UBIQUITIN AND UBIQUITIN-LIKE PROTEINS IN CELL CYCLE REGULATION

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology in the School of Medicine.

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ABSTRACT

Christine A. Mills: Ubiquitin and Ubiquitin-Like Proteins in Cell Cycle Regulation
(Under the direction of Michael J Emanuele)

Cell cycle is a tightly regulated process; however, it is mis-regulated in many cancers, leading to increased proliferation. Our lab is interested in better understanding cell cycle regulation, in particular, regulation by the ubiquitin system, which controls targeted protein degradation. The following work focuses on the modular ubiquitin E3 ligase composed of SKP1/CUL1/F-box protein (SCF) with its substrate adapter Cyclin F. Cyclin F is a unique F-box protein in that it is highly cell cycle regulated, and has been revealed as a key regulator of cell cycle progression despite few of its substrates having been identified. Our lab aims to identify novel Cyclin F substrates, and determine how these substrates regulate cell cycle processes.

The Cyclin F substrate, Nucleolar and Spindle Associated Protein 1 (NUSAP1), is a microtubule binding protein implicated in mitotic spindle stability and chromosome segregation, however, how it functions is unknown. I have identified a novel interaction between NUSAP1 and a small ubiquitin-related modifier (SUMO) E3 ligase composed of Ran Binding Protein 2 (RanBP2), Ran GTPase Activating Protein 1 (RanGAP1) and the SUMO E2 conjugating enzyme, UBC9. This work provides evidence that NUSAP1 may function in the SUMO pathway to promote faithful chromosome segregation.
Cell cycle and metabolic regulation are critical to the growth and proliferation of normal cells, and these systems can be rewired in cancer to promote proliferation. Better understanding how these processes are integrated could provide key insights into how cancers proliferate. The following work identifies Sirtuin 5, a mitochondrial deacetylating enzyme, as a novel Cyclin F substrate. Sirtuin 5 is a known regulator of key metabolic processes including gluconeogenesis and urea production, among others. To date, identified Cyclin F substrates are all involved in significant cell cycle processes, however, Sirtuin 5 has never been connected to cell cycle. This data reveals a new role for Sirtuin 5 as a regulator of G1 progression and suggests a possible role in quiescence. Furthermore, this data provides a link between cell cycle progression and metabolism. Additional research is needed to understand what possible metabolites are involved in this regulation, and how it is mis-regulated in cancer.
To my mentors, friends and parents, whose limitless support I have relied upon along the way. Special thanks to Charles Jervis, the teacher who ignited my love of science, Dr. Tara Phelps-Durr, who encouraged me to go farther than I ever imagined, and finally, Dr. Michael J Emanuele, who has helped me grow into an independent scientist and given me confidence in my work. I could not have come this far without you all.
ACKNOWLEDGEMENTS

Special thanks to the Emanuele laboratory and my thesis committee for critical feedback throughout the course of this research. This work was done in part at the University of North Carolina Flow Cytometry and Proteomics Core Facilities.
# TABLE OF CONTENTS

**LIST OF TABLES** .................................................................................................................. x

**LIST OF FIGURES** ................................................................................................................ x i

**LIST OF ABBREVIATIONS** ................................................................................................. x iii

**CHAPTER 1: INTRODUCTION** ............................................................................................... 1

1.1 Cell Cycle ............................................................................................................................. 1

   Cell cycle Checkpoints ........................................................................................................... 2

   The Ubiquitin system ............................................................................................................. 4

   Ubiquitin E3 ligases ............................................................................................................... 6

   Ubiquitin-Like Modifications ............................................................................................... 8

1.2 Cyclin F in Cell Cycle Control ........................................................................................... 10

   Identified Cyclin F substrates ............................................................................................. 10

1.3 Cell Cycle mis-regulation in Cancer .................................................................................. 12

**CHAPTER 2: NUCLEOLAR AND SPINDLE ASSOCIATE PROTEIN 1 (NUSAP1) INTERACTS WITH A SUMO E3 LIGASE COMPLEX**

**DURING CHROMOSOME SEGREGATION** .......................................................................... 17
2.1 Introduction ........................................................................................................................................... 17

2.2 Results .................................................................................................................................................. 20

NUSAP1 localizes to dynamic spindle microtubules near chromatin .................. 20
Identification of NUSAP1 interacting proteins using mass spectrometry .......... 23
NUSAP1 does not control RanBP2 localization during mitosis ......................... 25
RanBP2 depletion impairs the response to taxol ............................................. 27

2.3 Discussion .......................................................................................................................................... 28

2.4 Materials and Methods ...................................................................................................................... 31

Mammalian cell culture ......................................................................................... 31
Immunoblotting and immunoprecipitations ....................................................... 32
Immunological reagents ...................................................................................... 33
Mass Spectrometry analysis ............................................................................... 34
Gel filtration chromatography .......................................................................... 35
Chromatin Fractionation ................................................................................... 36
Immunofluorescence Imaging .......................................................................... 36
Flow Cytometry .................................................................................................. 38

CHAPTER 3: IDENTIFICATION OF A NOVEL SCF\(^\text{CYCLIN F}\) TARGET,

SIRTUIN 5, LINKING PROLIFERATION AND METABOLIC REGULATION .......... 50

3.1 Introduction ....................................................................................................................................... 50

3.2 Results ............................................................................................................................................... 53
Sirtuin 5 stability is increased in the absence of Cyclin F.......................... 54
Sirtuin 5 interacts with Cyclin F.................................................................... 55
SCF
\text{Cyclin F} \text{ can ubiquitinate Sirtuin 5} ..................................................... 56
Sirt5 protein levels influence G1 timing ............................................................... 57
Sirt5 protein levels increase with G0 arrest....................................................... 58
3.3 Discussion ..................................................................................................... 58
3.4 Materials and Methods.................................................................................. 61
  Mammalian cell culture....................................................................................... 61
  Immunoblotting and immunoprecipitations ................................................... 61
  Immunological reagents ................................................................................... 63
  Flow Cytometry ............................................................................................... 63
CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS .............................. 73
REFERENCES .................................................................................................... 76
LIST OF TABLES

Table 2.1. siRNA oligonucleotides used in Chapter 2. .............................. 49
Table 2.2. Antibodies used in Chapter 2. ..................................................... 49
Table 3.1. siRNA oligonucleotides used in Chapter 3. ................................. 72
Table 3.2. Antibodies used in Chapter 3. ...................................................... 72
LIST OF FIGURES

Figure 1.1. Cyclin levels through the cell cycle. ...................................................... 16

Figure 1.2. Cyclin F and its targets promote cell cycle progression.................. 16

Figure 2.1. NUSAP1 is a cell cycle regulated microtubule binding protein. ................................................................. 39

Figure 2.2. NUSAP1 is cell cycle regulated. ................................................................. 40

Figure 2.3. NUSAP1 mitotic localization. ................................................................. 41

Figure 2.4. NUSAP1 interacts with the RRU in a cell cycle dependent manner. ........................................................................ 42

Figure 2.5. RanBP2 co-precipitates with NUSAP1 in both Nocodazole and Taxol arrested cells, and does not influence complex assembly........................................................................ 43

Figure 2.6. NUSAP1 depletion does not affect mitotic localization of the RRU complex................................................................. 44

Figure 2.7. NUSAP1 depletion in U2OS cells does not alter RanBP2 localization. ........................................................................ 45

Figure 2.7. NUSAP1 depletion in U2OS cells does not alter RanGAP1 localization. ........................................................................ 46

Figure 2.9. NUSAP1 and RanBP2 interact in the cytosol of mitotic cells........... 47

Figure 2.10. RanBP2 knockdown sensitizes cells to taxol treatment. ............... 47

Figure 2.11. NUSAP1 contains a SAP domain in its N-terminus....................... 48

Figure 3.1. Schematic representing results of DRYGIN screen for potential Cyclin F substrates. ......................................................... 64

Figure 3.2. Sirt5 stability is increased in the absence of Cyclin F. ................. 65

Figure 3.3. Sirt5 and Cyclin F co-immunoprecipitate. ..................................... 66

Figure 3.4. Sirt5 and Cyclin F interact in cells. ..................................................... 67
Figure 3.5. Sirt5 is ubiquitinated in the presence of Cyclin F. .......................... 68

Figure 3.6. Sirt5 CRISPR KO cells exhibit redistribution of cell cycle phases. .......................................................... 68

Figure 3.7. Sirt5 protein expression influences G1 timing. ................................. 69

Figure 3.8. Sirt5 KO cells exhibit activated DNA damage response.................... 70

Figure 3.9. Sirt5 protein levels are increased in G0 cells................................. 70

Figure 3.10. Proposed model for Sirt5 role in cell cycle progression. ............... 71
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-[2Aminoethyl] benzenesulfonfyl fluoride</td>
</tr>
<tr>
<td>AKT</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase Promoting Complex/Cyclosome</td>
</tr>
<tr>
<td>apc5</td>
<td>Anaphase-promoting complex subunit 5</td>
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<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>ATR</td>
<td>Ataxia telangiectasia and Rad3-related protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C/EBP-β</td>
<td>CCAAT/Enhancer-Binding Protein beta</td>
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<td>Cell division cycle protein 20 homologue</td>
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<td>Centromere-associated protein C</td>
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<td>Description</td>
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<td>Checkpoint Kinase-1</td>
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<td>Carbamoyl phosphate synthase 1</td>
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<td>CRISPR</td>
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<td>Cytoskeletal buffer</td>
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<td>Dulbecco's Modified Eagle Media</td>
</tr>
<tr>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DRYGIN</td>
<td>Data Repository of Yeast Genetic Interactions</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>DUB</td>
<td>Deubiquitinating enzyme</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2'-deoxyuridine</td>
</tr>
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<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>Exo1</td>
<td>Exonuclease 1</td>
</tr>
<tr>
<td>FASP</td>
<td>Filter-aided sample preparation</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FF</td>
<td>Firefly luciferase</td>
</tr>
<tr>
<td>FoxM1</td>
<td>Forkhead box protein M1</td>
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<tr>
<td>HA</td>
<td>Human influenza hemagglutinin epitope</td>
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<td>HEC1</td>
<td>Kinetochore protein NDC80 homolog</td>
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<td>HECT</td>
<td>Homologous to E6-AP carboxy terminus</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HIS</td>
<td>Hexahistidine tag</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>hst3</td>
<td>NAD-dependent histone deacetylase hst3</td>
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<td>IBR</td>
<td>InBetweenRING domain</td>
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<td>IF</td>
<td>Immunofluorescence</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>INCENP</td>
<td>Inner Centromere Protein</td>
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<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KD</td>
<td>Knock-down</td>
</tr>
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<td>kDa</td>
<td>Kilodalton</td>
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<td>KIF4</td>
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<td>Keyhole Limpet Hemocyanin</td>
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<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
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<td>MCC</td>
<td>Mitotic Checkpoint Complex</td>
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<tr>
<td>mcm3</td>
<td>DNA replication licensing factor MCM3</td>
</tr>
<tr>
<td>MDM2</td>
<td>E3 ubiquitin-protein ligase Mdm2</td>
</tr>
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<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
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<td>MgCl2</td>
<td>Magnesium chloride</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
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<tr>
<td>Nedd8</td>
<td>Neural precursor cell expressed developmentally down-regulated protein 8</td>
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<tr>
<td>NETN</td>
<td>20mM Tris-Cl, pH 8.0, 100mM NaCl, 0.5mM EDTA, 0.5% Nonidet P-40</td>
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<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>NSLC</td>
<td>Non small-cell lung cancer</td>
</tr>
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<td>NUSAP1</td>
<td>Nucleolar and Spindle Associated Protein 1</td>
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<tr>
<td>orc3</td>
<td>Origin recognition complex subunit 3</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline, 0.05% tween-20</td>
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<td>p-Chk1</td>
<td>phosphorylated Chk1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PIAS</td>
<td>Protein Inhibitor of Activated STAT2</td>
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<tr>
<td>PIPES</td>
<td>piperazine-N-N'-bis(2-ethanesulfonic acid)</td>
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<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
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<tr>
<td>PRC1</td>
<td>Protein Regulator of Cytokinesis 1</td>
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<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
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<td>Full Name</td>
</tr>
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<td>-----------</td>
</tr>
<tr>
<td>RanBP2</td>
<td>Ran Binding Protein 2</td>
</tr>
<tr>
<td>RanGAP1</td>
<td>Ran GTPase Activating Protein 1</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma protein</td>
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<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
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<td>Ribonucleic acid</td>
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<td>RNA interference</td>
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<td>Ribonuclease A</td>
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<td>Ribonucleoside-diphosphate reductase subunit M2</td>
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<td>SAC</td>
<td>Spindle Assembly Checkpoint</td>
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<td>Scaffold Attachment Factor A/B</td>
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<td>SAF-A/B/Acinus/PIAS protein domain</td>
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<td>SCF</td>
<td>Skp1/Cul1/F-box</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>Sirt5</td>
<td>NAD-dependent protein deacetylase sirtuin-5, mitochondrial</td>
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<td>SLBP</td>
<td>Stem-Loop Binding Protein</td>
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<td>sli15</td>
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<td>Cu/Zn superoxide dismutase</td>
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<td>SUMO</td>
<td>Small ubiquitin-related modifier</td>
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<td>TOP2B</td>
<td>DNA topoisomerase 2-beta</td>
</tr>
<tr>
<td>TSC</td>
<td>Total spectral counts</td>
</tr>
<tr>
<td>UBC9</td>
<td>SUMO-conjugating enzyme</td>
</tr>
<tr>
<td>UBD</td>
<td>Ubiquitin Binding Domain</td>
</tr>
<tr>
<td>UBL</td>
<td>Ubiquitin-Like proteins</td>
</tr>
<tr>
<td>WCE</td>
<td>Whole cell extract</td>
</tr>
<tr>
<td>Wee1</td>
<td>Wee1-like protein kinase</td>
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CHAPTER 1: INTRODUCTION

1.1 Cell Cycle

Cell growth and division is a highly regulated process during which one cell becomes two daughter cells. This process, known as the cell cycle, is broken up into four phases; G1, S, G2 and Mitosis (M). During G1, cells monitor their surroundings and nutrient availability, assessing whether it is safe to proceed through the cell cycle. If the cell proceeds forward in the cell cycle, G1 acts as a preparatory phase for S, where DNA replication occurs. G1 cells contain only one copy of each chromosome, but to divide, the DNA must be duplicated to ensure that each daughter cell receives the same DNA. To prepare for DNA replication, cells must make nucleotides and proteins, and license DNA replication origins. At the beginning of S, origins fire and DNA replication begins. Once DNA replication is complete and the cells are equipped with two copies of each chromosome, the cells proceed into G2. During G2, the cell ensures that the DNA has been properly replicated and that it is of adequate size to proceed through mitosis. Once it is ready, the cell proceeds to mitosis, where it segregates sister chromatids equally, resulting in two genetically identical daughter cells. Mitosis is composed of six stages; prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis.

