Changes in Protein Abundance are Essential for Proper Cell Cycle Regulation

Karen Reidy Lane

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Genetics and Molecular Biology.

Chapel Hill
2012

Approved By

Jeanette Gowen Cook, PhD

William Marzluff, PhD

Robert Duronion, PhD

Cyrus Vaziri, PhD

Yue Xiong, PhD

Abstract

KAREN REIDY LANE: Changes in Protein Abundance are Essential for Proper Cell Cycle Regulation
(Under the direction of Jeanette Gowen Cook)

In order for a cell to successfully complete the cell cycle, the cell must accurately duplicate its DNA in a timely and precise manner. One way the cell controls this process is by regulating the formation of the pre-replication complex (pre-RC) and S phase entry through fluctuations in protein abundance. Two proteins essential for pre-RC formation, Cdc6 and Cdt1, peak at different phases of the cell cycle; this results in two small windows when pre-RC formation can occur. While this regulation of the cell cycle has been very well studied, how other processes are regulated during the cell cycle are not well known. We used SILAC mass spectrometry to identify biological processes whose regulation may have a cell cycle component. We showed that RNA processing, in particular alternative splicing, is regulated during S phase. Additionally, we looked for genes whose transcription is altered when cells undergo re-replication, as regulation of these proteins may play a role in transformation, genome stability, or tumorigenesis. By identifying a re-replication gene expression signature, we can identify tumor types that are undergoing re-replication. Identification of this signature in human tumors may enhance diagnostics and prognosis, as these tumor types may benefit from a particular type of chemotherapeutic. Because a

successful division involves the cooperation of many biological processes, the identification of proteins that are regulated in a cell cycle manner or in response to a particular form of DNA damage, re-replication, could result in novel targets for chemotherapeutics and biomarkers.

Acknowledgements

First and foremost, I would like to thank my mentor, Dr. Jeanette Gowen Cook. While it has not been the easiest journey, you have stuck with me the entire way, and I sincerely thank you for all of your help and guidance.

I would also like to thank the members of my committee: Dr. Cyrus Vaziri, Dr. Bob Durunio, Dr. Yue Xiong, and Dr. Bill Marzluff. Thank you for your continued support and guidance. I would also like to thank Dr. Xian Chen and Dr. Yanbao Yu for their expertise and help with the mass spectrometry. I would also like to acknowledge Dr. Zefeng Wang, Daniel Dominguez, and Dr. Mariano Garcia-Blanco for their helpful suggestions and expertise.

I would not have made it this far without the past and present members of the Cook Lab. Every member of the lab has influenced me as a scientist and as a person. To Kat Nevis and Elizabeth Dorn, thank you for welcoming me into your science family; without you, the Cook Lab would never have truly been home. To Sri Chandrasekaran, Kim Raiford, and Candice Carlile, thank you for always being a sounding board for ideas and a shoulder to cry on when they didn't work out. And most importantly, to my current lab members, particularly Kate Coleman and Lindsay Rizzardi, thank you for keeping me in touch with reality. Thank you for the long talks (science or otherwise). Thank you for always being there with a

quick joke. I could continue this way forever, so it's best I just say thank you for everything.

I would also like to thank my parents and my brother for their patience and support. I would also like to thank my North Carolina family, both at UNC and in Raleigh. To the members of GMB, thank you for being my second family when I first moved down here. I will always remember our dinners and shenanigans. I would also like to thank my Raleigh family, affectionately known as Those Guys. I consider myself truly blessed to call all of you friends; without you, my life would be completely different.

And last, but definitely not least, I would like to thank my loving husband, Cliff. Words cannot describe how much you mean to me. Thank you for being the best husband a girl could ask for. Thank you for your understanding and patience. I love you so much, and thank you for everything.

Table of Contents

List of Tables

2. Global Proteomics Reveal Unexpected Cell Cycle Regulated Processes

List of Figures

1. Introduction

2. Global Proteomics Reveal Unexpected Cell Cycle Regulated Processes

List of Abbreviations

AAA+ – ATPase-associated with various cellular activities

AACT – Amino Acid-Coded mass Tagging

ABC – Ammonium bicarbonate

ACN – Acetonitrile

AEBSF – 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride

APC/C – Anaphase Promoting Complex/Cyclosome

ATM – Ataxia Telangiectasia Mutated

ATR – Ataxia Telangiectasia and Rad3 related

BrdU – Bromodeoxyuridine

BSA – Bovine serum albumin

CBP – Calmodulin binding peptide

Cdc6 – Cell division cycle 6

Cdc20 – Cell division cycle 20

Cdk – Cyclin dependent kinase

Cdt1 – Cdc10 dependent transcript 1

Cdt2 – Cdc10 dependent transcript 2

Chk2 – Checkpoint kinase 2

CHO – Chinese hamster ovary

CID – Collision-Induced Dissociation

CKI – Cyclin dependent kinase inhibitor

CRL4 – Cullin 4

DAPI – 4’,6-diamidino-2-phenylindole

DAVID – Database for Annotation, Visualization, and Integrated Discovery

Dec – Decrease

Dec MG – Decreases with MG132

dFBS – Dialyzed Fetal Bovine Serum

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic acid

DNMT – DNA methyltransferase

DP – Dimerization partner

DT – Double thymidine block

DTT – Dithiothreitol

ECl – Enhanced chemoluminescence

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethylene glycol tetraacetic acid

Emi1 – Early mitotic inhibitor 1

FBS – Fetal bovine serum

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GFP – Green fluorescent protein

GO – Gene ontology

GST – Glutathione S-transferase

HAT – Histone acetyltransferase

Hbo1 – Histone acetyltransferase binding to Orc1

HDAC – Histone deacetylase

HMEC – Human mammary epithelial cell

hnRNP – Heterogeneous nuclear ribonucleoprotein

HPLC – High performance liquid chromatography

HRP – Horseradish peroxidase

IF – Immunofluorescence

Inc – Increase

Inc MG – Increases with MG132

IP – Immunoprecipitation

LC-MS/MS – Liquid chromatography-tandem mass spectrometry

Mad2 – Mitotic arrest deficient 2-like protein 1

MARCKSL1 – Marcks-related protein 1

Max – Myc associated factor X

MCM – Minichromosome Maintenance complex

MDM2 – Double minute 2 protein

MG132 – Z-Leu-Leu-Leu-al

miRNA – MicroRNA

Miz1 – Myc-interacting zing finger protein 1

MOI – Multiplicity of infection

mRNA – Messenger ribonucleic acid

NC – No Change

NC MG – No Change with MG132

NEDD8 – Neural precursor cell expressed developmentally down-regulated protein 8

NEM – *N*-Ethylmaleimide

NHF – Normal human fibroblast

NRS – Normal rabbit serum

NSB – Non-specific band

ORC – Origin recognition complex

PAGE – Polyacrylamide gel electrophoresis

Palmd – Palmdelphin

PBS – Phosphate buffered saline

PCNA – Proliferating Cell Nuclear Antigen

PCR – Polymerase chain reaction

PFA – paraformaldehyde

PIP motif – PCNA interacting protein motif

Plk1 – Polo-like kinase 1

pre-RC – Pre-replication complex

PTM – Post-translational modification

PVDF – Polyvinylidene fluoride

Rb – Retinoblastoma protein

RNA – Ribonucleic acid

RRM2 – Ribonucleoside-diphosphate reductase subunit M2

SBP – Streptavidin binding peptide

SCF – Skp, Cullin, F-box containing complex

SDS – Sodium dodecyl sulfate

SILAC – Stable Isotope Labeling of Amino acids in Cell culture

siRNA – Small interfering RNA

Set8 – SET domain-containing protein 8

Skp2 – S-phase kinase-associated protein 2

SLBP – Stem loop binding protein

SRSF – Serine rich splicing factor

Tmod3 – Tropomodulin 3

TRRAP – Transformation/transcription domain associated protein

UV – Ultraviolet radiation

Chapter 1

Introduction[1]

Introduction

{DNA replication is a fundamental biological process that serves to create two copies of the genetic material during each cell division. Complete and precise replication enables identical sets of genes to be faithfully delivered to daughter cells during each cell division. To achieve rapid duplication of the entire genome, eukaryotic cells initiate DNA replication at multiple locations on each chromosome termed origins of DNA replication. Origin DNA is unwound and complementary DNA is then synthesized from bi-directionally moving replication forks. The replication forks eventually merge to form two identical chromosomes.

The cell expends tremendous energy ensuring that a single origin of replication does not initiate replication twice within the same cell cycle. One of the most highly regulated steps in DNA replication is assembly of pre-replication complexes (pre-RCs). Pre-RC assembly begins as cells exit mitosis and continues through G1 phase, culminating in chromosomes poised for replication by the end of G1. At the onset of S phase, origins fire and replication begins.

[1]**{Portions of the following appear in: Regulation of DNA Replication Origin Licensing** Chandrasekaran, S., Reidy, K.T., and Cook, J.G. (2011) *in* Fundamental Aspects of DNA Replication, J. Kusic-Tisma, ed. (Rijeka, Croatia; Intech Open Access Publisher).}

During this time, several overlapping mechanisms prevent pre-RC assembly on origins that have already fired to avoid utilizing any origins twice.

An abnormal situation in which replication is triggered multiple times from the same origin during a single cell cycle is termed re-replication (Figure 1.1). Re-replication is detrimental to genome stability in part, because it generates multiple replication forks on the same DNA strand. Ultimately such structures result in double strand breaks, genome instability, and in some cases, tumorigenesis [2-5].}

Pre-RC formation is limited to G1 phase of the cell cycle

{To faithfully replicate its genomic information in a timely manner, a cell must initiate replication at thousands of sites across the genome. These origins of replication are prepared for replication through assembly of pre-RCs beginning in late mitosis and continuing through G1 phase of the cell cycle. Origins with a fully assembled pre-RC are said to be “licensed” for replication. It is essential that origins assemble pre-RCs only in G1 because assembly of pre-RCs in S or G2 can lead to re-replication.

Pre-RC assembly begins when the six-subunit origin recognition complex (ORC) binds to an origin of replication (Figure 1.2). ORC is composed of the constitutively expressed subunits Orc2-6, as well as the cell cycle-regulated Orc1 protein, and acts as an ATPase [6-9]. Once bound to origins, ORC recruits the remaining licensing factors Cdc6 and Cdt1 to origins [10,11].

Cdc6 was discovered in *Saccharomyces cerevisiae* and is essential for

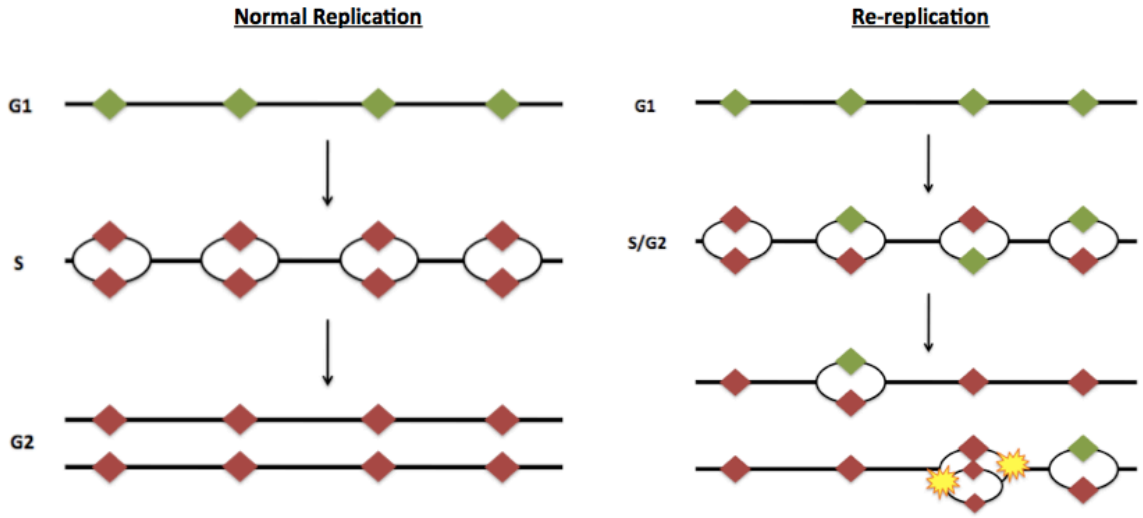

Figure 1.1. Re-replication leads to double strand breaks. In a normal replicating cell (left), origins are licensed only in G1 phase (indicated by the green diamonds). Replication begins in S phase, and origins are inhibited from firing again (indicated by the red diamonds); replication is completed by G2 phase. In re-replicating cells [1], origins are licensed again in either S or G2 phase, leading to origin re-firing and re-replicated stretches of DNA. The consequences of re-replication include DNA damage, genome instability, and tumorigenesis.

DNA replication; if Cdc6 is absent, yeast cells not only fail to replicate but also undergo reductional anaphase in which mitosis initiates without genome replication [12-14]. Cdc6 is a member of the AAA+ ATPase family, and is closely related to Orc1 [15,16]. ATP hydrolysis by Cdc6 and ORC is needed to load the helicase complex onto DNA [8,17-21]. Due to its tight association with ORC and its partially conserved DNA binding domain, it has been suggested that Cdc6 may also play a role in defining ORC binding sites [22].

Cdt1 was first discovered in *Schizosaccharomyces pombe* and, while possessing no enzymatic activity, is essential for the licensing reaction [11,23]. Cdt1 binds the core replicative helicase Mini-Chromosome Maintenance (MCM) complex and recruits MCM to origins through direct interactions with ORC and Cdc6 [24-27]. While both Cdc6 and Cdt1 are needed to load the MCM complex, they bind in a sequential manner; Cdt1 can only bind to chromatin-bound Cdc6 and ORC [28]. Both Cdc6 and ORC hydrolyze ATP to load MCM complexes onto DNA [17]. ATP hydrolysis by Cdc6 also releases Cdt1 to recruit additional MCM complexes [17]. Once MCM complexes are loaded, the origin is licensed and can initiate replication once the MCM helicase is activated in S phase. After MCM complexes have been loaded, ORC, Cdc6, and Cdt1 are no longer needed, and replication can continue in their absence [20,29,30]. This property of the loaded MCM complex is key to preventing re-replication because, as will be discussed below, ORC, Cdc6, and Cdt1 are inactivated beginning in S phase.

At each origin, at least two MCM hexamer complexes are loaded at a time, with multiple rounds of loading at each origin [31-34]. The exact mechanism of

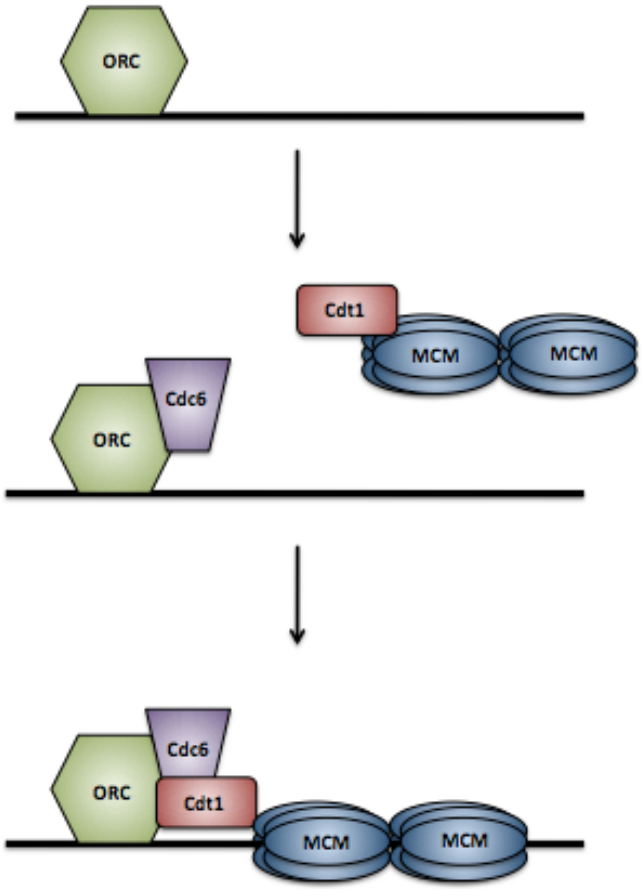

Figure 1.2. Pre-RC assembly in G1 phase. Pre-RC assembly begins when the Origin Recognition Complex (ORC) binds to origin DNA. ORC recruits Cdc6, which in turn recruits Cdt1 bound to the Mini-chromosome Maintenance (MCM) core helicase complex. Through the ATPase activity of ORC and Cdc6, the MCM complex is loaded onto DNA and the origin is licensed for replication.

MCM loading is not currently understood, but electron microscopy images suggest ORC and Cdc6 form a structure similar to known clamp loaders such as RFC [35,36]. While multiple MCM complexes can be loaded at each origin, perhaps as many ten copies per origin, the majority of the MCM complexes that associate with chromatin do not travel with the replication fork suggesting that they are not normally activated [33,37,38]. These additional MCM complexes may be loaded as a backup mechanism to ensure that a sufficient number of origins fire in S phase [39].

MCM loading is highly regulated by multiple overlapping mechanisms. Cdc6 and Cdt1 protein levels peak at different stages of the cell cycle; Cdt1 levels peak in G1 phase whereas Cdc6 peaks in S/G2 phase in mammalian cells [11,40]. Additionally, a member of the ORC complex, Orc1, is degraded or inactivated at the onset of S phase [9,41,42].

Due in part to these alternating protein levels, there are only two short windows in the cell cycle when pre-RC formation can occur. Pre-RC assembly begins at the end of mitosis, before the Anaphase Promoting Complex/Cyclosome (APC/C) becomes active in G1, and targets Cdc6 for degradation. The second round of pre-RC assembly occurs in late G1 phase when activated Cdk2 stabilizes Cdc6 but before Cdt1 is degraded at the onset of S phase [43]. Furthermore, the MCM subunits undergo post-translational modifications that facilitate MCM complex formation as well as their ability to be loaded onto DNA [44,45]. These mechanisms will be discussed in depth in the

subsequent sections with specific emphasis on the regulation of metazoan pre-RC assembly.}

Pre-RC assembly is regulated by the Cyclin dependent kinases

{Cyclin-dependent kinases (Cdks) are a family of serine-threonine protein kinases essential for timely and appropriate progression through different stages of the cell cycle. Cdks are activated by association with cyclins whose expression and stability are cell cycle-regulated. In budding and fission yeast, a single Cdk controls the G1/S and G2/M transitions, while in metazoans different Cdks are active in different phases of the cell cycle (Figure 1.3, reviewed in [46]). In metazoans, passage through G1 phase is governed by cyclin D/Cdk4 (or cyclin D/Cdk6) and cyclin E/Cdk2 [47,48]. S phase, and therefore DNA replication, is regulated by cyclin A/Cdk2 complexes [49]. Finally, mitotic entry is triggered by Cdk1 first binding to cyclin A and then cyclin B.

DNA replication is both positively and negatively governed by Cdk activity. High Cdk2 and Cdk1 activities, which are found from early S phase through mid-mitosis, block pre-RC assembly; thus pre-RC assembly begins as cells exit from mitosis when these kinases are not active. Cyclin E protein peaks in early S phase, triggering Cdk2 activity and replication initiation from licensed origins; replication begins also with the help of a dedicated replication kinase, Cdc7/Dbf4 [32,50]. Simultaneously, S phase Cdk activity inhibits pre-RC formation at origins that have already fired. Premature expression of cyclins E or A during G1 phase blocks normal pre-RC assembly [49,51]. Thus S phase Cdks promote replication

initiation but block pre-RC assembly after G1 resulting in one genome duplication per cell cycle.}

As previously mentioned, the control of pre-RC formation is tightly regulated by the abundance of the licensing factors Cdc6 and Cdt1, as well as the activity of cyclin/Cdk complexes. While this is only a single step in the long process of cell division, it is crucial to maintain the genomic stability of the cell. In order for this process to happen at the appropriate time, the correct proteins must be available and active at precisely the correct time and in the correct quantity. To ensure that this happens, these proteins are regulated at every stage of expression, from gene transcription to protein modification and degradation.

Regulation of Cell Cycle Genes by Transcription

Many of the gene products involved in cell cycle control and pre-RC formation are themselves transcribed in a cell cycle manner. The transcription of these genes are regulated by two major families of transcription factors, the E2F family and the myc family. E2F transcriptional targets are crucial for S phase entry and mitosis, whereas myc transcriptional targets are essential for general cell proliferation. Because pre-RC formation must be tightly regulated, this section focuses primarily on how these transcription factor families promote the G1/S transition.

The Rb/E2F pathway controls the expression of cell cycle genes

As one of the major regulators of the cell cycle, the Rb/E2F pathway is

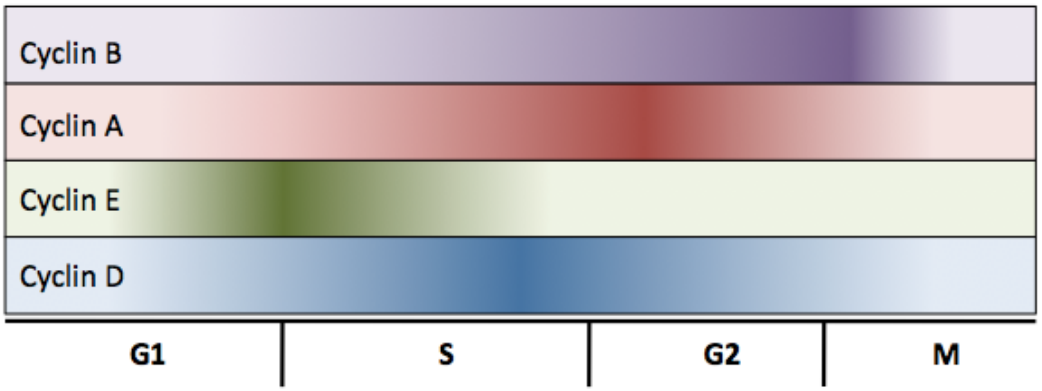

Figure 1.3. Cyclin levels fluctuate throughout the cell cycle. Cell cycle progression is regulated through the action of cyclin/Cdk complexes. In G1 phase, the cyclin D/Cdk4 and cyclin D/Cdk6 complexes are nuclear and active and regulate the transcription of genes for pre-RC components. Cyclin E/Cdk2 activity stimulates replication in S phase while simultaneously inhibiting relicensing of origins. Cyclin A/Cdk controls S phase progression and also inhibits relicensing. Entry into mitosis is triggered by cyclin B/Cdk1 complexes. Darker shading indicates peak protein expression or activity.

responsible for the transcription of genes needed to complete a successful S phase. There are 8 members of the E2F family of transcription factors (reviewed in [52] and [53]). This family contains both transcriptional activators (E2Fs 1, 2, and 3a) and transcriptional repressors (E2Fs 3b, 4, 5, 6, 7, and 8). In order to bind DNA, E2F family members bind to a dimerization partner (DP) protein [54]. E2F1 was first characterized through its association with its inhibitor protein, the Retinoblastoma (Rb) protein [55,56]. Three different proteins belong to the Rb family: pRb/p105 (hereafter referred to as pRb), which is primarily bound to the activator E2Fs, and pRb2/p130 and p107, which are primarily bound to the repressor E2Fs [53,57-59].

To keep E2F1 inactive, pRb binds the transactivation domain of the transcription factor; this interaction can occur at the target gene promoter [60-65]. The pRb/E2F complex can then recruit several additional complexes to either repress or activate transcription of its target genes (discussed below). The release of E2F constitutes a “restriction point”; once the cell has released E2F and transcription of S phase genes has begun, the cell has committed to completing the cell cycle [66,67].

During the cell cycle, pRb is expressed at steady levels, however its ability to bind E2F is regulated by the actions of the cyclin-dependent kinases [68-72]. pRb2/p130, on the other hand, is highly expressed in quiescent cells, but these levels decrease as cells are restimulated into the cell cycle; this decrease corresponds with an increase in activator E2F transcription [73]. Early in G1 phase, both pRb and pRb2/p130 are phosphorylated when cyclin D/Cdk4/6

complexes become active (Figure 1.4) [74,75]. At this time, the repressor E2F complexes, pRb2/E2F4, are found at the promoters of E2F target genes, resulting in the silencing of these genes [76,77]. As cells progress into late G1, cyclin/Cdk2 complexes further phosphorylate the Rb proteins, resulting in the release of the E2F transcription factors [78]. Due to a positive feedback loop (discussed later), E2F mRNA levels begin to rise, and pRb2/p130 levels begin decrease; at this point, the third Rb protein, p107, replaces pRb2/p130 at the promoters of genes that need to be silenced, typically at the promoters of genes needed for apoptosis [77,79]. As cells transition into S phase, cyclin E/Cdk2 complexes become fully active, resulting in the dissociation of E2F activators from the pRb, thereby allowing the transcription of genes needed for S phase [54,80-82]. Following S phase entry, E2F proteins are downregulated; this is essential for cell survival as prolonged expression of E2F proteins can result in apoptosis [83-87].

The Rb proteins can become highly phosphorylated; pRb alone contains 16 consensus Cdk phosphorylation sites [88-90]. However, no single phosphorylation event can disrupt the interaction between pRb and E2F [89,90]. Instead, multiple sections of the Rb proteins, particularly the spacer and the C-terminal domains, must be phosphorylated in order to release the E2F proteins [89]. The activity of cyclin D/Cdk4/6 or cyclin/Cdk2 alone cannot result in hyper-phoshphorylated Rb. For complete activation of the E2F proteins, Rb must be phosphorylated by cyclin D/Cdk4/6 complexes and cyclin/Cdk2 complexes;

indeed, cyclin/Cdk2 complexes can only phosphorylate Rb proteins that have previously been acted on by the cyclin D/Cdk4/6 complexes [91].

As SCF^{Skp2} levels rise, hyper-phosphorylated pRb2/p130 proteins can be ubiquitinated and targeted for proteasomal degradation [92-94]. Additionally, high levels of Cdk activity can result in the phosphorylation of serine 567 on pRb; this modification can target the protein for cleavage by a protease [95]. In addition to destruction, the Rb proteins can be dephosphorylated at the end of mitosis; this process is performed by protein phosphatases 1 and 2 [96-99].

The control of the Rb/E2F pathway is essential for proper replication. The misregulated expression of E2F is sufficient to stimulate quiescent cells to reenter the cell cycle [83]. Additionally, overexpression of any of the three Rb proteins can result in a G1 arrest [54,80-82,100-102]. Taken together, this indicates that these proteins must be tightly regulated in order for a cell to properly divide.

Rb/E2F represses target genes during G1 and S phase

In the E2F family of transcription factors, there are two types of repressors, the canonical E2F proteins 3b, 4, and 5, and the atypical repressors, E2Fs 7 and 8. The canonical E2F family members are bound by pRb2/p130 and p107. During quiescence and G1, pRb2/p130 binds to E2F4 and E2F5 to help repress target genes; during G1, p107 has also been found bound to E2F4 [61-64,103-105]. Interestingly, E2F4 and E2F5 do not have a nuclear localization signal and cannot be imported into the nucleus unless they are bound to either pRB2/p130

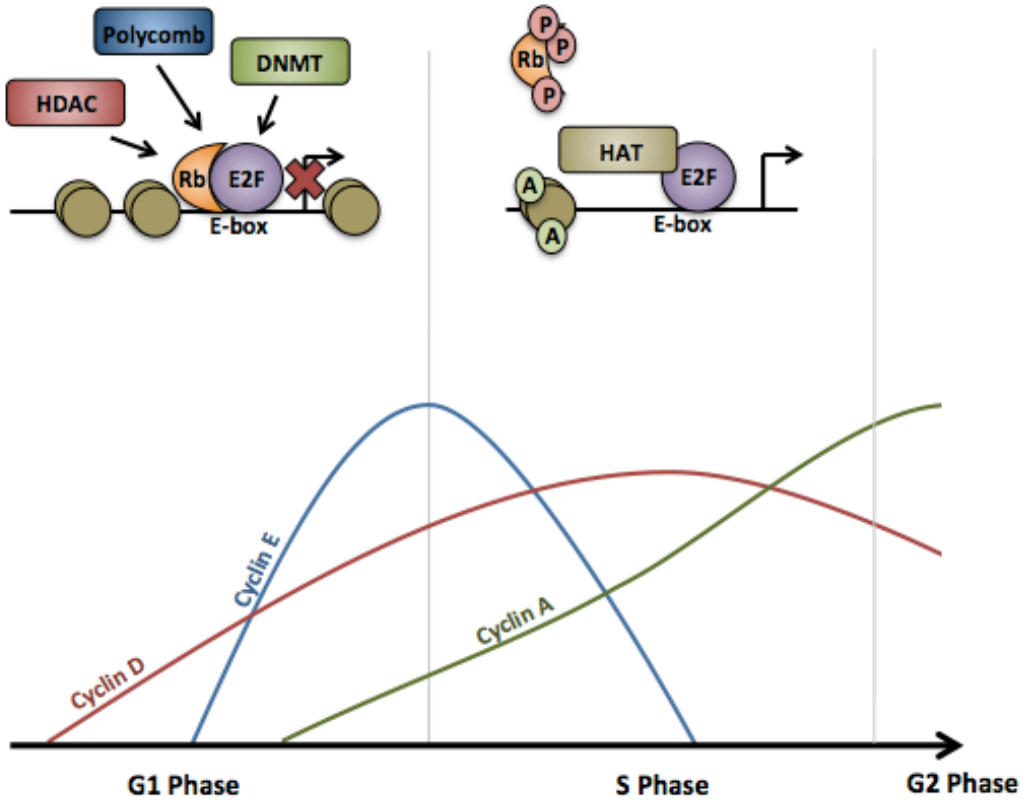

Figure 1.4. The Rb/E2F pathway regulates the transcription of genes needed for S phase progression. During G1 phase of the cell cycle, Rb is hypo-phosphorylated and can bind to E2F. The binding of these repressive E2F complexes recruits histone deacetylases (HDAC), DNA methyltransferases (DNMT), and chromatin modifying enzymes to change the chromatin environment surrounding the promoter of target genes. Once cyclin D/Cdk complexes and cyclin E/Cdk2 complexes become active, Rb is phosphorylated and releases E2F proteins, which then recruit histone acetyltransferases (HAT) to promoters to open the chromatin environment, facilitating transcription.

or p107 [106-110].

In order to repress transcription of target genes, the pRb2/E2F4 complexes recruit chromatin remodeling factors, histone deacetylases, Polycomb proteins, or DNA methyltransferases to physically change the chromatin environment surrounding the promoter [78,111-117]. In some instances, inhibiting the activity of histone acetyltransferases can modulate the repression of E2F target genes, suggesting that the modification of the surrounding chromatin is essential for proper silencing [118]. Important targets of E2F repression include the Cdt1 inhibitor, geminin, whose expression blocks pre-RC assembly, and ribonucleoside-diphosphate reductase subunit M2 (RRM2), which is needed for nucleotide synthesis during S phase [119,120].

The atypical E2Fs are highly conserved, with homologous proteins found in *Arabidopsis thaliana* [121-129]. These E2F proteins cannot be regulated in an Rb-dependent fashion, and do not need to bind to DP in order to bind to DNA [126]. This heterodimerization with DP is not necessary due to the duplication of the DNA binding domain found in canonical E2F family members [122-124,126,127]. Instead, E2F7 and E2F8 can homodimerize or heterodimerize with each other [122,126,130]. These E2F proteins are induced at the G1/S transition, and their protein levels peak in late S/G2 phase [121-124,126]. The regulation of these atypical E2Fs is controlled, both positively and negatively, by other E2F proteins; E2Fs 1, 3, 4 and 7 have been found at the promoters of both E2F7 and E2F8 [122,125]. Additionally, E2F7 and E2F8 are unstable proteins that are regulated by ubiquitin-mediated degradation [125,131-133].

Importantly, when these proteins were overexpressed, there was a marked effect on cell proliferation [121-124,126]. On the other hand, when these genes were deleted in mice, the mice exhibited widespread apoptosis [130]. These phenotypes can be explained by examining the targets of E2F7/8 repression. Perhaps the most important target of E2F7/8 repression is E2F1 itself; deletion of both of these proteins leads to a drastic change in the E2F1 transcription profile [130,134]. E2F7/8 repression is necessary for the normal downregulation of E2F1, even when the ubiquitin-mediated degradation pathway, dependent on SCF^{Skp2}, is still intact [135].

Rb/E2F activates transcription of cell cycle genes at the G1/S transition

Once the cell has passed the restriction point and committed to completing the cell cycle, E2F proteins are released from their Rb binding partners and are able to activate transcription of their downstream target genes; this activation is partially due to the recruitment of histone acetyltransferases, such as p300, which leads to a more favorable chromatin environment [136]. These targets include genes that are essential for cell cycle progression, such as *cyclins E* and *A*, *PCNA*, *DNA Polymerase* α, and genes needed for nucleotide biosynthesis [76,77,137]. In the case of the *cyclin E* promoter, the activator E2Fs (1, 2, and 3a), in conjunction with pRb, act as repressors and work to recruit histone deacetylase complexes to silence transcription [138-140]. While these E2F family members are typically thought of in an activating role, the role that

they play at the *cyclin E* promoter shows that activity of these complexes is most likely context dependent [141-144].

Importantly, E2F activators also control the levels of the E2F and Rb proteins in a positive feedback loop [76,77,137]. The promoter of the E2F1 gene has been shown to be regulated by all activator E2Fs [77,137,145]. E2F1 also controls the transcription of the atypical E2Fs, E2F7 and E2F8; because these repressor proteins can target the promoter of E2F1, this is one way that the cell limits the amount of E2F present [130]. Additionally, E2F can induce the transcription of the F-box protein Skp2, which is a subunit of the SCF^{Skp2} ubiquitin ligase; the activity of this ubiquitin ligase leads to the proteasomal degradation of E2F [146].

While E2F has many well-known targets, the list of genes affected by E2F activation is ever expanding. Recent evidence has shown that some E2F target genes may affect mRNA stability or even the splicing of certain transcripts by altering the levels of alternative splicing factors present [147-150]. These alternative splicing factors have been shown to be important for the proper transcription of certain apoptotic genes, which are also E2F targets [150]. E2F1 plays a unique role with respect to pro-apoptotic genes. An alternative pRb/E2F1 complex has been found bound to the promoters of pro-apoptotic genes, resulting in the silencing of these transcripts and continued cell proliferation [151]. These pRb/E2F1 complexes are Cdk resistant; even though pRb is hyper-phosphorylated, E2F1 remains bound, albeit with an altered DNA binding [151-155].

