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Defining the roles of YAP/TAZ in controlling cell fate decisions following abnormal mitosis

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DEFINING THE ROLES OF YAP/TAZ IN CONTROLLING CELL FATE DECISIONS FOLLOWING ABNORMAL MITOSIS

by

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DEDICATION

I would like to dedicate this work to all of my past science teachers. Thank you for instilling your passion and love for science onto me. You all were truly the beacons of light in my journey through science.

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DEFINING THE ROLES OF YAP/TAZ IN CONTROLLING CELL FATE DECISIONS FOLLOWING ABNORMAL MITOSIS

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ABSTRACT

Mitosis is a critically important and time sensitive cellular process that proceeds rapidly, typically completing in 15-45 minutes. Mechanisms have evolved to measure the duration of mitosis, resulting in the identification of aberrant cells that spend too long in mitosis. If non-transformed cells undergo a mitosis that exceeds 90 minutes, then the resulting daughter cells activate a durable G_1 arrest and cease proliferating. The underlying mechanism acting to time the duration of mitosis is unknown. Here, we demonstrate that cells activate the Hippo pathway upon entry into mitosis, which initiates degradation of the pro-growth transcriptional co-activators YAP and TAZ. Consequently, prolonged mitosis leads to decreased YAP/TAZ levels in the following G_1, thus enforcing cell cycle arrest. We reveal that inactivation of the Hippo pathway, which is common in solid tumors, is sufficient to restore YAP/TAZ levels following a prolonged mitosis, and cells born from this prolonged mitosis can progress through the cell cycle.

We also demonstrate that Hippo pathway inactivation alters cell fate decisions in response to mitotic arrest. Antimitotics (e.g. Taxol) have long been used to permanently arrest cells in mitosis, which frequently results in mitotic cell death. It has long been

recognized that some cancer cells are resistant to antimitotics; this resistance can arise from cells escaping mitosis into the G_1 phase in a process termed mitotic slippage. The mechanisms underlying these cell fate decisions are poorly understood. Here, we demonstrate that inactivation of the Hippo pathway promotes mitotic slippage and overall survival in cells treated with antimitotics by increasing antiapoptotic protein expression. Our data suggest that inactivation of the Hippo pathway may promote resistance to antimitotic therapies by favoring the survival and proliferation of cells that have experienced a prolonged mitosis. Interestingly, we find that restoring Hippo signaling to cancer cells that are resistant to antimitotic therapies sensitizes them to antimitotics and promotes mitotic cell death. Overall, we illuminate a broad role for Hippo signaling in determining cell fate during mitosis and identify a novel mechanism by which resistance to antimitotic therapies can arise.

PREFACE

Sections of Chapter I in this dissertation are reproduced from a review article from which I am an author on and published in *Chromosome Research* (doi: 10.1007/s10577-015-9502-8), its reproduction here is done so with permission from the publisher. Chapter V is also reproduced from an article in which I was a first author on and is published in *Journal of Visualized Experiments* (doi: 10.3791/57383). Portions of chapter I-IV are derived from a manuscript in which I am the first author on and is in preparation for submission to a peer-reviewed journal.

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LIST OF ABBREVIATIONS

2N …… Diploid Somatic Number

4N …… Tetraploid

53BP1 ……Tumor Suppressor p53-Binding Protein 1

A ……. Adenine

ABC ……..…. ATP-binding Cassette Transporter

Abl ……... Abelson Murine Leukemia Viral Oncogene Homolog 1

AMOTL1/2 ……..... Angiomotin Like 1/2

ANOVA ……..... Analysis of Variance

APC/C ……..... Anaphase-Promoting Complex Activator Protein CDH1

APC^{Cdh} ……...…. Anaphase-Promoting Complex Activator Protein CDH1

ARF …….. Alternative Reading Frame

ATP ……...… Adenosine Triphosphate

BAX ……..……..….. Bcl-2-Associated X

Bcl-2 …….. B-cell lymphoma 2

Bcl-w ……...… Bcl-2-Like Protein 2

Bcl-xL ……..… B-cell Lymphoma-Extra Large

BH3 ……..... Bcl-2 Homology 3

BJ..... Human Foreskin

BUB …….. Budding Uninhibited by Benzimidazoles Proteins

BubR1 ……... Bub1-Related Protein Kinase

C ……...…….. Cysteine

cAMP ……...…….. cyclic Adenosine Monophosphate

CAS9 ……..…… CRISPR Associate Protein 9

Cdc20 ……..…. Cell Division Cycle Protein 20

CDK1 ……... Cyclin-Dependent Kinase 1

CDKN1A ……..……. Cyclin-Dependent Kinase Inhibitor 1A

CIN …… Chromosome Instability

C-Nap1 ……. Centrosomal Protein 250

CRISPR …… Clustered Regularly Interspaced Short Palindromic Repeats

CYP3A4 …….. Cytochrome P450 3A4

diap1 …… Death-Associated Inhibitor of Apoptosis

DKO ……..……..Double Knock Out

DMEM ……..……... Dulbecco's Modified Eagle's Medium

DMSO …….. Dimethyl Sulfoxide

DNA ……... Deoxyribonucleic Acid

ECM …….……... Extracellular Matrix

Eg5 …… Kinesin 5

EMT …… Epithelial-to-Mesenchymal Transition

F:12 …….. Ham’s F-12 Nutrient Mixture

FBS …….……. Fetal Bovine Serum

FDA ……..... Food and Drug Administration

FT293 ……. High Transfection Efficiency SV40 Large T Antigen 293 Clone

FUCCI …….. Fluorescent Ubiquitination-based Cell Cycle

g ……... Gravity

G.....Glycine

G0 ……. Gap 0 Phase

G1….....Gap 1 Phase

G2…….....Gap 2 Phase

G_{α} ……. Guanine Nucleotide Binding Proteins

Gadd45 …….. Growth Arrest and DNA Damage-inducible 45 (GADD45)

GAPDH …… Glyceraldehyde 3-phosphate Dehydrogenase

GFP ……. Green Fluorescent Protein

GPCR ……. G-Protein Coupled Receptor

GTPase ……. GTPase-Activating Protein

hCdt1 ……... Human Chromatin Licensing and DNA Replication Factor 1 Homologue

hrs ……... Hours

HSET ……..… Human Kinesin-14 Motor KifC1

hTERT ……..…… Human Telomerase Reverse Transcriptase

INK4A …….. Inhibitors of CDK4

K5I ……. Kinesin-5 Inhibitor

K-fibers ……..… Kinetochore Fibers

Kif11 …… Kinesin Family Member 11

Kip2 ……. Kinase Inhibitory Protein

KO ……... Knock-out

KSP …… Kinesin Spindle Protein

LATS …….. Large Tumor-Suppressor Kinases

LPA ……..… Lysophosphatidic Acid

MAD ……... Mitotic Arrest Deficient Proteins

MCF10A ……. Michigan Standard Foundation 10A

Mcl-1 ……..... Myeloid Cell Leukemia-1

MDA-MB-231 …… M.D. Anderson Metastatic Breast Cancer 231

MDM2 …….. Mouse Double Minute 2 Homolog

MDR1 …… Multi-Drug Resistance Gene

Mer …… Merlin

min ……..…. Minutes

miR ……. microRNA

miRNA ……... microRNA

MOB1A/B …… Human Mps One Binder Kinase Activators 1A and 1B

MPF ……...…… Maturation Promoting Factor

Mps1 ……. Monopolar Spindle 1

MRP ……. Multidrug-Associated Protein

MST1/2 ……...……...…… Macrophage Stimulating Kinases 1 and 2

NaCl …….. Sodium Chloride

NDR …….. Nuclear Dbf2-Related

Nek2A ……..... NIMA-Related Kinase 2A

NF2 ……...……... Neurofibromatosis 2

NIMA ……... Never in Mitosis Gene A

P ……... Protein

PBABE ……. Plasmid Harold Babe Morgenstern

PBS ……..……. Phosphate-Buffered Saline

PCM ……. Pericentriolar Material

PCP ……... Planar Cell Polarity

PEI …….. Polyethyleneimine

P-gp ……... Permeability Glycoprotein

PKA …….. Protein Kinase A

PLK4 …… Polo-like kinase 4

PLVX …….. Plasmid Lentiviral Vector

pRb ……. Retinoblastoma Protein

Puro …… Puromycin

PVDF ……..……..... Polyvinylidene Difluoride

PY …….. Proline Tyrosine

Rac1 …….. Ras-related C3 Botulinum Toxin Substrate 1

Rb …… Retinoblastoma

RhoA …….. Ras Homolog Gene Family Member A

RNA …… Ribonucleic Acid

RNAi ……..……...… RNA Interference

ROCK …… Rho Kinase

RPE …...……... Retinal Pigment Epithelium-1

RPM ……...…… Rotations per Minute

RPMI …….……. Roswell Park Memorial Institute

S Phase …….…….. Synthesis Phase

S1P …… Sphingosine 1-Phosphophate

S ……. Serine

SA ……..… Serine Alanine

SAC ……. Spindle Assembly Checkpoint

SAS6 …….. Spindle Assembly Abnormal Protein 6 Homolog

SAV1 …….…….. Salvador1

SCF …….. Skp, Cullin, F-box Containing Complex

SDS ……..…….. Sodium Dodecyl Sulfate

siNC …….. siRNA Non-Targeted Control

siRNA …….……. Small Interfering RNA

siYAP ……... siRNA YAP

T ……...… Threonine

TAZ ……..……..... Transcriptional Co-Activator with PDZ-Binding Motif

TBS …….…… Tris-Buffered Saline

TEAD …… Transcriptional Enhancer Activator Domain

Tris-HCl ……. Trisaminomethane Hydrochloride

USP28 ……... Ubiquitin Specific Peptidase 28

v2 ……...…… Vector 2

W/V...…….. Weight/Volume

Waf1 ……...…… Wild-type Activating Fragment-1

Wnt ……..……... Wingless

WT ……..……. Wild Type

WW ……..... Tryptophan Tryptophan

YAP ……..…….. Yes-Associated Protein

Yki …… Yorkie

CHAPTER ONE: INTRODUCTION

Cell Cycle

Overview

In order for a cell to divide it must properly replicate and segregate its DNA through the different stages of the cell cycle. The cell cycle is a highly regulated complex process. It consists of five different stages where the cell will grow, replicate its DNA and divide into two daughter cells. These stages include gap 0 (G_0), gap 1 (G_1), synthesis (S), gap 2 (G_2), and mitosis (M). Cyclins interact with cyclin dependent kinases to drive the progression of cells from one stage of the cell cycle to the next, cyclins will be further described later in this chapter (Fisher and Nurse, 1996, Nurse and Thuriaux, 1980, Dutcher and Hartwell, 1982). While driving the progression of the cell through this cycle is important, halting the proliferation of abnormal cells is equally vital. The cell cycle has distinct checkpoints to ensure the proliferation of cells free of DNA damage that also have readily available nutrients.

Phases of the Cell Cycle

Cells in gap 0 (G_0) phase are categorized as either quiescent, senescent or differentiated, the former category is reversible while the latter two are typically irreversible. Quiescent cells have low cell turnover and have decreased proliferation markers (Huttmann et al., 2001, Fukada et al., 2007). In contrast to quiescent cells, senescent cells do not enter the cell cycle again. Cells can become senescent if they experience DNA damage that would make the daughter cells nonviable. Though the cell is

no longer able to proliferate in senescence, it is still able to perform regular cellular functions (Hayflick and Moorhead, 1961, Campisi, 2013, Rodier and Campisi, 2011, Burton and Krizhanovsky, 2014, Bodnar et al., 1998). Cells that are differentiated and are not actively dividing can remain in G_0 for the entirety of their existence.

G_1 is the phase directly following mitosis, during this time is when the cell assesses whether to divide (Figure 1.1). If the cell has enough nutrients, enough space to grow and is free of DNA damage the cell will continue on to S phase from G_1. The G_1/S transition is highly regulated and will be mentioned in its own section later in this chapter. Briefly, the S-phase requires the transcription of numerous genes necessary for DNA replication, when those genes are transcribed and proteins are synthesized in G_1 then the cell will enter S phase.

Once the cell enters S phase, the DNA is faithfully replicated. During replication, one original DNA molecule will yield two complementary strands of DNA. DNA is replicated in a semiconservative manner, meaning that each strand of the original DNA strand will be used for the template of one replica. DNA replication has three stages: initiation, elongation, and termination. During initiation, the DNA replication begins at specific loci along the DNA known as the origins of replication. The proteins that assemble the pre-replication complex are recruited once the origin of replication is found. Then DNA replication will enter the elongation phase. DNA is highly condensed and spooled around histone octamers creating a chromosome. During elongation, topoisomerase unwinds the DNA creating a replication fork. DNA helicases then create breaks in the DNA to relieve the tension caused by its unwinding. Different proteins are critical in the creation and

continuation of the replication fork in order to continue DNA synthesis, including single-stranded binding proteins that bind to DNA to prevent premature re-annealing. A primase, which is either a DNA or RNA primer is required to initiate DNA synthesis. DNA polymerases binds freely floating nucleotides (adenine, thymine, guanine and cytosine) that are complementary bases to the mother strand to the RNA primer (Abdel-Monem et al., 1976, Abdel-Monem and Hoffmann-Berling, 1976, Bessman et al., 1958). Since the original DNA strand is used as a template and DNA is replicated in a 5' to 3' fashion there will be one leading and one lagging strand. The leading strand is the strand of DNA where the template strand is in the 5' to 3' direction. Replication of this strand will be continuous following the replication fork. While the lagging strand's template is in the 3' to 5' direction, which is opposite of the replication fork. Because of this 3' to 5' directionality replication is much more difficult and DNA is synthesized in short segments known as Okazaki fragments. These Okazaki fragments are then rejoined into one continuous strand by DNA ligase. Once replication is complete the cell will now have twice as much DNA. There are many error correction mechanisms and proofreading mechanisms to ensure faithful replication during this phase of the cell cycle. Once the elongation phase has completed, DNA replication will begin the termination phase. Since human DNA is linear, the ends of the DNA are unable to be replicated as the replication fork will stop before reaching the end. So the DNA becomes processively shorter following each replication. Telomeres are repetitive DNA localized to the ends of chromosomes to prevent the cell from losing genes.

During S phase not only DNA is replicated, but key mitotic elements known as centrioles are also duplicated. Centrioles along with the pericentriolar material (PCM) form the poles that segregate chromosomes known as centrosomes (reviewed extensively in (Doxsey et al., 2005, Godinho and Pellman, 2014)). The centrosome is the microtubule organizing center, the microtubules formed at the centrosome help segregate chromosomes during mitosis. Microtubules consist of alpha-beta tubulin dimers and are important not only in the search and capture of chromosomes but also in the cytoskeleton of a cell. It is important that there are only two centrosomes because more than two would result in DNA segregation errors. The replication is highly regulated so that both DNA and centrioles are replicated once and only once (Firat-Karalar and Stearns, 2014, Nigg and Stearns, 2011, Sluder, 2014, Tsou and Stearns, 2006). At the end of S phase the cell will have duplicated its centrioles.

Gap 2 (G_2) is the final growth step before mitosis (Figure 1.1). During this phase there is rapid protein synthesis and cell growth. Cyclin B1 levels and CDK1 activity will begin to rise until their threshold is reached at the end of G_2, this accumulation of Cyclin B1/CDK1 activity is regulated to ensure the proper entry of the cell into mitosis (Porter and Donoghue, 2003, Sha et al., 2003). CDK1 complexed with Cyclin B drives the cell into mitosis.

Mitosis

Mitosis is the stage where the mother cell partitions its replicated DNA into two daughter cells. Mitosis consists of five phases: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1.2). During prophase the cell rearranges its cytoskeleton

and rounds up. Additionally, the two centrosomes separate and begin to migrate to opposing poles to form the bipolar mitotic spindle and DNA condenses (Hinchcliffe, 2014).

In prometaphase, the nuclear envelope breaks down and microtubules emanating from the centrosomes search for and attach to a proteinaceous structure located on the centromere of the chromosome known as the kinetochores (Figure 1.3)(Ottaviano and Gerace, 1985). Mitotic microtubules are highly dynamic, they grow from their plus end and depolymerize from their minus end, this dynamicity is critical for their function of binding to and moving chromosomes (Maiato et al., 2004, Allen and Borisy, 1974). Once all of the chromosomes have properly attached to the kinetochore, they align at the equatorial plane of the cell in metaphase. The proper attachment is termed amphitelic attachment. Amphitelic attachment occurs when the microtubules from opposing centrosomes are attached at the proper kinetochore (Gregan et al., 2011a). This attachment allows for the chromosome to have pulling forces from both microtubules resulting in the alignment of the chromosome at the equatorial plate, known as bi-orientation (Tanaka, 2002). Once all of the chromosomes are properly attached and aligned at the equatorial plate then the cells can enter anaphase.

During anaphase the two sister chromatids begin to separate by the shortening of the kinetochore microtubules. The sister chromatids migrate to opposing poles of the cell. In the last phase of mitosis, telophase, the nuclear envelope reforms around each individual set of chromosomes and chromosomes decondense.

Cytokinesis spans multiple stages of cell division, during cytokinesis the cytoplasm divides into two daughter cells. Cytokinesis is not a phase of mitosis though it happens

simultaneously. The cytokinetic process begins during anaphase and completes after the cell undergoes telophase. During cytokinesis, an indentation of the cells surface, known as the cleavage furrow, forms with an actin-myosin contractile ring at the equatorial plane of the cell; as the ring contracts it separates the two nuclei into two different cells. If cytokinesis is not successful this means the parent cell fails to divide into two separate daughter cells. A consequence of this is that the parent cell now has twice as much DNA as a normal cell. This condition is called tetraploidy. Tetraploid cells emerging from failed cytokinesis contain two distinct nuclei with a full complement of DNA in each of them (Figure 1.7).

Cyclins & Cyclin Dependent Kinases

Cyclins are named aptly, as their levels fluctuate rapidly through the cell cycle (Figure 1.1). The oscillation of cyclins drives the cell through the cell cycle. This is achieved by highly regulated fluctuations in cyclin synthesis and degradation (Darzynkiewicz et al., 1996, Luca et al., 1991, Markiewicz et al., 1994, Rechsteiner and Rogers, 1996). Cyclins are tagged for degradation via an ubiquitin-mediated proteolysis (Peters, 2006). The degradation of cyclins is critical for preventing the cell from falling back to its previous phase, thus the cell cycle can only progress forward and not backwards. Since cyclins are only present during certain times in the cell cycle they add another layer of regulation and control to the progression of the cell through the cell cycle.

Cyclins operate by effecting the activation of cyclin dependent kinases (CDKs). Cyclins bind to and activate specific CDKs. CDK activity is highly regulated as CDKs must bind to the proper cyclin and have phosphorylation at their activation loop in order to

be activated. There are four different CDKs: CDK1, CDK2, CDK4 and CDK6. In early G_1, Cyclin D interacts with CDK4 and CDK6. In late G_1, Cyclin E-CDK2 activity is critical for the G_1/S transition. Once in S phase Cyclin A binds with CDK2 and then towards the end of S phase, Cyclin A begins to interact with CDK1 until it peaks at the end of G_2. In late G_2, Cyclin B begins to replace Cyclin A's interaction with CDK1, so Cyclin B-CDK1 activity begins to rise, where it peaks before the onset of anaphase and then Cyclin B is degraded and the holoenzyme levels decrease as the cell enters G_1 again where the cycle is repeated. CDKs can only become active during stages of the cell cycle when the cyclin is present (Darzynkiewicz et al., 1996, Pines, 1991).

Active CDKs play important roles for each stage of the cell cycle. During G_1, mitogenic signaling increases the transcription of Cyclin D. Cyclin D interacts with CDK4/6 resulting in the phosphorylation of retinoblastoma (Rb), this phosphorylation results in the progression of the cell to S phase (see below for more information). Cyclin E-CDK2 complex is also critical for the G_1/S transition as the complex also phosphorylates Rb.

The increase in Cyclin A occurs in S phase, resulting in the progression of the cell into S phase. Cyclin A competes with Cyclin E for CDK2. As Cyclin A increases in S phase more of Cyclin A binds to CDK2. Cyclin A-CDK2 complexes phosphorylate substrates critical for the progression through S phase. Cyclin A-CDK2 function to turn off the transcription factors present at the G_1/S checkpoint that induced Cyclin E expression. Later in S phase and early G_2 Cyclin A begins to interact with CDK1.

Following early G_2, Cyclin B will increase to drive the cell into M thus controlling the direction of the cell cycle. The Cyclin A-CDK1 complex prepares for the activation of Cyclin B-CDK1, in doing so it causes the shutdown of Cyclin A. When CDK1 is bound to Cyclin B it phosphorylates targets resulting in the condensation of chromosomes and breakdown the nuclear lamina, the degradation of Cyclin B and thus the inactivation of the Cyclin B-Cdk1 complex is required for mitotic exit.

CDKs can also be negatively regulated. CDKs can be inhibited or stimulated by phosphorylation on their threonine and tyrosine residues(Schafer, 1998). Additionally, CDK/cyclin complexes can be inhibited by binding to an inhibitor enzyme, examples of these CDK inhibitors include $p16^{INK4A}$, $p57^{Kip2}$ and p21. These CDK inhibitors have the ability to stop the progression of the cell through the cell cycle when activated. For example p16 is activated by DNA damage, in the presence of DNA damage p16 will bind to and inhibit CDK4/6 from binding to Cyclin D, this will halt the cell at G_1/S. The cell now has time to correct DNA damage. Once the damage is fixed, p16 will no longer be transcribed and CDK4/6 will be available to bind to Cyclin D to progress the cell through the cell cycle. The inhibitory regulation of CDKs allows for a fine-tuned control of the cell cycle.

The G1/S Checkpoint

Checkpoints can result in cell-cycle arrest which allow for the cell to correct any errors it may have accumulated in the process; if the errors are too catastrophic these cell cycle checkpoints can trigger cell death to prevent the propagation of errors (Malumbres and Barbacid, 2009).

The G_1/S checkpoint ensures that all the DNA in G_1 is error free before entering S phase where the DNA is replicated. Cyclin D and Cyclin E are critical for driving the cell from G_1 to S phase (Figure 1.4) (Ohtsubo and Roberts, 1993, Quelle et al., 1993, Resnitzky et al., 1994). The retinoblastoma (Rb) protein normally restrains passage through the G1/S transition by sequestering a transcription factor, E2F. When E2F is active it transcribes genes required for S phase that are involved in DNA replication and synthesis. Rb is a substrate of Cyclin D-CDK4/6 kinase activity as well as Cyclin E-CDK2 kinase activity. When Rb is phosphorylated it releases the transcription factor E2F. E2F is then able to transcribe genes necessary for entering S phase (Connell-Crowley et al., 1997, Mudrak et al., 1994, Arroyo and Raychaudhuri, 1992, Harbour et al., 1999).

(G_2/M Checkpoint

The G_2/M checkpoint is the second road block in the cell cycle that has the ability to prevent cells from continuing through. The G_2/M checkpoint prevents cells with activated p53 from entering mitosis. CDK1 is inhibited by p53's transcriptional targets p21, Gadd45 and 14-3-3σ when the cell has DNA damage and p53 is activated. These transcriptional targets of p53 have distinct mechanisms when inhibiting CDK1. p21 inhibits CDK1 directly, Gadd45 dissociates CDK1 from Cyclin B1, and 14-3-3σ sequesters CDK1 in the cytoplasm where it cannot function to prompt mitosis (Taylor and Stark, 2001). The inhibition of CDK1 by p53's transcriptional targets halts the cells in G_2 (Charrier-Savournin et al., 2004). The G_2/M checkpoint is critical in preventing chromosomes from missegregating and is also vital for preventing chromosomes with DNA damage from entering mitosis (Charrier-Savournin et al., 2004).

Spindle Assembly Checkpoint

The spindle assembly checkpoint (SAC) exists to ensure the proper segregation of sister chromatids to two daughter cells in order to maintain the genomic integrity of cells during proliferation. The SAC is activated in prometaphase and causes the cell to halt until all of the sister chromatids have proper attachment to microtubules on their kinetochore. As mentioned earlier, the kinetochore is a proteinaceous structure located at the centromere. The SAC proteins localize transiently to the kinetochore during mitosis. To ensure genomic integrity key proteins are actively localized to the kinetochore until the SAC is satisfied.

While the SAC is activated, mitotic arrest deficient proteins (Mad2), Bub1-related protein kinase 1 (BubR1), budding uninhibited by benzimidazoles proteins 3 (Bub3) and cell division cycle protein 20 (Cdc20) are continuously being recruited and released from the kinetochore (Figure 1.5a). The monopolar spindle 1 (Mps1) is a kinase essential for the recruitment of SAC proteins to kinetochores that are not attached to microtubules (Lauze et al., 1995). Mps1 is also crucial for error correction in the cell; when Mps1 is inhibited it results in aneuploid cells(Colombo et al., 2010, Hewitt et al., 2010, Jemaa et al., 2013, Kwiatkowski et al., 2010). Mad1 is recruited to the kinetochore and then signals for the recruitment of Mad2, though sometimes these two proteins are recruited together. Mad2 is recruited in its closed form and forms a complex with BubR1, Mad3 and Bub3 (Sharp-Baker and Chen, 2001, Chen et al., 1998). This complex of Mad1, Mad2, BubR1, Mad3 and Bub3 results in the inhibition of Cdc20 (Sharp-Baker and Chen, 2001). The SAC

inhibits the anaphase promoting complex/cyclostome (APC/C). The active SAC is able to inhibit the APC/C as Cdc20, the protein that activates APC/C, is sequestered by Mad2 at the kinetochore.

In order for the SAC to become satisfied, chromosomes need to have proper attachments which is facilitated by the proper alignment of chromosomes. The chromosomes need to bi-orient properly when they are aligned at the equatorial plate during metaphase (Musacchio and Salmon, 2007). For a chromosome to bi-orient kinetochore fibers (k-fibers) (consisting of about 20 microtubules) emanating from the centrosome will attach to the kinetochore of one sister chromatid. K-fibers emanating from the opposite centrosome will then attach to the opposite kinetochore of the sister chromatid. This attachment is known as amphitelic attachment and will allow for the chromosome to align properly at the equatorial plate. When cells are treated with antimitotics such as paclitaxel proper bi-orientation of the chromosome does not occur as tension is imperfect across sister chromatids (Pinsky and Biggins, 2005).

Once all of the sister chromatids are properly attached to microtubules the SAC is satisfied. When the SAC is satisfied Mad1, Mad2, BubR1, Bub3 and Cdc20 are displaced from the kinetochore (Figure 1.5b). Cdc20 is now able to activate APC/C. APC/C is an E3 ubiquitin ligase. When active, APC/C will degrade the maturation promoting factor (MPF) Cyclin B1/$p34^{cdc2}$. The degradation of the MPF allows for the degradation of Cyclin B1 and securin(Fang et al., 1998, Hwang et al., 1998, DeAntoni et al., 2005, McGrogan et al., 2008, Musacchio and Salmon, 2007, Nasmyth, 2005). The degradation of Cyclin B1 results in the progression of the cell cycle to G_1. The degradation of securin results in the

activation of separase (Kops et al., 2005, Musacchio and Salmon, 2007, Nasmyth, 2005). Separase is an enzyme that as the name suggest separates the sister chromatid. Separase functions by cleaving the cohesion link so the sister chromatids are able to segregate into two daughter cells(Sun et al., 2009). The cell is able to proceed to anaphase once the SAC is satisfied.

The SAC is an important checkpoint in maintaining chromosomal stability and preventing cancer. If any of the key components of the SAC are deleted (such as Mad2, BubR1, and Bub3) this results in increased chromosomal instability (CIN) and increased tumor development(Dobles et al., 2000, Baker et al., 2004, Kalitsis et al., 2000, Dai et al., 2004, Michel et al., 2001). When the SAC is not functioning correctly it results in the segregation of chromosomes that are not properly attached. This results in an increase of chromosomes missegregation leading to CIN. Many cancer cells have a weakened SAC (Kops et al., 2005, Weaver and Cleveland, 2006, Lapenna and Giordano, 2009, Perez de Castro et al., 2007). Breast, lung, colon and nasopharyngeal carcinoma are associated with mutations in the SAC leading to aneuploidy and CIN.

Chromosomal Attachment

Chromosomes can attach to microtubules in many different ways, there can be an amphitelic, monotelic, syntelic or merotelic attachments(Gregan et al., 2011b). Each sister chromatid has its own kinetochore. The microtubule captures the chromatid by attaching to the kinetochore. Amphitelic attachment occurs when both sister chromatids are attached to microtubules emanating from opposing poles of the cell via the kinetochore and the SAC is satisfied then the sister chromatids are pulled in opposite directions toward the poles.

Monotelic attachment occurs when only one sister chromatid is attached at the kinetochore to a microtubule. If both sister chromatids are attached to microtubules emanating from the same pole, then this is known as syntelic attachment. Merotelic attachement occurs when one sister chromatid is attached to microtubules emanating from opposing poles.

Merotelically attached chromosomes do not activate the SAC and can result in DNA damage. A sister chromatid that is merotelically attached will undergo a tug of war between the two opposing centrosomes. If merotelic chromosomes persist through anaphase they will lag behind the other chromosomes. This lag occurs because the other chromosomes are attached to shortening microtubules bringing the chromosome to the poles while the merotelic chromosome is still in the middle of the cell being pulled by two opposing forces. The nuclear envelope will begin to form around the bulk of the chromosomes when the lagging chromosome is too far away. A nuclear envelope will form around the lagging chromosome resulting in a micronuclei. Micronuclei are prone to rupture. When the micronuclei ruptures the chromosome undergoes chromothripsis and thus breaks and rearrangements can occur resulting in DNA damage(Stevens et al., 2007, Zhang et al., 2015a, Forment et al., 2012, Stephens et al., 2011, Storchova and Kloosterman, 2016).

Molecular Mechanisms Timing Mitosis

Mammalian somatic cells require 12 to 30 hours to prepare to divide. Mitosis is a very important stage in the faithful separation of genomic information. Surprisingly, during mitosis DNA can be quickly separated to daughter cells, only taking between 15-45

minutes (Yang et al., 2008, Thompson and Compton, 2008, Thompson and Compton, 2011) .

The process of mitosis is very stressful to cells (Ganem and Pellman, 2012). During this stage of the cell cycle the chromosomes are condensed, the nuclear envelope is ripped apart, and the cell undergoes a drastic restructuring (Ganem and Pellman, 2012) Since mitosis is such a destructive process, it is important for the cell to rapidly finish mitosis.

A longer than normal mitosis may mean an error occurred in this process. Preventing the propagation of this error can be beneficial to the cell's DNA integrity. It is not surprising that the cell has evolved mechanisms to limit the proliferation and survival of cells born from an abnormal mitosis.

One of the mechanisms that has evolved to limit proliferation involves timing mitosis. There is a timing mechanism that senses when a cell has been in mitosis for more than 90 minutes. Daughter cells born from a mother cell that has been kept in mitosis for more than 90 minutes will arrest in the G_1 phase, even upon normal completion of mitosis (Uetake and Sluder, 2010). The daughter cells arrest because they have an increased amount of p53 in the G_1 following the prolonged mitosis. The cell has a molecular mechanism in place to prevent the proliferation of cells that have been in mitosis for too long by preventing the daughter cells born from this mitosis from proliferating.

The mechanism of this mitotic timer is starting to become unraveled. Recently, three groups have found USP28-53BP1-p53-p21 signaling axis is responsible for arresting cells after a prolonged prometaphase (Lambrus et al., 2016, Meitinger and Anzola, 2016, Fong et al., 2016). The USP28-53BP1-p53-p21 signaling axis also results in p53

accumulation due to centrosome loss or centrosome amplification (Lambrus_Holland 2016). We have shown that the Hippo pathway also regulates this mitotic clock and this will be discussed more in subsequent chapters.

Antimitotics

Targeting mitosis is a good therapeutic to pursue for dividing cells. Antimitotic drugs are effective chemotherapeutics as they result in the death of proliferating cells. Antimitotic chemotherapeutics, such as Taxol, have been used for the treatment of many different cancers including breast cancers, ovarian cancers, non-small cell lung cancer, as well as head and neck cancers[(Weaver, 2014)]. Antimitotics are effective therapies because cells are very vulnerable when in mitosis as transcription (Gottesfeld and Forbes, 1997, Blagosklonny, 2006) and translation are slowed down. Prolonging cells in this vulnerable state will make the cell more sensitive to different insults resulting in cell death(Rieder and Maiato, 2004).

One way in which antimitotics can operate is through targeting microtubules, which are essential for carrying out mitosis. Antimitotic microtubule targeting drugs are classified into two major groups, microtubule stabilizing and microtubule-destabilizing agents. Microtubule stabilizing agents stop the generation of tension across the kinetochores. Different type of taxanes including taxalere (breast) and braxane (ovarian nano-particle) are microtubule stabilizing agents. Paclitaxel and docetaxel, both FDA approved chemotherapeutics, stabilize microtubules (Figure 1.6) (Noguchi, 2006). Microtubule-destabilizing agents depolymerize microtubules and prevent microtubules from attaching

to the kinetochore. Microtubule destabilizing agents include the vinca alkaloids such as vincristine, vinblastine, vinorelbine and estramustine (Dominguez-Brauer et al., 2015). Nocodazole, a common laboratory tool, destabilizes microtubules. Targeting microtubules by either stabilizing or destabilizing them is an effective chemotherapeutic treatment as it prevents microtubules from making the proper attachment to chromatids via the kinetochore causing the cell to activate the SAC.

There are also antimitotic drugs that target non-microtubule proteins in order to activate the spindle assembly checkpoint. A group of these antimitotics work by targeting Eg5 (also known as K5I, KSP or Kif11). Eg5 is a motor kinesin protein that acts by pushing the centrosomes in opposing directions. When Eg5 is inhibited by compounds such as Monastrol, it creates a monaster, or a star like object when observing the cell under the microscope (Mayer et al., 1999, Kapoor et al., 2000, Dominguez-Brauer et al., 2015, Cochran et al., 2005, Jiang et al., 2006).

Microtubule stabilizing, microtubule destabilizing, and non-microtubule targeting antimitotics all have differing mechanism for halting the cell in mitosis but all result in mitotic arrest. In each case the kinetochores lack the proper tension and chromosomes are not aligned at the equatorial plane. Since there is a lack of tension on the kinetochore and the chromosomes are not properly aligned, the SAC is not satisfied and cells are halted in mitosis.

Variable Response to Antimitotic Drugs

Cells treated with an antimitotic can undergo a variety of cell fates following the treatment. Cells can’t arrest in mitosis forever. They show variability in arrest times. Cells

can undergo mitotic arrest until the antimitotic is cleared, or the cell can pump out the antimitotic or not have a functioning SAC; in these cases the cell can continue to divide. In the presence of the antimitotic the cell can also die in mitosis or it can exit mitosis without undergoing anaphase thus producing a tetraploid cell (Shi et al., 2008, Rieder and Maiato, 2004, Blagosklonny, 2007, Weaver and Cleveland, 2005). Cells that exit mitosis without undergoing anaphase, a process termed slippage, after treatment with an antimitotic can arrest in G_1. Alternatively these tetraploid cells can undergo death after slipping or the cells can be death insensitive and continue to proliferate. Some antimitotics such as paclitaxel not only cause cell death from mitosis, but also can result in cell death in the following G_1 when a cell slips from mitosis into G_1(Zhu et al., 2014). What determines these cell fates following antimitotic treatment is still not well characterized.

The mechanisms resulting in cell death in mitosis following antimitotic treatment are beginning to unravel. Previous studies have shown that this death can be induced by classic apoptosis with caspase activation (Shi et al., 2008, Tao et al., 2005, Wang et al., 1999, Bergstralh and Ting, 2006). However, there is variability in death induction based on the cell line used. Certain non-adherent cell lines undergo a caspase-independent cell death, as caspase inhibitors do not alter its death kinetics (Shi et al., 2008). Cell-line specific death mechanisms are one confounding factor making it hard to characterize mitotic cell fate following antimitotic treatment.

Antiapoptotic protein levels in a cell add another layer of variability that determines whether a cell undergoes apoptosis or exits mitosis without cell division. Antiapoptotic proteins are implicated in apoptosis following a prolonged mitosis (Terrano et al., 2010,

Harley et al., 2010, Shi et al., 2011). Antiapoptotic proteins are critical in keeping the cell alive during mitosis by inhibiting proapoptotic proteins from creating pores in the mitochondria that prime the mitotic cell for death. The mechanism in which antiapoptotic keep the cell alive will be described later in this chapter. The role of antiapoptotic proteins in staving off death in antimitotic cells is well characterized (Shi et al., 2008, Salah-Eldin et al., 2003, Basu and Haldar, 2003).

