) H1299 cells were transfected with the indicated siRNAs for 48 hours followed by exposure to paclitaxel for 24 hours. Cells were fixed and stained with β-tubulin and DAPI (left panel) followed by quantification of micro and multinucleated cells (right panel). Error bars represent SD, n=3. (B) Immortalized HBEC3 cells were transfected with the indicated siRNAs for 48 hours followed by exposure to paclitaxel for 24 hours. Error bars represent SD, n=3. (C) H1299 cells were infected for 5 days with lentivirus targeting control (GFP) or SYMPK shRNAs were followed by staining and quantification of micro and multinucleated cells. Errors bars represent SD, n=3. (D) H1155 cells were treated as in (C). Representative images (left panel) and quantification of percentage of total cells in mitosis (right panel) is shown. Error bars represent SD, n=3. (E) HCC515, HCC366 and BJ fibroblasts were treated as in B, except cells were fixed 7 days post infection. Cells were co-stained with β-tubulin/DAPI and scored microscopically for micronucleation. Error bars represent SD, n=3. P-values were calculated using a Student's t-test.

We next sought to determine the impacts of prolonged suppression of symplekin expression in NSCLC cells. To this end, we stably repressed symplekin expression in H1299 cells using an shRNA-mediated system where we pooled two effective shRNAs targeting symplekin (Figure 2.3C). Stably repressing symplekin expression in the H1299 NSCLC line led to an increase in micronucleation in the absence of paclitaxel (Figure 2.3C). In our original screening cell line, H1155, stable repression of symplekin led to an increase in mitotic figures in the absence of paclitaxel (Figure 2.3D). Extending this analysis to two additional NSCLC lines and immortalized BJ fibroblasts revealed that the generation of micronucleated cells following prolonged symplekin depletion is a common phenomenon in NSCLC but not normal diploid fibroblasts immortalized with hTERT (Figure 2.3E). Thus, while the transient impacts of symplekin depletion are observable only in the presence of a microtubule disrupting agent, prolonged symplekin depletion alone increases the frequency of aberrant mitosis and mitotic arrest in tumor, but not normal, cells.

Loss of symplekin impairs tumor formation in a mouse xenograft model - To directly test if the mitotic dysfunction we observe in multiple NSCLC backgrounds could result in reduced neoplastic potential, we evaluated the ability of H1299 cells stably depleted of symplekin to form xenograft tumors in nude mice. Luciferase-expressing H1299 cells were injected into nude mice 5 days following infection with shRNAs targeting symplekin or GFP. Tumor development was monitored for 4 weeks by twice weekly imaging of tumors. While 100% of control-infected cells formed sub-cutaneous tumors, only 50% of mice injected with symplekin depleted cells established tumors (Figure 2.4A). Furthermore, at 5 weeks post-injection, the symplekin hairpin tumors were significantly smaller than control cells (Figure 2.4B), suggesting that the asymmetric divisions we observe in vitro may accumulate over time to reduce tumor cell proliferation.

Figure 2.4

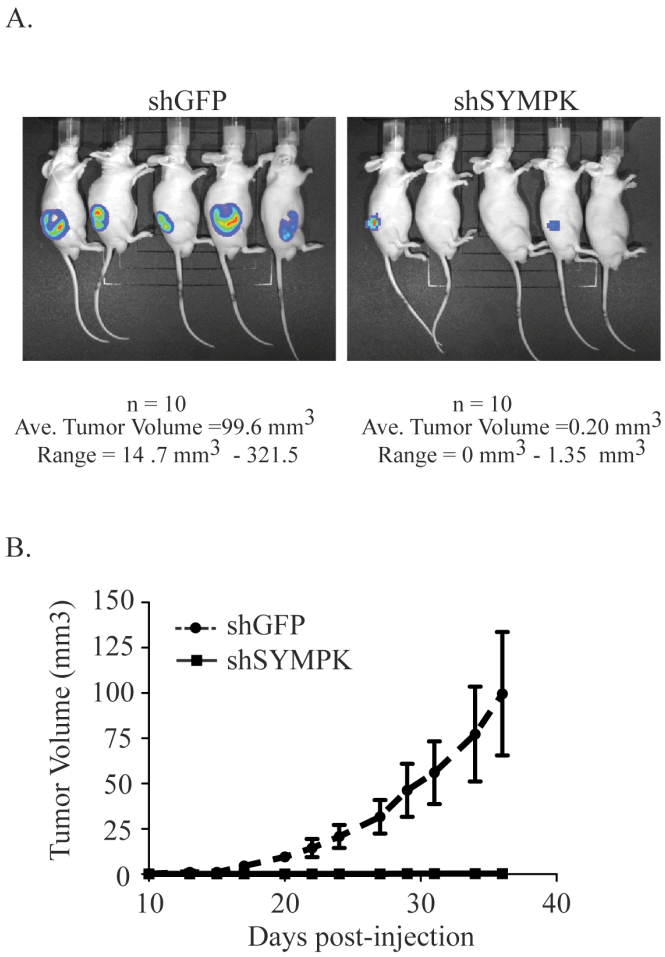


Figure 2.4 Depletion of SYMPK impairs tumor growth in vivo

(A) 2 million luciferase expressing H1299 cells infected with lentivirus harboring control or SYMPK shRNA's were injected in the right flank of Harlan Nude mice. Picture depicts bioluminescence imaging of representative tumor burden at 35 days post injection. (B) Mice in (A) were subjected to BLI imaging twice weekly following injection of H1299 cells. Line graph of growth curves for indicated groups is shown. Error bars represent SEM for 10 mice in each group.

Symplekin modulates microtubule polymerization - The formation of a normal, bipolar spindle apparatus is exquisitely dependent on proper microtubule function, which is significantly altered in the presence of chemotherapeutic drugs such as paclitaxel (43). Given the symplekin-paclitaxel synthetic lethal phenotype we observe in NSCLC, we probed microtubule polymerization efficiency in H1299 cells following transient depletion of symplekin using a microtubule regrowth assay. Here, H1299 cells transfected with control or symplekin siRNAs were exposed to a high dose of nocodazole to induce microtubule depolymerization. Microtubules were depolymerized to a similar degree in both control and symplekin transfected samples (Figure 2.5A and 2.5B). However, after 10 minutes of recovery, symplekin depleted cells displayed little microtubule regrowth from their centrosomes. A similar trend was observed in mitotic cells, where growth of microtubules from both the spindle poles and the kinetochores was significantly attenuated in symplekin depleted samples (Figure 2.5A and 2.5B). Similar phenotypes were observed in both H1299 and H1155 cells when we performed the experiment by an in vitro microtubule stability assay where polymerized tubulin is sedimented after the depolymerization and recovery steps and analyzed by immunoblotting (Figure 2.5C and 2.5D). Thus, depletion of symplekin significantly alters microtubule polymerization, a process that is essential for normal spindle formation.

SYMPK depletion leads to loss of CKAP5 - Symplekin is a multifunctional protein implicated in transcription and translation as well as signaling at tight junctions (94, 98, 108, 124). The localization pattern of symplekin has previously been described at the tight junctions and in the nucleus (94), the latter of which is a pattern we also observe in H1299 cells (Figure 2.6A). During mitosis, symplekin is distributed diffusely throughout the nucleoplasm and does not appear to localize to a specific mitotic structure at the microscopic resolution employed in this study (Figure 2.6A), suggesting that symplekin does not impact mitosis through a direct association with the mitotic machinery. Given the role of symplekin in gene expression (96, 108, 195) and the errors observed in spindle integrity, mitotic progression and microtubule nucleation,

Figure 2.5

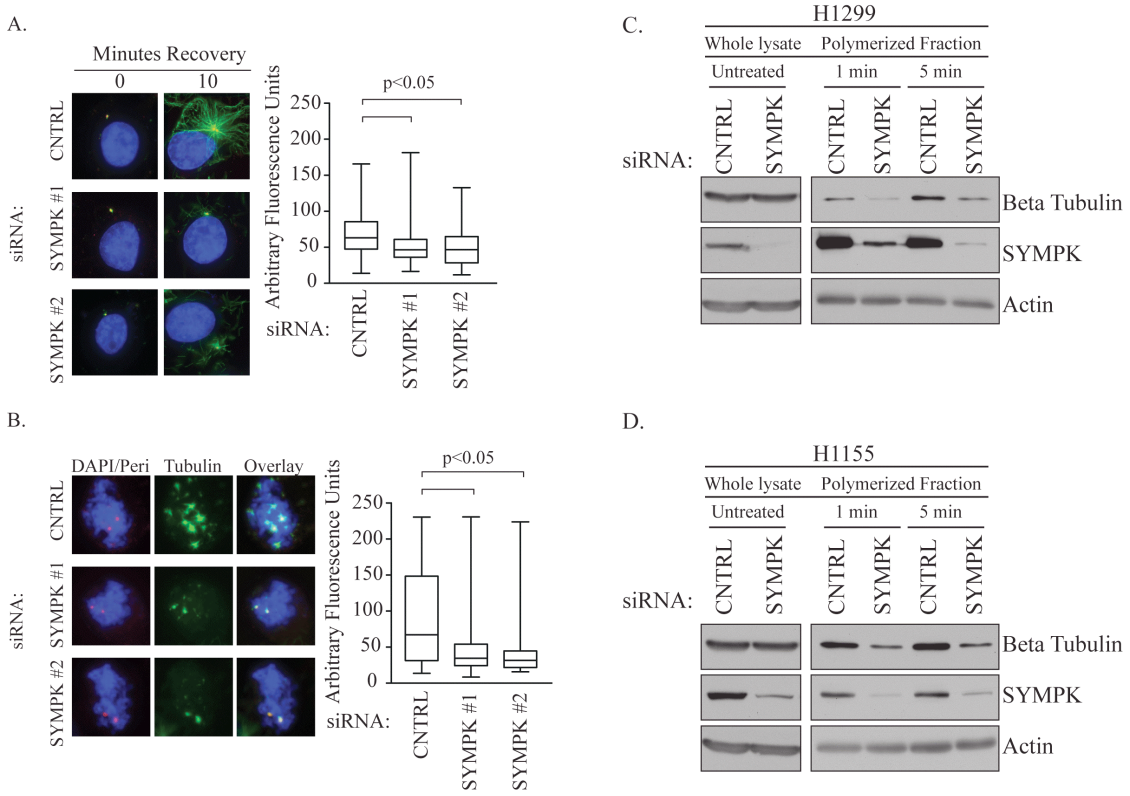


Figure 2.5 Depletion of SYMPK reduces microtubule stability

(A) H1299 cells transfected with indicated siRNAs for 96 hours were treated with 11 uM nocodazole for 2 hours. Cells were fixed at 0 and 10 minutes post nocodazole washout and stained with β-tubulin (green), pericentrin (red) and DAPI. A representative image (left panel) of interphase cells at 0 and 10 minutes post-recovery and box plot quantification of fluorescence intensity (right panel) is shown. The box plot was derived from quantifying a minimum of 75 interphase cells per condition across 8 independent fields of view. (B) Microtubule recovery in mitotic cells under the conditions described previously. A representative image (left panel) of mitotic cells and a box blot quantification of fluorescence intensity (right panel) is shown. The box plot was derived from quantifying a minimum of 45 mitotic cells per condition. All p-values are by Mann-Whitney test. (C) In vitro microtubule stability assay in H1299 cells. Cells were transfected with the indicated siRNAs for 72 hours, treated with nocodazole for 30 minutes and the polymerized tubulin fraction was harvested by scraping into cold microtubule stabilizing buffer followed by high-speed centrifugation. (D) Same assay as in (C) but performed in H1155 cells.

Figure 2.6

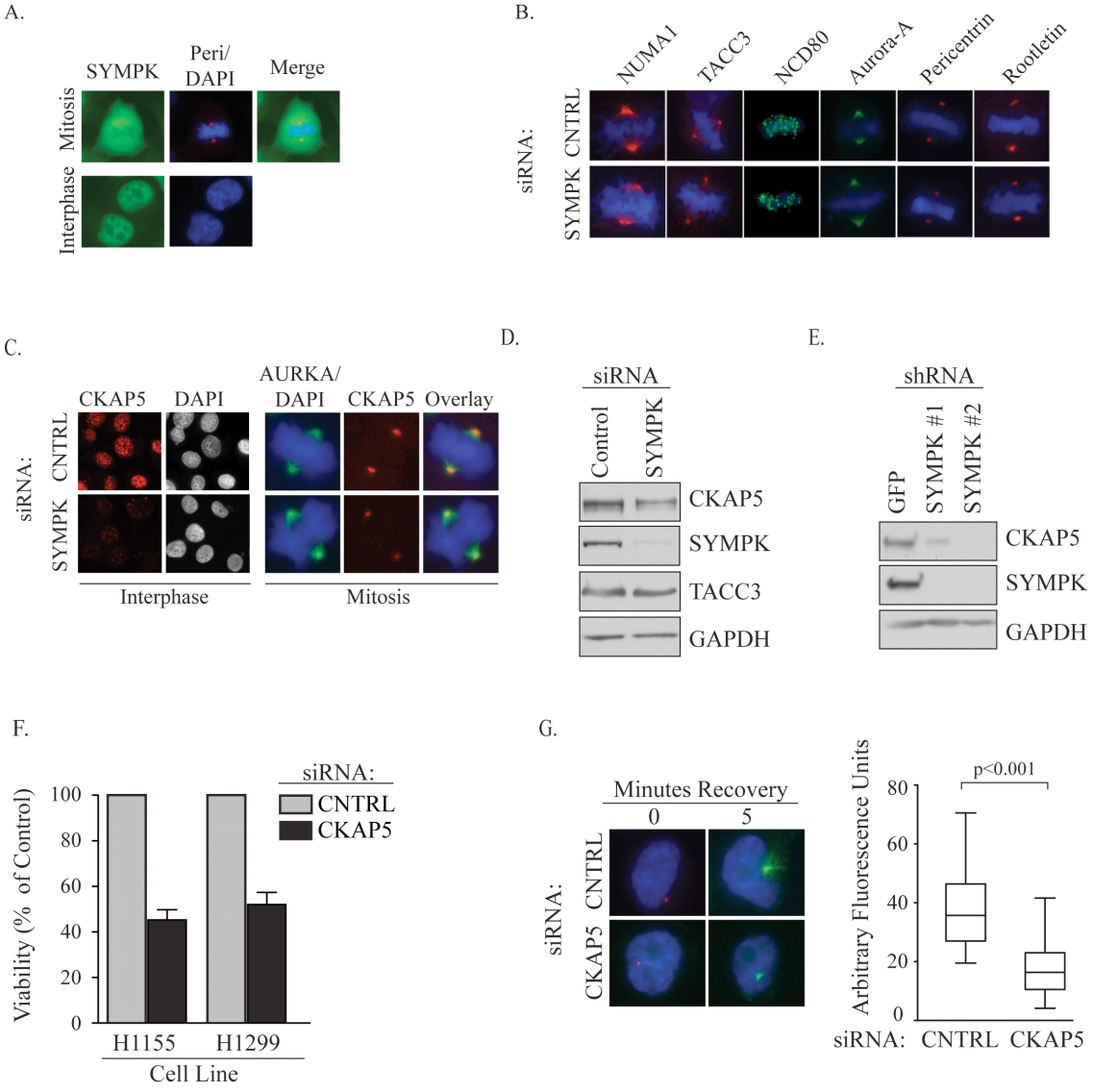

A.
SYMPK
Peri/ DAPI
Merge
Mitosis
Interphase
B.
NUMA1
TACC3
NCD80
Aurora-A
Pericentrin
Rootletin
siRNA:
CNTRL
SYMPK
C.
CKAP5
DAPI
AURKA/ DAPI
CKAP5
Overlay
siRNA:
CNTRL
SYMPK
Interphase
Mitosis
D.
siRNA
Control
SYMPK
CKAP5
SYMPK
TACC3
GAPDH
E.
shRNA
GFP
SYMPK #1
SYMPK #2
CKAP5
SYMPK
GAPDH
F.
siRNA:
CNTRL
CKAP5
Viability (% of Control)
100
80
60
40
20
0
H1155
H1299
Cell Line
G.
Minutes Recovery
0
5
siRNA:
CNTRL
CKAP5
Arbitrary Fluorescence Units
p<0.001
80
60
40
20
0
siRNA: CNTRL CKAP5