The Rb/E2F pathway is essential for a successful mitosis

While the Rb/E2F pathway plays a crucial role in the G1/S transition, E2F proteins target a wide range of genes. When cells are depleted of all three activator E2Fs, the cells arrest in all phases of the cell cycle, suggesting that E2F target genes are needed for more than just the transition from G1 to S phase [139]. While most E2F target genes are necessary to successfully complete S phase, there are several E2F target genes that are needed for a successful division. When E2F activity is misregulated, or when Rb is depleted from cells, both Mad2, a protein involved in the spindle checkpoint, and Emi1, an inhibitor of a critical ubiquitin ligase, are overexpressed; alterations in the levels of these proteins leads to mitotic defects and aneuploidy [156,157]. These defects are partially due to the inability of condensin II to load onto the chromatin, resulting in chromatin condensation defects [158-160].

Mutations in the Rb/E2f pathway are prevalent in cancer

Mutations in the Rb/E2F pathway are prevalent in a variety of tumor types, including small cell lung cancer, non-small cell lung cancer, retinoblastomas, and prostate cancer (reviewed in [161] and [162]) [163,164]. The Rb portion of the pathway is inactivated or deleted in retinoblastomas [165,166]. The mutations in Rb that occur in human tumors result in an inability of Rb to bind and regulate the E2F transcription factors [167]. Rb has also been shown to be inactivated by the E7 oncoprotein in cervical cancers [168]. Additionally, changes in E2F proteins have also been linked to cancer development. For example, E2F3 is amplified and overexpressed in bladder cancers [169,170]. Also, while E2F7 is an atypical

member and not subject to Rb-mediated regulation, low levels of E2F7 have been found in ovarian tumors and gliomas; low expression levels also correlate with a poor prognosis and decreased survival rates [171]. Misregulation of this pathway can result in double strand breaks and genome instability [172].

The Myc Family of Transcription Factors

The c-Myc protein was first identified through its homology to the viral oncogene encoded by the avian myelocytomatosis retrovirus, v-Myc [173]. The c-Myc protein contains an N-terminal transactivation domain but cannot bind DNA by itself; in order to regulate its downstream target genes, Myc has to be bound to the basic helix-loop-helix (bHLH) protein, Max (reviewed in [174] and [175]). When bound together, Myc/Max heterodimers bind to a specific DNA sequence (CACGTG) known as an E-box [176]. Myc's ability to bind DNA and affect transcription is necessary for the oncogenic activity that has been associated with Myc deregulation; mutations in either the transactivation domain or the DNA binding domain can abolish cell proliferation and affect the cell's ability to undergo apoptosis [177,178].

It has been estimated that more than 15% of human genes can be controlled by the c-Myc protein [179,180]. However, a small fraction of the identified target genes are consistently regulated; most of the identified target genes appear to be regulated in a cell type or species-specific manner [181]. Myc protein levels are low in quiescent cells, but are rapidly induced following a mitogenic stimulus, such as serum addition [182-185]. Once induced, Myc can

affect the transcription of proteins involved in many biological processes, such as glucose and iron metabolism, cell adhesion, and protein synthesis [186-196].

Myc can act as both a transcriptional activator by associating with histone acetyltransferases and chromatin modifiers, as well as a transcriptional repressor through its interactions with DNA methyltransferases [197-202]. When bound to target E-box sequences, the Myc/Max heterodimer can recruit TRRAP, a member of a histone acetylase complex, that can lead to acetylation of the local nucleosomes and activation of target genes [203,204]. In addition to the recruitment of TRRAP, Myc/Max heterodimers have also been shown to recruit the acetyltransferase Gcn5 [205].

While Myc can induce the transcription of a multitude of genes, it plays an important role in the induction of genes needed for cell proliferation and cell cycle progression. C-Myc has been found at the promoters of *cyclins D1*, *D2*, and *B1* in humans and *cyclins A* and *B* in *Drosophila melanogaster* [186,203,206]. Myc has also been found at the promoter of *Cdk4* [207]. Therefore, mitogenic stimuli can lead to the induction of cyclin D2/Cdk4 activity, which is turn sequesters the CKI p27; this allows cyclin E/Cdk2 complexes to be free of CKIs and become active, leading to S phase entry [208-210]. While Myc can influence cyclin and Cdk levels, perhaps the most important Myc target gene is *E2F1* [211]. As described previously, this family of transcription factors controls the transcription of a variety of cell cycle genes.

Perhaps even more important than its role as a transcriptional activator is its role as a transcriptional repressor. The repression of some Myc target genes,

particularly following terminal differentiation, is through its competition with the protein Mad [212]. Mad binds to Max, and these Mad/Max heterodimers compete with Myc/Max heterodimers for E-box binding. Once bound, Mad/Max can recruit chromatin-modifying complexes that include histone deacetylases, thereby shutting down transcription of some Myc target genes [213,214]. However, this mode of repression is not responsible for the downregulation of all Myc target genes. Myc/Max heterodimers have been found at the promoters of both active and repressed genes, suggesting that the Myc/Max interaction is needed to repress a certain subset of target genes [193]. Additionally, it has also been shown that the portion of Myc needed to bind TRRAP and other histone acetylase complexes is needed for the repression of certain target genes [215,216].

Myc can also repress transcription by antagonizing the transcriptional activator Miz1. Myc can bind to Miz1 and disrupt the interaction between Miz1 and the histone acetyltransferase p300; this method of transcriptional repression has been found at the promoters of the Cyclin dependent kinase inhibitor (CKI) genes *p16*, *p15*, and *p21* [217-222]. At the *p21* promoter, Myc binds to Miz1 and recruits the DNA methyltransferase DNMT3a, resulting in downregulation of p21 transcription [202].

Myc and cancer

The importance of the Myc family of transcription factors in cell proliferation is evident by the fact that deletion of the Myc genes results in

embryonic lethality in mice at days e9.5-e10.5 [223]. Interestingly, overexpression of the protein does not induce DNA replication or division, suggesting that the oncogenic effects of Myc are due, at least in part, to its effects on other pathways [194,224]. Indeed, the c-Myc transcription factor is one of the most frequently mutated oncogenes; it is estimated to be mutated and/or deregulated in about 20% of all cancers (reviewed in [225] and [185]). Changes in its expression patterns correlate with aggressive, poorly differentiated tumors and a poor patient prognosis. Currently, evidence of c-Myc deregulation can be found in many tumor types, including colon, breast, glioblastomas, melanomas, and lung cancers [185,225-227]. C-Myc has also been implicated in Burkitt's lymphoma; in this case, a chromosomal translocation leads to the fusion of c-Myc with one of three antibody loci, IGH@, IGK@, and IGL@ [228].

Interestingly, the entire Myc family of transcription factors (c-Myc, n-Myc, and l-Myc) has been implicated in lung cancer progression; these factors have been shown to be amplified and/or overexpressed in both small cell and non-small cell lung cancers [229-231]. This overexpression leads to increased activation of the E2F family of transcription factors [224,232,233]. It is important to note that E2F1 levels will only increase when Myc is expressed at levels similar to the level of Myc present after mitogenic stimuli; therefore, overexpression of Myc does not directly lead to overexpression of E2F1 [234]. However, recent evidence has shown that cells expressing c-Myc at high levels contain two or three times more total RNA than cells expressing low levels of c-

Myc; this suggests that previous gene expression studies may actually be misleading [235-237].

Regulation of Translation by miRNAs

After genes are transcribed, the rate at which the mRNA is transcribed can be controlled by the action of microRNAs (miRNAs), which were first described in *Caenorhabditis elegans* [238,239]. miRNAs are short, non-coding RNAs, typically between 18 and 25 nucleotides, that can affect the stability and translation of mRNAs (reviewed in [240]). These RNAs are transcribed by RNA polymerases II or III, processed into small hairpins, and exported to the cytoplasm; there, these hairpins are processed further and loaded into an RNA induce silencing complex (RISC) [241-243]. These complexes can bind to the 3' untranslated region of an mRNA, resulting in either cleavage and destruction of the message or blockage of translation [244].

miRNAs control cyclin/Cdk complexes directly and indirectly

The importance of miRNAs in controlling translation is becoming more apparent. Mice depleted of Dicer, the enzyme responsible for the final processing step of the miRNA synthesis, are embryonically lethal, suggesting that this process is essential for cell proliferation [245]. According to the miRBase Sequence Database (release 19), there have been over 2,000 mature miRNA sequences identified in humans [246,247]. It is estimated that between 30 and 60% of the human genome is regulated through miRNAs [248-250].

Because the cyclin/Cdk complexes are the master regulators of the cell cycle, it is not surprising that these proteins are heavily regulated at the post-transcriptional level as well (Figure 1.5). The translation of these proteins can be directly affected by the transcription of certain miRNAs or the activity of these proteins can be indirectly affected through downregulation of a secondary target. Cyclin D can be targeted by a multitude of miRNAs, including members of the let-7 and the miR-15 families, as well as miR-17, miR-19a, miR-20a, and miR-34 [251-258]. Several of these miRNAs target additional cyclins or Cdks. For example, miR-34a also targets cyclin E and Cdk4/6; overexpression of this miRNA results in the downregulation of cyclin D/Cdk4/6 complexes and a G1 arrest [252,259,260]. Members of the let-7 family can also downregulate cyclins E, A, and B [254]. Cyclins A and B can also be regulated by miR125b and miR-24, whereas cyclin E can be regulated by miR-16 [261-263].

Both Cdk4 and Cdk6 are heavily regulated by miRNAs. The translation of these proteins can be altered by miR-24, miR-34a, miR-124, miR-125b, miR-129, miR-137, miR-195, miR-449, and let-7 family members [252-254,259,264-269]. Interestingly, both miR-124 and miR-137 are silenced by hypermethylation in certain tumor types, resulting in Cdk6 activation and subsequent E2F activation [264,265,270]. Additionally, miR-372, which is frequently downregulated in cervical cancer, can target Cdk2 [271].

In addition to the direct downregulation of cyclin/Cdk complexes, the activity of these complexes can be modulated by the downregulation of associated proteins. The activity of cyclin/Cdk complexes is directly tied to the

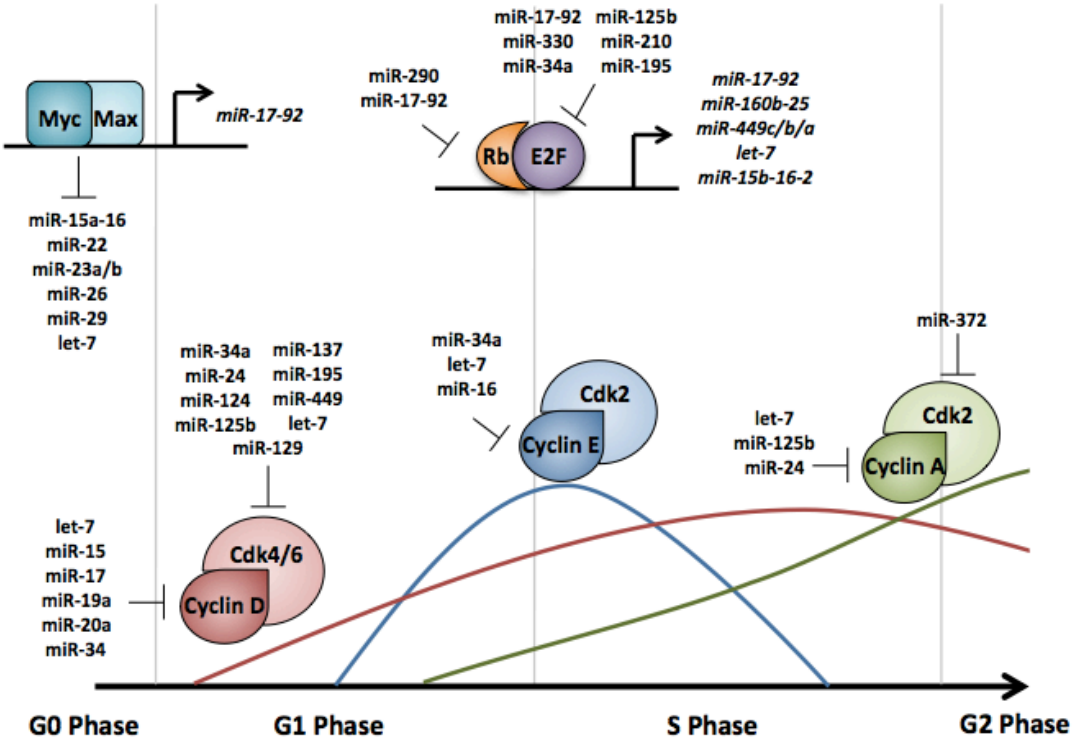

Figure 1.5. miRNAs regulate translation of proteins needed for S phase entry. Overall, the Myc/Max transcription factors repress miRNA transcription, whereas E2F transcription factors promote miRNA transcription. The cyclin/Cdk complexes are also heavily regulated by miRNAs.

amount of CKIs present. These inhibitor proteins are also highly regulated. A member of the CIP/KIP family, p21, is downregulated by the miR-17-92 miRNA cluster and by miR-106b [272,273]. Of note, miR-106b is overexpressed in many cancers [272]. Another family member, p27, is targeted by miR-221 and miR-222, leading to the activation of cyclin/Cdk complexes in a multitude of cancers [273] [274-282]. A second class of CKI, the INK4A family, can target only cyclin D containing complexes; one of the members of this family, p16, is targeted for downregulation by both miR-24 and miR-31 [266,283]. Additionally, Wee1, a negative regulator of cyclin B/Cdk1 is downregulated by several miRNAs [284,285]. Similarly, Plk1, which functions to activate cyclin B/Cdk1 through Cdc25c, is targeted by miR-100 [286].

miRNAs target the Rb/E2F pathway

Both E2F and Rb family members are targets of miRNAs. Of note, E2F1 can be targeted by several miRNAs of the miR-17-92 cluster, as well several other miRNAs, including miR-330 and miR34a [287-292]. Other members of the E2F family are also targeted. E2F3, another activator E2F, is targeted my miR-125b, miR-210, and miR-195 [253,261,293]. Additionally, the repressor E2F6 can be downregulated by miR-193a [265].

Two distinct clusters of miRNAs, miR-290 and miR-17-92, target the Rb proteins themselves, particularly pRb2/p130 and p107 [273,294-299]. Interestingly, downregulation of the miR-290 cluster results in more pRb2/p130,

which in turn represses the DNMT genes, leading to decreased methylation throughout the genome [294].

In addition to being targeted by many miRNAs, the Rb/E2F pathways can induce the transcription of some miRNA clusters [299,300]. E2F activators can bind directly to the promoter of the miR-17-92 cluster, inducing its transcription; miRNAs in this cluster are then able to downregulate E2F expression [287,300,301]. This autoregulatory loop also occurs through the induction of the miR-160b-25 cluster [299,302]. E2F1 and E2F3 can induce the transcription of the miR-449c-b-a, the let-7a-d, the let-7i, and the miR-15b-16-2 clusters as well [303,304]. The miR-449c-b-a cluster can in turn inhibit Cdk6 and Cdc25a expression, leading to a decrease in Rb phosphorylation [303].

Myc downregulates the expression of several miRNA clusters

While myc expression has been shown to induce the miR-17-92 cluster, myc has been shown to globally repress the transcription of miRNAs [262,287,304,305]. Several miRNA, including the miR-15a-16 cluster, miR-22, miR-23a/b, miR-26, miR-29, and members of the let-7 family, are direct targets of c-myc repression [305]. To repress the let-7 family, c-myc induces the RNA binding proteins Lin 28 and Lin28b, which are negative regulators of let-7 maturation [306,307]. The let-7 miRNAs, in turn, can downregulate myc expression [308]. It was observed that miRNA expression is reduced in tumor samples, and it has been suggested that the oncogenic properties of myc

overexpression is due, in part, to its repression of the let-7 miRNA family [305,309].

miRNA expression is frequently altered in cancer

In recent years, it has been found that the expression patterns of miRNAs are frequently altered in cancer [310,311]. These alterations range from deletion of specific miRNA clusters to amplification of certain miRNAs [312,313]. In particular, chronic lymphocyte leukemia (CLL) was the first cancer type to be linked with changes in miRNA expression [312]. Chromosomal aberrations lead to the deletion or downregulation of the miR-15a-16-1 cluster in ~70% of the CLL cases; recently, this cluster has also been linked to other forms of cancer, including pituitary adenomas, prostate cancer, and gastric cancer [251,312,314-316].

Amplification of the miR-17-92 cluster has been found in B-cell lymphomas, and has been implicated in the angiogenesis of some solid tumors [310,317-319]. The let-7 family is downregulated in many cancer types, including breast, colon, and lung cancers [320-323]. Levels of let-7 miRNAs have also been used as a prognostic tool for lung cancer [311,324]. Additional miRNAs have been implicated in neuroblastomas, pancreatic, colon and gastric cancers [299,310,325-329].

Post-Translational Control of Cell Cycle Proteins

While protein abundance can be controlled at both the transcriptional and the translational stage, by far the most regulation occurs post-translationally. This

can be due to post-translational modifications (PTMs) of the protein itself that affect the stability or localization of the protein, the binding of an inhibitor or enhancer protein, and most importantly in the case of cell cycle control, the timed degradation of the protein. Because nearly every protein in the cell undergoes some form of post-translational control, this section focuses on the proteins that are essential for proper origin licensing.

PTMs affect the stability and activity of pre-RC components

{Re-licensing of origins during S phase is prevented, in part, by Cdk2 in association with cyclin A [49]. Cdt1 interacts with the S phase cyclin A/Cdk2 complex, which results in Cdt1 phosphorylation at threonine 29 [42,330]. Phosphorylated Cdt1 binds to the F-box protein, Skp2, the substrate receptor for the ubiquitin ligase SCF^{Skp2}. Cdt1 is polyubiquitinated by SCF^{Skp2} and targeted for degradation by the 26S proteasome, thus reducing the pool of Cdt1 protein available to participate in origin licensing [331-333].}

{Cdc6 protein levels are very low in both quiescent cells and in early/mid-G1 phase cells due to ubiquitin-mediated proteolysis, but Cdc6 protein accumulates in late G1 just prior to a burst of MCM loading. In late G1, cyclin E/Cdk2 phosphorylates human Cdc6 on serine 54, which protects Cdc6 from the ubiquitin E3 ligase, APC/C^{Cdh1} [334].} Additionally, {Cdc6 is acetylated by Gcn5 on lysines 92, 105, and 109 which promotes cyclin A/Cdk2 phosphorylation on Cdc6 at serine 106 [334,335]. Serine 106 phosphorylation results in exclusion of Cdc6 protein from the nucleus, preventing re-replication [335-341]. The small

amount of Cdc6 that remains nuclear throughout S phase is chromatin-bound and likely participates in the ATR-dependent intra-S phase checkpoint by mechanisms that are not yet understood [342,343].}

{Additionally, the Orc1 subunit of ORC is phosphorylated by cyclin A/Cdk1 during S phase, and this phosphorylation promotes Orc1 degradation in HeLa cells [9]. The same phosphorylation on Orc1 in Chinese Hamster Ovary cells (CHO) does not affect Orc1 stability, but lowers the affinity of Orc1 for chromatin [42]. In both HeLa and CHO cells, Orc1 phosphorylation allows the export of Orc1 to the cytoplasm [344]. Over-expression of cyclin A from Kaposi's Sarcoma-associated herpes virus also facilitates re-localization of Orc1 to the cytoplasm. These results show that Orc1 is subject to phosphorylation by cyclin A/Cdk1, and this event modulates the stability and/or localization of Orc1, thereby contributing to the prevention of re-replication.

Recent evidence from *S. cerevisiae* suggests that Orc2 and Orc6 may also be targets of cyclin/Cdk inhibition. Phosphorylation of these subunits leads to a marked decrease in MCM loading [345-347]. Interaction between Orc6 and the S phase Cdk, Clb5, is needed to prevent MCM loading outside of G1 phase; this interaction occludes the Cdt1 binding site on the ORC complex [27,346,348]. In addition to steric hindrance, Clb5 phosphorylates Orc6; this modification also partially blocks the Cdt1 binding site and prevents MCM loading [27]. It remains to be determined if similar mechanisms also apply to Cdk regulation of mammalian ORC [349].}

{In addition to regulating Cdc6, Cdk complexes also facilitate the formation and loading of the MCM helicase complex. Mcm3 is phosphorylated on serine 112 by Cdk1, which triggers MCM complex assembly [44]. Cdk activity affects MCM loading in an indirect fashion as well. The activity of cyclin E/Cdk2 is required for the accumulation of Cdc7 mRNA, which in turn functions in origin firing [45,340,350-352]. In quiescent cells, phosphorylation of Mcm2 at serine 5 is necessary to promote MCM loading [45,353].}

Cdt1 is regulated by the accumulation of geminin

{As another layer of regulation, metazoan S phase cells accumulate the protein geminin, which binds to Cdt1 and blocks the Cdt1-MCM interaction [354-356]. Geminin is expressed throughout the S, G2 and M phases of the cell cycle when origin licensing is inhibited [355,357]. Geminin is targeted for degradation in G1, but begins to accumulate at the end of G1 when the ubiquitin ligase responsible for its degradation (APC/C^{Cdh1}) is inactivated [357,358]. In S phase, geminin binds to the residual Cdt1 that escaped degradation and renders it unavailable to relicense origins.

Recent biochemical evidence has suggested that geminin-Cdt1 complexes exist in several forms [359]. These forms include a licensing-inhibitory heterohexamer that consists of two Cdt1 molecules and four geminin molecules, and a licensing-permissive heterotrimer, comprised of one Cdt1 molecule and two geminin molecules [360]. Binding of geminin to Cdt1 in a heterohexamer can tether several Cdt1 molecules together, creating chromatin-bound foci that may

cooperatively inhibit licensing [361]. Depending on the amount of geminin in the cell, geminin may switch from being an inhibitor of origin licensing to a heterotrimeric activator when levels of geminin are low.}

APC/C regulates pre-RC components as well as mitosis

{The cell spends a significant amount of energy to ensure that the correct proteins are expressed at the appropriate time. Before one cell cycle phase begins, cells ensure that the previous step has been properly completed and in many cases inactivated by controlling protein activity abundance. One mechanism for enforcing the proper order of events is through regulated protein degradation. The Anaphase Promoting Complex/Cyclosome (APC/C) is uniquely tied to cell cycle progression and control of DNA replication as evidenced by the fact its regulation and activity are modulated in every phase of the cell cycle. APC/C is a RING-type E3 ubiquitin ligase originally discovered though its association with its substrates, the mitotic cyclins [362,363]. Two activator subunits, Cdc20 and Cdh1, interact dynamically with the APC/C holoenzyme to influence substrate recognition} (Figure 1.6).

APC/C^{Cdh1} targets in G1 include Skp2, a member of the SCF ubiquitin ligase complex, the licensing factor Cdc6, and the inhibitor protein geminin [40,357,364]. Degradation of Skp2 results in accumulation of the Cdk2 inhibitors p21 and p27, and delay prior to S phase entry due to low Cdk2 activity [364]. APC/C^{Cdh1} also acts to limit the amount of Cdc6 that is available in the cell.

As cells progress through S phase, Cdh1 is phosphorylated by cyclin A/Cdk2 complexes; since hyper-phosphorylated Cdh1 cannot interact with APC/C, the ubiquitin ligase complex is inactive [365]. This inactivation allows geminin to accumulate and bind any remaining Cdt1 [357]. During S phase, APC/C^{Cdh1} is also bound by its inhibitor protein, Emi1 [366]. Interestingly, Emi1 accumulation is not needed to begin S phase but is needed to signal the stop of replication and mitotic entry, even though APC/C^{Cdc20} can still ubiquitinate its targets if Emi1 is present in mitosis [367]. Emi1 remains bound until prophase, when it is phosphorylated by Plk1 [368]. While Emi1 accumulation is not needed for S phase entry, it is essential to inhibit re-replication. Depletion of Emi1 leads to re-replication in human cells, due to the untimely activation of APC/C^{Cdh1} [369,370]. This stabilization allows geminin levels to drop when Cdt1 levels are high; at the same time, increased activity of cyclin A/Cdk2 allows Cdc6 to become stabilized. With both licensing factors present, origins are licensed outside of G1 and re-replication occurs.}

$CRL4^{Cdt2}$ controls the degradation of many cell cycle proteins

{Cdk-independent mechanisms also prevent re-replication by targeting Cdt1. Non-phosphorylatable (Cdk-resistant) Cdt1 mutants are degraded during S phase despite being unable to bind to Skp2 [331,371,372]. An alternate, DNA-dependent, mechanism for Cdt1 degradation was subsequently uncovered [371-377]. Proliferating Cell Nuclear Antigen (PCNA) is a cofactor required to increase the processivity of DNA polδ during leading strand synthesis [378]. Thus,

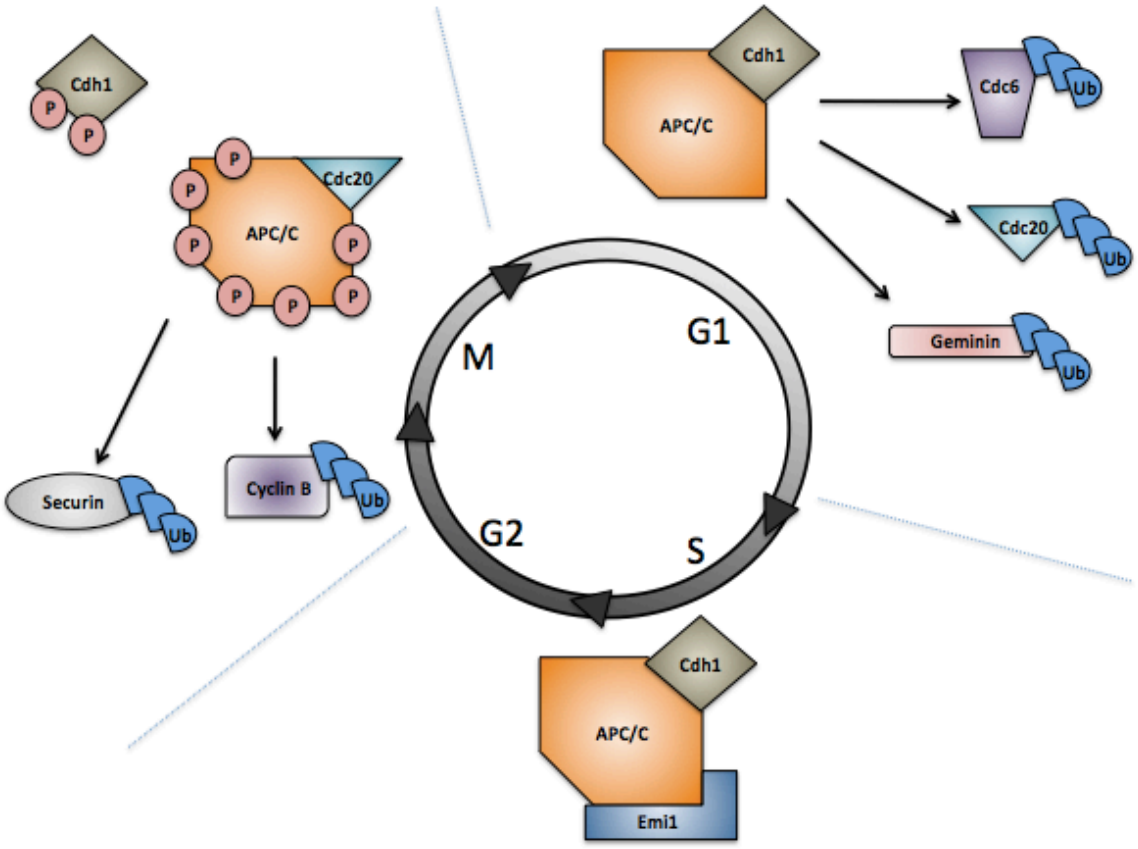

Figure 1.6. APC/C is cell cycle regulated and controls several important pre-RC proteins. In G1 phase, APC/C is bound to the adaptor protein Cdh1 and APC/C^{Cdh1} ubiquitinates the licensing factor Cdc6 and the Cdt1 inhibitor geminin. During this time, it also targets another APC/C adaptor protein, Cdc20, for degradation. As cells progress into S phase, APC/C^{Cdh1} is bound by its inhibitor protein, Emi1. At the beginning of mitosis, APC/C and Cdh1 become hyper-phosphorylated leading to dissociation of Cdh1 from APC/C. Phosphorylated APC/C can then bind the adaptor protein, Cdc20. The APC/C^{Cdc20} complex is responsible for degrading cyclin B and securin, thereby promoting sister chromatid separation and mitotic exit.

PCNA travels with active replication forks. Cdt1 interacts with PCNA through a highly conserved region called the PIP box during S phase (Figure 1.7). Cdt1 binding to DNA-loaded PCNA is essential for Cdt1 recognition by the $CRL4^{Cdt2}$ ubiquitin E3 ligase. CRL4 associates with Cdt1 via direct binding to the Cdt2 substrate adapter which links to the Cul4 scaffold. Since Cdt1 only binds PCNA on chromatin, it is only ubiquitinated by chromatin-associated $CRL4^{Cdt2}$. In this manner, Cdt1 degradation is directly coupled to DNA synthesis. A Cdt1 mutant that cannot bind either PCNA or cyclin/Cdk is stable during S phase and causes re-replication [371]. Likewise, Cdt2 depletion stabilizes Cdt1 in S phase, causing re-licensing of fired origins, and extensive re-replication [373,375,377].}

In addition to ubiquitinating Cdt1 at the onset of S phase, $CRL4^{Cdt2}$ also targets the histone methyltransferase Set8/PR-Set7 for degradation during S phase [379-382]. Set8/PR-Set7 monomethylates histone H4 on lysine 20, a chromatin mark that promotes origin licensing [383]. Stabilization of Set8/PR-Set7 during S phase results in re-replication and a lack of chromatin condensation during mitosis [379,383,384].

Pre-RC components are frequently overexpressed in cancer

{Each of the genes encoding pre-RC components is transcriptionally regulated by the Rb/E2F pathway. Given that tumor cells frequently exhibit high-level expression of E2F target genes, (*Rb* or *p16* loss, cyclin overproduction, etc.) it is not surprising that Cdt1 and Cdc6 are overproduced in many cancers [3,385-387].} {Many cancer cells have both high cyclin E/Cdk2 activity and high

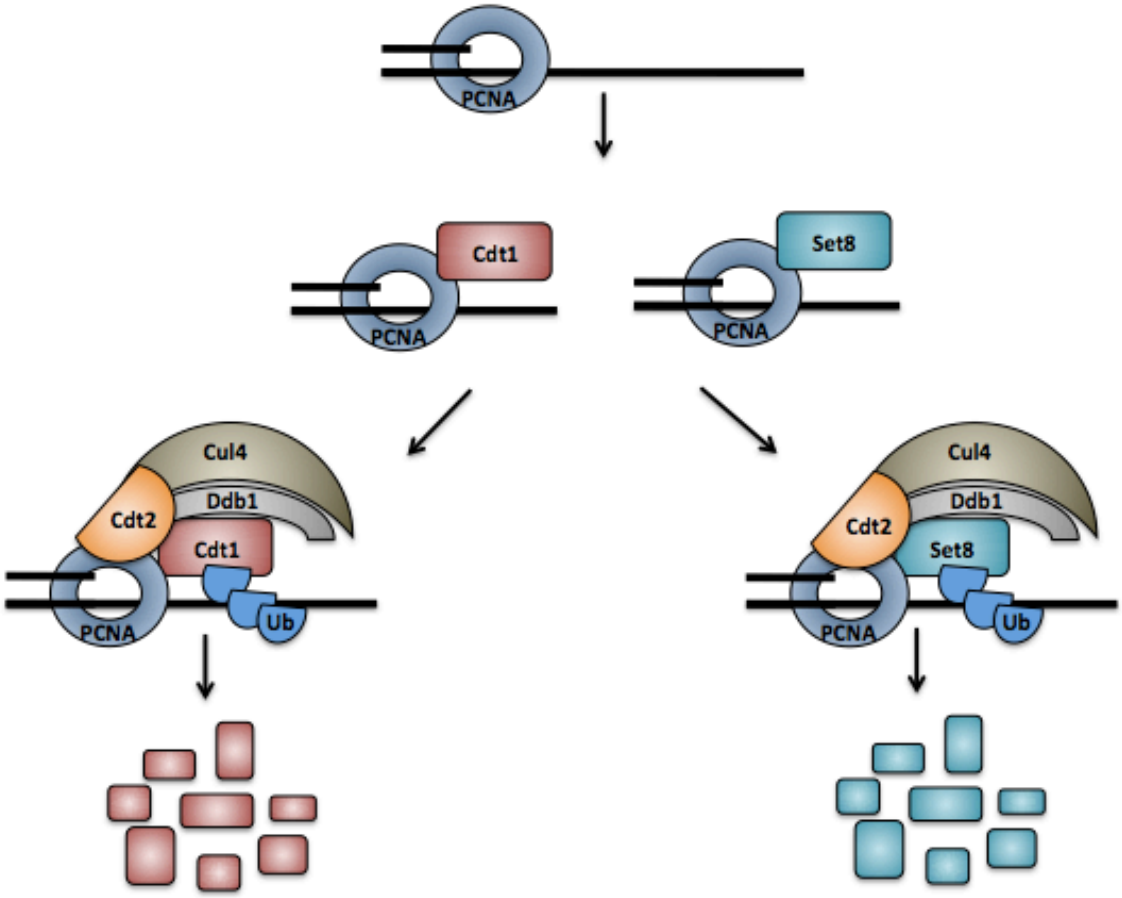

Figure 1.7. Cdt1 and Set8 are degraded during S phase. During replication, PCNA is loaded onto the chromatin. Cdt1 and Set8 are recruited to chromatin bound PCNA through their PIP motifs. Once bound, the CRL4Cdt2 ubiquitin ligase can then polyubiquitinate the substrate, leading to degradation by the 26S proteasome.

Cdc6 protein levels which may reflect not only the transcriptional up-regulation of *cyclin E* and *Cdc6* genes in tumors but also the stabilizing effect of cyclin E/Cdk2 on Cdc6 protein [388].}

{Overproduction of Cdt1 or Cdc6 in cultured human cells induces re-replication, raising the possibility that tumor cells also re-replicate *in vivo*. Recently it has been suggested that cancer cells “hyper-replicate” and that this form of replication stress is a driving force in oncogenesis. It has also been suggested that excessive pre-RC assembly may even downregulate expression of the INK4/ARF tumor suppressor locus due to interference between a nearby origin and the INK4 promoter [389]. Recently, mutations in genes for several components of the pre-RC, including *Orc1*, *Orc4*, *Orc6*, *Cdt1*, and *Cdc6* have been linked to the autosomal recessive primordial dwarfism syndrome, Meier Gorlin syndrome [1,390]. This report is the first implicating impaired licensing in a developmental disorder. Taken together there are now clear links between pre-RC formation, normal human development, and tumorigenesis.}

Conclusions

As shown above, the cell is able to transition from G1 to S phase of the cell cycle by modulating the abundance of certain key proteins. This regulation occurs transcriptionally, translationally, and most importantly, post-translationally. As shown, every stage of regulation is crucial because mutation in any number of proteins involved in this process can lead to re-replication, genome instability, or

tumorigenesis. This chapter has focused primarily on the control needed to properly regulate one step during the process of cell division, origin licensing.