Resistance to antimitotic chemotherapies

Antimitotic therapeutics have held much promise, however many patients develop treatment resistance (Weaver, 2014, Cabral, 2001). Phase II trial of paclitaxel (Taxol™) in 1991 showed that 8% of patients didn't respond to this treatment as their disease progressed (Holmes et al., 1991). In a multicenter Phase II trial, 28% patients had evidence of disease progression after paclitaxel treatment (Nabholtz et al., 1996). Resistance mechanisms need to be better understood in order to increase the efficacy of paclitaxel with combinatorial therapies.

There are a handful of well-studied antimitotic resistance mechanisms, however these resistance mechanisms do not account for all of the resistance occurring. Common resistance to antimitotic drugs occur when there are mutations in the drug binding site (Sharma et al., 2007). Additional resistance mechanisms have been noted in the way the drug is metabolized. Paclitaxel and docetaxel are metabolized by the liver enzyme CYP3A4. Studies have shown that patients treated with these antimitotics have increased CYP3A4 in their breast cancer tissues (Kapucuoglu et al., 2003). Another common mechanism of antimitotic drug resistance is the upregulation of drug efflux pumps of the

ATP-binding cassette (ABC) family(Dumontet and Jordan, 2010). The *multidrug resistance gene 1 (MDR1)* translates the P-gp pump, the P-gp pump is activated by hydrophobic drugs. When activated, P-gp will export these hydrophobic drugs out of the cell's cytoplasm (Dumontet and Jordan, 2010, Nobili et al., 2012). Vinca alkaloids and taxanes are hydrophobic drugs recognized by multidrug-associated protein 1 (MRP1) and MRP2/MRP7 respectively(Dumontet and Jordan, 2010). Resistance to antimitotic drugs is still poorly understood because the variation in drug response cannot be entirely explained by upregulation of drug efflux pumps, mutations in tubulin or upregulation of CYP3A4 (Noguchi, 2006).

Another resistance mechanism to antimitotics occurs when cells do not undergo apoptosis and instead fail mitosis (Cabral, 2001, Yamada and Gorbsky, 2006). These resistant cells enter G_1 phase without completing cytokinesis, an event termed slippage (this is one mechanism of resistance)(Yamada and Gorbsky, 2006). This slippage event creates tetraploid cells with twice as much DNA as a normal cell and duplicated centrosomes (Figure 1.7) (Yamada and Gorbsky, 2006, Vitale et al., 2011, Potapova et al., 2013). Cells that have slipped can continue to proliferate and thus re-enter the cell cycle as tetraploid cells. These tetraploid cells become more genomically unstable following each division (Weaver and Cleveland, 2005, Potapova et al., 2013). Tetraploid cells also result in additional problems for the patient as they are more invasive. have the twice the number of genes, so they buffering capability to acquire more mutations (Kuukasjarvi et al., 1997, McClelland et al., 2009). Because of this, antimitotic chemotherapeutic resistance in these cancers is important to understand.

Factors that Determine whether a cell slips or dies

Determining whether a cell undergoes slippage or mitotic cell death when treated with an antimitotic drug is crucial for understanding antimitotic resistance. When cells are halted in mitosis for a prolonged time transcription and translation are slowed down. However, natural protein turnover is still occurring, therefore a protein degradation race determines the fate of the mitotic cell. This protein degradation race is two armed, the fate of the mitotic cell depends on Cyclin B and antiapoptotic protein levels. Cyclin B is crucial for keeping the cell in mitosis, when Cyclin B levels drop below a critical threshold the cell will undergo slippage and enter the following G_1 without undergoing cytokinesis (Gascoigne and Taylor, 2009). Antiapoptotic proteins are crucial for keeping the cell alive and preventing the activation of the intrinsic apoptotic pathway during mitosis (Craig, 2002). If antiapoptotic proteins drop below a certain threshold they will be unable to stave off cell death and the cell will undergo mitotic cell death. It has been shown that cancer cells increase antiapoptotic proteins such as Mcl-1, Bcl-2 and Bcl-xL (Kaufmann et al., 1998, Khoury et al., 2003). Understanding how the Cyclin B levels and antimitotic levels vary from tumor to tumor will eventually help when determining if antimitotic therapeutics are the correct treatment options.

Tetraploidy and cancer

Overview

Though majority of human cells are diploid, some mammalian cells become tetraploid as part of the normal aging process (Guidotti et al., 2003, Fausto and Campbell,

2003). Human diploid cells are defined as having two complete sets of chromosomes totaling 46 chromosomes. As a zygote, humans inherit 23 chromosomes from the ovum and 23 chromosomes from the sperm. Liver cells are an example of cells that become tetraploid as they age. Tetraploidy in this context may function to control organ size (Otto, 2007). Tetraploidy is defined as cells having twice the number of chromosomes, these cells have 92 chromosomes instead of 46. Miscarriages and cell transformation can occur in cells that incorrectly become tetraploid (Carr and Gedeon, 1978, Eiben et al., 1990, Hassold et al., 1980, Neuber et al., 1993, Fujiwara et al., 2005). About 2% of all miscarriages are caused by the fetus being tetraploid (Jenderny, 2014). Tetraploid cells are more invasive (Kuukasjarvi et al., 1997, McClelland et al., 2009). Having twice as many genes also results in a potential proliferative advantage because tetraploid cells have the ability to buffer mutations, thus they can acquire more beneficial mutations and still have a second gene present to keep the cell alive if the cells would have otherwise undergone a deleterious mutation.

Almost 40% of cancer cells have passed through a tetraploid intermediate (Zack et al., 2013b). The notion that tetraploidy results in cancer is not a new one. Over 100 years ago Theodore Boveri postulated that tetraploidy resulting from mitotic defects promotes cancer.(Boveri, 2008, Boveri, 1914). Tetraploidy can arise in multiple manners. Cells that exit mitosis without undergoing cytokinesis(Mullins and Biesele, 1977, Shi and King, 2005, Reverte et al., 2006) have twice as much DNA and are tetraploid (Figure 1.7) . Tetraploid cells can also arise when two of the same cells containing one nuclei fuse together to become one cell via cell fusion (Duelli et al., 2005, Duelli et al., 2007, Migeon

et al., 1974). Additionally, if a cell repeatedly replicates its genome without undergoing cell division, termed endoreduplication, the cell will become tetraploid. As previously described, if a cell undergoes mitotic slippage it becomes tetraploid (Brito and Rieder, 2009, Lanni and Jacks, 1998, Sotillo et al., 2007, Elhajouji et al., 1998).

Paradoxically, tetraploid cells typically will die or arrest unless they also accrue other mutations such as loss of p53(Andreassen et al., 2001a, Cross et al., 1995, Fujiwara et al., 2005). Cells that are tetraploid can have oncogenic potential. Fujiwara and colleagues' elegant experiment demonstrated when tetraploid cells without p53 are injected into mice they form tumors where the p53 null diploid cells do not (Fujiwara et al., 2005). p53 and its transcriptional target p21 are critical for arresting tetraploid cells and preventing their proliferation. Thus many human and mouse tumor cells that are tetraploid have also lost p53(Galipeau et al., 1996, Ramel et al., 1995, Donehower et al., 1992, Jacks et al., 1994). Other tumor suppressors also play a major role in suppressing the proliferation of tetraploid cells by acting through the p53-p21 signaling axis, these include ARF, LATS2, and pRb (Andreassen et al., 2001b, Ganem et al., 2014, Margolis et al., 2003).

There are many checkpoints throughout the cell cycle to prevent tetraploid cells from proliferating. The first checkpoint is in G_1. When a cell exits mitosis without undergoing cytokinesis and thus slips from mitosis, it now has twice as much DNA. This cell that has undergone mitotic slippage will often arrest in G_1. The G_1 arrest is p53 dependent as tetraploid cells without a functional p53 are able to continue through the cell cycle (Borel et al., 2002, Casenghi et al., 1999, Cross et al., 1995, Hirano and Kurimura, 1974, Khan and Wahl, 1998, Lanni and Jacks, 1998, Stewart et al., 1999).

In addition to activating p53, tetraploid cells also have a high level of CIN (Fujiwara et al., 2005, Mayer and Aguilera, 1990, Storchova et al., 2006). CIN occurs when the cell either has structural errors (chromosomal damage) or persistent changes to its number of chromosomes. Cells that are CIN are constantly changing and rearranging their chromosome numbers and thus cells don't keep the same chromosome number through the generations of cell division.

Through CIN, tetraploidy is often a precursor to aneuploidy. During processive cell cycles different chromosomes are more readily lost or gained as the cell has twice as many chromosomes to segregate m(Figure 1.9). Depending on how the tetraploid cell arises it may have supernumerary centrosomes. Extra centrosomes can undergo a multipolar spindle intermediate. The cell then cluster the supernumerary centrosomes which can result in merotelic attachment (described in more detail later). This merotely results in lagging chromosomes. The lagging chromosome can either be missegregated or could rupture in a micronuclei (Ganem et al., 2009, Basto et al., 2008, Boveri, 2008, Brinkley and Goepfert, 1998, Quintyne et al., 2005, Nigg, 2002, Gisselsson et al., 2008, Maciejowski et al., 2015, Stevens et al., 2007). If a lagging chromosome is far away from the bulk of the chromosomes when the nuclear envelope reforms during telophase, then a separate nuclear envelope will form around the single chromosome, known as a micronuclei. Chromosomes in micronuclei are more vulnerable to many breaks and rearrangements, known as chromothripsis, if the micronuclear envelope undergoes rupture (Zhang et al., 2015a, Forment et al., 2012, Stephens et al., 2011, Storchova and Kloosterman, 2016). Thus

tetraploidy results in CIN through many different mechanisms eventually leading to aneuploidy.

Cells that undergo a multipolar mitosis undergo a longer mitosis in comparison to a bipolar mitosis. Multipolar mitosis are longer because the cell is taking an extended time to satisfy the spindle assembly checkpoint (Basto et al., 2008, Gisselsson et al., 2008, Kwon et al., 2008, Yang et al., 2008). There is an increased amount of syntelic attachment in tetraploid yeast cells. Syntelic attachment occurs when both sister chromatids are attached to microtubules emanating from the same pole. An increase in syntelic attachment in yeast tetraploid cells has been attributed to the change in size of yeast tetraploids. Yeast tetraploid cells have increased syntelic attachment because the cell has an increased size but the spindle length does not increase (Storchova et al., 2006). The microtubules emanating from the spindles in tetraploid yeast cells are unable to properly find and attach to chromosomes because they are too short and cannot span the larger cytoplasm. Syntelic attachments activate the SAC. The SAC is not satisfied until these attachments are corrected. A tetraploid cell with increased syntelic attachments will therefore take a longer time to go through mitosis as the SAC is activated.

Chromosome Instability and Centrosome Amplifications

While maintenance of centrosome number is highly regulated in non-transformed cells, errors do occasionally arise that lead to centrosome amplification. Extra centrosomes primarily arise via two distinct, yet non-mutually exclusive, mechanisms (Figure 1.1). Extra centrosomes can be generated by mitotic or cytokinetic failures, which produce tetraploid cells with twice the normal number of centrosomes (Davoli and de Lange, 2011,

Ganem et al., 2007). Alternatively, if the mechanisms that regulate centriole duplication go awry they can result in centriole over duplication (Firat-Karalar and Stearns, 2014, Nigg and Stearns, 2011, Sluder, 2014).

Regardless of the mechanisms underlying centrosome amplification, it is now well recognized that excess centrosomes are detrimental to the viability of non-transformed cells. Most notably, extra centrosomes greatly disrupt mitotic spindle formation and accurate chromosome segregation. Cells that enter mitosis with more than two centrosomes are predisposed to forming multipolar spindles (Brinkley, 2001, Nigg, 2002). Unless resolved prior to anaphase onset, multipolar spindles lead to catastrophic multipolar anaphase, the generation of grossly aneuploid daughter cells, and cell death (Ganem et al., 2009). This has been demonstrated *in vivo*, as induced centrosome amplification in the mouse brain promotes significant cell death through multipolar division, ultimately leading to microcephaly (Marthiens et al., 2013).

Extra centrosomes also appear to disrupt normal cell proliferation through mechanisms that are independent of abnormal mitosis and aneuploidy. Remarkably, it has been demonstrated that the presence of even a single extra centrosome in non-transformed cells is sufficient to activate the p53 tumor suppressor pathway and impede further cell proliferation (Ganem et al., 2014, Holland et al., 2012). This holds true irrespective of whether extra centrosomes are generated by cytokinesis failure or centriole over duplication. Unsurprisingly, non-transformed cells with extra centrosomes are selected against in long-term culture experiments (Chiba et al., 2000, Ganem et al., 2009, Godinho et al., 2014).

While centrosome number is strictly regulated in non-transformed cells, the opposite is true for many tumor cells. It is firmly established that centrosome amplification is a hallmark of human cancers (D'Assoro et al., 2002, Ganem et al., 2009, Ghadimi et al., 2000, Godinho and Pellman, 2014, Lingle et al., 2002, Sluder and Nordberg, 2004). This raises an obvious paradox: If extra centrosomes are so poorly tolerated by non-transformed cells, then why are they so common in human malignancies? One possibility is that there are positive selective pressures that promote the accumulation of extra centrosomes in cancer cells. For example, extra centrosomes are known to promote CIN (reviewed below) (Ganem et al., 2009, Silkworth et al., 2009). CIN is defined by the persistently elevated rate of whole chromosome missegregation during cell division (Lengauer et al., 1997). CIN generates significant genetic heterogeneity within tumors and can enable the outgrowth of cells that have acquired growth advantages (Nowell, 1976, Sheltzer et al., 2011, Sotillo et al., 2010, Thompson and Compton, 2010, Chen et al., 2015). Thus, acquisition of a CIN phenotype may be a major positive selective pressure for cancer cells to accumulate extra centrosomes. Extra centrosomes have also been shown to disrupt cell polarity, promote asymmetric cell division, alter cellular signaling, and promote tumor cell invasiveness, any or all of which may promote tumor growth and progression (Godinho and Pellman, 2014).

Another reason why extra centrosomes are more common in cancer cells could be that they are simply better tolerated. It is now obvious that cancer cells acquire several characteristics that make them more permissive of the negative effects imparted by extra centrosomes. One clear adaptation made by cancer cells to cope with extra centrosomes is to reduce the propensity of extra-centrosomal cells to undergo chaotic multipolar cell

divisions. To accomplish this, most cancer cells cluster excess centrosomes into two spindle poles during mitosis, enabling a relatively normal bipolar anaphase (Basto et al., 2008, Kwon et al., 2008). While this mechanism saves cells from catastrophic mitosis and likely cell death, it is not without consequences. Extra-centrosomal cancer cells pass through a transient 'multipolar spindle intermediate' prior to centrosome clustering, during which merotelic kinetochore-microtubule attachment errors accumulate (Ganem et al., 2009, Silkworth et al., 2009). Merotelic attachments (defined as single kinetochores attached to two spindle poles) are known to promote chromosome missegregation (Cimini et al., 2001, Cimini et al., 2003, Compton, 2011, Salmon et al., 2005, Thompson and Compton, 2008, Thompson and Compton, 2011). Thus, extra centrosomes, even if clustered into two poles to preserve cell viability, continue to promote whole chromosome segregation errors with resultant CIN.

Additional adaptive mechanisms made by cancer cells to tolerate extra centrosomes remain less well defined. For example, although extra centrosomes are known to stimulate the p53 pathway and limit the proliferation of non-transformed cells, the mechanisms underlying this response are recently unraveling. The USP28-53BP1-p53-p21 signaling axis results in p53 accumulation due to centrosome loss or centrosome amplification (Lambrus_Holland 2016). However, it remains unknown how cancer cells adapt to overcome the stress imparted by extra centrosomes that activates p53 in the first place.

One adaptive mechanism tetraploid cells use is to cluster their extra centrosomes (Ganem et al., 2009). If a tetraploid cell doesn't cluster its supernumerary centrosomes and undergoes a multipolar division, the daughter cells born from this division undergo cell

death(Ganem et al., 2009). HSET is a kinesin motor protein responsible for clustering extra centrosomes (Chavali et al., 2016).

HSET inhibition could work in combination with antimitotic chemotherapy. Originally it was thought that targeting HSET in cancer cells would result in selective inhibition and cell death in tetraploid cells. Targeting HSET would not result in any toxicity to the normal diploid cells in the human body as they have normal number of centrosomes. In theory, inhibiting HSET seems like an ideal targeted therapy. Inhibitors of HSET have been made. However, most of the tetraploid cancer cells have evolved and only have 2 centrosomes, so HSET inhibition would have to be a prophylactic therapeutic option (Ganem et al., 2009, Kawamura et al., 2013, Raab et al., 2012). However, using HSET inhibitors with antimitotic therapeutics seems like a promising idea. Any cells that do not die in mitosis when treated with this combinatorial therapy would slip from mitosis and become tetraploid. Any tetraploid cell born from this slipped mitosis would have to cluster its chromosomes effectively in order to proliferate. If HSET is inhibited in these slipped cells centrosome clustering would be impossible and the daughter cells would undergo multipolar division and thus die in the following G_1.

Not only do tetraploid cells have extra centrosomes, but cells with aberrant centrosomes also results in tetraploidy. When centrosomes are removed from cells tetraploidy occurs and p53 is translocated to the nucleus of these cells. Upon further study the p53 in these cells was shown to be phosphorylated at serine 33. Typically, p53 is phosphorylated at serine 15 when the cell experiences DNA damage. Serine 33 phosphorylation on p53 is known to induce p38 (Kishi, 2001). The centrosomes may act

as a signaling platform for p38-p53-p21 to ensure centrosome fidelity during mitosis (Aylon and Oren, 2011, Minn et al., 1996, Shinmura et al., 2007). Interestingly, p38 is activated and kills tetraploid cells by signaling to p53 and then p21(Toledo et al., 2011).

Cells with an active spindle assembly checkpoint that arrest in mitosis for a prolonged period of time also activate p38, p53 and p21 thus arresting the cell in the following G_1 (Uetake and Sluder, 2010). Since tetraploid cells have twice as many chromosomes and occasionally have twice as many centrosomes it takes these cells longer to satisfy the spindle assembly checkpoint by making the proper amphitelic attachment to each chromosome. The idea that tetraploid cells undergo a prolonged mitosis makes it attractive to suggest that tetraploid cells undergo a G_1 arrest because they underwent a prolonged mitosis (Gao et al., 2009, Vogel et al., 2004). p53 levels are known to increase in daughter cells born from a prolonged mitosis, this could be yet another mechanism halting the proliferation of tetraploid cells (Uetake and Sluder, 2010).

Since p53 plays such an important role in arresting tetraploid cells, it would seem that activating p53 would be a promising target. However, activating p53 as a therapeutic target has not been successful. This idea has been thought of and failed. Nutlin is an MDM2 inhibitor. Nutlin results in the increase of p53 by preventing MDM2 from inhibiting p53. In the presence of Nutlin, p53 accumulates in tetraploid cells and arrests these cells (Efeyan et al., 2007, Kumamoto et al., 2008). However, Nutlin has to be continuously present in these patients. If Nutlin is washed out the tetraploid cells will automatically replicate the genome again without going through mitosis, thus it will undergo endoreduplication. This endoreduplicated cell will continue through the cell cycle resulting in tetraploid cells that

are potentially more resistant to chemotherapy induced cell death since they now have the buffering capabilities of having twice the genome (Shen and Maki, 2010). A better understanding of the induction of p53 in tetraploid cells needs to be grasped in order to make better therapies at targeting these tetraploid cells.

Perhaps targeting a protein that modulates p53 upstream would be a more beneficial strategy than targeting and activating p53 directly. One promising tumor suppressor protein, LATS2, plays a role in regulating p53 (Aylon et al., 2006). LATS2 can be phosphorylated and activated in mitosis by Aurora A (Toji et al., 2004, Finkin et al., 2008) and Mps1 in response to mitotic stress (Huang et al., 2009). When LATS2 is activated it will bind to and inhibit MDM2 resulting in the accumulation of p53 (Aylon et al., 2006). In cells lacking LATS2 there is an increased amount of centrosome fragmentation and cytokinesis defects (McPherson et al., 2004, Yabuta et al., 2007). It has also been shown that knocking down LATS2 allows tetraploid cells to proliferate (Ganem et al., 2014). Intriguingly, recent studies demonstrate that centrosome amplification activates the Hippo tumor suppressor pathway, which indirectly stabilizes p53 (Ganem et al., 2014). This suggests that Hippo pathway inactivation, which is a widespread feature of human cancers, may represent one common adaptation that enables cancer cells to better tolerate tetraploid cells with extra centrosomes. This information suggests that there is indeed a connection between normal mitosis, centrosome integrity as well as LATS2 and p53 activation in tetraploid cells (Aylon and Oren, 2011). However, the mechanisms through which extra centrosomes trigger Hippo pathway activation remain poorly understood.

The Hippo Tumor Suppressor Pathway

Overview

The Hippo tumor suppressor pathway is a conserved regulator of cellular proliferation, differentiation, and death. Canonically, the major function of the Hippo pathway is to restrain organ size (Pan, 2010, Yu and Guan, 2013a), as loss of Hippo pathway activity is well known to promote tissue overgrowth and tumor development (Camargo et al., 2007, Dong et al., 2007, Lu et al., 2010, Song et al., 2010, Zhou et al., 2009a). However, recent studies have demonstrated that the Hippo pathway is also regulated by complex inputs that monitor cell-cell adhesion, cell-matrix adhesion, G-protein coupled receptor (GPCR) signaling, and contractile tension from the actin cytoskeleton (Aragona et al., 2013, Dupont et al., 2011, Ganem et al., 2014, Halder et al., 2012, Paramasivam et al., 2011, Wada et al., 2011a, Yu et al., 2012, Zhao et al., 2012a, Zhao et al., 2007). Indeed, several conditions known to induce cell cycle arrest, including contact inhibition, loss of cell attachment, and mitotic failure are now known to activate the Hippo pathway (Aragona et al., 2013, Aylon et al., 2006, Ganem et al., 2014, Zhao et al., 2012a, Zhao et al., 2007).

The core components of the Hippo pathway, which are conserved in mammals, were first identified in genetic screens for tumor suppressors in *Drosophila melanogaster*. These include *Hippo* (*Mammalian sterile 20-like kinase 1 (MST1)* and *Mammalian sterile 20-like kinase 2 (MST2)* orthologues in mammals) (Harvey et al., 2003, Jia et al., 2003, Pantalacci et al., 2003, Udan et al., 2003, Wu et al., 2003), *Warts* (*Large tumor suppressor kinase 1* (*LATS1)* and *Large tumor suppressor kinase 2* (*LATS2)* orthologues in mammals) (Justice et al., 1995, Xu et al., 1995), *Salvador* (*SAV1* or *WW Domain-Containing Adaptor*

45 (WW45) orthologues in mammals) (Kango-Singh et al., 2002, Tapon et al., 2002), and *MOB as tumor suppressor (Mats)* (*Mps1 binder* (*MOB)* family orthologues in mammals) (Lai et al., 2005). Loss of function mutations in these genes leads to increased cell proliferation, reduced cell death, and increases in organ size in flies (*e.*g. overgrown eyes and wings). Similarly, loss of Hippo pathway activity promotes tissue overgrowth and tumor development in mice (Camargo et al., 2007, Dong et al., 2007, Lu et al., 2010, Song et al., 2010, Zhou et al., 2009a). For simplicity, the mammalian gene/protein names will be used for the purposes of this dissertation.

The main function of the Hippo pathway is to negatively regulate the oncogenic transcriptional co-activators yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) (Pan, 2010, Yu and Guan, 2013a). This regulation is primarily accomplished through activation of the kinases LATS1 and LATS2, which phosphorylate YAP and TAZ to promote their inactivation (Figure 1.10) (Yu and Guan, 2013a, Zhao et al., 2010a). When LATS1 is phosphorylated at threonine 1079 it becomes active. When active, LATS will phosphorylate YAP at five sites: S61, S109, S127, S164, S381. The phosphorylation of YAP at S127 results in the binding of YAP to 14-3-3. The phosphorylation of YAP at site S381 primes CK1δ/ε resulting in the ubiquitination and degradation of YAP. Additionally, S61 and S109 phosphorylation on YAP interrupts YAP from binding to TEAD. When active, LATS will phosphorylate TAZ at four sites: S66, S89, S117, and S311. The phosphorylation of TAZ at S89 results in the binding of TAZ to 14-3-3. The phosphorylation of TAZ at site S311 primes CK1δ/ε resulting in the ubiquitination and degradation of TAZ. Additionally, S66 and S89 phosphorylation on

TAZ interrupts TAZ from binding to TEAD. In summary, phosphorylated YAP and TAZ bind to 14-3-3, which sequesters YAP/TAZ in the cytoplasm where they are subsequently proteasomally degraded (Hong and Guan, 2012). YAP/TAZ can also be sequestered at both tight and adherens junctions through direct binding to proteins that localize there (Avruch et al., 2012, Bertini et al., 2009, Zhao et al., 2010b, Zhao et al., 2011, Oka et al., 2008). YAP and TAZ are transcriptional co-activators of the transcription factor TEAD. Ultimately, activation of the Hippo pathway prevents YAP and TAZ from entering the nucleus and activating the TEAD-family of transcription factors to initiate the expression of genes important for cell growth and survival (Pan, 2010, Yu and Guan, 2013a, Zhao et al., 2010a, Zhao et al., 2010b, Zhao et al., 2011).

The upstream regulatory pathways that mediate LATS1/2 phosphorylation and activation are complex and not yet fully understood. In the classical signaling cascade (reviewed in (Pan, 2010, Yu and Guan, 2013a, Zhao et al., 2010a, Zhao et al., 2010b, Zhao et al., 2011), MST1 and MST2 kinases form a heterodimer with the adaptor protein SAV1, which enhances MST1/2 kinase activity. The MST/SAV1 complex then directly phosphorylates and partially activates LATS1/2 kinases. MST1/SAV1 also phosphorylates MOB1, enabling it to bind to the auto-inhibitory regions of LATS1/2. MOB binding releases LATS1/2 of their inhibitory state and enables them to be further phosphorylated on their activation loops. Together, the coordinated actions of MST1/2 and MOB fully activate LATS1/2 (Figure 1.10). Loss of function of any of these components can inactivate the Hippo pathway. It is not surprising then, that all core members of this signaling pathway (MST1/2, LATS1/2, SAV1, and MOB) have tumor suppressive activities in mammals (Cai

et al., 2010, Lee et al., 2010, McPherson et al., 2004, Nishio et al., 2012, Song et al., 2010, St John et al., 1999, Yabuta et al., 2007, Zhou et al., 2009b, Zhou et al., 2011).

In addition to the classical, linear MST1/2 signaling cascade, it is now recognized that regulation of YAP/TAZ can be achieved through MST1/2-independent processes in certain contexts. For example, disruption of the actin cytoskeleton and/or reduced RhoA activity, which occur upon cell detachment, serum starvation, tetraploidy, and contact inhibition, all activate LATS1/2 in an MST1/2-independent manner (Ganem et al., 2014, Mo et al., 2012, Wada et al., 2011a, Yu et al., 2012, Zhao et al., 2012a). This implies that additional regulatory mechanisms exist to activate LATS1/2 and inactivate YAP/TAZ. However, the mechanistic basis for this alternative regulation, and the key regulatory components and kinases involved, remain to be elucidated.

In addition to negatively regulating YAP/TAZ, activation of the Hippo pathway can also engage the p53 pathway. Phosphorylated, active LATS2 (but not LATS1) can bind and inhibit the E3 ubiquitin ligase MDM2, which normally targets p53 for destruction (Aylon et al., 2006). Consequently, LATS2 activation leads to p53 stabilization and the expression of downstream target genes that reinforce cell cycle arrest (*e.g. CDKN1A*). *LATS2* itself is a p53 target gene, and its activation thus initiates a feedback loop to enforce Hippo pathway activity (Aylon et al., 2006). Activation of the Hippo pathway therefore limits cellular proliferation in at least two ways: by inactivating YAP/TAZ and by stabilizing p53.

Activation of the Hippo Pathway

The Hippo pathway is regulated in a variety of ways including apical-basal polarity, planar cell polarity (PCP), G-Protein Coupled Receptor (GPCR) signaling, and the

contractile state of the actin cytoskeleton(Yu and Guan, 2013b). Apical-basal polarity activates upstream regulators of the Hippo pathway through tight junction and adherens junctions, resulting in the phosphorylation of MST1/2 and LATS1/2 respectively. Merlin is an upstream regulator of MST1/2 that is localized at the apical domain of the epithelial cells; it may recruit the Hippo complex to the apical plasma membrane for activation(Yu and Guan, 2013b). PCP allows for the orientation and coordination of cells to be in the same direction. PCP is controlled in part by the Fat/Dachsous signaling system; Dachsous activates Zyxin which inhibits Hippo in *Drosophila*, the pathways connection with the mammalian pathway is less understood (Simons and Mlodzik, 2008).

YAP/TAZ activation can also be regulated either positively or negatively by GPCR signaling based upon which heterotrimeric G-alpha protein is activated(Yu and Guan, 2013b). For example, the hormones sphingosine 1-phosphophate (S1P) and lysophosphatidic acid (LPA) act through the $G_{12/13}$-couple receptors to inhibit the Hippo pathway kinases LATS1/2 and activating the coactivators YAP/TAZ (Yu et al., 2012). YAP/TAZ is also influenced by the extracellular matrix (ECM) and cytoskeleton geometry**;** when cells are round and compact such as during mitosis as well as when trypsinized in cell culture, YAP/TAZ is inactive (Wada et al., 2011b). A specific type of apoptosis, anoikis, occurs when the cell is detached from the surrounding extracellular matrix; this results in inhibition of YAP/TAZ by its phosphorylation(Zhao et al., 2012b). YAP/TAZ can be regulated by mechanical stress. When cells are at a low confluence the cells are more spread out and thus have a larger strain on their ECM. The ECM of sparse

cells inactivates the Hippo pathway resulting in nuclear localized YAP/TAZ (Aragona et al., 2013).

Implications in Development and Organ Size

The activation of the Hippo pathway also plays a role in controlling organ size. Every organ reaches its own particular size through a delicate balance of cell proliferation and cell death. MST1/2 is known to activate caspase dependent cleavage, activating cell death (Graves et al., 1998). When MST is knocked out it also prevents phosphorylation of YAP, leading to constitutive YAP activity in the nucleus. Consequently, MST loss or overexpression of YAP in certain tissues results in larger than normal organ size as seen in the liver and the heart(Pan, 2010). Oversized organs are very dangerous and can lead to cancer, organ malfunction, or affect nearby organs. LATS1/2 and YAP phosphorylation are not exclusively MST1/2 phosphorylation and activation dependent. In order for cells in tissue to stop proliferating, apoptotic signaling must be activated and cell proliferation signaling needs to be shut off.

Regulation of the Cell Cycle by Hippo Signaling

In addition to regulating organ size, the Hippo pathway also plays a role in the regulation of the cell cycle. LATS1/2 activation can arrest the cell cycle in G_2 before it enters mitosis through CDK1 inhibition (Hergovich and Hemmings, 2012). The signaling plays a very important part in preventing cell cycle progression and maintaining DNA integrity before it divides. Additionally, YAP-TEAD interactions increases the expression

of Cyclin E allowing for the progression of the cell cycle from G_1 to S phase(Takeuchi et al., 2017, Shu and Deng, 2017).

The Hippo pathway is also implicated in controlling the cell's ability to survive. When the Hippo pathway is turned off or inactive YAP enters the nucleus and activates the transcription factor TEAD. This YAP-TEAD interaction results in the transcription of genes necessary for stimulation of cell proliferation while inhibiting apoptosis. The Hippo signaling pathway also plays a large role in apoptosis. Yki the *Drosophila* homolog of YAP is a well-known inhibitor of apoptotic proteins, specifically *diap1* (Wu et al., 2008, Zhang et al., 2008, Huang et al., 2005). MST2 is another component of the Hippo pathway that plays a role in apoptosis. MST2 is regulated by c-Abl tyrosine kinase; c-Abl phosphorylates MST2 promoting neuronal apoptosis in response to oxidative stress(Liu et al., 2012).

Activation of the Hippo Pathway Impairs the Proliferation of Cells with Extra Centrosomes

Extra centrosomes primarily arise from cytokinetic failures that give rise to tetraploid cells or from deregulation of mechanisms that control centriole over duplication (Figure 1.8). It has long been recognized that tetraploid cells activate the p53 pathway and fail to proliferate (Andreassen et al., 2001b, Fujiwara et al., 2005, Ganem and Pellman, 2007). Interestingly, a recent study by Holland et al. demonstrates that centrosome amplification alone, independent of tetraploidy, similarly activates the p53 pathway and impairs long-term cell growth (Holland et al., 2012). This suggests that extra centrosomes, *per se*, can impair cell proliferation.

To induce extra centrosomes in diploid cells, Holland *et al.* transiently overexpressed wild-type or degradation-resistant forms of the centriole duplication regulator Polo-like kinase 4 (PLK4) (Habedanck et al., 2005, Kleylein-Sohn et al., 2007). Clonogenic growth assays

revealed that the resulting diploid cells, now containing numerous supernumerary centrosomes, exhibited severely impaired proliferation. Upon closer inspection, it was revealed that the cells had activated the p53 pathway. This p53 stabilization was not simply an indirect effect of deregulated PLK4 kinase phosphorylating unidentified substrates, as expression of a non-degradable version of spindle assembly abnormal protein 6 homolog (SAS6) similarly led to centrosome amplification and reduced cell proliferation. Inactivation of the p53 pathway restored proliferation to the cells with extra centrosomes (Holland et al., 2012).

Recently, it has been shown that activation of the p53 pathway in both tetraploid cells and cells with centrosome amplification can be explained, at least in part, by activation of the Hippo pathway (Ganem et al., 2014). Extra-centrosomal cells display increases in the phosphorylation of LATS2 and subsequent inactivation of YAP. As described above, phosphorylated LATS2 binds to and inhibits MDM2, thus indirectly promoting the accumulation of p53 (Aylon et al., 2010). Importantly, depletion of LATS2 in cells with extra centrosomes mitigates p53 accumulation and activation of the p53 pathway (Ganem et al., 2014).

Defining the mechanism through which extra centrosomes activate the Hippo pathway remains an important area of investigation. Several recent studies have demonstrated that disruption of the actomyosin cytoskeleton and/or reduction in the activity of small G-protein RhoA have a major role in activating LATS in a MST-independent manner (Aragona et al., 2013, Dupont et al., 2011, Halder et al., 2012, Mo et al., 2012, Wada et al., 2011a, Yu et al., 2012, Mana-Capelli et al., 2014). Indeed, cells with extra centrosomes exhibit a significant

reduction in active RhoA (Ganem et al., 2014, Godinho et al., 2014). This reduction in RhoA is triggered, at least in part, by the indirect effects of increased microtubule nucleation from extra centrosomes. Dynamic microtubules are known to stimulate the activity of the small G-protein Rac1, and it has been reported that the increased microtubule nucleation from extra centrosomes hyperactivates Rac1 (Godinho et al., 2014). As active Rac1 antagonizes RhoA (Sander et al., 1999), increased Rac1 activity provides one molecular explanation for the observed loss of RhoA activity in cells with extra centrosomes.

An alternative possibility is that the centrosome may act as a scaffold that mediates LATS2 activation in the cell by physically localizing regulatory components of the Hippo pathway to a distinct subcellular space, and centrosome amplification would enhance this activation. Supporting this view, many core members of the Hippo pathway, including MST, MOB, SAV1 and LATS all localize to the centrosome (Morisaki et al., 2002, McPherson et al., 2004, Nishiyama et al., 1999, Mardin et al., 2010, Guo et al., 2007, Wong et al., 2015, Hergovich et al., 2006, Hergovich et al., 2007, Hergovich et al., 2009). This localization is similar in principle to how LATS2 is activated at the plasma membrane, where it is recruited by NF2 so that it may more efficiently interact with MST/SAV complexes (Yin et al., 2013). The idea of the centrosome as a signal platform is not new; centrosomes are known to anchor hundreds of regulatory proteins that mediate activation of diverse cellular networks (Jackman et al., 2003, Sluder, 2005). It is interesting to speculate that the subcellular localization of LATS2 may dictate which regulatory proteins activate it. For example, while it is known that LATS2 is phosphorylated and activated by MST1/2 at the plasma membrane, centrosomal-localized LATS2 may be responsive to different regulatory proteins. To date, the kinases

responsible for activating LATS in response to cytoskeletal defects remain unknown. It is possible that such kinases are specifically recruited or localized to centrosomes.

