A Dissertation

Entitled

The Regulation of Sororin by Phosphorylation

by

Megan Renee Dreier

Submitted to the Graduate Faculty as partial fulfillment of the
requirements for the Doctor of Philosophy Degree in Biology

___________________________________
William Taylor, Ph.D., Committee Chair

________________________________
Max Funk, Ph.D., Committee Member

________________________________
Song-Tao Liu, Ph.D., Committee Member

________________________________
John Plenefisch, Ph.D., Committee Member

________________________________
Kam Yeung, Ph.D., Committee Member

________________________________
Patricia Komuniecki, Ph.D., Dean
College of Graduate Studies

The University of Toledo

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An Abstract of

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Cell cycle control mechanisms are altered in cancer cells, often leading to chromosomal instability; however, these mechanisms are not fully characterized. Cohesin, a protein complex consisting of Smc1, Smc3, Scc1, and Scc3, protects sister chromatids from separating at inappropriate times. In addition, certain accessory proteins to cohesin, including Sororin, are required for cohesion maintenance. Sororin is phosphorylated in mitosis; however, the role of this modification is unclear. Here we show that mutation of potential Cdk1 phosphorylation sites leaves Sororin stranded on chromosomes and bound to cohesin throughout mitosis. These results were obtained by mutating nine Cdk1 consensus sites to alanine to create Sororin$^{9A}$. The nine phosphorylation sites are evolutionarily conserved in vertebrates. Interestingly, recombinant Cdk1/Cyclin B kinase can poorly phosphorylate Sororin$^{9A}$ in vitro, but highly phosphorylates Sororin$^{WT}$. Also, Sororin$^{WT}$ exhibits a reduced electrophoretic mobility in vivo as a result of phosphorylation. Sororin$^{9A}$ does not exhibit a reduced electrophoretic mobility when isolated from cells. This suggests that Cdk1 is the kinase responsible for phosphorylating Sororin in vivo. Sequence analysis uncovered a potential
cyclin interaction motif in Sororin. Key amino acids of the cyclin interaction motif were also mutated but this did not cause a significant phenotype. Sororin can be precipitated from cell lysates with DNA-cellulose, and only the hypophosphorylated form of Sororin shows this association. These results further suggest that phosphorylation of Sororin causes its release from chromatin in mitosis. Knocking-down Sororin with shRNA causes premature loss of cohesion whereas overexpressing Sororin \textsuperscript{9A} increases cohesion between sister chromatid arms. Altogether, these results suggest that the role of mitotic phosphorylation of Sororin by Cdk1 is to remove Sororin from the cohesion complex thereby disrupting its cohesin-stabilizing function, to allow removal of cohesion from chromosome arms. Phosphorylation-deficient Sororin can alleviate the mitotic block that occurs upon knock-down of endogenous Sororin indicating that phosphorylation is not required for Sororin to stabilize cohesion. The mitotic block that occurs upon Sororin localization is abrogated by the Aurora kinase inhibitor ZM447439, indicating that prematurely separated sister chromatids activate the spindle assembly checkpoint via an Aurora kinase-dependent pathway. Future work will focus on creating and analyzing phosphomimics of sororin.
For Norman, my grandpa. You lost the battle to cancer, but I am still waging your war.
Wow, what a journey! I cannot believe my pre-doctoral training is complete. My research experience first began in May 2004 as an undergraduate in the laboratory of my dear advisor, Dr. William R. Taylor, affectionately known as BT. I had just finished my freshman year of college and having very little basis of biology, BT graciously allowed me to work in his lab. I excitedly began my work on Sororin, which was at the time known as CDCA5. About two years into my fruitless research, BT gave me the option to abandon the Sororin project and start something new. I stubbornly declined his offer, and the rest is, well, history (aka my dissertation). Thank you BT, as I am forever indebted to you. Also, I would like to thank Drs. Max Funk, Song-Tao Liu, John Plenefisch, and Kam Yeung for serving as my committee members and providing constructive criticism to my dissertation, and current and former members of BT’s laboratory. Finally, I want thank my family, especially my parents, Gary and Suzanne, and brother, Douglas, for being my support system and helping me through some of the most difficult times of my life.
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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APC/C</td>
<td>Anaphase Promoting Complex/Cyclosome</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CDCA5</td>
<td>Cell Division Cycle Associated 5</td>
</tr>
<tr>
<td>CPC</td>
<td>Chromosomal Passenger Complex</td>
</tr>
<tr>
<td>CARs</td>
<td>Cohesin-Attachment Regions</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDK1-AF</td>
<td>Cyclin dependent kinase T14A Y14F</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Break</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>E2F</td>
<td>E2F promoter binding factor</td>
</tr>
<tr>
<td>ECO</td>
<td>establishment of mitotic (sister) chromatid cohesion</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>G1/2</td>
<td>Gap1/2</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S Transferase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>INCENP</td>
<td>Inner Centromeric Protein</td>
</tr>
<tr>
<td>NOC</td>
<td>Nocodazole</td>
</tr>
<tr>
<td>NB1</td>
<td>Nuclear Cyclin B1</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Plk1</td>
<td>Polo-like kinase 1</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polvinyldifluoride</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>RFC</td>
<td>Replication Factor C</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin Ribonucleic Acid</td>
</tr>
<tr>
<td>SGO</td>
<td>Shugoshin</td>
</tr>
<tr>
<td>SCC</td>
<td>Sister Chromatid Cohesion</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium Fluoride</td>
</tr>
<tr>
<td>SAC</td>
<td>Spindle Assembly Checkpoint</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>SMC</td>
<td>Structural Maintenance of Chromosomes</td>
</tr>
<tr>
<td>S phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>OA</td>
<td>Okadaic acid</td>
</tr>
<tr>
<td>Wapl</td>
<td>Wings apart-like</td>
</tr>
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The Regulation of Sororin by Phosphorylation

Introduction

1.1 Cancer and Cell Cycle

Cancer kills over half a million people per year in the United States and is second to heart disease as the leading cause of death. Cancerous cells are different from normal cells in that they are characterized by uncontrolled cell division, and the ability to invade areas that are typically reserved for other cells. Uncontrolled growth leads to large masses that are referred to as tumors or neoplasms. Neoplasms can be either benign, in that they are not invasive, or malignant, in that they invade the surrounding tissues. Many cell cycle control mechanisms are altered in cancer cells, leading to chromosomal instability, but are poorly characterized.

The cell cycle consists of two phases, which are referred to as interphase and mitosis. Interphase is subdivided into Gap 1 (G1), DNA synthesis (S), and Gap 2 (G2). G1, which is between mitosis and S phase, allows the cell to gauge its internal and external environment. Interestingly, if conditions are unfavorable, the cell enters a rest period known as G0. The cell can spend days, months, and even years in the G0 state and can even resume proliferation as conditions become favorable. S phase is where
DNA is synthesized, resulting in the duplication of chromosomes. Therefore, for a proliferating cell, S phase takes up half the cell cycle time. G2 occurs between S phase and mitosis and is where the cell makes the appropriate preparations for division, consisting of cell growth and protein synthesis. Mitosis consists of prophase, prometaphase, metaphase, anaphase, and telophase. Prophase is where chromosomes condense and the mitotic spindle is assembled. Prometaphase marks the breakdown of the nuclear envelope and the attachment of chromosomes to microtubules via the kinetochores. Also, the centrosomes are at the spindle poles. During metaphase, chromosomes align along the equator between the spindle poles via kinetochore-microtubule attachments. At anaphase, the sister chromatids become daughter chromosomes and are each pulled to opposite ends of the spindle, shortening kinetochore-microtubule attachments. These actions cause the segregation of chromosomes. Telophase is where the chromosomes arrive at the spindle poles and decondense. Also, the nuclear envelope reassembles around each set of chromosomes, the contractile ring, which consists of actin and myosin filaments, begins to contract. During cytokinesis, the cytoplasm separates, pinching the cell into two daughters, each with an intact nucleus.
1.2 Control of cell cycle events by CDKs

Cyclin-dependent kinases (Cdks) are protein kinases involved in the regulation of the cell cycle and are activated by Cyclins. There are four classes of cyclins that include Cyclins D, E, A, and B. Cyclin D activates Cdk4 and Cdk6 in G1 and helps Cyclin E activate Cdk2, causing the cell to enter the cell cycle. There are three D Cyclins in mammals, D1, D2, and D3. Cyclin A activates Cdk2 in S phase to help with chromosome duplication, and Cyclin A activates Cdk1 in early mitosis to help with mitotic events, before being degraded in prometaphase. Cdk1/Cyclin B initiates chromosome condensation and mitosis at the G2/M transition and is then degraded at the metaphase-anaphase transition. The levels of cyclin A and cyclin B are similar.
1.3 Rb and E2F Regulation

In order to drive cell cycle events, Cdk5 phosphorylate a protein in the Rb family (p130, p107, or p105 Rb) or one of many other Cdk substrates. The Rb family coordinates the transcription of many cell cycle regulated genes by binding to E2F. E2F is a family of proteins, specifically known as transcription factors, that bind to DNA as a heterodimer with DP1 or DP2. E2Fs act as either activators (E2F1, 2, and 3) or repressors (E2F4, 5, 6, 7, and 8). The activators interact with pRb/p105, while the repressors E2F4 and E2F5 interact with pRb/p107 and pRb2/p130 [1]. When Cdk4/Cyclin D phosphorylates Rb, E2F is released. Free E2F binds to the promoter region of E2F target genes, such as Cdk1, and induces transcription. When cyclin-Cdks
are inactive, Rb proteins remain bound to E2F and transcription of E2F target genes does not occur.

During quiescence (G0) and early G1, pRb/p107 and pRb2/p130 primarily interact with E2F4 and bind to the promoter region of genes needed for entry into S phase. This complex recruits the chromatin-remodeling factors SWI/SNF and HDAC (histone deactylase), which leads to chromatin condensation and histone deactylation, repressing transcriptional activity of genes required for entry into S phase [2]. Also, pRb/p105 associates with E2F activators to prevent transcriptional activity. In the middle of G1 the expression of pRb/p130 decreases, while Cdk4,6/Cyclin D hyperphosphorylates members of the Rb family. In late G1, Cdk2/Cyclin E phosphorylates pRb/p105, pRb/p107, and pRb2/130, releasing pRb/p105 from the activator E2Fs and pRb/p107 and pRb2/130 from E2F4,5 to allow entry into S phase [3]. Interestingly, Sororin, which was originally characterized as a substrate of the anaphase-promoting complex/cyclosome (APC/C), is encoded by a gene that is a target of E2F [4].
Figure 3: Rb/E2F Pathway.
CDKs respond to many signals to properly coordinate cell cycle progression. For example, when there is DNA damage, p53, a tumor suppressor gene, will be activated to stimulate DNA repair, arrest the cell in the cell cycle, and/or initiate apoptosis. p53 binds to the promoter of the p21 gene, and induces the transcription of p21. p21 protein binds to and inhibits the Cdk/Cyclin complex to block the cell cycle at the G1/S transition. Phosphorylation of the N-terminal portion of p53 causes its activation. Many protein kinases are responsible for this phosphorylation event including MAPK, ATM/ATR, and CHK1/2, and this activation greatly increases the amount of p53 in the cell. Cells that are not damaged have low levels of p53. This is because hDM2, a ubiquitin ligase, is able to covalently attach ubiquitin to p53, allowing for p53 to be degraded by the proteasome [5].

1.4 Mitotic Entry and Kinases

Entry into mitosis depends upon the activation of Cdk1. Cyclin B associates with Cdk1, leading to partial activation of the complex. Then, Cdk activating kinase (CAK) phosphorylates Cdk1 at Thr-161, leading to a change in the T loop of Cdk1 that makes it accessible to ATP thereby activating the kinase [6]. During G2, Wee1 and Myt1 kinases phosphorylate Cdk1 at Thr-14 and Tyr-15 resulting in the inhibition of Cyclin B/Cdk1. Wee1 is nuclear but can also associate with the centrosomes, while Myt1 is attached to membrane structures in the cytoplasm. When Thr-14 and Tyr-15 are dephosphorylated, Cyclin B/Cdk1 is active. Then, Cyclin B/Cdk1 phosphorylates Wee1 and Myt1 rendering them inactive [6]. Cyclin B/Cdk1 activates the Cdc25 phosphatase family, which dephosphorylates Thr-14 and Tyr-15 of Cdk1, which allows the cell to enter mitosis. There are three isoforms of Cdc25 in humans, and they are A, B, and C. The Cdc25
family shuttles back and forth between the nucleus and the cytoplasm. Interestingly, the localization of Cdc25A and Cdc25B is nuclear and the localization of Cdc25C is cytoplasmic [7] [8].