Recent high-throughout analyses have shown that many proteins are in fact cell cycle regulated, either at the mRNA level or the protein level [391,392]. Many of these proteins are not involved in canonical cell cycle regulated processes, such as DNA replication and mitosis (Chapter 2) [392]. With the advent of mass spectrometry analysis, we are able to take a more global view of how proteins are regulated, and how this regulation is affected as the cell moves through the cell cycle.

Chapter 2

Global Proteomics Reveal Unexpected Cell Cycle Regulated Processes[2]

Introduction

The cell cycle is highly regulated to ensure accurate duplication and segregation of chromosomes. Perturbations in cell cycle control can result in genome instability, cell death, and oncogenesis [2-5]. Critical transition points in the cell cycle reflect "points of no return" that are difficult or impossible to reverse. For example, the G1 to S phase transition, marked by the onset of DNA replication, is an essentially irreversible step, as is mitosis. For this reason, the major cell cycle transitions into and out of S phase and mitosis are under particularly complex and robust control. The mechanisms that govern such cell cycle transitions include changes in protein abundance that are driven by combinations of regulated gene expression and protein stability control (reviewed in ref. [393]). Though decades of genetic and biochemical studies have given great insight into such mechanisms, much remains to be learned about the overall impact of cell cycle transitions on intracellular physiology.

To date, cell cycle studies have focused primarily on the regulation of DNA

[2] **Modified from: Cell cycle-regulated protein abundance changes in synchronously proliferating HeLa cells include regulation of pre-mRNA splicing proteins**
Lane, K.R., Yu, Y., Lackey, P.E., Chen, X., Marzluff, W.M., and Cook, J.G. *Submitted to* PLoS One. Accepted February 4, 2013.

replication (S phase), chromosome segregation (M phase), and cytokinesis. A few recent unbiased analyses of cell cycle-associated changes in human mRNA abundance suggest that other biological processes are also cell cycle-regulated [391,394]. Nevertheless, the full spectrum of cellular changes at the major cell cycle transitions is still unknown. In particular, the mRNA changes during the cell cycle in continuously growing cells are unlikely to reflect the rapid changes in concentrations of critical proteins. A 2010 study by Olsen *et al.* analyzed both changes in protein abundance and phosphorylation events in the human cell cycle, focusing primarily on changes in mitosis [392]. In this current study, we investigated protein abundance changes associated with S phase relative to both G1 and G2 in highly synchronous HeLa cells (human cervical epithelial carcinoma). In parallel, we have catalogued changes in the proteome in response to inhibition of ubiquitin-mediated degradation in synchronous cells. In addition to finding some of the previously-described changes related to DNA metabolism and mitosis, we also uncovered changes in many proteins involved in alternative pre-mRNA splicing.

Materials and Methods

Cell Culture and Synchronization

HeLa cells were originally obtained from ATCC and were cultured in three different media. “Light” cells were grown in depleted Dulbecco’s Modified Eagle Medium (DMEM; UCSF Cell Culture Facility) reconstituted with 145 mg/L L-lysine (UCSF Cell Culture Facility) and 84 mg/L L-arginine (UCSF Cell Culture Facility).

"Medium" cells were grown in depleted DMEM reconstituted with 798 mM L-lysine ($^{4,4,5,5}D_4$) and 398 mM L-arginine ($^{13}C_6$). "Heavy" cells were grown in depleted DMEM reconstituted with 798 mM L-lysine ($^{13}C_6$; $^{15}N_2$) and 398 mM L-arginine ($^{13}C_6$; $^{15}N_4$). All three media were supplemented to 10% dialyzed fetal bovine serum (dFBS; Gibco) and 2 mM L-glutamine (UCSF Cell Culture Facility). All modified isotopes were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All HeLa cell cultures were grown in the SILAC media for a minimum of 5 passages to ensure that the amino acids had been fully incorporated. Labeling efficiency was checked by examination of the tubulin and actin proteins using LC-MS/MS (details of sample preparation and analysis follow). T98G cells were originally obtained from ATCC and were cultured in DMEM (Sigma Aldrich) supplemented with 10% FBS (Sigma Aldrich) and 2mM L-glutamine (Gibco). Cells were synchronized by serum starvation for 72 hr and stimulated with a final concentration of 10% FBS [77].

To determine the protein changes between G1 and S phase, simultaneously cultured biological replicates of HeLa cells were subjected to double-thymidine synchronization as previously described in ref. [391] with minor modifications. Ten hours after release from the second thymidine block, the medium was removed, and a mitotic shake-off was performed. Mitotic cells were replated and collected at 3 hr (G1 sample) and 10 hr (S sample). To capture proteins degraded after S phase onset, one separately-labeled culture was treated with 20 μM MG132 (Sigma Aldrich) for 2 hr prior to harvest (8 hrs after shakeoff). To determine the protein changes between S and G2 phase,

simultaneously cultured biological replicates were harvested 3 hr following release from the second thymidine treatment (S sample) and 8 hr after release (G2 sample); one separately-labeled culture received 20 μM MG132 2 hr prior to harvesting in G2. Cells were harvested by trypsinization, collected by centrifugation, and cell pellets were stored at -80°C prior to the preparation of cell lysates. A small fraction of cells was fixed with ethanol, stained with propidium iodide, and analyzed by flow cytometry to confirm cell cycle phase.

Cell Lysis and Sample Processing

Frozen cell pellets were lysed in 50 μL high salt lysis buffer (10 mM HEPES-KOH, pH 7.5, 350 mM KCl, 3 mM $MgCl_2$, 1% Triton-X100, 1 mM EDTA (Fisher Scientific), pH 8.0) and incubated on ice for 10 min. Lysis buffers were supplemented with 1 mM DTT, 0.1 mM AEBSF (Roche), 0.5 mM $NaOV_4$, 2 mM β-glycerolphosphate, 2 mM NaF, 200 nM trichostatin A, 2.5 mM sodium butyrate, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin A. Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich. Lysates were cleared by centrifugation for 2 min at 4°C; the supernatant was transferred to a new tube and cleared by centrifugation at full speed for 15 min at 4°C. Protein concentrations were determined according to Bradford assay instructions (Biorad). Samples were mixed 1:1:1 (70 μg each) and subjected to SDS-PAGE on a 15% polyacrylamide gel. The gel was stained with Coomassie blue (Amresco), and sample lanes were continuously excised into 25 slices.

The following steps, including destaining, dehydration, reduction and

alkylation, and overnight in-gel trypsin digestion, were performed following a standard protocol [395]. Briefly, gel slices were dissected into ~1 mM cubes and transferred to Axygen tubes (Axygen). HPLC-grade water [206] was added to each slice and shaken at room temperature for 5 min. The water was removed, and the slices incubated overnight at 4°C with Destain Solution (1:1 acetonitrile/ 100 mM ammonium bicarbonate (ABC)) (Sigma Aldrich). Once the gel slices were completely destained, the pieces were covered with acetonitrile and shaken at room temperature until they were white and hard; the pieces were then incubated at 50°C until all the acetonitrile had evaporated. The slices were rehydrated in 10 mM DTT and incubated at 37°C for 30 min in an Eppendorf Thermomixer R. The DTT was removed and replaced with 55 mM iodoacetamide (Sigma Aldrich) and shaken at room temperature for 45 min. Following this incubation, the gel slices were washed with 100 mM ABC for 10 min. After washing, the slices were again dehydrated with acetonitrile and chilled on ice for 10 min. Porcine trypsin (Promega) was diluted to 10 ng/μL in 50 mM ABC and added to the dehydrated gel slices; the slices were incubated at 37°C until the slices were completely rehydrated. Any excess trypsin solution was removed, and the slices were covered with Peptide Extraction Buffer (50% acetonitrile, 50% HPLC-grade water, 0.1% formic acid) [206] and incubated overnight in the thermomixer at 37°C. Acetonitrile was added and incubated at 37°C for 10 min to inactivate the enzyme. Peptides were extracted with 4 treatments of Peptide Extraction buffer at 37°C for 10 min. and lyophilized in a Savant Speed-Vac Plus at medium heat.

Desalting and LC-MS/MS

After digestion, the peptides were extracted using C18 ziptips (Millipore), lyophilized, and resuspended in buffer A (0.1% formic acid in H_2O) prior to LC separation. MS analyses were performed on an LTQ Orbitrap Velos (Thermo Scientific, Bremen, Germany) coupled with a nanoLC-Ultra system (Eksigent, Dublin, CA). Samples (5 mL) were loaded onto an IntegraFrit column (C18, 75 mm × 15 cm, 300Å, 5 μm, New Objective, MA). The peptides were eluted at a flow rate of 200 nl/min with a linear gradient from 2% to 40% buffer B (0.1% formic acid in acetonitrile) over the course of 110 min, followed by 80% buffer B for another 10 min. At the end of the gradient, the column was equilibrated for 10 min with 2% buffer B before starting another LC/MS run. The mass spectrometer was programmed to acquire spectra in a data-dependent and positive ion mode at a spray voltage of 2.1 kV using the XCalibur software (version 2.1, Thermo Scientific). Survey scans were performed in the Orbitrap analyzer at a resolution of 15,000 over a mass range between m/z 300-2,000. For each cycle, the top five most intense ions were subjected to CID fragmentation in the LTQ with normalized collision energy at 35% and activation Q 0.25; dynamic exclusion was enabled. Selected ions were repeated once and then excluded from further analysis for 45 sec. Unassigned ions or those with a charge of 1+ were rejected. Maximum ion accumulation times were 200 ms for each full MS scan and 100 ms for MS/MS scans. One microscan was acquired for each MS and MS/MS scan. The mass spectrometry data from this publication have been submitted to the Proteome Commons Tranche (www.proteomecommons.org). The data from the

G1 to S dataset can be found using the following hash code: ytUg3dJ7npt665b/ZRSADaIKbwhAbVLfVjOiV1qw0zUjr1f7rr+cJk6txiV+2CDE3cQEnKErNJ/mV6edECVH1yf4r70AAAAAAAAM5Q==. The data from the S to G2 dataset can be found using the following hash code: Pfr5X84wSDM2MuckUXaXkFAqfoq2r94aKYgVm7NCTmz4L/pd5OpHEfoz3CxrMJfnZe86hl8j2lJMDVZjSUkc1Du8hcQAAAAAAAAOuQ==.

Database Search

The raw files were processed using the MaxQuant software suite (version 1.2.0.34) [396]. The MS/MS spectra were used to interrogate the UniProt human database (release date of November 30, 2010. 20248 entries) using the Andromeda search engine [397] with the precursor and fragment mass tolerances set to 6 ppm and 0.5 Da, respectively. Up to two missed cleavage sites were allowed per peptide. Methionine oxidation and protein N-terminal acetylation were chosen as variable modifications, and cysteine carabamidomethlyation was set as a fixed modification for database searching. Only peptides with a minimum length of 6 amino acids were considered for identification. Both peptide and protein identifications were filtered to a maximum 1% false discovery rate. Proteins identified from only a single peptide were manually checked by direct visualization of the spectra and quantified using the XCalibur software. Finally, the lists of identified proteins were filtered to eliminate reverse hits and known contaminants.

As a complement to MaxQuant the Proteome Discoverer software (version

1.3, Thermo Scientific), configured with an in-house Mascot server (v2.3, Matrix Science), was also used to search the same set of MS/MS data. A built-in workflow and a "Quantification" module were used for protein identification and quantitation. All the search parameters were the same as the MaxQuant search, but were filtered at a false discovery rate of 5% to quantify a similar number of proteins as had been identified with MaxQuant. Both search strategies generated overlapping protein lists (77%). Once results were gathered from both programs, the results were combined. When proteins were identified by both programs, the quantification calculated by the MaxQuant software was reported. If the ratios were such that one program defined a protein as changed whereas the second program did not, the ratios were manually calculated through integration of the peak areas using the XCalibur software.

Proteins were divided into subsets based on their SILAC ratios using a 1.5-fold change as the cutoff threshold. That is, a ratio of 1.5 or higher was scored as an increase whereas a ratio of 0.666 or less was scored as a decrease; ratios that fell between these values were reported as no change. These ratios, as well as the $\log_2$ transformations, are reported in Supplementary Tables 1 and 2.

Dataset Comparison and GO Term Analysis

The $\log_2$ transformed data from Whitfield et al. (2002) was downloaded from www.cyclebase.org. Based on the calculated p-value of periodicity, mRNA data were separated according to mRNA peak time [398,399]. These lists were compared to our lists of increased and decreased proteins, and p-values were

calculated using Fisher's exact test; a p-value less than 0.01 was considered significant. The same strategy was applied to comparisons to the ubiquitome [400], a published ATM/ATR substrate list [401], a published phosphoproteome [392], a Cyclin A/Cdk2 substrate list [402], and a dataset that determined the subcellular localization of proteins [403]. GO term analysis was performed using the DAVID search engine [404,405]. Analysis was performed on the individual lists, and the reported p-value was calculated using a modified Fisher's exact test. When GO terms overlapped, terms were collapsed to the highest level (i.e., RNA splicing was collapsed into RNA processing).

Immunoblot Validation

Samples were subjected to SDS-PAGE on a 12% polyacrylamide gel and transferred to PVDF (Thermo Scientific). Blots were probed with the following antibodies: anti-Cyclin B1 (V152, Thermo Scientific), anti-Cyclin A (C-19, Santa Cruz Biotechnology), anti-Cdc6 (D-1, Santa Cruz Biotechnology), anti-Cdt1 [25], anti-Geminin (FL-209, Santa Cruz Biotechnology), anti-SLBP [406], anti-α-tubulin (DM1A, Sigma Aldrich), anti-RRM2 (Aviva Systems Biology), anti-MARCKSL1 (Aviva Systems Biology), anti-Palmdephin (Aviva Systems Biology), anti-Prelamin A/C (N-18, Santa Cruz Biotechnology), anti-Tropomodulin-3 [407], anti-MCM2 (46/BM28, BD Pharmingen), anti-Rbmx/hnRNPG (Aviva Systems Biology), anti-hnRNPA1 (K350, Cell Signaling), anti-hnRNPA3 (Y25, Santa Cruz Biotechnology), anti-hnRNPD0 (T10, Santa Cruz Biotechnology), anti-hnRNPL (Sigma Aldrich), and anti-β-actin (N-21, Santa Cruz Biotechnology). All HRP-

conjugated secondary antibodies were purchased from Jackson Immunoresearch. Proteins were visualized following incubation with ECL prime reagent (Amersham).

Results

Synchronous HeLa cells progress through the G1/S and S/G2 transitions.

We sought to investigate the proteome changes between G1 and S phase and between S and G2 phase. Our goal was to achieve very tight cell cycle synchrony while simultaneously avoiding strong checkpoint effects that could be induced in chemically-arrested cells. To facilitate accurate quantification of peptides by mass spectrometry, we labeled cultures for more than 5 cell divisions with three different stable isotope mixtures of lysine and arginine (i.e. amino acid-coded mass tagging/AACT or stable isotope labeling with amino acids in culture/SILAC) prior to synchronization [408-410].

To obtain populations of isotope-labeled tightly-synchronous cells progressing from G1 to S phase, we modified the Whitfield *et al.* (2002) double-thymidine block and release protocol (Materials and Methods) [391]. We released HeLa cells from the second thymidine block ("DT Block" = early S phase) to allow checkpoint recovery and normal passage through the subsequent transitions and allowed them to progress into mitosis without further chemical perturbation. We collected mitotic cells using a "shake-off" method, a procedure that takes advantage of the tenuous attachment of HeLa cells as they round up during mitosis. We replated mitotic cells in fresh dishes, and 3 hrs after mitosis, the

cells were a relatively pure population of G1 cells; by 10 hrs after mitosis they were in early-S phase (Figure 2.1A and 2.1B show a full time course from cells grown in normal isotope medium). Note that these cell cycle times reflect a moderate delay compared to cells grown under standard conditions due to the requirement for dialyzed fetal bovine serum for efficient metabolic labeling.

To facilitate the detection of proteins that may be rapidly degraded in S phase we treated another culture of cells with the proteasome inhibitor MG132 8 hrs after the mitotic shake-off (just prior to the G1/S transition) and harvested the cells 2 hrs later in early S phase. To quantify proteins that change between S phase and G2 phase, we released cells into S phase from the double-thymidine block rather than from a mitotic shake-off. These cells progressed through S phase and entered G2 phase synchronously; we harvested 3 hrs (S phase) and 8 hrs (G2 phase) after release from the second thymidine block (Figure 2.1D and 2.1E show a full time course from cells grown in normal isotope medium). We also treated cells with MG132 6 hrs after release (just prior to the S/G2 transition) and harvested them 2 hrs later (G2 phase).

For the G1/S comparison, the G1 culture contained normal isotopes (light), the early-S phase culture was metabolically labeled with intermediate isotopes (medium), and the early-S phase culture treated with MG132 at the G1/S transition had been cultured in the heaviest isotopes (heavy). For the S/G2 comparison, mid-S phase cells were cultured in the normal isotope medium (light), the G2 cells were cultured in the intermediate isotope medium, and the G2 cells that had been treated with MG132 at the S/G2 transition were labeled in

heavy isotope medium. In this manner, we generated synchronous metabolically-labeled cell populations naturally passing from one phase to the next without the potentially confounding issue of harvesting cells from a strong checkpoint arrest.

We confirmed cell cycle position by immunoblotting whole cell lysates for established cell cycle-regulated proteins. For example, we confirmed that both the Cdc6 and geminin proteins, two targets of the Anaphase Promoting Complex/ Cyclosome (APC/C) E3 ubiquitin ligase which is active from anaphase through late G1, were substantially more abundant in the S phase lysates than in the G1 lysates (Figure 2.1C, compare lanes 2 and 3 to lane 1) [40,357,362,363,371]. In contrast to Cdc6 and geminin, the Cdt1 protein is targeted for degradation at the onset of S phase by the $CRL4^{Cdt2}$ E3 ubiquitin ligase [373,374]. As expected, we detected very little Cdt1 in the early-S phase cells compared to the G1 cells (Figure 2.1C, compare lanes 1 and 2), but Cdt1 protein levels were high in the S phase cells treated with MG132 (Figure 2.1C, compare lanes 2 and 3). Moreover, we observed higher levels of Cdt1 in the G2 samples compared to the mid-S phase samples as expected because $CRL4^{Cdt2}$ can only target Cdt1 during active DNA replication (Figure 2.1F, compare lanes 1 and 2) [372,374,376].

Previously, we identified two proteins (SLBP and E2F1) that are degraded at the end of S phase as a result of Cyclin A/Cdk1 activation. Their degradation is blocked by MG132 treatment [411-413]. We detected not only the down-regulation of SLBP in G2 phase but also its stabilization in cells treated with MG132 (Figure 2.1F). Finally we confirmed that MG132 did not prevent S phase entry or exit as determined by flow cytometry and immunoblot analysis of marker

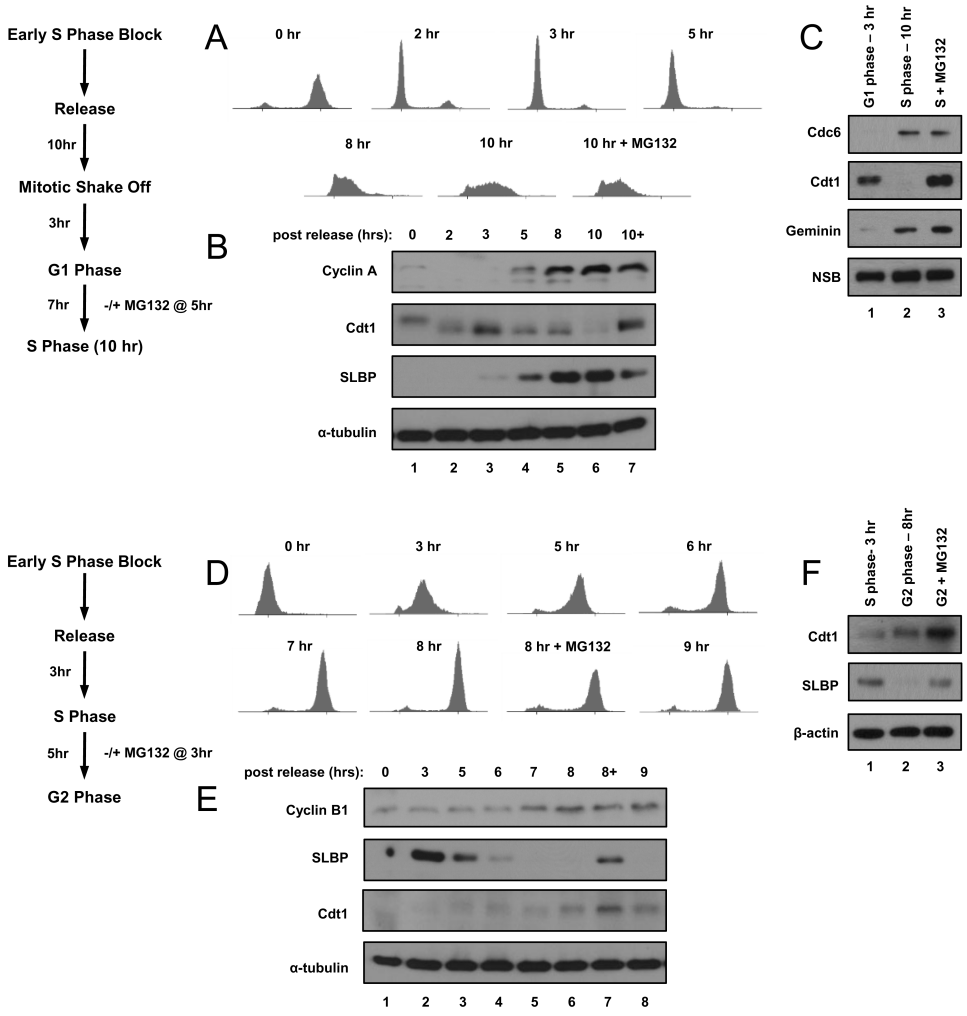

Figure 2.1. HeLa cell synchronization. A) Cells were synchronized by a modified double-thymidine block then released by re-plating and harvested at the indicated time points. Synchrony was determined by flow cytometric analysis of DNA content. B) Immunoblot analysis of endogenous Cyclin A, Cdt1, SLBP, and tubulin proteins in whole cell lysates from portions of the same cells used in A. C) Cells were metabolically labeled with stable isotopes and then synchronized as in A and B. Immunoblot analysis of endogenous Cdc6, Cdt1, and geminin in whole cell lysates used for subsequent mass spectrometric tests. A non-specific band (NSB) serves as a loading control. D) Cells were synchronized by double-thymidine block, released into S phase, and harvested at the indicated timepoints. Synchrony was determined by flow cytometric analysis of DNA content. E) Immunoblot analysis of endogenous Cyclin B, SLBP and Cdt1 in whole cell lysates from portions of the same cells used in D. F) Cells were metabolically labeled and as in D and E. Immunoblot analysis of endogenous Cdt1 and SLBP in whole cell lysates used for subsequent mass spectrometric analysis; β-actin serves as a loading control.

proteins Figures 2.1A and 2.1D). We conclude therefore that these protocols generated synchronous populations that display the expected differences in protein abundance of known cell-cycle regulated proteins at the G1/S and S/G2 transitions.

Protein abundance changes at the G1/S and S/G2 transitions.

Using these validated samples from synchronous cells, we prepared whole cell lysates, combined the three lysates representing the G1/S comparison and the three lysates representing the S/G2 comparison, and subjected them to SDS-PAGE. We divided the gel into slices from which we generated tryptic peptides for liquid chromatography separation and tandem mass spectrometry (LC-MS/MS), as described in Materials and Methods. Using both MaxQuant and Proteome Discoverer software, we analyzed peptide spectra from a total of 50 gel slices. We identified 28,684 unique peptides corresponding to 2,842 unique proteins (allowable false discovery rate of 5%). Spectra were of sufficient quality to accurately quantify 2,410 of these proteins. A recent very comprehensive analysis of the HeLa proteome detected a total 10,237 proteins from lysates of asynchronous cells indicating that our analysis covers approximately 28% of the currently detectable HeLa proteome [414]. Note that quantitation requires detection of at least two isotopically labeled forms of the peptide, so any protein that was clearly detectable in only one of the three cultures was excluded from our analysis. Our dataset is also approximately 43% as extensive as another recent proteome analysis of HeLa cells that focused on changes during mitosis

[392]. Interestingly, we detected 324 proteins not found in either previous report; these could reflect proteins that are only abundant enough for detection at specific cell cycle stages or could reflect random sampling differences among the three studies (Figure 2.2A). Therefore, our proteome analysis of the G1/S and S/G2 transitions complements and extends other investigations of the HeLa cell proteome.

To focus specifically on proteins that change in abundance from G1 to S phase, we compared the 1,611 quantifiable proteins (of 1,843 identified) from cells harvested in G1 to those from the subsequent early-S phase time point. We chose a 1.5-fold change in protein abundance as the threshold to score a protein as increased or decreased; these changes were calculated using the mean of all peptides from the same protein. Between these two cell cycle phases, two-thirds (67.3%) of the proteins neither increased nor decreased in abundance, whereas 32.7% either accumulated or decreased between G1 and S phase (Figure 2.2B and C). We quantified 1,640 proteins from the S/G2 comparison (of 1,913 identified). In contrast to the G1/S comparison, a higher proportion (84.7%) of these proteins did not change by more than 1.5-fold from S to G2 phase. Of the total quantifiable proteins, 15.3% either increased or decreased in their abundance (Figure 2.2B and 2.2D). These protein lists are provided in Supplementary Tables 1 and 2, and the individual peptide lists are provided in Supplementary Table 6 (found online at http://www.plosone.org).

The pharmacological inhibitor MG132 blocks the activity of the 26S proteasome, leading to the accumulation of proteins targeted for

polyubiquitination [415,416]. Since many cell cycle transitions are driven by ubiquitin-mediated protein degradation, we reasoned that we could identify some of these proteins based on altered abundance in the presence of MG132. It is important to note that MG132 was added close to the cell cycle transition under investigation. Overall, ~1% of S phase proteins and 8% of G2 proteins were induced by MG132 treatment for 2 hrs compared to untreated early-S phase and G2 cells, respectively (Figure 2.2B, 2.2E and 2.2F, and Supplementary Tables 3.1 and 4.1). We also detected proteins that were induced by treatment with MG132 that had not shown changes between cell cycle phases. These proteins could have short half-lives and be subject to continuous ubiquitin-mediated degradation at many or all cell cycle phases. Interestingly, more proteins were down-regulated after MG132 treatment than were induced - 13% of S phase and 10% of G2 proteins (Figure 2.2B, and Supplementary Tables 3.2 and 4.2). A similar phenomenon has been reported previously; one study reported that 15% of proteins were down-regulated at least 2-fold after treating asynchronous cells with MG132 for 4 hrs [417]. The complete list of protein changes in response to MG132 treatment for both datasets is provided as Supplementary Tables 3 and 4.

Some of the protein changes observed from one cell cycle phase to the next, such as cyclin B induction in G2, are well known. All the known cell cycle-regulated proteins that we detected changed as expected, although several relatively low abundance proteins were not detected. For example, the average abundance of peptides derived from ribonucleoside-diphosphate reductase subunit M2 (RRM2) increased 4.8-fold in S phase. This protein is regulated both

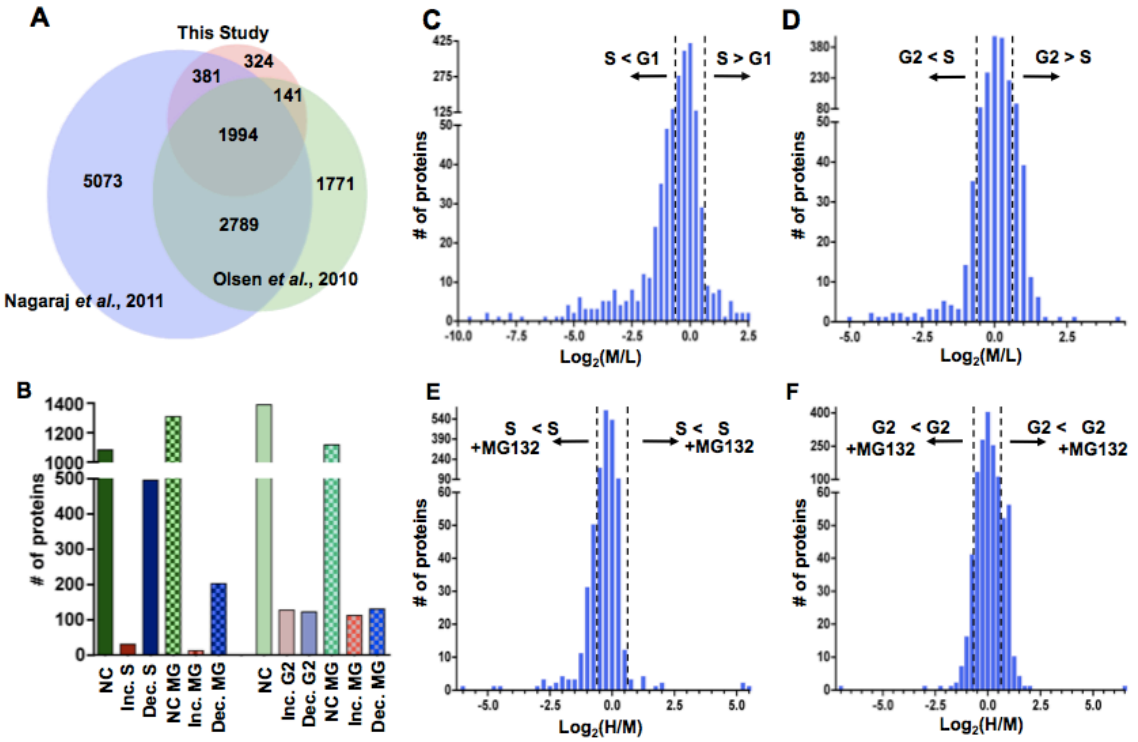

Figure 2.2. Cell cycle-regulated proteins from G1 to S and S to G2 detected by mass spectrometry. A) Comparison of the total number of proteins detected in this study (2,842 proteins) to two other studies of the HeLa cell proteome: Nagaraj et al., 2011 (10,237 proteins) [414] and Olsen et al., 2010 (6,695 proteins) [392]. B) Quantified proteins from this study were divided into lists based on their fold and direction of change; the total protein count for each list is plotted. "NC" denotes proteins that did not change. "NC MG," "Inc MG," and "Dec MG" denote proteins that either did not change, increased, or decreased in response to MG132 treatment, respectively. C) All quantifiable proteins in the G1 to S dataset plotted by their $\log_2$ transformed isotope ratios (medium S phase/light G1 phase). Dotted lines denote the 1.5-fold change threshold. D) All quantifiable proteins identified in the S to G2 dataset plotted by their $\log_2$ transformed isotope ratios (medium G2 phase/light S phase); dotted lines denote the 1.5-fold change threshold. E) Proteins identified in early-S phase cells compared to early-S phase cells treated with MG132 plotted by their $\log_2$ transformed isotope ratios (heavy S phase plus MG132/medium S phase minus MG132). Dotted lines denote the 1.5-fold change threshold. F) Proteins identified in G2 phase cells compared to G2 phase cells treated with MG132 plotted by their $\log_2$ transformed isotope ratios (heavy G2 plus MG132/medium G2 phase minus MG132). Dotted lines denote the 1.5-fold change threshold.

at the transcriptional level, as a target of E2F4 repression, and at the protein level, as a target of the APC/C ubiquitin ligase [120,418,419].

Our data also predicted changes in protein abundance that have not been previously identified. We selected several of these proteins for immunoblot validation on the original lysates of synchronized HeLa cells. Most of the proteins (17 out of 28) we selected for this validation showed changes in abundance that were consistent with the mass spectrometry quantification. For example, MARCKS-related protein (MARCKSL1) and palmdelphin (Palmd) increased in S phase compared to G1 phase by 2.9-fold and 2.0-fold, respectively, and we observed increases in band intensities for these proteins by immunoblotting (Figure 2.3A, compare lanes 1 and 2). Furthermore, mass spectrometry indicated that prelamin A/C protein levels decreased 4.7-fold in S phase compared to G1, and immunoblot analysis supported this finding (Figure 2.3A). As an example of a protein that does not change between G1 and S phase, we found that tropomodulin-3 (Tmod3) protein levels did not change significantly, in agreement with the mass spectrometry analysis. The total number of proteins that changed (increased or decreased) between S and G2 was smaller than the number of proteins that changed between G1 and S phase. We selected several proteins for validation by immunoblot analysis as above. For example, the average peptide abundance derived from prelamin A/C and cyclin B1 increased in G2 phase compared to mid-S phase by 1.7-fold and 2.1-fold, respectively; we observed changes in band intensities consistent with these mass spectrometry results (Figure 2.3B, compare lanes 1 and 2).

Frequent discordance of mRNA and protein abundance.

Changes in protein abundance can often be explained by corresponding fluctuations in mRNA abundance. A landmark study by Whitfield et al. (2002) catalogued changes in mRNA expression through multiple synchronous cell cycles in HeLa cells [391]. The primary data from this extensive analysis is readily available for interrogation (cyclebase.org), and we sought to determine the relationship between mRNA expression in the Whitfield study with the protein changes we detected in this study. We divided the mRNA data into groups based on peak cell cycle phase of abundance [398,399]. We then determined which of the proteins that changed from one cell cycle phase to the next in our study were also the products mRNAs whose abundance changed in the same way. Somewhat surprisingly, there was no significant overlap between the mRNAs that peak in S phase and the detected proteins that increased in S phase; likewise, proteins that decreased in S phase were unlikely to be the products of mRNAs that decreased in S phase (Figure 2.4A, first two bars). This poor correlation also existed when we compared proteins that increased in S phase to mRNAs that peaked in G1. As pointed out by Whitfield et al., there were fewer changes in mRNA levels between G1 and S phase than there were between S and M phase; only 19.5% of transcripts peak in S phase whereas 45% peak in G2/M [391].