Regulation of Centrosome Number and Function by Hippo Pathway Components

In addition to acting as signaling conduits to regulate cell proliferation, many key members of the Hippo pathway moonlight as regulators of various aspects of centrosome biology. For example, MST1 and MOB1A/B play keys roles in centriole duplication (Hergovich et al., 2009). Depletion of either MST1 or MOB1A/B results in impaired centriole duplication, while overexpression of MOB1A/B promotes centriole amplification (Hergovich et al., 2009). Mechanistically, MOB1A/B bind to NDR1/2 kinases, which facilitates their subsequent phosphorylation and activation by MST1 (Hergovich et al., 2009). Active NDR1/2 have been shown to be important for centriole duplication, though the mechanisms remain to be defined (Hergovich et al., 2009, Hergovich et al., 2007, Cook et al., 2014).

MST kinases, in complex with SAV1, also play an essential role in centrosome disjunction (Mardin et al., 2010). Replicated centrosomes are bound to one another during interphase by the linker proteins C-Nap1 and rootletin. Upon entry into mitosis, these linker proteins are phosphorylated by the kinase NIMA-related kinase Nek2A, which leads to their disassembly (Fry, 2002). This process, termed centrosome disjunction, enables centrosome separation and bipolar mitotic spindle assembly. A complex of MST2/SAV1 mediates this process by directly phosphorylating and activating Nek2A (Mardin et al., 2010). Thus, loss-of-function of MST2 or SAV1 impairs normal centrosome disjunction and has the capacity to promote abnormal mitosis (Mardin et al., 2010).

LATS1 and LATS2 kinases also localize to centrosomes, yet their function there remains poorly understood (Morisaki et al., 2002, McPherson et al., 2004, Nishiyama et al., 1999, Guo et al., 2007, Wong et al., 2015, Toji et al., 2004). Cells depleted of LATS1/2 show no defects in centriole duplication or centrosome disjunction. However, it has been reported that depletion of LATS2 impairs the recruitment of γ-tubulin to centrosomes, thus limiting microtubule nucleation during mitosis and hindering chromosome alignment (Abe et al., 2006).

Most strikingly, loss of Hippo pathway components indirectly deregulates centrosome numbers by disrupting normal cytokinesis. Knock-out studies in mice demonstrate that loss of Hippo pathway components LATS1, LATS2, MOB1A and MOB1B all promote cytokinesis failure and the production of tetraploid cells with extra centrosomes (McPherson et al., 2004, Nishio et al., 2012, St John et al., 1999, Yabuta et al., 2007). Tetraploid cells, by virtue of their extra centrosomes, are genetically unstable and are known to be significant contributors to tumorigenesis (Boveri, 1914, Davoli and de Lange, 2012, Fujiwara et al., 2005, Ganem et al., 2007, Zack et al., 2013a). Combatting this potentially oncogenic effect of tetraploidy, it has been demonstrated that activation of the Hippo pathway in tetraploid cells limits their growth (Ganem et al., 2014). Thus, inactivation of Hippo pathway components not only promotes the generation of oncogenic tetraploid cells, but also imparts them with the ability to proliferate. Unsurprisingly, deletion of *LATS1* and *LATS2* is significantly more common in high-ploidy tumors than near-diploid tumors (Ganem et al., 2014).

Role in Cancer

The oncogenes, *YAP* and *TAZ*, are elevated in many cancers. In addition to overexpression of *YAP* and *TAZ*, deletion or epigenetic silencing of genes that regulate its nuclear localization, such as *MST1/2* and *LATS1/2*, are also found in cancer. Genetic changes resulting in the activation of YAP and TAZ allows for cells to overcome contact inhibition, avoid death, and facilitate tumor growth. The Hippo pathway is commonly inactivated in cancers resulting in over active YAP and TAZ.

Neurofibromatosis type 2 (NF2), also known as Merlin (Mer), is a tumor suppressor; inactivating mutations in this gene result in nonmalignant brain tumors(Yu and Guan, 2013b). Mer is an upstream regulator of the Hippo pathway and induces MST1/2 and LATS1/2 phosphorylation, which leads to the downstream phosphorylation of YAP and TAZ. Almost all patients with mutations in NF2 have lesions in their nervous system, such as nervous system tumors, by the age of 60, implicating the severity deregulation of this pathway (Asthagiri et al., 2009).

YAP acts primarily as an oncogene but also plays a small role as a tumor suppressor. In order for cells to metastasize, the cells must lose their cell polarity and adhesion, this process is known as epithelial-mesenchymal transition (EMT). YAP plays an oncogenic role as its overexpression results in induction of EMT, anchorage independent growth, and avoidance of apoptosis (Overholtzer et al., 2006). YAP is also implicated as a tumor suppressor as it is a coactivator of p73 along with other transcription factors; p73 is known to mediate apoptosis after DNA damage (Basu et al., 2003). YAP's role as a tumor suppressor is much less clear. Cytoplasmic YAP inhibits Wnt signaling,

independent of the Wnt destruction complex, resulting in growth-suppressive functions (Barry et al., 2013). The importance of YAP's dysregulation in cancer is emphasized by its role as potentially both an oncogene and a tumor suppressor.

The Hippo pathway's role in cancer can also be mediated by miRNA. The Hippo pathway is a tumor-suppressing pathway mediated by microRNA (miRNA). This non-coding RNA can prevent the translation of coding mRNA by base pairing with complementary sequences on mRNA molecules and preventing their recognition by ribosomes. miRNA silences proteins in the tumor suppressor pathway resulting in different human cancers. LATS2 is suppressed by miR372 and miR373 (Voorhoeve et al., 2006) and NF2 is suppressed by mir24 (Vittoria et al., 2018); this results in less phosphorylation and inactivation of YAP. The deregulation of YAP resulting from the miR inhibition of LATS2 leads to testicular tumor cells as well as other tumors that have wild-type p53 present and are sensitive to DNA damage (Voorhoeve et al., 2006).

Hepatocellular carcinoma (HCC) is a liver cancer closely linked with the Hippo signaling pathways dysregulation. YAP functions as an oncogene and is upregulated in HCC (Xu et al., 2009). The ablation of tumor suppressor MST1 and MST2 has also lead to massive HCC (Zhou et al., 2009b). The removal of MST1/2 results in an impaired Hippo pathway and thus YAP activation. NF2, the same protein that results in lesions in the nervous system, can also result in HCC when it is inactivated (Zhang et al., 2010).

Pharmacological Treatments and Targets of the Hippo Pathway

In wild-type cells, the Hippo pathway is activated when there is a high density of cells, this leads to the termination of cell proliferation (Aragona et al., 2013). When Hippo

pathway is activated, YAP/TAZ is sequestered in the cytoplasm and cannot effect transcription or proliferation. Overexpression of YAP results in a loss of contact inhibition as well as defects in cell polarity (Park and Guan, 2013). The Hippo tumor suppressor pathway plays a critical role in tumorigenesis and is thus an interesting pharmacological target.

Pharmacologically targeting YAP and TAZ could be beneficial in the regulation of tumor growth. Tumors have stiff matrices that surrounds them; the interaction of these matrices with actin and the cytoskeleton can result in YAP/TAZ inactivation. The inactivation of YAP does not occur through the canonical Hippo/LATS cascade. Instead, Rho GTPase activity interacts with YAP to overcome physical constraint (Dupont et al., 2011).These mechanical cues in tension and proliferation signaled through Rho GTPases play a role in YAP inactivation. For example, cells that are in very dense areas, and thus contact inhibited, are not able to spread out so there is less mechanical stress on the ECM. In these contact inhibited cells YAP/TAZ are localized to the cytoplasm thus stopping proliferation (Aragona et al., 2013). Since Rho regulates actin bundle formation and is involved in cell movement and cytoskeletal dynamics it could potentially be used to inhibit YAP/TAZ activity in order to regulate its cytoskeletal and mechanical forces in tumor growth.

The regulation of YAP/TAZ translocation into the nucleus has important implications for transcription of proteins necessary for cellular proliferation. Regulating and decreasing YAP/TAZ nuclear translocation is of pharmacological interest. YAP and TAZ are also regulated by their C-terminal motifs. These motifs are recognized by PDZ;

and are involved in YAP nuclear translocation (Oka et al., 2010). The protein structure of YAP/TAZ has been of interest to researchers as well. The WW structure of YAP/TAZ can interact with the PY motif of its negatively regulating proteins (LATS1/2, AMOTL1/2)(Yu and Guan, 2013b). The WW domain's interaction with the proline-rich PY motif could potentially be enhanced or stabilized by small molecules; this could lead to increased inhibition of YAP/TAZ by its upstream modulators (Park and Guan, 2013).

The Hippo pathway has proven to be a hard pathway to drug. Part of the issue with drug development is that this pathway's signaling cascade is composed of non-enzymatic proteins. Recently, a small molecule inhibitor Verteporfin has been shown to increase YAP binding to 14-3-3σ (Liu-Chittenden et al., 2012, Wang et al., 2016b). The binding of YAP to 14-3-3σ results in the sequestration of YAP in the cytoplasm where it cannot activate TEAD. By inhibiting YAP from binding to TEAD the activation of the Hippo pathway is mimicked. Verteporfin has shown promise in inhibiting YAP-induced liver growth and inhibiting the YAP oncoprotein. Recently, CA3, a small-molecule drug has also been created to prevent YAP from binding to TEAD (Song et al., 2018). Targeting the last steps of the Hippo pathway that affects YAP's transcriptional co-activating function seem like a promising therapeutic.

G-protein coupled receptors are of pharmaceutical interest as many drugs target this pathway. As previously mentioned, GPCRs work upstream of the Hippo tumor suppressor pathway. The Gα proteins affect the Hippo pathway in different ways. G$\alpha_{12/13}$-Rho GTPase activation results in the inhibition of LATS1/2 through GPCR signaling. This inhibition of LATS1/2 by GPCR signaling has been exploited by LPA and sphingosine kinase 1

antagonists in order to reduce tumor metastasis (Yu et al., 2012). PKA results in the phosphorylation and activation of LATS1/2, consequently activating the Hippo pathway and decreasing cellular proliferation (Kim et al., 2013). $G\alpha_S$ stimulates cAMP which activates PKA. $G\alpha_S$ agonists are also potential pharmacological targets for modulating Hippo pathway activity. Understanding Hippo signaling has led to new therapeutic targets for preventing uncontrolled cell growth and metastasis.

The tumor suppressor p53

Besides regulating YAP and TAZ, LATS2 can also stabilize p53 by binding to its inhibitor MDM2. p53 is a well-known tumor suppressor, colloquially known as the guardian of the genome. This protein is important because it plays a key role in cell cycle arrest and apoptosis(Longley and Johnston, 2005). p53 arrests cells in G_1 and G_2 if the cell is exposed to DNA damage. Not surprisingly, over half of cancer cells have mutated p53(Levine, 1997).

Proteins that regulate p53 are often mutated or silenced in cancers. p53 is continuously being transcribed and degraded. MDM2 is an E3 ligase that tags p53 for degradation with ubiquitin. In cancers, MDM2 can become over active preventing p53 from accumulating. Additionally, ARF, a tumor suppressor that inhibits MDM2 is frequently lost or mutated, this results in the upregulation of MDM2 and a decrease of p53. LATS2 is another tumor suppressor that binds to and inhibits MDM2 (Aylon et al., 2006), resulting in an accumulation of p53. LATS2 is frequently silenced in cancer too.

Dysregulation of the proteins that regulate p53 in cancer often result in a decrease of p53 expression.

p53 transcribes genes important for restraining uncontrolled proliferation in cells. When p53 is present it enters into the nucleus where it acts as a transcription factor for multiple genes such as *p21, MDM2, Bcl-2* and *BAX*(Vogelstein et al., 2000, el-Deiry et al., 1993) (El-Diery Cell 1993, Volgelstein 2000 Nature). p21 downregulates gene transcription by binding to the promoter regions of several genes. The genes in which p21 downregulates include the cyclins such as *Cyclin B1*, *Cyclin D* and *Cyclin E*. Therefore p21 can prevent cell cycle progression through its regulation of the cyclins (Lohr et al., 2003). Since p53 is upstream of p21, mutant p53 can override the G_1/S checkpoint(Wang et al., 2000). Having a functional p53 is key to preventing uncontrolled cellular growth. p53 is commonly mutated in cancers so the cells can overcome this barrier(Levine, 1997).

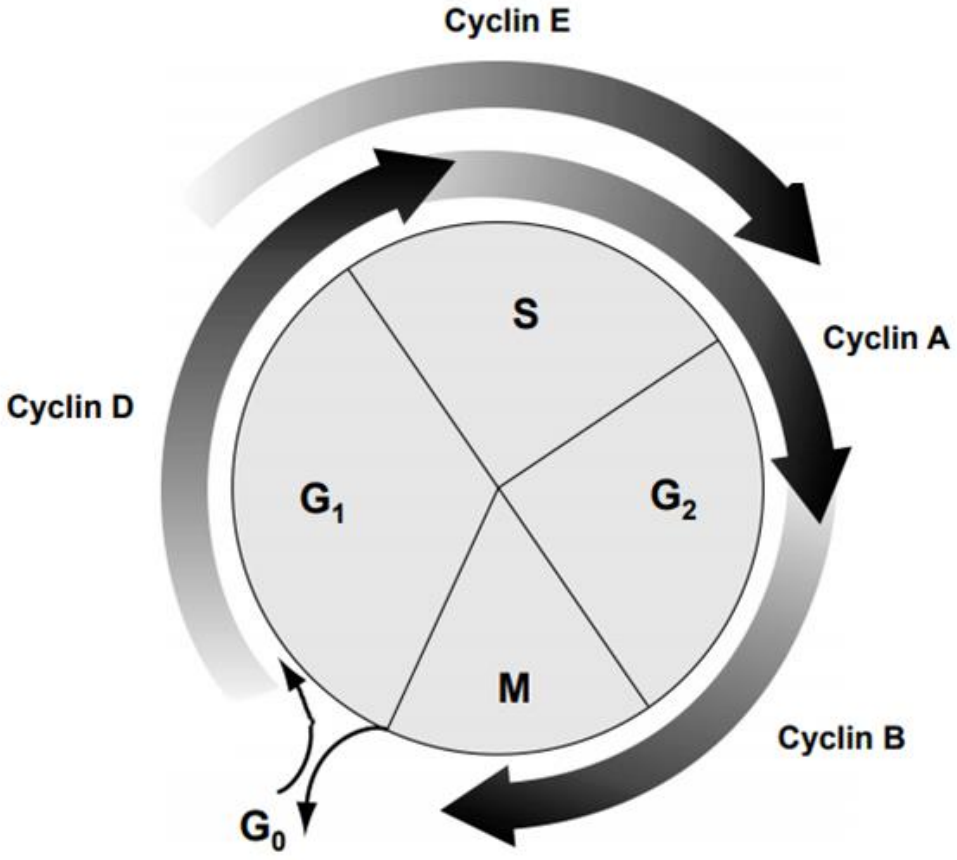

Figure 1.1: Cyclins drive the cell cycle

Cyclins are transcribed and translated in a cell stage dependent manner. Cyclins are also tagged for degradation in a cell stage dependent manner. Thus the cyclins increase and decrease throughout the cell cycle depending on which stage the cell is in. cyclins interact with cyclin dependent kinases to drive the cell through the progression of the cell cycle adapted from (Coller, 2007).

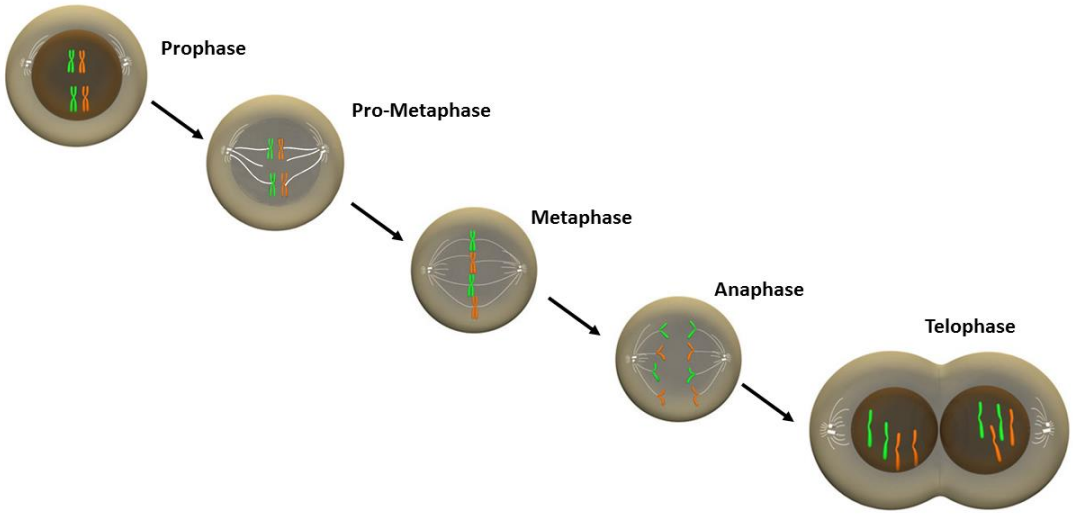

Figure 1.2 Stages of mitosis

There are five phases of mitosis: prophase, pro-metaphase, metaphase, anaphase, and telophase. During prophase the cell will round up and the DNA will condense into chromosomes. In prometaphase, the nuclear envelope breaks down and microtubules begin to attach to chromosomes. At metaphase, the chromosomes are bi-oriented and are aligned at the equatorial plate. Once all of the chromosomes have the proper alignment and the SAC has been satisfied, the sister chromatid will segregate to opposing poles during anaphase. In telophase the nuclear envelope reforms around the segregated chromosomes and the chromosomes decondense.

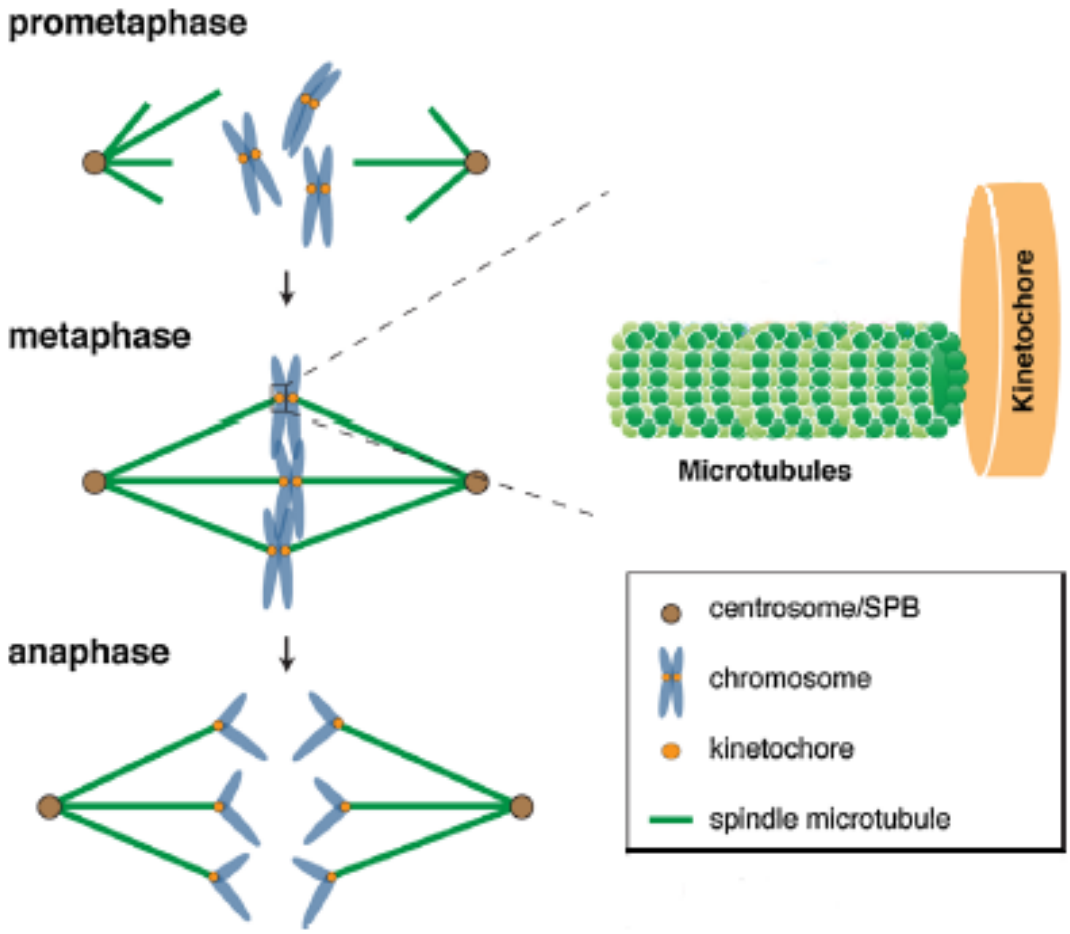

Figure 1.3 Microtubule attachment

Microtubules emanating from the centrosome search for and bind to chromosomes at the kinetochore located on the centromere (Tang and Toda, 2015).

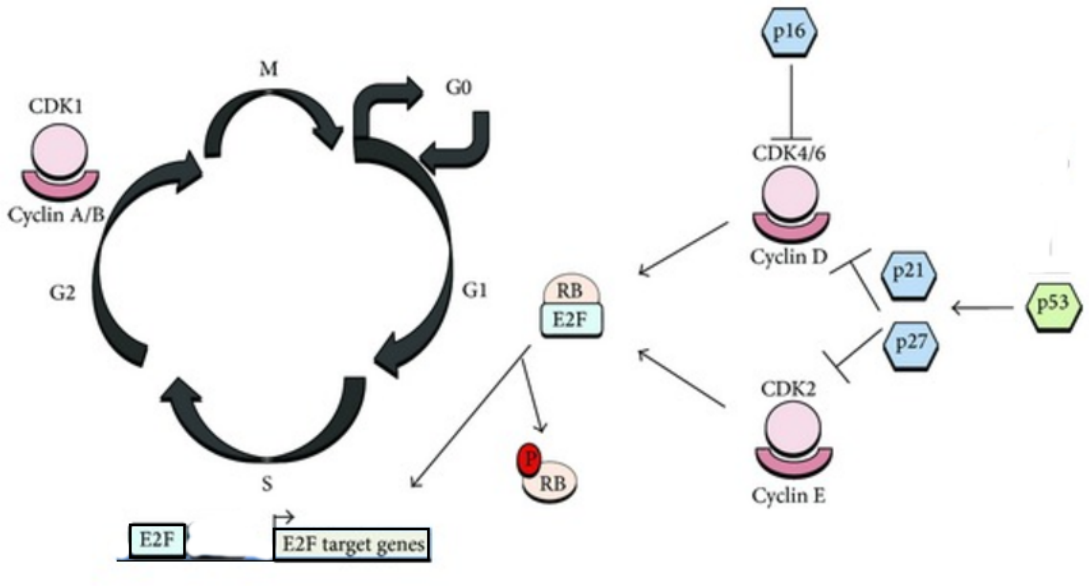

Figure 1.4 G1/S Transition

In G_1 the retinoblastoma (Rb) protein sequesters the transcription factor E2F. E2F transcribes genes necessary for the transition of the cell into S phase. Active Cyclin D/CDK4/6 and Cyclin E/CDK2 phosphorylate RB. The phosphorylation of Rb results in the disassociation of Rb from E2F. This allows for E2F to translocate to the nucleus and act as a transcription factor. p53 transcribes p21 and p27 which act as inhibitors of Cyclin D/CDK4/6 and Cyclin E/CDK2 respectively. p16 acts as a CDK inhibitor by binding to CDK4 and CDK6 and preventing their interaction with cyclins (Salas et al., 2014).

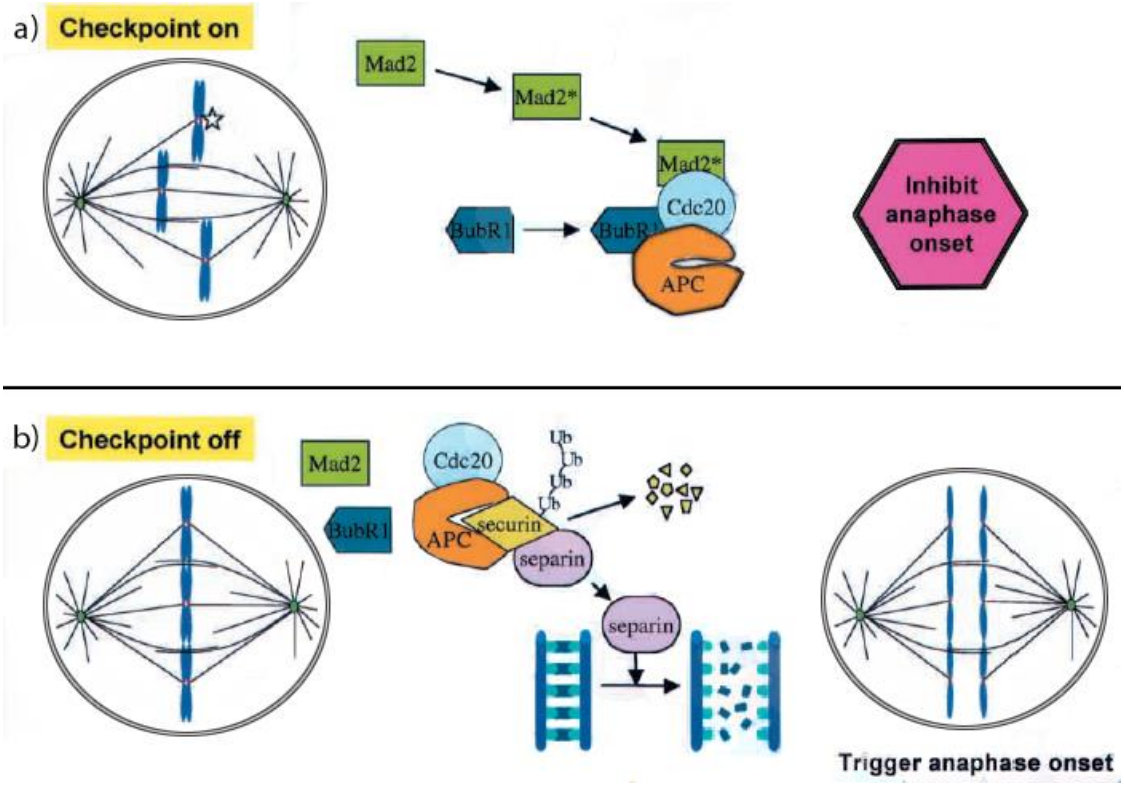

Figure 1.5 The SAC

a) The SAC is activated when one sister chromatid is not attached. While the SAC is activated Mad2, BubR1, Bub3 and Cdc20 are continuously being recruited and released from the kinetochore. The SAC inhibits the anaphase promoting complex/cyclosome (APC/C) as Cdc20, the protein that activates APC/C, is sequestered by Mad2 at the kinetochore while the SAC is activated. **b)** Once all of the sister chromatids are properly attached to microtubules, Mad1, Mad2, BubR1, Bub3 and Cdc20 are displaced from the kinetochore and the SAC is satisfied. Cdc20 is now able to activate APC/C. APC/C is an E3 ubiquitin ligase, when active, APC/C will degrade securing and Cyclin B1, allowing the sister chromatids to be separated and the cell to enter anaphase (Zhou et al., 2002).

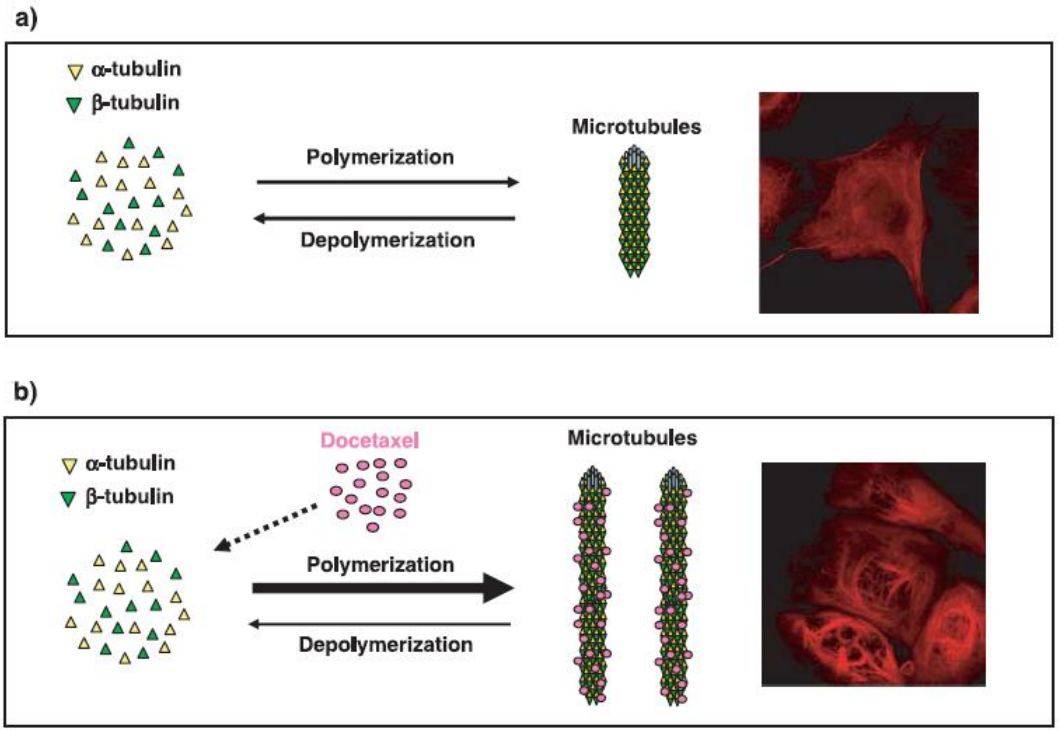

Figure 1.6 Microtubule stabilization

a) Microtubules consist of α-tubulin and β-tubulin dimers, these dimers polymerize into microtubules and depolymerize back into α-tubulin and β-tubulin dimers, this dynamicity is critical for their function to search for and capture chromosomes. b) Paclitaxel and docetaxel, both FDA approved chemotherapeutics, stabilize microtubules and increase the microtubules polymerization, the microtubules are no longer dynamic and cannot efficiently search for, capture, and align chromosomes (Noguchi, 2006).

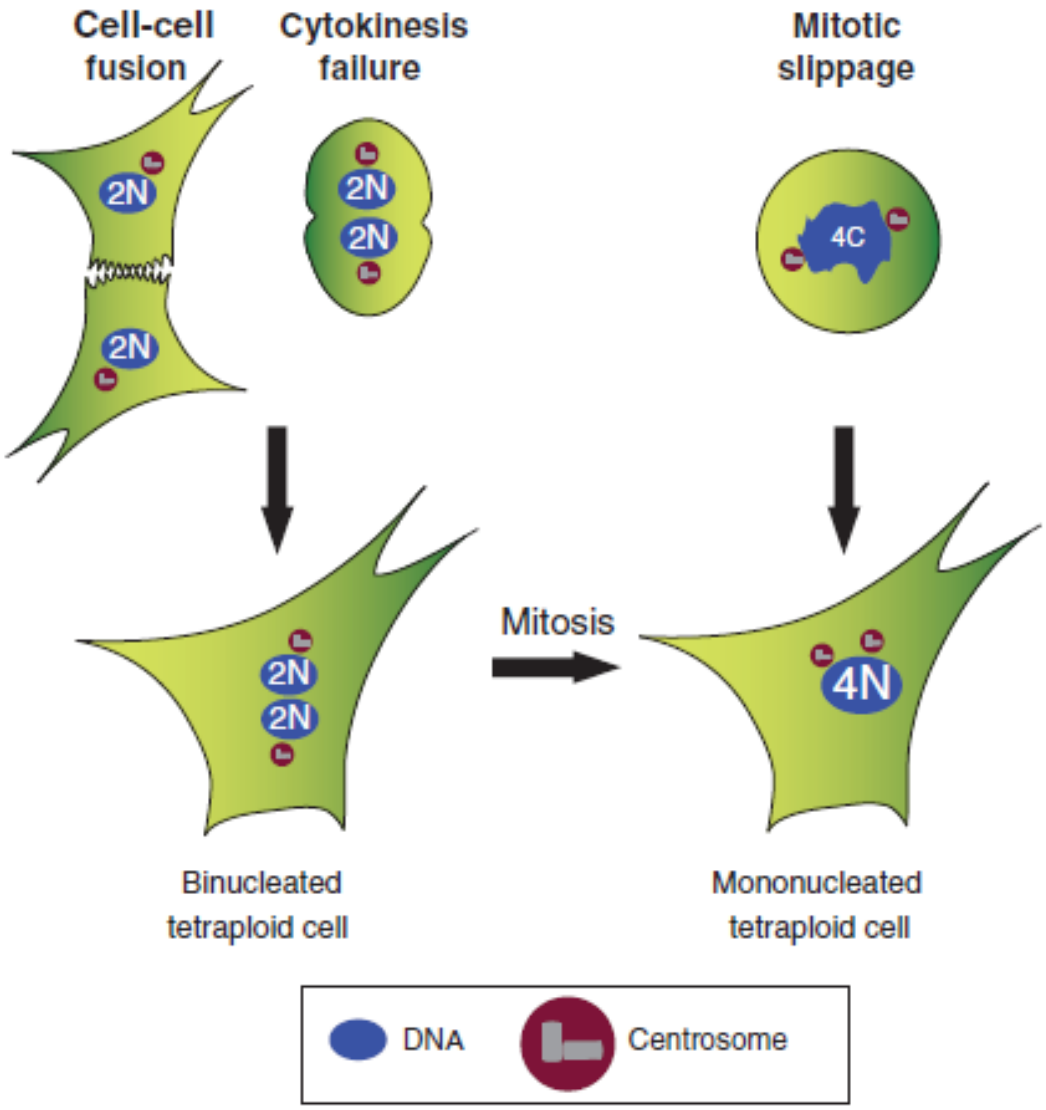

Figure 1.7 Tetraploid cell creation

If a cell fails cytokinesis, it will be binucleate as the cell will have underwent telophase and the nuclear envelope would have reformed around the separated DNA. If a cell has slipped from mitosis, this cell would have been halted at prometaphase and the cell would have only one nucleus, though this nucleus may be deformed (Storchova and Kuffer, 2008).

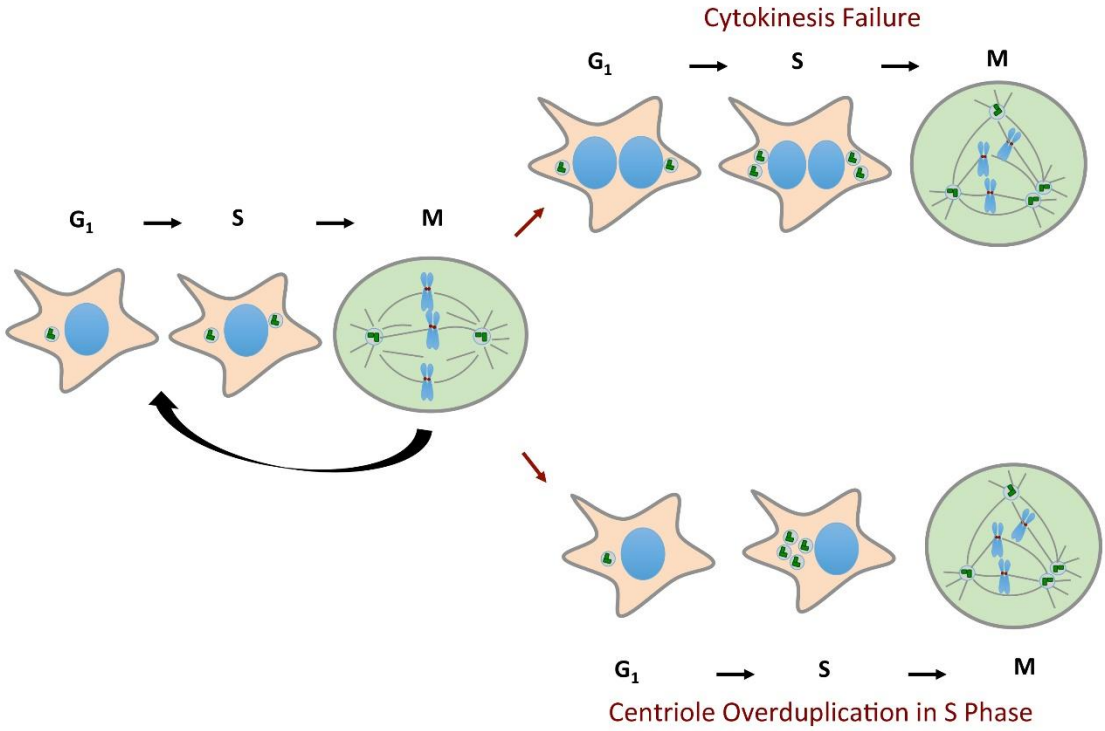

Figure 1.8 Centrosome amplification

Extra centrosomes predominantly arise from cytokinesis failure or centriole over duplication. Following cytokinesis failure, two centrosomes are present in G_1 interphase cells. These centrosomes replicate during S-phase and the resulting 4 centrosomes promote multipolar cell division during the next mitosis. Abnormal centriole duplication arises during S-phase and can produce a variable number of centrosomes in the subsequent mitosis (Bolgioni and Ganem, 2016).