1.5 Spindle Assembly Checkpoint

Equal chromosome segregation to daughter cells depends upon biorientation/bipolar attachment, which occurs when kinetochores on sister chromatids attach to microtubules from opposite spindle poles. Cohesion between sister chromatids is essential for bipolar attachment, and the timing of dissolution of cohesion at the metaphase to anaphase transition is strictly controlled to ensure proper chromosome segregation [9]. There are two mechanisms to make sure that sister chromatids split only after bipolar attachment, and they are the wait-anaphase signal and error correction. The wait-anaphase signal, also referred to as the mitotic checkpoint, delays anaphase until all of the chromosomes undergo bipolar attachment. The mitotic checkpoint is triggered by unattached kinetochores and consists of many proteins including Bub1, BubR1, Mps1, and Mad1/2. Unattached kinetochores cause the formation of the mitotic checkpoint complex (MCC), consisting of BubR1, Bub3, Mad2, and Cdc20, which prevents anaphase onset by inhibiting APC/C\textsuperscript{Cdc20} to block degradation of securin and Cyclin B. Even when there is bipolar attachment, sometimes there is insufficient tension between kinetochores. Interestingly, the error correction mechanism senses tension deficits and facilitates proper attachment of chromosomes to the spindle [10].

The complex senses tension and control the wait-anaphase signal is the chromosomal passenger complex (CPC). The CPC consists of Aurora B, INCENP (inner
centromere protein), Survivin, and Borealin. The CPC localizes to the inner centromere until anaphase. When there is no tension, Aurora B phosphorylates outer kinetochore protein and microtubule motors, thereby destabilizing kinetochore-microtubule attachments. Once biorientation has been achieved, kinetochores are physically moved by the spindle away from Aurora B; dephosphorylation of Aurora B substrates occurs by PP1 to stabilize attachments [10].

1.6 The Anaphase Promoting Complex/Cyclosome

The APC/C is a multisubunit E3 ubiquitin ligase activated at the end of metaphase to initiate anaphase. The APC/C covalently attaches ubiquitin to a lysine residue on a target protein. Then, the target protein is degraded by the 26S proteasome. Cdc20 and Cdh1 activate the APC/C and belong to the Cdc20 protein family. Each member has seven WD-40 repeats in their C terminus that form a seven-bladed propeller structure to mediate protein-protein interactions [11]. Cdc20 accumulates in S phase and peaks in mitosis, causing the activation of the APC/C<sup>Cdc20</sup>. APC/C<sup>Cdc20</sup> leads to the degradation of Securin, an inhibitor of Separase. Separase, which is a cysteine protease, initiates anaphase by hydrolyzing cohesin. In late mitosis, APC/C<sup>Cdh1</sup> degrades mitotic cyclins, leading to the inactivation of Cdk1, further activating the APC/C<sup>Cdh1</sup>[12] [13]. The APC/C<sup>Cdh1</sup> ubiquitinates Cdc20 at its KEN box located in the N-terminus, leading to proteasomal degradation of Cdc20 and the inhibition of APC/C<sup>Cdc20</sup> [14].

The APC/C is regulated during S and G2 when Emi1 binds to and inhibits APC/C<sup>Cdc20</sup>. In prometaphase Emi1 is degraded; however, the SAC prevents the APC/C<sup>Cdc20</sup> from targeting Cyclin B and Securin by partially inhibiting the APC/C [15]
The SAC-inhibited APC/C spares the ubiquitination of several prometaphase targets, including cyclin A. When sister chromatid pairs are attached to the spindle, the SAC becomes inactivated. The activated APC/C<sup>Cdc20</sup> causes the destruction of Cyclin B and Securin in metaphase. When Cyclins are destroyed this leads to Cdk inactivation, but dephosphorylation and activation of another APC activator, Cdh1. APC/C<sup>Cdh1</sup>, which causes the degradation of Cdc20, Plk1, Aurora A, and Aurora B, is active in anaphase, telophase and G1 until Cdk2 is activated. Once Cdk2 is activated, Cdk2 and Cdk1 inhibit APC/C<sup>Cdh1</sup> until anaphase [17].

Alternatively, if there is DNA damage, PKA phosphorylates Cdc20 at the metaphase/anaphase transition and causes the inhibition of APC/C<sup>Cdh1</sup> [18]. In late anaphase and G1, APC/C<sup>Cdh1</sup> targets Cdc20 for proteasomal degradation, and Cdc20 is not expressed again until S phase. Throughout interphase, the Cdns phosphorylate Cdh1 and prevents Cdh1 from associating with the APC/C. In metaphase Rae1, which is an mRNA export factor that anchors the nucleoporin Nup98 to the nuclear pore complex, inhibits ubiquitination of Securin by APC/C<sup>Cdh1</sup> [19]. After release from spindle checkpoint-mediated metaphase arrest, Rae1-Nup98 dissociates from the APC/C<sup>Cdh1</sup> as BubR1 dissociates from the APC/C<sup>Cdc20</sup>, however, the connection between the nuclear pore complex and the APC/C is unclear [20].

1.7 Mitotic Exit and Phosphatases

In order for mitotic exit to occur, three cyclins are degraded in mitosis. Cyclin A is degraded in prometaphase, right after the nuclear envelope is broken down [21]. Cyclin B is degraded in metaphase, once the sister chromatid pairs are bi-orientated on
the spindle. The third mitotic cyclin, Cyclin B3, is destroyed in anaphase, but its role in mitosis is unclear. The destruction of these cyclins eliminates Cdk activity, causing phosphatases to dephosphorylate Cdk substrates in late mitosis. Certain substrates, like Separase, must be dephosphorylated in metaphase before anaphase can begin. Other substrates, like INCENP, must be dephosphorylated in early anaphase in order to control chromosome and spindle behavior during anaphase [22]. Finally, some substrates, like Cdh1, are dephosphorylated in late anaphase, leading to Cdk inactivation and spindle disassembly. Interestingly, even in telophase, Cdk dephosphorylation is necessary for chromosome decondensation, nuclear envelope formation, and cytokinesis [23].

Two important phosphatases regulating mitotic events in mammals are PP1 and PP2A, and they are members of the PPP superfamily that also includes calcineurin and PP4-7. PP1 functions as a heterodimeric complex containing PP1α, β, or γ1-2. When Cdk1 activity is high in mitosis, protein kinase A (PKA) phosphorylates inhibitor 1, allowing inhibitor 1 to bind to PP1, inhibiting PP1 activity. When there is inactivation of Cdk1, inhibitor 1 and PP1 are auto-dephosphorylated, which leads to PP1 activation. However, PP1 probably does not directly dephosphorylate Cdk1 substrates [24].

Interestingly, PP2A, a heterotrimeric complex containing a structural scaffold subunit, a catalytic subunit, and a regulatory subunit, most likely dephosphorylates Cdk1 substrates in animals. In humans, the catalytic subunit consists of two isoforms, PP2Aα and β, the regulatory subunit consists of 15 isoforms categorized into 4 subfamilies (B55, B56, B’’, and B’’’). The phosphorylation levels of Cdk1 substrates are controlled by Cdk1 and PP2A-B55. When the SAC is satisfied, the APC/C and its co-activator Cdc20 inactivate Cdk1, decreasing the activity of Greatwall kinase, a Cdk1 substrate. Greatwall
kinase is also an indirect regulator of PP2A-B55 and phosphorylates α-endosulphine (ensa) and cyclic AMP-regulated phosphoprotein 19 (arpp19), allowing them to bind and inhibit PP2A-B55 [24].

**Figure 4:** Mitotic Entry and Exit.

### 1.8 The SMC Family Structure

During the prophase/metaphase transition of mitosis, interphase chromatin is converted to rod-shaped chromosomes in a process referred to as chromosome condensation. At the metaphase/anaphase transition, sister chromatids separate. Interestingly, the SMC (structural maintenance of chromosome) proteins are part of a family of chromosomal ATPases that are involved in chromosome condensation (condensins) and the segregation of sister chromatids (cohesins). Condensins and cohesins have similar structures, each consisting of five distinct domains, which include two nucleotide-binding motifs (Walker A and B), two long coiled-coil regions, and a
nonhelical hinge. The six members of the SMC family are classified into three distinct groups: SMC2-SMC4, SMC1-SMC3, and SMC5-SMC6 [25].

Condensins, which contain the core subunits SMC2 and SMC4, are large protein complexes that are essential in chromosome assembly and segregation. There are two types of condensin complexes, condensin I and condensin II. Condensin I is composed of two SMC subunits (SMC2-SMC4) and three non-SMC subunits (CAP-D2, CAP-G, and CAP-H). Condensin II is composed of two SMC subunits (SMC2-SMC4) and three non-SMC subunits (CAP-D3, CAP-G2, and CAP-H2) [26]. Condensin II is in the cytoplasm during interphase and interacts with the chromosomes when the nuclear envelope breaks down at the end of prophase. However, condensin I is located in the nucleus during interphase and is necessary for the beginnings of chromosome condensation in the prophase nucleus. During prometaphase and metaphase, condensin I and II are important in the assembly of condensed chromosomes. Even after the sister chromatids separate in anaphase, condensin I and II stay associated with the chromosomes [27] [28].

Cohesion of the sister chromatids is maintained by the four-subunit SMC complex, which consists of Smc1, Smc3, the kleisin family protein Scc1, and the accessory subunit Scc3. Additional proteins including Pds5 associate weakly with the Cohesin complex and regulate the interaction of Cohesin with chromatin. Smc1 and Smc3, have ABC-like ATPases at one end of the 30 nm coiled coils. At the other end are pseudosymmetrical hinge domains that interact to create V-shaped Smc1/Smc3 heterodimers. Scc1 binds to the Smc3 and Smc1 ATPase heads and creates a tripartite ring that is 45 nm in diameter [9].
The SMC5/6 complex, which consists of SMC5, SMC6, and six non-SMC proteins (Nse 1-6), is required for DNA repair and cell survival. The architecture of the complex resembles that of other SMC complexes with the Nse proteins associating with the SMC heads [29]. During S-phase, the SMC5/6 complex interacts with duplicated chromosomes and DNA damage induced by UV and γ-ray irradiation [30]. When the SMC5/6 complex is absent during replication, there is a delay in sister chromatid separation. In G2, this complex localizes to the centromere and at certain sites along the chromosome arms. The SMC5/6 complex is removed from chromosomes sometime between G2 and G1, but exactly when this occurs and how it is regulated remains unknown [31].

Both the Cohesin complex and the SMC5/6 complex are necessary for repairing double-strand breaks (DSBs). Cohesin associates with the DSB in S and G2, which leads to *de novo* establishment of cohesion in G2 cells. Interestingly, Scc2, the DNA damage response kinases (ATM, ATR, and Chk2), phosphorylated histone H2AX, and the damage–sensing protein Mre11 are required for the interaction of cohesin with DSBs [32]. In addition, SMC5 and Nse2 are needed in the recruitment of cohesin to DSB. Even the SMC5/6 complex associates with DSBs, but in the duplicated genome specifically. Contrary to Cohesin, the SMC5/6 complex requires Mre11 but not Chk2, ATR, and Scc2 for DNA repair [33].

In the absence of DNA damage, the conserved protein Eco1/Ctf7/Eso1 plays a key role in establishing the cohesive state. Eco1 is not necessary for cohesin to bind to chromatin in S phase, at cohesin-attachment regions (CARs), or for a DSB, but it is essential for the establishment of cohesion. Once cohesion is established, the inactivation
of Eco1 in G2 causes Eco1 to be dispensable in cohesion maintenance. Eco1 contains a zinc finger and acetyl transferase activity. *In vitro*, Eco1 can acetylate itself, as well as Mcd1, Scc3, and Pds5, but not histones or PCNA [34].

1.9 Regulation of Cohesin

Sister chromatids are held together by intertwining (catenation) and by cohesin [35]. There are many proposed models for how the sister chromatids are entrapped by the cohesin complex. The three most well-known models are the embrace model, the snap model, and the bracelet model. The embrace model claims that the sister chromatids are held together by a cohesin ring and that the ATP hydrolysis of the heads causes the hinge region to open. However, a problem with this model is that the diameter of the ring is only 45 nm; therefore, two 30 nm fibers could not be embraced, but two 10 nm fibers (where DNA is in the form of beads on a string) could. In the snap model, a ring forms around each sister chromatid. The bracelet model does not actually entrap sister chromatids. Instead, two rings will become one and the ATPase heads will bind to the sister chromatids.

In vertebrates, cohesins bind to DNA in telophase and continue to bind until anaphase; however, in yeast, cohesins bind to DNA during the G1/S transition [36] [37]. In yeast, Scc2 and Scc4 load cohesin onto chromosomes, and in vertebrates Scc2 is known as NIPBL and Scc4 is also known as Mau-2. Cohesin occurs at a high density around each centromere and at a lower density along the chromosome arms. In yeast, cohesins bind on the chromosome arms at 15-kb intervals referred to as cohesin-
associated regions (CARs). CARs are located in AT-rich regions between transcribed genes, and CARs span approximately 1 kb of DNA [38] [39].