Cell cycle progression is tightly controlled to ensure that cells are prepared to proceed before moving to the next phase. At the center of that control are Cyclin Dependent Kinases (CDKs) and their cyclin binding partners, which, when bound,
form active kinase complexes. There are a number of different CDKs and cyclins, which combine in specific pairs. While CDK protein levels remain constant throughout the cell cycle, cyclin protein levels oscillate. When a particular cyclin is expressed, it binds its preferred CDK to promote signaling and cell cycle progression (Figure 1.1). It is important to note, that while CDK/Cyclin pairs are considered the core of cell cycle regulation, abundance and activity of hundreds of proteins cycle throughout cell cycle and contribute to proper progression (1).

**Cell cycle Checkpoints**

There are many checkpoints during the cell cycle to prevent cells from prematurely beginning irreversible processes. These checkpoints are always "active" and monitoring the cellular state. For a cell to proceed past these checkpoints it must satisfy them by fulfilling a number of requirements, with each checkpoint having a unique set of requirements. Cell cycle checkpoints include the restriction point, DNA damage checkpoint, and Spindle Assembly Checkpoint (SAC). Weakened checkpoints can allow the cell to proceed through the next phase of the cell cycle despite being unprepared, which can result in damage to the cell.

The first checkpoint is called the restriction point (2). This checkpoint ensures that cells have enough resources, such as space, growth factors, or nutrients, to proceed safely through the next cell cycle (2). If the cell does not detect enough resources, it may exit the cell cycle during G1 into a state referred to as G0, or quiescence. Quiescence is a suspended state, during which the cell does not cycle and maintains an early G1-like state. If a quiescent cell senses that it has sufficient resources to cycle again, it can re-enter the cell cycle in G1.
The DNA-damage checkpoint, which arrests cell so that damaged DNA can be repaired, is controlled by either of two large kinases. Ataxia telangiectasia mutated (ATM) or Ataxia telangiectasia and Rad3-related protein (ATR) signaling, depending on which stage of the cell cycle the cell it is in at the time of damage. Both ATM and ATR phosphorylate hundreds of target proteins. If DNA damage is sensed in G1, the ATM signaling pathway is activated, preventing cells from entering S-phase before the damage is repaired, ultimately preventing replication of damaged DNA. ATM does this, in part, by phosphorylating and activating Checkpoint Kinase 2 (Chk2), which inhibits M-Phase inducer phosphatase 1 (Cdc25A) (3–7). Normally, Cdc25A dephosphorylates and activates CDK2/Cyclin E to promote S-phase entry, and phosphorylation by ATM in turn prevents activation of CDK2/Cyclin E (8). ATM also phosphorylates p53, releasing it from its inhibitor E3 ubiquitin-protein ligase Mdm2 (MDM2), so it can induce DNA repair proteins as well as the CDK2/Cyclin E and CDK2/Cyclin A inhibitor p21 (9–18).

If DNA damage occurs during S or G2, signaling goes through a similar signaling pathway mediated by ATR, to arrest cells and give the cell time to repair the damage before proceeding through mitosis. At the core of ATR damage response, ATR phosphorylates and activates Checkpoint Kinase 1 (Chk1), which, like Chk2, also inhibits Cdc25A (19–21). During S-phase however, Cdc25A promotes CDK1/Cyclin B activity (22, 23). ATR signaling also activates the CDK1 inhibitor Wee1-like protein kinase (Wee1) (24, 25). If cells slip through the damage checkpoint and into mitosis, cells undergo mitotic catastrophe and die.

Once cells enter mitosis they must satisfy the Spindle Assembly Checkpoint (or SAC). The SAC ensures that cells do not attempt to enter anaphase, where the
cell segregates mitotic chromosomes, until they have made the correct attachments to the mitotic spindles, meaning each sister of a chromosome pair is stably attached to opposite spindle poles (26). This checkpoint is controlled by the Mitotic Checkpoint Complex (MCC) which targets the E3 ubiquitin ligase complex known as the Anaphase Promoting Complex/Cyclosome (APC/C) (27). The MCC sequesters the APC/C substrate adapter protein Cell division cycle protein 20 homologue (Cdc20), preventing the APC/C from being active (28). Once each pair of sister chromatids has been stably attached to opposite poles, the checkpoint is satisfied and APC/C is activated. Once active, APC/C targets Cyclin B for degradation, as well as Securin, the protein that sequesters Separase (29–33). Once Separase is released, it can cleave Cohesin, the proteins holding sister chromatids together, and the cell can enter anaphase where sister chromatids are pulled to opposite poles of the mitotic cell (34, 35).

The Ubiquitin system

Targeted protein degradation is a major component of cell cycle regulation and allows the cell to degrade proteins within a few minutes of degradation onset, offering a quick switch-like mechanism for cells to release from, or even activate checkpoints. For example, once the SAC has been satisfied, cells trigger degradation of Cyclin B via the Anaphase Promoting Complex/Cyclosome (APC/C) and within minutes, Cyclin B is degraded, allowing cells to progress into anaphase.

This process of targeted protein degradation is controlled by the ubiquitin system. Ubiquitin is a small protein (~8.5 kDa), that once conjugated to a substrate, acts as a post-translational modification (PTM) (36, 37). Ubiquitin is highly conserved in eukaryotes, with paralogues identified in prokaryotes. Ubiquitin
is added specifically to substrate lysines through an enzyme cascade composed of E1, E2 and E3 enzymes (37, 38). The first step to this cascade is ATP-dependent ubiquitin activation by the E1, or activating enzyme. This activation results in a thioester linkage between the C-terminus of the ubiquitin and a cysteine in the E1. The ubiquitin molecule is then passed to the catalytic cysteine of an E2, or conjugating enzyme (39). Finally, the E2 interacts with an E3 ubiquitin ligase (discussed in detail below), to ligate the ubiquitin molecule to a substrate lysine via an isopeptide bond with the C-terminal glycine of ubiquitin (39). Alternatively, ubiquitin can be conjugated to the amino-terminal of a target substrate (40).

Ubiquitin can be added to substrates a number of different ways. Mono-ubiquitination refers to a single ubiquitin molecule added to a substrate on a single lysine. Multi-mono-ubiquitination occurs when multiple lysines of the substrate have a single ubiquitin modification. Furthermore, because ubiquitin is itself a protein that contains lysines, it too can be modified by other PTMs or other ubiquitin molecules, resulting in ubiquitin chains. There are many possible chain linkages due ubiquitin containing seven lysines, with different lysine linkages between ubiquitin molecules resulting in different chain topologies, which determine the outcome for the substrate (36, 41, 42).

Polyubiquitination of proteins with K11/K48, (with K denoting the lysine in ubiquitin), linked ubiquitin chains are known to target proteins for degradation through the proteasome (43, 44). The 26S proteasome is a large, multi-subunit machine composed of two primary complexes; the 20S proteasome core and the 19S regulatory cap (45). The ubiquitinated proteins first bind the 19S cap, which regulates their unfolding and entry into the 20S proteasome, which contains a
number of proteolytic enzymes that hydrolyze peptide bonds, ultimately degrading proteins (46, 47). Deubiquitinating enzymes (DUBs) associated with the proteasome cleave ubiquitin from target proteins, allowing it to be recycled (48, 49).

Apart from ubiquitin’s role in promoting degradation through the proteasome, ubiquitin also regulates cellular processes such as endocytosis, DNA repair and other signaling pathways (41, 50, 51). Ubiquitination of some proteins may regulate binding partners through mechanisms such as steric hinderance, or even promote complex assembly (52). Ubiquitin can also be conjugated to histones or transcription factors to regulate transcription (52).

**Ubiquitin E3 ligases**

As described above, ubiquitin is added to substrates via an enzyme cascade, with the last member of the cascade is the E3 ubiquitin ligase. E3 ligases typically fall into one of three families; RING-type, HECT-type or RING-between-RING (RBR) type (53–55). The largest family of E3 ligases, RING-type ubiquitin ligases, are classified by the Really Interesting New Gene (or RING) domain or protein, which recruits an E2 to the enzyme complex (53). In RING-type ligases, the E3 binds a substrate protein and the E2 transfers ubiquitin directly to the substrate, with the E3 acting more as a scaffold and never contacting the ubiquitin directly (53). For HECT-type E3 ubiquitin ligases, which contain a Homologous to E6-AP carboxy terminus (HECT) domain, the E2 binds the E3 and transfers the ubiquitin to a catalytic cysteine on the E3 ligase (54). The E3 then directly transfers ubiquitin to a target substrate. The RING-between-RING enzymes contain two RING domains with an InBetweenRING (IBR) domain between them (55). Similar to HECT-type E3’s,
the ubiquitin is first transferred from the E2 to the E3, which then transfers it to a substrate lysine (55).

E3 ligase families can be further broken down into subfamilies, for example, the RING-type ligases include Cullin E3 ligases (56). These are modular E3 ubiquitin ligases based on a Cullin backbone, and were first discovered for their role in the cell cycle (example in Figure 1.2) (56, 57). There are up to nine Cullin proteins, and each uses a specific family of substrate adapter proteins to recruit substrates to the complex. In the following research, the Cullin 1 (CUL1) based E3 ubiquitin ligase, called the SCF (Skp1/CUL1/Fbox protein) is of particular interest. This E3 ligase was the first Cullin based E3 ligase to be identified, and is composed of a CUL1 backbone, which binds a RING protein on its C-terminus and Skp1 (Suppressor of Kinetochore Protein 1) on its N-terminus (58, 59). The Skp1 protein binds an F-box protein, which then recruits specific substrates to the E3 complex for ubiquitination while the RING protein recruits the E2 (58, 59). Humans possess ~70 different F-box proteins, each enabling the SCF unique target specificity.

E3 Ubiquitin ligases are antagonized by DUBs, which remove ubiquitin modifications from targets. There are approximately 100 DUBs, divided up into two different classes; cysteine proteases and metalloproteases (60, 61). While some DUBs have specific activity against certain ubiquitin chain types, some are non-specific and can cleave multiple ubiquitin linkages (60, 61). Some DUBs also have activity against ubiquitin-like proteins, which are discussed further below (61).
Ubiquitin-Like Modifications

Ubiquitin is a member of the ubiquitin family of proteins, which primarily act as PTMs. Ubiquitin-Like proteins (UBLs) are classified into two types; type I have been identified as being conjugated to substrates, while type II have not, with ubiquitin being a type I UBLs. All UBLs undergo the same basic enzyme cascade for activation and conjugation to substrates, but so far, the best characterized of these type I UBLs, besides ubiquitin, include Neural precursor cell expressed developmentally down-regulated protein 8 (Nedd8) and Small Ubiquitin-related Modifier (SUMO).

Nedd8 is the UBL most similar to ubiquitin (62). The role of neddylation in the cell is most closely tied to regulation of Cullin E3 ligases. Neddylation of Cullin backbones aids in recruitment of the ubiquitin loaded E2 and promotes a structural change that brings the E2 and substrate closer together (63–66). Furthermore, it helps stabilize the transition state, during which the E2 and substrate are interacting, allowing chain elongation to occur (63).

While SUMOylation occurs through a similar enzyme cascade as ubiquitin, there are a few key differences (67, 68). The first being that there is only one E2 for the SUMO system compared to the almost 40 E2s for ubiquitin. The SUMO E2, SUMO-conjugating enzyme UBC9, (also referred to as UBE2I), appears more promiscuous than many ubiquitin E2 enzymes (67, 69–71). UBC9 can SUMOylate proteins in vitro without an E3 ligase, and it is poorly understood how its activity is regulated by SUMO E3 ligases in vivo. Furthermore, little is known about how SUMO E3 ligases identify and interact with substrates. There are also three isoforms of SUMO, SUMO1 and SUMO2/3 (with SUMO2 and SUMO3 being indistinguishable at
the protein level), which can both be conjugated to substrates, however only SUMO2/3 can form SUMO chains (67). Currently, it is unclear what the differences between SUMO1 and SUMO2/3 modification of substrates are, although it has been suggested that SUMO2/3 conjugation plays more of a role in stress response (72, 73). It is also unclear if either paralog is preferred by particular SUMO E3 ligases, although research has suggested that SUMO1 modifications compose the majority of preferential SUMO modifications in vivo (74).