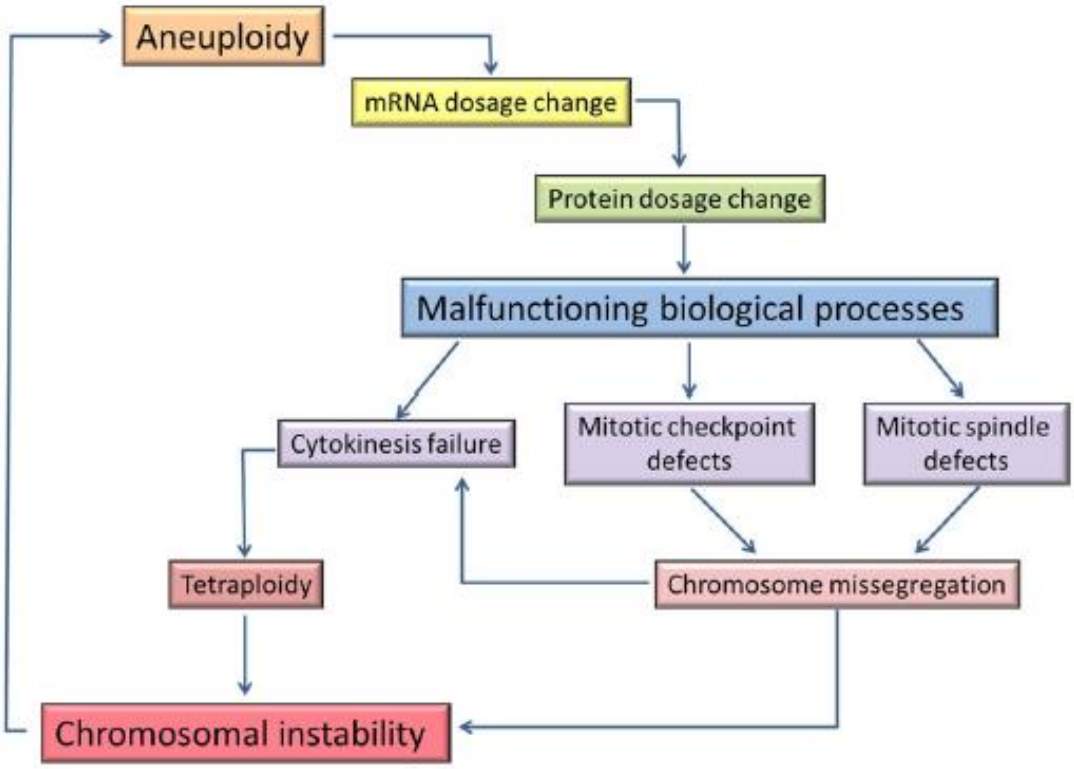

Figure 1.9 Tetraploid to aneuploid cycle

Through CIN, tetraploidy is often a precursor to aneuploidy, during processive cell cycles different chromosomes are more readily lost or gained as the cell has twice as many chromosomes to segregate thus increasing the error burden. The aneuploidy resulting from this can propagate errors in biological processes resulting in increased CIN and continuing this cycle (Potapova et al., 2013).

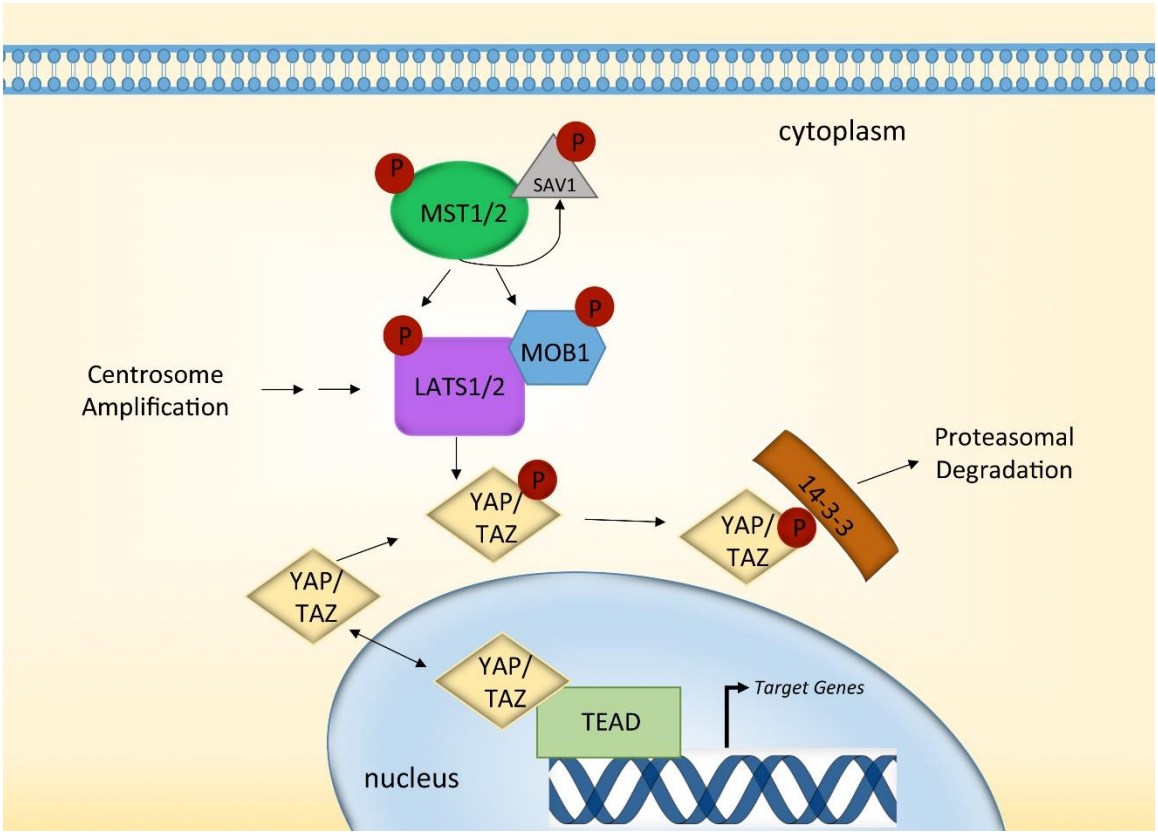

Figure 1.10 Hippo Pathway Signaling

MST1/2 kinases, in complex with the scaffolding protein SAV1, directly phosphorylate LATS1/2 kinases and MOB1. Phosphorylated MOB1 binds to the auto-inhibitory regions of LATS1/2. This binding releases LATS1/2 of their inhibitory state and enables phosphorylation on their activation loops. Together, the coordinated actions of MST1/2 and MOB fully activate LATS1/2. Active LATS1/2 then phosphorylate YAP and TAZ. Phosphorylated YAP and TAZ are sequestered in the cytoplasm through binding to 14-3-3, where they are subsequently proteasomally degraded. Thus, activation of the Hippo pathway prevents YAP and TAZ from entering the nucleus and activating the TEAD-family of transcription factors to initiate the transcription of genes important for cell growth and survival. Centrosome amplification leads to LATS1/2 activation in an MST1/2-independent manner (Bolgioni and Ganem, 2016).

Chapter II: ELUCIDATING THE HIPPO PATHWAY'S ROLE IN TIMING MITOSIS

2.1 Introduction

Mitosis is a very quick and stressful process. The cell is undergoing stress during mitosis because the DNA is condensed, the nuclear envelope is ripped apart and the whole cells is undergoing massive rearrangements. On average, a proliferating human cell takes about 24 hours to grow and divide. Of those 24 hours, mitosis comprises only 20-60 minutes (Yang et al., 2008). During mitosis the cell has to faithfully segregate and divide all replicated chromosomes into two daughter cells. In order to do this efficiently, chromosomes must tightly condense. This condensation prohibits normal transcription (Taylor, 1960b, Prescott and Bender, 1962) and drastically slows translation(Prescott and Bender, 1962). During this stage of the cell cycle, the nuclear envelope is broken down (Gerace et al., 1978). Other organelles such as the Golgi apparatus and endoplasmic reticulum are drastically rearranged (Robbins and Gonatas, 1964), thus affecting vesicle trafficking (Sager et al., 1984). The cytoskeleton radically changes shape and rounds up (Saxton et al., 1984, Kunda and Baum, 2009). During mitosis, the decrease of transcription and translation, the ablation of the nuclear envelope and the rearrangement of organelles is all extremely stressful to the cell. The cell wants to quickly and efficiently get through mitosis to avoid prolonging this stress to the cell and its genetic material.

Because mitosis is stressful, cells have a mechanism to time mitosis to avoid the proliferation of cells that last too long in the stressful stage of mitosis. In 2010, it was

demonstrated that if prometaphase lasts over 90 minutes, the cells born from this prolonged mitosis will arrest in the following G_1(Uetake and Sluder, 2010). Cells may have evolved this timing mechanism because a prolonged mitosis could indicates that the cell had a hard time attaching all of its chromosomes, suggesting some underlying defect. Developing a mechanism to time mitosis and arrest cells that take too long to satisfy the SAC would prevent these cells from continuing to proliferate and propagate said errors.

Mechanistically, daughter cells born from a prolonged mitosis undergo a p38- and p53-dependent G_1 arrest (Uetake and Sluder, 2010). The increase of p53 and p38 results in a subsequent translation and stabilization of p21, which prevents the cells from satisfying the G_1/S transition (Lafarga et al., 2009). Chromosome abnormality and DNA damage would be the typical culprits resulting in the increase of this p53. However, neither chromosome abnormality nor DNA Damage resulted in the increase of p53 caused by a prolonged mitosis (Uetake and Sluder, 2010). The p53 accumulation is through a third and unknown contrivance.

While the mechanisms causing p53 accumulation following a prolonged mitosis are poorly defined, recent findings have begun to unravel this mystery. USP28 and 53BP1 were discovered to stabilize p53 during a prolonged mitosis(Lambrus et al., 2016). When either USP28 or 53BP1 are knocked out, the daughter cells born from a prolonged prometaphase are able to proliferate. In 2016, USP28 and 53BP1 were found to act as part of the centrosome surveillance pathway. It was demonstrated that when centrosomes were depleted in cells, both USP28 and 53BP1. USP28 and 53BP1 stabilize p53 resulting in the increase of p21 when the cell loses centrosomes (Lambrus et al., 2016, Meitinger and

Anzola, 2016). Thus, the centrosome surveillance pathway signals through the USP28-53BP1-p53-p21 axis (Meitinger and Anzola, 2016, Lambrus et al., 2016). Cells without centrosomes take longer to go through mitosis and so the authors confirmed that the ablation of USP28 and 53BP1 rescued proliferation of cells after undergoing a prolonged mitosis by destabilizing p53.

A pathway that is important during mitosis is the Hippo pathway. One of the most obvious changes that occurs when a cell enters mitosis is that the cell rounds up. For adherent cells, the actin cytoskeleton is dismantled and reorganized so the cell can round (Figure 2.1). Interestingly, the Hippo pathway is activated when cells lose their attachment and round up (Halder 2011, Low 2014).

The Hippo pathway was originally found in *Drosophila melanogaster* to control organ size, and this function is broadly conserved (Halder 2011, Low 2014). When the Hippo pathway is activated, upstream kinases phosphorylate and activate LATS1/2. When active, LATS1/2 phosphorylate YAP and TAZ. YAP and TAZ are transcriptional co-activators that bind to TEAD resulting in the transcription of proliferative genes. However, when YAP and TAZ are phosphorylated they are sequestered in the cytoplasm by 14-3-3 and tagged for degradation. In addition to regulating YAP and TAZ, LATS2 also acts as a regulator of p53 (Figure 2.2). When LATS2 is phosphorylated and activated, it can bind and inhibit MDM2. MDM2 is an E3 ubiquitin ligase that tags p53 for degradation. p53 is continuously transcribed and translated and then tagged for degradation for MDM2. When MDM2 is inhibited by LATS2, it is no longer able to tag p53 for degradation. Thus, when LATS2 is active, p53 accumulates in the cell restraining

growth of cells (Aylon et al., 2006). These mechanisms work in concert to effect Hippo signaling whose ultimate output is to halt cellular proliferation.

Because of the Hippo pathway's ability to arrest cellular proliferation and its intrinsic ties to mitosis, we hypothesized that the unknown mechanism by which cells are able to time mitosis was through Hippo signaling. We proposed that when the cells round up and enter mitosis, the Hippo pathway is activated, leading to both decreased YAP and TAZ levels and a concomitant stabilization of p53 levels. Together, these outputs will prevent the subsequent proliferation of the resulting daughter cells, thus providing a molecular mechanism to functionally time mitotic duration.

2.2 Method:

2.2.1 Cell Culture

hTERT-RPE-1 (RPE)and FT293 cells were grown and maintained below 90% confluence in phenol red free DMEM F:12 (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (ThermoFisher) with 100 IU/mL Penicillin and 100 µg/mL Streptomycin (ThermoFisher). All control cells and CRISPR cells were passage matched. CRISPR LATS1/2 cells were passaged at 1:2, and RPE cells and CRISPR empty vector cells were passaged 1:10. Cells were grown at 37ºC with a 5% CO_2 atmosphere.

2.2.2 Generation of Cell Lines

FT293 cells were transiently transfected with either YAP-5SA PBABE-PURO, TAZ-4SA PLVX-PURO, CRISPR Empty Vector lentiCRISPR v2 plasmid expressing

CAS9, LATS2 lentiCRISPR v2 plasmid expressing CAS9 (guide sequence: GTGCCCGATTCATTAGCAAA), or LATS1 lentiCRISPR v2 plasmid expressing CAS9 (guide sequence: GGTTTCATGCTGGCATTAAT) viral vectors along with the appropriate packaging plasmids and PEI. 48 hours post transfection the supernatant of FT293 cells was collected and filtered. Polybrene (10 µg/mL) (Santa Cruz Biotechnology) was added to the filtered supernatant containing the virus. The virus containing the polybrene was added to the RPE cells or FUCCI RPE cells for 14 hours. The virus was then removed from the cells and the replaced with normal media to allow the cells to recover for 24 hours. Following the 24 hour recovery period selection media was added to the cells containing Puromycin (10 µg/mL) (Santa Cruz Biotechnology. Selection media was kept on the cells for 1 week and the media was replaced every 3 days. Cells were single cell cloned in 96-well plates

2.2.3 RNAi transfection

Cells were counted and plated at 7,000 on a 12-well glass bottom dish (Mattek). The next day cells were transfected with 50nM siRNA using Lipofectamine RNAiMax according to manufacturer's instructions. Videos were started 72 hours post transfection.

2.2.4 Mitotic Shake-off:

RPE Empty Vector and LATS1/2 CRISPR cells were plated on 10cm dishes and grown to 80% confluence. Cells were treated with RO-3306 (7µg/mL) for 12 hours. RO-3306 was washed off in 5 minute washes in DMEM repeated four times. Then washed into Monastrol (100uM) containing complete media. Cells were in Monastrol for 1 or 4

hours. Following the time in Monastrol, mitotic cells were vigorously shaken off the plate (hearing protection is recommended for this step). Following the shake off cells were counted. The mitotic cells in media was split 5 ways, one part was placed in a 15mL conical tube and the other 4 parts were placed in a 50 mL conical tube and centrifuged at 1200 RPM for 5 minutes. The mitotic sample was then collected in 1X cell Lysis Buffer (2% w/v SDS, 10% Glycerol, 60 mM Tris-HCl) supplemented with 1X HALT protease and phosphatase dual inhibitor cocktail (ThermoFisher) and immediately stored at -80ºC. The remaining 4 parts were then resuspended in 5 mL complete media and washed 3 times by centrifugation (1200 RPM for 5 minutes), ensuring to add fresh media and use new conical tubes each time. Following the washes, cells were resuspended in 4 mL complete media. Cells (in 1 mL media) were plated on a 6-well poly-D-lysine coated plate. Once plated the 6-well dish was centrifuged at 700 RPM for 3 minutes. Cells were collected at 1.5, 3, 6, and 9 hours after centrifugation. For collection cells were rinsed twice with ice-cold PBS and lysed immediately with 1X Cell Lysis Buffer (2% w/v SDS, 10% Glycerol, 60 mM Tris-HCl) supplemented with 1X HALT protease and phosphatase dual inhibitor cocktail (ThermoFisher) and immediately stored at -80ºC.

2.2.5 Western Blot:

Protein samples stored in -80C were put on ice and sonicated for 20 seconds at 20 kHz. Then 4X Laemmli buffer (Boston Bioproducts) was added to the protein sample and diluted to 2X. Following the dilution the samples were touch vortexed then centrifuged for 20 seconds at max speed. The samples were then boiled at 95ºC for 5 minutes. After the samples cooled, the mitotic samples were pre-cleared. Pre-clearing involved

centrifuging the samples at 17,000 x g at 4ºC for 30 minutes. The supernatant resulting from the centrifuge was collected and used to assess via western blot.

The protein samples (8-10 µL) were then loaded on a 10% acrylamide gel. Loading was assess by GAPDH or actin and was adjusted based on variability in quantitative densitometry after running the first gel. The BioRad electrophoresed slowly at 25 mA per gel, until the samples migrated through the resolving gel.

The gel was then transferred to a PVDF membrane (Bio-Rad). The blot was transferred overnight at 30 mA at 4ºC. Following transfer, membranes were blocked in TBS-0.5% Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.5% Tween-20) with 5% non-fat dried milk for 1 hour. Following blocking the membrane was then incubated with primary antibodies diluted in 1% non-fat dried milk in TBS-0.5% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated species-specific secondary antibodies (Cell Signaling) and ECL Prime (GE Amersham), Clarity ECL blotting substrate (Bio-Rad) or Clarity Max ECL blotting substrate (Bio-Rad). The blots were then imaged using the ChemiDoc XRS+ imaging system (Bio-Rad). The blots were quantitated using ImageLab software (Bio-Rad).

2.2.6 Live Cell Imaging

RPE-FUCCI 2.0 (including CRISPR LATS1/2 and empty vector CRISPR) cells were grown in individual wells on glass-bottom 12-well tissue culture dish (Mattek). To assess proliferation after a prolonged mitosis, cells were treated with paclitaxel (1nM), Monastrol (100µM) or DMSO. Multiple points of cells in each condition were imaged every 10 minutes for 72 hours on a Nikon TE2000-E2 inverted microscope equipped with

the Nikon Perfect Focus system. The Nikon was enclosed in an environmental chamber so the cells were kept at 37ºC and 5% humidified CO_2 for the entirety of the experiment. Fluorescence and phase contrast images were captured through a 10X 0.5 NA Plan Fluor objective. After 4 hours, the video was paused while each well underwent a series of washes 5 minutes with 2 mL complete and warm media repeated four times. Once the washes were complete the video was restarted. Following the completion of the video, the files were blindly quantified using NIS-Elements software.

2.3 Results

2.3.1 The Hippo Pathway is Activated Upon Mitotic Entry

We set out to identify the molecular mechanisms by which cells time mitotic duration. Because one of the most obvious changes that occur when cells enter mitosis is they round up and lose attachment to their surrounding cells and underlying substrate (Figure 2.1), a process that activates Hippo signaling, we asked whether Hippo pathway activation could provide a mechanistic basis for timing mitosis. We therefore assessed Hippo pathway activity upon entry into mitosis.

To assess Hippo pathway activity in mitotic cells, we first arrested RPE cells in mitosis for 1 or 4 hours through the addition of the antimitotic drug Monastrol. Mitotic cells were then collected by a shake off (Figure 2.3). We also collected interphase cells. Mitotic and interphase samples were immunoblotted for phosphorylated downstream Hippo pathway components LATS and YAP to determine Hippo pathway activation in mitosis. Our results revealed that cells in mitosis for 1 hour have a significant increase of

phosphorylated LATS when compared to interphase RPE cells (Figure 2.4) indicating strong activation of the Hippo pathway. This Hippo pathway activation is sustained during prolonged mitosis, as cells in mitosis for 4 hours maintain a significant increase of phosphorylated LATS in comparison to interphase RPE (Figure 2.4).

We then assessed whether YAP, a downstream target of LATS kinase activity, is also phosphorylated in mitosis. Indeed, cells in mitosis for 1 hour exhibited a 5-fold increase in the ratio of phosphorylated YAP (S127) to total YAP relative to interphase cells (Figure 2.5). Phosphorylation of YAP, similar to LATS, is sustained for the duration of mitosis, as cells in mitosis for a prolonged period of time (4 hours) have increased phosphorylated YAP to total YAP ratios when comparing to interphase cells and the phosphorylation is sustained from 1 hour to 4 hours in mitosis (Figure 2.5).

2.3.2 Prolonged Mitosis Reduces G_1 Levels of YAP/TAZ

As phosphorylated YAP is known to be tagged for proteasomal degradation, we aimed to determine if YAP levels decreased following prolonged mitosis, relative to mitosis of a normal duration. We performed a mitotic shake off and then collected cells that were in mitosis for a normal time (1 hour), or a prolonged mitosis (4 hours), and then replated them so that they would complete division and re-enter G1 phase. We subsequently assessed YAP levels in the G1 interphase cells at 1.5, 3, 6, and 9 hours post-mitosis (Figure 2.3). Since the Hippo pathway is active in mitosis we hypothesize that daughter cells entering G_1 following a prolonged mitosis will have less YAP and TAZ than daughter cells born from a normal mitosis.

Indeed, our results revealed that while YAP/TAZ levels in cells born from a normal mitosis (lasting 1 hour) increase as cells progress through G1 phase, cells born from a prolonged mitosis do not show increases in YAP and TAZ levels as cells progress through G1 phase (Figure 2.6).

2.3.3 Restoring Active YAP/TAZ Rescues Cell Cycle Progression after a Prolonged Mitosis

Our data reveal that cells born from a prolonged mitosis do not progress through the cell cycle and exhibit significant reduction in YAP and TAZ levels. We therefore tested whether restoring active YAP/TAZ is sufficient to overcome the arrest and enable proliferation following a prolonged mitosis. We performed live-cell imaging of cells expressing the FUCCI system. The FUCCI system is an ubiquitin-based cell cycle indicator that indicates what phase of the cell cycle the cell is in based on the expression of fluorescently tagged proteins (mcherry-hCdt1 or GFP-Geminin) (Figure 2.7)(Sakaue-Sawano et al., 2008). These proteins are expressed at different times during the cell cycle. hCdt1 is present at high levels during G_1 phase where it acts to license DNA for replication. hCdt1 is ubiqutinated by the E3 ubiquitin ligase SCF^{Skp2} and degraded during $S/G_2/M$ phases to prevent re-replication of DNA (Sakaue-Sawano et al., 2008). By contrast, hGeminin is an inhibitor of Cdt1 whose levels peak during S/G2/M, but is ubiquitinated by the E3 ubiquitin ligase APC^{Cdh1} (Sakaue-Sawano et al., 2008). When a cell bypasses the G_1/S checkpoint and enters S phase it will fluoresce green. The cell will stay green through G_2 and mitosis. Once the cell has divided and enters G_1, it will fluoresce red. If the cell does not proliferate and thus arrests in G_1 the cell will remain red

for the entirety of the video. This red/green readout of proliferation facilitates single-cell tracking in live-cell imaging proliferation studies.

We used multiple approaches to restore YAP/TAZ activity to cells. First, we used RNAi to knock-down LATS1 and/or LATS2. Decreasing the amount of LATS1/2 limits the amount of YAP and TAZ that are phosphorylated and marked for degradation, resulting in more active YAP/TAZ and mimicking Hippo pathway inactivation. Second, we expressed forms of YAP or TAZ (YAP-5SA and TAZ-4SA) that cannot be phosphorylated by LATS (as explained previously) and are thus constitutively active. We wanted to determine whether activation of YAP and TAZ allow daughter cells to proliferate after a prolonged mitosis. Cells with active YAP or TAZ that were also expressing the FUCCI system were treated with an antimitotic agent for 4 hours, then the drug was washed out, and progression through the cell cycle was tracked in each condition (Figure 2.9). As expected, control cells showed significantly impaired proliferation following a prolonged mitosis that was p53-dependent, as p53 depletion was sufficient to restore proliferative capacity (Figure 2.9). Interestingly, cells with active YAP and/or TAZ showed increased proliferation following a prolonged mitosis. Cells with less LATS1 or LATS2 showed a higher percent progression after a prolonged mitosis when compared to control siRNA (Figure 2.9), and there was synergistic response in proliferation when both LATS1 and LATS2 were knocked-down together. Cells with constitutively active YAP or TAZ also showed a robust proliferation following a prolonged mitosis (Figure 2.9). Therefore Hippo inactivation/activation of YAP and TAZ enables proliferation of daughter cells born from a prolonged mitosis.

To validate the proliferation observed in LATS1 and LATS2 knock-down cells, and to control for potential siRNA-mediated off-target effects, we deleted both LATS1 and LATS2 with CRISPR. We created CRISPR LATS1-/- and CRISPR LATS2-/- double knock-out cells. LATS1 and LATS2 CRISPR knock-out cells were treated with an antimitotic for 4 hours, the drug was then washed out, and cells were tracked for the remaining 68 hours. Compared to CRISPR empty vector RPE control cells, both LATS1 -/- and LATS2 -/- cells had at least three times higher proliferation following a prolonged mitosis (Figure 2.7). LATS2 knock-out cells had a close to 30% increased proliferation rate over LATS1 knock-out cells.

2.3.4 Knocking Out LATS1/2 Stabilizes YAP and TAZ Levels Following a Prolonged Mitosis.

We wanted to see what the YAP/TAZ proteins levels looked like in LATS1/2 knock out cells since we saw that these cells proliferated more following a prolonged mitosis. We demonstrated that YAP and TAZ are more stable in the CRISPR LATS1/2 cells following a prolonged mitosis (Figure 2.11A). We showed that following a prolonged mitosis, there is no decrease in YAP or TAZ protein levels when compared to cells born from a normal mitosis (Figure 2.11 B, C and D). Therefore, knocking out LATS1/2 is sufficient to stabilize YAP and TAZ protein levels following a prolonged mitosis. This stabilization of YAP and TAZ resulted in the ability of the cells to proliferate following a prolonged mitosis.

2.3.5 LATS1/2 Knock-out Prevents p53 Accumulation Following a Prolonged Mitotic Arrest

We next determined the effect LATS1/2 knock-out has on p53 levels following a prolonged mitosis. Cells that have undergone a prolonged mitosis are known to accumulate p53 (Lambrus et al., 2016, Uetake and Sluder, 2010). Previous research from our lab and others has demonstrated that LATS2 inhibits MDM2 resulting in the accumulation of p53 (Aylon et al., 2006, Ganem et al., 2014). We therefore hypothesized that activated LATS acts to arrest cells born from a prolonged mitosis by also stabilizing p53 in addition to destabilizing YAP/TAZ. (Figure 2.2).

We found a time-dependent increase in p53 levels in cells following both a normal and prolonged mitosis. Empty vector RPE cells show an increase of p53 at 3 hours in populations of daughter cells born from 1-hour and 4-hour mitoses (Figure 2.12). In daughter cells born from a normal mitosis the level of p53 decreases the longer the cells are in G_1 whereas p53 levels in cells born from a prolonged mitosis continue to increase. In contrast to control cells, LATS1/2 CRISPR RPE cells exhibited lower levels of p53 following prolonged mitosis (Figure 2.11). Thus, our data suggest that mitosis activates the Hippo pathway, and prolonged mitosis leads to destabilization of YAP/TAZ as well as stabilization of p53. We further demonstrated that inactivation of the Hippo pathway is sufficient to restore proliferation after prolonged mitosis.

2.4 Discussion

We have demonstrated that when a cell enters mitosis the Hippo pathway is activated. LATS is phosphorylated upon entry into mitosis, and this results in the subsequent phosphorylation and inactivation of YAP. LATS is not the only upstream kinase phosphorylating YAP in mitosis. Interestingly it has been shown that YAP and TAZ are also phosphorylated by CDK1 during mitosis (Yang et al., 2013, Zhang et al., 2015b, Bui et al., 2016).

To that end, the robustness in proliferation we see in the YAP-5SA and TAZ-4SA FUCCI cell lines following a prolonged mitosis could suggest that LATS1/2 are not the only proteins modulating the phosphorylation and degradation of YAP and TAZ during mitosis. As CDK1 also can phosphorylates YAP and TAZ (Zhang et al., 2015b, Yang et al., 2013, Bui et al., 2016)

Not only are there multiple potential regulators of YAP/TAZ, but our experiments also revealed that LATS1/2 are capable of compensating for one another and responded to our assays in a dose dependent fashion. As the proliferation in LATS1 or LATS2 knock-down cells following a prolonged mitosis were not as robust as the LATS1/2 double knocked down cells. This makes logical sense: when LATS1 is knocked down, LATS2 can compensate and vice versa, so knocking down both LATS1 and LATS2 would prevent one of the proteins from compensating for the other (Yabuta et al., 2013). In a similar assay, there was a dramatic increase in proliferation when comparing CRISPR knock-out of LATS1 and LATS2 versus RNAi knock-down of LATS1, LATS2,

or LATS1/2 in FUCCI RPE cells which could be attributed to the lack of efficiency in knocking down these proteins.

LATS1/2 CRISPR knock-out RPE cells have a lower basal level of p53 than their passage matched empty vector cells. CRISPR-CAS9 creates double strand breaks to remove targeted portions of the genome. The double-strand breaks initiated by CRISPR-Cas9 results in the activation of a DNA damage response resulting in the accumulation of p53. CRISPR-Cas9 in RPE cells causes a p53-mediated DNA damage response resulting in cell cycle arrest (Haapaniemi et al., 2018). This arrest selects against RPE cells with a functional p53 pathway(Haapaniemi et al., 2018). CRISPR-Cas9 selects cells with lower if not inefficient p53, which could explain the decrease in p53 levels we see in the LATS1/2 DKO cell lines(Haapaniemi et al., 2018).

We conclude that cells born from a prolonged mitosis have decreased YAP and TAZ protein levels. This result is not surprising, as the Hippo pathway is active for the duration of mitosis. The longer the cell is in mitosis, the longer the Hippo pathway is active, and the more YAP and TAZ are phosphorylated and tagged for degradation. This means when the cells enter the following G_1 after a prolonged mitosis they stand at a proliferative disadvantage due to diminished YAP and TAZ and increased p53.

2.5 Conclusion

We have demonstrated the robust activation of the Hippo tumor suppressor pathway upon entry into mitosis. This occurs as a result of cells rounding up and rearranging their actin cytoskeleton when they enter mitosis. Cells in mitosis also lose

attachment to surrounding cells. Both the rearrangement of the cytoskeleton and loss of attachment results in the activation of the Hippo pathway(Wada et al., 2011b).

We performed a mitotic shake-off and found that RPE cells born from a prolonged mitosis have altered protein expression in comparison to cells born from a normal mitosis. Cells born from a prolonged mitosis have decreased YAP and TAZ protein levels. In addition to depleted YAP/TAZ, these RPE cells born from a prolonged mitosis also have increased amounts of p53. Cells born from a prolonged mitosis are being halted by two mechanisms: a diminished pro-growth signal due to loss of YAP and TAZ as well as an increased arrest due to the stabilization of p53.

We found that inactivation of the Hippo pathway both by depleting LATS1/2, or by overexpressing constitutively active forms of YAP and TAZ, are sufficient to restore proliferation to cells born from a prolonged mitosis. We tracked the proliferation of individual daughter cells through the FUCCI system, and found that restoring YAP and TAZ activity in cells allowed them to proliferate after undergoing a prolonged mitosis. We also verified that YAP and TAZ were indeed stabilized after a prolonged mitosis in LATS1/2 CRISPR RPE cells.

It has been previously shown that cells born from a mitosis lasting longer than 90 minutes arrest in the following G_1. However, the molecular mechanism of this arrest is not well characterized. We believe that the physical rounding of a cell acts as the initiation event to begin the timing of mitosis via Hippo pathway activation. The longer the cell is in mitosis, the longer the Hippo pathway will be sustained resulting in decreased amounts of YAP and TAZ in the following G_1 phase. Additionally, the longer

the cell is in mitosis sustained activation of LATS2 results in increased amounts of p53 in the following G_1. Thus, activation of the Hippo pathway in a prolonged mitosis results in two barriers which the cell must overcome to proliferate and bypass the G_1/S transition.

Through single-cell tracking and population-based protein analysis, we have found that inactivation of the Hippo pathway enables the proliferation of cells born from a prolonged mitosis. Hippo pathway inactivation in these cells results in stabilization and activation of YAP and TAZ which drives cellular proliferation (Figure 2.12). This data validates that the Hippo pathway plays a role in the molecular mechanism of timing mitosis.

These findings have broad and significant implications in cancer cells, which often experience prolonged mitosis, and may explain one of the selective pressures to inactivate the Hippo pathway in tumor cells. Since many cancer cells undergo a prolonged mitosis, a potential therapeutic option would be to activate the Hippo pathway in cancer cells so they would no longer proliferate following their prolonged mitosis. This is a novel biological role for Hippo signaling that provides another mechanism for a previously unexplained phenomenon occurring in the timing of mitosis.

2.6 Figures

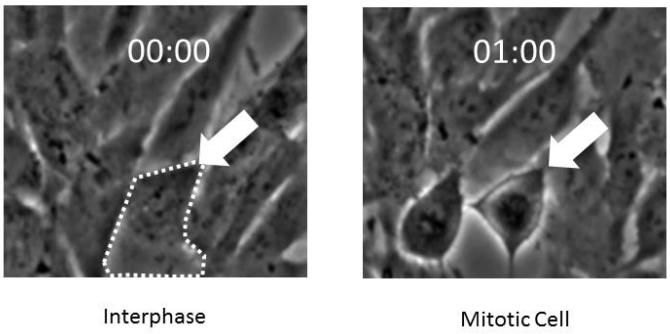

Figure 2.1 Mitotic cells round up

When a cell is in interphase (outlined in the dotted line) the cell is flat and has contact with other cells. As the same cell (indicated by the white arrow) enters mitosis 1 hour later, the actin-cytoskeleton rearranges and the cell rounds.

