Regulation of Death and Survival Signals in Mitosis

Mohamed Moustafa-Kamal

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Department of Biochemistry
McGill University
Montreal, Quebec, Canada

Preface

This thesis is a manuscript-based thesis. It contains one published manuscripts and one manuscript that is in preparation. The thesis is divided into five chapters:

1. A general introduction and literature review.

2 - 3. Manuscripts, each with their own abstract, introduction, materials and methods, results, discussion and figures

4. A general discussion of all the results

5. Bibliography

Summary

Mitosis is a highly coordinated phase of the cell cycle, an inter-dependent set of tasks that needs to be executed correctly and in a specific order: entry into mitosis, separation of chromosomes and cytokinesis. The commitment to mitosis has a serious implication; if cells arrest there for longer than usual they either become susceptible to death, or they exit without division with the risk of becoming aneuploid and cancer -promoting. Here I aimed at investigating the mitotic regulation of a death pathway, involving the proapoptotic BH3-only protein BimEL, and a survival pathway, comprising the mTORC1 complex, a promoter of cell growth and proliferation.

In the first part of the thesis we studied the regulation of Bim during mitosis. I observed that BimEL, the dominant splice variant of Bim, is phosphorylated by the mitotic kinase Aurora-A early in mitosis and dephosphorylated by PP2A phosphatase after mitotic exit. Aurora-A phosphorylation stimulated binding of BimEL to the F-box protein βTrCP1 and promoted ubiquitination and degradation of BimEL. These findings describe a novel mechanism by which the oncogenic kinase Aurora-A promotes cell survival during mitosis by downregulating proapoptotic signals. Inhibitors of Aurora-A are currently under investigation as cancer chemotherapeutics. Notably, we observed that knockdown of Bim significantly increased the resistance of cells to an Aurora-A inhibitor, MLN8054. Our findings suggest that efficacy of this class of drugs may function in part by enhancing the apoptotic activity of BimEL.

In the second part of the thesis, we addressed the regulation of Raptor. Raptor is the substrate binding subunit of the mTORC1 complex, a key regulator of many cellular processes including mRNA translation. Raptor is phosphorylated during mitosis but how this contributes to regulation of mitosis is not clear. We show that the signaling downstream of the mTORC1 complex is suppressed in mitotic HeLa cells and across many other cancer and normal cell lines. Mitotic phosphorylation of raptor affects its binding to the complex and probably the integrity of the complex. A phosphorylation-

deficient mutant of raptor was able to reverse this decreased activity of mTORC1 and extend survival time in response to Taxol in a rapamycin-sensitive manner. The mechanism of extended survival in response to Taxol seems to be through destabilization of the Programmed Cell Death protein 4 (PDCD4) downstream the mTORC1/S6K1 axis. PDCD4 is a known inhibitor of the eIF4A helicase and interfering with the mitotic eIF4A activity using a drug, Hippuristanol, synergize with Taxol to kill otherwise resistant cancer cells. From these results, we conclude that the mTORC1/S6K1/PDCD4 /eIF4A axis have a pivotal role in death vs. slippage decision under mitotic arrest and thus may be exploited to gain a clinical benefit in treating cancers resistant to drugs targeting mitosis like Taxol.

Résumé

La mitose est une phase hautement coordonnée du cycle cellulaire, un ensemble interdépendant de tâches qui doivent être exécutées correctement et ce dans un ordre précis: l'entrée en mitose, la séparation des chromosomes et la cytocinèse. Le choix de la cellule d'entrer en mitose peut avoir différentes conséquences. En effet, s'il y a dérégulation et que cette dernière reste dans cette phase plus longtemps qu'à l'habitude, le cellule s'expose soit à la mort ou à un défaut de cytodiérèse qui résulte en une aneuploïdie ce qui favorise le cancer. Ici, je cherche à étudier, pendant la mitose spécifiquement, la régulation d'une voie menant à la mort cellulaire impliquant la protéine pro-apoptotique BH3 : Bim_{EL} et d'une voie de survie comprenant le complexe mTORC1, promoteur de la croissance et de la prolifération cellulaire.

Dans la première partie de la thèse, nous avons étudié la régulation de Bim pendant la mitose. Je observé que Bim_{EL}, l'isoforme prédominant, est phosphorylé par la kinase Aurora A tôt dans la mitose et déphosphorylé par la phosphatase PP2A après la sortie de la mitose. La phosphorylation par Aurora A stimule la liaison de Bim_{EL} au domaine F-box de la protéine contenant des répétitions de la béta transducin (β-TrCP1) ce qui favorise l'ubiquitination et la dégradation de Bim_{EL}. Ces découvertes décrivent un nouveau mécanisme par lequel la kinase oncogénique Aurora A favorise la survie cellulaire pendant la mitose en régulant négativement des signaux pro-apoptotiques. Des inhibiteurs d'Aurora A sont actuellement à l'étude comme agents chimiothérapeutiques pour le traitement du cancer. Nous avons d'ailleurs observé que la perte de fonction de Bim faisait augmenter significativement la résistance des cellules à un des inhibiteurs d'Aurora A soit le MLN8054. Nos résultats suggèrent que l'efficacité de cette classe de médicaments fonctionnerait, en partie, par l'augmentation de l'activité apoptotique de Bim_{EL}.

Dans la deuxième partie de la thèse, nous avons abordé la régulation de Raptor. Raptor est la sous-unité de liaison au substrat du complexe

mTORC1, un régulateur clé de nombreux processus cellulaires, telle la traduction d'ARNm. Raptor est phosphorylé au cours de la mitose, mais nous ne savons pas comment cela contribue à la régulation de la mitose. Nous montrons que la signalisation, en aval du complexe mTORC1, est supprimée dans les cellules HeLa en mitose, dans de nombreux autres cancers ainsi que dans des lignées cellulaires normales. La phosphorylation de Raptor lors de la mitose affecte sa liaison au complexe et probablement l'intégrité du complexe lui-même. Cette observation a pu être renversée à l'aide d'une forme mutante de Raptor ne pouvant pas être phosphorylée. De plus, ce mutant a prolongé le temps de survie des cellules lorsque mises en présence du Taxol et ce d'une manière sensible à la rapamycine. Cette résistance au Taxol semble être due à la déstabilisation de PDCD4 (Programmed Cell Death Protein 4), protéine se trouvant en aval de l'axe mTORC1 / S6K1. PDCD4 est un inhibiteur connu de l'hélicase eIF4A. Lorsque l'on interfère avec l'activité mitotique de eIF4A en utilisant un médicament, comme l'Hippuristanol, il y a un effet de synergie avec le Taxol qui permet de tuer les cellules cancéreuses autrement résistantes. De ces résultats, nous concluons que l'axe mTORC1 / S6K1 / PDCD4 / eIF4A joue un rôle crucial dans la décision de mort ou de défaut de cytodiérèse lors d'une irrégularité pendant la mitose. Il est donc possible de prendre avantage de cette situation pour obtenir un bénéfice clinique dans le traitement des cancers résistants aux médicaments ciblant la mitose comme le Taxol.

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Publications

Related to thesis

1- **M Moustafa-Kamal**, I Gamache, Y Lu, S Li and J G Teodoro. **BimEL is phosphorylated at mitosis by Aurora A and targeted for degradation by βTrCP1**. Cell Death Differ 2013 20: 1393-1403 .

2- **M Moustafa-Kamal**, Wisal El-Asssaad, Yazan Abbas , Valentina Gandin, Thomas Kucharski, Yazan Abas , Bhushan Nagar , Jerry Pelletier, Ivan Topisirovic and J G Teodoro. **mTORC1 Promotes Survival During Mitotic Arrest**[manuscript in preparation].

Not related to thesis

3-**M. Moustafa-Kamal**, R. Towers and J.G. Teodoro. "Targeting tumor cells through the APC/C: lessons learned from viral proteins" in "Proteins Killing Tumor Cells". Transworld Research Network , 2009.

4-Thomas J Kucharski, Paul Minshall**, Mohamed Moustafa-Kamal**, Andrew S. Turnell and Jose Teodoro. Reciprocal regulation between 53BP1 and the Anaphase-Promoting Complex/Cyclosome is required for genomic stability in response to mitotic stress [Cell Reports, 2017 18: 1982–1995].

5- Ester Castellsague1, Owen J. Chen, Javad Nadaf, Barbara Rivera, Somayyeh Fahiminiya, Lai Jiang, Jian Carrot-Zhang, Isabelle Gamache, **Mohamed Moustafa-Kamal**, Leora Witkowski, Albert M. Berghuis, Stefan Schoenberger, Dominik Schneider, Susanne Bens, Reiner Siebert, Colin J. R. Stewart, Ziguo Zhang, William C. Chao, Celia M.T. Greenwood, David Barford, Marc Tischkowitz, Jacek Majewski1, Jose G. Teodoro , William D. Foulkes. Germline mutations in CDC20 result in familial malignant ovarian germ cell tumors and aberrant mitotic progression [manuscript in revision]

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Authors' Contribution

1- MMK performed all the experiments in this manuscript, made the figures, and devised the experiments together with JGT. IG provided helpful advice with DNA cloning. SL prepared GST-BimEL under IG supervision. YL helped with cloning of BimEL, BimL, and BimS. JGT supervised the project, helped devise the experiments and wrote the manuscript.

2- MMK performed all the experiments in this manuscript, made the figures, wrote the first draft of the manuscript and devised the experiments together with JGT. WE did the RT-PCR. VG helped with the S35 protocol. TK helped in making the time-lapse figures. YA did the FPLC experiment. JP provided Hippuristanol. IT provided helpful advice, protocols and reagents and assisted in writing the manuscript. JGT supervised the project, helped devise the experiments and assisted in writing the manuscript.

Table of Contents

List of Abbreviations

AP-1	activator protein 1
APC/C	anaphase promoting complex/cyclosome
S6K1	70 kDa ribosomal S6 kinases1
AML	acute myeloid leukemia
AIF	apoptosis inducing factor
APAF-1	apoptotic protease activating factor-1
AGO	Argonaute
Bcl-2	B-cell lymphoma 2
BAD	BCL-2 antagonist of cell death
BAK	Bcl-2 homologous antagonist/killer
BH	Bcl-2 homology
BIK	BCL-2 interacting killer
BMF	BCL-2 modifying factor
BOK	BCL-2 related ovarian killer
Bim	Bcl-2-interacting mediator of cell death
A1	BCL-2-related gene A1
Bcl-xL	BCL-2-related gene, long isoform
BAX	Bcl-2–associated X protein
βTRCP	Beta-transducin repeat containing E3 ubiquitin protein ligase
BRCA1	breast cancer 1
Bub	budding uninhibited by benzimidazole
JNK	c-Jun N-terminal kinase 1
CARD	caspase recruitment domain
CDC20	cell-division-cycle 20 homologue
CENPE	centromere protein E
CIN	chromosomal instability
CDK1	Cyclin-dependent Kinase 1
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DEPTOR	DEP domain containing mTOR interacting protein

DLC1	dynein light chain
4E-BPs	eIF4E-binding proteins
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ECM	Extracellular Matrix
ERK	Extracellular signal-regulated kinase
FTIs	farnesyl transferase inhibitors
FADD	Fas-associated protein with death domain
FOXO	Forkhead box protein O
GST	Glutathione S-transferase
GSK3	glycogen synthase kinase 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Grb10	growth factor receptor-bound protein 10
GEFs	guanine nucleotide exchange factors
HRK	harakiri
IP	Immunoprecipitation
Mcl-1	Induced myeloid leukemia cell differentiation protein 1
INCENP	inner centromere protein
IRS-1	insulin receptor substrate-1
IRES	Internal-Ribosome Entry Site
KSP	kinesin spindle protein
mLST8	mammalian lethal with Sec13 protein 8
MTA	Microtubule-targeting agents
MOMP	mitochondrial outer membrane permeabilization
MAPK	mitogen-activated protein kinase
Mad	mitotic arrest deficient
MCC	mitotic checkpoint complex
Mps1	monopolar spindle 1
MVA	Mosaic variegated aneuploidy
NOC	Nocodazole
NS	Non-Silencing
NSCLC	non-small cell lung cancer

OA	Okadic Acid
OMM	outer mitochondrial membrane
PUMA	p53-upregulated modulator of apoptosis
PTP	permeability transition pore
PTEN	phosphatase and tensin homologue
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PH	pleckstrin homology
PlK-1	Polo-like kinase 1
PARP-1	Poly [ADP-ribose] polymerase 1
PDCD4	programmed cell death protein 4
PRAS40	proline-rich Akt substrate of 40 kDa
PKC	protein kinase C
PP2A	Protein phosphatase 2A
PP2B	protein phosphatase type 2B
Rictor	rapamycin insensitive companion of mTOR
Rheb	Ras homolog enriched in brain
RTK	Receptor Tyrosine Kinase
Raptor	regulatory protein associated with mTOR
RB1	retinoblastoma 1
RSK	Ribosomal S6 Kinase
SGK1	serum/glucocorticoid regulated kinase 1
SCF	Skp1-Cullin1-F-Box
SAC	Spindle Assembly Checkpoint
TOR	target of rapamycin
TPX2	Targeting Protein for Xenopus kinesin-like protein 2
Thym	Thymidine
TM	trans-membrane
TSC	Tuberous Sclerosis Complex
TKIs	tyrosine kinase inhibitors
UB	Ubiquitin

Chapter 1

Review of Literature

1.1. Cell Cycle: an overview

The cell division cycle is an evolutionarily conserved process necessary for the growth and development of the mammalian cell. The successive activation and deactivation of proteins regulate progression through the various phases of the cell cycle. The cell cycle consists of four phases: G1 (gap 1), S (synthesis), G2 (gap2), and M (mitosis). G1, S, and G2 are collectively referred to as interphase. After mitosis cells may stop cycling and go into G0 (gap 0). The G0 is a resting state in which cells are not dividing and may maintain it for the rest of their lifespan. External growth factors stimulate some cells to enter G1, during which the cells prepare to grow by making the mRNAs and proteins necessary for DNA replication in S phase. In G2, replicated DNA is checked for fidelity, and the cell is ready to undergo mitosis. In mitosis, the cell divides its chromosomes and cytoplasm into two daughter cells (cytokinesis). Mitosis itself is divided into five phases: prophase, prometaphase, metaphase, anaphase, and telophase [1, 2].

The activity of cyclin-dependent kinases (CDKs) is crucial to the progression of the cell cycle. The activity of each CDK can be controlled by the availability of a particular cyclin partner and the expression of a specific CDK inhibitor (CKI) [3]. Two types of post-translational protein modification— phosphorylation and ubiquitination, regulate this network of proteins. Phosphorylation provides a qualitative layer of control, while ubiquitination provides a quantitative one. Ubiquitination is the covalent attachment of multiple ubiquitin molecules to the protein substrate and degradation of the poly-ubiquitinated protein by the 26S proteasome complex[4]. The formation of ubiquitin conjugates requires the activity of three enzymes. A ubiquitin-activating enzyme (E1) covalently attaches to the small protein (76 amino acids) ubiquitin and transfers it to an E2 or ubiquitin-conjugating enzyme. The E2 transfers ubiquitin to a substrate protein with the help of a third enzyme, the ubiquitin ligase (E3). Multiple rounds of this cycle lead to polyubiquitination of the substrate protein [5]. Specificity of this process is provided by the E3 ubiquitin ligase. Two types of ubiquitin ligases have crucial roles in regulating cell cycle transitions, the

Skp1–Cul1–F-box protein (SCF) complex family and the anaphase promoting complex/cyclosome (APC/C). The SCF and APC/C complexes belong to a broader family of cullin–RING ubiquitin ligases (CRLs). RING domain-containing proteins are important for interacting with E2 enzymes, whereas cullin proteins often serve as scaffolds to assemble multi-subunit E3 complexes. Despite the similarities between the SCF complex and the APC/C, their cellular functions are distinct. More specifically, the timing of action of each E3 complex is different: the APC/C is active from mid-mitosis (anaphase) to the end of G1 phase, whereas the SCF complex is active from late G1 to early M phase. The net result of their orchestrated actions provides directionality to the process of cell division [6].

To ensure healthy progeny, cells have evolved checkpoints that induce cell-cycle arrest in response to defects that may have occurred during DNA replication or other steps during interphase. Cell cycle arrest allows repair of these errors so that an intact genome can be inherited by each daughter cell. If the error is not repaired, cell-cycle checkpoints can trigger processes (e.g., apoptosis, mitotic catastrophe, and senescence) to prevent the propagation of damaged or high-risk cells. The mechanisms of several checkpoints are well established. The G1/S checkpoint restricts cells with damaged DNA from entering S phase until the damage is removed, or it triggers cell death or senescence. The intra-S checkpoint delays the firing of replication origins or slows down DNA replication during S phase to minimize replication errors. The G2/M checkpoint prevents cells from premature entry into mitosis, and thus it reduces chromosome missegregation [1, 7, 8]. The spindle assembly checkpoint (SAC, also known as the mitotic checkpoint) is the primary cell-cycle control mechanism in mitosis. It is responsible for the production of genetically identical daughter cells by ensuring the fidelity of chromosome segregation[9]. There also exists a p53-dependent, post-mitotic checkpoint to prevent daughter cells of abnormal mitosis from entering the next interphase [10]. These checkpoints are vital to reducing genomic instability during cell-cycle progression, which may predispose them to cancer.

1.2. Mitosis and Cancer

Loss of normal cell-cycle control is a hallmark of human cancer. Tumor cells accumulate alterations that result in unscheduled proliferation and genomic instability. The correlation between cancer and mitosis dates back to von Hansemann who observed many abnormal mitotic figures in samples from various carcinomas in 1890. Later, this observation led Theodor Boveri to postulate that such misdistribution of chromosome might be a cause for tumor development[11]. This phenomenon was termed aneuploidy (having a chromosome number other than 46), and since Boveri's prediction, it has been shown that most solid tumors are not only aneuploid but have also had structural alteration of chromosomes including deletions, duplications, inversions, translocations. Additionally, those tumors are characterized by a number of mutations in oncogenes like KRAS and p53) and tumour-suppressor genes such as RB1 (retinoblastoma 1), PTEN (phosphatase and tensin homologue), BRCA1 (breast cancer 1) and others[12]. The fact that the consequences of improper separation and distribution of the chromosomes during mitosis can lead to aneuploidy and later to chromosomal instability rationalized targeting mitosis to kill cancer cells and prompted the search for more mitosis-specific drug targets. However, before we introduce those targets, we need to understand first how the checkpoint works during mitosis, and how a failure of this mechanism can lead to aneuploidy and what are the consequences of aneuploidy.

1.2.1.The Mitotic Checkpoint

Accurate chromosome segregation during mitosis is critical for maintaining genomic stability. The kinetochore (a protein structure found at the center of a chromatid) functions as the docking site for spindle microtubules and a signaling center for the spindle assembly checkpoint (SAC). The SAC controls the activity of the Anaphase Promoting Complex/Cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase. APC/C activity requires a substrate-specificity co-activator, CDC20 (cell-division-cycle 20 homologue), in order to recognize its mitotic substrates. Proteins that are targeted for degradation by APC/C include cyclin B1, as well as

securin. Degradation of securin leads to the activation of separase, which, in turn cleaves the cohesin links that hold together sister chromatids. Degradation of cyclin B1, however, causes the inactivation of CDK1 (cyclin-dependent kinase 1) and initiates mitotic exit [13].

The checkpoint prevents premature advance to anaphase and, in vertebrates, is activated every cell cycle immediately upon entry into mitosis or meiosis. The signal generators of this checkpoint are unattached kinetochores. A single unattached kinetochore generates a saturating 'wait anaphase' signal that can delay anaphase for as long as multiple unattached kinetochores can delay anaphase. This signals recruits the mitotic checkpoint components and catalytically convert and release some of them in a form that inhibits the activity of the APC/C^{CDC20}-dependent recognition of cyclin B and securin [14, 15].

Genetic screens in budding yeast helped to identify the first components of the checkpoint signal which included Bub (budding uninhibited by benzimidazole)1–3, Mad (mitotic arrest deficient) 1–3 and Mps1(monopolar spindle 1)[16, 17]. Vertebrate orthologues of Mad1, Mad2, Bub3 and the kinases Bub1 and Mps1 have all been implicated in mitotic checkpoint control. Besides, the vertebrate mitotic checkpoint requires another two kinases, BUBR1 and Aurora B, the ZW10–ROD–zwilch protein complex, and the microtubule motor protein, centromere protein E (CENPE)[15]. Following nuclear envelope breakdown (which marks the entry into mitosis), the checkpoint proteins are recruited to the outer kinetochore surface of all unattached chromosomes. Direct binding of the kinetochore-bound microtubule motor protein CENPE to its binding partner BUBR1 activates the BUBR1 kinase activity. BUBR1 kinase activity is required for the recruitment of a stable MAD1–MAD2 heterodimer, which, in combination with other essential checkpoint components recruits and modifies MAD2 into an active conformation. Activated MAD2 and BUBR1, form a complex with BUB3, tightly associate with CDC20, preventing it from activating the APC/C and thereby inhibiting ubiquitination of securin and cyclin B [18, 19]. Once proper alignment of the chromosomes has been achieved, the cell deactivates the

SAC to finish mitosis. The SAC proteins are cleared off the kinetochore, followed by dephosphorylation of the checkpoint proteins that has been phosphorylated by Mps1 and Bub1. In yeast, the PP1 family of phosphatases accomplishes this, and it is likely that a similar mechanism exists in vertebrates. Lastly, the SAC complex on Cdc20 must be disassembled, and this is achieved by $p31^{comet}$ protein, which physically competes for Mad2 binding to BubR1 releasing cdc20 which then binds and activates the APC/C [20].

1.2.2. Mitosis and Aneuploidy

Aneuploidy is the state in which one or more chromosomes of a normal set are missing or exist in more than their usual number of copies. Unlike euploidy, aneuploid karyotypes will not be a multiple of the haploid number. Aneuploidy is not synonymous with chromosomal instability (CIN): some tumors are stably aneuploid, with a highly abnormal but relatively uniform karyotype. In other tumors, an increased rate of CIN generates diverse karyotypes within a tumor. The former represents 'state' of the karyotype and the later represents the 'rate' of karyotypic change[21, 22]. Aneuploidy can occur as a result of aberrant mitotic divisions caused by defects in the kinetochore-microtubule attachments and dynamics, centrosome (duplication, maturation or segregation), chromosome cohesion and spindle-assembly checkpoint [22].

Improper attachments of chromosomes to spindle microtubules like merotelic attachment can cause aneuploidy. Merotelic attachments occur when a single kinetochore attaches to microtubules that arise from both poles of the spindle. They occur frequently in cancer cell lines and can lead to the missegregation of chromosomes. Two main mechanisms have been suggested to explain why tumor cells have an increased frequency of merotelic attachments: an increased number of centrosomes and hyperstabilized kinetochore–microtubule attachments [23]. Extra centrosomes are produced either by deregulation of the centrosome duplication cycle or as a by-product of cells that become tetraploid. For example, centrosome amplification has been shown to arise through

overexpression of Aurora A kinase or viral infection. Tetraploidy can arise through viral-induced cell fusion, overexpression of Eg5, or cytokinesis failure. Microtubule attachment to kinetochores is reversible and the repeated association and dissociation of individual microtubules from kinetochores generates a dynamic kinetochore–microtubule attachment that is necessary to promote error correction. Inhibition of an attachment-error-correction mechanism that includes the aurora kinase B, borealin, survivin and inner centromere protein (INCENP) leads to increase in the rate of the merotelic attachment [23, 24].

Chromosome cohesion defects might also contribute to aneuploidy in human cancer cells. Resolution of sister-chromatid cohesion at the onset of anaphase depends on separase, a protease that is inhibited by securin. Inactivation of the securin or separase homologues in fission yeast (Cut2p and Cut1p, respectively) results in chromosome loss [25]. In human cancer cells, high level of CIN was observed after removing securin by homologous recombination, [26]. Also, recent work from Solomon *et al.* identified inactivating mutations and reduced protein expression of stromal antigen 2 (*STAG2*) gene in human cancer cell lines, xenografts, and primary tumours. *STAG2* encodes one of the two human orthologues of the yeast SCC3 cohesin subunit, which is a component of the cohesion complex that may form a ring structure around sister chromatids. Additionally, inactivation of *STAG2* in human cell lines leads to a defective sister chromatid cohesion and an increase in aneuploidy[27].

Finally, aneuploidy can also result from chromosome missegregation produced by defects that weaken the SAC signaling. In mammals, the SAC is essential for cell division, and homozygous null mutations in SAC genes are generally lethal [28]. However, heterozygous mutations or hypomorphic mutations in SAC genes can predispose mice to cancer. For example, mice heterozygous for mutations in Mad2 develop spontaneous lung tumors after 18 months [29], and mice with hypomorphic Bub1 alleles display an increase in hepatocellular carcinomas, lung adenocarcinomas, sarcomas, and lymphomas after 19 months [30]. Germline mutations in the mitotic

checkpoint protein BUBR1 have been identified in Mosaic variegated aneuploidy (MVA) patients —a rare disorder characterized by high levels of mosaic aneuploidy and a significantly increased risk of cancer— providing substantial evidence that mitotic checkpoint defects can cause aneuploidy in humans[31].

1.2.3. Aneuploidy and Cancer

Cancer cells are characterized by both aneuploidy and structural alterations in chromosomes. As discussed above, aneuploidy is caused by chromosome separation errors in mitosis, whereas structural chromosomal changes are produced by improper repair of DNA double-strand breaks. Although thought to be different pathways, recent evidence showed that they could be mechanistically linked, with chromosome missegregation promoting additional genomic instability through a direct and an indirect pathway.

Firstly, aneuploidy caused by lagging anaphase chromosomes trapped in the cleavage furrow during cytokinesis, could lead to double strand breaks [32]. Lagging chromosomes, including those that are not missegregated, can also form micronuclei, which often accumulate high levels of DNA damage due to errors in replication [33].

Secondly, aneuploidy creates imbalances in the levels of proteins required for DNA replication, repair or mitosis which increases the DNA mutation rate facilitating the development of genetic alterations that drive cellular growth and transformation[34]. Indeed, a single-chromosomal aneuploidy in yeast produces a modest, but significant, elevation in the rates of point mutations and mitotic recombination, and many aneuploid yeast strains have increased rates of whole-chromosome missegregation[35]. This shows that aneuploidy can induce CIN and therefore act as a self-propagating form of instability. Since cancer cells are characterized by more complex aneuploidies involving alterations in the copy number of several chromosomes, they are thus predicted to show even higher levels of genomic instability [34].

1.2.4.Targetting Mitosis in Cancer

On the premise that tumors have a larger fraction of actively dividing cells than do normal tissues it follows that they should be more vulnerable to agents aimed at cell division, or mitosis. Despite being the shortest phase of the cell cycle, mitosis orchestrates major changes in multiple cellular components. Some signaling pathways are activated, and some are silenced and the timely degradation of key regulators ensures the directionality of the process. Structural and functional Interference with the normal progression of mitosis leads to activation of SAC, which puts a break on progression and induces a prolonged mitotic arrest. Mitotic arrest likely signals the induction of a death program, which is widely used as an antiproliferative strategy to kill cancer cells. Early targets included targeting the dynamics of the microtubules, but recently drugs inhibiting more mitosis-specific targets such as Aurora kinases (AK), Polo-like kinases (PLK), and kinesin spindle protein (KSP) have emerged as targets for cancer therapeutics [36].

1.2.4.1.Targetting the Microtubules

The proper movement of the chromosomes and their adequate segregation to daughter cells requires rapid dynamics of microtubules polymerization. The polymerization of microtubules happens by a nucleation-elongation mechanism in which a nucleus of short microtubules is slowly formed, followed by a rapid elongation of the microtubules at its ends by a reversible, non-covalent addition of tubulin dimers. These rapid dynamics make mitosis particularly sensitive to drugs targeting microtubules. Microtubule-targeting agents (MTAs) have long been used in the clinic and still represent a frontline therapy for many cancers. This class of drugs disrupts proper microtubule dynamics, leading to abnormal spindle formation, chromosome misalignment and the activation of SAC[37]. MTAs can be categorized into (i) microtubule-stabilizing agents, like Taxanes and Epothilones, which stimulate polymerization (ii) and microtubule-destabilizing agents, like Vinca alkaloids, which prevent microtubule polymerization. MTAs have shown antitumor activity in a wide range of tumors, particularly breast, ovarian, non-small-cell-lung and head-and-neck cancers [38].

Microtubule stabilizers, such as Taxol bind to β-tubulin with high affinity, inducing a conformational change in the tubulin. This leads to an increased and stabilized interaction with neighboring tubulin molecules. Although mitotic chromosomes can attach to Taxol-stabilized microtubules, proper tension is not achieved across sister chromatids, and chromosome bi-orientation doesn't happen[39, 40].

On the other hand, Vinblastine causes microtubule depolymerization and targets both tubulin monomers and microtubules by binding to β-tubulin at a region adjacent to the GTP binding site known as the vinca domain. The subsequent conformational change in tubulin then promotes self-association and prevents microtubule formation [41, 42]. The mode of action may be different from the microtubule stabilizers, but it is the SAC-dependent mitotic delay that enhances cell susceptibility towards mitotic death or death after mitotic slippage.

Although MTAs were developed to be selective to actively dividing cells leveraging on the rapid dynamics of the spindles during mitosis, interphase cells may be targeted too. As a consequence, side effects to non-dividing cells are observed. This happens by disrupting physiological processes such as vesicular trafficking, axonal transport, and maintenance of cytoskeleton functions. Normal cells like cycling bone marrow cells and neuronal cells are affected leading to myeloid toxicity and neurotoxicity. In addition, resistance to MTAs compounds the challenges. Therefore, developing novel drugs that do not affect microtubule structures and yet can specifically inhibit the progression of mitosis attracted a lot of interest[36, 43].

1.2.4.2.Targetting the Motor Proteins

Kinesin motor proteins are essential regulators of the spindle as they participate in spindle assembly, chromosome congression and segregation. One of these proteins is Eg5, a plus-end directed motor protein, is required for centrosome separation and hence for building a bipolar spindle. Kinesins drew much interest as possible cancer targets after the identification of Monastrol, small-molecule inhibitor of Eg5[44, 45]. Inhibition of Eg5 functions results in monopolar spindles that lead to mitotic arrest in a SAC-

dependent manner. This mitotic arrest eventually causes cell death. This anti-proliferative activity was shown in a broad range of tumor cell lines, both in cell culture and xenografts. [46]. Another essential mitotic target of the kinesin family is the Centromeric protein E (CENP-E). It is localized at the kinetochores and is required for proper chromosome congression during metaphase. By binding and regulating the activity of BubR1, it acts as a sensor of SAC. It is also responsible for the stabilization kinetochore–microtubule interaction, required for proper bi-orientation of chromosomes during mitosis [47]. CENP-E inhibitors are subdivided into two categories: (i)ATPase antagonist of the motor domain or (ii) farnesyl transferase inhibitors (FTIs). The first group inhibits the ATPase activity of the CENP-E motor domain, making it tightly bound to the microtubules thus preventing proper chromosome alignment during metaphase leading to mitotic arrest and death. [48]. By contrast, inhibiting the farnesylation of CENP-E disturbs the normal assembly and function of the kinetochore complex, thereby weakening the kinetochore–microtubule interactions, activating the SAC leading to arrest and death[49].

1.2.4.3.Targetting the Mitotic Kinases

Kinases belonging to the Aurora kinase and polo-like kinase (Plk) families are considered genuine mitotic kinases based on their peak expression in mitosis, with almost no detection in G0, G1 and S phases (due to proteasomal degradation). These two kinases are overexpressed in cancer cells, often correlating with worse prognosis, which rationalized their targeting[50].

Aurora kinases A, B, and C (AURKA, B, C) play important roles in centrosome/centromere function and spindle assembly during mitosis. Aurora-A role starts early in mitosis by regulating centrosome maturation and separation, thereby playing a role in establishing the bipolar mitotic spindle. Aurora-B is a component of the chromosome passenger complex (CPC) which, by being at kinetochores, plays a role correcting improper spindle attachments contributing to SAC function[51, 52]. Although the role of Aurora-C remains to be fully clarified, functional redundancy with Aurora-B

has been suggested based on its association with the CPC and the fact that it can compensate for Aurora-B loss[53]. Abnormal Aurora kinase activity is associated with defects in cell division and aneuploidy. Aurora-A is amplified in cancers of the breast, ovary, lung, bladder, stomach, pancreas, head-and-neck, and colon, whereas Aurora-B is overexpressed in breast cancer, NSCLC, glioblastoma, and prostate cancer[54]. No obvious role in tumorigenesis has been yet ascribed for Aurora-C.

Polo-like kinases (PLKs) family is a group of serine/threonine kinases that includes PLK1-4, of which PLK1 has been the focus of the majority of PLK research. PLK1 is activated by Aurora-A and has several regulatory roles in the cell cycle, including mitotic entry, centrosome maturation, spindle assembly, anaphase-promoting complex/cyclosome (APC/C) regulation, and cytokinesis[55]. Similar to the Aurora kinases, Plk1 expression is upregulated in a variety of cancers, including breast, ovarian, gastric, colon, head and neck, melanomas and gliomas and often correlates with poor prognosis [56]. The spindle pole localization of both Plk1 and Aurora A during early mitosis is necessary to direct centrosome maturation and separation. Targeting both kinases leads to monopolar spindle formation SAC activation, mitotic arrest, and increased cell death [50]. On the other hand, Aurora B localizes to the kinetochore to regulate kinetochore–microtubule attachment during metaphase. Aurora B inhibitors work differently at the end of mitosis by disrupting cytokinesis and causing polyploidy cells with restricted viability[57].

1.2.5.Fate of cells arrested in mitosis

Although the mechanisms by which anti-mitotic drugs cause a mitotic arrest are now well understood, little is known about how cells respond to this prolonged mitotic delay. During normal mitosis, cyclin B1 degradation triggers mitotic exit. However under prolonged arrest, SAC is activated, cyclin B1 degradation is delayed, and cells eventually die. Initial observations were done in cell culture systems with the results averaged to total populations. However, in those studies, the exact role of the SAC in determining the cell fate or the correlation between the length of mitotic

arrest and cell fate was not clear. Some studies showed that, in response to mitotic poisons, cells exit mitosis without cytokinesis and remain in a tetraploid state followed by death in the subsequent interphase, or following one or more additional cell cycles [58, 59]. Other studies observed that cell death occurs directly during mitosis [60]. Additionally, the mechanism of cell death was uncertain: whereas some studies suggest that a classical apoptotic response occurs [61] others suggest a caspase-independent cell death[62]. Recent advances in imaging techniques allowed us to observe in real time the response of different cell lines to different kinds of anti-mitotic drugs on the level of single cell. This helped, to a certain extent, exclude some of the older hypotheses and to build a new model of the fate of mitotic cells under prolonged arrest.

Firstly, one group showed that there was a wide variation between different cancer line in response to the same drug and that death from mitosis is caspase-dependent[63]. Another group showed that the length of mitotic arrest is drug independent and that the duration of mitotic arrest was not determined by the presence or absence of microtubules [64]. Finally, in a more comprehensive study, Gascoigne and Taylor confirmed the above results in terms of the wide variation in the response of cells, both within cell lines (intraline variation) and between cell lines (interline variation) [65]. This study also observed that the anti-mitotic drug and its concentration are the major factors that affect the cell fate not the length of mitotic arrest and hence the state of the checkpoint. A new model was thus proposed; whereby two competing networks determine cell fate, one is driven by the accumulation of cell death signals, and another is driven by the degradation of cyclin B1 and thus exit from mitosis. Both networks have thresholds, and whichever threshold is breached first determines the fate (Figure 1.1). It was also concluded from the study that the two networks work independently, with no cross talk. However, more recently, this model was updated by work from Hongtao Yu's laboratory, which showed that the two networks could actually interact and affect each other. Using a genome-wide RNAi screen and following the fate of Taxol-treated HeLa cells, Hongtao Yu's laboratory

identified a number of known and novel candidate genes involved in mitotic cell death and slippage [66]. For instance, Knockdown of mitochondrial proapoptotic (Bad, Noxa, and Bax) and SAC fidelity (Mps1, Mad2, and BubR1) proteins increased survival of HeLa, as did depletion of factors that delayed mitotic entry. In contrast, knockdown of APC/C components (ANAPC1/5/13, CDC23, and CDC26), which take part in the slippage network, reduced survival, as did knockdown of the mitotic regulator Plk1 or the MAD2-inhibitor $p31^{comet}$[66]. $p31^{comet}$ prevents conformational activation of MAD2, a key component of the mitotic checkpoint complex (MCC), and thus favors the activation of the APC/C ^{cdc20} and mitotic exit [67]. In the same study, it was shown that in the slippage-prone U2OS cells, $p31^{comet}$ knockdown significantly reduced slippage and increased rates of mitotic cell death. Not only that, but $p31^{comet}$ knockdown in cell death-prone HeLa cells significantly shortened the time to cell death, and accelerated caspase activation. Several other studies have already highlighted possible molecular crosstalk between the cell death and cell cycle machineries: For example, the SAC protein BubR1 is cleaved by caspases during apoptosis [68]. Importantly, CDK1 have been shown to phosphorylate and inactivate pro-survival Bcl-2 family members and upstream cell death- initiating caspases [69].