In contrast, proteins that increased in G2 were somewhat more likely to be the products of mRNAs that also increased in G2 (Figure 2.4A, third bar). For example, the prelamin A/C mRNA peaks in G2/M, and the protein also modestly

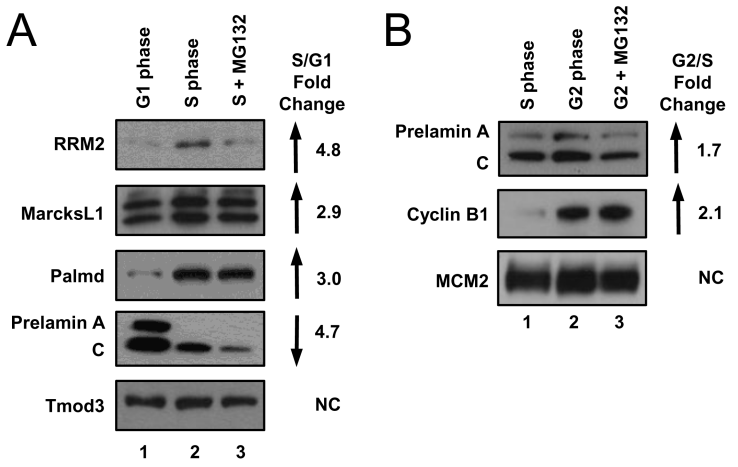

Figure 3. Validation of selected cell cycle-regulated protein predicted by mass spectrometry. The same cell lysates analyzed by mass spectrometry were subjected to immunoblot analysis for the indicated endogenous proteins in the A) G1 to S lysates or B) S to G2 lysates. Reported fold change ratios from mass spectrometry are listed to the right.

increased in our G2 samples compared to S phase (Figure 2.3B, compare lanes 1 and 2). In contrast, proteins that decreased in G2 were not well-predicted by mRNAs that also decreased in G2 (Figure 2.4A, fourth bar). Furthermore, when we compared the proteins that did *not* change in either of our datasets to the mRNAs that are constitutively expressed throughout the cell cycle, more than 60% of the genes/proteins were in agreement (Figure 2.4B, first two bars). When some of the proteins whose abundance did not change by mass spectrometry are the products of mRNAs that do change; these proteins may be long-lived and thus not fully reflective of corresponding mRNA changes.

Since mRNA abundance could not fully account for the protein changes we observed, we considered the possibility that the changes in protein abundance were correlated with ubiquitination and thus, regulated protein degradation. We compared our lists of proteins that change from G1 to S or from S to G2 to a recently-published list of ubiquitinated proteins identified in asynchronously growing HCT116 (human colon carcinoma) cells [400]. Strikingly, a high proportion of the proteins that either increased (56.7%) or decreased (62.6%) between G1 and S also appeared in the list of 4,462 ubiquitinated proteins (Figure 2.5A, first two bars). Moreover, proteins whose abundance was affected by MG132 treatment in S phase (either increased or decreased) were also highly represented in the reported list of total ubiquitinated proteins. In contrast, proteins that changed from S to G2 were not as enriched in the “ubiquitome,” regardless of MG132 treatment with the exception of proteins that increased from S phase to G2 (Figure 2.5A). Both nuclear and cytoplasmic proteins were

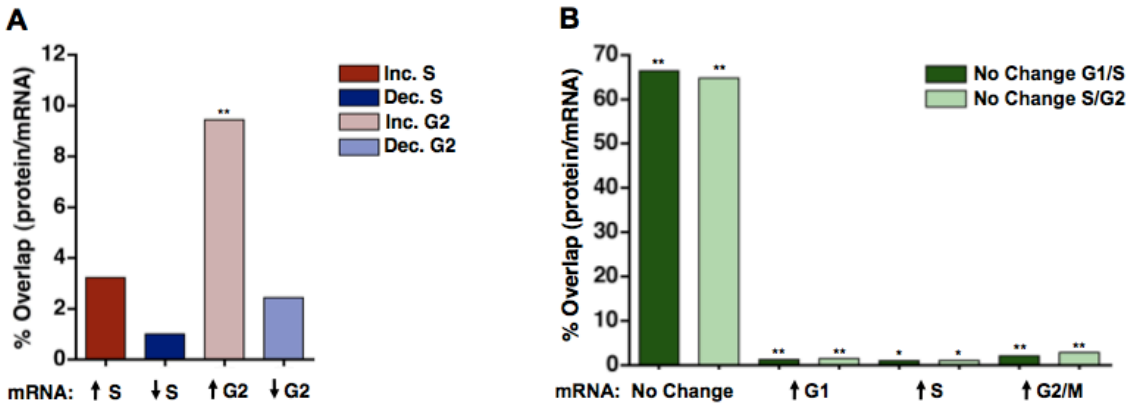

Figure 2.4. Discordance between mRNA and protein abundance. A) Individual lists of proteins that changed by at least 1.5-fold were compared to the mRNA data for those same proteins in synchronized HeLa cells from Whitfield et al. 2002 [391]. The percentage of proteins whose corresponding mRNA also changed is graphed for both S phase and G2 phase. ** p <0.001. B) Proteins that did not change in either the G1 to S or the S to G2 dataset were compared to mRNAs that were ubiquitously expressed or peaked at the indicated cell cycle phases [391]. * p<0.01; ** p <0.001.

present in all of our datasets, and we detected no differences in nuclear-cytoplasmic localization among proteins that changed from one cell cycle phase to the next (Figure 2.5B and 2.5C).

A strikingly large proportion of proteins whose abundance changed from G1 to S or from S to G2 have been detected as phosphoproteins, consistent with the notion that many protein abundance changes are controlled by phosphorylation (Figure 2.6A). This enrichment was true both for proteins that changed from G1 to S and for those that changed from S to G2.

Since the cyclin-dependent kinases (Cdks) govern many cell cycle transitions, we compared our sets of regulated proteins with a list of candidate Cdk substrates [402]. Many proteins that increased (6 of 31) or decreased (28 of 496) in S phase appear on this list of Cdk substrates (Figure 2.6B, first two bars). Moreover, a statistically significant number of proteins that increased in G2 phase are also putative Cdk substrates (Figure 2.6B, fifth bar). A significant number of proteins that changed with MG132 treatment at the S/G2 transition are also putative Cdk substrates (Figure 2.6B, last two bars). In contrast, proteins that changed in response to MG132 treatment at the G1/S transition were not enriched for putative Cdk substrates (Figure 2.6B, third and fourth bars).

Like Cdks, the ATR kinase is active during S phase [420]. ATR activity is also stimulated by DNA damage, and this property was used to identify candidate ATR substrates. Putative ATR kinase substrate lists were developed by Stokes et al. (2007) from phosphopeptides detected following UV irradiation, an activator of ATR [401]. A subset of our regulated proteins also appeared in these lists of

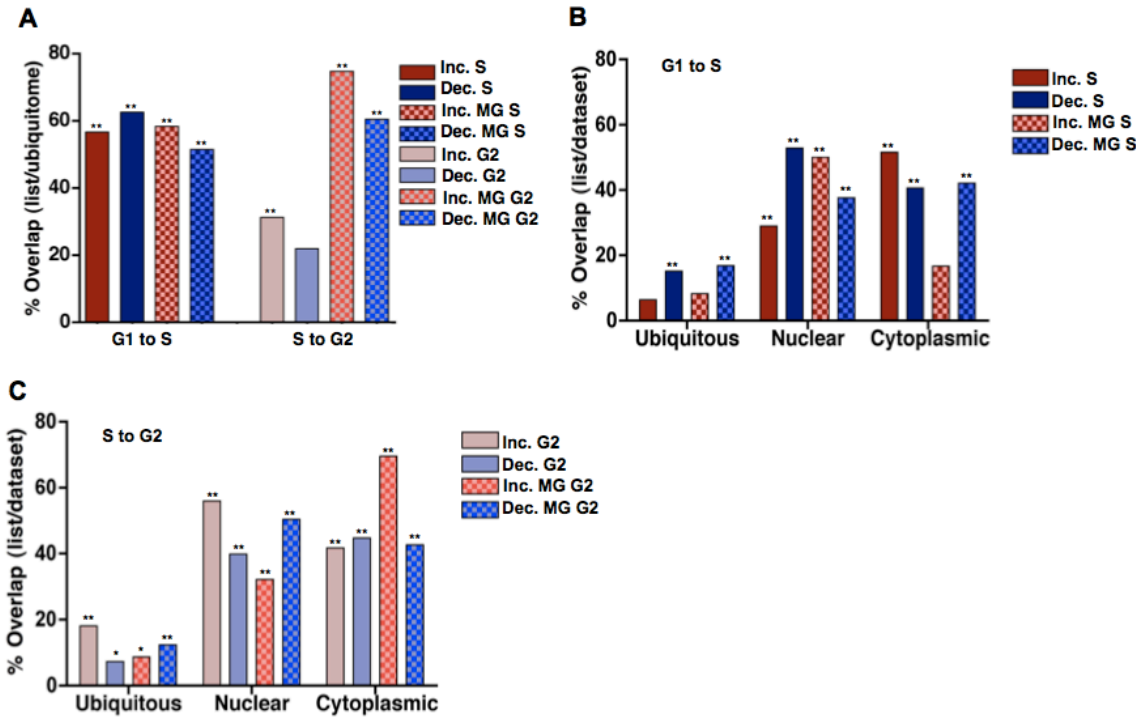

Figure 2.5. Proteins at both the G1/S and S/G2 transitions are ubiquitinated. A) Individual lists of proteins that changed by at least 1.5-fold were compared to proteins predicted to be ubiquitinated in asynchronous HCT116 cells [400]. * p <0.01; ** p <0.001. Individual list of proteins that changed by at least 1.5-fold in either the B) G1/S dataset or the C) S/G2 dataset were compared to proteins with established subcellular localizations [403]. “Ubiquitous” denotes proteins that were found in both the nuclear and cytoplasmic fractions, whereas “Nuclear” or “Cytoplasmic” proteins were found only in that compartment. Data are represented as the percentage of the individual list that overlaps with the published dataset. * p <0.01; ** p <0.001.

potential ATR substrates (Figure 2.6C). The majority of proteins that change with MG132 treatment, (both lists), were not ATR substrates, but proteins that decreased with MG132 treatment at the S/G2 transition were significantly enriched in ATR substrates (Figure 2.6C). Taken together, these comparisons are consistent with the prevailing model that many changes in protein abundance between G1 and S phase and between S and G2 phase are associated with both protein ubiquitination and protein phosphorylation, but this analysis also underscores the idea that only some changes, particularly as cells progress from G1 to S phase in continuously growing cells, are due solely to mRNA fluctuations.

Unanticipated cell cycle-regulated proteins include alternative splicing factors.

To determine which biological processes might be cell cycle-regulated, we analyzed the Gene Ontology (GO) enrichment of each of our lists. As expected, "cell cycle" was enriched in our sets of cell cycle-regulated proteins (increase in G2). The three most highly-enriched terms for each list are shown in Table 1, and the full list is provided in Supplementary Table 5. Proteins involved in cell morphogenesis increased from G1 to S phase, whereas proteins assigned to the GO term "protein folding" decreased (Table 1) from S to G2 phase. Surprisingly, proteins involved in RNA processing and ribonucleoprotein complex biogenesis were significantly represented in the set of proteins that decreased from G1 to S phase and the set that increased from S to G2 phase. (The proteins that decreased from G1 to S phase are not necessarily the same proteins that were increased in the S to G2 dataset.) Both sets of MG132-sensitive proteins were

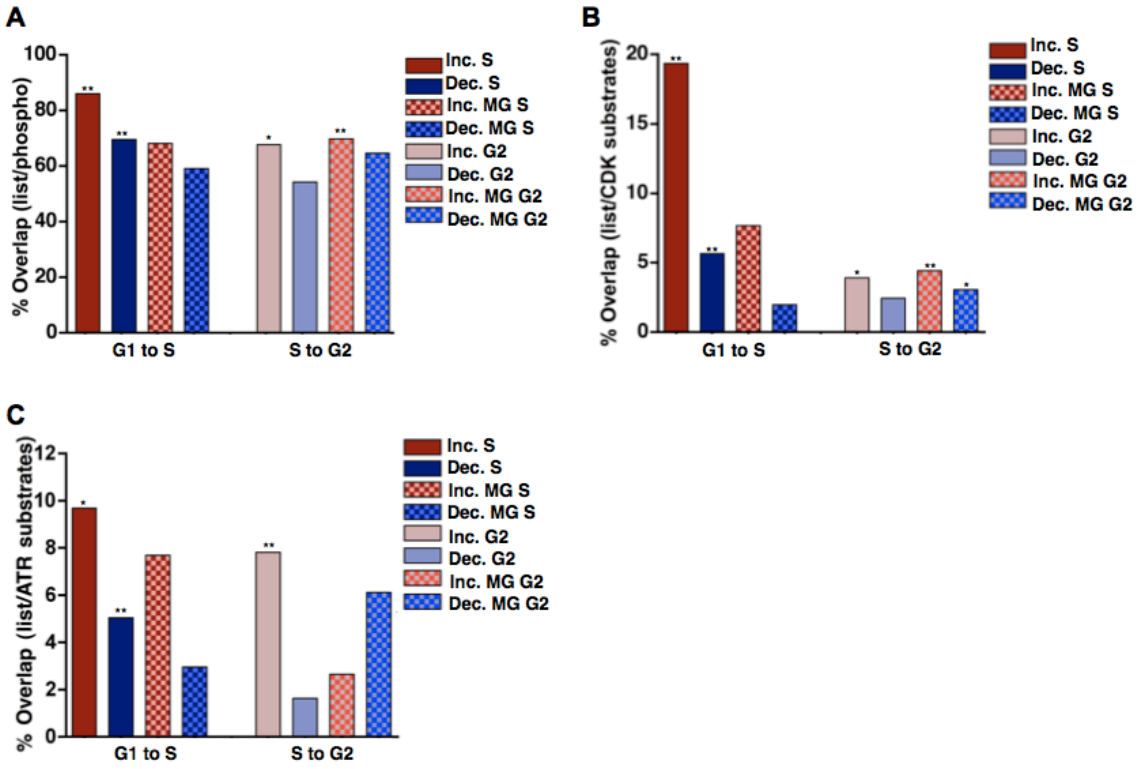

Figure 2.6. Proteins at transitions are phosphorylated. Individual lists of proteins that changed by at least 1.5-fold were compared to proteins predicted to be proteins A) phosphorylated in HeLa cells [392], B) substrates of Cyclin A/Cdk2 [402], and C) substrates of the ATR kinase [401]. The percentage of each list that overlaps with the published dataset is plotted. .* p <0.01; ** p <0.001.

also enriched for RNA processing and ribonucleoprotein complex biogenesis proteins (Table 2).

The striking enrichment of pre-mRNA processing proteins in the collection of proteins that were down-regulated in S phase prompted us to analyze those proteins more directly. In particular, the enriched GO terms included nuclear pre-mRNA splicing, and more specifically, alternative splicing (Figure 2.7A). Of the 244 known splicing factors, we detected 72 core proteins and 65 non-core proteins (Supplementary Table 7) [421]. Overall, we detected 31.9% of the core spliceosome proteins, of which 46.7% decreased in S phase (Figure 2.7B, first bar). Of note, proteins in the U2 complex decreased, suggesting that a specific part of the core machinery may be regulated during S phase. Additionally, we detected 58.7% of the non-core spliceosome machinery, and 62.3% of these subunits decreased in S phase (Figure 2.7B, second bar). Strikingly, we quantified almost all (95.7%) of the known heterogeneous nuclear ribonucleoproteins (hnRNPs), and 72.7% of these proteins decrease in S phase (Figure 2.7B, third bar). These proteins are important in determining exon inclusion, suggesting that alternative splicing is particularly affected during S phase [422-425].

We probed several of the alternative splicing factors by immunoblotting to determine if the changes observed by mass spectrometry were valid. As shown in Figure 2.7C, several hnRNPs decreased between G1 and S phase, such as hnRNPG, hnRNPA1, and hnRNPL (compare lanes 1 and 2). For two other proteins, hnRNPA3 and hnRNPD0, we detected multiple isoforms that clearly

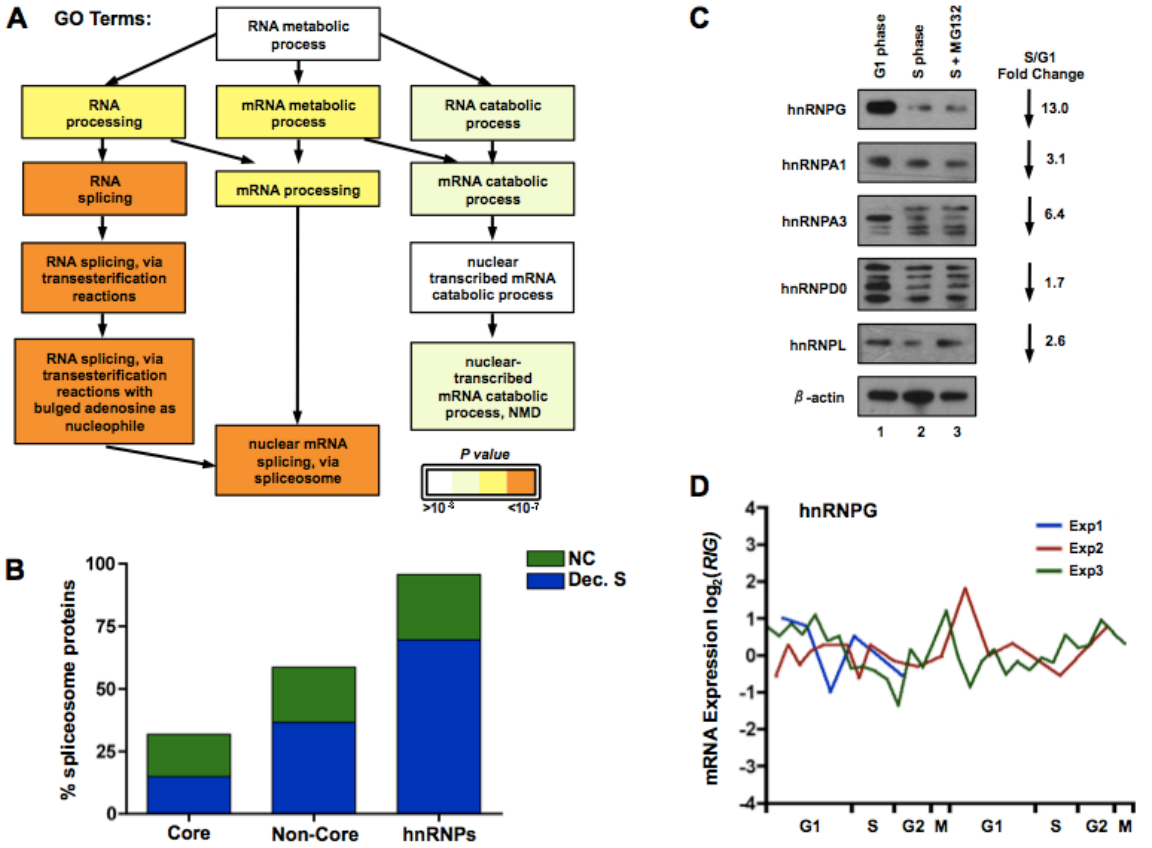

Figure 2.7. pre-mRNA alternative splicing factors are enriched among proteins that decrease from G1 to S phase. A) The GO term analysis tree of a branch of RNA metabolism is shaded to indicate decreasing p-values for the enrichment in the protein datasets of this study. B) Spliceosome proteins were designated as either core or non-core proteins; hnRNPs represent a subset of the non-core spliceosome proteins [421]. The total percentage of the category of splicing proteins is plotted. The portion of the bars shaded blue represents the percentage that decreased between G1 phase and S phase, and the portion shaded green represents the fraction that did not change between G1 and S phase. The full list of splicing proteins quantified is provided in Supplementary Table 7. C) Whole cell lysates from synchronized cultures (Figure 1C) were analyzed for the indicated endogenous hnRNP proteins; the fold change ratios from mass spectrometry are listed to the right. β-actin serves as a loading control. D) mRNA abundance for the hnRNPG gene was extracted from the Whitfield *et al.* (2002) dataset [391]; expression data from 3 double-thymidine block and release experiments are shown as a function of cell cycle phase.

changed between G1 and S phase. Some isoforms decreased in abundance but new isoforms accumulated in the S phase samples (Figure 2.7C, compare lane 1 with lanes 2 and 3). Of note, the hnRNPA3 protein has been reported to be heavily phosphorylated, raising the possibility that the decrease observed by mass spectrometry was due to cell cycle regulated post-translational modifications [426-433]. Indeed, a number of hnRNPs, including hnRNPD0, were identified as Cyclin A/Cdk2 substrates [402]. Moreover, we confirmed S phase downregulation of hnRNPG in biological replicates of synchronized HeLa cells (Figure 2.8A) and S phase downregulation of hnRNPA3 in another line, T98G (Figure 2.8B). Additionally, none of the splicing proteins that decreased in S phase were the products of mRNAs that also decreased in S phase (for example, hnRNPG is shown in Figure 2.7D), suggesting that their regulation must be posttranscriptional.

Discussion

Previous unbiased analyses of the human transcriptome and proteome have generated an appreciation for the interconnectedness of different biochemical pathways. Inspired by such findings, we considered it likely that the human cell cycle includes changes not only in the well-studied processes of chromosome replication, mitosis, and cell division, but also changes in other cellular processes. This hypothesis was supported by our discovery that proteins involved in alternative pre-mRNA splicing are down-regulated in S phase. The reason for this apparent systemic regulation of pre-mRNA splicing has yet to be

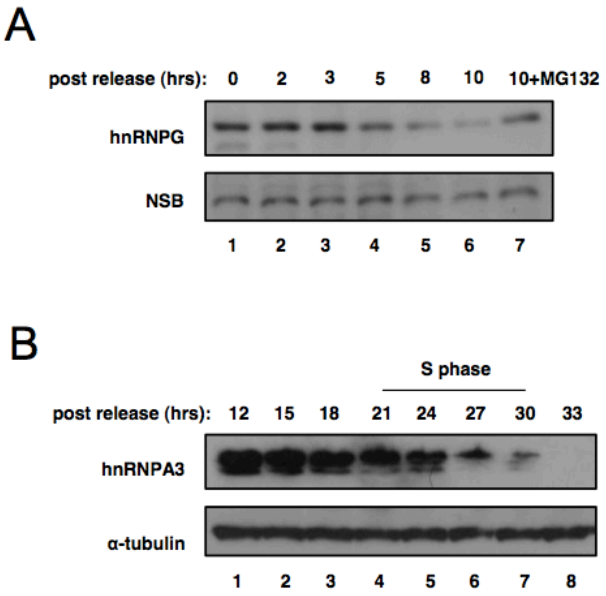

Figure 2.8. Cell cycle changes in pre-mRNA splicing factors are found in different cell lines. A) HeLa cells were synchronized as in Figure 1A and the endogenous levels of hnRNPG were examined. B) T98G cells were synchronized in quiescence by serum starvation and stimulated to re-enter the cell cycle with 10% FBS; S phase entry begins at 20 hr. post-serum addition [77]. Lysates were analyzed for levels of endogenous hnRNPA3.

elucidated, but could reflect a need to rapidly alter the isoforms of a cohort of proteins from one cell cycle phase to the next. The depth of our proteome coverage likely reflects changes in the most abundant and readily detectable proteins; thus these fluctuations indicate novel biological pathways and processes that are cell cycle-regulated even when the rarest proteins were not quantified.

Alternative splicing, particularly the production of different isoforms of specific mRNAs at different times in the same cell, is determined by *cis* elements (splicing enhancers and splicing silencers) and the relative concentrations of the *trans* factors, splicing activators and repressors (reviewed in ref. [434]). Changes in the relative concentrations of these regulatory proteins are responsible for most of the changes observed in alternative splicing. Thus, relatively small changes in the concentrations of these common splicing regulatory proteins, particularly the hnRNPs and SRSF proteins, can result in changes in a number of coordinately regulated alternative splicing events [435-438].

This study extends and complements the cell cycle proteome analysis by Olsen et al. [392]. Our cells were not only very tightly synchronized in early S phase by the double-thymidine and mitotic shakeoff protocol, but importantly, we collected cells as they progressed synchronously through the cell cycle *after* release from the block. This protocol is distinct from other popular synchronization methods in which cells were harvested while chemically arrested with replication or mitotic inhibitors or were harvested very shortly after release from such inhibitors. Likely due to these differences, a comparison of proteins

that change from G1 to S or from S to G2 in our dataset to those reported by Olsen et al. (using a single block and release or nocodazole block and release) showed little overlap. Nevertheless, the alternative splicing factors we detected were also reported in the Olsen dataset, although the amplitudes of those changes were less than those we measured. These differences may be due to technical variations in culture conditions (for example, adherent vs. suspension cultures) or to differences in the degree of cell cycle synchrony. One area of close agreement between the two studies, however, is the conclusion that only a subset of cell cycle-regulated changes in protein abundance can be accounted for by changes in mRNA abundance.

Although many protein changes detected in this study did not match corresponding changes in mRNA levels, we noted a clear difference between the degree of concordance of the mRNA changes and protein changes between the two G1-to-S and S-to-G2 datasets. Proteins that increased from S to G2 were more likely to be the products of mRNAs that showed similar cell cycle-dependent changes, though these mRNA changes were only able to predict ~10% of these G2-inducible proteins (Figure 2.4A). This relationship is consistent with the finding that 45% of the cell cycle regulated mRNAs peak in G2/M [391]. Strikingly, more than half of the proteins that changed – either increased or decreased – from G1 to S phase are among those reported to be polyubiquitinated, but this enrichment was much less or non-significant for proteins that changed from S to G2 (Figure 2.5A). Taken together, our analysis is consistent with the notion that protein changes from S to G2 are somewhat

reflective of changes in mRNA levels, but proteins that change from G1 to S are reflective of ubiquitin-mediated protein degradation and phosphorylation.

Given the importance of ubiquitin-mediated protein degradation in cell cycle transitions, and that a number of cell cycle regulators change concentrations rapidly without concomitant changes in mRNA concentrations, we included analysis of cells treated with the proteasome inhibitor MG132. A relatively small number of proteins that increase after MG132 treatment at the G1/S transition were detected, whereas a larger number of MG132-inducible proteins were detected in cells treated at the S/G2 transition (Figure 2.2B and Supplementary Tables 3.1 and 4.1). Interestingly, at least as many proteins were MG132-*repressible* as were MG132-inducible in both experiments (Figure 2.2B and Supplementary Tables 3.2 and 4.2). Given the mechanism of action of MG132 as a competitive inhibitor of the 26S proteasome, we interpret these changes as a reflection of indirect cellular responses to the accumulation of polyubiquitinated proteins or the prevention of degradation of specific proteins. Some of the MG132-repressible proteins may themselves be targets for negative regulation by MG132-inducible repressors. Those targets of negative regulation would therefore be indirectly repressed by MG132. In addition, the loss of proteasome function may trigger a cellular stress response that is reflected in the proteome as down-regulation of a cohort of proteins. Of note, proteasome inhibitors are a chemotherapeutic strategy for anti-cancer treatment [439,440], and prolonged treatment of HeLa cells with MG132 (e.g. 24 hrs) results in apoptosis [441]. Our report here of proteins whose levels change in response to MG132 at two

specific cell cycle phases sheds additional light on the biological responses to such strategies.

A major challenge in this type of study is the detection of relatively low abundance proteins, many of which are critical regulators of cellular processes. Many of the previously defined cell cycle regulated proteins, often regulated by proteolysis, were not detected. These include SLBP, a critical regulator of histone mRNA metabolism, the E2F1-3 transcription factors, which are essential for the transcription of S phase genes, and many proteins needed for the formation of the pre-replication complex (Orc subunits, Cdc6, Cdt1, etc.). Detection of these low abundance proteins will require further advances in proteomics technology, perhaps through some method that removes the most abundant proteins, similar to how “ribo-minus” technology removes the most abundant RNAs to allow the detection of very low abundance RNAs by high-throughput sequencing.

Studies such as the one presented here add to our general knowledge of the global changes that can occur during the cell cycle. We expect that the combination of this analysis with other studies focused on mitosis, the phosphoproteome, the transcriptome, the ubiquitome, cell cycle changes in model organisms, etc. will facilitate a complete systems-level understanding of the cell cycle.

Table 1. Top three significant GO terms enriched in individual lists of cell cycle-regulated proteins.

Increase in S phase		
GO Term	**p-value**	**Protein Count**
Regulation of cell morphogenesis	0.001	4
Negative regulation of cellular component organization	0.024	3
Negative regulation of cell projection organization	0.047	2
Decrease in S phase		
GO Term	**p-value**	**Protein Count**
RNA processing	3.96e-34	83
Ribonucleoprotein complex biogenesis	1.98e-20	38
Translational elongation	2.46e-18	28
Increase in G2 phase		
GO Term	**p-value**	**Protein Count**
RNA processing	2.25e-05	16
Cell cycle	0.001	16
Cellular protein localization	0.002	11
Decrease in G2 phase		
GO Term	**p-value**	**Protein Count**
Protein folding	0.007	6
Macromolecular complex assembly	0.015	11
Positive regulation of anti-apoptosis	0.018	3

Table 2. Top three significant GO terms enriched in the individual lists of MG132-sensitive proteins.

Increase in S phase following MG132 treatment		
GO Term	**p-value**	**Protein Count**
Signal complex assembly	0.009	2
Cell migration	0.011	3
Cellular macromolecular complex assembly	0.014	3
Decrease in S phase following MG132 treatment		
GO Term	**p-value**	**Protein Count**
Ribonucleoprotein complex biogenesis	2.59e-10	17
Ribosome biogenesis	1.57e-07	12
RNA processing	3.09e-07	23
Increase in G2 phase following MG132 treatment		
GO Term	**p-value**	**Protein Count**
Translational elongation	5.44e-130	68
Ribosome biogenesis	1.01e-14	16
Ribonucleoprotein complex biogenesis	2.13e-13	17
Decrease in G2 phase following MG132 treatment		
GO Term	**p-value**	**Protein Count**
Protein transport	1.45e-05	20
Protein localization	3.30e-05	21
mRNA processing	7.03e-05	12

Chapter 3

Identification of a Re-replication Induced Gene Expression Signature

Introduction

For a cell to successfully complete a division, it must replicate its genomic material in a timely and accurate manner. In order for this to happen, replication must initiate at thousands of sites across the genome known as origins of replication. To exactly duplicate the genome, it is essential that replication initiates only once at every origin; if re-replication, or replication of a previously replicated region of the genome, occurs, this can lead to DNA damage and subsequent genome instability (reviewed in [43]). One mechanism by which the cell prevents re-replication is through coordinating the inhibition of the replication protein Cdt1 with the onset of S phase, thereby ensuring that origins cannot become re-licensed. Once S phase begins, Cdt1 binds to the PCNA that has been loaded onto the DNA, allowing Cdt1 to associate with the $CRL4^{Cdt2}$ ubiquitin ligase; this association results in the polyubiquitination and subsequent degradation of the bulk of Cdt1 protein [371-376,442]. The remaining Cdt1 protein is bound by the inhibitor protein geminin, thereby preventing Cdt1 from performing its origin licensing function [354-356].

Deregulation of origin licensing has been shown to promote genome instability; in fact, many studies have shown that aberrant Cdt1 expression alone can result in re-replication [2,5,443-445]. Re-replication can lead to replication fork collision and collapse, resulting in DNA double strand breaks [443,446-448]. In yeast, re-replication has been shown to induce gene amplification, suggesting that prevention of re-replication contributes to overall genomic stability [449]. DNA damage has long been linked to tumor formation and genomic instability. Therefore, DNA damage, and perhaps re-replication specifically, may contribute to the process of tumorigenesis. Indeed, it has been shown that HeLa cells constitutively undergo re-replication, whereas normal fibroblasts do not experience this stress [450]. Tumors that are driven by oncogenic stressors, such as Ras activation, are prone to both DNA damage and genomic instability [387,451,452]. Furthermore, proteins involved in origin licensing are controlled by pathways, such as the Rb/E2F pathway, that are frequently deregulated in a multitude of cancers [163-166,168-170,172].

These observations led us to hypothesize that re-replication contributes to the genomic instability observed in multiple tumor types and may thereby contribute to cancer development. On a molecular level, we hypothesize that the presence of re-replication results in altered patterns of gene expression. To determine if re-replication results in a gene expression signature, we set out to induce re-replication in normal mammary epithelial cells by modulating Cdt1 activity.

Materials and Methods

Cell Culture and siRNA transfection

Normal human fibroblasts immortalized with human telomerase (NHF-hTERT) were obtained from the Kaufmann lab (University of North Carolina) and were cultured in DMEM (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich). Human mammary epithelial cells immortalized with human telomerase (HMEC-hTERT) were acquired from the Perou lab (University of North Carolina) and the MCF10 series of breast cancer cell lines (MCF10a, MCF10AT1, and MCF10DCIS) was acquired from the Troester lab (University of North Carolina); all four cell lines were grown in supplemented HuMEC media (Gibco). As a positive control, the colorectal carcinoma HCT116 cell line was obtained from ATCC and was grown in McCoy's media (Sigma Aldrich) supplemented with 10% FBS (Sigma Aldrich).

Cells were seeded in 6-cm dishes and allowed to attach overnight. siRNA oligonucleotides were synthesized by Invitrogen. The following oligonucleotides were used: GFP control, 5'-GGC UAC GUC CAG GAG CGC AC CTT; geminin siRNA, 5'-CUU CCA GCC CUG GGG UUA UTT; Cdt2 siRNA, 5'-GAA UUA UAC UGC UUA UCG ATT; and p53 siRNA, AAG GAA GAC UCC AGU GGU AAU TT. Unless otherwise noted, transfections were performed with a total of 100 nM siRNA using the Dharmafect 1 reagent (Dharmacon), according to the manufacturer's guidelines. In the case of dual knockdowns, 50 nM of each siRNA was used. Twenty-four hours after transfection, cells were washed with PBS and trypsinized. Cell were then divided and replated either in a 6-cm dish or on glass

slides for immunofluorescence (IF) analysis (see below). For longer experiments (72 hr and 96 hr), cells were redosed with siRNA after 48 hr.