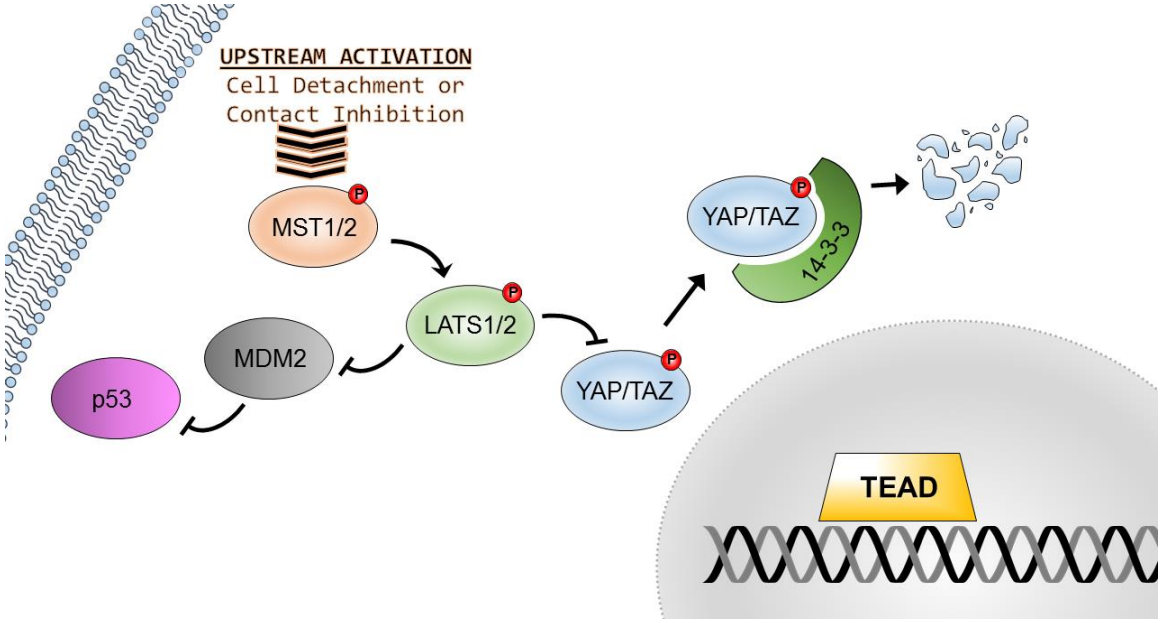

Figure 2.2 LATS role in p53 accumulation

When LATS2 is phosphorylated and activated, it can inhibit MDM2. MDM2 is an E3 ubiquitin ligase that tags p53 for degradation. P53 is continuously transcribed and translated and then tagged for degradation for MDM2. When MDM2 is inhibited by LATS2, it is no longer able to tag p53 for degradation. Thus when LATS2 is active, p53 accumulates in the cell restraining growth of cells.

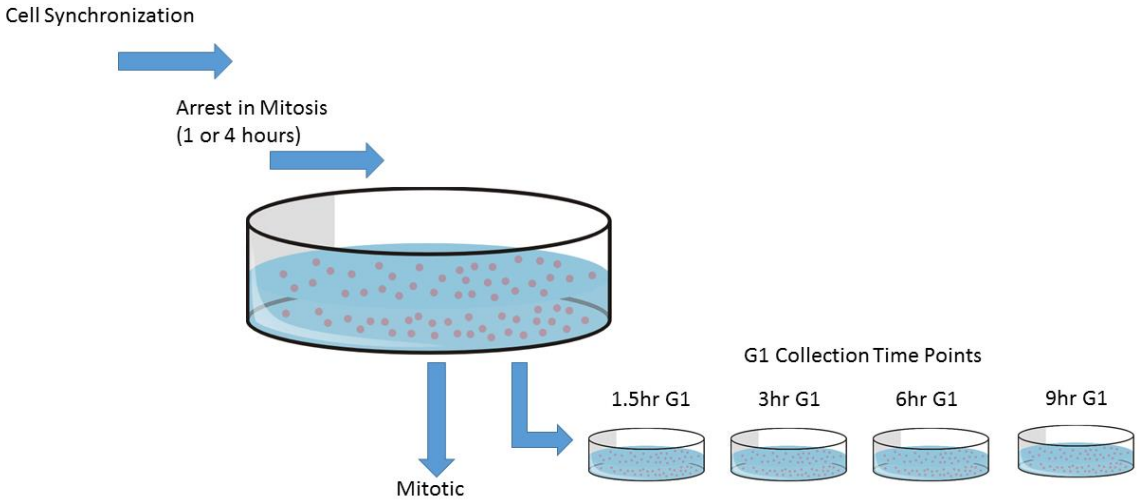

Figure 2.3 Mitotic shake off protocol

Cells were treated with a CDK1 inhibitor and arrested at the G2/M border to synchronize the cells. The CDK1 inhibitor was washed off. Then the cells were treated with an antimitotic. Cells were treated with the antimitotic for 1 or 4 hours. Following the time in antimitotic, mitotic cells were vigorously shaken off the plate. The collected mitotic cells were split 5 ways. Part of the mitotic cells were collected directly. The antimitotic was washed off the remaining cells and these cells were replated to be collected at different time points during G_1.

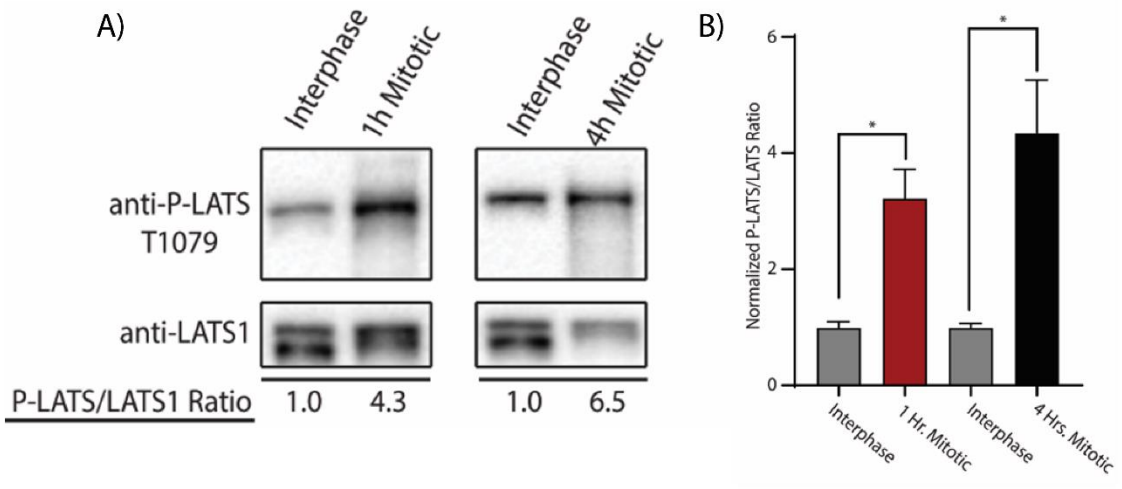

Figure 2.4 LATS mitotic phosphorylation

A) Cells in mitosis for 1 hour have a significant increase of phosphorylated LATS (Threonine 1079) when compared to interphase RPE cells. The phosphorylation of LATS is sustained during a prolonged mitosis as cells in mitosis for 4 hours also have a significant increase of phosphorylated LATS in comparison to interphase RPE. B) Quantification of immunoblots normalizing pLATS to LATS1. Quantitative immunoblots were run in triplicate. The ratio paired t-test showed statistical significance ($p=0.0138$).

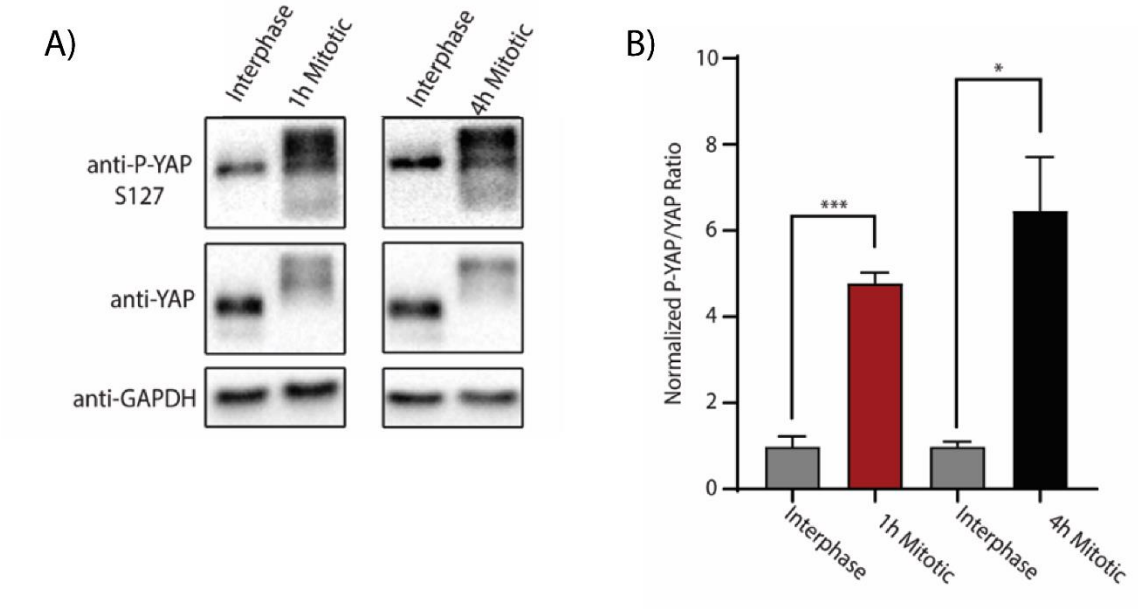

Figure 2.5 YAP mitotic phosphorylation

A) Cells in mitosis for 1 hour have a significant increase in phosphorylated YAP (S127) in comparison to RPE interphase cells. Cells in mitosis for 1 hour have about a 5 fold increase of phosphorylated YAP to YAP when comparing to interphase cells. The phosphorylation of YAP is sustained for the duration of mitosis as cells in mitosis for a prolonged period of time (4 hours) have increased phosphorylated YAP to YAP ratios when comparing to interphase cells. B) Quantitation of normalized pYAP/YAP ration. Quantitative immunoblots were run in triplicate. The ratio paired t-test showed statistical significance (p=0.0014).

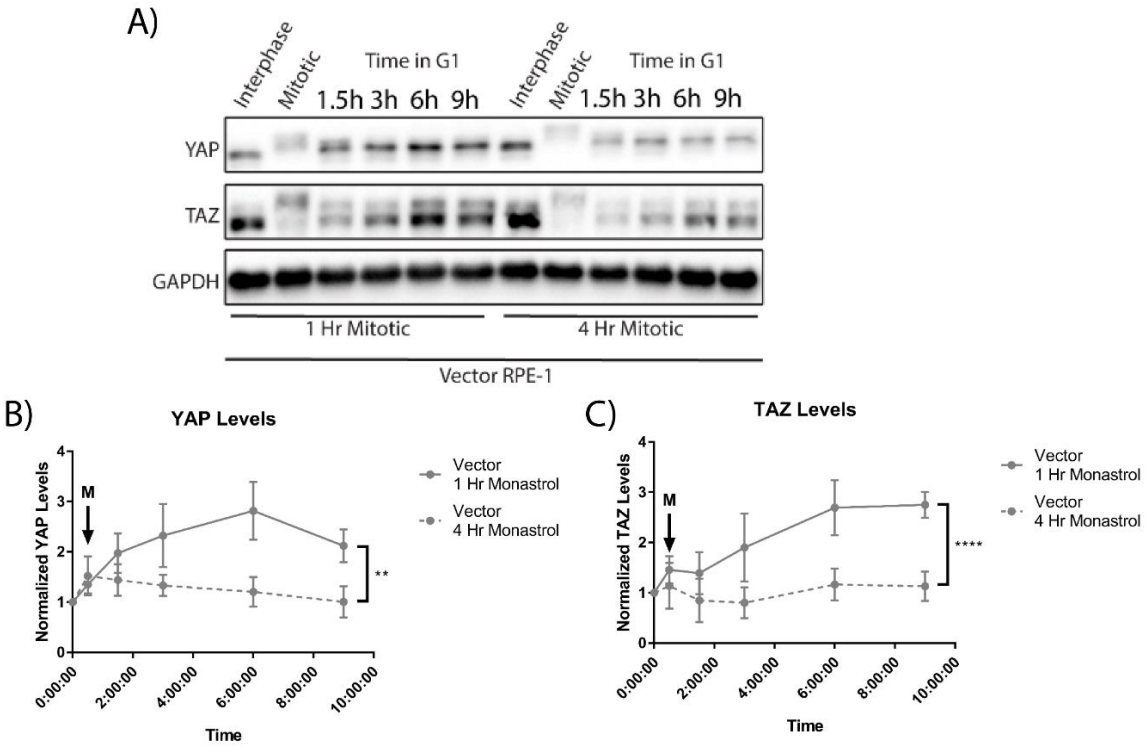

Figure 2.6 Long mitosis deplete YAP&TAZ

A) Cells born from a normal mitosis do not have a decrease in YAP or TAZ protein levels in the following G_1. However, cells born from a prolonged mitosis have decreased amounts of YAP and TAZ in the following G_1. B) Quantification of YAP protein levels following a normal and prolonged mitosis. Cells born from a prolonged mitosis (4 hours) have significantly less YAP than cells born from a normal mitosis (1 hour). C) Quantification of TAZ levels following mitosis. Cells born from a prolonged mitosis (4 hours) have significantly less TAZ then cells born from a normal mitosis (1 hour). A 2-way ANOVA with multiple comparison and a Holm-Sidak post-hoc test was run for significance.

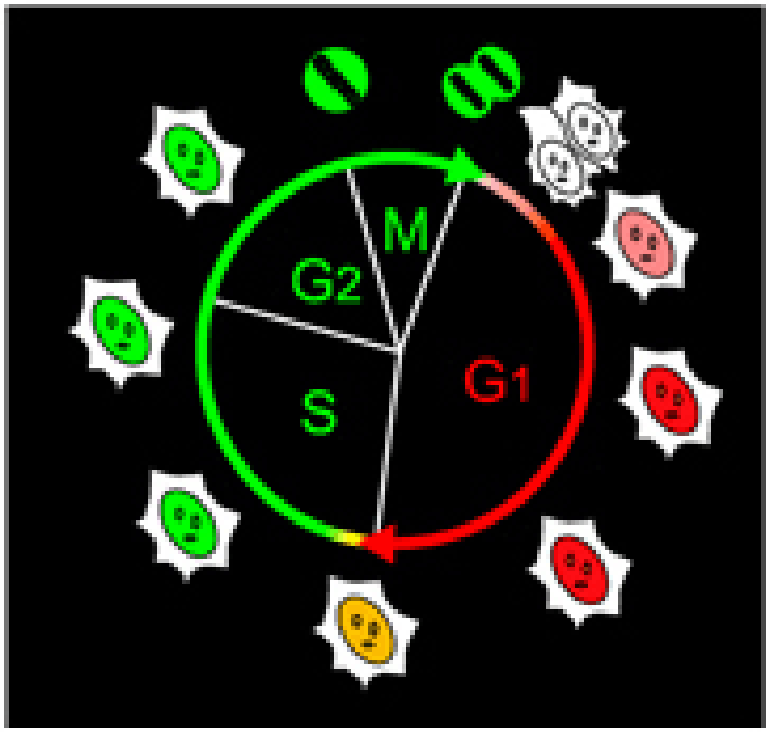

Figure 2.7 The FUCCI System

The FUCCI system is an ubiquitin-based cell cycle indicator that makes the cells express mcherry or GFP depending on the stage of the cell cycle they are in. When a cell bypasses the G_1/S checkpoint and enters S phase it will fluoresce green. The cell will stay green through G_2 and mitosis. Once the cell has divided and entered G_1 it will fluoresce red (Sakaue-Sawano et al., 2008).

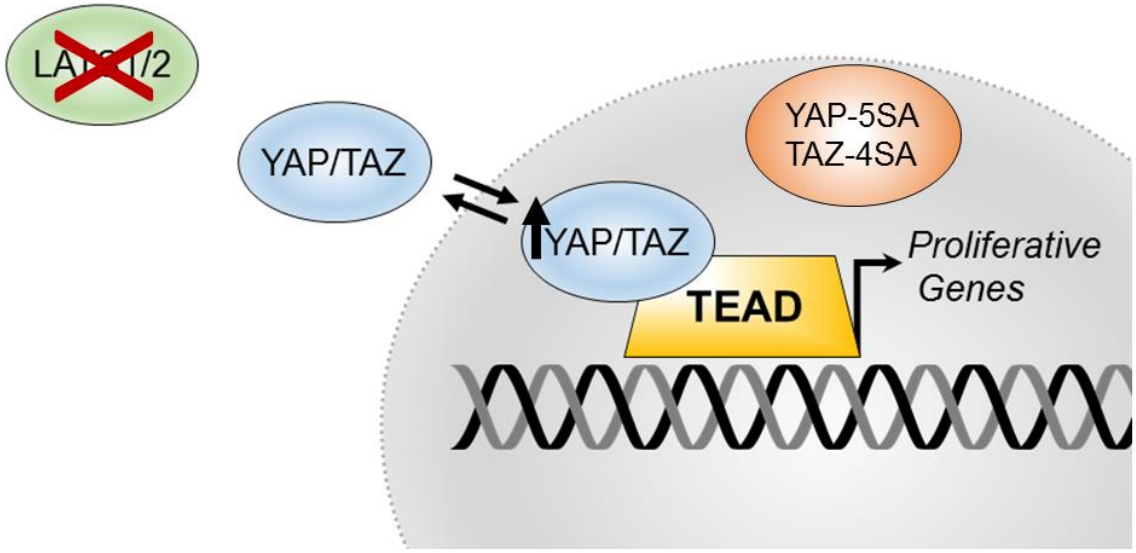

Figure 2.8 Inactivating Hippo pathway

Decreasing the amount of LATS1/2 will limit the amount of YAP and TAZ that are phosphorylated and marked for degradation. Additionally, constitutively active forms of YAP and TAZ where sites in which upstream kinases would phosphorylate can be mutated. The mutation of five serine sites on YAP and four serine sites on TAZ to alanine prevent these proteins from being sequestered in the cytoplasm and degraded. In these cases, more YAP and TAZ will be active and enter the nucleus. When YAP and TAZ are in the nucleus they will act as transcriptional co-activators and transcribe pro-growth and pro-proliferation genes.

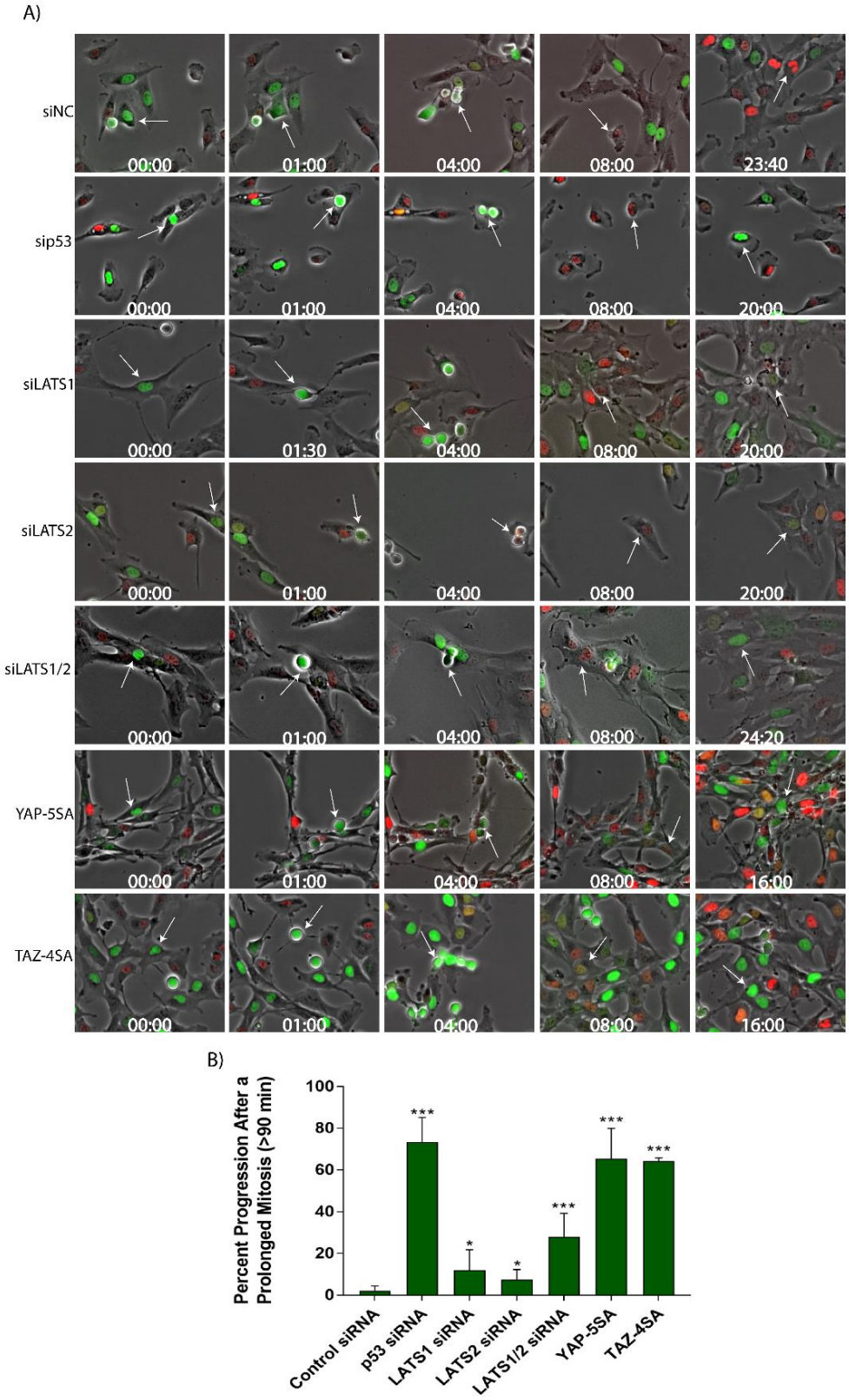
A)
siNC
00:00
01:00
04:00
08:00
23:40
sip53
00:00
01:00
04:00
08:00
20:00
siLATS1
00:00
01:30
04:00
08:00
20:00
siLATS2
00:00
01:00
04:00
08:00
20:00
siLATS1/2
00:00
01:00
04:00
08:00
24:20
YAP-5SA
00:00
01:00
04:00
08:00
16:00
TAZ-4SA
00:00
01:00
04:00
08:00
16:00
B)
Percent Progression After a Prolonged Mitosis (>90 min)
100
80
60
40
20
0
***
*
*
***
***
***
Control siRNA
p53 siRNA
LATS1 siRNA
LATS2 siRNA
LATS1/2 siRNA
YAP-5SA
TAZ-4SA

Figure 2.9 Active YAP/TAZ proliferation

A) RPE-FUCCI cells transfected with control, p53, LATS1, LATS2, or LATS1/2 siRNAs along with cells expressing YAP-5SA and TAZ-4SA were treated with Monastrol to induce mitotic arrest. Cells were held in mitosis for >3hr before the Monastrol was removed to enable anaphase. Proliferation of the daughter cells was monitored over the next 16 hr. White arrows mark individual cells immediately before mitosis (00:00), during mitosis (01:00), and at the onset of anaphase (04:00). White arrows then track one of the two daughter cells through the next cell cycle. Entry into S-phase is monitored by red-green fluorescence. Time, hrs: min. B) Quantification of the percent of cells that turned green following a mitosis lasting longer than 90 minutes.

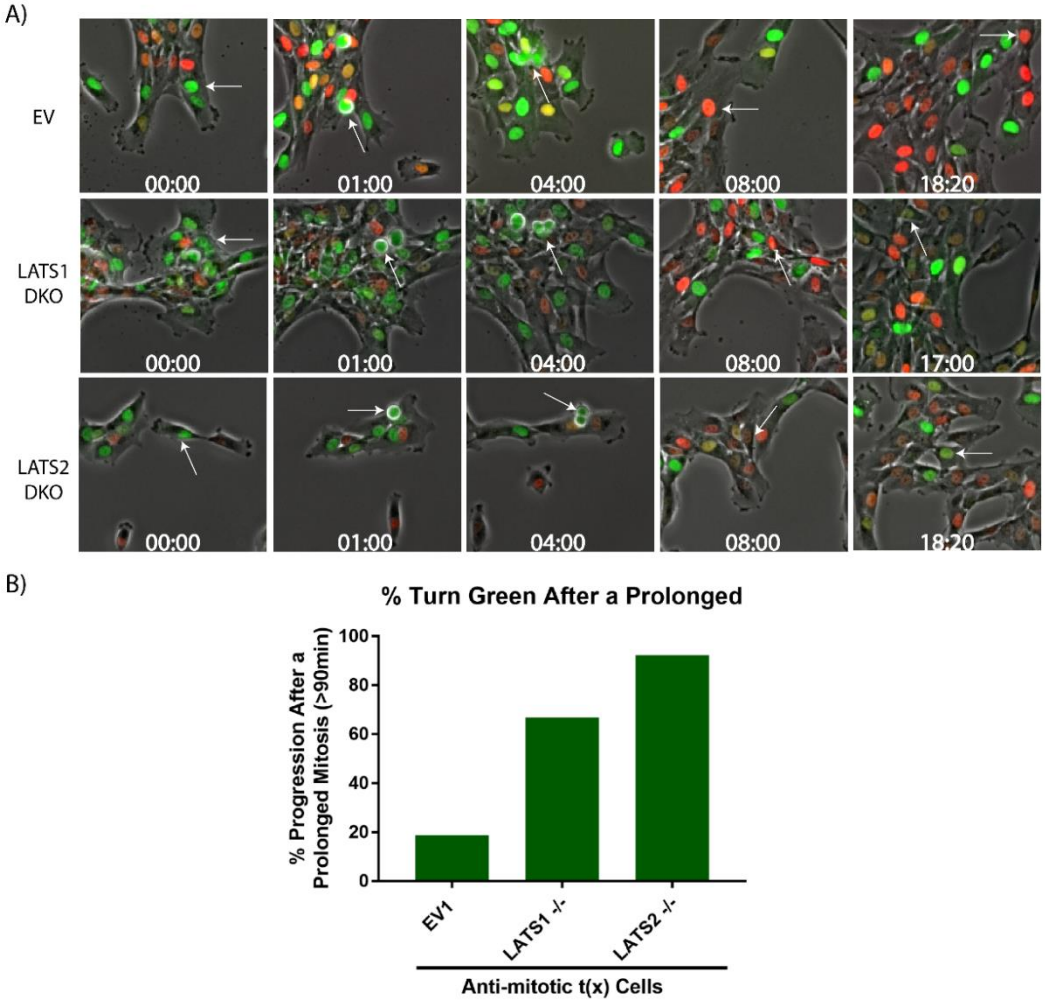

Figure 2.10 CRISPR LATS proliferation

A) LATS1 or LATS2 CRISPR CAS9 knock out RPE-FUCCI cells were treated with Monastrol to induce mitotic arrest. Cells were held in mitosis for >3hr before the Monastrol was removed to enable anaphase. Proliferation of the daughter cells was monitored over the next >16 hr. White arrows mark individual cells immediately before mitosis (00:00), during mitosis (01:00), and at the onset of anaphase (04:00). White arrows then track one of the two daughter cells through the next cell cycle. Entry into S-phase is monitored by red-green fluorescence. Time, hrs: min. B) Quantification of the percent of LATS1 or LATS2 knock out cells that turned green following a mitosis lasting longer than 90 minutes.

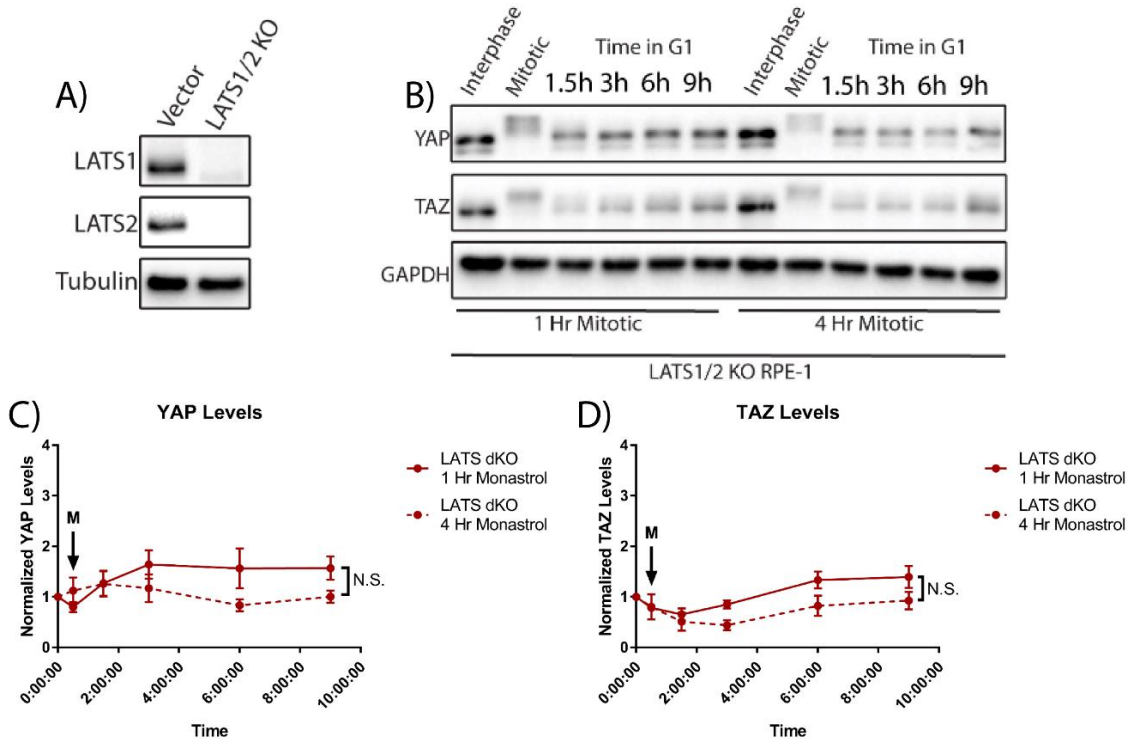

Figure 2.11 LATS KO effect on G1 YAP/TAZ

A) LATS1 and LATS2 were knocked out of RPE cells. B) A representative immunoblot of YAP and TAZ protein levels following a normal mitosis (1hour) and a prolonged mitosis (4 hours) in LATS1/2 RPE CRISPR cells. C) Protein quantification of YAP in n=3 immunoblots, There is no significant difference between YAP levels following a prolonged mitosis (4 hour) or a normal mitosis (1 hour). D) Protein quantification of TAZ in n=3 immunoblots. There is no significant difference in TAZ levels born from a normal (1 hour) versus a prolonged (4 hour) mitosis.

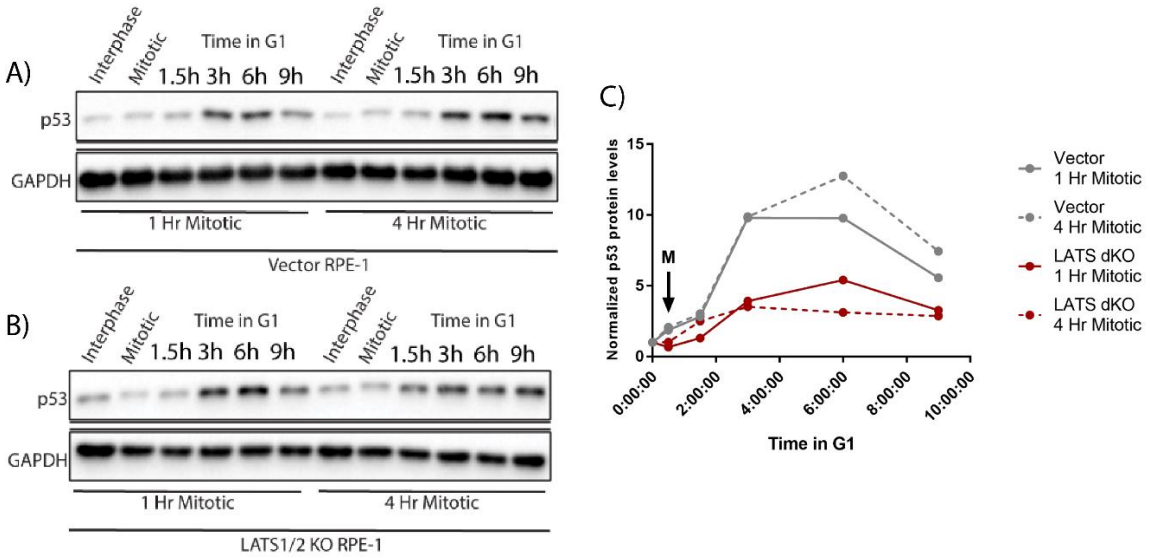

Figure 2.12 LATS KO effect on G1 p53

A) p53 protein levels from Empty Vector RPE cells born from a mitosis lasting 1 hour or 4 hours. B) p53 protein levels from LATS1/2 CRISPR Cas9 knock out RPE cells born from a mitosis lasting 1 hour or 4 hours. C) Quantification of p53 levels in Empty Vector and LATS1/2 knock out cells born from normal and prolonged mitosis.

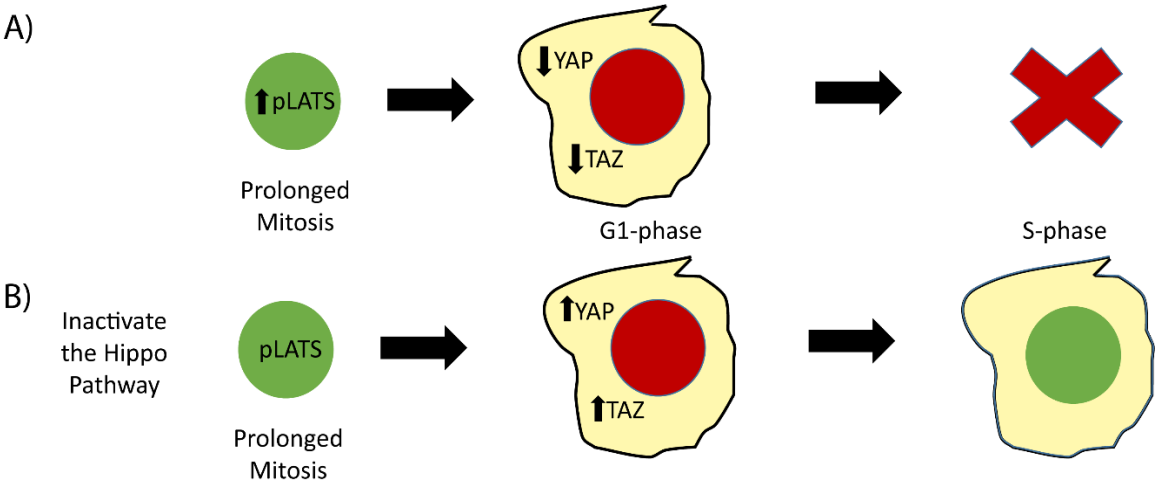

Figure 2.13 Long mitosis proliferation

A) Cells with an active Hippo pathway born from a prolonged mitosis have decreased YAP and TAZ levels in the following G_1 phase and do not continue through the cell cycle. B) If the Hippo pathway is inactive, cells born from a prolonged mitosis will have increased amounts of YAP and TAZ and will continue through the cell cycle.

CHAPTER III: DETERMINING THE ROLE OF YAP AND TAZ'S ACTIVITY IN MITOTIC SLIPPAGE FROM CELLS TREATED WITH ANTIMITOTICS

3.1 Introduction

Antimitotic drugs are highly effective therapeutic agents. When cells are treated with antimitotics such as paclitaxel (Taxol^{TM}), proper bi-orientation of the chromosome does not occur as tension is imperfect across sister chromatids (Pinsky and Biggins, 2005). These therapies halt cells in mitosis until the spindle assembly checkpoint is satisfied. Paclitaxel is an example of an antimitotic chemotherapeutic agent that is used in breast, ovarian, lung, and many other types of cancers (McGuire et al., 1989, Weaver, 2014). While Taxol can be highly efficacious, resistance can emerge (Holmes et al., 1991). Instead of dying in mitosis following treatment of antimitotic drugs, cells can exit mitosis and enter G_1 without undergoing cytokinesis (Figure 3.1) (Gascoigne and Taylor, 2008, Gascoigne and Taylor, 2009). This exit from mitosis is termed cell slippage. When a cell slips from mitosis, it has the capability to continue to proliferate which is a concern for tumor recurrence.

Cells treated with antimitotics can either undergo cell slippage or cell death (Gascoigne and Taylor, 2008, Brito and Rieder, 2009). What determines whether a cell slips or dies from antimitotic treatment is based upon a two-armed degradation race (Gascoigne and Taylor, 2009). During mitosis transcription is halted and translation is slowed down (Taylor, 1960a, Prescott and Bender, 1962). Consequently, there is limited production of new proteins. Since there is no new protein being synthesized when the cell

is arrested in mitosis by antimitotic therapeutics, degradation determines the fate of the cell.

The degradation between two groups of proteins, Cyclin B and antiapoptotic proteins, determines whether a cell slips or dies (Figure 3.2)(Gascoigne and Taylor, 2009). When CDK1 is bound to Cyclin B it phosphorylates targets resulting in the condensation of chromosomes and the breakdown of the nuclear lamina, the degradation of Cyclin B and thus the inactivation of the Cyclin B-Cdk1 complex is required for mitotic exit. Therefore, Cyclin B is critical for keeping cells in mitosis, and when it is degraded past a certain threshold level, cells exit mitosis. If this occurs when the cell is arrested in prometaphase by an antimitotic, then the cell will undergo slippage, meaning it will exit mitosis without dividing.