Cohesin-bound chromatin is found in two states: noncohesive and cohesive. Since cohesin is established prior to S-phase where there is only one chromatid, cohesin first binds to chromatin in a noncohesive state. Moreover, even when sister chromatids are in close proximity, cohesin-bound chromatin is not sufficient to generate cohesion. Once a chromosome suffers a double-strand break in G2/M, cohesin moves from a noncohesive to a cohesive state. Therefore, cohesion can be regulated by DNA damage [40] [41]. In the absence of DNA damage, cohesin is established during S phase, allowing for the passage of the replication complex through the cohesin ring, resulting in two daughter DNA molecules held together by the cohesin ring.

In yeast, to establish the tethering of sister chromatids a protein referred to as establishment of cohesion 1 (Eco1) acetyltransferase acetylates Smc3 at K112 and K113 in S phase. Interestingly, Eco1 is not needed in G2/M for binding or maintenance of cohesion. In vertebrates, the Eco1 orthologues, known as ESCO1 and ESCO2, acylate Smc3 at K105 and K106. As described more fully below, acetylation of Smc3 during S phase is essential to recruit Sororin to the cohesin complex where it stabilizes the association of the cohesin ring with the sister chromatids.

During prophase and prometaphase, the majority of cohesin dissociates from the chromosome arms in a process referred to as the prophase pathway. Polo-like kinase 1 (Plk1) phosphorylates an isoform of Scc3 (SA1 or SA2) causing release of cohesin from the arms. Also, the conserved protein Wapl is required for the removal of cohesin in the prophase pathway. When Wapl is depleted, the removal of cohesin is blocked and
phosphorylated Scc3 remains bound to the metaphase chromosomes. Therefore, phosphorylated Scc3 and Wapl work in concert to remove cohesin from the chromosomal arms [42]. At the metaphase/anaphase transition, the APC/C causes the degradation of Securin, releasing Separase. Plk1 phosphorylates Scc1 to facilitate its cleavage by Separase, which leads to inactivation of cohesin and the dissolution of cohesion [43].

However, the Shugoshin (Sgo) - Protein Phosphatase 2A (PP2A) complex protects centromeric cohesin by counteracting Plk1 until the kinetochores are captured by the spindle microtubules. Sgo recruits PP2A to the centromere through Bub1, and the B56 subunit of PP2A dephosphorylates cohesion subunits, preventing cleavage of cohesin at the centromere. At anaphase, the inactivation of Sgo may cause cohesin to delocalize from the centromeres or cohesin to be cleaved by Separase [44].

**Cohesin Pathway**

Adapted from Xiong and Gerton (2010)

**Figure 5:** Cohesin Complex Schematic.
1.10 Sororin

Sororin is a substrate of the APC/C and is involved in sister chromatid cohesion. Sororin is highly conserved in vertebrates and Sororin-related proteins are also found in invertebrates. Understanding how Sororin regulates cohesion may provide insight into cancer. Sororin was originally identified using a small pool expression screen for proteins degraded by the APC/C in *Xenopus laevis* extracts [45]. This protein localizes to the nucleus in interphase but not to the nucleoli, and in mitosis it disperses from the chromatin. Sororin is not needed for the association of cohesin with chromatin but is needed for cohesion and DNA repair in G2. Suppression of Sororin with small interfering RNAs causes loss of sister chromatid cohesion, implicating Sororin in the establishment and maintenance of cohesion in S phase and G2, respectively [45-47]. In fact, Sororin is recruited to the cohesin complex by Esco2 during S phase and binds once DNA replication is complete [48] [49]. Once at the cohesin complex, Sororin displaces Wapl from Pds5 [49]. When Wapl is knocked-down Sororin is no longer necessary to maintain cohesion; however, when Wapl is present, Sororin prevents Wapl from dissociating cohesin from DNA. Since Wapl is a cohesion-destabilizer, the recruitment of Sororin to DNA during S phase helps to solidify sister chromatid cohesion until mitosis.

The stabilizing effect of Sororin must be overcome during prophase to allow removal of cohesin from chromosome arms. Our studies indicate that Sororin is phosphorylated in response to Cdk1 *in vitro* and *in vivo* [50]. Furthermore, mutation of potential Cdk1 phosphorylation sites in Sororin creates a protein that is unable to dissociate from chromosomes in mitosis. This was visualized by inserting mutants into a
GFP vector and performing live-cell imaging. Also, we transfected HeLa M cells with the GFP constructs and H2A-RFP to show that the Sororin mutant localizes to the chromatin. Interestingly, we discovered that Sororin$^{\text{WT}}$ increases the length of mitosis compared to mutants and that is because Sororin$^{\text{WT}}$ increases the number of lagging chromosomes. Sororin$^{\text{WT}}$ associates with the cohesin complex in G2, but the mutant Sororin associates with the cohesin complex even in mitosis. Mutant Sororin and the faster migrating form of Sororin$^{\text{WT}}$ were able to bind to DNA cellulose. Therefore, the hypophosphorylated form of Sororin binds to DNA cellulose. Overexpression of the mutant Sororin increases cohesion yet is still able to rescue a mitotic arrest triggered by knock-down of the endogenous protein. Altogether, these observations suggest that phosphorylation of Sororin by Cdk1 inhibits the ability of Sororin to stabilize cohesion upon entry into mitosis.
II. Hypothesis

Many genes have been discovered that are important in the regulation of cell cycle, including Sororin. Understanding how Sororin regulates cohesion may provide insight into cancer. The main hypothesis is that phosphorylation of Sororin by Cdk1 plays a key role in regulating sister chromatid cohesion.
III. Materials and Methods

3.1 Cell Line and Culture Conditions

Parental HCT116 cells, originally derived from a human colon carcinoma, contain wild type p53 and were compared to HCT116 cells in which both p53 alleles had been inactivated by homologous recombination [51]. HeLa M, a subline of Hela [52] and HCT116 cells were incubated in a humidified atmosphere containing 10% CO₂ in Dulbecco’s Modified Eagle’s Medium (Mediatech, Inc.) supplemented with 10% fetal bovine serum (Atlanta Biologicals). All chemicals were from Sigma unless otherwise noted. To induce DNA damage, cells were treated with Adriamycin at 0.2 µg/ml or Etoposide at 10 µM. Colonies were visualized by staining with a saturated solution of methylene blue in 50% ethanol. We used 100 ng/ml nocodazole, 2 mM thymidine, and 2.5 µM ZM447439 (AstraZeneca). Transfections were carried out using Expressfect (Denville), Fugene (Roche), or polyethyleneimine (Polysciences). A typical experiment in a 6-well plate would include 2-5 µg DNA that was mixed with 50-100 µl of DMEM and 6-15 µl of PEI (1mg/ml) followed by a 10 minute incubation before adding to cells. This was scaled up accordingly.

3.2 Immunoblotting

HeLa M cells were collected by scraping into PBS followed by centrifugation (16000 x g, 4 °C) for 5 minutes and stored at -80 °C. Pellets were lysed by adding RIPA Buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS (supplemented with 1 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM
DTT, and 0.1 M PMSF) for 20 minutes on ice and centrifuged at high speed for 20 minutes at 4 °C [53]. To ensure equal loading, the protein concentration of each lysate was determined by using the BSA Protein Assay Kit (Pierce). Proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were then blocked for 1.5 hours with Blocking buffer, which consists of 5% (w/v) non-fat dry milk dissolved in PBST (1X PBS containing 0.05% [v/v] Tween 20). Antibodies to p53 directly conjugated to horse-radish peroxidase were obtained from Santa Cruz Biotechnology, to γH2A.X were obtained from Upstate Cell-Signaling Solutions, to serine 15 phosphorylated p53 were from Cell Signaling Technologies, to Rabbit-V5 or SMC3 were obtained from Millipore, and to actin were obtained from NeoMarkers. The primary antibody was incubated on the membrane for 1.5 hours at room temperature. The membranes were then washed three times for 10 minutes in PBST. Goat anti rabbit secondary antibodies conjugated to horse-radish peroxidase were used at a dilution of 1:10,000 in blocking buffer for 1 hour (Sigma). Membranes were washed again three times for 10 minutes in PBST. Antibody binding was detected using enhanced chemiluminescence (Pierce).

### 3.3 Immunofluorescence

Immunofluorescence was carried out as we have described [54]. Briefly, cells on coverslips were washed with PBS, fixed with 2% formaldehyde for 15 minutes, followed by permeabilization [150 mM NaCl, 10 mM Tris (pH 7.7), 0.1% Triton X-100, and 0.1% BSA] for 9 minutes, and blocked with PBS containing 0.1% BSA for 1 hour at RT.
Lagging chromosomes were quantified in cells transfected with V5-tagged Sororin$^\text{WT}$ or Sororin$^\text{9A}$. Immunofluorescence was used to stain H2A phosphorylated at T121 (H2A pT121; AssaybioTech), and lagging chromosomes were identified by H2ApT121 positive centromeres associated with Hoechst 33342-stained chromatin. Only V5-positive cells were included in the analysis. Interkinetochore distances were obtained on cells transfected with V5-tagged Sororin. Cells were simultaneously stained with antibodies to Hec1 (Abcam) to mark the kinetochore, H2ApT121, to mark the inner centromere, and V5 to identify Sororin. Hec1-positive dots on either side of a H2ApT121 positive dot belong to sister kinetochores. Phosphorylated S7 CENP-A was detected with a polyclonal antibody (Cell Signaling). Magnifications of images were kept the same within each figure panel.

3.4 Microscopy

For time lapse, cells were maintained in a sealed flask in medium equilibrated to 10% CO$_2$, placed on a microscope stage pre-heated to 37 °C, and viewed using phase contrast optics. Images were captured using either an Olympus C740 digital camera connected to a Motic inverted microscope or a Spot camera connected to an inverted Leitz Diavert microscope. For immunofluorescence, an Olympus IX 81 inverted microscope connected to a cool snap HQ$_2$ digital camera was used to capture images. Chromosome drops were imaged by a Zeiss Axiophot microscope. Images were converted to stacks and navigated using ImageJ software.
3.5 Cloning of Sororin

mRNA was isolated from HeLa M cells by using Trizol (Invitrogen) according to the manufacturer’s instructions and reverse transcription-polymerase chain reaction (RT-PCR) was carried out using enzymes from Promega with the primers found in Table 1. To construct a C-terminal V5-tagged Sororin protein, the PCR products were cloned into the pcDNA 3.2 expression vector using topo cloning (Invitrogen). Gateway cloning by Invitrogen was used to create the GFP constructs (we used the pcDNA-DEST47 vector).

All plasmids were used to create the GFP constructs (we used the pcDNA-DEST47 vector).

Table 1: Primers

<table>
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<th>Primer Name</th>
<th>Primers Used</th>
<th>Primer Sequence</th>
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</tr>
<tr>
<td>Reverse Primer-pcDNA3.2</td>
<td>5'TTTCAGGAGAGATCACATGTC-3'</td>
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<tr>
<td>WT forward primer-pGEX3X</td>
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3.6 Co-Immunoprecipitation and Flow Cytometry

Sororin was co-immunoprecipitated with cohesin from chromatin essentially as described [47]. Sororin\textsuperscript{WT-V5} and Sororin\textsuperscript{9A-V5} were transiently transfected into HeLa cells that were then treated with 2 mM thymidine for 24 hours arresting the cells in S phase. After thymidine was washed off, cells were left either for 6 hours so that they could progress through S phase or blocked in mitosis with nocodazole for 14 hours. Then, the cells were harvested and lysed on ice for 20 minutes in IP buffer (20 mM Tris pH 7.7, 100 mM NaCl, 5 mM MgCl\(_2\), 0.1% Triton X-100, 10% glycerol) containing 1 mM DTT, 1 mM NaF, and protease inhibitors (1 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A). To pellet the chromatin, samples were spun for 10 minutes at high speed. Pellets were solubilized by sonication and spun at high speed for 10 minutes. The supernatant was digested with 0.6 U/µl DNase (Fisher BioReagents) for 20 minutes at 37 °C [47]. The immunoprecipitation was performed with an antibody to SMC3 (Millipore) coupled to protein A beads and immunoblotted with an antibody to V5 directly conjugated to horseradish peroxidase (Bethyl). In order to confirm cell cycle positions, cells were analyzed by flow cytometry for DNA content and by chromosome dropping (described below) to quantify mitotic cells. For flow cytometry, cells were collected by trypsinization and fixed in 70% ethanol. Cells were resuspended in PBS and stained with a mixture of propidium iodide (50 µg/ml) and RNase A (5 µg/ml).

3.7 Analysis of Truncated forms of Sororin

We identified three exact matches to the Cdk consensus phosphorylation site and one potential cyclin interaction motif (CIM) in the Sororin sequence. Sororin contains
another six serines/threonines followed by proline that might be Cdk sites. We engineered three different GST fusion proteins, each containing the CIM but lacking a different group of potential phosphorylation sites. PCR using primers shown in Table 1 was used to engineer the GST fusions and the fragments were cloned into pGEX-3X that was digested with EcoR1 and BamH1. All plasmids were confirmed by sequencing.