Collectively, these observations clearly disclosed a level of complexity regarding the role of specific proteins in directly or indirectly affecting the wiring of the other network and argue against the complete independency of their actions. Further analysis is needed to dissect the exact the role of, for example, the Bcl-2 family of proteins and other mitochondrial proteins in affecting the fate of different cell lines. A more detailed understanding of the underlying networks should open up new opportunities to tip the balance towards death, thereby achieving better outcomes using antimitotic in cancer therapeutics.

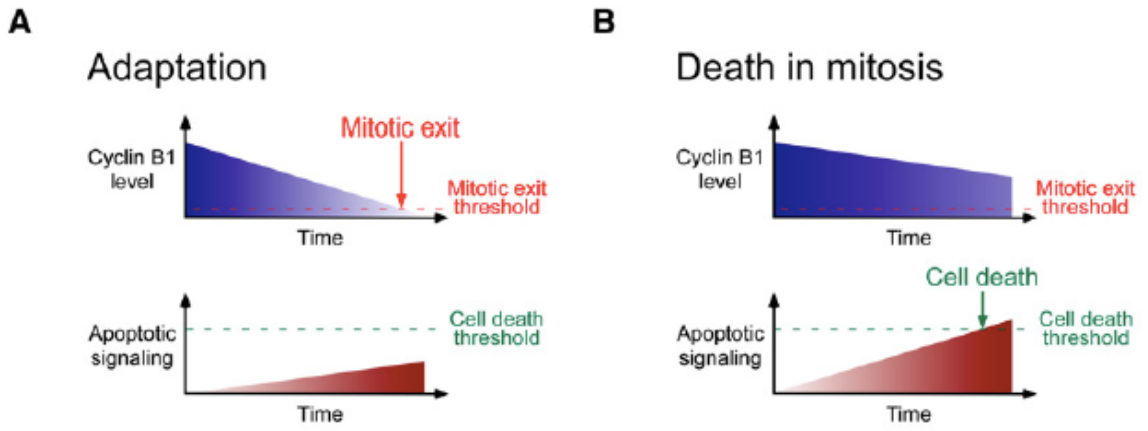

Figure 1.1. The Two Competing Networks Model that Dictates Mitotic Cell Fate
(A) Cyclin B1 (blue) is continuously destroyed during a mitotic arrest, and at the same time, a death signal (red) accumulates. Eventually, cyclin B1 levels fall bellow a threshold required to maintain mitotic arrest, and the cell undergoes adaptation (slippage). (B) Cyclin B1 levels decrease during mitotic arrest but fail to reach the threshold required to allow mitotic exit. Instead, the apoptotic signal reaches a level sufficient to promote cell death in mitosis. Adapted from Holland and Cleveland [70]

1.3. Bim and Bcl2 Family in Apoptosis and Cancer

B cell/lymphoma-2 (Bcl-2) and its relatives comprise the Bcl-2 family of proteins; play a central role in controlling outer mitochondrial membrane integrity and apoptosis. Recently other functions, such as a role in autophagy, have been discovered, but here we will discuss the mechanisms and functions of the BCL-2 family in the context of mitochondria, its role in cell fate decision, the interaction of its members, and its aberrant regulation in cancer.

1.3.1 Apoptosis: an overview

Apoptosis, or programed cell death, is a suicide program essential for tissue homeostasis, development, and immunity. This systematic self-destruction involves a cascade of events that culminates in the activation of a class of cysteine proteases called caspases, which in turn execute the degradation of cellular constituents with minimal impact on neighboring cells. Too little apoptosis can promote cancer and autoimmune diseases and too much apoptosis can enhance neurodegenration[71]. The term "apoptosis" was first coined by Kerr et al. to describe the morphological processes observed in controlled cell suicide [72]. Some of the key morphological features include cell shrinkage, chromatin condensation, and margination to the nuclear periphery, plasma membrane blebbing and fragmentation of the cell into multiple, compact, membrane-bound 'apoptotic bodies' [72, 73].

On the molecular level, once a death signal accumulates, apoptosis is triggered through common cell death machinery that involves activation of caspases. Caspases are evolutionarily conserved and can be found in mammals, all the way down to insects and nematodes [74-76]. Like most proteases, caspases are synthesized in the cell as inactive zymogens called pro-caspase, which consists of a pro-domain at the N-terminal, followed by a large (~20 kDa), p20 and small (~10 kDa), p10 subunit. They can be activated rapidly by proteolytic cleavage of the region between the p20 and p10 domains and removal of the pro-domain, to form the mature and active caspase which is usually a heterotetramer consisting of two p20/p10 heterodimers [77, 78]. All known caspases possess a cysteine residue at

their active-sites which is crucial for their catalytic activity, and they cleave their substrate after an aspartic acid residue (Asp-X sites) within a tetrapeptide recognition motif, hence the name 'caspase' (cysteine aspartate-specific proteases) [79].

Caspases involved in apoptosis can be divided into two groups: the 'initiator' caspases which include caspase-2, -8, -9 and -10 with long prodomains (more than 90 amino acid residues); and the 'effector' caspases, including caspase-3, -6, and -7 with short prodomains (20-30 amino acid residues). The long prodomain in initiator caspases contains structurally related motifs; the DED (death effector domain) in caspase-8 and -10, and the CARD (caspase recruitment domain) in caspase-2 and -9. During apoptosis, interactions between DED or CARD motifs on the initiator caspase prodomain with similar motifs on adaptor proteins lead to activation of the procaspase. Activated initiator caspases then proteolytically cleave and activate downstream effector caspases (caspase-3, -6, -7) which proceed to execute the apoptosis program by cleaving vital cellular proteins. Caspase substrates include the following: cytoplasmic proteins like actin, β-catenin; nuclear structural proteins like Lamin A, B; RNA-binding and ribonucleoprotein-associated proteins including hnRNP proteins C1 and C2; proteins Involved in DNA metabolism and repair including PARP, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), human RAD51; proteins Involved in regulation of cell cycle and proliferation include p21Cip1/Waf1, p27Kip1, the retinoblastoma susceptibility protein (pRB) and many other substrates. [77, 80].

Initiation of apoptosis occurs through either an intrinsic or an extrinsic pathway. In the extrinsic pathway death signals originate at the plasma membrane where an extracellular ligand (e.g., FasL) binds to its transmembrane death receptor on the cell's surface (e.g., Fas receptor), which induces oligomerization of the receptor. This oligomerization leads to recruitment of an adaptor protein, the Fas-associated protein with death domain (FADD) via interaction of the death domain (DD) located on both FADD and the cytoplasmic tail of the death receptor [81]. FADD also contain

another important domain, the death effector domain (DED), through which it recruits procaspase-8 via homologous interaction with another DED in procaspase-8's prodomain region [82]. Together they form the death-inducing signaling complex (DISC), which serves as a platform to bring procaspase-8 in proximity of each other [83]. This induced proximity results in procaspase-8 dimerization and activation due to their low intrinsic proteolytic activity. Active caspase-8 in turn cleaves effector caspases like caspase-3, activating the downstream caspase-signaling cascade [84, 85].

On the other hand, the intrinsic pathway is activated down stream signaling from intracellular stress, DNA damage, growth factor-withdrawal or irregular oncogene expression [86]. It proceeds through the mitochondria and involves loss of mitochondrial membrane potential and release of cytochrome c from the intermembrane space of the mitochondria into the cytoplasm[87]. Once in the cytoplasm, cytochrome c interacts with the adaptor protein, Apaf-1 (apoptotic protease activating factor-1), to form the heptameric backbone of the apoptosome complex. This complex then recruits and activates caspase-9 by inducing its dimerization[88, 89]. Active caspase-9 cleaves and activates downstream effector caspases, such as caspase-3 and caspase-7 [85].

1.3.2 Bcl2 Family Proteins and Intrinsic Pathway of Apoptosis

The Bcl2 family of proteins is a central regulator of the mitochondrial pathway of apoptosis leading to caspase activation, and the opposing actions of its anti- and pro-apoptotic members dictates survival or commitment to apoptosis. The major event in the intrinsic pathway of apoptosis is the mitochondrial outer membrane permeabilization (MOMP). When MOMP happens, it leads to the release of proteins normally found in the space between the inner and outer mitochondrial membranes like cytochrome c, apoptosis inducing factor (AIF), and others. Two models have been proposed to explain the mechanism of MOMP. The first model involves the opening of a permeability transition pore (PTP) in the inner mitochondrial membrane that allows water and solutes up to 1.5 kDa to pass through [90, 91]. Certain pro-

apoptotic stimuli such as increased Ca2+ levels or oxidative stress induces the opening of PTP, allowing influx of water and ions into the mitochondria matrix, causing the loss of mitochondrial membrane potential (ΔΨm), uncoupling of oxidative phosphorylation and matrix swelling. This leads to mechanical disruption of the mitochondrial outer membrane and subsequent release of apoptogenic factors into the cytosol [87, 92]. The second model for MOMP does not involve a PTP. It appears to be mediated by members of the Bcl-2 family of apoptosis-regulating proteins acting directly on the outer mitochondrial membrane. BCL-2 and its relatives are functionally classified as either anti-apoptotic or pro-apoptotic (Figure 1.2). Anti-apoptotic Bcl-2 family members function to block MOMP, whereas the various pro-apoptotic members promote it [93, 94].

Anti-apoptotic BCL-2 proteins contain four BCL-2 homology domains (BH1–4) and are generally integrated within the outer mitochondrial membrane (OMM), but may also be in the cytosol or ER membrane. BCL-2, BCL-2-related gene A1 (A1), BCL-2-related gene, long isoform (BCL-xL), BCL-w, and myeloid cell leukemia 1 (MCL-1) are the major members of the antiapoptotic BCL-2 family and preserve OMM integrity by inhibiting direct activator BH3-only proteins, activated effector BCL-2 proteins or both (discussed later) [95-97].

The pro-apoptotic BCL-2 members are divided into the effector proteins and the BH3-only proteins. The effector proteins includes BCL-2 antagonist killer 1 (BAK), BCL-2-associated x protein (BAX) and possibly BCL-2 related ovarian killer (BOK). For a long time they were described to contain only BH1-3; however recent structure-based alignment of globular BCL-2 family proteins revealed a conserved BH4 motif [98, 99].

The BH3-only proteins share only the third BCL-2 homology (BH) domain and are subdivided based on their ability to interact with the antiapoptotic or proapoptotic-effector BCL-2 members. BH3-only proteins that only bind to the antiapoptotic repertoire are referred to as ‘‘sensitizer’’ and/or ‘‘derepressor’’ BH3-only proteins; [e.g., BCL-2 antagonist of cell death (BAD), BCL-2 interacting killer (BIK), BCL-2 modifying factor (BMF), harakiri

(HRK), and Noxa. They have the ability to occupy binding sites in anti-apoptotic proteins, or disrupt existing anti-apoptotic complexes without directly causing MOMP[100]. The second group includes proteins like BCL-2 interacting domain death agonist (BID), BCL-2 interacting mediator of cell death (BIM), and p53-upregulated modulator of apoptosis (PUMA), interacts with the anti-apoptotic as well as the effectors, and can directly induce BAX and BAK oligomerization and MOMP. These BH3-only proteins are referred to as ‘‘direct activators’’. The net result of the interactions between the antiapoptotic, direct activator/sensitizer/derepressor BH3-only proteins and effectors determine MOMP and apoptosis [96, 101, 102].

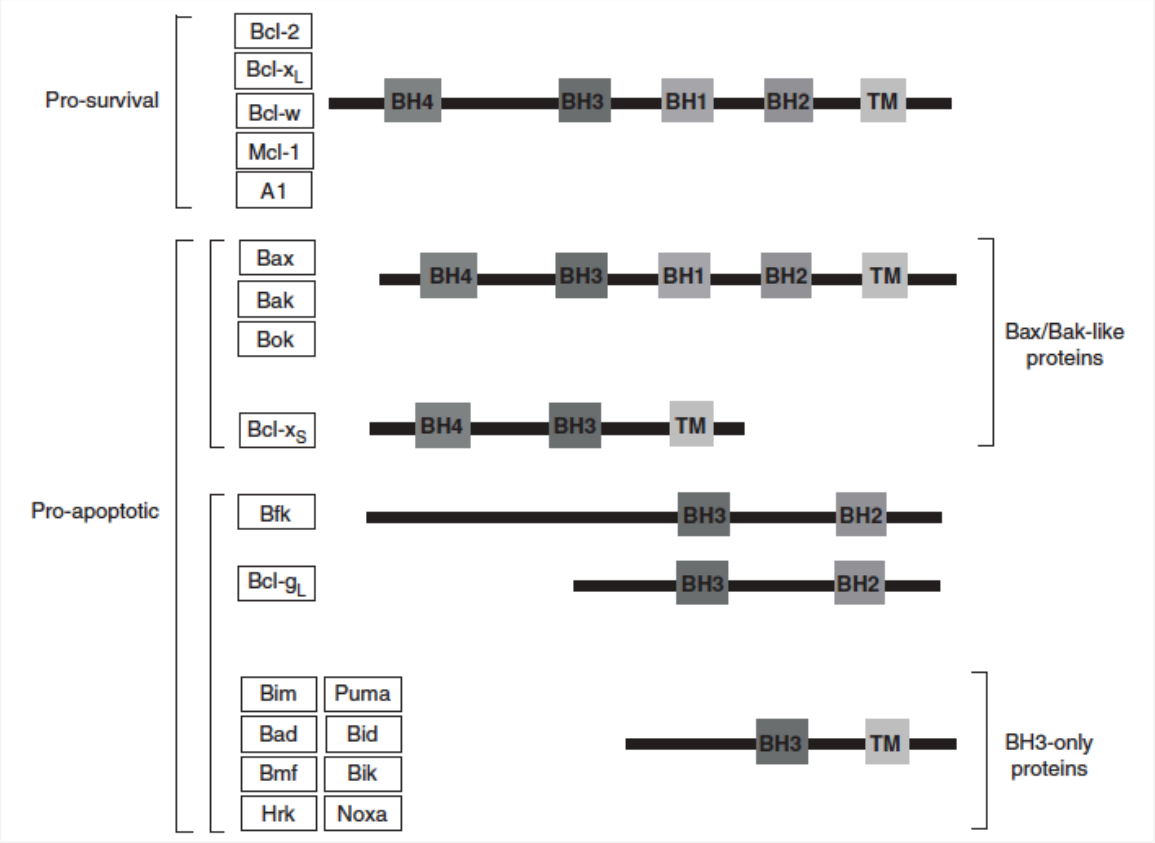

Figure 1.2. The mammalian Bcl-2 protein family

Bcl-2 family members share regions of homology called Bcl-2 homology (BH) domains, and in some cases contain a trans-membrane (TM) domain that mediates insertion into the outer membrane of the mitochondria and to the endoplasmic reticulum. Adapted from Kelly and Strasser [103]

1.3.2.1 Initiation of apoptosis

BAX/BAK activation at the OMM is central to the initiation of apoptosis. In unstimulated cells, BAK resides on the mitochondria, with its transmembrane domain (α9) spanning the outer membrane. By contrast, BAX is primarily cytosolic, with its transmembrane domain unexposed within its canonical hydrophobic groove. Cytotoxic signals promote the accumulation of BAX on the mitochondria [104, 105]. This step, though, is not enough to induce BAX /BAK oligomerization; direct activation by the BH3-only members (e.g., BID and BIM) is still needed to induce critical conformational changes. Interestingly, BID and BIM BH3 peptides can also induce BAK and BAX oligomerization and pore-forming activity with isolated mitochondria [106]. Without this step, Pro-survival proteins can bind through the BH3 domains of activated BAX and BAK, and thereby restrain their pro-apoptotic activity. BAX activity can be restrained by all of the pro-survival proteins, but BAK seems to be restrained mainly by BCL-xL, MCL1 and A1[107].

Other BH3-only proteins, such as BAD, BIK, HRK, Noxa, and PUMA function predominantly by binding to the antiapoptotic members and act through a ''sensitization'' or ''derepresstion'' mechanism (Figure 1.3). Each sensitizer/derepressor BH3-only protein has a unique binding profile towards the antiapoptotic members, which allows for differential regulation. In a sensitization scenario, a complex is formed between an antiapoptotic protein and a sensitizer BH3-only protein, which releases the inhibition on subsequent direct-activators, thus lowering the threshold for BAK and BAX activation without causing apoptosis. For example, if BCL-2 is associated with PUMA, any induced-BIM is not inhibited, and MOMP proceeds. In the absence of PUMA, BIM would be sequestered by one of the antiapoptotic Bcl2 members and the cell may survive [106]. For derepression, a direct activator is already bound by an antiapoptotic BCL-2 protein, and a "derepressor" BH3-only protein releases the direct activator to promote MOMP. For example, stress induces BIM expression, but this extra BIM is inhibited by the antiapoptotic proteins and the cell survives. If a derepressor BH3-only

protein is induced on top, this can lead to release of BIM, and MOMP proceeds[96, 102, 108]. The multiplicity of mammalian BH3-only proteins seems to have evolved to allow more subtle control over the initiation of cell death. Not only differential affinity towards the multiple-homology members, but also differential pattern of expression between tissues, differential localization and differential sensitivity to upstream stress signaling sets the balance between survival and death [109, 110].

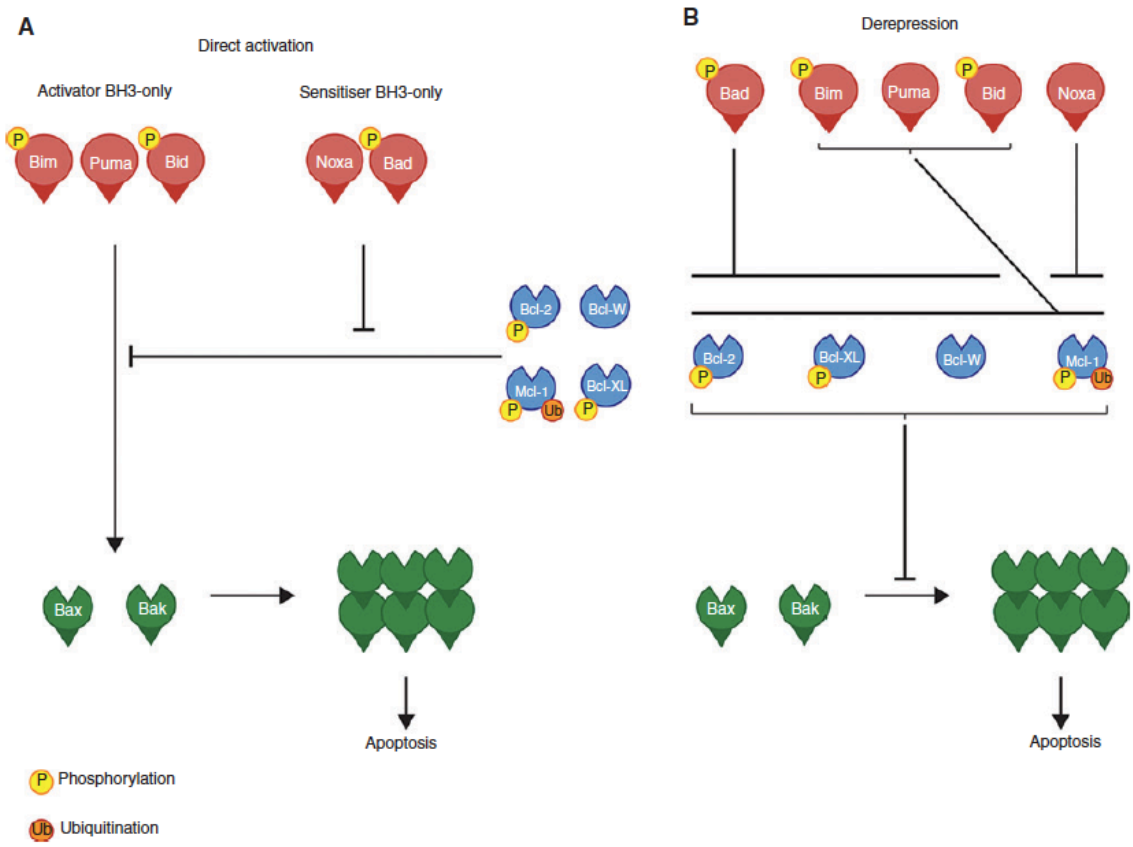

Figure 1.3: Models for regulation of mitochondrial apoptosis.
(A) *Direct activation model*: Activator BH3-only proteins are able to directly bind and activate Bax and Bak, inducing MOMP and this can be prevented by sequestration to anti-apoptotic BH1–4 proteins. Sensitisers act by displacing the activators that have been sequestered by anti-apoptotic proteins. B) *Derepression model*: anti-apoptotic Bcl-2 proteins bind to Bax and Bak preventing them from oligomerising, thus preventing MOMP and apoptosis. BH3-only proteins are able to bind to anti-apoptotic proteins and displace Bax or Bak, allowing them to permeablise the mitochondrial outer membrane. Adapted from Pedley and Gilmore [111].

1.3.2.2 Regulation of the Bcl2 family

As discussed above the ultimate decision to apoptosis is dictated by the net activity of all members of the Bcl2 family. Depending on the context, different layers of regulation of gene expression exist starting with transcriptional down to posttranslational regulation.

Although basal expression of BAX and BAK are enough to induce MOMP, they could also be transcriptionally induced downstream p53 [112]. On the post-translational level, Akt-mediated Bax phosphorylation at Ser-184 residue (located in the C-terminal hydrophobic domain), inactivates Bax and prevent apoptosis in neutrophils after stimulation with Granulocyte-macrophage colony-stimulating factor (GM-CSF) [113]. Additionally, the prosurvival ERK kinase was shown to phosphorylate a ubiquitously expressed b isoform of human BAX resulting in its degradation and inhibition of its BH3 only-independent apoptotic function[114].

The prosurvival BCL-2 protein can also be transcriptionally upregulated by the ERK1/2 pathway as has been reported for BCL-2, BCL-xL, and MCL-1 in pancreatic cancer cells [115]. The anti-apoptotic function of these proteins can also be affected by posttranslational modification like phosphorylation and ubiquitination. A notable example to mention is MCL-1 due to its tight regulation on almost every possible level including its transcription, translation, and stability. In particular, phosphorylation plays a significant role in the regulation of Mcl-1 protein function. Mcl-1 has a shorter half-life (30 minutes) compared to Bcl-2 and Bcl-xL, due to the presence of a PEST region upstream of its BH3 domain[116]. Indeed, MCL-1 turnover is regulated by phosphorylation at two sites within this PEST domain. ERK1/2-dependent phosphorylation of Thr163 within the PEST domain stabilizes MCL-1[117]. However, it has also been reported that phosphorylation at Thr163 'primes' MCL-1 for phosphorylation at Ser159 by GSK3β, which enhances MCL-1 polyubiquitination and degradation by βTrCP1, which can also regulate BIM-EL stability[118]. Additionally, MCL1 can be targeted for degradation by other ubiquitin ligases such as MULE (downstream genotoxic stress)[119],

FBW7 (downstream mitotic kinases) [120] and, conversely, is stabilized by the deubiquitylase USP9X [121].

1.3.2.3 Regulation of the Bim and BH3-only proteins

The BH3-only proteins are the major sensors of stress in the cell, and their multiplicity is essential for the cellular response to diverse stimuli. The BH3-only proteins that are produced constitutively are kept in check until released by diverse mechanisms. For example, BAD is sequestered by 14-3-3 scaffold proteins after phosphorylation on Ser-136 by Akt/PKB [122]. As a result, its activation requires dephosphorylation by Ca^{2+}-dependent protein phosphatase type 2B (PP2B) or calcineurin[123]. The proapoptotic function of the BID BH3 domain is uncovered through a different mechanism that involves the proteolytic cleavage of the large unstructured loop that joins its inhibitory N terminus and the BH3-containing C terminus by a different of proteases like caspase-8 (activated via death receptors)[124], and caspase-2 (activated via heat shock) [125] to give the active form t-BID.

In healthy cells, the proapoptotic activity of both predominant forms of Bim (the splice variants BimEL and BimL) is held in check through sequestration to the dynein motor complex on microtubules via the dynein light chain DLC1 (also known as LC8)[126]. UV irradiation of cells releases Bim via phosphorylation of the conserved DLC binding motif by JNK on Thr-112 [127]. In some cell types, however, Bim is instead bound predominantly to the pro-survival Bcl-2 members on the mitochondria [128]. This pool of Bim experience two fates; the first includes displacement by BMF or PUMA and unleashing of its pro-apoptotic potential [129]; the second involves phosphorylation-dependent displacement from Bcl-xL or Mcl-1 and degradation. The kinase responsible for the second scenario is ERK1/2, which phosphorylates Bim on Ser-59, Ser-69 and Ser-104 [130-132]. Phosphorylation of BimEL on Ser-69 primes it for further phosphorylation on Ser93, 94, 98 by ribosomal S6 kinase (RSK) allowing binding of the F-box proteins β-TrCP1/2, which promotes BimEL poly-ubiquitination and proteasomal degradation [133]. In contrast to ERK phosphorylation that

leads to Bim degradation, JNK phosphorylation of Bim in neurons downstream trophic factor deprivation leads to phosphorylation of mouse BimEL at Ser-65 (the equivalent of human Ser-69) that potentiates its pro-apoptotic activity [134]. Compared to non-neuronal cells where ERK-mediated phosphorylation is mainly detected, JNK-mediated phosphorylation of Bim in neurons leads to its binding to the mitochondrial-enriched prolyl-isomerase Pin1, which stabilizes Bim [135]. This shows the complexity of Bim phosphorylation, with phosphorylation on the same site (Ser 69) leading to degradation (ERK1/2 phosphorylation) or stability (JNK phosphorylation).

Bim and other BH3-only proteins are also regulated transcriptionally. Cytokine deprivation leads to induction of *bim* transcription by the forkhead box O transcription factor FOXO3A downstream of AKT. In cytokine–supported cells, FOXO3A is inactivated and cytoplasmically retained by AKT phosphorylation. Cytokine deprivation leads to inactivation of AKT, dephosphorylation of FOXO3A and to its nuclear entry [136]. Interestingly, ERK-mediated phosphorylation of FOXO3A targets it for proteasomal degradation as an additional way of reduction of Bim expression and promotion of cell survival [137].

In addition to transcriptional and posttranslational regulation, Bim is also regulated post-transcriptionally. The gene locus of Bim has six exons and undergoes alternative splicing to form at least 18 different variants (Figure 1.4). The three major alternative splice variants of Bim described in human and mouse are BimEL (Extra Long), BimL (Long) and BimS (Short) where BimEL is the most abundant[138-140]. All of the major isoforms contain a consensus BH3 domain of 9 amino acids (LRRIGDEFN) that forms an amphipathic α helix, but lack other BH domains (BH1, BH2 and BH4) found in multiple homology Bcl-2 family proteins [138]. BimS is encoded by exon 2, exon 5 (which includes the BH3 domain) and exon 6 (which includes the hydrophobic tail, required for insertion into the outer mitochondrial membrane). BimL further includes exon 4, which encodes a binding site for dynein light chain 1 (DLC1)[126]. BimEL includes exons 2, 4, 5 and 6, but additionally includes exon 3. All 3 isoforms induce apoptosis; the shortest

being the most potent. The latter can be explained by the ability of BimS to directly bind the pro-apoptotic BAX protein, together with the absence of sequestration to the microtubules and post-translational regulation. As discussed above, most of the regulatory phosphorylation targets Exon 3, which is unique to BimEL [130, 141-143].

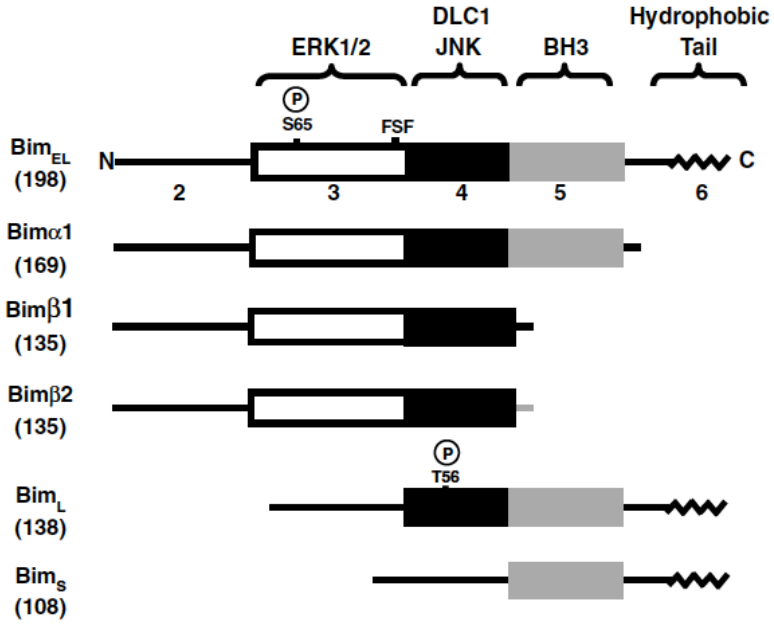

Figure 1.4: Bim splice variants that are subject to post-translational regulation
The numbers beneath each Bim protein represent the number of constituent amino acids (according to the human sequence), while the numbers below the BimEL figure represent the different exons. Adapted from Ley, Ewings [144]

Finally, Bim expression can also be negatively regulated by a variety of microRNAs. Embryonic stem cells deficient in the Argonaute (Ago) 1-4 proteins (defective in microRNA silencing), show upregulated expression of the three isoforms of Bim (BimEL, BimL, and BimS) and undergo apoptosis that can prevented by simultaneous expression of activated Akt [145]. The 3'-UTR of Bim mRNA is targeted by the oncogenic miR-17~92 cluster [146]. Genetically engineered mice with higher expression of miR-17~92 in lymphocytes develop lympho-proliferative disease and autoimmunity and die prematurely. Lymphocytes from these mice showed more proliferation and less sensitive to cell apoptotic stimuli [147]. Conversely, mice deficient for miR-17~92 die shortly after birth with lung hypoplasia and a ventricular septal defect as a result of increased Bim expression [148]. Interestingly, dexamethasone treatment of lymphocytes led to reduced expression of the miR-17~92 cluster, with concomitant up-regulation of Bim and apoptotic sensitization [149].

1.3.2.4 Normal physiological functions of The Bcl2 family

Studies using gene deletion and gene targeting in mice have revealed the functions of many BCL-2 family members in both health and disease. As expected, these studies confirmed to an essential protective role for the anti-apoptotic members and its involvement in tissue homeostasis. The first member of this family, BCL2*,* was identified in human follicular lymphoma as a translocation to the human immunoglobulin heavy-chain locus that causes its constitutive overexpression [150]. Later studies using constitutive or conditional gene deletion of different members of the Bcl2 showed that in some case they have a redundant role and in other cases their absence cause a tissue specific lesions that can't be rescued by other members. For example, BCL-2 deletion causes fatal polycystic kidney disease due to the death of renal epithelial progenitor cells and depletion mature lymphoid cells. Remarkably, these defects can be reversed by concomitant loss of the BH3-only protein BIM [151]. On the other hand, loss of BIM can only rescue some of the defects associated with BCL-xL deletion like testicular and

erythroid defects but not the excessive neuronal death [152]. The ability of *bim* deletion to rescue the phenotypes observed with *bcl-2* deletion seems to be attributed to its unique BH3 domain since replacing it with the BH3 domain of PUMA, Noxa or BAD failed to rescue *bcl-2*-deficient animals [153].

Bax and Bak appear to be redundant in function as evidenced by the fact that the loss of either gene has, in general, little effect on tissue development. However, combined deletion of both genes is embryonic lethal due to failure of elimination of excess cells in several tissues [154]. Though, cells from Bax/Bak double knockout mice that survive birth are completely resistant to diverse apoptotic stimuli including enforced expression of BH3-only proteins, which confirm the absolute requirement for Bax/Bak to execute the apoptotic signal downstream the BH3-only proteins [99].

Genetic manipulation of the BH3-only proteins revealed that the loss of the "activator" type of proteins like BIM, BID and PUMA has a generally detrimental effect compared to the loss of the "sensitizer" type of proteins like BAD, BIK, HRK, and Noxa which, when deleted, shows a limited effect. For instance, loss of BIM shows a deleterious effect on the hematopoietic, lymph and immune system [155]. Loss of Bim renders lymphocytes refractory to Ionomycin, Taxol, and cytokine deprivation, and partially resistant to glucocorticoids[156]. Under normal conditions, Bim is needed to delete autoreactive lymphocytes and antigen-activated T cells during the shutdown of immune responses[157].

1.3.3 Bcl2 Family Proteins and Cancer

Studies on the B-cell lymphoma in the 1980s provided early insights into the role of the Bcl-2 in tumor development. Now considered an oncogene, BCL-2 overexpression confers protection against death to tumor cells rather than promoting proliferation [103]. In transgenic mice, overexpression of pro-survival BCL-2 causes a low incidence of lymphoma. However, combining BCL-2 overexpression with MYC overexpression in (Eμ-bcl-2/c-myc) model led to marked acceleration in B-cell lymphoma development compared to expressing either transgene alone [158]. The

mechanism behind this synergy is likely because of the ability of BCL-2 to balance the apoptosis induced by c-myc oncogene. Myc is a transcription factor and controls cell size, proliferation and differentiation and its overexpression leads to induction of apoptosis [159].

The fact that the antiapoptotic Bcl-2 family members have oncogenic potential of suggests that proapoptotic members could be tumor suppressors. Indeed, both *bim* alleles are deleted in 17% of mantle cell B lymphomas [160]; and *bim* expression is decreased in a variety of renal cell carcinoma due to promoter hypermethylation [161]. Loss, of even one bim allele in Eμ-myc mice, accelerates tumorigenesis, mostly due to early onset of acute B-cell-leukemia[162]. Finally, BIM degradation has been shown to have a role in mediating metastasis, another hallmark of cancer. Normally, epithelial cells when detached from substratum, undergo apoptosis through a process termed anoikis as a result of loss integrin signals required for survival. A metastatic cell needs to acquire anchorage independence to overcome anoikis and be able to survive. Detachment leads to rapid degradation of Mcl-1 via a GSK-3β–dependent proteasomal pathway and reduced ERK activity (through attenuated integrin signaling) which culminates Bim induction. Importantly, this process is deregulated in metastatic tumors. Overexpression of EGFR results in the maintenance of ERK activity following detachment, thus preventing Bim induction and anoikis, and overexpression of Mcl-1 can directly antagonize BIM function [161, 163].

1.3.4. Deregulated BIM function downstream oncogenic signaling

The Bcl-2 family of proteins including BIM is involved with a variety of oncogenic signaling that mediates many of the hallmarks of cancer [164]. In addition to the involvement in the ability to resist cell death, deregulation of the Bcl-2 family can be also be involved with sustaining proliferative signal, evading growth suppressors and activating invasion and metastasis (discussed above). In the case of BIM, its expression is regulated by at least two signal pathways, Raf-MEK-ERK1/2 and PI3K-PDK-AKT, which are

deregulated in cancer [164]. The redundancy in using two pathways help cancer cells acquire selective advantage over time, which, of course, has implications for how we target cancer cells. In some cases, as I will discuss, tumor cells activate either the ERK1/2 or the PI3K pathway, and in some case both get activated downstream a common oncogene which leads eventually to a decrease in BIM expression level.

1.3.4.1 Sustaining Proliferative Signaling: EGFR signaling and Bim

Cancer cells have the ability to maintain growth and proliferation in the absence of extracellular signaling molecules. Epidermal growth factor (EGF) is the key regulatory factor in promoting cell survival. When EGF is withdrawn from the extracellular environment, normal cells undergo apoptosis [165]. EGF binds to a family of receptors (ErbB receptor family) and elicit specific cellular responses through downstream signaling cascades. The EGF receptor (ErbB) family consists of four closely related tyrosine kinase transmembrane receptors: ErbB1/ EGFR, ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4. EGFR has been shown to be involved with many tumours of epithelial origin, including cancers of the lung, breast, head and neck and bladder, and is associated with a poor prognosis[166]. Particularly, in non-small cell lung carcinoma, EGFR overexpression, kinase-activating mutation and in-frame deletion of the extracellular domain have been observed. Treatment of sensitive NSCLC cells with EGFR-selective tyrosine kinase inhibitors (TKIs), like erlotinib, increased the expression of BIM. Erlotinib can also inhibit growth of lung cancer xenografts in nude mice associated with increased BIM expression [167].