Figure 2.6 SYMPK depletion leads to loss of CKAP5

(A) H1299 cells growing on glass coverslips were fixed and immunostained with anti-symplekin (green), pericentrin (red) and DAPI. Images depict SYMPK localization in both interphase and mitotic cells. (B) Representative immunofluorescence images of H1299 cells depleted of SYMPK and immunostained for a panel of centrosome and tubulin-associated proteins at 80 hours post-transfection. (C) H1299 cells transfected with indicated siRNAs were fixed at 80 hours post-transfection and immunostained for CKAP5 (red), Aurora-A (green) and DAPI. (D) Whole cell lysates from H1299 cells transfected with indicated siRNAs for 96 hours or infected with indicated shRNAs for 5 days were immunoblotted to assess CKAP5 expression. TACC3 is included as a control for effects on global protein expression following transient depletion. (E) H1299 cells transduced with lentivirus from control or two independent SYMPK-targeting shRNAs were blotted for CKAP5 expression at 9 days post-infection. (F) Cell-titer Glo iability assay in H1155 and H1299 cells depleted of indicated siRNAs for 96 hours. Error bars represent Standard Error of the Mean (SEM). (G) H1299 cells were transfected with control and CKAP5 siRNAs for 96 hours and subjected to a microtubule regrowth assay as described previously. Representative images (left panel) and quantification of fluorescence intensity (right panel) is shown. Box plot quantification is from a minimum of 40 cells across 11 independent fields of view.

we hypothesized that symplekin could be impacting expression of proteins required for mitotic spindle formation. To assess this possibility, we analyzed a panel of proteins required for proper centrosomal maturation and microtubule nucleation (176, 196-201). Symplekin depletion alone had no detectable effect on the localization and expression of almost all proteins studied (Figure 2.6B). However, symplekin depletion had a profound effect on the expression of CKAP5 as detected by both immunofluorescence (Figure 2.6C) and immunoblot analysis (Figure 2.6D and 2.6E). CKAP5 displays an elevated expression pattern in tumor cells (56) and localizes to the centrosome where it enhances microtubule polymerization and nucleation (57, 178). CKAP5 depletion significantly decreases viability of H1155 and H1299 cells (Figure 2.6F). This effect is likely due to the potent impact of CKAP5 on microtubule polymerization and stability in H1299 and other cell types (Figure 2.6G) (176, 202, 203).

SYMPK alters CKAP5 levels post-transcriptionally and independently of protein degradation - Given symplekin's role in transcription and translation, we evaluated CKAP5 transcript levels in symplekin-depleted cells. Symplekin depletion did not affect the level of CKAP5 mRNA, suggesting a post-transcriptional mode of regulation (Figure 2.7A). To determine whether the effects on CKAP5 were mediated by increased degradation, we evaluated CKAP5 protein levels in symplekin depleted cells exposed to the proteosome-inhibitor, MG-132. CKAP5 levels were globally increased in MG-132 treated cells, however, proteosome inhibition was not sufficient to rescue the reduced CKAP5 levels observed in symplekin depleted cells (Figure 2.7B). Since CKAP5 stabilizes microtubules primarily by opposing the depolymerizing activity of MCAK, we evaluated the impact of codepletion of SYMPK and MCAK on the microtubule network. In H1299 cells, MCAK depletion impairs microtubule depolymerization by nocodazole as has previously been reported (44). Importantly, co-depletion of MCAK and SYMPK results in the depolymerization of microtubules in the presence of nocodazole. (Figure 2.7C).

Multiple polyadenylation components collaborate with paclitaxel - Symplekin is a core component of the polyadenylation machinery, which has recently been implicated in

Figure 2.7

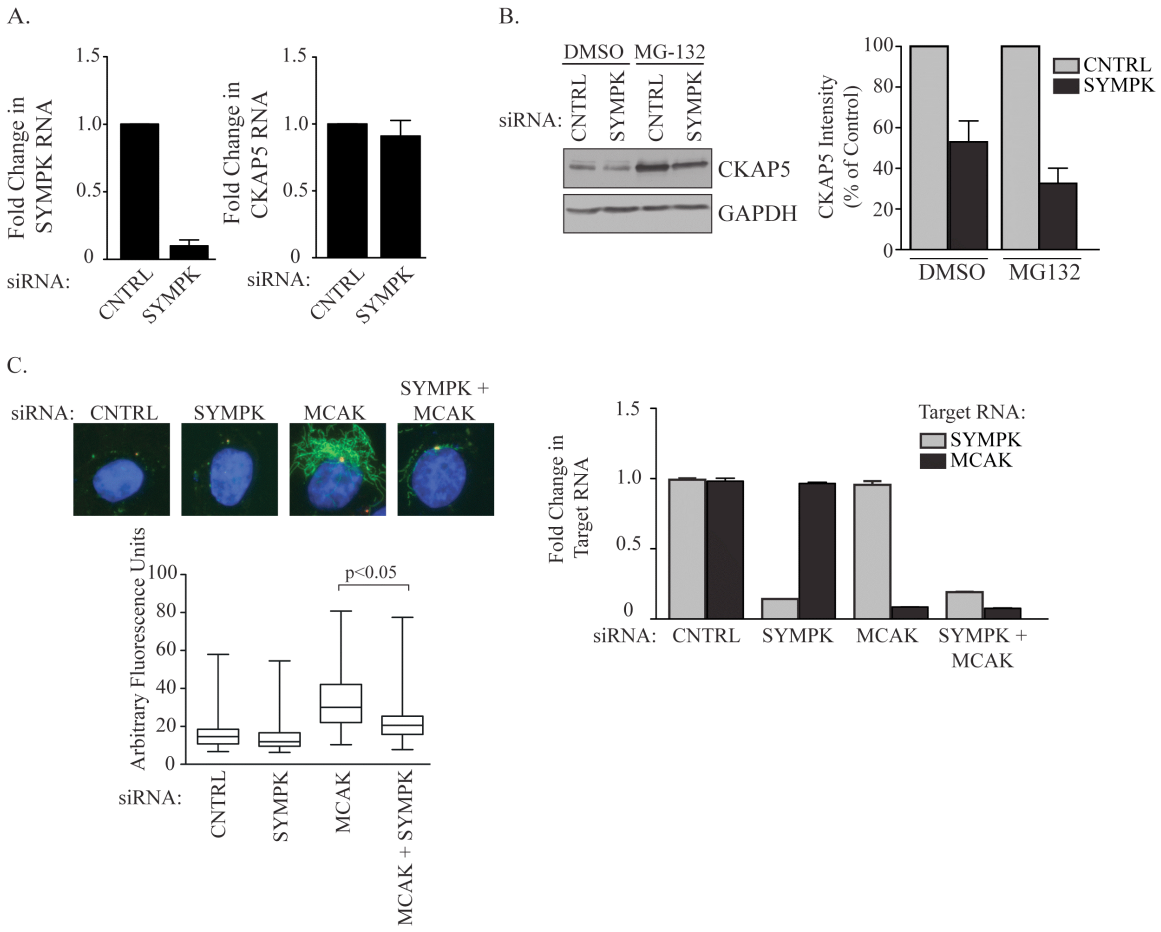


Figure 2.7 SYMPK alters CKAP5 levels post-transcriptionally

(A) qPCR analysis of CKAP5 mRNA levels in H1299 cells depleted of SYMPK at 96 hours post-transfection. Error bars represent SD, n=4 biological replicates. (B) Whole cell lysates of H1299 cells transfected with indicated siRNAs for 96 hours and exposed to vehicle or MG-132 for 14 hours were immunoblotted for CKAP5 expression (left panel). The graph (right panel) depicts densitometry analysis with error bars representing SEM from n=2 biological replicates. (C) H1299 cells were transfected with the indicated siRNAs for 96 hours followed by depolymerization of microtubules with 11 uM nocodazole for 2 hours. Representative images at 0 minutes recovery (top panel) and quantification of a minimum of 120 cells from a representative experiment (bottom panel) is shown. Gene knockdown was confirmed by RT-PCR (right panel) with error bars representing SEM from triplicate analysis.

regulating mitotic progression through phase-specific changes in poly(A) tail length (194). To determine if attenuation of the polyadenylation complex in general can collaborate with paclitaxel, we retrospectively examined the impact of the 14 core polyadenylation proteins in our original genome-wide paclitaxel sensitivity screen (4). In addition to symplekin, depletion of the polyadenylation proteins CPSF1, CSTF2 and CPSF3 all enhanced paclitaxel sensitivity to some degree in our primary screen. In H1299 cells, depletion of both CPSF1 and CPSF3 reduced CKAP5 protein levels as detected by immunoblot analysis (Figure 2.8A), suggesting that CKAP5 expression is exquisitely sensitive to perturbations of the polyadenylation complex. Additionally, H1299 cells depleted of CSTF2, CPSF1 or CPSF3 and exposed to paclitaxel demonstrated a significant increase in the occurrence of multi and micronucleated cells (Figure 2.8B). To determine if these subunits impacted mitotic progression in a similar manner to symplekin, we employed our time-lapse imaging system in H1155 GFP-H2B cells to evaluate mitotic outcomes. As with symplekin, CPSF1, CPSF3 and CSTF2 depletion increased the frequency of abnormal mitotic exits (Figure 2.8C). Thus, altered expression of multiple polyadenylation components has acute effects on mitotic fidelity.

Discussion

We have uncovered a functional connection between the polyadenylation machinery and formation of a bipolar spindle needed for accurate segregation of chromosomes. These mitotic defects are due, at least in part, to attenuation of the microtubule polymerization machinery and loss of microtubule dynamicity, which is essential for chromosome capture and alignment. The observation that depletion of multiple components of the polyadenylation complex leads to mitotic defects demonstrates an unanticipated and critical contribution of this complex to mitotic progression by supporting microtubule dynamics. Our results, taken together with the identification of symplekin depletion as a significant sensitizer to paclitaxel, suggest that polyadenylation machinery is tightly coupled to mitotic progression.

Figure 2.8

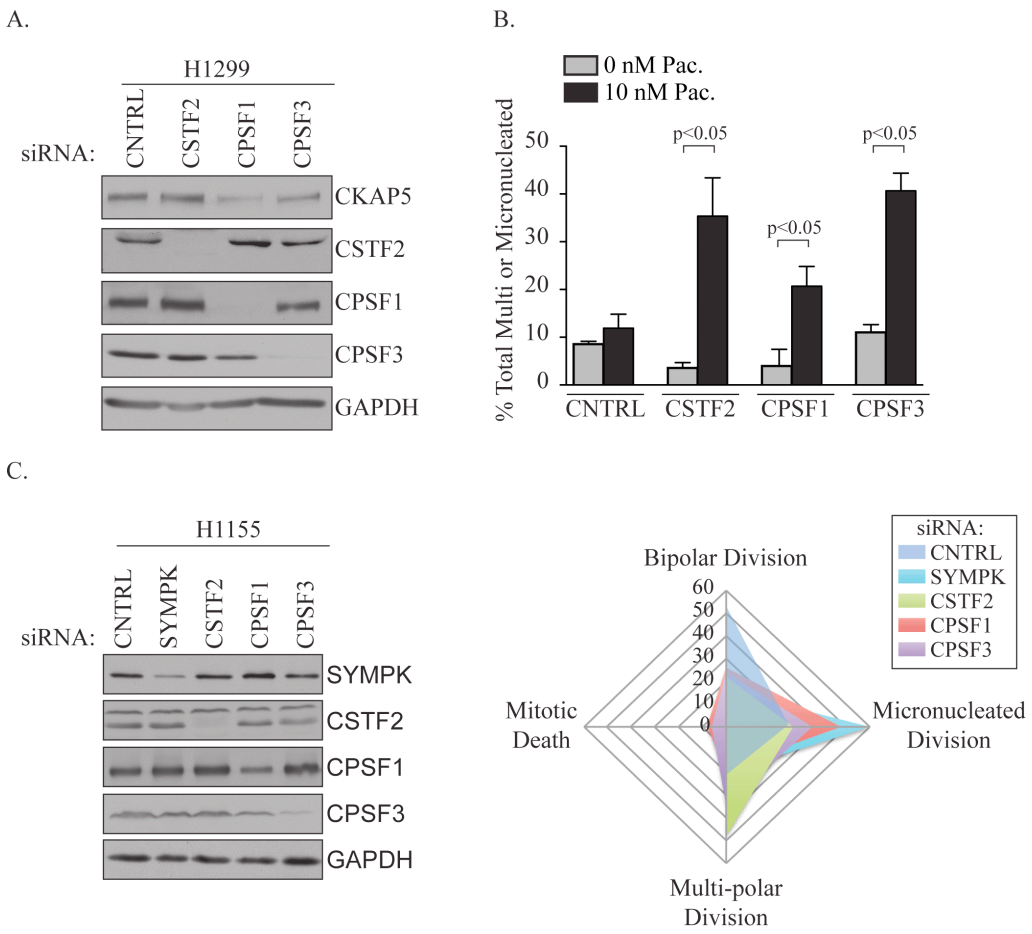


Figure 2.8 Polyadenylation is required for CKAP5 expression and mitosis

(A) Whole cell lysates from H1299 cells transfected with indicated siRNAs for 96 hours were immunoblotted for CKAP5 expression. (B) H1299 cells were transfected with the indicated siRNAs for 48 hours followed by exposure to paclitaxel for an additional 24 hours. Cells were stained for β-tubulin and DAPI followed by quantification of multi and micronucleated cells. Error bars represent SD, n=3. (C) H1155 GFP-H2B cells were transfected with the indicated siRNA's to mediate gene knockdown (left panel), treated with 10 nM paclitaxel and imaged under the conditions described previously. Radar plot (right panel) depicts mitotic exit phenotype with each line indicating a 10% increase in exit type. Results are from a minimum of 50 cells per condition.