Immunoblot Analysis

Cells were washed with phosphate buffered saline (PBS) and harvested by trypsinization. Cells were collected by centrifugation, and a portion was removed for flow cytometric analysis (see below). The remaining cells were resuspended in CSK buffer (10 mM PIPES, pH 7.0, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$) supplemented with 0.5% Triton X-100, 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 μg/mL pepstatin A, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 5 μg/mL phosvitin, 1 mM β-glycerol phosphate, and 1 mM orthovanadate, and were incubated on ice for 30 min. Protein concentration was determined using the Bradford assay (Biorad). Samples were subjected to SDS-PAGE and transferred to PVDF (Thermo Scientific). Blots were probed with the following antibodies: anti-Cdt2 [453], anti-phospho p53 (serine 15, Cell Signaling), anti-phospho γ-H2Ax (serine 139, Millipore), anti-α-tubulin (T9026, Sigma Aldrich), anti-Geminin (FL-209, Santa Cruz Biotechnology), and anti-phospho Chk2 (threonine 68, Cell Signaling). All HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch. Proteins were visualized following incubation with ECL Prime reagent (Amersham).

Flow Cytometry Analysis and Immunofluorescence

Cells were fixed in ethanol and stained with propidium iodide. Cells were analyzed using either a Dako CyAn flow cytometer (Beckman-Coulter) or an Accuri C6 flow cytometer (BD Biosciences). Cell cycle distribution was determined using Summit v4.3 software (DakoCytomation) or FCS Express v4 software (De Novo Software), respectively.

Slides were removed from the dish, washed twice with PBS, and treated with CSK buffer supplemented with 0.5% Triton X-100 for 2 min at room temperature. Slides were then washed three times with PBS and fixed in 3% paraformaldehyde [454] at room temperature for 10 min. Slides were washed three times with PBS and then blocked with 10% FBS at room temperature for 1 hr. Slides were washed three times, for 5 min each, at room temperature with PBS. Slides were then incubated with anti-phospho γ-H2Ax (serine 139, Millipore) in a 1% bovine serum albumin (BSA) solution at room temperature for 3 hr. After three washes, for 5 min each, with PBS, slides were incubated with an anti-mouse secondary antibody conjugated to the DyLight-594 fluorophore (Jackson Immunoresearch) at 37°C for 1 hr. Slides were washed twice with PBS for 5 min each, and counterstained with 4',6-diamidino-2-phenylindole (DAPI). To quantify γ-H2Ax staining, at least 200 cells were counted per slide.

RNA preparation and microarray analysis

Total RNA was prepped using the RNeasy kit (QIAgen), according to the manufacturer's instructions. RNA quality was checked by the UNC Genomics

and Bioinformatics Core Facility prior to labeling and hybridization to the microarray. Two experimental samples (one 5 nM set and one 10 nM set) were submitted to the Genomics and Bioinformatics Core Facility. The siCdt2 sample was labeled with Cy3 and compared to its matching siGFP sample labeled with Cy5. Samples were also dye swapped to control for labeling errors. Samples were hybridized to a Human GE 4x44K V2 Microarray with SurePrint Technology (Agilent Technologies), and data were analyzed using SAM 4.0 software (Stanford University).

cDNA preparation and qPCR analysis

Total RNA was prepped using the RNeasy kit (QIAgen), according to the manufacturer's instructions. One microgram total RNA was converted to cDNA using an Oligo dT primer and the SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Quantitative PCR was performed using the following primers: *Cdt2 F*, 5'-TGG TCT TCA CAA TAC CCT CTT CA; *Cdt2 R*, 5'-CTT CAT TGG CAA CTG CTA GTA CA; *p21 F*, 5'-GCA GAC CAG CAT GAC AGA TTT; *p21 R*, 5'-GGA TTA GGG CTT CCT CTT GGA; *MDM2 F*, 5'-CAG TAG CAG TGA ATC TAC AGG GA; *MDM2 R*, 5'-CTG ATC CAA CCA ATC ACC TGA AT; *Geminin F*, 5'-GAG ATC CAG ATG CAG AAG GC; *Geminin R*, 5'-TGA ATC CCA GTA TGA AGC AGA A; *p53 F*, 5'-GCT CGA CGC TAG GAT CTG AC; *p53 R*, 5'-GCT TTC CAC GAC GGT GAC; *GAPDH F*, 5'-AAG GTG AAG GTC GGA GTC AAC; *GAPDH R*, 5'-GGG GTC ATT GAT GGC AAC AAT A. Reactions were performed with 150 nM each primer, 20 ng

template, and the Maxima SYBR Green/Rox qPCR Master Mix (Fermentas), according to the manufacturer's instructions.

Results

Depletion of Cdt2 results in a G2/M arrest

Cdt1 is targeted for degradation at the onset of S phase through the action of the $CRL4^{Cdt2}$ ubiquitin ligase [371-376]. In order to induce re-replication, we depleted cells of the adapter protein Cdt2, which confers substrate specificity to the CRL4 ligase [373,375,455]. In addition to regulating Cdt1 degradation, $CRL4^{Cdt2}$ is also responsible for the S phase degradation of the Cdk inhibitor p21 and the histone methyltransferase Set8 (PR-Set7) [380,382,383,453,456,457]. Set8/PR-Set7 is also essential for proper origin function; monomethylation of lysine 20 on histone H4 promotes the loading of the pre-RC components, and degradation of Set8/PR-Set7 during S phase helps prevent re-replication [380,383]. Therefore, by depleting cells of Cdt2, both Cdt1 and Set8/PR-Set7 are stabilized and robust re-replication should occur.

We sought to induce re-replication in two normal immortalized cell lines: human mammary epithelial (HMEC-hTERT) cells and normal human fibroblast (NHF-hTERT) cells. HMEC cells were chosen for this project because of the wide breadth of microarray data that is available for different breast cancer subtypes [458-462]. NHF cells were chosen because they have been used extensively to study the DNA damage checkpoint [463-466]. The HCT116 colorectal carcinoma cell line was used as a positive control for induced re-replication; previous work

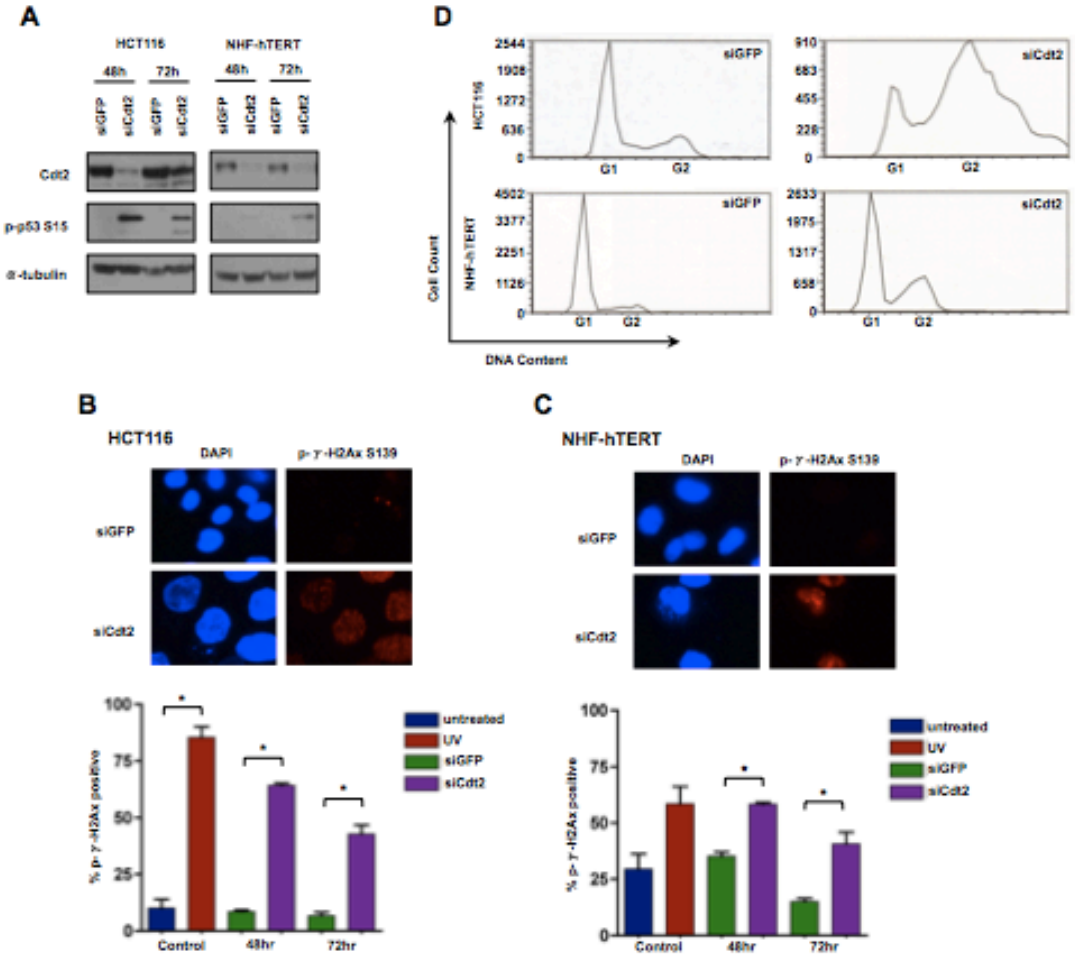

Figure 3.1. Depletion of Cdt2 results in DNA damage and a G2/M arrest. A) HCT116 and NHF-hTERT cells were transfected with 100 nM siGFP or siCdt2 and incubated for either 48 hr or 72 hr. A) Whole cell extracts were analyzed for the presence of Cdt2 and activated p53 (phosphorylated serine 15). α-tubulin was used as a loading control. B) HCT116 and C) NHF-hTERT cells treated with 100nM siRNA were stained for phoshphorylated γ-H2Ax (serine 139). Results from multiple experiments are quantified below. * $p<0.05$. D) The DNA content of HCT116 (top) and NHF-hTERT cells (bottom) treated with 100 nM siRNA were analyzed by flow cytometry.

had shown that Cdt1 misregulation, either through overexpression or due to geminin depletion, can induce re-replication in these cells [467]. As shown in Figure 3.1A, Cdt2 could successfully be depleted in HCT116 and NHF-hTERT cells. When depleted, the DNA damage response was activated as evident by the activating phosphorylation on p53 (serine 15). Since p53 could become activated for a number of reasons, cells were stained for the presence of the DNA damage marker, phosphorylated γ-H2Ax (serine 139, Figure 3.1B and 3.1C). Upon DNA damage, γ-H2Ax is rapidly phosphorylated and deposited at the sites of double strand breaks and is therefore an early marker for the DNA damage response [468]. Interestingly, cells depleted of Cdt2 result in a G2/M arrest with no apparent accumulation of greater than 4C DNA content (Figure 3.1D).

Because the goal of the project was determine a gene expression signature that was specific to cells undergoing re-replication and not a general DNA damage response, we focused on inducing re-replication in the NHF cell line so we could compare directly to the DNA damage gene signatures. Initial experiments were performed using 100 nM siRNA. As seen in Figure 3.2A, both 5 nM and 10 nM siRNA was sufficient to partially deplete cells of Cdt2. This partial depletion was enough to stabilize the $CRL4^{Cdt2}$ target p21, as well as activate p53 (Figure 3.2A) and form γ-H2Ax foci (Figure 3.2B). Importantly, when treated with either 5 nM or 10 nM siRNA, the cells did not arrest at the G2/M border (Figure 3.2C).

Total RNA was extracted from NHF cells treated with either 5 nM or 10 nM siRNA for 72 hr and hybridized to a 4x44K V2 Microarray to examine the

changes in gene expression when Cdt2 was depleted. Unfortunately, the microarray results were inconclusive. Even though Cdt2 depletion was confirmed by immunoblot analysis, microarray analysis showed no changes in Cdt2 mRNA levels. We therefore concluded that the level of re-replication that was being induced was not sufficient enough to lead to changes in gene expression.

When treated with the full dose (100 nM) siCdt2, cells arrested at the G2/M border (Figure 3.1D). This arrest could be the result of a full activation of the DNA damage checkpoint due to extensive re-replication. In order to bypass the checkpoint, cells were depleted with both Cdt2 and p53. Co-depletion of p53 was able to alleviate the G2/M arrest in both HCT116 (Figure 3.3A and HMEC-hTERT cells (Figure 3.3B), however cells did not accumulate greater than 4C DNA content. Additionally, arresting depleted cells with nocodazole did not lead to accumulation of re-replicated DNA (data not shown). These experiments were also performed in the pre-cancerous MCF10a series of breast cancer cell lines; we hypothesized that these cells may be predisposed to accumulating DNA damage. However, siRNA knockdown could not be achieved in these cell lines (data not shown).

These observations suggest that cells depleted of Cdt2 undergo enough stress to activate p53 but are not, in fact, re-replicating. This could be due to the combined stabilization and induction of the Cdk inhibitor p21. As mentioned previously, p21 is targeted for degradation at the onset of S phase by the $CRL4^{Cdt2}$ ubiquitin ligase [453,456,457]. p21 has also been shown to be induced by p53 during a cell cycle arrest [469-472]. Additionally, p21 has been shown to inhibit DNA

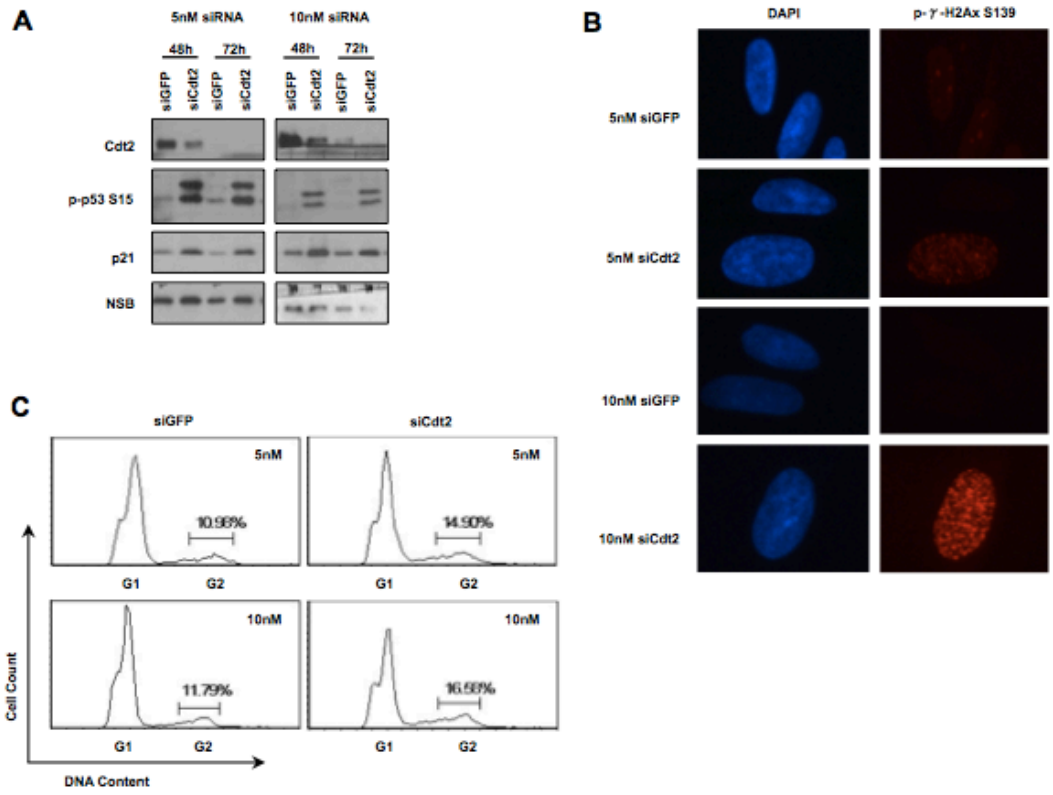

Figure 3.2. Treatment with 10 nM siCdt2 causes DNA damage but does not induce a G2/M arrest. NHF-hTERT cells were treated with 5 nM or 10 nM siGFP or siCdt2 and were incubated for 48 hr or 72 hr. A) Whole cell extracts were analyzed for the presence of Cdt2, activated p53 (phosphorylated serine 15), and p21. A non-specific band (NSB) was used as a loading control. B) Cells treated with 5 nM or 10 nM siRNA were stained for phoshphorylated γ-H2Ax (serine 139). C) The DNA content of NHF-hTERT cells treated with 5 nM (top) or 10 nM (bottom) siRNA were analyzed by flow cytometry.

replication through its binding to PCNA; this inhibition is achieved through by blocking the loading of PCNA onto DNA or by disrupting the loading of polymerase δ onto PCNA *in vitro* [473-475]. We examined the levels of p21 mRNA and found them to be induced in the Cdt2 depleted cells (data not shown), perhaps contributing to the G2/M arrest in these cells.

Depletion of geminin leads to re-replication in HMEC cells

To bypass the arrest, we attempted to induce re-replication by targeting Cdt1 function specifically by depleting cells of the inhibitor protein geminin. Depleting HCT116 cells of geminin induces significant amounts of re-replication [467]. We confirmed that geminin could be depleted through RNAi in HMEC cells (Figure 3.4A). This depletion also leads to an increase in the activation of the checkpoint kinase Chk2, suggesting that these cells are undergoing DNA damage. Indeed, DNA damage was confirmed through the formation of γ-H2Ax foci (Figure 3.4B). It has also been shown in *S. pombe* that DNA damage can induce the levels of Cdt2 [476,477]. Indeed, when we deplete HMEC cells of geminin, we see a 3-fold induction of Cdt2 mRNA, suggesting that the DNA damage checkpoint has been activated (data not shown). Most importantly, however, HMEC cells that are depleted of geminin do not arrest, but instead accumulate greater than 4C DNA content (Figure 3.4C).

Discussion

Cells depleted of Cdt2 accumulated multiple markers of DNA damage, but

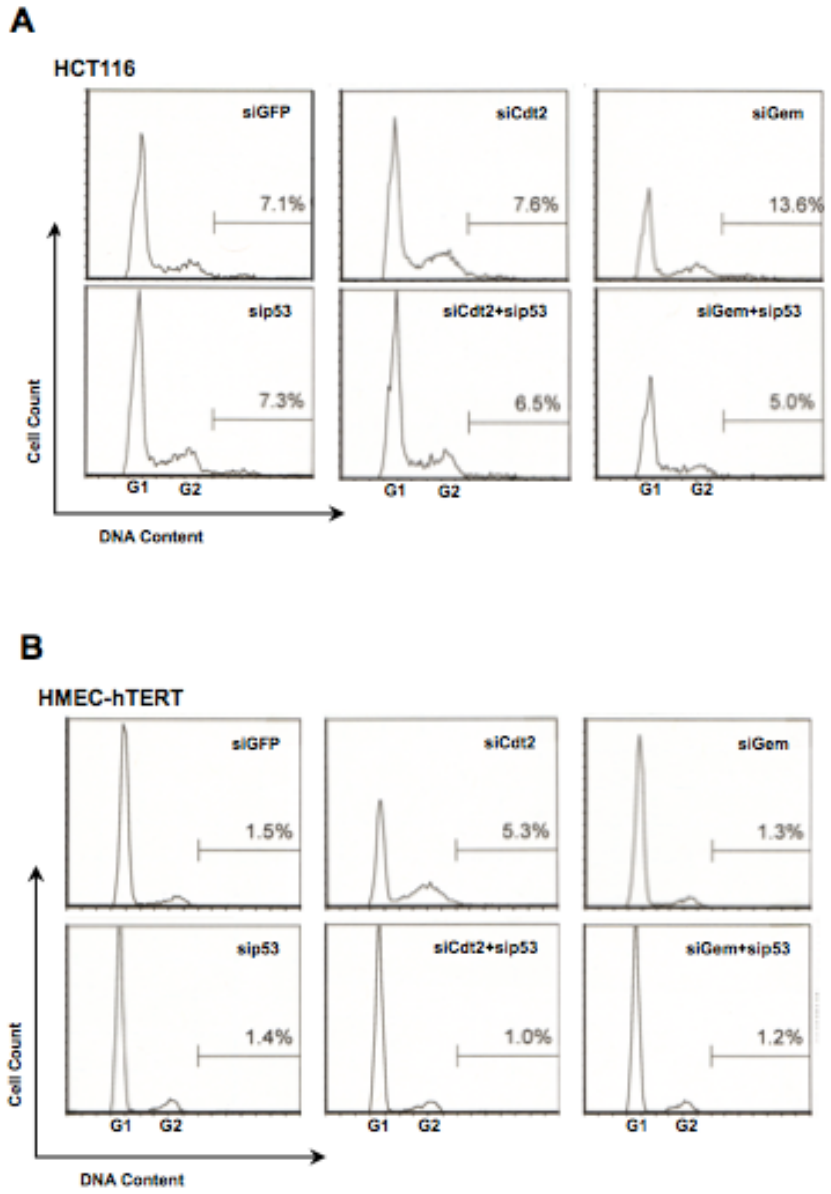

Figure 3.3. Co-depletion of Cdt2 and p53 abrogates the G2/M arrest. A) HCT116 or B) HMEC-hTERT cells were transfected with a total 100 nM of the indicated siRNA. The DNA content of the cells was analyzed 72 hr later by flow cytometry.

evidence of re-replication was not observed by flow cytometry; instead, cells arrested in G2/M phase (Figure 3.1). We hypothesized that depletion of Cdt2 was actually causing re-replication during S phase, leading to what appeared to be 4C content. To exclude this possibility, we treated Cdt2 depleted cells with nocodazole to arrest cells in mitosis, thereby allowing the extra re-replicated DNA to appear as greater than 4C content by flow cytometry. However, treatment with nocodazole did not result in accumulation of re-replicated DNA, suggesting that while these cells are damaged, the G2/M checkpoint prevents re-replication from occurring. This checkpoint could be overcome by concurrently depleting cells of Cdt2 and p53, but re-replication was still not observed (Figure 3.3). Recent studies have stabilized $CRL4^{Cdt2}$ substrates by treating cells with MLN4924, a neddylation inhibitor that blocks the activation of the cullin ubiquitin ligases through the inhibition of NEDD8 [478,479]. When HCT116 cells were treated with MLN4924, re-replication was observed, as well as an increase in apoptosis [478]. Interestingly, when $HCT116^{p21-/-}$ cells were treated with this inhibitor, they saw an increased sensitivity to MLN4924, suggesting that co-depletion of p21 and Cdt2 may increase the chances of inducing re-replication in normal cells.

Because Cdt2 depletion leads to large perturbations of the cell by affecting multiple $CRL4^{Cdt2}$ substrates, we decided to make a targeted approach and affect Cdt1 function only. We therefore depleted HCT116 and HMEC cells of the Cdt1 inhibitor, geminin. Previously, depletion of geminin in NHF-hTERT cells resulted in a normal flow cytometry profile; however, by using DNA fiber spreading, re-replication was evident at low levels [450]. When HMEC cells were depleted of

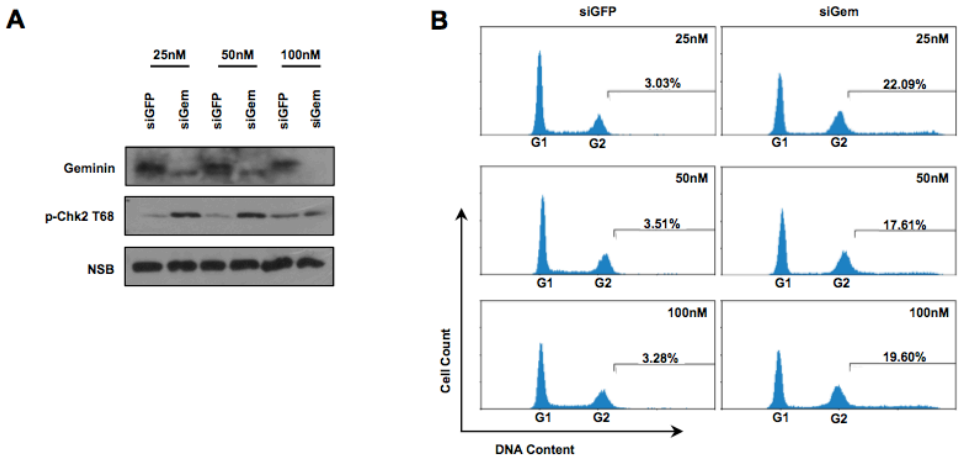

Figure 3.4. Depletion of geminin results in re-replication. HMEC-hTERT cells were treated with either 25 nM, 50 nM, or 100 nM siGFP or siGem for 72 hr. A) Whole cell extracts were analyzed for geminin and activated Chk2 (phosphorylated threonine 68) levels. B) DNA content was analyzed by flow cytometry.

geminin, markers of DNA damage accumulated, as well enough re-replication to be observed by flow cytometry (Figure 3.4). However, these results varied from experiment to experiment. Phosphorylation of Chk2 was consistently observed, suggesting that even though re-replication could not be observed by flow cytometry, it may still be occurring. In order to see changes in gene expression, however, the phenotype may have to be robust enough to be observed by flow cytometry. This could be achieved by combining geminin depletion with transient overexpression of Cdt1, either through adenoviral infection or lentiviral transduction.

Once re-replication is confirmed, a re-replication gene expression signature can be generated. This signature can then be compared to the gene expression profiles of various cancer types, particularly the established subtypes of breast cancer and oncogene driven tumors. This comparison will allow us to correlate cancer subtype, degrees of genomic instability, or potentially patient prognosis with re-replication.

Chapter 4

Conclusions and Future Directions

Conclusions

The work in this dissertation provides evidence that a successful cell cycle is driven by changes in protein abundance. Prior to the work presented here, the majority of the known cell cycle abundance changes were detected through mutagenic, single gene studies. These studies have led to the discovery of many proteins essential for cell cycle progression, such as Cdc6, Cdt1, etc. However, single gene studies limit the scope of what can be observed. By taking a mass spectrometry approach, we have shown that other biological processes, such as mRNA alternative splicing, are regulated in a cell cycle dependent manner.

Future Directions

Repeat mass spectrometry to detect low abundance proteins

While we were able to detect about a third of the known proteome, surprisingly, we could not detect many of the known cell cycle regulated proteins. These proteins included origin licensing proteins, such as Cdc6 and Cdt1, and the E2F transcription factors. Since our screen only identified the most abundant proteins in the cell, it is possible that we were unable to detect critical mediators

of other signaling pathways are indeed cell cycle regulated but are expressed at a low level. This could be circumvented by performing mass spectrometric analysis on a larger amount of protein. Also, the cells could first be fractionated into nuclear and cytoplasmic lysates in order to decrease the complexity of the mixture. Alternatively, the whole cell extract could be separated further, either through SDS-PAGE or a longer elution from the LC separation procedure.

Due to our experimental approach, it is possible that post-translational modifications may have been mistaken as a decrease in protein abundance. In order to elucidate these proteins, we could perform a phosphopeptide enrichment to enhance our detection of phosphorylation events that may occur in a cell cycle dependent manner. However, detection of other modifications, such as acetylation or methylation, typically involved immunoprecipitating proteins from a complex mixture with a modification specific antibody. While this would be possible, we may also be able to detect these modifications through modified database searches. The protocols mentioned previously (i.e., fractionation, longer elution times, etc.) would also boost the possibility of identifying these modifications.

Determine the effects of hnRNP protein changes on alternative splicing

Several alternative splicing factors, both hnRNPs and SR proteins, were found to be differentially regulated during S phase. This suggests that splicing of certain mRNA targets changes during S phase. This has major implications for how protein abundance and function can be regulated throughout the cell cycle.

Recently, changes in alternative splicing have been confirmed for some cell cycle proteins, such as Chk2 (Z. Wang, personal communication), though the cell cycle regulation of alternative splicing has not yet been examined on a large scale. This question can be answered using high-throughput RNA-seq. By sequencing the mRNAs that are present during different stages of the cell cycle, we can determine whether certain isoforms are more abundant during particular cell cycle phases.

Splicing factors are notoriously promiscuous; for example, some target mRNAs, particularly the hnRNP mRNAs themselves, can be regulated by several different splicing factors [438]. In our datasets, we see the majority of the hnRNP proteins decrease during S phase and recover during G2. With such a large scale change in splicing factor abundance and the promiscuity of these factors, overall splicing efficiency may be affected during S phase. In this scenario, the splicing sites that are closest to the consensus site will be bound, and splicing will occur as normal. However, binding to these consensus sites will sequester the limited amount of protein away from the weaker binding sites. To test this, the Garcia-Blanco lab (Duke University) has developed a series of reporter plasmids that vary in their splice site efficiencies. As controls, the 5' consensus site is constitutively spliced, whereas the 15d site is rarely spliced (~10%). Another construct, designated as C, is spliced about ~50% and is therefore contingent on the availability of splicing factors [480]. These constructs could provide insight into whether overall splicing efficiency is affected by cell cycle position.

Determine the gene expression profile of re-replicating HMEC cells

In order to induce re-replication, HMEC cells may need to be depleted of geminin and overexpress Cdt1. This overexpression can be achieved through either adenoviral expression or lentiviral transduction. Due to the possible off-target consequences of adenoviral infection, we are currently testing if transient lentiviral transduction can result in re-replication. Additionally, because normal cells are efficient at repairing re-replication, this damage should be confirmed through both flow cytometry and DNA fiber spreading, as well as the appearance of DNA damage markers, such as γ-H2Ax staining and Chk2 phosphorylation. Gene expression profiling of these cells will reveal an expression pattern that is induced by re-replication, but is, at least partially, different from a DNA damage signature. This re-replication signature can then be compared to the multitude of tumor gene expression profiles that are available.

Because genome instability is a hallmark of cancer, re-replication may be more prevalent in the most aggressive tumors. Therefore, there may be a correlation between re-replication and poor patient prognosis. Cancers that are prone to re-replication may also benefit from treatment with certain types of chemotherapeutics that target DNA replication.

Appendix A: Protocols optimized for Cdc6 isolation

Project Rationale

To further understand the mechanisms by which pre-RC assembly is controlled, we sought to further elucidate the mechanisms by which Cdc6 is regulated. As mentioned in Chapter 1, Cdc6 is phosphorylated in late G1 phase by cyclin/Cdk2 complexes; this phosphorylation blocks the association of Cdc6 with APC/C, resulting in increased protein levels [334]. Recently, it was discovered that Cdc6 is also acetylated on three lysine residues [335]. Acetylation of these residues promotes the phosphorylation of serine 106, which in turn promotes the export of Cdc6 from the nucleus during S phase [335,336,338-341]. While the bulk of Cdc6 is exported, a small fraction of the protein remains chromatin-bound throughout S phase; it is thought that this population of Cdc6 protein participates in the intra-S phase checkpoint [337,342,343]. In addition to this regulation, upon DNA damage, Cdc6 is released from chromatin and targeted for degradation by the ubiquitin ligase Huwe1 [481]. However, under conditions of cellular stress, Cdc6 is not degraded, but pre-RC formation is still blocked [482].

The mechanism by which some Cdc6 is exported while a fraction remains chromatin-bound is still unknown. Additionally, it is unknown why Cdc6 is degraded following DNA damage but is stabilized when cells are stressed. We hypothesized that additional post-translational modifications of Cdc6 may modulate its activity during these conditions. This hypothesis was supported by

the finding that the acetyltransferase Hbo1 associates with many members of the pre-RC complex and is essential for MCM loading [483]. Mass spectrometric data also suggested that Cdc6 was acetylated on lysines 57 and 531 (Y. Xiong, personal communication). Given the proximity of one of these sites, lysine 57, to a known Cdk phosphorylation site, we hypothesized that this modification may regulate the accessibility of Cdc6 to the cyclin/Cdk complexes.

In order to confirm these acetylation sites, as well as identify other modifications that may be present on Cdc6, we sought to immunoprecipitate (IP) endogenous Cdc6 from asynchronously growing HeLa cells to analyze by mass spectrometry.

Cdc6 Isolation Protocols

Isolation of Endogenous Cdc6 with UNC274 or UNC275 sera

HeLa cells were grown asynchronously in DMEM supplemented with 10% FBS. Cells were washed with PBS and harvested by trypsinization. Cells were collected by centrifugation and snap frozen. Pellets were resuspended in Co-IP3 buffer (50 mM HEPES, pH 7.2, 33 mM KAc, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% NP-40, 10% glycerol) supplemented with 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 μg/mL pepstatin A, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 5 μg/mL phosvitin, 1 mM β-glycerol phosphate, 1 mM orthovanadate, 200 nM trichostatin A, and 2.5 mM sodium butyrate. Lysates were sonicated for 10 pulses on the lowest setting, at 20% duty cycle. Following sonication, 5 mM $CaCl_2$ and S7 nuclease was added to release chromatin bound

proteins, and the lysates were incubated on ice for 20 min. Lysates were cleared by centrifugation, and protein concentration was determined using the Bradford assay (Biorad). Samples were divided and incubated with either normal rabbit serum (NRS) or the anti-Cdc6 sera, UNC274 or UNC275 overnight at 4°C. Protein A beads were washed with Co-IP3 buffer, added to the IPs, and rotated at 4°C for 1 hr. Following incubation, the protein A beads were washed three times with Co-IP3 buffer and resuspended in SDS loading buffer supplemented with DTT. Samples were subjected to SDS-PAGE on an 8% polyacrylamide gel and transferred to PVDF (Thermo Scientific). Blots were probed with anti-Cdc6 (180.2, Santa Cruz Biotechnology). As shown in Figure A.1A and A.1B, both UNC274 and UNC275 sera were able to immunoprecipitate Cdc6, whereas NRS was not.