To make a large amount of GST fusion protein, 2 ml of 2x YTA media was inoculated with a colony and shaken at 37 °C for 12 hours. Then, 1 ml of culture was inoculated into 100 ml of 2x YTA media, and shaken at 37°C until the OD$_{600}$ was between 0.5 and 2.0. Then, 100 mM IPTG was added to the culture and shaken at 37 °C for 2 hours. The culture was transferred to a tube that could withstand high centrifugal forces (oakridge tube). The sample was spun at 8000 x g for 10 minutes at 4 °C, and the supernatant was discarded. Then, the pellet was drained and the tube was placed on ice. The cells were resuspended in 50 µl of cold 1x PBS per 1 ml of culture (in this case, 5 ml) with protease inhibitors (1 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A) and 1% Triton X-100. The solution was transferred to a 15 ml centrifuge tube and kept on ice. The sample was sonicated for 10 s at 10% 5x. The solution was transferred to an oakridge tube and spun at 12,000 x g for 10 minutes at 4 °C. The supernatant was transferred to 15 ml falcon and kept on ice. The glutathione sepharose beads were equilibrated by using 700 µl of the 75% slurry and adding 5 ml of 1x PBS, followed by centrifugation of this mixture at 1000 x g. After the supernatant was aspirated, the solution was incubated with the equilibrated beads with gentle agitation for 30 minutes at 4°C. The suspension was centrifuged at 1000 x g for 5 minutes at 4 °C. The supernatant was removed and the pellet was washed with 1x PBS. The suspension was centrifuged at
1000 x g for 5 minutes at 4°C, and then the supernatant was discarded, which was repeated two additional times. 1 volume of storage buffer (10% glycerol) was added to the beads. We flicked the tube to get the beads into solution. Aliquots of 50 µl each were stored at -80°C. In vitro kinase assays were conducted with each GST fusion protein purified from E. coli using glutathione sepharose beads. GST fusions were incubated with 32P-ATP and purified Cdk1/Cyclin B1 (Cell Signaling). The beads were washed and loaded onto an SDS-PAGE gel that was dried and exposed to film.

3.8 Generation of Sororin Point Mutants

QuikChange Multi Site-Directed Mutagenesis (Stratagene) was carried out with the primers shown in Table 1 to create point mutations in Sororin that we had previously inserted into pcDNA3.2. Gateway cloning (Invitrogen) was used to make GFP constructs with all the point mutants. For this purpose, the mutants were first transferred into the donor vector pDONR 221 from pcDNA3.2. Then, mutants were transferred into destination vector pcDNA-DEST47 to create C-terminal GFP fusions. All constructs were confirmed by sequencing. In some experiments Sororin was knocked down by using shRNA. For this purpose, a previously defined target sequence, GCCTAGGTGTCCTTGAGCT, was cloned into pSUPER [47].

3.9 Chromosome Drops

HeLa M cells were blocked in mitosis with nocodazole, swelled with 0.075 M KCl, and fixed with methanol/acetic acid (3:1, v/v) [55]. The cells were dropped onto slides, briefly exposed to steam, and stained with Giemsa. Chromosome morphology
was determined in a blinded manner and includes data from at least two independent experiments performed in triplicate.

3.10 DNA Binding Assay

DNA-cellulose binding assays were performed essentially as described [56]. HeLa M cells were transfected with Sororin$^{WT-V5}$, Sororin$^{9A-V5}$, or pBABEpuro as a negative control using Expressfect (Denville). One-day post transfection the cells were treated with nocodazole (100 ng/ml) for 16 hours. Cells were lysed (Lysis buffer: 1x PBS, 10% glycerol, 0.5% NP-40) and protein concentrations determined using a Bradford assay (Pierce). DNA-cellulose at 12 mg was suspended in 400 µl of protein binding buffer [50 mM Tris-Cl, pH 8.0, 4 mM MgCl$_2$, 1 mM DTT, 150 mM NaCl, and 0.1% (vol/vol) Triton X-100], spun, and washed twice. The DNA-cellulose was resuspended in 150 µl of protein binding buffer and distributed into three 50 µl aliquots. Equilibrated lysate was added to each tube of DNA-cellulose and incubated 16 hours on a rocker at 4°C. Then, samples were spun at 4000 rpm for 2 minutes, supernatant was aspirated, and the pellet was resuspended in 0.5 ml of protein binding buffer. This wash was repeated three times. Bound proteins were separated by SDS-PAGE and analyzed by Western blotting with an antibody to the V5 tag (Millipore).
IV. Results

4.1 Cdk1 phosphorylates Sororin.

The full consensus for Cdk1 phosphorylation is S/T-P-X-K/R, although some substrates simply contain a serine or threonine followed by proline [57]. Sororin contains three sites that conform to the full consensus and six others that are serines/threonines followed by prolines (Fig. 6). Phospho-proteomic mapping data indicates that all nine of the potential Cdk1 sites could be phosphorylated in vivo [58-64]. Also, we identified a potential cyclin interaction motif (CIM) in the Sororin sequence. In other Cdk substrates, Cyclin binds to the CIM and recruits Cdk allowing more efficient phosphorylation.
Figure 6: Diagram of wild-type and mutant forms of Sororin. Each one of these sites appears to be phosphorylated *in vivo*, based upon proteomic analysis of phosphopeptides isolated from cells (information obtained from phosida and phosphosite websites) [58-64]. Each of these nine sites has a serine or threonine followed by proline (minimal Cdk consensus) while sites marked with an asterisk conform to the full Cdk consensus (S/T-P-X-K/R). RDLEM = potential cyclin interaction motif (CIM). Below wild-type Sororin are the various truncation and multi-site mutants of Sororin that were generated. In addition, a single mutant at each of the nine sites was generated (not shown). Throughout this dissertation, superscripts are used to indicate which form of Sororin is used, for example Sororin$^{WT}$ = wild-type; Sororin$^{9A}$ = mutant in which all nine serines or threonines followed by proline were mutated to alanine.
Sororin exhibits a slower electrophoretic mobility during mitosis as a result of phosphorylation [45]. We investigated the role of Cdk1, a kinase highly active in mitosis, in this phosphorylation event. Two truncated forms of Sororin were fused to glutathione-S-transferase (GST) and used for *in vitro* kinase reactions. Mutation of one of the full-consensus sites (S21) to alanine had no effect on phosphorylation. Full-length, as well as both truncated forms of Sororin were phosphorylated by recombinant Cdk1/Cyclin B1 *in vitro* (Figs. 6, 7A). Therefore, we mutated all nine serines/threonines followed by prolines to alanines to further investigate the role of phosphorylation in Sororin function. As expected, GST-Sororin9A was poorly phosphorylated by Cdk1/Cyclin B1 *in vitro* (Fig. 7B). In Fig. 7b, some of SororinWT got degraded. Degradation could have been prevented by working in a cold environment.

**Figure 7:** Phosphorylation of Sororin by Cdk1 *in vitro.* A.) Recombinant Cdk1/Cyclin B1 was mixed with GST-Sororin and phosphorylation reaction was carried out *in vitro* with 32P-ATP. Reactions were separated by SDS-polyacrylamide gel and analyzed by autoradiography. B.) Effect of serine to alanine mutations on Sororin phosphorylation. Recombinant SororinWT or Sororin9A with an N-terminal GST tag was phosphorylated in an *in vitro* reaction with Cdk1/Cyclin B1 and 32P-ATP. Reactions were analyzed by autoradiography with Histone H1 serving as a positive control. CBB: Coomassie brilliant blue stained gels.
To analyze Sororin phosphorylation \textit{in vivo}, we added a V5 tag to the C-terminus of the Sororin mutants shown in Figure 6 and used recombinant adenoviruses to overexpress a nuclear targeted Cyclin B1 (NB1) and a constitutively active mutant CDK1-AF (Cdk1 T14A Y15F) \cite{65}. HeLa M cells were blocked in S phase with hydroxyurea and then infected with adenovirus to express NB1 and Cdk1AF. Overexpression of Cdk1/Cyclin B1 reduced the mobility of Sororin\textsuperscript{WT-V5} but not Sororin\textsuperscript{9A-V5} (Fig. 8). This result suggests that Sororin is phosphorylated in response to Cdk1/Cyclin B1.

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\textbf{Figure 8:} Overexpression of Cdk1/Cyclin B induces a Sororin mobility shift. HeLa M cells were transiently transfected with Sororin\textsuperscript{WT-V5} or mutant forms of Sororin and analyzed by Western blot to detect a mobility shift. HeLa M cells were blocked in S phase with hydroxyurea and infected with recombinant adenovirus that express Cdk1 T14A Y15F (Cdk1AF) and Cyclin B1 fused to the nuclear targeting signal of SV40 T antigen (NB1). Cell lysates were analyzed by Western blotting with an antibody to the V5 tag on Sororin.

Next, we tested the effect of mutating potential phosphorylation sites on the mobility of Sororin during mitosis. We mutated each of the nine Cdk1 sites to alanine individually as well as in various combinations. Ryann Sohaney mutated the CIM in Sororin\textsuperscript{WT}. HeLa M cells were transfected with Sororin cDNAs and treated with
nocodazole to block the cells in mitosis. Sororin$^{\text{WT-V5}}$ migrated more slowly in mitosis compared to interphase, while Sororin$^{\text{9A-V5}}$ failed to exhibit a mobility shift (Fig. 9A).

Every single and multiple mutant (minus Sororin$^{\text{9A-V5}}$) demonstrated a mobility shift (Fig. 9B, C). Thus, phosphorylation of multiple sites is responsible for reducing the electrophoretic mobility of Sororin during mitosis. Note, there is a faint band above the unphosphorylated form of Sororin$^{\text{9A}}$ that is also present in the untrasfected lane. This is indicative of a nonspecific band. Interestingly, many of the single and multiple mutants are overexpressed and exhibit multiple bands. The fact that there are multiple bands means that the Sororin protein needs to be phosphorylated at more than one site. When one runs these samples using a phos-tag gel, Sororin$^{\text{WT}}$ separates into five separate bands, suggesting that there are five phosphorylated forms of Sororin (our unpublished data).
Figure 9: Electrophoretic mobility of Sororin. A, B, C). HeLa M cells were transiently transfected with Sororin<sub>WT-V5</sub> or mutant forms of Sororin and analyzed by Western blot to detect a mobility shift. Transfected cells were either left untreated or treated with nocodazole for 20 hours to block them in mitosis. Cell lysates were separated on a 12.6% SDS-polyacrylamide gel and analyzed by Western blotting with an antibody to the V5 tag. See legend of Fig. 6 for details.
To further investigate the notion that Cdk1 is the kinase that phosphorylates Sororin, inhibitor studies were performed. HeLa M cells were transfected with Sororin$^{WT-V5}$ and treated with nocodazole in the presence or absence of purvalanol A to inhibit Cdk1. Cells treated with nocodazole exhibited a mobility shift compared to untreated cells (Fig. 10). Cells treated with both nocodazole and purvalanol A contained less of the slower migrating species (Fig. 10). These results provide further evidence that Cdk1/Cyclin B1 is responsible for the phosphorylation of Sororin in vivo. However, it is possible that purvalanol A is driving the cells out of mitosis and that is why there is a lack of a mobility shift. What we need to know is if the cells are still in mitosis after treatment with both nocodazole and purvalanol A. To test this we could treat the cells with MG132, which is an inhibitor of the proteasome, to prevent the cells from exiting mitosis. Also, before we harvest the cells we could have examined the petri dish to make sure the cells are blocked in mitosis. Alternatively, we could have probed the immunoblot for cyclin B. When cells are in mitosis, they express high levels of cyclin B. Therefore, even though we treat the cells with purvalanol A, which inhibits Cdk1, cyclin B would not be inhibited and thus we could find out if the cells are indeed in mitosis.
Figure 10: Purvalanol A inhibits Sororin mobility shift. HeLa M cells were transiently transfected with Sororin\textsuperscript{WT-V5}, treated with nocodazole (noc) with and without purvalanol A (purv) for 16 hours, and analyzed by Western blot. Un: untreated.

4.2 Sororin\textsuperscript{9A} fails to release from chromatin in mitosis

To better understand the importance of phosphorylation, we fused a GFP tag to the C-terminus of wild-type and mutant Sororin. GFP-tagged Sororin\textsuperscript{WT} and Sororin\textsuperscript{9A} were first compared by transfecting the constructs into HeLa M cells and performing live-cell imaging. As previously observed, Sororin\textsuperscript{WT-GFP} was displaced from the nucleus and localized to the cytoplasm in prometaphase but appeared to re-localize to the chromatin in anaphase [45]. In contrast, a population of Sororin\textsuperscript{9A-GFP} appeared to remain localized to the chromatin throughout mitosis (Fig. 11). Sororin is a substrate of APC/C\textsuperscript{Cdh1}, which means that Sororin remains active into G1 and is known to be degraded four hours after the end of mitosis [45] and hence is degraded in G1. If we had extended time points in Fig. 11, we would have noticed that Sororin is degraded later in G1.
Figure 11: Phosphorylation of Sororin releases it from chromatin during mitosis. H2B-GFP, Sororin\textsuperscript{WT-GFP}, and Sororin\textsuperscript{9A-GFP} were transiently transfected into HeLa M and then was monitored by time-lapse fluorescence microscopy. Images of cells progressing through mitosis are shown (image interval: 12 minutes).