SUMOylation may influence many protein characteristics, including localization, dimerization/interactions, activity, and stability. While a few SUMO substrates have been well characterized, there is still debate on the function of the SUMOylation. So far, studies aimed at identifying SUMOylation substrates have relied on stress induced SUMOylation or overexpression of SUMO or the promiscuous E2, UBC9. Using overexpression methods in the identification of SUMO targets is difficult because the SUMO can be used by other UBL pathways, for example the ubiquitin pathway, which results in ubiquitin targets being modified by SUMO instead of ubiquitin. These caveats make it unclear whether the substrates identified in many of these studies are real, or relevant, in normal physiological conditions. Furthermore, many of the methods used to probe the SUMO pathway are methods developed to probe the ubiquitin pathway. While SUMO appears very similar to ubiquitin, these methods may be insufficient to elucidate details of the SUMO pathway. Further research is needed to better understand the role of SUMO in normal, or unstressed, cellular state.
1.2 **Cyclin F in Cell Cycle Control**

Some members of the ubiquitin pathway are becoming more recognized for key roles in cell cycle progression. One such protein is Cyclin F (Figure 1.2). Cyclin F is the founding member of the F-box containing family of proteins, most of which are substrate adapters for the SCF E3 ubiquitin ligase complex (75–77). Cyclin F is a non-traditional cyclin, as it does not bind to and activate a CDK (78). However, like cyclins, Cyclin F protein levels are highly cell cycle regulated, and it is the most cell cycle regulated F-box protein (75, 78). Cyclin F was first identified as a protein that could rescue the yeast Cdc4 mutant phenotype, which causes a G1 arrest and subsequent death (75). While many of the known Cyclin F substrates alluded to its importance for cell cycle progression, more recent studies have shown that feedback between the APC/C\textsuperscript{Cdh1} and SCF\textsuperscript{Cyclin F} is important for promoting the G1-S transition (79, 80). During early G1, when the APC/C substrate adapter Cdh1 is around, the APC/C\textsuperscript{Cdh1} targets Cyclin F for ubiquitination and degradation (79). However, as Cyclin F protein levels slowly accumulate, a switch occurs and the SCF\textsuperscript{Cyclin F} targets Cdh1 for ubiquitination and degradation, promoting the transition into S-phase (79). This switch has been shown to be controlled by growth factor signaling through the RAC-alpha serine/threonine-protein kinase (AKT) pathway, which promotes the recruitment of Cyclin F into the SCF complex, resulting in the degradation of Cdh1 (80).

*Identified Cyclin F substrates*

Identified Cyclin F substrates to date include Centriolar coiled-coil protein of 110 kDa (CP110), Nucleolar and Spindle Associated Protein 1 (NUSAP1), Ribonucleoside-diphosphate reductase subunit M2 (RRM2), Exonuclease 1 (Exo1),
Stem-Loop Binding Protein (SLBP), Cell division control protein 6 homolog (Cdc6), and Fizzy-related protein homolog Cdhl (79, 81–85). All of these substrates have roles in cell cycle progression and are involved in highly regulated processes.

RRM2, Cdc6 and Exo1 are all important in G1-S transition and DNA replication. RRM2 is a subunit of ribonucleoside-diphosphate reductase complex, which is important for nucleotide synthesis needed for DNA replication. Cdc6 is important for pre-replication complex loading, an event critical to proper DNA replication (86, 87). Degradation of Cdc6 by Cyclin F is important for preventing re-replication (85). Exo1 is required for mismatch repair during DNA replication and strand resection during homologous recombination (88). Exo1 is targeted for degradation by Cyclin F in response to UV-induced DNA damage in G2, likely to prevent excessive strand resection (84).

CP110, NUSAP1 and Cdc6 are all important for G2-M transition and mitotic progression. Along with its role in DNA-replication initiation, Cdc6 prevents premature mitotic entry when un-replicated DNA is present by regulating phosphorylation of Chk1, promoting arrest until DNA replication is completed (86, 87, 89). CP110 controls centrosome duplication, an event critical for the establishment of a bipolar spindle during mitosis, however, centrosome duplication normally only occurs one time in a cell cycle (90). If cells duplicate centrosomes more than once it can lead to multipolar spindles. In cells with reduced levels of Cyclin F, multipolar spindles are more common, leading to mis-segregation phenotypes (81). NUSAP1 is not only implicated in the G2-M transition, but is important for maintenance and stability of the mitotic spindle (91–93). NUSAP1 levels must be tightly controlled however, because overexpression of NUSAP1
results in microtubule bundling which inhibits normal mitotic progression (93). SLBP is also targeted by Cyclin F for degradation during G2, preventing the translation and accumulation of H2A.X and γH2A.X (94). Upon high genotoxic stress, SLBP accumulates and promote the induction of H2A.X and γH2A.X (94).

Finally, Cdh1 plays important roles in a number of cell cycle transitions. As one of the substrate adapter for the APC/C, it is especially important for mitotic exit, where it targets Cyclin B for degradation (30). As mentioned in the previous section, Cyclin F mediated degradation of Cdh1 is also important for the G1-S transition (79, 80).

Despite only a handful of Cyclin F substrates having been identified to date, the evidence is clear that Cyclin F plays key roles in regulating cell cycle progression. Identification of more Cyclin F substrates is needed, and will help further clarify our understanding of cell cycle regulation.

1.3 Cell Cycle mis-regulation in Cancer

Cancer is a disease consisting of over proliferative cells, indicating disruptions in normal cell cycle regulation. Over the years, many cell cycle components have been shown to be mis-regulated in cancers, including CDK4/6/Cyclin D, p53 and mitotic machinery.

Many cancers, including some breast cancers, exhibit amplification of the CDK4/6/Cyclin D pathway. Mitogenic factors, estrogen signaling, or oncogenes can promote the expression of Cyclin D in quiescent cells, promoting their re-entry into the cell cycle (95). This is often associated with amplification of CDK4/6 genes, as well as decreases in the CDK4/6 inhibitor p16 and the downstream CDK4/6/Cyclin D
target; Retinoblastoma protein (RB). All of these changes result in an increase in E2F-mediated transcription of S-phase promoting genes, ultimately promoting proliferation. Several drugs have been developed to target this pathway, including the CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib (96–98).

Cancers commonly have misregulated or weakened cell cycle checkpoints, and will not arrest properly in response to stress such as DNA damage. One clear misregulation of the DNA damage response pathway includes the mutation or loss of p53. p53 is a key player in promoting cell cycle arrest in response to DNA damage, which is the most commonly mutated gene among cancers. For example, p53 is mutated in >90% of triple negative breast cancer (99). Drugs currently in clinical trials include inhibitors of the ubiquitin E3 ligase MDM2, such as idasanutlin, that allow p53 accumulation and activation, which induces cell cycle arrest and/or apoptosis (100, 101) (clinical trials.gov, Dec 2017). The Wee1 inhibitor MK1775 is also currently in clinical trials for treatment of p53 deficient cancers, in which it is shown to improve efficacy of DNA damaging agents (102) (clinicaltrials.gov, Dec 2017). Finally, ATR and Chk1 inhibitors have been developed and are being tested in combination with DNA damaging agents, preventing DNA damage checkpoint-induced cell cycle arrest (103–105) (clinicaltrials.gov, Dec 2017).

Mitosis is a tightly controlled series of events that results in segregation of chromosomes equally into daughter cells. Any failures in this process may result in aneuploidy. Aneuploidy is when a cell contains too many or too few chromosomes, which has serious implications for the resulting daughter cells. Physiologically, cycling aneuploid cells are rare in the body because it typically results in cell death, however many cancer cells are highly aneuploid, and may reach a stable state of
aneuploidy, which in some cases is thought to promote survival (106–108). In recent years, it has been shown that low levels of aneuploidy may promote tumorigenesis (108). In an attempt to cause even higher rates of mis-segregation in cancer cells, with the goal of inducing cell death, mitotic spindle assembly has been targeted by traditional chemotherapeutic agents for years, using spindle poisons. Spindle poisons fall into two categories; 1) microtubule depolymerizing agents or 2) microtubule stabilizing agents (103, 109, 110). While these drugs are commonly used, cells can become resistant to spindle poisons via different mechanisms (111, 112). Cells have a number of checks in place to ensure proper chromosome segregation, including the SAC, and maintenance of spindle stability. Much is known about establishment and maintenance of spindle stability, and it’s known that these are processes that are regulated by hundreds of proteins, further research is needed to understand the precise functions of many of those proteins, or how they may promote resistance, or sensitivity to spindle poisons.

The ubiquitin system has also been a target of cancer therapeutics. Bortezomib (PS-341), the reversible proteasome inhibitor, has been approved for treatment of multiple cancers, having been shown to increase the cytotoxic effects of both radiation and chemotherapy (113–118). The NEDD8-activating enzyme inhibitor MLN4924 (or pevonedistat), which inhibits function of CRLs, is currently under clinical trials for use in treatment in a number of cancer types (119) (clinicaltrials.gov, Dec 2017). More specific E3 ligase targeted drugs have also been developed, including CC-220, which specifically targets cerablon (120) (clinicaltrials.gov, Dec 2017). Cerablon is a substrate adapter for the CUL4 based
E3 ubiquitin ligase complex, and treatment with CC-220 promotes E3 ligase activity, leading to increased degradation of certain substrates (120).

While cell cycle and the ubiquitin system have proven to be useful targets for cancer treatment, there is still much to be learned about how these processes are controlled in normal cells, and how they may be mis-regulated in cancer. Better understanding of how cancers re-wire these key programs could provide the insight needed to develop more effective chemotherapeutic agents. The following research has been performed with the intention of better understanding how the ubiquitin system, particularly through the E3 ubiquitin ligase SCF<sub>Cyclin F</sub>, function to promote cell cycle progression.
Figure 1.1. Cyclin oscillations through the cell cycle. Each cyclin has precise cell cycle regulated expression, and bind to specific CDKs, forming active kinase complexes that promote cell cycle progression.

Figure 1.2. Cyclin F and its targets promote cell cycle progression. Cyclin F targets all play key roles in highly regulated cell cycle events. Proper regulation of those events, in part by Cyclin F targeting proteins for degradation, helps ensure proper cell cycle progression.
CHAPTER 2: NUCLEOLAR AND SPINDLE ASSOCIATE PROTEIN 1 (NUSAP1) INTERACTS WITH A SUMO E3 LIGASE COMPLEX DURING CHROMOSOME SEGREGATION

2.1 Introduction

The accurate partitioning of chromosomes during cell division is essential for cell survival and preventing chromosome instability. The movement of chromosomes during mitosis requires the assembly and organization of a bipolar array of microtubules termed the mitotic spindle. Spindle dynamics are controlled by numerous microtubule associated proteins, and the molecular function of many of these remains to be characterized.

Nucleolar and Spindle Associated Protein 1 (NUSAP1) is a mitotic phosphoprotein that binds microtubules and which has been implicated in cell division (91–93, 121–123). NUSAP1 is highly conserved among higher eukaryotes and genetic knockout in mice is embryonic lethal due to chromosome segregation defects (92). NUSAP1 is overexpressed in numerous malignancies, and high levels correlate with poor prognosis in aggressive triple-negative breast cancer (124). A central domain in NUSAP1 directly interacts with microtubules in vitro and in vivo, and its association with the mitotic spindle is controlled by phosphorylation (91, 124).

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NUSAP1 and has been implicated in mitotic progression, spindle formation and stability (91, 93, 121). In addition, NUSAP1 depletion sensitized a variety of cell types to the chemotherapeutic agent taxol, consistent with its role in spindle formation and stability (82, 125). Furthermore, studies in frog egg extracts have suggested a potential role for NUSAP1 in tethering microtubules to chromatin in a kinetochore independent manner (121).

Mass spectrometry based analysis of spindle associated factors demonstrated that NUSAP1 is among a small group of proteins, that includes PRC1/Ase1 and KIF4, whose binding to microtubules increases after anaphase compared to earlier stages of mitosis (126). Consistent with this observation, NUSAP1 phosphorylation by CDK1/Cyclin B, which is active in early mitosis, displaces it from microtubules (123). Together, these studies point to a crucial role for NUSAP1 is regulating both early and late mitotic events. Importantly, they strongly suggest that there exists a pool of microtubule-free NUSAP1 in early mitosis that could contribute to its function during cell division.

We previously identified NUSAP1 as a substrate for a cell cycle regulated, SCF-type E3 ubiquitin ligase during S/G2 phase (82). NUSAP1 is also targeted for degradation during late mitosis and in early G1 by a second E3 ligase, the Anaphase Promoting Complex/Cyclosome (APC/C) (127). In addition to its regulation by ubiquitin, NUSAP1 was also recovered in large-scale cell cycle phospho-proteomic studies (128, 129). However, the role of NUSAP1 in mitosis remains largely unknown, as does the network of proteins to which it binds during cell division. To gain mechanistic insights into how NUSAP1 regulates cell division, we applied mass spectrometry based proteomics to identify endogenous NUSAP1
interacting proteins. This analysis identified a cell cycle regulated interaction between NUSAP1 and a SUMO (small ubiquitin like modifier) E3 ligase complex.