Two pathways are downstream of EGFR are involved in BIM deregulation: the RAS/RAF/MEK/ERK and PI3K/AKT pathways. EGF activates ERKs through activating RAS. In normal cells, the activity of RAS is controlled by the ratio bound GTP to GDP. In order to be active, RAS have to be in close proximity to guanine nucleotide exchange factors (GEFs) such as SOS, which itself is activated downstream EGFR. Activation of RAS leads to the activation of the MKKK (MAP Kinase Kinase Kinase) Raf-1. Raf-1 kinase then

phosphorylates and activates the MKK MEK1/2. Activated MEK1/2 then phosphorylates and activates ERK1/2 [168, 169] Active ERK1/2 phosphorylation of BimEL prime for further phosphorylation by 90 kDa ribosomal S6 protein kinase (Rsk) creating a binding site for the E3 ligase βTrCP1/2 which targets BimEL for proteasomal degradation [133] Activation of Rsk also causes phosphorylation and inactivation of the pro-apoptotic Bcl-2 family member BAD [170] .

PI3K/AKT pathway is activated by EGF through binding of PI3K's SH2 domain to tyrosine phosphorylated EGFR. PI3K activation catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 act as binding sites for proteins with pleckstrin homology (PH) domains like the serine/threonine kinase AKT. AKT's PH domain binds to phosphatidylinositols generated by PI3K leading to its translocation to the plasma membrane where it becomes phosphorylated and activated [171, 172]. AKT promotes cell survival by directly phosphorylates two apoptotic proteins BAD and caspase-9 inhibiting their apoptotic activity and phosphorylating FOXO3a preventing its translocation to the nucleus and thus transcriptional induction of BIM [173].

In addition to activating mutations of EGFR that are observed in lung cancer, mutations in other down stream targets like RAS and RAF are involved in multiple other types of cancer. The *ras* gene is mutated in almost 30% of all human tumors, with high incidences found in adenocarcinomas of the pancreas (90%), the colon (50%), the lung (30%), in thyroid tumors (50%) and myeloid leukemia (30%) [174] with highest frequency in KRAS (about 85% of total), then NRAS (about 15%), then HRAS (less than 1%). These mutations keep RAS locked in a GTP-bound state and thus constitutively active [169]. Downstream RAS is RAF, which exist in three isoforms, ARAF, BRAF, and CRAF. BRAF is commonly mutated in melanoma, thyroid and colorectal cancer [175]. Specifically, $BRAF^{V600E}$ mutation in melanoma cells protects against apoptosis induced by cisplatin or actinomycin D [176]. In $BRAF^{V600E}$-positive colorectal cancer cell lines serum withdrawal and MEK inhibition by U0126 or AZD6244 leads to a strong

increase in BIM expression. Interestingly, death under these conditions is significantly reduced by siRNA mediated Knockdown of BIM [177] In another example, activation of the ERK1/2 pathway by expression of mutant HRAS or mutant CRAF was shown to reduces sensitivity of baby mouse kidney epithelial cells to paclitaxel by promoting BIMEL phosphorylation and turnover. Treatment with the proteasome Bortezomib restored BIM induction, abrogated H-ras-dependent paclitaxel resistance, and promoted BIM-dependent tumor regression [178].

The rational behind targeting the ERK1/2 pathway in the above examples is that some cancers, like the one with BRAF mutation, are addicted to this pathway. However in tumors with mutations in RAS or RTKs, targeting ERK1/2 may not be enough due to the redundancy of survival signals. In these cases targeting RTKs, PI3K, or AKT may improve the outcome of the treatment [179-181].

1.3.5 Targeting the Bcl-2 Family in cancer

Cancer cells have evolved different mechanisms to escape or limit oncogene and stress-induced apoptosis. Most common is the loss of the tumor suppressor p53, which removes an important response to DNA damaging agents. Alternatively, tumors may use a different mechanism by increasing expression of antiapoptotic regulators (Bcl-2, Bcl-xL) or by downregulating proapoptotic factors (Bax, Bim, Puma), through the maintenance of survival signals downstream RTKs, RAS, PI3K and other oncogenes. As shown before, γ-irradiation, DNA damage-inducing chemotherapeutic drugs (e.g., etoposide) or glucocorticoids (e.g., dexamethasone) can't induce death in BCL-2-overexpressing lymphoma cells [182]. So it followed that a reasonable strategy to bypass such resistance mechanisms is to target the prosurvival-BH3 only proteins' interaction using **'**BH3-mimetics**'** which led to the development of ABT-737 and its clinical analogue ABT-263 (Navitoclax) by Abbott Laboratories [183, 184]. This compound binds strongly (low nM affinity) to Bcl-2, Bcl-xL and Bcl-w (but not to Mcl-1 or A1) displacing the endogenous BH3-only proteins, which can then

bind to MCL-1, A1 or BAX/BAK. This causes killing of tumour cells via a BAX/BAK-dependent mechanism [185]. ABT-737 was shown to enhance the killing of NSCLC cell lines expressing mutant EGFR in combination with Gefitinib (which induces BIM accumulation), confirming the need to target pro-survival proteins for optimal therapeutic cell killing [186]. Additionally, ABT-737 has been found to potently synergize with various chemotherapeutic drugs in the killing cancer cells. Colon cancer cells with BRAF mutation show only a modest response when treated with a MEK inhibitor. In these cells, upregulation of Bim was insufficient to induce apoptosis and was countered by overexpression of Bcl-2. Addition of ABT-737 led to a switch in the effect of MEK inhibition from cytostatic to a cytotoxic, causing long-term tumor regression in mice xenografted with human tumor cell lines [187]. Likely, ABT-737 synergies with Docetaxel in breast cancer cell lines overexpressing BCL-2 [188].

One of the major problems facing BH3 mimetics like ABT-737 and ABT-263 is that they don't target MCL-1. Indeed, cells expressing Mcl-1, which the drug does not bind, proved resistant to ABT-737, whereas those with low Mcl-1 are highly sensitive [185], a property that can be used to predict the sensitivity of tumors to the BH3 mimetics. Alternatively, a more comprehensive approach called "BH3 profiling" is now used to predict the priming of a tumor to apoptosis [189] and to predict sensitivity to small molecule BH3 mimetics [190, 191]. In BH3 profiling, mitochondria isolated from the cell of interest (a tumor sample) is incubated with standardized amounts of peptides derived from the BH3 domains of BH3-only proteins (activators and sensitizers) and observe which peptides cause MOMP as measured by either cytochrome c release or depolarization across the inner mitochondrial membrane. The premise is that different cancers have addictions to different members of the antiapoptotic Bcl2 members and hence different sensitivities to apoptosis [190]. For example, HRK BH3 peptide binds with high affinity only to BCL-XL. Thus, MOMP following HRK peptide incubation indicates dependency on BCL-xL. This technique was used recently to test (ex vivo) for a population of acute myeloid leukemia (AML)

cell lines that are sensitive to ABT-199, a selective BCL-2 antagonist[191]. Collectively, these finding about the effects of BH3 mimetics support a strong proof of principle that targeting the Bcl-2 prosurvival protein will have a significant use in cancer therapy, especially in combination with other chemotherapeutics and probably in case of cancer resistance.

1.4 Regulation of the Apoptotic machinery in mitosis

Despite the extensive use of anti-mitotic drugs in the treatment of numerous malignancies including breast cancer, ovarian cancer, non-small cell lung cancer (NSCLC) and leukemia, they have poor predictability in addition to their toxicities and the development of resistance. One of the reasons behind this is the complex fate of cells treated with these agents after chronic activation of the mitotic checkpoint. As discussed earlier in this introduction, even within the same cell line the fate of a seemingly homogenous population depends on the type of the drug, its concentration and other factors that differ even between daughter cells of the same genetic background [63, 192, 193]. The "two competing networks" model proposed by Gascoigne and Taylor [65] comprises a death network and an exit network. Concerning the death signal, several lines of evidence suggest that death in mitosis is mainly through a mitochondrial-dependent intrinsic pathway requiring mitochondrial outer membrane permeabilization (MOMP) to activate the downstream caspase-dependent death machinery [65, 194]. I will focus on reviewing the current understanding of the dynamic regulation of the death machinery including the Bcl-2 family, the mitochondria and the cross talk between the death network and the exit (slippage) network.

During mitosis, transcription is heavily suppressed (chromosomal condensation) and general translation is reduced except for essential proteins (discussed later). So, the regulation of mitotic progression is governed largely by post-translational modifications, in particular protein phosphorylation and proteasome-dependent degradation. Indeed, during mitosis, a significant amount of the proteome undergoes mitosis-specific phosphorylation as a result of the surge in CDK-1 kinase and other mitosis

specific kinases [195, 196]. Many proteins of the Bcl-2 family and the apoptotic machinery are phosphorylated during mitosis; for example, MCL-1 Bcl-xL, Bcl-2, BIM, BID, Caspase-2 and Caspase-9, are phosphorylated. We will discuss in detail the current knowledge about posttranslational regulation of these proteins.

1.4.1 Mcl-1

Compared to other multi-domain Bcl-2 proteins, Mcl-1 has a shorter half-life due to proteasomal degradation. Mitotic degradation of Mcl1 was first reported by Harley *et al.,* who showed that Mcl1 levels decline during a prolonged mitotic arrest as a result of proteasomal degradation. This mitosis specific degradation requires phosphorylation on threonine 92 (T92) by Cdk1. They also identified the APC/C as the relevant E3 ligase. Importantly, expressing a phosphorylation-deficient Mcl1 T92A mutant protected U2OS cells from death during a prolonged mitotic arrest [197]. Subsequent reports from two different groups confirmed that Mcl1 was degraded in mitosis but through a different kinase- E3-ligase network. They showed that Mcl1 was stabilized in FBW7-deficient cells arrested in mitosis, implicating the SCF ^{FBW7} as the relevant E3 ligase. Since SCF ^{FBW7} recognizes a phospho-degron in its targets, accordingly the authors identified phosphorylation of serine 121, 159 and threonine 163 as being required for SCF ^{FBW7} binding and also identified JNK, p38, and CKII as well as Cdk1 as the relevant kinases [120, 198]. Importantly, they showed that FBW7- deficient HCT116 cells were more likely to slip out of mitosis when treated with Taxol (high MCL-1 levels), while reducing the MCL1 protein levels in these cells with shRNA decreased mitotic slippage, enhanced Taxol- or vincristine-induced apoptosis [120]. Recently, the role of Mcl-1 as an important mitotic survival factor was also shown in RKO cells; overexpressing Mcl-1 delays mitotic death time and RNAi-mediated inhibition accelerates it[199].

1.4.2 Bcl-2 and Bcl-xL

During normal or prolonged mitosis, BCL2 and BCLXL become highly phosphorylated on multiple serine/threonine sites, without being degraded as in the case of MCL. CDK1 phosphorylates BCL2 at the Thr56 residue, which

can be detected on mitotic chromosomes or in the mitochondrial fraction. The phosphorylation is claimed to inhibit the Bcl2 anti-apoptotic activity although it exist within a nonstructural loop that links the BH3 and BH4 domains and thus should not affect the binding to the BH3 partners [200, 201]. In addition, overexpressed Bcl-2 seems to bypass the effect of this phosphorylation and partially prevent death in response to Taxol [194]. CDK1 was also shown to phosphorylate BCL-xL on Ser62 thereby inhibiting its function. A Ser62 phospho-deficient mutant showed higher binding to BAX and a significant protection from death induced by anti-mitotic drugs [201-203]. Bcl-xL levels seem to have an upper hand in determining the cell fate during mitosis, with Mcl-1 as a second determinant. As was recently shown, Mcl-1 degradation in cells with higher levels of Bcl-xL is insufficient to induce mitotic cell death, compared to cells with low levels of Bcl-xL [204].

1.4.3. BH3 only proteins

Mitotic regulation of pro-survival Bcl2 family proteins necessitates the presence of a counter regulation on the proapoptotic BH3 only proteins side to set a balance for apoptosis. Of particular importance are BIM, BID and NOXA. BIM and BID have the ability to neutralize both MCL-1 and BCL-xL, while NOXA can target MCL-1 only [203, 205]. Noxa is subject to transcriptional regulation down stream myc oncogene and is not regulated on the posttranslational level. BIM and BID, in addition to their regulation by myc, undergo posttranslational regulation in mitosis[206].

Early reports have shown that BimEL is phosphorylated during mitosis and suggested that this phosphorylation is catalyzed by ERK1/2[207] and ERK5[208] most likely as a pro-survival signal. However, more recent studies have found that mitotic phosphorylation of BimEL is not dependent on ERK1/2 or ERK5 signaling;[209] but rather upon cyclin-dependent kinase 1 (CDK1) activity [210, 211] and is associated with the polyubiquitination and proteasome-dependent turnover of BimEL [210]. Although there is now considerable evidence that Bim levels are regulated by phosphorylation and proteasome-mediated proteolysis during mitosis, the mechanism remains

unknown (discussed in details in Chapter 2 which features the phosphorylation-dependent degradation of BimEL in mitosis).

Contrary to Bim, Bid mitotic phosphorylation activates its proapoptotic activity. Bid is phosphorylated on Ser67 as cells enter mitosis. RKO cells (an apoptosis-prone cell line) expressing a non-phosphorylatable version of Bid (Bid-D67A) or a BH3-domain mutant were resistant to mitotic-arrest-induced apoptosis. Phosphorylation of Bid in mitosis seems to suffice for its activation with no need for caspase 8-induced cleavage and formation of t-Bid. Indeed, the non-cleavable Bid-D59E mutant was shown to be still phosphorylated in mitosis and able to restore sensitivity to Taxol in RKO cells following endogenous Bid knockdown[212].

Noxa, another BH3 only that is known previously for its pro-apoptotic role in DNA-damage response downstream p53, came up in genome-wide siRNA screen as an important regulator of mitotic death in response to Taxol. In HeLa cells, Noxa siRNAs decreased the percentage of apoptotic cells, similar to that of the siBax-1 and siBak-1 mixture. Additionally, depletion of Noxa increased the duration of taxol-induced mitotic arrest and delayed the onset of apoptosis [66]. U2OS cells, in which Mcl-1 is not critical for mitotic death, were not affected by Noxa siRNAs, which confirm the thesis that different cell lines depend on different anti-apoptotic Bcl2 members for protection and hence will be sensitive to the activity of different BH3 only proteins[111].

1.4.4 Caspases

CDK1–cyclin B1 phosphorylates and inactivates two important caspases, one is upstream of the mitochondria; caspase 2 and the other is downstream, caspase 9. Caspase-2 is an initiator caspase and has been implicated in response to DNA damage, heat shock-induced death, and mitotic catastrophe-induced death. Cdk1 phosphorylates caspase-2 within an evolutionarily conserved sequence at Ser340 in the caspase-2 interdomain thus preventing its activation[213]. Caspase-2 activation involves caspase-2 binding to an adaptor protein named RAIDD leading to caspase-2 dimerization, binding of an additional protein, PIDD, and formation of pro-

apoptotic multiprotein complex known as the PIDDosome [214]. Interestingly, the mitotic checkpoint protein, BubR1, was shown recently to inhibit PIDDosome-mediated apoptosis after ionizing radiation due to competition with RAIDD recruitment [215]. Additionally, CDK-1 phosphorylates caspase-9 on Thr125, inhibiting its proapoptotic activity. Induction of apoptosis is enhanced in U2OS cells when a non-phosphorylatable mutant replaces endogenous caspase-9[61]. This site is an inhibitory site targeted during interphase by ERK1 and ERK2 mitogen-activated protein (MAP) kinases [216]. The implication of this inhibition is that it sets a threshold for the induction of apoptosis, since even in case of accidental MOMP and cytochrome c release this will not be able to promote apoptosome formation and hence caspase 9 activation. As shown previously, caspase activation initiated in mitosis can cause cleavage of spindle assembly checkpoint proteins like BubR1, resulting in inactivation of the checkpoint and exiting mitosis due to the rapid decline of CDK-1-cyclin B1 activity [68].

Although CDK1 activity seems to tip the balance towards cell death by inhibiting the anti-apoptotic Bcl2 activity, it readjusts it by a counter-inhibition of the pro-death BH3 only proteins and the initiator caspases. As discussed earlier, the final outcome of death in mitosis or exiting without death (slippage) will depend on how fast a mitotic cell reaches its death threshold. Most of the effects on the apoptotic machinery observed thus far in mitosis are due to post-translational modifications. However, recent data from different laboratories suggest that active translation is needed to maintain the mitotic state [217-219], which adds another important level of regulation to the death vs slippage decision during prolonged mitotic arrest.

1.5. mTOR Kinase and Pro-survival Signaling

The mechanistic (formerly ‘‘mammalian’’) target of rapamycin (mTOR) is a serine/threonine protein kinase that belongs to the family of phosphoinositide-3-kinase (PI3K)-related kinases (PIKKs) together with ATM, ATR, and DNA-PK. All the members share C-terminal protein kinase domains similar to the lipid kinase PI3K, thus giving the family its name. While the members of this family respond to genotoxic stresses, mTOR responds, additionally, to diverse other stresses, including those related to nutrient, energy, and oxygen availability and growth factor signaling, thus coordinating eukaryotic cell growth and proliferation with environmental conditions. On the intracellular level, mTOR signaling regulates many fundamental cell processes, like protein synthesis, metabolism and autophagy, and its deregulated signaling is involved with cancer, diabetes and aging process [220].

mTOR was discovered as the intracellular target for rapamycin-FKBP12 complex in mammals and found to be the homolog of the yeast TOR genes (TOR1 and TOR2) that had previously been identified in genetic screens for rapamycin resistance [221]. Rapamycin- FKBP12 complex binds to the FRB (FKBP12–rapamycin binding) domain inhibiting the kinase activity of mTORC1[222]. mTOR, the kinase, nucleates two distinct protein complexes, known as mTOR Complex 1(mTORC1) and 2 (mTORC2). mTORC1 has three core components: mTOR, Raptor (regulatory protein associated with mTOR), and mLST8 (mammalian lethal with Sec13 protein 8, also known as GßL)[223, 224]. Raptor, through binding to the TOR signaling (TOS) motif found on several mTORC1 substrates, facilitates their phosphorylation by mTORC1[225]. mLST8 binds to the catalytic domain of mTORC1 and may stabilize the kinase activation loop[226]. In addition to these three core components, mTORC1 also contains two inhibitory subunits PRAS40 (proline-rich Akt substrate of 40 kDa) [227]and DEPTOR (DEP domain containing mTOR interacting protein) [228]. On the other hand, mTORC2 is characterized by its insensitivity to acute rapamycin treatment. Like mTORC1, mTORC2 also contains mTOR and mLST8. Instead of Raptor,

however, mTORC2 contains Rictor (rapamycin insensitive companion of mTOR) [229]mTORC2 also contains DEPTOR, the regulatory subunits mSin1 and Protor1/2 [230].

mTORC1 and mTORC2 regulate different cellular functions by phosphorylating diverse sets of substrates. mTORC1 substrates include the eIF4E-binding proteins (4E-BPs), 70 kDa ribosomal S6 kinases 1 and 2 (S6Ks), PRAS40, Ser**/**Thr kinase Ulk1(also known as hATG1), and growth factor receptor-bound protein 10 (Grb10). These substrates regulate a variety of cellular processes including translation, growth, proliferation and autophagy. mTORC2 phosphorylates AGC kinase family members, like AKT, protein kinase C (PKC) and serum**/**glucocorticoid regulated kinase 1(SGK1) and thus controls cytoskeletal organization and cell survival [220, 231]. In this introduction we will be focusing on the regulation of mTORC1 activity.

1.5.1 Signaling to the mTORC1

mTORC1 activity can be regulated by two kinds of upstream signaling: one that directly modifies the mTORC1 subunits (mainly through phosphorylation) and another through the Rheb (Ras homolog enriched in brain). Rheb is a small GTPase that, when bound to GTP, activates mTORC1 through direct interaction [232]. Amino acid signaling to mTORC1 occurs through a different pathway that requires Rheb, and the Rag GTPases [233] (not discussed here).

1.5.1.1 Indirect Regulation

Availability of growth factors is a signal for cells to grow and proliferate and to achieve this, cells must double their components and increase in size in every division cycle to double their components and increase in size. mTORC1, through its regulation of general translation, responds to signals from hormones and growth factors. Hormones (e.g. insulin) and growth factors (e.g. insulin–like growth factors) activate receptor Tyr kinases and associated adaptor molecules (e.g., IRS-1 and -2), which, in turn, activate several key signal transduction pathways like the PI3K–AKT pathway and the Ras–ERK pathway[234]. The two pathways stimulate mTORC1 signalling by inhibiting the tumour suppressor Tuberous Sclerosis Complex (TSC), which is

a negative regulator of mTORC1. TSC is a heterotrimeric complex consisting of TSC1 (hamartin) and TSC2 (tuberin)[235] and functions as a GTPase activating protein (GAP) for the small GTPase Rheb [232]which directly binds and activates mTORC1 through yet to be identified mechanism [236]. Inhibition of TSC1–TSC2 is mediated mainly through the phosphorylation of TSC2 by several upstream kinases, including AKT, ERK and the 90kDa ribosomal S6 kinase (RSK).

AKT phosphorylates TSC2 on five residues (S939, S981, S1130, S1132, and T1462), which leads to inhibition of its GAP activity and reduction of the Rheb-GTP hydrolysis, accumulation of active Rheb and activation of mTORC1[237, 238]. Similarly, ERK1/2 was found to phosphorylate TSC2 down stream RTK/RAS/MAPK signaling leading to the dissociation of TSC1 from TSC2 and inactivation of the complex [239]. Contrary to the above, phosphorylation of TSC2 by AMPK leads to its activation. AMPK can be activated under conditions of energy depletion in which there is an increase of the level of AMP or the ratio of AMP to ATP. In response, AMPK turns on ATP generating pathways while shutting down ATP consuming processes like translation. Activated AMPK phosphorylates TSC2 on Ser1345 and enhances its GAP activity towards Rheb leading to mTORC1 inhibition, energy level to the protein synthesis machinery[240].

1.5.1.2 Direct Regulation

Direct regulation of the mTORC1 activity can be achieved by phosphorylation of its core subunits, namely PRAS40, mTOR and Raptor. PRAS40-mTOR interaction has two possible outcomes. When phosphorylated on Thr246 via PI3K**/**AKT, PRAS40 inhibits the mTORC1 kinase activity [241]. On the other hand, PRAS40 can be phosphorylated by mTORC1 at multiple sites, including rapamycin-sensitive (Ser183 and Ser221) and -insensitive (Ser212) residues, which leads to its release from the complex and increases substrate accessibility [242]. Active mTORC1 phosphorylates itself at Ser2481 [243] and can be phosphorylated by the downstream kinase S6K1 at T2446/S2448 [244]. Raptor can be phosphorylated by mTOR on several

sites (including Ser863 in human Raptor) that, in turn, increases mTOR activity toward downstream substrates [245]. Additionally, raptor is directly phosphorylated by ERK1/2 on three proline-directed residues within Ser8, Ser696 and Ser863. Expression of phosphorylation-deficient mutants of Raptor revealed that phosphorylation of these sites by ERK1/2 boosts mTORC1 activity[246]. In TSC2 null cells, depleting cellular energy can still inhibit mTORC1 activity. This was found to be through direct AMPK inhibition of mTORC1 by raptor phosphorylation. Under conditions of low energy (and low oxygen, too), AMPK phosphorylates raptor on two well conserved Ser residues, Ser722 and Ser 792 resulting in its sequestration by 14-3-3 σ proteins and inhibition of mTORC1 kinase activity[247].

1.5.2 Signaling from the mTORC1

In general, mTORC1 stimulates anabolic processes that help build up a growing and a dividing cell like protein and lipid synthesis and inhibits catabolic processes like autophagy. It also affects other processes like glucose metabolism and protein turnover [230]. We will be focusing on mTORC1 role in translation. mTORC1 stimulates global translation, as well as translation of a specific subset of mRNAs. This is achieved by phosphorylating two important downstream effectors, the eIF4E-binding proteins (4E-BPs) and the ribosomal protein S6 kinases (S6K1 and S6K2) [248-251].

1.5.2.1. mTORC1 and the 4EBPs

Translation start when the eIF4F complex assembles on the 5'-mRNA cap structure, a step known as of cap-dependent translation initiation. The eIF4F complex comprises the cap binding protein, eIF4E, the large scaffolding protein eIF4G and the helicase eIF4A, which unwinds secondary structure within the 5'-untranslated region (5'UTR) of the mRNA [252]. The 4E-BPs are a family of inhibitory proteins (4E-BP1, 2 and 3 in mammals) that negatively control the assembly of the eIF4F complex by competing with eIF4G for binding to eIF4E. [253] The binding of the 4E-BPs to eIF4E is regulated by mTORC1-mediated phosphorylation. Hypophosphorylated 4E-BP1 (the best characterized member of the 4E-BPs) binds to eIF4E with high affinity and inhibits the formation of the eIF4F complex, which leads to a

suppression of cap-dependent translation. When activated, mTORC1 directly phosphorylates human 4E-BP1 on Thr37 and Thr46, which act as priming sites for the phosphorylation of Ser65 and Thr70. Phosphorylation of 4E-BPs on these four residues, leads to their dissociation from eIF4E, allowing for the recruitment of eIF4G and eIF4A to the 5′ end of an mRNA in addition to eIF3, the small ribosomal subunit and the ternary complex (comprising eIF2, Met-tRNA and GTP) resulting in the assembly of the 48S translation pre-initiation complex, ribosome scanning and translation initiation [254, 255]. eIF4E is the least abundant translation initiation factor in mammalian cells and over-expression of eIF4E results in transformation and oncogenesis [256]. This oncogenic function of eIF4E was found to be a consequence of the selective upregulation of translation of mRNAs thought to be "eIF4E-sensitive," as a majority of them have long and highly structured 5′ UTRs, making them more dependent on the unwinding activity of eIF4A helicase (discussed later). These mRNAs include cell cycle regulators (e.g., cyclin D3, Ornithine Decarboxylase), survival promoting proteins (BclxL, survivin), pro-angiogenic factors (e.g. VEGF) and oncogenes (e.g.,Myc). Other "eIF4E-insensitive" mRNAs such as those encoding housekeeping proteins (e.g., actins and tubulins) usually have short 5′UTRsand are not as sensitive to changes in eIF4F level[257-259].

1.2.5.2. mTORC1 and the S6Ks

In contrast to the 4EBPs, mTORC1- mediated phosphorylation of the S6Ks leads to their activation. As a result, this promotes mRNA translation via several substrates, such as ribosomal protein S6(rpS6), eukaryotic initiation factor 4B (eIF4B), programmed cell death 4 (PDCD4), and eukaryotic elongation factor 2 kinase (eEF-2K) [234, 249]. Two S6 kinase proteins (S6K1 and S6K2) are expressed in mammalian cells, and both proteins are phosphorylated (on a conserved site) and activated by mTORC1. S6K1, the best characterized of the two kinases, is involved in the regulation of cell growth in Drosophila and human cells. Complete activation of S6K1 requires initial Phosphorylation at Thr389 by mTORC1, which prime it for a subsequent phosphorylation of Thr229 by PDK1 [260].

rpS6 was the first identified S6K substrate, however the functional significance of rpS6 phosphorylation is remains obscure. S6K1 phosphorylates rpS6 at five phosphorylation sites (Ser235, Ser236,Ser240, Ser244, and Ser247) clustered in the carboxyl terminus of both human and rodent rpS6 [261]. Previously, it was thought that phosphorylation of rpS6 is necessary for the translational activation of mRNAs that contain a 5' terminal oligopyrimidine tract (TOP) like mRNAs of the translational machinery itself. However, studies in cells lacking S6K1 and 2 demonstrated that neither the loss of S6Ks nor the phosphorylation status of rpS6 influences translation of 5'-TOPmRNAs [262].

eIF4B, an RNA binding protein, is another important target for S6K1 phosphorylation. S6K1 phosphorylates eIF4B on Ser422, leading to an increase in cap-dependent translation [263]. One possible mechanism for this increase is likely due to the enhanced interaction between phosphorylated eIF4B and eIF3 [264]. Phosphorylation may also increase the activity of eIF4B towards the helicase eIF4A, leading to more efficient translation of mRNAs containing complex 5' UTR secondary structure [265].

S6K1 may also indirectly affect eIF4F activity via regulation of the eIF4A inhibitor PDCD4 (programmed cell death protein 4). PDCD4 plays a known role in apoptosis and has been suggested to have tumor suppressor properties [266]. PDCD4 binds to eIF4A through two conserved MA-3 domains (also found in eIF4G) and competes with eIF4G for eIF4A binding preventing its incorporation into the eIF4F complex, consequently leading to repression of cap-dependent translation[267, 268]. PDCD4 is phosphorylated on Ser67 and Ser457 by S6Ks and AKT respectively leading to its degradation by the E3-ubiquitin ligase $SCF^{\beta TrCP}$. Furthermore, a PDCD4 mutant that cannot bind the ubiquitin ligase $SCF^{\beta TrCP}$ inhibited translation of an mRNA with a complex 5' UTR. Importantly, expression of this mutant reduces cell growth and proliferation, suggesting that PDCD4 degradation via S6K1-mediated phosphorylation is necessary for efficient protein synthesis. [269]. Lastly, S6K1 was shown to affect the regulation of translation elongation via phosphorylation of eEF2 kinase. eEF2 is a GTPase that

promotes translocation of the nascent polypeptide chain from the A-site to the P-site of the ribosome . eEF2 kinase phosphorylates and inactivates eEF2, leading to inhibition of the translocation step in translation elongation and thus act as a negative regulator of protein synthesis as it [270]. Active S6K1 phosphorylates eEF2 kinase on Ser366, which inhibits its kinase activity towards eEF2 and alleviate the suppression of translation elongation[271]. Figure 1.5 summaries signaling to and from mTORC1.

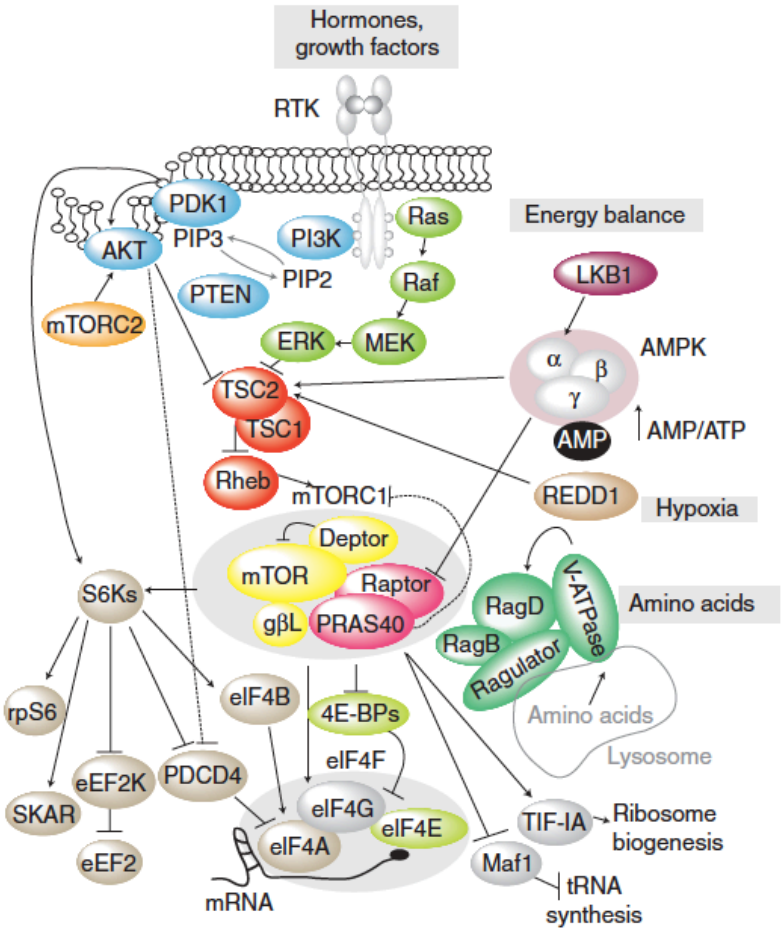

Figure 1.5: Schematic representation of mTORC1 signaling to the translational machinery. T-bars represent inhibitory signals, whereas arrows indicate stimulatory signals. Adapted from Roux and Topisirovic [249]

1.5.3. mTOR targeting in cancer

Both upstream oncogenic "gain of function" or tumor suppressive "loss of function" can cause over-activation of mTORC1 signaling resulting in increased phosphorylation of 4E-BP1 and S6K and a cellular environment that enhance transformation and oncogenesis. For instance, oncogenic pathways, including the PI3K/Akt pathway as well as the Ras/Raf/Mek/Erk (MAPK) pathway are frequently mutated in a high percentage of human cancers. Furthermore, mTOR can be negatively regulated downstream of three important tumor suppressive pathways: PTEN (a phosphatase that negatively regulated PI3K pathway) LKB1 (a serine/threonine kinase responsible for phosphorylating and activating AMPK), and TSC1/TSC2 [248, 272].

The first drug to be used as an mTOR inhibitor was rapamycin. Rapamycin was initially used as an immunosuppressant, but then due to its anti-cancer properties in a variety of cancer cell lines and synergistic effects with chemotherapeutics, it emerged as a potential anticancer agent [272-274]. Cells treated with rapamycin undergo a G1/S phase cell cycle arrest. The growth arrest may be due in part to a decrease in the synthesis of cell cycle regulator proteins, such as D-type cyclins that are required for cell cycle propagation [275]. Rapamycin is generally cytostatic, under certain conditions it has been known to induce apoptosis through induction of the pro-apoptotic protein Bad and reduction the expression of Bcl2 [276]. Rapamycin analogues ''rapalogs", like temsirolimus (Pfizer) and everolimus (Novartis), were developed to improve its stability and water solubility. In clinical trials, however, these agents showed limited efficacy except for a small subset of cancers such as endometrial carcinoma and renal cell carcinoma [277]. At least two explanations for this lack of efficacy have been proposed. First, acute treatment with rapamycin or its derivatives doesn't inhibit mTORC2 (rapamycin/FKBP12 complex can not bind to the FRB domain of mTOR in mTORC2). As a result mTORC2 is free to perform signaling in the presence of rapamycin. mTORC2 phosphorylates AKT on Ser473 leading to its activation. The continued activation of AKT by mTORC2 in the presence of rapamycin has, paradoxically, a prosurvival and anti-apoptotic effect; a

serious effect in the context of cancer [278]. Indeed, increased Akt signaling has been observed in biopsies of cancer treated with everolimus, which may help explain why rapalogs have cytostatic, but not cytotoxic, effects on tumors [279]. Second, rapamycin inhibition of S6K1 activation removes a negative feedback loop that is normally activated by S6K1. S6K activates a negative feedback loop by inhibiting the insulin receptor substrate-1 (IRS-1). Phosphorylation of IRS-1 by S6K marks it for degradation leading to a reduction in PI3K /AKT signaling. However, upon treatment with rapamycin or its derivatives, no S6K cause a decrease in IRS-1 degradation, an increase in IRS-1 mediate signaling, and consequently elevated PI3K and AKT activity [280]. Additionally, since mTORC1 was shown to negatively regulate growth factor signaling by directly phosphorylating IRS1 [281]and the RTK inhibitor growth factor receptor-bound protein 10 (Grb10) [282], it follows that rapamycin treatment will also remove this negative feedback loop. Finally, since rapalogs are allosteric inhibitors, they don't directly inhibit the kinase activity of mTORC1 and were found to block the phosphorylation of some but not all mTORC1 substrates. In particular, the phosphorylation of 4EBP is largely insensitive to rapamycin[283, 284].

To solve some of the drawbacks of the rapalogs, second-generation, ATP-competitive catalytic inhibitors have been developed like PP242 and Torin1. These compounds directly inhibit the catalytic activity of mTOR and therefore fully suppress both mTORC1 and mTORC2. These compounds are more potent than rapamycin in terms of their effect on 4E-BP1 dephosphorylation, cell proliferation and protein synthesis [285]. Although these second-generation mTOR inhibitors initially suppress Akt signaling due to inhibition of mTORC2, the PI3K/AKT eventually get reactivated following long-term treatment. The proposed solution for this is the use of dual PI3K/mTOR inhibitors, which inhibit a closely related catalytic domains of both PI3K and mTOR, thereby more fully blocking the prosurvival PI3K and mTOR pathways [286].