In order to confirm that Sororin\textsuperscript{9A-GFP} co-localizes with chromatin in mitosis, we co-transfected HeLa M cells with Sororin-GFP and histone H2A-RFP. Sororin\textsuperscript{9A-GFP}, but not Sororin\textsuperscript{WT-GFP}, co-localized with H2A-RFP in prometaphase, suggesting that mitotic phosphorylation of Sororin is necessary to remove the protein from the chromatin (Fig. 12).
Figure 12: Sororin$^{9A-GFP}$ localizes to DNA in mitosis. Histone H2A-RFP and Sororin$^{GFP}$ were transiently transfected into HeLa M cells. An example of a cell showing co-localization of Sororin$^{9A-GFP}$ and H2A-RFP is shown.

To quantify GFP fluorescence levels as cells progressed through mitosis, we determined the ratio of Sororin$^{GFP}$ on the chromatin versus the cytoplasm using time-lapse movies obtained as described in Figure 11. Sororin$^{9A-GFP}$ was significantly more enriched on chromatin than Sororin$^{WT-GFP}$ from G2 through metaphase, while at anaphase similar staining was observed for both Sororin$^{WT-GFP}$ and Sororin$^{9A-GFP}$ (Fig. 13). For both forms of Sororin, we observed a large drop in chromatin enrichment as cells entered prophase consistent with the release of Sororin into the cytoplasm at nuclear envelope breakdown (Fig. 13). This observation also indicates that a proportion of Sororin$^{9A-GFP}$ is released from chromatin at mitosis.
**Figure 13**: Enrichment of Sororin$^{\text{GFP}}$ on chromatin. Time-lapse frames were used to quantify mean pixel intensities in a defined region of the chromatin and compared to cytoplasmic intensity of the same sized region. Since cells spent different amounts of time in metaphase, only the first two metaphase frames were quantified, and hence the dashed lines. Also, the onset of anaphase occurred at a different time for each cell; this effect is indicated by the dashed line. Averages of at least eight cells with standard errors are shown.

Further analysis of time-lapse movies of HeLa M cells transfected with Sororin$^{\text{GFP}}$ indicated that overexpression of Sororin$^{\text{WT-GFP}}$ increased the length of mitosis when compared to cells transfected with H2B-GFP alone (Fig. 14). Sororin$^{9\text{A-GFP}}$ also increased the length of mitosis, but not quite as efficiently as Sororin$^{\text{WT-GFP}}$. 
Figure 14: Sororin$^{\text{WT-V5}}$ and Sororin$^{\text{9A-V5}}$ increase the length of mitosis. HeLa M cells were transiently transfected with Sororin$^{\text{WT-GFP}}$ or Sororin$^{\text{9A-GFP}}$. Time-lapse frames obtained as described in figure 11 were used to quantify the length of mitosis for each cell. In order to measure the length of mitosis, entry into mitosis was indicated by release of Sororin-GFP from the nucleus (NEBD: nuclear envelope break-down). End of mitosis was indicated by anaphase separation of chromatids. As a control, cells were transiently transfected with H2B-GFP to visualize DNA. In this case, entry into mitosis was taken as the first frame that DNA condensation was visible.

Interestingly, we observed a subtle but significant increase in the percentage of cells with lagging chromosomes after overexpression of Sororin$^{\text{WT}}$ compared to Sororin$^{\text{9A}}$ (Fig. 15A, B).
Figure 15. A.) Quantitation of lagging chromosomes. Hela M cells were transfected with either Sororin$^{WT-V5}$ or Sororin$^{9A-V5}$, fixed and analyzed by immunofluorescence with antibodies to the V5-tag and to H2A phosphorylated at T121 to indicate centromeres. V5-positive cells in metaphase were assessed for the presence of chromosomes that had not aligned at the metaphase plate. Average percent of cells showing lagging chromosomes with standard errors are shown. B.) Images used to measure lagging chromosomes. Example of a Hela M cells stained for H2A phosphorylated at T121 (H2ApT121) and Hoechst 33342 to visualize DNA. Lagging chromosomes were assessed in V5-positive cells in metaphase. The antibody to H2ApT121 also reacts with spindle poles. Therefore, lagging chromosomes were assessed by looking at both H2ApT121 and Hoechst images.
The average pixel intensities of Sororin\textsuperscript{WT-GFP} and Sororin\textsuperscript{9A-GFP} in the live-cell image series were similar (Fig. 16). Therefore, the increase in lagging chromosomes, and not expression level, may be responsible for the increased mitotic length in cells overexpressing Sororin\textsuperscript{WT}.

![Bar graph](image)

**Figure 16.** Average expression level of either Sororin\textsuperscript{WT-GFP} or Sororin\textsuperscript{9A-GFP} in live cells. Hela M cells were transfected with GFP-tagged Sororin and visualized by time-lapse fluorescence microscopy as in Figure 11. Single frames of GFP-positive cells in metaphase were used to measure average pixel intensities within the whole cell (au: arbitrary units). Average values from at least 28 cells for each condition with standard errors are shown.

We considered the possibility that overexpression of Sororin might alter the ratio of phosphorylated to unphosphorylated endogenous Sororin, thereby altering chromosome dynamics and lengthening mitosis. After transfecting HeLa M cells with Sororin and treating cells with nocodazole, immunoblotting was performed. There was no apparent difference in the ratio of phosphorylated to unphosphorylated Sororin when Sororin was overexpressed (Fig. 17).
Figure 17: Overexpression does not alter the phosphorylation ratio of Sororin. HeLa M cells were transiently transfected with Sororin WT-GFP, Sororin 9A-GFP, Sororin WT-V5, or Sororin 9A-V5, blocked in mitosis with nocodazole, and probed with an antibody from Santa Cruz Biotechnology raised against endogenous Sororin (Sororin endo). The membrane was probed with β-Actin as a loading control. The position of phosphorylated (phos.) and nonphosphorylated (nonphos.) Sororin is indicated. Ratios were determined using ImageJ software to analyze scanned films.

Extended imaging of HeLa M transfected with Sororin GFP revealed that the intensity of Sororin WT-GFP was rapidly extinguished after telophase, consistent with recognition by the APC/C (Fig. 18A) [45]. We observed no significant difference in the kinetics of protein decay when comparing Sororin 9A-GFP (Fig. 18B). This observation suggests that phosphorylation of Sororin does not have a major effect on recognition by the APC/C.
Figure 18: Sororin$^{\text{WT}}$ and Sororin$^{\text{9A}}$ are degraded with similar kinetics. A.) Sororin$^{\text{WT-GFP}}$ was transiently transfected into HeLa M cells. Time lapse was performed at 12 minute intervals. The time begins with when the cell enters mitosis. GFP intensity of a single cell is shown. After anaphase, Sororin$^{\text{WT}}$ was completely degraded by 3 hours. NEBD = Nuclear Envelope Breakdown B.) Sororin$^{\text{WT-GFP}}$ and Sororin$^{\text{9A-GFP}}$ were transiently transfected into HeLa M cells. Time lapse was performed and images collected at 12 minutes intervals. The time is indicative of the number of hours of cell progression after anaphase. Average GFP intensities from 4 Sororin$^{\text{WT}}$ cells and 5 Sororin$^{\text{9A}}$ cells are compared over time. No significant differences between the curves were observed.
4.3 Multiple sites of phosphorylation release Sororin from chromatin.

In order to identify the phosphorylation sites required for the removal of Sororin from chromatin, HeLa M cells were transiently transfected with a number of single and multiple point mutants. Cells were then treated with nocodazole and the localization of the Sororin-GFP fusions was determined in live cells. Sororin\textsuperscript{8A-GFP} and Sororin\textsuperscript{9A-GFP} both localized to the chromatin (Sororin\textsuperscript{8A} is identical to Sororin\textsuperscript{9A} except that S209 is wild-type). Similar results were obtained when we created N-terminal GFP constructs of Sororin\textsuperscript{WT} and Sororin\textsuperscript{9A}, suggesting that this effect is not due to the C-terminal tag (our unpublished data). Fixed cells expressing V5 or GFP-tagged Sororin\textsuperscript{9A} showed only minimal co-localization of Sororin with chromatin. Therefore, analysis of subcellular localization was carried out using C-terminal GFP tags in live cells. All the single point mutants localized to the cytoplasm in mitotic cells (Fig. 19A). Cells transfected with additional multi-site mutants of Sororin showed intermediate patterns of localization between Sororin\textsuperscript{WT-GFP} and Sororin\textsuperscript{9A-GFP} (Fig. 19B).
Figure 19: Multiple sites of phosphorylation release Sororin from chromatin. Sororin WT-GFP and mutant forms of Sororin were transiently transfected into HeLa M cells. Transfected cells were exposed to nocodazole to block them in prometaphase. A.) Localization of single point mutants of Sororin. Representative cells transfected with the indicated single point mutants of Sororin are shown. B.) Localization of Sororin-GFP with multiple S/T →A mutations. Hela M cells expressing the indicated forms of Sororin are shown. Specific mutations in each of the constructs are shown in Figure 5.

In order to quantify the various patterns of localization of WT and mutant forms of Sororin, we captured digital images of live GFP-transfected cells and measured the standard deviation (SD) of pixel intensities within the bounds of a cell (cells with uniform Sororin staining have lower SDs [66]). The SD was then corrected by mean pixel
intensity of the same cell and the corrected SD derived from multiple cells was presented as an average value (Fig. 19). One of the Sororin mutants with two Cdk1 sites mutated (2Aα) showed a slightly higher corrected SD compared to wild-type Sororin. Sororin3A-GFP and Sororin6A-GFP showed intermediate corrected SDs between SororinWT-GFP and Sororin9A-GFP (Fig. 20). We also measured the fold enrichment as described for Figure 9, which indicated similar trends of staining with the multiple mutants (our unpublished data). Overall, these results suggest that multiple sites of phosphorylation, possibly acting in an additive manner, are required to remove Sororin from the chromosomes.

**Figure 20:** Staining uniformity of various forms of Sororin. To measure the uniformity of staining, digital images were captured of live transfected cells. This corrected SD was averaged over multiple cells and is shown with standard errors indicated by bars. The various mutants were compared to either SororinWT or Sororin9A using a students t-test. p-values are indicated in the table.

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<td>CIM</td>
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4.4 Sororin9A increases association with cohesin

To further investigate the mechanism by which phosphorylation controls the release of Sororin from chromosomes, we investigated the interaction between Sororin and the cohesin complex. We compared cells progressing through S/G2 phase to those blocked in prometaphase with nocodazole (Fig. 21C). Using co-immunoprecipitation, we observed that in prometaphase, more Sororin9A-V5 than SororinWT-V5 was
immunoprecipitated with SMC3 (Fig. 21A). In S/G2, more Sororin$^{WT-V5}$ was associated with SMC3 than in prometaphase (Fig. 21A). Sororin did not detectably change SMC3 levels associated with chromatin (Fig. 21B). Flow cytometry confirmed the mitotic and S/G2 cell cycle positions of synchronized populations (Fig. 21C). These results suggest that Sororin$^{WT-V5}$ is associated with the cohesin complex through S phase, but dissociates in prometaphase. The fact that more Sororin$^{9A-V5}$ than Sororin$^{WT-V5}$ is associated with SMC3 in prometaphase suggests that dissociation from the cohesin complex might be triggered by Cdk1-mediated phosphorylation of Sororin.
**Figure 21:** Phosphorylation of Sororin reduces its association with the cohesin complex. Sororin WT (“WT”) or Sororin 9A (“9A”) which contain C-terminal V5 epitope tags, were transiently transfected into HeLa M cells. Some samples were untransfected (“UNT”). The cells were then synchronized in S phase with 2 mM thymidine for 24 hours. Thymidine was removed and nocodazole was added for 14 hours to synchronize the cells in mitosis. To prepare an S/G2 population, cells were released from the thymidine block for 6 h. Chromatin was prepared as described in the materials and methods. A.) Sororin 9A associates with chromatin in mitosis. Cohesin was immunoprecipitated with an antibody to SMC3. Immune complexes were loaded on 12.6% SDS-polyacrylamide gels and analyzed by Western blotting with an anti-V5 antibody. B.) Sororin does not alter the levels of SMC3. Samples were prepared as in Fig. 5A and loaded on a 12.6% SDS-polyacrylamide gels and analyzed by Western blotting with SMC3 and anti-V5 antibodies. UN: untransfected. C.) Verification of the cell cycle stage for each of the cell populations. Flow cytometry was performed using propidium iodide-stained cells. Numbers of cells in G1, S or G2/M were determined from flow cytometry. “M” indicates mitotic index of parallel cultures prepared by the chromosome dropping method. AYSN: asynchronous, UNT: untransfected.
In order to obtain more insight into the association of Sororin with chromatin, we tested whether the protein could be precipitated from cell lysates with DNA-cellulose. For those experiments, HeLa M cells were transfected with wild-type or 9A forms of Sororin bearing C-terminal V5-tags. Cell lysates were incubated with DNA-cellulose and the bound fractions were analyzed by Western blotting. Both Sororin\textsuperscript{WT-V5} and Sororin\textsuperscript{9A-V5} associated with DNA-cellulose (Fig. 2A). Interestingly, only the fast migrating form of Sororin\textsuperscript{WT-V5} was found in the bound fraction suggesting that phosphorylation reduces the association of Sororin with DNA-cellulose (Fig. 2B).
Figure 2: Nonphosphorylated form of Sororin binds to DNA-cellulose. A.) Association of Sororin with DNA-cellulose. HeLa M cells were transiently transfected with either Sororin\textsuperscript{WT-V5} or Sororin\textsuperscript{9A-V5}. UNT: untransfected. Cells were blocked in mitosis by exposing the cells to nocodazole for 16 hours. Cell lysates were incubated with DNA-cellulose for 16 hours, and the bound fraction washed extensively. Proteins remaining associated with the DNA-cellulose were analyzed by Western blotting. An aliquot of the lysate used for the binding reaction (“input”) was also analyzed for comparison. B.) Desitometric scans of the lanes. The fastest migrating nonspecific band was used to register the scans.
4.5 Effect of Sororin\textsuperscript{9A} on sister chromatid cohesion

