A Thesis Entitled

cdca8: A Target of p53/Rb Dependent Repression

by

Cara Jacob

Submitted as partial fulfillment of the requirements for the Master of Science in Biology

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Advisor- Dr. William Taylor

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Graduate School

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An Abstract of

*cdca8*: A Target of p53/Rb Dependent Repression

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One of the hallmarks of cancer is uncontrolled cell division. Our goal is to find and characterize novel genes that are important for cellular division. Characterizing novel cell cycle genes is an important step in gaining insight into mechanisms that regulate the cell cycle. Rb/E2F is a complex that controls the expression of genes that regulate the cell cycle. Previous research that utilized Affymetrix microarrays identified a potential novel E2F target. The sequence that was detected was an EST that corresponded to the mouse predicted protein D4Ertd421e. This sequence is 76% identical to a human protein, Cdca8. Cdca8, or cell division cycle associated 8, was given this name because its expression, based on EST frequencies, correlated with other cell cycle genes.

*cdca8* is regulated in a cell cycle-dependent manner, characteristic of Rb/E2F targets. *cdca8* is also repressed in a p53-dependent manner, but basal levels of *cdca8* are not solely controlled by p53. This suggests there are multiple pathways that control the expression of *cdca8*. The expression of *cdca8* is also over represented in cancerous tissues. Two splice variants of *cdca8* were identified, but they appear to have the same protein product and function the same in cells. Localization studies show a dynamic
localization pattern for Cdca8, implicating it as a possible component of cellular division. These studies have provided further insight into the mechanisms of cellular division. The understanding of the molecular basis of the cell cycle is required for greater knowledge of cancer formation, prevention, and treatment.
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<td>chronic myelogenous leukemia</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>Cdk</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin dependent kinase inhibitor</td>
</tr>
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<td>Rb</td>
<td>retinoblastoma</td>
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<td>GTP</td>
<td>guanosine 5’-triphosphate</td>
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<td>Plk1</td>
<td>polo-like kinase 1</td>
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<td>GDP</td>
<td>guanosine 5’-diphosphate</td>
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<td>INCENP</td>
<td>inner centromere protein</td>
</tr>
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<td>telophase disk-60</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis family</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<td>Cdca8</td>
<td>cell division cycle associated 8</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryo fibroblast</td>
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<tr>
<td>RLC</td>
<td>repressed like Cdk1</td>
</tr>
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<td>Hdm2</td>
<td>human Mdm2</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>MEGM</td>
<td>mammary epithelial growth medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s medium</td>
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<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney 293</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
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<td>Lurea Broth</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>picomole</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>TBE</td>
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<td>tris EDTA</td>
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<td>µl</td>
<td>microliter</td>
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<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>BES</td>
<td>N, N-Bis(2-hydroxyethyl)-2aminoethanesulfonic acid</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<td>w</td>
<td>weight</td>
</tr>
<tr>
<td>v</td>
<td>volume</td>
</tr>
<tr>
<td>gapdh</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>µCi</td>
<td>micro curie</td>
</tr>
<tr>
<td>dCPT</td>
<td>2’-deoxycytosine 5’-triphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<td>--------------------------------------------------</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline plus Tween</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>CGAP</td>
<td>cancer genome anatomy project</td>
</tr>
<tr>
<td>shp53</td>
<td>short hairpin p53</td>
</tr>
<tr>
<td>shp21</td>
<td>short hairpin p21</td>
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<tr>
<td>LVSV40 ERN</td>
<td>LVSV40 empty reverse neomycin</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<tr>
<td>ADR</td>
<td>adriamycin</td>
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<td>NPAT</td>
<td>nuclear protein mapped to the AT locus</td>
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<td>D1/Cdk2</td>
<td>Cyclin D/ Cdk2</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>Mut</td>
<td>mutant</td>
</tr>
<tr>
<td>cdca8L</td>
<td>cdca8 long form (with intron 1)</td>
</tr>
<tr>
<td>cdca8S</td>
<td>cdca8 short form (intron 1 spliced)</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S transferase</td>
</tr>
<tr>
<td>MCAK</td>
<td>mitotic centromere-associated kinesin</td>
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I. Introduction

A hallmark of cancer is uncontrolled cell division, which entails duplication of all cellular contents, nuclear division and cytokinesis, processes that are coordinated by the cell cycle. According to the American Cancer Society, approximately 10 million people worldwide are diagnosed with cancer every year and approximately 500,000 people in the United States die from cancer each year. Understanding the molecular mechanisms responsible for cancer formation is crucial for prevention and treatment. Molecular techniques are being used as preventative measures to test for the reoccurrence of cancers, such as in the breast. In particular a test has been developed that is based on the level of RNA expression of a variety of cancer-related genes. Using this test has proven useful in determining what patients can benefit the most from chemotherapy [1]. Molecular biology research has also led to a new treatment for chronic myelogenous leukemia (CML). The drug Gleevec is a selective inhibitor of the BCR-ABL tyrosine kinase which is overactive in CML. Unlike most other drugs used for cancer treatment, Gleevec kills only leukemic cells [2]. Molecular biology research has also led to treatments for people with breast cancer. The receptor for epidermal growth factor is frequently over expressed in breast cancer and there have been many proposals to target this receptor for anti-tumor therapy. One such means is to target the EGF receptor by
using an EGFR specific toxin. This toxin was produced by fusing sequences for the enzymatic activity and membrane translocation domains of diphtheria toxin plus sequences from human epidermal growth factor [3].

**Regulation of the Cell Cycle by Cdks and Cyclins**

Nuclear division, as well as cytokinesis, is coordinated by the cell cycle. Cyclins, cyclin dependent kinases (Cdks), and cyclin dependent kinase inhibitors (CDKIs) tightly regulate the cell cycle, which consists of G0, G1, S, G2, and M phases and concludes with cytokinesis [4]. Cdks are found in a complex with a cyclin protein. All cyclins contain a common region of homology called the cyclin box, a domain used to bind and activate cdks. Eukaryotic cells in G1 express low levels of Cdk activity, but as cells progress through G2 and M, the levels increase. After M phase, Cdk activity is low again (Arellano, 1997 #112). Cyclin E/ Cdk2 and Cyclin D/ Cdk4, 6 function at the G1/S boundary; whereas progression into S phase is regulated by Cyclin A/ Cdk2. Cyclin B1/ Cdk1 and Cyclin A/ Cdk1 are important for regulation at the G2/M boundary.

The D-type cyclins (D1, D2, and D3) and their catalytic partners Cdk4 and 6 are important during early G1 phase and then are no longer required until cells complete mitosis and re-enter the following G1 phase [5, 6]. Mitogen induced signal transduction pathways are crucial in promoting activation of Cyclin D-Cdk complexes at the transcriptional, translational, and assembly level. These pathways are also important for import of the Cyclin/ Cdk holocomplex into the nucleus where they phosphorylate their substrates [5]. Once the Cyclin D/ Cdk complex is assembled, it can promote cell
division by inactivating two classes of cell cycle inhibitors: negative regulators of S-phase gene expression and a class of stoichiometric Cdk inhibitory proteins.

One such substrate of the Cyclin D/Cdk complex that negatively regulates cell cycle dependent gene expression is the Rb family members. Phosphorylation of the family members (Rb, p130, p107) helps to inactivate their transcriptional co-repressor activities [7]. Phosphorylation of Rb frees E2F, a transcription factor that can then bind to the promoters of genes that are required for the cell cycle and DNA synthesis, such as Cyclins E and A, Cdk1, DNA polymerase α, and thymidine kinase. Cyclin D/ Cdk4 has also recently been found to phosphorylate Smad3; this phosphorylation negatively regulates the functions of transcriptional complexes that mediate cell growth inhibition by TGF-β family proteins [8]. The Cyclin D/ Cdk complex also plays a role in inactivating two CDK1’s, p27Kip1 and p21Cip1 by sequestering them away from their substrates. p27 and p21 are potent inhibitors of Cdk2 [9] [10] [5]. Binding of CIP/KIP family members by Cyclin D1/ Cdk4 stabilizes the complex and results in nuclear import [11] [12].

The Cyclin E/ Cdk2 complex is functional during the same phase in the cell cycle (G1) as Cyclin D/ Cdk 4 and 6, but is activated after the D type cyclins. The activity of the Cyclin E complex is maximal at the G1 to S phase transition [13] [14]. The activity of Cyclin E/ Cdk2 is mitogen dependent like the Cyclin D complex and also has similar substrates such as Rb and p27Kip1, but inactivation of these substrates occurs through different mechanisms. p27Kip1 is not sequestered by Cyclin E, but instead is phosphorylated on a single threonine by Cyclin E/ Cdk2 [15]. The Cyclin E complex does inactivate Rb through phosphorylation, but phosphorylation occurs at a site different
than where the Cyclin D complex has its effects [16]. It is also believed that Cyclin E/Cdk2 phosphorylates substrates that play a more direct role in cell duplication leading to downstream effects such as a change in histone gene expression by phosphorylation of nuclear protein mapped to the AT locus (NPAT), centrosome duplication by phosphorylation of nucleophosmin, and replication origin firing by phosphorylating MCM proteins [17-19].

Cdk2 is not only found in a complex with Cyclin E, but is also found bound to Cyclin A. Levels of Cyclin A/ Cdk2 increase as cells begin to replicate their DNA and then decrease when Cyclin A is degraded in early mitosis [20]. During S phase, Cyclin A/ Cdk2 triggers the start of DNA replication from already assembled replication initiation complexes and also inhibits the assembly of new complexes [21]. The dual function of Cyclin A ensures that G1 ends before DNA synthesis begins, thereby preventing re-initiation until the next cell cycle [22]. Cyclin A is found in a complex with Cdk2, but can also be found bound to Cdk1 during late S phase where it is important for the completion of S phase and entry into mitosis [23-25]. Some members of the E2F family of transcription factors are negatively regulated by Cyclin A. Cyclin-associated kinases can phosphorylate DPI, a binding partner of E2F. This results in a loss of E2F DNA-binding activity. The activities of Cyclin A in the regulation of E2F are opposite to what is found with Cyclin E. Cyclin E has the ability to positively regulate E2F activity [26] [27].

The Cyclin A/ Cdk1 complex is important in regulating mitosis along with Cyclin B1, B2/ Cdk1. These complexes phosphorylate cytoskeletal proteins, such as lamins, histone H1, and components of the mitotic spindle. In order for cells to exit mitosis, both
Cyclin A/ Cdk1 and Cyclin B/ Cdk1 complexes must be degraded. The most important activity is the degradation of Cyclin B1, leading to a loss of Cdk activity [26] [27]. Figure 1 depicts the regulation of progression through the cell cycle by Cyclin/ Cdk complexes.

**Control of Cell Division by p53 and the Rb Family**

As previously mentioned, Rb is a substrate for Cyclin/ Cdk complexes and its tight regulation by these complexes suggests it has a crucial role in the cell cycle. The role that Rb plays in the cell cycle is that of a tumor suppressor. The level of phosphorylation controls the activities of Rb. During late G1, Rb is hyperphosphorylated and remains so throughout S, G2 and M. At this level of phosphorylation, Rb and E2F do not interact and E2F is free to activate transcription. Cells in G0/G1 contain hypophosphorylated Rb that is bound to E2F 4 and 5, which causes transcriptional repression [28-31]. Constitutive heterozygosity for functional Rb in humans greatly increases the frequency of retinoblastoma, osteosarcoma as well as other types of tumors. When Rb is functional it promotes apoptosis or cell cycle arrest in cells with harmful defects. Cell cycle arrest occurs by Rb acting as a histone deacteylase and consequently causing chromatin to be condensed and causing transcription of genes required for the cell cycle to be inhibited.

p53, another tumor suppressor, can regulate Rb activity. In a normal cell, p53 levels are kept low by Mdm2, which adds ubiquitin to lysine residues on p53. In a stressed cell, p53 is activated by phosphorylation of serine and threonine residues and acetylation or sumoylation of lysines [32]. In cells that have encountered DNA damage,
**Figure 1.** Cyclin/ Cdk complexes regulate progression through the cell cycle. The cell cycle consists of G1, S, G2, and M phases. Each phase of the cell cycle is under tight control by Cyclin/ Cdk complex. During G1, Cyclin D/ Cdk4,6 and Cyclin E/ Cdk2 complexes are active. During S phase, Cyclin A/ Cdk2 has maximal activity as DNA is duplicated. Cyclin A/ Cdk1 is important for entry into M phase, where as Cyclin B1/ Cdk1 is important throughout M phase and must be degraded for cells to exit mitosis.
levels of p53 are higher compared to normal cells. p53 can halt the cell cycle at either G1 or G2 phases of the cell cycle. G1 arrest occurs through upregulation of p21\textsuperscript{waf1} by p53, which inhibits Cyclin D1/ Cdk4 activity, thereby reducing the phosphorylation of Rb. Arrest at the G2 phase is through an indirect pathway in which p53 upregulates p21\textsuperscript{waf1}, which inhibits Cdk activity, mainly that of Cdc2/ Cdk1. This in turn increases the level of hypophosphorylated Rb that can bind to E2F and repress transcription [33]. When p53 or Rb is mutated, the cell cycle can continue even in the presence of DNA damage or errors. Unchecked cell cycle progression errors may result in aneuploidy, cell death, or oncogenesis [4].