In particular, we find that the protein expression level of a critical mitotic component, CKAP5, is sensitive to depletion of polyadenylation machinery. While we have not yet determined if CKAP5 protein expression can be directly regulated by polyadenylation, we find that symplekin depletion does not appear to affect CKAP5 transcript abundance or protein turnover. Thus, the changes we observe in CKAP5 protein expression could be due to an alteration in translation initiation or mRNA stability, which could be a direct result of the depletion of key polyadenylation components. Alternatively, perturbations in polyadenylation machinery, which may be impacting a large set of set of transcripts (194), could alter endogenous mechanisms that regulate CKAP5 protein levels. In either case, we have revealed that mitosis, microtubule dynamics and CKAP5 levels are sensitive to alterations in the polyadenylation machinery.

An emerging paradigm is that polyadenylation may play an important role in tumorigenesis. Most transcripts have alternative polyadenylation (APA) sites, which allows for regulation of the 3'UTR length. APA has been correlated with proliferation, as activation of T-cells induces a global shortening of 3'UTRs (204), and with tumorigenesis, where truncation of the 3'UTR is widespread, potentially conferring resistance to miRNA-mediated silencing to support oncogenic phenotypes (132, 205). In addition, symplekin expression in lung and colon cancer cells is elevated as compared to normal cells (96, 128), indicating that an upregulation of polyadenylation machinery could be a frequent event during tumorigenesis. Our findings that symplekin depletion induces mitotic defects in tumor cells, but not in normal cells, suggest that the demand for cell division in tumor cells may heighten the dependency on polyadenylation machinery to maintain the molecular framework that supports mitosis. Collectively, these results suggest that polyadenylation may be a vulnerability in tumor cells. Anti-mitotics, such as paclitaxel, are first-line chemotherapeutics whose effectiveness is limited by significant toxicity and acquired resistance. Thus, therapeutic regimens that combine anti-mitotics with polyadenylation inhibitors may have an enhanced effectiveness on cytotoxicity in tumor cells,

while decreasing adverse events in normal tissues.

Experimental Procedures

Cell culture - H1155, H1299, HCC366 and HCC515 cells were a gift from John Minna. All cells lines had recently been genotyped using SPR analysis. Cells were maintained in RPMI (Gibco) with 5% FBS as described (4). BJ fibroblasts immortalized with hTERT were a gift from Fred Grinnell (UT-Southwestern). BJs were maintained in DMEM + 10 % FBS.

Cell Titer Glo Assays - Cell Titer Glo assays were performed using independent siRNAs from the siGENOME SMART pool targeting symplekin as previously described (4).

siRNA Transfections - Transfections were performed as described with siGENOME SMART pools (ThermoFisher). Cells were transfected for either 72 or 96 hours as indicated in the figure legends. As a control, either a mismatch siRNA or an siRNA targeting DLNB14, which has no detectable impact in our assay system, was used.

High-Content Imaging - H1155 GFP-histone 2B-expressing cells were obtained by retroviral transduction. Retrovirus was produced by Fugene (Roche) transfection of 293gp cells with pCLNCX-GFP-H2B (a gift from Gray Pearson, UT-Southwestern) and VSV-G and virus was harvested at 48 hours post-transfection. H1155 cells at 50% confluency were transduced with virus in 4 ug/mL Polybrene and stably expressing cells were selected using 600 ug/mL Geneticin (Gibco). For imaging, cells were reverse transfected with the indicated siRNAs, plated in a 96 well format and exposed to paclitaxel at 48 hours post-transfection. 24 hours post-paclitaxel treatment, the cells were imaged on a BD Pathway 855 bio-imager using a 40X or 20X high-NA objective. Images were taken every 15 minutes for the next 48 hours and an image sequence was generated using Image J. Manual quantification was used for the indicated parameters.

Flow cytometry - H1155 cells were fixed in 50% ethanol/PBS, washed and resuspended in propidium iodine (BD) for 30 minutes. A minimum of 10,000 cells were collected for each condition by Summit 4.3 (Dako) and cell cycle distribution was determined using the ModFit

software package (Verity Software House).

Lentivirus production - shRNA clones in the PLKO1 vector were obtained from The RNAi Consortium (Open Biosystems). Lentivirus targeting SYMPK was produced by Fugene-mediated transfection of 293T cells with plasmids for VSV-G, Δ8.9 and shRNA's targeting SYMPK or GFP (SYMPK clones TRCN0000141511 and TRCN0000144902 were effective). Virus was harvested at 48 hours post-transfection and used to infect cells at 50% confluency in conjunction with 5 ug/uL Polybrene. Infection rates based on GFP performed in parallel were over 90 %.

Quantitative real-time RT-PCR - Total RNA was collected from H1299 cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). cDNA was synthesized from 2 ug total RNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Real-time RT-PCR used inventoried TaqMan gene expression assays designed to detect mRNA exclusively and the 7500 Fast real-time PCR system (Applied Biosystems). Actin or GAPDH was used as the endogenous control and cells transfected with control siRNA were used for calculating differences in expression by the $2^{-\Delta\Delta CT}$ method. For CKAP5 levels following SYMPK reduction, results are from pooling of 3 individual experiments performed in duplicate in which the average endogenous control Ct values between conditions never varied more than 0.3. For measurement of transcript knockdown, experiments were performed in triplicate with Ct values between conditions never varying more then 0.6.

Immunoblotting - Cells were lysed directly in boiling sample buffer (100 mM Tris-Cl, 4% SDS, 20% glycerol, 0.2% bromophenol blue), subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membrane (Sigma). For MG-132 experiments, 20 uM MG-132 (Sigma) was added for 12 hours prior to harvesting. Primary antibodies used include anti-SYMPK (BD Biosciences), anti-GAPDH (Santa Cruz), anti-cyclin B1 (Cell Signaling), anti-TACC3 (Santa Cruz), anti-CKAP5 (Abcam), anti-CSTF2 (Abcam), anti-CPSF1 (Santa Cruz) and anti-CPSF3 (Santa Cruz). Secondary antibodies used

include peroxidase-conjugated anti-mouse and anti-rabbit IgG (Jackson ImmunoResearch). Densitometry analysis was performed in ImageJ.

Immunofluorescence - Cells were grown on glass coverslips and fixed at 72 or 96 hours post-transfection in 3.7% formaldehyde. Cell were processed for immunofluorescence as described previously (4, 155). To visualize centrosomal proteins (CKAP5, TACC3, c-NAP1, Rootletin and NUMA1), cells were extracted with 0.5% Triton for 30 seconds, prior to fixation in cold methanol. Primary antibodies used include anti-beta tubulin (Sigma), pericentrin (Abcam), rootletin (Santa Cruz), Aurora-A (Sigma), Ncd80 (Abcam), TACC3 (BioLegend), NUMA1 (Novus) and CKAP5 (Abcam). After washes in PBTA, slips were placed in AlexaFluor conjugated secondary antibody (Invitrogen). Slides were imaged on an Axioimager upright microscope (Zeiss) equipped with a CCD camera.

Microtubule Regrowth Assay - H1299 cells were treated with 11 uM nocodazole (Calbiochem) at 96 hour post-transfection. After 2 hours of treatment, cells were placed in fresh media and allowed to recover for the indicated time period. Cells were washed in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2.0 mM $MgCl_2$, 1.0 uM paclitaxel) and depolymerized tubulin was extracted with 0.2% Triton in PHEM buffer for 1 minute. Extracted cells were washed in 1X PBS and fixed in 3.7% formaldehyde for 15 minutes. Immunofluorescence for β-tubulin and pericentrin was performed after permeabilization in 0.5% Triton and blocking in PBTA. Quantification of mean ß-tubulin fluorescence intensity in the region of the centrosome was measured in ImageJ. For the measurement, pericentrin staining was used to identify the centrosomes of each cell and a circle of constant diameter across all samples was drawn around the centrosome to measure the intensity of the ß-tubulin fibers emanating from the centrosome. ImageJ was utilized to measure the ß-tubulin fluorescence in the circle with a minimum of 40 cells per treatment group.

In Vitro Microtubule Stability Assay - H1299 cells were treated with 11 uM nocodazole for 2 hours and allowed to recover in fresh media for the indicated time period. Cells

were washed twice in PBS and then harvested by scraping into cold microtubule stabilizing buffer (180 mM PIPES, 13M glycerol, 1M $MgCl_2$, 25 mM EGTA, 0.5% Triton, 50 mM paclitaxel, protease inhibitors). Polymerized microtubules were pelleted by centrifugation at 12,000g for 10 minutes at 4 degrees. The supernatant containing the de-polymerized microtubule fraction was removed and the polymerized microtubules were resuspended in microtubule stabilizing buffer and boiling sample buffer prior to analysis by western blotting for beta tubulin.

Tumor xenografts - H1299 cells stably expressing luciferin (H1299_luc) were a gift from John Minna (UT-Southwestern). H1299_luc cells were transduced with lentivirus targeting shGFP or SYMPK and viable cells were harvested at 5 days post-infection for injection into nude mice. Two million cells/mouse were injected subcutaneously in the right flank and mice were treated with 10ul/gram luciferin and imaged bi-weekly with Bioluminescent Imaging until tumor development. Tumor growth was thereafter monitored bi-weekly by BLI imaging. Tumor volume was calculated as $l \times w^2 \times 0.5$ from BLI measurements. All animals were treated in accordance with *Institutional Animal Care and Use Committee (IACUC)* guidelines instituted at The University of North Carolina at Chapel Hill.

CHAPTER III

TACC3 AND MULTIPLE GAMETOGENIC GENES SUPPORT THE CANCER CELL MITOTIC SPINDLE

Summary

Increased proliferation rates in tumors, coupled with abnormalities in spindle architecture, places tumor cells under increased mitotic stress. Previously, we performed a genome-wide paclitaxel chemosensitizer screen to identify targets whose depletion sensitizes lung cancer cells to this mitotic stress. The screen uncovered multiple cancer-testis antigens and gametogenic proteins whose depletion increases the efficacy of paclitaxel. The gametogenic landscape is emerging as an important player in tumorigenesis. Here, we demonstrate that multiple gametogenic proteins identified in our screen impact paclitaxel sensitivity by altering mitotic spindle formation. Using a tumor progression model, we show dependence of the mitotic spindle on the gametogenic gene TACC3 can be detected in the early stages of oncogenic transformation but not in normal bronchial epithelial cells. This dependency may derive from alterations in microtubule dynamics and mitotic progression that accompany cellular transformation and which are observed in our tumor progression model. Finally, we show that the gametogenic protein TACC3 can targeted in vitro using a small molecule. Together, these results suggest mitotic roles for the gametogenic program in mammalian tumorigenesis and suggest therapeutic efficacy in targeting gametogenic genes.

Introduction

Numerous biological processes are corrupted in tumor cells to allow for increased proliferation rates, evasion of apoptosis and metastases (1). While these corrupted networks drive tumor growth, they also put the cell under heightened stress (2). One of the most common stress phenotypes in cancer cells is mitotic stress, which presents several barriers to a successful division. These include aneuploidy, which forces the cell to contend with an altered amount of genetic material during mitosis and may itself arise from aberrant cellular division (9, 18, 22, 24). Additionally, cancer cells frequently display centrosomal amplification, making it necessary for the cell to cluster the redundant centrosomes to achieve a bipolar mitosis (26, 28, 29, 33, 41). Finally, cancer cells have altered microtubule stability and this has been postulated to drive genomic instability (44-46). These mitotic stress phenotypes represent vulnerabilities in the cancer cell that can be exploited therapeutically, as demonstrated by the success of anti-mitotics such as paclitaxel.

The ability to query the entire genome now presents the opportunity to identify pressure points within these stressed processes to reveal ideal therapeutic entry points. Previously, we have used this technique to identify a cohort of gene products whose depletion sensitizes cancer cells to a sublethal dose of paclitaxel (4). Our genome-wide loss of function chemosensitizer screen uncovered several cancer-testis (CT) antigens and gametogenic proteins that alter the response to paclitaxel. Additionally, we have shown that expression of the CT antigen ACRBP, originally recovered in our genome-wide screen, correlates with survival of ovarian cancer patients and modulates the responsiveness of lung and ovarian cancer cells to paclitaxel (155).

Cancer cells and germ cells share several important features including rapid proliferation, invasive ability, immortalization, and genome hypomethylation (93). Recently, overexpression of several germline genes has been shown to drive malignant tumor grown in *Drosophila* (206) and a somatic to germline transition was documented in long-lived *C. elegans* mutants (207). These results suggest a functional role for the germline program in controlling proliferative capacity that

may apply to mammalian tumors. Several groups have demonstrated widespread expression of CT-antigens in a variety of human cancers (143, 145, 146). However, little is known about the functional relevance of the gametogenic program in supporting mammalian tumor growth. Due to their limited expression in normal adult tissues, the CT-antigens and gametogenic proteins represent theoretically ideal drug targets that may be associated with minimal off-target effects on normal tissues.

Here, we examine the intersection between the gametogenic program and response of cancer cells to mitotic poisons. We first demonstrate that multiple CT-antigens and gametogenic proteins are required for formation of a normal bipolar mitotic spindle in the presence of paclitaxel. We then use a lung cancer progression model to catalog the changes in microtubule stability and mitotic progression that accompany the steps of cellular transformation. Finally, we use the protein TACC3 as a model dependency to demonstrate that some gametogenic players are more required in tumor cells then normal cells and can be targeted both in vitro and in vivo. Together, these results establish the gametogenic machinery, and TACC3 in particular, as a unique target to increase the effectiveness of current anti-mitotic therapeutics.

Results

Multiple gametogenic genes identified in genome-wide screen - Previously, our genome-wide screen identified a number of CT-antigens that, when depleted, synergize with low-dose paclitaxel (4). A retrospective analysis of our dataset revealed additional gametogenic genes that, while not classified as CT-antigens, demonstrate an expression pattern showing enrichment in the organs of gametogenesis (Table 3.1). Although these genes share a common enrichment in gametogenic tissue and often are over-expressed in tumor cells, little is known about their cellular function in gametogenesis and tumorigenesis. The identification of these genes as hits that reduce viability in a genome-wide paclitaxel chemosensitizer screen suggests a functional role for the gametogenic program in mammalian tumorigenesis.

Table 3.1 Characteristics of gametogenic proteins identified in screen

Gene	Full gene name	Domains	Role in spermatogenesis	Role in tumorigenesis	CT antigen?	Overexpessed in tumors
ACRBP	Acrosin binding protein	Signal peptide	Binds to acrosin, a proteolytic protein in sperm that allows penetration of the zona pellicuda of the oocyte (208)	Interacts with NuMa to support mitotic spindle formation (155)	Yes	Yes
FMR1NB	Fragile X mental retardation neighbor 1	Signal peptide, trefoil, transmembrane	Unknown	Unknown	Yes	Yes
NXF2	Nuclear RNA export factor 2	Unknown	Promotes mRNA export from nucleus, NXF2-deficient mice are infertile and demonstrate meiotic errors (209, 210)	Unknown	Yes	Yes
FATE1	Fetal and adult testis expressed 1	Transmembrane	Unknown	Overexpression promotes proliferation of hepatocellular carcinoma cells (211)	Yes	Yes
MAGEA5	Melanoma antigen family A5	MAGE	Unknown	MAGE family proteins can bind RING E3 ligases to direct degradation of target proteins (212)	Yes	Yes
STARD6	START domain containing protein 6	START	May control lipid and sterol transport (213)	Unknown	No, enriched in germ cells (213, 214)	Unknown
TACC3	Transforming acidic coiled-coil containing protein 3	TACC	Unknown	Control of microtubule stability during mitosis (177, 178)	No, enriched in germ cells (162)	Yes
FSIP1	Fibrous sheath interacting protein 1	Coiled-coil	Interacts with sperm fibrous sheath, a cytoskeletal structure in the flagellum that may support motility (215)	Unknown	No, enriched in germ cells (215)	Unknown
BDG29	Zinc finger CCHC domain containing 14	ZINC finger, SAM	Unknown	Unknown	No	Unknown

Information in this table is derived from the Human Protein Reference Database (216), CTPedia (142), and from the individual sources listed above.