SUMO is an ubiquitin related protein that is post-translationally appended to substrates, contributing to various aspects of signaling. SUMOylation has been linked transcriptional activation, protein stability, and regulating protein-protein interactions (130, 131). The first described SUMO E3 ligase is composed of three proteins; Ran Binding Protein 2 (RanBP2), Ran GTPase Activating Protein 1 (RanGAP1) and the SUMO E2 conjugating enzyme, UBC9 (69, 132, 133). During interphase, this complex is part of the nuclear pore where it functions in Ran mediated nuclear import and export (134, 135). However, following nuclear envelope breakdown at mitotic entry, the RanBP2 SUMO E3 ligase dissociates from the nuclear pore complex and SUMOylates proteins important for chromosome segregation (132, 136–138). The DNA decatenating enzyme TOP2A is SUMOylated at the metaphase to anaphase transition by the RanBP2 E3; SUMOylation directs TOP2A localization to centromeres, where it functions in sister chromatid disjunction (137). Failure to SUMOylate TOP2A during mitosis has been linked to severe chromosome mis-segregation (137–139). Another RanBP2 SUMO E3 ligase target is Borealin, a member of the Chromosome Passenger Complex (CPC), whose functions are critical to kinetochore-microtubule attachment and chromosome segregation (136, 138). The CPC is composed of Borealin, Survivin, INCENP and Aurora B, and SUMOylation of CPC complex members is highly conserved (140). SUMOylation of the cell cycle transcription factor FoxM1 during G2/M regulates its activity (131, 141). Finally, the kinetochore associated microtubule motor CENP-E is SUMOylated, contributing to its kinetochore localization and function (142).
The most well characterized SUMO ligases described to date are the PIAS family of SUMO E3s. PIAS proteins are a family of conserved SUMO ligases involved in various aspects of cellular physiology, including cell cycle control. PIAS proteins contain an amino-terminal SAP (SAF-A/B, Acinus and PIAS) domain that has been implicated in both enzyme activation and substrate targeting (143). Despite the significant size of the RanBP2-RanGAP1-UBC9 complex (RanBP2 has a predicted molecular weight of 358kDa), it lacks a recognizable SAP domain. Notably, NUSAP1 has an obvious SAP domain in its amino terminus and we propose that NUSAP1 could facilitate RanBP2 ligase function through the amino-terminal SAP domain.

2.2 Results

*NUSAP1 localizes to dynamic spindle microtubules near chromatin*

NUSAP1 is a cell cycle regulated, microtubule binding protein whose expression has been shown previously, by us and others, to oscillate during the cell cycle (82, 91, 144). However, experiments performed to date were done on relatively short time scales after synchronization and release, making it difficult to know if its dynamics were due to the effects of chemical synchronization. To analyze NUSAP1 protein dynamics throughout an entire cell cycle we performed immunoblots on U2OS cells synchronized using nocodazole, isolated by shake-off, and followed for 28 hours after re-plating (Figure 2.1A). NUSAP1 levels are elevated in mitotic cells compared to asynchronous populations, concomitant with an increase in phosphorylated histone H3 (Ser10), a marker of mitosis. NUSAP1 levels decrease abruptly as cells enter G1-phase, consistent with degradation mediated by the APC/C. NUSAP1 levels remain low through early S-phase, when Cyclin E is expressed and Cdh1 is degraded, and then begin to accumulate after the
expression of Cyclin A, which marks the beginning of S-phase. NUSAP1 is also targeted by another E3 ligase, the SCF\textsuperscript{Cyclin F}, during S/G2 (82). Interestingly, abundance of the APC/C co-activator Cdc20, Cyclin F, and NUSAP1 are all abruptly diminished at mitotic exit, consistent with their coordinated degradation by APC/C and its other co-activator, Cdh1 (Figure 2.1A and Figure 2.2) (79).

We used high-resolution immunofluorescent (IF) imaging to interrogate the localization of NUSAP1 during mitosis, when its protein levels are at their highest. The specificity of the NUSAP1 antibody was confirmed by comparing anti-NUSAP1 stained cells treated with either control siRNA targeting firefly luciferase (FF) or oligonucleotides targeting NUSAP1. RNAi depletion of NUSAP1 completely eliminated staining, confirming antibody specificity for IF. In prometaphase, NUSAP1 staining was diffuse and localization to specific mitotic structures was not apparent (Figure 2.3). Later in mitosis NUSAP1 did not localize to the whole of the mitotic spindle, like the majority of known microtubule binding proteins in mitosis (Figure 2.1B). Instead, it localizes to the central spindle with the most concentrated area of NUSAP1 being near the chromatin (Figure 2.1B). Highly concentrated NUSAP1 staining in the vicinity of chromatin was visible during metaphase, anaphase and telophase, with the bulk of NUSAP1 appearing to localize to the spindles around chromatin. NUSAP1 localization is coincident with regions of anti-parallel, overlapping microtubules in the central spindle. Notably, this chromatin-centric spindle localization is highly unique among known microtubule binding proteins in mitosis. Interestingly, it is comparable, although not identical, to PRC1 and KIF4, which also show increased microtubule binding after anaphase and control anti-parallel microtubule assemblies in the central spindle (126, 145–147).
This suggests that NUSAP1 represents a unique class of microtubule binding protein that localize in the vicinity of inter-digitated microtubules and that tracks chromatin localization in both early and late mitosis.

The localization of a pool of NUSAP1 on spindle microtubules near chromatin prompted us to determine if NUSAP1 localization is microtubule dependent. Prior to fixation, cells were treated with either DMSO (control) or the microtubule depolymerizing drug nocodazole. NUSAP1 localization is lost when the spindle is depolymerized by nocodazole treatment, confirming that its localization is microtubule dependent (Figure 2.1C). To determine which population of microtubules NUSAP1 localizes to, we depolymerized dynamic spindle microtubules prior to fixation (Figure 2.1C). Cells were cold treated prior to fixation, which leads to the destabilization of microtubules that are not stably attached to kinetochores (k-fibers). NUSAP1 localization to the spindle was lost when non-kinetochore microtubules were depolymerized, suggesting that NUSAP1 localizes to dynamic microtubules during mitosis (Figure 2.1C). This observation, and the diffuse NUSAP1 staining in prometaphase cells, is consistent with the notion that NUSAP1 binds to overlapping spindle microtubules. Finally, we analyzed single focal planes of NUSAP1 and tubulin staining by confocal microscopy. We observed NUSAP1 localization along microtubules, but not at the centromere, centrosome or kinetochore (Figure 2.1D). Together, these data confirm that NUSAP1 is cell cycle regulated, and demonstrate its chromatin-centric localization to dynamic microtubules during mitosis.
Identification of NUSAP1 interacting proteins using mass spectrometry

NUSAP1 has a unique mitotic localization pattern compared to known microtubule binding proteins (Figure 2.1). Since NUSAP1 has been implicated in spindle stability and chromosome segregation we were interested in the mechanism by which NUSAP1 contributes to mitotic progression. To address this question, we analyzed protein interaction partners that bind NUSAP1 using endogenous NUSAP1 immunoprecipitation (IP) followed by protein identification using mass spectrometry (MS/MS). We performed IP experiments using control IgG and endogenous NUSAP1 antibodies in multiple cell lines (HeLa and HEK-293T). In addition, since NUSAP1 levels peak during mitosis (Figure 2.1A) we also performed IPs from both asynchronous and mitotic HEK-293T cells arrested using nocodazole. By performing endogenous IPs in multiple cell lines and physiological conditions we sought to identify the strongest interactors that are most likely to be physiologically relevant in controlling mitotic progression.

We filtered out non-specific interactions identified in control IgG IPs, which were performed in parallel with each experiment, and removed known contaminants based on the CRAPome dataset (148). We then overlapped the remaining interactions between the three IPs to identify the highest-confidence set of NUSAP1 interacting proteins (Figure 2.4A). This resulting list of 14 proteins included the known NUSAP1 interacting protein Importin-β (93).

This analysis identified all three members of the RanBP2 mitotic SUMO E3 ligase complex, which includes RanBP2, RanGAP1 and the SUMO E2 conjugating enzyme UBC9. We identified multiple RanBP2 and RanGAP1 peptides in all three experiments. Despite the fact that NUSAP1 is more abundant in mitotic cells, the
IPs were saturating in that we detected a similar number of NUSAP1 TSCs between asynchronous and mitotic 293T samples. This allowed us to compare the relative number of RanBP2, RanGAP1 and Ubc9 TSCs between asynchronous and mitotic experiments. Our data show an enrichment of all three proteins in the mitotic sample relative to asynchronous cells, indicating that their interaction is cell cycle regulated (Figure 2.4B). Further supporting an interaction between NUSAP1 and RanBP2, their binding was detected in a recent, large scale interactome study using a tagged version of NUSAP1 (93).

To confirm our IP-MS/MS findings we tested whether RanBP2 co-IPed with endogenous NUSAP1 in multiple cell lines. Importantly, isolated endogenous NUSAP1 precipitated from nocodazole arrested U2OS, HeLa, HEK-293T and HCT116 cell lines co-precipitated endogenous RanBP2 (Figure 2.4C). Similarly, when we precipitated endogenous RanBP2 from nocodazole arrested HEK-293T cells we co-precipitated endogenous NUSAP1, as well as its known interactor RanGAP1 (Figure 2.4D). This interaction was also detected in Taxol arrested cells, which prevents microtubule depolymerization, indicating that their interaction is not due to gross changes in microtubule dynamics (Figure 2.5).

To further confirm these findings, we analyzed mitotic HEK-293T cell lysates using size exclusion chromatography to separate proteins and complexes based on their size and shape, followed by endogenous NUSAP1 IP. In this experiment, RanBP2, RanGAP1 and UBC9 co-migrated in a high molecular weight complex (~1 mega-Dalton) (Figure 2.4E, lanes 2-4). There was a small, but detectable amount of NUSAP1 that also co-migrated with those fractions. Importantly, when we precipitated endogenous NUSAP1 from those fractions we co-precipitated both
RanBP2 and RanGAP1 (Figure 2.4F). Together this data strongly supports an interaction between a pool of available NUSAP1 and the RanBP2 SUMO E3 ligase complex.

Interestingly, only a subset of SUMOylated RanGAP1 co-migrated with RanBP2 based on the size exclusion chromatographic analysis. The majority of SUMOylated and unSUMOylated RanGAP1 eluted in fraction of ~500 kDa (Figure 2.4E). This demonstrates that there are RanBP2 bound and unbound pools of RanGAP1 in mitotic 293T cells and contrasts with a recent study suggesting that all of RanBP2 and RanGAP1 are complexed together in HeLa cells (136). The reason for this discrepancy is unknown, but could be cell line dependent. The peak elution of NUSAP1 partially overlapped with the peak elution of RanGAP1 that lacked RanBP2 and IPs from these fractions demonstrate that RanGAP1 and NUSAP1 interact in those fractions (lanes 7-10; Figure 2.4E and F). The full composition of these different NUSAP1 complexes remains unknown.

**NUSAP1 does not control RanBP2 localization during mitosis**

The RanBP2 complex regulates the SUMOylation of TOP2A and Borealin, both of which have distinct mitotic localization patterns (132, 136–138). In addition, RanBP2 localizes at the kinetochore and on the spindle (149). We hypothesized that NUSAP1 could recruit the RanBP2 SUMO E3 ligase to the spindle. We performed IF; probing for RanBP2 and RanGAP1 localization in control (FF) and NUSAP1 depleted cells. We observed the previously reported RanBP2 and RanGAP1 localization patterns in control depleted cells (150). However, in both U2OS and HeLa cells lines neither RanBP2 nor RanGAP1 localization was affected by NUSAP1 depletion (Figure 2.6A-C, Figure 2.7 and Figure 2.8). Due to previous reports suggesting that
SUMOylation of TOP2 regulates its centromeric localization, we also analyzed the localization of TOP2A and TOP2B on chromatin in control and NUSAP1 depleted cells using biochemical fractionation. Similarly, we observed no change in the localization of TOP2 on chromatin in control and NUSAP1 depleted cells (Figure 2.6D). We conclude that NUSAP1 is not involved in the localization of RanBP2 and RanGAP1, nor that of the RanBP2-RanGAP1-UBC9 SUMO substrate TOP2.

Since our IF staining was unable to distinguish clear co-localization of NUSAP1 with RanBP2 or RanGAP1 and there are soluble pools of NUSAP1, RanBP2 and RanGAP1 during mitosis, we determined where these proteins interact using a proximity ligation assay (PLA; Figure 2.9). PLA relies on the proximity of co-localizing antibodies during immune staining of fixed cells, which allows for the rolling circle amplification of a DNA probe that is detected using fluorescence hybridization. The result is a fluorescent foci at each site of interaction between the target proteins (151). Performing PLA in asynchronous cells with either NUSAP1 or RanGAP1 antibody alone produced a low background (Figure 2.9A), quantified in Figure 4B. Co-staining RanBP2 and RanGAP1 served as a positive control since they interact in both interphase and mitotic cells. Co-staining with NUSAP1 and RanGAP1 antibodies showed a strong increase in the number of foci in the cytosol of mitotic cells (Figure 2.9A and B). Intriguingly, the mitotic cells with the lowest number of foci in the NUSAP1 and RanGAP1 stained samples were in the late stages of mitosis (telophase and after; identified by red triangles). This suggests that the interaction between NUSAP1 and the RanBP2 E3 ligase complex decreases in late mitosis as the cells begin to rebuild their nuclear membranes/pores. Consistent with expression of NUSAP1 late in the cell cycle, and a cell cycle dependent interaction
between NUSAP1 and RanBP2-RanGAP1, the PLA signal was unchanged between single-antibody stained controls (NUSAP1 and RanGAP1 only) and dual-antibody (combined NUSAP1/RanGAP1) stained interphase cells. This supports the observation that NUSAP1 interacts with RanBP2-RanGAP1 in a cell cycle dependent manner, and suggest that NUSAP1 binds RanBP2-RanGAP1 independent of the mitotic spindle, consistent with the binding observed in nocodazole treated cells.