We hypothesized that constitutive binding of Sororin\textsuperscript{9A} to chromosomes might increase sister chromatid cohesion. To examine sister chromatid cohesion in prometaphase, HeLa M cells were transfected with V5-tagged Sororin. Transiently transfected cells were blocked in mitosis with nocodazole, and examined using chromosome spreads. Chromosome spreads were prepared after hypotonic swelling followed by fixation and drying of chromosomes on glass slides before staining. Cells transfected with Sororin\textsuperscript{9A}, but not Sororin\textsuperscript{WT} showed a significant increase in the number of cells with closed arms (Fig. 23A and B). If a majority of the chromosomes appeared to have closed arms, then the spread was given the label of “closed arms;” however, if a majority of the chromosomes appeared to have open arms, then the spread was given the label of “open arms.”
Figure 23: Sororin\textsuperscript{9A} alters sister chromatid cohesion. A.) Examples of chromosome spreads with closed or open arms. Sororin\textsuperscript{WT-V5} and Sororin\textsuperscript{9A-V5} were transfected into HeLa M cells. Then, the cells were treated for 24 hours with 2 mM Thymidine. After that, the Thymidine was washed off and 100 ng/ml of nocodazole was added for 24 hours. Then chromosome drops were performed. Images of giemsa-stained cells are shown. Arrows indicate sister chromatids. B.) Quantification of closed sister chromatids in prometaphase. Cells were prepared as in “A”, samples were then analyzed in a blinded manner to determine chromosome morphology. Percent of spreads where sister chromatids are closed are indicated. Average values with standard errors are shown.

To test whether cohesion is affected by Sororin\textsuperscript{9A-V5} in intact fixed cells, we measured interkinetochore distances using immunofluorescence (Fig. 24A, B). We observed no significant difference in interkinetochore distances in cells overexpressing
Sororin$^{9A-V5}$ compared to Sororin$^{WT-V5}$ (Fig. 24A). Overall, these observations suggest that overexpression of Sororin$^{9A-V5}$ increases sister chromatid cohesion. The fact that Sororin$^{9A-V5}$ did not alter interkinetochore distances may mean that Sororin$^{9A-V5}$ stabilizes the complex during chromosome preparation, but does not directly block the removal of cohesion. Alternatively, Sororin may be more important in regulating arm cohesion than pericentromeric cohesion. Recent evidence suggests that Sororin actually interacts with Plk1, which aids in removing cohesin from the arms. Interestingly, Sororin must first be phosphorylated by Cdk1/cyclin B in order for Plk1 to come in close proximity of SA2. Then, Plk1 is able to phosphorylate SA2, leading to the removal of cohesin from chromosome arms [67].
Figure 24: Measurement of interkinetochore distance. A. and B.) Hela M cells were transfected with Sororin^{WT-V5} or Sororin^{9A-V5} and analyzed by immunofluorescence using antibodies to Hec1 and H2A phosphorylated at T121 (H2ApT121). Antibodies to the V5-tag were also used to identify cells expressing the transfected Sororin proteins. H2ApT121 staining indicates which Hec1-positive dots belong to sister chromatids. Interkinetochore distance was measured on blinded samples. At least 10 Hec1 pairs per cell were analyzed and at least 14 cells were measured for each condition. Average distances with standard errors are shown.

4.6 Sororin rescues mitotic arrest induced by knock-down

To investigate the effect of phosphorylation on the function of Sororin, we generated an shRNA to the 3’UTR of Sororin. Sororin cDNA clones used in this study lack a 3’ UTR and are not targeted by the shRNA construct, allowing us to reconstitute
cells with either Sororin$^{\text{WT-V5}}$ or Sororin$^{\text{9A-V5}}$. Transiently transfecting HeLa M cells with Sororin-shRNA caused Sororin-knockdown (Fig. 25A) and resulted in a prometaphase arrest (Fig. 25B). Cells blocked in mitosis as a result of Sororin knockdown were characterized by misaligned chromosomes that did not form a metaphase plate (Fig. 20B), similar to previous reports [46, 47].
**Figure 25:** shRNA-mediated knock-down of endogenous Sororin. A.) Efficiency of shRNA knock-down of Sororin. HeLa M cells were transiently transfected either with a plasmid that produces an shRNA targeting the 3’ UTR of Sororin (shRNA Sororin) or empty pSUPER. Cell lysates were separated on a 12.6% SDS-polyacrylamide gel and analyzed by Western blotting with an antibody to endogenous Sororin. Actin served as a loading control. B.) Defective metaphase plates after Sororin knockdown. pSUPER, shRNA Sororin, or shRNA Sororin along with Sororin WT-V5 were transiently transfected into HeLa M cells. H2B-GFP was also transfected to visualize DNA. Examples of live mitotic cells are shown.

Reconstituting with either Sororin WT-V5 or Sororin 9A-V5 reduced the percent of cells in mitosis compared to cells transfected with shRNA alone (Fig. 26). Also, knocking down Sororin increased the percent of cells with multiple nuclei, presumably as
a result of progress through a defective mitosis. Sororin\textsuperscript{WT-V5} and Sororin\textsuperscript{9A-V5} were similarly able to suppress the formation of multinucleated cells when combined with the shRNA against Sororin (Fig. 26). These results suggest that phosphorylation-deficient Sororin retains those activities of wild-type Sororin required for cells to progress through mitosis.

\textbf{Figure 26:} Rescue of mitotic arrest by reconstituted Sororin. Hela M cells were transfected simultaneously with a mixture of three plasmids: i) shRNA against Sororin in pSUPER; ii) either Sororin\textsuperscript{WT-V5}, Sororin\textsuperscript{9A-V5}, or Sororin\textsuperscript{cim-V5}, in a mammalian expression construct; and iii) H2B-GFP to visualize the DNA and to mark transfected cells. Positively transfected cells were then quantified by microscopy three days later to determine whether they were in mitosis or interphase. Interphase cells with multiple nuclei were also counted. Living cells were quantified to avoid loss of mitotic cells during fixation.
4.7 Role of SAC in mitotic arrest after Sororin knockdown

The mitotic block that occurs upon Sororin knock-down is most likely triggered by sister chromatids that separate prematurely due to a lack of proper cohesion [45-47]. Consistent with this idea, transfecting HeLa M cells with Sororin shRNA increased the number of cells with separated sister chromatids (Figs. 27A, B). In addition, chromosomes were about half as long after Sororin knockdown when compared to control chromosomes (Fig. 27C). This shortening of chromosomes upon Sororin knockdown was previously attributed to hypercondensation during a prolonged mitosis [45].
Figure 27: Knockdown of Sororin disrupts sister chromatid cohesion. A.) Examples of connected and separated chromosomes. Giemsa-stained chromosomes are shown as examples. The “connected” spread was from mock-transfected HeLa M cells, while the “separated” spread was from HeLa M cells transfected with Sororin shRNA. B.) Chromatid separation after Sororin knock-down. HeLa M cells transfected with pBABEpuro (mock) or Sororin shRNA were analyzed by chromosome dropping 72 hours post-transfection. C.) shRNA Sororin decreases chromosome length. Cells were transfected as in Fig. 26B and analyzed by chromosome spreads. Images of chromosomes were analyzed using Slidebook software to measure chromosome lengths.
In order to test the role of the SAC in the arrest that occurs upon Sororin knock-down, we determined the effect of the Aurora kinase inhibitor, ZM447439, on the mitotic block. Cells transfected with Sororin shRNA spent ~10 hours in mitosis, with many cells dying before being able to exit the block (Fig. 28). When Sororin-shRNA transfected cells were exposed to ZM447439, the length of mitosis was reduced to ~1 hour (Fig. 28). These results suggest that single sister chromatids activate an Aurora kinase-dependent mitotic block.

**Figure 28:** Effect of ZM447439 on mitotic arrest induced by Sororin knock-down. HeLa M cells were co-transfected with H2B-GFP, used to mark transfected cells, and with Sororin shRNA. 72 hours later, the length of mitosis of GFP positive cells were determined by time-lapse microscopy. In the +ZM sample, 2.5 µM ZM447439 was added 72 hours post-transfection and filming began at the time of drug addition. Averages and standard errors are shown.

Consistent with the effect of ZM447439, we were able to detect punctate staining of CENP-A, phosphorylated at serine 7, in Sororin-shRNA transfected mitotic cells (Fig. 29, conducted by Michael Bekier). These areas of staining were closely associated with INCENP, a marker for the inner centromere. In the absence of the primary antibody
there was some modest cytoplasmic staining using the secondary antibody for the CENP-A detection (our unpublished data). Phosphorylation of CENP-A at serine 7 is catalyzed by Aurora kinase suggesting that chromatids that have prematurely separated are able to recruit this kinase to activate the SAC.

**Figure 29:** Phosphorylated CENP-A in Sororin knock-down cells. HeLa M cells were transfected with Sororin shRNA, and 72 hours later analyzed by immunofluorescence. Cells were co-stained with antibodies to INCENP and CENP-A phosphorylated at Serine 7. Examples of mitotic cells are shown.
4.8 Constitutive Phosphorylation and Mutating the FGF motif in Sororin

In order to determine the effect of constitutive phosphorylation on Sororin, HeLa M cells were transiently transfected with a number of multiple point mutants and treated with nocodazole to block them in mitosis. Then, the staining uniformity was calculated for each cell (Fig. 30b) in a similar manner as to Figure 20. The Sororin “E” mutants all localized to the cytoplasm (Fig. 30A). This makes sense because these glutamic acid mutations mimic a phosphorylated Sororin. As we know, phosphorylation of Sororin by Cdk1 removes Sororin from the chromatin. Interestingly, the FGF motif in Sororin interacts with Pds5 [45]; however, when we mutated the FGF motif to AGA in the 9A construct, Sororin was able to localize to the chromatin. If Pds5 is the only binding partner to Sororin, then we would expect AGA-9A to be unable to localize to the chromatin and instead localize throughout the cytoplasm. However, our results indicate otherwise. Therefore it is probable that Sororin has another binding partner besides Pds5. In a massive phosphorylation screen, it was suggested that Sororin also interacts with Rad21 [68].
Figure 30: Localization of phospho-mimic and FGF-mutated Sororin. Sororin$^{WT-GFP}$ and mutant forms of Sororin were transiently transfected into HeLa M cells. Transfected cells were exposed to nocodazole to block them in prometaphase. A.) Localization of mutants of Sororin. Representative cells transfected with the indicated mutants of Sororin are shown. B.) Staining uniformity of constitutively phosphorylated forms of Sororin. The various mutants were compared to Sororin$^{WT}$ using a students t-test. p-values are indicated in the table.