**Multiple Pathways Coordinate Progression Through Mitosis**

If a cell has properly duplicated its DNA content, it must then divide all cellular components. Mitotic spindle formation is essential for chromosome segregation and positioning of the cleavage furrow in order for cytokinesis to occur. The spindle is a dynamic structure composed of microtubules that connect to the chromosomes [34]. Spindle dynamics are controlled by a number of factors including Ran-GTP, Plk1, Cdk1, and the chromosomal passenger complex [34-37]. Ran-GTP plays an essential role in nuclear transport, but is also required for microtubule formation and stabilization [35]. Ran-GTP is found at high levels near chromosomes and induces microtubule nucleation [34, 35]. Microtubule assembly is promoted by increasing local concentrations of tubulin, which is regulated by stathmin [38]. During mitosis, Plk1 phosphorylates stathmin; this
action inhibits the ability of stathmin to bind to tubulin [34]. Stathmin destabilizes microtubules by sequestering tubulin heterodimers [38]. The length of microtubules is determined by active Cdk1 kinase and also by changes in the ratio of Ran-GTP to Ran-GDP. During interphase, microtubules are able to self-assemble, but in mitotic extracts only a few microtubules are able to self assemble and centrosomes are able to nucleate only a few microtubules. Upon addition of Cdk1 to mitotic extracts, a transition between the two states of microtubule assembly can be achieved. It is not known exactly how Cdk1 and Ran-GTP work together in determining the length of microtubules but local changes in phosphorylation seem to be important. [34].

During anaphase and telophase, microtubules undergo vast changes in their organization. During anaphase, microtubules become organized in an antiparallel and interdigitating fashion. A number of regulatory proteins associate with the microtubules and become organized into bundles in the spindle midzone, which is the region between separated sister chromatids. Upon completion of anaphase, the cleavage furrow forms and actin and myosin become organized into a ring called the actinomyosin ring [39]. The ring then contracts, which creates a membrane barrier between the cytoplasmic contents of the two daughter cells. The ingressing furrow and contracting ring causes midzone microtubules to condense into a structure called the midbody [39, 40]. Cytokinesis is then thought to initiate sometime in anaphase by signals from polar microtubules at the midzone [41].
The Chromosomal Passenger Complex

Cytokinesis, as well as spindle assembly and chromosome segregation and condensation are regulated by the chromosomal passenger complex, which consists of INCENP, Aurora B kinase, Survivin, TD-60, and Cdca8 [4, 37] [42] [43] [44]. The dynamic localization of the passenger complex suggests it plays a role in coordinating chromosomal and cytoskeletal events during mitosis. One possibility is that the chromosomal passenger complex helps to stabilize the spindle midzone, allowing delivery of proteins such as actin to the actomyosin ring [39]. The release of the passenger complex from centromeres at anaphase after sister chromatid separation, may ensure that the actomyosin ring, or other structures needed for cleavage do not assemble prematurely. The complex is associated with chromosomes during early mitosis and then moves to the centromeres during metaphase. At the metaphase/anaphase transition, the complex disassociates from chromosomes and associates with the antiparallel microtubules at the spindle midzone where it remains throughout cytokinesis [43, 45]. Also, the passenger complex promotes proper chromosome alignment and segregation by phosphorylating histone H3 through Aurora B [4] [46].

INCENP belongs to a class of proteins that localizes to the inner centromere. Through metaphase, INCENP is localized between the sister chromatids and is concentrated at the centromere. During anaphase, INCENP leaves the inner centromere and becomes highly concentrated at the spindle midzone [41]. INCENP is a 95 kDa
protein with distinct regions for microtubule binding [45] [47]. Intact, dynamic microtubules are required for INCENP localization to the spindle midzone. If microtubules are depolymerized during anaphase by the addition of taxol, INCENP becomes distributed throughout the entire cell as was shown through immunofluorescence. Yeast two-hybrid and *in vitro* binding assays suggest that targeting of INCENP to the midzone is mediated by a direct interaction between INCENP and microtubules [47]. An N-terminal domain of INCENP associates with centromeres, while a C-terminal domain is required for association with interphase microtubules [45, 47]. Over expression of INCENP lacking amino acids 1-450 interferes with metaphase chromosome alignment and cytokinesis [45]. Consistent with a role in mitosis, the expression of INCENP mRNA is maximal during G2 and lowest during G1 [41].

INCENP is phosphorylated at the C-terminus by Aurora B kinase, another member of the chromosomal passenger complex [48] [49]. The Aurora family of kinases consists of three proteins, Aurora A, B, and C, each of which has been implicated in different aspects of mitotic spindle dynamics [46]. Only Aurora B shows the localization characteristic of the chromosomal passenger proteins. CSC-1, a *C. elegans* subunit of Aurora B kinase, binds to ICP-1 (INCENP) and also BIR-1 (Survivin), another member of the chromosomal passenger complex. Localization of AIR-2 (Aurora B) requires CSC-1, BIR-1, and ICP-1 to localize to chromosomes in *C. elegans* [50]. In mammalian cells, the localization of Aurora B also depends on INCENP and Survivin as was shown in RNAi studies [51] [52] [53]. Removal or disruption of Aurora B from the complex causes defects in chromosome alignment, loss of phosphorylation of histone H3, failure to localize spindle checkpoint components, and a lack of cytokinesis [51] [52]. Interestingly,
Aurora A and Aurora B kinase levels are elevated in a number of human cancers and are oncogenic when over-expressed [54].

The chromosomal passenger complex also contains Survivin, which has been implicated as a member of the inhibitor of apoptosis family (IAP). Survivin’s role in apoptosis has come into question recently and it is suggested that it has a more important role as a chromosomal passenger protein. For example, RPE and IMR-90 cells are not immediately killed when Survivin is knocked down by siRNA. Death does occur but only after aberrant mitosis and induction of DNA damage and p53 signaling [55]. Survivin, like the other complex members, is regulated in a cell-cycle dependent manner [56]. Survivin influences microtubule dynamics and promotes increased stability of microtubules by regulating growth and catastrophe rates by recruiting motor proteins [57]. Mammalian cells lacking Survivin, due to siRNA, are unable to align their chromosomes, fail to recruit Aurora B to kinetochores, and become polyploid at a high frequency [58]. Interestingly, consistent with this hypothesis, survivin mRNA is over-expressed in chemoresistant cancerous cells [57].

The role of TD-60 in the chromosomal passenger complex is not clear because of the lack of research that has been conducted on the protein. TD-60 does interact with INCENP, but an interaction with any other passenger proteins has not been proven. A link between the other chromosomal passengers has not been proven through co-immunoprecipitation experiments. TD-60 does co-localize with INCENP, and demonstrates the characteristic passenger complex localization pattern throughout G2 and M phases (25). TD-60, as well as INCENP, are located at the spindle midzone, a structure that arises during late telophase and makes contact with the cell cortex at the site that
predicts where the cleavage furrow will form [59]. Therefore, TD-60 may assist INCENP to signal the induction of cytokinesis in cells, supporting the possibility of TD-60 as a chromosomal passenger protein.

Our studies have focused on cell division cycle associated 8 (Cdca8) also known as DasraB and Borealin, a recently discovered fifth member of the chromosomal passenger complex [44] [37]. cdca8 was discovered by Affymetrix microarrays as a gene repressed in response to DNA damage (Taylor, unpublished data). To obtain Affymetrix data, mouse embryo fibroblasts (MEFs) lacking p130 and p107 and wild-type MEFs were treated with adriamycin for 12 and 24 hours in order to find genes repressed in wild-type cells but not p130/p017 null cells. In response to adriamycin, cdca8 was repressed in wild-type cells, but not in p130/p107-null MEFs, suggesting that cdca8 is a target of Rb-dependent repression. A number of genes in addition to cdca8 were found to be repressed at least 2 fold in an Rb-dependent manner. Some genes identified are required for entry into mitosis, such as cyclin B1, cdc25, and survivin. Others identified are important for spindle formation such as bub1 and mad2. A third set of genes identified are important for DNA synthesis (Table 1). Finally, 10 novel genes including cdca8 and 14 miscellaneous genes were also identified (Table 2). Our lab termed these genes RLC1-5 because their expression is similar to Cdk1 (repressed like Cdk1). cdca8 was given the name because expression of cdca8 EST’s correlated with other cell cycle genes (Jackson et al., unpublished data). A statistical method for co-expression analysis also identified cdca8 as a gene whose expression is under cell cycle control [60].

In the present studies we have investigated the regulation of human cdca8 expression during cell cycle progression, or in response to p53 activation, and in multiple
normal and cancer derived cell lines and tissues. Also, we have begun to investigate the
effect of disrupting the cytoskeleton on the dynamic localization of Cdca8 protein. Our
studies have shown that cdca8 is 1.) regulated in a cell cycle-dependent manner, 2.)
repressed in response to p53, and 3.) highly expressed in cancerous tissues. Our studies
have also shown that disrupting microtubules and the actin cytoskeleton in telophase cells
releases Cda8 from the midbody. When the cytoskeleton is disrupted in early mitotic
cells, Cdca8 remains localized to the chromosomes.
Table 1. Genes repressed in a p130/p107 dependent manner. A number of genes are regulated in an Rb-dependent manner (Jackson et al., unpublished data). Genes found were divided into five categories, including mitotic genes, spindle checkpoint genes, genes required for DNA synthesis, novel genes, and finally miscellaneous genes.
<table>
<thead>
<tr>
<th>Function</th>
<th>Number of Genes</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitosis</td>
<td>24</td>
<td>Cdk1, Cyclin B1, Cdc25c, Survivin</td>
</tr>
<tr>
<td>Spindle checkpoint</td>
<td>3</td>
<td>bub1, mad2</td>
</tr>
<tr>
<td>DNA synthesis</td>
<td>10</td>
<td>Mcm5, Mcm3</td>
</tr>
<tr>
<td>Novel</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>14</td>
<td>racgap1</td>
</tr>
</tbody>
</table>
Table 2. Novel genes identified by Affymetrix. Ten novel genes were identified by Affymetrix to be repressed at least 2 fold in response to adriamycin in p130/p107 null MEFs. Our lab has focused on five of the ten novel genes. The present studies focus on *cdca8*, which is presented in red. Our lab gave the name RLC 1-5 (repressed like Cdk1) to the five novel genes because their expression is similar to Cdk1.
<table>
<thead>
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</tr>
</thead>
<tbody>
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<td>-8</td>
<td>-2</td>
</tr>
<tr>
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<td>+1.2</td>
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</tr>
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II. Materials and Methods

1. Cell Lines and Culture Conditions
1.1 Cell Lines

All cell lines were previously derived from various human tissues. NARF2 cells were derived from U2OS osteosarcoma cells that contain wild-type p53 and Hdm2, by transfection with a construct that encodes an IPTG-inducible p14ARF [61]. A number of leukemia cell lines were used in expression analysis. The cell lines K562, KT-1, and BV173 were all previously derived from chronic myelogenous leukemias. The Jurkat cell line was derived from T cells, while the Daudi cell line was derived from B cells. Finally, the TF-1 cell line was derived from myeloid leukemia. The leukemia cell lines were a gift from Dr. Fan Dong. Prostate cancer derived cell lines were also used for expression analysis. Du145 and PC-3 cells are androgen-independent human prostate cancer cell lines, while LNCaP cells are androgen-dependent. The prostate cell lines were a gift from Dr. Lirim Shemshedini. HCT 116 cell lines were derived from a human colon carcinomas. Two different variants of HCT 116 were used, the parental cell line contained wild type p53 (p53+/+), while the other had both p53 alleles knocked out (p53-
by homologous recombination. The HT1080 cell line was derived from a fibrosarcoma and contains wild type p53. HT1080 GSE cells were derived by infecting HT1080 with a retrovirus that expressed GSE 56, a dominant-negative version of p53. HT1080 LX are a control cell line infected with the empty retrovirus vector [62]. The HeLaM cell line was used in transfections as well as expression analysis, and was previously derived from cancerous cervical tissue. The HeLaM cell line was a gift from Dr. Douglas Leaman. The HEK293 cell line was derived from human embryonic kidney cells. The cell lines MCF7 and MCF10A were both derived from breast tissue. MCF7 was derived from cancerous mammary epithelium, whereas MCF10A was derived from the corresponding non-cancerous tissue. The MCF10A cell line was a gift from Dr. Deborah Vestal. Finally, the MDAH041 cell line was derived from p53 negative immortalized fibroblasts.