To confirm the gametogenic expresssion pattern of these genes, we performed real-time PCR analysis of human tissue RNA samples (Figure 3.1A). All the identified genes demonstrated enrichment in testis tissue relative to several other normal human tissues. Most of the CT-antigens (ACRBP, FMR1NB, NXF2, FATE1 and MAGEA5) were expressed at very low levels in normal tissues but the ACRBP was expressed at a higher level in normal brain tissue, consistent with previous reports of enrichment of CT-antigens in brain (143). In contrast, the gametogenic genes (STARD6, TACC3, FSIP1, BDG29) were expressed at higher levels in normal tissues. Given our identification of these genes in a viability screen, we tested whether gene depletion in combination with paclitaxel led to increased apoptosis. Strikingly, depletion of these genes in H1155 cells (Figure 3.1B) markedly increased induction of the apoptotic marker cleaved caspase 3 (Figure 3.1C) Importantly, gene depletion alone had little effect on apoptosis, with the exception of the CT-antigen FATE1, which showed marked induction of cleaved caspase 3 following gene depletion. These genes share a common enrichment in gametogenic tissue but have diverse cellular functions, making it unclear how depletion of most of these genes alters paclitaxel sensitivity.

Loss of gametogenic genes impairs formation of the bipolar mitotic spindle – Previously, we have observed that paclitaxel chemosensitizer screens often return components which impact mitotic spindle formation (4, 92, 155). There is only limited knowledge about the cellular function of the gametogenic genes studied here and the knowledge that does exist have implicated these genes in everything from regulation of mRNA transport to protein degradation. Given these diverse cellular functions but common enrichment in our paclitaxel sensitizer screen, we hypothesized that many of these genes may alter mitotic spindle formation to increase paclitaxel sensitivity. To test this hypothesis, we depleted these genes in H1155 cells, treated with paclitaxel and stained the mitotic spindle with gamma tubulin and pericentrin (Figure 3.2A). FATE1 was excluded from this analysis due to the potent induction of apoptosis following

Figure 3.1

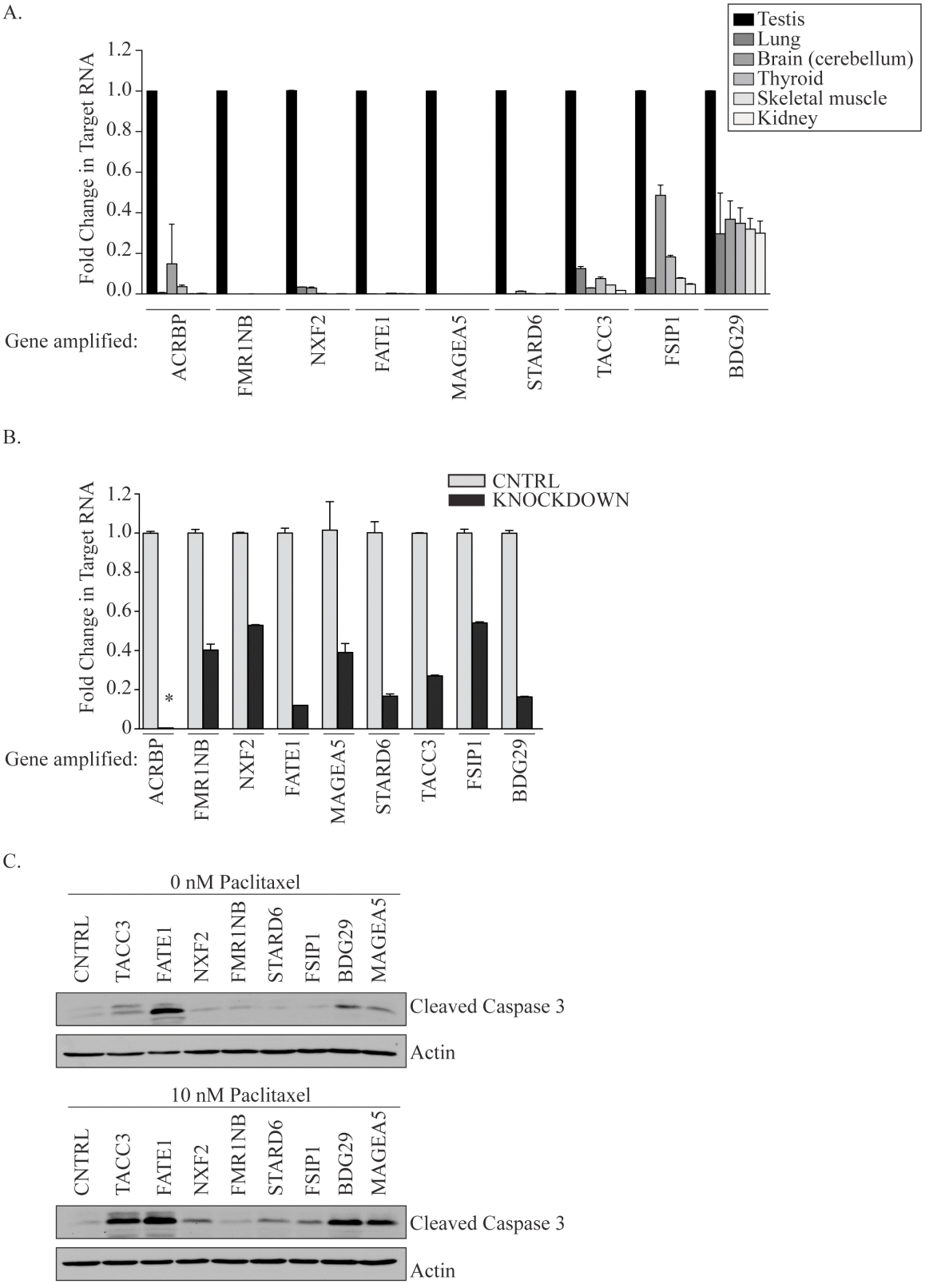
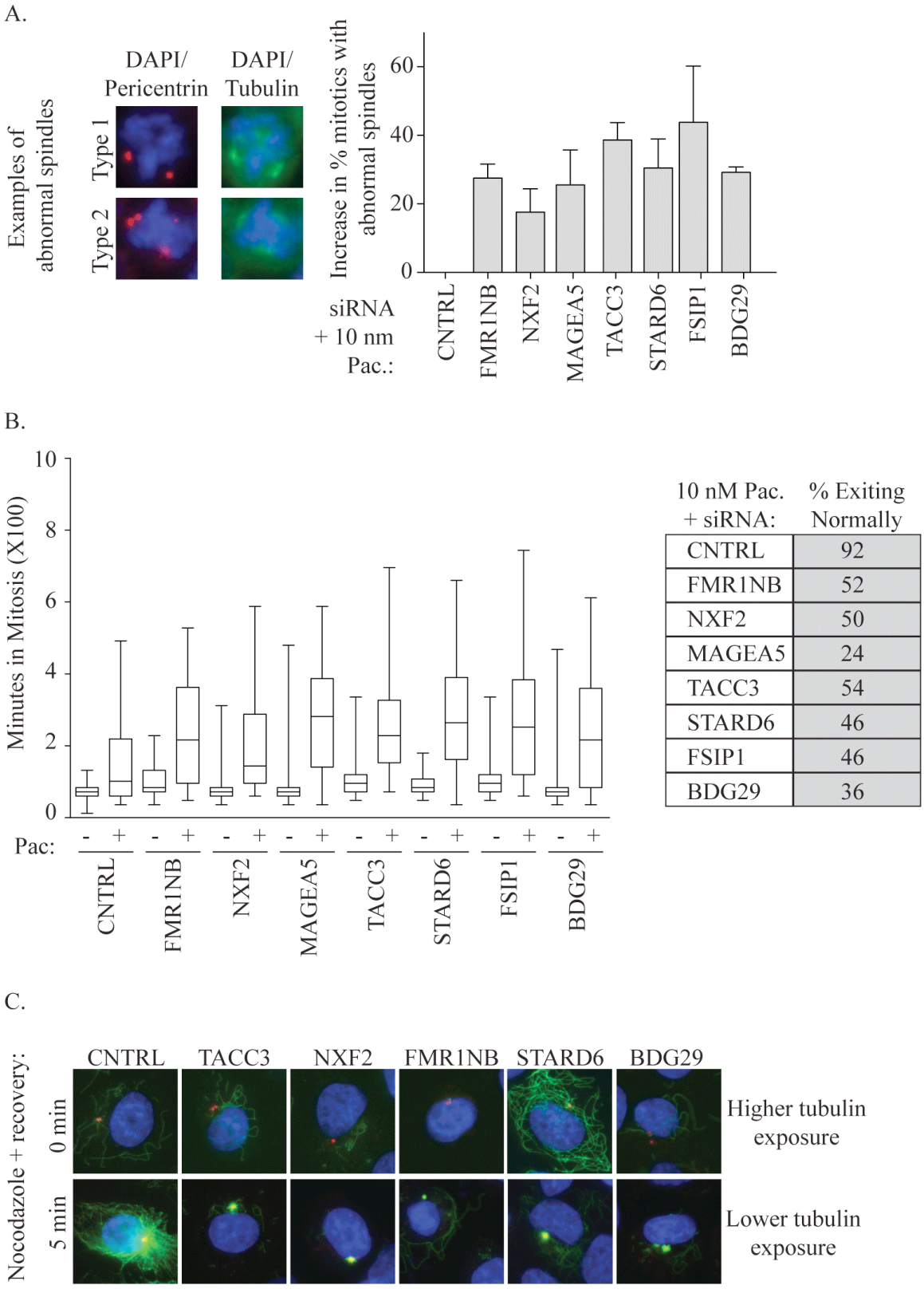

A.
Testis
Lung
Brain (cerebellum)
Thyroid
Skeletal muscle
Kidney
Fold Change in Target RNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Gene amplified:
ACRBP
FMR1NB
NXF2
FATE1
MAGEA5
STARD6
TACC3
FSIP1
BDG29
B.
CNTRL
KNOCKDOWN
Fold Change in Target RNA
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Gene amplified:
ACRBP
FMR1NB
NXF2
FATE1
MAGEA5
STARD6
TACC3
FSIP1
BDG29
C.
0 nM Paclitaxel
CNTRL
TACC3
FATE1
NXF2
FMR1NB
STARD6
FSIP1
BDG29
MAGEA5
Cleaved Caspase 3
Actin
10 nM Paclitaxel
CNTRL
TACC3
FATE1
NXF2
FMR1NB
STARD6
FSIP1
BDG29
MAGEA5
Cleaved Caspase 3
Actin

Figure 3.1 Multiple gametogenic genes sensitize H1155 NSCLC cells to paclitaxel

(A) Real-time PCR analysis of gene expression profiles in pooled human tissue mRNA samples. Gene expression is normalized to testis and is represented by the 2^-ddCT method with error bars representing standard deviation (n=2). (B) Real-time PCR analysis of gene expression following transfection of the indicated siRNA for 72 hours. Error bars represent standard error of the mean (n=3). (C) Lysates from siRNA transfected H1155 cells treated for 24 hours with paclitaxel (Pac.) were immunoblotted for cleaved caspase 3 and GAPDH.

Figure 3.2



10 nM Pac. + siRNA:	% Exiting Normally
CNTRL	92
FMR1NB	52
NXF2	50
MAGEA5	24
TACC3	54
STARD6	46
FSIP1	46
BDG29	36

Figure 3.2 Loss of gametogenic genes impairs formation of the bipolar mitotic spindle

(A) H1155 cells were transfected with the indicated siRNA's, treated with paclitaxel for 24 hours and then stained with gamma tubulin/pericentrin to visualize the mitotic spindle. Examples of abnormal spindles observed are shown on the left. Error bars represent standard deviation (n=3). (B) Live cell imaging results from H1155 GFP-H2B cells transfected and then exposed to paclitaxel for 24 hours prior to imaging on a high content scope. All numbers are from a minimum of 50 cells per condition. (C) Microtubule recovery assay in H1299 cells depleted of the indicated components. The microtubule network was depolymerized with nocodazole, allowed to recover in warm media for the indicated time period and then visualized with beta tubulin/pericentrin immunostaining.

FATE1 depletion. Depletion of all the gametogenic genes led to an increase in abnormal spindle architecture. Two main types of abnormal spindles were observed; type 1 abnormal spindles had only two pericentrin-positive poles but exhibited gamma tubulin bundles while type 2 spindles had multiple pericentrin positive poles (Figure 3.2A). Abnormal spindle architecture can lead to abnormal mitotic progression or may be successfully repaired by the cell to allow for a normal division. To examine the consequence of these abnormal spindles on mitotic progression, we performed high-content live cell imaging in H1155 cells stably expressing the chromatin marker GFP-histone 2B. Strikingly, depletion of these cancer-testis antigens and gametogenic genes in the presence of paclitaxel increased the percent of cells exiting abnormally from mitosis and led to an increase in mitotic delay (Figure 3.2B).

Abnormal mitotic spindle formation and mitotic progression can arise from errors in many cellular pathways. In the past, we have observed that multiple genes identified in our genome-wide paclitaxel screen, including the CT-antigen ACRBP and the polyadenylation scaffold SYMPK, modulate the function of microtubule associated proteins or microtubule stability (92, 155). The finding that many screen hits modify microtubule stability is not surprising given the known impact of paclitaxel on microtubule dynamics (43, 217). Therefore, we tested whether these additional gametogenic gene products affect microtubule stability. While several genes had no effect on microtubule stability, the genes TACC3, FMR1NB, NXF2, STARD6 and BDG29 significantly decreased the ability of the microtubule network to regrow following depolymerization with nocodazole (Figure 3.2C). Importantly, different genes had unique effects on microtubule recovery. For example, STARD6 depletion impaired microtubule depolymerization while depletion of FMR1NB enhanced depolymerization. Even with these different effects on depolymerization, all genes led to a significantly slower recovery of organized microtubule asters after nocodazole treatment, indicating an impaired ability of the microtubule network to reorganize following mitotic stress. Taken together, these results show an unanticipated reliance of the cancer cell mitotic spindle on several gametogenic proteins for

microtubule dynamics, formation of a bipolar mitotic spindle, and mitotic progression.