*RanBP2 depletion impairs the response to taxol*

Previous reports have shown that NUSAP1 depletion sensitizes cells to spindle poisons, such as taxol or nocodazole (82). To determine if RanBP2 depletion would show a consistent phenotype, we depleted cells of RanBP2 using siRNA and treated them with increasing doses of taxol overnight. RanBP2 was effectively depleted by siRNA based on immunoblot analysis (Figure 2.10B). Propidium iodide staining for DNA content in control depleted cells shows a progressive increase in G2/M phase cells in response to taxol, indicating an increased number of cells arresting in response to spindle checkpoint activation (Figure 2.10A). RanBP2 depleted cells had substantially reduced numbers of cells in G2/M phase at all doses of taxol tested, consistent with a defect in maintaining their mitotic arrest in response to checkpoint activation. Consistent with a slippage through mitosis, there was also a reduction in Cyclin B levels in RanBP2 depleted cells compared to controls. At higher doses of taxol the number of surviving cells at time of harvest was also reduced. These data are consistent with our previous studies showing that NUSAP1 depleted cells are sensitive to spindle poisons that activate the spindle checkpoint (82).
2.3 Discussion

NUSAP1 is an important regulator of mitotic progression and chromosome segregation. NUSAP1 is essential for mouse development, and its inactivation by RNAi leads to defects in chromosome segregation (92). The NUSAP1 protein is tightly controlled post-translationally during the cell cycle. Its stability is controlled by at least two E3 ubiquitin ligases: SCFCyclin F during S/G2 phase and by APC/C in G1 (82, 127). Furthermore, NUSAP1 phosphorylation is upregulated during cell cycle progression on upwards of 20 different residues (128, 129). Nevertheless, little is known about where NUSAP1 fits mechanistically in the mitotic spindle apparatus.

We used confocal imaging to determine the precise localization of NUSAP1 on the mitotic spindle, providing a high-resolution snap-shot of NUSAP1 localization at each stage of mitosis. Interestingly, NUSAP1 exhibits a prominent, chromatin-centric localization pattern during metaphase and anaphase that is unique among microtubule binding proteins. We demonstrate here that NUSAP1 is localized on microtubules, and that its localization is dependent on dynamic spindle microtubules, indicating a unique role for NUSAP1 in the process of cell division. The localization of NUSAP1 is most consistent with that of overlapping, interdigitated spindle microtubules. Consistent with this, we see no significant NUSAP1 staining in prometaphase cells where the spindle poles have not yet separated. We are unaware of another microtubule binding protein with a localization that is fully coincident with chromatin during metaphase and anaphase of mitosis. The PRC1 and KIF4 proteins show the most consistent localization with that of NUSAP1 during metaphase, but localize to the spindle mid-zone at anaphase. However, NUSAP1,
PRC1 and KIF4 all showed increased microtubule binding after anaphase, suggesting a potential relationship between these factors in controlling spindle integrity (126).

To further define the role of NUSAP1 we examined endogenous binding partners using mass spectrometry. Through this analysis we identified and validated a cell cycle regulated interaction between NUSAP1 and the RanBP2-RanGAP1-UBC9 SUMO E3 ligase. Their interaction was identified first using endogenous NUSAP1 pulldown followed by mass spectrometry and was validated by co-IP of both endogenous proteins in multiple cell lines. We were surprised not to identify a larger set of overlapping conditions between datasets, and predict that inter-cell lines differences could be explained by variances in the oncogenic repertoire of the different cell types. An interaction between NUSAP1 and RanBP2 was also detected in a large scale study that globally mapped protein-protein interaction networks, providing further validation for their interaction (152). RanBP2-RanGAP1-UBC9 is a critical SUMO ligase involved in cell division. However, little is known about which substrates it targets, how those substrates are recognized, how its activity is regulated, and how its localization is controlled.

PIAS proteins, the most well characterized family of SUMO E3 ligases, all share a SAP domain (SAFA/B, Acinus, PIAS protein domain) at their N-terminus. While the SAP domain of PIAS proteins has been shown to be involved in nuclear import and DNA binding, PIAS protein SAP domains also mediate substrate interactions. For example, PIAS1 interacts with its substrate C/EBP-β via its SAP domain, with deletion of its SAP domain resulting in failure to SUMOylate C/EBP-β (143). Interestingly, none of the RanBP2-associated SUMO ligase components
contain an identifiable SAP domain. NUSAP1, however, has a well conserved SAP domain at its N-terminus, with nearly all of the key, conserved residues found in the PIAS protein SAP domains (Figure 2.11). Like the PIAS proteins and other SAP domain containing proteins, the NUSAP1 SAP domain has been shown to be important for its interactions with DNA, however, this may not be its only function (153). It is unknown how RanBP2 SUMO ligase is activated and how it specifies substrates for SUMOylation. We speculate that NUSAP1 could be a regulatory subunit for the complex, mediating substrate interactions and/or complex activation, similar to the role of substrate adapters in cullin E3 ligases. Importantly, depletion of NUSAP1 using multiple siRNA reagents does not interfere with RanBP2-RanGAP1 complex assembly (Figure 2.5B). It is noteworthy that despite being the first discovered SUMO E3, little is known about the enzymology of the intact complex, due in large part to the size of RanBP2 (136, 154).

Despite the prominent localization of NUSAP1 during metaphase and anaphase to microtubules in the vicinity of chromatin, its binding to RanBP2-RanGAP1 is cytoplasmic. Thus, NUSAP1 could contribute to mitotic progression through multiple mechanisms: at the site overlapping microtubules on the mitotic spindle and through interactions with RanBP2 in the cytoplasm.

Recent large-scale studies have sought to identify targets of SUMOylation and have even examined cell cycle dependent changes in SUMOylation. However, NUSAP1 has not been identified in any of these large-scale SUMO substrate screens, suggesting it is not a target for SUMOylation, despite these screens often being conducted using mitotic cells (155–158). While this does not rule out NUSAP1
as a SUMO substrate, we currently lack evidence supporting it as target of
SUMOylation and were unable to detect NUSAP1 in SUMO pulldowns.

Little is known about how SUMO E3 ligases interact with, and subsequently
SUMOylate their targets and how these interactions are regulated. If NUSAP1 did
mediate enzymatic activity of the RanBP2 SUMO E3 ligase, this would provide
important insight into the functions of not only the RanBP2 complex, but possibly
how other SUMO E3 ligases are regulated as well. Further study of the interaction
between NUSAP1 and the RanBP2 SUMO E3 ligase, and possibly the SUMO
pathway, could elucidate the mechanisms involved in the regulation of other SUMO
E3 ligases.

2.4 Materials and Methods

Mammalian cell culture

HEK-293T, U2OS, HCT116 and HeLa cells were grown in Dulbecco's Modified
Eagle's Medium (DMEM; Gibco) supplemented with 10% FBS (Atlanta Biologicals)
and Pen/Strep (Gibco). For live cell imaging, cells were imaged in Fluorobrite DMEM
(Gibco) + 10% FBS. Nocodazole (Sigma 487928) was used at 150 ng/mL for U2OS
and 200 ng/mL for 293T. All siRNA transfections were performed using
Lipofectamine RNAiMax (Thermo) following manufacturers protocol. Control, non-
specific siRNA targeted firefly luciferase (siFF). Three different siRNAs against
NUSAP1 were used, each at a concentration of 20nM. The siRNA oligonucleotide
sequences used in this study are detailed in Table 2.1.
Immunoblotting and immunoprecipitations

Samples analyzed by immunoblot were lysed in NETN (20mM Tris-Cl, pH 8.0, 100mM NaCl, 0.5mM EDTA, 0.5% Nonidet P-40 (NP-40)) supplemented with 1ug/mL apoprotinin, 1ug/mL pepstatin, 10ug/mL leupeptin, 1mM Na3VO4, 1mM NaF and 1mM AEBSF (4-[2Aminoethyl] benzenesulfonyl fluoride). Protein concentration was estimated using the Bradford assay (Bio-Rad). Laemmli buffer was added to samples, which were then separated by SDS-PAGE gel electrophoresis using home-made or commercially available gels (Bio-Rad). Gels were transferred to nitrocellulose membranes and blotted using standard immunoblotting procedures.

NUSAP1 interacting proteins were identified using endogenous immunoprecipitation followed by tandem mass spectrometry. The mass spectrometry analysis was carried out by the UNC Hooker Proteomics Facility (described below). As a source of starting material, we used asynchronous HEK-293T and HeLa cells, or HEK-293T cells that were arrested in mitosis by overnight incubation in nocodazole. Whole cell extracts (WCE) were prepared on ice in the aforementioned NETN lysis buffer. Protein A/G agarose beads were covalently coupled to control IgG or anti-NUSAP1 antibodies using dimethyl pimelimidate (74). WCE was clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C in a benchtop centrifuge. Clarified lysates were mixed with antibody coated beads on a rotary mixer for 4 hours at 4°C. Samples were quickly washed three times with lysis buffer, eluted using 100mM Glycine, pH 2.5 and neutralized with Tris buffer (pH7.5). Elutions were then digested with trypsin and analyzed by mass spectrometry (see below for details).
For the co-IP experiments in Figure 2, cells were lysed in hypotonic lysis buffer (10mM HEPES, pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.5mM DTT), supplemented with 1ug/mL apoprotinin, 1ug/mL pepstatin, 10ug/mL leupeptin, 1mM Na3VO4, 1mM NaF and 1mM AEBSF (4-[2Aminooethyl] benzenesulfonyl fluoride). Protein A/G DynaBeads (Thermo) were bound to control rabbit IgG, NUSAP1 or RanBP2 antibodies overnight at 4°C. Samples were incubated with beads for 4 hours at 4°C, which were subsequently washed three times in lysis buffer and eluted with 2X Laemmli sample buffer at 95°C for 10 minutes.

**Immunological reagents**

Commercially available antibodies used in this study, including their use (immunoblotting, immunofluorescence, etc.), catalog numbers and specific dilutions are included in Table 2.2.

An antibody against RanBP2 was generated in-house for these studies. The DNA sequence encoding amino acids 1000-1200 was cloned into the pET28A using traditional PCR amplification to generate an amino-terminally tagged hexahistidine tagged version of the fragment. The cloning was verified by Sanger sequencing and resulting plasmid DNA was introduced into BL21 (DE3) E.coli for recombinant protein production. The expression of the 6HIS-tagged RanBP2 fragment was induced by the addition of IPTG for 22 hours at 18°C. Bacterial pellets that had been frozen and then thawed on wet ice, were diluted in 6HIS purification buffer (20mM Tris pH7.9, 500mM NaCl, 0.5% NP-40, 5mM Imidizole, 0.5mg/ml lysozyme, 0.5mM AEBSF, 1ug/mL apoprotinin, 1ug/mL pepstatin, 10ug/mL leupeptin, 1mM DTT). Cells were sonicated for five minutes and lysates was centrifuged at 15,000 rpm for 30 minutes at 4°C in a SS-34 fixed angle rotor. Soluble extracts were
incubated in batch with Ni-NTA agarose (Thermo) on a rotary mixer for 90 minutes at 4°C. Beads washed extensively with 20mM Tris pH7.5, 500mM NaCl, 0.5% NP-40, 30mM Imidizole and then eluted in 20mM Tris pH7.5, 200mM NaCl, 300mM Imidizole. Eluted samples were analyzed by Coomassie blue staining, combined, and tested by Bradford. 6HIS-RanBP21000-1200 was conjugated to KLH and injected into rabbits for antiserum production by Pocono Rabbit Farm & Laboratory (PRF&L, Canadensis, PA). The serum was affinity purified over a column of recombinant protein using described protocols and dialyzed into PBS (159).

For immunoblotting, antibodies were diluted in a solution of 5% nonfat dry milk in phosphate buffered saline, 0.05% tween 20 (PBST). Antibodies were either incubated at room temperature for 2 hours or overnight at 4°C. Detection was performed using HRP conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc; 1:10000), ECL reagent (Pierce), and exposure to film.

Mass Spectrometry analysis

Samples provided in solution were digested using the FASP (Filter assisted sample preparation) protocol. This includes reduction, alkylation, and digested with trypsin. The peptides were extracted, lyophilized, and resuspended in 2% acetonitrile/98% (0.1% formic acid). The peptides were loaded onto a 2 cm long X 360 µm o.d. × 100 µm i.d. microcapillary fused silica precolumn packed with Magic 5 µm C18AQ resin (Michrom Biosciences, Inc.). After sample loading, the precolumn was washed with 95% Solvent A (0.1% formic acid in water) /5% Solvent B (0.1% formic acid in Acetonitrile) for 20 min at a flow rate of 2 uL/min. The pre-column was then connected to a 360 µm o.d. × 75 µm i.d. analytical column packed with 22 cm of 5 µm C18 resin. The peptides were eluted at a flow
rate of 250 nL/min by increasing the percentage of solvent B to 40% with a Nano-Acquity HPLC solvent delivery system (Waters Corp.). The LC system was directly connected through an electrospray ionization source interfaced to an LTQ Orbitrap Velos ion trap mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was controlled by Xcalibur software and operated in the data-dependent mode in which the initial MS scan recorded the mass to charge (m/z) ratios of ions over the range 400–2000. The 10 most abundant ions were automatically selected for subsequent collision-activated dissociation. All files were searched using MASCOT (Matrix Science, Ver. 2.3.02) via Proteome Discoverer (Thermo., Ver. 1.3.0.339) against a recently downloaded human FASTA database. The search parameters included peptide mass tolerance of 10 ppm, fragment ion tolerance of 0.6 mass unit. The search allowed variable modifications for methionine oxidation and carbamidomethylation of Cys.