A number of derivatives of the NARF2 cell line were obtained for expression analysis. The cell lines were created by first infecting NARF2 cells with a retrovirus containing pBabe-Bleo-Eco vector. Infected cells were pooled and then cells were individually infected with retroviruses with the following vectors: pLXSN, pLXSN-GSE56, pLXSN-Cyclin E, pLXSNE7, pBabe-Puro, pBabe-puro-Cyclin D1/Cdk fusion or were infected with lentiviral vectors encoding Hdm2 cDNA or short hairpin RNA targeting p53 or p21 (Jackson et al., unpublished data) These cells lines were a generous gift from Mark Jackson (CWRU, Cleveland, OH)

1.2 Culture Conditions

Cells were grown in plastic culture plates in an atmosphere of 37°C and 10% carbon dioxide. Leukemia derived cell lines were grown in 25cm² culture flasks in suspension.
All cells were grown in media supplemented with 10% fetal calf serum (FCS) (Gibco and Atlanta Biologicals), penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml) (Cambrex biosciences). Leukemia derived cell lines, BV173, KT-1, Jurkat, Daudi, TF-1, and K562 as well as prostate cancer derived lines LNCaP and PC-3 were maintained in RPMI media (HyClone). The prostate cancer derived cell line DU145 was grown in F12K media (gift from Shemshedini lab). Mammary epithelial growth media (MEGM) (Cambrex biosciences) was used to maintain the non-cancerous derived breast cell line MCF10A. All other cell lines were maintained in Dulbecco’s minimal essential medium (DMEM) (Gibco).

2. Isolation and Preparation of DNA Plasmids

The vector pcDNA3.2/V5/Gw/D-TOPO purchased from Invitrogen was used to construct Flag-tagged Cdca8. This vector contains genes that confer resistance to ampicillin and neomycin.

The vector pcDNA3.1/myc-His(-)A MCS from Invitrogen was used for construction of GFP-Cdca8. This plasmid contains a myc epitope as well as a polyhistidine tag which were replaced with EGFP cDNA by Shaun Rosebeck. EGFP was cloned into the restriction enzyme cites Kpn1 and Bfr1, and retains all other restriction sites. The pcDNA 3.1 vector contains a neomycin resistance gene and an ampicillin resistance gene.
2.1 Propagation of Purified Plasmid DNA

2.1.2 Transformation of \textit{E.coli}

To propagate pcDNA3.2/V5/Gw/D-TOPO and pcDNA 3.1/\textit{myc}-His plasmids, 20 ng of vector DNA was transformed into competent \textit{E. coli} cells. Vector DNA and \textit{E. coli} were incubated on ice for 30 minutes, then 42°C for 90 seconds, and then back on ice for 2 minutes. One ml of Lurea Broth (LB) was added to the mixture and incubated for 1 hour at 37°C. The transformed \textit{E.coli} was spread on LB agar plates with (50 µg/ml) penicillin and incubated at 37°C for 16 hours. Individual colonies were picked and grown in liquid LB cultures on a shaker at 37°C for 16 hours and used for large-scale plasmid preparation.

2.1.3 Construction of Flag-tag and GFP-Cdca8

To construct the Flag-tag DNA, the tag was incorporated into the reverse primer (5’CGCGAATTCTCACTTGTCGTCGTCCTTGTAGTCTTTGTGGGTCCGTATGCTGCT 3’) The forward primer for the PCR amplification of Flag-tag DNA was constructed with a Bcl1 restriction enzyme site on the 5’end for future manipulations (5’CACCTGATCACAGCGAGGTTTTGCTCAGCC 3’). Also, a “CACC” overhang was added to allow directional cloning into pcDNA3.2. RNA from HT1080 cells was made into cDNA and Flag-tag DNA was amplified first by completing 30 cycles of PCR using Taq polymerase, followed by 35 cycles of PCR with VENT polymerase at an annealing temperature of 58°C. To construct Flag-tag Cdc8, pcDNA3.2/V5/Gw/D-TOPO vector
was used for topoisomerase-mediated ligation according to the manufacturer’s instructions (Invitrogen).

The plasmid containing cdca8 cDNA called BTTOP4 was used as a template to amplify the insert to construct the GFP vector. A forward primer was constructed that contains an EcoR1 restriction enzyme site on the 5’end which leaves a 5’overhang when cut (5’GCGGAATTCCAGCGAG GTTTT GCTCAGCC 3’). The reverse primer was constructed with a Kpn1 site on the 5’end that leaves a 3’overhang after restriction digest (5’ GCGGGTACCTTTGTGGGTCCGTA TGCTGCT 3’). One hundred ng of genomic DNA and 100 pmole of primer were used in PCR amplification. The proofreading polymerase VENT was used in the 50 µl reaction. Genomic DNA was amplified for 30 cycles, with a 1 minute denaturation time at 95°C, 1 minute annealing at 58°C, and an extension time of 3 minutes at 72°C.

To construct GFP-Cdca8, pcDNA 3.1/myc-His was cut with EcoR1 and Kpn1 for 1 hour at 37°C. Insert DNA was also digested with the same restriction enzymes that corresponded to the appropriate plasmid. Digests were run on a 0.7% agarose gel in 0.5 TBE (5.4%Tris, 2.75% Boric Acid, 0.5 M EDTA, pH 8.0) and bands were excised and purified using QIAEX II Gel Extraction Kit according to manufacturer’s instructions (QIAGEN Sciences).

A three to one molar excess of insert to vector was used for ligations, along with 10X ligase buffer, ligase, and water. The mixture was incubated at room temperature for 3 hours. The ligation reaction was then added to competent E. coli and transformed as described above for transformation of the TOPO and GFP vectors, with the exception that the final solution was resuspended in 250 µl of LB instead of 1 ml. All colonies were
picked and grown at 37°C in LB broth on the shaker for small scale and large-scale plasmid preparation. All clones were confirmed by sequencing.

PCR products were purified using phenol/chloroform. 200 µl of 100 mM Tris, 10 mM EDTA pH 8.0 (TE) was added to PCR products as well as 300 µl phenol/chloroform/isoamylalcohol. The solution was vortexed briefly and then centrifuged at 16,100 x g for 5 minutes. The top aqueous layer was removed and sodium acetate was added to a final concentration of 0.3 M at pH 5.2, followed by adding 2 volumes of ethanol. The solution was mixed and placed at –20°C for 20 minutes and centrifuged at 4°C for 20 minutes at 16,100 x g. The supernatant was then removed and washed with 200 µl of 70% ethanol and centrifuged for 5 minutes at 5,400 x g. The supernatant was once again removed and the DNA pellet was resuspended in 40 µl of TE.

2.1.4 Small Scale Plasmid Preparation

Isolation of Flag-tag Cdca8 and GFP-Cdca8 plasmids was conducted by pelleting transfected bacteria and resuspending the pellet in 200 µl of STET which contains 10mM Tris-Cl, pH 8.0, 0.1 M NaCl, 1mM EDTA pH 8.0, 5% Triton X 100 and 10 mg/ml lysozyme and then placed into boiling water to kill DNAse and precipitate protein and chromosomal DNA. The solutions were then centrifuged at 16,100 x g for 10 minutes in a 4°C microcentrifuge. A large clot formed that was removed with a toothpick. DNA was precipitated with 200 µl of isopropanol and left at room temperature for 10 minutes, and then centrifuged for 5 minutes at 16,100 x g. The supernatant was removed and the pellet was washed with 70% ethanol. After centrifuged for 5 minutes at 16,100 x g, the ethanol
was removed and the pellet was air dried and resuspended in 50 µl of TE ([63] and modified by Koelle).

2.1.5 Large Scale Plasmid Preparation

Large quantities of plasmid were prepared by growing individual colonies in 5 ml LB containing ampicillin (50 µg/ml). After overnight incubation at 37°C the suspension was added to 70 ml of LB plus ampicillin. The 70 ml culture was incubated for 16 hours on a shaking incubator at 37°C. Isolation of plasmids was conducted following manufacturer’s instruction for Perfectprep Plasmid Midi prep kit (Eppendorf).

3. Transfection of Plasmid DNA

3.1 Transient Transfection

For transfection of Flag-tag Cdca8 and GFP-Cdca8, 3 µg of DNA was added to 500 µl of sterile DMEM. In a separate tube, 15 µl of GenePorter (Gene Therapy Systems) was added to 500 µl of DMEM. The two solutions were combined and incubated for 30 minutes at room temperature. The mixture was then added to growing HEK 293 cells, and then incubated at 37°C and 3% carbon dioxide (CO₂) for 7 hours, then incubated at 10% CO₂ overnight. The media on the transfected cells was changed the next day.

3.2 Stable Transfection

GFP-Cdca8 construct was stably inserted into HeLaM cells using 50 µl CaCl₂, 500 µl N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 500 µl water, and 10
µg GFP-Cdca8 DNA. The solution was incubated for 15 minutes and added to HeLaM cells for 6 hours at 3% CO₂ and then incubated at 10% CO₂ for 16 hours. Clones stably expressing GFP-Cdca8 were selected by adding G418 (0.2 mg/ml). Clones of G418 resistant cells were picked by scraping the colony with a pipette tip and expanding the colony in a 6 well plate. Stable GFP-Cdca8 clones were screened by immunofluorescence. Colonies expressing moderate and high levels of GFP-Cdca8 were selected, expanded onto larger plates, and used for localization experiments.

4. Imaging

4.1 Immunofluorescence

Cultures for immunofluorescence with Flag-tagged Cdca8 were grown on coverslips and harvested at 50-75% confluence. Cells grown on coverslips were washed twice with phosphate buffered saline (PBS) (8% NaCl, 0.2% KCl, 1.44% sodium phosphate, 0.2% potassium phosphate, pH 7.4) and fixed in 100% methanol at –20°C for 10 minutes, followed by 100% acetone at –20°C for 1 minute. Cells were blocked in a solution containing PBS and 1% bovine serum albumin for 15 minutes followed by specific antibodies. The Flag-tag was detected using rabbit polyclonal antibodies from Sigma, Ki67 was detected with mouse monoclonal antibodies from Oncogene Research Products, and α-tubulin was detected with mouse monoclonal antibodies from Sigma. Antibodies were detected using secondary reagents conjugated to either rhodamine (Jackson ImmunoResearch Laboratories) or fluorescein isothiocyanate (FITC) (Sigma).
Cultures for immunofluorescence with GFP-Cdca8 were plated onto coverslips and then harvested at 50-75% confluence. At harvesting, the media was removed and then coverslips were washed twice with PBS. Two ml of fixative which consisted of 2% formaldehyde in PBS (w/v) was added to the coverslips and incubated for 20 minutes at room temperature. The formaldehyde fixative was removed and 2 ml of absolute methanol was added for 20 minutes. After addition of methanol, the coverslips were washed three times with PBS and were placed onto slides. Immunofluorescence and confocal microscopy were used to view Cdca8 localization.

4.2 Real Time Imaging

To capture images of cells as they progress through the cell cycle, real time imaging was performed using immunofluorescence in conjunction with ImagePro Plus Software. GFP-Cdca8 stable clones were grown in 25 cm² culture flasks with 5 ml of phenol red free DMEM supplemented with 10% FCS and penicillin/streptomycin. At the time of imaging the appropriate drugs were added. Drugs used were nocodazole (200 ng/ml) and cytochalasin D (0.002 ng/ml). Experimental time frames varied between 2 hours and 6 hours and images were captured at various time points.

5. Expression Analysis

5.1 Preparation of Cells for RNA Isolation

5.1.1 Double Thymidine Block
To synchronize cells in S phase by targeting ribonucleotide reductase, HeLaM cells were subject to a double thymidine block. Cells were counted with a hemocytometer and 1 X 10^6 cells were plated onto 15 cm culture plates. Twenty-four hours later thymidine (0.2 mM) was added to each plate. Eighteen hours post treatment, cells were washed twice with PBS and fresh media was added: nine hours later thymidine was re-added. After eighteen hours, the media was removed and cells were washed again with PBS. At this time, fresh media was added and cells were free to synchronously progress through the cell cycle. Cells were collected for RNA at 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 hours after released from the thymidine block.

5.1.2 Treatment of Cells with Mitotic Inhibitors

HCT 116 p53+/+ and HCT 116 p53-/- cells were treated with nocodazole (200 ng/ml) and thymidine (0.2 mg/ml) for 16 hours to arrest cells in M phase and S phase respectively. RNA was then collected for Northern blot analysis.

5.1.3 Treatment of Cells with p53 Inducing Agents

HCT116 cells were treated with adriamycin to cause DNA damage and activate p53, while NARF2 cells were treated with IPTG to induce p53. HCT116 cells were treated with 0.2 mg/ml adriamycin for 24, 48, and 72 hours and then RNA was collected for Northern blot analysis. NARF2 cells were treated for 24, 48, and 72 hours with IPTG (100 mM) and RNA was once again collected for Northern Blots.
Derivatives of NARF2 cells were also used for expression analysis. Confluent cells were split in half and plated onto two plates. The following day untreated cells were collected for RNA and the second plate of cells was treated with IPTG (100 mM) and RNA was collected 48 hours later.