Reconstituting with Sororin$^{9D-V5}$ and Sororin$^{9E-V5}$ reduced the percent of cells in mitosis and suppressed the formation of multinucleated cells when combined with the shRNA against Sororin (Fig. 31). These results suggest that phospho-mimic Sororin retains those activities of wild-type Sororin required for cells to progress through mitosis.
Also, Sororin\textsuperscript{AGA-WT-V5} and Sororin\textsuperscript{AGA-9A-V5} reduced both the percent of cells in mitosis and suppresses the formation of multinucleated cells when combined with the shRNA against Sororin (Fig. 31). Our previous experiments indicated that phosphorylation of Sororin removes it from the cohesin complex in mitosis. We hypothesized that the phospho-mimic Sororin\textsuperscript{9D} and Sororin\textsuperscript{9E} mutants would not be able to bind cohesion and would inefficiently antagonize Wapl leading to premature chromatid separation in the absence of endogenous Sororin. The fact that cells transfected with Sororin shRNA, Sororin\textsuperscript{9D}, and Sororin\textsuperscript{9E} do not get blocked in mitosis suggests that Sororin\textsuperscript{9D} and Sororin\textsuperscript{9E} are still able to stabilize the cohesin complex. Several possibilities are described in the discussion.

![Figure 31](image)

**Figure 31**: Rescue of mitotic arrest by additional forms of reconstituted Sororin. Hela M cells were transfected simultaneously with a mixture of three plasmids: i) shRNA against Sororin in pSUPER; ii) Sororin\textsuperscript{V5} in a mammalian expression construct; and iii) H2B-GFP to visualize the DNA and to mark transfected cells. This experiment was analyzed in a manner similar to figure 25.
4.9 Evidence that PP2A may dephosphorylate Sororin

Interestingly, Sororin is known to be dephosphorylated by 4 h after nocodazole release; however, the phosphatase responsible for this dephosphorylation event is unknown [45]. Therefore, we transfected HeLa M cells with Sororin WT-V5, performed a nocodazole release, and treated the cells with Okadaic acid for up to 4 h. Okadaic acid is capable of inhibiting PP2A, a serine/threonine phosphatase that can target Cdk1 substrates upon mitotic exit [69]. Okadaic acid delayed the dephosphorylation of Sororin WT-V5 upon nocodazole release (Fig. 32). This result suggests that PP2A or another okadaic acid-sensitive phosphatase targets Sororin for dephosphorylation upon mitotic exit.

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**Figure 32:** Okadaic acid inhibits Sororin dephosphorylation. HeLa M cells were transiently transfected with Sororin WT-V5 and released from a nocodazole block in the presence or absence of okadaic acid (OA). Western blotting with an antibody to the V5-tag was used to detect a mobility shift of transfected Sororin. The blot was reprobed with an antibody to actin to indicate loading.
V. Discussion

Sister chromatid cohesion is essential for faithful chromosome segregation, keeping sister chromatids together until the exact time at the metaphase-anaphase transition when the duplicated genome is equally segregated to daughter cells. Chromosomes that fail to disjoin contribute to aneuploidy, a condition commonly found in tumor cells. The exact role of aneuploidy in tumor progression is under debate [70]. It is clear that without sister chromatid cohesion, segregation of complex genomes would be impossible. Sister chromatid cohesion in animal cells is mediated by mechanisms that include chromatin catenation as well as the proteinaceous ring cohesin [71-73]. Sororin is a substrate of the anaphase-promoting complex/cyclosome (APC/C) with a key role in sister chromatid cohesion [45]. High levels of Sororin added to *Xenopus laevis* extracts cause an increase in cohesin association with metaphase chromosomes, which leads to failed segregation of the sister chromatids [45]. In human cells, Sororin is essential for cohesion after S phase [47]. Depletion of Sororin causes mitotic arrest and failed sister chromatid cohesion, which is comparable to the phenotypes observed upon depletion of Shugoshin, a protein that protects centromeric cohesion [46].

Sororin is phosphorylated during mitosis, resulting in a reduced electrophoretic mobility. Here we have investigated the role of Cdk1 in Sororin phosphorylation and have uncovered a function of this modification in regulating the subcellular localization of the protein. We expressed a GFP-tagged version of Sororin and found that it localizes to the nucleus of HeLa M cells, and as previously observed, in mitosis disperses from the chromatin and localizes throughout the cytoplasm. Sororin contains nine residues that match the minimal Cdk phosphorylation consensus sequence, and one potential CIM.
GST-Sororin is phosphorylated by Cdk1, and mutating the nine serines/threonines followed by proline to alanines severely reduces this phosphorylation. When expressed in HeLa M cells, all of the Sororin mutants tested exhibited a reduced mobility after nocodazole treatment except for Sororin\textsuperscript{9A-V5}. This suggests that multiple phosphorylation sites contribute to the reduced electrophoretic mobility of Sororin. Interestingly, Sororin\textsuperscript{9A-GFP} remained associated with the chromosomes and the cohesin complex throughout prometaphase, while Sororin\textsuperscript{WT-GFP} was dispersed from chromosomes during mitosis. Also, we found that hypophosphorylated Sororin precipitates with DNA-cellulose. These results suggest that Cdk1 phosphorylation of Sororin releases Sororin from the chromosomes by weakening its interaction with the cohesin complex. The precipitation of Sororin with DNA-cellulose may indicate an ability to bind to DNA. Alternatively, since these experiments were carried out with cell lysates, Sororin may indirectly bind to DNA via the cohesin complex. In either case, phosphorylation appears to influence this association. Sororin is targeted for destruction by the APC/C. Consistent with this, we observed a loss of Sororin-GFP intensity after anaphase. Based on the behavior of Sororin\textsuperscript{9A-GFP}, loss of phosphorylation at the nine sites does not appear to alter the kinetics of degradation.

In order to narrow down the phosphorylation sites that are needed to remove Sororin from the chromatin, we analyzed a number of multi-site mutants of Sororin fused to GFP and analyzed their subcellular localization. Interestingly, Sororin\textsuperscript{3A-GFP} and Sororin\textsuperscript{6A-GFP} showed intermediate phenotypes between Sororin\textsuperscript{WT-GFP} and Sororin\textsuperscript{9A-GFP}. By increasing the number of sites that were blocked from phosphorylation, we observed a graded increase in the association of Sororin with chromosomes. This observation
suggests that each site of phosphorylation may contribute in an additive manner to the release of Sororin from chromosomes. Despite the ability of Cdk1 to induce Sororin phosphorylation, mutations that are expected to disrupt the putative CIM (R134A; L134A) had little effect on the Sororin protein. Sororin\textsuperscript{R134A;L134A} was released from chromatin in mitosis, still rescued the mitotic arrest induced by Sororin knock-down and also exhibited an electrophoretic mobility shift. These observations suggest that Cdk1 phosphorylates Sororin in a CIM-independent manner.

Since Sororin is involved in sister chromatid cohesion, high levels of a non-phosphorylatable protein might cause defects in cell division, such as impairing the segregation of chromosomes. However, we observed that although overexpressing Sororin\textsuperscript{9A-V5} increased the length of mitosis, Sororin\textsuperscript{WT-V5} increased the length of mitosis even more. This difference might be related to the fact that a higher frequency of Sororin\textsuperscript{WT} transfected cells contain lagging chromosomes than Sororin\textsuperscript{9A} expressing cells.

It is not known how Sororin overexpression increases the frequency of lagging chromosomes. One possibility is that high levels of phosphorylated Sororin bind to a cytoplasmic target to interfere with chromosome alignment. Sororin\textsuperscript{9A} may also induce this effect by displacing a population of endogenous Sororin from chromosomes. In either case it appears that phosphorylation-competent Sororin is linked to the persistence of lagging chromosomes in our studies. The lagging chromosomes we observed were mainly found as one or two chromosomes that had not yet aligned to the metaphase plate. This phenotype may be a result of delayed attachments or failure to resolve inappropriate attachments of chromosomes to the spindle.
Sororin appears to antagonize the cohesin-destabilizer Wapl [49]. Phosphorylation of Sororin by Cdk1 during prometaphase may be required to neutralize Sororin, allowing Wapl to coordinate prophase removal of cohesin from chromosome arms. Consistent with this idea, overexpression of Sororin\(^9A\) increased sister chromatid cohesion as assessed in chromosome spreads. We also expected that the Sororin\(^9A\) mutant might increase the frequency of chromosome nondisjunction at anaphase, however this defect was very rare and did not appear to be exacerbated by overexpression of Sororin\(^9A\) (our unpublished data). The fact that cells are able to progress through anaphase in the presence of Sororin\(^9A\) might be taken as evidence that prophase removal of cohesin is not essential for cells to separate chromosomes at anaphase. In support of this idea is the observation that most cells in which Wapl levels are reduced with RNAi still progress from prometaphase to anaphase with normal kinetics, and show no major defects in chromosome separation [74]. This raises the question of the physiological significance of the prophase pathway. Importantly, cells transfected with Wapl RNAi, show a significant increase in the percentage of multi-lobed nuclei suggesting that some cells do have defective mitosis. This suggests that cleavage of Scc1 by separase is a dominant activity, allowing chromosome segregation even under conditions of suboptimal prophase chromosome resolution. Prophase removal therefore, may be most important in supporting high fidelity chromosome segregation needed to avoid aneuploidy.

Phosphorylation by Cdk1 does not appear to be required to activate Sororin to carry out its role in cohesion establishment. This latter point is supported by our observation that Sororin\(^9A-V5\) can rescue the mitotic block that occurs upon Sororin
knock-down. This is also consistent with the fact that Sororin establishes cohesin during interphase, when Cdk1 activity is low. The mitotic block induced by Sororin knockdown is likely triggered by single sister chromatids that accumulate [46, 47]. These single chromatids presumably trigger the SAC. Along these lines, we have observed that inhibiting Aurora kinases with ZM447439 abrogates the mitotic block induced by Sororin knock-down. Aurora B plays an essential role in sensing tension defects at the inner centromere and in triggering the SAC [75]. Aurora B may be acting at the prematurely separated sister chromatids to invoke the SAC-dependent arrest. Along these lines, INCENP still localizes to a region near the centromere after suppressing Scc1 with RNAi, although the localization pattern is altered compared to control cells [76]. This localization of the chromosomal passenger complex to separated sister centromeres may allow Aurora B to constitutively phosphorylate kinetochore targets to destabilize monotelic attachments and activate the SAC. Consistent with this, CENP-A was still phosphorylated at Serine 7 in Sororin knock-down cells. The fact that Sororin$^{9A-V5}$ can relieve the mitotic block induced by Sororin knock-down would be consistent with an ability to establish sister chromatid cohesion.

Since Sororin is involved in sister chromatid cohesion, non-phosphorylatable forms of Sororin might cause defects in cell division. When serine/threonine are replaced with the phosho-mimic glutamic acid or aspartic acid, this Sororin may fail to associate with cohesin, leading to premature sister separation. We engineered Sororin$^{9D}$ and Sororin$^{9E}$ with either a GFP or a V5-tags. The localization of Sororin$^{9D-GFP}$ and Sororin$^{9E-GFP}$ is cytoplasmic when Hela M cells are blocked in mitosis with nocodazole. This result is expected because Sororin proteins mimicking phosphorylation should theoretically not
bind to chromatin because phosphorylation removes Sororin from chromatin. With this model in mind, when endogenous Sororin is knocked-down with shRNA and replaced with Sororin\textsuperscript{9D-V5} and Sororin\textsuperscript{9E-V5}, we would expect the cells to remain blocked in mitosis. However, when we performed a mitotic index to quantitfy the number of cells in mitosis, both Sororin\textsuperscript{9D-V5} and Sororin\textsuperscript{9E-V5} behaved like Sororin\textsuperscript{WT-V5}. There could be many reasons to explain this unexpected phenomenon. First and foremost, phosphorylation of Sororin is likely more complicated than we originally thought. Perhaps phosphorylation only releases Sororin from mitotic chromosomes in that phosphorylated Sororin may still bind during S phase. S phase is when Sororin competes with Wapl. To address this concern, future studies are aimed at conducting a DNA cellulose binding assay using cells that were transfected with the various Sororin mutants and treated with hydroxurea, to examine the S phase population.

Another possible reason why Sororin\textsuperscript{9D} and Sororin\textsuperscript{9E} replace Sororin\textsuperscript{WT} is the mutants are overexpressed and may mask subtle effects of constitutive phosphorylation. Also, aspartic acid and glutamic acid are not the same as phosphates, and may not properly mimic the phosphorylated state. Regardless, our experiments uncover an important role for Cdk1 phosphorylation of Sororin in inhibiting its association with the cohesin complex and inactivating its stabilization of cohesion during prometaphase.
VI. Conclusions

From this project, we have increased our understanding of Sororin phosphorylation. We demonstrated that Cdk1 phosphorylates Sororin$^{\text{WT}}$ in vitro and in vivo, while Sororin$^{9A}$ is poorly phosphorylated. Also, Sororin$^{9A}$ localizes to chromatin, and these multiple sites of phosphorylation are necessary to remove Sororin from chromatin, reducing its association with the cohesin complex. Interestingly, Sororin$^{9A}$ increases arm cohesion but not centromeric cohesion. Sororin knock-down induces a mitotic arrest that can be reconstituted with the various mutant forms of Sororin. ZM447439 is able to override the mitotic block produced by Sororin shRNA, suggesting that single chromatids are able to recruit aurora kinase and activate the SAC. These experiments helped to uncover the role of Sororin phosphorylation in sister chromatid cohesion. Since many cancers are aneuploid, the understanding of how chromatids are linked together and separate at the right time may help to define how cells become aneuploid and may shed new light on the origins of cancer.
VII. References


Appendix

Introduction

Certain anti-cancer drugs target proteins required for cell cycle processes, including the Aurora kinases [77-80]. Aurora kinases are needed to resolve merotelic attachments to form daughter cells. Mammals contain Aurora A, B, and C kinases. Aurora A functions at the spindle pole to ensure integrity of the centrosomes. Aurora B and C function as part of the CPC, which also consists of INCENP, Survivin, and Borealin [81, 82]. One particular Aurora kinase inhibitor is ZM447439.