1.5.4. mTOR, protein synthesis and mitotic arrest

In response to stress, cells alter their gene expression patterns. One way to achieve this is through changes in translation. Translation is considered to be the most energy demanding cellular processes, consuming at least 20% of cellular ATP[287]. Stressed cells tend to reduce their global translation as a way of saving energy and being more selective in translating proteins required for cell survival under stress. Prolonged mitotic arrest, which happens as a result of chronic activation of the mitotic checkpoint, is one way of inducing stress. Early observations noticed that the rate of protein synthesis in mitotic cells is significantly reduced to 25–30% compared to interphase cells[288]. More specifically, cap dependent translation is inhibited under conditions of prolonged mitotic arrest. Phosphorylation of eIF4E, the cap binding protein, is reduced in mitosis due to a decrease in the activity of its kinase MNK1. On the other hand, phosphorylation of eIF4G2 add to the decreased phosphorylation of eIF4E by preventing its access to the eIF4F complex [289]. Moreover, Cdk1/cyclin B phosphorylates Ser1232 on eIF4G1 during mitosis. The C-terminal portion of eIF4G1, where Ser1232 is located, coordinates assembly of the eIF4G/-4A/-4B helicase complex and binding of MNK1. Although this phosphorylation seems to enhance the interactions of eIF4A with HEAT domain 2 of eIF4G, it leads to a decreased association of eIF4G/-4A with RNA and inhibit translation[290]. Additional mechanisms were proposed to account for the suppression of mitotic translation, for instance, 14-3-3 binding to eIF4B [291], hyper-phosphorylation of elongation factor 2 (eEF2) [292] or mitotic phosphorylation of eEF1D (decreasing tRNA delivery to elongating ribosomes)[293]. Altogether, these data showed that during mitotic arrest, various steps of translation are inhibited and that this happening downstream CDK-1, the major mitotic kinase.

Since cap-dependent translation is inhibited during mitotic arrest, and since mitotic cells still need a basic level of translation to main this stressed state, it was proposed that translation in mitosis is cap-independent or more specifically IRES (Internal-Ribosome Entry Site)-mediated [289, 291, 294,

295]. This hypothesis was strengthened by the observation that poliovirus mRNA which translation is IRES-dependent is still translated efficiently during mitosis [288]. Following this, some cellular mRNAs were found to be translated from their IRES in mitosis like for example ornithine decarboxylase [294], the CDK-related kinase p58PITSLRE[296], BCL-2 and CDK1 itself [218].

The role of mTORC1 in translation during mitotic arrest seems to be complex and controversial. Early reports showed a decrease in cap-dependent translation during prolonged mitotic arrest as evidenced by hypophosphrylation of 4EBP1 (from which it's inferred that mTORC1 activity is low) [289]. Later, using phospho-specific antibodies, it was noted that 4E-BP1 accumulate as one hyper phosphorylated, δ-4E-BP, in mitosis [297-300]. This phosphorylation was thought initially to be an indication of high mTOR activity though this phosphorylation didn't seem to enhance cap-dependent translation. As a result, a role for mTORC1 in cap-independent translation was proposed. The problem with this model is that this phosphorylation was shown to be rapamycin-resistant, which occurred at the same time a rapamycin-sensitive phosphorylation of S6K [297]. Another recent report, however, has shown that cyclin-dependent kinase 1 (CDK1) substitutes for mTOR kinase activity toward 4EBP1 and phosphorylates 4E-BP1 at canonical sites (T37, T46, S65, and T70) and the noncanonical S83 site, resulting in a mitosis-specific hyper-phosphorylated δ isoform. This extra phosphorylation on S83 seems to have little effect on cap-dependent translation but plays a significant role in 4EBP1 localization at the centrosomes[301]. The picture is no less complicated with S6K1 phosphorylation with reports showing its phosphorylation S6K1 at multiple Ser/Thr, Pro (S/TP) sites, including Ser371, Ser411, Thr421, and Ser424 concomitant with reduced phosphorylation of the hydrophobic motif site, Thr389, resulting in a decrease in the specific activity of S6K1[302-304]. Conversely, other reports have shown an increase in phosphorylation on Thr389, an indication of S6K1 activity downstream mTORC1 [297].

Taken together, it's clear that under drug-induced, prolonged mitotic arrest, global translation is suppressed but not completely inhibited. This response seems to be directly or indirectly related to CDK1 activity, similar to what has been shown to occur with the apoptotic machinery.

1.6. Rationale and Objectives of the Study

The overall aim of this study is to gain insight into the regulation of two important signaling pathways in the context of mitosis and prolonged mitotic arrest. The first comprises one of the most important pro-apoptotic proteins, Bim and the second involves a key prosurvival signaling hub, the mTOR kinase and its critical subunit raptor.

Previous work has shown that Bim protein level is positively correlated with Taxol in lung and breast cancer, but the exact phase in the cell cycle where Bim exerts its pro-apoptotic role remained elusive. Additionally, different research groups reported the phosphorylation and in some case the ubiquitination of Bim in mitosis, but the nature of the kinase or the E3 ligase remained to be discovered. Bim is a potent activator and sensitizer of intrinsic pathway of apoptosis, so discovering its regulators in mitosis will potentially help make full use of its activity during chemotherapeutic treatment.

Multiple factors may affect the fate of cells arrested in mitosis after treatment with anti-mitotic drugs but at the end the decision to die or to exit stills down to two competing (sometimes collaborating) networks; a death and a survival network. We hypothesized that the prosurvival mTORC1 kinase might have a role in tipping the balance of death vs slippage in mitosis. Prior research has reported the phosphorylation of raptor, the substrate recruitment subunit of mTORC1, but the significance of this phosphorylation was not clear. The goal of my research is to investigate the effect of this phosphorylation on raptor and on the mTORC1 activity in mitosis and during mitotic arrest. I also wanted to examine the status of mTORC1 in mitosis and how it affects its main function, enhancing translation, bearing in mind that general translation is known to be compromised in arrested cells. The findings of this research may help us understand the details of the networks controlling cell fate and to better choose a drug target that helps achieve maximum efficiency in killing cancer cells.

Chapter 2

BimEL is phosphorylated at mitosis by Aurora A, and targeted for degradation by βTrCP1*

Mohamed Moustafa-Kamal[1,2], Isabelle Gamache[1], Yiqing Lu[1,2], Stephan Li[2] and Jose G. Teodoro[1,2,3]

[1]Goodman Cancer Research Center, McGill University, Montréal, Québec, Canada
[2]Department of Biochemistry, McGill University, Montréal, Québec, Canada
[3]Department of Microbiology and Immunology, Montréal, Québec, Canada

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2.1. Abstract

Bim is a pro-apoptotic Bcl-2 family member implicated in numerous apoptotic stimuli. In particular, Bim is required for cell death mediated by antimitotic agents, however mitotic regulation of Bim remains poorly understood. Here we show that the major splice variant of Bim, BimEL, is regulated during mitosis by the Aurora A kinase and phosphatase PP2A. We observed that BimEL is phosphorylated by Aurora A early in mitosis and dephosphorylated by PP2A after mitotic exit. Aurora A phosphorylation stimulated binding of BimEL to the F-box protein bTrCP1 and promoted ubiquitination and degradation of BimEL. These findings describe a novel mechanism by which the oncogenic kinase Aurora A promotes cell survival during mitosis by downregulating proapoptotic signals. Notably, we observed that knockdown of Bim significantly increased resistance of cells to the Aurora A inhibitor MLN8054. Inhibitors of Aurora A are currently under investigation as cancer chemotherapeutics and our findings suggest that efficacy of this class of drugs may function in part by enhancing apoptotic activity of BimEL.

2.2. Introduction

Apoptosis is triggered by many types of cell stress including DNA damage, oncogene activation, virus infection and metabolic stress. Induction of apoptosis is regulated by the family of Bcl-2 proteins that have either pro- or anti-apoptotic activities. The family is characterized by the presence of up to four Bcl-2 homology (BH1-4) domains. A subset of the pro-apoptotic Bcl-2 family members containing only the BH3 domain are induced in response to a wide range of cell stresses. BH3-only proteins induce apoptosis by activating the pro-apoptotic Bcl2 family members, Bax and Bak, and by antagonizing anti-apoptotic members such as Bcl-2 and Mcl-1 [305].

The Bim protein is a BH3-only member of the Bcl-2 family that is an important initiator of apoptosis in lymphocytes in response to growth factor withdrawal [156, 306, 307] and elimination of autoreactive T-cells in the thymus [157]. Deletion of a single allele of Bim can accelerate the progression of lymphomas in the Eμ-myc model, suggesting the apoptotic activity of Bim has tumour suppressor activity *in vivo* [162]. Expression of Bim is regulated by several transcriptional and post-transcriptional mechanisms. At the transcriptional level, the promoter of the Bim gene is activated by stress-inducible transcription factors including AP-1 and FOXO [307-309]. Post-transcriptional mechanisms that have been shown to control Bim expression levels include both mRNA stability [310] and protein stability [131, 132, 311].

The stability of the Bim protein has been shown to be regulated by several mechanisms. Signaling downstream of receptor tyrosine kinases including EGFR have been shown to induce the proteasomal degradation of Bim [312-315]. There are three splice variants of Bim: BimEL, BimL and BimS. The longest form, BimEL, is generally the most highly expressed amongst the variants [316]. Phosphorylation by Erk1/2 on Serine-69 (S69) of human BimEL has been shown to induce proteosomal degradation of the protein [132, 142, 143]. More recently, phosphorylation at S69 was shown to promote phosphorylation at additional sites on Serines 93/94/98

(S93/94/98) by Ribosomal S6 Kinase (Rsk1/2) within a conserved phosphodegron motif recognized by the F-Box protein βTrCP [133].

In addition to being phosphorylated in response to growth factor signaling, BimEL has also been shown to be phosphorylated during mitosis. Early reports suggested that this phosphorylation is catalyzed by ERK1/2 [207] and ERK5 [208] most likely as a pro-survival signal. However, more recent studies have found that mitotic phosphorylation of BimEL is not dependent on ERK1/2 or ERK5 signaling [317]; but rather upon CDK1 activity [210, 211] and is associated with the poly-ubiquitination and proteasome-dependent turnover of BimEL [210]. Although there is now considerable evidence that Bim levels are regulated by phosphorylation and proteasome-mediated proteolysis during mitosis, the mechanism remains unknown.

In the current study we show that stability of BimEL is regulated during progression through mitosis. BimEL is targeted for proteasome-mediated degradation following phosphorylation of the protein on a conserved phosphodegron recognized by βTrCP1. We further show that the kinase responsible for the mitotic phosphorylation of BimEl at S93/94/98 is Aurora A kinase. Since Aurora A inhibitors are being pursued in the clinic as anti-mitotic cancer drugs, we tested if the efficacy of Aurora A inhibition was dependent upon expression of Bim. The Aurora A inhibitor, MLN8054, induced stabilization of BimEL and knockdown of Bim expression rendered cells more resistant to the inhibitor. These data define a previously unknown mechanism controlling the activity of Bim at mitosis that has implications in the design of anti-mitotic cancer therapies.

2.3. Results

2.3.1. BimEL is phosphorylated and targeted for degradation at mitosis.

In order to examine expression of BimEL during mitosis, HeLa cells were synchronized using thymidine (Thy) followed by nocodozole (Noc) and analyzed by immunoblot at various times post release. Figure 2.1A shows that after release of cells from Thy/Noc a slower migrating form of BimEL was apparent which gradually shifted downward as cells progressed through mitosis (Figure 2.11A, lanes 1-6). Figure 2.1B shows that synchronization of HeLa cells using the cdk1 inhibitor RO3306 obtained similar results as with Thy/Noc with one important difference. RO3306 arrests cells at G2/M and BimEL in RO3306 arrested cells shows that the protein is present in a faster migrating form that immediately shifts upward upon release from the drug and entry into mitosis (compare Fig 2.1B lanes 2 and 3). Interestingly, as cells progressed through mitosis, we observed that BimEL levels were decreased and reached a low point approximately 90 minutes following release (compare Fig2.1A, lanes 1 and 6). In order to test that loss of BimEL expression was at the level of protein degradation, HeLa cells were synchronized as in Figure 2.1A and then treated with the proteasome inhibitor MG132 for 90 minutes post post-release. Figure 2.1C shows that MG132 treatment resulted in accumulation of BimEL in mitosis and that the stabilized form of BimEL appears as a slower migrating band. In order to test that the slower migrating form of BimEL was due to phosphorylation, cell extracts were treated with λ phosphatase. Figure 2.1D shows that phosphatase treatment resulted in a downward shift of BimEL, demonstrating that the protein is phosphorylated in mitosis. These data suggest that BimEL is regulated during mitosis by coupled phosphorylation and proteolytic steps.

2.3.2. BimEL is phosphorylated on Serine 69 and Serines 93/94/98 and degraded at mitosis in a mechanism independent of the spindle assembly checkpoint.

Since BimEL degradation occurred concomitantly with phosphorylation, we examined two phosphorylation sites on BimEL previously shown to regulate proteolysis. Erk 1 and 2 were shown to phosphorylate BimEL on S69 [131, 132, 142] and promote degradation and phosphorylation on S93/94/98 has been shown to induce degradation through the SCF complex [133]. In order to assess the importance of these sites for BimEL degradation in mitosis, phosphospecific antibodies were used to monitor these modifications during mitosis. 293T cells were synchronized as shown in Figure 2.1A with the exception that cells were then left in Nocodozole. Figure 2.2A (lane2 6-10) shows that BimEL becomes phosphorylated on S69 and S93/94/98 in mitosis concomitant with it's degradation.

To further investigate the mitotic degradation of BimEL, HeLa cells were synchronized as depicted in Figure 2.1A and released into four different conditions, MG132, Taxol, RO3306, or control (Figure 2B). As expected, release of cells into MG132 stabilized levels of BimEL as well as several other proteins known to undergo proteolysis during mitosis including: cyclin B, securin, and Wee1 (Figure 2.2B, lanes 10-18). Taxol interferes with tubulin dynamics during mitosis and thereby activates the spindle assembly checkpoint (SAC). SAC activation then inhibits the anaphase promoting complex/cyclosome (APC/C), which normally mediates degradation of key mitotic substrates such as securin and cyclin B. Figure 2.2B (lanes 19-27) show that taxol treatment stabilized the APC/C substrates cyclin B and securin but did not affect stability of BimEL or Wee1, suggesting that degradation of BimEL was APC/C independent. Interestingly, treatment with RO3306 stabilized BimEL (compare lanes 1-9 with lanes 28-36), suggesting that cdk1 activity was required for degradation. Immunoblots using phosphospecific antibodies against S69 and S93/94/98 confirmed that phosphorylation at both of these sites occurred in mitosis and decreased as cell progressed through mitosis (Figure 2.2B, lanes 1-9). Furthermore,

whereas treatment with MG132 and Taxol maintained phosphorylation of both S69 and S93/94/98, RO3306 treatment prevented phosphorylation at these sites. Similar results were obtained using 293T cells (Supplemental Figure 2.1). Taken together these data show that phosphorylation sites on BimEL that regulate stability are phosphorylated during mitosis in a cdk1 dependent mechanism. In addition, degradation of BimEL during mitosis was not affected by the SAC suggesting that the APC/C is not involved in this process.

2.3.3. BimEL is ubiquitinated at mitosis and requires phosphorylation on Serine 93/94/98.

In order to determine if degradation of BimEL during mitosis is ubiquitination-dependent, FLAG-tagged Bim was co-transfected with HA-tagged Ubiquitin (Ub). Bim conjugated with Ub was detected by immunoprecipitation (IP) of Bim using a-FLAG antibody followed by immunoblot for HA. Figure 2.3A (lane 4) shows that BimEL was ubiquitinated during mitosis and displayed the characteristic laddering in the presence of MG132. Transfection of the two other major splice variants, BimL (lane 6) and BimS (lane 8), did not result in appreciable ubiquitination suggesting that the degron required for ubiquitination was specific to BimEL. Phosphorylation sites at S69 and S93/94/98 have been previously shown to affect protein stability and are located in the N-terminal region specific to BimEL. We therefore tested ubiquitination of BimEL mutants in which these phosphorylation sites were converted to alanine. Figure 2.3B shows that mutation of S69 to alanine (S69A) did not affect ubiquitination (lane 5), however, mutation of S94/98 (S94/98A) was severely defective (lane 7). In order to determine if phosphorylation of BimEL was essential for degradation at mitosis, 293T cells were transfected with FLAG-tagged BimEL and immunoblot performed under asynchronous or mitotic conditions. Figure 2.3C shows that, as expected, wild-type Flag-BimEL displayed greatly reduced steady state levels during mitosis (compare lanes 1 and 2). However, the S94/98A mutant was stabilized at mitosis (compare lanes 7 and 8) and the

stabilized form of the protein appeared to migrate slower. Serine 104 of BimEL has been previously shown to be phosphorylated by cdk1 during mitosis [211], but mutation of this site had no affect on stability (lanes 11 and 12). The S94/98A mutation is clearly still phosphorylated at other sites in mitosis since the mutated protein migrates slower in mitosis and may be due to phosphorylation by cdk1 as previously reported [210, 211]. We further mutated four other potential cdk phosphorylation sites on BimEL but none displayed major effects on mitotic stability of the protein. These data indicate that BimEL is actively ubiquitinated and targeted for degradation during mitosis using a mechanism that requires phosphorylation at S93/94/98.

2.3.4. Phosphorylation on Serine 93/94/98 of BimEL creates a binding site for bTrCP1.

Treatment with phorbol ester has been shown to induce phosphorylation of BimEL on S93/94/98 through the ERK/MAPK pathway, which creates a binding site for βTrCP1 and targets BimEL for degradation through the Skp1-Cullin1-F-Box (SCF) complex [133]. βTrCP1 is also known to mediate degradation of Wee1 during mitosis[318]. Since our results in Figure 2.2B indicated that degradation of BimEL occurred with similar timing and properties as Wee1, we tested if βTrCP1 can also target BimEL during mitosis. Figure 2.4A shows that siRNA knockdown of βTrCP1 resulted in elevated levels of both Wee1 and BimEL. Since binding of βTrCP1 requires phosphorylation of the degron, we determined if binding of BimEL to bTrCP1 occurred specifically during mitosis. Figure 2.4B shows that HeLa cells synchronized in G2/M using RO3306 showed a dramatic increase in Serine 93/94/98 phosphorylation at 30 minutes post-release. Furthermore, interaction between BimEL and βTrCP1 occurred only when S93/94/98 phosphorylation was apparent (Fig 2.4B, lanes 5 and 6). We then determined if S93/94/98 is essential for βTrCP1 interaction with BimEL during mitosis. Figure 2.4C (lanes 7 and 8) shows that a point mutation of Serines 94/98 to alanines (S94/98A) dramatically reduced binding to βTrCP1. Point mutation

of S69 to alanine (S69A), however, had no effect on βTrCP1 binding (lanes 5 and 6). Furthermore, two well characterized point mutations of βTrCP1, R474A and Y488A [319], that are known to be defective for substrate binding failed to interact to BimEL at mitosis (Figure 2.4D). Deletion of the F-box, which mediates interaction of βTrCP1 with the SCF was not required for binding to BimEL. Taken together these data show that activation of the βTrCP1 phosphodegron on BimEL occurs during mitosis and targets the protein for degradation by the SCF complex.

2.3.5. PP2A dephosphorylates BimEL on S93/94/98 and prevents degradation.

BimEL was previously shown to be dephosphorylated by the phosphatase PP2A and thereby inhibiting its ubiquitination and degradation through an unknown mechanism [320]. As shown in Figure 2.1B BimEL is rapidly phosphorylated as cells enter mitosis and is then gradually dephosphorylated after mitotic exit when BimEL levels increase. We therefore asked if PP2A dephosphorylates S93/94/98 during mitosis and if this regulates mitotic stability of BimEL. Figure 2.5A(lanes 5-8) shows that treatment of Thy/Noc synchronized HeLa cells with Okadaic acid (OA), a specific inhibitor of PP2A, resulted in a dramatic dose-dependent increase of S93/94/98 phosphorylation on endogenous BimEL. Interestingly, levels of BimEL were dramatically reduced in the presence of OA. Similar results were obtained by IP of 293T cells transfected with FLAG-BimEL followed by western blot to detect S93/94/98 (Fig 2.5B). Conversely, addition of the PP2A activator, C2-Ceremide, resulted in stabilization of BimEL relative to vehicle controls (Figure 2.5A, compare lanes 3 and 9). Figure 5C shows that knockdown of PP2A using siRNAs targeting the catalytic subunit also resulted in elevated phosphorylation on S93/94/98. In order to determine if phosphorylation of S93/94/98 was required for OA-induced degradation, *wild-type* BimEL or phosphorylation site point mutants were transfected into 293T cells synchronized using Thy/Noc. Figure 2.5D shows that whereas wild-type BimEL and the mutant at S69 were still degraded in response to OA

treatment, mutation of S93/94/98 rendered the proteins resistant. Similarly, the two other major splice variants of Bim, BimL and BimS, that lack S93/94/98 were also unaffected by OA (Fig 2.5D, lanes 10-15). Taken together, these data show that BimEL phosphorylation and stability are dynamically regulated during mitosis with PP2A mediated dephosphorylation of S93/94/98 stabilizing levels of BimEL after mitosis.

2.3.6. Phosphorylation of the βTrCP1 degron in BimEL is mediated by Aurora A kinase during mitosis.

Previous studies have shown that the βTrCP phosphodegron at S93/94/98 is phosphorylated by Rsk1/2 in response to treatment with phorbol ester. Rsk1/2 is activated downstream of receptor tyrosine signaling which does not explain how this site becomes phosphorylated during mitosis in a cdk1-dependent manner. Moreover, we show in Figure S2.2 that addition of the Rsk1/2 inhibitor BI-D1870 did not significantly affect phosphorylation of S93/94/98 during mitosis. Inhibition of several other kinases downstream of RTKs also had little or no effect on S93/94/98 (Figure S2.2) suggesting that RTK signaling is dispensable for S93/94/98 phosphorylation during mitosis. Although the cdk1 inhibitor RO3306 completely prevented S93/94/98 phosphorylation, these sites do not have the required context of a cdk1 site suggesting another mitotic kinase downstream of cdk1 is required. The S93/94/98 phosphodegron includes the amino acid sequence 91-RSSSG-95, which contains the consensus for Aurora A kinase (R-X-X-S/T-f, where f is any hydrophobic amino acid [321]). Intriguingly, mitotic activation of Aurora A has been shown to be dependent upon cdk1 activity [322]. We therefore determined if S93/94/98 is phosphorylated during mitosis by Aurora A. 293T cells were arrested using Thy/Noc and released into increasing concentrations of the Aurora kinase inhibitor MLN8054. Figure 2.6A (lanes 4-9) show that MLN8054 prevented mitotic phosphorylation of S93/94/98 in a dose dependent manner. Furthermore, Figure 2.6B shows that addition of MLN8054 to mitotic HeLa cells stabilized levels of BimEL

during mitosis. Under these conditions treatment with MLN8054 also prevented phosphorylation on a well-characterized Aurora A site on Polo-like kinase (T210) [323, 324]. Genetic inhibition of Aurora A activity using siRNAs also resulted in elevated levels of BimEL in mitosis (Fig 2.6C). Since phosphorylation of the βTrCP1 degron is required for interaction with BimEL, we determined if MLN8054 affects this interaction. Figure 2.6D shows that BimEL failed to co-IP with βTrCP1 in cells treated with MLN8054.

In order to determine if Aurora A is able to directly phosphorylate BimEL, we combined purified Aurora A and GST-BimEL *in vitro*. Figure 2.6E shows that Aurora A was indeed able to directly phosphorylate BimEL at S93/94/98 *in vitro*. It has been suggested that phosphorylation at S69 is required for effective phosphorylation at S93/94/98, however, a S69A mutant was still effectively phosphorylated by Aurora A *in vitro* (Fig 2.6F). BimEL has been shown to form complexes with kinases that phosphorylate it [133, 143, 211]. Similarly, in figure 2.6G we show that that GST-BimEL was able to pull down Aurora A from mitotic extracts derived from HeLa or 293T cells.

Numerous clinical trials are ongoing testing Aurora kinase inhibitors as cancer therapeutics [325]. Since our data shows that Aurora A phosphorylates BimEL on S93/94/98, triggering the proteolysis of the protein, the efficacy of Aurora A inhibitors may be dependent on stabilization of BimEL. In order to test this hypothesis we determined if levels of Bim expression could affect response to MLN8054. Figure 2.7A shows that HeLa cells with stable knockdown of Bim were significantly more resistant to treatment with MLN805 relative to non-silencing control. Quantitation of this effect shows that after 3 days of treatment with MLN8054, HeLa cultures with Bim knockdown survived the treatment and contained nearly four fold the number of cells compared to non-silencing control (sh-NS) (Figure 2.7B). MLN8054 has been previously shown to induce apoptosis in cancer cells [326] so we therefore determined if treatment with MLN8054 induces death in a Bim-dependent mechanism. Figure 2.7C shows that treatment of sh-NS cells with MLN8054 resulted in caspase-3 and PARP1 cleavage that was

inhibited by Bim knockdown. Moreover, NLN8054 treatment of sh-NS cells induced significant levels of apoptosis that was partially reversed in cell lines with stable knockdown of Bim (Fig 2.7D).

Taken together, these data show that S93/94/98 on BimEL is phosphorylated by Aurora A, creating a binding site for βTrCP1 and inducing degradation of the protein. Furthermore, inhibition of cell growth and induction of apoptosis by Aurora A inhibition is at least partially dependent upon Bim expression levels.

2.4.Discussion

The levels of Bim protein are tightly regulated at the level of protein stability and are responsive to many cues including growth factor and stress signaling. Previous studies have suggested that Bim is regulated by phosphorylation during mitosis but the molecular details of these observations have not been elucidated. In the current study we show that proteolysis of BimEL is induced during mitosis following phosphorylation by Aurora A kinase. Phosphorylation of BimEL on the phosphodegron at S93/94/98 creates a binding site for the F-box protein βTrCP. This mechanism of BimEL inactivation is similar to that observed after growth factor stimulation with the exception that the βTrCP phosphodegron is activated by Rsk1/2 downstream of growth factors rather than Aurora A [133]. We observed that the phosphorylation of S93/94/98 is dynamic and reversed by the activity of PP2A leading to stabilization of BimEL proteins levels after mitosis. Aurora A is itself degraded late in mitosis by the APC/C and may also explain how BimEL levels return to normal state [327, 328].

The biological functions of BimEL degradation during mitosis need further study but are likely due to the mechanism of how the apoptotic activity of Bim is normally kept in check. In healthy cells, BimEL remains sequestered to the microtubule network, which neutralizes its apoptotic activity [126, 329]. Stress activated signaling such as the Jnk pathway can phosphorylate Bim, resulting in release from microtubules and induction of apoptosis [127]. Since the onset of mitosis triggers the global disassembly of

the microtubule network in preparation for assembly of the mitotic spindle, this would also result in widespread release of sequestered Bim. Induction of Bim degradation may therefore be a mechanism to cope with the sudden release of cytoskeleton associated Bim during mitosis.

Downregulation of Bim and other core components of the apoptotic machinery may be a general property of cells entering mitosis. Caspase-2, 8 and 9 are phosphorylated by Cdk1/Cyclin B1 during mitosis and these modifications inhibit pro-apoptotic activity [61, 213, 330]. A general feature of mitotic cells is the loss of attachment to extracellular matrix as the cytoskeleton is remodeled in preparation for spindle formation. When anchorage-dependent cells remain in a detached state for extended periods they undergo a process termed anoikis [331]. Interestingly, Bim is required for cell death induced by anoikis [163] suggesting that downregulation of Bim during mitosis may prevent cell death in response to ECM detachment.

Our data show that Aurora A phosphorylates BimEL during mitosis and promotes degradation of the protein. The Aurora kinase family consists of 3 paralogues that control key events during mitosis including mitotic entry, centrosome regulation and cytokinesis [332]. The Aurora kinases become activated during mitosis and are subsequently shut down late in mitosis by being targeted for degradation by the APC/C. As would be expected, we observe that levels of BimEL begin to increase late in mitosis when activity of Aurora A is inhibited. Aurora A and B are each required for multiple events during mitosis and inhibition of either activity through chemical or genetic methods leads to severe mitotic defects [332]. Although we cannot completely rule out the possibility that Aurora B may also target BimEL, several lines of evidence suggest that Aurora A is the major kinase targeting BimEL in mitosis. First, we observe that mitotic phosphorylation of BimEL on S93/94/98 is dependent upon Cdk1/Cyclin B. Only Aurora A has been shown to definitively require Cdk1/Cyclin B for activation during mitosis [322]. Second, Aurora A has been shown to act as a bona fide oncogene and induction of BimEL degradation is consistent with this activity. Third, we observe that the Aurora A inhibitor MLN8054 prevents mitotic

phosphorylation of BimEL on S93/94/98 and stabilizes the protein. MLN8054 inhibits Aurora A with 40 fold selectivity over Aurora B [333], indicating that Aurora A is the main kinase being affected in our experiments.

Aurora A is overexpressed in a wide range of tumour types and has been demonstrated to enhance tumour cell growth both *in vivo* and *in vitro* [332]. Aurora A has been shown to downregulate several tumor suppressor mechanisms. The p53 tumor suppressor was shown to be phosphorylated by Aurora A leading to destabilization and degradation of the p53 protein in a mechanism reminiscent of what we have observed for BimEL [334, 335]. Similarly, Aurora A was shown to phosphorylate BRCA1 and promote G2/M cell cycle progression [336]. Thus, in addition to the wide range of substrates targeted by Aurora A regulating chromosome and spindle dynamics during mitosis, it can also phosphorylate and deactivate tumor suppressor pathways including Bim.

Numerous Aurora kinase inhibitors are currently in clinical trials for a wide range of cancer types [325]. Our results suggest that BimEL expression is required for efficacy of Aurora A inhibition. Several reports have shown that pharmacologic inhibition of Aurora A leads to apoptosis and our results suggest that this may due in part to mitotic stabilization of Bim [326, 337, 338]. Interestingly, Li et al. conclusively demonstrated that apoptosis induced by Aurora A inhibition occurs through the intrinsic mitochondrial pathway requiring the activity of either Bax or Bak [326]. This is consistent with activation of a BH3-only protein such as BimEL, which functions in part by activating pro-apoptotic Bcl-2 family members Bax and Bak [305]. Since Aurora A is upregulated in many tumor types it may contribute to the transformed phenotype by maintaining low levels of BimEL.

2.5.Materials and Methods

Antibodies : Mouse monclonal antibodies to the following proteins were purchased from the indicated manufacturers and used for immunoblotting according to standard protocols: Cdc20, cyclin B1, cyclin A2 (Santa Cruz Biotechnology), Cdc27 (BD Biosciences),, EGFP (Clontech), Plk1 (Zymed), Securin (Abcam), anti-Flag M2, (Sigma), anti-HA (Covance), PP2A-C subunit (Cell Signaling), Phospho Ser-Thr-Pro MPM2 (Millipore). The following rabbit polyclonal antibodies were purchased from the indicated maufacturers: Bim (Calbiochem), b-actin (Sigma), APC2 (Biolegend), Phospho BimEL(S69) and Phospho-(Ser) CDK substrates (Cell Signaling) PARP-1 and Caspase 3 (Santa Cruz Biotechnology). Rabbit monoclonal antibodies were from Cell Signaling (bTrCP, Phospho Plk1-T210 and Aurora A). The phospho Histone H3 Ser10 rat polyclonal was prchased from Sigma. Anti-Phospho-BimEL (S93/94/98) has been described previously [133] and was a kind gift from Dr. Michele Pagano. Mouse TrueBlot, secondary anti-mouse IgG antibody was from eBioscience.

Plasmids and Cloning: The 3 major splice variants of Bim were obtained by RT-PCR from form HeLa total RNA and cloned into p3XFLAG-myc-CMV™-26 Expression Vector (Sigma). Stop sequences were generated to exclude the C-terminal myc tag. HA bTrCP1 was a kind gift from Dr. Michele Pagano. pRK5-HA-Ubiquitin-WT was from Addgene (plasmid 17608) [339]. BimEL and bTrCP1 point-mutations were generated using site-directed mutagenesis. To generate the GST-BimEL fusion protein, BimEL WT or phosphorylation mutants were sub-cloned into pGEX-6P1 (GE Healthcare). All constructs were verified by restriction digest analysis and DNA sequencing.

Cells and drug treatments: Hela and 293T cells were obtained from ATCC. All cells were maintained in Dulbecco's modified Eagle medium (Wisent Inc., Canada) supplemented with 10% fetal bovine serum (HyClone/Thermo Scientific) and 0.1% gentamicin (Wisent Inc., Canada). Mitotic cells were

prepared by incubating with 2 mM thymidine for 20 hours followed by three hours release into normal medium before the addition of 100ng/ml Nocodazole (Sigma). Rounded cells were collected after treatment for 12 hours. RO3306 (Alexis Biochemicals) synchronization was performed by treating Hela cells for 19 hours with 9μM RO3306. The drug was then washed out and cells released into fresh media for the specified time points. For proteasome inhibition studies, cells were incubated with 10 μM MG132 (Calbiochem) for 3 h before further treatments. Other drugs that were used were: PMA (10 ng/ml) for 3 hrs, MLN8054 (Selleckchem), Okadaic acid (Calbiochem), C2-Ceramide (Sigma) for the indicated times and concentrations. Treatment of cell extracts with l phosphatase (New England BioLabs) was performed by adding 50 μg of total protein extract to the 1×phosphatase buffer (supplied by manufacturer) supplemented with 2 mM $MnCl_2$ and incubated with 10 U of l phosphatase at 30°C for 30 minutes. The reactions were stopped by the addition of 1x Laemmli sample buffer.

Extract preparation, immunoprecipitation, and immunoblotting: For HA βTrCP 1 or 3XFlag BimEL IPs, transfected 293T or Hela cells were lysed in 1 ml IP Lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM PMSF and protease inhibitor (Roche) per 10^7 cells on ice for 20 min. Cell debris was pelleted by centrifugation at maximum speed for 15 min at 4°C. The supernatant was then incubated with 30 μl anti-Flag or anti-HA affinity gel (Sigma) for 2 h at 4°C. The beads were then pelleted and washed 4 times with the same buffer. Following the washes, the beads were pelleted and resuspended in 1× Laemmli sample buffer, boiled for 5 min, and stored at −20°C until further use. Immunoblots were performed using standard protocols and visualized by enhanced chemiluminescence (PerkinElmer).

***In vivo* ubiquitination assay:** *In vivo* ubiqutination assays were performed as previously described [340]. Briefly, 293T cells were co-transfected with pRK5-HA ubiquitin (Addgene) and 3Xflag Bim constructs using the

Lipofectamine 2000 protocol. 24 hours post-transfection the standard Thy/Noc protocol was applied. Drug treatment was performed in the last 2-3 hours of the experiment. Cells were washed twice with PBS before re-suspending the in 4-5 volumes of 50mM Tris-HCl, pH 7.4, 0.25 M NaCl, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1 mM Na_3VO_4, protease inhibitor tablet and 2mM N-ethylmaleimide. The regular IP protocol was followed as described above.

Gene silencing using RNAi and shRNA: The siRNA oligonucleotide sequence targeting βTRCP1/2 has been described previously [341] and corresponds to nt 515-535 of human βTRCP1 and nt 262-282 of human βTRCP2. The following siRNA sequences were used to knock down Aurora A [342]: ^{725}AUG CCC UGU CUU ACU GUC A^{743} and ^{155}AUU CUU CCC AGCGCG UUC C^{173}. To knock down PP2A catalytic subunit we used the following siRNA sequences Hs_PPP2CA_5 ATGGAACTTGACGATACTCTA and Hs_PPP2CA_6 CAAACAATCATTGGAGCTTAA [343]. The non-silencing control siRNA sequence used was AATTCTCCGAACGTGTCACGT (Qiagen). Cells were transfected with 50 nM siRNAs using the Lipofectamine 2000 protocol as provided by the manufacturer. Cells were subjected to two rounds of transfection (24 and 48 hours after plating) before Thy/Noc or RO3306 synchronization as described above. Lentivirus shRNA constructs targeting Bim were obtained from Sigma (Mission shRNA pLKO.1-puro, NM_138621.x-541s1c1 and NM_138621.x-522s1c1). SHC002 MISSION Non-Target shRNA was used as the non-silencing control. Lentivirus were packaged as previously described [344].