5.2 Analysis of RNA Levels
5.2.1 RNA Isolation and Electrophoresis

RNA was isolated using Trizol reagent (Invitrogen) according to manufacturers instructions. For Northern blotting, RNA samples containing 15 µg of RNA were added to a denaturation buffer containing 12.5 µl formamide, 2.5 µl 10 X MOPS buffer (200 mM MOPS, 50mM sodium acetate, pH 5.2, and 10mM EDTA, pH 8.0), and 4 µl formaldehyde. Samples were heated to 65°C for 5 minutes and then incubated on ice for 2 minutes, 2.5 µl of RNA loading dye (50% glycerol, 0.5 M EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol) was subsequently added. Denatured samples were loaded on a 1% agarose gel containing 6% formaldehyde and 1 X MOPS and separated by electrophoresis in 1 X MOPS buffer for 2 hours at 100 Volts. RNA was transferred for 16 hours onto Hybond-N+ nylon membrane (Amersham Biosciences) in 10 X SSC. RNA was cross-linked onto membrane using a SPECTRO linker under optimal cross-link conditions. Membranes were pre-hybridized in Church buffer (0.5M Na₂HPO₄, 0.5M NaH₂PO₄, 0.5M EDTA, BSA, SDS) for 2 hours at 65°C.
5.2.2 Preparation of Probe

To probe transferred membranes for \textit{cdca8}, the plasmid BTTOP2 containing \textit{cdca8} cDNA which has all introns spliced, was digested with the restriction enzymes EcoRV and BamH1. A band of 500 nucleotides pairs was excised from the gel and purified under manufacturers instructions using the QIAEX II Gel Extraction Kit (QIAGEN Science). DNA was then quantified. The p21 probe was constructed by digesting pblWaf4 plasmid, which contains a human p21 (waf1) insert. The plasmid was digested using the enzymes Apa1 and EcoR1. A band of 320 nucleotides was cut from the gel and purified using the QIAEX II Gel Extraction Kit (Qiagen Science). DNA was then quantified. In order to control for loading, membranes were probed with \textit{gapdh}. The \textit{gapdh} probe was previously prepared (Taylor, W.R, unpublished data).

5.2.3 Labeling DNA Probe

To label the probe for Northern blotting, 50 ng of DNA was added to water for a final volume of 50 µl. The sample was mixed and boiled for 95°C for 2 minutes, incubated for 2 minutes on ice, and then spun briefly at 16,100 x g. The DNA/water mixture was then added to Ready-To-Go DNA Labeling beads (Amersham Biosciences) and \textsuperscript{32}P dCTP (50 µCi) was subsequently added, except when probing for p21 in which 25 µCi of \textsuperscript{32}P dCTP was added. The solution was incubated for 1 hour at 37°C. The labeled probe and 50 µl of TE was added to a sepharose G50 column and spun for 3 minutes at 16,100 x g to remove unlabeled nucleotides, and then boiled for 3 minutes. Labeled probe was then added to the pre-hybridization buffer.
5.2.4 Northern Hybridization and Washing

Northern blots were hybridized for 16 hours at 65°C. After hybridization, the membrane was washed with 65°C, 2 X SSC (15 minutes), 1 X SSC (10 minutes), and 0.1 X SSC (10 minutes). After washing, the blot was wrapped in laboratory wrap and placed into a cassette with a BioMax intensifying screen. Film was developed at various time points to obtain the optimal exposure. cdca8 expression was determined using Scion image software.

5.2.5 Methylene Blue Staining of 28S RNA

To determine the amount of 28S RNA on blots, probed membranes were stained with methylene blue (0.2% methylene blue, 0.3 M sodium acetate, pH 5.2) for a few seconds. The methylene solution was removed and membranes were rinsed with distilled water for 48 hours to remove excess stain.

5.2.6 Reverse Transcriptase PCR

To analyze how abundant splice variants of cdca8 are in cells, 14 different cell lines were assayed by reverse transcriptase PCR (RT-PCR). The cell lines K562, KT-1, BV173, Jurkat, Daudi, TF-1, DU145, PC3, MDAH041, MCF7, HeLaM, HEK293, HT1080, and HCT116 were grown to 75-80% confluency and RNA was collected as indicated above and made into cDNA following the manufacturer’s instructions (Promega). To detect expression of cdca8 that contains intron 1, a forward primer was designed within intron one (5’ CGTCCCTACCCAGTTTCTTG 3’) to use in PCR reactions. To detect expression of unspliced cdca8, a forward primer was designed that
was complementary to a sequence within exon 1 (5’ TTTTGCTCAGCCCTTGTCTC 3’).
The reverse primer was the same for both sets of reactions (5’ GGATGGAGGACAC CTTTT GA 3’). PCR reactions were prepared by using 25 µl JumpStart Taq ReadyMix (Sigma-Aldrich), 5 µl cDNA, 20 pmole of primer, and water (Fisher Scientific) in a final volume of 50 µl. DNA was amplified for 30 cycles by denaturing at 95°C for 1 minute, annealing for 1 minute at 57°C, and an extension period of 5 minutes at 72°C. To control for loading, gapdh cDNA was also amplified (forward primer 5’AAATCCCATCACCA TCTTCC 3’) (reverse primer 5’ GTCCACCACCCCTGTTGCTGC 3’). PCR products from reactions containing the primer within intron and gapdh were separated on a 0.7% agarose gel stained with ethidium bromide and detected with UV light. PCR products amplified with the primer complimentary to exon 1 were separated on an acrylamide gel (0.5 X TBE, 5% acrylamide) stained with ethidium bromide.

5.3 Western Analysis

5.3.1 Preparation of Cells for Western Analysis

NARF2 cell lines with various constructs inserted into the genome were used for Western blotting. Cells were prepared the same as described in section 5.1., with the exception that at the time of harvesting, cells were washed with PBS, and then scraped in a volume of 1 ml PBS.

5.3.2 Western Blotting

Whole cell extracts were prepared by incubating cell pellets in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1.0% NP40, 10 µg/ml aprotinin, 100
µg/ml phenylmethane sulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml pepstatin, and 1mM sodium metavanadate [62]. Extracts with equal amounts of protein, determined by Bradford method were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide) for one hour at 120 V and then 5 hours at 200 V. Gels were transferred to polyvinylidene difluoride membranes overnight at 20 V (Millipore). Membranes were blocked for 3 hours at room temperature in blocking buffer (5% non-fat dried milk, PBST). Antibodies to p53 directly conjugated to horse-radish peroxidase were obtained from Santa Cruz Biotechnology and used at a dilution of 1:1000. Antibodies to actin from NeoMarkers were used at a dilution of 1:1000. Goat anti mouse secondary antibodies conjugated to horse-radish peroxidase were obtained from Santa Cruz Biotechnologies and used at a dilution of 1:1000. Antibodies were diluted in PBS containing 3% (w/v) non fat dry milk and 0.2% Tween20. Membranes were incubated in the antibody solution for 1 to 2 hours at room temperature or for 16 hours at 4°C. Membranes were washed 3 x 10 minutes in PBST (PBS plus 0.2% 500 X Tween 20) and bound antibody was detected using enhanced chemiluminescence (Dupont).

6. Flow Cytometry

HeLaM cells were synchronized by double thymidine block as previously described in section 5.1.1. Cells were released from the block to progress through the cell cycle. Media was removed from cells; cells were washed with 4 ml of PBS. PBS was removed and 0.5 ml of trypsin was added to cells, when cells detached from the plate, 0.9 ml of growth media was added to wash cells from the plate. Cells were then centrifuged
at 2,000 x g, for 7 minutes in a 4°C centrifuge. The supernatant was removed and the cell pellet was washed with 1 ml PBS and centrifuged again as described above. The cell pellet was resuspended in 400 µl of PBS and the solution was added drop-wise to 1 ml pre-chilled 100% ethanol. Cells were transferred to an Eppendorf tube and spun as previously described. The supernatant was removed and the cell pellet was resuspended in 1 ml PBS and spun once again. After spinning, the supernatant was removed and the cells were resuspended in 300 µl of PBS. To stain the cells for DNA content, 0.5 mg/ml propidium iodide (PI) along with 5 µg/ml RNaseA was added to the cell solution and incubated for 30 minutes. Cells were then analyzed by flow cytometry using CellQuest software. Cylchred software was used in analyzing the percentage of cells in each phase of the cell cycle.
III. Results

1. Characteristics of Cdca8

The human gene for cdca8 resides on chromosome one and consists of eleven exons encoding a basic protein of 280 amino acids (Figure 2). The closest match to cdca8 within the human genome is a DNA sequence found within an intron of a gene on chromosome 7. The gene on chromosome 7 codes for a predicted septin-like protein. Septin were initially identified in yeast and are essential for cytokinesis in yeast. The cdca8-like sequence from chromosome 7 may be transcribed but it not present in mature mRNA, therefore is not represented in EST databases supporting the idea that this region is a pseudogene. Cdca8 is a 31 kDa protein with two potential nuclear localization signals that span amino acids 4-21 and amino acids 150-167, as well as a potential Cdk1 phosphorylation site at amino acids 105-109 and a potential PLK1 phosphorylation site at amino acids 178-181 (Figure 3). Also, interestingly Cdca8 has a string of seven glutamic acids. The importance of these glutamic acids is unknown at this time. Orthologues of Cdca8 are found in mice and rats. The mouse orthologue is 76% identical to human Cdca8 and has 289 amino acids. The rat is 77% identical to human Cdca8 and has 288 amino acids.
Figure 2. Genomic Structure of human *cdca8*. (A) *cdca8* is located on chromosome 1 position 34.3. (B) *cdca8* is composed of ten exons, but intron 1 is inefficiently spliced. Exons are represented as red blocks, whereas introns are represented as red lines. The 5’ and 3’ untranslated region is represented as blue blocks. Schematics of chromosome 1 and the genomic sequence were obtained from http://www.ncbi.nlm.nih.gov.
Human Chromosome 1

A.

B.

- Coding sequence
- Non-coding sequence
Figure 3. Amino Acid Sequence of Cdca8. Cdca8 is a 280 amino acid protein. Two potential nuclear localization signals have been identified at positions 4-21 and 150-167. These regions are highlighted in purple. A potential Cdk1 phosphorylation site spanning amino acids 105-109 as well as a potential Plk1 phosphorylation site at amino acids 178-81 were also identified and are highlighted in red and green respectively. Phosphorylation sites were determined using the Prosite website (www.expasy.org).
MAPRKGSSRVANTNSLRRRKLASFLKDFDREVEIRIKQIESDRQNLLKEVDNL
YNIEILRLPKALREMNWLDYFALGGGNKQALEEAATADLDITEINKLTAEAIQTP
LKSASKTRKVIQVDEMIVEEEEEEENERKLNQTARVKRCPPSKKRTQSIQQKKG
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YNISGNGSPLADSKEIFLTVPVGGGESLRLLASDLQRHSAQLDPEALGNIKKLSNRLAQICSSIRTHK
2. Expression of *cdca8*

2.1 Comparison of *cdca8* Expression in Cancerous and Non-cancerous Tissues

Several genes regulated by the Rb/E2F system such as *plk1* and *ki67* are highly expressed in various cancers [64, 65]. To determine if the expression of *cdca8* is highly expressed in cancerous tissues, the cancer genome anatomy project (CGAP) was explored to determine *cdca8* expression. EST frequencies from 274 libraries were compiled and analyzed by hierarchical cluster analysis from a variety of cancerous and non-cancerous tissues suggest that *cdca8* is more highly expressed in the cancerous tissues as compared to non-cancerous tissues (Figure 4). This suggests that the expression of *cdca8* is correlated with high rates of proliferation in human cancer. For comparison, the expression of *birc5* (*survivin*), *plk1*, and *ki67* which have been previously shown to be over expressed in cancer were also determined through CGAP queries (Taylor, W.R., unpublished data). Like *cdca8*, *birc5*, *plk1*, and *ki67* are more highly expressed in cancerous tissues (Figure 4).

2.2 Cell Cycle Regulation of *cdca8*

Genes regulated by the Rb/E2F system are regulated in a cell cycle-dependent manner; therefore it was important to determine if *cdca8* was cell cycle regulated as well. HeLaM cells were synchronized by a double thymidine block and were then released to progress through the cell cycle. Cells were collected at various time points and the level of *cdca8* mRNA was determined by Northern blotting. As seen in Figure 5, the steady state level of *cdca8* mRNA is low at zero hours post release, which represents S
**Figure 4.** *cdca8* Expression in Cancer. The CGAP was searched to determine expression of *cdca8* as well as *birc5* (*survivin*), *plk1*, and *ki67* in various cancerous tissues. EST frequencies were compiled and analyzed by hierarchical cluster analysis. Red bars indicate a high level of expression of the gene, whereas black bars represent basal levels of expression (unpublished data, Taylor, 2004).
Figure 5. Cell-Cycle Regulation of *cdca8*. HeLaM cells were synchronized by a double thymidine block and released to progress through the cell cycle for 18 hours. Fifteen µg of RNA was loaded in each lane. Steady state mRNA levels were assessed using the Northern method. Transfers were stripped and reprobed for *gapdh* to act as a loading control. Methylene blue staining was used to visualize 28S RNA and also to control for loading. Results represented are from two experiments.
Hours post thymidine release

0  2  4  6  8  10  12  16  18

- cdca8
- gapdh
- 28S RNA
phase. Steady state levels continue to increase until 10 hours after release where levels were maximal (Figure 5). Flow cytometric analysis showed that between 2 and 10 hours, the percentage of cells in S phase gradually decreased while the percentage of cells in G2/M increased. At 10 hours, the maximum percentage of cells are in G2/M (Figure 6A & B) Visual examination indicated that many rounded mitotic cells were evident at 10 hours.