**Gel filtration chromatography**

Mitotically arrested 293T cells were analyzed by gel filtration chromatography. Cells were arrested overnight in nocodazole and lysed in hypotonic buffer as described above. The cell extract was clarified via centrifugation followed by filtration through a 0.22 µm syringe filter. Protein complexes in the clarified lysate were then separated using a size exclusion column (Superose 6 10/30, G.E. Healthcare) that had been pre-equilibrated in hypotonic lysis buffer. During separation, 0.4 mL fractions were collected and later analyzed by immunoblot and endogenous NUSAP1 IP.
Chromatin Fractionation

Cells were lysed in CSK buffer (10mM PIPES, pH 7.0, 300mM sucrose, 100mM NaCl, 3mM MgCl2, 0.1% triton X-100) supplemented with 1ug/mL apoprotinin, 1ug/mL pepstatin, 10ug/mL leupeptin, 1mM Na3VO4, 1mM NaF and 1mM AEBSF (4-[2Aminoethyl] benzenesulfonyl fluoride). Protein concentration was determined using Bradford and a portion of the lysate was taken for WCE samples. Samples were then pelleted at 3,000rpm for 5 minutes at 4°C. Supernatant was saved as the soluble fraction (S). Each pellet was washed with CSK buffer on ice and pelleted. The supernatant was removed and the pellet was resuspended in Laemmli buffer diluted in CSK and boiled for 5 minutes before the DNA was sheared using a needle to produce the insoluble fraction (I).

Immunofluorescence Imaging

Cells were plated on poly-L-Lysine coated coverslips approximately one day before fixation. Cells were fixed in PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM MgCl2, adjusted to pH 7.0 using KOH) + 3% PFA for 13 minutes at 37°C. Cells were washed with PHEM buffer and permeabilized using PHEM + 0.5% NP-40 for 15 minutes at room temperature. Cells were washed in PBS before blocking in PBS + 5% BSA. All antibodies were subsequently diluted in PBST + 5% BSA. Primary antibodies and their dilutions used: α-NUSAP1 (1:500), α- RanGAP1 (1:100), α-RanBP2 (1:100), α-tubulin (1:200), mouse anti-HEC1 (abcam ab3613; 1:500), guinea pig anti-CENP-C (MBL; 1:1000). Samples were incubated in primary antibody solution for 1h at 37°C. All fluorescent secondary antibodies (anti-mouse Alexa594, anti-rabbit Alexa488, anti-mouse Alexa488, anti-guinea pig Cy5) were diluted 1:200 dilution and incubated for 1h at 37°C. DNA was counter-stained with
1µg/mL Hoechst 33342 for 5 minutes at room temperature. All samples were mounted onto glass slides in ProlongGold media.

The cold stability assay was conducted as detailed in Suzuki et al. Nat Comm 2015 (160). Briefly, cells were treated with ice cold media for 10 minutes before fixation and staining. Proximity Ligation Assay (PLA) was performed using the Sigma Duolink In Situ Red Starter Kit Mouse/Rabbit (DUO92101 Sigma). Cells were plated and fixed as described above. Staining was performed following the DuoLink kit protocol, with primary antibodies against NUSAP1, RanBP2 and RanGAP1 being used at the concentrations described above. Tubulin counterstaining was performed using AlexaFluor488 conjugated α-tubulin at a dilution of 1:100 for 40 min at 37°C.

For image acquisition, 3D stacked images were obtained sequentially at 200 nm steps along the z-axis through the cell using MetaMorph 7.8 software (Molecular Devices) and a Nikon Ti inverted microscope equipped with the Orca-ER cooled CCD camera (Nikon) and an 100x/1.4 NA PlanApo objective (Nikon). X, Y, and Z stage movement was controlled by piezo MS2000-500 (ASI). Solid state laser (Andor) illumination at 488, 568, 647 nm were projected through Borealis (Andor) for uniform illumination before a spinning disc confocal head (Yokogawa CSU-10, Perkin Elmer) (161).
**Flow Cytometry**

Cells were collected and fixed in 70% ethanol. Cells were then washed in 1mL PBS twice, and then resuspended in a solution of PBS containing a final concentration of 25ug/mL propidium iodide (sigma) and 100ug/mL RNase A. Cells were sorted using a Beckman Coulter CyAn ADP. Data was analyzed using FlowJo software.
Figure 2.1. NUSAP1 is a cell cycle regulated microtubule binding protein. A) U2OS cells were synchronized by overnight treatment with nocodazole and released by mitotic shake-off. Samples were analyzed by immunoblot as cells progress through the cell cycle. B) NUSAP1 localization to the mitotic spindle analyzing by immunofluorescent imaging of mitosis in U2OS cells. (Scale bars = 10μM.) C) NUSAP1 localization was analyzed in nocodazole treated cells and following incubation with ice-cold buffer to destabilize non-kinetochore microtubules. (Scale bars indicate 5μM.) D) Single plane confocal imaging of NUSAP1 localization on the spindle during metaphase. Insets highlight two kinetochore-microtubule attachments. (Scale bars indicate 5μM.)
Figure 2.2. **NUSAP1 is cell cycle regulated.** U2OS Nocodazole release. Cells were arrested in 150ng/mL Nocodazole overnight and released by mitotic shake-off. Time points were taken every 2 hours until 10h.
Figure 2.3. NUSAP1 mitotic localization. NUSAP1 localization through mitosis. NUSAP1 localization to the spindle during mitosis in U2OS cells. Metaphase and Anaphase images are replicated from those shown in Figure 1B. Scale bars indicate 10um.
**Figure 2.4 NUSAP1 interacts with the RRU in a cell cycle dependent manner.** A) Venn diagram showing overlap of IP-MS/MS experiment results. B) Total Spectral Counts (TSC) for each of the RRU complex members determined by mass spectrometry. C) Endogenous NUSAP1 IPs were performed in four different nocodazole arrested cells and analyzed for RanBP2. D) Endogenous RanBP2 IP performed in nocodazole arrested 293T cells. E) Size exclusion chromatography was performed on extracts from nocodazole arrested 293T cells. Extracts were analyzed on a Superose 6 column. Previously tested size markers migrated in the indicated fractions. F) Endogenous NUSAP1 IPs were performed using each of the gel filtration fractions from (E). (*) indicates SUMOylated RanGAP1.
Figure 2.5 RanBP2 co-precipitates with NUSAP1 in both Nocodazole and Taxol arrested cells, and does not influence complex assembly. A) U2OS cells were either arrested using 200ng/mL Nocodazole or 500nM Taxol overnight before collection. Cells were then used for endogenous NUSAP1 IP. B) HEK-293T cells were depleted of NUSAP1 using one of two different siRNAs or a pool of siRNAs. Endogenous RanBP2 IPs were then performed, indicating RanBP2 is still able to associate with SUMOylated RanGAP1 when NUSAP1 is depleted.
Figure 2.6 NUSAP1 depletion does not affect mitotic localization of the RRU complex. A) Endogenous RanBP2 and RanGAP1 localization in both interphase and metaphase HeLa cells. B) Endogenous RanBP2 localization in either control or NUSAP1 depleted HeLa cells. C) Endogenous RanGAP1 localization in either control or NUSAP1 depleted HeLa cells. D) Chromatin fractionation in U2OS cells. Cells were transfected with either control or NUSAP1 targeting siRNA and split for overnight treatment with either DMSO or nocodazole. (WCE= whole cell lysate; S=soluble (cytoplasmic); I= insoluble (nuclear/chromatin). All scale bars indicate 10μM.)
Figure 2.7. NUSAP1 depletion in U2OS cells does not alter RanBP2 localization. Control and NUSAP1 depleted cells were fixed and stained with RanBP2, Tubulin, and CENP-C. (Scale bars indicate 10μM.)
Figure 2.8. NUSAP1 depletion in U2OS cells does not alter RanGAP1 localization. Control and NUSAP1 depleted cells were fixed and stained with RanGAP1, Tubulin and NUSAP1. (Scale bars indicate 10μM.)
Figure 2.9 NUSAP1 and RanBP2 interact in the cytosol of mitotic cells. A) PLA in U2OS cells using endogenous against NUSAP1, RanGAP1, RanBP2, or control IgG. Tubulin is shown in green with PLA signal in red. (Scale bars indicate 10μM) B) Average number of foci/cell for each PLA condition shown in A. Foci were counted using ImageJ.

Figure 2.10 RanBP2 knockdown sensitizes cells to taxol treatment. A) U2OS cells were transfected with control of RanBP2 targeting siRNA and then treated overnight with increasing doses of taxol. Cell cycle was analyzed by propidium iodide staining and flow cytometry. B) Immunoblot analysis of cells from (A)
Figure 2.11. NUSAP1 contains a SAP domain in its N-terminus. A) Schematics of NUSAP1 and PIAS1 as a representation of the PIAS family members, which all contain SAP domains approximately 10 AA away from their N-termini. B) Alignment of SAP domains of NUSAP1 and four PIAS protein family members.
### Table 2.1. siRNA oligonucleotides used in Chapter 2.

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### Table 2.2. Antibodies used in Chapter 2.

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CHAPTER 3: IDENTIFICATION OF A NOVEL SCF<sub>CYCLIN F</sub> TARGET, SIRTUIN 5, LINKING PROLIFERATION AND METABOLIC REGULATION

3.1 Introduction

Precise cell cycle progression is important for maintaining genomic integrity in dividing cells. Cell cycle is highly regulated, in part, by the ubiquitin-proteasome system, which targets proteins for degradation. One Cullin E3 ubiquitin ligase, a modular complex composed of Skp1, CUL1 and F-box protein (SCF) with the substrate adapter Cyclin F, has been identified as an important cell cycle regulator. Most recently, it has been recognized as having an important role in G1-S transition via feedback with the Anaphase Promoting Complex/Cyclosome with its substrate adapter Cdhl (APC/C<sup>Cdh1</sup>) during late G1 (79, 80).

Cyclin F is the founding member of the F-box containing family of proteins, most of which act as substrate adapters for the SCF E3 ubiquitin ligase (75–77). As a substrate adapter, Cyclin F recruits substrates to the SCF E3 ubiquitin ligase so that they may be ubiquitinated, which typically results in degradation via the proteasome (76, 77). Cyclin F contains a cyclin homology domain, and is the most highly cell cycle regulated F-box protein with expression during S, G2 and M, and is the only identified F-box protein in all cell cycle transcriptomic profiles performed to date (1, 75, 76). Despite its name, and the presence of a cyclin homology domain, Cyclin F is not a traditional cyclin and does not bind and activate a cyclin dependent kinase (CDK) protein (75, 78).
Identified Cyclin F targets to date include Centriolar coiled-coil protein of 110 kDa (CP110), which is involved in centrosome duplication, an event important for bipolar spindle establishment during mitosis (81, 90). Another substrate of Cyclin F is Nucleolar and Spindle Associated Protein 1 (NUSAP1), a protein implicated in G2-M transition, spindle stability and chromosome segregation (82, 91, 121, 125). Cyclin F also targets ribonucleoside-diphosphate reductase subunit M2 (RRM2), exonuclease 1 (Exo1) and cell-division control protein 6 homolog (cdc6) during S, all of which are important for DNA replication (83–85). RRM2 is important for biosynthesis of deoxyribonucleotides while Cdc6 is key for DNA replication initiation, and ensuring that the DNA is completely replicated before entering mitosis (86, 87). Exo1, as well as being important for DNA double strand break repair, is important for mismatch repair during DNA replication (88). Targeting of Stem-Loop Binding Protein (SLBP) for degradation by Cyclin F is also important in genotoxic stress response. SLBP promotes translation and accumulation of DNA damage response histones H2A.X and γH2A.X (94). In cycling cells with low genotoxic stress Cyclin F degradation of SLBP helps promote mitotic entry (94). Finally, Cyclin F has been shown to regulate G1-S transition via regulation of another E3 ubiquitin ligase, the APC/C, specifically through regulation of its substrate adapter, Fizzy-related protein homolog Cdh1 (79). During early G1, APC/C<sup>Cdh1</sup> targets Cyclin F for degradation, however, as Cyclin F protein levels rise, AKT-mediated phosphorylation of Cyclin F promotes its assembly into the SCF, which then targets Cdh1 for degradation (79, 80). This switch is important for promoting the G1-S transition (79, 80).
While a number of Cyclin F substrates have been characterized, using traditional protein-protein interaction, mass spectrometry (MS) based approaches to identify novel Cyclin F substrates has proven challenging. This is, in part, due to the transient nature of F-box-substrate interactions, a feature common to E3 ubiquitin ligase and substrate interactions. Furthermore, the substrate is typically being targeted for degradation, making it difficult to capture. Finally, these experiments are often performed in asynchronous cells, which is problematic because Cyclin F, and many of its substrates, are cell cycle regulated, making it difficult to detect in asynchronous cell extracts. In this report, based on genetic yeast interactions and conservation in the ubiquitin system, we examined whether a human sirtuin could be regulated by Cyclin F.