Antiapoptotic proteins are the other group of proteins critical to deciding cell fate after a prolonged mitosis. Antiapoptotic proteins compete and bind to proapoptotic proteins preventing proapoptotic proteins from creating pores in the mitochondria (Letai et al., 2002, Kuwana et al., 2005, Certo et al., 2006). These pores release cytochrome c resulting in the onset and activation of caspase-dependent apoptotic signaling. The balance of proapoptotic and antiapoptotic proteins is heavily dependent on transcription. BH3 profiling has shown that cells in a prolonged mitosis are primed for cell death (Bah et al., 2014). Additionally, prolonged mitosis has shown to trigger partial apoptosis as cytochrome c is released from the mitochondria activating caspases and thus resulting in DNA damage (Orth et al., 2012). Since cells are primed for death during a prolonged mitosis, they become dependent on antiapoptotic proteins such as Mcl-1, Bcl-xL and Bcl-

w for their survival. Antiapoptotic proteins normally keep cells alive during prolonged mitosis. Thus, if antiapoptotic proteins degrade past a certain threshold then the cell will activate cell death pathways and undergo apoptosis. If a cell has a higher level of antiapoptotic proteins, then the cell will undergo slippage and evade death when treated with an antimitotic. Interestingly, cancer cells often have increased levels of antiapoptotic proteins which could explain the evasion of cell death during prolonged mitosis (Kaufmann et al., 1998, Khoury et al., 2003).

The Hippo pathway is known to regulate antiapoptotic protein levels (Huo et al., 2013, Chen et al., 2018). When the Hippo pathway is off or inactive, YAP and TAZ enter into the nucleus and act as transcriptional co-activators of TEAD, resulting in the transcription of pro-survival and pro-growth genes including antiapoptotic genes Bcl-2, Bcl-xL and Mcl-1(Pan, 2010, Zhao et al., 2010b, Zhao et al., 2011, Yu and Guan, 2013b).

We hypothesized that functional inactivation of the Hippo pathway would therefore increase the levels of antiapoptotic proteins prior to mitosis, thus promoting cell slippage in cells held in prolonged mitosis through antiapoptotic signaling. Indeed, we find that YAP and TAZ activation leads to increased antiapoptotic levels resulting in increased slippage from mitosis in cells treated with antimitotic chemotherapeutics. Importantly, we demonstrate that inhibition of YAP and TAZ in Taxol-resistant cancer cells sensitizes these cells to antimitotic treatment by increasing mitotic cell death.

3.2 Methods:

3.2.1 Cell Culture

All cells were grown and maintained below 90% confluence. RPE, BJ Fibroblasts, MDA-MB-231 and FT293 were grown in phenol red free DMEM F:12 (GIBCO) and were passaged at 1:10 and 1:20 dilutions. ZR7530 and ZR751 cells lines were grown in RPMI (ThermoFisher) and passaged at 1:2 and 1:5 dilutions. HCC1954 cells were grown in RPMI (ThermoFisher) and passaged at 1:5 and 1:10 dilutions. All media except for MCF10A cell media was supplemented with 10% Fetal Bovine Serum (FBS) with 100 IU/mL Penicillin and 100 µg/mL Streptomycin (ThermoFisher). MCF10A cells were grown in DMEM F:12 (GIBCO) supplemented with 5% Horse Serum (Life Technologies), Insulin (10µg/mL)(Gibco), EGF (20ng/mL)(Thermo Fisher), Hydrocortisone (0.5mg/mL), Cholera Toxin (100ng/mL)(Sigma), 100 IU/mL Penicillin and 100 µg/mL Streptomycin (ThermoFisher). After all of the supplements were added to the MCF10A media, the media was sterile filtered (0.2µ) (Millipore). All cells were grown at 37ºC with a 5% CO_2 atmosphere.

3.2.2 Generation of Cell Lines

FT293 cells were transiently transfected with either YAP-5SA PBABE-PURO, YAP-WT PBABE-PURO, TAZ-4SA PLVX-PURO, or TAZ-WT PLVX PURO viral vectors along with the appropriate packaging plasmids and PEI. 48 hours post transfection the supernatant of FT293 cells was collected and filtered. Polybrene (10 µg/mL) (Santa Cruz Biotechnology) was added to the filtered supernatant containing the

virus. The virus containing the polybrene was added to the RPE cells or FUCCI RPE cells for 14 hours. The virus was then removed from the cells and the replaced with normal media to allow the cells to recover for 24 hours. Following the 24 hour recovery period selection media was added to the cells containing Puromycin (10 µg/mL) (Santa Cruz Biotechnology. Selection media was kept on the cells for 1 week and the media was replaced every 3 days. Cells were single cell cloned in 96-well plates

3.2.3 RNAi transfection

Cells were counted and plated at 7,000 on a 12-well glass bottom dish (Mattek). The next day cells were transfected with 58nM siRNA using Lipofectamine RNAiMax (ThermoFisher) according to manufacturer's instructions. Videos were started 48 hours post transfection.

For protein collection cells were counted and plated at 30,000 cells per well in a 6-well dish (Falcon). The next day cells were transfected with 58nM siRNA using Lipofectamine RNAiMax (ThermoFisher) according to manufacturer's instructions. Protein was collected 48 hours post transfection.

3.2.3 Protein Collection

For protein collection, media on cells was aspirated then the cells were rinsed twice with ice-cold PBS and lysed immediately with 1X Cell Lysis Buffer (2% w/v SDS, 10% Glycerol, 60 mM Tris-HCl) supplemented with 1X HALT protease and phosphatase dual inhibitor cocktail (ThermoFisher). Immediately following lysing, 4X Laemmli buffer (Boston Bioproducts) was added to the protein sample and diluted to 1X.

Following the dilution the samples were touch vortexed then centrifuged for 20 seconds at max speed. The samples were then boiled at 95ºC for 5 minutes.

3.2.4 Western Blot

The protein samples (8-10 µL) were then loaded on a 10% acrylamide gel. Loading was assess by GAPDH or actin and was adjusted based on variability in quantitative densitometry after running the first gel. The BioRad box was run at 100V, until the samples migrated through the resolving gel then the speed was increased to 200V.

The blot was then transferred to a PVDF membrane (Bio-Rad). The blot was transferred semi-dry (BioRad) at 1.0A, 25V for 60 minutes. Following transfer, membranes were blocked in TBS-0.5% Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.5% Tween-20) with 5% non-fat dried milk for 1 hour. Following blocking the membrane was then incubated with primary antibodies diluted in 1% non-fat dried milk in TBS-0.5% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated species-specific secondary antibodies (Cell Signaling) and ECL Prime (GE Amersham), Clarity ECL blotting substrate (Bio-Rad) or Clarity Max ECL blotting substrate (Bio-Rad). The blots were then imaged using the ChemiDoc XRS+ imaging system (Bio-Rad). The blots were quantitated using ImageLab software (Bio-Rad).

3.2.5 Live Cell Imaging

RPE, BJ Fibroblast, MCF10A, ZR751, MDA-MB-231, ZR7530, and HCC1954 cells were grown in individual wells on glass-bottom 12-well tissue culture dish (Mattek).

To assess whether a cell slips or dies during a prolonged mitosis cells were treated with paclitaxel (5µM), Monastrol (200µM) or DMSO once for the entirety of the video. Multiple points of cells in each condition were imaged every 10 minutes up to 72 hours on a Nikon TE2000-E2 inverted microscope equipped with the Nikon Perfect Focus system. The Nikon was enclosed in an environmental chamber so the cells were kept at 37ºC and 5% humidified CO_2 for the entirety of the experiment. Phase contrast images were captured through a 10X 0.5 NA Plan Fluor objective. Following the completion of the video, the files were blindly quantified using NIS-Elements software.

3. Results

3.3.1 Hyperactive YAP/TAZ promotes cell slippage from a prolonged mitosis in non-transformed RPE cells.

We sought to examine whether the Hippo pathway regulates cell fate decisions following treatment with antimitotic drugs. To do this, we made RPE cell lines that stably expressed constitutively active forms of YAP and TAZ or overexpressed wild-type versions of YAP and TAZ. These stable cell lines have more active YAP and TAZ that will enter the nucleus (Figure 3.3). We determined the rate of slippage in control cells to be around 70% when treated with an antimitotic (Figure 3.4B). We then inactivated the Hippo pathway and found the rate of slippage to go up by about 20%. Cells with active YAP and TAZ are dying during prolonged mitosis 10-15% less than control cells upon treatment with antimitotic drugs (Figure 3.4C). Increased YAP and TAZ promotes slippage and decreases cell death.

3.3.2 Activation of YAP and TAZ results in increased antiapoptotic levels leading to increased slippage from mitosis.

We determined the molecular mechanism resulting in the increased slippage observed in active YAP and TAZ cell lines. RPE cells expressing constitutively active forms of YAP (YAP-5SA) and TAZ (TAZ-4SA) have a higher expression of antiapoptotic proteins Mcl-1, Bcl-xL and Bcl-w than Empty Vector RPE cells (Figure 3.5A and Figure 3.5B). YAP-5SA had a ~2-fold increase of both BCL-W and MCL-1, with a drastic fourfold increase of BCL-xL over control cells (Figure 3.5B). TAZ-4SA had a ~4-fold increase of BCL-w and MCL-1 with a 5-fold increase of BCL-xL over control cells (Figure 3.5B). These data strongly suggest that cells with active YAP and TAZ have increased slippage rates because they have increased antiapoptotic proteins.

3.3.3 Inhibiting YAP/TAZ or using antiapoptotic inhibitors induce death from a prolonged mitosis in YAP active cells.

We verified that indeed antiapoptotic proteins are critical for causing the YAP active cells to slip instead of undergo cell death when treated with antimitotics by treating the cells with an antiapoptotic inhibitor (Figure 3.6). We found that Navitoclax (ABT-263), which inhibits the Bcl-2, Bcl-xL and Bcl-w proteins(Gandhi et al., 2011, Rudin et al., 2012, Chen et al., 2011), caused a near complete reversal in the number of the cells undergoing cell slippage from mitosis (Figure 3.7A and Figure 3.7B). This validates that the increase in cell slippage in YAP active cells is indeed from the increase in antiapoptotic proteins.

We have shown that cells with an active YAP and TAZ have increased slippage from mitosis, we also demonstrated that decreasing YAP and TAZ will decrease slippage from cells treated with antimitotics (Figure 3.8B). We knocked down YAP and TAZ by treating RPE PLVX Puro (empty vector control) cells with siRNA targeting YAP and TAZ. Depleting YAP and TAZ in these RPE cells resulted in decreased cell slippage by about 50% and increased cell death by about 50 %(Figure 3.8B and Figure 3.8C).

3.3.5 Breast cancer cells are sensitized to antimitotic therapeutics by targeting YAP and TAZ.

We acquired several breast cancer cell lines and performed live cell imaging to determine their sensitivity to Taxol. We found that there was a breast cancer cell line variation in response to the antimitotic Taxol (Figure 3.9). Some breast cancer cells such as ZR7530 and MDA-MB-231 slipped the majority of the time (over 60%) (Figure 3.9). Other breast cancer cells such as HCC1954 never slipped and only died upon Taxol treatment (Figure 3.9). We hypothesized that this variation may be due to Hippo pathway activity. To test this we asked whether the Taxol-resistant breast cancer lines could be sensitized to antimitotic therapeutics by depleting YAP and TAZ. We depleted YAP and TAZ using RNAi in MDA-MB-231 cells (Figure 3.10), which we found slipped more than 60% of the time in response to Taxol (Figure 3.9). When YAP and TAZ are knocked down in these cells, their slippage decreases about 30%, and death during mitosis increases (Figure 3.11 A-C). MDA-MB-231 cells are sensitized to Taxol when YAP and TAZ are depleted more than 60% of MDA-MB-231 cells undergo cell death under these

conditions (Figure 3.11C). MCF10a cells are also sensitized to Taxol when YAP and TAZ is depleted in them, there is a 20% increase in cell death.

3.4 Discussion

When we halt cells in mitosis using antimitotics, we have demonstrated that Hippo pathway activity determines cell fate during mitosis. We have shown that RPE cells expressing an abundance of active YAP or TAZ have increased slippage from the halted mitosis with a corresponding decrease in mitotic cell death. Additionally, cells with decreased YAP and TAZ have a decrease in slippage from mitosis induced by antimitotics accompanied by an increase in mitotic cell death. We have shown that YAP and TAZ activity play a key role in determining whether a cells slips or dies from mitotically arrested cells.

Navitoclax is an orally available antiapoptotic inhibitor that has recently been used for small cell lung cancer to increase cell death (Rudin et al., 2012). However, Navitoclax alone was not an effective therapy, as there was low patient response of 2.6% (Rudin et al., 2012).We have demonstrated that Navitoclax works potently in combination with antimitotic therapeutics in inducing cell death (Figure 3.7). We showed that cell slippage decreases in cells with active YAP by decreasing antiapoptotic proteins, a downstream transcriptional target of active YAP (Figure 3.6). The concern with targeting only a subset of antiapoptotic proteins (Navitoclax targets Bcl-2, Bcl-xL and Bcl-w) is that other antiapoptotics, such as Mcl-1, will result in resistance. Indeed, Mcl-1 inhibitors are currently in the research and development phase for this exact reason

(Leverson et al., 2015, Kotschy et al., 2016). If YAP and TAZ were inhibited, then this could prevent the transcription of pro-survival antiapoptotic proteins and there wouldn't be a need for targeting each individual antiapoptotic protein.

Investigating some of the breast cancer cell line's responses to Taxol after manipulating YAP and TAZ levels in the cells proved to be challenging. ZR7530 had the greatest percentage of cell slippage from mitosis when treated with Taxol; however, we experienced technical difficulties in decreasing YAP and TAZ by RNAi, so we were not able to track the effects of YAP/TAZ depletion in cell fate from Taxol treatment. We hypothesized that expressing YAP and TAZ in cell lines that were prone to cell death such as HCC1954 and ZR751 would result in an increase in cell slippage. However, HCC1954 and ZR751 cells lost their expression of YAP-5SA and TAZ-4SA over time in culture (data not shown), so the experiments of increasing cell slippage in these cells that were prone to cell death were unable to be completed.

In future studies, it will be important to develop and use a therapeutic *in vivo* that can mimic Hippo pathway activation in cells that have inactivated the Hippo pathway. One way would be to target and deplete YAP and TAZ. This could be done by creating nanoparticles that could deliver siRNA targeting YAP and TAZ directly to the tumor in a mouse or patient. Another potential avenue could be to impair the function of YAP and TAZ as transcriptional co-activators of TEAD. Off-label use of Verteporfin is an example of decreasing YAP and TAZ's functionality by increasing 14-3-3 expression, a protein that sequesters YAP and TAZ in the cytoplasm(Wang et al., 2016a). Recently, a group has created a YAP/TEAD inhibitor, CA3. This prevents YAP from binding to and acting

as a transcriptional co-activator with the transcription factor TEAD (Song et al., 2018). Using these tools *in vivo* would allow for further characterization of the Hippo pathways role in mitosis both physiologically and in disease.

Hippo pathway activity could also be used as a biomarker for antimitotic therapeutics. Assessing Hippo pathway activity could be tested before assigning patients to a regimen including antimitotics. To do this, sections of the solid tumor could be biopsied and complete immunohistochemistry for YAP and TAZ localization could be tested for. When cells are in contact with each other, as they are in tumors, they are unable to stretch out and will have decreased stress fibers, this decrease in stress fibers will lead to the loss of inhibition on the Hippo pathway; in other words, the Hippo pathway will be activated (Wada et al., 2011b). When the Hippo pathway is active we would expect the majority of YAP and TAZ to be cytoplasmic. If the patient has an abundance of YAP and TAZ in the nucleus, this would be indicative of Hippo pathway inactivation. Since tumors are heterogeneous, multiple biopsies from different locations will need to be taken in order to determine if any of the clones have inactivated the Hippo pathway. If patients have inactivated the Hippo pathway then alternative treatment options should be assessed. Alternative therapeutic treatments could be a combinatorial therapy with a Hippo activator used in conjunction with antimitotics. This could serve as an alternative to a previously failed Phase I clinical trial where antiapoptotic inhibitors and antimitotics were used in combination due to hematological and non-hematological toxicities (Vlahovic et al., 2014).

Other proteins have been implicated in effecting cell fate in mitosis induced by antimitotics such as Myc which is also a key contributing factor of cell fate (Topham et al., 2015). Instead of affecting antiapoptotic proteins like YAP and TAZ, Myc upregulates clusters of pro-apoptotic BH3-only proteins (Topham et al., 2015). Having increased proapoptotic proteins has a similar effect as having decreased antiapoptotic proteins: the cell will die during mitosis when treated with antimitotics. Understanding the factors that cause cell slippage versus death from mitosis will be critical in creating effective biomarkers. For example, if we know that cells with active YAP and TAZ result in increased slippage, but cells with more MYC increase cell death; biopsies could stain for these proteins to determine if antimitotics are the correct treatment regimen for the patient. If the tumor cells have high MYC and low YAP and TAZ in the nucleus, then antimitotics would potentially be beneficial to the patient. Knowing the basic cell biology will also be crucial for creating new targets for future combinatorial therapeutics to use with antimitotics.

3.5 Conclusion

Cells with hyperactive YAP and TAZ undergo increased slippage from mitosis when treated with antimitotic therapeutics. The increase of slippage in these cells is a result of the increased antiapoptotic proteins Mcl-1, Bcl-xL and Bcl-w. Inhibiting the antiapoptotic proteins by using Navitoclax, a Bcl-xL and Bcl-w antiapoptotic inhibitor, sensitizes the constitutively active YAP cells to antimitotic therapeutics.

Expressing higher levels of YAP and TAZ not only affects cell fate from antimitotic treatment, but depleting YAP and TAZ has the reverse effect. We have demonstrated that depleting YAP and TAZ in RPE cells shifts their fate toward increased cell death and thus sensitize them to antimitotic therapeutics. This sensitization is not RPE cell specific. We have also seen this effect in breast cancer cells treated with Taxol. Indeed, MDA-MB-231 cells often slip from mitosis when treated with Taxol. However, when MDA-MB-231 have YAP and TAZ depleted via RNAi, the cells become sensitized to Taxol. MCF10a cells, which are non-cancerous breast epithelial cells are also sensitized to Taxol when YAP and TAZ are depleted.

We have found that cells with hyperactive YAP and TAZ, and thus an inactive Hippo pathway, do not undergo cell death in response to antimitotic therapeutics. We have shown that if we target and deplete YAP and TAZ in cells with an inactive Hippo pathway that we can sensitize them to antimitotic therapeutics. This research has important implications for antimitotic therapies, as the Hippo pathway is inactive in many cancers. Therefore, Hippo pathway activity status should be assessed before beginning an antimitotic regimen.

3.6 Figures

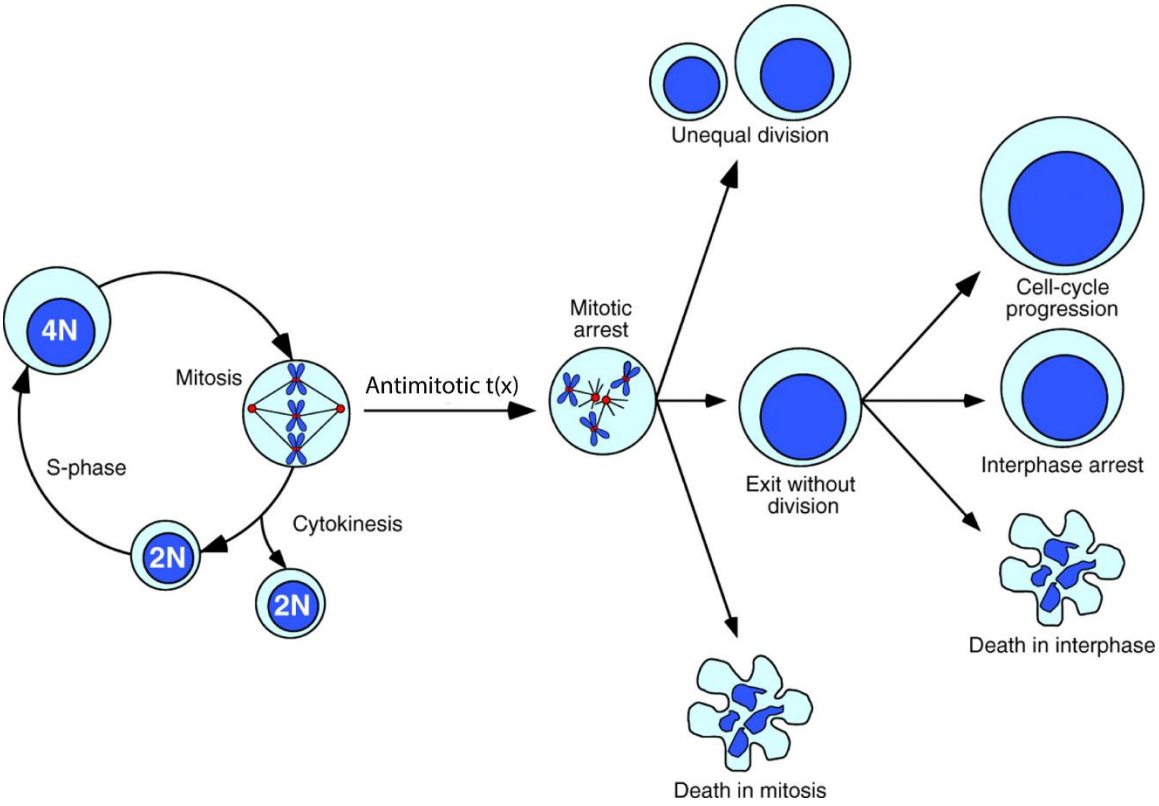

Figure 3.1 Fates post antimitotic t(x)

Antimitotic drugs arrest cycling cells in pro-metaphase. The cell will then arrest in mitosis until it either slips, dies or undergoes an unequal division. When cells undergo slippage they can either die in interphase, arrest in interphase and not continue through the cell cycle, or progress through the cell cycle. Adapted from (Gascoigne and Taylor, 2009).

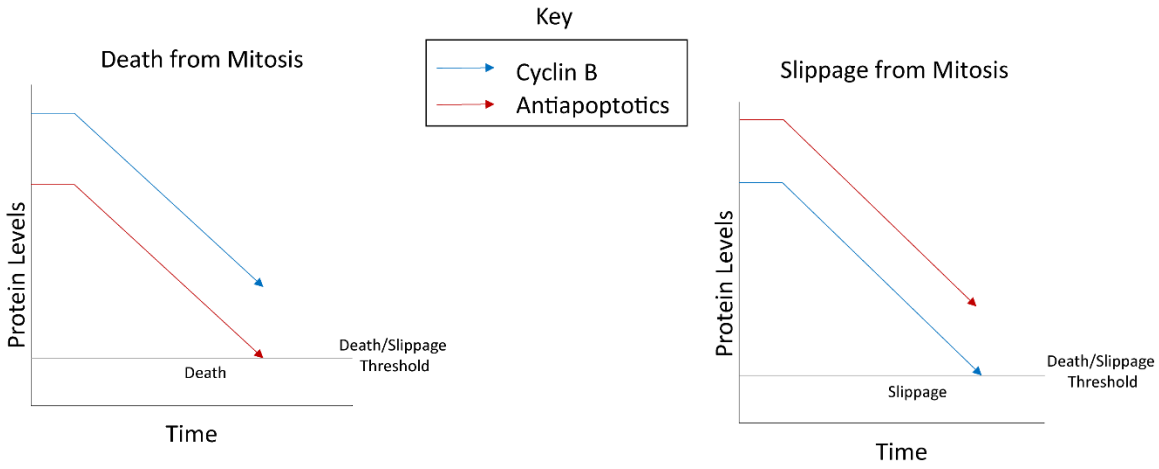

Figure 3.2 Protein degradation race

During mitosis transcription is halted and translation is slowed, this drastically decreases new protein turnover. A protein degradation cell occurs in the cell which determines the cell fate from a prolonged mitosis. This is a two-armed race between Cyclin B and antiapoptotic proteins. Antiapoptotics keep the cell alive, when their protein levels decrease past a certain threshold than the cell will undergo cell death. Cyclin B keeps the cell in mitosis, when Cyclin B decreases past a certain threshold the cells will exit mitosis and slip into G_1.

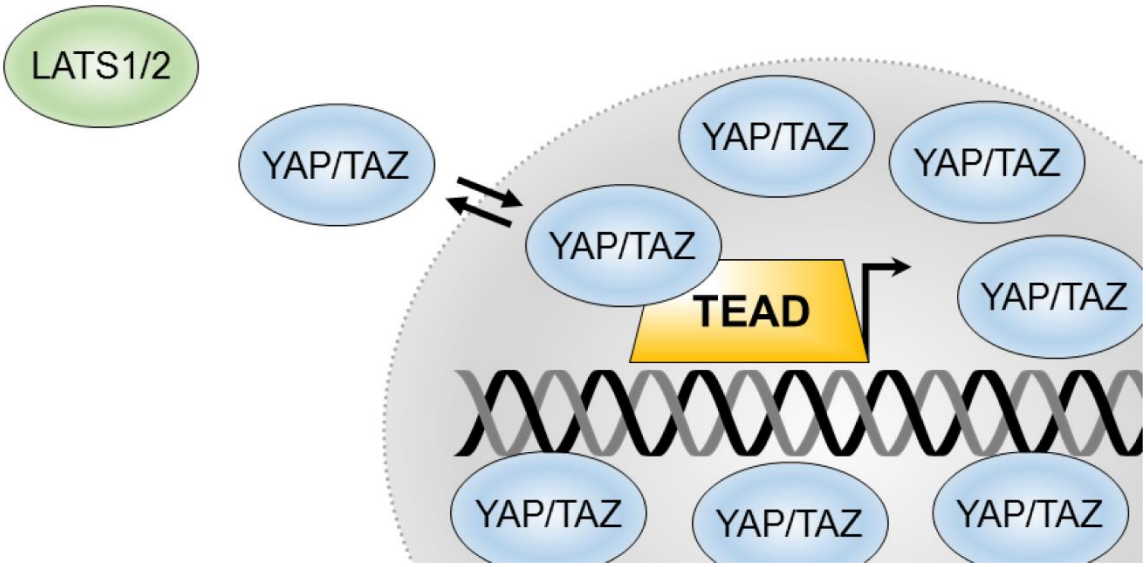

Figure 3.3 Active YAP & TAZ manipulation

Cells expressed constitutively active forms of YAP and TAZ or overexpressed wild-type versions of YAP and TAZ in RPE cells were used to create cells with more active YAP and TAZ that will enter the nucleus

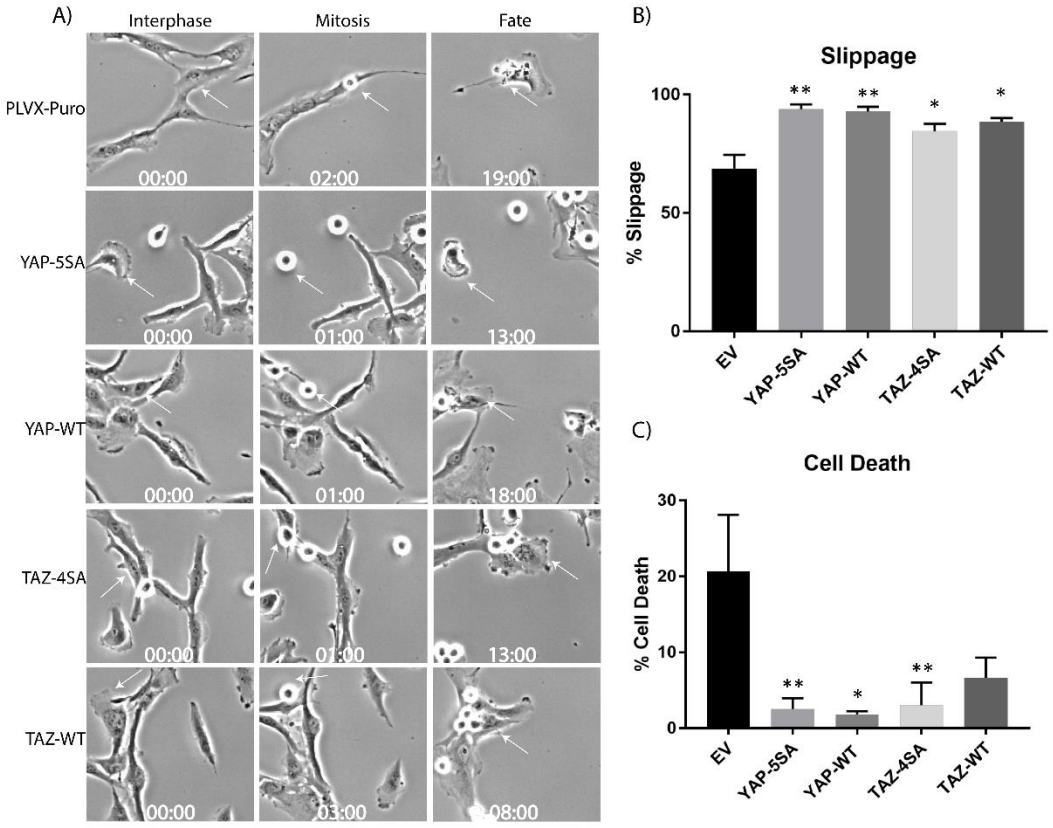

Figure 3.4 YAP/TAZ promotes slippage

A) RPE cells expressing PLVX-Puro (Empty Vector (EV)), YAP-5SA, YAP-WT, TAZ-4SA and TAZ-WT were treated with Monastrol to induce mitotic arrest. Cells were arrested in mitosis for the duration of the video. Cell fate of the single cells was monitored over the 48 hours. White arrows mark individual cells immediately before mitosis (00:00), during mitosis (01:00), and at the onset of cell fate. White arrows track one cell through the duration of the video. The still frames of PLVX-Puro track on cell undergoing cell death from mitosis. The still frames of YAP-5SA, YAP-WT, and TAZ-4SA and TAZ-WT cells show the cells undergoing slippage from mitosis. Time, hrs: min. B) Quantification of the percent of antimitotic treated cells slipping from mitosis. C) Quantification of the percent of antimitotic treated cells undergoing cell death from mitosis. A 1-way ANOVA using multiple comparisons Friedman's test determined significance, n=3.

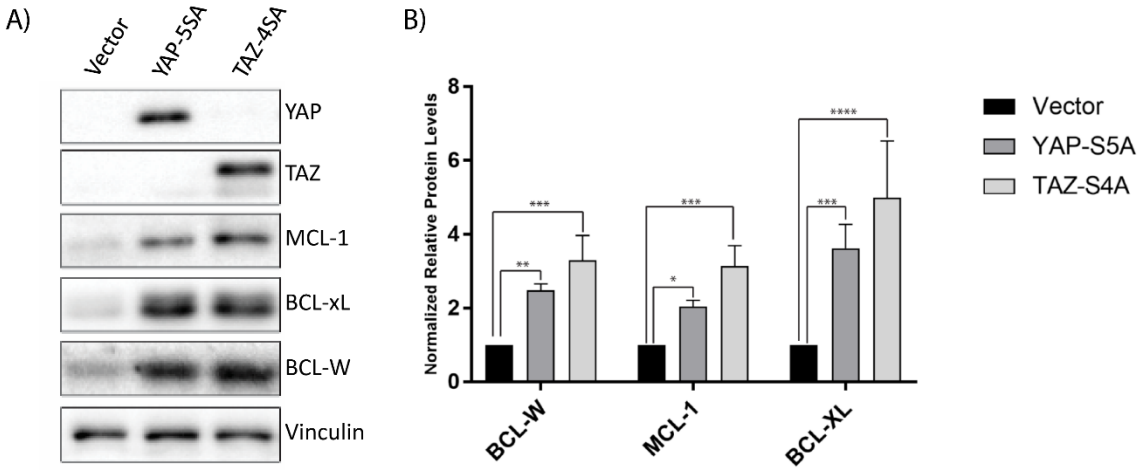

Figure 3.5 Antiapoptotic protein levels

A) Immunoblot of antiapoptotic proteins in PLVX-Puro (Vector), YAP-5SA and TAZ-4SA RPE cells. B) Quantification of antiapoptotic proteins normalized to loading control vinculin. A 2-way ANOVA with Holm-Sidak's multiple comparison test post-hoc test was completed for statistical significance, n= 5.

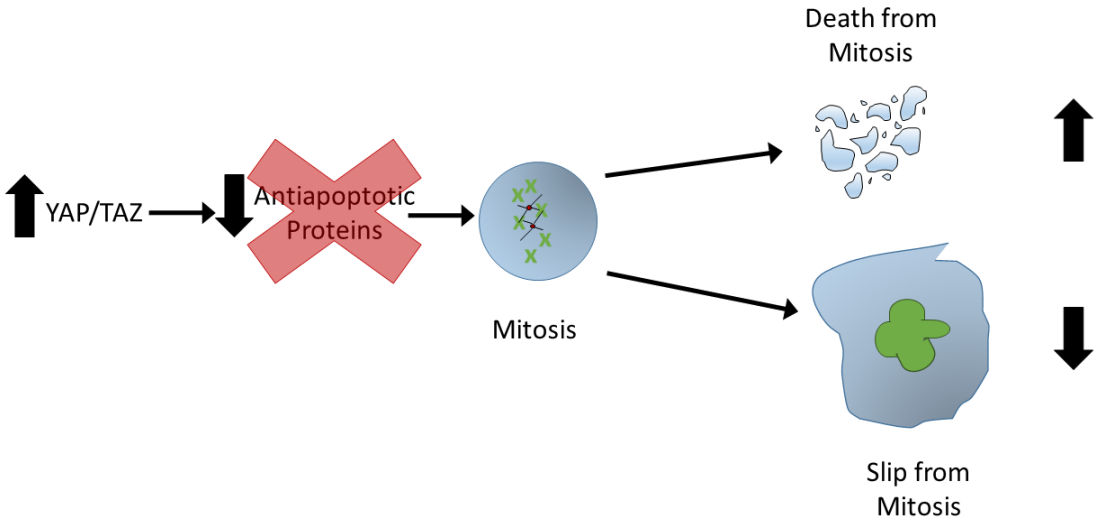

Figure 3.6 Inhibiting antiapoptotics

Active YAP and TAZ leads to the transcription of antiapoptotic protein resulting in increased slippage from mitosis of cells treated with antimitotic therapeutics. However, if the antiapoptotic proteins downstream of YAP and TAZ are inhibiting using therapeutic agents such as Navitoclax in combination with antimitotic therapeutics there will be an increase in cell death and a decrease in cell slippage from mitosis.