ZM447439 does not stop cells from entering mitosis but instead causes defects in chromosome segregation. Although cells exposed to ZM447439 exit mitosis, they are unable to divide, creating polyploid cells that undergo apoptosis. Cells lacking p53 are able to re-replicate DNA when Aurora kinase inhibitors block cytokinesis. We studied the effects of Aurora kinase inhibitors in isogenic pairs of cells that only differed in p53 status. Although p53 slowed cell cycle progression after treatment with ZM447439, this cell cycle block was not completely penetrant. Induction of the p53 response is correlated with the appearance of localized DNA damage after inhibition of Aurora kinases. Removal of the drug after several days allowed some cells to evade killing by the Aurora kinase inhibitor. These clones were not resistant to the drug upon re-exposure and commonly showed alterations in ploidy. The origin of some of these colonies may involve the asymmetric division of multinucleated giant cells.
Materials and Methods

Cell Lines and Culture Conditions

Parental HCT116 cells, originally derived from a human colon carcinoma, contain wild type p53 and were compared to HCT116 cells in which both p53 alleles had been inactivated by homologous recombination [51]. The HT1080 cell line was originally derived from a human fibrosarcoma and contains wild type p53. The HeLaM cell line is a subclone of the HeLa cervical carcinoma cell line [52]. Cells were grown at 37°C with 10% CO₂. All cells were grown in Dulbecco’s minimal essential medium (Gibco) supplemented with 10% fetal bovine serum (Gibco and Atlanta Biologicals), penicillin (10,000 units/ml), and streptomycin (10,000 µg/ml) (Cambrex biosciences). ZM447439 was dissolved in DMSO and was obtained from Astra Zeneca. To induce DNA damage, cells were treated with Adriamycin (Sigma) at 0.2µg/mL or Etoposide (Sigma) at 10µM.

Immunoblotting

Immunoblotting was carried out in section 3.2. An antibody to p53 (DO1) directly conjugated to horse-radish peroxidase was obtained from Santa Cruz Biotechnology. An antibody to Actin was obtained from NeoMarkers. Goat anti-mouse secondary antibodies conjugated to horse-radish peroxidase were obtained from Santa Cruz Biotechnology.
Immunofluorescence

Immunofluorescence was carried out as described in section 3.3. We used p53 and γH2A.X antibodies, and they were detected using secondary reagents conjugated to fluorescein isothiocyanate or rhodamine (Sigma). Hoechst 33342 was used to stain nuclei and coverslips were mounted with Vectashield (Vector Laboratories).

Time-lapse microscopy

Time-lapse microscopy was carried out as described in section 3.4.

Growth Rate Assay

To quantify cell proliferation, cells were plated in 12-well plates, and fixed and stained with a saturated solution of methylene blue in 50% ethanol (v/v). Plates were rinsed and dried. Then the bound stain was extracted with 1xPBS containing 0.1 M HCl. Absorbance of samples was analyzed at 630 nm with a spectrophotometer.
Results

Induction of p53 by the Aurora kinase inhibitor ZM447439

Western blotting indicated that p53 levels were increased by 8 hours after treatment with ZM447439 and remained elevated up to 7 days in the continued presence of the drug (Fig. 33 and our unpublished data). ZM447439 treatment also led to an increase in the steady state levels of p53 phosphorylated at serine 15 (Fig. 33). This phosphorylation event is commonly induced by cellular stress such as DNA damage. Similar levels of serine 15 phosphorylation and total p53 levels were observed with either 2.0 or 2.5µM ZM447439 suggesting that these two doses induce a similar level of cellular stress. Interestingly, co-treatment of cells with ZM447439 and the CDK1 inhibitor purvalanol resulted in lower levels of serine 15 phosphorylation and total p53 levels as compared to ZM447439 alone (Fig. 33). This suggests that cells need to enter mitosis in the presence of ZM447439 in order for p53 to be upregulated.
Figure 33. Induction of p53 by ZM447439. HCT116 p53+/+ cells were treated with ZM447439 for the indicated times followed by analysis of p53. Effect of ZM447439 on p53. The top panel shows HCT116 p53+/+ cells exposed to two different concentrations of ZM447439 (“ZM”) and then analyzed by the Western method for the levels of p53 phosphorylated at serine 15 (p53-pS15) and total p53. Cells were also analyzed after 24 hours of simultaneous treatment with ZM447439 and 10µM purvalanol (ZM+PURV). The membrane was stripped and probed for β-actin to control for loading. Adriamycin (ADR; 0.2µg/ml) was added for 16 hours as a positive control. Cells left untreated (“UNT”) or exposed to dimethyl sulfoxide (DMSO; vehicle for ZM) were included as negative controls.

Induction of p53 by Aurora kinase inhibitors involves DNA damage

ZM447439 induces localized γH2A.X staining and heterogeneous accumulation of p53. Interestingly, although γH2A.X was distributed throughout the nucleus in cells exposed to Etoposide, cells exposed to ZM447439 showed high local concentrations of this modified histone (Fig. 34). In some cells, γH2A.X was confined to single micronuclei within a cell while being excluded from others (Fig. 34). In other cells, γH2A.X was found in localized regions of a single nucleus (Fig. 34). The frequency of these γH2A.X positive regions was relatively rare (5 out of 119 cells, 4%) but they were reproducibly observed in multiple experiments. Cells exposed to ZM447439 also showed a non-uniform distribution of p53 among different nuclei within the same cell (Fig. 34).
Figure 34. Nuclear distribution of p53 and γH2A.X in cells with ZM447439. HCT116 +/+ cells were left untreated (UNT), exposed to 2.5µM ZM447439 (ZM) for 72 hours or to 10 µM Etoposide (ETOP) for 16 hours, fixed and analyzed by immunofluorescence. Cells were stained simultaneously with antibodies for both p53 and γH2A.X.

Avoidance of cell death in cultures exposed to ZM447439

Tumor cells can resume proliferation and form clones after ZM447439 removal. One explanation for the appearance of clones after the removal of ZM447439 was that these cells were resistant to the drug. Cell division in untreated emergent clones occurred similarly to parental cells (our unpublished observations). However, when exposed to 2.5µM ZM447439, all clones tested entered mitosis, but most failed to form a cleavage furrow and exited mitosis without dividing (Fig. 35). The clones analyzed (clones 1, 2, 4, C, F, and J) were derived from HCT116 cells initially exposed to 2.5µM ZM447439. These results suggest that these clones are not resistant to this dose of ZM447439.
Figure 35. Effect of ZM447439 on mitosis in emergent clones. Clones of HCT116 cells that emerged after 1 week exposure to ZM447439 were re-exposed to the drug and analyzed by time-lapse phase contrast microscopy. The percent of cells that undergo cytokinesis (divide) into two daughter cells and the percent of cells that fail in cytokinesis (regress) in the presence and absence of ZM447439 are shown.

Another reason that non-resistant colonies might arise after drug removal was the original presence of a subpopulation of cells that could evade the effects of the drug due to having a long cell cycle. Therefore, it is possible that ZM447439 selects for slow-growing clones. However, clones that arose after drug treatment proliferated at a similar rate as parental HCT116 cells in the absence of treatment (Fig. 36).
Figure 36. Growth rates of emergent clones. A and B.) Cells were plated in 96 well plates in the absence of drugs and stained with methylene blue at the times indicated. Methylene blue was extracted with 0.1N HCl and measured by spectrophotometry (absorbance at 630nm). Samples in “A” were stained, rinsed, extracted, and analyzed on a different day than the samples in “B”. Therefore, absorbance units in “A” may not be directly comparable with those in “B”.

A

B

Figure 36.
Interestingly, colonies that arose from both p53+/+ and p53−/− HCT116 cells exposed to the drug contained an excess of chromosomes with some carrying a tetraploid complement (Fig. 37). This suggested that at some point in their origin these clones had failed to complete mitosis, or had re-replicated their DNA.

Another potential scenario for the origin of clones after removal of ZM447439 is that a small subpopulation of cells may arrest in the cell cycle after a single failed attempt at mitosis. Resumption of cell cycle progression after removal of the drug may allow colonies to form. Analysis of two clones (p53−/− clone J and p53+/+ clone 2) indicated that at least 80% of cells (similar for both clones) were able to enter mitosis twice in the presence of the ZM447439 (Fig. 38). This suggests that these clones are not characterized by a stable preference to arrest after one failed mitosis in the presence of

![Figure 37. Chromosome numbers in emergent clones. Chromosome spreads were prepared from clones of HCT116 cells that emerged after treatment with ZM447439, and stained with propidium iodide to allow counting of chromosomes. Standard deviations are shown by the bars.](image)
ZM447439. This does not preclude the possibility that this may have occurred during the original isolation of the clones. Interestingly, more cells from the clone 2 cell line were able to enter mitosis a second time compared to the parental HCT116 cells. The basis of this difference is now known.

Figure 38. Clones that emerge after ZM447439 treatment attempt mitosis. Mitosis in the presence of 2.5μM ZM447439 was analyzed by time-lapse microscopy for the cell lines indicated. Multiple mitotic attempts in clone J derived from HCT116 p53−/− cells (A) and clone 2 derived from HCT116 p53+/+ (B) are shown.
The presence of p53 slows down re-replication and appeared to reduced the number of colonies after ZM447439 treatment [53]. Therefore, we analyzed p53 responses in some of the cell lines that arose after treatment of HCT116 p53+/+ cells with ZM447439. All but one cell line showed a normal induction of p53 protein in response to Etoposide and ZM447439 (Fig. 39A). The defect in Clone #1 does not appear to be due to alteration of the hDM2-mediated degradation of p53 since the hDM2 inhibitor Nutlin3 was able to induce p53 (Fig. 39A). Also, p53 in Clone #1 was still phosphorylated at serine 15 in response to Etoposide indicating that DNA damage signaling pathways upstream of p53 may be intact (Fig. 39B). Therefore, the emergence of colonies is only rarely associated with the alteration of p53 signaling pathways.

**Figure 39.** p53 signaling in cells that evade killing by ZM447439. p53, p21/waf1 and serine 15 phosphorylation of p53 (pS15-p53) were analyzed in clones that emerged after exposure of HCT116 cells to 2.5 µM ZM447439. Western blots were probed with the antibodies indicated, stripped and reprobed with antibodies to β-actin to control for loading. HCT116 p53+/+ cells were used as a positive control for Western blots. A). Effect of Etoposide and Nutlin 3 on the levels of p53. Cells were left untreated (“Unt”), treated with Etoposide at 10µM for 16 hours (“Etop”), or treated with Nutlin 3 at 10µM for 18 hours (“Nut”) and then analyzed by Western blotting. B.) Effect of Etoposide and Nutlin 3 on the phosphorylation of p53 at serine 15. Cells were exposed to Etoposide or Nutlin 3 as described above and analyzed by Western blotting with antibodies that recognized p53 phosphorylated at serine 15.
Discussion

Our studies were aimed at understanding the cellular responses to Aurora kinase inhibition. A number of Aurora kinase inhibitors are currently in various stages of development. Three inhibitors, Hesperadin, ZM447439, and MK-0457 have received the most attention and all are capable of preventing cell division [80]. Aurora kinase inhibitors can kill tumor cells and there is additional evidence that killing may be more efficient in cells lacking p53 [83]. Our studies were aimed at further characterizing the role of p53 in the response of human tumor cells to Aurora kinase inhibitors and analyzing the long-term effects of these drugs in vitro.

In summary, our studies indicate that ZM447439 induces DNA damage and up-regulates p53 through a pathway that relies on the ATM/ATR protein kinases. In addition, the cells that evade killing by Aurora kinase inhibitors in our study were not resistant to the drug. In a clinical setting, it is possible that a higher dose, more prolonged treatment, or sequential treatments with Aurora Kinase inhibitors may generate resistant cells. At least one report has shown that mutations in Aurora B can occur in cell lines and can confer resistance to a panel of Aurora B inhibitors [84]. However, if tumor cells can evade these inhibitors during chemotherapy in a manner similar to what we have observed, we predict that the resulting cells could be sensitive to subsequent treatments with the same agent.