Isolation of Endogenous Cdc6 with H304 antibody

In addition to the rabbit serum raised against Cdc6, we were able to immunoprecipitate Cdc6 using a commercial antibody, H304 (Santa Cruz Biotechnology). Asynchronous HeLa cells were washed with PBS and harvested by trypsinization. Cells were pelleted and resuspended in HNNG buffer (15 mM HEPES, pH 7.5, 250 mM NaCl, 1% NP-40, 5% glycerol) supplemented with 0.1 mM AEBSF, 1 μg/mL pepstatin A, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 5 μg/mL phosvitin, 1 mM β-glycerol phosphate, 1 mM orthovanadate, 200 nM trichostatin A, and 2.5 mM sodium butyrate. Lysates were incubated on ice for 20 min and were cleared by centrifugation. Protein concentration was determined using the

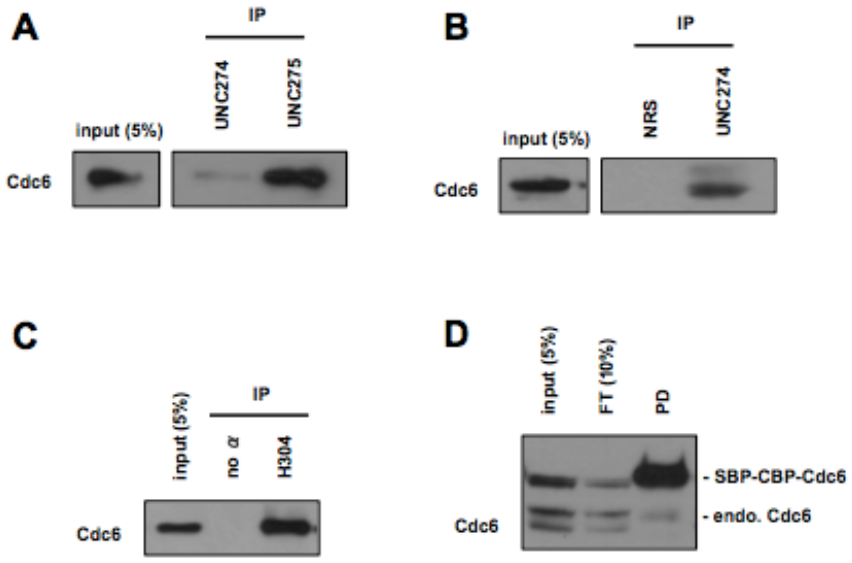

Figure A.1. Cdc6 isolation from HeLa cell extracts. Asynchronous HeLa cell extracts were subjected to immunoprecipitation with either A) UNC 274 serum and UNC275 serum, B) normal rabbit serum (NRS) and UNC275 serum, or C) the commercial H304 antibody. D) Asynchronous HeLa cells were treated with adenovirus encoding SBP-CBP-Cdc6 and were harvested 48 hr after infection. SBP-CBP-Cdc6 was precipitated (PD) using streptavidin beads.

Bradford assay (Biorad). Samples were divided and incubated with either no antibody or H304 overnight at 4°C. Protein A beads were washed with HNNG, added to the IPs, and rotated at 4°C for 1 hr. Following incubation, the beads were washed three times with HNNG buffer and resuspended in SDS loading buffer supplemented with DTT. Samples were subjected to SDS-PAGE on an 8% polyacrylamide gel and transferred to PVDF (Thermo Scientific). Blots were probed with anti-Cdc6 (180.2, Santa Cruz Biotechnology). As shown in Figure A.1C, H304 was able to efficiently pull down endogenous Cdc6, whereas Cdc6 was not detected in the control reaction.

Isolation of SBP-CBP-Cdc6

Asynchronous HeLa cells were infected with an adenovirus expressing GFP or SBP-CBP-Cdc6 at an MOI of 500. Twenty-four hours later, cells were washed with PBS and harvested by trypsinization. Cells were collected by centrifugation and resuspended in SBB-L buffer (40 mM Tris HCl, pH 7.4, 100 mM KCl, 5 mM β-mercaptoethanol, 2 mM EDTA, 0.1% Triton X-100). All buffers were supplemented with 0.1 mM AEBSF, 1 μg/mL pepstatin A, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 5 μg/mL phosvitin, 1 mM β-glycerol phosphate, 1 mM orthovanadate, 200 nM trichostatin A, and 2.5 mM sodium butyrate. Cells were sonicated for 10 pulses at the lowest setting, at 20% duty cycle. Lysates were supplemented with 5 mM $CaCl_2$ and S7 nuclease to release the chromatin-bound proteins and were incubated on ice for 30 min. An equal volume of SBB-H buffer (40 mM Tris HCl, pH 7.4, 500 mM KCl, 5 mM β-mercaptoethanol, 2 mM EDTA

0.1% Triton X-100) was added, and the lysates were incubated for an additional 10 min on ice. Lysates were cleared by centrifugation, and protein concentration was determined using the Bradford assay (Biorad). Streptavidin resin (Stratagene) was washed in SBB buffer (40 mM Tris HCl, pH 7.4, 250 mM KCl, 5 mM β-mercaptoethanol, 2 mM EDTA, 0.1% Triton X-100) and added to the cell lysates. The beads were incubated with the lysates at 4°C for 2 hr and then washed three times with SBB buffer. The beads were resuspended in SDS loading buffer and subjected to SDS-PAGE on an 8% polyacrylamide gel. Proteins were transferred to PVDF (Thermo Scientific) and probed with anti-Cdc6 (180.2, Santa Cruz Biotechnology). As shown in Figure A.1D, SBP-CBP tagged Cdc6 can be efficiently pulled down used streptavidin resin.

Project Status and Future Directions

Unfortunately, even though endogenous or tagged Cdc6 was successfully immunoprecipitated from asynchronous HeLa cell extracts, we were unable to isolate enough Cdc6 to perform mass spectrometry analysis. Endogenous Cdc6 was immunoprecipitated using the UNC274 sera, the UNC275 sera, or the H304 commercial antibody; unfortunately, given the size of Cdc6 (~63kD), it was nearly impossible to isolate Cdc6 from IgG heavy chain peptides following SDS-PAGE separation. To circumvent this problem, we treated extracts with *N*-ethylmaleimide [484] to prevent the breakdown of the IgG complex or attempted to conjugate the antibody to a resin column. In both cases, however, separation of the antibody and Cdc6 was not successful. Following NEM treatment and

SDS-PAGE, both Cdc6 and the antibody shifted in the gel. To completely eliminate IgG from the SDS-PAGE separation entirely, we attempted to conjugate all three antibodies to agarose resin using the CarboLink Immobilization kit [206]. While the antibodies were successfully cross-linked to the resin, Cdc6 could not be eluted from the column in quantities sufficient for mass spectrometry analysis.

Because endogenous immunoprecipitations would not work, we attempted to tag Cdc6 with several different epitopes (see Appendix B for a full list). The best results were achieved using an N-terminal combination streptavidin binding peptide (SBP)-calmodulin binding peptide (CBP) epitope tag. The addition of this epitope allowed us to pull down Cdc6 under a variety of salt and detergent conditions (Figure A1.D). Unfortunately, while we could express this tagged protein using an adenovirus expression system, we were unable to stably express this protein in a variety of cell types. While we would be able to purify sufficient Cdc6 to perform mass spectrometry analysis using this system, it is possible that overexpression of a tagged protein could result in skewed results.

These issues could perhaps be overcome by the use of a smaller epitope tag or tagging the protein on its C-terminus. Our lab has observed that at least one epitope tag, the 5x myc tag, partially stabilizes Cdc6 when the protein is tagged on the N-terminus. The SBP-CBP tag is of a similar size and could result in a more stable protein, thereby adding additional replication stress to cells that are constantly expressing this protein. Use of a smaller epitope, such as an *in vivo* biotinylation system, may alleviate that stress, allowing for stable expression

of the tagged protein. The stabilization effect of the 5x myc tag may also be due to its placement at the N-terminus, close to where the Cdk phosphorylation site and APC/C binding site are located; relocating the SBP-CBP tag to the C-terminus may help promote stable expression of the protein.

Appendix B: List of plasmids generated

Cdc6 mutants:

pENTR vectors:

1. pENTR1A HsCdc6-stopless

   Description: Entry vector encoding an untagged human Cdc6 gene with a mutated stop codon.

   Strategy: Stopless codon was amplified from pENTR1A (primers: cdc6.stopless, K531R 5'). PCR was digested (BglII) and ligated into pENTR1A HsCdc6 (BglII & EcoRV).

   Note: A consensus kozak sequence was added to this plasmid.

2. pENTR1A HsCdc6-GST

   Description: Entry vector encoding a C-terminal Glutathione-S-transferase (GST) tagged human Cdc6 gene.

   Strategy: HsCdc6-GST was cut from pDEST24 HsCdc6-GST (SalI & EcoRV) and ligated into pENTR1A (SalI & EcoRV).

3. pENTR1A HsCdc6-strep

   Description: Entry vector encoding a C-terminal StrepTagII tagged human Cdc6 gene.

   Strategy: The StrepTagII tag was added to HsCdc6 via PCR (primers: cdc6.strep, K531R 5') using pENTR1A HsCdc6 as a template. Tagged Cdc6 PCR was cut (BglII) and ligated into pENTR1A HsCdc6 (BglII & EcoRV).

   Note: A consensus kozak sequence was added to this plasmid.

4. pENTR2B sbp-HsCdc6#

   Description: Entry vector encoding an N-terminal streptavidin binding protein (SBP)-calmodulin binding protein (CBP) tagged human Cdc6 gene, resistant to the D5 hairpin.

   Strategy: SBP-CBP tagged Cdc6 was cut from pNTAP3C HsCdc6# (NotI & PvuI, blunted) and ligated between EcoRI sites (blunted) in pENTR2B.

   Note: In order to successfully create adenovirus, this plasmid was digested with PacI and blunted with T4 polymerase to destroy the site.

5. pENTR3C myc-cdc6 K57R

   Description: Entry vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K57R mutation.

   Strategy: Mutation was created by megaprimer PCR (primers: K57R muta, K57R 5' #2, K57R 3' Rev). PCR was cut (KpnI & NdeI) and ligated into pENTR3C myc-cdc6 (KpnI & NdeI).

6. pENTR3C myc-cdc6 K57Q

   Description: Entry vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K57Q mutation.

   Strategy: Mutation was created by megaprimer PCR (primers: K57Q muta, K57R 5' #2, K57R 3' Rev). PCR was cut (KpnI & NdeI) and ligated into pENTR3C myc-cdc6 (KpnI & NdeI).

7. pENTR3C myc-cdc6 K531R

   Description: Entry vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K531R mutation.

Strategy: Mutation was created by megaprimer PCR (primers: K531R muta, K531 3' Rev, K531R 5'). PCR was cut (BglII & NotI) and ligated into pENTR3C myc-cdc6 (BglII & NotI).

8. pENTR3C myc-cdc6 K531Q

Description: Entry vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K531Q mutation.

Strategy: Mutation was created by megaprimer PCR (primers: K531Q muta #2, K531 3' Rev, K531R 5'). PCR was cut (BglII & NotI) and ligated into pENTR3C myc-cdc6 (BglII & NotI).

pDEST/expression vectors:

9. pcDNA3 HsCdc6-strep

Description: Expression vector encoding a C-terminal StrepTagII tagged human Cdc6 gene.

Strategy: HsCdc6-strep was cut from pDEST40 HsCdc6-strep (SmaI & EagI) and ligated into pcDNA3 (KpnI-blunted & NotI).

10. pcDNA3 myc-cdc6-strep

Description: Expression vector encoding an N-terminal 5x myc tagged and a C-terminal StrepTagII tagged human Cdc6 gene.

Strategy: C-terminal tag was cut from pDEST40 HsCdc6-strep (EagI & EcoRI) and ligated into pcDNA3 myc-cdc6 (NotI & EcoRI).

11. pDEST24 HsCdc6-GST^

Description: Bacterial expression vector encoding a C-terminal GST tagged human Cdc6 gene.

Strategy: Tagged Cdc6 was moved into pDEST24 from pENTR1A HsCdc6-stopless via LR Gateway reaction.

Note: This plasmid contains a frame shift. The pENTR1A HsCdc6-stopless plasmid has been corrected, however this plasmid was not remade.

12. pDEST40 HsCdc6-GST

    Description: Expression vector encoding a C-terminal GST tagged human Cdc6 gene.

    Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR1A HsCdc6-GST via LR Gateway reaction.

13. pDEST40 HsCdc6-His

    Description: Expression vector encoding a C-terminal 6x His tagged human Cdc6 gene.

    Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR1A HsCdc6-GST via LR Gateway reaction.

    Note: A consensus kozak sequence was added to this plasmid. A frame shift mutation was corrected by digesting with XhoI and blunted with T4 polymerase.

14. pDEST40 HsCdc6-strep

    Description: Expression vector encoding a C-terminal StrepTagII tagged human Cdc6 gene.

    Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR1A HsCdc6-strep via LR Gateway reaction.

15. pDEST40 myc-cdc6

Description: Expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene.

Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR3C myc-cdc6 via LR Gateway reaction.

16. pDEST40 myc-cdc6 K57R

Description: Expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K57R mutation.

Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR3C myc-cdc6 K57R via LR Gateway reaction.

17. pDEST40 myc-cdc6 K57Q

Description: Expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K57Q mutation.

Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR3C myc-cdc6 K57Q via LR Gateway reaction.

18. pDEST40 myc-cdc6 K531R

Description: Expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K531R mutation.

Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR3C myc-cdc6 K531R via LR Gateway reaction.

19. pDEST40 myc-cdc6 K531Q

Description: Expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K531Q mutation.

Strategy: Tagged Cdc6 was moved into pDEST40 from pENTR3C myc-cdc6 K531Q via LR Gateway reaction.

20. pNTAP3C HsCdc6

    Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene.

    Strategy: HsCdc6 was cut from pENTR1A HsCdc6 (BamHI & XhoI) and ligated into pNTAP3C (BamHI & XhoI).

21. pNTAP3C HsCdc6#

    Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin.

    Strategy: Resistant Cdc6 was created using the QuikChange mutagenesis kit (primers: D5 muta #2, D5 muta 3').

22. pNTAP3C cdc6 K57R

    Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene containing the K57R mutation.

    Strategy: Cdc6 K57R was cut from pDEST40 myc-cdc6 K57R (BamHI & EcoRI) and ligated into pNTAP3C (BamHI & EcoRI).

23. pNTAP3C cdc6 K57R#

    Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the K57R mutation.

    Strategy: Resistant Cdc6 was created using the QuikChange mutagenesis kit (primers: D5 muta #2, D5 muta 3').

24. pNTAP3C cdc6 K57Q

Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene containing the K57Q mutation.

Strategy: Cdc6 K57Q was cut from pDEST40 myc-cdc6 K57Q (BamHI & EcoRI) and ligated into pNTAP3C (BamHI & EcoRI).

25. pNTAP3C cdc6 K57Q#

Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the K57Q mutation.

Strategy: Resistant Cdc6 was created using the QuikChange mutagenesis kit (primers: D5 muta #2, D5 muta 3').

26. pNTAP3C cdc6 K531R

Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene containing the K531R mutation.

Strategy: Cdc6 K531R was cut from pDEST40 myc-cdc6 K531R (EcoRI & HindIII) and ligated into pNTAP3C (EcoRI & HindIII).

27. pNTAP3C cdc6 K531R#

Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the K531R mutation.

Strategy: Resistant portion of Cdc6 was cut from pNTAP3C HsCdc6# (BamHI & EcoRI) and ligated into pNTAP3C cdc6 K531R (BamHI & EcoRI).

28. pNTAP3C cdc6 K531Q

Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene containing the K531Q mutation.

Strategy: Cdc6 K531Q was cut from pDEST40 myc-cdc6 K531Q (EcoRI & HindIII) and ligated into pNTAP3C (EcoRI & HindIII).

29. pNTAP3C cdc6 K531Q#

Description: Expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin containing the K531Q mutation.

Strategy: Resistant portion of Cdc6 was cut from pNTAP3C HsCdc6# (BamHI & EcoRI) and ligated into pNTAP3C cdc6 K531Q (BamHI & EcoRI).

Viral vectors:

30. pAD-DEST sbp-cdc6#

Description: Adenoviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin.

Strategy: Tagged Cdc6# was moved into pAD-DEST/CMV/V5 from pENTR2B sbp-cdc6# via LR Gateway reaction.

31. pBABEhy MmCdc6-GFP

Description: Retroviral expression vector encoding a C-terminal Green Fluorescence Protein (GFP) tagged mouse Cdc6 gene.

Strategy: MmCdc6 was cut from pLL5.0x MmCdc6 (ClaI) and ligated into pBABEhy [452].

32. pBABEhy MmCdc6 GFP IRES

Description: Retroviral expression vector encoding a mouse Cdc6 gene and GFP, separated by an internal ribosomal entry site [117].

Strategy: MmCdc6 GFP IRES was cut from pLL5.0x MmCdc6 GFP IRES (ClaI) and ligated into pBABEhy [452].

33. pBABEpu myc-cdc6-strep

Description: Retroviral expression vector encoding an N-terminal 5x myc and a C-terminal StrepTagII tagged human Cdc6 gene.

Strategy: Tagged Cdc6 was cut from pcDNA3 myc-cdc6-strep (KpnI & XhoI, blunted) and ligated into pBABEpu (EcoRI, blunted).

34. pBABEpu sbp-cdc6#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin.

Strategy: Tagged Cdc6# was cut from pNTAP3C sbp-cdc6# (NotI & PvuI, blunted) and ligated into pBABEpu (EcoRI, blunted).

35. pCLXSN myc-cdc6 K57R

Description: Retroviral expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K57R mutation.

Strategy: Tagged Cdc6 K57R was cut from pDEST40 myc-cdc6 K57R (KpnI & NotI, blunted) and ligated into pCLXSN (XhoI, blunted).

36. pCLXSN myc-cdc6 K57Q

Description: Retroviral expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K57Q mutation.

Strategy: Tagged Cdc6 K57Q was cut from pDEST40 myc-cdc6 K57Q (KpnI & NotI, blunted) and ligated into pCLXSN (XhoI, blunted).

37. pCLXSN myc-cdc6 K531R

Description: Retroviral expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K531R mutation.

Strategy: Tagged Cdc6 K531R was cut from pDEST40 myc-cdc6 K531R (KpnI & NotI, blunted) and ligated into pCLXSN (XhoI, blunted).

38. pCLXSN myc-cdc6 K531Q

Description: Retroviral expression vector encoding an N-terminal 5x myc tagged human Cdc6 gene containing the K531Q mutation.

Strategy: Tagged Cdc6 K531Q was cut from pDEST40 myc-cdc6 K531Q (KpnI & NotI, blunted) and ligated into pCLXSN (XhoI, blunted).

39. pCLXSN sbp-cdc6

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene.

Strategy: Tagged Cdc6 was cut from pNTAP3C HsCdc6 (SacII & XhoI, blunted) and ligated into pCLXSN (XhoI, blunted).

40. pCLXSN sbp-cdc6#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin.

Strategy: Tagged Cdc6# was cut from pNTAP3C HsCdc6# (PmlI & AleI) and ligated into pCLXSN sbp-cdc6 K57Q# (PmlI & AleI).

41. pCLXSN sbp-cdc6 S45A#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S45A mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S45A muta R, S45 3'R #2). PCR was cut (DraIII) and ligated into pCLXSN sbp-cdc6# (DraIII).

42. pCLXSN sbp-cdc6 S45D#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S45D mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S45D muta R, S45 3'R #2). PCR was cut (DraIII) and ligated into pCLXSN sbp-cdc6# (DraIII).

43. pCLXSN sbp-cdc6 S54A#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S54A mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S54A muta R, S45 3'R #2). PCR was cut (EcoRI) and ligated into pCLXSN sbp-cdc6# (EcoRI).

44. pCLXSN sbp-cdc6 S54D#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S54D mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S54D muta R, S45 3'R #2). PCR was cut (EcoRI) and ligated into pCLXSN sbp-cdc6# (EcoRI).

45. pCLXSN sbp-cdc6 K57R#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the K57R mutation.

Strategy: Cdc6 K57R# was cut from pNTAP3C cdc6 K57R# (PmlI & AleI) and ligated into pCLXSN sbp-cdc6 K57Q# (PmlI & AleI).

46. pCLXSN sbp-cdc6 K57Q#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the K57Q mutation.

Strategy: Cdc6 K57Q# was cut from pNTAP3C cdc6 K57Q# (NotI & PvuI, blunted) and ligated into pCLXSN (XhoI, blunted).

47. pCLXSN sbp-cdc6 S74A#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S74A mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S74A muta R, S45 3'R #2). PCR was cut (EcoRI) and ligated into pCLXSN sbp-cdc6# (EcoRI).

48. pCLXSN sbp-cdc6 S74D#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S74D mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S74D muta R, S45 3'R #2). PCR was cut (EcoRI) and ligated into pCLXSN sbp-cdc6# (EcoRI).

49. pCLXSN sbp-cdc6 S106A#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S106A mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S106A muta R, S45 3'R #2). PCR was cut (EcoRI) and ligated into pCLXSN sbp-cdc6# (EcoRI).

50. pCLXSN sbp-cdc6 S106D#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the S106D mutation.

Strategy: Mutation was created by megaprimer PCR (primers: cdc6 S45 F, S106D muta R, S45 3'R #2). PCR was cut (EcoRI) and ligated into pCLXSN sbp-cdc6# (EcoRI).

51. pCLXSN sbp-cdc6 K531R#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the K531R mutation.

Strategy: Cdc6 K531R# was cut from pNTAP3C cdc6 K531R# (NotI & PvuI, blunted) and ligated into pCLXSN (XhoI, blunted).

52. pCLXSN sbp-cdc6 K531Q#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin, containing the K531Q mutation.

Strategy: Cdc6 K531Q# was cut from pNTAP3C cdc6 K531Q# (NotI & PvuI, blunted) and ligated into pCLXSN (XhoI, blunted).

53. pCLXSN PIP-sbp-cdc6#

Description: Retroviral expression vector encoding an N-terminal PIP motif (from Cdt1), SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin.

Strategy: HsCdc6# was cut from pNTAP3C HsCdc6# (PvuI-blunted & SacII) and ligated into pCLXSN PIP (XhoI-blunted & SacII).

54. pCLXSN PIPm-sbp-cdc6#

Description: Retroviral expression vector encoding an N-terminal mutated PIP motif, SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin.

Strategy: HsCdc6# was cut from pNTAP3C HsCdc6# (PvuI-blunted & SacII) and ligated into pCLXSN PIPm (XhoI-blunted & SacII).

Note: Sequencing confirmed that the PIP motif is not actually mutated.

55. pLL5.0x MmCdc6-GFP

Description: Lentiviral expression vector encoding a C-terminal GFP tagged mouse Cdc6 gene.

Strategy: MmCdc6 was amplified from pcDNA3 MmCdc6 (primers: SacII MmCdc6, BamHI MmCdc6). PCR was cut (BamHI & SacII) and ligated into pLL5.0x (BamHI & SacII).

56. pLL5.0x MmCdc6 IRES

Description: Lentiviral expression vector encoding the mouse Cdc6 gene and GFP, separated by an IRES.

Strategy: IRES was amplified from pLL5.5x (primers: BamHI IRES R, SphI stop IRES F). PCR was cut (SphI & BamHI) and ligated into pLL5.0x MmCdc6-GFP (SphI & BamHI).

57. pLXIN sbp-cdc6#

Description: Retroviral expression vector encoding an N-terminal SBP-CBP tagged human Cdc6 gene, resistant to the D5 hairpin and the neomycin gene separated by an IRES.

Strategy: Sbp-cdc6# was cut from pNTAP3C HsCdc6# (NotI & PvuI, blunted) and ligated into pLXIN (XhoI, blunted).

Cdt1 Plasmids:

58. pLL5.0x HA-Cdt1*

Description: Lentiviral expression vector encoding an N-terminal 2x HA tagged human Cdt1 gene, resistant to siCdt1.

Strategy: HA-Cdt1* was amplified from pENTR3C HA2 HsCdt1* (primers: 5' HA Cdt1, 3' HA Cdt1). PCR was cut (EcoRV) and ligated into pLL5.0x (SacII, blunted).

59. pLL5.0x PIPm-Cdt1*-V5

Description: Lentiviral expression vector encoding the human Cdt1 gene with a mutated PIP motif, resistant to siCdt1.

Strategy: PIPm-Cdt1*-V5 was amplified from pDEST40 PIPm-Cdt1*-V5 (primers: 5' PIPm-Cdt1, 3' PIPm-Cdt1). PCR was cut (HpaI) and ligated into pLL5.0x (SacII, blunted).

Note: Sequencing confirmed that the PIP motif is not actually mutated.

Splicing Reporter Plasmids:

60. pENTR2B C

Description: Entry vector encoding the splicing reporter construct C (50% inclusion).

Strategy: C construct was amplified from pI-12 C (primers: SalI C5'F, SalI C3'R).

61. pENTR2B 15d

Description: Entry vector encoding the splicing reporter construct 15d (10% inclusion).

Strategy: 15d construct was amplified from pI-12 15d (primers: SalI C5'F, SalI C3'R).

62. pENTR2B 5' consensus

Description: Entry vector encoding the splicing reporter 5' consensus (100% inclusion).

Strategy: 5' consensus was amplified from pI-12 5' consensus (primers: SalI C5'F, SalI C3'R).

63. pENTR2B ISS-KK5

Description: Entry vector encoding the GFP splicing reporter sensitive to SF2.

Strategy: ISS-KK5 was cut from pZW2C KK5 (BamHi-blunted & NheI) and ligated into pENTR2B (XbaI & EcoRV).

64. pENTR2B ISS-KK6

Description: Entry vector encoding the GFP splicing reporter sensitive to hnRNPA3.

Strategy: ISS-KK6 was cut from pZW2C KK6 (BamHI-blunted & NheI) and ligated into pENTR2B (XbaI & EcoRV).

65. pLX302 C

Description: Lentiviral expression vector encoding the splicing reporter construct C (50% inclusion).

Strategy: C was moved into pLX302 from pENTR2B C via LR Gateway reaction.

66. pLX302 15d

Description: Lentiviral expression vector encoding the splicing reporter construct 15d (10% inclusion).

Strategy: 15d was moved into pLX302 from pENTR2B 15d via LR Gateway reaction.

67. pLX302 5’ consensus

Description: Lentiviral expression vector encoding the splicing reporter construct 5’ consensus (100% inclusion).

Strategy: 5’ consensus was moved into pLX302 from pENTR2B 5’ consensus via LR Gateway reaction.

68. pLX302 ISS-KK5

Description: Lentiviral expression vector encoding the GFP splicing reporter sensitive to SF2.

Strategy: ISS-KK5 was moved into pLX302 from pENTR2B ISS-KK5 via LR Gateway reaction.

69. pLX302 ISS-KK6

Description: Lentiviral expression vector encoding the GFP splicing reporter sensitive to hnRNPA3.

Strategy: ISS-KK6 was moved into pLX302 from pENTR2B ISS-KK6 via LR Gateway reaction.

Other plasmids:

70. pCLXSN PIP

Description: Retroviral expression vector encoding the PIP motif from the human Cdt1 gene.

Strategy: PIP motif was amplified from pENTR3C Cdt1 5SA (primers: Cdt1 PIP 5', Cdt1 PIP linker 3'). PCR was cut (EcoRI & XhoI) and ligated into pCLXSN (EcoRI & XhoI).

71. pCLXSN PIPm

Description: Retroviral expression vector encoding a mutated PIP motif from the human Cdt1 gene.

Strategy: PIPm was amplified from pENTR3C Cdt1 PIPm (primers: Cdt1 PIP 5', Cdt1 PIP linker 3'). PCR was cut (EcoRI & XhoI) and ligated into pCLXSN (EcoRI & XhoI).

Note: Sequencing confirmed that the PIP motif is not actually mutated.

References

1. Bicknell LS, Bongers EM, Leitch A, Brown S, Schoots J, et al. (2011) Mutations in the pre-replication complex cause Meier-Gorlin syndrome. Nat Genet 43: 356-359.

2. Arentson E, Faloon P, Seo J, Moon E, Studts JM, et al. (2002) Oncogenic potential of the DNA replication licensing protein CDT1. Oncogene 21: 1150-1158.

3. Karakaidos P, Taraviras S, Vassiliou LV, Zacharatos P, Kastrinakis NG, et al. (2004) Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability--evidence of E2F-1 transcriptional control over hCdt1. Am J Pathol 165: 1351-1365.

4. Xouri G, Lygerou Z, Nishitani H, Pachnis V, Nurse P, et al. (2004) Cdt1 and geminin are down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines. Eur J Biochem 271: 3368-3378.

5. Liontos M, Koutsami M, Sideridou M, Evangelou K, Kletsas D, et al. (2007) Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. Cancer Res 67: 10899-10909.

6. Dhar SK, Yoshida K, Machida Y, Khaira P, Chaudhuri B, et al. (2001) Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. Cell 106: 287-296.

7. Vashee S, Simancek P, Challberg MD, Kelly TJ (2001) Assembly of the human origin recognition complex. J Biol Chem 276: 26666-26673.

8. Bowers JL, Randell JC, Chen S, Bell SP (2004) ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. Mol Cell 16: 967-978.

9. Mendez J, Zou-Yang XH, Kim SY, Hidaka M, Tansey WP, et al. (2002) Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell 9: 481-491.

10. Cocker JH, Piatti S, Santocanale C, Nasmyth K, Diffley JF (1996) An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. Nature 379: 180-182.

11. Nishitani H, Lygerou Z, Nishimoto T, Nurse P (2000) The Cdt1 protein is required to license DNA for replication in fission yeast. Nature 404: 625-628.

12. Hartwell LH (1976) Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. J Mol Biol 104: 803-817.

13. Zwerschke W, Rottjakob HW, Kuntzel H (1994) The Saccharomyces cerevisiae CDC6 gene is transcribed at late mitosis and encodes a ATP/GTPase controlling S phase initiation. J Biol Chem 269: 23351-23356.

14. Piatti S, Lengauer C, Nasmyth K (1995) Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae. Embo J 14: 3788-3799.

15. Gaudier M, Schuwirth BS, Westcott SL, Wigley DB (2007) Structural basis of DNA replication origin recognition by an ORC protein. Science 317: 1213-1216.

16. Liu J, Smith CL, DeRyckere D, DeAngelis K, Martin GS, et al. (2000) Structure and function of Cdc6/Cdc18: implications for origin recognition and checkpoint control. Mol Cell 6: 637-648.

17. Randell JC, Bowers JL, Rodriguez HK, Bell SP (2006) Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Mol Cell 21: 29-39.

18. Weinreich M, Liang C, Stillman B (1999) The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. Proc Natl Acad Sci USA 96: 441-446.

19. Herbig U, Marlar CA, Fanning E (1999) The Cdc6 nucleotide-binding site regulates its activity in DNA replication in human cells. Mol Biol Cell 10: 2631-2645.

20. Donovan S, Harwood J, Drury LS, Diffley JF (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. Proc Natl Acad Sci U S A 94: 5611-5616.

21. Cook JG, Park CH, Burke TW, Leone G, DeGregori J, et al. (2002) Analysis of Cdc6 function in the assembly of mammalian prereplication complexes. Proc Natl Acad Sci USA 99: 1347-1352.

22. Mizushima T, Takahashi N, Stillman B (2000) Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. Genes Dev 14: 1631-1641.

23. Hofmann JF, Beach D (1994) cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. Embo J 13: 425-434.

24. Tanaka S, Diffley JF (2002) Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. Genes Dev 16: 2639-2649.

25. Cook JG, Chasse DA, Nevins JR (2004) The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells. J Biol Chem 279: 9625-9633.

26. Bruschi CV, McMillan JN, Coglievina M, Esposito MS (1995) The genomic instability of yeast cdc6-1/cdc6-1 mutants involves chromosome structure and recombination. Mol Gen Genet 249: 8-18.

27. Chen S, Bell SP (2011) CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6. Genes Dev 25: 363-372.

28. Tsuyama T, Tada S, Watanabe S, Seki M, Enomoto T (2005) Licensing for DNA replication requires a strict sequential assembly of Cdc6 and Cdt1 onto chromatin in Xenopus egg extracts. Nucleic Acids Res 33: 765-775.

29. Rowles A, Tada S, Blow JJ (1999) Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. J Cell Sci 112 (Pt 12): 2011-2018.

30. Maiorano D, Moreau J, Mechali M (2000) XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. Nature 404: 622-625.

31. Evrin C, Clarke P, Zech J, Lurz R, Sun J, et al. (2009) A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. Proc Natl Acad Sci U S A 106: 20240-20245.

32. Remus D, Beuron F, Tolun G, Griffith JD, Morris EP, et al. (2009) Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell 139: 719-730.

33. Edwards MC, Tutter AV, Cvetic C, Gilbert CH, Prokhorova TA, et al. (2002) MCM2-7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in Xenopus egg extracts. J Biol Chem 277: 33049-33057.

34. Lei M, Kawasaki Y, Tye BK (1996) Physical interactions among Mcm proteins and effects of Mcm dosage on DNA replication in Saccharomyces cerevisiae. Mol Cell Biol 16: 5081-5090.

35. Chen Z, Speck C, Wendel P, Tang C, Stillman B, et al. (2008) The architecture of the DNA replication origin recognition complex in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 105: 10326-10331.

36. Speck C, Chen Z, Li H, Stillman B (2005) ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. Nat Struct Mol Biol 12: 965-971.

37. Krude T, Musahl C, Laskey RA, Knippers R (1996) Human replication proteins hCdc21, hCdc46 and P1Mcm3 bind chromatin uniformly before S-phase and are displaced locally during DNA replication. J Cell Sci 109 (Pt 2): 309-318.

38. Dimitrova DS, Todorov IT, Melendy T, Gilbert DM (1999) Mcm2, but not RPA, is a component of the mammalian early G1-phase prereplication complex. J Cell Biol 146: 709-722.

39. Ge XQ, Blow JJ (2010) Chk1 inhibits replication factory activation but allows dormant origin firing in existing factories. J Cell Biol 191: 1285-1297.

40. Petersen BO, Wagener C, Marinoni F, Kramer ER, Melixetian M, et al. (2000) Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev 14: 2330-2343.

41. Li CJ, DePamphilis ML (2002) Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. Mol Cell Biol 22: 105-116.

42. Li CJ, Vassilev A, DePamphilis ML (2004) Role for Cdk1 (Cdc2)/cyclin A in preventing the mammalian origin recognition complex's largest subunit (Orc1) from binding to chromatin during mitosis. Mol Cell Biol 24: 5875-5886.

43. Diffley JF (2004) Regulation of early events in chromosome replication. Curr Biol 14: 778-786.

44. Lin DI, Aggarwal P, Diehl JA (2008) Phosphorylation of MCM3 on Ser-112 regulates its incorporation into the MCM2-7 complex. Proc Natl Acad Sci USA 105: 8079-8084.

45. Chuang LC, Teixeira LK, Wohlschlegel JA, Henze M, Yates JR, et al. (2009) Phosphorylation of Mcm2 by Cdc7 promotes pre-replication complex assembly during cell-cycle re-entry. Mol Cell 35: 206-216.

46. Malumbres M, Barbacid M (2009) Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 9: 153-166.

47. Braden WA, McClendon AK, Knudsen ES (2008) Cyclin-dependent kinase 4/6 activity is a critical determinant of pre-replication complex assembly. Oncogene 27: 7083-7093.

48. van den Heuvel S, Harlow E (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262: 2050-2054.

49. Wheeler LW, Lents NH, Baldassare JJ (2008) Cyclin A-CDK activity during G1 phase impairs MCM chromatin loading and inhibits DNA synthesis in mammalian cells. Cell Cycle 7: 2179-2188.

50. Bochman ML, Schwacha A (2009) The Mcm complex: unwinding the mechanism of a replicative helicase. Microbiol Mol Biol Rev 73: 652-683.