Flow cytometry: Cells were collected by scraping, washed twice with cold PBS and resupended in Nuclear Isolation and Stain (NIM-DAPI 10) from Beckman Coulter to obtain an average concentration of 1×10^6 cells/ml. DNA content was determined by flow cytometry on a Cell Lab Quanta SC flow cytometer (Beckman Coulter). Apoptosis assays were performed in Hela cells treated with MLN8054 or vehicle control (DMSO). Cells were collected by scraping and washed twice with ice cold PBS then resupended in 100 1ml of

AnnexinV binding buffer (2.5 mM CaCl2, 140 mM NaCl, 7.75 mM HEPES [pH 7.4]) and then incubated with PE-AnnexinV (BD Biosciences) and7-amino-actinomycin D (7AAD) (A.G. Scientific) according to the manufacturing protocol. Cells were then analysed on the same Flow cytometer as above for staining with PE-Annexin alone or PE-Annexin plus 7AAD staining. At least 10,000 cells were analyzed per sample.

***In vitro* kinase and in vitro binding assays:** 500 ng of recombinant GST-BimEL (WT or mutant) purified from *E. coli* were incubated at 30°C for 30 minutes with 500 ng recombinant His Aurora A (Millipore) in a 30ml of kinase buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) plus 100 μM ATP. Reaction was stopped by addition of 4X laemmli buffer. For *in vitro* binding assays, 5mg of Hela or 293T lysates were incubated with 1mg of GST alone or GST BimEL for 2 hrs at 4°C. Samples were then processed as described above for IPs.

2.6. Acknowledgements

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2.7. Figure legends & Figures

Figure 2.1. BimEL is phosphorylated and targeted for degradation at mitosis.

(**A**) Immnoblot analysis of HeLa cells synchronized using the Thymidine/Nocodozole (Thy/Noc) protocol as shown (**top**). Whole cell extracts were analyzed for expression of BimEL following release from Thy/Noc for the indicated times. Immunoblots for three other proteins known to be degraded during mitosis, cyclin B1, Securin, and Polo-like kinase-1 (Plk-1) were performed for comparison. Anti-phospho histone H3 (P-H3) is included as a marker for onset of mitosis and APC2 as a loading control. Flow cytometry was used to confirm cell cycle phase (**bottom**).

(**B**) Immunoblot analysis of HeLa cells synchronized with the cdk1 inhibitor RO3306 and released for the indicated length of time or left asynchronous (Asyn). Whole cell extracts were analyzed by immunoblot analysis for expression of BimEL and three other proteins known to be degraded during mitosis: cyclin B1, cyclin A2, and cdc20. (**C**) Immunoblot of HeLa cells synchonized as in panel A and released for 90 minutes in the presence of DMSO (vehicle control) or MG132. (**D**) Immunoblot analysis of endogenous BimEL in whole cell extracts treated with λ phosphatase (+) or control (-) prepared from Nocodozole treated HeLa cells.

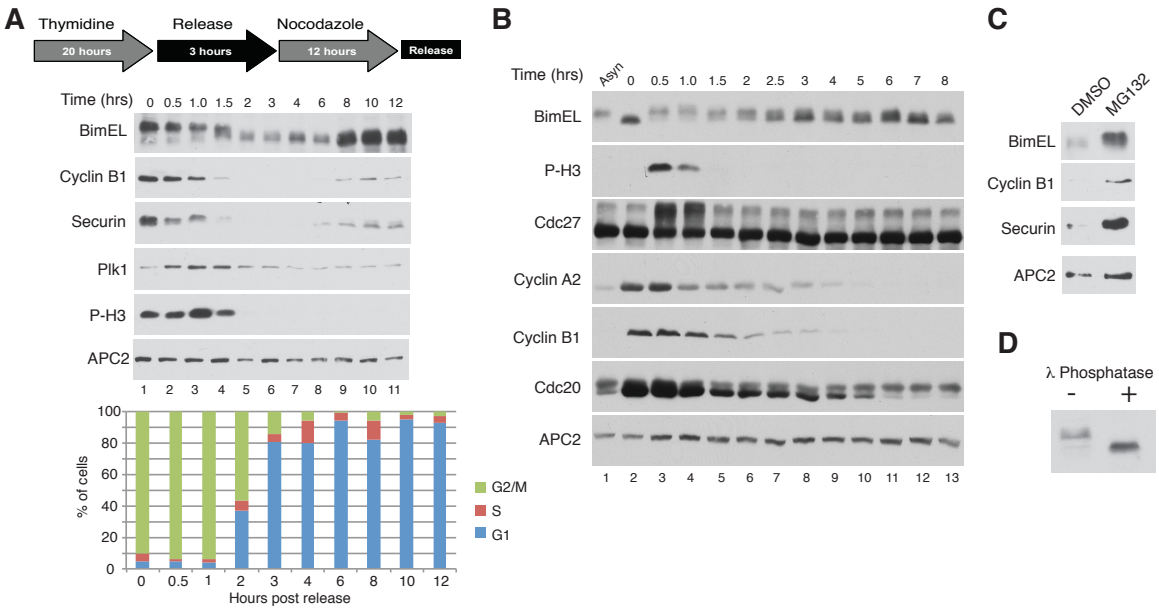


Figure 2.1. BimEL is phosphorylated and targeted for degradation at mitosis

Figure 2.2. BimEL is phosphorylated and degraded at mitosis in a mechanism independent of the spindle assembly checkpoint.

(**A**) Immunoblot analysis of 293T cells synchronized as in Figure 1A with the exception that cells were left in Nocodozole for the indicated times. Immnoblots were performed for total BimEL and phosphorylated BimEL on serine-69 (P-S69) and serines 93/94/98 (P-S93/94/98). Immunoblots for two other proteins known to be degraded during mitosis, wee1 and cyclin A2 were performed. Anti-phospho histone H3 (P-H3) is included as a marker for onset of mitosis and b-actin as loading control.

(**B**) Immunoblot analysis of HeLa cells synchronized at mitosis as in Figure 1A and released into 4 different conditions: normal media (control), MG132 (10 μM), Taxol (100nM) and RO3306 (10 μM). Whole cell extracts were prepared at the indicated times post release. Immnoblots were performed for total BimEL, P-S69 and P- S93/94/98. Immunoblots for three other proteins known to be degraded during mitosis, Wee1, cyclin B1, and Securin, were performed for comparison. P-H3 is included as a marker for onset of mitosis and APC2 as loading control.

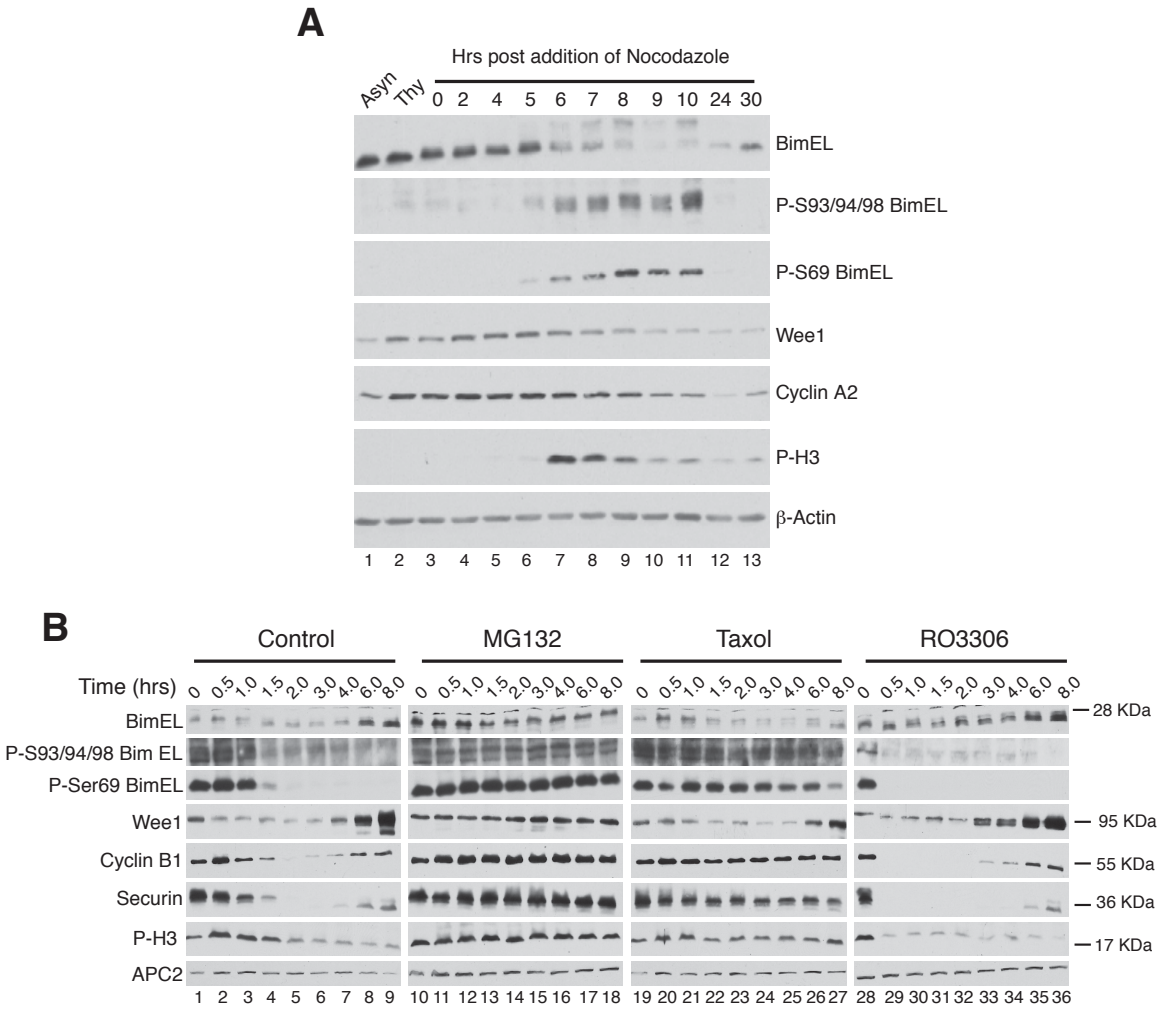


Figure 2.2. BimEL is phosphorylated and degraded at mitosis in a mechanism independent of the spindle assembly checkpoint.

Figure 2.3. BimEL is ubiquitinated at mitosis and requires phosphorylation on Serine 93/94/98.

(**A**) 293T cells were co-transfected with HA-tagged Ubiquitin (Ub) and Flag-tagged BimEL, BimL BimS or empty vector control (EV). Cells were synchronized as shown in figure 2.1A. Asynchrous (Asyn) cells treated with PMA and MG132 was used as a positive control for BimEL ubiqutination (lanes 1 and 9). Cells were treated with MG132 (+) or vehicle control (-) as indicated. (**Left**) Anti-Flag immunoprecipitation (IP) followed by immunoblot for HA and FLAG epitopes. (**Right**) Immunoblots were performed on cell extracts used as input for IPs using anti-Flag and HA antibodies to detect Bim and Ub expression. Anti-Cdc27 immunoblot was used to confirm mitotic state of cells and β-actin was monitored as loading control.

(**B**) Nocodozole-treated 293T cells were co-transfected with HA-Ub and Flag-tagged *wild-type* BimEL, S69A, S94/98A or EV control. Anti-Flag IP was performed as in panel A. Resulting IPs were analyzed by immunoblot using anti-Flag, anti-HA, and the phoshospecific BimEL antibodies (P-S93/94/98 and P-S69). Cells were treated with MG132 (+) or vehicle control (-) as indicated. Immunoblots were performed on cell extracts used as input for IPs using anti-Flag to confirm expression of BimEL constructs and and β-Actin was monitored as loading control.

(**C**) 293T cells were transfected with wild-type (WT) BimEL or phosphorylation site mutants as indicated. In all cases EGFP was cotransfected to monitor transfection effeciency. Cell extracts were prepared from either asynchronous (A) or after nocodozole (N) (100ng/ml) treatment. Immunblots were performed on WCE using anti-Flag to detect BimEL, β-Actin as loading control, EGFP to monitor transfection efficiency and cdc27 to confirm the mitotic state of the cell.

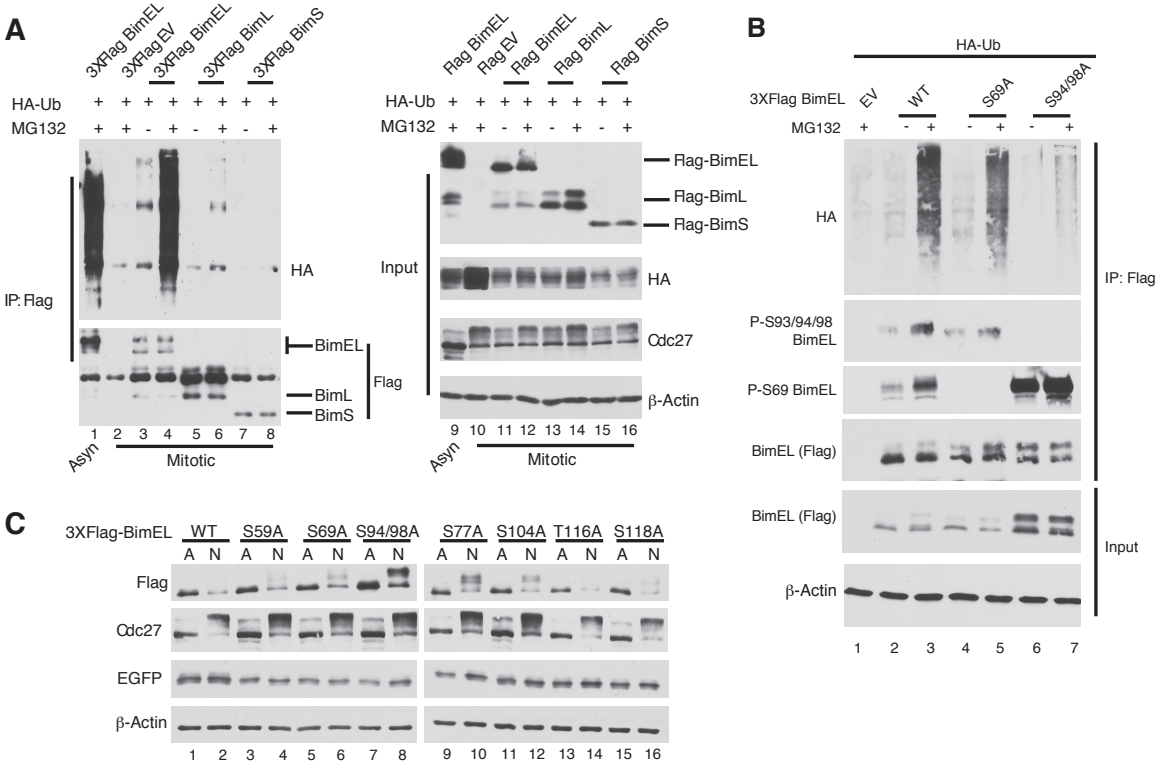


Figure 2.3. BimEL is ubiquitinated at mitosis and requires phosphorylation on Serine 93/94/98.

Figure 2.4. Phosphorylation on Serine 93/94/98 of BimEL creates a binding site for bTrCP1.

(**A**) Knockdown of βTrCP1 expression in HeLa cells using siRNA at the indicated concentrations. A non-silencing siRNA (Ctl) was used as control. Immunoblot analysis was performed against endogenous BimEL and Wee1. β-Actin was monitored as loading control. (**B**) HeLa cells were transfected with Flag-tagged bTrCP1 or empty vector control (EV). Cells were synchronized in mitosis as in Figure 2.1A except that cells were released in the presence of MG132. Cell lysates prepared at the indicated times post-release. Immunoprecipitation (IP) was performed using anti-Flag antibody and immunoblot analysis used to detect endogenous BimEL, Flag (βTrCP1) and phosphorylated BimEL on serines 93/94/98 (P-S93/94/98). Cell extracts used as input for IP were analyzed by immunoblot to measure expression of total BimEL, Flag (bTrCP1), and b-Actin as loading control.

(**C**) 293T cells were transfected with EV, wild-type (WT) BimEL or phosphorylation site mutants as indicated. Cells were synchronized in mitosis as in figure 2.1A. Asynchrous (Asyn) cells treated with PMA and MG132 was used as a positive control (lane 1). left asynchronous (Asyn). IP was performed using anti-FLAG antibody and immunoblot analysis used to detect endogenous βTrCP1, Flag (BimEL) and phosphorylated BimEL on serines 93/94/98 (P-S93/94/98). Cell extracts used as input for IP were analyzed by immunoblot to measure expression of total βTrCP1 and Flag (BimEL). Cdc27 immunoblots were used to confirm mitotic state of cells and b-Actin as loading control.

(**D**) 293T cells were transfected with EV, wild-type (WT) βTrCP1 or the indicated βTrCP1 mutant. Cells were synchronized as in figure 2.1A. Asynchronous cells transfected with HA bTrCP1 WT and treated with PMA and MG132 for three hours was used as a positive control (lane 1). IP was performed using anti-HA antibody and immunoblot analysis used to detect IPed BimEL and HA (βTrCP1). Cell extracts used as input for IPs were analyzed by immunoblot to measure expression of total Bim and HA (βTrCP1). Cdc27 immunoblots were used to confirm mitotic state of cells and β-Actin as loading control.

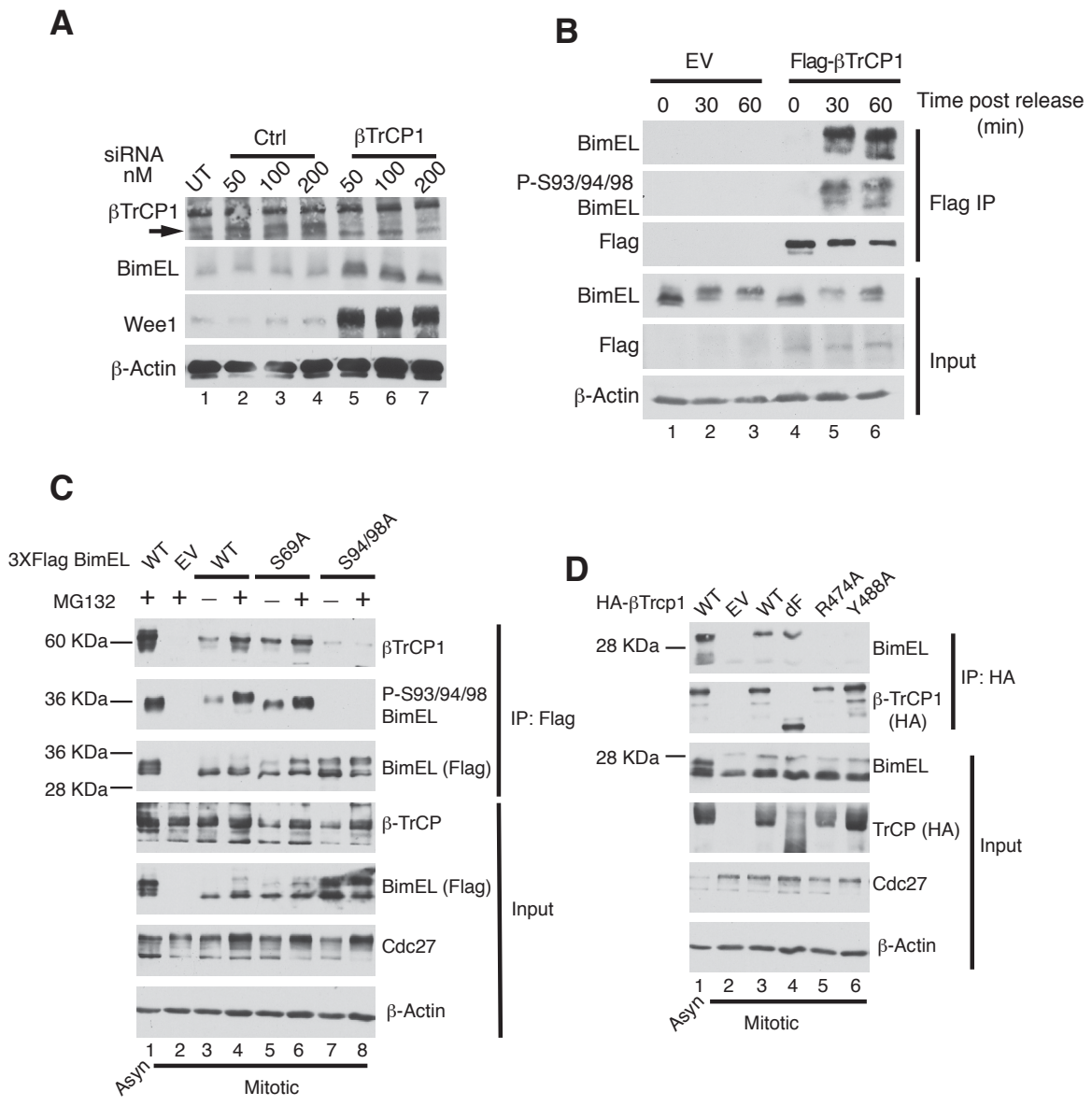


Figure 2.4. Phosphorylation on Serine 93/94/98 of BimEL creates a binding site for bTrCP1.

Figure 2.5. PP2A dephosphorylates BimEL on Ser93/94/98 and prevents degradation.

(**A**) HeLa cells were synchronized in G2/M with RO3306 and released into fresh media plus vechicle (DMSO), MG132 , Okadaic acid (OA) or C2-ceramide at the indicated concentrations and harvested 90 minutes post-release. Whole cell extracts were analyzed by immunoblot to detect endogenous BimEL, phosphorylated BimEL on serines 93/94/98 (P-S93/94/98) and Wee1. Cdc27 immunoblots were used to confirm mitotic state of cells and β-Actin as loading control.

(**B**) 293T cells were transfected with wild-type (WT) Flag-tagged BimEL or left untransfected (UT). Cells were synchronized in mitosis as in figure 1 and released into vehicle control (DMSO), MG132 (10 μM), RO3306 (9μM) and OA (500nM) as indicated. IP was performed using anti-FLAG antibody (BimEL) and immunoblot analysis used to detect IPed BimEL (FLAG) and P-S93/94/98. b-Actin immunoblot was used as the input control for IPs.

(**C**) Knockdown of the PP2A catalytic subunit in HeLa cells using two different siRNA PPP2CA_5 and PPP2CA_6. A non-silencing siRNA (Ctl) was used as control. Immunoblot analysis was performed against endogenous BimEL, phosphorylated BimEL (P-S93/94/98) and the PP2A-C subunit. Cdc27 immunoblots were used to confirm mitotic state of cells and β-Actin as loading control.

(**D**) 293T cells were transfected with WT Flag-tagged BimEL, BimL, BimS or phosphorylation site mutants as indicated. Transfected cells were synchronized in mitosis as shown in figure 2.1A and treated with vehicle control (DMSO), MG132 (10 μM) , or OA (500nM) as indicated. Whole cell extracts were analyzed by immunblot using anti-Flag to detect Bim and β-Actin as loading control.

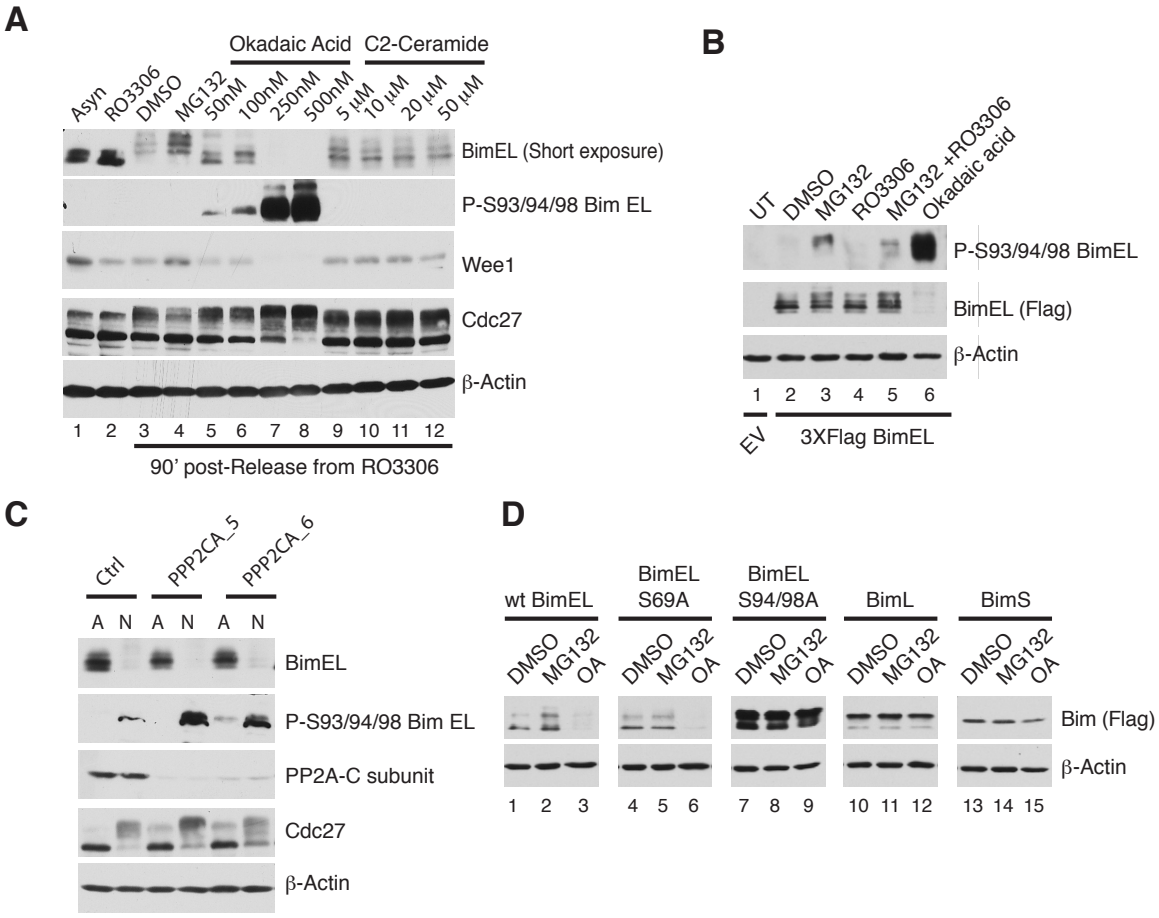


Figure 2.5. PP2A dephosphorylates BimEL on Ser93/94/98 and prevents degradation.

Figure 2.6. Phosphorylation of the BimEL degron is mediated by Aurora A kinase during mitosis.

(**A**) 293T cells were synchronized in mitosis as in figure 2.1A and released into vehicle control (DMSO), MG132, RO3306, or the indicated concentration of MLN8054. Whole cell extracts were analyzed by immunoblot to detect endogenous BimEL and phosphorylated BimEL on serines 93/94/98 (P-S93/94/98). Cdc27 immunoblots were used to monitor mitotic state of cells and β-Actin as loading control. (**B**) HeLa cells were synchronized in G2/M with RO3306 and released into fresh media plus vechicle (DMSO), MG132 (10 μM) or MLN8054 (5 μM) and harvested 90 minutes post-release. Whole cell extracts were analyzed by immunoblot to detect endogenous BimEL . P-T210 Plk1 and total Plk1 were used as controls to confirm the downstream effects of the aurora A Inhibitor MLN8054. (**C**) Knockdown of Aurora A expression in HeLa cells using two different siRNA. A non-silencing siRNA (Ctl) was used as control. Immunoblot analysis was performed against endogenous BimEL and Aurora A. P-T210 Plk1 and total Plk1 was used as a control to confirm the downstream effects of the knockdown. Cdc27 immunoblots were used to confirm mitotic state of cells and b-Actin as loading control.

(**D**) 293T cells were transfected with HA-tagged βTrCP1 or empty vector control (EV). Cells were synchronized in mitosis as in Figure 1A and released into vehicle control (DMSO), MG132 (10 μM), RO3306 (9μM), MLN3306 (5 μM) or left untreated (UT). Immunoprecipitation (IP) was performed using anti-HA antibody. Immunoblot analysis was perfored on IPs to detect endogenous BimEL and transfected bTrCP1 (HA). Cell extracts used as input for IP were analyzed by immunoblot to measure expression of total BimEL, HA (βTrCP1), and β-Actin as loading control. (**E** and **F**) Recombinant GST-BimEL or phosphorylation site mutants produced in *E. coli* were incubated with increasing amounts of purified Aurora A kinase (E) or 500 ng purified Aurora A kinase (F). Reactions were analyzed by immunoblot to detect GST-Bim, P-S93/94/98 GST-BimEL and Aurora A. (**G**) Recombinant GST or GST-BimEL produced in *E. coli* were incubated with either mitotic Hela or 293T extracts prepared using the standard thymidine /nocodazole protocol. Immunoblot analysis was perfored to detect Co-precipetated

endogenous Aurora A. Mitotic Hela or 293T cell extracts were used as an input to measure expression of endogenous Aurora A. Image of Red Ponceau stained membrane used to show GST proteins.

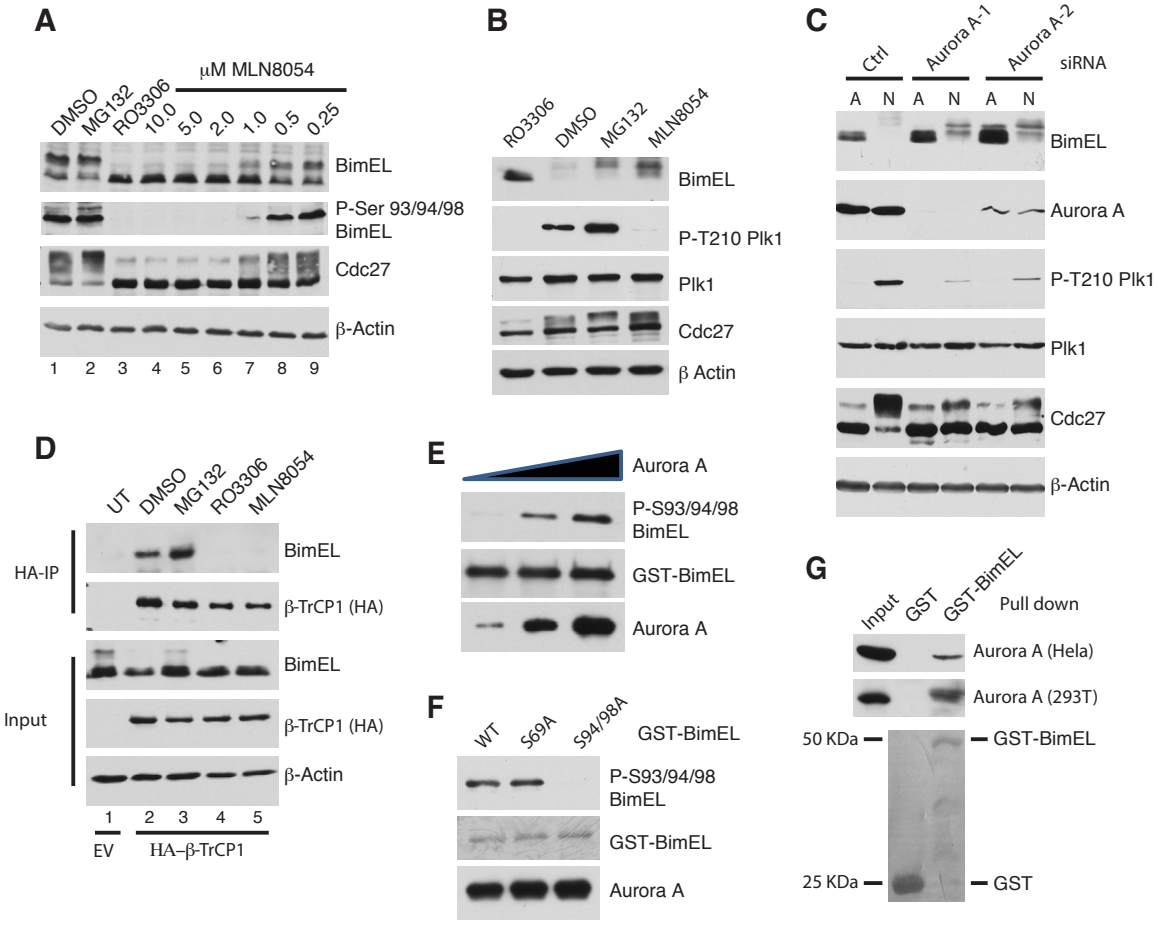


Figure 2.6. Phosphorylation of the BimEL degron is mediated by Aurora A kinase during mitosis.

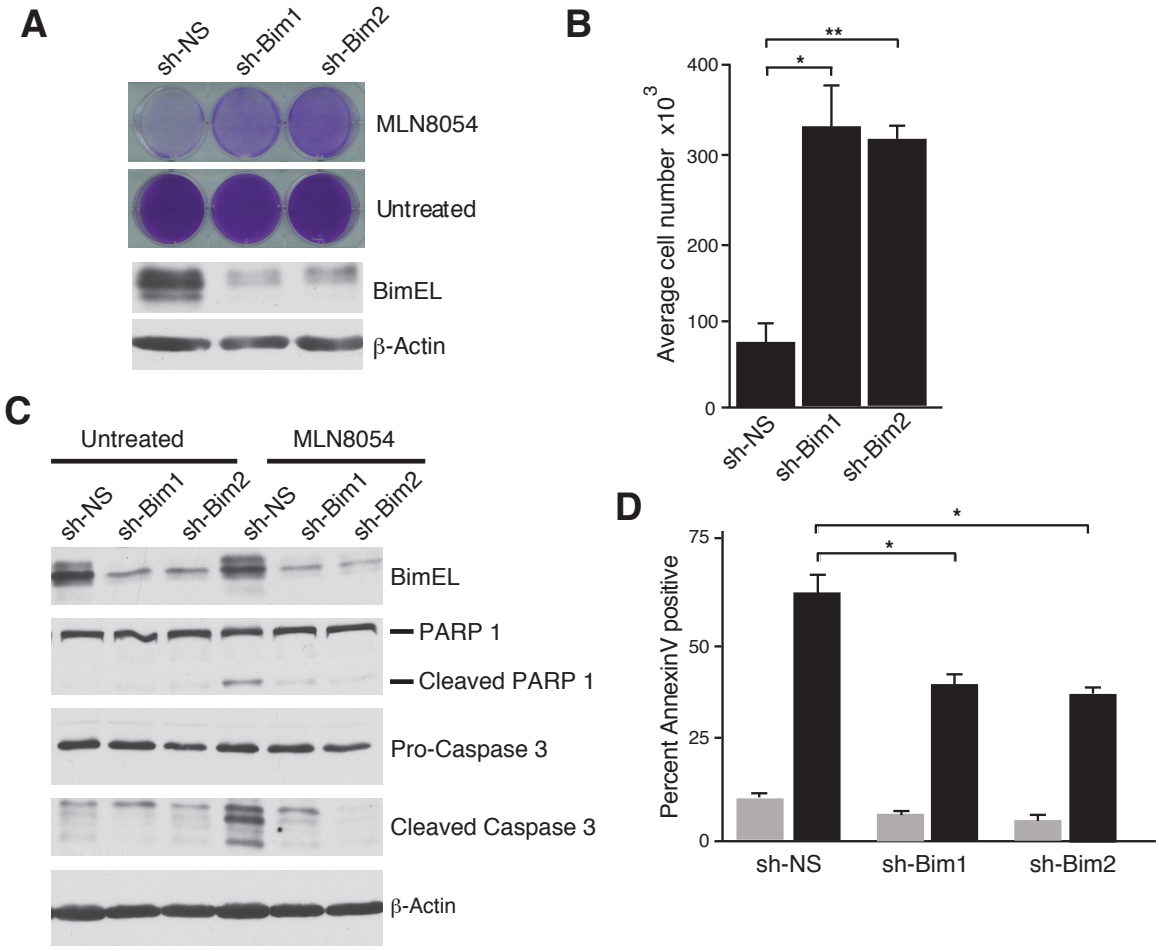
2.7. Knockdown of Bim protects cells from apoptosis induced by Aurora A inhibition.

(**A**) Bim expression was knocked down in HeLa cells using two different lentivirus delivered shRNAs (sh-Bim1 and shBim2). A non-silencing shRNA (sh-NS) was used as control. (**top**) Knockdown and control cells were treated with 5μM MLN8054 for 72 hours. Resulting tissue culture plates were stained with crystal violet. (**bottom**) Immunoblots of whole cell extracts confirming knockdown in Bim shRNA expressing cells. β-Actin is shown as loading control.

(**B**) Quantitation of cell numbers from experiment in panel A Students t-test was used to determine statistical significance. (* indicates $p \leq 0.05$, ** indicates $p \leq 0.01$).