Oncogenic changes alter mitotic properties - Given our observation that multiple gametogenic genes, many of which are upregulated in cancer cells, impact microtubule dynamics, we hypothesized that cancer cells may become dependent on these genes to support microtubule stability. Other groups have observed increased microtubule stability in cancer cells (44, 45) but it is unclear how most oncogenic changes alter microtubule stability. To address this question, we obtained a cancer cell progression model where the immortalized human bronchial epithelial cell line, HBEC3, has been engineered to lose the tumor suppressor p53 and/or over-express a constitutively active KRas V12 mutant, or wild-type EGFR (218). The lines expressing KRas or EGFR combined with loss of p53 are more tumorigenic as indicated by their increased propensity to form colonies in soft agar (218). To probe whether these oncogenic changes alter microtubule stability, we performed microtubule recovery assays in each line of the progression model (Figure 3.3A). Loss of p53 expression makes cells more sensitive to nocodazole depolymerization. However, over-expresssion of constitutively active KRas and to a lesser extent wild-type EGFR significantly increased the rate at which the microtubule network recovered from nocodazole treatment. Importantly, depletion of KRas by siRNA decreased the rate of recovery of the microtubule network, indicating that the effects on microtubule stability are dependent on expression of KRas (Figure 3.3B) Together, these results indicate that oncogenic changes profoundly alter microtubule dynamics.

To examine the consequence of these changes on mitotic progression, we engineered each line of the progression model to express GFP-histone 2B and then observed the lines as they progress through an unperturbed mitosis (Figure 3.3B). Interestingly, loss of p53 led to a significant mitotic delay and an increase in the percent of cells exiting abnormally from mitosis. Expression of mutant KRas or wild-type EGFR rescued this mitotic delay and restored normal exit phenotype. Overall, these results indicate that oncogenic changes can profoundly alter both microtubule dynamics and mitotic progression.

Figure 3.3

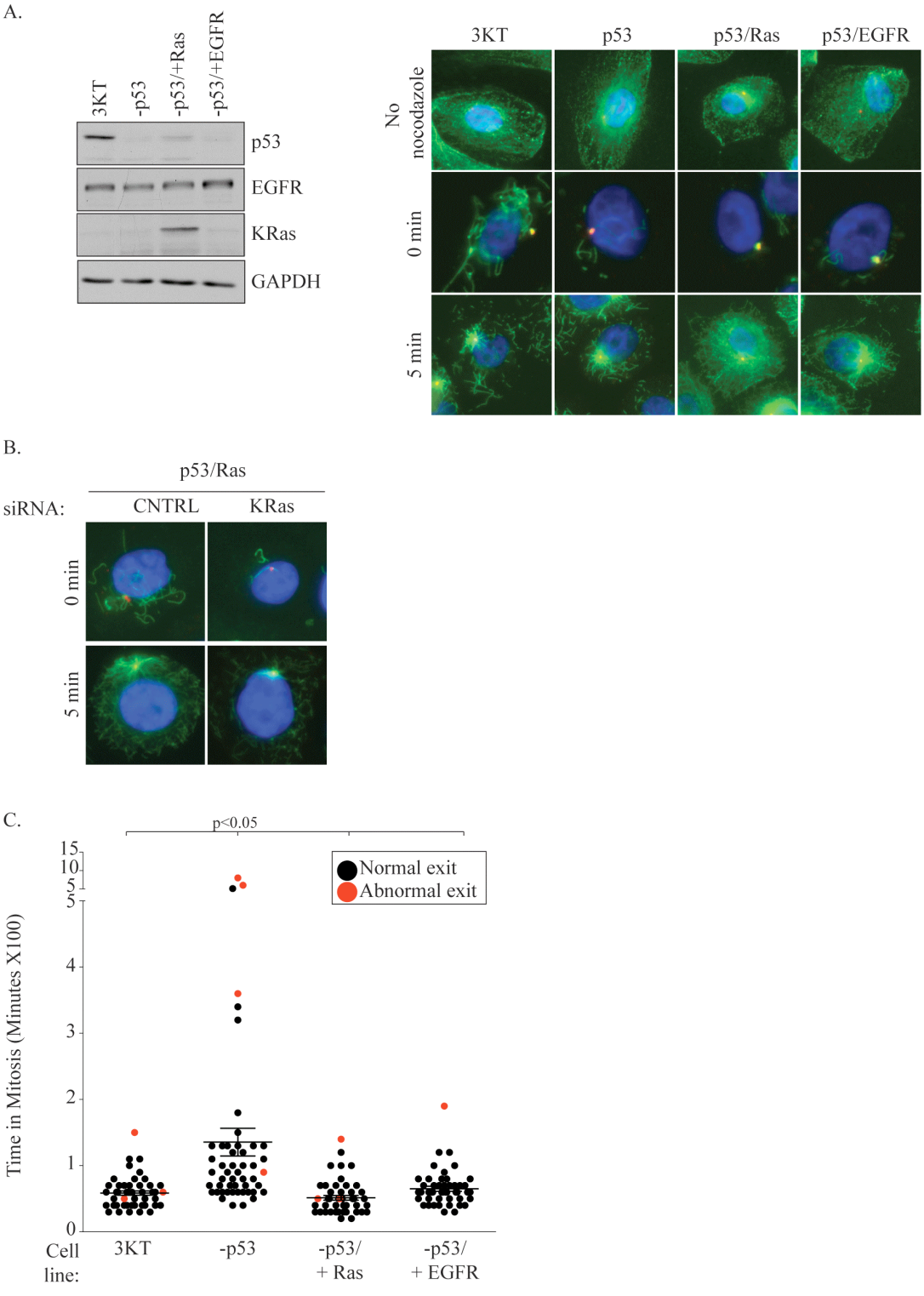

A.
3KT
-p53
-p53/+Ras
-p53/+EGFR
p53
EGFR
KRas
GAPDH
3KT
p53
p53/Ras
p53/EGFR
No nocodazole
0 min
5 min
B.
p53/Ras
siRNA:
CNTRL
KRas
0 min
5 min
C.
p<0.05
Normal exit
Abnormal exit
Time in Mitosis (Minutes X100)
15
10
5
5
4
3
2
1
0
Cell line:
3KT
-p53
-p53/ + Ras
-p53/ + EGFR

Figure 3.3 Oncogenic changes alter mitotic properties

(A) Microtubule recovery assay in each line of the lung cancer progression model. The cell lines were depolymerized with nocodazole and allowed to recover in warm media for the indicated time period. Immunoblot for p53, KRas, p-EGFR and GAPDH is to confirm line identities. (B) Microtubule recovery assay performed as in (A) following transfection of siRNA for 72 hours into the p53/Ras line. (C) Live cell imaging in the 3KT, p53, p53Ras and p53EGFR lines engineered to stably express GFP-H2B. Manual analysis for mitotic timing and exit phenotype is represented for 50 cells per condition. P value is derived from a Mann-Whitney test of medians comparing mitotic timing in the p53 line to all other lines individually.

Oncogenic changes drive a dependency on TACC3 - The observation that oncogenic changes alter microtubule stability and mitotic progression in this tumor progression model suggest the presence of networks uniquely supporting mitosis in transformed as compared to normal immortalized cells. To test this hypothesis, we depleted TACC3 in a panel of three normal immortalized lines and three tumor lines (Figure 3.4A). We chose TACC3 for this analysis because, unlike many of the gametogenic genes we are studying, the mechanism by which TACC3 impacts microtubule dynamics is well known (176). Additionally, TACC3 is expressed, albeit at lower levels, in normal immortalized lung cancer lines, in contrast to the classical CT-antigens, which are often not expressed in normal immortalized lines (55, 93, 166, 167). Strikingly, TACC3 depletion had minimal impact on all the normal lines analyzed but significantly increased micronucleation response in each tumor line (Figure 3.4A).

The increased sensitivity of tumor lines to TACC3 depletion could arise from a myriad of changes in the mitotic spindle that accompany transformation. The isogenic HBEC3 tumor progression model allows identification of the transformation events that drive a dependency on TACC3. Depletion of TACC3 using a pool of two effective shRNAs in this model system led to increased micronucleation in both transformed lines (p53Ras and p53EGFR) while having minimal effects in the p53 line and no effect in the normal immortalized 3KT line (Figure 3.4B). These differential effects on micronucleation could be due to a unique effect of TACC3 in transformed lines or could derive from experimental differences between the lines such as differential rates of division or altered apoptotic signaling in response to mitotic damage. To exclude the latter two possibilities, we utilized our GFP-histone 2B expressing lines and performed live-cell imaging in the context of TACC3 depletion in each line. Strikingly, depletion of TACC3 in this system had no effects on the normal immortalized 3KT line, some effect on the p53 line and the strongest effects on the p53Ras and p53EGFR lines (Figure 3.4C). TACC3 depletion occurred at a similar rate in both the 3KT and p53Ras lines, indicating that these effects

Figure 3.4

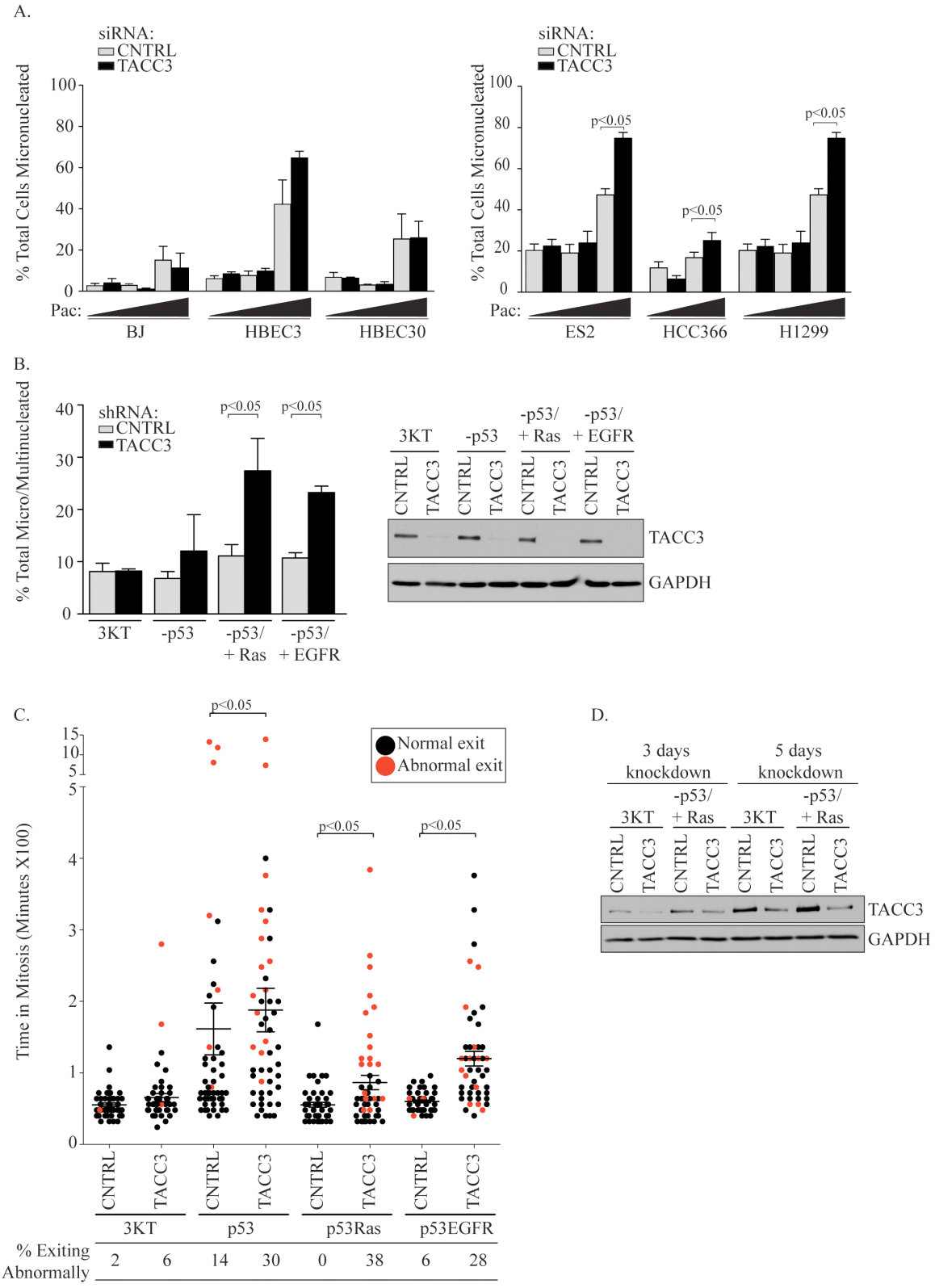

A.
siRNA:
CNTRL
TACC3
% Total Cells Micronucleated
Pac:
BJ
HBEC3
HBEC30
ES2
HCC366
H1299
p<0.05
B.
shRNA:
% Total Micro/Multinucleated
3KT
-p53
-p53/ + Ras
-p53/ + EGFR
TACC3
GAPDH
C.
Normal exit
Abnormal exit
Time in Mitosis (Minutes X100)
p53
p53Ras
p53EGFR
% Exiting Abnormally
2
6
14
30
0
38
6
28
D.
3 days knockdown
5 days knockdown

Figure 3.4 Oncogenic changes drive a dependency on TACC3

(A) Counts of micro/multinucleated cells after knockdown of TACC3 by siRNA for 48 hours followed by treatment of increasing doses of paclitaxel. Doses of paclitaxel utilized were: BJ (0, 1, 10 pM), HBEC30 (0, 0.1, 1 nM), HBEC3, ES2 and H1299 (0,1,10 nM) and HCC366 (0 and 1 nM). Error bars represent standard deviation, n=3 biological replicates for normal lines and n=1 for tumor lines. (B) 3KT, p53, p53Ras and p53EGFR lines were depleted of TACC3 by shRNA for 7 days, fixed and stained for beta tubulin/pericentrin. Counts for multi/micronucleated are from n=2 biological replicates with error bars representing standard deviation. Immunoblot is for confirmation of knockdown at Day 7 post-transduction. (C) 3KT, p53, p53Ras and p53EGFR lines stably expressing GFP-H2B were depleted of control or TACC3 by shRNA for 5 days prior to live cell imaging for an additional 48 hours. Counts of mitotic timing and exit phenotype are from a manual analysis of 50 cells per condition. P-values are from a Mann-Whitney test of medians. (D) Immunoblot depicting degree of gene knockdown at 3 and 5 days post-transduction in the 3KT and p53Ras lines.

are unlikely due to differential rates of gene knockdown (Figure 3.4D). Together, these results indicate that cancer cells may be more sensitive to depletion of microtubule stabilizing proteins such as TACC3.

TACC3 dependency is targetable with KHS101 - A conceptually ideal anti-mitotic agent would target components that are only required for division of tumor cells. Several of the CT-antigens studied here may represent these ideal targets but no small molecules targeting these proteins have been described to date. Our findings that TACC3 is specifically required in tumor cells and observations from several groups that TACC3 is upregulated in tumor tissue relative to normal tissue (55, 166, 167), suggest that TACC3 may also represent an ideal target. Recently, the TACC3 inhibitor KHS101 was identified in a screen for compounds that drive differentiation of neurons (182). These authors treated rats with the TACC3 inhibitor and saw no toxicity associated with the compound. Further, the effects of the TACC3 inhibitor on mitotic spindle formation were not examined.