51. Ekholm-Reed S, Mendez J, Tedesco D, Zetterberg A, Stillman B, et al. (2004) Deregulation of cyclin E in human cells interferes with prereplication complex assembly. J Cell Biol 165: 789-800.

52. Attwooll C, Lazzerini Denchi E, Helin K (2004) The E2F family: specific functions and overlapping interests. Embo J 23: 4709-4716.

53. Dimova DK, Dyson NJ (2005) The E2F transcriptional network: old acquaintances with new faces. Oncogene 24: 2810-2826.

54. Helin K, Harlow E, Fattaey A (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol Cell Biol 13: 6501-6508.

55. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR (1991) The E2F transcription factor is a cellular target for the RB protein. Cell 65: 1053-1061.

56. Bagchi S, Weinmann R, Raychaudhuri P (1991) The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. Cell 65: 1063-1072.

57. Lees JA, Saito M, Vidal M, Valentine M, Look T, et al. (1993) The retinoblastoma protein binds to a family of E2F transcription factors. Mol Cell Biol 13: 7813-7825.

58. Cam H, Dynlacht BD (2003) Emerging roles for E2F: beyond the G1/S transition and DNA replication. Cancer Cell 3: 311-316.

59. Cobrinik D (2005) Pocket proteins and cell cycle control. Oncogene 24: 2796-2809.

60. Weintraub SJ, Prater CA, Dean DC (1992) Retinoblastoma protein switches the E2F site from positive to negative element. Nature 358: 259-261.

61. Beijersbergen RL, Kerkhoven RM, Zhu L, Carlee L, Voorhoeve PM, et al. (1994) E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev 8: 2680-2690.

62. Ginsberg D, Vairo G, Chittenden T, Xiao ZX, Xu G, et al. (1994) E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev 8: 2665-2679.

63. Hijmans EM, Voorhoeve PM, Beijersbergen RL, van 't Veer LJ, Bernards R (1995) E2F-5, a new E2F family member that interacts with p130 in vivo. Mol Cell Biol 15: 3082-3089.

64. Vairo G, Livingston DM, Ginsberg D (1995) Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev 9: 869-881.

65. de la Luna S, Burden MJ, Lee CW, La Thangue NB (1996) Nuclear accumulation of the E2F heterodimer regulated by subunit composition and alternative splicing of a nuclear localization signal. J Cell Sci 109 (Pt 10): 2443-2452.

66. Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81: 323-330.

67. Yao G, Lee TJ, Mori S, Nevins JR, You L (2008) A bistable Rb-E2F switch underlies the restriction point. Nat Cell Biol 10: 476-482.

68. Buchkovich K, Duffy LA, Harlow E (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58: 1097-1105.

69. Chen PL, Scully P, Shew JY, Wang JY, Lee WH (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58: 1193-1198.

70. DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, et al. (1989) The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell 58: 1085-1095.

71. Mihara K, Cao XR, Yen A, Chandler S, Driscoll B, et al. (1989) Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science 246: 1300-1303.

72. Claudio PP, De Luca A, Howard CM, Baldi A, Firpo EJ, et al. (1996) Functional analysis of pRb2/p130 interaction with cyclins. Cancer Res 56: 2003-2008.

73. Smith EJ, Leone G, DeGregori J, Jakoi L, Nevins JR (1996) The accumulation of an E2F-p130 transcriptional repressor distinguishes a G0 cell state from a G1 cell state. Mol Cell Biol 16: 6965-6976.

74. Dowdy SF, Hinds PW, Louie K, Reed SI, Arnold A, et al. (1993) Physical interaction of the retinoblastoma protein with human D cyclins. Cell 73: 499-511.

75. Mudryj M, Hiebert SW, Nevins JR (1990) A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. Embo J 9: 2179-2184.

76. Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ (2000) Target gene specificity of E2F and pocket protein family members in living cells. Mol Cell Biol 20: 5797-5807.

77. Takahashi Y, Rayman JB, Dynlacht BD (2000) Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev 14: 804-816.

78. De Luca A, MacLachlan TK, Bagella L, Dean C, Howard CM, et al. (1997) A unique domain of pRb2/p130 acts as an inhibitor of Cdk2 kinase activity. J Biol Chem 272: 20971-20974.

79. Young AP, Longmore GD (2004) Differential regulation of apoptotic genes by Rb in human versus mouse cells. Oncogene 23: 2587-2599.

80. Hiebert SW, Chellappan SP, Horowitz JM, Nevins JR (1992) The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev 6: 177-185.

81. Flemington EK, Speck SH, Kaelin WG, Jr. (1993) E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc Natl Acad Sci U S A 90: 6914-6918.

82. Zamanian M, La Thangue NB (1993) Transcriptional repression by the Rb-related protein p107. Mol Biol Cell 4: 389-396.

83. Johnson DG, Schwarz JK, Cress WD, Nevins JR (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature 365: 349-352.

84. Shan B, Lee WH (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol Cell Biol 14: 8166-8173.

85. Qin XQ, Livingston DM, Kaelin WG, Jr., Adams PD (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc Natl Acad Sci U S A 91: 10918-10922.

86. Kowalik TF, DeGregori J, Schwarz JK, Nevins JR (1995) E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J Virol 69: 2491-2500.

87. Sun B, Wingate H, Swisher SG, Keyomarsi K, Hunt KK (2010) Absence of pRb facilitates E2F1-induced apoptosis in breast cancer cells. Cell Cycle 9: 1122-1130.

88. Adams PD, Li X, Sellers WR, Baker KB, Leng X, et al. (1999) Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-cdk complexes. Mol Cell Biol 19: 1068-1080.

89. Knudsen ES, Wang JY (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol Cell Biol 17: 5771-5783.

90. Brown VD, Phillips RA, Gallie BL (1999) Cumulative effect of phosphorylation of pRB on regulation of E2F activity. Mol Cell Biol 19: 3246-3256.

91. Lundberg AS, Weinberg RA (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 18: 753-761.

92. Tedesco D, Lukas J, Reed SI (2002) The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2). Genes Dev 16: 2946-2957.

93. Bhattacharya S, Garriga J, Calbo J, Yong T, Haines DS, et al. (2003) SKP2 associates with p130 and accelerates p130 ubiquitylation and degradation in human cells. Oncogene 22: 2443-2451.

94. Kalejta RF, Shenk T (2003) Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein. Proc Natl Acad Sci U S A 100: 3263-3268.

95. Ma D, Zhou P, Harbour JW (2003) Distinct mechanisms for regulating the tumor suppressor and antiapoptotic functions of Rb. J Biol Chem 278: 19358-19366.

96. Ludlow JW, Glendening CL, Livingston DM, DeCarprio JA (1993) Specific enzymatic dephosphorylation of the retinoblastoma protein. Mol Cell Biol 13: 367-372.

97. Nelson DA, Krucher NA, Ludlow JW (1997) High molecular weight protein phosphatase type 1 dephosphorylates the retinoblastoma protein. J Biol Chem 272: 4528-4535.

98. Yan Y, Mumby MC (1999) Distinct roles for PP1 and PP2A in phosphorylation of the retinoblastoma protein. PP2a regulates the activities of G(1) cyclin-dependent kinases. J Biol Chem 274: 31917-31924.

99. Cicchillitti L, Fasanaro P, Biglioli P, Capogrossi MC, Martelli F (2003) Oxidative stress induces protein phosphatase 2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. J Biol Chem 278: 19509-19517.

100. Dynlacht BD, Brook A, Dembski M, Yenush L, Dyson N (1994) DNA-binding and trans-activation properties of Drosophila E2F and DP proteins. Proc Natl Acad Sci U S A 91: 6359-6363.

101. Dynlacht BD, Flores O, Lees JA, Harlow E (1994) Differential regulation of E2F transactivation by cyclin/cdk2 complexes. Genes Dev 8: 1772-1786.

102. Hamel PA, Gill RM, Phillips RA, Gallie BL (1992) Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene. Mol Cell Biol 12: 3431-3438.

103. Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, et al. (1995) E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc Natl Acad Sci U S A 92: 2403-2407.

104. Moberg K, Starz MA, Lees JA (1996) E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol Cell Biol 16: 1436-1449.

105. Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, et al. (2000) E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. Mol Cell 6: 729-735.

106. Muller H, Moroni MC, Vigo E, Petersen BO, Bartek J, et al. (1997) Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol Cell Biol 17: 5508-5520.

107. Chestukhin A, Litovchick L, Rudich K, DeCaprio JA (2002) Nucleocytoplasmic shuttling of p130/RBL2: novel regulatory mechanism. Mol Cell Biol 22: 453-468.

108. Magae J, Wu CL, Illenye S, Harlow E, Heintz NH (1996) Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. J Cell Sci 109 (Pt 7): 1717-1726.

109. Puri PL, Cimino L, Fulco M, Zimmerman C, La Thangue NB, et al. (1998) Regulation of E2F4 mitogenic activity during terminal differentiation by its heterodimerization partners for nuclear translocation. Cancer Res 58: 1325-1331.

110. Macaluso M, Montanari M, Giordano A (2006) Rb family proteins as modulators of gene expression and new aspects regarding the interaction with chromatin remodeling enzymes. Oncogene 25: 5263-5267.

111. Gunawardena RW, Siddiqui H, Solomon DA, Mayhew CN, Held J, et al. (2004) Hierarchical requirement of SWI/SNF in retinoblastoma tumor suppressor-mediated repression of Plk1. J Biol Chem 279: 29278-29285.

112. Ferreira R, Magnaghi-Jaulin L, Robin P, Harel-Bellan A, Trouche D (1998) The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc Natl Acad Sci U S A 95: 10493-10498.

113. Stiegler P, De Luca A, Bagella L, Giordano A (1998) The COOH-terminal region of pRb2/p130 binds to histone deacetylase 1 (HDAC1), enhancing transcriptional repression of the E2F-dependent cyclin A promoter. Cancer Res 58: 5049-5052.

114. Iavarone A, Massague J (1999) E2F and histone deacetylase mediate transforming growth factor beta repression of cdc25A during keratinocyte cell cycle arrest. Mol Cell Biol 19: 916-922.

115. Luo RX, Postigo AA, Dean DC (1998) Rb interacts with histone deacetylase to repress transcription. Cell 92: 463-473.

116. Dahiya A, Wong S, Gonzalo S, Gavin M, Dean DC (2001) Linking the Rb and polycomb pathways. Mol Cell 8: 557-569.

117. Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, et al. (2001) Rb targets histone H3 methylation and HP1 to promoters. Nature 412: 561-565.

118. Zhang HS, Gavin M, Dahiya A, Postigo AA, Ma D, et al. (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell 101: 79-89.

119. Markey M, Siddiqui H, Knudsen ES (2004) Geminin is targeted for repression by the retinoblastoma tumor suppressor pathway through intragenic E2F sites. J Biol Chem 279: 29255-29262.

120. Chabes AL, Bjorklund S, Thelander L (2004) S Phase-specific transcription of the mouse ribonucleotide reductase R2 gene requires both a proximal repressive E2F-binding site and an upstream promoter activating region. J Biol Chem 279: 10796-10807.

121. de Bruin A, Maiti B, Jakoi L, Timmers C, Buerki R, et al. (2003) Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem 278: 42041-42049.

122. Di Stefano L, Jensen MR, Helin K (2003) E2F7, a novel E2F featuring DP-independent repression of a subset of E2F-regulated genes. Embo J 22: 6289-6298.

123. Logan N, Delavaine L, Graham A, Reilly C, Wilson J, et al. (2004) E2F-7: a distinctive E2F family member with an unusual organization of DNA-binding domains. Oncogene 23: 5138-5150.

124. Logan N, Graham A, Zhao X, Fisher R, Maiti B, et al. (2005) E2F-8: an E2F family member with a similar organization of DNA-binding domains to E2F-7. Oncogene 24: 5000-5004.

125. Christensen J, Cloos P, Toftegaard U, Klinkenberg D, Bracken AP, et al. (2005) Characterization of E2F8, a novel E2F-like cell-cycle regulated repressor of E2F-activated transcription. Nucleic Acids Res 33: 5458-5470.

126. Maiti B, Li J, de Bruin A, Gordon F, Timmers C, et al. (2005) Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem 280: 18211-18220.

127. Kosugi S, Ohashi Y (2002) E2Ls, E2F-like repressors of Arabidopsis that bind to E2F sites in a monomeric form. J Biol Chem 277: 16553-16558.

128. Mariconti L, Pellegrini B, Cantoni R, Stevens R, Bergounioux C, et al. (2002) The E2F family of transcription factors from Arabidopsis thaliana. Novel and conserved components of the retinoblastoma/E2F pathway in plants. J Biol Chem 277: 9911-9919.

129. Vandepoele K, Raes J, De Veylder L, Rouze P, Rombauts S, et al. (2002) Genome-wide analysis of core cell cycle genes in Arabidopsis. Plant Cell 14: 903-916.

130. Li J, Ran C, Li E, Gordon F, Comstock G, et al. (2008) Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. Dev Cell 14: 62-75.

131. Ohta T, Xiong Y (2001) Phosphorylation- and Skp1-independent in vitro ubiquitination of E2F1 by multiple ROC-cullin ligases. Cancer Res 61: 1347-1353.

132. Hofmann F, Martelli F, Livingston DM, Wang Z (1996) The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. Genes Dev 10: 2949-2959.

133. Campanero MR, Flemington EK (1997) Regulation of E2F through ubiquitin-proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. Proc Natl Acad Sci U S A 94: 2221-2226.

134. Zalmas LP, Zhao X, Graham AL, Fisher R, Reilly C, et al. (2008) DNA-damage response control of E2F7 and E2F8. EMBO Rep 9: 252-259.

135. Moon NS, Dyson N (2008) E2F7 and E2F8 keep the E2F family in balance. Dev Cell 14: 1-3.

136. Frolov MV, Dyson NJ (2004) Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J Cell Sci 117: 2173-2181.

137. Hurford RK, Jr., Cobrinik D, Lee MH, Dyson N (1997) pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev 11: 1447-1463.

138. Herrera RE, Sah VP, Williams BO, Makela TP, Weinberg RA, et al. (1996) Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. Mol Cell Biol 16: 2402-2407.

139. Wu L, Timmers C, Maiti B, Saavedra HI, Sang L, et al. (2001) The E2F1-3 transcription factors are essential for cellular proliferation. Nature 414: 457-462.

140. Magnaghi-Jaulin L, Groisman R, Naguibneva I, Robin P, Lorain S, et al. (1998) Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature 391: 601-605.

141. Murga M, Fernandez-Capetillo O, Field SJ, Moreno B, Borlado LR, et al. (2001) Mutation of E2F2 in mice causes enhanced T lymphocyte proliferation, leading to the development of autoimmunity. Immunity 15: 959-970.

142. Zhu JW, Field SJ, Gore L, Thompson M, Yang H, et al. (2001) E2F1 and E2F2 determine thresholds for antigen-induced T-cell proliferation and suppress tumorigenesis. Mol Cell Biol 21: 8547-8564.

143. Chong JL, Tsai SY, Sharma N, Opavsky R, Price R, et al. (2009) E2f3a and E2f3b contribute to the control of cell proliferation and mouse development. Mol Cell Biol 29: 414-424.

144. Chong JL, Wenzel PL, Saenz-Robles MT, Nair V, Ferrey A, et al. (2009) E2f1-3 switch from activators in progenitor cells to repressors in differentiating cells. Nature 462: 930-934.

145. Rayman JB, Takahashi Y, Indjeian VB, Dannenberg JH, Catchpole S, et al. (2002) E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev 16: 933-947.

146. Marti A, Wirbelauer C, Scheffner M, Krek W (1999) Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat Cell Biol 1: 14-19.

147. Li Z, Kreutzer M, Mikkat S, Mise N, Glocker MO, et al. (2006) Proteomic analysis of the E2F1 response in p53-negative cancer cells: new aspects in the regulation of cell survival and death. Proteomics 6: 5735-5745.

148. Ma Y, Croxton R, Moorer RL, Jr., Cress WD (2002) Identification of novel E2F1-regulated genes by microarray. Arch Biochem Biophys 399: 212-224.

149. Young AP, Nagarajan R, Longmore GD (2003) Mechanisms of transcriptional regulation by Rb-E2F segregate by biological pathway. Oncogene 22: 7209-7217.

150. Merdzhanova G, Edmond V, De Seranno S, Van den Broeck A, Corcos L, et al. (2008) E2F1 controls alternative splicing pattern of genes involved in apoptosis through upregulation of the splicing factor SC35. Cell Death Differ 15: 1815-1823.

151. Cecchini MJ, Dick FA (2011) The biochemical basis of CDK phosphorylation-independent regulation of E2F1 by the retinoblastoma protein. Biochem J 434: 297-308.

152. Dick FA, Dyson N (2003) pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. Mol Cell 12: 639-649.

153. Calbo J, Parreno M, Sotillo E, Yong T, Mazo A, et al. (2002) G1 cyclin/cyclin-dependent kinase-coordinated phosphorylation of endogenous pocket proteins differentially regulates their interactions with E2F4 and E2F1 and gene expression. J Biol Chem 277: 50263-50274.

154. Ianari A, Natale T, Calo E, Ferretti E, Alesse E, et al. (2009) Proapoptotic function of the retinoblastoma tumor suppressor protein. Cancer Cell 15: 184-194.

155. Wells J, Yan PS, Cechvala M, Huang T, Farnham PJ (2003) Identification of novel pRb binding sites using CpG microarrays suggests that E2F recruits pRb to specific genomic sites during S phase. Oncogene 22: 1445-1460.

156. Hernando E, Nahle Z, Juan G, Diaz-Rodriguez E, Alaminos M, et al. (2004) Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature 430: 797-802.

157. Margottin-Goguet F, Hsu JY, Loktev A, Hsieh HM, Reimann JD, et al. (2003) Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. Dev Cell 4: 813-826.

158. Longworth MS, Herr A, Ji JY, Dyson NJ (2008) RBF1 promotes chromatin condensation through a conserved interaction with the Condensin II protein dCAP-D3. Genes Dev 22: 1011-1024.

159. Manning AL, Longworth MS, Dyson NJ (2010) Loss of pRB causes centromere dysfunction and chromosomal instability. Genes Dev 24: 1364-1376.

160. Coschi CH, Martens AL, Ritchie K, Francis SM, Chakrabarti S, et al. (2010) Mitotic chromosome condensation mediated by the retinoblastoma protein is tumor-suppressive. Genes Dev 24: 1351-1363.

161. Nevins JR (2001) The Rb/E2F pathway and cancer. Hum Mol Genet 10: 699-703.

162. Salgia R, Skarin AT (1998) Molecular abnormalities in lung cancer. J Clin Oncol 16: 1207-1217.

163. Gorgoulis VG, Zacharatos P, Mariatos G, Kotsinas A, Bouda M, et al. (2002) Transcription factor E2F-1 acts as a growth-promoting factor and is associated with adverse prognosis in non-small cell lung carcinomas. J Pathol 198: 142-156.

164. Foster CS, Falconer A, Dodson AR, Norman AR, Dennis N, et al. (2004) Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. Oncogene 23: 5871-5879.

165. Knudson AG, Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68: 820-823.

166. Bignon YJ, Rio P (1993) [The retinoblastoma gene: will therapeutic use of its tumor suppressive properties be possible?]. Bull Cancer 80: 704-712.

167. Qian Y, Luckey C, Horton L, Esser M, Templeton DJ (1992) Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. Mol Cell Biol 12: 5363-5372.

168. Cobrinik D, Whyte P, Peeper DS, Jacks T, Weinberg RA (1993) Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev 7: 2392-2404.

169. Feber A, Clark J, Goodwin G, Dodson AR, Smith PH, et al. (2004) Amplification and overexpression of E2F3 in human bladder cancer. Oncogene 23: 1627-1630.

170. Oeggerli M, Tomovska S, Schraml P, Calvano-Forte D, Schafroth S, et al. (2004) E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer. Oncogene 23: 5616-5623.

171. Reimer D, Sadr S, Wiedemair A, Stadlmann S, Concin N, et al. (2007) Clinical relevance of E2F family members in ovarian cancer--an evaluation in a training set of 77 patients. Clin Cancer Res 13: 144-151.

172. Bester AC, Roniger M, Oren YS, Im MM, Sarni D, et al. (2011) Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell 145: 435-446.

173. Vennstrom B, Sheiness D, Zabielski J, Bishop JM (1982) Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J Virol 42: 773-779.

174. Pelengaris S, Khan M, Evan G (2002) c-MYC: more than just a matter of life and death. Nat Rev Cancer 2: 764-776.

175. Cole MD, McMahon SB (1999) The Myc oncoprotein: a critical evaluation of transactivation and target gene regulation. Oncogene 18: 2916-2924.

176. Blackwood EM, Eisenman RN (1991) Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 251: 1211-1217.

177. Amati B, Brooks MW, Levy N, Littlewood TD, Evan GI, et al. (1993) Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell 72: 233-245.

178. Amati B, Littlewood TD, Evan GI, Land H (1993) The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. Embo J 12: 5083-5087.

179. Cawley S, Bekiranov S, Ng HH, Kapranov P, Sekinger EA, et al. (2004) Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell 116: 499-509.

180. Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, et al. (2003) A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc Natl Acad Sci U S A 100: 8164-8169.

181. Zeller KI, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV (2003) An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. Genome Biol 4: R69.

182. Lau LF, Nathans D (1987) Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc Natl Acad Sci U S A 84: 1182-1186.

183. Lanahan A, Williams JB, Sanders LK, Nathans D (1992) Growth factor-induced delayed early response genes. Mol Cell Biol 12: 3919-3929.

184. Obaya AJ, Mateyak MK, Sedivy JM (1999) Mysterious liaisons: the relationship between c-Myc and the cell cycle. Oncogene 18: 2934-2941.

185. Dang CV (1999) c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 19: 1-11.

186. Menssen A, Hermeking H (2002) Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. Proc Natl Acad Sci U S A 99: 6274-6279.

187. Osthus RC, Shim H, Kim S, Li Q, Reddy R, et al. (2000) Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem 275: 21797-21800.

188. Kim JW, Zeller KI, Wang Y, Jegga AG, Aronow BJ, et al. (2004) Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. Mol Cell Biol 24: 5923-5936.

189. Bowen H, Biggs TE, Baker ST, Phillips E, Perry VH, et al. (2002) c-Myc represses the murine Nramp1 promoter. Biochem Soc Trans 30: 774-777.

190. Wu KJ, Polack A, Dalla-Favera R (1999) Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC. Science 283: 676-679.

191. Yang BS, Geddes TJ, Pogulis RJ, de Crombrugghe B, Freytag SO (1991) Transcriptional suppression of cellular gene expression by c-Myc. Mol Cell Biol 11: 2291-2295.

192. Yang BS, Gilbert JD, Freytag SO (1993) Overexpression of Myc suppresses CCAAT transcription factor/nuclear factor 1-dependent promoters in vivo. Mol Cell Biol 13: 3093-3102.

193. Mao DY, Watson JD, Yan PS, Barsyte-Lovejoy D, Khosravi F, et al. (2003) Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. Curr Biol 13: 882-886.

194. Kim S, Li Q, Dang CV, Lee LA (2000) Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc in vivo. Proc Natl Acad Sci U S A 97: 11198-11202.

195. Poortinga G, Hannan KM, Snelling H, Walkley CR, Jenkins A, et al. (2004) MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation. Embo J 23: 3325-3335.

196. Boon K, Caron HN, van Asperen R, Valentijn L, Hermus MC, et al. (2001) N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. Embo J 20: 1383-1393.

197. O'Connell BC, Cheung AF, Simkevich CP, Tam W, Ren X, et al. (2003) A large scale genetic analysis of c-Myc-regulated gene expression patterns. J Biol Chem 278: 12563-12573.

198. McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD (1998) The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 94: 363-374.

199. Cheng SW, Davies KP, Yung E, Beltran RJ, Yu J, et al. (1999) c-MYC interacts with INI1/hSNF5 and requires the SWI/SNF complex for transactivation function. Nat Genet 22: 102-105.

200. Eberhardy SR, Farnham PJ (2002) Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. J Biol Chem 277: 40156-40162.

201. Kanazawa S, Soucek L, Evan G, Okamoto T, Peterlin BM (2003) c-Myc recruits P-TEFb for transcription, cellular proliferation and apoptosis. Oncogene 22: 5707-5711.

202. Brenner C, Deplus R, Didelot C, Loriot A, Vire E, et al. (2005) Myc represses transcription through recruitment of DNA methyltransferase corepressor. Embo J 24: 336-346.

203. Bouchard C, Dittrich O, Kiermaier A, Dohmann K, Menkel A, et al. (2001) Regulation of cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. Genes Dev 15: 2042-2047.

204. Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B (2001) Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. Genes Dev 15: 2069-2082.

205. McMahon SB, Wood MA, Cole MD (2000) The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol Cell Biol 20: 556-562.

206. Orian A, van Steensel B, Delrow J, Bussemaker HJ, Li L, et al. (2003) Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. Genes Dev 17: 1101-1114.

207. Hermeking H, Rago C, Schuhmacher M, Li Q, Barrett JF, et al. (2000) Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci U S A 97: 2229-2234.

208. Bouchard C, Thieke K, Maier A, Saffrich R, Hanley-Hyde J, et al. (1999) Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. Embo J 18: 5321-5333.

209. Perez-Roger I, Kim SH, Griffiths B, Sewing A, Land H (1999) Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). Embo J 18: 5310-5320.

210. Muller D, Bouchard C, Rudolph B, Steiner P, Stuckmann I, et al. (1997) Cdk2-dependent phosphorylation of p27 facilitates its Myc-induced release from cyclin E/cdk2 complexes. Oncogene 15: 2561-2576.

211. Leung JY, Ehmann GL, Giangrande PH, Nevins JR (2008) A role for Myc in facilitating transcription activation by E2F1. Oncogene 27: 4172-4179.

212. Ayer DE, Eisenman RN (1993) A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. Genes Dev 7: 2110-2119.

213. Alland L, Muhle R, Hou H, Jr., Potes J, Chin L, et al. (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature 387: 49-55.

214. Ayer DE, Lawrence QA, Eisenman RN (1995) Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell 80: 767-776.

215. Lee TC, Li L, Philipson L, Ziff EB (1997) Myc represses transcription of the growth arrest gene gas1. Proc Natl Acad Sci U S A 94: 12886-12891.

216. Conzen SD, Gottlob K, Kandel ES, Khanduri P, Wagner AJ, et al. (2000) Induction of cell cycle progression and acceleration of apoptosis are two separable functions of c-Myc: transrepression correlates with acceleration of apoptosis. Mol Cell Biol 20: 6008-6018.

217. Wu S, Cetinkaya C, Munoz-Alonso MJ, von der Lehr N, Bahram F, et al. (2003) Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. Oncogene 22: 351-360.

218. Seoane J, Le HV, Massague J (2002) Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature 419: 729-734.

219. Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, et al. (2001) Repression of p15INK4b expression by Myc through association with Miz-1. Nat Cell Biol 3: 392-399.

220. Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, et al. (2001) TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. Nat Cell Biol 3: 400-408.

221. Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, et al. (2000) Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. Proc Natl Acad Sci U S A 97: 3260-3265.

222. Claassen GF, Hann SR (2000) A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest. Proc Natl Acad Sci U S A 97: 9498-9503.

223. Davis AC, Wims M, Spotts GD, Hann SR, Bradley A (1993) A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev 7: 671-682.

224. Leone G, DeGregori J, Sears R, Jakoi L, Nevins JR (1997) Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. Nature 387: 422-426.

225. Nesbit CE, Tersak JM, Prochownik EV (1999) MYC oncogenes and human neoplastic disease. Oncogene 18: 3004-3016.

226. Henriksson M, Luscher B (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv Cancer Res 68: 109-182.

227. Schlagbauer-Wadl H, Griffioen M, van Elsas A, Schrier PI, Pustelnik T, et al. (1999) Influence of increased c-Myc expression on the growth characteristics of human melanoma. J Invest Dermatol 112: 332-336.

228. Spencer CA, Groudine M (1991) Control of c-myc regulation in normal and neoplastic cells. Adv Cancer Res 56: 1-48.

229. Nau MM, Brooks BJ, Battey J, Sausville E, Gazdar AF, et al. (1985) L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318: 69-73.

230. Wong AJ, Ruppert JM, Eggleston J, Hamilton SR, Baylin SB, et al. (1986) Gene amplification of c-myc and N-myc in small cell carcinoma of the lung. Science 233: 461-464.

231. Broers JL, Viallet J, Jensen SM, Pass H, Travis WD, et al. (1993) Expression of c-myc in progenitor cells of the bronchopulmonary epithelium and in a large number of non-small cell lung cancers. Am J Respir Cell Mol Biol 9: 33-43.

232. Jansen-Durr P, Meichle A, Steiner P, Pagano M, Finke K, et al. (1993) Differential modulation of cyclin gene expression by MYC. Proc Natl Acad Sci U S A 90: 3685-3689.

233. Sears R, Ohtani K, Nevins JR (1997) Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. Mol Cell Biol 17: 5227-5235.

234. Wong JV, Yao G, Nevins JR, You L (2011) Viral-mediated noisy gene expression reveals biphasic E2f1 response to MYC. Mol Cell 41: 275-285.

235. Lin CY, Loven J, Rahl PB, Paranal RM, Burge CB, et al. (2012) Transcriptional amplification in tumor cells with elevated c-Myc. Cell 151: 56-67.

236. Nie Z, Hu G, Wei G, Cui K, Yamane A, et al. (2012) c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell 151: 68-79.

237. Loven J, Orlando DA, Sigova AA, Lin CY, Rahl PB, et al. (2012) Revisiting global gene expression analysis. Cell 151: 476-482.

238. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 843-854.

239. Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75: 855-862.

240. Fabian MR, Sonenberg N, Filipowicz W (2010) Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79: 351-379.

241. Cai X, Hagedorn CH, Cullen BR (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10: 1957-1966.

242. Borchert GM, Lanier W, Davidson BL (2006) RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13: 1097-1101.

243. Lee Y, Kim M, Han J, Yeom KH, Lee S, et al. (2004) MicroRNA genes are transcribed by RNA polymerase II. Embo J 23: 4051-4060.

244. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123: 631-640.

245. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, et al. (2003) Dicer is essential for mouse development. Nat Genet 35: 215-217.

246. Kozomara A, Griffiths-Jones S (2011) miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res 39: D152-157.

247. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res 36: D154-158.

248. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92-105.

249. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, et al. (2005) Phylogenetic shadowing and computational identification of human microRNA genes. Cell 120: 21-24.

250. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20.

251. Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, et al. (2008) The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med 14: 1271-1277.

252. Sun F, Fu H, Liu Q, Tie Y, Zhu J, et al. (2008) Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. FEBS Lett 582: 1564-1568.

253. Xu T, Zhu Y, Xiong Y, Ge YY, Yun JP, et al. (2009) MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. Hepatology 50: 113-121.

254. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, et al. (2007) The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res 67: 7713-7722.

255. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, et al. (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 137: 1005-1017.

256. Schultz J, Lorenz P, Gross G, Ibrahim S, Kunz M (2008) MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. Cell Res 18: 549-557.

257. Yu Z, Wang C, Wang M, Li Z, Casimiro MC, et al. (2008) A cyclin D1/microRNA 17/20 regulatory feedback loop in control of breast cancer cell proliferation. J Cell Biol 182: 509-517.

258. Qin X, Wang X, Wang Y, Tang Z, Cui Q, et al. (2010) MicroRNA-19a mediates the suppressive effect of laminar flow on cyclin D1 expression in human umbilical vein endothelial cells. Proc Natl Acad Sci U S A 107: 3240-3244.

259. He L, He X, Lim LP, de Stanchina E, Xuan Z, et al. (2007) A microRNA component of the p53 tumour suppressor network. Nature 447: 1130-1134.

260. Tarasov V, Jung P, Verdoodt B, Lodygin D, Epanchintsev A, et al. (2007) Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle 6: 1586-1593.

261. Huang L, Luo J, Cai Q, Pan Q, Zeng H, et al. (2011) MicroRNA-125b suppresses the development of bladder cancer by targeting E2F3. Int J Cancer 128: 1758-1769.

262. Lal A, Navarro F, Maher CA, Maliszewski LE, Yan N, et al. (2009) miR-24 Inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to "seedless" 3'UTR microRNA recognition elements. Mol Cell 35: 610-625.

263. Wang F, Fu XD, Zhou Y, Zhang Y (2009) Down-regulation of the cyclin E1 oncogene expression by microRNA-16-1 induces cell cycle arrest in human cancer cells. BMB Rep 42: 725-730.

264. Pierson J, Hostager B, Fan R, Vibhakar R (2008) Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma. J Neurooncol 90: 1-7.

265. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68: 2094-2105.

266. Lal A, Kim HH, Abdelmohsen K, Kuwano Y, Pullmann R, Jr., et al. (2008) p16(INK4a) translation suppressed by miR-24. PLoS One 3: e1864.

267. Shi L, Zhang J, Pan T, Zhou J, Gong W, et al. (2010) MiR-125b is critical for the suppression of human U251 glioma stem cell proliferation. Brain Res 1312: 120-126.

268. Wu J, Qian J, Li C, Kwok L, Cheng F, et al. (2010) miR-129 regulates cell proliferation by downregulating Cdk6 expression. Cell Cycle 9: 1809-1818.

269. Feng M, Yu Q (2010) miR-449 regulates CDK-Rb-E2F1 through an auto-regulatory feedback circuit. Cell Cycle 9: 213-214.

270. Lujambio A, Ropero S, Ballestar E, Fraga MF, Cerrato C, et al. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67: 1424-1429.

271. Tian RQ, Wang XH, Hou LJ, Jia WH, Yang Q, et al. (2011) MicroRNA-372 is down-regulated and targets cyclin-dependent kinase 2 (CDK2) and cyclin A1 in human cervical cancer, which may contribute to tumorigenesis. J Biol Chem 286: 25556-25563.

272. Ivanovska I, Ball AS, Diaz RL, Magnus JF, Kibukawa M, et al. (2008) MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol 28: 2167-2174.

273. Kim YK, Yu J, Han TS, Park SY, Namkoong B, et al. (2009) Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. Nucleic Acids Res 37: 1672-1681.

274. Visone R, Russo L, Pallante P, De Martino I, Ferraro A, et al. (2007) MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. Endocr Relat Cancer 14: 791-798.

275. Wang X, Gocek E, Liu CG, Studzinski GP (2009) MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D3. Cell Cycle 8: 736-741.

276. Miller TE, Ghoshal K, Ramaswamy B, Roy S, Datta J, et al. (2008) MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. J Biol Chem 283: 29897-29903.