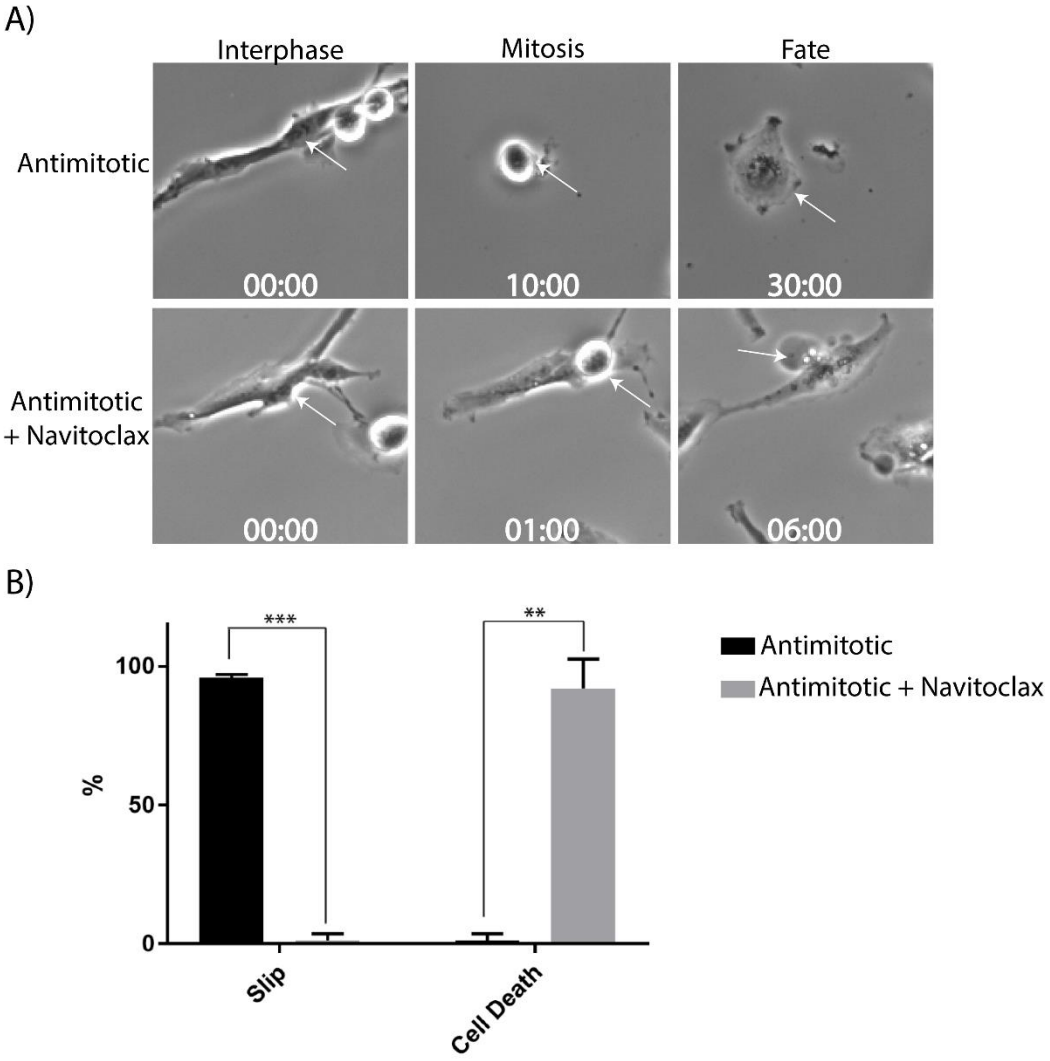

Figure 3.7Navitoclax cause mitotic death

A) RPE cells expressing YAP-5SA were treated with an antimitotic to induce mitotic arrest. Cells were arrested in mitosis for the duration of the video. Cell fate of the single cells was monitored over the 48 hours. White arrows mark individual cells immediately before mitosis (00:00), during mitosis (01:00), and at the onset of cell fate (slippage or death from mitosis). White arrows track one cell through the duration of the video. The top panel shows YAP-5SA RPE cells undergoing slippage from mitosis when treated with antimitotic alone. The bottom panel shows YAP-5SA RPE cells undergoing cell death from mitosis when treated with a combination of antimitotic and antiapoptotic inhibitor Navitoclax. Time, hrs: min. B) Quantification of the percent of cells that underwent slippage or cell death from mitosis when treated with antimitotic alone or antimitotic and Navitoclax. A t-test was run for statistical significance, n=3.

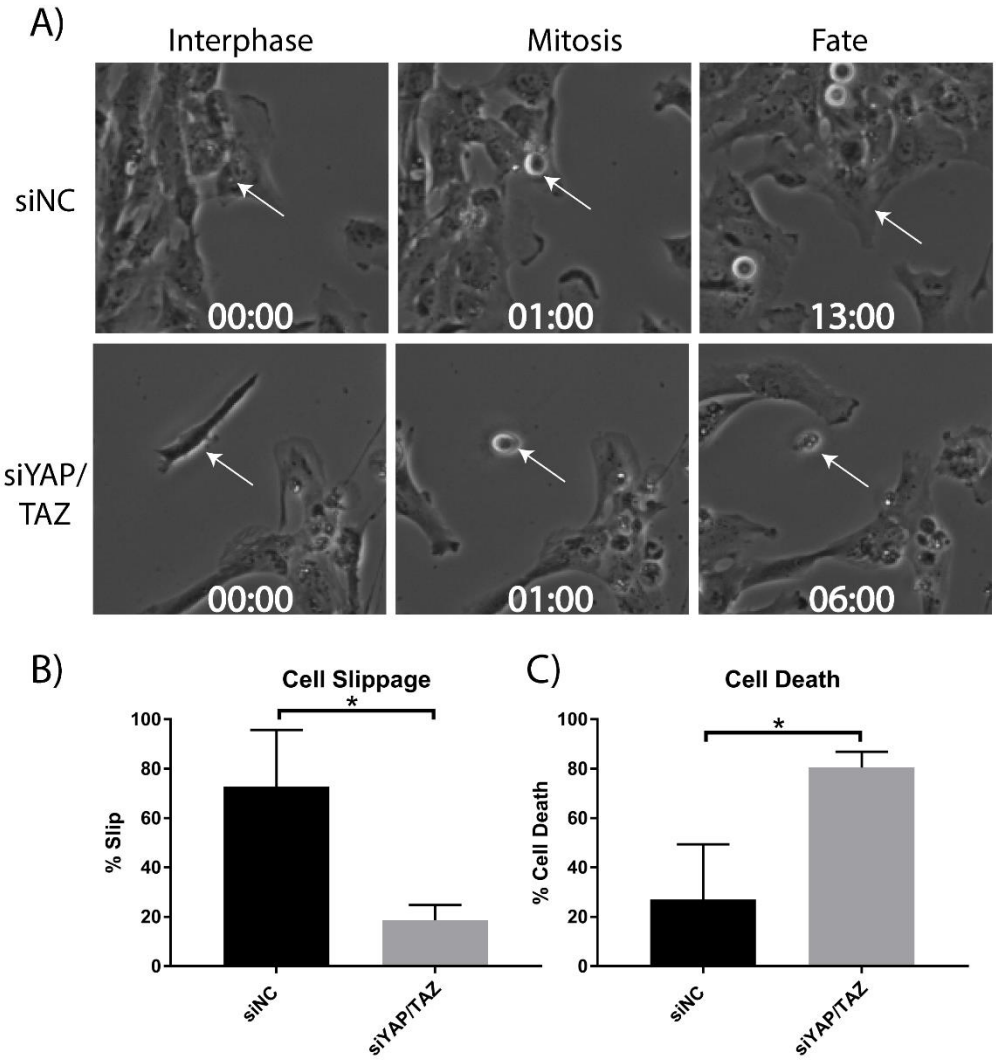

Figure 3.8 RNAi YAP/TAZ in RPE cells

A) RPE cells treated with RNAi YAP/TAZ and the antimitotic Monastrol to induce mitotic arrest. Cells were arrested in mitosis for the duration of the video. Cell fate of the single cells was monitored over the 48 hours. White arrows mark individual cells immediately before mitosis (00:00), during mitosis (01:00), and at the onset of cell fate (slippage or death from mitosis). White arrows track one cell through the duration of the video. The top panel shows RNAi non-target control (siNC) treated RPE cells undergoing slippage from mitosis when treated with antimitotic. The bottom panel shows RNAi YAP/TAZ (siYAP/TAZ) treated RPE cells undergoing cell death from mitosis when treated with the antimitotic Monastrol. Time, hrs: min. B) Quantification of the percent of siNC and siYAP/TAZ cells slipping from mitosis when treated with Monastrol. C) Quantification of the percent of siNC and siYAP/TAZ cells undergoing cell death from mitosis when treated with Monastrol. Statistics was determined using an unpaired t-test with Welch's correction, * = $p<0.05$, n=3.

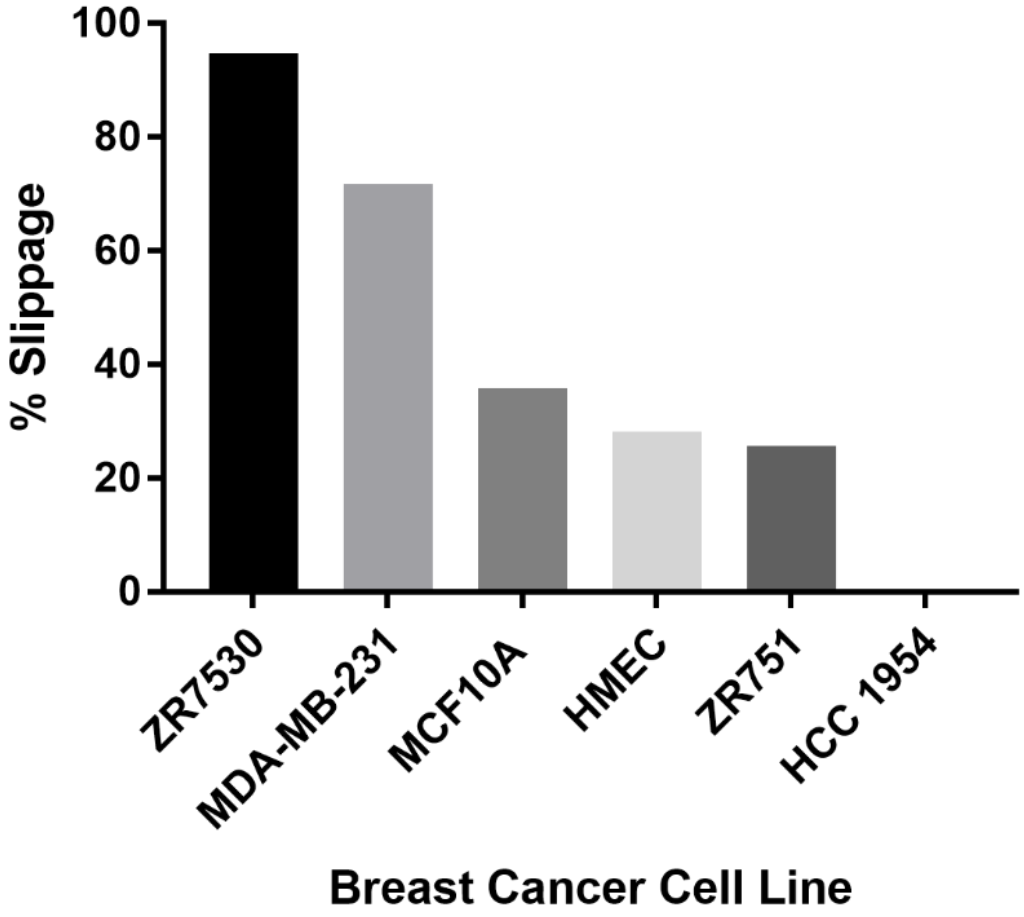

Figure 3.9 Breast cancer Taxol response

Breast cancer cell lines have a variation in response to Taxol. Cell lines were treated with Taxol for the duration of the video. Single cells were tracked until they underwent cell death or slippage, n=100.

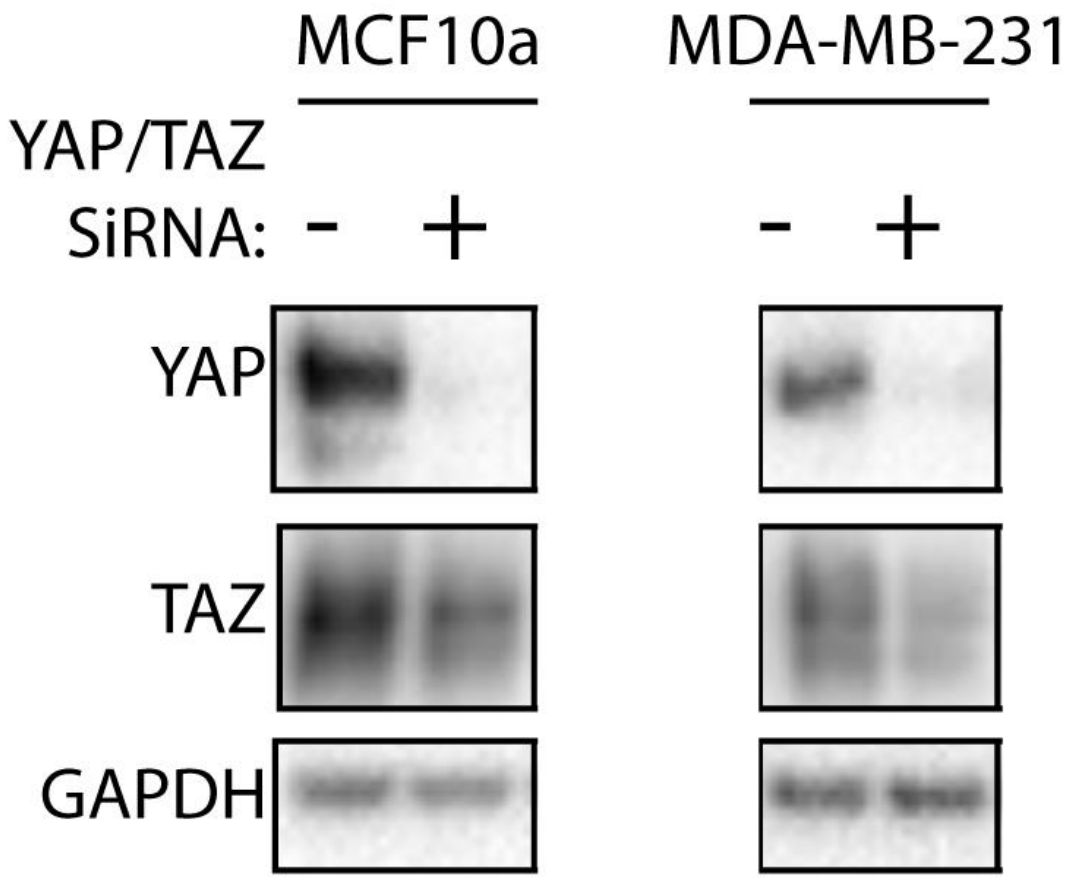

Figure 3.10 RNAi YAP/TAZ validation

Representative immunoblots of YAP and TAZ protein levels in MCF10a and MDA-MB231. Cells were collected post video imaging analysis to assess knock-down efficiency of YAP and TAZ in cell lines.

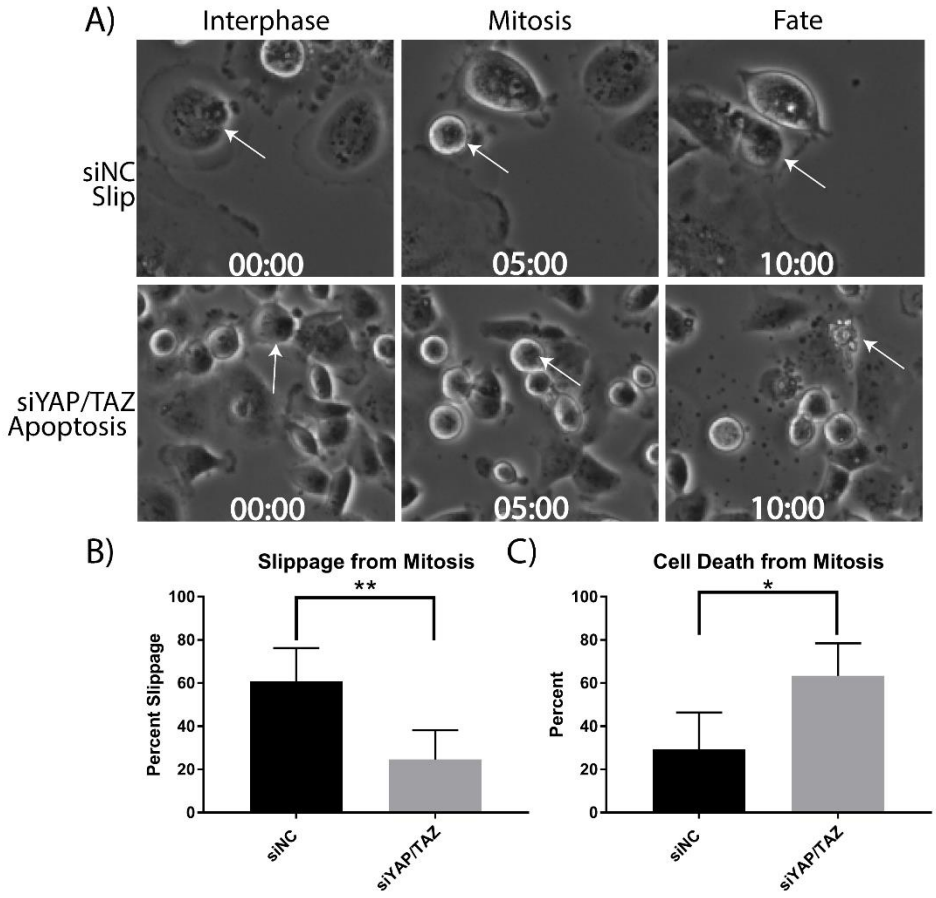

Figure 3.11 MDA-MB-231 Taxol response

A) MDA-MB-231 cells treated with RNAi YAP/TAZ and the antimitotic Taxol to induce mitotic arrest. Cells were arrested in mitosis for the duration of the video. Cell fate of the single cells was monitored over the 48 hours. White arrows mark individual cells immediately before mitosis (00:00), during mitosis (01:00), and at the onset of cell fate (slippage or death from mitosis). White arrows track one cell through the duration of the video. The top panel shows RNAi non-target control (siNC) treated MDA-MB-231 cells undergoing slippage from mitosis when treated with antimitotic. The bottom panel shows RNAi YAP/TAZ (siYAP/TAZ) treated MDA-MB-231 cells undergoing cell death from mitosis when treated with the antimitotic Taxol. Time, hrs: min. B) Quantification of the percent of siNC and siYAP/TAZ cells slipping from mitosis when treated with Taxol. C) Quantification of the percent of siNC and siYAP/TAZ cells undergoing cell death from mitosis when treated with Taxol. Statistics was determined using paired t-test with, * = $p<0.05$, ** = $p<0.01$, n=3.

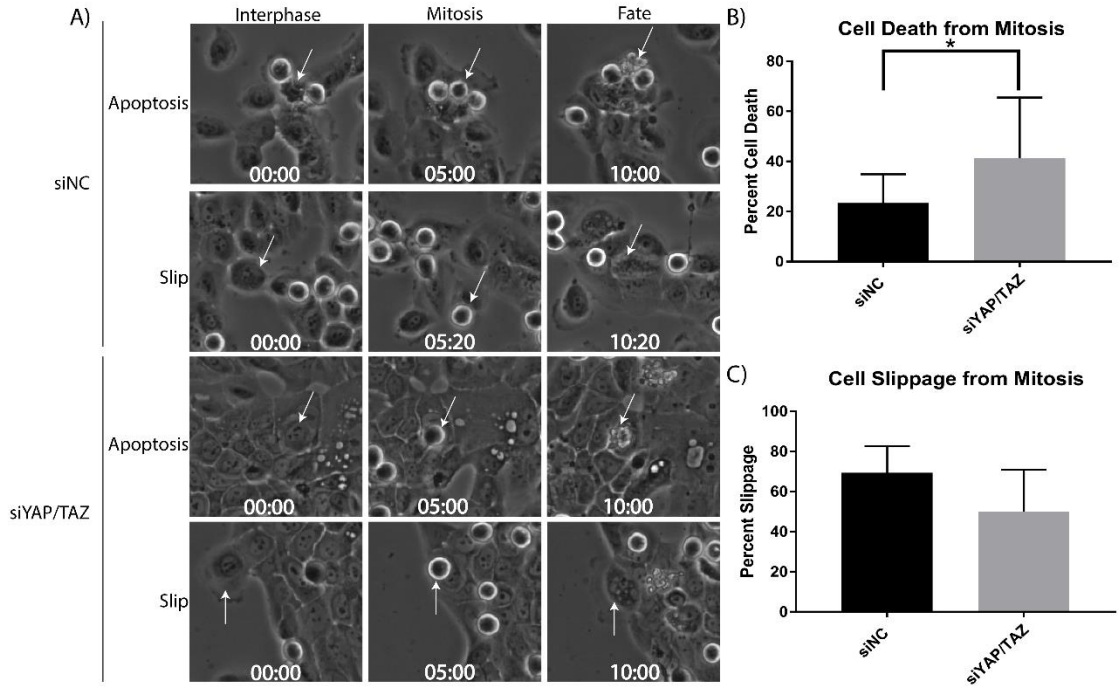

Figure 3.12 MCF10a response to Taxol

A) MCF10a cells treated with RNAi YAP/TAZ and the antimitotic Taxol to induce mitotic arrest. Cells were arrested in mitosis for the duration of the video. Cell fate of the single cells was monitored over the 48 hours. White arrows mark individual cells immediately before mitosis (00:00), during mitosis (01:00), and at the onset of cell fate (slippage or death from mitosis). White arrows track one cell through the duration of the video. The top panel shows RNAi non-target control (siNC) treated MCF10a cells undergoing slippage from mitosis when treated with antimitotic. The bottom panel shows RNAi YAP/TAZ (siYAP/TAZ) treated MCF10a cells undergoing cell death from mitosis when treated with the antimitotic Taxol. Time, hrs: min. B) Quantification of the percent of siNC and siYAP/TAZ cells slipping from mitosis when treated with Taxol. C) Quantification of the percent of siNC and siYAP/TAZ cells undergoing cell death from mitosis when treated with Taxol. Statistics was determined using a ratio paired t-, * = $p<0.05$, n=3.

CHAPTER IV: LONG-TERM LIVE-CELL IMAGING TO ASSESS CELL FATE IN RESPONSE TO PACLITAXEL

4.1 Introduction

Antimitotic drugs have long been used in the chemotherapeutic regimens of various types of solid tumors and often show great efficacy (van Vuuren et al., 2015, Chan et al., 2012, Jackson et al., 2007). Mechanistically, these drugs disrupt normal mitotic progression and promote mitotic arrest in rapidly proliferating cancer cells. However, cell fate in response to mitotic arrest is highly variable: while a fraction of cells undergo cell death directly from mitosis, others exit out of mitosis and return to interphase as tetraploid cells (a process termed mitotic slippage) (Gascoigne and Taylor, 2009, Rieder and Maiato, 2004, Gascoigne and Taylor, 2008, Huang et al., 2010, Shi et al., 2008). These interphase cells can execute apoptosis, undergo permanent cell cycle arrest, or even re-enter the cell cycle(Gascoigne and Taylor, 2009, Gascoigne and Taylor, 2008, Rieder and Maiato, 2004, Huang et al., 2010, Ganem and Pellman, 2007, Ganem et al., 2007, Shi et al., 2008, Ganem and Pellman, 2012). Cells that evade mitotic cell death by slipping into interphase, only to re-enter the cell cycle following drug removal, may therefore contribute to the re-emergence of cancer cell populations. Moreover, cells that slip from mitosis are tetraploid, and tetraploidy is known to promote chromosome instability (CIN) that drives tumor relapse (Sotillo et al., 2010, Sotillo et al., 2007, Ganem et al., 2009, Silkworth et al., 2009). Defining the factors that control cell fate in response to antimitotic drug treatments is therefore critical to optimize current thefrapeutics.

In this protocol, we describe methods to directly observe and study the fate of cells that undergo prolonged mitotic arrest in response to the antimitotic drug paclitaxel. Paclitaxel is an established therapeutic in the clinic and has proven highly efficacious in many tumors types, including those of the breast, ovaries, and lungs(McGuire et al., 1996, McGuire et al., 1989, Ettinger, 1993, Weaver, 2014, Jordan and Wilson, 2004). Paclitaxel, which is a plant alkaloid derived from the bark of the Yew tree, stabilizes microtubules and thus prevents their dynamicity (Wall and Wani, 1995, Schiff and Horwitz, 1980). While dampening of microtubule dynamics by paclitaxel does not affect cell cycle progression from G_1 through G_2, the drug does lead to sustained activation of the spindle assembly checkpoint during mitosis by hindering kinetochore-microtubule attachment (reviewed in depth here(Lara-Gonzalez et al., 2012, Musacchio, 2015))(Uetake et al., 2007). As a consequence, anaphase onset is prevented in paclitaxel-treated cells and results in a prolonged mitotic arrest.

This protocol will first describe approaches to identify mitotic cells in live-cell imaging experiments. Mitosis can be visualized in adherent tissue culture cells due to two noticeable cell biological changes. First, chromosomes become highly condensed immediately prior to nuclear envelope breakdown. While often detectable by standard phase-contrast microscopy, chromosome condensation can be more clearly detected using fluorescent tags that label chromosomes (*e.g.* fluorescently-labeled histone proteins). Second, mitotic cells can also be identified by the dramatic morphological changes that result from cell rounding.

This protocol will then demonstrate how to use live-cell imaging approaches to track the fates of cells experiencing prolonged mitotic arrest. Cells arrested in mitosis undergo one of three distinct fates. First, cells can undergo cell death during mitosis. This phenomenon is readily visualized by light microscopy, as dying cells are observed to shrink, bleb, and/or rupture. Second, cells can exit from mitosis and return back to interphase without chromosome segregation or cytokinesis, a process termed mitotic slippage. The decondensation of chromosomes and/or the flattening of the mitotic cell readily identifies this process. Cells that slip from mitosis also often display irregular, multi-lobed nuclei and frequently harbor several micronuclei(Rieder and Maiato, 2004). Third, cells arrested in mitosis can initiate anaphase and proceed through mitosis after a long delay. While uncommon at higher drug concentrations, this behavior suggests that the arrested cells may have satisfied the spindle assembly checkpoint, or that the spindle assembly checkpoint is partially weakened or defective. Anaphase onset can be visualized by chromosome segregation and subsequent cytokinesis using live-cell imaging.

Live-cell imaging methods to track the fate of cells that evade mitotic cell death by undergoing mitotic slippage will also be described. Cells that undergo mitotic slippage either die in the subsequent interphase, trigger a durable G_1 cell cycle arrest, or re-enter the cell cycle to initiate a new round of cell division(Gascoigne and Taylor, 2009). An approach using the FUCCI (fluorescent ubiquitination-based cell cycle indicator) system to determine the fraction of cells that re-enter the cell cycle following mitotic slippage will be described. FUCCI allows for the direct visualization of the G_1/S transition and can be used in conjunction with long-term live-cell imaging both *in vitro* and *in vivo* (Sakaue-

Sawano et al., 2008, Chittajallu et al., 2015). The FUCCI system takes advantage of two fluorescently labeled proteins, truncated forms of hCdt1 (chromatin licensing and DNA replication factor 1) and hGeminin, whose levels oscillate based on cell cycle position. hCdt1 (fused to a red fluorescent protein) is present at high levels during G_1 phase where it acts to license DNA for replication, but is ubiquitinated by the E3 ubiquitin ligase SCF^{Skp2} and degraded during $S/G_2/M$ phases to prevent re-replication of DNA(Sakaue-Sawano et al., 2008). By contrast, hGeminin (fused to a green fluorescent protein), is an inhibitor of hCdt1 whose levels peak during $S/G_2/M$, but is ubiquitinated by the E3 ubiquitin ligase APC^{Cdh1} and degraded at the end of mitosis and throughout G_1 (Sakaue-Sawano et al., 2008). Consequently, FUCCI delivers a straightforward fluorescence readout of cell cycle phase, as cells exhibit red fluorescence during G_1, and green fluorescence during $S/G_2/M$. The FUCCI system is a significant advance over other approaches (such as bromodeoxyuridine staining) to identify proliferative cells, because it does not require cell fixation and allows for single cell imaging without the need for additional pharmacological treatments to synchronize cell populations. Though not discussed in this protocol, additional live-cell sensors have also been developed to visualize cell cycle progression, including a helicase B sensor for $G_{1(Gu\ et\ al.,\ 2004)}$, DNA ligase-RFP(Easwaran et al., 2005) and PCDNA-GFP(Hahn et al., 2009) sensors for S-phase, and the recent FUCCI-4 sensor, which detects all stages of the cell cycle(Bajar et al., 2016).

Finally, a live-cell imaging method to detect nuclear envelope rupture will be described. Recent studies have revealed that the nuclear envelopes of cancer cells are unstable and prone to bursting, thereby allowing the contents of the nucleoplasm and

cytoplasm to intermix. This phenomenon, termed nuclear rupture, can promote DNA damage and stimulation of the innate immune response(Gekara, 2017, Chow et al., 2012, Crasta et al., 2012, Hatch et al., 2013, Maciejowski et al., 2015, Zhang et al., 2015a, Denais et al., 2016, Raab et al., 2016, Bernhard and Granboulan, 1963, de Noronha et al., 2001, Vargas et al., 2012, Sieprath et al., 2012, Mitchison et al., 2017). While the underlying causes of nuclear rupture remain incompletely characterized, it is known that deformations in nuclear structure correlate with an increased incidence of nuclear rupture (Vargas et al., 2012). One well-known effect of paclitaxel treatment is the generation of strikingly abnormal nuclear structures following mitosis; as such, a method using live-cell imaging to quantify nuclear rupture will be described, while also exploring if paclitaxel treatment increases the frequency of nuclear rupture events. Nuclear rupture can be detected by the observed leakage of a nuclear-targeted fluorescent protein into the cytoplasm (*e.g.* a tandem dimer repeat of RFP fused to a nuclear localization signal, TDRFP-NLS). This leakage is distinctly visible by eye, which enables simple quantitation of rupture events.

This protocol requires a wide field epifluorescence microscope that is equipped with an encoded stage and autofocusing software. The encoded stage allows for precise automated movement to defined X-Y coordinates, while autofocus software maintains cells in focus for the duration of the imaging period. In addition, this protocol requires equipment to maintain cells at 37 °C with humidified 5% CO_2 atmosphere. This can be achieved by enclosing the entire microscope within a temperature and atmosphere controlled enclosure, or by using stage-top devices that locally maintains temperature and environment. The phase-contrast objective used in this protocol is a plan fluor 10x with a

numerical aperture of 0.30. However, 20X objectives are also sufficient to identify both rounded mitotic and flattened interphase cells in a single focal plane. If performing phase-contrast imaging (as described in this method), the cover can be either glass or plastic. If differential interference contrast (DIC) microscopy is used, it is imperative to use a glass cover to prevent depolarization of light.

4.2 Methods:

Note: This protocol focuses on using the non-transformed and chromosomally stable RPE cell line for live-cell imaging experiments. However, this protocol can be adapted to any adherent cell line so long as cell culture conditions are adjusted as necessary. All procedures must adhere to institutional biosafety and ethical guidelines and regulations.

4.2.1 Preparing Cells for Live-Cell Imaging

1.1. Use freshly thawed and early passage RPE cells expressing either human histone H2B fused to a fluorescent protein (*e.g.* H2B-GFP), or the FUCCI system (a detailed protocol on how to generate FUCCI-expressing cells can be found in ref. (Shenk and Ganem, 2016)) to assess mitotic cell fate.

1.1.1. To measure the frequency of nuclear rupture, use RPE cells expressing both H2B-GFP and a tandem dimer of red fluorescent protein fused to a single nuclear localization signal (TDRFP-NLS).

Note: Constructs of three green fluorescent proteins fused in tandem to a single nuclear localization signal (GFP_3-NLS) have also been used to demonstrate rupture (as in ref. (Vargas et al., 2012)).

1.1.2. Maintain cells on 10 cm tissue culture plates in the appropriate growth medium. RPE cells are maintained in phenol red-free, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM:F12) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin.

1.2. Aspirate medium from the cells, wash the tissue culture dish with 10 mL of sterile, room temperature phosphate buffered saline (PBS) to remove remaining medium, and then aspirate the PBS.

1.2.1. Add 2 mL of 0.25% Trypsin with ethylenediaminetetraacetic acid (EDTA) to the cells and incubate at 37 °C for 3 min or until the majority of cells have detached from the plate.

Note: Do not keep cells in trypsin longer than needed.

1.3. Add 10 mL of complete medium to collect the trypsinized cells with a 10 mL serological stripette and dispense in a 15 mL conical tube.

1.3.1. Pellet the cells by centrifugation at 180 x *g* for 3 min at room temperature.

1.4. Aspirate the supernatant, being careful not to disrupt the pellet, and then thoroughly resuspend the pellet in 10 mL of fresh medium by gently pipetting the medium up and down in the serological stripette.

1.5. Use a hemocytometer or automated cell counting machine to count cells (Morten et al., 2016).

1.5.1. Dilute cells in a new conical tube to 30,000 cells/1 mL of complete medium.

1.5.2. Plate 30,000 RPE cells (1 mL volume) per well of a 12-well glass bottomed (#1.5 thickness) imaging dish to achieve the required cellular density the next day (30-50% confluent).

Note: Always handle the plate with gloves and be careful not to touch the glass-bottom.

1.6. Allow cells to grow in a 37 °C tissue culture incubator until they fully attach and flatten on the glass-bottomed imaging plate. While cells can attach in as little as 4 h, it is recommended that cells are not treated with drugs and imaged until the following day.

1.7. Add paclitaxel (dissolved in dimethyl sulfoxide (DMSO)) to the desired final concentration in complete medium and mix thoroughly.

1.7.1. Prepare complete medium with an equal volume of DMSO alone to use as a control.

1.7.2. Warm the medium containing drug or DMSO to 37 °C before adding to cells. This will prevent focal drift due to a sudden temperature change.

1.7.3. Add 1 mL of medium containing either paclitaxel or DMSO alone to individual wells of the 12-well imaging dish.

4.2.2 Setting Up the Microscope for Live-Cell Imaging

1.1. Clean the glass-bottom of the imaging dish with optical cleaner to remove any fingerprints or dust that may interfere with imaging. Use sufficient optical cleaner to wet the entire glass surface.

1.2. Place the glass-bottom dish on the microscope stage in the imaging dish adaptor, remove the plastic cover from the dish, and cover the dish with a glass-topped chamber. Ensure the chamber has a valve to allow a steady flow of air consisting of 5% CO_2.

Note: To humidify the 5% CO_2, the gas is flowed through tubing inserted into a sterile water bath housing. This allows for the gas to become equilibrated to 95% humidity.

1.3. Initiate/calibrate the encoded stage using the software program controlling the microscope. This will ensure accurate X-Y coordinates and prevent focal drift.

1.4. Focus on the cells using phase-contrast optics and perform Koehler illumination (described in detail in ref. (Salmon and Canman, 2001)) to focus the light and provide optimal contrast.

1.5. Use acquisition software to determine the optimal exposure times for white light and all fluorescent channels being used (exposures that give 75% pixel saturation on the camera are ideal, provided this amount of light is not toxic to cells).

1.5.1. Use acquisition software to select several, non-overlapping fields of view from each well for imaging. Select imaging regions where cells have adhered well to the glass-bottom and are between 50% and 70% confluent. Avoid areas of clumped cells, as this will make subsequent analysis difficult.

Note: It is important to have non-overlapping fields of view from each well to avoid tracking the same cells twice. If cells are highly motile, it may be necessary to acquire several images radiating out from a central point and stitch them back together to make one large field of view.

1.6. Activate the microscope's autofocusing feature to ensure all points are maintained in focus for the duration of the experiment.

1.7. To assess mitotic cell fate, set the imaging software to collect images from each selected field of view every 10 min for up to 96 h. Unperturbed mitosis lasts 20-40 min,

and thus 10 min intervals will provide enough sampling to identify when cells divide. To identify nuclear rupture, which is a transient event, acquire images every 5 min.

Note: Make sure that the computer driving the image acquisition software has auto-updating, screensavers, and energy-savings modes disabled, as these can often interfere with image acquisition over long experiments.

1.8. Initiate the imaging experiment. Periodically confirm that image acquisition is running smoothly over the course of the video.

4.2.3 Video Analysis to Identify Cell Fate in Response to Paclitaxel

1.1. Confirm that imaged cells are healthy throughout the course of the imaging experiment. Control cells treated with DMSO should be viable and actively proliferating.

Note: If cells exhibit signs of stress, do not quantitate the video and instead focus on optimizing imaging conditions(Cole, 2014). Signs of imaging stress include blebbing/dying cells, cells that fail to attach/spread on the plate, and cells that show a low mitotic index and/or prolonged mitosis. Possible sources of stress include fluctuations in temperature or CO_2 levels inside the imaging chamber or phototoxicity(Cole, 2014).

1.2. When imaging an entire field of view with a 10X or 20X objective, hundreds of individual cells may be present. Therefore, to assist with quantitation, divide the field of view into smaller quadrants using available software tools. Score cells within each quadrant separately (as described in steps 3.3.1.-3.6.2.).

1.3. When analyzing the video, track each cell in a merged view using phase-contrast and/or fluorescent images. Use software analysis tools to create the merged view.

1.4. . Starting at the beginning of the video, identify a single interphase cell and track its progress through the cell cycle using phase optics. To track a single cell, observe the cell from frame to frame by eye. Identify interphase cells due to their flattened morphology (as assessed by phase contrast) and their lack of DNA condensation (as assessed by H2B-GFP) (Figure 4.1A).

1.5. Identify cells that enter mitosis by observation of cell rounding (using phase-contrast optics) and/or chromosome condensation (using H2B-GFP) (Figure 4.1A-4.1C). Both cell rounding and chromosome condensation are readily visualized by eye.