2.3 The Effect of DNA Damaging Agents on cdca8 Expression

Affymetrix data suggested that cdca8 is down-regulated in response to adriamycin. To confirm this finding, we tested the effect of DNA damage on cdca8 expression in human cells that contain wild type p53. HCT 116 cells were treated with adriamycin for 24, 48, and 72 hours and RNA was collected for Northern blotting to measure cdca8 levels. The expression of cdca8 was undetectable after 24 hours of treatment and remained undetectable throughout the course of treatment (Figure 7). Thus, DNA damage leads to decreased expression of cdca8 in HCT 116 cancer cells.

2.4 cdca8 Expression is p53 Dependent

Previous data using Affymetrix microarrays indicated that cdca8 is a target of the transcription factor E2F (Taylor, W.R., unpublished). The effect of the tumor suppressor p53 was determined because p53 triggers a pathway that inactivates E2F activity. To test the role of p53 in the regulation of cdca8, NARF2 cells, which have an IPTG inducible p14ARF, were treated with IPTG for 24, 48, and 72 hours. The addition of IPTG causes an increase in p14ARF protein, which in turn causes p53 to accumulate by blocking its degradation
Figure 6. Cell-Cycle Distribution of HeLaM cells. HeLaM cells were synchronized by a double thymidine block and released to progress through the cell cycle for 18 hours. Cells were fixed in ethanol and the DNA content of propidium iodide-stained cells was determined by FACS. The peaks shown represent G1, S, and G2 phases of the cell cycle. (A) Cell cycle distribution of cells at various time points after thymidine release. (B) Graph representing the percentage of HeLaM cells in different phases of the cell cycle as determined by Cyclchred software. Results are a representation of two trials.
A.

Cell number

DNA content

G1

0h

G2

8h

G1

10h

G2

2h

S

12h

G1

4h

S

G2

16h

G1

6h

S

18h
B.

The graph shows the percentages of cells in each phase (G1, G2, S) over time (hours post thymidine release). The x-axis represents the hours post thymidine release, ranging from 0 to 18 hours. The y-axis represents the percent in each phase, ranging from 0 to 90%. The graph includes lines for G1, G2, and S phases, each represented by different symbols and colors.

- G1 phase: represented by blue diamonds.
- G2 phase: represented by red squares.
- S phase: represented by green triangles.

The graph illustrates the dynamic changes in cell phase distribution over the time period.
Figure 7. The Effect of Adriamycin on cdca8 Expression in HCT 116 Cells. HCT 116 cells were treated with adriamycin for 24, 48, and 72 hours. cdca8 mRNA levels were determined by the Northern method. To control for loading, the transfer was stripped and probed for gapdh. Numbers represent units of cdca8 expression after correcting for loading. Results are a representation of at least two trials.
HCT 116

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by Hdm2. High levels of p53 in NARF2 cells induce cell cycle arrest [61]. We observed that after 24 hours of treatment with IPTG, cdca8 mRNA was very low and remained so through 72 hours (Figure 8). These results suggest that p53 can cause downregulation of cdca8 possibly through regulating activity of E2F.

2.5 Confirmation of Cell Cycle Regulation of cdca8

In order to confirm that cdca8 is cell cycle regulated, HCT 116 p53+/+ and p53-/- cells were treated with nocodazole and thymidine for 16 hours. Nocodazole was used to block cells in mitosis, whereas thymidine was used to block cells in S phase by targeting ribonucleotide reductase. RNA was collected after 16 hours for Northern blotting. Figure 9 shows that after addition of nocodazole or thymidine to HCT 116 p53+/+ cells, mRNA levels increased as compared to untreated cells but higher expression was achieved in cells treated with nocodazole. However, thymidine did not change the expression level of cdca8 mRNA in HCT 116 p53-/- when compared to untreated cells. We currently do not have an explanation as to why the expression level of cdca8 does not change in HCT 116 p53-/- cells exposed to thymidine.

2.6 Contribution of p53 to Basal Levels of cdca8

The role of p53 on the basal levels of cdca8 was explored in HT1080 LX and GSE cells and HCT 116 p53+/+ and p53-/- cells. LX cells have a functional p53, whereas GSE cells contain a dominant negative p53 [62]. Figure 10 shows that the basal expression level of cdca8 is slightly increased in asynchronously
**Figure 8.** Expression of *cdca8* in p53-Inducible NARF2 Cells. NARF2 cells were treated with IPTG for 24, 48, and 72 hours to induce p53. mRNA levels were assessed using the Northern method. To control for loading, the transfer was stripped and probed for *gapdh*. Numbers represent units of *cdca8* expression after correcting for loading. Results are a representation of at least two experiments.
NARF2

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Hours post IPTG
Figure 9. The Effect of Thymidine and Nocodazole on cdca8 Expression in HCT 116 cells. HCT 116 p53 +/- and HCT 116 p53-/- cells were treated with either thymidine (thy) to block cells in S phase or with nocodazole (noc) to arrest cells in mitosis. Cells were treated for 16 hours. mRNA levels were assessed using the Northern method. The transfers was stripped and probed for gapdh to determine loading. Numbers represent the units of cdca8 expression when corrected for loading. Results are a representation of two experiments.
HCT 116 p53+/+

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HCT 116 p53-/-

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Figure 10. Expression of cdca8 in HT1080 LX and GSE cells. Steady state cdca8 mRNA levels from asynchronously growing HT1080 LX and GSE cells were analyzed using the Northern method. gapdh served as a loading control. Numbers represent units of cdca8 expression when corrected for loading. Results represent at least three trials.
HT1080

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growing GSE cells as compared to LX cells. The units of cdca8 expression show a very modest increase of expression in GSE cells. The basal expression level of cdca8 was also higher in HCT 116 p53-/- cell compared to HCT 116 p53+/+ cells (Figure 9). These results suggest that basal levels of cdca8 may be regulated by p53.

2.7 Expression of cdca8 in NARF2 Variants Expressing Cell Cycle Proteins

To further address the regulation of cdca8, variants of a NARF2 cell line were assayed by the Northern method for expression of cdca8. The NARF2 variants were previously created by first pooling NARF2 cells that express the Ectrophic receptor by infecting parental NARF2 cells with an amphotropic retrovirus containing a pBabe-Bleo-Eco vector. The pool was then individually infected with ecotropic retroviruses produced in Bosc cells using the following vectors: pLXSN, pLXSN GSE56, pLXSN-Cyclin E, pLXSN E7, pLXSN E7Δ, pBabe-Puro, pBabe-puro-Cyclin D1/Cdk fusion or lentiviral vectors encoding Hdm2 cDNA or short hairpin RNA targeting p53 or p21 [66]. For expression analysis, cells were collected after treatment with IPTG for 48 hours. Untreated cells were collected to assess basal levels. We also analyzed the expression of the p21 mRNA. The levels of p53 protein were determined by Western blotting. Shscr cells served as a control for shp53 and shp21. shp53 and shp21 are cell lines with short hairpin RNA directed at p53 and p21. After addition of IPTG to shscr, p21 mRNA levels increased as compared to untreated cells, while cdca8 levels decreased (Figure 11A). In the knockdown p53 cell line, a high level of cdca8 mRNA was detectable in both untreated and treated cells, while the levels of p21 were low. In the p21 knockdown cell line, cdca8 levels became undetectable after the addition of IPTG to induce p53. The
level of p21 though remains high, suggesting that p21 was not efficiently knocked down in this cell line.

The NARF2 variant, LVSV40 empty reverse neomycin (LVSV40 ERN) contains an empty lentiviral vector and is a control for the cell line that over expresses Hdm2. After addition of IPTG to LVSV40, there is an increase in the expression of p21 mRNA, while the expression of cdca8 mRNA was decreased. When Hdm2 was over expressed, high levels of cdca8 were detected in both untreated and treated cells, while the level of p21 increased after the addition of IPTG. NARF2 cells were also infected with a retrovirus that contained a pBabe-Puro vector. This construct was a control for a NARF2 variant infected with the pBabe-puro retrovirus which also contains a Cyclin D1/Cdk fusion protein. After addition of IPTG to pBabe-Puro cell line, p21 levels increased while cdca8 was no longer detectable. The NARF2 variant with the Cyclin D1/Cdk fusion expressed higher levels of cdca8 than the control cell both in the presence and absence of IPTG. As expected, the levels of p21 increased after addition of IPTG.

The cell line LXSN was also created by infecting NARF2 cells with a retrovirus. This cell line serves as a control for the GSE 56, E7, E7Δ, and Cyclin E cell lines. Each of these cell lines was also infected with a retrovirus derived from the LXSN vector that expressed the GSE 56, E7, E7Δ, and Cyclin E genes. Addition of IPTG to LXSN cells caused an increase in p21 levels and a decrease in cdca8 mRNA levels as compared to untreated cells. When a dominant negative form of p53, GSE 56, was overexpressed, levels of cdca8 remained high in both IPTG treated and untreated cells. p21 mRNA levels were very low in both treated and untreated cells, but there was a slight increase in expression after the addition of IPTG. In the cell line E7, cdca8 levels were slightly
decreased after the addition of IPTG, while \(p21\) levels increased as compared to untreated cells. E7 is from the human papiloma virus and knocks out expression of all three Rb family proteins. The E7∆ construct has a mutated E7 that does not bind Rb proteins. The expression of \(cdca8\) and \(p21\) were very similar in E7∆ as compared to the E7 construct. \(cdca8\) mRNA levels decreased after addition of IPTG, while \(p21\) levels increased. The NARF2 cells over expressing of Cyclin E plus showed a decrease in \(cdca8\) expression and an increase in \(p21\) levels when IPTG was added.

Addition of IPTG to the NARF2 variants in most cases should cause an increase in p53 expression. In order to determine if the cell lines were appropriately expressing p53, a Western blot was performed to assay for p53 levels before and 48 hours after the addition of IPTG. A select set of cell lines tested show an increase in p53 after the addition of IPTG, except for the p53 knockout line, shp53 (Figure 11B). This suggests that p53 is being induced upon addition of IPTG. Northern and Western blotting results combined further support the idea that \(cdca8\) is a target of p53 dependent repression.

2.8 Expression of \(cdca8\) in Cancer

Fourteen different cell lines derived from a number of different cancerous as well as a non-cancerous tissue were surveyed for the basal level of \(cdca8\) expression which was then correlated with the p53 status of the cell lines. Asynchronously growing cells were used to collect RNA and the expression of \(cdca8\) was detected using the Northern method. As seen in Figure 12, there is a detectable level of \(cdca8\) in all cells derived from cancerous tissues. In contrast, in the non-cancerous breast cell line MCF10A there is a low level of \(cdca8\) expression when compared to the corresponding cancerous breast cell
Figure 11. Expression of *cdca8* in Various NARF2 Constructs. NARF2 cells infected with various constructs were treated with IPTG for 48 hours. Shscr cell line is a control from shp21 and shp53, which have short hairpin RNA directed at p21 and p53 respectively. LVSV40 cell line contains an empty lentiviral vector and serves as a control for the cell line that overexpresses human Mdm2 (Hdm2). The cell line pBabe-Puro contains an empty retrovirus vector and is a control for the cell line that over-expresses CyclinD/Cdk1 (D1/Cdk1). LXSN cell line was also created by infecting cells with a retrovirus. This cell line serves as a control for the cell lines over-expressing GSE56, E7, E7Δ, and Cyclin E. (A) mRNA levels for *cdca8* and *p21* were assayed using the Northern method. Transferred membranes were also assessed for loading using *gapdh*. (B) Western blotting was used to assay the levels of p53 in a select set of NARF2 constructs. Actin was used to control for loading. Results are representative of two experiments.
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**Image:**
- **p53**
- **actin**
Figure 12. Comparison of cdca8 and p53 Expression in Various Cell Lines. The steady state level of cdca8 mRNA was analyzed in 13 cancerous and 1 non-cancerous cell line by Northern blotting. The p53 status of each cell line was also surveyed. (A) Leukemia cell lines. (B) Various cancer cell lines and the non-cancerous cell line MCF10A. (C) Cell lines derived from prostate cancers. To assess loading, transfers were stripped and probed for gapdh. Numbers represent the units of cdca8 expression when corrected for loading. Results are representative of at least two experiments.
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<th>Du145</th>
<th>LNCaP</th>
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line MCF7, although much of the under representation of cdca8 in MCF10A was due to loading. The p53 status of each cell line was also surveyed to determine if p53 expression has an effect on expression of cdca8. The level of cdca8 is high even in cancer cell lines expressing wild type p53. For example in the p53 +/-, BV173 (3.40 units of cdca8 expression) and Jurkat (1.64 units) cell lines, cdca8 expression levels are similar to the p53 null cell line K562 (2.10 units). Daudi (p53+/+) and TF-1 (hemizygous for p53, p53-/+ ) cell lines indicate similar expression of cdca8 when the amount of RNA when loading was considered (1.95 and 1.90 units respectively). HT1080 LX, HCT 116 p53 +/-, and MCF7 all have wild type p53 and the steady state mRNA expression of cdca8 was easily detected. In the mutant p53 cell line Du145, there was a higher level of cdca8 (1.45 units) as compared to the p53 wild type cell line LNCaP (0.95 units) and the null p53 cell line PC-3 (0.74 units). This data suggests that p53 is not the major factor in controlling the basal levels of cdca8 in the cell lines tested.