Here we identify Sirtuin 5 (Sirt5) as a novel SCF\textsuperscript{Cyclin F} target important for the G1-S transition. Sirtuins are a class of deacylating enzymes involved in regulating a variety of processes including epigenetic regulation, DNA damage response and metabolism (162, 163). This is the first described role for Sirt5, a mitochondrial sirtuin, in cell cycle control. Sirt5 has specific deacylating activity towards succinyl, malonyl, glutaryl and acetyl post-translational modifications (PTMs) (164–167). Sirt5 is one of three mitochondrial sirtuins, (Sirtuins 3-5), which are classified by their ability to be imported into the mitochondria, but are not restricted to the mitochondria. Sirt5 also localizes to the cytoplasm and nucleus, however its roles outside of the mitochondria are less clear. Sirt5 is best known for its role in regulating metabolic enzymes, such as carbamoyl phosphate synthase 1 (CPS1), which catalyzes the production of carbamoyl phosphate from ammonia and bicarbonate, which is the first step of the urea cycle (168–170). Sirt5 is also known
to desuccinylate and activate Cu/Zn superoxide dismutase (SOD1), which functions in reactive oxygen species response (171, 172). Sirt5 has been implicated in a number of other metabolic processes as well, including glycolysis, purine metabolism, fatty acid oxidation, as well as the citrate cycle (173). While it is known that Sirt5 is localized to the mitochondria as well as the nucleus and cytoplasm, it has yet to be determined whether it has different activities based on localization.

Here we describe the interaction between Cyclin F and Sirt5, and its impact on the G1-S transition. These data provide a link between cell cycle ubiquitin machinery, and metabolic regulation, a connection of which little has been described.

3.2 Results

Cyclin F was originally identified in a gain-of-function cDNA screen searching for human genes that could rescue the yeast Cdc4 temperature sensitive mutant, which caused G1 arrest and subsequent death (75). Cyclin F was identified as a gene that rescued the G1 arrest phenotype, and based on the fact that it cycled and contained a cyclin homology domain, Cyclin F was classified as a traditional Cyclin (75). However, subsequent studies identified Cyclin F as a substrate adapter for the SCF E3 ubiquitin ligase, and Cdc4 is a yeast F-box substrate adapter (174). Furthermore, Cyclin F can target yeast proteins for degradation, including yeast Cdh1. Knowing this information, we hypothesized that Cyclin F rescued the G1 arrest phenotype in the original Cdc4 mutant screens by targeting a protein for degradation, particularly a protein which prevents S-phase entry. Based on this hypothesis, we interrogated the Data Repository of Yeast Genetic Interactions
(DRYGIN), a database containing data from global synthetic genetic interactions in yeast, for mutants or deletions that also rescue the Cdc4 mutant phenotype as a tool to identify new potential Cyclin F targets (175). To narrow our list down we also compared this to a list of deletions that rescue the Cdc53 mutant G1 arrest phenotype as well (Figure 3.1). Cdc53 is a yeast cullin, which complexes with the F-box protein, Cdc4, to form an E3 ubiquitin ligase. A list of ~40 genes that rescued the G1 arrest and lethality of both the Cdc4 and Cdc53 mutations was identified, which we narrowed down by first eliminating all the genes that had no connection to cell cycle, either directly or through genetic interactions. We then eliminated apc5, orc3 and mcm3 due to their incorporation into large protein complexes with low turnover rates. The yeast specific transcription factor, swi5, was eliminated because no human homologue has been identified. Finally, we chose not to interrogate sli15 because it is a component of the Aurora B complex, which is only active in mitosis. This eliminated all but one gene; hst3, a yeast sirtuin family deacetylase. When we looked at genes with similar genetic interactions to hst3, what we identified was a list of genes important for DNA replication and DNA damage response, indicating a potential role in S-phase entry and progression. Interestingly, hst3 is ubiquitinated by the yeast SCF\textsuperscript{Cdc4} ligase.

\textit{Sirtuin 5 stability is increased in the absence of Cyclin F}

Since our screen identified a yeast sirtuin as a potential Cyclin F target, we analyzed human sirtuin levels in Cyclin F CRISPR KO HeLa cells, with the expectation that if a sirtuin were a Cyclin F substrate, there would be more sirtuin protein in the Cyclin F KO cells compared to control. Immuno-blotting for human sirtuins in Cyclin F CRISPR KO HeLa cells showed an increase in the mitochondrial
sirtuin, Sirt5 as well as a slight increase in Sirt7 (Figure 3.2A). Elevated Sirt5 protein levels, as well as the known Cyclin F targets Cdh1 and CP110, was observed in both U2OS and 293T cells treated with multiple Cyclin F targeted siRNAs compared to control (firefly luciferase targeted) (Figure 3.2B and C).

To establish whether this elevation in protein level was due to an increase in protein stability, we performed cycloheximide chase in both the control and Cyclin F CRISPR KO HeLa cells (Figure 3.2D). Cycloheximide prevents translation of new protein from mRNA, allowing us to measure half-life of the proteins present at time of treatment. Cells were treated with cycloheximide and samples were collected every two hours, for eight hours, and samples were analyzed via immunoblot. Cycloheximide treatment of control cells showed that both Sirt5 and Sirt7 have a half-life between 3-6 hours, consistent with previously published data for both Sirt5 and Sirt7 (Figure 3.2D) (176, 177). No difference in Sirt7 half-life was observed in Cyclin F CRISPR KO HeLa cells, however a marked increase in the half-life of Sirt5 protein was observed, with the protein becoming so stable the half-life was greater than eight hours (Figure 3.2D). Together, these data show that Sirt5 protein is more stable in the absence of Cyclin F.

**Sirtuin 5 interacts with Cyclin F**

To determine if Cyclin F and Sirt5 can interact, we performed Myc and Flag immuno-precipitations (IPs) from cells co-expressing a Flag-Sirt5 together with Myc-Cyclin F in 293T cells, in the presence or absence of the proteasome inhibitor Bortezomib. In the Myc-Cyclin F IPs, Flag-Sirt5 signal was detected in the Bortezomib treated sample, indicating an interaction (Figure 3.3A). This was confirmed by the reverse Flag-Sirt5 IP, using the same samples, showing Myc-
Cyclin F co-precipitating in both the co-expressed samples (Figure 3.3A).

Furthermore, there was an increase in the amount Myc-Cyclin F protein pulled down in the Bortezomib treated sample compared to co-expression alone (Figure 3.3A).

To further confirm this interaction, we transiently expressed a Flag-Cyclin F and HA-Sirt5 in U2OS cells for 48h. The cells were fixed and analyzed by Proximity Ligation Assay (PLA), using primary antibodies against Flag and HA epitopes. PLA allows for fluorescent detection of each site of interaction. In negative controls, which were transfected with empty Flag or HA vectors, or empty vector with either Flag-Cyclin F or HA-Sirt5, no PLA signal (red) was detected (Figure 3.4). In cells co-expressing Flag-Cyclin F and HA-Sirt5, PLA signal was detectable throughout the cell. These data confirm an interaction between Cyclin F and Sirt5, and suggests that it occurs in the cytoplasm of cells (Figure 3.5). This conclusion is supported by mitochondrial fractionation data, which shows Cyclin F is excluded from the mitochondria while Sirt5 is found in both the mitochondria and cytoplasm (data not shown).

**SCF\(^\text{Cyclin F}\) can ubiquitinate Sirtuin 5**

To establish whether Cyclin F could ubiquitinate Sirt5 we performed and *in vivo* ubiquitination assay. For this assay, we express a hexa-HIS-ubiquitin construct in cells, together with our potential substrate and substrate adapter. Ubiquitin is covalently linked to substrates, so the use of HIS-Ubiquitin allows for lysis and subsequent pulldowns under denaturing conditions, to distinguish between ubiquitinated and ubiquitin-interacting proteins. If a protein is ubiquitinated, a higher molecular weight species will appear in the HIS pulldown sample when immuno-blotted. Expression of HA-Sirt5 with HIS-ubiquitin alone did not produce any signal in the HIS pulldown, however, the addition of Myc-Cyclin F resulted in a
multiple banding pattern of HA signal in the HIS pulldown, indicating that Sirt5 is ubiquitinated in the presence of Cyclin F (Figure 3.5). Further experiments, such as *in vitro* ubiquitination assays, are needed to confirm this result.

**Sirt5 protein levels influence G1 timing**

To date, identified Cyclin F targets have been shown to play key roles in cell cycle progression. However, while Sirt5 has been implicated in a number of metabolic processes, it has no obvious role in cell cycle. To determine whether Sirt5 could play a role in cell cycle progression, particularly G1-S transition, we performed flow cytometry analysis of propidium iodide (PI) stained Sirt5 CRISPR KO 293 cells. Sirt5 CRISPR KO 293 cells exhibit a notable decrease in the G1 population (~20%) compared to control, which is compensated for by an overall increase in S/G2/M (Figure 3.7). When compared to controls, the Sirt5 CRISPR KO cells also had elevated Cyclin A protein levels, a cyclin that is expressed during S/G2/M (Figure 3.7). To confirm that this cell cycle alteration was due to Sirt5 loss, we re-expressed wild type Flag-Sirt5, and the catalytically dead mutant, Flag-Sirt5<sup>HY</sup>, and performed PI staining and flow analysis. Re-expression of the Flag-Sirt5<sup>WT</sup> for either 48h or 72h, rescued the cell cycle phenotype, while re-expression of Flag-Sirt5<sup>HY</sup> rescued to a lesser degree (Figure 3.7). Together, this data indicates that Sirt5 protein levels influence G1-S transition timing.

While a clear redistribution of cell cycle phases occurs in the absence of Sirt5, Sirt5 KO cells appeared to have the same doubling time as control cells. This suggests that Sirt5 CRISPR KO cells spend less time in G1, but more time in S/G2/M. To determine why cells were spending more time in subsequent phases we examined DNA damage pathway activation. If cells exit G1 prematurely, we would
expect these cells would undergo replication stress, and possibly accumulate DNA
damage, which would slow S-phase progression. To test this hypothesis, control
and Sirt5 CRISPR KO cells were immuno-blotted for phospho-Chk1 (p-Chk1
(S345)), a protein that is phosphorylated by ATR in response to DNA damage.
Analysis showed that p-Chk1 was elevated in Sirt5 CRISPR KO cells compared to
controls, indicating an activated DNA damage response (Figure 3.8). Further
experiments are needed to examine activation of other DNA damage response
proteins, and to determine whether this damage is linked to replication stress.

*Sirt5 protein levels increase with G0 arrest*

Since Sirt5 expression increases the number of cells prior to the start of DNA
replication, we analyzed Sirt5 protein levels in proliferating and quiescent cells. We
arrested normal human fibroblast (NHF) cells in G0, using either serum depletion or
contact inhibition. Interestingly, G0 arrest resulted in an increase in Sirt5 protein,
as well as a decrease in Cyclin F protein levels, compared to cycling cells (Figure
3.9A). Furthermore, RPE1 cells grown in varying amounts of serum show a clear
correlation between Sirt5 protein level and serum concentration, with more Sirt5
accumulation in cells grown in lower concentrations of serum (Figure 3.9B). This
further supports that as cells enter G0 Sirt5 protein levels increase, however, more
data is needed to determine whether Sirt5 plays a role in establishing and
maintaining G0 arrest.

**3.3 Discussion**

Cyclin F has proven to be a key regulator of cell cycle progression, even
though only a handful of substrates have been identified. Cyclin F regulation of
Cdh1 is important for G1-S transition, while its regulation of CP110 and NUSAP1 is important for G2/M progression.

During G1, both Cyclin F levels and activity, are tightly regulated by both APC/C\textsuperscript{Cdh1} and AKT signaling. This regulation is important for G1-S transition timing, however, only a couple of Cyclin F substrates have been linked to G1 exit/S-phase entry. In an attempt to identify Cyclin F targets that are important in G1-S transition, we utilized the DRYGIN database to identify potential substrates based on conservation of the ubiquitin system in yeast. Ultimately, we identified human Sirt5, a mitochondrial deacetylating enzyme, as a potential Cyclin F substrate.

Sirt5 has been linked in numerous ways to metabolic regulation, however, it has not been previously linked to cell cycle regulation. Our data indicate that Sirt5 protein levels influence G1-S transition timing, with Sirt5 depletion resulting in premature G1 exit and extended S/G2/M. This phenotype is common to other key G1-S transition regulators, including Cdh1 depletion or Cyclin E overexpression (178, 179). Importantly, using EdU incorporation assays, it has previously been shown that when Cyclin F protein is depleted, cells spend longer in G1 compared to control cells (79). In addition, Cyclin F null MEFs are slow to enter S after release from serum withdrawal (77). We hypothesize, that along with targeting other substrates, Sirt5 is an important SCF\textsuperscript{Cyclin F} target for G1 exit, and Sirt5 levels must dip below a certain threshold for cells to enter S phase (Figure 3.10). Furthermore, maintaining higher Sirt5 levels may be important for maintaining a G0 arrest. This could be linked to metabolic reprogramming that occurs in G0 cells. For instance, the urea cycle, which Sirt5 has been shown to promote, is increased in quiescent cells (180). Future research will focus on whether particular Sirt5 targets, and
possible affected metabolites, are most important for G1-S transition, as well as which pool of Sirt5 is being regulated by Cyclin F (i.e. cytoplasmic vs mitochondrial). As it is possible that Sirt5 regulated metabolites involved in G1-S transition are different from those involved in G0 regulation, experiments will also focus on G0 establishment and maintenance.