1.5.1. Annotate the time when the cell enters mitosis. Continue tracking the cell until it reaches its fate (anaphase, cell death, or mitotic slippage).

1.6. Control cells should efficiently align their chromosomes and enter anaphase within 1 h (Figure 4.1D). Visualize anaphase by phase contrast optics as the cell begins to pinch into two or through visualization of poleward-moving chromosomes labeled with H2B-GFP (Figure 4.1A). Annotate the time when the cell undergoes anaphase.

1.7. By contrast, cells treated with paclitaxel will remain rounded with condensed chromosomes for several hours (from 3-40 hours) (Figure 4.1B-4.1D).

1.7.1. Identify mitotically–arrested cells that undergo cell death.

Note: Cells that die during mitosis are visualized by phase-contrast microscopy, as cells will bleb, shrink, and/or rupture (Figure 4.1B). If imaging H2B-GFP, the chromosomes will also fragment during cell death.

1.7.2. Identify mitotically-arrested cells that undergo cell slippage.

Note: Cells that undergo mitotic slippage are observed by phase contrast microscopy, as they flatten back out into interphase and decondense chromosomes without undergoing anaphase (Figure 4.1C). Cells that undergo mitotic slippage give rise to large tetraploid cells that are often multinucleated (Figure 4.1C).

1.8. Continue tracking cells from the original field of view. Once a whole field of view is tracked, move to a separate field of view acquired from the same well and continue tracking cells.

4.2.5 Video Analysis to Identify Cell Fate Following Mitotic Slippage

1.1. Assess cell fate following mitotic slippage (as described in step 3.6.2) using RPE cells expressing the FUCCI system. In addition to phase-contrast imaging, it is necessary to acquire both red fluorescence (indicative of G_1 phase) and green fluorescence (indicative of S/G_2/M) images).

1.1.1. Track FUCCI RPE cells as described above.

1.1.1.1. To confirm that the FUCCI system is working properly, validate that control cells alternate expression of the red and green fluorescent proteins appropriately from analysis of the live-cell imaging data. Control cells should transition from exhibiting entirely nuclear red fluorescence to exhibiting entirely nuclear green fluorescence during interphase as cells progress from G_1 to S phase. Cells should continue exhibiting green fluorescence throughout the completion of mitosis. Immediately following mitosis, cells should once again exhibit entirely red fluorescence during interphase.

Note: While methods exist to quantify both RFP and GFP fluorescence intensities from the FUCCI system in single cells using fluorescent traces(Burke and Orth, 2016), this is often not necessary as the fluorescence color change is robust and visible by eye.

1.2. Identify cells arrested in mitosis using phase contrast microscopy and track them until they undergo mitotic slippage, as described previously. Cells that slip out of mitosis and back into interphase will change from exhibiting green fluorescence during mitosis to red fluorescence during G_1 phase (Figure 4.2A-4.2C).

1.2.1. Track these slipped cells using phase-contrast and epifluorescent imaging by eye to assess their cell fate (Fig. 4.2D).

1.2.1.1. Identify cells that re-enter the cell cycle. These cells are identified by the red-to-green change in fluorescence expression using the FUCCI system that indicates G_1/S transition (Figure 4.2A).

1.2.1.2. Identify cells that undergo G_1 cell cycle arrest. These cells are identified by expression of red fluorescence that persists for > 24 h (Figure 4.2B).

1.2.1.3. Identify cells that die in interphase. These cells are identified by cell rupture/blebbing/shrinking using phase-contrast imaging (Figure 4.2C).

4.2.6 Video Analysis to Identify Frequency of Nuclear Envelope Rupture

1.1. Use RPE cells expressing H2B-GFP and TDRFP-NLS to assess nuclear envelope rupture. In addition to phase contrast imaging, acquire both red fluorescence (TRITC) and green fluorescence (FITC) images. Acquire images every 5 min to visualize rupture.

Note: It is critical to generate cell lines in which the TDRFP-NLS is efficiently imported into the nucleus with minimal cytoplasmic fluorescence.

1.2. Image cells as described previously. The TDRFP-NLS fluorescence signal and H2B-GFP should co-localize during interphase. Upon mitosis (as visualized by cell rounding using phase optics and/or chromosome condensation by H2B-GFP), the nuclear envelope will break down and the TDRFP-NLS signal will become cytoplasmic (Figure 4.3A). Following mitosis, the nuclear envelope will reform in the daughter cells and the TDRFP-NLS signal will become nuclear.

1.3. Track daughter cells throughout the subsequent interphase by live-cell imaging. Identify nuclear rupture events by observing a transient burst of nuclear-localized TDRFP-NLS into the surrounding cytoplasm. Within minutes, the nuclear envelope will be repaired and the TDRFP-NLS will be relocalized to the nucleus (Figure 4.3A).

Note: Often, the nuclear DNA, as visualized by H2B-GFP, will be seen to protrude outside the rupture site as a small bleb.

1.4. Score the fraction of nuclei that undergo a rupture event during interphase. To score this fraction, count the number of cells that undergo a rupture event over the total number of cells tracked.

4.3 Results:

Using the protocol described above, RPE cells expressing H2B-GFP were treated with an antimitotic or vehicle control (DMSO) and analyzed by live-cell imaging. Analysis revealed that 100% of control mitotic RPE cells initiated anaphase an average of 22 min after entering mitosis (Figure 4.1A, 4.1D). By contrast, RPE cells treated with paclitaxel exhibited a profound mitotic arrest lasting several hours (Figure 4.1D). Imaging revealed

that 48% of these mitotically arrested cells underwent mitotic cell death (Figure 4.1B, 4.1E), while the remaining 52% underwent mitotic slippage (Figure 4.1C, 4.1E).

To assess the fate of RPE cells that underwent mitotic slippage, RPE cells expressing the FUCCI system were treated with either low-dose 50 nM paclitaxel, high-dose 5 μM paclitaxel, or vehicle control (DMSO) and analyzed by long term live-cell imaging. The data revealed that of the RPE cells that underwent mitotic slippage following treatment with 50 nM paclitaxel, 22% died in the subsequent cell cycle (Figure 4.2C, 4.2D), 72% induced cell cycle arrest (Figure 4.2B, 4.2D), and only 5% re-entered S-phase (Figure 4.2A, 2D). By contrast, of the RPE cells treated with high-dose 5 μM paclitaxel that underwent mitotic slippage, 35% died in the subsequent cell cycle, 65% induced cell cycle arrest, and none re-entered S-phase (Figure 4.2D).

Interestingly, low-dose paclitaxel-treatment promotes nuclear envelope rupture in RPE cells following mitosis. After mitotic completion daughter cells were tracked and scored for any nuclear rupture events, revealing that only ~4% of control (DMSO-treated) RPE daughter cells ruptured, whereas ~23% of daughter cells treated with 10 nM paclitaxel exhibited rupture (Figure 4.3A, 4.3B).

4.4 Discussion:

The most critical aspect of long-term live-cell imaging is ensuring the health of the cells being imaged. It is essential that cells be exposed to minimal extraneous environmental stressors, such as substandard conditions regarding temperature, humidity, and/or CO_2 levels. It is also important to limit photo damage from fluorescent excitation

illumination, as imaging stress is known to affect cell behavior (Douthwright and Sluder, 2017). Limiting photo damage can be achieved in multiple ways as detailed elsewhere (Cole, 2014). In general, it is best to use the shortest exposure time necessary to produce a sufficiently bright enough fluorescence signal to efficiently track cells, thus limiting phototoxicity. However, if cells are imaged only once every 10-20 min, longer exposures (that approach pixel saturation) are typically well tolerated (though this must be determined empirically for each fluorophore and cell type). Because imaged cells are sensitive to both environmental and imaging stresses, it is imperative that control cells are always included in live-cell imaging experiments and monitored to confirm normal proliferation.

One limitation to long-term live-cell imaging is that it requires equipping an existing microscope with either an environmental chamber that maintains both temperature and 5% humidified CO_2 atmosphere, or a stage-top chamber that is also capable of supporting the appropriate temperature and atmosphere. While flowing CO_2 is optimal, cells can also be imaged for up to 48 h using CO_2-independent medium if an environmental chamber with 5% humidified CO_2 is not available. However, many cell types are intolerant of such medium, and this must be determined empirically by measuring cell growth and viability. Overlaying the cells with mineral oil can also be used to prevent medium evaporation.

A second major limitation to this method is that manually tracking cell fates is highly laborious and time-intensive. However, cell-tracking programs are available and may be optimized to automate image analysis (Hilsenbeck et al., 2016, Skylaki et al., 2016, Jones et al., 2009). A further limitation with this method is that highly motile cells are often

difficult to track over extended periods of time. If this poses a significant problem, the entire well can be imaged and the images stitched together to form one large field of view. Many software programs support this method.

Autofluorescence is another constraint that must be addressed with this protocol. Phenol red free medium reduces background autofluorescence during live cell imaging. It has also been demonstrated that two vitamins commonly found in growth medium, riboflavin and pyridoxal, can decrease photostability of fluorescent proteins. Thus, medium lacking these vitamins can also be used during fluorescence imaging to enhance signal to noise rations. While plastic bottom dishes are less expensive and may provide adequate imaging results, they also produce a greater amount of autofluorescence that negatively affects signal-to-noise ratios. In addition, plastic bottom dishes have greater variation in thickness, which can disrupt the autofocus feature of many microscopes. The thickness of the glass bottomed imaging dish in this protocol is 0.17 mm, which is the ideal glass for use with modern microscopes.

Lastly, long-term movies encompassing multiple fields of view and acquiring several fluorescence channels produces massive data files (tens of gigabytes to even terabytes each), which pose a problem for data storage and back-up. To mitigate this issue, it is recommended that all images be acquired using 2 x 2 or 4 x 4 binning, provided the resulting loss in resolution is acceptable. Binning not only limits exposure times (*i.e.* cells stably expressing H2B-GFP or the FUCCI system should have exposures less than 500 ms), but also drastically reduces the size of data files (an image that is binned 2 x 2 is one-fourth the file size of a non-binned image).

Despite these limitations, long-term live-cell imaging represents the best method to track individual cell fates from whole populations, especially when cell fates are highly heterogeneous. As more live-cell fluorescent markers and sensors become available, live-cell imaging approaches will be expanded to visualize and quantitate several additional aspects of cell biology. For example, live-cell sensors currently exist to quantitate DNA damage foci, p53 levels, cell cycle position, and apoptosis(Sakaue-Sawano et al., 2008, Lukas et al., 2003, Bekker-Jensen et al., 2005, Loewer et al., 2010, Lekshmi et al., 2017, Jullien et al., 2002). Thus, imaging experiments have the capacity to reveal the underlying cellular properties that dictate how and why single cells respond variably to chemotherapeutic agents.

4.5 Figures:

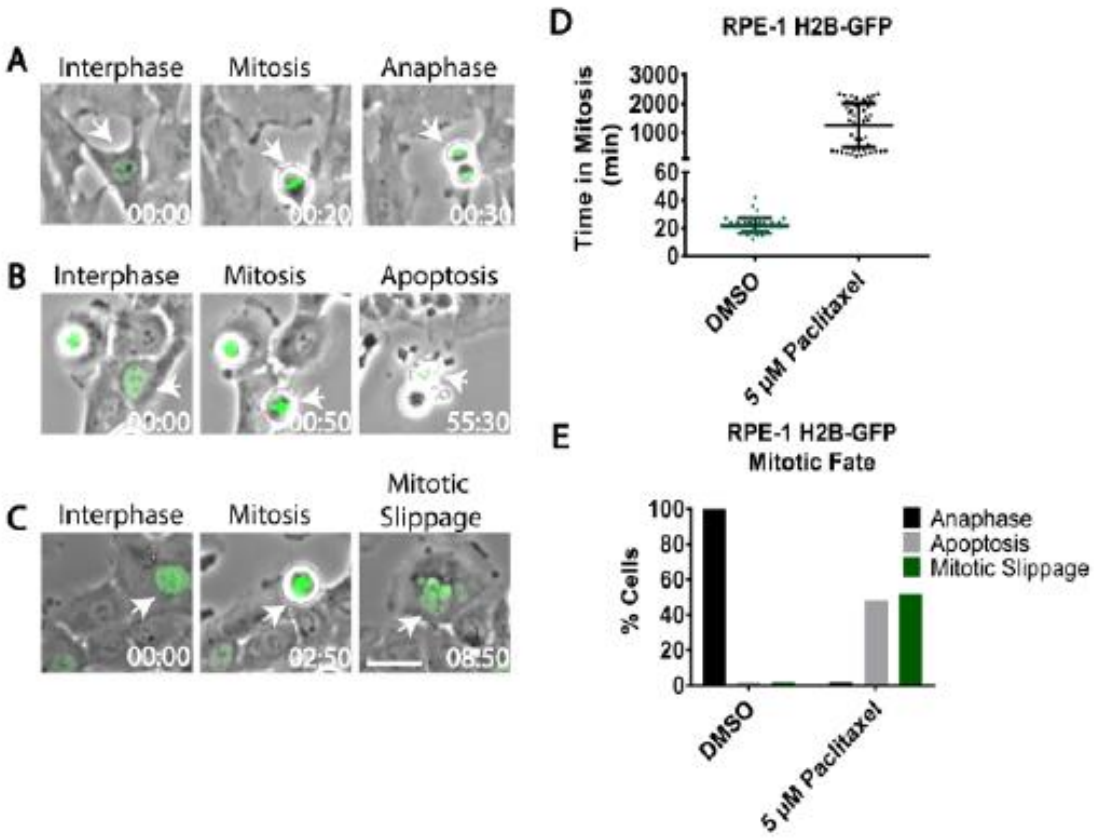

Figure 4.1 Taxol induced mitotic fate

RPE cells stably expressing H2B-GFP were treated with DMSO vehicle control (A) or 5 μM paclitaxel (B, C) and imaged using time-lapse phase-contrast and wide field epifluorescence microscopy to assess mitotic cell fate. Interphase cells (left panels) were tracked as they entered mitosis (middle panels) and until they either initiated anaphase (A), underwent mitotic cell death (B), or slipped from mitosis back to interphase (C). White arrows indicate tracked cells. (D) The amount of time that control cells (DMSO-treated) and antimitotic-treated cells spent in mitosis, as quantitated by live-cell imaging (n=100 cells for each condition). (E) The fraction of cells (n=100) that underwent anaphase, mitotic cell death, or mitotic slippage in DMSO-treated cells or cells treated with 5 μM paclitaxel. Time, h: min. Scale Bar, 50 μm.

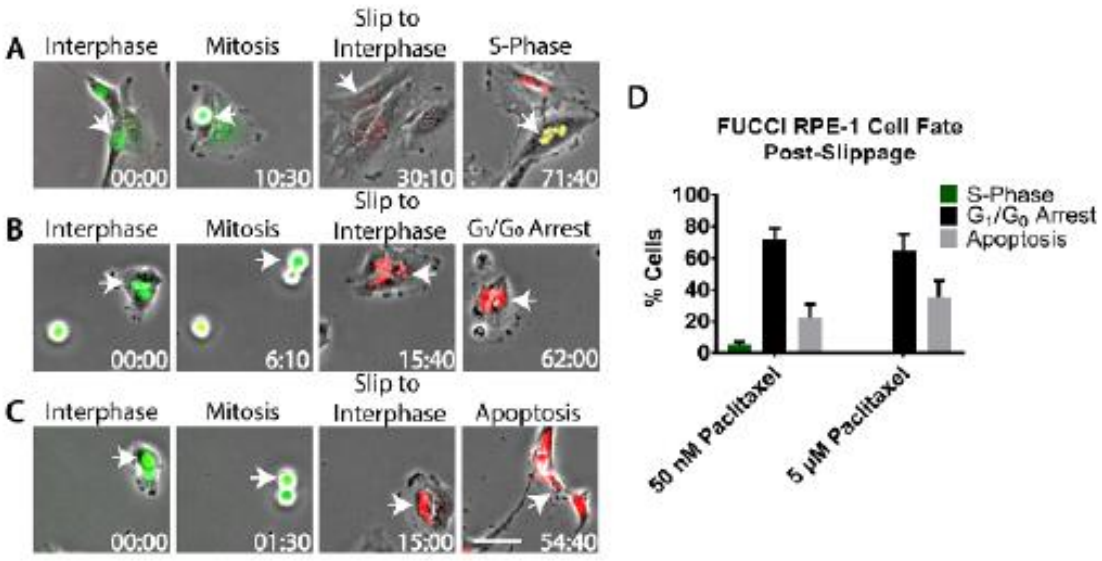

Figure 4.2 Fate of slipped cells

RPE cells stably expressing the FUCCI system were treated with 50 nM paclitaxel or 5 μM paclitaxel and imaged using time-lapse phase-contrast and wide field fluorescence microscopy to quantitate cell fate following mitotic slippage. Cells were scored as either re-entering the cell cycle, as judged by a red-to-green fluorescence change in the FUCCI system (A); arresting in G_1 phase, as judged by persistent red fluorescence for > 24 h (B); or dying during the subsequent mitosis, as judged by cellular blebbing/rupture (C). White arrows indicate tracked cells. (D) The fraction of cells (n=100) that underwent each fate. Error bars represent the standard deviation from the mean from two independent experiments. Time, h: min. Scale Bar, 50 μm.

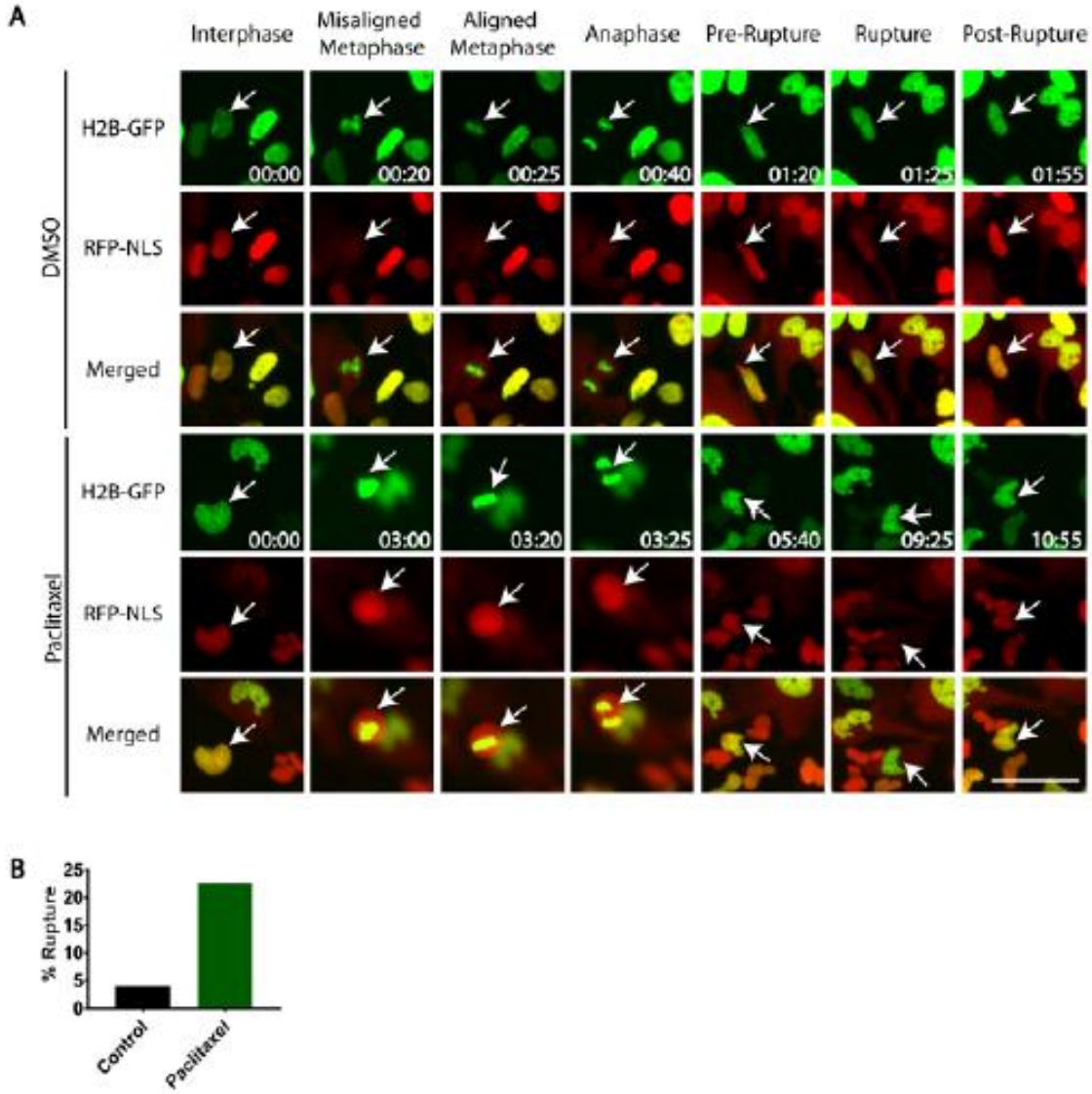

Figure 4.3 Taxol induced nuclear rupture

RPE cells stably expressing TDRFP-NLS and H2B-GFP were treated with 10 nM paclitaxel or vehicle control (DMSO) and imaged using time-lapse phase-contrast and wide field epifluorescence microscopy (A). During mitosis (Metaphase/Anaphase) the nuclear envelope is broken down and TDRFP-NLS becomes cytoplasmic. Following mitosis, the TDRFP-NLS relocalizes to the nucleus of interphase cells (Pre-Rupture). Nuclear rupture events are identified by the delocalization of TDRFP-NLS from the nucleus to the cytoplasm in interphase cells (Rupture), followed by the relocalization of TDRFP-NLS to the nucleus following nuclear envelope repair (Post-Rupture). White arrows indicate tracked cells. (B) The fraction of cells (n>100 per condition) that show nuclear envelope rupture following mitosis in the presence of paclitaxel or vehicle control. Time, h: min. Scale Bar, 50 µm.

CHAPTER V: DISCUSSION AND FUTURE DIRECTIONS

Overall, we have illuminated a broad role for Hippo signaling in determining cell fate during mitosis and identified a novel mechanism by which resistance to antimitotic therapies can arise. YAP and TAZ represent targetable proteins that can be exploited to sensitize cells to antimitotic therapy. Depleting YAP and TAZ works in two distinct mechanisms in sensitizing cells to antimitotic therapeutics. First, depleting YAP and TAZ from cells prevent cellular proliferation after escaping an antimitotic induced prolonged mitosis. Second, targeting YAP and TAZ increases cell death during treatment with antimitotics.

5.1 The Hippo pathway times mitosis

Through single-cell tracking and population based protein analysis we have found that cells with an inactive Hippo pathway proliferate after they are born from a prolonged mitosis. We have shown when the Hippo pathway is inactivated in cells born from a prolonged mitosis, these cells stabilize YAP and TAZ levels and are able to proliferate (Figure 2.12). This data validates that the Hippo pathway plays a role in the molecular mechanism of timing mitosis. This has disease relevance because cancer cells often take longer than normal cells to complete mitosis. One reason for this delay in mitosis is because cancer cells often have extra chromosomes and sometimes extra centrosomes which makes it harder for the cell to correctly align and segregate its DNA. Thus, disabling the mechanism that times mitosis may explain one of the selective pressures to inactivate the Hippo pathway in cancer cells.

The mechanisms behind what times mitosis are only beginning to unravel. The Hippo pathway provides the answer to the mechanism behind what is timing mitosis. The role of LATS and its downstream targets YAP and TAZ in timing the duration of mitosis is a novel finding. This adds to the understanding of the basic cell biology of mitosis. Eventually this knowledge could be used to create therapeutic targets to prevent cells from proliferating after undergoing mitosis.

To expand on the data demonstrated here, future studies could elucidate the exact mechanism by which LATS and YAP are phosphorylated when cells enters mitosis. We expect that as cells round up, the decrease in stress fibers result in the activation of the Hippo pathway as previously shown (Wada et al., 2011b). This activation of the Hippo pathway by the loss of stress fibers results in the phosphorylation of LATS and YAP (Wada et al., 2011b). We believe that the rearrangement of actin occurring with the rounding of the cell as it enters mitosis activates the Hippo pathway by phosphorylating LATS1/2 (Aragona et al., 2013).

The natural next steps from this project will be to test to see if indeed a decrease in stress fibers or the rearrangement of actin are the mechanism that activates the timing of mitosis. To test this in the future, we will need to prevent cells from rounding up by overlaying soft agar on the cells as well as by depleting the ezrin/radixin/moesin (ERM) proteins as moesin is a protein critical for cell rounding during mitosis(Dumont and Mitchison, 2009, Kunda et al., 2008).

Future directions will also include testing if activation of the Hippo pathway in a mother cell for a prolonged period of time at any point in the cell cycle can result in arrest

of daughter cells born from that cell. One way to test this is to use trypsinized cells. When cells in tissue culture are tripsinized, the cells lose adherence to the plate and round up, and this rearrangement of the cytoskeleton activates the Hippo pathway. Single-cell tracking of asynchronous populations of trypsinized Fucci RPE and Hippo inactivated cells can help determine whether daughter cells whose mothers were exposed to Hippo activation for prolonged periods of time will proliferate.

In summary we have determined that activation of the Hippo pathway acts as one of the molecular mechanisms that times the duration of mitosis. Inactivation of the Hippo pathway in many cancers may be selected for as a path for cells to disable the mechanism that times mitosis. Future experiments include determining whether prolonged Hippo pathway activation in other contexts during different times in the cell cycle can also prevent daughter cell proliferation.

5.2 YAP and TAZ activity determines cell fate following antimitotic treatment

Using live-cell imaging and other protein based assays we have found that cells with hyperactive YAP and TAZ, and thus an inactive Hippo pathway, undergo decreased cell death in response to antimitotic therapeutics. We have shown that if we target and deplete YAP and TAZ in cells with an inactive Hippo pathway that we can sensitize them to antimitotic therapeutics. This research has important implications for antimitotic therapies, as the Hippo pathway is inactive in many cancers. Therefore, Hippo pathway activity status should be assessed before beginning an antimitotic regimen. Assessing Hippo pathway activity status could provide insight into how tumors will respond to

antimitotic therapy. For example, if cells have increased nuclear YAP and TAZ then they are less likely to undergo cell death when treated with antimitotic therapeutics.

Using a therapeutic that could mimic Hippo pathway activation in cells that have inactivated the Hippo pathway will be important to create and implement *in vivo*. Current therapeutics that prevent YAP from functioning as a transcriptional co-activator include Verteporfin and CA3. Verteporfin functionality by increasing 14-3-3 expression, a protein that sequesters YAP and TAZ to the cytoplasm, thus Verteporfin decreases the amount of YAP and TAZ that can enter into the nucleus and bind to and activate TEAD (Wang et al., 2016a). CA3 was recently developed and it prevents YAP from binding to activating transcription factor TEAD (Song et al., 2018). Using these tools *in vivo* would allow for further characterization of the Hippo pathways role in mitosis both physiologically and in cancer.

Since we determined that Hippo pathway inactivation results in resistance to antimitotic chemotherapeutics a key next step would be to test this finding in an animal model. We would inject YAP-WT overexpressing cells subcutaneously into severe combined immunodeficient (SCID) mice (Paine-Murrieta et al., 1997). We would first establish the tumor volume growth curves and survival for this cell line, then we would observe the chemotherapeutic drug effect on tumor volumes and survival. A coformulation of Taxol and YAP siRNA would be used as one of the chemotherapeutic drug treatments in this experiment (Overholtzer et al., 2006, Salzano et al., 2015). An exciting possibility would be a regression in tumor growth and metastasis in the mouse tumors compared to Taxol treatment alone. We would expect a decrease in tumor size at a faster rate in mice

treated with the coformulation of Taxol and YAP siRNA. Additionally, we would expect to see a decrease in tumor recurrence since less cells would be slipping from mitosis. Since there would be less cells slipping from mitosis, this means that there will be even fewer slipped cells that can re-enter the cell cycle and continue to proliferate. Overall, we believe this combinatorial approach would be a successful therapeutic option.

Besides targeting the Hippo pathway as a therapeutic approach, this pathways activity could also be assess when determining therapeutic regimens. Hippo pathway activity could also be used as a biomarker for antimitotic therapeutics. Assessing Hippo pathway activity could be tested before assigning patients to a regimen including antimitotics. In order to assess Hippo signaling activity, the solid tumor could be biopsied and complete immunohistochemistry for YAP and TAZ localization could be tested for. We would anticipate tumor cells to have active Hippo signaling due to the dense three-dimensional nature in which tumor cells grow together. When cells are in contact with each other they are unable to stretch out and will have decreased stress fibers, this decrease in stress fibers will lead to the loss of inhibition on the Hippo pathway; the Hippo pathway will be activated(Wada et al., 2011b). When the Hippo pathway is active, we would expect the majority of YAP and TAZ to be cytoplasmic. If the patient has an abundance of YAP and TAZ in the nucleus then that would be indicative of Hippo pathway inactivation. Multiple biopsies from different locations will need to be taken in order to determine if any of the clones have inactivated the Hippo pathway since tumors are heterogeneous. If patients have inactivated the Hippo pathway than alternative treatment options should be assessed. Using a Hippo pathway activator in conjunction

with antimitotics could prove to be a successful therapeutic option. However, the combination of antiapoptotic inhibitors and antimitotics would not be recommended as a Phase I clinical trial had to be stopped prematurely because of hematological and non-hematological toxicity (Vlahovic et al., 2014).

To expand upon the research it would be beneficial to verify our findings *in vivo* and determine what other resistance mechanisms occur in antimitotic therapy. To do this we could take biopsies of tumors that have relapsed from antimitotic therapies. We would expect to see an increase in nuclear YAP and TAZ localization in these biopsies of patients who have relapsed on Taxol.

Additionally, a future study would be to keep cells in low dose Taxol for a prolonged time to evolve a population of cells that have become resistant to Taxol. Through GSEA we could identify different mechanisms resulting in the resistance and ideally validate that Hippo pathway inactivation is one route resulting in resistance to Taxol. We would also expect to see additional resistance mechanisms in the way the drug is metabolized. Taxol is metabolized by the liver enzyme CYP3A4. Studies have shown that patients treated with these antimitotics have increased CYP3A4 in their breast cancer tissues (Kapucuoglu et al., 2003). Another common mechanism of resistance we would expect to see is the upregulation of drug efflux pumps of the ATP-binding cassette (ABC) family (Dumontet and Jordan, 2010). The multidrug resistance gene 1 (MDR1) produces the P-gp pump that is activated by hydrophobic drugs and export these drugs from the cytoplasm of the cell out of the cell (Dumontet and Jordan, 2010, Nobili et al., 2012). In addition to validating known mechanisms of resistance to Taxol, it would also be

interesting to discover unknown resistance mechanism. This information would help the medical profession better understand resistance and non-responders to antimitotic therapeutics.

We have determined that cells with an inactive Hippo pathway die from mitosis less when treated with antimitotics. In the future we hope target YAP and TAZ *in vivo* to increase cell death response to antimitotic therapeutics. Additionally we would like to assess Hippo pathway activity in human patients as a biomarker for resistance to antimitotic therapy. Finally, we would like to assess other antimitotic resistance pathways through GSEA.

LIST OF ABBREVIATIONS

Am J Cancer Res ……..... American Journal of Cancer Research

Am J Hum Genet…….……..... American Journal of Human Genetics

Ann Hum Genet……...…Annals of Human Genetics

Ann Intern Med……...…Annals of Internal Medicine

Annu Rev Cell Dev Biol…… Annual Review of Cell and Developmental Biology

Annu Rev Genet……...…… Annual Review of Genetics

Biochem Biophys Res Commun ….. Biochemical Biophysical Research Communications

Biochem J ……..... Biochemical Journal

Biochim Biophys Acta ……... Biochimica et Biophysica Acta

Can J Genet Cytol ……..… Canadian Journal of Genetics and Cytology

Cancer Biol Ther …….…. Cancer Biology & Therapy

Cancer Chemother Pharmacol ……... Cancer Chemotherapy and Pharmacology

Cancer Lett ……..……. Cancer Letters

Cancer Metastasis Rev …….…. Cancer and Metastasis Reviews

Cancer Res …… Cancer Research

Cancer Sci ……. Cancer Science

Cancer Treat Rev ……. Cancer Treatment Reviews

Cell Adh Migr ……..…… Cell Adhesion & Migration

Cell Death Differ ……...…… Cell Death & Differentiation

Cell Death Dis ……...…… Cell Death & Disease

Cell Growth Differ …….. Cell Growth & Differentiation

Cell Mol Life Sci ……..... Cellular and Molecular Life Science

Cell Motil Cytoskeleton …….…….. Cell Motility and the Cytoskeleton

Chromosome Res ……Chromosome Research

Clin Cancer Res ……. Clinical Cancer Research

Clin Invest …..……. Journal of Clinical Investigation

Curr Biol ……...……. Current Biology

Curr Opin Cell Biol …….. Current Opinion in Cell Biology

Curr Opin Genet Dev……..…….. Current Opinion in Genetics & Development

Curr Protoc Cell Biol ……. Current Protocols in Cell Biology

Dev Cell ……..……. Developmental Cell

Drug Resist Updat ……..……. Drug Resistance Updates

EMBO J …….. The EMBO Journal

Eur Biophys J ……...……. European Biophysics Journal

Eur J Biochem …….…….. European Journal of Biochemistry

Exp Cell Res …… Experimental Cell Research

Exp Hematol ……..…… Experimental Hematology

FEBS Lett …….…….. FEBS Letters

Genes Dev..…….. Genes & Development

Human Genet …… Human Genetics

Int J Cancer …… International Journal of Cancer

Int J Radiat Oncol Biol Phys……...……International Journal of Radiation Oncology

Int Rev Cell Mol Biol ……... International Review of Cell and Molecular Biology

Invest New Drugs …….…… Investigational New Drugs

J Biol Chem …….…….. The Journal of Biological Chemistry

J Cell Biochem …… Journal of Cellular Biochemistry

J Cell Biol ……...……. The Journal of Cell Biology

J Cell Physiol……...…… Journal of Cellular Physiology

J Cell Sci ……...…….. Journal of Cell Science

J Cell Science …….. Journal of Cell Science

J Clin Oncol ……..……. Journal of Clinical Oncology

J Diabetes Res ……...… Journal of Diabetes Research

J Mol Biol ……...…….. Journal of Molecular Biology

J Natl Cancer Inst …….. Journal of the National Cancer Institute

J Natl Cancer Inst Monogr….…….. Journal of the National Cancer Institute Monographs

J Pathol ……..…….. The Journal of Pathology

J Vis Exp …….…….. Journal of Visualized Experiments

Mech Dev …….……. Mechanisms of Development

Methods Mol Biol …..……. Methods in Molecular Biology

Mini Rev Med Chem …….……. Mini-Reviews in Medicinal Chemistry

Mol Biol Cell ……...…… Molecular Biology of the Cell

Mol Cancer Ther ……...……. Molecular Cancer Therapeutics

Mol Cell ……...…… Molecular Cell

Mol Cytogenet ……...……..... Molecular Cytogenetics

Mol Oncol ……..……. Molecular Oncology

Mutat Res …….…….. Mutation Research

Nat Biotechnol …….……. Nature Biotechnology

Nat Cell Biol …….……..……. Nature Cell Biology

Nat Chem Biol …… Nature Chemical Biology

Nat Commun ……...…….. Nature Communications

Nat Genet……...……...…….. Nature Genetics

Nat Methods……...……...…….. Nature Methods

Nat Rev Cancer……..……...…….. Nature Reviews Cancer

Nat Rev Drug Discov ……..…… Nature Reviews Drug Discovery

Nat Rev Mol Cell Biol ……..……..…… Nature Reviews Molecular Cell Biology

Nucleic Acid Res……...…….. Nucleic Acid Research

Oncol Rep …….…….. Oncology Reports

Open Biol ……..…… Open Biology

Philos Trans R Soc Lond B Biol Scie …….……. Philosophical Transactions of the Royal Society B: Biological Sciences

Proc Natl Acad Scie USA ……. Proceedings of the National Academy of Sciences of the United States of America

Prog Cell Cycle Res …….…… Progress in Cell Cycle Research

Sci Signal …… Science Signaling

Semin Cell Dev Biol ……...…… Seminars in Cell and Developmental Biology

Trends Biochem Sci ……...….. Trends in Biochemical Sciences

Trends Cell Biol ……..…… Trends in Cell Biology

Trends Pharmacol Sci …..……. Trends in Pharmacological Sciences

Vet Pathol …….……. Veterinary Pathology

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