2.9 Expression of Splice Variants of cdca8

Upon cloning and sequencing of cdca8, two splice variants were isolated. One variant contained intron one, while the other variant had all introns removed (Figure 13). To determine if both splice variants were abundant in cells, 14 different cell lines were assayed by semi-quantitative RT-PCR for their expression of cdca8. In the RT-PCR reactions using a forward primer that annealed to a sequence within intron 1, we were able to detect an unspliced version of cdca8 in all cell lines tested. To detect the completely spliced version, we used a primer that annealed to a sequence within exon 1 and then ran the PCR products on an acrylamide gel. We were able to detect both spliced
Figure 13. Splice Variants of cdca8. A forward primer was designed that annealed to a region of exon 1 (represented as arrow A), while a second forward primer was complementary to a sequence in intron 1 (represented as arrow B). A reverse primer was designed that annealed to a sequence in exon 7. Two separate PCR reactions were performed in order to detect the expression of each splice variant. Results represent at least two trials for each set of PCR reactions.
Coding Sequence/Exon

Intron 1
Figure 14. Expression of Splice Variants of cdca8. Steady state mRNA levels were assayed from 14 different cell lines to determine if the expression of cdca8 splice variants was variable in different cell lines. Several types of cells were analyzed by RT-PCR. To assess loading, gapdh cDNA was made and amplified along with cDNA specific for the splice variants. cdca8L represents the splice variant with intron 1 present, while cdca8S represents the variant with all introns removed. (A) PCR reaction using intronic primer. (B) PCR products using primer complimentary to a region in exon 1.
A.

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The gel images show bands for cdca8L and gapdh.
an inefficiently spliced \textit{cdca8} in all cell lines using this primer. The larger band represents \textit{cdca8} with intron 1, a second band approximately 100 nucleotides smaller represents \textit{cdca8} with intron 1 removed. The BTTOP4 plasmid contains a cDNA from the completely spliced form of \textit{cdca8}, while BTTOP2 contains cDNA from \textit{cdca8} with intron 1 and both were used as controls (Figure 14). RT-PCR results suggest that both \textit{cdca8} transcripts are abundant in cells. Western blots were also used to determine the abundance and size of the Cdca8 protein derived from the two splice variants. Flag-tagged \textit{cdca8} DNA containing intron 1 as well as Flag-tagged \textit{cdca8} that had all introns removed was transiently transfected into HEK293 cells. Western blots were probed for the Flag tag. These blots showed that protein derived from both splice variants is abundant and is of the same size as expected because a kozak consensus sequence is present in exon 2 (Stiff, A & Taylor, W.R., unpublished data).

3. Subcellular Localization of Cdca8

3.1 Localization of Flag-tagged Cdca8

In an attempt to determine the function of Cdca8, we next assessed the subcellular localization. Since Cdca8 has no known functional domains, we generated a Flag-tagged version to observe its subcellular localization. Two versions of carboxy-terminal Flag-tag Cdca8 were constructed, one using cDNA containing the first intron and one version with intron one removed. The localization experiments shown use the version with intron one present, but similar patterns of localization were seen with the other construct.

HEK293 cells were transiently transfected with Flag-tagged Cdca8 and the localization of Cdca8 was observed using immunofluorescence. In interphase cells, Cdca8 localized to the nucleus in punctate regions (Figure 15A). Throughout early stages
Figure 15. Subcellular Localization of Flag-tagged Cdca8. HEK 293 cells were transiently transfected with a Flag-tagged Cdca8 construct. Localization of Cdca8 was determined by immunofluorescence.
anti-Flag (Cdca8-Flag)  Hoescht (DNA)

Interphase

Early Mitosis

Anaphase/Telophase
**Figure 16.** Co-Localization of Flag-tagged Cdc8 and a known nucleolar protein, Ki67.

HEK 293 cells were transiently transfected with Flag-tagged Cdc8. Antibodies to the Flag-tag and Ki67 were used to determine co-localization.
of mitosis, Cdca8 was found throughout the nucleus (Figure 15C). Interestingly, during late anaphase and early telophase, Cdca8 localized to the cleavage furrow where a large number of fibers were seen that traverse the furrow (Figure 15E). Localization of Cdca8 to the cleavage furrow indicated that it may play a role in cytokinesis.

During interphase, Cdca8 is localized within punctate regions of the nucleus. Next we tested whether the punctate regions that contained Cdca8 during interphase were nucleoli. Flag-tagged Cdca8 was transiently transfected into HEK293 cells and was observed to co-localize with a known nucleolar protein, Ki67. During interphase, Cdca8 co-localized with Ki67 (Figure 16A & B), but cells with a high level of punctate staining for Cdca8 showed low Ki67 expression (Figure 16D & E). The mean pixel intensity of Ki67 was used as a measure of the level of Ki67 in the cells examined. We observed a range of Ki67 staining levels in cells not expressing Cdca8 (from a mean pixel intensity of 12 to 36). In a cell expressing Cdca8 in the same field, the staining intensity of Ki67 was fourfold lower than the lowest level of Ki67 observed in untreated cells (Figure 17). We currently do not have an explanation for this phenomena. During mitosis, Cdca8 is found throughout the nucleus, whereas Ki67 is present at the ends of chromosomes (Figure 17G & H). These results suggest that during interphase Cdca8 is located to the nucleoli.

The localization of Cdca8 was also observed in cells that had undergone faulty mitosis. NARF2 cells were stained with antibodies for anti-Flag Cdca8 as well as antibodies to anti-α tubulin. Cdca8 was highly concentrated in the center of the chromatin mass in a fibrous pattern (Figure 18A). The fibrous nature of Cdca8 suggests
Figure 17. Expression of Cdca8 and Ki67. HEK 293 cells were transiently transfected with Flag-tagged Cdca8. Antibodies to Flag-tag and Ki67 were used in co-localization studies. (A) Whole field image of localization and expression of Cdca8 (B) Same whole field image as shown in A, but showing localization and expression of Ki67 (C) Hoescht staining (D) Comparison of cells expressing low levels of Cdca8 and high levels of Ki67 to cells expressing high levels of Cdca8 and low levels of Ki67. Numbers represent the mean pixel intensity of each cell. Ten total cells were measured for the mean pixel intensity. Results of expression and localization are representative of at least two separate experiments.
Cdca8

Ki67

Hoescht (DNA)

Ki67 Mean Pixel Intensity
**Figure 18.** Localization of Cdca8 in a Defective Mitotic Cell. NARF2 cells were transiently transfected with Flag-tagged Cdca8 and visualized by immunofluorescence.
anti-Flag (Cdca8-Flag)  Hoescht (DNA)
that this region may be the remnants of the midbody in a cell in which the cleavage furrow regressed due to a chromatin bridge.

3.2 Localization of GFP-Cdca8

To determine if the observed localization of Cdca8 was a by-product of the Flag-tag, we constructed a GFP fusion using cdca8 cDNA which contained intron one. Interphase cells, showed a punctate pattern of localization for Cdca8 (Figure 19A). During telophase, GFP-tagged Cdca8 was present at the spindle midbody (Figure 19B). The phenotypes observed with the GFP-tagged Cdca8 were identical to the Flag-tagged construct, suggesting that the localization observed is the natural subcellular location of Cdca8.

3.3 Real Time Imaging of Cdca8

To address the dynamic localization of Cdca8 that was seen in still immunofluorescence images, real time imaging was performed. Stable clones of GFP-Cdca8 derived from HeLaM cells were produced and analyzed by time-lapse fluorescence microscopy. Cells in early mitosis were identified and were monitored as they progressed through mitosis. A cell that was in prophase when the experiment began is seen in Figure 20A. This cell enters into metaphase and remains there for at least 24 minutes. During the time spent in metaphase, we were able to visualize dots oscillating around the majority of GFP fluorescence at the cell equator. These dots may represent kinetochores of unattached chromosomes (See Discussion). As seen in Figure 20B, a cell in metaphase entered into anaphase and after 9 minutes, the ingress of the cleavage
**Figure 19.** Subcellular Localization of GFP-Cdca8. HeLaM cells were stably transfected with GFP-Cdca8 construct. (A) Localization of GFP-Cdca8 (B) Localization of α-tubulin (C) Hoescht staining of DNA. The cell shown is a telophase cell depicting staining at the midbody. Figure is representative of cells visualized by immunofluorescence.
GFP-CDCA8  \(\alpha\)-tubulin

A.  

B.  

C.  

Hoescht (DNA)
Figure 20. Dynamic localization of Cdc8 in mitosis. Real time imaging was used to capture the dynamic localization of Cdc8 during mitosis. Stably transfected GFP-HeLaM cells were cultured in a 25 cm² culture flask and imaged as they progressed through the cell cycle. Cells were imaged using an immunofluorescence microscope in conjunction with ImagePro Plus imaging software. Images address the timing of relocation of Cdc8 as a cell progress through the cell cycle. (A) A cell in early prophase progressing into metaphase. The top panel shows images in 6 minute increments, whereas the bottom two panels show images in 3 minute increments. Arrows represent “dots” oscillating around condensed DNA at the cell equator. (B) A cell in metaphase progresses to telophase. All images are in 3 minutes increments.
furrow becomes visible and Cdca8 became localized to the midzone, a region between
the chromosomes that is rich in tubulin. Just fifteen minutes after cleavage furrow
formation, a distinct midbody was recognizable. The midbody forms from the ingression
of the spindle midzone [39].

3.4 Confocal Image of Cdca8

In order to better localize Cdca8, we used confocal microscopy to observe cell in late
telophase and early anaphase (Figure 21). During telophase, Cdca8 was localized to the
spindle midbody as expected. Cdca8 appeared to localize to two distinct regions of the
midbody with a space between the two areas. During anaphase, Cdca8 was present at the
spindle midzone as was seen in earlier localization studies. Cdca8 was also localized to
the DNA (Figure 21)

3.5 The Effects of Nocodazole on Cdca8

To determine when Cdca8 is released from the chromosomes to the spindle
midzone, cells were treated with nocodazole, an anti-mitotic agent that has its effects by
inhibiting microtubule polymerization by binding to β-tubulin. Cells are arrested in G2/M
after the addition of nocodazole. Cells should be able to enter into metaphase but will
progress no further in the cell cycle because of the inability to segregate DNA.
Nocodazole was added to GFP-Cdca8 stable clones at the time of imaging. Cells were
followed for 6 hours after the addition of nocodazole to determine the effects of
nocodazole on the localization of Cdca8. Interestingly, we found a cell that was in
telophase when the nocodazole was added. After 40 minutes of nocodazole treatment, the
telophase cell was still unable to divide, however Cdca8 was still present at the midbody
**Figure 21.** Confocal Image of Cdca8. HeLaM cells were stably transfected with GFP-Cdca8. Cells were asynchronously grown on coverslips and were fixed with formaldehyde. Cells were analyzed by confocal microscopy. Arrow A represents a cell in telophase with midbody staining. Arrow B represents an anaphase cell with Cdca8 present at the spindle midzone.
Figure 22. The effects of Nocodazole on the Subcellular Localization of Cdc8. HeLaM cells stably expressing GFP-Cdc8 cells were treated with nocodazole at the time of imaging. (A-D) A cell in telophase was followed for 6 hours by time lapse microscopy after the addition of nocodazole. (E) Phase image of nocodazole treated early mitotic cells. (F) HeLaM cells stably expressing GFP-Cdc8 cells fixed on coverslips. Cells were treated with nocodazole and were also fixed to view by immunofluorescence. (G) Hoescht staining of nocodazole treated cells. Results are representative of many cells visualized with the same phenotype.
region (Figure 22B). Sixty-five minutes post treatment, Cdca8 staining at the midbody disappeared, but the cell’s cytoplasm had not divided (Figure 22C). This telophase cell treated with nocodazole for 120 minutes showed no Cdca8 at the midbody and the nuclei came closer together, still without the division of the two daughter cells (Figure 22D). The phenotype seen at 120 minutes remained the same throughout the duration of the experiment. Cells in early mitosis appeared to have Cdca8 concentrated into one area on the chromosomes after the addition of nocodazole (Figure 22F). Phase and hoescht images can also be seen (Figure 22E, 22G). The same phenotype was also seen in time lapse images taken (data not shown). Since Cdca8 was present on DNA after the addition of nocodazole, this suggests that Cdca8 is released from the chromosomes and travels to the spindle midzone some time after the point where nocodazole has its effects.