(**C**) Control (sh-NS) or sh-Bim1 and sh-Bim2 HeLa cells were treated with 5μM MLN8054 for 48hrs. Immunoblots of whole cell extracts were performed to confirm Bim knockdown and cleaved PARP-1 and Caspase 3 were used to demonstrate apoptotic effect of MLN8054. β-Actin is shown as a loading control.

(**D**) HeLa cells were treated as described for panel C, stained with AnnexinV and 7AAD, and analyzed by flow cytometry. The percentage of AnnexinV-positive cells is indicated for each treatment.



2.7. Knockdown of Bim protects cells from apoptosis induced by Aurora A inhibition.

2.7. Supplementary Figures

Fig. S2.1. **BimEL is phosphorylated and degraded at mitosis in a mechanism independent of the spindle assembly checkpoint.**

Immunoblot analysis of 293T cells synchronized at mitosis as in Figure 2.1A and released into 4 different conditions: normal media plus vehicle control (DMSO) , MG132 (10 μM), Taxol (100nM) or RO3306 (9 μM). Whole cell extracts were prepared at the indicated times post release. Immnoblots were performed for total BimEL, P-S69 and P-S93/94/98. Immunoblots for two other proteins known to be degraded during mitosis, Wee1 and cyclin B1 were performed for comparison. Cdc27 and Phospho-(Ser) CDK substrates is included as a marker for the mitotic state of the cell. β-Actin was monitored as loading control.

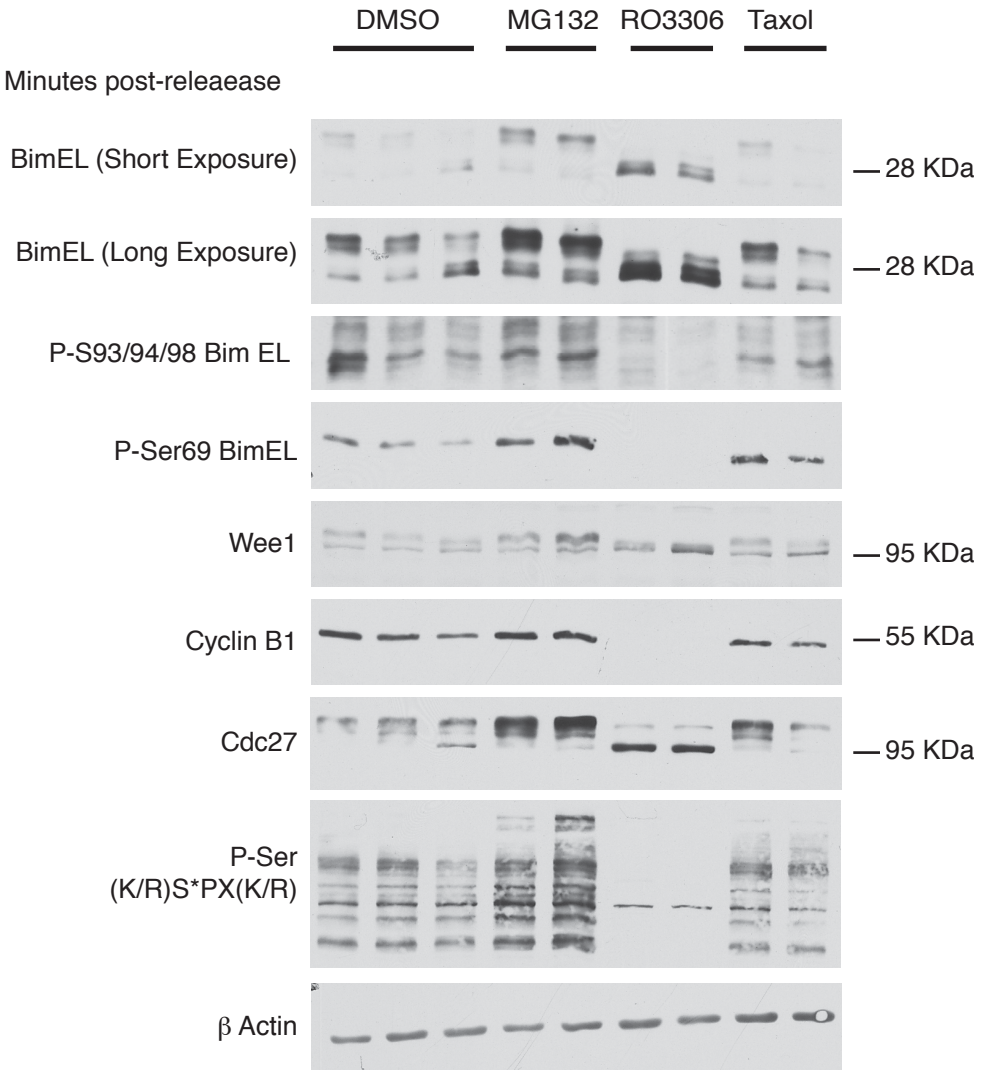


Fig. S2.1. **BimEL is phosphorylated and degraded at mitosis in a mechanism independent of the spindle assembly checkpoint.**

Fig. S2.2: Effect of kinase inhibitors on mitotic phosphorylation of BimEL.

293T cells were synchronized as in Figure 2.1A. Mitotic cells were collected and re-plated in the presence of DMSO (Control), MG132 or indicated kinase inhibitor. The following kinase inhibitors were placed on cells for 120 minutes: CDK1 inhibitor RO3306 (9μM), Rsk1/2 inhibitor BI-D1870 (10μM), Plk1 inhibitor BI2536 (100nM), GSK3β inhibitor LiCl (25mM), MKK1-MKK5 inhibitor U0126 (20μM), MKK1 inhibitor PD098059 (50μM), MKK1 inhibitor PD184352 (10μM), JNK1/2 inhibitor SP-600125 (10μM), p38 inhibitor SB-202190 (5μM), PI3K inhibitor LY-294002 (50μM), PI3K inhibitor Wortmannin (100nM). λ phosphatase treatment was performed as in Figure 1D. Whole cell extracts were analyzed by immunblot to detect endogenous Bim, phosphorylated BimEL on serine-69 (P-S69) and serines 93/94/98 (P-S93/94/98). Phospho-(Ser) CDK substrates is included as a marker for the mitotic state of the cell. β-Actin was monitored as loading control.

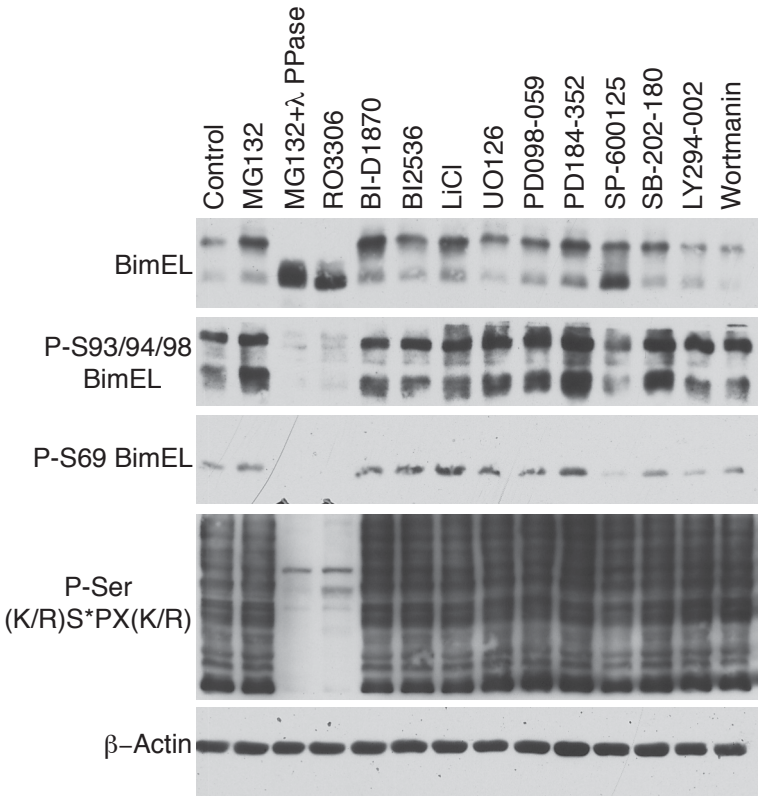


Fig. S2.2: Effect of kinase inhibitors on mitotic phosphorylation of BimEL.

Chapter 3

mTORC1 Promotes Survival During Mitotic Arrest

Mohamed Moustafa-Kamal[1,2], Wissal Alassad[1], Valentina Gandin[4] , Thomas Kucharski[1,2], Yazan Abas [2], Bhushan Nagar[2] , Jerry Pelletier[1,2], Ivan Topisirovic [2,4] and Jose G. Teodoro[1,2,3]

1-Goodman Cancer Research Center, McGill University, Montréal, Québec, Canada
2-Department of Biochemistry, McGill University, Montréal, Québec, Canada
3-Department of Microbiology and Immunology, Montréal, Québec, Canada
4-Lady Davis Institute for Medical Research, Sir Mortimer B. Davis- Jewish General Hospital, and Department of Oncology, McGill University, Montréal, Québec, Canada

3.1. Abstract

mTOR is a serine/threonine kinase which acts a master regulator of cell growth and proliferation. Raptor, a scaffolding protein that recruits substrates to mTOR complex 1 (mTORC1), is known to be phosphorylated during mitosis, but the significance of this phosphorylation remains largely unknown. Herein we show that raptor expression and mTORC1 activity are dramatically reduced in mitotic cells across a variety of cancer and normal cell lines. Loss of phosphorylation stabilized raptor during mitosis, which resulted in reactivation of mTORC1 in a rapamycin-sensitive manner. Expression of the phosphorylation-deficient raptor mutant caused a dramatic reduction in cytotoxicity of the spindle poison taxol, which was mediated via degradation of the Programmed Cell Death protein 4 (PDCD4), a tumour suppressor protein that inhibits eIF4A activity and is negatively regulated by the mTORC1/S6K1 axis. Accordingly, pharmacological inhibitors of eIF4A synergized with taxol, whereby this combination is cytotoxic even in taxol resistant cells. These findings indicate that the mTORC1/S6K1/PDCD4/eIF4A axis has a pivotal role in death vs. slippage decision under mitotic arrest and thus may be exploited to gain a clinical benefit in treating cancers resistant to drugs targeting mitosis including taxol.

3.2. Introduction

In order to achieve tissue homeostasis, cells need to coordinate both their growth (increase in cell mass) and proliferation (increase in cell number). The two processes are linked via the evolutionarily conserved TOR (Target Of Rapamycin) signaling pathway, which integrates a variety of extracellular signals and intracellular cues including hormones, growth factors and nutrients to coordinate growth and proliferation with metabolic activity in the cell. In mammals, mechanistic/mammalian TOR (mTOR) nucleates two different large signaling complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of mTOR, raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with sec-13), PRAS40 (proline-rich AKT substrate 40 kDa), and DEPTOR (DEP domain-containing mTOR interacting protein). It stimulates anabolic processes such as protein synthesis and energy production. mTORC2 is composed of mTOR, rictor (raptor independent companion of mTOR), mLST8, mSIN1 observed with rictor-1) and controls cytoskeletal organization and cell survival [345, 346].

In yeast, TOR primarily regulates cell growth and secondarily impacts on proliferation [347]. In mammals, however, mTORC1 impacts on both cell growth, by phosphorylating the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs), and proliferation, by phosphorylating the ribosomal protein S6 kinases (S6Ks)([348]). During cap-dependent translation initiation, mRNA is recruited to the ribosome via the eIF4F complex, which comprises a cap-binding subunit eIF4E, large scaffolding protein eIF4G and DEAD box RNA helicase eIF4A, that facilitates scanning of the ribosome towards the initiation codon. Phosphorylation of 4E-BPs by mTORC1 stimulates release of 4E-BPs from eIF4E, which allows eIF4E-eIF4G association and the assembly of the eIF4F complex, thereby increasing translation initiation rates [252]. S6Ks phosphorylate a number of components of the translational machinery and related regulators such as ribosomal protein S6, eIF4B, eEF2K and PDCD4, an inhibitor of eIF4A [220].

Previous studies indicated that raptor has a role in mediating mTORC1

assembly, recruiting substrates, and regulating mTORC1 activity [222, 223]. Recent studies have demonstrated the importance of phosphorylation of raptor on various sites in the regulation of mTOR signaling by pro- and anti-proliferative signals. Phosphorylation by Rsk at S721 [349] as well as by mTOR at S863 [245] have been shown to enhance mTORC1 activity, whereas phosphorylation at S722 and S792 by AMPK create 14-3-3σ binding sites and suppress mTORC1 activity [247]. Raptor has also been shown to be heavily phosphorylated in mitosis on at least 9 conserved sites downstream of cyclin-dependent kinase 1 (cdk1) and glycogen synthase kinase 3 (GSK3) [297, 350]. These reports showed that mTORC1 activity is needed for mitotic progression despite the reportedly decreased mitotic activity of two of the upstream activators of the mTORC1 pathway, AKT and MAPK pathways [297, 351]. The significance of mitotic phosphorylation of raptor and the role of mTORC1 during mitosis state thus remains largely underexplored.

In the current study we observed that, unexpectedly, mTORC1 activity in mitosis is dramatically suppressed. Furthermore, we provide evidence that multisite mitotic phosphorylation of raptor leads to reduction in its stability and mTORC1 activity. Hypo-phosphorylated raptor reactivates the mTORC1 complex and promotes extended survival of cells challenged with taxol. Finally, we demonstrate that mTORC1 delays cell death under mitotic arrest by inducing degradation of the pro-apoptotic protein, PDCD4, and subsequently bolstering eIF4A activity. These results highlight the previously unappreciated role of the mTORC1/S6K/PDCD4/eIF4A axis in mitosis.

3.3 Results

3.3.1. mTORC1 activity is decreased during mitotic arrest

In order to examine the activity of mTORC1 complex in the context of prolonged mitosis (mitotic arrest), HeLa cells were synchronized using thymidine followed by release into nocodazole (Noc), and analyzed by immunoblotting at indicated time points post release (Figure 3.1A). This experiment revealed that the cells that progress into mitosis, as evidenced by the appearance of phosphorylated cdc27 and phosphorylated geminin, gradually decrease S6K1 phosphorylation at Thr-389, which is a well-established mammalian/mechanistic Target of Rapamycin (mTOR) complex 1 (mTORC1)-specific phosphorylation site (Figure 3.1A). Concomitantly, we observed an upward electrophoretic mobility shift and a decrease in raptor levels (Figure 3.1A). We confirmed that the electrophoretic mobility shift of raptor is at least in part caused by phosphorylation using λ phosphatase [Supp. figure 3.1A]. In contrast to S6K1, phosphorylation of 4E-BP1 was increased. This observation is consistent with a previous report showing that CDK1, not mTORC1,phosphorylates 4E-BP1 in mitosis [301] which, in addition to our observation, contradicts earlier reports that suggested a normal and essential activity of mTORC1 in mitosis [297, 350]. We speculate that this discrepancy may be due to different protocols for harvesting mitotic cells. When using HaCat cells, a mitotic shake off protocol was essential to prevent contamination with non-mitotic cells. Figure 3.1B shows that collection of only non-adherent cells after Thym/Noc treatment was required to prevent contamination with G2 cells (compare cdc27 profiles for each). Importantly, we detected comparable dowregulation of mTORC1 mitotic activity in other cell lines including 293T, PC3, HT29, A549, and an immortalized normal cell line, BJ-tert (Figure 3.1C). To exclude the possibility that decrease in S6K1-Thr 389 phosphorylation is caused by an increase in activity of a phosphatase [e.g. PP2A has been reported to dephosphorylate some of the mTORC1 kinase substrates [352]], and not by decrease in mTORC1 activity, we employed a phosphatase inhibitor, okadic acid, which did not rescue S6K1 phosphorylation in mitosis, as compared to control

interphase cells (Figure 3.1D). To exclude the possibility that the observed changes in S6K1 phosphorylation status stem from inadvertent effects of nocodazole treatment, HeLa cells were released from mitotic arrest into fresh media and collected over time as they exit mitosis into G1-phase. Strikingly, S6K1 phosphorylation at Thr-389 started to increase only after cells completely exited mitosis (decrease in cyclin B1 levels, lane 5), which was paralleled by a loss of electrophoretic mobility shift and accumulation of raptor protein (Figure 3.1E). Finally, we used the same protocol to collect mitotic HeLa cells after treatment with other agents that cause prolonged mitotic arrest including the APC/C inhibitor, pro-TAME and Taxol, which produced shifts in electrophoretic mobility of raptor and decrease in S6K1 phosphorylation that were comparable to those observed using nocodazole (Figure 3.1F). Taken together, these results suggest that mTORC1-dependent phosphorylation of S6K1 is diminished during prolonged mitotic arrest, which correlates with phosphorylation of raptor and reduction in its levels.

3.3.2. Raptor protein is downregulated during mitotic arrest

To further characterize the regulation of raptor during mitosis, we set out to distinguish whether the above observed effects occur under conditions of unperturbed mitosis or are due to cellular response to prolonged mitotic arrest. To address this question, HeLa cells were arrested at G2/M using the cdk1 inhibitor, RO3306, and released into fresh media containing either nocodazole (Noc) or vehicle (DMSO). These experiments revealed that under conditions of normal mitosis, raptor is phosphorylated as efficiently as in the presence of nocodazole (Figure 3.2A). However, the mitotic decrease in raptor protein levels occurred only in the presence of Noc and under conditions of sustained mitotic arrest. The observation that raptor levels are decreased acutely and in a manner that is coupled with phosphorylation suggest that the protein is actively degraded in a cell cycle dependent manner. We therefore investigated whether raptor is degraded by the proteasome. Strikingly, treatment with the proteasome inhibitor, MG132,

failed to rescue mitotic levels of raptor, but was able to stabilize other mitotic targets of the Ub-proteome systems including wee-1 and Cyclin A2 (Figure 3.2B). We further investigated caspase cleavage or lysosomal degradation as plausible causes of reduction in mitotic raptor levels. As with the proteasome inhibitor, pan-caspase (zVad-FMK) or lysosome activity inhibitors (chloroquine, Baffylomycin A, or NH4Cl) were also unable to restore mitotic raptor levels (Fig. 3.2C and 3.2D). The possibility that we failed to detect raptor due of technical artifacts caused by epitope masking through phosphorylation or protein insolubility were also ruled out as neither lysing mitotic HeLa cells in Laemmli buffer nor treatment of lysates with λ-phosphatase could rescue the raptor levels (Supplemental Fig. 3.1A and B). Finally, the possibility of the presence of a strong inhibitory mitotic signal was also excluded by the use of TSC2 Null MEFs. Figure 3.2E shows that even in the absence of TSC2, a condition expected to give higher mTORC1 activity, we were still unable to rescue neither Raptor's level nor mTORC1 activity. We then assessed the level of raptor mRNA using RT-PCR which showed a modest decrease (approximately 25%) as cells progress in mitosis which is insufficient to account for the sharp decrease in raptor protein levels (Supplemental Fig. 3.1C).

The above data show that there is a near complete loss of mTORC1 activity at mitosis paralleled with simultaneous phosphorylation and dramatic reduction in raptor protein levels. We therefore determined whether expression of exogenous raptor, which is driven by a CMV promoter and devoid of UTRs could rescue mTORC1 activity in mitosis. Although transfection of raptor cDNA was able to rescue levels of mitotic raptor, it failed to rescue the mTORC1 activity as evidenced by the continued absence of Thr-389 S6K1 signal (Figure 3.2F). Moreover, transfected myc-tagged raptor appeared to be almost entirely in a slower migrating phosphorylated form. Taken together these data show that mTORC1 activity is inhibited at mitosis through a phosphorylation dependent mechanism. Furthermore, conditions of prolonged mitotic arrest appear to negatively regulate levels of raptor through a non-canonical, unknown mechanism. Ectopic expression of

raptor could not rescue mTORC1 activity suggesting that phosphorylation of raptor is sufficient to inhibit mTORC1 activity during mitosis.

3.3.3. Raptor phosphorylation regulates mTORC1 dimerization.

mTORC1 is thought to act as an obligate dimer with raptor having a crucial function in mediating and maintaining the higher-order organization of mTORC1[222, 353]. Mitotic phosphorylation of raptor may therefore regulate binding to other mTORC1 subunits and/or mTORC1 dimerization. To test both hypotheses, we expressed flag-tagged and myc-tagged mTOR in asynchronous and mitotic HeLa cells followed by Flag-IP. Flag-mTOR pulled down much less total raptor and phosphorylated raptor from mitotic HeLa lysates as compared to asynchronous lysates (Figure 3.3A). Additionally, less myc-mTOR was pulled down with flag-mTOR, suggesting a compromise of mTORC1's dimerization in mitosis. similar effects were observed by immuno-precipitating PRAS40, which is an additional mTORC1-specific component [297, 354] (Figure 3.3B). We further examined mTORC1 dimerization during mitosis by co-transfecting Flag-tagged and myc-tagged raptor. Whereas Flag-tagged raptor was able to efficiently IP co-transfected myc-tagged Raptor from asynchronous cell extracts, it was unable to do so from mitotic extracts (Figure 3.3C). Moreover, IPed raptor appeared to be predominantly in the unphosphorylated form, which further suggests that raptor phosphorylation may affect the integrity of mTORC1 dimers. To further confirm these observations, the mTORC1 complexes from asynchronous and mitotic HeLa cell extracts were analyzed using size-exclusion chromatography. mTOR eluted in two major peaks, centered at fractions 3 and 8 both in asynchronous and mitotic extracts. Based on the elution position of standards, we estimated that the peak at fraction 8 is corresponds to mTORC1 dimers, whereas the peak corresponding to higher MW complexes is likely representative of mTOR multimers [355]. Interestingly, in asynchronous cell extracts raptor co-eluted with mTOR in fraction 8, whereas phosphorylated raptor in mitotic extracts eluted at fractions corresponding to lower molecular weights (9 and 10). In turn, PRAS40 showed no difference in

elution pattern between asynchronous and mitotic extracts. Taken together, these data suggest phosphorylation of raptor might affect the dimeric nature of the mTORC1 complex in mitosis.

3.3.4. Mutation of raptor phosphorylation sites activates the mTORC1 kinase in mitosis

Since we observed that raptor phosphorylation appears to negatively regulate mTORC1 in mitosis, we next identified phosphorylation sites on raptor that are responsible for this effect. As shown in Fig 3.4A, the primary structure of raptor shows that its central region contains no structured domains compared to the n-terminal and c-terminal mTOR binding sites or the c-terminal WD40 domains. Previous studies have shown that at least 9 serine and threonine residues—that cluster to two regions located between the HEAT domain and the WD40-domain of raptor—are phosphorylated in mitosis including the AMPK phosphorylation sites (Ser722, Ser792). Mutation of the AMPK phosphorylation sites in conjunction with other reported sites (Ser696, Thr706) to alanine, however, did not exert a major effect on the electrophoretic mobility of raptor or mTORC1 activity in mitosis [Supplemental Fig. 3.1D]. We thus mutated all of the phosphorylation sites on raptor that were reported in mitosis. These sites included Ser696, Thr706, Ser711 located before the central HEAT domain (designated raptor-3A), and Ser855, Ser859, Ser863 (raptor-3A*) after the HEAT domain. In addition, we mutated both of the latter clusters simultaneously (raptor-6A) (Figure 3.4A). HeLa cells were transfected with WT myc-Raptor and corresponding mutants and asynchronous or thymidine-nocodazole synchronized cells were analyzed by western blotting. Raptor-6A mutation prevented the mitotic change in mobility and was accompanied with a modest increase in P-Thr389 S6K1 signal (Figure 3.4B, compare lane 2 to lane 8). Strikingly, mutation of two additional residues (Ser771 and Ser877) to generate the raptor-8A mutant abolished the mitotic mobility shift of raptor and dramatically bolstered mitotic mTORC1 activity as evidenced by an elevated P-Thr389 S6K1 signal (Figure 3.4B compare lane 8 to lane 16). To confirm that the observed

effects are mediated via the mTORC1 complex, we repeated the experiments in the presence of the allosteric mTOR inhibitor rapamycin, which in acute treatment selectively inhibits mTORC1, but not mTORC2 (Figure 3.4C). Rapamycin treatment was able to completely abolish the raptor 8A-dependent rescue of S6K1 phosphorylation in mitosis. Furthermore the raptor-8A effects are mitosis-specific since it did affect the asynchronous signal of P-Thr389 S6K1 (Figure 3.4C, compare lane 1 and 3). Finally, to confirm the role of raptor phosphorylation in suppressing mTORC1 activity we co-expressed Flag mTOR and EGFP-Raptor WT and 8A mutant in HeLa cells and performed Flag IP on lysates from asynchronous and Thym/Noc arrested cells. Flag-mTOR pulled down much less total EGFP-Raptor and phosphorylated raptor from mitotic HeLa lysates compared to asynchronous lysates and compared to EGFP-Raptor 8A which shows no change in binding to mTOR (Figure 3.4D). Furthermore, we noticed that the activity of the mutant extended down to increase phosphorylation of ribosomal protein S6, which is downstream, the S6K1. Taken together, these results show that mitotic phosphorylation of raptor acts as a phosphoswitch, which is necessary and sufficient to release it from the complex and to suppress the mTORC1 activity during this phase of the cell cycle.

3.3.5. Raptor 8A mutant promotes survival in response to Taxol

Several studies have shown that under prolonged mitotic arrest global rates of protein synthesis are strongly reduced [289-291]. Since mTORC1 increases the rates of mRNA translation, we next assessed the effect of raptor-8A mutant overexpression on the overall protein synthesis in HeLa cells during mitotic arrest. In mitotic HeLa cells, the raptor 8A mutant increased global mRNA translation rate significantly ($p < 0.001$) compared to WT raptor (Figure 3.5A). In order to determine whether the raptor-8A mutant affects normal mitotic progression, myc-raptor WT and the 8A mutant were transfected into HeLa cell and synchronized with the Thym/Noc, followed by release into fresh media (Figure 3.5B). As expected, compared to WT, myc-raptor 8A showed a minimal change in electrophoretic mobility and

a much higher signal for p-Thr389 S6K1, starting in mitosis and maintained throughout exit and entrance into G1 (as monitored by the presence of cdc27 phosphorylation). However, the timing for cyclin-B1 degradation (a marker for mitotic exit) showed no major differences between WT and 8A myc-raptor expressing cells. In addition, time-lapse microscopy for cells transfected with either empty vector, myc-raptor WT, or myc-raptor 8A did not display any conspicuous differences in the average time cells spent to complete one cell cycle (Figure 3.5C). Since mTORC1 signaling is known to promote survival by stimulating translation and inhibiting autophagy, we hypothesized that the raptor 8A mutant may affect cell fate during mitotic arrest. To this end, we transfected HeLa cells with EGFP-empty vector, EGFP-Raptor WT, and EGFP-Raptor 8A. 24 hours post transfection cells were synchronized in G2/M using RO3306 and then released into fresh media containing 100nM Taxol +/- 200nM Rapamycin and time-lapse imaging using GFP filter was initiated. The behavior of 100 (green) mitotic cells from each transfection was compared using time-lapse microscopy. In response to taxol the average time cells remained arrested in mitosis before dying was significantly higher in cells transfected with EGFP-raptor 8A compared to empty vector or EGFP-raptor WT (Figure 3.5D). This effect was mTORC1 dependent since addition of rapamycin reversed the pro-survival effects induced by raptor-8A mutant Figure 3.5E. Altogether, these data show that reactivating mTORC1 kinase activity in mitosis enhances cell survival during prolonged mitotic arrest.

3.3.6. PDCD4-eIf4A axis mediates the survival signaling downstream of the Raptor 8A mutant in response to Taxol

To further investigate the mechanism(s) by which raptor 8A attenuates cell death during mitotic arrest, HeLa cells transfected with either EGFP empty vector, EGFP-raptor WT, or EGFP-Raptor 8A were maintained under asynchronous and Taxol-arrested mitotic conditions. Subsequent analysis of known mTORC1-regulated proteins which are key players in the regulation of survival revealed that PDCD4 expression decreased and Bcl-xL expression increased in EGFP-Raptor 8A transfected as compared to EGFP-raptor WT

transfected or control cells (Figure 3.6A). PDCD4 is a tumor suppressor [356] that functions as an inhibitor of eIF4A function [268]. PDCD4 is phosphorylated by the mTORC1/S6K1 axis and degraded via the SCF^{bTrCP1} E3 Ligase [269]. To confirm the correlation between PDCD4 and active mTORC1 in mitosis we depleted endogenous PDCD4 using two different siRNA (PDCD4_505, PDCD4_1260). Cells were synchronized as in figure 3.5D, released into 100nM taxol and analyzed using time-lapse imaging. Strikingly, depletion of PDCD4 phenocopies that of expressing the raptor-8A mutant in terms of a significant prolongation of the time of mitotic survival in response to taxol ($p<0.001$ for both siRNAs) (Fig 3.6.B.). Since PDCD4 is known to inhibit the activity of eIF4A helicase, so we hypothesized that eIF4A might be involved in mediating the prosurvival activity of raptor 8A mutant. To test this hypothesis directly we repeated the same experiment as in figure 3.5D but in the presence of Hippuristanol, a selective eIF4A inhibitor [357] As shown in figure 3.6C, Hippuristanol akin to rapamycin reduced the prosurvival phenotype of raptor 8A mutant –expressing cells (mean death time with the mutant is not significantly different from EV or WT as in the absence of Hippuristanol).

Taken together, these data show that in response to a prolonged mitotic arrest cells deactivate mTORC1 complex through the phosphorylation of its substrate-binding subunit raptor. Overexpression of a phosphorylation-deficient mutant of raptor (raptor-8A mutant), reactivates the complex, and interferes with the death signal that triggers mitotic death in response to drugs that compromise the dynamics of regular mitosis, which at least in part is mediated by down-regulation of PDCD4 levels and consequently upregulating the downstream target, eIF4A.

3.3.7.The role of CDK1-mTOR-S6K1-PDCD4-eIF4A axis in regulating the death vs. slippage decision upon prolonged mitotic arrest

The observation that Hippuristanol can abolish the prosurvival phenotype of an over-active mTORC1 complex prompted us to test if this pathway, downstream the major mitotic kinase CDK1, is involved as a timer for the

death threshold during mitotic arrest. The model we have been using so far for Taxol-induced death in HeLa cells after release from RO3306 block allowed us to target eIF4A in a similar way to conditional knockdown to check its role exclusively in mitosis. To test this we treated HeLa cells released from RO3306 block with 100nM taxol alone or taxol and increasing concentrations of Hippuristanol. Hippuristanol acted in a dose dependent manner to speed up the time for cell death at higher concentration in combination with Taxol. The mean time of death for a hundred cells moved from 448 minutes (taxol alone) to only 188 minutes when taxol was combined with 500 nM Hippuristanol (Figure 3.7A). The current model for cell fate upon exposure to a mitotic poison like Taxol proposes a competition between a death and a slippage (adaption) network, with each having its own threshold. If the cell accumulates enough death signals, the death threshold is reached first and the cell dies. Conversely, if the cells cannot maintain the mitotic state and the slippage threshold is surpassed, cells exit and escape mitosis [65]. To test whether inhibiting that residual eIF4A activity in mitosis with hippuristanol is able to affect this fate we treated HeLa cells released from RO3306 block with a much lower dose of taxol (10 nM) that is known to cause mainly slippage as compared to 100 nM taxol. Figure 3.7B shows that in fact there is a synergy between 10nM taxol and 200 nM hippuristanol. Hippuristanol alone caused mixed phenotypes with mainly G2 arrest and only 27% cell death. Taxol alone resulted in mainly slippage with just 24% cell death. On the other hand, combing both drugs not only increases the percentage of cell death (71%) but also shifts the slippage phenotype we see with Taxol to a death phenotype, which confirms our hypothesis that eIF4A may be involved in the death vs. slippage decision. To emphasize this finding we extended the investigation to two other different cancer cell lines: H1299, a small cell lung carcinoma, known to exhibit slippage in response to taxol, and MCF-7, an invasive breast ductal carcinoma known to be resistant to taxol. In response to 300 nM taxol, H1299 main response was slippage (74%) with just 22 % cell death. Adding 500nM Hippuristanol increases the death to 68% and reduces slippage to just 12% as shown in figure 3.7 C.

Similarly MCF7 cells—though more sensitive to Taxol compared to H1299—showed increase in the percentage of death when treated with the drug combination (Taxol alone 42%, Taxol plus Hippuristanol 68%). In both cell lines, Hippuristanol alone at the doses used caused mainly a G2 arrest (H1299), or a mixed G2 arrest and death during the arrest(MCF-7). This was expected for a drug that inhibits a core component of the translation initiation machinery to affect mitotic entry. In summary, the findings here, confirms our observation that in response to prolonged mitotic arrest cells needs to shut down the mTORC1 activity in order to, at least, fine tune the translation initiation pathway, through regulating eIF4A activity. We speculate that aberrantly increased eIF4A activity in mitosis may interfere with the natural fate of cells exposed to mitotic poison. We propose a model in which perturbation of the mTORC1/PDCD4/eIF4A axis may affect the death threshold in mitosis and may thus interfere with the death vs. slippage decision in response to a prolonged mitotic arrest (Figure 3.7D).

3.4. Discussion

Previous studies have shown that raptor is phosphorylated during prolonged mitotic arrest but the significance of this phosphorylation has been largely unknown. In the current study we show that phosphorylation of raptor at multiple sites, plays a crucial role in deactivating the mTORC1 complex during prolonged mitotic arrest. A phosphorylation deficient mutant of raptor, raptor-8A, can restore the activity of mTORC1 complex and prolongs survival of mitotic cells when exposed to mitotic poisons.

This mechanism of mTORC1 inactivation is similar to that observed after of energy deprivation and activation of the AMPK pathway in which AMPK-mediated phosphorylation of raptor induces 14-3-3 σ binding and inhibition of mTORC1 [247]. However, in the present case this happens downstream at least two mitotic kinases, CDK1/GSK3β on multiple conserved sites on raptor as shown by Ramirez-Valle et al. [297]. The role of AMPK in inhibiting the mTORC1 complex was ruled out by the use of raptor Ser 722/792 mutant and the use of TSC2 null MEFs which uncouples mTORC1

from any of the canonical pathways that activate/deactivate it in interphase including LKB/AMPK, Insulin/PI3K/AKT, and Ras/ERK all of which reported to have low mitotic activity [249, 297].

It's important here to distinguish between two different but confusing terms; mitosis and prolonged mitotic arrest. We show here that under normal mitosis raptor is still phosphorylated which, using raptor 8A mutant as an evidence, inhibits the mTORC1 activity. Similarly, under prolonged mitotic arrest, not only is raptor phosphorylated, but its expression level also seems to be reduced through an unknown mechanism that is universal to all cancer and normal cell lines we tested. Thus the results presented here clearly dismiss any role for mTORC1 as an important kinase during prolonged arrest, but do not rule out a role for phosphorylated raptor during normal, unperturbed, mitosis. This may help explain the seemingly contradictory results showing the involvement of raptor in spindle assembly [358]or cytokinesis [359]. In both reports mTOR inhibitors were not used so as to exclude any mTOR-independent function of raptor. Additionally, a possible pre-mitotic role for mTORC1/raptor is likely to interfere with any conclusion about its role in mitosis. This problem is hard to control except with the use of a conditional knock down like the one recently used to conditionally knock down cdc20 [360].

Translation is one of the most energy consuming processes in the cell [287], which may explain the reason behind its inhibition during prolonged mitotic arrest as a way to cope with this energy stress. Inhibition of translation core components like eIF4E and eIF4G have been previously reported [288, 290]. Since mTORC1 kinase is a positive regulator of both general and specific cap-dependent-translation, inhibition of mTORC1 fits into the general theme of limiting the translation activity of cells during this time. Our data also show that the level of the tumor suppressor PDCD4 is sensitive to the mTORC1's activity in mitosis, which is in agreement with former reports showing its degradation through the mTORC1/S6K1 axis [269]. PDCD4 is a known inhibitor to eIF4A [268], and our data shows that eIF4A still retains some activity during prolonged mitotic arrest. The absence of

mTORC1 activity during mitosis doesn't refute the observation that cap-dependent translation is at least reduced during mitotic arrest. Recent reports showed that CDK1 actually phosphorylates the 4EBPs on a mitosis-specific site that is resistant to Rapamycin inhibition[301]. As previously reported, during mitotic arrest cells still need to maintain a reduced constitutive level of translation [219, 361]. This residual translation activity may be involved in determining the fate of the cell under prolonged arrest which is determined by the competition between two networks; one regulates the buildup of apoptotic signal and the other promotes exit without cytokinesis, or slippage [65]. The observation that targeting eIF4A activity in the presence of Taxol not only leads to a shift from a slippage-phenotype to a death-phenotype but also caused a quicker death in a dose-dependent manner, may help us solve the puzzle about how cells set the death timer during arrest. For example Bcl-xL, an anti-apoptotic protein has been shown to be an "eIF4E-sensitive" target and is selectively up-regulated when eIF4E activity is increased [362]. Bcl-xL can interfere with the fate of mitotic cells under arrest, thus by lowering eIF4A activity we might be able to interfere with the level of Bcl-xL and other death-preventing proteins involved in the death network.