To test whether the phenotypes we observe with TACC3 depletion can be recapitulated with this inhibitor, we examined the impact of co-treatment of H1155 cells with KHS101 and paclitaxel. Combined drug treatment significantly enhanced the ability of paclitaxel to drive apoptosis as indicated by the induction of cleaved caspase 3 (Figure 3.5A). Additionally, the combination of paclitaxel and KHS101 impaired the ability of H1155 cells to form colonies in soft agar (Figure 3.5B). As observed with siRNA to TACC3 and paclitaxel, co-treatment of H1155 cells with paclitaxel and KHS101 led to an increased proportion of cells in mitosis accompanied by a marked increase in the frequency of micro and multi-nucleated cells (Figure 3.5C). Closer examination of the mitotic spindle apparatus by staining with beta-tubulin and pericentrin revealed the spindle in KHS101 and paclitaxel treated cells was grossly abnormal with cells frequently displaying multiple pericentrin-positive poles (Figure 3.5D). Previously, KH101 was shown to interact with TACC3 in vitro and in vivo (182) but how this interaction impairs TACC3 activation is unknown. Given the known requirement for TACC3 phosphorylation prior

Figure 3.5

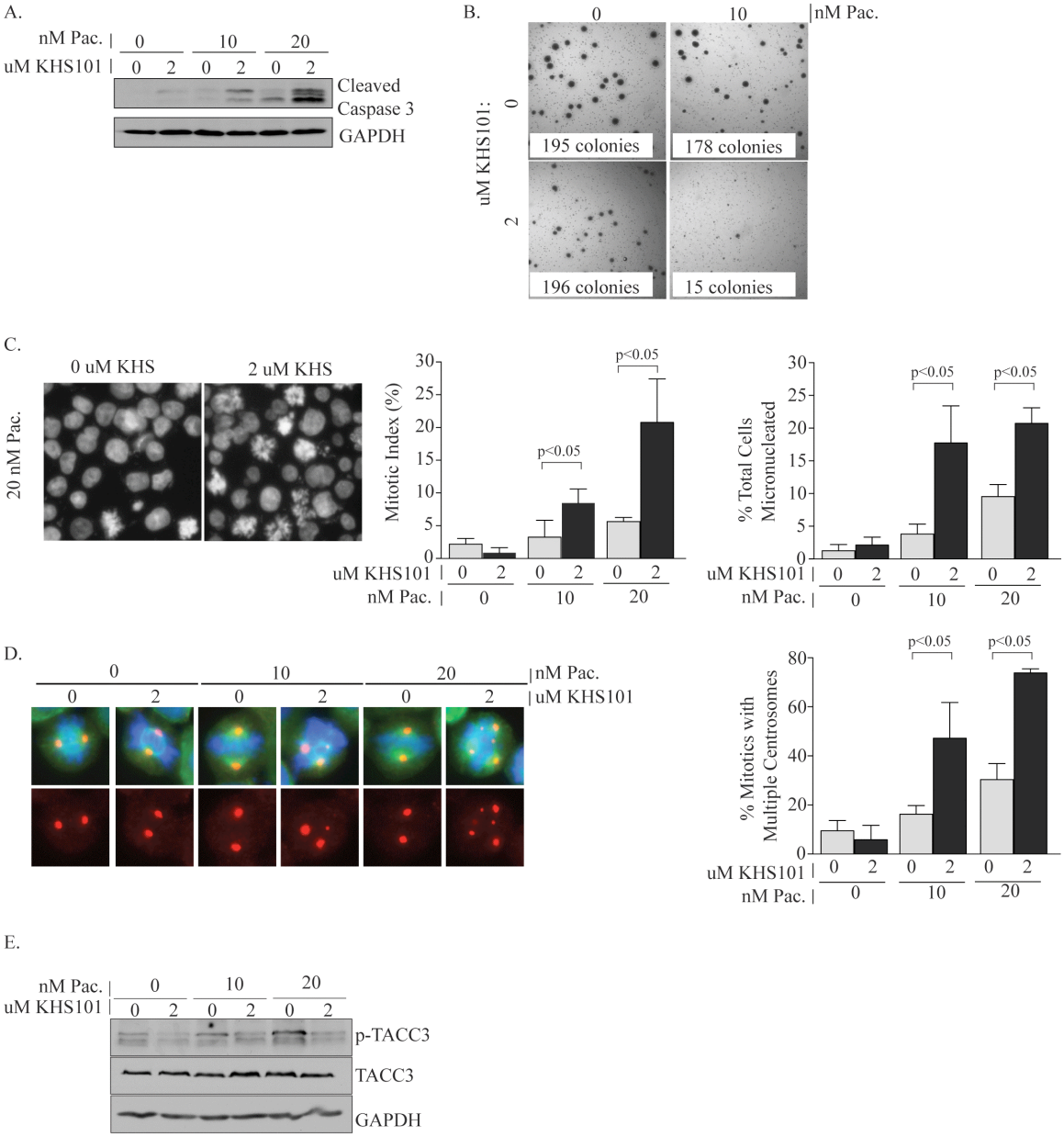

A.
nM Pac.
0
10
20
uM KHS101
0 2 0 2 0 2
Cleaved Caspase 3
GAPDH
B.
0
10
nM Pac.
uM KHS101:
0
2
195 colonies
178 colonies
196 colonies
15 colonies
C.
0 uM KHS
2 uM KHS
20 nM Pac.
Mitotic Index (%)
p<0.05
p<0.05
uM KHS101
nM Pac.
% Total Cells Micronucleated
p<0.05
p<0.05
D.
nM Pac.
uM KHS101
% Mitotics with Multiple Centrosomes
p<0.05
p<0.05
E.
nM Pac.
uM KHS101
p-TACC3
TACC3
GAPDH

Figure 3.5 TACC3 dependency is targetable with KHS101

(A) Immunoblot of whole cell lysates from H1155 cells treated with paclitaxel for 24 hours followed by 48 hours of combined treatment with KHS101. (B) Soft agar assay of H1155 cells maintained in paclitaxel/KHS101 media for 2 weeks. Counts are of total colonies in a 6-well dish from a representative experiment. (C) H1155 cells were treated with paclitaxel for 24 hours followed by 24 hours of combined treatment with KHS101 and fixed prior to staining with beta tubulin/pericentrin. Quantification is from manual counting of mitotic index and micronucleated cells. Error bars represent standard deviation (n=3). (D) H1155 cells treated as in (C) were stained with beta tubulin/pericentrin and mitotic cells were analyzed for normal spindle formation. Quantification is by manual counting of cells with multiple pericentrin positive poles. Error bars represent standard deviation (n=3). (E) Whole cell lysates from H1155 cells treated with paclitaxel for 24 hours followed by 24 hours of combined treatment with KHS101 were immunoblotted for p-TACC3, TACC3 and GAPDH.

to its activation and direction to the spindle (174), we probed the ability of KHS101 to impact phosphorylation of TACC3. Treatment of H1155 cells with KH101 significantly decreased the phosphorylation of TACC3 both with and without the addition of paclitaxel (Fig. 4G). Taken together, these data suggest the TACC3 dependency observed in tumor cells may be targetable through combined treatment with KHS101 and paclitaxel.

Discussion

This study establishes the functional relevance of multiple CT-antigens and gametogenic proteins in supporting cancer cell growth and mitosis. Beyond being mere byproducts of aberrant protein expression patterns in tumor cells, these proteins appear to be important dependencies that have developed in the tumorigenic environment. Understanding the functional role of the other CT antigens in supporting tumor growth is an important future direction. Further, it will be important to understand how the gametogenic proteins interface with the normal cellular and mitotic machinery. We have previously demonstrated the ability of the CT-antigen ACRBP to alter the activity of the normal mitotic component NuMA (155). A recent study also demonstrated that the CT-antigen MAGEA4 interacts with TACC3 in tumor cells (219). Therefore, there appear to be important connections between the normal and gametogenic machinery that require further study.

Previously, several other groups have described alterations in mitosis that accompany tumor suppressor loss or activation of oncogenes (47, 48, 50, 220-222). To our knowledge, our study is the first that catalogs the sequential impact of these changes in a tumor progression model system. From our study it appears that oncogene activation can rescue some of the defects in mitotic progression that accompany tumor suppressor loss. Part of this ability may stem from alterations in microtubule dynamic instability that accompany oncogenic changes and may also account for the potent efficacy of microtubule-targeted drugs in cancer cells. We hypothesize these changes in the mitotic spindle may drive *conditional dependencies* on components that are

normally expressed in all cells and also *emergent dependencies* on components that are aberrantly upregulated in tumor cells. The requirement for TACC3 expression specifically in the transformed lines supports the postulation that there exist mitotic dependencies that are unique to tumor cells.

Inhibition of these mitotic dependencies in tumor cells may provide a route to more effective and tumor-targeted chemotherapies. While TACC3 is more widely expressed in multiple tissues then a true CT-antigen, we observed no toxicity in mice treated with KHS101 even in combination with paclitaxel and a previous study that identified the compound observed no toxicity in rats (182). KHS101 appears to be truly synthetic lethal with paclitaxel on mitosis, as we observed no mitotic defects with in vitro single agent treatment of KHS101 even at doses up to 10 uM. Notably, we did observe changes in pH in the media of cells treated with KHS101, a phenotype that is consistent with the effects of TACC3 on hypoxic response (183). The lack of single agent activity with KHS101 alone on mitotic cells may account for why a previous group never identified mitotic spindle defects with the drug (182). This synthetic lethal phenotype could be useful if combined with paclitaxel formulations that are specifically directed to tumor cells.

More broadly, this study establishes the relevance of pharmacologically targeting gametogenic proteins to treat cancer. Current efforts are focused on therapeutic vaccines directed against the CT-antigens to raise an immune response against the cancer cell (153, 154). The establishment of a functional role of these proteins in supporting tumor growth suggests another avenue for intervention may utilize pharmacological targeting of these antigens independent of their immunogenic properties.

Experimental Procedures

Cell culture - H1155, H1299 and HCC366 cells were maintained in RPMI medium (Gibco) with 5% fetal bovine serum. ES2 cells were maintained in RPMI supplemented with

10% fetal bovine serum and BJ fibroblasts were grown in DMEM supplemented with 10% fetal bovine serum. 3KT, p53, p53/Ras and p53/EGFR lines were generated as described previously (218) and were maintained in keratinocyte media (Gibco) with provided supplements. Paclitaxel (LC laboratories) was resuspended in DMSO.

KHS101 treatment - KHS101 was a kind gift from Peter Schultz (182). Additional KHS101 was synthesized by the UNC Center for Integrative Chemical Biology and Drug Discovery. KHS101 was suspended in DMSO to a concentration of 10 mM and diluted in media to 2 uM final concentration.

siRNA transfection - Transfection conditions were as described previously (4) using siGENOME Smart pools (ThermoFischer) and Dharmefect transfection reagent. Control oligonucleotide was either a mismatch siRNA or a siRNA targeting DLNB14.

shRNA transductions - Lentivirus was produced as described previously (92) using short hairpin RNA clones in the PLKO1 vector from the RNA Consortium (Open Biosystems).

High-content live cell imaging - Cell lines expressing GFP-histone 2B were generated by viral transduction as described previously (92). A stable population of GFP-H2B expressing cells was obtained through fluorscence activated cell sorting by the UNC Flow Cytometry Core Facility. The stably expressing lines were then treated as described in the figure legends and imaged on the BD Pathway 855 imager using a 20X high-numerical aperature objective. Images were taken approximately every 15 minutes for the indicated time period and movies were generated using ImageJ. Results are from manual quantification for a minimum of 50 cells per condition.

Quantitative real-time RT PCR - For confirmation of gene knockdown, RNA was harvested from cells transfected with siRNA for 72 hours using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). cDNA was generated using 2 ug of total RNA and the High-Capacity cDNA reverse transcription kit (Applied Biosystems). Real-time RT PCR detection used inventoried TaqMan gene expression assays purchased from Applied Biosystems that are

designed to exclusively detect mRNA. Amplification was performed on the 7500 Fast Real Time PCR machine (Applied Biosystems). The ribosomal subunit RPL27 was used as the endogenous control and differences in expression were calculated using the $2^{-\Delta\Delta CT}$ method. For evaluation of gene expression in various human tissues, total human RNA samples pooled from multiple individuals were obtained from Clontech.

Immunoblotting - Cells were lysed in boiling sample buffer as described previously (92). The primary antibodies used were from Santa Cruz (KRas, GAPDH, TACC3) or Cell Signaling (p53, EGFR, p-EGFR, cleaved caspase 3, p-AURKA). p-TACC3 antibody was a kind gift from Kazuhisa Kinoshita (174). Secondary antibodies were peroxidase-conjugated anti-mouse and anti-rabbit IgG (Jackson ImmunoResearch).

Immunofluorescence - Conditions for immunofluorescence were described previously (92). Briefly, cells were grown on glass coverslips and fixed at the indicated time point in 3.7% formaldehyde. Primary antibodies used were anti-beta tubulin (Sigma) and pericentrin (Abcam). Secondary antibodies were Alexa Fluor conjugated mouse or rabbit antibodies (Invitrogen).

Soft agar assay - 1X10^4 H1155 cells were resuspended in 0.5% bacto agar in complete media and overlaid on solidified 0.5% bacto agar in 60 mm dishes. After solidification, a top layer of complete media was added. For drug treatments, half the media from the top layer was removed and replaced with media containing 2X the final drug concentration and 2X fetal bovine serum. Colonies were grown for 2 weeks and then stained with 0.005% crystal violet in PBS overnight.

Microtubule regrowth assay - Cells grown on glass coverslips were depolymerized with 11 uM nocodazole (Calbiochem) for 30 minutes. After depolymerization, cells were washed and allowed to recover in warm media for the indicated time period. Depolymerized microtubules were extracted in 0.2% Triton-X100 in PHEM buffer (60 mM PIPES pH 7.0, 25 mM HEPES, 100 mM EGTA, 2 mM MgCl2, 1 uM paclitaxel) and slips were then fixed in 3.7% formaldehyde. The microtubule network was stained with beta-tubulin (Sigma) and pericentrin (Abcam).

CHAPTER IV

CLINICAL RELEVANCE AND FUTURE DIRECTIONS

Summary

Understanding the networks controlling response of cells to mitotic stress can reveal important insights into tumorigenesis, mitosis and anti-mitotic chemotherapy. This work has uncovered two methods by which NSCLC cells can be sensitized to mitotic stress induced by paclitaxel treatment: inhibition of the polyadenylation machinery and altered expression of gametogenic genes.

In Chapter 2, we revealed important links between the polyadenylation machinery and formation of the bipolar mitotic spindle. Specifically, we demonstrated that SYMPK depletion combined with paclitaxel treatment leads to a G2/M delay, formation of abnormal mitotic spindles, and altered mitotic progression. We further showed SYMPK depletion leads to mitotic errors in multiple tumor, but not normal, cell lines and that depletion of SYMPK impairs tumor formation in vivo. The mitotic errors observed with SYMPK depletion are likely due to altered microtubule stability and reduced expression of the microtubule stabilizing protein CKAP5. Finally, we showed depletion of multiple polyadenylation subunits leads to similar errors in mitosis. Together, these findings suggest that polyadenylation is essential for both mitosis and response to mitotic stress.