3.6 The Effects of Cytochalasin D on Cdca8

The effects of cytochalasin D on GFP-Cdca8 mitotic cells was also explored in order to determine if blocking cytokinesis and disrupting the actin cytoskeleton would have an effect on the localization of Cdca8. Cytochalasin D disrupts actin filaments and inhibits actin polymerization. Polymerization of actin is important in forming the actinomyosin ring, which is essential for cytokinesis. At the time of imaging cells were treated with cytochalasin and followed for 3 hours after the addition of the drug. Cells in metaphase were able to segregate their DNA and progress into anaphase after 50 minutes of cytochalasin treatment (Figure 23A). Cdca8 was found to travel to the spindle midzone even in the presence of cytochalasin, suggesting this relocation does not require the actin
Figure 23. The Effects of Cytochalasin D on the Localization of Cdca8. GFP-Cdca8 was stably transfected into HeLaM cells and cultured in 25 cm² flasks. Images of cells were captured using time lapse microscopy. Cytochalasin D was added to the culture media at the time of imaging. Images were captured for a three hour duration after the addition of cytochalasin D. (A) A cell in metaphase with Cdca8 localized to the DNA is imaged (B) A cell progressed into anaphase after 50 minutes of treatment with cytochalasin D. (C) Cell remains in anaphase and all Cdca8 staining at the spindle midzone has been depleted. (D) Cells in early and late telophase are imaged just after addition of cytochalasin D. (E-G) After 30 minutes of treatment all spindle midzone and midbody staining has disappeared. (H-I) Phase images showing characteristic blebbing of cells after addition of cytochalasin D. Results are representative of many cells visualized with the same phenotype.
cytoskeleton (Figure 23B). After 75 minutes of treatment, the cells failed to form a cleavage furrow, have not entered into telophase and all spindle midzone staining had disappeared (Figure 23C). In telophase cells arrested with cytochalasin D, the cells failed to complete mitosis and after approximately 30 minutes of treatment most spindle midzone staining was absent. After 60 minutes, spindle midzone staining has become completely depleted as Cdca8 became delocalized from the midbody (Figure 23E & F). In contrast, the average time of 15 untreated cells to progress from anaphase to the formation of a cleavage furrow is approximately 9 minutes. A midbody will then form within another 10 minutes, and finally two completely divided daughter cells form approximately 20 minutes after midbody formation. Therefore, it appears that the transition to the spindle midzone is independent of actin. It is apparent that cytochalasin was affecting the mitotic progression of the cells due to the longer length of time spent in mitosis. Also, blebbing of cells, which is a characteristic of cytochalasin treatment, can be seen in the phase images also confirming that cytochalasin was having an effect on cells (Figure 23H).

4. Knocking Down cdca8

4.1 siRNA of cdca8

The localization of Cdca8 seen by immunofluorescence studies suggested that Cdca8 may function in cytokinesis. We predicted that knocking down cdca8 by siRNA would cease cellular division, but allow nuclear division to occur. This would cause a phenotype of multinucleation in cells depleted of Cdca8. HEK293 cells were transfected with cdca8 siRNA plus Histone GFP. As a control, cells were transfected with siRNA buffer and Histone GFP. Samples were collected 4 and 6 days post transfection. Cells
were stained with anti-\(\alpha\) tubulin antibodies and the number of nuclei were counted. Four days post transfection, approximately 20% of siRNA treated cells had more than one nuclei, whereas less than 10% of control treated cells had multiple nuclei. Six days post transfection of siRNA; approximately 30% of siRNA treated cells had multiple nuclei, whereas only about 10% of control samples were multinucleated (Hennessey, B & Taylor, W.R, unpublished data). Therefore, the absence of \(Cdca8\) in cells does cause a phenotype of multinucleation.
IV. Discussion

Uncontrolled cell proliferation is a hallmark of cancer. The mechanisms of nuclear and cytoplasmic division are important to understand in order to identify causes and potential cures for cancer and other diseases. The cell cycle consists of interphase, mitosis, and cytokinesis, resulting in two identical daughter cells. Each phase of the cell cycle is tightly regulated by a number of factors including cyclins, Cdk, and CDKIs. These factors activate or deactivate many key components of the cell cycle. The goal of our lab is to study the mechanisms of the cell cycle in order to find means to prevent or treat people with cancer.

The studies described in this thesis were aimed at understanding the function and regulation of cdca8, a novel gene identified using Affymetrix microarrays as a gene repressed in a p130/p107-dependent manner in response to adriamycin. Many genes identified by these studies were already known to be important in various phases of the cell cycle (Jackson et. al, unpublished data). p130/p107 are Rb family members that play an important role in repressing cell cycle-regulated genes [16]. Therefore, these studies sought out to characterize the expression, localization, and function of cdca8 in order to determine if it too is an important factor in the cell cycle.
Human cdca8 is found on chromosome one at position 34.3 and consists of eleven exons, which encodes a basic protein of 280 amino acids (Figure 2). Rat and mouse orthologues of human cdca8 are 77% and 76% identical respectively. Orthologues in frogs, zebrafish, orangutan, and chicken have also recently been identified. No orthologues to cdca8 are found in plants or yeast. Plants may not contain cdca8 because they do not form a cleavage furrow like mammalian cells; they instead form a cell plate. Yeast are unicellular organisms that undergo a different type of cellular division, such as budding. The different mechanisms to divide cellular contents by plants and yeast as well as the unicellularity of yeast may be related to the fact that they do not have cdca8. Two putative nuclear localization signals, and potential Cdk1 and Plk1 phosphorylation sites can be found within the 31 kDa protein. These sites may be important in the regulation of Cdca8 (Figure 3).

**Rb/E2F Regulates Cell Cycle Genes**

Tumor suppressors are important factors that control the cell cycle. Rb and p53 cause a halt to the cell cycle when DNA damage or other cellular problems have occurred. Mutations in p53 or Rb allow the cell cycle to progress even in the presence of cellular mistakes; this can lead to aneuploidy, cell death, or oncogenesis [4]. The Rb/E2F system regulates a number of genes in a cell cycle-dependent manner. Many of these genes are also highly expressed in cancer [64]. Through queries of the CGAP we found cdca8 expression to be higher in a number of cancerous tissues as compared to non-cancerous tissues (Figure 4). The results suggest that the expression of cdca8 is likely
correlated with the percent of cells in the cell cycle and it is possibly a cell proliferation marker. These data were an early indication that \textit{cdca8} is important in the cell cycle.

Our previous Affymetrix data suggested that \textit{cdca8} is regulated in an Rb/E2F-dependent manner. The expression of E2F target genes is often variable depending on the phase of the cell cycle. Our Northern blotting analysis indicates that the expression of \textit{cdca8} is maximal during G2 and then decreases as cells exit mitosis (Figure 5). Flow cytometry was used to determine what phase of the cell cycle cells were in. When \textit{cdca8} was maximally expressed, 75% of cells were in G2 phase of the cell cycle (Figure 6A & B). These data show that \textit{cdca8} is cell cycle-regulated and is consistent with our results with p130/p107-null cells. After G2, Rb becomes dephosphorylated and is bound to E2F, making E2F inactive. During G2, Rb is phosphorylated and E2F is free to bind to promoters of cell cycle genes, resulting in an increase in expression of these genes [28-31]. Therefore we predict that \textit{cdca8} is low during S phase when there is less phosphorylation of Rb and it is high in G2 because Rb is phosphorylated to a higher extent. We are unable to tell exactly at what point in G2 the maximal expression of \textit{cdca8} is reached because propidium iodide solely stains for DNA content. All cells past the end of S phase will have a 4N content of DNA and appear the same on DNA histograms.

Previous research by Mansur indicates that \textit{gapdh} is also cell cycle-regulated [67], which is consistent with our data. To further address loading issues, we stained the ribosomal RNA with methylene blue and confirmed that \textit{cdca8} is cell cycle regulated, though the 8, 10, and 12 hour time points appear to be overrepresented.

To re-address the cell cycle regulation of \textit{cdca8}, we found that levels of \textit{cdca8} were higher in nocodazole treated cells when compared to cells treated with thymidine
(Figure 9). This data confirms that \textit{cdca8} levels are maximal during G2, possibly due to the extent of phosphorylation of Rb. In HCT 116 p53 +/- cells treated with thymidine and nocodazole, significant \textit{cdca8} expression was detected. These data suggest that there may be a p53-independent pathway that also controls \textit{cdca8} expression.

\textbf{p53-Dependent Regulation of Genes Required for the Cell Cycle}

Like Rb, p53 is also regulated by phosphorylation. When active, p53 is phosphorylated on serine and threonine residues. p53 can also be activated through acetylation or sumoylation [32]. p53 triggers a pathway that inactivates E2F activity [33]. Therefore in cells expressing p53 we would expect to see low \textit{cdca8} mRNA expression compared to cells in which p53 is absent. Northern blots show that in HCT 116 cells treated with adriamycin to induce DNA damage, \textit{cdca8} steady state mRNA levels are not detected after 48 hours of treatment. DNA damage leads to activation of p53 by phosphorylation, sumoylation, or acetylation leading to activation or repression of p53 target genes. One explanation for our data was that \textit{cdca8} was being down-regulated in a p53-dependent manner. p53 dependence on \textit{cdca8} regulation was confirmed using NARF2 cells. After inducing p53 with the addition of IPTG, the expression of \textit{cdca8} was downregulated.

Confirmation of p53-dependent regulation and cell cycle regulation of \textit{cdca8} was also shown in HCT 116 p53 +/- cells treated with thymidine and nocodazole. Northern blotting confirmed that \textit{cdca8} expression is higher in untreated, thymidine and nocodazole treated HCT 116 p53-/- cells when compared to the same treatments in HCT
116 p53 +/- cells (Figure 9). These data show that in HCT 116 cells with wild type p53 the levels of cdca8 are lower as compared to when p53 is absent or non-functional.

We also found that the basal levels of cdca8 are regulated by p53 in HT1080 cells although this was a modest effect. HT1080 LX cells expressing wild-type p53 had lower cdca8 expression compared to HT1080 GSE cells expressing a dominant negative p53. We confirmed this finding in untreated HCT 116 p53 +/- and HCT 116 p53/- cells. These experiments show that the basal levels of cdca8 mRNA are higher in p53 null cells. All these data when combined, suggest that the regulation of cdca8 expression is p53 dependent.

Next we determined if the expression of cdca8 in various cancer cell lines was correlated with the functional status of p53. For example, in the cell line PC-3, which does not have functional p53, cdca8 expression level was lower compared to LNCaP cells, which have a functional p53. Also, in all cell types with functional p53, a significant amount of cdca8 expression was detected (Figure 12). This indicates that p53 status is not the sole factor in determining the basal levels of cdca8 in a larger number of samples. The pattern of expression may be due to other factors being altered in these cells. We are unsure what the differences in the cells are because we are using a non-isogenic system.

A series of NARF2 cells containing various constructs were obtained to assay cdca8 expression. Addition of IPTG to NARF2 cells will cause an accumulation of p53, but we also wanted to determine if over expression of other cell cycle genes such as Cyclin E and Cyclin D/ Cdk complex would alter cdca8 expression because these complexes are important regulators of cell cycle genes. cdca8 levels decreased after
addition of IPTG, except when p53 was knocked out by short hairpin RNA or when Hdm2 was over expressed. Hdm2 causes degradation of p53 by adding ubiquitin to lysine residues of p53. Over expressing a dominant negative form of p53 (GSE 56) did abrogate p53-dependent repression of cdca8. Expression of a Cyclin D/ Cdk fusion did reduce the repression caused by IPTG on cdca8 mRNA levels, but some repression was still observed. Therefore, cdca8 is repressed in a p53 dependent manner, but may also be regulated to some extent by the Cyclin D/ Cdk complex.

E7 is a protein from the human papillomavirus that binds to Rb and decreases its ability to act as a tumor suppressor. We expected that over-expression of E7 would eliminate repression of cdca8 because we hypothesize that cdca8 repression is Rb-dependent; however we still observed a decrease in the expression of cdca8 after the addition of IPTG. One possible explanation is that the LXSN vector used to drive expression of E7 does so through the LTR; therefore it may not be expressing genes at a high level, thus there is residual repression due to residual Rb function. Also, it is possible that there is an Rb-independent pathway that causes repression of cdca8; this explanation is consistent with our results with the NARF2 cells expressing the Cyclin/ Cdk fusion protein data explained above. When an E7 mutant (E7Δ) was over expressed, we saw an almost complete loss of cdca8 expression. This suggests that cdca8 is repressed at least to some extent in an Rb/p53 dependent manner. The short hairpin p21 construct also did not show the expected results. If p21 is knocked out we would expect to see a high level of cdca8 expression because p21 would no longer be able to inhibit Cdks that are needed for Rb phosphorylation. We were able to see a high level of p21 expression in both treated and untreated cells. It appears that the short hairpin RNA is not
efficiently targeting p21. Western blotting analysis confirmed that the level of p21 protein was high (data not shown).

**Alternative splicing of cdca8**

Cloning and sequencing of cdca8 revealed two cDNAs that appeared to represent alternatively spliced variants. One variant has intron one present, while the other form had all introns removed. We were able to detect the inefficiently spliced version of cdca8 in a survey of 14 different cell lines using semi-quantitative RT-PCR (Figure 14). The intense staining in every lane suggests that the PCR reactions had exceeded past the linear range, nonetheless, we conclude that cdca8 with intron one is present in multiple cell types. To compare the relative amounts of both forms of cdca8 mRNA we used a forward primer that annealed to a region within exon 1 and then separated intronic cdca8 from cdca8 without the intron on an acrylamide gel. Our studies show that both splice variants are detectable in all cell lines tested. The band which represents the spliced version of cdca8 is lighter when compared to the unspliced version. This is likely due to the spliced version being approximately 100 nucleotides shorter, therefore resulting in less DNA and a lighter band. Both splice variants were transfected and used for localization studies and showed the same localization pattern, suggesting there is no difference in the function of the two types. Western blots indicate that Cdca8 from both splice variants is the same size. This is consistent with the presence of a consensus initiator methionine is located in exon 2.
Subcellular Localization of Cdca8

Localization studies revealed a dynamic localization pattern for cdca8. The localization pattern suggested that Cdca8 played a role in cytokinesis because anaphase and telophase cells contained Cdca8 at the cleavage furrow, which then regresses to the midbody as cells begin to divide (Figure 15,19, & 21). Our confocal image analysis, showed in more detail the exact location of Cdca8 at the midbody. It appears that when Cdca8 is at the midbody, it is present in two distinct areas with a gap separating the two (Figure 20). The chromosomal passenger complex, which includes Cdca8, is known to bind to the ends of anti-parallel microtubules, resulting in what appears to be two distinct locations of Cdca8 as the cell begins to divide [43, 45].