Although drugs targeting the microtubules like Taxol and Vincristine are considered front line therapy for many cancers, their toxicity represents a large limitation. Our results show that it is possible to obtain the same sensitivity of cancer cells to these drugs but using a much lower dose. Combining Taxol with other drugs targeting the translation machinery like eIF4A inhibitors may thus provide a clinical use in cancers that are otherwise resistant to Taxol and other anti-mitotics.

3.5. Experimental Procedures

Cell lines and treatments

Cells were maintained in Dulbecco's modified Eagle medium (Wisent Inc., QC, Canada) supplemented with 10% fetal bovine serum (HyClone; Thermo Scientific) and 0.1% gentamicin (Wisent Inc., QC, Canada). Cells synchronized in mitosis were obtained by 20 hour treatment with 2.5 mM thymidine (Sigma), 4 hours of release before adding 100 ng/ml nocodazole(Sigma). RO3306 synchronization was performed by treating HeLa cells for 20 hours with 9mM RO3306 (Enzo). proTAME (Boston Biochem) was used at 10 to 20 μM. Taxol (Sigma) was used at 100nM unless indicated. MG132 was purchased from Sigma and used for 4-hour treatments. Other drugs that were used were: Chloroquine , Baffylomycin, ammonium chloride, rapamycin(200nM) and Hippuristanol (Dr. Jerry Pelletier). Treatment of cell extracts with λ phosphatase (New England BioLabs) was performed by adding 50 μg of total protein extract to the 1×phosphatase buffer (supplied by manufacturer) supplemented with 2 mM $MnCl_2$ and incubated with 10 U of λ phosphatase at 30°C for 30 minutes. The reactions were stopped by the addition of 1x Laemmli sample buffer.

Plasmids and Cloning

pRK5-Myc-empty vector was purchased from Clontech. The following vectors were purchased from Addgene : Raptor wt (Plasmid #1859), myc-Raptor S722A/S792A (Plasmid #18118), pcDNA3-Flag mTOR wt (Plasmid #26603), myc-mTOR (Plasmid #1861), pRK5 Flag PRAS40 (Plasmid #14950), pRK5-myc-PRAS40 (Plasmid #15476). Raptor point mutations were generated using site-directed mutagenesis. To generate the EGFP-Raptor fusion protein, Raptor was sub-cloned into pEGFPC1 (Clontech). All constructs were verified by restriction digest analysis and DNA sequencing.

Extract Preparation and Immuno-precipitation

Cell extracts were prepared by lysing cells in Lysis Buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM β-

glycerophosphate, 1 mM PMSF and protease inhibitor (Roche)), followed by protein quantification using the Bradford assay (Bio-rad). For Flag-mTOR, Flag PRAS40 or Flag-Raptor immunoprecipitations, transfected HeLa or Hela cells were lysed in 1 ml IP Lysis buffer (40 mM HEPES pH7.4, 100mM NaCl, 0.3% CHAPS, 2mM EDTA, 10 mM Sodium pyrophosphate and protease inhibitor (Roche)) per 10^7 cells on ice for 20 min. Cell debris was pelleted by centrifugation at maximum speed for 15 min at 4°C. The supernatant was then incubated with 30 µl anti-Flag (Sigma) for 2 h at 4°C. The beads were then pelleted and washed 4 times with the same buffer. Following the washes, the beads were pelleted and resuspended in 1× Laemmli sample buffer, boiled for 5 min, and stored at −20°C until further use.

Antibodies and Blotting

Mouse monclonal antibodies to the following proteins were purchased from the indicated manufacturers and used for immunoblotting according to standard protocols: cyclin B1, cyclin A2 (Santa Cruz Biotechnology), Cdc27 (BD Biosciences), EGFP (Clontech), anti-Flag M2, (Sigma), anti-myc (Bioshop), Cdk1, ribosomal S6(Cell Signaling), Bcl2 (Zymed). The following rabbit polyclonal antibodies were purchased from the indicated maufacturers: Raptor (Millipore), b-actin (Sigma),p70-S6K(SC), phospho-4EBP1Thr37/46, Phospho-S6K1-Thr389, Geminin, P-Ser2448 mTOR, eIF4B, P-Ser240/244 rPS6(Cell Signalling). Rabbit monoclonal antibodies were from Cell Signaling (mTOR,4EBP1,TSC2,PRAS40 and PDCD4). Western blotting was performed using standard protocols for SDS-PAGE and wet transfer for at least 24 hours at 30V onto nitrocellulose membranes (Bio-Rad). Conditions for Western blots were the use of 5% nonfat dry milk in TBS-T (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Tween 20). The bands were visualized by enhanced chemiluminescence (Western Lightning [PerkinElmer] or SuperSignal™ West Femto [ThermoFisher Scientific]) and exposure on film.

Gel Filtration Chromatography

About 4.0 x10^7 HeLa cells were lysed with 0.3 ml of lysis buffer on ice for 20 min. After centrifugation at 13,000 g for 10 min, the supernatant was passed through a 0.22-m filter (Millipore Corp., Bedford, MA). About 1.5–2.0 mg of proteins in 0.3-ml volume were loaded onto a Superose 6 HR10/30 column (GE HealthCare) pre-equilibrated with lysis buffer. The proteins were eluted at 0.2 ml/min, and 0.5-ml fractions were collected. Sixteen microliters of each were then analyzed by immunoblotting with the indicated antibodies.

siRNA transfection

150,000 HeLa cells were seeded in 6-well plate and then transfected overnight using 5µl Lipofectamine 2000 (Invitrogen-Life Technologies) per well and 50 nM siRNA in Opti-MEM reduced-serum medium (Invitrogen-Life Technologies). All siRNAs were purchased from Sigma. The cells were allowed to recover and then treated as indicated.

PDCD4_505 5'CAC CAA UCA UAC AGG AAU A dTdT3' [363]

PDCD4_1260- 5'CAU UCA UAC UCU GUG CUG G dTdT3' [364]

Non-silencing 5’-AAT TCT CCG AAC GTG TCA CGT dTdT-3’ (Qiagen)

[35S]methionine-cysteine pulse-chase

HeLa cells were transfected and synchronized as above. 15 hours post Nocodazole floating cell were collected by shake off, cells pelleted and resupsended in DMEM without methionine and cysteine and supplemented with 10% dialyzed serum (both from Gibco) for 2 h and 100ng/ml nocodazole and replated. Cells were then labeled with a 10 µCi/ml mixture of [35S]methionine-cysteine (Amersham) for 10 min. Cell lysis was performed as previously described, and 10µl of supernatant was precipitated by trichloroacetic acid (TCA) on a filter paper. Filter papers were soaked in scintillation fluid, and radioactivity was measured using a scintillation counter [365].

Real Time Quantitative PCR analysis

Total RNAs were extracted from HeLa cells using Trizole protocol.1ug RNA was reverse-transcribed to cDNA (QuantiTect Reverse Transcription Kit, Qiagen). Primers used are:

Raptor-Fwd:5'GCCTGCTGTACATAGTGAAGCT 3',

Raptor-Rev:5'TGGATGCTGGTGCTCAGTGGG3',

18S-Frd 5'GTAACCCGTTGAACCCCATT3' and

18S-Rev 5' CCATCCAATCGGTAGTAGCG 3'.

QRTPCR analysis was performed on the Eppendorf Realplex using the QuantiTect SYBR Green (Qiagen). Gene expression analysis was determined using the delta CT method and normalized to 18S.

Time-Lapse Microscopy

100,000 HeLa cells were seeded on 6-well plates and then either transfected or treated with drugs as indicated. Synchronization was performed as described above. Following the addition of Taxol or Hippuristanol, the cells were placed in an incubation chamber on the microscope to maintain temperature and CO_2 levels. Images were taken every 10 minutes at 10X total magnification. For analysis, 100 cells were followed for each condition and the outcome of mitosis recorded.

3.6 Figure Legends & Figures

Figure 3.1: mTORC1 activity is decreased during mitotic arrest

A- Immunoblot analysis HeLa cells synchronized using Thymidine followed by release into Nocodazole. Whole cell extracts were analyzed for expression of Raptor and signalling pathway targets downstream mTORC1, like phospho-Thr389 and phospho-4E-BP1, following release from Thymidine into Nocodazole at the indicated times. Anti-Cdc27 and anti-Geminin are included as a marker for onset of mitosis and b-Actin as a loading control. **B-** Immunoblot of HaCat cells that were either left unsynchronized (Asyn) or synchronized using Thymidine-Nocodazole protocol. 15 hours post-Nocodazole mitotic cells were collected by shake-off (Floating) or scraped from their plate (Adherent). **C-** Immunoblot analysis of different cell lines that were either left asynchronous (A) or synchronized with Thymidine/Nocodazole protocol (N). Floating mitotic cells were collected by shake-off. **D-** Immunoblot of HeLa cells synchronized as in **C**. Two hours before harvesting cells were treated with Okadic acid (OA). Phospho-Ser2448 mTOR and eIF4B were used as controls for the phosphatase treatment. **E-** Immunoblot analysis of HeLa cells synchronized using Thymidine-Nocodazole protocol then Nocodazole was washed of mitotic cells and released into fresh media and followed at the indicated times. Cyclin B1 was used as a marker for mitotic progression and exit. **F-** HeLa cells were synchronized with Thymidine for 20 hours, and then released into media with RO3306 (CDK1 inhibitor), pro-TAME (an APC/C inhibitor), Taxol, and Nocodazole for 12 hours. Anti-Cyclin A2 was used as a control for pro-TAME.

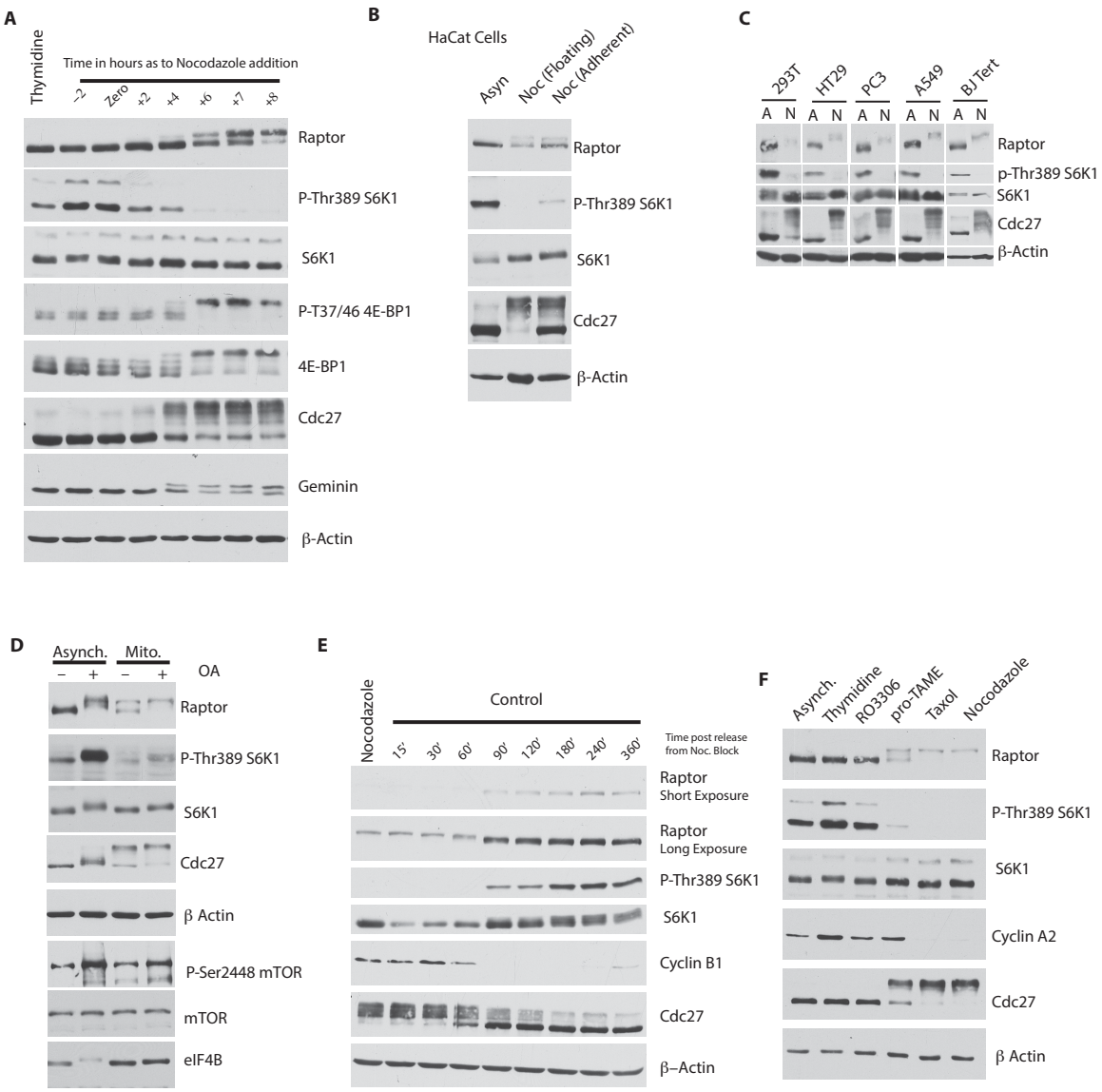


Figure 3.1: mTORC1 activity is decreased during mitotic arrest

Figure 3.2: Raptor protein is downregulated during mitotic arrest

A- Immunoblot analysis of HeLa cells synchronized with the cdk1 inhibitor RO3306 and released for the indicated length of time in vehicle control (DMSO) or in Nocodazole . Cyclin B1 was used as a marker for mitotic exit. Phosphorylated Cdc27 and BubR1 were used as markers for mitosis. **B-** HeLa cells were either left untreated (Asyn) or treated with RO3306 for 19 hours (G2) and then released into fresh media containg Nocodazole to trap them in mitosis (M). One set of all plates was treated with MG132 for 3hours. immunoblot analysis for expression of Wee1 and cyclin A2 known to be degraded during mitosis were used as controls. **C.** Immunoblot analysis of asynchronous or Thym/Noc synchronised HeLa then treated with vehicle control or the pan-caspase inhibitor zVad-FMK for the last 12 hours of treatment. **D.** HeLa cells were treated as in C, but then released into fresh media containg Nocodazole plus vehicle control or three different lysosome inhibitors: cloroquine (CQ), Baffylomycin A (Baff A), and ammonium chloride (NH_4Cl). **E-** WT and TSC2 Null MEFS were synchronized as in A except that they were released in 100nM Taxol and harvested by shake-off 60' after release. **F-**HeLa cells were transfected with pRK5-myc empty vector or Raptor WT and synchronized as in C.

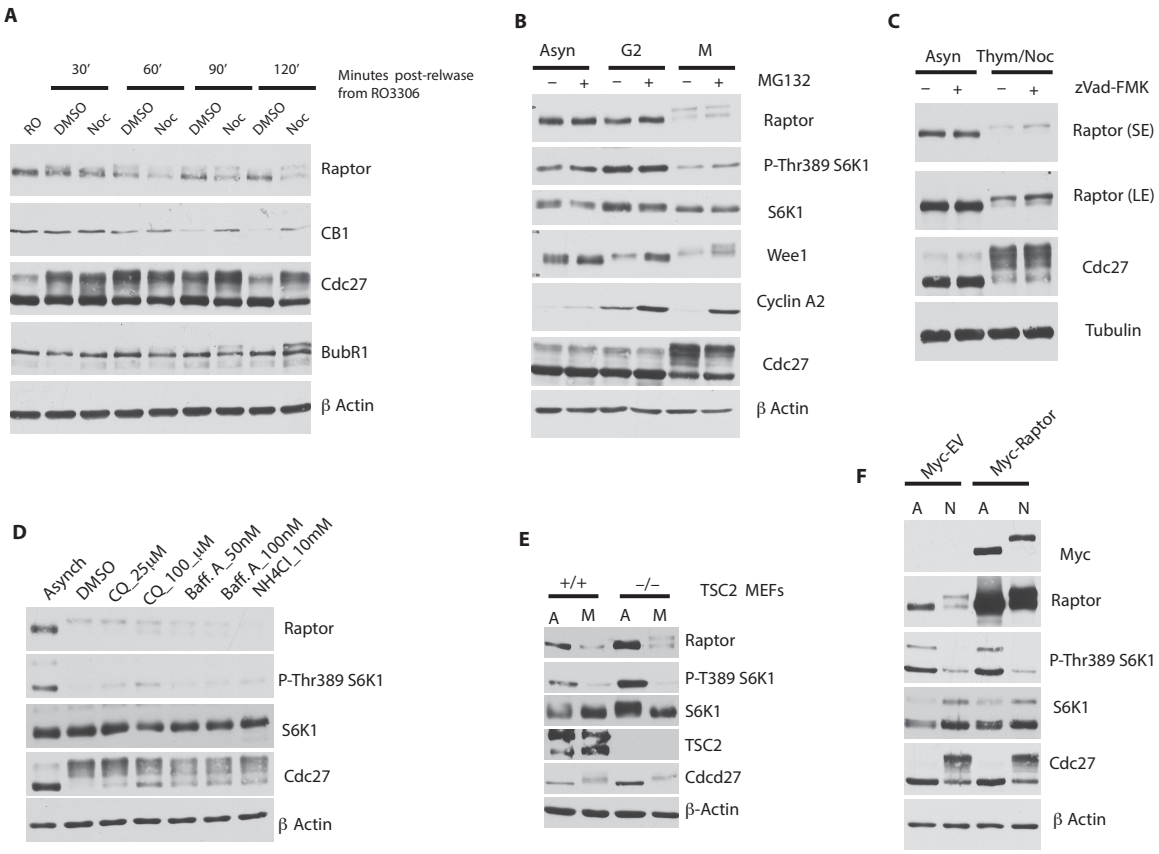


Figure 3.2: Raptor protein is downregulated during mitotic arrest

3.3: Raptor phosphorylation regulates mTORC1 dimerization.

A, B, and C- HeLa cells were co-transfected with Flag and Myc-tagged mTOR (A), Flag and Myc-tagged PRAS40 (B), and Flag and Myc-tagged Raptor (C). Cells were synchronized as in Figure 1, cell lysates were prepared and immune-precipitation (IP) was performed using anti-Flag antibody followed by Immunoblot analysis. **D-** HeLa cells were synchronized as in A. Lysates from asynchronous (Asyn), or Thym/Noc synchronized (Mito) were fractionated on a Superose 6 HR 10/30 column. Fractions were analyzed by immunoblotting with the indicated antibodies. The elution positions of molecular mass markers are shown on the top.

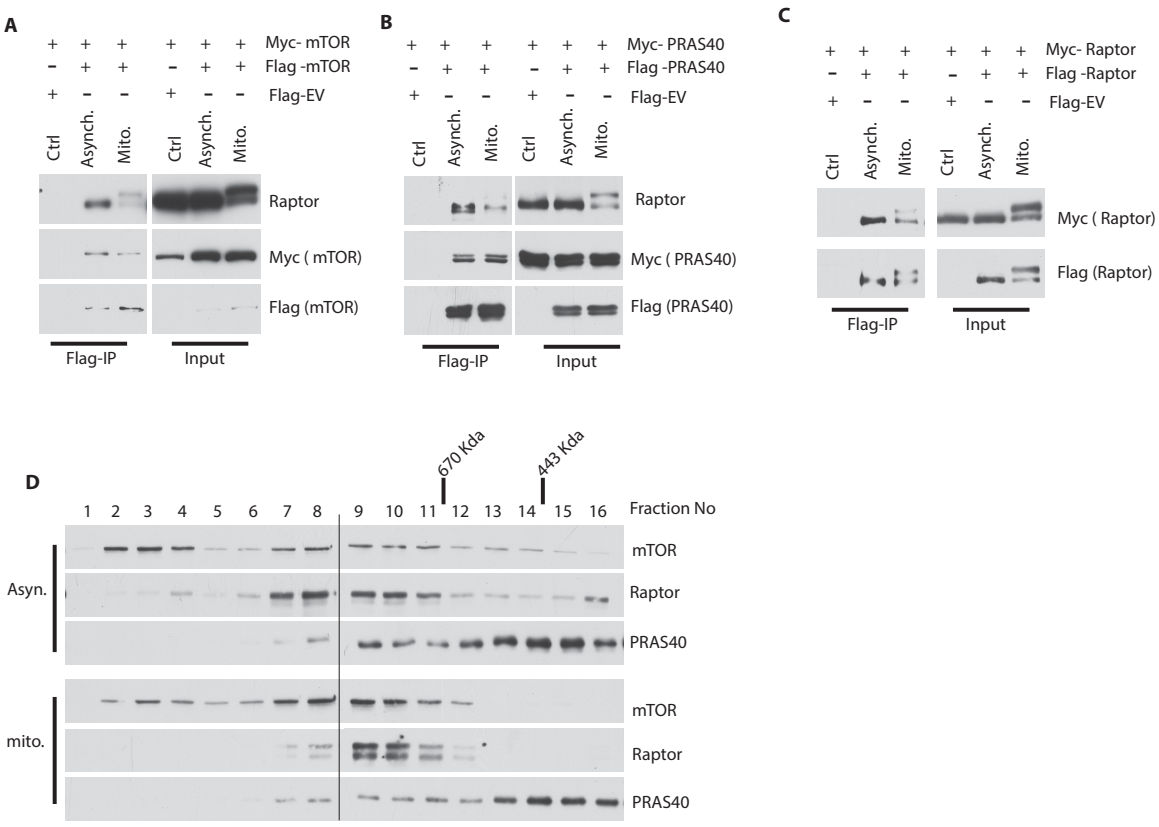


Figure 3.3: Raptor phosphorylation regulates mTORC1 dimerization.

Figure 3.4: Mutation of raptor phosphorylation sites activates the mTORC1 kinase in mitosis

A- Diagram of Raptor's protein sequence showing the mitosis-specific phosphorylation sites. **B-** Immunoblot analysis of HeLa cells that were transfected with different myc-raptor's point mutants and were either left unsynchronized (A)or synchronized with Thymidine/Nocodazole protocol (N). Floating mitotic cells were collected by shake-off. **C-** HeLa cells were transfected with either Raptor WT or 8A mutant, synchronized as in B, and then treated with 200nM Rapamycin for 3 hours to inhibit the mTORC1 activity. **D-** HeLa cells were co-transfected with Flag mTOR (A), Flag and EGFP-Raptor WT or 8A mutant and synchronized as in B. Cell lysates were prepared and immune-precipitation (IP) was performed using anti-Flag antibody followed by Immunoblot analysis.

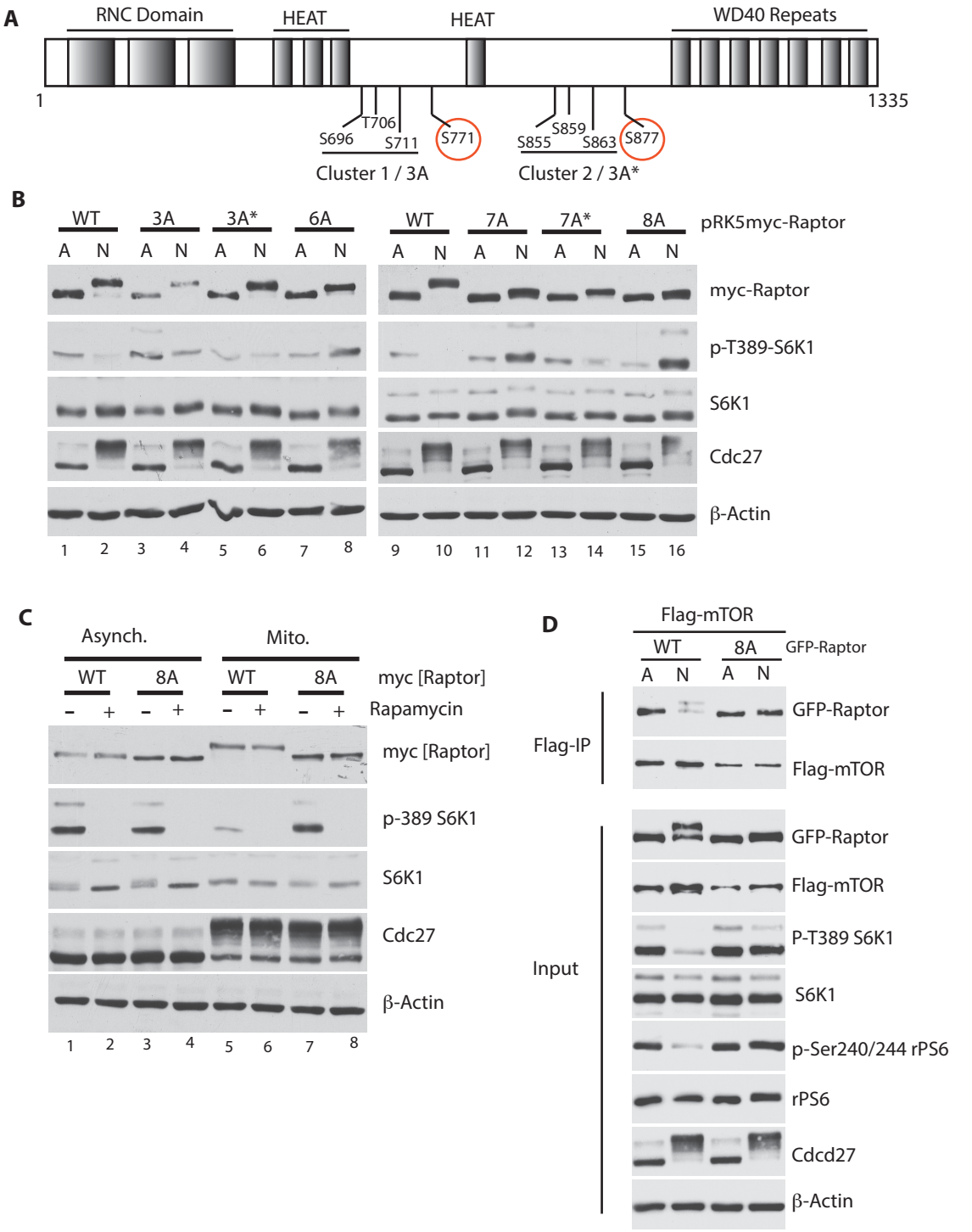


Figure 3.4: Mutation of raptor phosphorylation sites activates the mTORC1 kinase in mitosis

Figure 3.5: Raptor 8A mutant promotes survival in response to Taxol

A- HeLa cells were transfected in triplicates with either pRK5- myc raptor WT or 8A mutant and synchronized with Thym/Noc protocol then labeled with [S^{35}] methionine and the specific activity of incorporation into equal amounts of protein was determined by trichloroacetic acid (TCA) precipitation and scintillation counting. **B-** Immunoblot analysis of HeLa cells that were transfected with pRK5- myc raptor WT or 8A synchronized with Thym/Noc protocol. Nocodazole arrested cells were collected and released into fresh media. Degradation of cyclin B1, and phosphorylation of cdc27 are used as marker for exit from mitosis. **C-** The length of one whole cell cycle (between two mitosis) was measured for 50 cells (transfected with, pRK5- myc raptor WT, or 8A) using time-lapse microscopy. **D.** HeLa cells were transfected overnight with either EGFP-empty vector or Raptor WT or 8A mutant. Twenty-four hours following transfection, cells were synchronized by RO3306. After 20 hours cells were washed twice with PBS and released into fresh media containing either 100 nM Taxol (Top panel) or 100 nM Taxol + 200nM Rapamycin, and time-lapse imaging started. The length of time spent by 100 cells from mitotic entry until death was plotted.

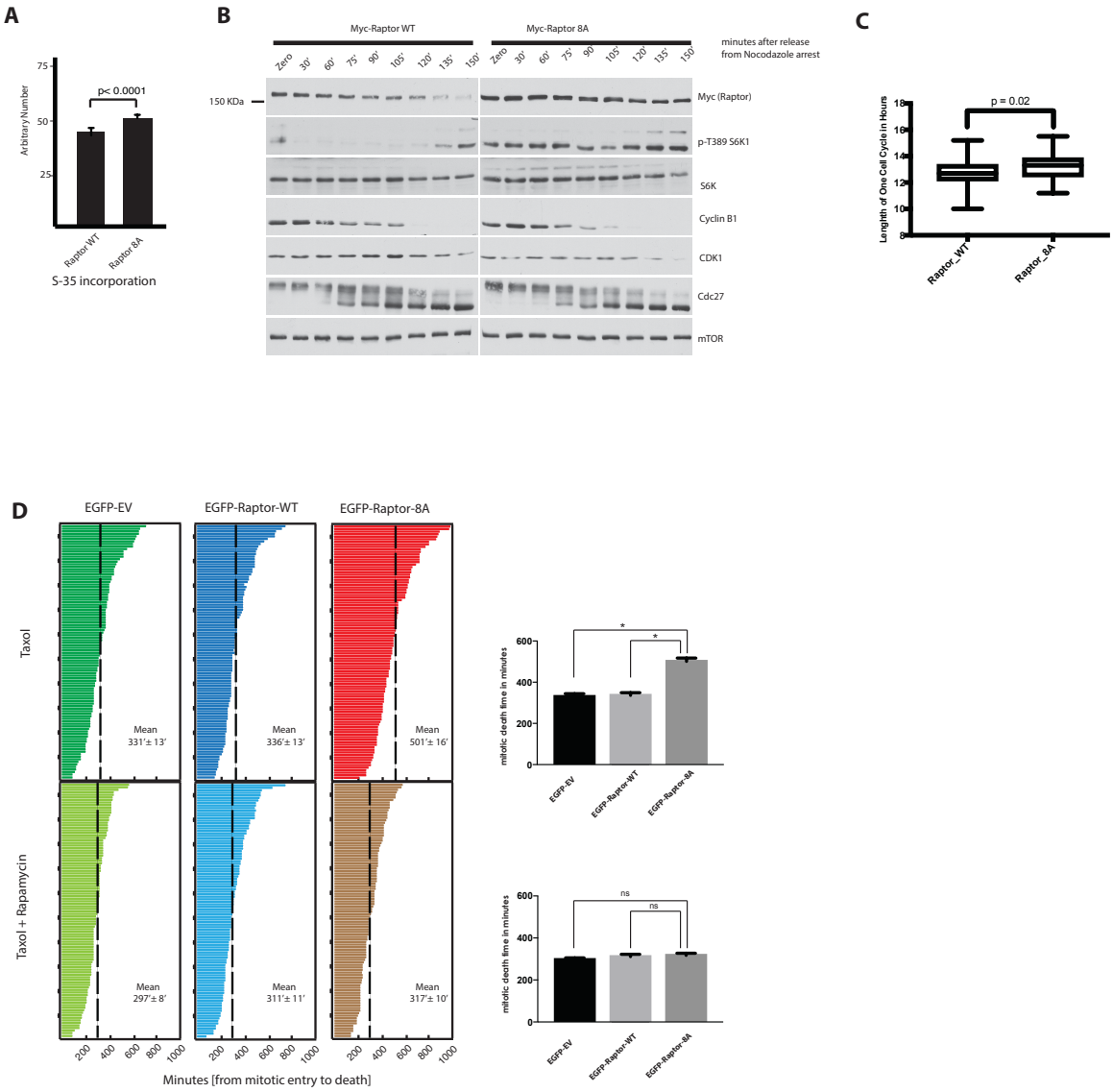


Figure 3.5: Raptor 8A mutant promotes survival in response to Taxol

Figure 3.6: PDCD4 and eIf4A axis mediates the survival signaling downstream of the Raptor 8A mutant in response to Taxol

A- Immunoblot analysis of HeLa cells transfected with either EGFP-empty vector or Raptor WT or 8A mutant. Twenty-four hours following transfection, cells were synchronized using Thym/Noc protocol. Cells harvested after 15 hours and lysates immunoblotted for the indicated antibodies. **B-** HeLa cells were transfected overnight with 50 nM siRNAs as indicated. Twenty-four hours following transfection, cells were synchronized by RO3306. After 20 hours cells were washed twice with PBS and released into fresh media containing either 100nM Taxol, and time-lapse imaging started. The length of time spent by 100 cells from mitotic entry until death was plotted. The knockdown was confirmed by immunoblotting using PDCD4 antibody. **C.** HeLa cells were transfected overnight with either EGFP-empty vector or Raptor WT or 8A mutant. Twenty-four hours following transfection, cells were synchronized by RO3306. After 20 hours cells were washed twice with PBS and released into fresh media containing either 100 nM Taxol (Top panel) or 100 nM Taxol + 20nM Hippuristanol, and time-lapse imaging started. The length of time spent by 100 cells from mitotic entry until death was plotted.

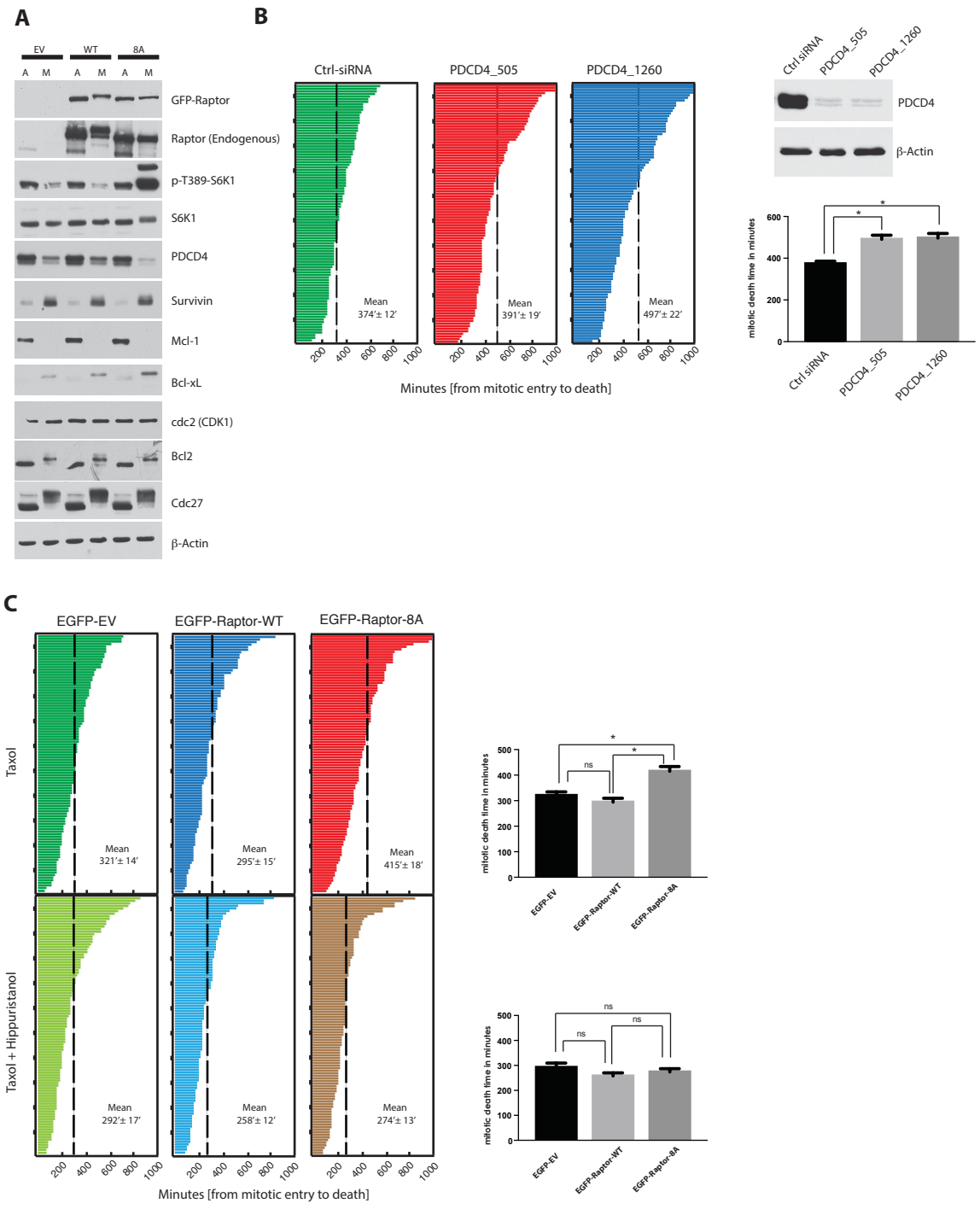


Figure 3.6: PDCD4 and Bcl-xL mediate the survival signaling downstream of the Raptor 8A mutant in response to Taxol

Figure 3.7: The role of CDK1-mTOR-S6K1-PDCD4-eIF4A axis in regulating the death vs. slippage decision upon prolonged mitotic arrest

A- HeLa cells were synchronized by RO3306. After 20 hours cells were washed twice with PBS and released into fresh media containing either 100 nM Taxol (Top panel) or 100 nM Taxol plus increasing concentrations of Hippuristanol as indicated, and time-lapse imaging started. The length of time spent by 100 cells from mitotic entry until death was plotted (left panel). A box plot comparing the range and the mean death time for all of the treatment is shown to the left. **B.** HeLa cells were synchronized as in A but then released into either Hippuristanol (200nM), Taxol (10nM), or a combination of both drugs and then time-lapse analysis started as in A. C. H1299 cells (top panel) and MCF-7 cell (bottom panel) were synchronized and treated as in B. Time lapse imaging was then started. The fate of 100 cells is shown. **D-** A proposed model explaining the change we have observed in death time after a prolonged mitotic arrest under different conditions of mTORC1 activity or PDCD4 protein level or eIF4A activity.