In Chapter 3, we examined the intersection of the gametogenic program with mitotic spindle formation. Here, we demonstrated that depletion of a panel of gametogenic genes in combination with paclitaxel leads to apoptosis, mitotic errors, abnormal mitotic progression, and altered microtubule stability. We then used a tumor progression model to demonstrate that both microtubule dynamics and mitotic progression are altered following loss of tumor suppressors or overexpression of oncogenes. We also showed these oncogenic changes drive a dependency on the gametogenic protein TACC3 such that mitotic errors are only observed following TACC3 depletion in the more transformed lines. Finally, we demonstrate that inhibition of TACC3 using the small molecule KHS101 synergizes with paclitaxel. These findings provide evidence that these gametogenic proteins are functionally important in tumorigenesis and suggest efficacy in

targeting the gametogenic program in combination with current anti-mitotic drugs.

In this final chapter, I discuss the clinical significance of our findings and potential future directions. First, I discuss the potential existence of unique tumor-specific dependencies in the cancer cell mitotic spindle that arise in the context of mitotic stress. Second, I discuss emerging links between polyadenylation and mitosis. Third, I examine the parallels between the gametogenic machinery and tumorigenesis. Finally, I discuss the clinical implications of this work in terms of therapeutic targeting of polyadenylation, TACC3, and gametogenic proteins.

Future directions

Unique dependencies of the cancer cell mitotic spindle – We have demonstrated a dependence of the cancer cell mitotic spindle on polyadenylation and expression of gametogenic genes. Interestingly, the mitotic defects we observe following depletion of SYMPK or TACC3 are seen only in tumor, but not normal immortalized, cell lines. This absence of phenotype in the normal immortalized lines suggests that unique networks may support mitosis in tumor cells. We propose that tumor cells demonstrate two types of mitotic dependencies: *conditional dependencies* on processes such as polyadenylation that are present in both tumor and normal cells and *emergent dependencies* on processes such as the gametogenic program that are reactivated in tumor cells. Inhibition of these tumor-specific dependencies may provide a route to more effective anti-mitotic chemotherapies that have fewer effects on normal tissues.

Mitotic stress phenotypes that are unique to tumor cells may drive the development of conditional and emergent dependencies. Mitotic stress phenotypes are commonly observed in tumor cells but absent in normal immortalized cells and include aneuploidy (5), supernumerary centrosomes (26-29), and alterations in microtubule stability (44-46). To support proliferation in the presence of these mitotic stressors, tumor cells may develop enhanced dependencies on certain processes (Figure 4.1). Specifically, cancer cells may develop alterations in gene expression that allow for a bipolar division. Over time, there may be competitive selection for

Figure 4.1

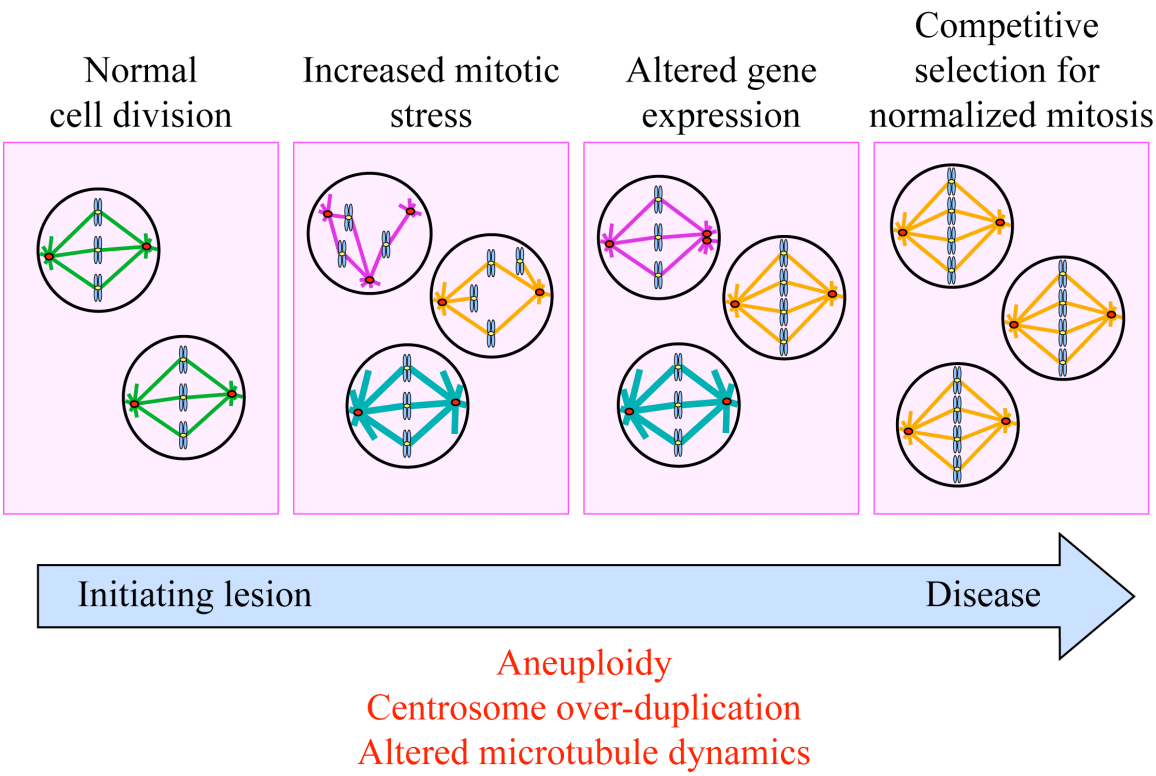


Figure 4.1 Model of adaptation to mitotic stress in tumor cells

In normal cellular division, there is little mitotic stress. During tumorigenesis, alterations including aneuploidy, centrosome over-duplication, and altered microtubule dynamics increase mitotic stress. To adapt to this stress, tumor cells may exhibit altered expression of genes that support division under mitotic stress. Over-time, there is competitive selection for cancer cells that have acquired these alterations to normalize mitosis. These selected cells may exhibit conditional and emergent dependencies on the genes that are supporting division in the presence of mitotic stress.

tumor cells within the population that have acquired these alterations. These tumor cells have enhanced dependencies that allow for a normal mitosis but which also represent a potential therapeutic target. An important future direction will be to uncover the networks specifically supporting response to mitotic stress. Two recent studies have initiated this search by identifying genes required specifically in aneuploid cells (7) and cells with supernumerary centrosomes (41). These studies showed both CKAP5 and TACC3 are genes required for centrosomal clustering (41, 42). A unique requirement for centrosomal clustering could account for some of the tumor-specific phenotypes we observe following SYMPK or TACC3 depletion. The other genes uncovered in these studies may represent additional tumor-specific dependencies that could eventually be targeted for improved chemotherapy. Further efforts to uncover genes uniquely supporting mitotic stress phenotypes will likely reveal additional dependencies.

Another important future direction is gaining a better understanding of how mitotic stress develops in tumor cells. In Chapter 3, we used a tumor progression model to catalog the changes in mitotic progression and microtubule stability that accompany cellular transformation. Here, we showed loss of the tumor suppressor p53 increases the amount of time that cells spend in mitosis. The increased time spent in mitosis may be due to an increased level of genomic instability because p53 inhibits the proliferation of aneuploid cells (223). Interestingly, the mitotic timing defects we observe with p53 loss are rescued by overexpression of either a constitutively active KRas mutant or wild-type EGFR. This suggests that oncogenic signaling events can compensate for the errors in mitosis induced by tumor suppressor loss. It is not currently known how these oncogenic signaling events compensate for mitotic defects seen with tumor suppressor loss. Interestingly, we have observed changes in microtubule stability that accompanies loss of p53 and overexpression of mutant KRas or EGFR. These changes in microtubule stability may provide a mechanism by which oncogenic signaling alter mitotic progression. Additionally, altered microtubule stability could account for the enhanced dependency on TACC3 for normal mitotic progression in the transformed cell lines. An

important future direction is testing the effects of tumor suppressor loss or oncogene activation on mitotic progression and microtubule stability in an independent tumor progression model. This study will establish whether the effects we observe are cell-line specific and whether similar effects can be observed with loss of other tumor suppressors or activation of other oncogenes. Unraveling these connections will lead to a better understanding of the molecular drivers of mitotic stress in tumor cells.

A final important future direction is to understand the influence of mitotic stress phenotypes on tumorigenesis. As observed with many of the stress phenotypes of tumor cells, mitotic stress appears to have a dual role in both driving tumorigenesis and sensitizing tumor cells to mitotic stressors. In particular, aneuploidy can lead to an increase in tumor relapse in a mouse model of lung cancer (19). Additionally, the presence of supernumerary centrosomes in tumor cells correlates with tumor aggressiveness (26, 29, 224). Therefore, therapies designed to induce mitotic stress in tumor cells may have the unintended effect of driving tumorigenesis. A better understanding of the influence of mitotic stress on tumorigenesis will help predict the likelihood of such adverse outcomes. Importantly, microtubule-targeted agents such as paclitaxel or the Vinca alkaloids have not been associated with a worsening of tumorigenesis, even though altered microtubule stability can lead to aneuploidy (44-46). The clinical success of these agents suggests that targeting mitotic stress may be a plausible therapeutic strategy without increasing tumorigenesis. The success of drugs that induce mitotic stress may relate to their ability to cause catastrophic mitotic errors rather then sub-acute mitotic errors that allow for continued proliferation and potential worsening of tumorigenesis.

Emerging links between polyadenylation and mitosis- In addition to our own work, several new studies suggest ties between polyadenylation and the mitotic spindle. First, a recent study also observed a G2/M delay following shRNA-mediated SYMPK depletion in HeLa cells (109). Importantly, these authors were able to rescue the mitotic phenotypes seen with SYMPK depletion by overexpression of wild-type SYMPK. This data suggests SYMPK is required for

normal mitotic progression in cervical cancer in addition to lung cancer. Second, another study performed a genome-wide screen to identify genes regulated by cytoplasmic polyadenylation in mammalian cells (194). This study also observed mitotic defects following depletion of the cytoplasmic polyadenylation components CPEB1 and CPEB4. Together with our own work, these studies link the polyadenylation machinery to formation of the mitotic spindle.

A critical next step in our understanding of how polyadenylation interfaces with mitosis is identifying mitotic genes that are regulated by polyadenylation. During *Xenopus* meiosis, only a subset of genes that encode a cytoplasmic polyadenylation element is regulated by polyadenylation (119, 121, 122). For several reasons I suspect dysregulated expression of a similar subset of genes accounts for the mitotic errors we observe following depletion of polyadenylation components. First, we identified CKAP5 as a gene affected by SYMPK depletion by taking a candidate approach to examine expression of multiple genes important for spindle formation and microtubule dynamics. While CKAP5 depletion may account for many of the phenotypes we observe with SYMPK depletion, it is unlikely that we would have identified the only gene affected by polyadenylation by taking a candidate approach alone. Second, the impacts on mitosis and tumorigenesis we observe following loss of polyadenylation components seem more consistent with alterations in a subset of genes. This subset of genes must be limited in size because we see little effect on cell viability with siRNA targeting SYMPK alone. However, the strong effects of SYMPK depletion on tumorigenesis and mitosis suggest more then loss of CKAP5 expression may be involved. Finally, a recent paper that identified genes affected by depletion of the cytoplasmic polyadenylation component CPEB in mammalian cells identified a relatively small subset of genes showing altered polyadenylation following CPEB depletion (194). This suggests that, similarly to *Xenopus* meiosis, only some genes are regulated by cytoplasmic polyadenylation. An important future direction will be to perform a similar study in mammalian cells depleted of SYMPK to identify mitotic genes regulated by polyadenylation.

An additional future direction is determining whether components of the polyadenylation

machinery associate with the mitotic spindle. Recently, a study demonstrated localization of a subset of mRNAs to mitotic microtubules in *Xenopus* oocytes and in human cells (225). Association of mRNAs with the mitotic spindle could allow for localized and temporal control of mRNA expression, similar to that observed in *Xenopus* (120). Additionally, we have observed an interaction between SYMPK and TACC3 by endogenous co-immunoprecipitation in H1155 and H1299 NSCLC cells (Figure 4.2). These data suggest the intriguing hypothesis that the polyadenylation machinery may associate with the mitotic spindle. This association could be an additional mechanism by which mitotic cells detect spindle damage and control mitotic progression. Unraveling these physical connections between the polyadenylation machinery and the mitotic spindle will be an interesting direction for future study. In particular, immunoprecipitation-mass spectrometry studies of SYMPK binding partners should be performed in mitotic cells to identify other mitotic components that may interact with SYMPK. Additionally, it will be important to verify that TACC3 interacts with other components of the polyadenylation machinery, such as CSTF2, that are necessary for formation of the complete polyadenylation complex.

Parallels between gametogenesis and tumorigenesis- Commonalities between gametogenesis and tumorigenesis have been noted for decades. Most famously, John Beard proposed the trophoblastic theory of cancer over a century ago that suggested cancer arises from a "vagrant primary germ cell" that fails to migrate to the gametogenic tissue (226). Although subsequent findings do not support this theory for the origin of most cancers, there continue to exist important parallels between germ cell development and tumorigenesis. These similarities were recently reviewed by Lloyd Old and colleagues and include immortalization, invasive ability, asymmetric cell divisions, migratory ability, genomic hypomethylation, angiogenesis, immune evasion, and expression of the CT-antigens (93). These parallels between gametogenesis and cancer suggest the same processes that support gametogenesis may likewise support cancer growth. In addition to our own work, two recent studies support this hypothesis. First, a soma-

to-germline transition has been documented in *Caenorhabditis elegans* mutants that demonstrate increased longevity (207). The finding that germline genes increase longevity may indicate a role for these genes in maintaining proliferative capacity that could be relevant to cancer cell proliferation. Second, a recent report demonstrated overexpression of multiple germline genes in *Drosophila* embryos stimulates growth of brain tumors (206). This suggests germline genes can initiate cellular transformation to drive tumor growth. The contribution of most germline genes to mammalian tumorigenesis, however, remains unknown.