During interphase, Cdca8 was localized to punctate regions in the nucleus. We found that these regions are likely nucleoli because they contain Ki67, a known nucleolar protein. Interestingly, when Cdca8 is highly expressed, Ki67 expression is very low. We currently do not have an explanation for this phenomena. One possibility is that high Cdca8 causes cells to arrest in the cell cycle. Since Ki67 is a proliferation marker, when cells are arrested the level of Ki67 would decrease. A second possibility is that Cdca8 interferes with Ki67 going to the nucleoli. Our results using overexpression need to be interpreted with caution since previous studies have shown that the localization of Cdca8 to the nucleoli is likely an artifact [37, 44].

Time-lapse microscopy was carried out to determine the amount of time it takes for Cdca8 to localize to different regions of the cell. We discovered that Cdca8 spends the majority of mitosis localized to the chromosomes, while the transition from the spindle midzone to the midbody occurs rapidly (Figure 20). The mechanism behind the dynamic
localization of Cdca8 at present is unknown, but changes in its phosphorylation may play a role. As mentioned previously, cdca8 has a potential Cdk1 phosphorylation state. The Cyclin A/ Cdk1 complex and the Cyclin B1, B2/ Cdk1 complex are known to phosphorylate components of the mitotic spindle [26, 27]. Also, Aurora B kinase is known to phosphorylate members of the mitotic spindle in vitro, including Cdca8 [44]. Therefore, phosphorylation by one of these complexes may cause Cdca8 to relocalize to the spindle midzone from the chromosomes.

During the course of the time-lapse photography we were able to see dots oscillating around the majority of chromosomal staining (Figure 20). One possibility is that these dots represent centromere staining of chromosomes that have not yet made bipolar attachment to the spindle. Once the cell reaches late metaphase and enters into anaphase, all kinetochores become associated with spindle microtubules. We were only able to visualize single oscillating dots. If the dots were kinetochores, we would expect to see a doublet of dots. The presence of only single dots leads us to believe these dots represent Cdca8 at the inner centromere. The centromere can be viewed as the primary constriction of metaphase chromosomes and the site of kinetochore formation. The inner centromere is the region that spans the area between the kinetochores on the mitotic chromosomes. Scc1, a cohesion protein found at the inner centromere, is known to be important for the recruitment of the chromosomal passenger proteins Aurora B, INCENP, and Survivin to this region. Cells lacking Scc1 are unable to localize the chromosomal passenger complex to this region. [68].
Changes in Cdca8 Localization Due to Cell Cycle Arrest

Nocodazole was used to arrest cells in mitosis, in order to determine at what point Cdca8 relocalized to the spindle midzone from the chromosomes. During these experiments, we found an interesting phenotype. A cell arrested at telophase with midbody staining of Cdca8 was unable to divide its cellular contents and after 65 minutes of treatment Cdca8 was no longer found at the midbody (Figure 22 A-D). Nocodazole inhibits microtubule polymerization, but not microtubule depolymerization. It is possible that a majority of the microtubules have become depolymerized by this time, therefore Cdca8 would no longer be present at the midbody, since it is bound to microtubules. Another consequence of the nocodazole treatment was the cells were unable to complete cytokinesis due to the lack of microtubules.

Images of cells in early mitosis were also captured after treatment with nocodazole. We found that Cdca8 localized to one distinct region of the cell in a circular pattern (Figure 22 F), which corresponded to condensed chromosomes based on Hoechst staining. We were unable to see the release of Cdca8 from the chromosomes; therefore the majority of Cdca8 must be relocalized sometime after the point at which nocodazole has its effects. Nocodazole inhibits microtubule polymerization and blocks progression of cells from metaphase to anaphase. These results are consistent with our time-lapse images, in which Cdca8 becomes relocalized late in metaphase or early anaphase.

Treatment of cells with cytochalasin D disrupts the actin cytoskeleton. Consistent with the role for the actomyosin ring in division, cytochalasin D treated cells were not able to form a cleavage furrow. Disrupting the actin cytoskeleton appeared to have no effect on the kinetics of re-localization of Cdca8 from the chromosomes at metaphase to
the spindle midzone at anaphase. However, cells that were trapped in telophase with cytochalasin D gradually showed a loss of Cdc8 staining at the midzone. This loss of Cdc8 is the same as what was seen when cells in the middle of cytokinesis were exposed to nocodazole. However, the delocalization from the midbody in the presence of cytochalasin is not due to an absence of microtubules. One explanation for the results is that cells arrested in telophase for an extended period of time undergo changes in phosphorylation that release Cdc8 from the midbody or release another member of the chromosomal passenger complex which leads to delocalization of the whole complex. Alternatively, the actomyosin ring may have a more direct role in ensuring that Cdc8 is bound to the spindle midzone and midbody. For example, it is possible that actin physically interacts with proteins associated with the midzone microtubules. However, such interactions have not been described.

**Cdc8: A Member of the Chromosomal Passenger Complex**

While completing our studies, Cdc8 was identified as a new member of the chromosomal passenger complex [37] [44]. Along with Cdc8, the passenger complex includes INCENP, Aurora B, and Survivin and has been implicated in processes such as spindle assembly, chromosome condensation and segregation, and cytokinesis [4, 37, 42-44]. The majority of the work published focused on localization of *cdca8*, deletions of *cdca8*, siRNA directed at *cdca8*, and immunoprecipitations to determine the association of Cdc8 with other members of the complex. For the most part, our localization studies of Cdc8 coincided with the published data from two separate groups [37, 44]. However, we did see localization of GFP-Cdc8 on the DNA and on the spindle pole during
mitosis. These findings were not reported in the publications, suggesting our findings may be a result of over expression of cdca8. Future work will be aimed at determining if these results represent the natural localization of Cdca8.

Cdca8 was identified as a member of the chromosomal passenger complex based on co-immunoprecipitations with Aurora B, INCENP, Survivin, and TD-60 as well as immunofluorescence studies showing the predicted localization during the cell cycle [37]. Cdca8 does not co-immunoprecipitate with TD-60, suggesting TD-60 binding may be specific to INCENP or simply may not be a part of the complex [37]. GST-Cdca8 binds INCENP, Survivin, and itself, but not to Aurora B or TD-60 [37]. Therefore, the arrangement of the chromosomal passenger proteins is complex, with the multimeric Cdca8 binding specifically to INCENP and Survivin, while the interaction of Aurora B with Cdca8 is likely mediated through other members, such as INCENP (Figure 24). The stoichiometries of each member of the complex are currently unknown.

In anaphase, the deletion of Cdca8 amino acids 144-280 did not change its localization; however, a more extensive deletion of amino acids 88-280 disrupted localization to the spindle midzone. Similarly, deleting the C-terminus resulted in failure of Cdca8 localization to the spindle midzone. INCENP, Survivin, Aurora B, and TD-60 were delocalized from centromeres during interphase, but not from the spindle midzone during anaphase in cells expressing N-terminal deletions of Cdca8. Anaphase cells expressing GFP-Cdca8 1-88, expressed low levels, indicating that this Cdca8 mutant may
Figure 24. Chromosomal Passenger Protein Interactions. Localization studies show that the chromosomal passenger complex is located at the centromere of chromosomes. Through co-immunoprecipitations the interactions of each member of the complex has been predicted. An N-terminal domain of INCENP binds to centromeres [45] [47]. The localization of Aurora B is dependent on INCENP and Survivin indicating that Aurora B directly binds Survivin [52] [53, 69]. GST-Cdca8 binds INCENP, Survivin, and itself, but not Aurora B. Therefore, it is predicted that Cdca8 binds specifically to INCENP and Survivin, but the association with Aurora B is likely mediated through INCENP, Survivin, or both.
Cdca8

Cdca8

Survivin

Incenp

Aurora B

TD-60
be a toxic protein. Over all, experiments with Cdca8 deletion mutants suggest that the N-terminus interacts with chromosomal passenger proteins and recruits them to the centromere, while the C-terminus is required for targeting of Cdca8 to the spindle midzone [44]. During interphase, all deletion mutants of Cdca8 tagged with GFP were nuclear, but all failed to localize to centromeres. Deletions in Cdca8 can lead to delocalization of other members of the chromosomal passenger complex leading to detrimental effects in the cell such as aneuploidy.

Treatment of metaphase arrested HeLa cells with cdca8 siRNA leads to misaligned chromosomes [44]. Also, further analysis of cdca8 depleted cells revealed defective interactions between kinetochores and the mitotic spindle, as well as spindle abnormalities. Cells that contained spindle abnormalities were able to progress through early stages of mitosis and form a normal metaphase plate. However, as cells entered anaphase, sister chromatids began to separate from the metaphase plate in many different directions toward multiple spindle poles. The addition of siRNA to normal growing cells also induces multiple nuclei during interphase [44]. We were also able to observe multiple nuclei in cells treated with siRNA directed at cdca8 (Hennessy, B & Taylor, W.R., unpublished data). The same results are observed when cells are depleted of Survivin or Aurora B kinase by siRNA, along with the delocalization of Cdca8 [37, 44]. Treatment of cells with cdca8 siRNA also leads to reduced Survivin levels and phosphorylated INCENP, but unchanged levels of Aurora B [44]. Although, the levels of Aurora B are unchanged, Aurora B was no longer targeted to the chromosomes, suggesting that Cdca8 is specific for targeting of the complex to the centromere [44]. Therefore, the presence of Cdca8 is necessary for proper cell division and formation of
the mitotic spindle as well as to target Aurora B to the chromosomes in order to phosphorylate INCENP.

The Chromosomal Passenger Complex is Implicated in Microtubule Nucleation

High levels of Ran-GTP near the chromosomes induces microtubule nucleation [34] [35]. Once microtubules are assembled, they are constantly polymerizing and depolymerizing. Cdk1 and Ran-GTP are believed to play a role in these activities by altering local phosphorylation states [34].

The chromosomal passenger complex may also have a role in microtubule nucleation along with Ran-GTP. In recently published studies, DNA-coupled beads were used as a substrate for spindle assembly, since they have been shown to be sufficient to promote microtubule assembly and bipolar spindle formation [37]. In mock-depleted extracts, microtubule nucleation was observed. In contrast, cell extracts depleted of INCENP were not able to assemble microtubules. This phenotype was rescued when cells were depleted of MCAK, an activator of microtubule depolymerization. Aurora B was able to phosphorylate and suppress microtubule-destabilization of MCAK activity in vitro, suggesting this may be the reason why microtubule nucleation was rescued when cells were devoid of MCAK. Chromosomes have the ability to induce microtubule nucleation in the absence of the chromosomal passenger complex, however under these conditions microtubules are highly unstable because of MCAK activity [37].

To determine if Ran-GTP and the chromosomal passenger complex worked in the same pathway to nucleate microtubules, extracts lacking INCENP and MCAK along with the addition of a dominant negative Ran (RanT24N) were visualized for microtubule
nucleation around chromatin beads. The addition of RanT24N prevents production of Ran-GTP by blocking its exchange factor RCC1. After the addition of RanT24N to the extract, no microtubule nucleation was observed, whereas in cell extracts lacking INCENP and MCAK microtubule nucleation was present. Therefore, Ran-GTP can stimulate nucleation of the microtubules from chromatin in the absence of the chromosomal passenger complex. Microtubule nucleation around chromatin can be prevented by the addition of RanT24N in extracts lacking only MCAK. MCAK is therefore required for the instability of microtubules in extracts lacking INCENP, but the lack of microtubule nucleation caused by RanT24N does not require MCAK activity. Addition of a hydrolysis deficient Ran to metaphase extracts is known to induce aster formation. After depletion of INCENP from *Xenopus* egg extracts and addition of the hydrolysis mutant Ran, aster formation was observed [37]. Altogether these studies show that the chromosomal passenger complex is not required for Ran-GTP induced aster formation and functions in a pathway separate from Ran-GTP in the nucleation of microtubules.
V. Conclusions

The studies reported here described several aspects of the novel gene \textit{cdca8}. We determined that Cdca8 has a dynamic localization throughout the cell cycle. Through our studies and those of two separate groups, Cdca8 was implicated as a member of the chromosomal passenger complex, where it plays a role in chromosomal segregation, mitotic spindle formation, and cytokinesis. We found that the expression of \textit{cdca8} is regulated in a cell cycle dependent manner, with maximal expression occurring during G2. \textit{cdca8} is also a target for Rb/E2F and p53 regulation and is highly expressed in many different cancerous cell lines. We were also able to detect two functional splicing products of \textit{cdca8}. Our observations help to provide insight into the processes of cell division and how these processes may be regulated. Understanding the regulation and process of cell division is important in the alleviation of cancer and may help in the treatment and prevention of many types of cancers.
VI. References