Surprisingly, Sirt5 protein levels are increased in some cancer types, including breast cancer and non-small cell lung cancers (NSLCs) (181, 182). It has been shown that increased Sirt5 protein levels in NSLCs may promote resistance to nucleoside analogs such as 5-fluorouracil. 5-fluorouracil is metabolized into an analog of uracil and can be incorporated into mRNA during transcription, which prevents that RNA from being read correctly by ribosomes. Furthermore, it inhibits production of thymidine triphosphate, which is needed for DNA synthesis. Cells with increased Sirt5 protein levels may be more resistant because they are spending more time in G1 or possibly even G0. However, Sirt5 has been implicated in de novo DNA synthesis, and it is possible that with increased Sirt5, there is increased nucleotide synthesis, preventing the 5-fluorouracil from being incorporated as much as it would be in Sirt5 low cells. Future experiments are needed to better understand the role of Sirt5 in cancer survival and cell cycle.

Together, our data describe a new Cyclin F target, Sirt5, and support a novel role for Sirt5 in cell cycle progression. This provides a novel link between metabolism and cell cycle progression. Future research is required to better understand which metabolites are being influenced by Sirt5 degradation, and how they promote or inhibit cell cycle.
3.4 Materials and Methods

Mammalian cell culture

HEK-293T, U2OS, RPE-1, NHF and HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% FBS (Seradigm, VWR) and Pen/Strep (Gibco). Sirtuin 5 CRISPR KO and control 293 cells, a generous gift from Matthew Hirschey, were grown in DMEM. Nocodazole (Sigma 487928) was used at 150 ng/mL for U2OS and 200 ng/mL for 293T. All siRNA transfections were performed using Lipofectamine RNAiMax (Thermo) following manufacturers protocol. Control, non-specific siRNA targeted firefly luciferase (siFF). Two different siRNAs against Cyclin F were used, each at a concentration of 30nM. The siRNA oligonucleotide sequences used in this study are detailed in Table 3.1. All plasmid transfections were performed using lipofectamine 2000 (Thermo) or lipofectamine 3000 (Thermo) according to manufacturer protocols.

Immunoblotting and immunoprecipitations

Samples analyzed by immunoblot were lysed in NETN (20mM Tris-Cl, pH 8.0, 100mM NaCl, 0.5mM EDTA, 0.5% Nonidet P-40 (NP-40)) supplemented with 1ug/mL apoprotinin, 1ug/mL pepstatin, 10ug/mL leupeptin, 1mM Na3VO4, 1mM NaF and 1mM AEBSF (4-[2Aminoethyl] benzenesulfonyl fluoride). Protein concentration was estimated using the Bradford assay (Bio-Rad). Laemmli buffer was added to samples, which were then separated by SDS-PAGE gel electrophoresis using home-made or commercially available gels (Bio-Rad). Gels were transferred to nitrocellulose membranes and blotted using standard immunoblotting procedures.

For the co-IP experiments in Figure 2, cells were lysed in NETN, supplemented with 1ug/mL apoprotinin, 1ug/mL pepstatin, 10ug/mL leupeptin,
1mM Na3VO4, 1mM NaF and 1mM AEBSF (4-[2Aminoethyl] benzenesulfonfyl fluoride). Protein A/G DynaBeads (Thermo) were bound to control rabbit IgG, Flag or Myc antibodies overnight at 4°C. Samples were incubated with beads for 4 hours at 4°C, which were subsequently washed three times in lysis buffer and eluted with 2X Laemmli sample buffer at 95°C for 10 minutes.

For the *in vivo* ubiquitination assay, cells were transfected with a combination of empty pcDNA3.1, 6XHIS-Ubiquitin, pcDNA-Sirt5-HA and Myc-Cyclin F (see figure for combinations) for a total of 5ug DNA/transfection using lipofectamine 2000 (Thermo). Cells were treated with 10 uM MG132 4h before harvesting. HIS pulldowns were performed as previously described in Choudhury et al. 2016, 80% cell suspension was lysed in denaturing conditions in buffer 1 (6M Guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl [pH 8.0], 15mM Imidizole, and 10mM β-mercaptoethanol [βME]). Lysates were sonicated and loaded onto pre-washed Ni2+-NTA resin and incubated for 4h, rotating, at room temperature. Samples were then washed three times, first using buffer 1, followed by buffer 2 (8M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCL [pH 8.0]), then buffer 3 (8M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCL [pH 6.3]) plus 0.2% Triton X-100, and finally with buffer 3 plus 0.1% Triton X-100. Samples were then eluted by incubating 20min in buffer 4 (200 mM imidazole, 0.15 M Tris/HCl [pH 6.7], 30% glycerol and 0.72M βME, 5% SDS). The remaining 20% of cell suspension was lysed in NETN supplemented with 1ug/mL apoprotinin, 1ug/mL pepstatin, 10ug/mL leupeptin, 1mM Na3VO4, 1mM NaF and 1mM AEBSF and protein content was measured using Bradford assay. Samples were analyzed by immune-blot.
**Immunological reagents**

Commercially available antibodies used in this study, including their use (immunoblotting, immunofluorescence, etc.), catalog numbers and specific dilutions are included in Table 3.2.

For immunoblotting, antibodies were diluted in a solution of 5% nonfat dry milk in phosphate buffered saline, 0.05% tween 20 (PBST). Antibodies were either incubated at room temperature for 2 hours or overnight at 4°C. Detection was performed using HRP conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc; 1:10000), ECL reagent (Pierce), and exposure to film.

**Flow Cytometry**

Cells were collected and fixed in 70% ethanol. Cells were then washed in 1mL PBS twice, and then resuspended in a solution of PBS containing a final concentration of 25ug/mL propidium iodide (sigma) and 100ug/mL RNase A. Cells were sorted using a ThermoFisher Attune Nxt. Data was analyzed using FlowJo software.
Figure 3.1. Schematic representing results of DRYGIN screen for potential Cyclin F substrates. To search for potential Cyclin F substrates, the DRYGIN database was interrogated for gene deletions or mutations that would rescue both the yeast Cdc4 and Cdc53 mutant cell cycle arrest phenotypes. Gray circles indicate genes with no connection to cell cycle regulation either directly, or through genetic interactions. Yellow circles indicate genes that are cell cycle relevant, but have been eliminated due to low turnover rate, no role in G1 or S, or having no human homolog. Green circle indicates potential Cyclin F target.
Figure 3.2 Sirt5 stability is increased in the absence of Cyclin F. A) Sirt5 protein levels are increased in Cyclin F CRISPR KO HeLa cells compared to controls. B) and C) Sirt5 protein levels, as well as known Cyclin F targets CP110 and Cdh1, are increased in both U2OS (B) and 293T (C) cells depleted of Cyclin F for 48h using 30-50nM siRNA. D) Cycloheximide treatment in both control and Cyclin F CRISPR KO HeLa cells. Sirt5 protein half-life is extended in Cyclin F CRISPR KO cells compared to controls while Sirt7 remains the same between cells lines.
Figure 3.3 Sirt5 and Cyclin F co-immunoprecipitate. Flag-Sirt5 and Myc-Cyclin F were co-expressed in 293T cells, with or without 150nM Bortezomib treatment. Cells were then lysed and split between Flag and Myc IPs. Exp=Exposure.
Figure 3.4 Sirt5 and Cyclin F interact in cells. U2OS cells were co-transfected with HA-Sirt5 and Flag-Cyclin F, fixed and used for PLA to detect site of interaction. PLA signal (red foci) is visible in cells throughout the cytoplasm.
Figure 3.5. Sirt5 is ubiquitinated in the presence of Cyclin F. 293T cells were transfected with a combination empty vector (pcDNA), 6HIS-Ubiquitin, HA-Sirt5 or Myc-Cyclin F. Cells were treated with MG132 and lysed under denaturing conditions, and used for HIS pulldowns.

Figure 3.6. Sirt5 CRISPR KO cells exhibit redistribution of cell cycle phases. A) Control and Sirt5 CRISPR KO cells were stained using propidium iodide and analyzed using flow cytometry. Sirt5 KO cells show clear decrease in G1 population in comparison to controls. B) Immunoblot analysis of control and Sirt5 CRISPR KO cells show that Sirt5 KO cells have elevated levels of the S/G2/M cyclin, Cyclin A.
Figure 3.7. Sirt5 protein expression influences G1 timing. A) Cell cycle distribution of control or Sirt5 CRISPR KO 293 cells using propidium iodide staining. Cells were transfected with either empty, Flag-Sirt5\textsuperscript{WT} or Flag-Sirt5\textsuperscript{HY} plasmids and cell cycle was analyzed both 48h and 72h post-transfection. B) Western blot of samples from A.
Figure 3.8. Sirt5 KO cells exhibit activated DNA damage response. Sirt5 CRISPR KO 293 cells have increased p-Chk1 (S345), which is phosphorylated by ATR in response to DNA damage, compared to control cells.

Figure 3.9. Sirt5 protein levels are increased in G0 arrested cells. A) NHF cells were grown under normal conditions, contact inhibited, or no serum (FBS) for 48h. B) RPE1 cells grown in normal media were transferred to media containing lower concentrations of FBS for 24h and then collected and analyzed via immune-blot.
Figure 3.10. Proposed model for Sirt5 role in cell cycle progression. Our data indicate that Sirt5 protein must decrease to a specific threshold for cells to exit G1, and this protein decrease is regulated by protein degradation mediated by SCF$^{\text{Cyclin F}}$. Cells with increase Sirt5 may not effectively exit G1, or it may promote G0 entry.
Table 3.1 siRNA oligonucleotides used in Chapter 3.

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Table 3.2 Antibodies used in Chapter 3.

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Table 3.1 siRNA oligonucleotides used in Chapter 3.

Table 3.2 Antibodies used in Chapter 3.
The SCF\textsuperscript{Cyclin F} is a key regulator of cell cycle progression. Substrate ubiquitination and degradation by Cyclin F is important in regulating key processes, including DNA replication and mitotic progression. The research reported here identifies further roles for both Cyclin F and its substrates in both G1/S progression and mitotic processes. We show that the Cyclin F substrate; Nucleolar and Spindle Associated 1 (NUSAP1) interacts with a SUMO E3 ligase during mitosis. Furthermore, we identify the metabolic enzyme, Sirtuin 5, as a new Cyclin F substrate and demonstrate that Sirt5 protein levels impact G1/S progression. While both of these projects shed new light on the functions of Cyclin F in proliferating cells, more work is needed to understand the impact of these regulations.

NUSAP1 promotes spindle stability and chromosome segregation in mitotic cells, however, it is unknown how it functions. The above works (Chapter 2) identify a cell cycle regulated interaction between NUSAP1 and a SUMO E3 ligase composed of Ran Binding Protein 2 (RanBP2), Ran GTPase Activating Protein 1 (RanGAP1) and the SUMO E2 conjugating enzyme, UBC9. Furthermore, we show this interaction occurs independent of microtubules, in the cytoplasm of mitotic cells. While we were able to characterize this interaction, the functional meaning of this interaction remains unclear. Further research is needed to understand how NUSAP1 interacts with the complex and completely rule out whether NUSAP1 is a SUMO substrate of
this complex. If it is not, the role of NUSAP1 in the RanBP2 SUMO E3 ligase needs to be determined.

Little is known about how SUMO E3 ligases identify and interact with substrates. Based on a SAF-A/B, Acinus, PIAS protein domain (SAP domain) within NUSAP1, we speculate that NUSAP1 could be important for identification, and subsequent SUMOylation of some RanBP2 SUMO E3 ligase substrates. SAP domains are present at the N-terminus in all PIAS proteins, the most highly characterized class of SUMO E3 ligases, and are important for the identification and SUMOylation of a subset of PIAS SUMO targets. The RanBP2 complex does not contain a SAP domain, however, NUSAP1, like PIAS proteins, contains a SAP domain at its N-terminus. If NUSAP1 is important for RanBP2 substrate identification in mitotic cells, it would greatly further our understanding of how the RanBP2 SUMO E3 ligase functions.

In Chapter 3 we identify Sirt5 as a novel SCF<sup>Cyclin F</sup> substrate. Sirt5 is a mitochondrial deacylating enzyme that regulates a number of metabolic processes. Interestingly, our data show that Sirt5 may also have a role in G1/S progression, and possibly quiescence establishment and maintenance. While this data is promising, more research is needed to better characterize Sirt5 regulation, as well as the cell cycle phenotypes associated with Sirt5 overexpression or depletion. Cell cycle synchronization experiments are needed to determine when Sirt5 is targeted for degradation. Furthermore, more ubiquitination assays are needed to confirm Cyclin F mediated ubiquitination of Sirt5.
Our data show the Sirt5 protein levels influence cell cycle distribution. We hypothesize that Sirt5 is likely regulating a metabolic process that feeds back into cell cycle regulation. A number of metabolic processes, that have been previously identified as cell cycle regulated, are good candidates for this regulation, including nucleoside and amino acid metabolism as well as glycolysis. These are processes Sirt5 has already been implicated in regulating. Metabolic assays performed in both Sirt5 and Cyclin F knockout or over expression are needed to determine whether this is true.
REFERENCES


APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J. Cell Biol.* **154**, 925–936


Functions of Ubiquitin in Endocytosis and Signaling. *Science*. 315, 201–205


RG7388, a Potent and Selective p53−MDM2 Inhibitor in Clinical Development. 