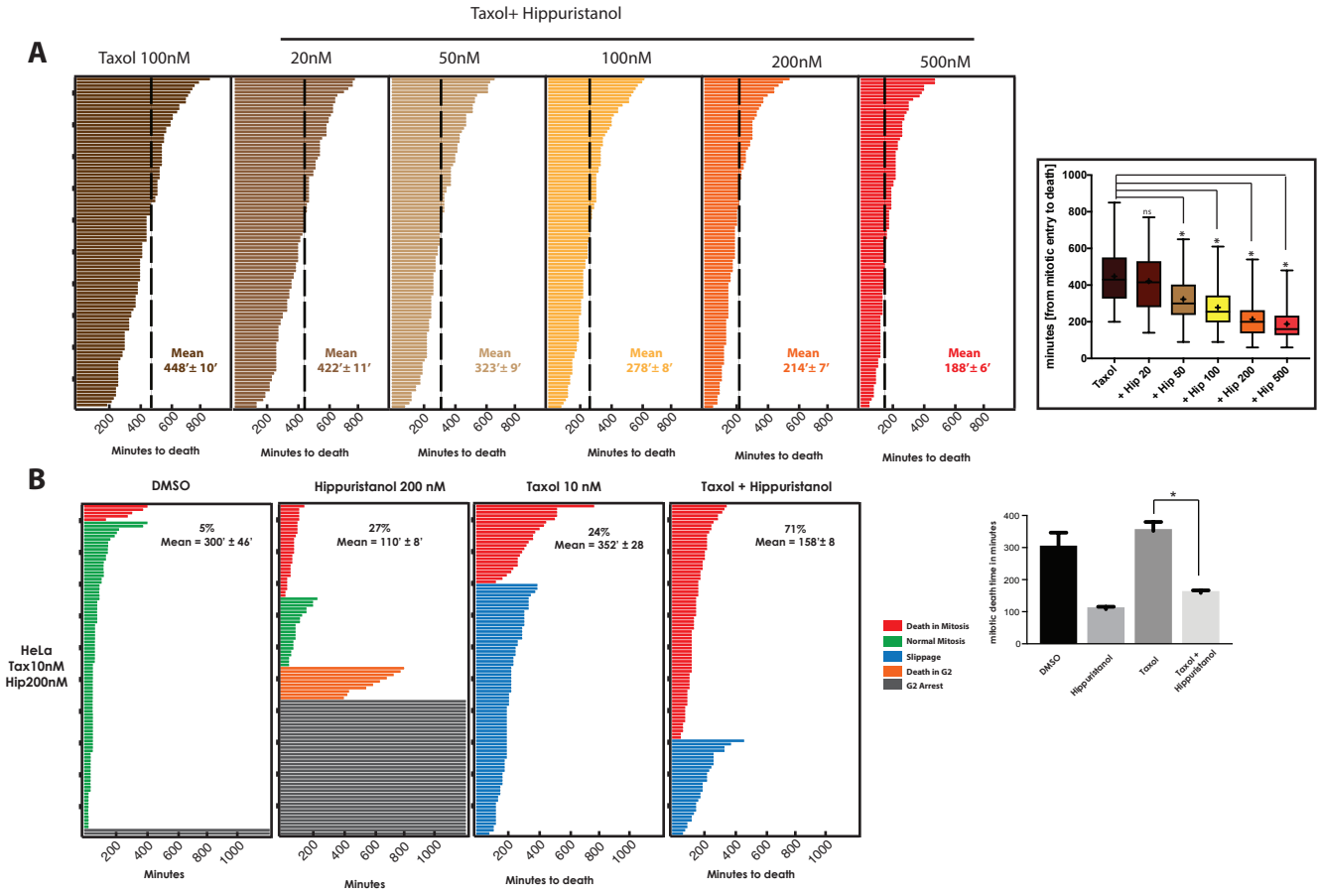


Figure 3.7: The role of CDK1-mTOR-S6K1-PDCD4-eIF4A axis in regulating the death vs. slippage decision upon prolonged mitotic arrest

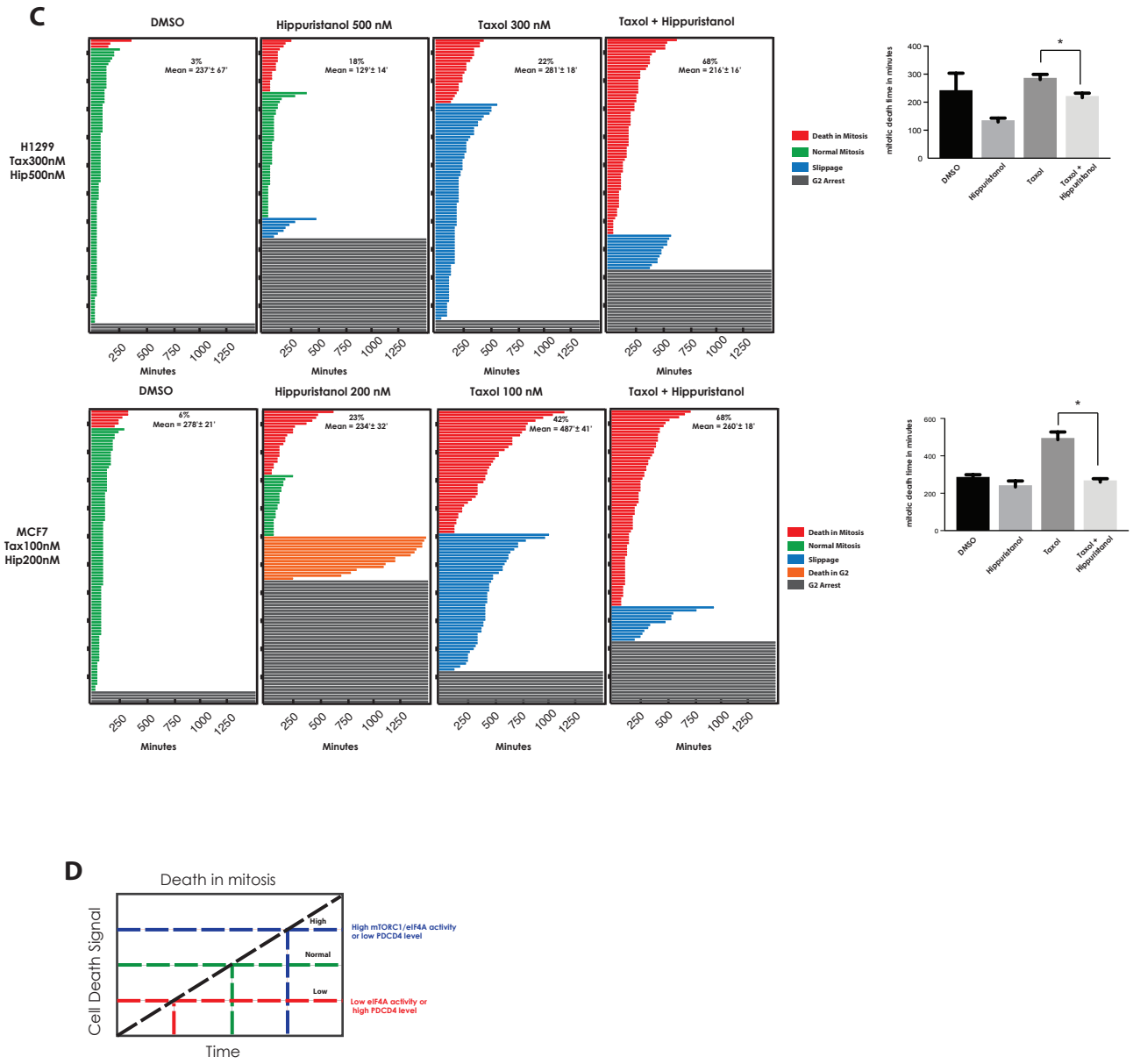
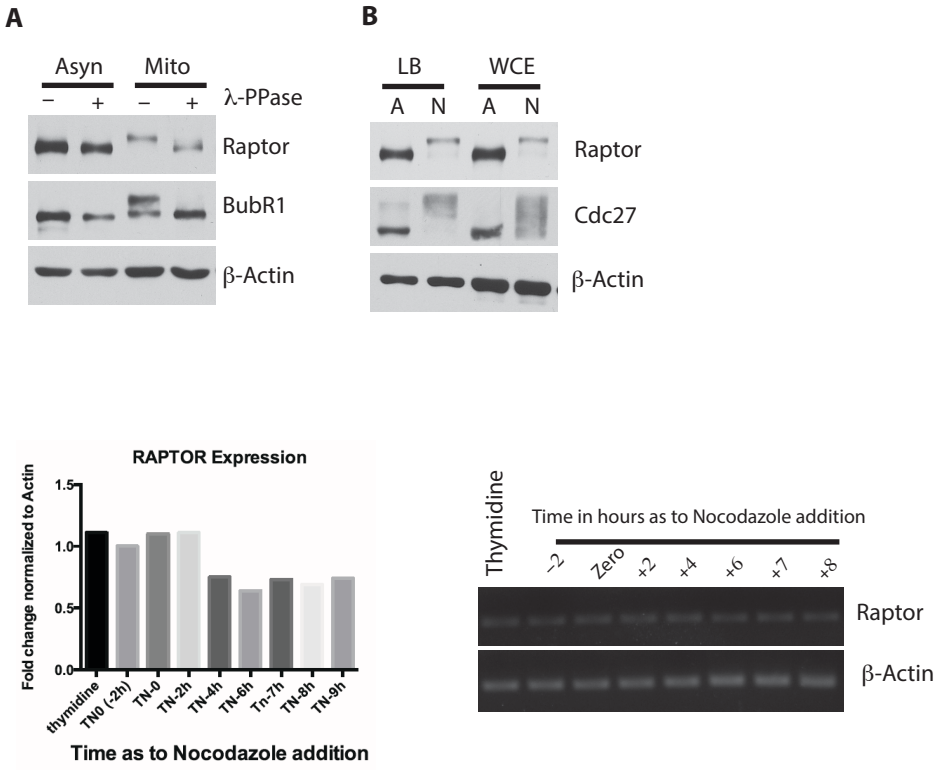


Figure 3.7 (Cont'd): The role of CDK1-mTOR-S6K1-PDCD4-eIF4A axis in regulating the death vs. slippage decision upon prolonged mitotic arrest

3.7. Supplementary Figures

Supplementary figure 3.1

A- Immunoblot analysis of endogenous Raptor in whole cell extracts treated with λ phosphatase (+) or control (-) prepared from asynchronous or Thymidine/ Nocodazole mitotic extracts. BubR1 phosphorylation was used as a control for the phosphatase. **B-** Immunoblot of HeLa cells that were either left unsynchronized (Asyn) or synchronized using Thymidine-Nocodazole protocol. 15 hours post-Nocodazole, mitotic cells were collected by shake-off and asynchronous cells by scraping. Cell pellet was either lysed in lysis buffer or whole cell extract was prepared in Laemmli buffer. **C.** Expression analysis of raptor's mRNA level in HeLa cells under Thymidine or released from thymidine into Nocodazole and Harvested at different time points. **D-**Immunoblot analysis of HeLa cells that were transfected with different myc-raptor's point mutants and were either left unsynchronized (A)or synchronized with Thymidine/Nocodazole protocol (N).



Supplementary figure 3.1

Chapter 4
General Discussion

4.1. Summary of findings

The primary goal of the research presented here is to gain a better understanding of the mitotic regulation of two important regulatory mechanisms of death and survival signals: the pro-apoptotic BH3 only protein Bim and the prosurvival mTORC1 kinase complex. The secondary goal is to use this knowledge to find a better way of targeting cancer cells based on the premise that mitotic targets are unique to dividing cells and that cancer cells are rapidly dividing.

In chapter 2 we demonstrated that during both normal mitosis and prolonged mitotic arrest the longer isoform of Bim, BimEL, is phosphorylated and targeted for proteasomal degradation. We identified the oncogene Aurora A kinase as the relevant kinase and SCF-βTrCP, another oncogene, as the E3 ligase. We show that upon mitotic entry BimEL is phosphorylated on at least two sites, S69 and S93/94/98; the later is necessary and sufficient to mediate BimEL degradation. Upon mitotic exit, normal or induced, BimEL is dephosphorylated and its level starts to build up. In addition, we found that, using pharmacological and genetic tools that BimEL phosphorylation on S93/94/98 (and hence degradation) are counteracted by protein phosphatase 2A (PP2A). Using a drug that is a selective Aurora-A inhibitor, we can rescue BimEL levels and we proved that the apoptotic response downstream of this drug is at least partially dependent on BimEL as a BH3-only activator of apoptosis.

In chapter 3 we unraveled a new mechanism by which prolonged mitotic arrest impinge on the translation machinery. We showed that the activity of the mTORC1 complex towards S6K1, a critical activator of cap-dependent translation, is completely diminished in response to mitotic poisons like nocodazole and Taxol. The mitotic phosphorylation of raptor, the substrate recognition subunit, is the reason behind this reduced activity. We showed that raptor's phosphorylation displaces it from the complex and may

affect the active dimeric state of the complex. We identified a raptor phospho-deficient mutant, raptor-8A, that is capable of rescuing the activity of mTORC1 towards S6K1 and induce the degradation of PDCD4, an inhibitor eIf4A, ultimately leading to enhancement of translation and extension of the survival signal in response to Taxol. Finally, we showed that cells stressed with mitotic poisons like Taxol maintain an essential level of eIF4A activity, which once nullified (by the use of a specific inhibitor like Hippuristanol), speeds up the death timer and in cells prone to undergo slippage without death, we observed a reversal of the phenotype towards death.

4.2. Significance of findings: degradation of Bim during mitosis and Taxol

The finding that Bim is degraded in mitosis was surprising since it was long thought that Bim level correlates with Taxol induced cell death like in lung, breast and prostate cancer cell lines [366]. In baby mouse kidney cells, Taxol was found to cause an increase in Bim protein levels that could be counteracted by H-ras expression. In cells that show resistance to Taxol due to H-ras overexpression, restoring Bim levels with the proteasome inhibitor Bortezomib, abrogates the resistance and promote Bim-dependent tumour regression in vivo [178]. If Bim doesn't mediate Taxol-induced death in mitosis, so when does this happen during the cell cycle? Two possible observations can explain this paradox. Firstly, recent observations using time-lapse analysis of single cell fate showed that death in mitosis is not the only fate in response to Taxol. Cells showed both interline and intraline variation in response to Taxol and other anti-mitotics. For example, in response to 100nM Taxol, 73% of HeLa cells exited a prolonged mitotic arrest and then died in the following interphase in contrast to RKO cells (colon cancer cell line), which died exclusively in mitosis. The fate profiles of cells can change dramatically depending on the drug concentrations. In H1703 (Lung carcinoma) cells, low dose of Taxol (100nM) kills almost all cells in mitosis while high dose (10mM) causes mitotic exit [65]. Secondly, in MCF7 breast cancer cell line, Taxol was shown to induce Bim transcriptionally

through the FoxO3a transcriptional factor[367]. So we may conclude that the role of Bim in mediating Taxol-induced death is not necessarily in mitosis. Death can happen in interphase especially in a cell line with a normal Ras/Raf/ERK pathway. Consequently, we think that Bim can still be used as a marker for Taxol sensitivity though the dynamics of death will differ between cell lines.

4.3. Significance of findings: Phosphorylation of Bim during mitosis and Aurora A kinase inhibitors

Although Taxol and other microtubules targeting agents (MTAs) are effective in many cancers, their use in cancer therapy is limited due to their severe side effects. MTAs affect organelle and protein trafficking in non-dividing cells like neurons, which explain why patients treated with MTAs experience neurotoxicities affecting their sensation, movement and organ function [368]. In addition, since MTAs don't differentiate between malignant and normal cells, they can target rapidly dividing normal cells such as platelets, erythrocytes, and leukocytes that leads to myelosuppression[37, 369]. This lead to the search for a more mitosis specific targets in the hope of reducing the side effects associated with MTAs. Mitotic kinases, such as the Aurora Kinases presented an appealing target since some of these kinases are overexpressed in cancers. For example, Aurora A kinase, function is exclusive to mitosis as it is involved in early preparation for mitosis by regulating centrosome maturation and separation, bipolar spindle assembly, chromosome alignment and cytokinesis [370]. Preclinical studies demonstrated the oncogenic potential of Aurora A hyper-activation which results in *in vitro* and *in vivo* transformation of rodent fibroblasts cells probably due to the formation of multipolar mitotic spindles that leads to genome instability and transformation. Additionally, Aurora A is commonly amplified in many epithelial malignant tumours, including breast, colon, ovarian and pancreatic cancers establishing Aurora-A as bona fide oncogene [371, 372]. The kinase activity of Aurora-A is tightly regulated during the cell cycle. It is activated through the auto-phosphorylation of T288 on its activation loop [373]. Dephosphorylation of T288 by protein phosphatase

1PP1) deactivate it [374]. Importantly, Aurora-A activation and localization to the mitotic spindle depends on the motor binding protein TPX2 (Targeting Protein for Xenopus kinesin-like protein 2), which acts as an allosteric activator of auto-phosphorylation [375]. Since targeting protein-protein interaction proved difficult, inhibiting Aurora-A activity was thus focused on selectively targeting the enzymatic activity of the kinase with small molecules that can occupy the catalytic ATP binding site.

One of the first Aurora-A selective inhibitors to be described is MLN8054. MLN8054 is highly specific to Aurora-A due to its ability to inhibit T288 phosphorylation but at a higher concentration can inactivate Aurora-B. Tumor cells treated with MLN8054 show a high incidence of abnormal mitotic spindles, often with unseparated centrosomes. Although this causes an arrest cells eventually divide and die through apoptosis probably as a result of detrimental aneuploidy [333, 376]. MLN8054-induced apoptosis was shown to be dependent on increasing the expression of p73 and its downstream pro-apoptotic genes, PUMA and NOXA; an advantage in killing p53 null tumor cells[377]. Similar effects were obtained with a related drug MLN8237 (Alisertib), which has more than 200 times selectivity to Aurora-A than Aurora B and less central nervous system side effects. Alisertib used as a single therapy showed tumour regression in preclinical mouse models of neuroblastoma, ALL and lymphoma. Cancer cells with Alisertib undergo mitotic arrest, polyploidy, and die by apoptosis [378, 379]

Using an in vitro kinase assay we showed that Aurora-A is able to phosphorylate BimEL specifically on Ser93/94/98, an event that is lost in BimEL S94/98A mutant and in the presence of MLN8054. Interfering with Aurora-A function using siRNA and MLN8054 in HeLa cells lead to inhibition of BimEL phosphorylation on this site, which consequently lead to inhibition of its interaction with β-TrCP and stabilization of BimEL protein in mitosis. The significance of this BimEL phosphorylation in normal mitosis could be explained by the fact that cell have to deal with a large pool of the pro-apoptotic Bim that is usually stored in the microtubules which undergo a dynamic organization during mitosis. We confirmed previous results that

showed that MLN8054 induces apoptosis in cancer cells using apoptotic markers like Annexin V staining, PARP cleavage and caspase 3 processing. Knocking down Bim with shRNA partially rescues these effects and protects from MLN8054 induced cell death. Interestingly, the correlation between Bim stabilization and cell death induced by Aurora-A inhibition was confirmed in glioblastoma cell lines. Glioblastoma neurosphere tumor stem–like cells that were isolated from a patient were treated with Alisertib. This treatment induced BimEL accumulation and cell death due to apoptosis. Furthermore, alisertib extended median survival of mice having intracranial human glioblastoma neurosphere tumor xenografts [380]. We showed also that high concentration of MLN8054 can indeed force cells to exit mitosis, which is likely due to Aurora-B inhibition. Aurora-B is a component of the chromosome passenger complex (CPC), which contributes to SAC function at kinetochores by correcting incorrect spindle attachments. Inhibition of aurora B activity causes abrogation of the SAC, cytokinesis failure and exit without division [381]. The specificity of MLN8054 toward Aurora-A is the reason why we think it is the relevant kinase for BimEL phosphorylation in mitosis. Taken together we think that Bim stabilization can be used as a marker for Aurora-A inhibition in cells and in tumors treated with MLN8054 or Alisertib.

4.4. Significance of findings: BH3 mimetics in combination with Aurora Kinase inhibitors and MTAs

In vitro and in vivo studies with Aurora kinase inhibitors have pointed to an alarming problem that my affect their long term use. Inhibiting a kinase like Aurora A or B that is required for faithful inheritance of chromosomes present a risk because of the probability of actually assisting tumor evolution and hence heterogeneity in case these agents fail to induce death[43]. The finding that Bim is degraded during prolonged mitotic arrest and that degradation can be rescued by the use of MLN8054 or Alisertib make it a logical step to combine Aurora-A inhibitors with BH3-mimetics. Our results in addition to previous research showed that the general state of the Bcl-2 family in mitosis is as follows: Blc-2 and Bcl-xL (role of phosphorylation is

controversial), Mcl-1 (phosphorylated and degraded), Puma and NOXA (induced downstream p53 or myc), BID (phosphorylated and probably active), BMF (active but weaker than Bim), and Bim (degraded). Since Bim is both an activator and sensitizer BH3-only member, rescuing its level with an Aurora-A inhibitor in the presence of a BH3-mimetic like ABT-263 (inhibits Bcl-xL) or ABT-199 (inhibits Bcl-2) will probably have a strong effect on the death profile. Indeed, this synergy was shown recently to have a lethal effect in treating neuroblastomas with amplification in the *MYCN* gene[382]. Building on the sensitivity of these cells to ABT-199 due to high expression of NOXA as result of MYCN amplification, adding MLN8237 (Alisertib) to the treatment regimen resulted in a synthetic lethality. The combined effect of mitotic arrest, which leads to accumulation of Bim, and the neutralizing effect of ABT-199 on the excess Bcl-2 in these cells gave the chance to Bim to exert its full action in activating Bax directly leading to induction of the mitochondrial death pathway. In different models of MYCN-amplified neuroblastoma, including a patient-derived xenograft model, this combination induced tumor shrinkage, and in some cases resulted in a complete tumor regression[382]. Combining Alisertib with others MTAs like Taxol (in patients with recurrent ovarian cancer) or with Docetaxel (castration-resistant prostate cancer) showed promising activity [383]. In the same way combining ABT-263 (Navitoclax) with Taxol have proven to be lethal in HeLa, U2OS, A549 and OVCAR-5 cancer cell lines. In this regimen the variation in response between cell lines was attributed to the difference in expression levels of Mcl-1 and Bcl-xL which, as we will discuss later, affect the priming state of the cell towards mitochondrial death [204].

4.5. Significance of findings: Mitotic phosphorylation of Raptor and its effect on the mTORC1 kinase activity

mTORC1 positively regulates key cellular processes such as growth, proliferation, mRNA translation and autophagy. Over activation of mTORC1 activity thus represents an asset for tumor cells characterized by faster growth and proliferation rates and the ability to maintain survival under

stress conditions. It is thus logical to think that tumor cells will maintain a high level of mTORC1 activity during the whole cell cycle. However, we observed a decrease in its activity during prolonged mitotic arrest. This decrease seems to be an essential part of the response of any cell to this kind of stress since it occurs both in transformed and non-transformed cells. These results seemed to contradict previous results showing no change or increase in activity during mitotic arrest and we think the reason behind this is a technical reason concerning the synchronization protocol. We don't believe that mTORC1 is involved in any activity promoting cap-independent translation in mitosis. Rather, this regulation of mTORC1 activity is a kind of fine-tuning of the core translation machinery activity during prolonged mitotic arrest or we propose it may represent a special kind of a "stress response".

In response to stress stimuli like hypoxia, viral infection, ER stress or nutrient deprivation, global translation is usually reduced. One of the most sensitive steps of translation is the initiation step where most of the regulation happens. To form the 43S preintiation complex three complexes must come together. The first is the 40S subunit associated with eIF1A and eIF3, the second is composed of the mRNA (to be translated) with its m^7G cap recognized by the eIF4F (regulated by mTORC1), eIF4B and eIF4H. The last complex is the ternary complex, which consists of eIF2–GTP and Met-tRNAi ^{Met}and is required for the loading of Met-tRNAi ^{Met}. Many different types of stress decrease global translation by triggering the phosphorylation of the **α**-subunit of eIF2 on Ser51. This phosphorylation inhibits the exchange of GDP for GTP on the eIF2 complex, and thus prevents the formation of the ternary complex [384, 385]. The phosphorylation of eIF2α is mediated by four distinct protein kinases and one of these, protein kinase RNA (PKR), has been shown to be active in mitosis. Nuclear envelope breakdown at the beginning of mitosis release Inverted Alu repeats (IRAlus) to form dsRNAs and activates PKR, which in turn phosphorylate and inactivate eIF2α. Additionally, eIF2B, which catalyzes the assembly of the 43S pre-initiation

complex, is itself inhibited in mitosis by phosphorylation and binding to 14-3-3 s [291].

The role of mTORC1 in enhancing translation as discussed above is through the binding of the cap complex to the 5'end cap structure. This happens by phosphorylating two critical targets; 4EBP-1 and S6K1. We observed that raptor phosphorylation is the critical step in regulating the mTORC1 activity in mitosis. Based on the reported phosphorylation sites on raptor, we created a phospho-deficient raptor mutant, raptor-8A that is capable of rescuing the mTORC1 activity at least towards S6K1. One of the ways that S6K1 activates translation is through phosphorylating PDCD4, an inhibitor of the eI4A activity[268]. This phosphorylation creates a binding site for the E3 ligase SCF-βTrCP1 and leads to PDCD4 degradation [269]. Indeed, we show that in mitosis, raptor 8A mutant is capable of wiping out any remaining PDCD4 thus relieving an inhibitory arm on eIF4A. Through this axis, mTORC1-S6K1-PDCD4-eIF4A, we show that this mutant can, extend the survival time in response to the death signal induced by Taxol. Interfering with the function of this pathway using Rapamycin (to inhibit mTORC1) or Hippuristanol (to inhibit eIF4A) completely reverses this phenotype. In order to explain these observations we need to answer two questions; firstly, how is raptor-8A activating translation under these conditions and secondly, how is this related to enhancement of survival in mitosis.

First, we showed that the raptor-8A mutant moderately enhances translation (around 17% increase), which seems just enough to maintain a survival signal in response to Taxol but still for a limited time. This is understandable especially in the background of inhibited cap-dependent translation mainly due to mitotic phosphorylation of different core components of the translation machinery[386]. Despite the fact that phosphorylation of eIF2α negatively affects general translation, it is not well-established that eIF2α-independent translation can occur. For example, translation of the inhibitor of apoptosis protein XIAP was shown to be unaffected by the inhibition of global translation after serum deprivation[387]

or glucose deficiency [388]. Other examples include c-myc during ER stress [389]and Bcl-xL during hypertonic stress [390]. The translation of these mRNAs do not depend on the recognition of a cap structure or the recruitment of the ternary complex, but instead on the direct recruitment of the 40S ribosome to an on Internal ribosome entry site (IRES) within the 5'UTR. IRESs were first described in viruses but then many cellular IRESs were later discovered. The recruitment of the 40S to an IRES can occur either in the complete absence of any other protein factors or with the aid of different combinations of classical initiation factors (such as eIF4G and eIF3) and accessory proteins in case of viral IRES [391]. Second, we found that Bcl-xL, an anti-apoptotic protein, is upregulated in mitotic HeLa cells transfected with raptor-8A mutant. We think that the combined effect of an IRES-dependent translation in addition to a boost in the activity of eIF4A, explain the increase observed with Bcl-xL expression level. Bcl-xL has a prominent role in maintaining survival during mitotic arrest. Indeed Bcl-xL depletion speeds up and increases the percentages of cell death in response to Taxol [203]. We believe that the reason why cells eventually die after release from G2/M block directly into Taxol, is that mitotic phosphorylation of Bcl-xL on Ser62 decreases its efficiency in sequestering Bax and protecting cells from Bax-dependent mitotic death [203].

4.6. Significance of findings: mTORC1 activity and Mitochondrial regulation of cell death during mitosis

High mTORC1 activity is known to inhibit catabolic processes like autophagy. The finding that mTORC1 activity is decreased during prolonged mitotic arrest implies that autophagy is at least not inhibited, and may in fact participate in mitotic death as Doménech et al previously reported [360]. They showed that autophagy indeed is not inhibited in mitosis (accumulation of the lipidated and autophagosome associated form of LC3, LC3-II) and they demonstrated that loss of the mitochondria through mitohpagy (a special form of autophagy related to the mitochondria) is crucial to the maintenance of the mitotic state. Mitophagy represents a simple way of removing old

mitochondria. The consequence of this is that a reduced mitochondrial mass during arrest, a reduction in ATP level leading to activation of AMPK and finally replacement of oxidative phosphorylation with glycolosis as an energy source. Interestingly, Morita *et al* showed that mTORC1 stimulates the synthesis of a number of mitochondrial regulators such as TFAM (transcription factor a, mitochondria), mitochondrial ribosomal proteins and components of complex I and V by enhancing the translation of corresponding mRNAs. Inhibition of mTORC1 activity by Torin 1 or PP242 signaling strongly decreases mitochondrial biogenesis and respiration (oxidative phosphorylation)[392]. Collectively, our data shows that inhibition of mTORC1 activity during prolonged mitotic arrest fits into the big picture of reduced translation, reduced mitochondrial fitness, progressive loss of energy and finally death; probably as fail safe mechanism in case a failure of cytokinesis or a faulty separation of chromosomes which may lead to aneuploidy.

4.7. Implication of findings: targeting eIF4A in mitotically arrested cancer cells

As discussed above, the inhibition of mTORC1 activity during mitotic arrest may be to fine-tune the activity of eIF4A. Under these conditions cells still maintain an essential level of translation and seem to be dependent on the activity of eIF4A. Using Hippuristanol, a natural product that inhibits eIF4A [393], we showed a synergy with the antimitotic drug Taxol in HeLa cells. We observed an inverse correlation between the dose of Hippuristanol and the time cells arrest in mitosis before they die. Furthermore, we showed that Hippuristanol is indeed capable of reversing a slippage phenotype (obtained with low dose Taxol) to a death phenotype. These results have two important implications: the first is that eIF4A activity may play a major role in controlling the death network under mitotic arrest and the second is that targeting eIF4A might be the better choice when combined with Taxol or other anti-mitotics.

As previous research has shown, tumor cells exhibit intraline and interline differences in response to anti-mitotics, and we think that differences in the eIF4A activity may explain such effects at least in part. High eIF4A activity lies under the big umbrella of a higher eIF4F activity observed in cancer. Targeting this oncogenic activity could be achieved through different ways like targeting mTORC1 activity, eIF4E, eIF4A or eIF4G. Interestingly, targeting eIF4A has been shown to have a much more profound effect on global translation compared to targeting mTOR (by rapamycin) or eIF4E (by LY2275796)[394]. In vitro and in vivo studies have shown a synergistic effect between Rapamycin and Taxol in rapamycin-sensitive breast cancer cells [395]. Additionally, a phase I clinical trial conducted against resistant HER2-positive metastatic breast cancer patients, treated with Everolimus (Rapamycin) in combination with Paclitaxel (Taxol) plus Trastuzumab (Anti-HER2 antibody) yielded an overall response rate of 44% and disease was controlled for at least 6 months in 74% of patients [396]. Accordingly, we think that combining anti-mitotics like Taxol with an eIF4A inhibitor might have a higher beneficial effect in patients with Taxol-resistant tumours. Indeed, another eIF4A inhibitor, Silvestrol, has shown activity in various pre-clinical cancer models. In one example, B cells derived from patients with chronic lymphocytic (CLL) leukemia are more sensitive to Silvestrol than B cells from healthy volunteers, in addition to lower toxicity towards T cells [397]. This later activity may prove beneficial if we use a combination of Silvestrol with Taxol since one major side effect during Taxol treatment is T cell toxicity and a consequent compromise of the patient immunity.

4.8. Summary & Conclusions and Future directions

Antimitotic drugs are frontline therapies for breast, lung, and ovarian cancer; and various hematological malignancies, but have many limitations due to side effects and the development of resistance. The goal of this research was to investigate the role of death and survival signals in mitosis in

hope of improving the profile of toxicity and the spectrum of tumors treated with these drugs.

The identification of Aurora-A as the major kinase that affects Bim stability during mitotic arrest has a great implication for cancer treatment especially when using combination therapy. Using an Aurora-A inhibitor in combination with other anti-mitotics like Taxol or with BH3-mimemtics like Navitoclax is predicted to tip the balance towards more mitotic death. Unlike Aurora-B, Aurora-A is an oncogene and is a commonly amplified in various epithelial tumors. Aurora-A expression is cell cycle regulated with peak expression in G2/M and degradation in G1-S phases. However, we don't know if in those tumors Aurora-A degradation is compromised and thus its activity extends beyond mitosis. In this case Bim degradation will be both during mitosis and during interphase providing a huge advantage for cancer cells to escape Bim-mediated death stimuli. We established the correlation between Aurora A-mediated BimEL phosphorylation at the phosphodegron (Ser93/94/98) and its stability but we still need to investigate the role of non-degron phosphorylation sites on BimEL function. Some of these sites like Ser104 are common between BimEL and BimL and some are unique to BimEL like Ser69. An important follow up is to check if these sites affect the proapoptotic function of Bim in terms of either neutralizing the Bcl-2 prosurvival family or directly activating Bax/Bak.

The unveiling of the role of raptor phosphorylation in switching off the mTORC1 activity during mitosis has solved the controversy of the true function of mTORC1 during mitotic arrest. Further research is required to define the mechanism behind disappearance of raptor during mitotic arrest, despite the fact that phosphorylation seems to be the more relevant factor in determining mTORC1 activity and the more common event between normal mitosis and prolonged mitotic arrest. Since we observed that eIF4A might be one of the few executioners of translation that still active under conditions of drug-induced mitotic arrest, we propose that inhibiting its activity in the presence of other antimitotic might be a superb strategy to kill cancer cells in mitosis. Since eIF4A activity regulates many of pro-survival signals, we think

that it might be involved in setting the threshold for the death network during mitotic arrest.

One of the greatest challenges in using anti-mitotics to kill cancer cells is the heterogeneity of the response; some cells die quickly in mitosis, some die after a long time, some slip and die in interphase and some slip and survive. Understanding the molecular mechanisms behind these mixed outcomes is essential for choosing the right drug or drug combinations. With our current knowledge we can say that some rules will still hold true in any kind of scenario. Firstly, CyclinB1/CDK1 activity is the endpoint of controlling slippage; no slippage can happen in the absence of cyclin B1 degradation or CDK1 inactivation[398]. Cyclin B1 degradation is regulated by the APC/C activity and CDK1 activity can counteract by phosphatase like PP2A, whose exact role in death/slippage decision is still not clear. Secondly, the mitotic state is dependent on an essential level of translation and whether it is cap-dependent or cap-independent needs further research. Finally, the mitochondria plays a pivotal role in the death/slippage decision as the workhouse of energy production, the residence of the anti-apoptotic proteins and the site where the BH3-only protein exerts their action and initiate apoptosis. It's likely that the oncogene/tumor suppressor balance, with its transcriptional/translational effects, affects the priming state of the mitochondria prior to mitosis, which in addition to the activity of the translation machinery will dictate the decision to either die in mitosis or escape the death peril and die another day.

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