An important future direction will be identifying the functions of the CT-antigens in tumorigenesis. This dissertation has focused on a subset of CT-antigens that were identified in a synthetic lethal screen with an anti-mitotic drug, so naturally we have found many of these genes support mitosis. However, other groups have shown CT-antigens to be involved in diverse cellular processes (212, 227). Several methods would elucidate the functions of the CT-antigens in tumorigenesis. First, identification of CT-antigen interaction partners is an important future direction. We have previously demonstrated that the CT-antigen ACRBP interacts with the mitotic spindle protein NuMA to impact mitosis (155). The gametogenic gene TACC3 also interacts with the CT-antigen MAGEA4 (219). Determining whether other CT-antigens connect to the normal cellular machinery is a necessary future direction. Second, an increased understanding of the role of the CT-antigens in gametogenesis may inform our understanding of their roles in tumorigenesis. For example, the CT-antigens MAGEA5 and NXF2 are expressed in the spermatogonial stage of gametogenesis, the stage in which germ cells undergo mitotic division (93). Our finding that both these genes impact mammalian mitosis suggests they may function similarly in both gametes and tumors. Finally, functional studies of the impact of CT-antigen depletion or overexpression on tumor growth are needed. These studies have not been performed on a large-scale with the CT-antigens as a group, probably due to their sporadic expression across cell lines. However, systematic studies of cell lines that do express CT-antigens will establish which are required for tumorigenesis. Overall, a better understanding of

Figure 4.2

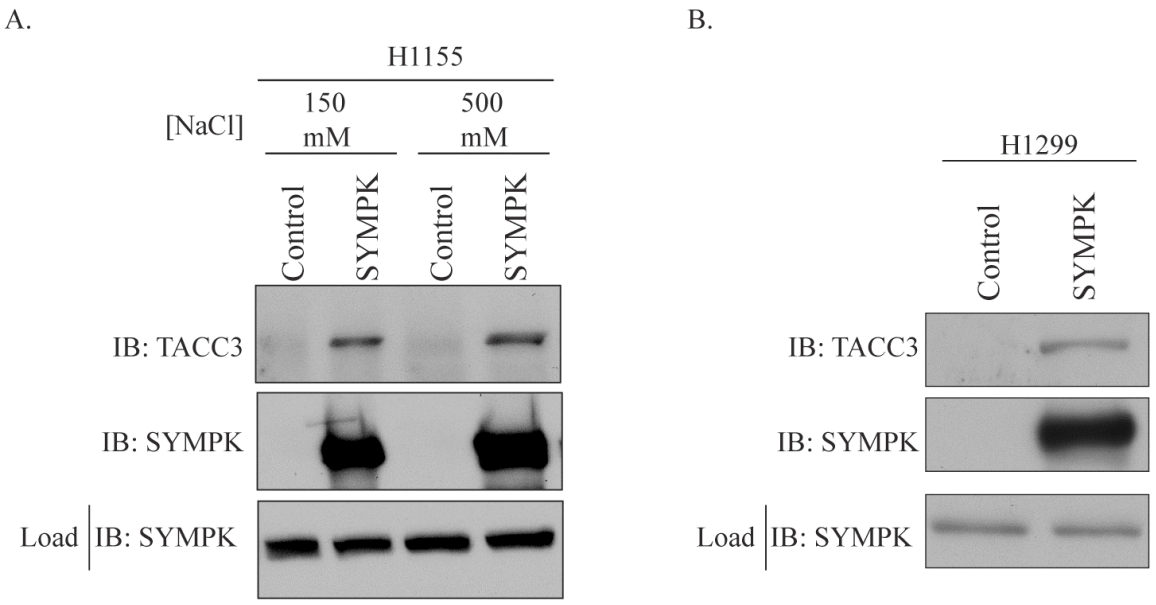


Figure 4.2 Interaction of SYMPK and TACC3

(A) Co-immunoprecipitation of SYMPK and TACC3 in the H1155 NSCLC line. SYMPK was immunoprecipitated using a monoclonal SYMPK antibody (Cell Signaling) from whole cell lysates (40 mM HEPES, 5% TritonX-100, 80 mM beta-glycerophosphate, 0.5% sodium deoxycholate, 1 mM EGTA, 150 mM or 500 mM NaCl, and protease inhibitor tablets). The immunoprecipitated fraction was blotted for TACC3 (Santa Cruz). (B) Co-immunoprecipitation of SYMPK and TACC3 in the H1299 NSCLC line. Conditions were as in (A) but using 150 mM NaCl in the lysis buffer.

the cellular functions of the CT-antigens may allow targeting of the gametogenic program in cancer cells.

The reasons for sporadic inter- and intra-tumoral expression of the CT-antigens also require further study. First, if the CT-antigens are indeed supporting tumorigenic phenotypes, it is unclear why some cancers require expression of many CT-antigens while other cancers require none at all. It is possible there is similar heterogeneity in requirements for CT-antigen expression as there is in the signaling pathways that are activated to drive tumor growth. Alternatively, only a subset of CT-antigens may actually increase tumor growth while the others are simply byproducts of aberrant gene expression. Second, the factors driving CT-antigen expression in most cancers are not clear. Genomic hypomethylation can drive expression of a subset of CT-antigens but expression of many CT-antigens is not induced even with high doses of the demethylating agent 5-aza-2'-deoxycytidine (146). This demonstrates that hypomethylation is not sufficient for expression of all CT-antigens and suggests additional unidentified factors may have a role in driving CT-antigen expression. Finally, further study is needed to uncover the reasons for CT-antigen expression in only a subpopulation of cells within the tumor. It is possible that CT-antigens confer stem-cell like properties to the tumor or support tumor growth by driving proliferation of other non-CT-antigen expressing cells within the tumor. For example, the CT-antigen FMR1NB encodes a signal peptide (216) and may be secreted into the extracellular space. This could allow FMR1NB to influence the properties of surrounding cells in a paracrine manner. Ultimately, an understanding of these factors controlling CT-antigen expression will require gene expression profiling coupled with functional studies of the CT-antigens at the single-cell level.

Clinical implications

As an MD/PhD student, the clinical implications of my dissertation research merit special emphasis. Here, I will discuss several potential impacts of this work on drug development

including the usage of polyadenylation inhibitors to treat cancer, the utility and limitations of the TACC3 inhibitor KHS101, and the potential for targeting other CT-antigens with small molecules.

Cordycepin to inhibit polyadenylation- Our results demonstrating synergy between depletion of polyadenylation components and treatment with paclitaxel suggest potential efficacy in combined treatment with anti-mitotic drugs and inhibitors of the polyadenylation machinery. Although no specific inhibitors of the polyadenylation machinery exist, several studies have been performed with an adenosine analog called cordycepin. Cordycepin treatment inhibits polyadenylation by chain termination of the poly(A) tail but also potently inhibits nucleic acid synthesis (228). In pre-clinical studies, cordycepin has shown efficacy in killing leukemia cells (137, 139-141) and is currently in clinical trials (136, 138). The impact of cordycepin on cell growth in these studies could derive from cordycepin's effects on polyadenylation or changes in nucleic acid synthesis. To test whether cordycepin treatment can synergize with paclitaxel, we have treated H1155 NSCLC cells with the combination of cordycepin and paclitaxel. In these studies, we observed no significant synergy with combination treatment (Figure 4.3). We attribute this lack of efficacy to a potent interphase block that we observe with cordycepin treatment, likely derived from the effect of cordycepin on nucleic acid synthesis. This interphase block likely inhibits the efficacy of paclitaxel because paclitaxel treatment is specific for mitotic cells. Importantly, we and others have not observed a similar interphase block following depletion of polyadenylation components (92, 109). This suggests that cordycepin does not mimic the cell biological effects of loss of the polyadenylation machinery. Therefore, polyadenylation inhibitors with increased specificity towards polyadenylation may demonstrate more significant synergy with paclitaxel.

Utility and limitations of the TACC3 inhibitor KHS101- The significant efficacy seen with combined paclitaxel/KHS101 treatment of NSCLC lines in this dissertation suggests utility of KHS101 in vitro. As a potential small molecule chemotherapeutic agent, three factors require

Figure 4.3

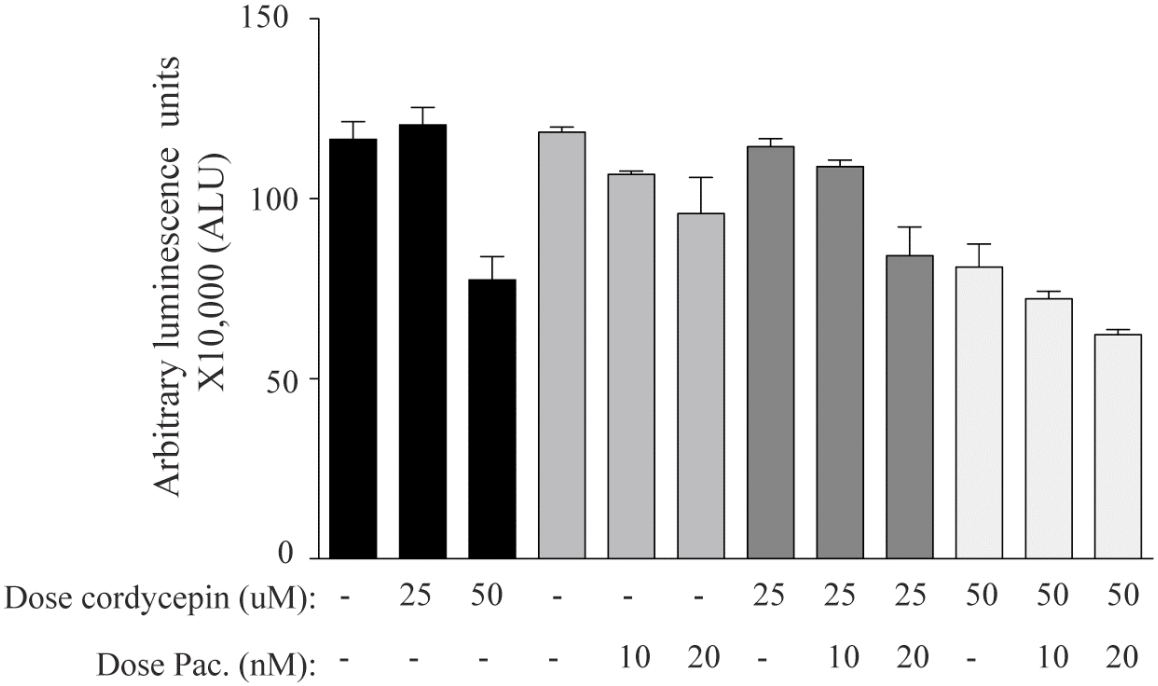


Figure 4.3 Co-treatment of H1155 cells with cordycepin and paclitaxel

CellTiter-Glo (CTG) viability assay in H1155 cells treated with cordycepin, paclitaxel, or combination treatment. H1155 cells were plated in a 96-well format, treated with paclitaxel for 24 hours, treated with cordycepin for an additional 48 hours, followed by addition of CTG reagent and luminescence readings. Increased arbitrary luminescence units (ALU) indicate the presence of more viable cells.

consideration including the ability of KHS101 to inhibit TACC3, the specificity of KHS101 for TACC3, and the potential utility of KHS101 treatment in vivo. First, it is important to consider whether KHS101 inhibits TACC3 function. The initial study that identified KHS101 demonstrated the ability of biotinylated KHS101 to bind TACC3 both in cellular lysates and using purified recombinant TACC3 protein (182). Additionally, these authors demonstrated that the KHS101-TACC3 interaction could be inhibited by addition of unlabelled KHS101 (182). Moreover, the cell biological phenotypes observed in this original study of KHS101 and in our own work with KHS101 are consistent with those expected following inhibition of TACC3. Together, these results suggest that KHS101 indeed targets TACC3. Second, it is important to consider the specificity of KHS101 for TACC3. Since KHS101 was only identified in 2010, there is as no extensive data addressing inhibitor specificity but the structure of the compound provides clues. KHS101 is a 4-aminothiazole compound and aminothiazoles can inhibit Aurora kinase activity (229). TACC3 is phosphorylated by AURKA (174) so it is possible that the effects we observe with KHS101 are due to AURKA inhibition rather then a specific effect on TACC3. However, the cell biological phenotypes we observe are inconsistent with a strong effect on AURKA activity. In particular, AURKA inhibitors cause massive mitotic defects without the addition of paclitaxel (230) whereas we do not see any mitotic defects with single agent KHS101 treatment even at high (10 uM) doses. This suggests KHS101 is not targeting AURKA. A more detailed analysis of the specificity of KHS101 is nevertheless needed. Finally, it is important to determine whether KHS101 has efficacy in vivo. A previous study achieved a plasma concentration of >1.5 uM following intravenous dosing of KHS101 at 6 mg/kg of body weight but observed poor oral bioavailability (182). The authors of this study also observed no toxicity with KHS101 treatment (182), a finding that is consistent with our own studies in mice (data not shown). An important future direction is testing the efficacy of KHS101 in treating a mouse model of NSCLC. Overall, forthcoming studies to examine the specificity and in vivo efficacy of KHS101 will elucidate the therapeutic utility of this compound.

Potential for targeting other CT antigens- In addition to the TACC3 inhibitor KHS101, it would be useful to identify drugs that may target other CT-antigens in a range of cancers. Identification of such drugs will hinge on a better understanding of the functional significance and expression pattern of all CT-antigens. Current knowledge points to two methods by which some CT-antigens may be targeted with small molecules including direct targeting of CT-antigens that have known "druggable" structures and indirect targeting of CT-antigens that are modified by processes which are druggable.

Directed targeting may be efficacious with CT-antigens that have known domains that can be targeted and is best illustrated with two CT-antigens: human monopolar spindle kinase 1 (MPS1) and bromodomain testis specific (BRDT). MPS1 (also known as TTK) is a dual-specificity kinase that can phosphorylate serine/threonine and tyrosine residues in target proteins and controls SAC activation (231, 232). MPS1 is a CT-antigen that is highly expressed in gametogenesis and also upregulated in gastric, lung, and bladder tumors (233, 234). As a kinase, MPS1 is highly targetable and a recent study identified an ATP-competitive inhibitor of MPS1 that reduces tumor growth in vitro and in vivo (235), suggesting potential efficacy in targeting this CT-antigen. In addition to MPS1, the CT-antigen BRDT may also represent a potential drug target. BRDT is expressed during gametogenesis (236), upregulated in tumors (237), and appears to function in the control of chromatin remodeling (238). BRDT mediates chromatin remodeling through two encoded bromodomains, a domain that the Bradner lab has recently been successful in selectively inhibiting with small molecules (239). Identification of additional targetable domains in CT-antigens will be an important future direction in the push for development of CT-antigen directed therapeutics.

In addition to targeting the CT-antigens directly, it may also be possible to indirectly target these proteins by targeting pathways that control CT-antigen function or expression. Indirect targeting may be advantageous because there are likely more compounds available that could modify CT-antigen function as compared to compounds that specifically target the

individual CT-antigen proteins. However, two disadvantages of indirect targeting are loss of specificity on tumor tissue and the requirement for significant knowledge about CT-antigen function to design an effective indirect targeting strategy. One method of indirect targeting would involve targeting proteins upstream of the CT-antigen that modify CT-antigen function. For example, the activity of members of the MAGE family of CT-antigens is regulated by phosphorylation (240), suggesting that control of these upstream phosphorylation events may be a pathway to modulate MAGE function. Another method of indirect targeting would be to modify pathways that control CT-antigen expression. Currently, it is known that expression of a subset of CT-antigens is driven by genomic hypomethylation but what drives expression of the remaining antigens is unclear (146). Future efforts to identify the pathways controlling CT-antigen function may therefore yield important insights into methods for indirect targeting. These studies should include analysis of the role of oncogenic signaling pathways and transcriptional networks in controlling CT-antigen expression.

Conclusions

In this work, we have revealed previously unrecognized connections between the polyadenylation machinery, gametogenesis, and formation of the mitotic spindle. Understanding the mechanisms in which polyadenylation components and gametogenic genes influence mitotic stress may have important implications for the design of next generation anti-mitotic chemotherapeutics. Additionally, this work has informed our understanding of the processes required for tumor cell response to mitotic stress. Perhaps more importantly, this research has opened up additional avenues for future study. In particular, characterizing the mitotic mRNAs affected by polyadenylation and probing the functional impact of all the CT-antigens on tumorigenesis are important directions for future work. Further study of these questions will doubtless have impact on our understanding of tumor cell biology and open up new in-roads for chemotherapy.

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