277. Medina R, Zaidi SK, Liu CG, Stein JL, van Wijnen AJ, et al. (2008) MicroRNAs 221 and 222 bypass quiescence and compromise cell survival. Cancer Res 68: 2773-2780.

278. Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, et al. (2007) miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem 282: 23716-23724.

279. Fornari F, Gramantieri L, Ferracin M, Veronese A, Sabbioni S, et al. (2008) MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene 27: 5651-5661.

280. le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, et al. (2007) Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. Embo J 26: 3699-3708.

281. Gillies JK, Lorimer IA (2007) Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. Cell Cycle 6: 2005-2009.

282. Lambeth LS, Yao Y, Smith LP, Zhao Y, Nair V (2009) MicroRNAs 221 and 222 target p27Kip1 in Marek's disease virus-transformed tumour cell line MSB-1. J Gen Virol 90: 1164-1171.

283. Malhas A, Saunders NJ, Vaux DJ (2010) The nuclear envelope can control gene expression and cell cycle progression via miRNA regulation. Cell Cycle 9: 531-539.

284. Qi J, Yu JY, Shcherbata HR, Mathieu J, Wang AJ, et al. (2009) microRNAs regulate human embryonic stem cell division. Cell Cycle 8: 3729-3741.

285. Butz H, Liko I, Czirjak S, Igaz P, Khan MM, et al. (2010) Down-regulation of Wee1 kinase by a specific subset of microRNA in human sporadic pituitary adenomas. J Clin Endocrinol Metab 95: E181-191.

286. Shi W, Alajez NM, Bastianutto C, Hui AB, Mocanu JD, et al. (2010) Significance of Plk1 regulation by miR-100 in human nasopharyngeal cancer. Int J Cancer 126: 2036-2048.

287. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435: 839-843.

288. Pickering MT, Stadler BM, Kowalik TF (2009) miR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression. Oncogene 28: 140-145.

289. Lin RJ, Lin YC, Yu AL (2010) miR-149* induces apoptosis by inhibiting Akt1 and E2F1 in human cancer cells. Mol Carcinog 49: 719-727.

290. Lee KH, Chen YL, Yeh SD, Hsiao M, Lin JT, et al. (2009) MicroRNA-330 acts as tumor suppressor and induces apoptosis of prostate cancer cells through E2F1-mediated suppression of Akt phosphorylation. Oncogene 28: 3360-3370.

291. Guo X, Guo L, Ji J, Zhang J, Chen X, et al. (2010) miRNA-331-3p directly targets E2F1 and induces growth arrest in human gastric cancer. Biochem Biophys Res Commun 398: 1-6.

292. Tazawa H, Tsuchiya N, Izumiya M, Nakagama H (2007) Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. Proc Natl Acad Sci U S A 104: 15472-15477.

293. Giannakakis A, Sandaltzopoulos R, Greshock J, Liang S, Huang J, et al. (2008) miR-210 links hypoxia with cell cycle regulation and is deleted in human epithelial ovarian cancer. Cancer Biol Ther 7: 255-264.

294. Benetti R, Gonzalo S, Jaco I, Munoz P, Gonzalez S, et al. (2008) A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 15: 268-279.

295. Wang Q, Li YC, Wang J, Kong J, Qi Y, et al. (2008) miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130. Proc Natl Acad Sci U S A 105: 2889-2894.

296. Cloonan N, Brown MK, Steptoe AL, Wani S, Chan WL, et al. (2008) The miR-17-5p microRNA is a key regulator of the G1/S phase cell cycle transition. Genome Biol 9: R127.

297. Venturini L, Battmer K, Castoldi M, Schultheis B, Hochhaus A, et al. (2007) Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. Blood 109: 4399-4405.

298. Inomata M, Tagawa H, Guo YM, Kameoka Y, Takahashi N, et al. (2009) MicroRNA-17-92 down-regulates expression of distinct targets in different B-cell lymphoma subtypes. Blood 113: 396-402.

299. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, et al. (2008) E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 13: 272-286.

300. Woods K, Thomson JM, Hammond SM (2007) Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. J Biol Chem 282: 2130-2134.

301. Sylvestre Y, De Guire V, Querido E, Mukhopadhyay UK, Bourdeau V, et al. (2007) An E2F/miR-20a autoregulatory feedback loop. J Biol Chem 282: 2135-2143.

302. Li Y, Tan W, Neo TW, Aung MO, Wasser S, et al. (2009) Role of the miR-106b-25 microRNA cluster in hepatocellular carcinoma. Cancer Sci 100: 1234-1242.

303. Yang X, Feng M, Jiang X, Wu Z, Li Z, et al. (2009) miR-449a and miR-449b are direct transcriptional targets of E2F1 and negatively regulate pRb-E2F1 activity through a feedback loop by targeting CDK6 and CDC25A. Genes Dev 23: 2388-2393.

304. Bueno MJ, Gomez de Cedron M, Laresgoiti U, Fernandez-Piqueras J, Zubiaga AM, et al. (2010) Multiple E2F-induced microRNAs prevent

replicative stress in response to mitogenic signaling. Mol Cell Biol 30: 2983-2995.

305. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, et al. (2008) Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet 40: 43-50.

306. Chang TC, Zeitels LR, Hwang HW, Chivukula RR, Wentzel EA, et al. (2009) Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. Proc Natl Acad Sci U S A 106: 3384-3389.

307. Dangi-Garimella S, Yun J, Eves EM, Newman M, Erkeland SJ, et al. (2009) Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7. Embo J 28: 347-358.

308. Sampson VB, Rong NH, Han J, Yang Q, Aris V, et al. (2007) MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res 67: 9762-9770.

309. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet 39: 673-677.

310. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, et al. (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 103: 2257-2261.

311. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065-7070.

312. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, et al. (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99: 15524-15529.

313. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, et al. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A 101: 2999-3004.

314. Bottoni A, Piccin D, Tagliati F, Luchin A, Zatelli MC, et al. (2005) miR-15a and miR-16-1 down-regulation in pituitary adenomas. J Cell Physiol 204: 280-285.

315. Takeshita F, Patrawala L, Osaki M, Takahashi RU, Yamamoto Y, et al. (2010) Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. Mol Ther 18: 181-187.

316. Xia L, Zhang D, Du R, Pan Y, Zhao L, et al. (2008) miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int J Cancer 123: 372-379.

317. Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, et al. (2004) Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res 64: 3087-3095.

318. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, et al. (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828-833.

319. Dews M, Homayouni A, Yu D, Murphy D, Sevignani C, et al. (2006) Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat Genet 38: 1060-1065.

320. Yu F, Yao H, Zhu P, Zhang X, Pan Q, et al. (2007) let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131: 1109-1123.

321. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, et al. (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64: 3753-3756.

322. Akao Y, Nakagawa Y, Naoe T (2006) let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. Biol Pharm Bull 29: 903-906.

323. Fang WJ, Lin CZ, Zhang HH, Qian J, Zhong L, et al. (2007) Detection of let-7a microRNA by real-time PCR in colorectal cancer: a single-centre experience from China. J Int Med Res 35: 716-723.

324. Eder M, Scherr M (2005) MicroRNA and lung cancer. N Engl J Med 352: 2446-2448.

325. Welch C, Chen Y, Stallings RL (2007) MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene 26: 5017-5022.

326. Sotillo E, Laver T, Mellert H, Schelter JM, Cleary MA, et al. (2011) Myc overexpression brings out unexpected antiapoptotic effects of miR-34a. Oncogene 30: 2587-2594.

327. Bagchi A, Mills AA (2008) The quest for the 1p36 tumor suppressor. Cancer Res 68: 2551-2556.

328. Bandres E, Cubedo E, Agirre X, Malumbres R, Zarate R, et al. (2006) Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol Cancer 5: 29.

329. Lanza G, Ferracin M, Gafa R, Veronese A, Spizzo R, et al. (2007) mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. Mol Cancer 6: 54.

330. Liu E, Li X, Yan F, Zhao Q, Wu X (2004) Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. J Biol Chem 279: 17283-17288.

331. Takeda DY, Parvin JD, Dutta A (2005) Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase. J Biol Chem 280: 23416-23423.

332. Sugimoto N, Tatsumi Y, Tsurumi T, Matsukage A, Kiyono T, et al. (2004) Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. J Biol Chem 279: 19691-19697.

333. Kim Y, Kipreos ET (2007) Cdt1 degradation to prevent DNA re-replication: conserved and non-conserved pathways. Cell Div 2: 18.

334. Mailand N, Diffley JF (2005) CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell 122: 915-926.

335. Paolinelli R, Mendoza-Maldonado R, Cereseto A, Giacca M (2009) Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle. Nat Struct Mol Biol 16: 412-420.

336. Saha P, Chen J, Thome KC, Lawlis SJ, Hou ZH, et al. (1998) Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. Mol Cell Biol 18: 2758-2767.

337. Coverley D, Pelizon C, Trewick S, Laskey RA (2000) Chromatin-bound Cdc6 persists in S and G2 phases in human cells, while soluble Cdc6 is destroyed in a cyclin A-cdk2 dependent process. J Cell Sci 113 (Pt 11): 1929-1938.

338. Kim J, Feng H, Kipreos ET (2007) C. elegans CUL-4 prevents rereplication by promoting the nuclear export of CDC-6 via a CKI-1-dependent pathway. Curr Biol 17: 966-972.

339. Fujita M, Yamada C, Goto H, Yokoyama N, Kuzushima K, et al. (1999) Cell cycle regulation of human CDC6 protein. Intracellular localization,

interaction with the human mcm complex, and CDC2 kinase-mediated hyperphosphorylation. J Biol Chem 274: 25927-25932.

340. Jiang W, Wells NJ, Hunter T (1999) Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci U S A 96: 6193-6198.

341. Petersen BO, Lukas J, Sorensen CS, Bartek J, Helin K (1999) Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. Embo J 18: 396-410.

342. Mendez J, Stillman B (2000) Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol 20: 8602-8612.

343. Lau E, Zhu C, Abraham RT, Jiang W (2006) The functional role of Cdc6 in S-G2/M in mammalian cells. EMBO Rep 7: 425-430.

344. Saha T, Ghosh S, Vassilev A, DePamphilis ML (2006) Ubiquitylation, phosphorylation and Orc2 modulate the subcellular location of Orc1 and prevent it from inducing apoptosis. J Cell Sci 119: 1371-1382.

345. Green BM, Morreale RJ, Ozaydin B, Derisi JL, Li JJ (2006) Genome-wide mapping of DNA synthesis in Saccharomyces cerevisiae reveals that mechanisms preventing reinitiation of DNA replication are not redundant. Mol Biol Cell 17: 2401-2414.

346. Tanny RE, MacAlpine DM, Blitzblau HG, Bell SP (2006) Genome-wide analysis of re-replication reveals inhibitory controls that target multiple stages of replication initiation. Mol Biol Cell 17: 2415-2423.

347. Nguyen VQ, Co C, Li JJ (2001) Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature 411: 1068-1073.

348. Wilmes GM, Archambault V, Austin RJ, Jacobson MD, Bell SP, et al. (2004) Interaction of the S-phase cyclin Clb5 with an "RXL" docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch. Genes Dev 18: 981-991.

349. DePamphilis ML (2005) Cell cycle dependent regulation of the origin recognition complex. Cell Cycle 4: 70-79.

350. Francis LI, Randell JC, Takara TJ, Uchima L, Bell SP (2009) Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation. Genes Dev 23: 643-654.

351. Masai H, Matsui E, You Z, Ishimi Y, Tamai K, et al. (2000) Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 bY Cdks. J Biol Chem 275: 29042-29052.

352. Sheu YJ, Stillman B (2006) Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell 24: 101-113.

353. Geng Y, Lee YM, Welcker M, Swanger J, Zagozdzon A, et al. (2007) Kinase-independent function of cyclin E. Mol Cell 25: 127-139.

354. Lee C, Hong B, Choi JM, Kim Y, Watanabe S, et al. (2004) Structural basis for inhibition of the replication licensing factor Cdt1 by geminin. Nature 430: 913-917.

355. Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, et al. (2000) Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 290: 2309-23012.

356. Tada S, Li A, Maiorano D, Mechali M, Blow JJ (2001) Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat Cell Biol 3: 107-113.

357. McGarry TJ, Kirschner MW (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell 93: 1043-1053.

358. Rape M, Reddy SK, Kirschner MW (2006) The processivity of multiubiquitination by the APC determines the order of substrate degradation. Cell 124: 89-103.

359. De Marco V, Gillespie PJ, Li A, Karantzelis N, Christodoulou E, et al. (2009) Quaternary structure of the human Cdt1-Geminin complex regulates DNA replication licensing. Proc Natl Acad Sci U S A 106: 19807-19812.

360. Lutzmann M, Maiorano D, Mechali M (2006) A Cdt1-geminin complex licenses chromatin for DNA replication and prevents rereplication during S phase in Xenopus. Embo J 25: 5764-5774.

361. Ode KL, Fujimoto K, Kubota Y, Takisawa H (2011) Inter-origin cooperativity of geminin action establishes an all-or-none switch for replication origin licensing. Genes Cells 16: 380-396.

362. Sudakin V, Ganoth D, Dahan A, Heller H, Hershko J, et al. (1995) The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. Mol Biol Cell 6: 185-197.

363. King RW, Peters JM, Tugendreich S, Rolfe M, Hieter P, et al. (1995) A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell 81: 279-288.

364. Wei W, Ayad NG, Wan Y, Zhang GJ, Kirschner MW, et al. (2004) Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. Nature 428: 194-198.

365. Zachariae W, Schwab M, Nasmyth K, Seufert W (1998) Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. Science 282: 1721-1724.

366. Hsu JY, Reimann JD, Sorensen CS, Lukas J, Jackson PK (2002) E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). Nat Cell Biol 4: 358-366.

367. Di Fiore B, Pines J (2007) Emi1 is needed to couple DNA replication with mitosis but does not regulate activation of the mitotic APC/C. J Cell Biol 177: 425-437.

368. Moshe Y, Boulaire J, Pagano M, Hershko A (2004) Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. Proc Natl Acad Sci U S A 101: 7937-7942.

369. Sivaprasad U, Machida YJ, Dutta A (2007) APC/C--the master controller of origin licensing? Cell Div 2: 8.

370. Machida YJ, Dutta A (2007) The APC/C inhibitor, Emi1, is essential for prevention of rereplication. Genes Dev 21: 184-194.

371. Nishitani H, Sugimoto N, Roukos V, Nakanishi Y, Saijo M, et al. (2006) Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. Embo J 25: 1126-1136.

372. Senga T, Sivaprasad U, Zhu W, Park JH, Arias EE, et al. (2006) PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination. J Biol Chem 281: 6246-6252.

373. Jin J, Arias EE, Chen J, Harper JW, Walter JC (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. Mol Cell 23: 709-721.

374. Arias EE, Walter JC (2006) PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. Nat Cell Biol 8: 84-90.

375. Higa LA, Banks D, Wu M, Kobayashi R, Sun H, et al. (2006) L2DTL/CDT2 interacts with the CUL4/DDB1 complex and PCNA and regulates CDT1 proteolysis in response to DNA damage. Cell Cycle 5: 1675-1680.

376. Hu J, Xiong Y (2006) An evolutionarily conserved function of proliferating cell nuclear antigen for Cdt1 degradation by the Cul4-Ddb1 ubiquitin ligase in response to DNA damage. J Biol Chem 281: 3753-3756.

377. Ralph E, Boye E, Kearsey SE (2006) DNA damage induces Cdt1 proteolysis in fission yeast through a pathway dependent on Cdt2 and Ddb1. EMBO Rep 7: 1134-9113.

378. Maga G, Hubscher U (2003) Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci 116: 3051-3060.

379. Abbas T, Shibata E, Park J, Jha S, Karnani N, et al. (2010) CRL4(Cdt2) regulates cell proliferation and histone gene expression by targeting PR-Set7/Set8 for degradation. Mol Cell 40: 9-21.

380. Centore RC, Havens CG, Manning AL, Li JM, Flynn RL, et al. (2010) CRL4(Cdt2)-mediated destruction of the histone methyltransferase Set8 prevents premature chromatin compaction in S phase. Mol Cell 40: 22-33.

381. Oda H, Hubner MR, Beck DB, Vermeulen M, Hurwitz J, et al. (2010) Regulation of the histone H4 monomethylase PR-Set7 by CRL4(Cdt2)-mediated PCNA-dependent degradation during DNA damage. Mol Cell 40: 364-376.

382. Jorgensen S, Eskildsen M, Fugger K, Hansen L, Larsen MS, et al. (2011) SET8 is degraded via PCNA-coupled CRL4(CDT2) ubiquitylation in S phase and after UV irradiation. J Cell Biol 192: 43-54.

383. Tardat M, Brustel J, Kirsh O, Lefevbre C, Callanan M, et al. (2010) The histone H4 Lys 20 methyltransferase PR-Set7 regulates replication origins in mammalian cells. Nat Cell Biol.

384. Houston SI, McManus KJ, Adams MM, Sims JK, Carpenter PB, et al. (2008) Catalytic function of the PR-Set7 histone H4 lysine 20 monomethyltransferase is essential for mitotic entry and genomic stability. J Biol Chem 283: 19478-19488.

385. Ohta S, Koide M, Tokuyama T, Yokota N, Nishizawa S, et al. (2001) Cdc6 expression as a marker of proliferative activity in brain tumors. Oncol Rep 8: 1063-1066.

386. Pinyol M, Salaverria I, Bea S, Fernandez V, Colomo L, et al. (2006) Unbalanced expression of licensing DNA replication factors occurs in a subset of mantle cell lymphomas with genomic instability. Int J Cancer 119: 2768-2774.

387. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, et al. (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 444: 638-642.

388. Nielsen NH, Arnerlov C, Emdin SO, Landberg G (1996) Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. Br J Cancer 74: 874-880.

389. Gonzalez S, Klatt P, Delgado S, Conde E, Lopez-Rios F, et al. (2006) Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. Nature 440: 702-706.

390. Guernsey DL, Matsuoka M, Jiang H, Evans S, Macgillivray C, et al. (2011) Mutations in origin recognition complex gene ORC4 cause Meier-Gorlin syndrome. Nat Genet 43: 360-364.

391. Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, et al. (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell 13: 1977-2000.

392. Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, et al. (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. Sci Signal 3: ra3.

393. Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. Annu Rev Biochem 71: 333-374.

394. Cho RJ, Huang M, Campbell MJ, Dong H, Steinmetz L, et al. (2001) Transcriptional regulation and function during the human cell cycle. Nat Genet 27: 48-54.

395. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1: 2856-2860.

396. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26: 1367-1372.

397. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, et al. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res 10: 1794-1805.

398. Gauthier NP, Larsen ME, Wernersson R, de Lichtenberg U, Jensen LJ, et al. (2008) Cyclebase.org--a comprehensive multi-organism online database of cell-cycle experiments. Nucleic Acids Res 36: D854-859.

399. Gauthier NP, Jensen LJ, Wernersson R, Brunak S, Jensen TS (2009) Cyclebase.org: version 2.0, an updated comprehensive, multi-species repository of cell cycle experiments and derived analysis results. Nucleic Acids Res 38: D699-702.

400. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, et al. (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell 44: 325-340.

401. Stokes MP, Rush J, Macneill J, Ren JM, Sprott K, et al. (2007) Profiling of UV-induced ATM/ATR signaling pathways. Proc Natl Acad Sci U S A 104: 19855-19860.

402. Chi Y, Welcker M, Hizli AA, Posakony JJ, Aebersold R, et al. (2008) Identification of CDK2 substrates in human cell lysates. Genome Biol 9: R149.

403. Boisvert FM, Ahmad Y, Gierlinski M, Charriere F, Lamont D, et al. (2012) A quantitative spatial proteomics analysis of proteome turnover in human cells. Mol Cell Proteomics 11: M111 011429.

404. Huang dW, Sherman B, Lempicki R (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 44-57.

405. Huang dW, Sherman B, Lempicki R (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37: 1-13.

406. Wang ZF, Whitfield ML, Ingledue TC, 3rd, Dominski Z, Marzluff WF (1996) The protein that binds the 3' end of histone mRNA: a novel RNA-binding protein required for histone pre-mRNA processing. Genes Dev 10: 3028-3040.

407. Fischer RS, Fritz-Six KL, Fowler VM (2003) Pointed-end capping by tropomodulin3 negatively regulates endothelial cell motility. J Cell Biol 161: 371-380.

408. Chen X, Smith LM, Bradbury EM (2000) Site-specific mass tagging with stable isotopes in proteins for accurate and efficient protein identification. Anal Chem 72: 1134-1143.

409. Zhu H, Pan S, Gu S, Bradbury EM, Chen X (2002) Amino acid residue specific stable isotope labeling for quantitative proteomics. Rapid Commun Mass Spectrom 16: 2115-2123.

410. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, et al. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple

and accurate approach to expression proteomics. Mol Cell Proteomics 1: 376-386.

411. Zheng L, Dominski Z, Yang XC, Elms P, Raska CS, et al. (2003) Phosphorylation of stem-loop binding protein (SLBP) on two threonines triggers degradation of SLBP, the sole cell cycle-regulated factor required for regulation of histone mRNA processing, at the end of S phase. Mol Cell Biol 23: 1590-1601.

412. Koseoglu MM, Graves LM, Marzluff WF (2008) Phosphorylation of threonine 61 by cyclin a/Cdk1 triggers degradation of stem-loop binding protein at the end of S phase. Mol Cell Biol 28: 4469-4479.

413. Koseoglu MM, Dong J, Marzluff WF (2010) Coordinate regulation of histone mRNA metabolism and DNA replication: cyclin A/cdk1 is involved in inactivation of histone mRNA metabolism and DNA replication at the end of S phase. Cell Cycle 9: 3857-3863.

414. Nagaraj N, Wisniewski JR, Geiger T, Cox J, Kircher M, et al. (2011) Deep proteome and transcriptome mapping of a human cancer cell line. Mol Syst Biol 7: 548.

415. Tsubuki S, Kawasaki H, Saito Y, Miyashita N, Inomata M, et al. (1993) Purification and characterization of a Z-Leu-Leu-Leu-MCA degrading protease expected to regulate neurite formation: a novel catalytic activity in proteasome. Biochem Biophys Res Commun 196: 1195-1201.

416. Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S (1996) Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. J Biochem 119: 572-576.

417. Wagner SA, Beli P, Weinert BT, Nielsen ML, Cox J, et al. (2011) A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. Mol Cell Proteomics 10: M111 013284.

418. Chabes A, Thelander L (2000) Controlled protein degradation regulates ribonucleotide reductase activity in proliferating mammalian cells during the normal cell cycle and in response to DNA damage and replication blocks. J Biol Chem 275: 17747-17753.

419. Chabes AL, Pfleger CM, Kirschner MW, Thelander L (2003) Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. Proc Natl Acad Sci U S A 100: 3925-3929.

420. Dart DA, Adams KE, Akerman I, Lakin ND (2004) Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase. J Biol Chem 279: 16433-16440.

421. Hegele A, Kamburov A, Grossmann A, Sourlis C, Wowro S, et al. (2012) Dynamic protein-protein interaction wiring of the human spliceosome. Mol Cell 45: 567-580.

422. Zhu J, Mayeda A, Krainer AR (2001) Exon identity established through differential antagonism between exonic splicing silencer-bound hnRNP A1 and enhancer-bound SR proteins. Mol Cell 8: 1351-1361.

423. Lam BJ, Hertel KJ (2002) A general role for splicing enhancers in exon definition. RNA 8: 1233-1241.

424. House AE, Lynch KW (2006) An exonic splicing silencer represses spliceosome assembly after ATP-dependent exon recognition. Nat Struct Mol Biol 13: 937-944.

425. Martinez-Contreras R, Fisette JF, Nasim FU, Madden R, Cordeau M, et al. (2006) Intronic binding sites for hnRNP A/B and hnRNP F/H proteins stimulate pre-mRNA splicing. PLoS Biol 4: e21.

426. Molina H, Horn DM, Tang N, Mathivanan S, Pandey A (2007) Global proteomic profiling of phosphopeptides using electron transfer dissociation tandem mass spectrometry. Proc Natl Acad Sci U S A 104: 2199-2204.

427. Imami K, Sugiyama N, Kyono Y, Tomita M, Ishihama Y (2008) Automated phosphoproteome analysis for cultured cancer cells by two-dimensional nanoLC-MS using a calcined titania/C18 biphasic column. Anal Sci 24: 161-166.

428. Cantin GT, Yi W, Lu B, Park SK, Xu T, et al. (2008) Combining protein-based IMAC, peptide-based IMAC, and MudPIT for efficient phosphoproteomic analysis. J Proteome Res 7: 1346-1351.

429. Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, et al. (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol Cell 31: 438-448.

430. Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, et al. (2008) A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci U S A 105: 10762-10767.

431. Gauci S, Helbig AO, Slijper M, Krijgsveld J, Heck AJ, et al. (2009) Lys-N and trypsin cover complementary parts of the phosphoproteome in a refined SCX-based approach. Anal Chem 81: 4493-4501.

432. Mayya V, Lundgren DH, Hwang SI, Rezaul K, Wu L, et al. (2009) Quantitative phosphoproteomic analysis of T cell receptor signaling reveals system-wide modulation of protein-protein interactions. Sci Signal 2: ra46.

433. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, et al. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325: 834-840.

434. Matlin AJ, Clark F, Smith CW (2005) Understanding alternative splicing: towards a cellular code. Nat Rev Mol Cell Biol 6: 386-398.

435. Adamson B, Smogorzewska A, Sigoillot FD, King RW, Elledge SJ (2012) A genome-wide homologous recombination screen identifies the RNA-binding protein RBMX as a component of the DNA-damage response. Nat Cell Biol 14: 318-328.

436. Dery KJ, Gaur S, Gencheva M, Yen Y, Shively JE, et al. (2011) Mechanistic control of carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) splice isoforms by the heterogeneous nuclear ribonuclear proteins hnRNP L, hnRNP A1, and hnRNP M. J Biol Chem 286: 16039-16051.

437. Wang Y, Ma M, Xiao X, Wang Z (2012) Intronic splicing enhancers, cognate splicing factors and context-dependent regulation rules. Nat Struct Mol Biol 19: 1044-1052.

438. Huelga SC, Vu AQ, Arnold JD, Liang TY, Liu PP, et al. (2012) Integrative genome-wide analysis reveals cooperative regulation of alternative splicing by hnRNP proteins. Cell Rep 1: 167-178.

439. McCormack PL (2012) Carfilzomib: In Relapsed, or Relapsed and Refractory, Multiple Myeloma. Drugs.

440. Vij R, Siegel DS, Jagannath S, Jakubowiak AJ, Stewart AK, et al. (2012) An open-label, single-arm, phase 2 study of single-agent carfilzomib in patients with relapsed and/or refractory multiple myeloma who have been previously treated with bortezomib. Br J Haematol 158: 739-748.

441. Han YH, Moon HJ, You BR, Park WH (2009) The effect of MG132, a proteasome inhibitor on HeLa cells in relation to cell growth, reactive oxygen species and GSH. Oncol Rep 22: 215-221.

442. Havens CG, Walter JC (2009) Docking of a specialized PIP Box onto chromatin-bound PCNA creates a degron for the ubiquitin ligase CRL4Cdt2. Mol Cell 35: 93-104.

443. Melixetian M, Ballabeni A, Masiero L, Gasparini P, Zamponi R, et al. (2004) Loss of Geminin induces rereplication in the presence of functional p53. J Cell Biol 165: 473-482.

444. Vaziri C, Saxena S, Jeon Y, Lee C, Murata K, et al. (2003) A p53-dependent checkpoint pathway prevents rereplication. Mol Cell 11: 997-1008.

445. Mihaylov IS, Kondo T, Jones L, Ryzhikov S, Tanaka J, et al. (2002) Control of DNA replication and chromosome ploidy by geminin and cyclin A. Mol Cell Biol 22: 1868-1880.

446. Davidson IF, Li A, Blow JJ (2006) Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision. Mol Cell 24: 433-443.

447. Archambault V, Ikui AE, Drapkin BJ, Cross FR (2005) Disruption of mechanisms that prevent rereplication triggers a DNA damage response. Mol Cell Biol 25: 6707-6721.

448. Zhu W, Chen Y, Dutta A (2004) Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. Mol Cell Biol 24: 7140-7150.

449. Green BM, Finn KJ, Li JJ (2010) Loss of DNA replication control is a potent inducer of gene amplification. Science 329: 943-946.

450. Dorn ES, Chastain PD, 2nd, Hall JR, Cook JG (2009) Analysis of re-replication from deregulated origin licensing by DNA fiber spreading. Nucleic Acids Res 37: 60-69.

451. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, et al. (2006) Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 444: 633-637.

452. Denko NC, Giaccia AJ, Stringer JR, Stambrook PJ (1994) The human Ha-ras oncogene induces genomic instability in murine fibroblasts within one cell cycle. Proc Natl Acad Sci U S A 91: 5124-5128.

453. Abbas T, Sivaprasad U, Terai K, Amador V, Pagano M, et al. (2008) PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdt2 ubiquitin ligase complex. Genes Dev 22: 2496-2506.

454. Legesse-Miller A, Elemento O, Pfau SJ, Forman JJ, Tavazoie S, et al. (2009) let-7 Overexpression leads to an increased fraction of cells in G2/M, direct down-regulation of Cdc34, and stabilization of Wee1 kinase in primary fibroblasts. J Biol Chem 284: 6605-6609.

455. Sansam CL, Shepard JL, Lai K, Ianari A, Danielian PS, et al. (2006) DTL/CDT2 is essential for both CDT1 regulation and the early G2/M checkpoint. Genes Dev 20: 3117-3129.

456. Kim Y, Starostina NG, Kipreos ET (2008) The CRL4Cdt2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing. Genes Dev 22: 2507-2519.

457. Nishitani H, Shiomi Y, Iida H, Michishita M, Takami T, et al. (2008) CDK inhibitor p21 is degraded by a proliferating cell nuclear antigen-coupled Cul4-DDB1Cdt2 pathway during S phase and after UV irradiation. J Biol Chem 283: 29045-29052.

458. Network CGA (2012) Comprehensive molecular portraits of human breast tumours. Nature 490: 61-70.

459. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, et al. (2000) Molecular portraits of human breast tumours. Nature 406: 747-752.

460. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98: 10869-10874.

461. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, et al. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 100: 8418-8423.

462. Hu Z, Fan C, Oh DS, Marron JS, He X, et al. (2006) The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 7: 96.

463. Zhou T, Chou JW, Simpson DA, Zhou Y, Mullen TE, et al. (2006) Profiles of global gene expression in ionizing-radiation-damaged human diploid fibroblasts reveal synchronization behind the G1 checkpoint in a G0-like state of quiescence. Environ Health Perspect 114: 553-559.

464. Lu X, Shao J, Li H, Yu Y (2010) Temporal gene expression changes induced by a low concentration of benzo[a]pyrene diol epoxide in a normal human cell line. Mutat Res 684: 74-80.

465. Belitskaya-Levy I, Hajjou M, Su WC, Yie TA, Tchou-Wong KM, et al. (2007) Gene profiling of normal human bronchial epithelial cells in response to asbestos and benzo(a)pyrene diol epoxide (BPDE). J Environ Pathol Toxicol Oncol 26: 281-294.

466. Ding LH, Shingyoji M, Chen F, Hwang JJ, Burma S, et al. (2005) Gene expression profiles of normal human fibroblasts after exposure to ionizing radiation: a comparative study of low and high doses. Radiat Res 164: 17-26.

467. Hall JR, Lee HO, Bunker BD, Dorn ES, Rogers GC, et al. (2008) CDT1 and CDC6 are destablized by rereplication-induced DNA damage. J Biol Chem: 25356-25363.

468. Rogakou EP, Boon C, Redon C, Bonner WM (1999) Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol 146: 905-916.

469. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, et al. (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817-825.

470. Di Leonardo A, Linke SP, Clarkin K, Wahl GM (1994) DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes Dev 8: 2540-2551.

471. Waldman T, Kinzler KW, Vogelstein B (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res 55: 5187-5190.

472. el-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, et al. (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 54: 1169-1174.

473. Waga S, Hannon GJ, Beach D, Stillman B (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369: 574-578.

474. Shivji MK, Grey SJ, Strausfeld UP, Wood RD, Blow JJ (1994) Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. Curr Biol 4: 1062-1068.

475. Podust VN, Podust LM, Goubin F, Ducommun B, Hubscher U (1995) Mechanism of inhibition of proliferating cell nuclear antigen-dependent DNA synthesis by the cyclin-dependent kinase inhibitor p21. Biochemistry 34: 8869-8875.

476. Liu C, Poitelea M, Watson A, Yoshida SH, Shimoda C, et al. (2005) Transactivation of Schizosaccharomyces pombe cdt2+ stimulates a Pcu4-Ddb1-CSN ubiquitin ligase. Embo J 24: 3940-3951.

477. Moss J, Tinline-Purvis H, Walker CA, Folkes LK, Stratford MR, et al. (2010) Break-induced ATR and Ddb1-Cul4(Cdt)(2) ubiquitin ligase-dependent nucleotide synthesis promotes homologous recombination repair in fission yeast. Genes Dev 24: 2705-2716.

478. Lin JJ, Milhollen MA, Smith PG, Narayanan U, Dutta A (2010) NEDD8-targeting drug MLN4924 elicits DNA rereplication by stabilizing Cdt1 in S phase, triggering checkpoint activation, apoptosis, and senescence in cancer cells. Cancer Res 70: 10310-10320.

479. Gong L, Yeh ET (1999) Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. J Biol Chem 274: 12036-12042.

480. Evsyukova I, Bradrick SS, Gregory SG, Garcia-Blanco MA (2012) Cleavage and polyadenylation specificity factor 1 (CPSF1) regulates alternative splicing of interleukin 7 receptor (IL7R) exon 6. RNA.

481. Hall JR, Kow E, Nevis KR, Lu CK, Luce KS, et al. (2007) Cdc6 stability is regulated by the Huwe1 ubiquitin ligase after DNA damage. Mol Biol Cell 18: 3340-3350.

482. Iizuka M, Sarmento OF, Sekiya T, Scrable H, Allis CD, et al. (2008) Hbo1 Links p53-dependent stress signaling to DNA replication licensing. Mol Cell Biol 28: 140-153.

483. Iizuka M, Matsui T, Takisawa H, Smith MM (2006) Regulation of replication licensing by acetyltransferase Hbo1. Mol Cell Biol 26: 1098-1108.

484. Wang C, Chen L, Hou X, Li Z, Kabra N, et al. (2006) Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. Nat Cell Biol 8: 1025-1031.